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UNIVERSITÀ
DEGLI STUDI
DI MILANO

DOCTORAL THESIS

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DEVELOPMENT OF COMBINED ANALYTICAL TECHNIQUES TO DETECT QUALITY AND AUTHENTICITY ATTRIBUTES IN ANIMAL PRODUCTS

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy and *Doctor Europaeus*

SCIENTIFIC SUPERVISOR

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SSD: AGR/19

AA2020/2021
XXXIV Doctoral Cycle

“No me arrepiento si me perdí,
fue cuestión de tiempo,
el camino llevó hasta aquí”

Abstract

Nowadays, consumers are reorienting themselves more and more toward the consumption of food products characterized by additional quality traits. Since consumers cannot always personally verify the attributes of foodstuffs, these have to be guaranteed by a robust quality assurance process acting along the whole supply chain. An important worldwide increase of the demand for animal source foods is occurring, leading to the arising of a phenomenon known as the "livestock revolution". However, animal food products are considered among the most frequently adulterated edible goods.

Many compounds naturally present in animal products may represent important biomarkers related to considerable *authenticity* and *quality* issues. In the experimental part of this PhD thesis, chromatographic analytical techniques were developed and employed with the aim to identify and characterize chemical markers of integrity in animal products. This approach was employed on two levels: on a **farm-scale**, collecting raw material directly in the farms, and on a **manufactory-scale**, collecting transformed products, in order to relate their quality to the production process. Research was conducted on two different animal products: a) dairy products and b) fish products, particularly sturgeon meat and fish roes.

a) Dairy products

In **Trial 1**, the fatty acid (FA) composition of goat milk yielded in three Italian farms, chategorized based on the production system employed, was investigated by means of gas-chromatography and flame ionization detection (GC-FID). Results allowed to detect significant differences among milk collected in the three farms, particularly enhanced for many fatty acids, which amount varied in relation to the livestock system. Odd and branched chain fatty acids (OBCFA), linoleic acid (LA), α-linolneic acid (ALA), elaidic acid (EA), total n3 and n6 series FA were identified as the most significant factors in the characterization of samples coming from low- or high-input livestock systems in goat milk.

In **Trial 2**, nutritional quality of goat cheese obtained by GC-FID analysis of the acidic profile was supplemented with animal welfare measurements performed in the farms involved in **Trial 1** by means of a standardized protocol. The so-called *extrinsic quality* of goat cheese samples analysed, represented by the chemical profile determined by laboratory analysis, showed a significant higher quality in cheese collected from low-input farms, confirming the results obtained in **Trial 1**. On the contrary, welfare parameters (referred to as *intrinsic quality*) showed that no clear relationship was observable between the level of the animal welfare and the livestock systems analysed. The results of this trial indicated that the *extrinsic* and the *intrinsic quality* of low-input farms did not always match, suggesting that the information on the livestock system is not always enough in order to provide consumers with complete awareness of the total product quality.

In **Trial 3**, fat quality in Alpine cow milk was characterized by means of GC-FID and related to the seasonal variations of diets. Two different feeding strategies were compared in two small mountain farms in Piedmont Alpine region, Italy. Particularly, during the summer season, one of the two farms was distinguished by the exclusive employment of Alpine pasture, assumed as the best way to improve the quality of FA profile in milk.

Milk samples obtained by the exclusive employment of alpine grazing during summer (farm A) were represented by a FA profile of higher quality. However, during the summer season, milk obtained by the integrated strategy (farm B) resulted in a more homogeneous composition, with higher concentration of polyunsaturated FA (PUFA). These outcomes confirmed that the integrated strategy, even if related to a slightly lower ability in improving milk FA profile, could represent a valid and cost-effective alternative for mountain farmers to obtain an overall superior quality of milk, being not strictly linked to the grazing practice.

b) Fish products – sturgeon meat and fish eggs

In **Trial 4**, sturgeon raw eggs, caviar and meat obtained from different species reared in an Italian production plant were evaluated for their chemical composition, in order to improve their appreciation on the market and to detect any eventual distinctness related to the species. Fatty acids profile was investigated by GC-FID and multivariate statistics. Important differences in the deposition of individual fatty acids in sturgeon meat and ovas were detected, principally based on their different biological role. Particularly, interesting differences were observed in the different FA profile that characterized the phospholipid and the neutral lipid fractions in sturgeon roes. Finally, colour parameters were measured on sturgeon fillets, characterizing the species-specific properties of sturgeon meat.

In **Trial 5**, quality, traceability and safety issues related to processed fish roe products from different species were investigated. FA was performed by GC-FID. Results showed a differentiation among eggs harvested from different fish species. Then, a discriminant model was applied including an external set of sturgeon roes samples, coming from **Trial 4,** that enabled a good discrimination among roes from sturgeon or other spescies. Additionally, food safety of fish roes products was investigated, proving a general good hygienic level for the products analysed. On the overall, results obtained by this trial suggested fish roes as safe food products, which can also provide human nutrition a valuable content of essential fatty acids.

In **Trial 6**, a multiple headspace solid phase microextraction (MHS-SPME) followed by GC and mass spectrometry detection (MS) was firstly developed and subsequently employed to identify and quantitatively estimate the presence of volatile compounds (VOCs) in white sturgeon caviar during 4 months of ripening. The method allowed the detection and the quantitative estimation of twenty-five VOCs, mainly represented by aldehydes and alcohols, already know as main representative of fish and seafood volatilome, without any severe alteration of the matrix before the analysis and in a short time. Mainly, an increase of the amount of total aldehydes was observed in 4 months of ripening; moreover, the arise of 3-hydroxy-2-butanone was observed at the last sampling time.

In **Trial 7**, a HS-SPME-GC-MS method was applied to evaluate the evolution of the volatile profile in white sturgeon caviar treated with different preservative mixtures in an extended storage time (up to 14 months). Results obtained for the VOCs profile were matched with microbiological analysis. Multivariate statistics allowed the identification of different clusters based on the time of ripening and the preservative treatment used.

Particularly, samples added just with salt were characterized by the highest viable counts and the greatest presence of VOCs driven by spoilage processes, strongly enhanced by the prolonged ripening time, pushed beyond the optimal maturation time generally considered in caviar production plants.

In **Trial 8**, a ultra high performance liquid chromatography and high resolution mass spectrometry (UHPLC-HRMS) method was developed and optimized in order to characterize the non volatile metabolome of white sturgeon caviar during the ripening. Both the optimization of the method and data analysis were performed by chemometrics techniques. Results showed a strong evolution of the non volatile compounds present in the aqueous phase of sturgeon eggs during the first 4 months of ripening and then a partial stabilization. Most of the compound tentatively identified were associated to a high nutritional value, comprising protein and non protein amino acids, phospholipids species made of long chain and unsaturated fatty acids and vitamins. Moreover, the presence of chemical species related to fundamental impact on the characteristic *umami* taste in food, such as glutamic acid and nucleotides, were detected.

Sintesi

I consumatori moderni sono sempre più orientati al consumo di prodotti alimentari caratterizzati da proprietà riferibili alla cosidetta qualità aggiunta. Poichè, però, essi non sono sempre in grado di verificare personalmente le caratteristiche dei prodotti che acquistano, queste devono essere garantite da un processo di assicurazione di qualità robusto e affidabile, in grado di agire efficacemente lungo tutta l'intera catena di approvvigionamento. A livello globale, attualmente, si sta verificando un importante aumento della domanda di alimenti di origine animale, fenomeno noto come '*livestock revolution*'. Al contempo, i prodotti di origine animale sono tra i prodotti più frequentemente coinvolti nei casi di frode e adulterazione alimentare.

Alcuni composti naturalmente presenti negli alimenti di origine animale possono essere considerati importanti bio-marcatori, ovvero indicatori di caratteristiche di *autenticità* e *qualità*. Nella parte sperimentale di questa tesi di dottorato, sono state sviluppate e impiegate tecniche analitiche di cromatografia con l'obiettivo di identificare e caratterizzare marcatori di integrità in prodotti di origine animale. L'approccio analitico è stato adottato su due livelli: a **livello primario**, campionando la materia prima direttamente nelle aziende agricole, e su **scala manifatturiera**, campionando i prodotti già trasformati, sul mercato, in maniera tale da mettere in relazione la qualità finale con il processo produttivo. La ricerca è stata condotta su due tipologie diverse di prodotti di origine animale: a) prodotti lattiero-caseari e b) prodotti ittici, in particolare carne di storione e uova di pesce.

a) Prodotti lattiero-caseari

Nella **Prova 1** è stata studiata la composizione in acidi grassi (AG) del latte di capra prodotto in tre diversi allevamenti italiani, classificati in base al sistema produttivo impiegato, mediante gas-cromatografia e rivelazione a ionizzazione di fiamma (GC-FID). I risultati hanno permesso di evidenziare differenze significative tra i campioni di latte raccolto nei tre allevamenti, differenze particolarmente accentuate per la quantità di alcuni acidi grassi, variabili in relazione al sistema zootecnico impiegato nella corrispettiva azienda. Gli acidi grassi a catena dispari e ramificata (OBCFA), l'acido linoleico (LA), l'acido alfa-linolenico (ALA), l'acido elaidico (EA), gli acidi grassi totali delle serie n3 e n6 sono stati identificati come i fattori più significativi nella caratterizzazione dei campioni di latte di capra provenienti da sistemi di allevamento a basso o alto input.

Nella **Prova 2**, le informazioni sulla qualità nutrizionale del formaggio di capra ottenuta mediante analisi GC-FID del profilo acidico sono state integrate con parametri ottenuti dalla valutazione del benessere animale negli allevamenti coinvolti nella **Prova 1**, mediante un protocollo standardizzato. La cosiddetta qualità *estrinseca* dei campioni di formaggio di capra analizzati, rappresentata dal loro profilo chimico determinato mediante analisi di laboratorio, ha mostrato una qualità significativamente più elevata nei formaggi raccolti da allevamenti a basso input, confermando i risultati ottenuti nella **Prova 1**. Al contrario, i parametri di benessere (cui si fa riferimento con *qualità intrinseca*) hanno mostrato che non esisteva una chiara relazione tra il livello di benessere animale ed i sistemi di allevamento analizzati nella prova. I risultati di questa sperimentazione suggeriscono che la qualità estrinseca e quella intrinseca del formaggio di capra prodotto in allevamenti a basso input non sempre coincidono, a prova del fatto che fornire informazioni sul sistema produttivo impiegato non è

sempre uno strumento sufficiente per dotare i consumatori di una piena consapevolezza nei confronti della qualità complessiva del prodotto che acquistano.

Nella **Prova 3**, la qualità del grasso del latte vaccino di montagna è stata caratterizzata mediante GC-FID e correlata alla variazione stagionale della composizione chimica della dieta fornita alle bovine. Sono state confrontate due strategie alimentari impiegate in due piccole aziende agricole di montagna della regione alpina piemontese, in Italia. In particolare, durante la stagione estiva, i due allevamenti si distinguevano per l'esclusivo impiego dell'alpeggio in una delle due aziende, considerato come la migliore strategia per ottimizzare la qualità del profilo acidico del latte. I campioni di latte ottenuti con l'impiego esclusivo dell'alpeggio durante l'estate (allevamento A) si sono caratterizzati per un profilo in AG generalmente di qualità superiore. Tuttavia, durante la stagione estiva, il latte raccolto nell'azienda dove si impiegava una strategia di alimentazione integrata (allevamento B) ha mostrato possedere una composizione più omogenea, con una concentrazione in media più alta di acidi grassi polinsaturi (PUFA). Questi risultati hanno confermato che la strategia integrata, anche se associata ad una capacità leggermente inferiore di migliorare il profilo in AG del latte, può rappresentare una valida ed economica alternativa per gli allevatori di montagna per ottenere un latte di qualità, pur non essendo strettamente vincolati alla pratica del pascolo.

b) Prodotti ittici – carne di storione e uova di pesce

Nella **Prova 4** è stata analizzata la composizione chimica di uova, caviale e carne di diverse specie di storione provenienti da uno stabilimento di acquacultura italiano, al fine di migliorarne l'apprezzamento sul mercato e di identificare eventuali differenze relazionate alla specie di provenienza. Il profilo in AG è stato analizzato mediante GC-FID e l'analisi dei dati è stata effetuata mediante tecniche di statistica multivariata. Sono state evidenziate importanti differenze nella deposizione dei singoli acidi grassi nei campioni di uova e di carne di storione analizzati, in linea con la funzione biologica ricoperta. In particolare, una differenza significativa nel profilo acidico è stata osservata comparando le frazioni lipidiche dei fosfolipidi e dei lipidi neutri nelle uova. Infine, sui filetti di storione sono stati misurati i parametri di colore, caratterizzandone le proprietà e le caratteristiche specie-specifiche.

Nella **Prova 5**, sono stati studiati aspetti di qualità, tracciabilità e sicurezza relativi ai prodotti trasformati di uova di pesce di diverse specie. L'analisi degli acidi grassi è stata eseguita mediante GC-FID. I risultati ottenuti hanno mostrato differenze tra uova provenienti da diverse specie ittiche. Consequentemente, l'applicazione di un modello discriminante che includeva un set esterno di campioni di uova di storione, analizzati nella **Prova 4**, ha permesso di distinguere le uova di diverse specie dal caviale di storione. Inoltre, sono stati misurati parametri di sicurezza alimentare dei prodotti campionati, dimostrando un buon livello generale di igiene. Nel complesso, i risultati ottenuti da questa sperimentazione hanno permesso di indicare i prodotti a base di uova di pesce come alimenti sicuri e in grado di fornire alla nutrizione umana un prezioso contenuto di acidi grassi essenziali.

Nella **Prova 6**, è stata inizialmente sviluppata una tecnica analitica basata su microestrazioni multiple in fase solida su spazio di testa (MHS-SPME) seguita da GC e spettrometria di massa (MS), conseguentemente applicata per identificare e stimare la quantità dei principali composti volatili (VOCs) presenti nel caviale di storione bianco, durante 4 mesi di maturazione. Il metodo ha permesso di identificare la presenza, e di stimare la quantità, di 25 composti volatili, rappresentati principalmente da aldeidi e alcoli, già noti in letteratura come responsabili del flavour del pesce e dei frutti di mare, senza incorrere in alterazioni significative della matrice prima delle analisi e in un tempo relativamente breve. Principalmente, è stato rilevato un aumento significativo della concentrazione di aldeidi totali in 4 mesi di maturazione; inoltre, si è osservata la comparsa del chetone 3-idrossi-2-butanone all'ultimo tempo di campionamento incluso nella prova.

Nella **Prova 7**, la tecnica di HS-SPME-GC-MS è stata applicata per valutare l'evoluzione del profilo volatile nel caviale di storione bianco addizionato con diverse miscele di conservanti in un tempo di conservazione prolungato (fino a 14 mesi). I risultati ottenuti per il profilo dei VOCs sono stati uniti con parametri di microbiologia prima di effettuare l'analisi dei dati. Le tecniche di statistica univariata e multivariata applicate hanno permesso di identificare diversi gruppi di campioni, in base al tempo di maturazione e alla miscela di conservanti utilizzata. In particolare, i campioni di caviale *purosale* sono risultati essere queli caratterizzati, nel lungo termine, dalle più alte cariche microbiologiche e dalla maggiore concentrazione di VOCs, la cui formazione è stata relazionata con i processi di deterioramento, fortemente esaltati dal tempo di maturazione prolungato, spinto ben oltre il tempo di maturazione ottimale generalmente utilizzato negli impianti di produzione di caviale.

Nella **Prova 8**, è stato sviluppato e ottimizzato un metodo di cromatografia liquida ad alta prestazione e spettrometria di massa ad alta risoluzione (UHPLC-HRMS) per caratterizzare il metaboloma non volatile del caviale di storione bianco durante la maturazione. Sia l'ottimizzazione del metodo che l'analisi dei dati utilizzata per l'interpretazione dei risultati sono state eseguite mediante tecniche di chemiometria. I risultati hanno mostrato una forte evoluzione dei composti non volatili presenti nella fase acquosa delle uova di storione durante i primi 4 mesi di maturazione, seguita poi da una parziale stabilizzazione. La maggior parte dei composti tentativamente identificati erano composti caratterizzati da un alto valore nutritivo, tra cui aminoacidi proteici e non proteici, specie fosfolipidiche di acidi grassi a catena lunga e insaturi, e vitamine. Inoltre, è stata rilevata la presenza di specie chimiche note per il fondamentale impatto generato sul caratteristico gusto *umami* negli alimenti, tra cui acido glutammico e nucleotidi.

Part 1

General introduction

Chapter 1

Quality and authenticity in the agri-food system:

a confidence history

The notion of *food quality* rests on a complex and multi-dimensional concept influenced by a wide range of situational and contextual factors. According to FAO (Food Agriculture Organization; World Health Organization (FAO), 2003), *food quality* "includes all the attributes that influence a product's value to the consumer. This includes negative attributes such as spoilage, contamination with filth, discoloration, off-odours and positive attributes such as the origin, colour, flavour, texture and processing method of the food."

Figure 1. The characteristics contributing to *food quality*. Graphic adapted from "Framework for selecting and testing of food products to assess quality related characteristics." by the European Commission Joint Research Centre (2018).

Actually, the concept of *food quality* could refer to the presence of desirable characteristics likely to justify an **added value** of a food product regarding, for example, the form of production (organic farming, environmental consideration, animal welfare), the production area (designation of origin) and the associated traditions (definition of quality by EU JRC, 2018). With the term 'added value', in this thesis we will refer to the economic value created for a product during the transformation process.

On the contrary, with the definition of *food safety,* the European law refer only to "all those hazards, whether chronic or acute, that may make food injurious to the health of the consumer" (FAO & WHO, 2003). However, it is clear that product integrity does not involve just the compliance with *food safety* standards and hazard prevention protocols, but it is also related to the existence of specific attributes that can be perceived by consumers (Davidson et al., 2017).

Actually, identify-preservation and authenticity issues, even if not directly related to sanitary hazards, have a primary importance in the *food quality* outlook and they concern with all people interacting in the entire food chain, from producers to consumers. In this context, in food industry, ensuring the authenticity of goods arises as an essential point of contact and trust between the producers and the consumers. Really, the formers lay their expectations on quality cues, that can be either intrinsic (e.g. the aspect of a foodstuff) or extrinsic to the product (e.g. advertising, brand image); the better the match between the 'expected quality' and the 'experienced quality', the higher the level of consumer satisfaction (EU JRC, 2018). The interest regarding these issues has enormously grown during last years (Bennet, 2009), accompanied by an increasing necessity for products differentiation. Actually, modern consumers have become more demanding, critical and fragmented in their food choices, leading to the need for producers to differentiate their product from the others, in order to endure on the market (Grunert, K. G., 2005).

Moreover, the international trade is growing rapidly and the modern food supply chains have been lengthened, complicated, and accelerated (Spink & Moyer, 2011). This fact exposes the global market to an extremely high risk that food frauds occur, leading to the necessity to develop and to change the actual food processing and marketing systems (Lupien, 2002). In the lack of safeguard over the guarantee of a food product, food adulterations and frauds can occur. Unfair practices are often fulfilled, leading to, for example, the transmission of incorrect information through the product label or its graphic representation or the addition of flavours or additives that are not allowed to improve the yield of the product while keeping production costs low, increasing the producer's earnings.

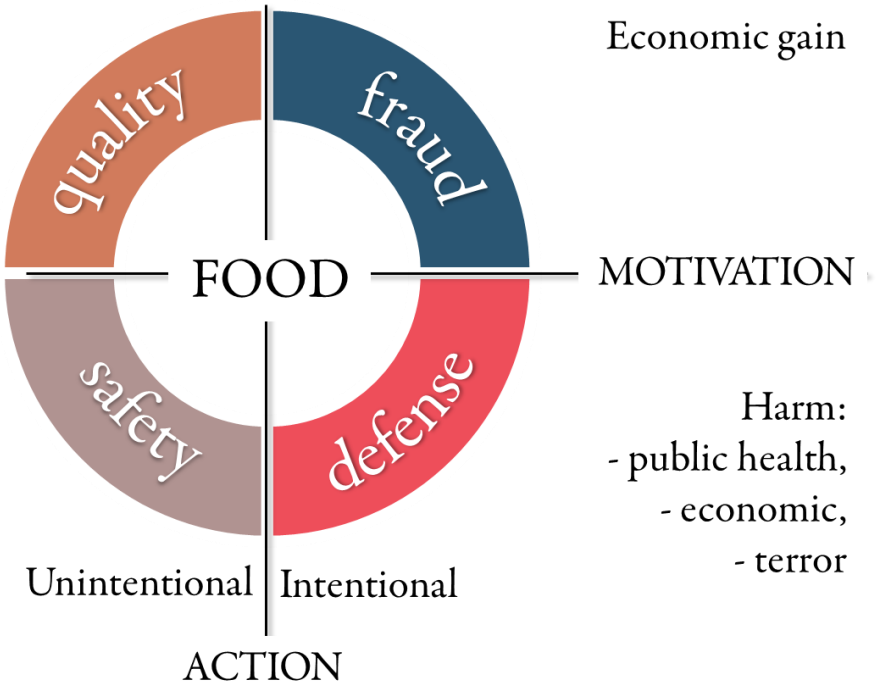

Figure 2. The food protection risk matrix, adapted from Spink and Moyer, 2011 (Spink & Moyer, 2011)

The EU food law was created in order to reach a harmonization of national product standards, thanks to the agreement about quality and identity of food products among the Member States (van der Meulen, 2013). However, even if EU is considered to have some of the toughest food safety regulations in the world, there is a strong necessity to enforce consumers' confidence toward its products and producers (Avery, 2014). This confidence is related to the concept of *product integrity* and, considering the value of food and beverage industry for EU (827.2 billion € in 2019, according to Eurostat, 2020), it appears clear the huge economic relevance of the public perception over the food system.

1.1 The European Food Law – from crisis to virtue

The concept of *food quality* in Europe has progressively evolved between the second half of the twentieth century and the early years of the twenty-first, in order to protect the Community market. In 1963, the *Codex Alimentarius* Commission, formed by members of FAO (Food and Agriculture Organization) and WHO (World Health Organization), was founded with the aim of protecting consumer health and promoting fair business practices. Still today, the *Codex Alimentarius* includes a series of general and specific safety standards that have been formulated with the scope of protecting the health of the consumer and ensuring the fairness of the food trade. The fundamental principle underlying all the indications is that the foods placed on the market for local consumption or for export must be safe and of good quality. The ultimate goal is to guide and promote the development and application of definitions and requirements for foods, encourage their harmonization and promote international trade.

The years following the establishment of the European Union as we know it today (EU, 1993) were years of epochal changes in the management of food, with the affirmation of innovative techniques for processing, packaging, transport and storage of the same. Following the main food crises that hit the European continent in the 90s (BSE, dioxins in poultry meat, etc.), then, a definitive turning point in the field of food safety was crossed. At the dawn of the 21st century, precisely on January 12, 2000, the "White Book" on food safety was published, as an official, proactive, informative document in which the EU Legislator specified the guidelines for compliance with food safety in 84 points. The top priority that emerged in this document was to ensure that the EU had the highest possible standards of food safety, assuming that the production and consumption of food was a central aspect for the society, with repercussions on the economic, social and environmental plan.

Consequently, many concepts were introduced for the first time in the White Paper that would have become fundamental, such as traceability, transparency of the supply chain, the use of risk analysis as a control and self-control tool, whose responsibility was assigned, respectively, to the operators interested in the supply chain and the competent authorities. Furthermore, the White Paper established the legal framework that would be developed in the immediately following years, and the foundation of the European Food Safety Authority (EFSA).

1.2 Food safety and traceability standards

In the general framework of the European food law developed in the last twenty years, there are several legal acts. A schematic summary of the relevant European legislation in force introduced in the following paragraphs is given in *Table 1* at the end of this chapter.

The Regulation (EC) no. 178/2002 represents the "springboard regulation" for food safety in the European Community, where the Commission and the European Committees laid the foundations to allow the free circulation of safe and healthy food. This objective was pursued both to safeguard the health and interests of citizens, intended as consumers, and to protect and encourage the **development of the internal market** in the Community, eliminating the differences linked to the principles and procedures between the laws of the Member States regarding food. These bases were created in order to hinder the free movement of goods and create conditions of an **even competition**. Furthermore, the EU Legislator expressed the intention to make consumers - and all interested parties– confident toward the whole supply chain, starting from the production of raw materials, passing through the feed supplied to farmed animals, up to the finished and processed product. In the Regulation (CE) No. 178/2002 the concept of *traceability* was also introduced, intended as the possibility for all the figures involved in the supply chain, including the consumers, to follow the path that all the ingredients that make up the food have carried out in the stages of production, transformation, distribution and marketing.

In 2004, the so-called Hygiene Package including the Regulations (CE) No. 852/2004, No. 853/ 2004, No. 854/2004 and 882/2004 was released among the act of the European Commission, laying down the general and specific rules for the hygiene of foodstuffs. This legal scaffold put the basis for the food business operators in terms of responsibility, requirements for the working and cleaning procedures, identification, prevention and management of the hazards, general traceability and documentations, correction activities and so on. Moreover, it designed the guidelines for the official control from the competent authority to check the compliance with food laws. Particularly, the Regulations (CE) No. 853/2004 and No. 854/2004 defined specific rules on the hygiene and the control on products of animal origin, with specific requirements for the animal identification and consequent traceability system, the correct management of transport and slaughter procedures, the HACCP based procedures for each class of animal and derived products, etc. Simultaneously, the Regulation (EC) No. 882/2004 established specific rules on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules in all the Member States. In this Regulation, the EU Legislator defined the rules to design the authorities, the responsibility of the Member States, the designation of the official laboratories and methods of analysis and so on.

The EU Regulations on food law are based on the assumption that "a strong **consumer protection system**, effective all over Europe, will benefit consumers as well as competitive producers and sellers; underlines the fact that this will create incentives for businesses to produce and sell more durable goods, resulting in more **sustainable growth**; underlines the fact that effective and improved consumer protection is necessary in order to achieve a better functioning internal market" (European Parliament Resolution of 20 May 2008 on EU Consumer Policy Strategy 2007-2013, 2009). Thus, in order to improve the traceability system, in 2011, the

European legislator published the Regulation (EC) no. 1169/2011 on the provision of food information to consumers. The ultimate goal was, once again, to ensure adequate consumer health protection. The label was defined in the Regulation as "any commercial or trademark, sign, image or other graphic representation written, printed, stamped, branded, embossed or imprinted on the packaging or container of a food or accompanying said packaging or container ". From the point of view of traceability, the label can therefore be considered as the last "imprint" that remains on the product, or on its container, showing the information that has been collected throughout the production process. Until the definition of Regulation (EC) no. 1169/2011, in EU food law, the mandatory provisions relating to origin had been developed on the basis of vertical approaches, with specific Regulations for certain products (honey, fruit and vegetables, beef, fish, olive oil ...). The Parliament and the European Council, with increasing pressure on the issue of food safety, decided that it was necessary to consider the need to extend mandatory origin labelling to other foods.

Specifically, the mandatory particulars that must appear on a label were identified as follows (*Article* 9):

a) the name of the food
b) the list of ingredients
c) eventual allergens
d) the quantity of certain ingredients
e) the net quantity of the food
f) the date of minimum durability
g) aby special storage and/or usage condition
h) the name and the address of the business operator responsible
i) the country of origin or place or provenance
j) instructions for use where it would be difficult to make appropriate use of the food
k) the alcoholic strength by volume in the case of beverages containing more than 1.2% (v/v) of alcohol
l) a nutrition declaration

Such an example, regarding the nutrition declaration (point l), the mandatory nutrition declarations to include in the label were identified in the energy value and the amount of fat, saturates, carbohydrate, sugars, protein and salt (*Article 30*), with the possibility to supplement the information with an indication of the amounts of one or more of the following:

a) mono-unsaturates;
b) polyunsaturates;
c) polyols;
d) starch;
e) fibre;
f) vitamins or minerals.

Moreover, the Regulation laid the groundwork for an intensive investigation on the presence of *trans* fats in foods and in the overall diet of the Union population, with the aim to “assess the impact of appropriate means

that could enable consumers to make healthier food and overall dietary choices or that could promote the provision of healthier food options to consumers, including, among others, the provision of information on *trans* fats to consumers or restrictions on their use" (*Article 30, point 7*). Once again, it is evident that the attention of the Legislator is focused on consumer protection and willingness to spread correct information, allowing more conscious consumption choices and, consequently, a fair trade.

1.3 Quality schemes

Beside the food law essentially directed to delineate traceability and safety standards for general food products, in the European legal framework we can also counts Regulations and Directives oriented to define the quality schemes for the production and trade of specific food products and the guidelines for their traceability. This is because, as stated before, *safety* is not the only relevant aspect related to the concept *food quality* that is worth to consider when the objective is to protect and encourage the development of the market in the EU Community.

1.3.1 Organic production

According to the Regulation (EC) No 834/2007, "organic production is an overall system of farm management and food production that combines:

- best environmental practices;
- a high level of biodiversity;
- the preservation of natural resources;
- the application of high animal welfare standards; and
- production methods in line with the preference of certain consumers for products produced using natural substances and processes."

The organic production method thus is considered to play a "dual societal role, where it on the one hand provides for a specific market responding to a consumer demand for organic products, and on the other hand delivers public goods contributing to the protection of the environment and animal welfare, as well as to rural development." In the Regulation (EC) No. 834/2007 and subsequent (No 889/ 2008, No. 710/2009 and No. 464/2020) the EU Commission established specific rules for the food sector operators at each level (from the production to the transformation and the distribution), regarding the production criteria and the official controls to follow for comply with the organic standards.

Among the assumption listed from the EU Legislator determining such standards, it is specified that the organic production has to be strictly regulated in order to promote the "**fair competition** and **proper functioning of the internal market** [...] and to mai**ntain and justify consumer confidence in products** labelled as organic. It

should further aim at providing conditions under which this sector can **progress** in line with production and market developments." It appears clear that consumers (and producers) protection and fair trade are considered pivotal points in the EU decision making process toward the agri-food sector legislation even in the case of the organic production. A particular attention was put on the labelling system in organic production, considered as the tool to keep a transparency link among all the members of the supply chain, from farm to fork. With this purpose, consumer confidence and perception toward organic production is considered of fundamental importance and its consideration is stressed in all the Regulations.

Organic production is maybe the most renowned example of opportunity for producers to diversify their products on the market, showing a growing trend in production of 4-5 folds in the last 15 years (Hurtado-Barroso et al., 2019), as a response to the demand for a more sustainable agriculture (Mzoughi, 2011). Consumers seem to accept to pay a higher price for organic products, mainly because of the conception of such products as healthier, more sustainable, more respectful of animal welfare and the environment (Hurtado-Barroso et al., 2019; Mzoughi, 2011; Rana & Paul, 2020; Shafie & Rennie, 2012). However, organic production leads to higher cost and lower productivity for producers (Hurtado-Barroso et al., 2019). Thus, a suitable communication strategy, either from a public policy or commercial perspective, is fundamental in order to allow the consumers to perceive the added value related to the concept of 'organic' and to sustain the willingness to pay the related price premium.

Figure 3. The EU organic logo. The logo can only be used on products that have been certified as organic by an authorised control agency or body and is compulsory for most organic products. The use of the organic logo is considered to prevent consumer confusion, help maintain trust in organic food and support the authorities in their inspection regimes, giving a coherent visual identity to EU produced organic products, making easier for consumers to identify organic products and helps farmers to market them across the entirety of the EU. Source: https://ec.europa.eu/info/food-farming-fisheries/farming/organic-farming/organic-logo_en

1.3.2 Quality schemes among geographical origin, production process and tradition

The Regulation (CE) No. 1151/2012 introduced quality schemes for agricultural products and foodstuffs, with the aim to "help producers of agricultural products and foodstuffs to communicate the product characteristics and farming attributes of those products and foodstuffs to buyers and consumers, thereby ensuring:

- a fair competition for farmers and producers of agricultural products and foodstuffs having value-adding characteristics and attributes;
- the availability to consumers of reliable information pertaining to such products;
- respect for intellectual property rights; and
- the integrity of the internal market."

In the Regulation, a scheme for protected designations of origin (PDO) and protected geographical indications (PGI) was introduced for the first time, with the aim of promoting and protecting a certain quantity of food products that, within the European Union, are still produced respecting and preserving traditions, while taking into account the evolution of production methods and materials. This Regulation defines as *traditional* the proven use on the national market for a period, at least thirty years, of a product that allows the pass on knowledge about its production from one generation to another. The ultimate goal of the Legislator was to protect the quality and variety of agricultural and livestock production in the European Union, which represents an important competitive advantage for producers and is an integral part of the cultural and gastronomic heritage of the EU citizens.

To date, in order to benefit from protection in the territory of the Member States, designations of origin and geographical indications must be registered at Union level. This protection process makes it possible to get PDO or PGI products protected from fraud in the food sector, usurpation or imitation, such as the indication of false or misleading information relating to provenance, origin, nature or the essential qualities of the products on their packaging or packaging, in advertising material or on documents relating to the products in question. The European Committee in the Regulation affirms, "The added value of geographical indications and traditional specialties guaranteed is based on **consumer confidence**. It is credible only if accompanied by effective checks and controls". For this reason, in order to guarantee the conformity of the branded productions linked to the territory and the continuity of the same, the producers, organized in groups, define the product in a technical specification. The specification contains requirements that the agricultural or food product bearing a protected geographical reference must satisfy; these requirements must be intended to protect the natural resources or the landscape of the production area, or to improve the welfare of the animals involved.

Actually, the existence of these protected marks makes it possible to guarantee farmers and producers a fair income for the quality and characteristics of the products or their production method when actually linked with the territory of production. In this way, it is possible to provide clear and specific information on products that possess such characteristics, allowing consumers to make more conscious choices. The product with a protected designation thus acquires an added value.

With regard to the labelling of the products with a geographical indication, the EU Legislator states: "Given their peculiarity, it is appropriate to adopt specific rules on labelling for protected designations of origin and protected geographical indications, which require producers to use the symbols of the Union or appropriate indications on the packaging. For Union names, the use of such symbols or indications should be made compulsory in order to make consumers better aware of this category of products and the guarantees they offer and to facilitate the identification of these products on the market, facilitating thus controls". In this case, labelling is configured as a fundamental tool, as it represents the means of communicating information relating to the "specialty" of the product.

The Regulation (EU) No. 1151/2012 also introduce to the Traditional Specialities Guaranteed (STG), namely food products considered to have an added value only because of the traditional methods of production and recipes involved. STG are specifically regulated by the Regulation (EC) No. 509/2006. An example of food product listed the register of STG is the '*Haymilk*' ('*Heumilch*'(de); '*Latte fieno*' (it); '*Lait de foin*' (fr); '*Leche de heno*' (es). In order to complain to the *haymilk* standards, cow's milk has to be produced by dairy farmers who have undertaken a series of rules, mainly referred to the feeding system (the use of fermented fodder, such as silage, moist hay and fermented hay is forbidden) and did not implied animals and feed which are to be identified as 'genetically modified'. According to the applicant group state (Austria), *haymilk* represents an historical link between the consumers and tradition. Actually, in the Commission Implementing Regulation (EU) No. 2016/304, it is specified that former dairy farming authority in Austria have always regulated certain production areas known as 'silage-free zones', in order to preserve the raw material 'haymilk' for cheese manufacturers reliant on raw milk. They clearly assert, "In alpine regions animals have always traditionally been fed according to the haymilk criteria". The safeguard and the promotion of the added value of such kind of products is critic and strategic, since "citizens and consumers in the EU increasingly demand quality as well as traditional products. They are also concerned to maintain the diversity of the agricultural production in the Union. Operating quality schemes for producers which reward them for their efforts to produce a diverse range of quality products can benefit the rural economy [...] particularly in less favoured areas, in mountain areas and in the most remote regions, where the farming sector accounts for a significant part of the economy and production costs are high" (Reg. No. 1151/2012).

1.3.3 Quality marks as a bridge between financial and social status: the example of 'mountain products' and 'traditional agri-food products'

Regulation (CE) No. 1151/2012 introduces other quality marks others than PDO, PGI and STG, referred to as *optional quality terms*. Adherence of a company to these quality schemes is absolutely optional and represents a surplus for the company itself: through this tool, a production process or product is valued voluntarily and spontaneously. Adherence to this type of schemes arises from the need felt by organizations operating in the agri-food sector to position, enhance and differentiate their product on the market, to guarantee the consumer the quality, safety and reliability of their product. The ultimate result is obtaining a trademark thanks to

compliance with certain requirements defined in the technical production regulations, drawn up with the consent of the interested parties.

The Commission Communication 2010/C 341/04, reporting the best practice guidelines for voluntary certification schemes for agricultural products and foodstuffs affirms, "Certification schemes for agricultural products and foodstuffs provide assurance that certain characteristics or attributes of the product or its production method or system, laid down in specifications, have been observed. It covers a wide range of different initiatives that function at different stages of the food supply chain (pre- or post-farm gate; covering all or part of the food supply chain; affecting all sectors or just one market segment, etc.). [...] The certification schemes can bring benefits:

- to intermediate actors in the food supply chain, by assuring standards and thereby **protecting liability and reputation for product and** label claims,
- to producers, by **increasing market access, market share and product margins** for certified products and also, potentially, by increasing efficiency and reducing transaction costs, and
- to consumers, by **providing reliable and trustworthy information** on product and process attributes."

Among the voluntary quality marks, we can list *mountain products,* officially regulated by the Regulation (EC) No. 665/2014. With the term 'mountain product', the European Commission refers to food products intended for human consumption in respect of which both the raw materials and the feedstuffs for farm animals come essentially from mountain areas and, in the case of processed products, the processing also takes place in mountain areas (delimited pursuant to Article 18(1) of Regulation (EC) No 1257/1999). In EU mountain areas, the dominant output is represented by animal production: 54% of the total turnover of mountain farms comes from livestock activities. According to the JRC Scientific Report on Labelling of agricultural and food products of mountain farming (Santini et al., 2013) 10.5% of total dairy livestock units (cows) are located in mountain, producing about 9.5% of the European milk. However, this production sector is affected by lower productivity (tons of milk per livestock unit 9.4% lower than the EU average) not fully compensated by higher milk price (+8.5% versus the EU average). This output makes clear how much the positive perception and willing to pay of consumers toward mountain products plays a fundamental role toward the endurance of this production sector that, other than furnishing high-quality products on the market, contributes to the sustainable development of mountain areas by providing a high level of positive environmental externalities and significant inputs for the local economies and societies. Actually, according to recent studies, the communication strategy around this quality system is often insufficient and promotion strategies need to be implemented to increase the worldwide visibility of these products (Finco et al., 2017; Martins & Ferreira, 2017). The result of consumer-targeted studies demonstrated that in general, there is a lack of sensitization about the real importance of farmers on society, and their role is often devalued and considered not important for communities, indicating the need to **promote an effective science-based dialogue** between the business operators of the sector, supported by governmental organizations, and consumers, especially in the case of urban citizens (Zuliani et al., 2018).

1.4 Health claims

An increasing number of foods labelled and advertised in the Community bear nutrition and health claims. In the Regulation (EC) No. 1924/2006 on nutrition and health claims made on foods, the European Parliament and the Council established a series of rules in order to ensure a high level of **protection for consumers** and to facilitate their choice, through a reliable labelling system even in the case of the nutritional and health claims. With 'claim' the EU Legislator refers to "any message or representation, which is not mandatory under Community or national legislation, including pictorial, graphic or symbolic representation, in any form, which states, suggests or implies that a food has particular characteristics" (Reg. (EC) No. 1924/2006 Art. 2, comma2). To date, a claim can accompany a food product only if authorized at Community level and listed in a specific register. The necessity to define specific guidelines toward such information spread by a claim was driven by the presence of huge differences between national provisions before the publication of the Regulation, thus falling into an impediment to the free movement of foods and unequal conditions of competition in the internal market.

According to the EU Commission, "The establishment of nutrient profiles should take into account the content of different nutrients and substances with a nutritional or physiological effect, in particular those such as fat, saturated fat, trans-fatty acids, salt/sodium and sugars, excessive intakes of which in the overall diet are not recommended, as well as poly- and mono-unsaturated fats, available carbohydrates other than sugars, vitamins, minerals, protein and fibre" (Reg. No. 1924/2006). Such an example, the admitted health claims related to the quantitative and qualitative fat content of a food product are reported in *Table 2* at the end of the chapter.

For sure, to put a claim on a food product, the nutrient object of the claim has to be present in sufficient quantities (or, on the contrary, lower quantities or absent) to produce the physiological or nutritional effect desired and has to be available to be used by the body. Actually, foodstuffs accompanied by a health and nutritional claim, thus related to functional properties, can generate an added value since consumers generally appears interested in buying food that can have a positive impact on their health. However, in consumer-directed surveys it appears clear that often it is difficult for the 'average consumer' to interpret all the information spread with a claim and researchers suggest that the EU strategies should take into account the heterogeneity of consumers receptiveness in both terms of product positioning and health education (Nocella et al., 2002; von Trijp et al., 2007). In order to guarantee a truthful declaration, the request for a claim has to be strongly supported by scientific data by weighing the evidence. Actually, "The application shall include all the studies, including, where available, independent, peer- reviewed studies, which have been carried out with regard to the health claim and any other material which is available to demonstrate that the health claim complies with the criteria provided for in this Regulation" (Reg. (EC) No. 1924/2006, Art 15). In this context, it is clear the relevance of the scientific effort toward the evidence of the superior quality of such a product, in order to increase consumers' awareness and to allow them to make conscious choices in food consumption, thus generating a benefit for the market.

Table 1. A brief summary of the relevant European food legislation, resolutions and communication mentioned in this chapter, in chronologic order of publication.

LAW	APPLICATION
Council Regulation (EC) No 1257/1999 on support for rural development from the European Agricultural Guidance and Guarantee Fund (EAGGF) and amending and repealing certain Regulations	Support for natural development
Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety	General Food Law
– **Regulation (EC) No 852/2004** on the hygiene of foodstuffs – **Regulation (EC) No 853/2004** laying down specific hygiene rules for on the hygiene of foodstuffs – **Regulation (EC) No 854/2004** laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption – **Regulation (EC) No 882/2004** on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules	Hygiene Pack
Regulation (EC) No 1924/2006 on nutrition and health claims made on foods	Health claims
Council Regulation (EC) No 509/2006 on agricultural products and foodstuffs as traditional specialities guaranteed	Traditional specialities guaranteed
- **Regulation (EC) No 834/2007** on organic production and labelling of organic products - **Regulation (EC) No 889/2008** laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control - **Regulation (EC) No 710/2009** laying down detailed rules on organic aquaculture animal and seaweed production	Organic production
European Parliament Resolution No. 2009/C - 279E/14 on rising food prices in the European Union and developing countries	
Commission Communication No 2010/C - 341/04 EU best practice guidelines for voluntary certification schemes for agricultural products and foodstuffs	Voluntary certification schemes
Regulation (EC) No 1169/2011 on the provision of food information to consumers	Labelling system
Regulation (EC) No. 1151/2012 on quality schemes for agricultural products and foodstuff	Quality schemes for agricultural products
Regulation (EC) No 665/2014 supplementing Regulation (EU) No 1151/2012 of the European Parliament and of the Council with regard to conditions of use of the optional quality term 'mountain product'	Mountain products
Regulation (EC) No 2016/304 entering a name in the register of traditional specialities guaranteed (Heumilch/Haymilk/Latte fieno/Lait de foin/Leche de heno (TSG))	Haymilk

Table 2. Admitted health claims related to the quantitative and qualitative fat content of a food product, extracted from the Annex of the Regulation (EC) No. 1924/2006.

FAT CONTENT		FATTY ACIDS PROFILE	
Claim	**Conditions**	**Claim**	**Conditions**
LOW-FAT	no more than 3 g of fat per 100 g for solids *or* 1.5 g of fat per 100 ml for liquids (1.8 g of fat per 100 ml for semi-skimmed milk)	**SOURCE OF n3 FATTY ACIDS**	at least 0.3 g alpha-linolenic acid per 100 g and per 100 kcal *or* at least 40 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal
FAT-FREE	no more than 0.5 g of fat per 100 g or 100 ml	**HIGH n3 FATTY ACIDS**	at least 0.6 g alpha-linolenic acid per 100 g and per 100 kcal *or* at least 80 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal
LOW SATURATED FAT	the sum of saturated fatty acids and trans-fatty acids in the product does not exceed 1.5 g per 100 g for solids or 0.75 g/100 ml for liquids *and* the sum of saturated fatty acids and trans-fatty acids must not provide more than 10 % of energy	**HIGH MUFA***	at least 45 % of the fatty acids present in the product derive from monounsaturated fat *and* monounsaturated fat provides more than 20 % of energy of the product
SATURATED FAT FREE	the sum of saturated fat and trans-fatty acids does not exceed 0.1 g of saturated fat per 100 g or 100 ml	**HIGH PUFA***	at least 45 % of the fatty acids present in the product derive from polyunsaturated fat *and* fat provides more than 20 % of energy of the product.
		HIGH UFA*	at least 70 % of the fatty acids present in the product derive from unsaturated fat *and* unsaturated fat provides more than 20 % of energy of the product

*SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids

References of Chapter 1

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Chapter 2

Food authentication of animal products

2.1 Animal products – why so important?

The first European food standards were adopted in 1964 and concerned the hygienic requirements for fresh meat. Just in a second time, following the development of industrial food production and the increase in the use of food additives for conservation and improvement purposes, the EU began to establish further rules regarding the hygiene requirements of other kinds of food. Actually, until 2005, in the EU market only some products were traceable, such as meat, fish and eggs, those considered most at risk for the consumer's health. From 1 January 2006, with the entry into force of the Hygiene Package written in 2004, the obligation of traceability was extended to all agri-food products, which made it possible to identify any product in each phase of the production cycle. Thus, we can say that the attention given to products of animal origin, considered as more dangerous, served as model for the European Commission to develop efficient traceability systems applicable to all food products. Moreover, as seen right now, animal products play a fundamental role in the wide range of foodstuffs linked to an added value.

Due to the rapid growth of population at a global level, jointed to the increasing incomes verified in many countries, we are now observing an important worldwide increase of the demand for animal source foods. This trend is particularly noticeable in the increasing-income countries, and it leads to the arising of such a phenomenon known as the "livestock revolution" (Food and Agriculture Organization, 2011). Estimations performed by the United Nations organisms assert that the global population will count 9.1 billion people in 2050 and that, consequently, the agricultural production will need to increase by 70% on average, in order to fulfil the increasing food demand (United Nations, 2008).

According to FAO data (Food and Agriculture Organization, 2011), the demand for livestock products from 2000 to 2030 will grow at different rates, depending by the food source and the geographical location. Global consumption will increase of 81% for beef, 97% for milk, 66% for pork, 70% for eggs and 170% for poultry meat. The highest rates have been estimated for the low- and middle- income countries. As an example, globally meat consumption per caput has been estimated to rise from 37 kg at present to 52 kg in 2050, and the increment in the developing countries will be from 26 to 44 kg (Bruinsma, 2014). Experts have explicated the discrepancy among countries by a "saturation effect" combined with the reduced consumption of particular animal-source foods (particularly beef) in the high-income countries. In other words, in many areas of the world, people already eat as much animal-source foods as they need and would like to, and the tendency to choose alternative nutritional style (such as vegetarianism, veganism) in such countries will increase (Food and Agriculture Organization, 2011).

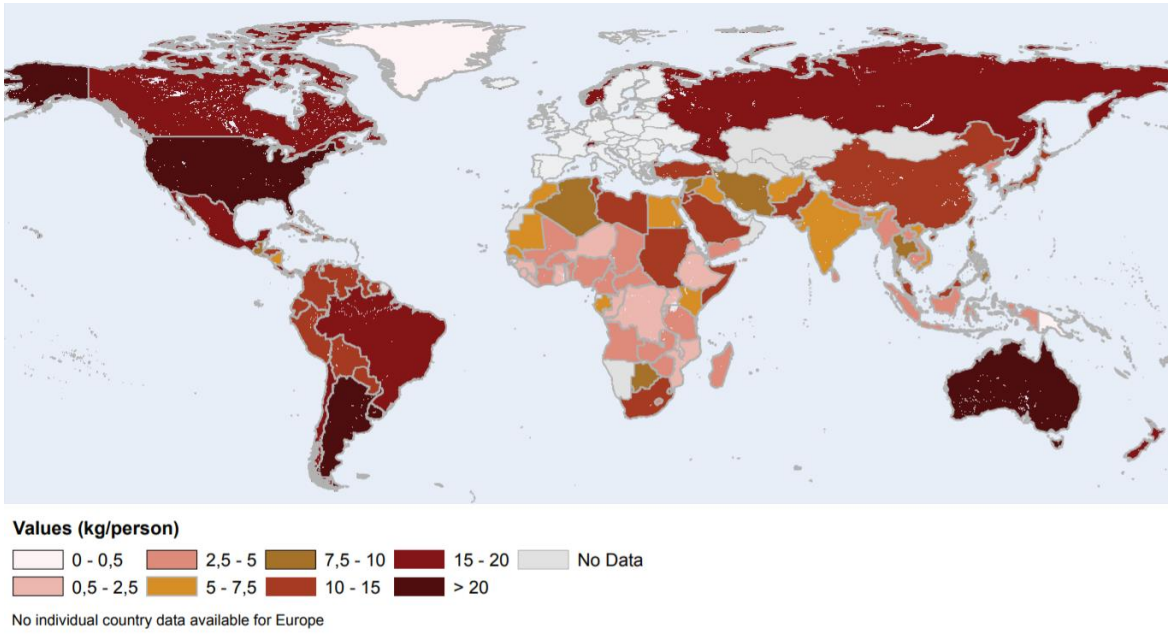

Figure 1. Global, projected consumption of protein from animal-source foods in 2030 per person. Figure source: Mapping supply and demand for animal-source foods to 2030 (Food and Agriculture Organization, 2011).

However, together with the need for a rapid increment of the production volumes, we can observe also a growing sensitivity and attention of the consumer toward to the origin of food and, therefore, to their quality and healthiness. This is especially true among citizens of the high-income countries, who can afford higher expenses to buy food. Hence, the importance to develop effective tools to detect authenticity and quality issues in animal origin products is straightforward. The animal origin food production is becoming more and more important and it undergoes a gradual change, represented by a global level "upscaling" phenomenon, that is the conversion of small livestock farms to large specialized farms. This can be linked to technological innovations occurred in the fields of genetics, animal nutrition, construction of innovative structures, disease prevention and processing, transport and marketing strategies.

2.1.1 The Italian case

Estimations for 2018 show that the Italian agri-food system, including the food and beverage industry, the retail trade and the restaurant services, covered the 15% of the total turnover of the national economy, for a value of 522 billion € and setting itself a strategic sector of the Italian economic system (CREA, 2020). The agriculture, forestry and fishery sector in 2018 produced an income of 59.2 billion €, and 33 billion € of added value. Though, the Italian scenario still appears characterized by a very fragmented picture. The most of the enterprises in the Italian agriculture industry are represented by non-professional farms, characterized by small economic dimension (less than 8,000 € measured in terms of standard output). Significant differences can be observed between the different regions, with the lowest values recorded in the southern regions and the highest in the northern (especially Lombardy, Emilia-Romagna and Veneto). Similar differences can be observed on the

territorial occupation of the factories, going from a minimum of 4.3 ha on average in Liguria to over 20 ha on average in Sardinia, Lombardy and Aosta-Valley. Regarding livestock only, most of the farms are specialised toward the farming of herbivores, including cattle and sheep and goats, particularly in Sardinia, Sicily, Piedmont, Lombardy and the province of Bolzano. However, observing the ALU (Adult Livestock Unit) data, it is remarkable that the regions of Piedmont, Lombardy and Veneto alone hold more than half of the entire national livestock population (CREA, 2020).

Table 1. Main livestock productions in Italy for 2018 (adapted by CREA, 2020).

	QUANTITY (000 t) live weight for meat	**VALUE (000 euro) production at basic prices**
Cow and buffalo milk (000hl)	121,104	4,521,534
Pigs	2,080	3,036,125
Cattle	1,180	2,978,648
Poultry	1,833	2,750,166
Eggs (million units)	13,150	1,421,915
Rabbits, game meat and small-scale breedings	285	762,449
Sheep and goat milk (000hl)	5,930	441,813
Goats and sheep	59	162,718
Horses	42	97,283
Honey	8	61,560

Contemporary, in 2018 Italy ranked first among the EU countries in the fishery sector, with a national fish value of 944 million € and a production of 191,000 tons. The Italian fishery market is mainly driven by preserved products and this places Italy as a key country in terms of supplying preserved fish on the global market and, in particular, in Europe. Simultaneously, fish farming produces 62,300 tons of fresh fish associated to a value of over 300 million €. The production is due to the presence of over 3,300 farms in the Italian territory, the half of them specialized for fattening fish, shellfish and crustaceans for consumption. The Italian pisciculture is mainly represented by the farming of trout in freshwater plants and sea bass and sea bream in marine and brackish waters. The farms are located mainly in Veneto (shellfish and fish), Emilia-Romagna and Puglia (CREA, 2020).

Table 2. Italian aquaculture: production and value for 2018 (adapted by CREA, 2020).

Species	**TOTAL PRODUCTION (t)**	**VALUE OF PRODUCTION (million euro)**
Bass	7,300	59.0
Bream	9,700	75.0
Ombrine	100	0.8
Eel	850	9.4
Mullet	2,500	9.5
Trout	37,500	120.0
Others	4,350	27.1
Total pisciculture	62,300	300.7

In the context of the protected designations of origin and geographical indications, the number of food products officially registered in the EU up to date is 1485. Among these, animal origin food products represent 862, divided in 323 PDO and 387 PGI. Over these amounts, up to date Italy registered 83 animal origin PDO products and 32 animal origin PGI products (data from European Commission Quality Products Register, accessed on 19th October 2021).

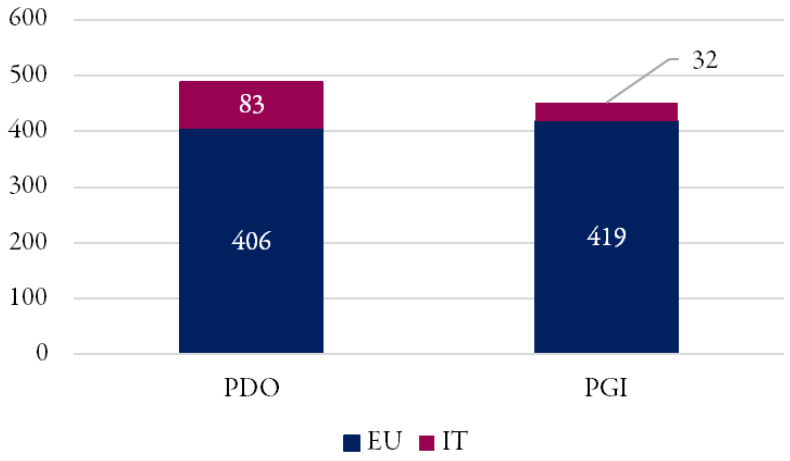

Figure 2. PDO and PGI food products of animal origin in Europe (blue) and Italy (purple). Data extrapolated from European Commission Quality Products Register, accessed on 19th October, 2021.

Manufacturing of food products associated with an added value represents a strategical segment in italian agri-food system even in the case of other quality marks than PDO and PGI. In relation to the product named 'haymilk' ('Latte fieno' in Italian), introduced in paragraph 1.2.2, two recent studies demonstrated that Italian consumers positively perceived haymilk as a high quality product, being related to the production place, the local culinary traditions and also to environmental issues (Bush et al., 2018; Palmieri et al., 2021). The authors suggested that people evaluate milk types positively according to many aspects such as their healthiness and sustainability. This outcome is very interesting in the concept of *food authenticity* since product differentiation appears as a critical strategy farmers and for the entire community. Actually, many authors (Leroy et al., 2018; Palmieri et al., 2021) suggested that the promotion of haymilk production in Italy could represent a way to:

a) restart the livestock sector and the local economy in geographical areas in economic need, and
b) maintain a connection with cultural heritage and traditional landscapes, being the most of the enterprises represented by small-scale and family-driven farms.

In addition to the horizontal European quality schemes, voluntary certifications standards and adherence to voluntary quality marks can be implemented at a national level, covering a wide range of initiatives and operating between businesses, or between businesses and consumers. Such an example, the Italian caption *Traditional Agri-food Products* (TAP) refers to a number of food products recognized to have an added value, being related to processing and conservation methods consolidated over time, or practiced in a specific territory according to

traditional rules and protracted over time (not less than 25 years) (Ministerial Decree No. 350/1999 of Ministero dell'Agricoltura). The list carried out by the Italian Ministry containing such kind of products is annually updated and published, with the task of promoting its knowledge at national and international level. In 2020, the Italian TPA registered were 5,266, with 1,648 (31%) represented by animal origin food (meat and fishery products, cheese and dairy products with the exclusion of butter) (Annex to the Italian Ministerial Decree 10 febbraio 2020 of Ministero delle politiche agricole alimentari e forestali). Really, the most of Italian TAPs can be considered as 'niche' products, for which the market is difficult be expanded beyond the current scale. However, since they represent the main source of income for the small producers involved, a science-based strategy for their valorisation appears fundamental, and would be reflected, more than in a mere economic value, in the importance that their intimate relation with the territory has, from both a social and an environmental point of view (Cafiero et al., 2020).

Figure 3. Logo of the Italian Traditional Agrifood Products

During last years, it has been evidenced that Italian consumers are positively directed toward the consumption of quality food products that represent an important share of the market, in economic terms equal to the one of unlabelled products. Actually, Italian consumers recognize the added value of animal origin products labelled for extrinsic (geographic origin, the rearing and feeding system) and intrinsic (animal welfare) parameters (Lazzaroni et al., 2013). This is an important outcome for the entire market. Particularly, the ability to differentiate the products by means of quality labels can provide an important source of income and competitiveness in the field of animal production for Italian farmers.

It is worth mentioning that the importance of the Italian agri-food system is not only related to economically measurable benefits. On the contrary, it is also driven by the fundamental function carried out by agriculture toward the environment, providing goods and services useful to the community and contributing to the development of the so-called bioeconomy. Actually, the Italian agri-food sector not only fulfil the needs of the European bioeconomy strategy defined in 2018, but it makes it possible to enumerate Italy among leader countries in the EU, together with Germany and France. The European bioeconomy approach is based on the pursuing of sustainable and circularity principles and Italy perfectly align to its strategy, mainly characterized

by strengthening the organic-based sector and developing local bio economies strongly linked to agricultural and rural context. Furthermore, agriculture play an irreplaceable mitigation role against the climate-change emissions, contributing to the absorption of CO_2 from the atmosphere thanks to the forests and shrub cover which affect more than half of the national territory (CREA, 2020).

2.2 Animal products and authenticity: analytical tools

The rapid globalization of food production and supply chains has led to an enormous expansion of global markets and even to changes in consumers' attitude toward their food behaviour. Actually, modern consumers are more and more concerned about the transparency of the information about the origin and the intrinsic quality of food (Hanus, 2018). Many consumers segments are reorienting themselves towards the consumption of food products characterized by additional quality traits, as in the case of local food products (Carzedda et al., 2018) and mountain food products (Martins & Ferreira, 2017), or their functional properties (Vicentini et al., 2016). In this context, the ability to correlate the effective features of quality and authenticity in food products to consumers' perception is extremely important. The occurrence of an exhaustive, accurate and reliable communication about the extrinsic and the intrinsic quality of foodstuffs can benefit not only consumers who access on the market, but also industry drivers: retailers, manufacturers, and importers. Since the consumers cannot always personally verify the attributes of foodstuff they acquire, these must be guaranteed by a quality assurance process along the whole supply chain, fulfilled by chemical, physical, microbiological, molecular and organoleptic analysis.

Animal food products are considered among the most frequently adulterated food in several ways: mislabelling of the provenance, species substitution, discrepancies in the production method and farming technique, addition of non-declared substances, as well as fraudulent treatments and non-declaration of processes (Hassoun, Måge, et al., 2020). Many molecules detected in animal origin food are molecular and chemical markers that allow the identification of the species of origin, the production and feeding system, the geographical origin and the effects of the production process. Generally, in food of animal origin these compounds arise from the diet supplied to animals and from the environment in which they live.

Up to date, many studies have been carried out and rapid scientific and technological advances have taken place in the determination of food authenticity, since the authentication system requires the use of highly sophisticated analytical techniques (Collomb et al., 2008; Coppa et al., 2019). However, it is often debated toward the efficiency of such techniques, because of their hard-working nature, especially regarding the time expense. Thus, the scientific community is still active and interested in the development of rapid analytical methods that would allow a reliable food authentication keeping a high level of accuracy and robustness.

2.2.1 DNA-based techniques

Undeclared species substitution in food products is one of the most frequently frauds encountered in the food production sector (Abbas et al., 2018). This kind of fraud has a double implication in *food safety* and *food authenticity*, respectively. First, it may represent a health treat for many consumers, because of the eventual

presence of protein related to an allergic reaction (i.e., molluscs in fish products). Second, it is strictly related to the deception of consumers. This second aspect, even if not directly related to health risk for the community, can have heavy repercussions on the entire sector, because a loss of consumers' confidence toward the supply chain means negative market development. Such an example, just think about the repercussions of the 2013 horse-meat scandal (Smith & McElwee, 2020).

The DNA-based analysis are highly employed in species identification studies (Böhme et al., 2019). Especially in seafood, these techniques represent the gold standard for species authentication (Chatterjee et al., 2019). These methods allow the identification of animals and all derivatives of animal origin at different taxonomic levels, from the single individual to the breed, population, species, providing an exceptionally powerful tool to verify the accuracy of traditional identification methods.

DNA-based method are mainly related to the polymerase chain reaction (PCR) and its numerous modification. PCR is a molecular technique mainly based on the amplification of specific DNA fragments, subsequentially separated by gel electrophoresis and visualized by the use of an intercalating agent (Schieber, 2018). PCR has a numerous advantages related to its high simplicity, rapidity, sensitivity and specificity. For this reason, this technique has been widely employed in authentication studies directed to the species-specific identification of animal food products up to date (Abbas et al., 2018).

Several advancement of PCR were developed until now, including, among others, the detection of the single nucleotide polymorphisms (SNPs), the restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), a single-strand conformation pattern (SSCP), real-time PCR, species-specific PCR and multiplex PCR (Abbas et al., 2018).

2.2.2 Enzymatic activity and enzymatic essays

Enzymatic-directed analysis are represented by chemical assay methods addressed to measure enzyme activities, frequently used for the determination of food components. An example of the direct measurement of enzymatic activity with authenticity purposes in animal origin product is the evaluation of the heath treatment performed on milk. In the dairy industry, enzymatic activity of alkaline phosphatase and peroxidase are analysed to measure the efficiency of pasteurization and sterilization, respectively (Ritota et al., 2017).

The advantage of enzymatic techniques lies in their high level of specificity, standardization and cheapness and in the minimum sample preparation requirements. On the other hand, their limit is related to the impossibility to simultaneously determine the presence and the concentration of multiple analytes, contrary to what happen with other analytical methods (Schieber, 2018).

Furthermore, the enzyme activity can be implied in species-authentication studies by means of enzyme-linked immunosorbent essays (ELISA). Briefly, this technique is based on the binding of the searched analyte (in this case, a muscular or serum animal protein) with a specific antibody, generally fixed to a solid surface, and then to the measurement of the activity of an enzyme bind to the antibody. In the case of meat, fish and milk products adulteration, enzymatic analysis are routinely performed order to detect the presence of not-declared species in complex processed product (Asensio et al., 2008).

2.2.3 Spectroscopic techniques

During last years, various spectroscopic techniques have been developed and improved to target issues related to food authentication. Several authors (Danezis et al., 2016; Hassoun, Heia, et al., 2020) resumed the main spectroscopic techniques employed to detect fraud markers in animal origin food products:

- **Vibrational spectroscopy**, including near (NIR) and mid (MIR) infrared-spectroscopy and Raman spectroscopy. Vibrational spectroscopy furnishes structural information regarding food samples: based on the measurement of the vibrational frequencies of the atoms chemical bonds in the electromagnetic spectrum, it provides a unique spectral fingerprint of the analytes (such an example, food proteins). The main advantage is related to the fact the vibrational spectroscopy enables a chemical analysis of an intact food samples in situ in real time (depicting it as a not disruptive technique).
- **Fluorescence spectroscopy**, based on measurement of the spectral distribution of the intensity of the light emitted by electronically excited molecules. The main advantage of fluorescence spectroscopy is its high sensitivity and selectivity, featuring very low detection limits as compared to other spectroscopic techniques. Fluorescence spectroscopy appears as a simple, non-destructive, non-invasive and relatively inexpensive analytical technique particular useful for studying minor and trace components in complex food matrices.
- **Nuclear magnetic resonance (NMR)**, a well-established methodology in food analysis, particularly suitable to obtain high-throughput spectroscopic and structural information on a wide range of molecular compounds with high analytical precision. This technique is frequently focused on lipid analysis, because it enables the deconvolution of the specificity of the lipid composition at molecular level.

Being linked to a broad spectrum of sensitivity, specificity and versatility, this set of techniques is employed in a wide range of food authenticity studies (Schieber, 2018). However, due to the huge size of data obtained by spectroscopic analysis, high-throughput data analysis are indispensable.

In the field of animal origin products, spectroscopic techniques are widely employed with several applications. Among the purposes, we can list (Abbas et al., 2018; Valdés et al., 2018):

- the prediction of the sensory quality and the chemical composition (fat, fatty acids…) of samples;
- the discrimination between products obtained by different feeding regimes (by means of metabolites like aromatic amino acids and nucleic acids, vitamins, etc.) and related to different geographical origin (by means of a set of metabolites such as hydroxyl groups, fatty acids, esters, amides of proteins, etc.);
- the detection of different types of adulteration (by means of species-specific metabolites or undeclared compounds in the label).

To report an effective example for the advantages of the development of spectroscopic techniques in food authenticity studies, we can report the case of the study by Jakes et al. (2015). The authors proposed a screening protocol to distinguish beef from horsemeat by means of the signature of triacylglycerols obtained by ^{1}H NMR spectroscopy. The authors demonstrated that through the combination of a simple extraction and rapid analysis (10min), plus a proper statistical approach, they would be able to screen the authenticity of raw meat.

2.2.4 Stable isotope analysis

Stable-isotope analysis represent an analytical approach successfully applied to detect authenticity markers in animal origin food, related to geographical origin or production systems (Vinci et al., 2012). The basis for the importance of isotopic pattern in animal origin food with authentication purposes is related to the fact that the ratios of the stable isotopes such as D/H, $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{16}O/^{14}O$ can vary depending on the origin of soil, water and feed used in the farm. In general, the isotopic ratio of C and N are related to the feeding system, while H and O are linked to regional climatic conditions, latitude, attitude, distance from the sea, precipitations and seasonality. Thus, a combined approach based on the determination of the isotopic pattern of these elements can provide useful indication toward the production system and the geographical provenance. Moreover, the composition of the $^{34}S/^{32}S$ ratio is useful to provide additional information on the geographical provenance of an animal origin products, being the sulphur isotopic pattern related to geological characteristic of the area of origin of feedstuffs (Vinci et al., 2012).

The main technique developed and employed in the determination of isotope ratios is isotope ratio mass spectrometry (IRMS), coupled with different types of preparative techniques, including elemental analysis (EA), gas chromatography (GC) and high-performance liquid chromatography (HPLC). In a review, Camin et al. (2016) reported several cases in which the determination of isotopic ratios by IRMS, combined or not with other analytical methods, allowed the identification of traceability and authenticity indicators in several animal products and particularly:

- **traceability** markers, related to the geographical origin, determined by the oxygen, hydrogen and sulphur isotopic pattern of the water extracted from tissues or milk;
- **authenticity** markers, related to the feeding regime and the livestock system, determined by the carbon and nitrogen isotopic pattern of proteins and lipids.

Related to authentication purposes, the isotopic analysis proved to be very interesting because it allowed the discrimination among matrices obtained from animals fed following different dietary patterns, such as vegetal vs animal origin proteins, different types of vegetal feedstuffs (C_3 vs C_4 plants) or different percentages of specific raw materials included in dietary formulations.

It is worth to highlight the particular relevance of the result obtained by the application of the IRMS technique on fish products, in which the isotopic ratios provided useful information toward the species (based on the trophic level played in the aquatic environment), the diet followed (different in wild and farmed fish) and the geographical origin of the fish (Camin et al., 2016). This outcome is particularly enchanting in consideration of the fact that the fish species, the production method (caught or farmed) and the area where the fish was caught or farmed (with a specific reference to the FAO zone or the water basin when caught) are mandatory information to supply to consumers as established by the Regulation (CE) No. 1379/2013 on the common organisation of the markets in fishery and aquaculture products.

2.2.5 Chromatographic techniques

Among the most important methods employed in food analysis, we can list chromatographic techniques. These analytical tools can allow the detection a big range of compounds, ranging from small organic molecules (<1000Da) to macromolecules (such as biopolymers), characterized by a wide range of polarities (Danezis et al., 2016). The principle of chromatography lays on the adsorption/partition phenomenon of analytes between a mobile and a stationary phase, based on the chemical affinity among them. The most employed chromatographic techniques are gas-chromatography (GC) and liquid chromatography (LC) coupled to several detection methods. Generally, GC can need a prior derivatization of analytes in order to make them volatile and suitable for gas-mediated chromatographic runs; on the other hand, liquid chromatography can be employed in the separation of several compounds, including polar and non-polar compounds, without derivatization (Esteki et al., 2018).

Authentication by chromatography is based on the profiling of specific compounds for each food products, which forms a characteristic chemical profile for food identity.

Thanks to their high sensitivity, reproducibility and robustness, chromatographic techniques are widely employed in with food authentication purposes even in the field of animal food production.

In two recent reviews, Esteki et al. (2018, 2020) reported many examples for the application of chromatographic analysis with authentication purpose in animal origin food, namely dairy products, meat, fish and seafood products. Particularly, in the studies reported in the reviews chromatographic techniques allowed:

- the characterization and authentication of the distinctive chemical profile;
- the detection of adulteration (species adulteration or addition of foreign proteins);
- the evolution of the aromatic profile and the evaluation of the maturity stage of foodstuffs;
- the authentication of the production method (organic or conventional, distinctive dietary pattern followed by animals);
- the influence of eventual heat treatments;
- the characterization of products coming from different countries (thus, related to different processing methods such as in the case of Spanish and French hams);
- the detection of many quality control markers (such in the case of fresh and frozen products).

A more detailed presentation of chromatographic techniques is taken on Chapter 3 of this thesis.

2.2.6 Non-chromatographic Mass Spectrometry

Non-chromatographic Mass Spectrometry (MS) is represented by several stand-alone techniques which advantage is represented by the fact that they provide a chemical fingerprint without the need of a previous chromatographic separation. Actually, the elemental or molecular profile (spectrum) is depicted quite directly by the ionization of a whole sample aliquot (Danezis et al., 2016). Obviously, this peculiarity confers to this set of techniques the beneficial property of the rapidity. Among the non-chromatographic mass spectrometry techniques most commonly employed with authentication purposes in food science, we can list Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) and ambient mass spectrometry such as Direct Analysis in Real Time (DART-MS).

As said before, the sample preparation step is null or minimum and, generally, the chemical fingerprint obtained even in complex matrices is determined with a high selectivity and sensitivity of the MS detection, represented by low limits of detection. Furthermore, in the case of the ambient mass spectrometry, another advantage is represented by the fact that it does not need vacuum condition and can be performed in an open atmosphere, increasing and simplifying the time of analysis (Danezis et al., 2016).

In recent years, non-chromatographic MS techniques demonstrated to be suitable in order to define authenticity or adulteration markers in animal origin food products. Cajka et al., (2013) showed that by an ambient MS approach (DART-MS) performed on ionisable polar and non-polar extracted of common carp muscle, the discrimination in response to dietary supplementation (fed with and without cereals) was feasible, thus providing a discrimination based on the production system. Contemporarily, Hrbek et al. (2014) obtained a reliable discrimination among cow and goat/sheep milk samples, also in mixtures prepared at adulteration level of 50%, by the triacylglycerol profile detected by DART-MS. In this case, the same analytical technique was judged to be suitable to detect a species-contamination case. Similarly, MALDI-TOF MS demonstrated to be a suitable tool for the detection of animal species of in several animal origin products, such as scallops (Stephan et al., 2014), meat (Flaudrops et al., 2015), fish (Stahl & Schröder, 2017), milk and cheese (Rau et al., 2020). In these studies, species identification was based on the identification of species-specific protein patterns and, in the case of Stephan et al. (2014) the results were validated by genomic analysis, showing a high reliability.

2.2.7 The FoodOMICs approach

During last years, the scientific community put a particular focus on the development of high-throughput, non-targeted and broad scale approaches to analyse features of food samples in the holistic view of the *food quality* concept. The 'omics' approach, developed at an inter-disciplinary level in the scientific world, is pointed toward the best identification possible of the pathways of an organism that led to the production and turnover of various metabolites. Particularly,

- **genomics**, addresses all genes and their inter relationships in order to identify their combined influence on the growth and development of the organism;
- **transcriptomics** (gene expression) studies the changes observable in the transcriptome of an organism, namely the entire complement of RNA produced by DNA transcription of a cell, tissue or organism at a particular time point;
- **proteomics** studies the total protein complement (the proteome); and
- **metabolomics** studies the entire complement of small molecules present in a biological system (low molecular weight, <1500 Da) (Akiyama, 2021; Davies, 2010).

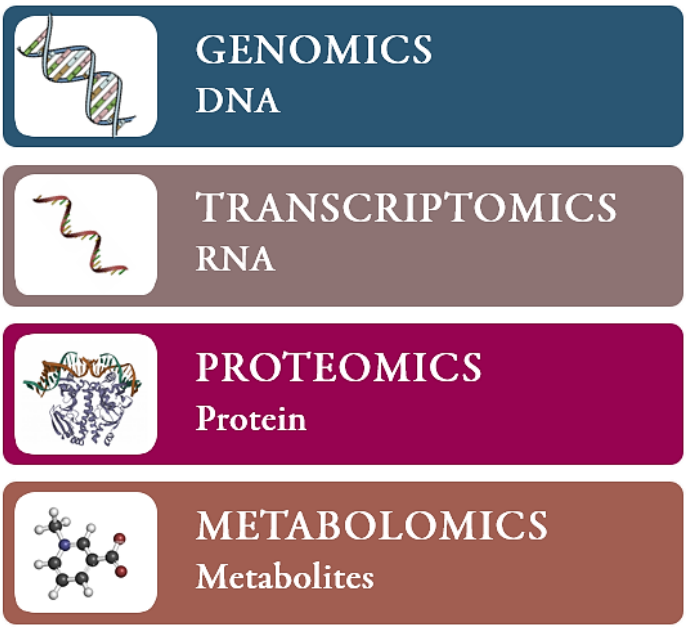

Figure 4. Overview of 'omics' data. Figure adapted and re-elaborated from Akiyama (2021).

The 'omics' approach, broadening the scope of analytical plans, is considered to have the potential to improve the knowledge and the standards concerning *food safety, food quality* and *public health* issues, extending the range of information collected from a single system (Cook & Nightingale, 2018). Such an example, the approaches related to genomics allow to increase the number of genes that can be studied in a single analysis. Actually, genomics and transcriptomics are mostly employed in *food safety* directed studies. Such an example, these 'omics' branches have been recently employed to understand the epidemiology of livestock-related microbial strains associated to antimicrobial resistance and thus representing critical virulence factors for public health because of the transmission via animal-borne food (Liu et al., 2021; Mthembu et al., 2021).

On the other hand, proteomics and metabolomics have been more intensively employed in *food authenticity* directed studies. Particularly, the **metabolomics** approach, as opposed to proteomics that rely largely on genome information, is mainly species-independent and can be applied to different biological matrices without huge re-optimisations of protocols of analysis. Even in food products, the metabolome is considered to represent the final downstream product of the genome and its interaction with the environment, being correlated to certain characteristics of the food sample (Ferri et al., 2015). Thus, changes observed in the metabolome better reflect the activities of the cell at the phenotypic (functional) level and in an amplified extent if compared to genes and proteins (Davies, 2010).

One of the main challenges in food metabolomics is discover and explain the presence of a complex network of molecules occurring in a particular food item, namely, sugars, amino acids, peptides, organic acids, phenols, terpenes, etc. Two main approaches are employed in the field of metabolomics (Davies, 2010):

- *Metabolite profiling*: a **targeted** strategy focused on the analysis of a specific group of related metabolites, often belonging to the same chemical class;

- *Metabolite fingerprinting*: an **untargeted** strategy based on comparing the whole patterns of metabolites among different samples using chemometric tools. The main aim of fingerprinting is not to identify all the involved compounds but to discover whole fingerprints. The fingerprinting approach enables the simultaneous detection of a wide class of metabolites.

Ortea et al. (2016) and Cubero-Leon et al. (2014) resumed in two reviews the wide range of application of these 'omics' approaches, even in animal origin food. The main applications in the studies reported by the authors conceived, among others, the differentiation of animal species and breed and the detection of eventual species-adulteration, the determination of the geographic origin, the rearing system and others quality traits (such as fresh/frozen state) in milk, cheese, meat, shellfish and fish products.

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Chapter 3

Chromatography

The science of separation

To date, several analytical techniques have been developed and implemented in order to comply with food authenticity and traceability queries. Among them, chromatographic techniques are represented by a broad spectrum of accurate and versatile analytical methods wide implicated in several research fields (Danezis, Tsagkaris, Brusic, et al., 2016; Danezis, Tsagkaris, Camin, et al., 2016; Fanali et al., 2016). These techniques have the capability to allow the separation and detection of a huge number of chemical compounds, characterized by different polarity, molecular weight and volatility. The outcome of a chromatographic analysis is represented by a chemical profile in which compounds or functional groups found in a sample have been separated and identified. This profile can be considered as a chemical fingerprint distinctive of the sample and potentially applied to differentiate samples one from the others. For this reason, chromatographic techniques are widely employed in animal science and food analysis, as part of authenticity and traceability investigations. Methods most frequently used in food analysis are high-performance liquid chromatography (HPLC) and gas chromatography (GC) (Fanali et al., 2016).

The separation power of chromatography is based on the partition processes of the molecules present in a complex mixture (analytes) between the particles of a chemical-bonded support, called *stationary phase,* and a flowing solvent, called *mobile phase.* In both LC and GC, the stationary phase is represented by a support packed with a material capable to interact (chemically and/or physically) with sample analytes, represented by the chromatographic *column*.

The characteristic features of a chromatographic separation are the **differential migration** and the **spreading** along the column of solutes present in the sample (Snyder & Kirkland, 1974). Since each compound is related to different chemical properties (molecular weight, polarity, shape, etc.), the interactions occurring with the stationary phase are different for each solute in the mixture. Compounds that interact more strongly with the stationary phase will move slowly in the mobile phase flow and, thus, will leave the column later. Thus, different analytes reach the end of the column with different times and then they are directed to a detector, where their passage generate a signal generally characterized by an intensity proportional to the concentration. The result is a *chromatogram*, a graph that represents the analytes and their concentration as a function of the analysis time. Compounds leave the column in the form of a symmetrical, bell-shaped band called *peak* (a Gaussian curve). Each peak is characterized by a *height* (H), a *width* (W) and a *retention time* (tR), as illustrated in Figure 1.

The retention time is a fundamental property, typical for each analyte, in order to identify unknown molecules separated in a chromatographic analysis. tR is measured as the time passing between sample injection and the maximum point of the peak detected after leaving the column. Separation is more efficient when peaks are narrower and when the distance between two adjacent peaks (in terms of tR) is maximum.

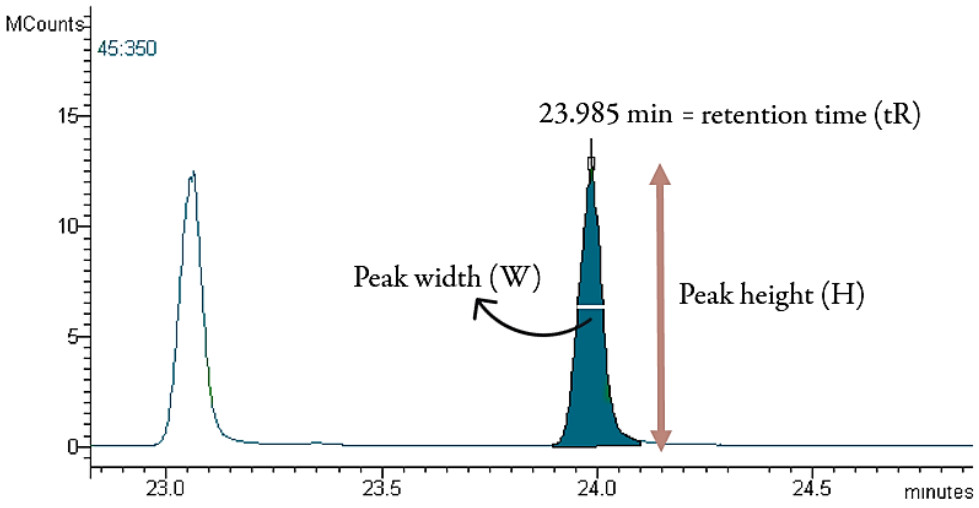

Figure 1. A brief representation of the characteristic features of a chromatographic peak. The peak width (W) is measured at half of the peak height (H).

In both LC and GC, the efficiency of a chromatographic analysis depends on:

a) **Column length**. The longer the column, the bigger the retention time, the better the separation.

b) **Column diameter**. The capacity of the column increases quadratically with respect to its diameter.

c) **Column pressure**. Smaller diameter particles cause a greater pressure drop. By halving the particle size, the drop in the column increases approximately 4 times. The pressure drop can be minimized by using low viscosity solvents, which also increase efficiency thanks to a higher diffusion coefficient.

d) **Column temperature**. Controlling the temperature of the column can play a fundamental role in improving the reproducibility of the separation. In some cases, in LC the temperature is increased to increase the solubility of the sample.

e) **Nature of the stationary phase.** The particles making up the stationary phase can be of different size and nature. Small packed particles offer a large interaction surface, making a fair resistance to flow and maximizing resolution and column capacity. Bigger particles offer a much smaller interaction surface, obtaining a lower capacity.

f) **Nature of the mobile phase.** The mobile phase must be component-selective, reduce pressure drops and improve molecule transfer.

g) **Mobile phase flow.** The mobile phase flow passing into the column is generally set to obtain feasible analysis times. The resolution increases as the flux decreases, and viceversa.

h) **Elution gradient.** Solvent (in LC) or temperature (in GC) gradients are used to decrease the duration of the analysis and to optimize the separation (in terms of time and resolution). A programmed condition is recommended for complex samples that contain a wide range of analytes characterized by different distribution coefficients.

3.1 High Performance Liquid Chromatography (HPLC)

Nowadays, the chromatographic technique most employed in food analysis is represented by High Performance Liquid Chromatography (HPLC), allowing the separation of a wide range of non-volatile compounds (such as proteins, amino acids, phenolic compounds, carbohydrates, additives, triglycerides, phospholipids, etc.) (Fanali et al., 2016). In HPLC, a pump provide the force necessary to overcome the pressure drop due to the passage of the solvent, that is in liquid state, in the chromatographic column. The stationary phase is coated in a packed column, consisting of a solid core (generally made of a silica matrix) plus a thin external coating of a chemical phase that can be represented by either porous or nonporous particles. The smaller the particle diameter, the greater is the resistance of the column to the flow of the mobile phase: columns with a particle size lower than 2 mm require special instrumentation, which are referred to as ultra-high-pressure liquid chromatography (UHPLC) instruments (Fanali et al., 2016).

Figure 2. Examples of packed columns employed in HPLC analysis. In the detail, a guard-column is shown. The guard-columns help preserving the analysis column by removing particulate contaminants and highly absorptive compounds from samples, prolonging column life. Columns represented in the photo were provided by Phenomenex (Torrance, California, USA) and Thermo Fisher Scientific (Waltham, Massachusetts, USA). Photo by Annalaura Lopez.

Column packing is what allows to achieve higher efficiency and capacity performances. Furthermore, given the variety of polarity that can be obtained using different materials, it makes possible to analyse a large amount of different compounds. Actually, various functional groups or polymeric layers can readily be attached to the silica surface, thus extending the utility of these particles for applications to any individual LC method. When the stationary phase is more polar than the mobile phase, it is called *normal phase* (NP) chromatography. On the other hand, when non-polar compounds have to be separated, a stationary phase less polar than the mobile one is used, and the technique is called *reverse phase* (RP) chromatography.

a) **NP chromatography**. In this technique, column is packed with a material characterized by the presence of polar groups, thus the stationary phase is more polar than the mobile phase. Solvents typically consist of mixtures of hydrocarbons, dichloromethane, tetrahydrofuran, etc., in which 1-2% of acetic or phosphoric acid, or 0.1-1% of ammonium or other amines can be added to inhibit ionization and the formation of peak tails.

b) **RP chromatography**. In reverse-phase chromatography, the mobile phase is more polar than the stationary phase. The stationary phases more commonly used are C8 and C18 silica-bonded stationary phases (Fanali et al., 2016).The selectivity is significantly different from the one obtained with a NP chromatography, in particular the elution order is inverse. Actually, it is related to the hydrophobic nature of the analytes; this means that the faster the solute breaks down in the aqueous phase, the faster it will elute in the chromatographic run. This inversion of the elution sequence can be exploited in an advantageous way, for example, in the case of peaks that in a NP elute very early and are difficult to separate: in RP, they will elute at the end of the run, making separation easier. The solvents most used in the reverse phase are water-methanol and water-acetonitrile.

The nature of the column for each analysis has to be selected based on the chemical properties of the compounds to separate. The ability of sample molecules (analytes) to interact with the chemical of the mobile and the stationary phase is referred to as the *polarity* of the compound. *Polar* solvents preferentially attract and dissolve *polar* solute molecules and it is common to refer to the *strength* of a solvent in relation to its polarity (Snyder & Kirkland, 1974).

In order to optimize peak resolution, the chromatographic condition in HPLC can be set in either isocratic or gradient elution mode:

- **Isocratic Elution (IE).** The sample is injected onto the column and the mobile phase is unchanged throughout the time required for the sample to elute from the column. In most cases, isocratic separation is not enough to achieve an optimal separation in complex mixtures, showing poor resolution of early-eluting bands, difficult detection of late-eluting bands, and unnecessarily long separation times.
- **Gradient Elution (GE)**. The mobile phase conditions are changed during the chromatographic run to allow better separation of the peaks. Gradient elution consists in the admixture of two (or more) solvents, a weak initial solvent and a strong solvent, so that the concentration of the strongest solvent in the mobile phase entering the column increases throughout the separation. At the conclusion of a gradient elution separation, the column must be re-equilibrated with the weakest solvent to remove all traces of the stronger before the next sample is injected. GE generally enhance the detection sensitivity in trace analysis, improve

the resolution of retained bands from large concentrations of unretained components and increases peak capacity and the resolving power for very complex samples.

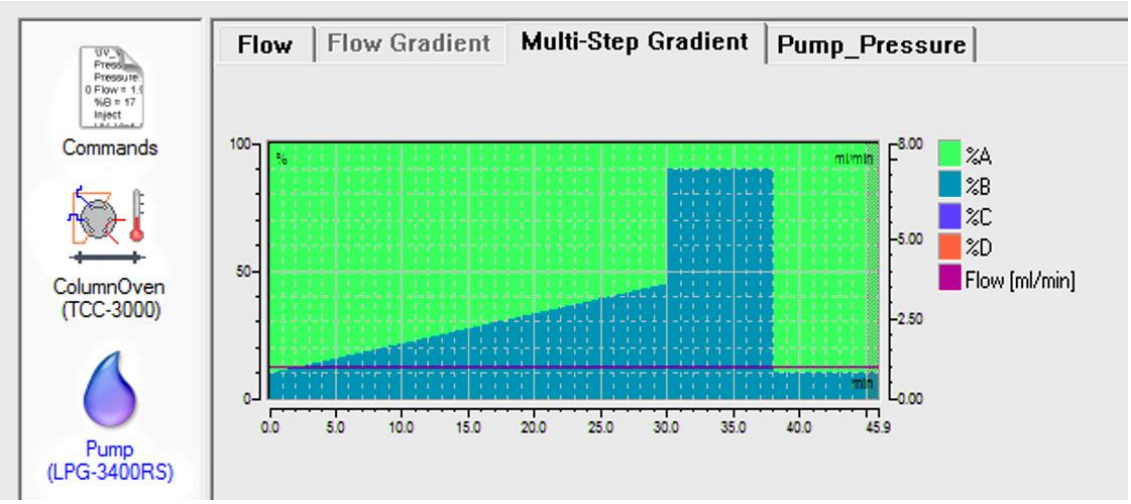

Figure 3. Example of a gradient elution employed in a RP-LC separation performed by UltiMate 3000 UHPLC by Thermo Fisher Scientific (Waltham, Massachusetts, USA). In the example, the organic solvent (B, 0.1 mM formic acid in MeOH) increased during the run and the initial condition (80% A, 0.1 mM formic acid in H_2O) were re-equilibrated at the end of the analysis.

The separation ability and the versatility of HPLC has allowed the application in several studies in the field of food analysis. HPLC technique has be reported to be employed to detect food components such as vitamins, carbohydrates, proteins, amino acids and triglycerides in mayor food products, in addition to minor bioactive substances like phenolic compounds and pigments (Fanali et al., 2016).

3.2 Gas Chromatography (GC)

In gas-chromatography (GC) the mobile phase is represented by an inert carrier gas, making this technique suitable for the analysis of volatile and semi-volatile compounds. The most commonly used approach in GC is represented by gas-liquid chromatography (GLC), in which the stationary phase of the column is represented by a thin layer of liquid covering a solid support (commonly made of synthetic fused-silica) (Fanali et al., 2016). Nowadays, capillary column are employed, characterized by typical column lengths between 5 and 60 m (although longer columns can be used), a film thickness variable between 0.1 and 5 µm and inner diameters (ID) of 0.10-0.53 mm (Engewald et al., 2014).

When liquid stationary phase is used as thin layer coating the internal surface of a GC column, it must fulfil some requirements (Engewald et al., 2014):

- It has to be **not volatile**
- It has to be **thermally stable**. Degradation-vaporization processes occurring at high temperatures can reduce the life of the column and decrease the sensitivity of the measurements.

- It has to be **inert.** The stationary phase cannot react in an irreversible manner with the carrier gas or the sample compounds. For this purpose, is fundamental that the carrier gas is of the highest purity grade possible.
- It has to be associated **to different solubility powers**. In order to separate the most of the analytes, the stationary phase has to be able to create intermolecular interactions with several compounds. As in the case of LC, in order to interact, the stationary phase and the analytes have to be chemically similar. The polarity of the stationary phase depends on the nature and the number of functional groups present.

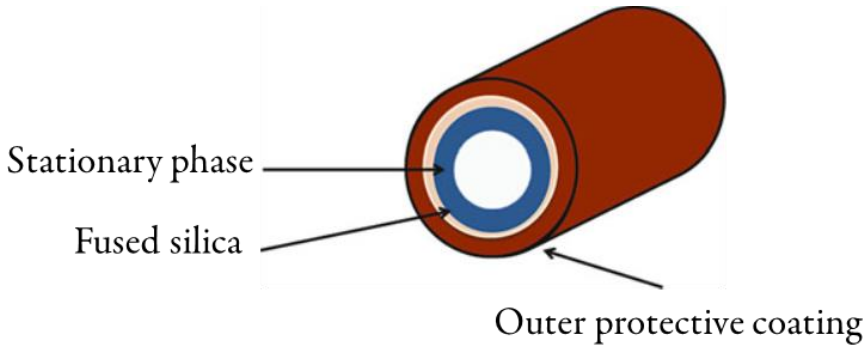

Figure 4. Structure of a fused-silica capillary column. Image adapted from Engewald et al. (2014)

GLC helpfully facilitates separation of analytes based on their different vapour pressures and their affinity for the chemical nature of the stationary phase. The partition process occurring between the mobile and the stationary phase depends on solubility of each compound at the given operating temperature. Highly volatile compounds tend to endure largely in the mobile phase and so they elute earlier than compounds with lower volatility, that interact with the stationary phase more strongly (Cordero et al., 2012). It has to be specified that the 'volatility' criteria in GLC does not necessarily mean that the column temperature has to be kept above the boiling point of the analytes, in order to keep them completely in the gaseous phase. Actually, to enable the distribution of analytes between the stationary and the mobile phase and their transport through the column at a given temperature, it is sufficient that a part of the analytes is in the gaseous phase (Engewald & Dettmer-Wilde, 2014).

Carrier gases more commonly employed in GC are hydrogen, helium, and nitrogen; the nature of the carrier gas affects the sensitivity of the detection and the separation performance.

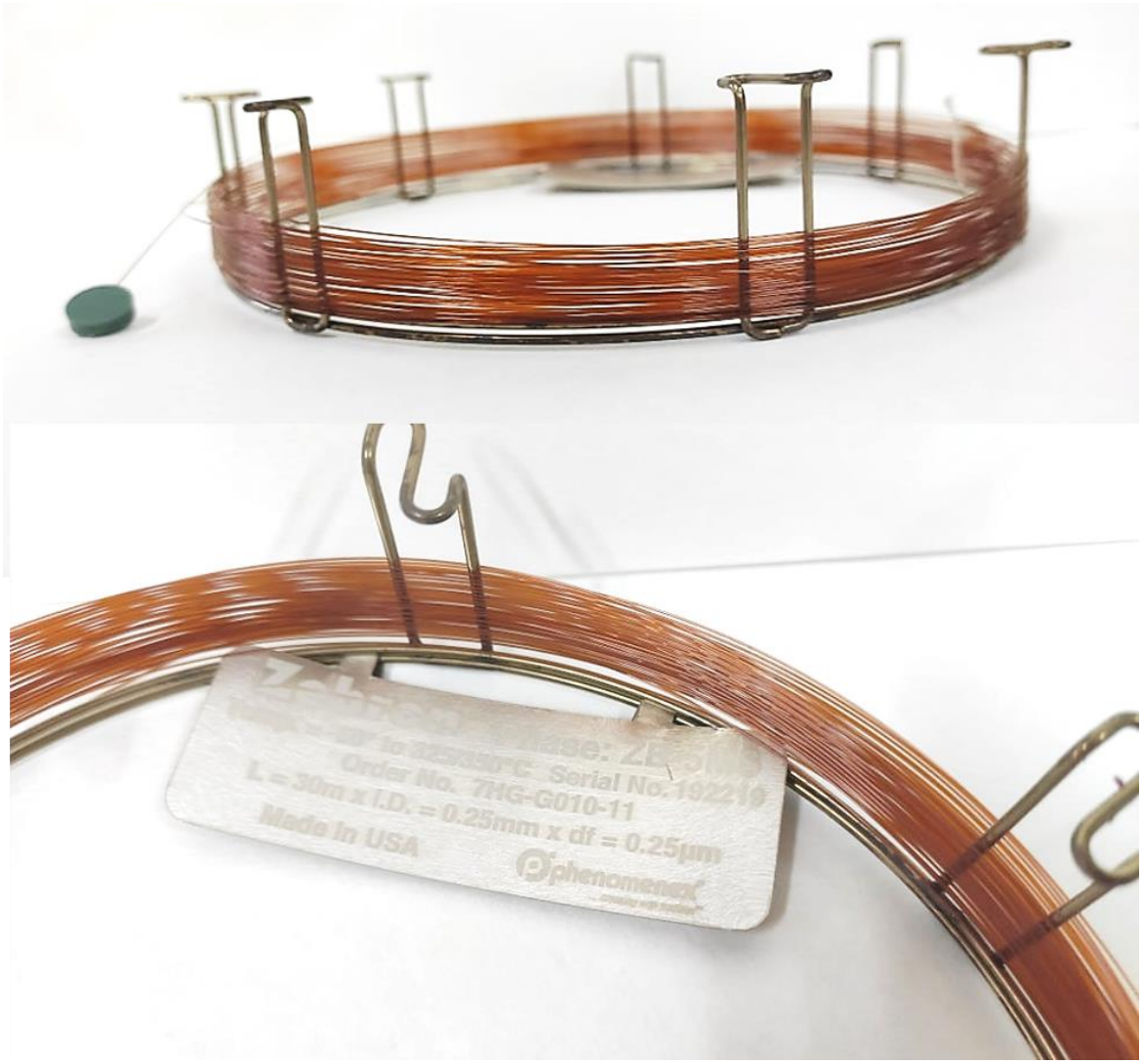

Figure 5. Examples of a packed column employed in GC analysis. In the detail, the property of the column (length, ID, stationary phase) are shown. The column, a Zebron ZB-5MS (30m x ID= 0.25mm x df= 0.25µm) was purchased by Phenomenex (Torrance, California, USA). Photo by Annalaura Lopez.

Even in GC, the chromatographic parameters can be set in order to maintain a constant or variable condition, in this case related to the temperature (Tolley et al., 2014):

a) **Isothermal separation**. The temperature of the oven containing the chromatographic column is kept constant for all the analysis time. This is the best option to apply when retention times of compounds are very similar, in order to resolve analyte bands. The drawback is that these operational conditions need long analysis time.

b) **Temperature gradient separation**. A gradient of temperature is applied during the chromatographic run, in order to speed up the separation. The disadvantage of applying a gradient of temperature lays in the fact that resolution of two analyte bands with similar retention times is reduced.

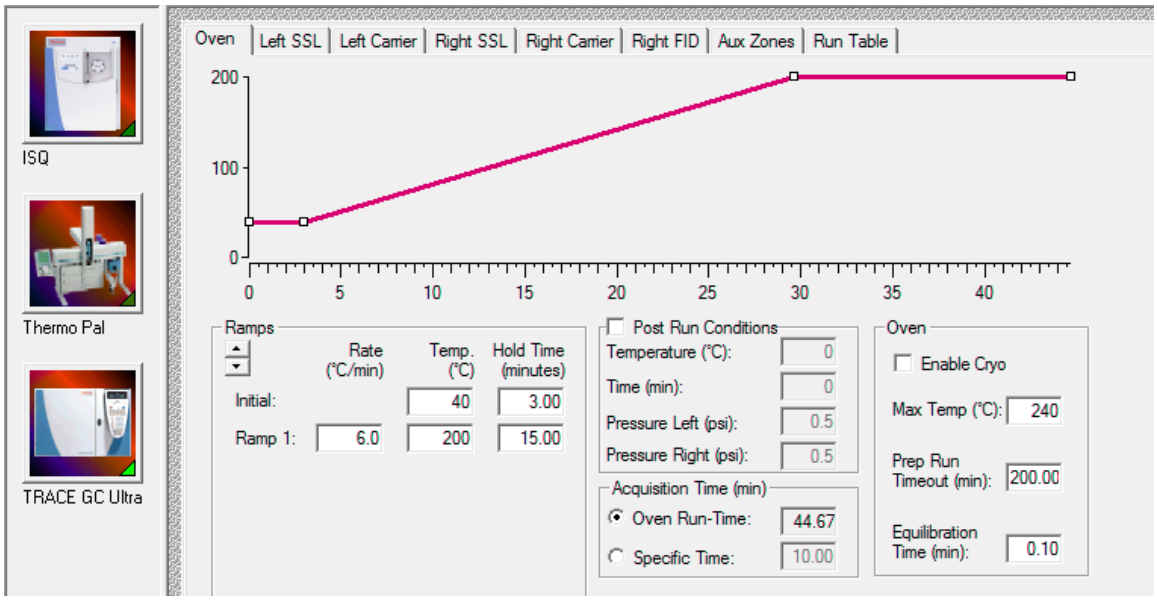

Figure 6. Example of a programmed temperature gradient employed in a gas-chromatographic analysis performed by a Trace GC Ultra (Thermo Fisher Scientific S.P.A., Rodano, Milan, Italy) equipped with a HP–FFAP (50 m × 0.320 mm × 0.50 µm film thickness) column (Agilent, Santa Clara, CA). Carrier gas was helium at a constant flow rate of 1.7 mL min^{-1}.

Gas-chromatography found applications in several application for the investigation of food composition: fatty acids, sterols, alcohols, aroma compounds, off-flavours can be detected by GC (Fanali et al., 2016). However, many molecules need a prior derivatization step. Derivatization in analytical chemistry represents the modification of the structure of an analyte by a chemical reaction, in order to obtain a better performance of the analysis (for example, to make it more soluble in the solvent or to allow a better detection) (Niwa, 2019). Particularly, compounds containing more than one polar group exhibit a low volatility and can be separated by GC only after chemical transformation into more volatile derivatives (Engewald & Dettmer-Wilde, 2014; Feng et al., 2019; Moldoveanu & David, 2015). Such an example, in lipid analysis the fatty acid components of lipids are most commonly converted to their simplest volatile derivative methyl esters (FAMEs) for GC analysis. Since the majority of fatty acids in nature are esterified to glycerol, cholesterol or long-chain aliphatic alcohols, a derivatization process is needed to generate their volatile counterpart, by esterification and transesterification reactions in acid or alkaline environment, and make them suitable for gas-chromatography (Christie, 2003).

3.3 Detectors

The detector is represented by an analytical equipment put at the end of the separation module in order to provide and measure a signal associated to the compounds present in the elute passing through it. The passage of the analytes in the detector generates a change that is revealed by the detector by the generation of an electric signal. Finally, the signal is processed in order to provide the qualitative and quantitative information on the composition of the sample (Andersson, 2014).

3.3.1 HPLC specific detectors: optical, luminescent and electrochemical

Among the most common detection methods coupled to liquid chromatographic separations in food analysis we can list (Zhang et al., 2008):

- **Optical detectors**, such as the Refractive Index (RI) detector and the photometric detectors working in the UV or visible region. The RI detector is universal but very sensitive to signal noise, so it is associated to high limit of detections (micromoles). The UV detectors, on the contrary, are less universal but are capable to reach lower limits of detection (nanomoles). The most commonly used UV detector is the Diode Array Detector (DAD), able to perform a complete spectral analysis of analytes on a continuous basis.
- **Luminescent detectors**, such as the fluorescence and chemiluminescence detectors. Fluorimeter detectors, particularly, are specific and sensitive, with limits of detection in the range of picomoles. For this reason, they are usefully employed in trace component analysis.
- **Electrochemical detectors**, such as the amperometric and conductometric detectors. This kind of detector is useful when the analytes can be oxidized (or reduced) and for detecting anions and cations, respectively. Thus, they are commonly employed in ion exchange chromatography.

With these kinds of detector, to ensure adequate sensitivity of the molecule detection after a LC separation, a derivatization is sometimes needed before or after the injection (pre- or post- column derivatization). In HPLC, the derivatization is performed mainly to enhance the detectability of analytes or to increase the sensitivity of detection. For example, the UV-vis detector needs the analyte to contain a chromophore group in order to generate a strong absorption band. Similarly, in the case of florescence detection, derivatization is frequently used with the purpose of adding chromophores or fluorescing moieties to the analytes (Moldoveanu & David, 2015). Such an example, for the determination of biogenic amines by LC a chemical derivatization is needed, because these compounds do not show adequate absorption properties in the visible, ultraviolet or fluorescence wavelength ranges (Ordóñez et al., 2016). Derivatization with dansyl chloride for the identification of biogenic amines in cheese by RP-HPLC-UV detection was reported (Innocente et al., 2007).

3.3.2 GC specific detectors: universal and selective

Detectors coupled to gas-chromatographic separation can be either universal or selective. Among the universal detectors, one of the most employed in the field of food science is the flame ionization detector (FID). The FID is a mass-sensitive and destructive detector, characterized by high robustness and low limit of detections (high sensitivity). The response of a FID is generally stable and linear with concentration of the analytes up to seven orders of magnitude (dynamic linear range up to 10^7). In this kind of detector, a flame (made up by a flow of hydrogen and air) reacts with the carbon present in the organic molecules of the sample passing in the detector through the mobile flow. The reaction produces the formation of ions, which movement is measured as electric current between two electrodes and generate a signal associated to each compound (Andersson, 2014). Other universal detectors employed in GC are the thermal conductivity detector (TCD) and the Pulsed Discharge Helium Ionization Detector (PDHID).

On the other hand, among the selective detectors coupled to GC, we can mention the electron capture detector (ECD), the sulfur and the nitrogen chemiluminescence detector (SCD and NCD), the nitrogen–phosphorus detector (NPD), the photoionization detector (PID) and others. Most of these detectors recognize the presence of particular chemical groups in the molecule of the sample, such as halogen, oxygen, nitrogen, phosphorus or sulphur containing groups(Andersson, 2014).

3.3.3 A versatile detector: the Mass Spectrometer (MS)

Nowadays, in order to obtain an optimal identification and quantification of food compounds, **mass spectrometry** (MS) is frequently coupled to LC and GC in a hyphenated separation-detection strategy characterized by high sensitivity and resolution.

Mass spectrometry is a well-established detection technique that works through the separation of molecules of a sample after their ionization, based on their characteristic m/z (mass to charge) ratio. The result obtained after the detection process is represented by the *mass spectrum*, a bar diagram in which the relative abundance of any ion is plotted against its m/z value. In a mass spectrum, the base peak is the most intense and it is used as reference (as 100%) to express the abundance of other ions (Maccoll, 2016).

As said before, in order to make compounds detectable by a mass spectrometer, they have to reach the detector in ionized form, because the MS is only capable to recognize the presence of charged species. Several ionization techniques can be employed, depending on the purposes of the application and the separation technique previously used. In LC, the most commonly used are Electron Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (Nollet, 2003; Vékey, 2001). In both cases, the liquid flow containing the sample is converted in vapour phase at elevated temperatures (around 350°C) and the ionization occurs in the vapour phase, respectively (Vékey, 2001):

- In ESI, the ionization occurs for complete desolvatation and protonation of the analyte molecules, often leading to the formation of multi-charged species, especially in presence of high mass molecules. This technique is particularly useful when working with RPLC, being particularly compatible with highly polar compounds that can be easily ionized. In ESI, ionization protonation, deprotonation or cation attachment is observed. The working range for high mass compounds in ESI is 500-2000 m/z. Mass spectra generated with ESI are generally simple and allow the easy attribution of molecular ions signals. The drawback of the ESI ionization technique is represented by the fact that it is extremely affected by the presence of contaminants in the samples, like salts, metals, etc. (matrix effect).
- In APCI, an ionized solvent reacts with the solute creating ionized species, generally mono-charged ions. With this technique, compounds characterized by a medium polarity are ionized better. Typically, singly charged protonated or cationized molecular ions are observed. The upper molecular mass limits in APCI is 1500-2000 m/z. The drawback of this technique is represented by the fact that it is very susceptible to the amount of solvent, being ineffective below certain flow rate values and that the hardware configuration are generally more complex for the analysts than in ESI mode.

In both cases, after the ionization of the analytes, the charged species resultant are directed to the mass detector. The advantage to apply MS detection after LC separation is the possibility to obtain structural information about

the molecules detected, in most of the cases without needing a prior derivatization of the analytes, and to increase the specificity (Vékey, 2001).

In combination with GC, MS is commonly employed providing substance-specific information for each separated component even when present at trace levels in a complex mixture. The sensitivity of the MS analyser is as high as, or even better than, the one of other detectors coupled to GC, like FID (Moeder, 2014). As seen before, the MS analyser is capable to detect and measure the compounds present in the mixture only after the ionization step. For GC-MS analysis, electron impact (EI) is the most used technique to produce ions from neutral molecules entering the mass spectrometer via the GC capillary. The EI is a hard ionization technique in which the gaseous molecules collide with a flow of electrons emitted by a glowing filament. Neutral molecules colliding with the electron flow generally lose one electron from the outer shell and a positively charged ion (molecular ion $M^{+\bullet}$) is formed. All the charged species created in this process are focused, accelerated, and transmitted through electric or magnetic fields of mass analysers, whereas radicals and neutral molecules are removed by the pumping system (Moeder, 2014). The fragmentation pattern is strictly dependent on the structure and the chemical-physical properties (weight, internal energy) of the single molecules and on the energy of the reaction. Most of the mass spectrometers integrated in a GC-MS system operate at a fixed energy of 70 eV, which guarantees reproducible high ionization efficiency (Moeder, 2014). Spectra generated at 70 eV electron energy show well-reproducible fragmentation pattern independent of the instrument. Thus, EI mass spectra can be compared with those collected in mass-spectral libraries in order to tentatively recognize the compounds present in the mixture, even if the presence of homologues, isomers or related structures can lead to error of identification in absence of analytical standards (Vékey, 2001).

Other ionization techniques are possible in GC-MS, even if they are less frequently employed, such as chemical ionization (CI), negative chemical ionization (NCI).

Several type of detectors exist, associated to different degree of sensitivity. The most simple and commonly employed is the single quadrupole (Q), consisting of four parallel metal rods electrically connected, with a radio frequency (RF) voltage applied between one pair of rods and the other. In the quadrupole, ions move between the rods following a specific trajectory dependent on the m/z ratio. When ions associated to a determined m/z ratio reach the detector, the signal is detected and recorded, while all the other ones are filtered out. A range of m/z values can be selected varying continuously the applied voltage. Other analysers are available, able to operate a separation of the ionized molecules, either spatial (as in the case of the time of flight detector - ToF) or temporal (as the linear and orbital ion trap detectors). The m/z scanning can be performed in *full scan* mode, recording the whole spectrum (at the expense of loss of sensitivity), or in *selected ion monitoring* (SIM) mode, detecting one specific m/z ratio intensity as a function of the elution time.

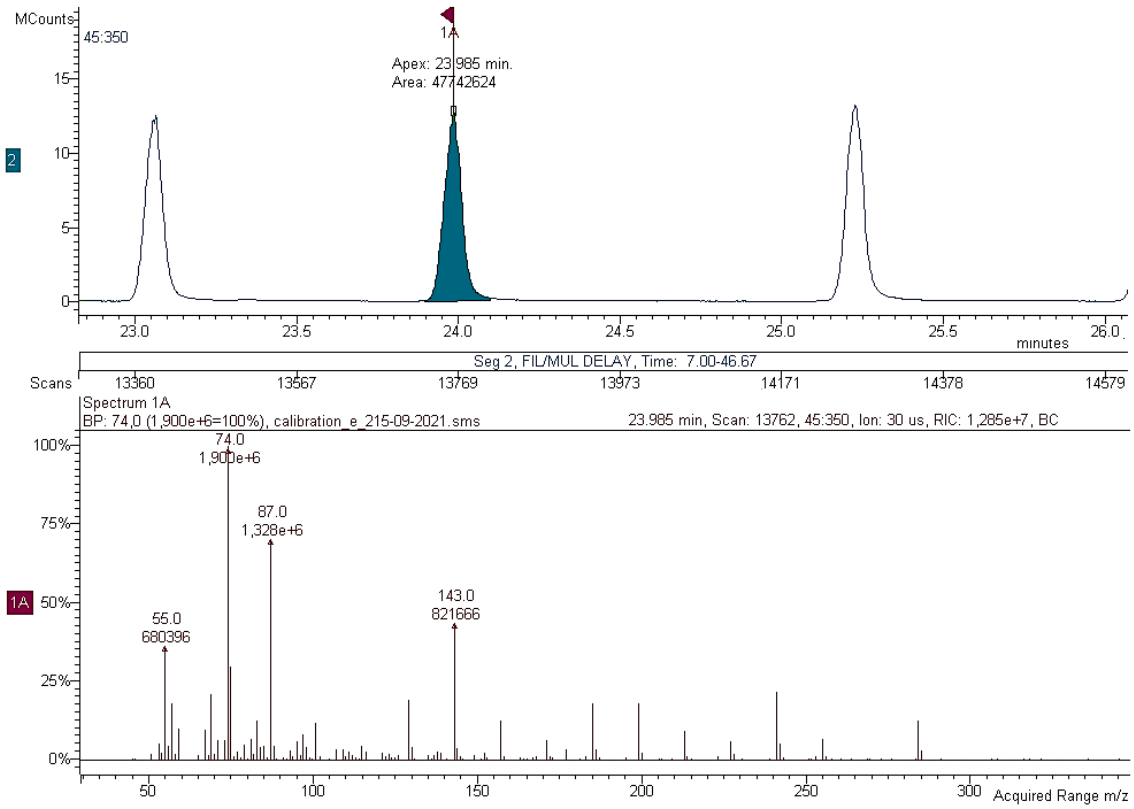

Figure 7. An example of the mass spectrum of a volatile compound (heptadecanoic acid methyl ester) eluting at the retention time 23.985 min of a GC run. The parameters of detection of the detector (4000 MS by Varian, Inc., Walnut Creek, CA, USA) were set in full scan mode (44-350 m/z range). The ionization mode was EI at 70 eV.

3.4 Chromatographic techniques in animal products authenticity

Chromatographic analysis have been extensively applied for authentication of several animal products. Such an example, HPLC separation of individual species-specific proteins, in both the casein and the whey fraction of dairy products, allowed to determine species identification and species-adulteration. The method employed showed good accuracy, precision, rapidity and sensitivity (Fanali et al., 2016). Similarly, the separation and identification of species-specific peptides resulted to be a feasible analytical approach in order to detect specific markers of the presence of horse and pork meat (and eventual adulteration) in different processed food samples (Von Bargen et al., 2014).

The determination of organic acids and carbohydrates in dairy products and meat by means of HPLC analysis was demonstrated to provide interesting outcomes (Pereira da Costa & Conte-Junior, 2015). Organic acids in food products are known to derive from the metabolism of large molecular mass compounds (such as carbohydrates, lipids and proteins). Their presence and eventual fluctuations detected in animal food can be indicative of the manufacturing process or even microbial growth occurred in the products. In dairy products, organic acids are formed primarily by lactose degradation operated by microbial starters, thus their identification appears interesting in order to provide information toward the composition and the quality of the final products

and the manufacturing process. In this view, carbohydrates and organic acids detected by HPLC were proposed as authenticity markers (Pereira da Costa & Conte-Junior, 2015). Even in fish products, HPLC separation was applied as methodological strategy with interesting outcomes for products authentication. Such an example, the determination of the isomer configuration ratio of carotenoids (particularly, astaxantine) allowed the differentiation of wild and farmed salmon, making this compounds markers related with the feeding pattern followed by fish, thus with the production system (Molkentin et al., 2015).

Gas chromatography is widely employed in authentication studies on animal products with the same extent. The target molecules for the determination of authenticity by GC are specific compound classes such as lipophilic compounds, like fatty acids, fatty alcohols, phytosterols, and triterpenes (Feng et al., 2019). The determination of the profile of fatty acids (FA) in the lipid component of a product of animal origin involves nutritional implications and can also provide information regarding the production method and farming technologies implied, being strictly related to the lipid sources furnished to animals by feedstuffs. In animal sciences, FA can be useful to investigate the multifactorial causes that influence lipid fingerprint of animal origin foodstuffs.

In dairy and meat products from ruminants, it has been demonstrated that the FA profile is strongly influenced by the type of diet provided to animals (Chilliard et al., 2007; Liu et al., 2016), making the lipid profile a reliable marker of the production system (extensive *vs* intensive, outdoor *vs* indoor, conventional *vs* organic) (Butler et al., 2008; Schwendel et al., 2015; Segato et al., 2017; Tsiplakou et al., 2010). Especially, cows reared on pasture produce milk with a lipid profile distinguishable from the one of traditionally reared cows, and the same occurs in the acidic composition of meat from beef cattle reared on pasture or fed with concentrates (Acciaro et al., 2020). Moreover, even if in a lesser extent, the FA profile in ruminant products can provide information toward their geographical origin, since it has been demonstrated that different pasture, containing different levels of plant metabolites, differently influence the metabolism of FA in ruminants (Collomb et al., 2008; Coppa et al., 2019).

Similarly, the fatty acids pattern on fish flash can provide information toward its origin, especially if coming from aquaculture or caught in the wild. It has been proved that the FA profiling can provide information with a great discrimination power toward the production system in several fish species, such as salmon (Molkentin et al., 2015), turbot (Busetto et al., 2008), sea bream (Lenas et al., 2011), trout (Strobel et al., 2012). Furthermore, Strobel et al., (2012), by means of GC-FID analysis, also evidenced a species-specific pattern in the FA profile of many fish species, allowing the identification of oily sea fish as associated to a higher nutritional value.

When coupled to MS, GC can be profitably employed even in volatolomics studies. Volatile Organic Compounds (VOCs) are represented by a series of molecules from different chemical classes, originating from primary and secondary metabolites in food matrices, which are generally associated with food aroma and flavour that can be perceived by the human noise. Even the volatile profile can be a useful tool to determine authenticity and traceability features in animal origin food. For instance, it is known that the volatile composition of milk and cheese is affected by the feeding constituents supplied to by ruminants (Bennato, Ianni, Innosa, et al., 2020; Bennato, Ianni, Martino, et al., 2020) and also by the type of pasture they are allowed to graze (Povolo et al., 2007). Actually, the typical aroma of cheese is known to derive both from endogenous factors, related to the catabolism of the native components of milk, and from exogenous factors, brought from the environment and

arriving at the unmodified milk. The same occurs for VOCs formed in meats during the Maillard reaction in the interaction between sugars and proteins in the cooking process or for those deriving from lipid self-oxidation in fish products. All these compounds, which presence varies mainly according to the lipid sources supplied to the animals through the diet, can be analysed and used to discriminate different farming systems (extensive *vs* intensive, fished *vs* farmed). Particularly, in fish products, generally VOCs generation is associated to the deterioration processes that occur during the storage (Alasalvar et al., 2005; Iglesias et al., 2009; Jørgensen et al., 2001; Nordvi et al., 2007), thus a number of volatile compounds identified by GC-MS methods were suggested as indicators of spoilage, useful to monitor the loss of freshness of seafood.

References of Chapter 3

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Chapter 4

Multivariate data analysis and chemometrics

4.1 Chromatographic fingerprint and chemometrics

The output of a chromatographic screening can be referred to as a chemical *fingerprint*, defined as a characteristic profile reflecting the complex chemical composition of an individual sample (Esteki et al., 2018, 2020). Chemical fingerprinting usually leads to the yield of large number of discrete data values for each sample. Moreover, sometimes different specimens are associated to very similar measurements, represented by slight differences quite observable by traditional data analysis methods (Kemsley et al., 2019). Multivariate methods are used with the purpose to properly treat large datasets containing high number of variates and for this reason are often employed in combined approach together with chromatographic fingerprinting (Esteki et al., 2018, 2020).

In food authenticity research, several multivariate techniques have been proposed, which can be divided in two principal groups: *unsupervised methods* and *supervised methods* (Bertacchini et al., 2013).

4.1.1 Unsupervised methods

In **unsupervised methods**, the data analysis is directed to the identification of clusters or relationships between samples without any prior knowledge of classes or groups (Esteki et al., 2018).

The unsupervised method most widely applied for data compression and visualization is the Principal Component Analysis (PCA). PCA is a technique used in order to strongly reduce the significant dimensionality of data matrix, allowing the analyst to more easily understand the structure of chemical data retaining a good amount of the original information (Kemsley et al., 2019). In PCA, the starting assumption is that the information lies in a systematic correlation structure underlying the data, and the PC algorithm helps to explain in which way each variable varies with respect to the other, rather than individually (Kemsley et al., 2019).

This mathematical procedure decomposes the independent variables of the original dataset into new independent variables, the principal components (PCs), which represent linear combination of the original variates. The PCs represent a system of orthogonal variables where the first principal component (PC1) is a vector (*eigenvector)* that accounts for the maximum of the original variance, associated to the highest *eigenvalue*. The second principal component (PC2) is orthogonal to the first one and stands in the direction of the largest remaining variation, and so on, until the total variance is covered (Kamal & Karoui, 2015). Each PC retains different sources of information, defining a singular direction of distribution in the data (Esteki et al., 2018). In this system, it is assumed that a large variance is associated to a great information content (great difference among difference observable among samples), so a strategy for data exploration after the transformation is to plot the pairs of the PCs one against another (i.e. PC1 *vs* PC2, as shown in Figure 1).

The projections of the points from the original dataset (individual samples) on the new hyperplane defined by the PCs on the x- and y- axes are called *scores* (Fig X a). The vector multipliers are called *loadings* (Fig X b) and represent the contribution of the original independent variables (measured parameters) on the whole variability of the new data matrix, calculated to maximize the variance (Kemsley et al., 2019). The scores that have similar values for the new PCs tend to cluster in the scatterplot, while different samples tend separate in the space.

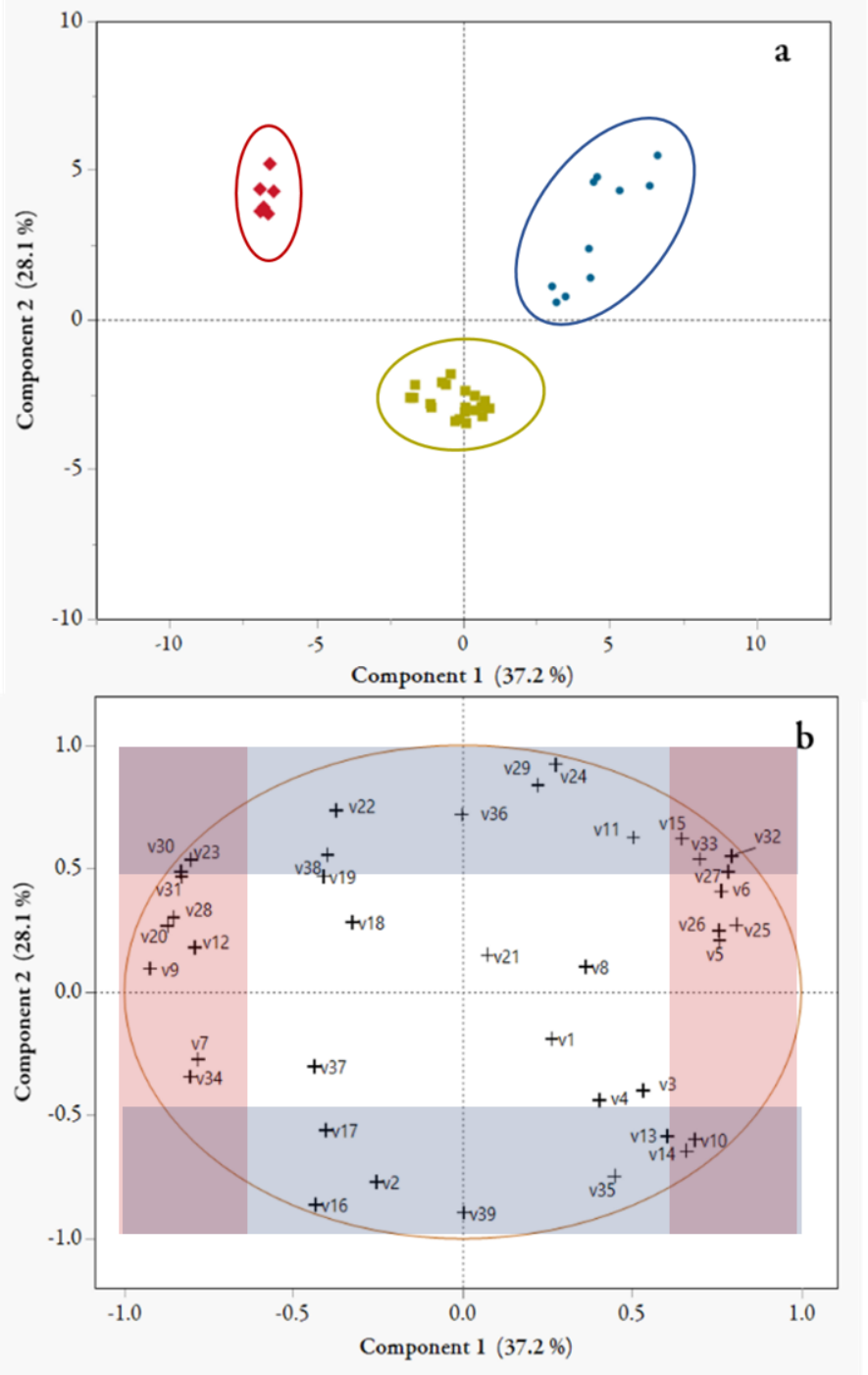

Figure 1. Example of the score plot (a) and loading plot (b) obtained plotting the first two PCs (PC1 *vs* PC2) calculated on the correlation matrix of a multivariate dataset of property of the author. In the score plot (a), the points that tend to cluster are associated to different colours and evidenced by an ellipse. In the loading plot (b), the variables associated to the higher loadings, at the borders of the ellipse, are the one that have greater weights on the distribution of data (clustering and separation). Such an example, the variables evidenced on the left and the right quarters (in red) are associated with the highest loadings for PC1; the variables evidenced on the higher and the lower quarter (in blue) are associated with the highest loadings for PC2. The model was developed by JMP Pro 15 (SAS Institute Inc., Cary, NC, USA)

4.1.2 Supervised methods

In authenticity studies, a typical requirement is represented by the classification of samples in classes characterized by different features (Kemsley et al., 2019). The classification is performed by the so-called **supervised multivariate methods**. In contrast to unsupervised methods, supervised methods require a series of information on class membership of samples *a priori,* before their development and construction. The classes and their attributes are known previously to the development of the algorithm, so the information are added to the classification methods by the analyst, in order to build a proper classification method (Esteki et al., 2018). The target of the classification approach is to assign the individual observations (samples) to one or more categories (classes) on the basis of the measured variables (Kemsley et al., 2019).

Different strategies, differing both in their approach and in the kind of outputs and information that they provide, have been developed with classification purposes in chemometrics. Among the supervised classification models, **discriminant methods** are among the most employed. The philosophy standing behind the discriminant algorithms is to build a model that can differentiate as best as possible the categories under investigation and in which each sample is attributed to one, and only one, of the classes. In order to reach this purpose, during the model-building step, the position and the boundaries among the classes are optimized, in order to have the minimum possible number of observation wrongly attributed. The complexity of the boundaries built among the classes is dependent by the nature of the discrimination method chosen (i.e. linear or non-linear) (Kemsley et al., 2019). However, all the supervised methods share a common strategy for developing the learning algorithms (Esteki et al., 2018):

a) Selection of a **training set** and a **validation set**. The training set is made up by a number of observation of the original dataset and is used to build the model with multiple parameter settings. The validation set is composed by other observations (that can be internal or external to the original dataset, as explained in point d) and is used to challenge the trained model.

b) **Variable selection**. The purpose of variable selection or feature is to eliminate irrelevant or redundant information, led by many variables not associated to a discriminating power, in order to enhance the performance of the classification algorithm. Variable selection is particularly useful when the number of observations is relatively small and the number of variables is large; in this case, a variable selection procedure is necessary in order not to get the overfitting problem. The variable selection method most commonly used is the *stepwise selection*, based on a process that sequentially adds or deletes variables from the pool of original features measured. Several stepwise strategies exist, such as *forward stepwise*, *backward stepwise* and others. In the *forward stepwise* selection, variables are progressively moved into the model, whereas the *backwa*rd options start with all the original variables in the model, and then progressively removing them. In both the strategies, the addition or removal of a variable is evaluated based on probability or Fisher criteria (p or F-values) to significantly influence the discriminatory power. Therefore, *forward stepwise* variable selection acts adding a new variable and removing of previously entered one. On the contrary, *backward stepwise* selection examines the removal of a new variable and the addition of a previously deleted one (Berrueta et al., 2007).

c) **Model construction**. A mathematical model is built up by a set of selected variables from the original data matrix belonging to the training set (model input) and their known categories (model output). Discrimination models are based on the determination of discriminant functions that maximize the ratio of *between-class* variance and minimize the ratio of *within-class* variance. The aim of a discriminant model is to select a combination of the original variables to achieve maximum separation among the given classes. This function is called *canonical variate*. The combination of the original variables can be linear, as in the case of linear discriminant analysis (LDA), or not.

d) **Verification** and **validation** of the model. The validation step involves running the model using input parameters measured for an independent test set of samples, in order to assess the quality and the reliability of the classification model. Model validation implies the evaluation of all the parameters selected in the previous steps, fundamental to characterize the dataset, and of the power of the model obtained performing the classification of unknown samples. This is usually done observing how successful the model is at classifying known objects, evaluating the percentage of samples in the training set correctly classified (*recognition ability*) and the percentage of samples in the validation set correctly classified by using the model developed in the training step (*prediction ability*). The validation of the model can be external or internal. It is called **external validation** when the dataset is big enough to create separate (independent) training and validation set, representative of each class, as subsets of the original data matrix. In this kind of validation, even if the validation set contains samples with known provenance, the model is built ignoring these classifications. Unfortunately, commonly in food analysis this situation, considered the ideal, is not possible to reach. Therefore, **cross-validation** (or **internal**) methods are generally used. In cross-validation, the prediction ability of the model is determined by splitting the original dataset in two or more subsets. In turn, each one of these subsets is used as validation set, while the remaining subsets as training set. The operation is repeated until all the subsets have been used as validation set. In this way, the model parameters are optimized several time, each time excluding one of the subsets created (Berrueta et al., 2007; Kemsley et al., 2019). In all the approaches, the aim of applying the model to a validation set is to assess model accuracy. Actually, based on the errors calculated on the validation set, the optimal model parameters are defined (Berrueta et al., 2007; Xu & Goodacre, 2018).

Even in this case, the discrimination can be visualized in a multivariate space, called *canonical plot*, in which observations belonging to different classes are separated and delineated on the basis of the power of the model. In Figure 2, an example of a canonical plot is reported.

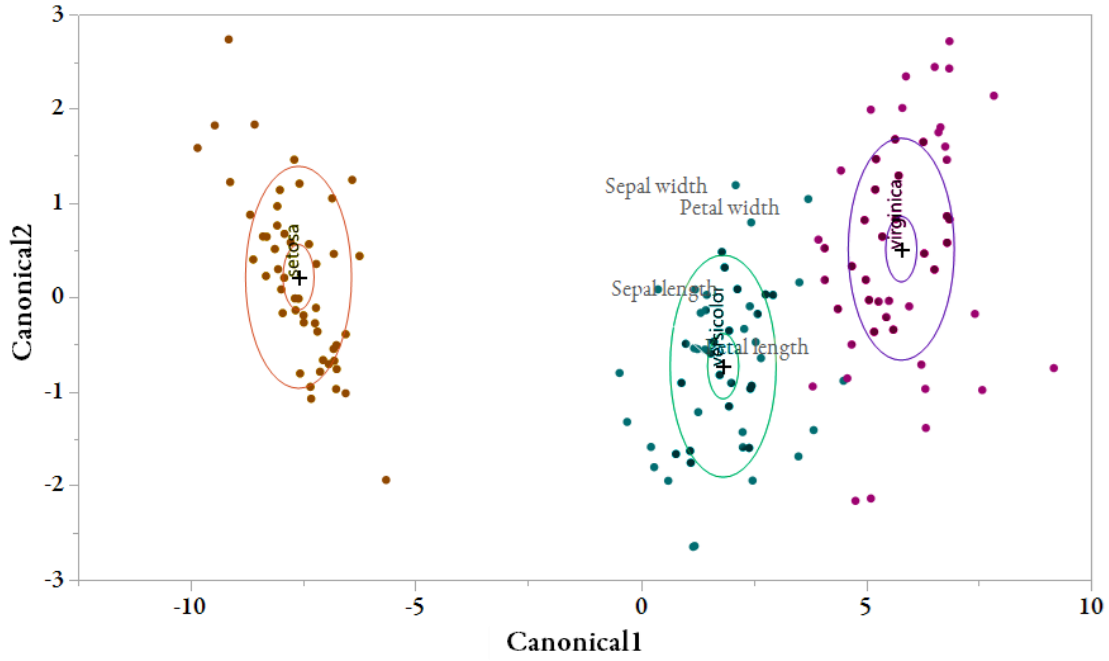

Figure 2. Example of a canonical plot obtained by a Linear Discriminant Analysis performed on the "iris.jmp" dataset furnished by JMP Pro15 (SAS Institute Inc., Cary, NC, USA). The entropy RSquare of the model was 0.96, with 3 samples over 150 misclassified over (2 *versicolor* and 1 *virginica*).

Other examples of discriminant algorithms developed and employed in food authenticity studies, include factorial discriminate analysis (FDA), partial least squares discriminant analysis (PLS-DA), quadratic discriminant analysis (QDA), and others (Esteki et al., 2018).

Often, a data compression step is performed before the construction of the classification model, in order to reduce the data matrix size and complexity, removing redundancy and noise while retaining the maximum of the information. For this purpose, exploratory data analysis and unsupervised pattern recognition are commonly used to simplify and gain better knowledge of data sets before developing supervised models (Berrueta et al., 2007).

4.2 Data pre-treatment

Generally, when working with datasets obtained by a chemical fingerprint on biological samples, many drawbacks can be present in the structure of data, making them not suitable for multivariate analysis in their original form. For example, differences among the magnitude of the concentration of the variables measured can be observed; moreover, each variable can be associated to a larger or smaller variability respect to the others. Generally, compounds that are more abundant in the biological systems exhibit higher concentration and, subsequently, larger differences among samples compared to the low-abundant compounds. Since many multivariate methods focus on maximizing the variance in data structures, the highly abundant variable dominantly contribute to the model, masking the importance of low-abundant compounds and biasing the statistical analysis (Karaman, 2017; van den Berg et al., 2006). Furthermore, an *uninduced biological variation* can be observed in the data structure: some variables can show larger fluctuations even under identical

experimental conditions, while others present a higher 'stability'. All these elements can be problematic and lead to conceal fundamental biological features. Really, data pre-treatment methods focus on emphasizing the biologically relevant information, reducing the technical and measurement errors and avoiding the biases related to data structure (Karaman, 2017; van den Berg et al., 2006). The choice of the suitable data pre-treatment method depends on the aim of an investigation and on the multivariate data analysis to be used.

4.2.1 Data centering and scaling

Data centering is a pre-treatment process usually employed when the aim of the investigation is to discover biomarkers in a complex data matrix. Centering allows to remove the offset from the data and to focus on the biological variation and on the similarities (or dissimilarities) among the samples. Thus, it is particularly useful when using unsupervised methods. Data centering is performed by subtracting to each value the mean of the observations for each parameter:

$$x_T = x_{NT} - \bar{x}$$

Equation 1

where x_T is the value of the observation after the treatment, x_{NT} is the value of the observation before the treatment and $\bar{x}$ is the average value for the variable x in all the observations. Thus, centering converts the measured values (concentrations) to fluctuation around zero and adjusts for differences in the offset between high and low abundant variables (Karaman, 2017; van den Berg et al., 2006).

Generally, the features that are present in higher concentrations show also the higher variability. When applying a statistical model aimed to enhance the variance of data distribution (such as PCA), the highly abundant compounds tend to dominantly contribute to the model, being related to the larger proportion of the total variance and centering could be not enough to adjust this bias. **Data scaling** are data pre-treatment approaches that aim to adjust for the high fold-differences observed between the variables, converting data into differences relative to a *scaling factor*. Scaling is performed dividing each variable by a factor (equation 2), which is different for each variable, and we can list several scaling strategies on the basis of the factor employed (Karaman, 2017; van den Berg et al., 2006):

$$x_T = \frac{x_{NT} - \bar{x}}{scaling\ factor}$$

Equation 2

The principal scaling methods are resumed in Table 1.

Table 1. Overview of the most used scaling methods, adapted from (van den Berg et al., 2006)

SCALING METHOD	SCALING FACTOR	GOAL	ADVANTAGE	DISADVANTAGE
AUTO SCALING	Standard deviation (*sd*)	Compare compounds based on correlations	All metabolites become equally important	Inflation of the measurement errors
RANGE SCALING	Range (*max-min*)	Compare metabolites relative to the biological response range	All metabolites become equally important. Scaling is related to biology	Inflation of the measurement errors and sensitive to outliers
PARETO SCALING	Square root of the standard deviation (√*sd*)	Reduce the importance of large values keeping data structure intact	Closer to the original measurement than autoscaling	Sensitive to large fold changes
VAST SCALING	Coefficient of variation ($sd/\bar{x}$)	Focus on metabolites that show small fluctuations	Aims for robustness	Not suited for large induced variations without group structure

4.2.2 Data transformation

Multivariate statistical analysis methods assume that the noise is consistent across all features (*homoscedastic*). However, fingerprinting data are generally subjected to the so-called *heteroscedastic noise*, in other words, the noise increases as a function of increased signal intensity. Therefore, the dataset often needs to be transformed in order to adjust the noise structure. Furthermore, the distributions of the variables can be skewed and may need to be converted to resemble normality prior to of statistical analysis. **Transformation** is the pre-treatment process that aims to correct for heteroscedasticity and skewness (van den Berg et al., 2006). Transformation also has a pseudo-scaling effect on the dataset, because the differences of the features with high and low abundances are substantially diminished. However, often is still necessary to apply centering and scaling treatments after transformation (Karaman, 2017). The most used transformation approaches are the logarithmic transformation and the power transformation, as summarized in Table 2.

Table 2. Overview of the most used transformation methods, adapted from (van den Berg et al., 2006).

TRANSFORMATION METHOD	MATHEMATICAL FUNCTION	GOAL	ADVANTAGE	DISADVANTAGE
LOGARITHMIC	Logarithm: $x_T = \log_{10} x_{NT}$	Correct for heteroscedasticity and pseudoscaling	Reduce heteroscedasticity and transform multiplicative effects in additive effects	Difficulties with values with large standard deviations and zeros
POWER	Square root: $x_T = \sqrt{x_{NT}}$	Correct for heteroscedasticity and pseudoscaling	Reduce heteroscedasticity without problems toward values equal to zero	Choice for square root is arbitrary

x_T = value of the observation after the treatment; x_{NT} = value of the observation before the treatment.

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Part 2

Experimental part

Chapter 5

Aims and scopes

The scope of this thesis involved the employment of appropriate analytical techniques with the aim to characterize chemical markers of *food quality* and *authenticity* in animal products. The selected approach was employed on two levels:

I. On a **farm-scale**, collecting raw material such as bulk milk and raw fish eggs, in order to relate their quality to intrinsic and extrinsic factors, such as the farming system or the species of origin;

II. On a **manufactory-scale**, collecting transformed products, such as ripened fish eggs and caviar, in order to relate their quality to the influence of the production process.

Particularly, in the case of **dairy products** (goat and cow milk and cheese), the principal aim was to identify features associated to their origin depending on the farming system and the feeding strategy employed in the farm, thus searching for suitable authenticity markers related to the production system (i.e. organic, conventional, grass-fed...). In the case of **fish products** (sturgeon meat and fish eggs), the scope was to identify features associated to both different origin and processing method. In the latter case, the aims of the investigation covered:

a) The characterization of the evolution of the chemical profile during the maturation; and

b) The identification of possible chemical markers to discriminate among different products.

The aim of the project was followed by developing and employing chromatographic techniques in *quality* and *authenticity* research. The objectives can be summarized as follows:

a) **Dairy products:** to select and apply a suitable and reliable GC-FID method for the identification of fatty acids as markers of the livestock system employed in the farm and conveying an added value, linked to higher nutritional quality, to the product.

b) **Fish products:** I. to apply a robust and standardized GC-FID technique to assess fatty acids as markers of origin (species) and to characterize the nutritional quality of sturgeon meat and fish roes products; II. to develop and optimize chromatographic methods coupled to mass spectrometry detection (HS-SPME-GC-MS and UHPL-HRMS) to characterize the whole metabolome (volatile and non-volatile) of white sturgeon caviar during its ripening and to likely detect compounds particularly relevant for the development of the taste and flavour.

Chapter 6

Authenticity and quality traits in *dairy products* obtained under different livestock systems

6.1 Authenticity and quality in *dairy products* obtained under different livestock systems – General introduction

Gas chromatographic techniques can be usefully employed in food quality studies for the separation and the characterization of food flavour and lipid profile. Food lipids are represented by several components, fatty acids (FA) being the major constituents. The most of FA are esterified on a glycerol molecule as triacylglycerols (TAG) but they are also part of the phospholipid membranes and can be present in the unesterified form (free fatty acids). These compounds are wide studied because of the ongoing discussion about the impact of different FA classes in human nutrition (Bernard et al., 2018; Hanus et al., 2018a). In animal sciences, particularly, FA profiling can be a useful approach to investigate the multifactorial causes that influence lipid fingerprint of animal origin foodstuffs.

In dairy products, FA profile is strictly related to both extrinsic and intrinsic factors able to influence the chemical composition of the final products, including genetic (species, breed), the production system employed in the farm (i.e. extensive *vs* intensive, conventional *vs* organic), the dietary regimen supplied to animals and, partly, also to the geographical origin, being different plants in pasture related to different levels of secondary metabolites that influence the metabolism of FA (Butler et al., 2008; Chilliard et al., 2007; Collomb et al., 2008; Coppa et al., 2019; Liu et al., 2016; Schwendel et al., 2015; Segato et al., 2017; Tsiplakou et al., 2010). In this context, some FA could represent interesting reliable chemical markers in a chemometric approach, providing for discrimination of the origin of milk fat and contributing in the characterization and protection of typical dairy products (Białek et al., 2020; Capuano et al., 2014). Mostly, milk and cheese from ruminants fed on grass have been demonstrated to naturally contain bioactive substances that help improving the FA composition of milk and the overall nutritional quality of dairy products. Diets based on grazing, or including fresh grass in the formulation, were demonstrated to induce a higher content of conjugated linoleic acid (CLAs) and polyunsaturated fatty acids in milk than hay-based diets and silage (Jahreis et al., 1997; Pajor et al., 2009; Stockdale et al., 2003).

FA profiling in dairy products is a theme of extreme interest in the scientific community, due to the impact that the different classes of FA have in human nutrition, mostly *cis* and *trans* monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), CLAs and branched chain fatty acids (BCFA) (Bernard et al., 2018; Hanus et al., 2018b). Particularly, BCFA including the *iso* and *anteiso* form, which are representative of the major lipids of rumen bacterial membranes, are known to act as important bioactive components, related to positive functions in the gut microbiota and to functional activities (Ran-Ressler et al., 2011). Dairy products obtained by a feeding strategy related to the consumption of fresh grass or pasture have been demonstrated to contain higher levels of BCFA. In all the ruminant species, the content of BCFA in milk is strictly related to forage and concentrates content of the diet supplied to animals in the farm (Serment et al., 2011). Diets rich in starch or represented by low amount of neutral detergent fibre (NDF) and, consequently, by a low forage to concentrate ratio (F/C), can promote the growth of amylolytic bacteria and the detriment of cellulolytic bacteria in the rumen with a consequent reduction of *iso* fatty acids in milk fat (Vlaeminck et al., 2006).

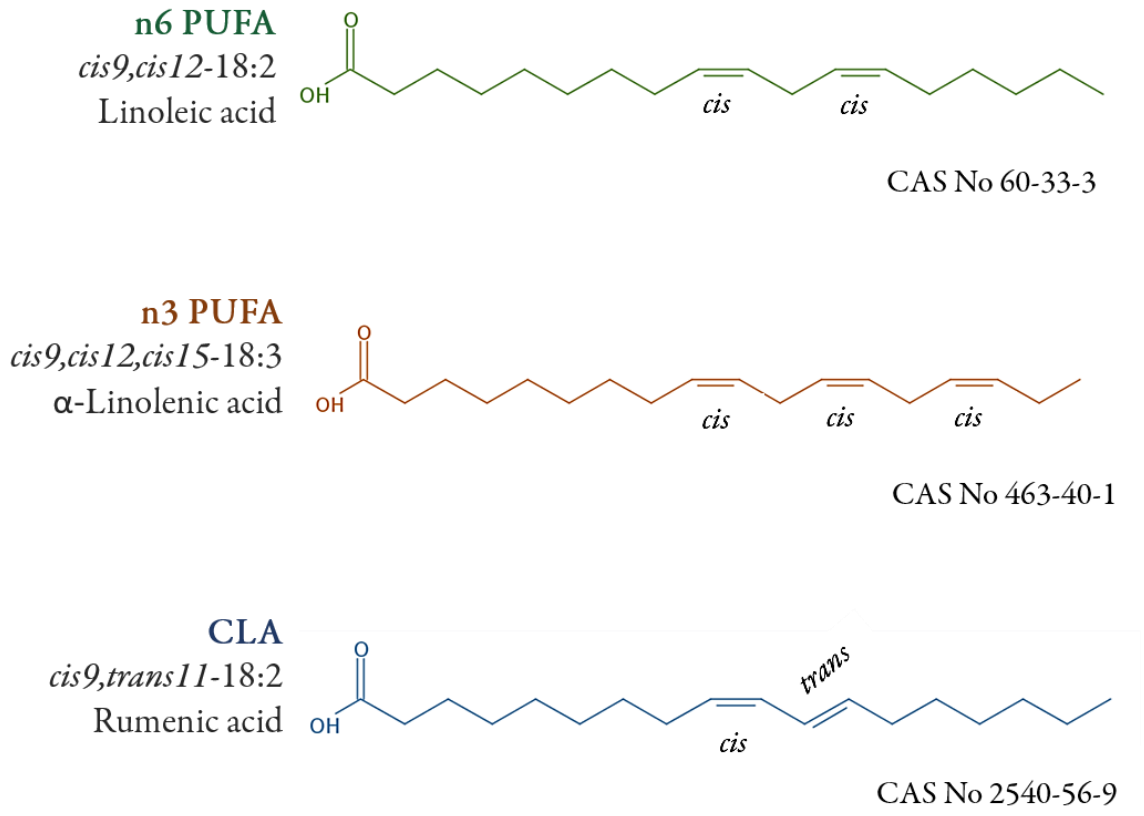

Figure 1. Linoleic and linolenic acid are reported as the most representative of the n6 and n3 series FA, respectively. Moreover, rumenic acid, the conjugated linoleic acid isomer (c9t11-CLA) is reported with the *cis* and *trans* configuration of the conjugated double bonds in position C9 and C11.

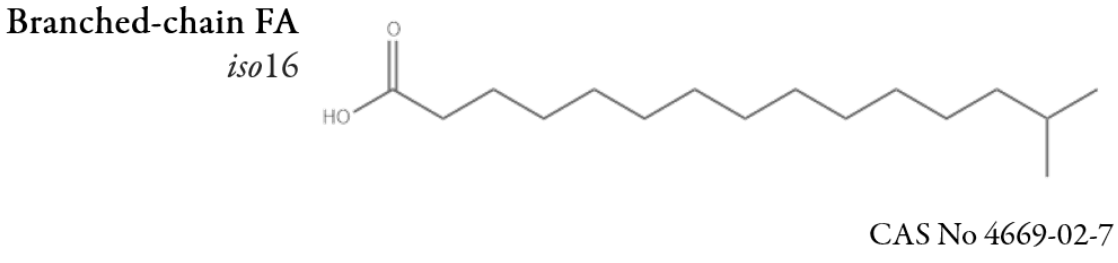

Figure 2. 14-methyl-heptadecanoic acid, common name *iso*16, as example of branched-chain fatty acid. The methyl branch is linked to the second to last carbon atom, and for this reason the BCFA is commonly known as *iso* form. On the contrary, a methyl branch linked to the third to last carbon atom give rise to the *anteiso* form of a BCFA.

Dairy products from small ruminants resulted to be enriched in this class of FA. Goat products, particularly, are widely considered as excellent source of nutrients, related to a lower allergenic potential and higher digestibility if compared to cow products (Ran-Ressler et al., 2014; Verruck et al., 2019). The presence of high biological value proteins, essential FA high mineral bioavailability and vitamin content makes goat dairy products particularly interesting for a wide consumers fragment that shows to be aware of food quality not just as 'basic nutrition' but also a fundamental part of the well-being (Albenzio et al., 2016; Haenlein, 2004; Sepe & Argüello, 2019). Moreover, dairy goat products are gaining a good reputation among consumers because of their perception to be linked to a more "ethic" livestock production, related to lower environmental impact and higher animal welfare standard in the farm, thus more sustainable (Massaglia et al., 2019).

Generally, small mountain farms make use of livestock systems conferring low-input to the environment and putting low breeding pressure toward the animals if compared to the conventional production system in the dairy sector. The quality of yielded milk in small mountain farm has shown to have a high nutritive and functional quality, represented by high content of nutritionally favourable fatty acids (Chung et al., 2018; Collomb et al., 2008; Malissiova et al., 2015; Schwendel et al., 2015; Tsiplakou et al., 2010). Moreover, these farms are usually associated to an on-farm cheese making and to the sale of the products directly in the farm or in local shops, representing a market segment generally related to high-quality and traditional products, strictly linked to the territory (Massaglia et al., 2019). All these factors contribute to the perception of a higher quality toward dairy products coming from small mountain farms. However, the functional benefits of many dairy products remain unexplored by the most of consumers (Verruck et al., 2019). Sepe and Arguello (2019) defined goats grazing in pastoral systems as 'almost unknown carriers of health promoting compounds'. Moreover, in many mountain areas, such as the Alpine region, the agricultural crisis led to the loss of fundamental ecosystem services, including the production of typical dairy products based on pasture livestock system (Hoffmann et al., 2014; MacDonald et al., 2000; Montrasio et al., 2020).

Thus, it is fundamental that scientific research in dairy sector could furnish solid knowledge toward the positive properties of such kind of products, information to be published and promulgated in order to support consumers appreciation and the policies of protection toward the mountain animal husbandry sector, related to environmental, social and cultural values. Actually, when positive perceptions toward the consumption of these kind of products are supported by scientific evidences, the results can be positive for the entire production chain, allowing consumers to be more informed about the real quality of products on the market and, consequently, small producers to gain market size as a consequence of the increase of consumers demand (Verruck et al., 2019).

In the studies presented in the following sections of this thesis, the aim was to employ a solid GC-FID method in order to characterize the lipid profile in dairy products obtained by different rearing systems, particularly focusing on the presence of many bioactive FAs, in order to:

a) evaluate the impact of different production systems in the extrinsic and intrinsic quality of goat dairy products;
b) trace the qualitative characteristic of grass-fed farming system in terms of uniqueness of mountain dairy products.

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Trial 1

Fatty Acid Profile in Goat Milk from High- and Low-Input Conventional and Organic Systems

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Abstract. According to the knowledge that the composition in fatty acids of milk is related to the production system, we determined the fatty acid composition of goat milk yielded in three different Italian farms. Two low-input system farms; one organic (LI-O) and one conventional (LI-C), and one high-input system conventional farm (HI-C) were involved in the study. Significant differences were detected among the different groups considering the fatty acid pattern of milk. Fatty acids (FA) strictly related to the rearing system, such as odd and branched chain fatty acids (OBCFA), linoleic acid (LA, 18:2 n6), alpha-linolenic acid (ALA, 18:3 n3), elaidic acid (EA, 18:1 n9), total n6 and total n3 FA, were identified as the most significant factors in the characterization of samples coming from low or high-input systems. OBCFA amounts were found to be higher ($p < 0.05$) in the LI-O milk (4.7%), followed by the LI-C milk (4.5%) and then by the HI-C milk (3.4%). The same trend was observed for n3 FAs, mainly represented by ALA (0.72%–0.81% in LI-O systems and 0.41% in HI-system), and the opposite for n6 FAs, principally represented by LA (2.0%–2.6% in LI-systems and 3.1% in HI-system). A significant ($p < 0.01$) discrimination among samples clusters coming from the different systems was allowed by the principal component analysis (PCA).

Results from this investigation have been published in the scientific journal *Animals*

Animals **2019**, 9, 452; doi:10.3390/ani9070452

1. Introduction

The fatty acid (FA) composition and related factors variability in milk fat are recently renewed due to the impact of different FA classes in human nutrition, particularly, cis and trans monounsaturated fatty acids (MUFA), odd and branched chain fatty acids (OBCFA), conjugated linoleic acid (CLA) and polyunsaturated fatty acids (PUFA) (Laurence Bernard et al., 2018; Hanus et al., 2018). Several studies aimed at discussing the linkage between animal diet and rumen microbiota, and the related effect on milk quality. The rumen bacteria population and mammary gland activity are the main responsible for biochemical mechanisms that include rumen biohydrogenation, mammary lipogenic and D9-desaturation in CLA and odd and branched chain formation (Fievez et al., 2003, 2012; Vlaeminck et al., 2006). Particularly the type of forage, forage to concentrate ratio (F/C), lipids supplementation and starch level, together with their interactions, significantly affect milk FA composition, including OBCFA. Bacterial OBCFA are major lipids of bacterial membranes (Kaneda, 1991; Mackie et al., 1991). A significantly different content of odd-chain (C15:0, C17:0, C17:1) fatty acids was reported in cow milk and cheese samples from different pasture vegetation type, contributing in the characterization and protection of typical dairy products (Falchero et al., 2010; Povolo et al., 2013). Branched chain fatty acids (BCFA), iso and anteiso forms relating to the methyl-group located on the penultimate carbon and on the antepenultimate carbon of the carbon chain, represent a lesser component of milk (about 2%–3% of total fatty acids). However, they are recognised as important bioactive components since their positive role in gastrointestinal microbial ecology and potential anti-cancer activity (Forouhi et al., 2014; Khaw et al., 2012; Rinat R. Ran-Ressler et al., 2011; Yang et al., 2000). Cytotoxicity of these fatty acids might be compared to that of CLA to which much more attention has been spent, despite milk BCFA being more represented than milk CLA. Recently, the contribution of BCFA from various food, prominently featured in the American diet, has been estimated. These studies have shown that BCFA may have a beneficial effect on proper gut functions; thus, their intake becomes relevant for human health (Rinat R. Ran-Ressler et al., 2011).

Grazing represents a right approach to improve healthful nutritional quality of milk lipid composition in ruminant species. Goat milk products seem to be enriched in OBCFA when compared to cow milk products (Rinat Rivka Ran-Ressler et al., 2014), and this could be one of the criterions useful in enhancing the appreciation of consumers toward goat products, and to take them into account as possible functional foods. In goat milk, the OBCFA content was significantly affected by lipid supplementation and its interaction with forage levels (Cívico et al., 2017; Mele et al., 2008). Moreover, different percentages of diet concentrate affect the relative amount of C13:0, iso-C15:0, and iso-C16:0 in goat fat milk (Serment et al., 2011). Comparisons of organic and conventional farming system characterized by grazing and a reduced amount of conserved forage generally show a higher proportion of nutritionally favourable FA such as PUFA n3, rumenic acid and branched FA (Chung et al., 2018; Collomb et al., 2008; Malissiova et al., 2015; Schwendel et al., 2015). Diets rich in starch or a decrease in F/C ratio and neutral detergent fibre (NDF) content promote the growth of amylolytic and go to detriment of cellulolytic bacteria with a consequent reduction of iso fatty acids in milk fat (Vlaeminck et al., 2006). Generally, the conventional livestock production system adopted in dairy goat breeding in Italy, particularly in small mountain farms, is considered as semi-intensive. This could be the reason for the higher amount of OBCFA detected in goat products. It is interesting to determine if any difference or similarities exists among milk yielded

in farms managed under different conditions. Particularly, in many goat farms, which we can consider as "low-input-system farms", the production system is not so different to that used in organic-certified farms, and this could lead to a similar nutritive and functional quality of yielded milk, even if it is not certified as organic (Gillian Butler et al., 2008). Thus, the aim of this work was to evaluate the impact of different production systems in Italy on milk fatty acids composition of goat milk, with a particular focus on OBCFA.

2. Materials and Methods

2.1 Animals, Housing and Feeding

The experiment involved three goat farms from the end of March until October 2017. Each farm was considered for different peculiarities distinguishing its livestock production system. With high-input (HI), authors refer to the conventional, typically intensive, dairy goat rearing system in Italy, in which animals are always reared in the barn and the diet is mainly based on concentrates, conserved forage and a low F/C ratio all the year around. A conventional lowland farm with Saanen breed goats (HI-C) was selected to represent the HI production and feeding system (HI-C). In the HI-C farm, goats were fed by local ryegrass hay offered ad libitum, alfalfa hay offered once a day (about 500 g/d) during the first period of lactation and commercial feed, consisting of a mixture of flaked and flour cereals, distributed individually twice a day (totally 1200 g/d) during milking. Two farms were considered representing the low-input (LI) production system. In the LI-system, goats are mainly allowed to graze. In the barn, their diet is integrated with conserved forage and concentrates, with a higher F/C ratio. A conventional mountain farm (altitude, 980 m) with Alpine breed goats, represented the not-organically-certified LI breeding system in our study (LI-C). In the LI-C farm, goats grazed depending on favourable climatic conditions and other feed supplements were also included in the diet. In more detail, goats were fed ad libitum with local polyphite grass hay (first harvest), alfalfa hay distributed once a day after the morning milking (about 500 g per goat per day) and concentrates (commercial mixtures) distributed twice a day during milking (totally 1000 g per goat per day). Finally, an organic Alpine breed goat farm (LI-O) was selected representing the organic-certified (Regulation (EU) 2018/848) system, in which, in addition to feeding, further organic-farming standard must be observed (LI-O). In this farm, lactating goats were systematically and daily grazed from spring to autumn (March–October); the access to fresh grass was controlled, starting after the morning milking and ending around noon (about 4 hours/day). Forage consisting of alfalfa hay (1000 g per goat per day) and polyphite grass hay (500 g) was distributed twice a day, firstly in the morning before grazing fresh pasture and after the second milking in the afternoon grains. A mixture of organically certified maize and barley grains (3:1, respectively), formulated in-farm, was supplied twice a day during milking (totally 800 g/d). In all three farms goats had free access to water and to salt integrators; no additional lipids were supplemented. Feeding (including access to pasture) and milk yield and composition data were recorded by information collected by farmers. The average daily feed intake per goat was evaluated on the basis of dry matter intake (DMI) prediction models for lactating goats reported by Pulina et al. (2013), taking into account the goat's average size and milk yield for the

lactation period involved in the study. Estimated intakes were calculated by fodder analysis of feedstuffs dry matter (European Commission, 2009).

2.2 Sampling

All farms had similar proportions of goats in lactation at all sampling dates (42, 39 and 45 in HI-C, LI-C and LI-O farms, respectively). Goats were machine milked twice a day in all farms. Bulk tank raw milk samples were sampled twice a month, from March (4 ± 1 week of lactation) to October, from two consecutive milking, corresponding to cheese making in farm. A total amount of 37 milk samples were stored at minus 20 °C until the analysis of fatty acid composition. The measurement of fat, protein and lactose were assumed from monthly official controls, determined by near infrared spectrophotometry (ISO 9622).

2.3 Analytical Procedure

To determine the fatty acids composition of milk, fat was extracted according to the method of Folch et al. (1957). Lipids were extracted from 10 mL of milk by chloroform methanol 2:1. Extracted milk fat was quantified and an aliquot (about 40–50 mg) was used for fatty acid composition analysis. Fatty acids were determined as methyl ester, prepared by base catalysed methanolysis of glycerides as described by Christie (2003). Lipids were dissolved in 1 ml of diethyl ether, then 50 μL of methyl acetate and 100 μL of 1 M sodium methoxide in methanol were added. The reaction was stopped after 5 min at room temperature by adding 50 μL of an oxalic acid in diethyl ether saturate solution. After centrifugation at 1500 g for 5 min, 200 μL of upper layer of solution was used directly for gas chromatography (GC) analysis. The injector was set in split mode (1:100 split ratio) at a constant temperature of 250 °C and 1 μL sample was injected. Fatty acid analysis was carried out on an Agilent gas-chromatograph (Model 6890 Series GC, Agilent Technologies, Santa Clara, CA, USA) fitted with an automatic sampler (Model 7683, Agilent Technologies, Santa Clara, CA, USA) and FID detector (Agilent Technologies, Santa Clara, CA, USA). The carrier gas was helium with a flow rate of 1.0 mL min^{-1} and an inlet pressure of 16.9 psi. A TRACE™ TR-FAME column (60 m length, 0.25 mm i.d., 0.25 μm film thickness; Thermo Fisher Scientific, Waltham, MA, USA) was used to separate fatty acid methyl esters. The oven temperature program for separation started with an isotherm of 6 min at 50 °C, then the temperature increased at a rate of 10 °C min^{-1} until 170°C and kept at this temperature for 30 min. Afterwards the temperature was increased from 4°C min^{-1} to the final temperature of 220°C and hold for 20 min.

Individual fatty acids methyl esters were identified by comparing sample peak retention times with standard mixtures (Supelco 37 FAME Mix, Supelco, Bellafonte PA, USA) and pure standard methyl esters from Sigma-Aldrich (Sigma-Aldrich, Saint Louis, MO, USA, cat n.CRM 47791) and expressed as percentage of total fatty acids. The identification of branched chain fatty acids was determined by preparing methyl esters from standards available (Sigma-Aldrich, Saint Louis, MO, USA), then analysed under the same instrumental condition. A gas-chromatographic correction factor has been applied to take into account the lower response of the flame ionization detector to the molecules with a lower number of carbon atoms for 4:0, 6:0, 8:0, 10:0 and 12:0 (Ulberth et al., 1999).

2.4 Statistical Analysis

The evaluation of different farming systems was calculated by the analysis of variance. Normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene test) were confirmed and comparison between means was performed by the ANOVA test when the normality and homoscedasticity assumptions were confirmed, and the Welch ANOVA F test when the assumptions were not confirmed. The Student Newman-Keuls was used as the post Hoc test for comparison of the means among different farming systems. Significance was declared at $p \leq 0.05$. Multivariate analysis (principal component analysis, PCA) was applied, taking into account all fatty acids of milk sampled as graphical projection technique, in order to study the distribution of samples in a two-dimensional space and to establish if any separation between different groups (LI-O, LI-C, HI-C) was feasible using measured variables. The statistical procedure was performed using JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). Data in the tables are reported as mean values _ standard deviation.

2.5 Ethical Approval

This article does not contain any experimental practice performed on animals by the authors. Only raw bulk milk sampling, considered a routine practice in farm, was performed in order to develop the present research. No biological material was collected. Authors guarantee that in the three farms involved in the study all the applicable guidelines for animal welfare established by harmonised EU rules were followed. No approval by the institutional ethics committee was requested by University of Milan for this kind of research.

3. Results

Table 1 discloses goat feed intake and milk proximate composition of farms involved in the study. Proximate composition did not show significant differences between the three groups, with the exception of protein concentration, which slightly increased in HI-C farm ($p<0.05$).

Table 1. Feed intake and milk yield (per goat per day) and composition in LI-O, LI-C, HI-C. Milk composition data are presented as mean ± standard deviation for each farm.

	FARM									
	LI-O LOW INPUT ORGANIC				**LI-C** LOW INPUT CONVENTIONAL			**HI-C** HIGH INPUT CONVENTIONAL		
	C [1]	**AH[2]**	**PGH[3]**	**P[4]**	**C[1]**	**AH[2] ***	**PGH[3]**	**C[1]**	**AH[2] ***	**RH[5]*****
Supplied *g/day*	800	1000	500	*ad libitum*	1000	500	*ad libitum*	1200	500	*ad libitum*
g/Kg DM	297.1	362.1	183.1	-	423	215.6	-	488.1	207.7	-
F/C ratio	70/30				60/40			50/50		
Milk yield *g/day*	2.55 ± 0.5				2.50 ± 0.6			2.60 ± 0.5		
Fat *%*	3.2 ± 0.7				3.3 ± 0.5			3.6 ± 0.55		
Protein *%*	3.2 ± 0.3 [a]				3.3 ± 0.3 [a]			3.7 ± 0.4 [b]		
Lactose *%*	4.4 ± 0.3				4.4 ± 0.3			4.6 ± 0.15		

[1] C - concentrates; [2] AH - alfalfa hay; [3] PGH - polyphite grass hay; [4] P - pasture; [5] RH - ryegrass hay. [a,b] = mean values for each group within a row with unlike superscript letters were significantly different ($p < 0.05$).

The results obtained from fatty acid analysis are reported in Table 2, expressed as g/100g of total FA.

Table 2. Fatty acids (g/100g of total FA) of bulk goat milk samples. Data are expressed as mean ± standard deviation.

	FARM		
Fatty Acid	**LI-O** (*N* = 14 *)	**LI-C** (*N* = 9 *)	**HI-C** (*N* = 14 *)
4:0, Butirric Acid	2.0±0.30	1.9±0.29	1.9±0.17
6:0, Caproic Acid	2.0±0.28	1.9±0.17	2.1±0.14
8:0, Caprylic Acid	2.7±0.36	2.6±0.19	2.9±0.16
10:0, Capric Acid	10.0±0.77 ^{a}	9.3±1.39 ^{a}	10.8±0.52 ^{b}
12:0, Lauric Acid	5.7±1.33	5.2±1.74	5.6±0.66
14:0, Myristic Acid	11.7±1.81	10.8±1.85	11.8±0.56
16:0, Palmitic Acid	26.3±2.05 ^{b}	23.2±1.97 ^{a}	26.9±1.07 ^{b}
18:0, Stearic Acid	8.5±2.66	10.6±3.42	8.3±1.38
20:0, Eicosanoic Acid $^{+}$	0.82±0.14 ^{a}	0.98±0.23 ^{b}	0.8±0.106 ^{ab}
22:0, Docosanoic Acid	0.09±0.02 ^{b}	0.10±0.03 ^{b}	0.06±0.01 ^{a}
Σ Saturated Fatty Acids	69.8±1.91 ^{b}	66.7 ±3.50^{a}	71.22±1.17 ^{b}
11:0, Undecanoic Acid	0.13±0.04	0.10±0.06	0.10±0.02
13:0, Tridecanoic Acid	0.13 ±0.02^{b}	0.11±0.03 ^{a}	0.10±0.01 ^{a}
iso 14:0	0.16 ±0.02^{c}	0.13±0.03 ^{b}	0.10±0.01 ^{a}
iso 15	0.23±0.02	0.24±0.05	0.25±0.03
15:0	1.14±0.09 ^{c}	1.07 ±0.08^{b}	0.91±0.05 ^{a}
anteiso 15:0	0.67±0.09 ^{b}	0.63 ±0.03^{b}	0.54 ±0.05^{a}
iso 16:0	0.34±0.05 ^{c}	0.29±0.08 ^{b}	0.25 ±0.02^{a}
iso 17:0	0.35±0.03	0.37±0.07	0.39±0.03
17:0	0.70±0.11 ^{b}	0.75±0.18 ^{b}	0.57 ±0.06^{a}
anteiso 17:0	0.82±0.21	0.76±0.08	0.72±0.08
17:1n-8	0.33±0.04 ^{b}	0.32±0.05 ^{b}	0.22±0.02 ^{a}
Σ Branched Chain Fatty Acids	2.6 ±0.23^{b}	2.4±0.18 ^{b}	2.2 ±0.13^{a}
Σ Odd Chain Fatty Acids	2.1±0.12 ^{b}	2.0±0.15 ^{b}	1.7±0.08 ^{a}
Σ Odd and Branched Chain Fatty Acids	4.7±0.24 ^{c}	4.4±0.32 ^{b}	3.9 ±0.17^{a}
14:1, Miristoleic Acid	0.28±0.21	0.22±0.14	0.20±0.07
16:1 c7+9, Palmitoleic Acid	0.79±0.31	0.64±0.19	0.61±0.13
18:1 t9, Elaidic Acid	0.29 ±0.05^{a}	0.41±0.09 ^{b}	0.48±0.06^{c}
18:1 t11, *trans*-Vaccenic Acid	1.0±0.38	1.4±0.22	1.2±0.41
18:1 c9, Oleic Acid	18.9±1.60 ^{a}	21.1±3.42 ^{b}	17.4±0.80 ^{a}
18:1 c11, cis-Vaccenic Acid	0.17±0.08 ^{a}	0.25 ±0.09^{b}	0.26±0.05 ^{b}
20:1n-9, Gondoic Acid	0.04±0.01 ^{a}	0.04±0.01 ^{a}	0.06 ±0.01^{b}
Σ Monounsaturated Fatty Acids	21.9±1.58 ^{a}	24.4±3.13 ^{b}	20.5±0.97 ^{a}
18:2n-6 t9t12	0.20±0.06 ^{a}	0.26±0.04^{b}	0.24±0.04 ^{ab}
18:2n-6 c9t12	0.11±0.01 ^{a}	0.17±0.07^{b}	0.14±0.04 ^{ab}
18:2 c9c12, Linoleic Acid	2.0±0.27 ^{a}	2.6±0.29^{b}	3.1±0.22 ^{c}
18:3n-6, γ-Linolenic Acid	0.03±0.01	0.04±0.02	0.04±0.00
18:3n-3 c9c12c15, α-Linolenic Acid	0.72±0.11 ^{b}	0.81±0.10^{b}	0.41 ±0.11^{a}
20:2n-6, Eicosadienoic Acid	0.07±0.01 ^{b}	0.07±0.02^{b}	0.06±0.01 ^{a}
20:3n-6, Dihomo-γ-Linolenic Acid	0.03±0.01 ^{a}	0.03±0.01^{a}	0.04±0.01 ^{b}
20:4n-6, Arachidonic Acid	0.15±0.01 ^{a}	0.17±0.02^{b}	0.18±0.02 ^{b}

Table 2 (cont.)			
20:5n-3, Eicosapentaenoic Acid	0.10±0.03 [b]	0.10±0.02[b]	0.06 ±0.03[a]
22:5n-3, Docosapentaenoic Acid	0.18±0.04 [b]	0.17±0.03[b]	0.11±0.03 [a]
n3	1.0±0.14 [a]	1.1±0.12[a]	0.6±0.15 [b]
n6	2.6±0.35 [a]	3.4±0.27[b]	3.8±0.27 [c]
n6/n3	2.6±0.27 [a]	3.1±0.35[a]	6.5±1.45 [b]
Σ Polyunsaturated Fatty Acids	3.6 ±0.46[a]	4.4±0.31[b]	4.4±0.31 [b]

[a, b, c] = values in the same row that have a different superscript are significantly different at $p \leq 0.05$, ANOVA and Student-Newman-Keuls post-hoc test. *LI-O and HI-C consisted in one sample in March, two samples per month from April to September and one sample in October (1 + 2 × 6 + 1), meanwhile LI-C consisted in two samples in April, one sample per month from May to September and two samples in October (2 + 1 × 5 + 2). [+]This peak can include CLA c9t11, accounting for 0.6% of total FA in hay fed goat milk as reported by Bernard et al. (2005).

Detected fatty acids were divided into four groups according to their chemical structure. For each group, the sum (∑) was made. Particularly, for the OBCFA group we calculated the amount of branched-chain fatty acids (∑BCFA), linear odd-chain fatty acids (∑OCFA) and the total amount of both groups (∑OBCFA). Oleic acid was found in higher concentrations in LI-systems samples (21.1% in the conventional farm and 18.9% in the organic farm) than in the HI-system samples (17.4%). Elaidic acid (EA) and linoleic acid (LA) were found to be higher ($p < 0.05$) in the HI farm milk (0.48% and 3.1%, respectively), followed by the LI-C farm milk (0.41% and 2.6%, respectively) and, then, by the LI-O milk (0.29% and 2.0%, respectively). Alpha-linolenic acid (ALA) showed differences ($p < 0.05$) between the LI farms milk (0.72% in the organic farm and 0.81% in the conventional farm) and the HI farm milk (0.41%). PUFA of the n3 series were found in higher ($p < 0.05$) amounts in LI-O milk (1%) and LI-C milk (1.1%) than in HI-C milk (0.6%). On the contrary, n6 PUFA were significantly higher ($p < 0.05$) in HI-C milk (3.8%), followed by LI-C milk (3.4%) and, then, by LI-O milk (2.6%). Consequently, the n6/n3 ratio showed differences ($p < 0.05$) between LI groups, with values of 2.6 in the organic farm and 3.4 in the conventional farm, and the HI group, in which it reached a value of 3.8. Long chain n3 PUFA, EPA and DPA, resulted in higher values ($p < 0.05$) in the LI-systems than in the HI-system. DHA (22:6 n3) was detected only in trace and in a small number of samples. Significant differences ($p < 0.05$) were found among the three groups in the whole content of OBCFA, which ranged from 4.7% in the LI-O farm to 3.9% in the HI-C farm, and with the LI-C farm it reached an intermediate value of 4.4%. Particularly, branched fatty acids iso14, iso16 and the linear-odd fatty acid 15:0 showed different ($p < 0.05$) amounts in LI-O, LI-C and HI-C samples. Branched-chain were the most representative, reaching values of 2.6%, 2.4% and 2.2% in LI-O, LI-C and HI-C, respectively, while linear-odd chain fatty acids ranged from 2.1% in LI-O milk to 1.7% HI-C milk.

In Figure 1, the results obtained by principal component analysis (PCA) of data are reported. PC-1 and PC-2 have been chosen as coordinates on the x- and y- axes, accounting for 34% and 32% of total variance in data distribution, respectively. This combination of PCs has been chosen as it allowed for the best separation of the groups. In the correlation loadings plot (Figure 1a) we can see that OBCFA, EA, LA, ALA, n3 FA, n6 FA and n6/n3 ratio could be identified as factors that more significantly contributed to the variance among samples, by PC-2 vector's direction. The scores plot (Figure 1b) shows three clusters, with the average point, variability within the groups and density ellipses set at 0.68 (mean ± 1 standard deviation) for each group. PC-2 allowed a

discrimination among samples coming from the three different farms. Both LI-O and LI-C groups were distributed in the area of the two-dimensional space where the "low-input factors" (green) are positively correlated with PC-2, in line with the higher values found in these samples by chemical analyses. On the contrary, HI-C samples are more characterized by the "high-input factors", which presented negative loadings on PC-2.

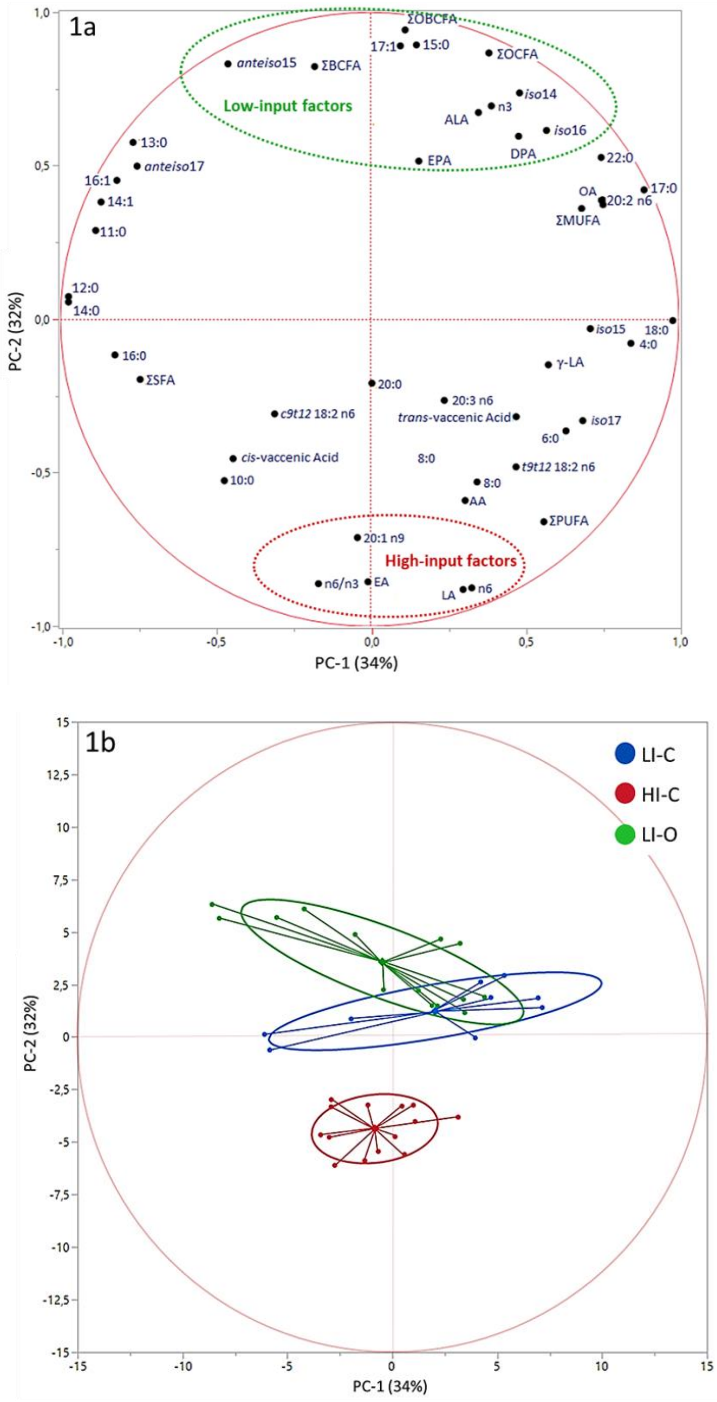

Figure 1. Principal Component Analysis Loadings Plot (a) and Scores Plot (b).

In Figure 2, the most influencing factors on clusters discrimination and their eigenvectors on the second component of PCA are reported.

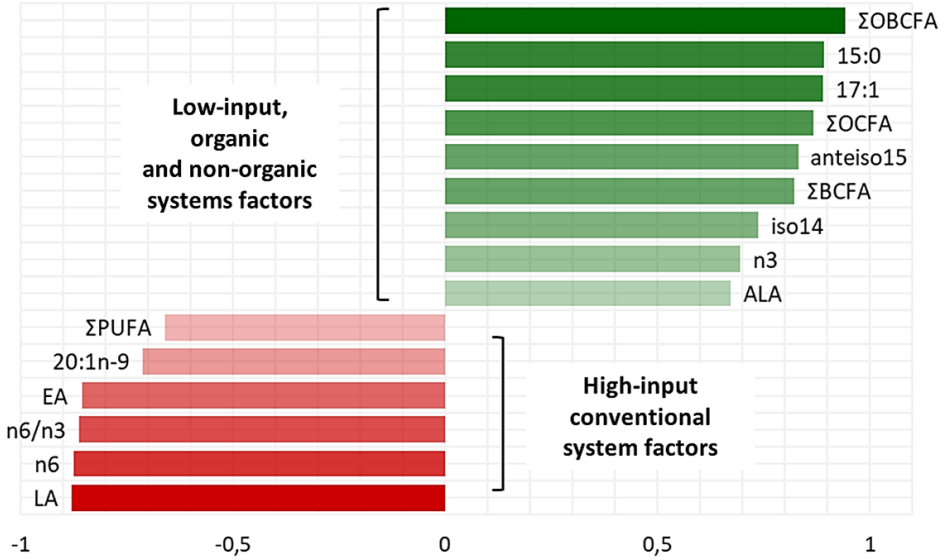

Figure 2. Principal Component-2 eigenvectors of factors considered as representative of the low-input and the high-input systems. Variables with absolute values of their eigenvectors equal or greater than 0.6 have been chosen. Factors with the higher absolute values of their eigenvectors are associated with a higher influence on data variability.

The factors taken into account could be divided in two groups, according to their positive or negative relation with PC-2. Variables selected in this model were total OBCFA, n3 and ALA, which showed the overall maximal values in LI-systems samples, while higher levels of LA, n6, EA, 20:1n9 and PUFA content were observed in the HI-system samples. In Table 3, results of the analysis of variance on PC-1 and PC-2 are reported. The ANOVA of the PC scores revealed that whereas PC-1 did not allow us to discriminate different samples, PC-2 effectively separated the three groups ($p < 0.01$) according to the livestock production system.

Table 3. ANOVA of the Principal Components (PCs) eigenvalues. Data are reported as mean ± SEM.

Principal Component	FARM						
	LI-O		LI-C		HI-C		
	Mean	SEM	Mean	SEM	Mean	SEM	P
PC-1	−0.47	1.05	2.04	1.31	−0.84	1.06	0.2123
PC-2	3.57 [a]	0.40	1.23 [b]	0.50	−4.36 [c]	0.40	<0.0001

[a, b, c] = values in the same row that have different superscript are significantly different at $p \leq 0.05$, ANOVA and Student-Newman-Keuls post-hoc test.

4. Discussion

No significant differences were found in the proximate composition of milk samples coming from the different farms, except for the slight differences in protein amounts. In all three groups, fatty acids showed the characteristic fatty acid pattern of caprine milk and values of fatty acids from 6:0 to 10:0 agree with values previously reported in literature (Alonso et al., 1999; L. Bernard et al., 2005; Serment et al., 2011). Fatty acids from 4 to 8 carbon atoms were not found to be significantly different in milk obtained from different farms and confirmed the previously observed results (Serment et al., 2011), proving that such differences in production systems did not modify volatile fatty acids proportion in goat milk. The proportion of oleic acid in milk is controlled by its plasma uptake and partly from desaturation of stearic acid by mammary Δ9-desaturase (Y. Chilliard et al., 2001).

The desaturation ratio 18:1 c9/18:0 in the mammary gland is influenced by the diet, since the increase in availability of either PUFA or trans-FA inhibits the Δ9-desaturase. Consequently, the proportions of oleic acid in goat milk decrease with the increasing percentage of concentrate (Serment et al., 2011). According to this, we detected the lowest values for oleic acid in samples of HI-C farm, in which goats were fed with the higher amount of concentrates (50% of DMI). Even the concentration of trans18:1 isomers (t9 + t11) is related to the percentage of concentrate in the diet, and it increases when the concentrate proportion is higher (Y. Chilliard et al., 2003). Rumen microbial population is considered directly involved in this process, since trans18:1 isomers are intermediary products of ruminal biohydrogenation of the dietary PUFA (Glasser et al., 2008). The main factor that affects the biohydrogenation rate is the percentage of the concentrate in the diet (Yves Chilliard et al., 2007). In literature, vaccenic acid is the major component (about 36.2%) of total trans18:1 isomers (LeDoux et al., 2002). In our study, vaccenic acid (18:1 t11) represented 70% to 80% of the detected trans18:1 isomers, without differences among different farms. However, the lowest amount of VA was detected in LI-O milk and the highest in HI-C and LI-C milk. Elaidic acid (18:1 t9) relative content resulted different ($p<0.05$) in the three groups of milk samples. The relative percentages of EA detected matched with the results obtained by Serment et al. (2011), who reported an effect of the percentage of concentrate on the fatty acid profile in milk goats that produced a relative amount of 0.3% of EA in a lower concentrate diet versus an amount of 0.4% of EA in a higher

concentrate one. These results are consistent with the knowledge that a decrease in the fiber content (or an increase of concentrate) in the goat daily ration would lead to

a higher content of the trans18:1 fatty acids in milk (except 18:1 t11) (Yves Chilliard et al., 2007; Haenlein, 2004). In ruminants, the unsaturated fatty acids are metabolized by microorganisms in the rumen and undergo biohydrogenation and double-bond migration to yield a mixture of structural isomers (cis-trans isomers and positional isomers)(Bickerstaffe et al., 1972). PUFA quantities in ruminant milk generally increase with PUFA dietary intake, even if the transfer efficiency of PUFA from the diet to milk is low because of the biohydrogenation process that occurs in the rumen (Y. Chilliard et al., 2001). In our research, similar amounts of PUFA were detected, with a difference ($p < 0.05$) between samples coming from the high-input system (4.41%) and samples coming from the low-input systems (4.43% in the conventional one and 3.6% in the organic one). It is known that the fresh forage intake increases dietary PUFA supply (Gillian Butler et al., 2008) (Bickerstaffe et al., 1972; G. Butler et al., 2011; D'urso et al., 2008). However, some studies (Kalscheur et al., 1997; Loor et al., 2004) have reported that a high amount of concentrates in diet can cause a decrease in biohydrogenation processes, with a consequent increase of PUFA amounts. Moreover, in Table 3 it is shown that the higher amount of PUFA in HI-C milk is mainly due to the higher amount of n6FA in these samples. The concentration of linoleic acid, the most representative FA among PUFA, was higher in HI-system milk compared to LI-systems milk. These results agree with values reported in literature, specifically in the absence of lipids added to diets, as the proportion of LA on goat milk FAs is between 2% and 3% (Alonso et al., 1999; Yves Chilliard et al., 2007; Cívico et al., 2017; Serment et al., 2011). Generally, all or most of LA in milk fat comes from dietary LA that escapes from rumen biohydrogenation activity, and its transfer to milk is related to the amount of this fatty acid that is ingested (Khiaosa-Ard et al., 2010). Most likely, we found higher LA values in HI-system milk because of the higher intake of concentrates by goats reared in this farm, according to the fact that by decreasing the F/C ratio the LA concentration increases (Yves Chilliard et al., 2007). Alpha-linolenic acid and long chain n-3 series in goat milk are influenced by a fresh grass-based diet that is a good source of ALA. Consequently, pasture induces an increase in ALA in ruminant milk (Yves Chilliard & Ferlay, 2004). In our results, ALA content was found to be significantly higher in LI-systems milk than in HI-system milk. The higher amount of ALA in LI-systems milk could be explained by the fact that goats at these farms had access to pastures normally enriched in ALA, and that were fed with a natural grassland hay, which had in the past showed a positive effect on ALA content in goat milk (Yves Chilliard et al., 2007). Furthermore, forage is rich in ALA, whereas cereals contain higher amounts of LA (Khiaosa-Ard et al., 2010). HI-C goats were fed with the highest amount of concentrates and the lowest F/C ratio. Similar amounts below or around the 1% of ALA were reported by other authors in milk from Alpine goats fed a hay-based diet at a low percentage of concentrate and without lipid supplementation (L. Bernard et al., 2005; Laurence Bernard et al., 2009; Serment et al., 2011). In the milk of Saanen goats fed with a high F/C ratio diet, Mele et al. (2008) showed a presence of ALA (0.49%) comparable to our results. In addition to total PUFA and ALA, CLA (LA-conjugated isomers) are generally considered as markers of fiber intake in ruminants (Chilliard et al., 2007). CLA c9t11, which is the most representative of total CLAs, shows higher concentrations when the proportion of fresh grass intake increases (Ricordeau, 1993). Unfortunately, with our analytical equipment, provided with a 60 m TR-FAME column, we were able to separate two LA-non conjugated isomers,18:2 t9t12

and 18:2 c9t12, but we had the coelution of the CLA c9t11 with 20:0. For this reason, the amount of 20:0 reported in Table 2 can include also CLA c9t11. EPA and DPA relative content in Alpine goat milk resulted in higher values than the ones reported by Bernard et al. (2009). The author studied fatty acids trend in Alpine goat milk, reporting values of 0.07% for EPA and 0.12% for DPA in milk goats fed with natural grassland hay. Differently, the relative amount of EPA we detected in HI-C milk (0.06%) was lower than the amount reported by other authors (Mele et al., 2008) in Saanen milk (0.11%). Since PUFA are not synthesized by tissues in ruminants, their concentration in milk is positively related to the dietary amount and to the decrease of rumen hydrogenation activity, such as a high forage/concentrate ratio. According to this, we found the highest amount of EPA and DPA in milk samples coming from the two LI-systems farms.

In our study, OBCFA showed differences ($p < 0.05$) between the HI-farm milk and the two LI-farms milk. In previous studies (Fievez et al., 2012) linear odd-chain fatty acids were reported as the majority of ruminant milk OBCFA, followed by anteiso-fatty acids. In the present work we found the higher values in branched-chain fatty acids. The most representative were anteiso15:0 and anteiso17:0, which reached the higher values in LI-O milk in both cases (0.7% and 0.8%, respectively), then followed by iso-forms. These results follow the trend observed by Alonso et al. (1999) who found that the most important OBCFA in quantitative terms in goat milk were the iso15:0 and anteiso15:0, iso17:0 and anteiso17:0 and iso16:0. We found the main differences in iso14:0, 15:0 and iso16:0 fatty acids, with the higher amount in LI-O milk, followed by LI-C milk and by HI-C milk. These differences may be relevant by a nutritional point of view. Indeed, 15:0 levels in subcutaneous adipose tissue and serum have been used as markers of intake of ruminant fat by humans (Smedman et al., 1999; Warensjö et al., 2004; Wolk et al., 1998). Meanwhile, it is known that iso14:0 and iso16:0 levels in milk fat are related to the rumen functionality, showing a positive association with the presence of cellulolytic bacteria and decreasing when diets rich in starch are supplied to animals (Nielsen et al., 2004; Shingfield et al., 2005; Vlaeminck et al., 2006). On the contrary, increasing the forage/concentrate (F/C) ratio in the diet resulted in a higher proportion of milk OBCFA, even if the effects of variation in the dietary F/C ratio on OBCFA are not uniform over all studies found in literature (Vlaeminck et al., 2006). In the present study, we found higher levels of total OBCFA in goats that had access to grazing and were fed with the higher amount of forages. These changes in milk OBCFA concentration might reflect the equilibrium in the rumen bacterial populations induced by the differences in dietary F/C ratio and by the quality of the fiber supplied (fresh grass vs conserved forage).

Also, the n6/n3 ratio, considered as a healthy balance index, was evaluated. We obtained a difference ($p < 0.05$) between samples collected in the LI farms (both organic and conventional) and samples collected in the HI farm. The lower amounts of n6/n3 were detected in the LI-O milk, followed by LI-C milk and then by HI-C milk. A lower n6/n3 ratio is indicative of a forage-based diet (Schwendel et al., 2015) and this is consistent with the fact that in LI farms goats were fed with a higher amount of forage. Values obtained in this research for the LI-systems products display lower values for n6/n3 ratio than values reported in literature for goat milk (5.0) (Markiewicz-Keszycka et al., 2013), suggesting a more favourable composition of products originated by these rearing systems.

According to our discussion regarding fatty acids composition in the different groups, variables associated with positive values of PC-2 eigenvectors (Figure 2, in green) could be considered as markers of the low-input systems,

both organic and non-organic, mainly related to the higher F/C ratio of the supplied diet and to the access to pastures. On the contrary, variables associated with negative values of PC-2 eigenvectors (Figure 2, in red) could be considered as markers of the high-input system, characterized by a lower F/C ratio in the diet and by the exclusive use of conserved forages supplied in the barn. Thus, even if a partial overlapping between LI-O and LI-C is observable in the scores plot (Figure 1b), the analysis of variance of PC eigenvalues (Figure 2) revealed that the combination of original variables obtained with PCA on the first two components allowed us to identify three different clusters. It suggests that the total pattern of milk fatty acids is significantly influenced by the considered production systems, more so than single fatty acids. Moreover, LI-O and LI-C goats milk composition showed the largest variability of data (Figure 1b), putatively linked to seasonal variations and to the quantity and the quality of feed in pasture-based systems, compared with the HI-C milk farm, where the diet had very few variation all over the research time.

5. Conclusions

The present study have mainly demonstrated that goat milk from farms managed under a low-input rearing system showed differences if compared to milk from a high-input system farm. A multivariate statistical technique supported results found by analytical means. Differences were particularly detectable between the LI and the HI farming systems, but few differences were found between the two LI-system farms. The differences were mainly imputable to different amounts of some fatty acids, primarily OBCFA, n3FA, n6FA, ALA, LA and EA, selected by the Authors as factors related to the farming system.

Author Contributions: A.L. took up the investigation, the formal and data analysis and the writing of the paper. M.V. performed the main investigation and developed the methodology. V.M.M. co-supervised the research, validated the results and had an input on the writing–review and editing–of the paper. F.B. conceived and supervised the study and the writing–first draft and reviews.

Funding: This work was supported by the University of Milan [Piani di Sostegno alla Ricerca 2016–2017, Linea 2].

Acknowledgments: The authors acknowledge all the farmers for their availability in samples collection.

Conflicts of Interest: The authors declare no conflict of interest.

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Trial 2

Intrinsic and Extrinsic Quality Attributes of Fresh and Semi-Hard Goat Cheese from Low- and High-Input Farming Systems

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Abstract: In this study, we investigated the lipid composition of fresh and semi-hard goat cheese produced in three Italian farms as well as the welfare assessment of goats reared in these farms. The fatty acid (FA) profile of cheese samples were found to be strictly related to the livestock system. Cheese collected from farms in which goats were allowed to graze and were fed diets with a higher forage/concentrate (F/C) ratio showed a FA profile represented by higher contents of health-promoting fatty acids. In the same samples, the health lipid indices showed the most favorable values. Conversely, cheese samples collected from a conventional-lowland farm, where goats were fed with higher amounts of concentrates and lower F/C ratio, presented a lower nutritional quality, characterized by the worst results for what concerns the health lipid indices. Then, we built a multivariate model able to discriminate samples coming from farms managed by a low-input system from those coming from farm managed by a high-input system. The comparison of animal welfare measurements and fatty acids data showed that a better intrinsic quality of low-input farms did not always correspond to better extrinsic quality, suggesting that the information on the livestock system is not always enough to provide consumers with complete awareness of the total product quality.

Results from this investigation have been published in the scientific journal *Animals*

Animals **2020**, 10, 1567; doi:10.3390/ani10091567

1. Introduction

Nowadays, a significant interest in dairy goat products has been growing among both the scientific community and consumers, due to spreading knowledge about their functional properties and high nutritional value (Albenzio et al., 2016; Haenlein, 2004; Sepe & Argüello, 2019). Moreover, dairy goat products finely match with the interests of present-day consumers, especially regarding the ethics of livestock production, environmental impact, and animal welfare issues. These aspects are included in a broader food quality concept that considers not only the product's intrinsic quality attributes, but also its extrinsic attributes. As stated by Massaglia et al. (2019) during recent years, goat products have gradually been associated with consumer perception of innate quality because of their status of being sustainable, traditional, and health products. These features have a fundamental importance for the assessment of these products to the "functional food" business, in which Italy is estimated to generate 11% of the total revenues of the total European market share (Vicentini et al., 2016). Nevertheless, Italian consumers are still confused about what functional food products are, even if they are strongly conscious of the existing link between the diet and their health and they have demonstrated increasing interest toward the health implications of their food choices (Annunziata & Vecchio, 2011).

According to FAOSTAT (Food and Agriculture Organization Corporate Statistical Database) data, Italy is the fifth largest European goat cheese producer recording an amount of 4500 tons produced in 2014 placing them after France, Greece, Spain, and Bulgaria (FAOSTAT, 2017). Lombardy is the second-largest Italian region for goat livestock number, with 98,766 female goats bred out of the total 827,418 in Italy (12%) (ISTAT, 2019). Half of the goat farms in Lombardy have small herds, from 20 to 60 lactating goats, represented 95% by Saanen or Alpine breeds (AIA, 2018). Of overall Italian goat milk production (43,444 tons), Lombardy contributes 16% (7076 tons) (ISTAT, 2018), being second only to Sardinia, with an average production of 549 ± 216 kg of milk per goat per lactation (AIA, 2018). The caprine livestock production systems adopted in dairy goat farms in Italy are very diversified, ranging from intensive, indoor, highly productive, and specialized productions, to extensive outdoor systems, with traditional local breeds and with seasonal productions (Manfredi et al., 2010; Sandrucci et al., 2019). As evidenced in a study conducted on 173 dairy goat farms in Lombardy, in almost 30% of the studied farms, goats had access to pasture during the spring and summer season, with advantages in terms of reducing feeding costs (Sandrucci et al., 2019).

In Italian small goat farms located in the mountains, the conventional production system can be considered a "low-input system" (LIS), characterized by feeding practices similar to those used in organic farming, but not complying with all the restrictions established by organic standards (Butler et al., 2008). The LIS is known to be strictly related to milk quality in ruminants (Cabiddu et al., 2019) and, specifically, in goats (Eleni Tsiplakou et al., 2010; Tudisco et al., 2014), mainly due to the access to pasture. Basically, LIS goats are forage-fed plus given concentrate supplementation mostly during a certain lactation period and physiological stages (Sepe & Argüello, 2019), whereas in the conventional "high-input system" (HIS) goats are fed diets richer in grain and characterized by a lower forage/concentrate (F/C) ratio. As a matter of fact, the concentrates and forage intake and the nature of individual foodstuffs have an impact on the characteristics of goat milk and milk products (Goetsch et al., 2011). Even if small mountain farms are not always organic-certified and do not always practice pasture, the yielded milk has shown to have a similar nutritive and functional quality to its organic counterpart.

The main differences between the LIS and the HIS are recorded in the milk production and content of nutritionally favorable fatty acids, such as n3-series PUFA (polyunsaturated fatty acids), CLA, and branched-chain fatty acids (BCFA) (Chung et al., 2018; Collomb et al., 2008; A. Lopez et al., 2019; Malissiova et al., 2015; Schwendel et al., 2015). Moreover, small mountain farms are usually associated with an on-farm cheese making process and the sale of the products directly on the farm or in local shops, comprising a market segment that is generally related to high-quality and traditional products, strictly linked to territory and seasonality (Massaglia et al., 2019). Typically, Goat cheese produced and sold in Italy are small-sized and soft, obtained by lactic or rennet coagulation. Fresh cheese obtained by a combination of a slow acidification through the addition of mesophilic starter culture combined with heat (Lucey, 2016) is characterized by 50–65% moisture, a lipid content up to 15–18 g/100 g and a protein content up to 16–29 g/100 g (Salvadori del Prato, 2013). Hard and semi-hard goat cheese, represented by the presamic curd obtained by the enzymatic action of kid rennet on casein micelle (Corredig & Salvatore, 2016), is characterized by 35–45% moisture, a lipid content up to 24–32 g/100 g and a protein content up to 20–26 g/100 g (Salvadori del Prato, 2013).

The on-farm cheese making appears to be fundamental since farmers can also benefit from the added value of the processing and sale, making the business more profitable (de Asís Ruiz Morales et al., 2019). Finally, LIS are often associated by consumers to the idea of high welfare levels, because outdoor and extensive farming systems allow animals to behave in a more natural way (Spigarelli et al., 2020). In goats, a study using qualitative behavior analysis (QBA) highlighted a better emotional state in animals on pasture that in animals housed indoors (Grosso et al., 2016).

Food products can also be described through descriptive factors that contribute to the definition of total quality. These factors can be divided into either intrinsic to the food itself, as chemical composition, aroma, and nutritional properties, or those that are extrinsic. Extrinsic attributes are aspects related to the product but not physically a part of it. They are linked to natural and cultural assets and are hidden into a broader food quality concept. In the case of animal origin products, examples of important extrinsic quality attributes are environmental sustainability and animal welfare, which may be related to the production process and to the place of origin.

To date, scientific studies about dairy goat products are fewer if compared to those regarding raw goat milk. The present study aimed to show the potential of the different production systems to affect some intrinsic (fatty acid composition of goat cheese) and extrinsic (animal welfare) quality attributes of goat products on three farms in Lombardy.

2. Materials and methods

2.1 Animals, Feeding and Housing Practices

Three farms from Lombardy were involved in this study, conducted from March (4 ± 1 weeks of lactation) to October 2017, covering three quarters of the milking season. The first farm (342 m above sea level, a.s.l.) was represented by an organic-certified farm (European Parliament & European Council, 2018), breeding 45 Alpine lactating goats. On this organic farm (O), goats had access to pasture for 224 days/year and 4 h/day after the morning milking. Goats were rationally grazed with mixed vegetative forages during the entire sampling season and the access to fresh grass at pasture was controlled. In the barn, goats received forages, consisting of alfalfa hay (1000 g/d per animal) and polyphite grass hay (500 g/d per animal) distributed twice a day, and a mixture of organically-certified maize/barley (3/1) grains, supplied twice a day during milking (800 g/d per animal). The second farm (M) was represented by a typical mountain farm (980 m a.s.l.), with 39 Alpine goats, where the production system can be considered a LIS, since goats were reared and fed primarily in the barn but a diet with a high F/C ratio and receiving local fresh grass when available on the farm. In farm M, feed supplements were provided to goats, including polyphite grass hay (first harvest) or fresh grass, when available, offered ad libitum plus alfalfa hay (500 g/d per animal) distributed during the morning milking and a commercial mixture of concentrates (1000 g/d per animal) distributed twice a day, during milking. Finally, the third farm (C) was represented by a conventional-lowland dairy goat farm (278 m a.s.l.) with 42 Saanen lactating goats, permanently reared in the barn. The diet included local ryegrass hay offered ad libitum, alfalfa hay offered once a day (500 g/d per animal) during the first part of lactation, and commercial feed, represented by a mixture of flaked and flaked cereals and flour, provided twice a day (1200 g/d per animal) during milking. In all three farms, goats had free access to water and to salt integrators. The F/C ratio for each farm was calculated on the basis of the average daily feed intake and it is reported in Table 1. Average feed intake was estimated by means of dry matter intake (DMI) prediction models for lactating goats as reported by Pulina et al. (2013), taking into account average goat size and the average milk yield during the sampling season of the study. Dry matter of feedstuffs was calculated by fodder analysis according to official methods (Commission, 2009).

Cheese samples consisted of two different dairy goat products typically produced and sold in Italy. The former was represented by fresh cheese samples, processed by lactic coagulation (about 120 g of weight), collected once a month from March to October in each farm, except for farm M that was lacking of one sample, for a total amount of 23 samples (8 in O, 7 in M, 8 in C). The latter was represented by semi-hard cheese blocks, processed by rennet coagulation (about 100 g of weight), obtained after 3 ± 1 weeks of ripening and collected once a month from April to October in each farm, with the exception of O farm in which one sample was lacking, for a total amount of 20 samples (6 in O, 7 in M, 7 in C). Both fresh and semi-hard cheese samples (a total amount of 43 samples) were stored at −20°C until fatty acids analysis was performed.

2.2 Fatty acids Analysis

Fat was extracted from cheese samples by the Folch method (1957), using a mixture of chloroform/methanol (2:1). After lipid quantification, an aliquot corresponding to 40–50 mg of fat was used for fatty acids analysis by

means of gas-chromatography (GC) and flame ionization detection (FID). In order to perform the GC-FID analysis, fatty acids were methyl-esterified by means of base-catalysed methanolysis of glycerides, following the method of Christie (2003). Briefly, lipids were dissolved in 1 mL of diethyl ether, then 50 µL of methyl acetate and 100 µL of sodium methoxide in methanol 1M were added. After 5 min at room temperature, the reaction was stopped by adding 50 µL of a saturated solution of oxalic acid in diethyl ether. The solution was centrifuged at 1500 g for 5 min, then 200 µL of the supernatant was taken and immediately injected in the GC system. The GC system was represented by an Agilent gas-chromatograph model 6890, fitted with an automatic sampler model 7683 and a FID detector (Agilent Technologies, Santa Clara, CA, USA). Chromatographic conditions were set following the method described in Lopez et al. (A. Lopez et al., 2019). Individual fatty acids methyl esters were identified by comparing sample peak retention times with standard mixtures and pure standard methyl esters from Sigma Aldrich (Saint Louis, MO, USA) and then were expressed as percentage of total fatty acids. A gas-chromatographic correction factor was applied in order to take into account the lower response of the FID for compounds represented by a lower number of carbon atoms (4:0, 6:0, 8:0, 10:0, and 12:0) (Ulberth et al., 1999).

2.3 Welfare assessment

Animal welfare was evaluated using the 1st-level AWIN (Animal Welfare Indicators) welfare assessment protocol for goats (AWIN Goat, 2015; Battini et al., 2015). In agreement with the AWIN protocol, in each farm the evaluation was carried out in a pen selected as having the highest risk for animal welfare, according to the following criteria: highest density, lower feeding space/animal ratio, lower drinking place/animal ratio, and presence of both horned and hornless goats in the same pen. Therefore, if more than one pen was present in a farm, the number of assessed goats per farm does not correspond to the total number of lactating goats, but to that of goats in the selected pen (number of goats assessed per farm: O = 36; M = 24; C = 39). The assessment included the collection of the following indicators when the animals were at the feed rack after the morning milking: incorrect disbudding, presence of abscesses, kneeling at the feed rack, queueing at feeding, queueing at drinking, hair coat conditions, oblivion (isolated goats), thermal stress (either heat or cold stress signs). After the collection of these indicators, the goats were observed from outside the pen for 10 min for the qualitative behaviour assessment (QBA; (Battini et al., 2018)). Then the assessor entered the pen to evaluate the quality of the human-animal relationship, performing the latency to first contact test, which consists in measuring the time until the first goat spontaneously gets in touch with the operator after his/her entrance into the pen (Bernard et al., 2005). Finally, the bedding quantity (sufficient, insufficient) and cleanliness (clean, dirty and/or wet) were assessed, as an indirect index of animal comfort, and the number of goats that presented severe signs of lameness was recorded (AWIN Goat, 2015).

2.4 Statistical Analysis

The difference among the three farms (O, M, C) for fatty acid analysis was evaluated by means of the analysis of variance. Normal distribution (Shapiro–Wilk test) and homogeneity of variances (Levene test) were confirmed and comparison among means was performed by the ANOVA test and the Welch ANOVA F-test. The Tukey-HSD test was used as the post-Hoc test for comparison of the means among different farms. Significance was declared at $p \leq 0.05$ (*) and $p \leq 0.01$ (**). Afterwards, a multivariate analysis was performed by means of a combined principal component analysis (PCA) and linear discriminant analysis (LDA) approach. In a first step, PCA was performed in order to reduce the dimensionality of the final data matrix. The PCA was performed including milk samples obtained in a previous study (Lopez et al., 2019) analyzing the bulk milk used to produce cheese samples analyzed in the present work. Variables were selected when PC loadings score were > |0.5|. In a second step, LDA was performed in a new matrix, including the variables selected by PCA. Milk samples were included in the model as training set and cheese samples as validation set, in order to verify if the discrimination among samples coming from different farms was satisfying by means of the multivariate approach chosen. Animal welfare indicators were expressed as the proportion of animals that do not present a specific welfare problem out of the number of animals observed, except for the latency to first contact, which was expressed in seconds, and the QBA, which was expressed as scores on a visual analogue scale and then submitted to PCA. Statistical analysis was performed using JMP Pro 14.0.0 (SAS Institute Inc., Cary, NC, USA).

2.5 Ethical approval

This research did not involve any experimental practice performed on living animals by the authors and no biological matter was collected. Only cheese samples were collected, after the on-farm production stage. Authors guarantee that in the three farms involved in the present study, all the applicable guidelines for animal welfare established by the harmonized EU rules were followed. No approval by the ethics committee of authors' institution (University of Milan) was requested.

3. Results and discussion

Results obtained by the fatty acid (FA) analysis on fresh and ripened cheese samples are reported in Table 1. Dairy goat products are generally enriched in the content of short-chain saturated fatty acids (sc-SFA) 6:0, 8:0, and 10:0, commonly known as caproic, caprylic, and capric acid, considered responsible for the characteristic flavor of goat products (Silanikove et al., 2010). SFA are known to be directly related to obesity, cardiovascular, and metabolic diseases in humans (Kratz et al., 2013), caproic, caprylic, and capric acid have been recognized to have a unique metabolic ability in humans, since they provide direct energy instead of being deposited in the adipose tissue and lower serum cholesterol. For this reason, they have already achieved an important role in the functional evaluation of goat products in human nutrition and medicine (Haenlein, 2004). No significant differences were found in the content of such fatty acids among the three farms involved in this study, with the exception of capric acid that was higher in semi-hard cheese samples collected in farm C (9.82%) than in samples collected in farm M (8.37%). Short- and medium-chain FA are synthesized in the mammary gland mainly by FA

synthase, starting from volatile fatty acids produced by rumen activity as precursors. Moreover, mammary delta-9-desaturase can also convert stearic acid (18:0) to oleic acid (18:1 n9). Stearic acid and oleic acid in dairy products could be derived from the feed intake, the body fat mobilization, or by the hydrogenation processes that occur in the rumen (Chilliard & Ferlay, 2004). Oleic acid, particularly, is known to have positive effects on human health, mainly toward the digestive and cardiovascular systems and the inflammatory response, and it seems to play a pivotal role in the management of the oxidative stress and in the development of the nervous system (Piccinin et al., 2019). Thus, in dairy products, it is considered advisable to keep the oleic acid/stearic acid proportion as high as possible, in order to improve the nutritional characteristics of milk and related products (Chilliard & Ferlay, 2004). The amount of oleic acid in caprine products is strongly influenced by the diet, since (i) it is inversely related to the amount of concentrates supplied to goats (Serment et al., 2011) and (ii) grazing practice increases oleic acid concentration in milk, through the hydrogenation and desaturation processes against PUFA present in the pasture (Chilliard & Ferlay, 2004). In agreement with these findings, we found the lowest concentration of oleic acid in cheese samples coming from farm C (17.78–18.09%), where goats were fed with the highest amount of concentrates. Conversely, the highest concentrations were found in samples from farm O (19.70–19.74%) and M (21.11–21.93%), where goats consumed less concentrate and more fresh grass or pasture browsing plants.

PUFA biohydrogenation in the rumen also leads to the formation of many intermediate products, such as trans (t9 + t11) 18:1 isomers (Glasser et al., 2008). It has been demonstrated that the consumption of trans fatty acids, in contrast to cis fatty acids, can lead to pleiotropic negative effect on human health, including pro-inflammatory activity, adverse lipid effects, endothelial dysfunctions, insulin resistance, etc. (Mozaffarian et al., 2009). At the same time, recent scientific opinions state that the consumption of trans FA by means of ruminant products in actual diets is very modest and that, at such levels, they do not appear to be major contributors to health risk (Mozaffarian et al., 2009). In our work, we found a significantly higher content of elaidic acid (t9, 18:1) in samples from farm C (0.50%), followed by farm M (0.39–0.41%) and then by farm O (0.31%). No significant differences were found in the three farms regarding the content of vaccenic acid (t11, 18:1) that is the main *trans* 18:1 isomer in dairy products. These results are in accordance with the scientific evidence stating that the concentrates proportion in goat diets is positively related to the content of the trans 18:1 isomers in milk, with the exception of vaccenic acid (Chilliard et al., 2003).

Table 1. Fatty acids composition of fresh and semi-hard goat cheese samples. Data are expressed as g/100 g of total fatty acids (mean ± SD).

	FRESH CHEESE				SEMI-HARD CHEESE			
	O	**M**	**C**		**O**	**M**	**C**	
N	8	7	8		6	7	7	
F/C [1]	70/30	60/40	50/50	***sign***	70/30	60/40	50/50	***sign***
SATURATED FATTY ACIDS (SFA)								
4:0	1.98 ± 0.22	1.87 ± 0.25	1.99 ± 0.17	ns	2.01 ± 0.25 ab	2.42 ± 0.41 a	1.93 ± 0.10 b	*
6:0	1.98 ± 0.21	1.88 ± 0.15	2.05 ± 0.18	ns	1.98 ± 0.20	2.15 ± 0.37	1.96 ± 0.08	ns
8:0	2.63 ± 0.28	2.12 ± 0.93	2.76 ± 0.24	ns	2.58 ± 0.23	2.01 ± 1.29	2.62 ± 0.08	ns
10:0	9.52 ± 0.85	9.05 ± 1.05	10.04 ± 0.65	ns	9.41 ± 0.47 ab	8.37 ± 2.52 b	9.82 ± 0.38 a	*
12:0	5.43 ± 1.71	5.65 ± 2.19	5.19 ± 0.70	ns	5.08 ± 0.89	4.08 ± 0.70	5.31 ± 0.78	ns
14:0	11.18 ± 2.12	11.20 ± 2.27	11.20 ± 0.59	ns	10.91 ± 1.53 ab	9.85 ± 0.70 b	11.63 ± 0.73 a	*
16:0	25.96 ± 2.34 ab	23.80 ± 1.49 a	26.95 ± 1.58 b	*	26.25 ± 1.91 a	23.68 ± 1.99 b	27.30 ± 0.93 a	**
18:0	9.16 ± 3.03	9.97 ± 3.49	8.96 ± 1.44	ns	9.37 ± 2.33 ab	11.92 ± 0.71 a	8.89 ± 1.62 b	*
20:0 [2]	0.84 ± 0.18	1.01 ± 0.15	0.86 ± 0.10	ns	0.93 ± 0.09	0.95 ± 0.37	0.88 ± 0.07	ns
22:0	0.10 ± 0.03 ab	0.12 ± 0.04 a	0.07 ± 0.02 b	**	0.11 ± 0.02	0.08 ± 0.05	0.07 ± 0.01	ns
ΣSFA	70.88 ± 3.04	68.74 ± 3.24	71.77 ± 1.02	ns	70.75 ± 1.66 a	67.58 ± 2.63 b	72.16 ± 1.01 a	**

Table 1 (cont.)

MONOUNSATURATED FATTY ACIDS (MUFA)								
14:1	0.26 ± 0.22	0.27 ± 0.18	0.19 ± 0.07	ns	0.22 ± 0.13	0.20 ± 0.29	0.18 ± 0.07	ns
16:1	0.77 ± 0.33	0.71 ± 0.21	0.60 ± 0.11	ns	0.71 ± 0.17	0.64 ± 0.27	0.59 ± 0.14	ns
T9 18:1	0.31 ± 0.07 a	0.39 ± 0.04 b	0.50 ± 0.06 c	**	0.31 ± 0.04 a	0.41 ± 0.12 ab	0.50 ± 0.04 b	**
T11 18:1	1.06 ± 0.39	1.31 ± 0.09	1.32 ± 0.21	ns	1.17 ± 0.32	1.60 ± 0.73	1.41 ± 0.45	ns
C9 18:1	19.70 ± 2.62	21.11 ± 3.22	18.09 ± 0.71	ns	19.74 ± 1.25 a	21.93 ± 1.79 b	17.78 ± 0.68 c	**
C11 18:1	0.41 ± 0.05	0.38 ± 0.06	0.40 ± 0.02	ns	0.41 ± 0.05 ab	0.47 ± 0.06 a	0.40 ± 0.02 b	*
20:1 n9	0.05 ± 0.00 a	0.04 ± 0.01 a	0.07 ± 0.01 b	**	0.05 ± 0.01 a	0.04 ± 0.02 a	0.07 ± 0.01 b	**
ΣMUFA	22.85 ± 2.60 ab	24.51 ± 2.88 a	21.33 ± 0.77 b	*	22.89 ± 1.39 a	25.59 ± 2.49 b	21.09 ± 0.83 a	**
POLYUNSATURATED FATTY ACIDS (PUFA)								
t9t12, 18:2n6	0.21 ± 0.07	0.26 ± 0.02	0.25 ± 0.04	ns	0.23 ± 0.06	0.26 ± 0.08	0.24 ± 0.04	ns
c9t12, 18:2 n6	0.12 ± 0.02 a	0.17 ± 0.06 b	0.15 ± 0.03 ab	*	0.11 ± 0.01	0.11 ± 0.07	0.13 ± 0.02	ns
c9c12, 18:2 n6	2.13 ± 0.41 a	2.47 ± 0.43 a	3.28 ± 0.29 b	**	2.14 ± 0.28 a	2.71 ± 0.12 b	3.17 ± 0.12 c	**
18:3n6	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.01	ns	0.02 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	ns
18:3n3	0.72 ± 0.13 a	0.76 ± 0.07 a	0.45 ± 0.13 b	**	0.78 ± 0.09 a	0.82 ± 0.13 a	0.43 ± 0.10 b	**
20:2n6	0.05 ± 0.02	0.04 ± 0.01	0.04 ± 0.02	ns	0.03 ± 0.02	0.02 ± 0.02	0.03 ± 0.00	ns
20:3n6	0.03 ± 0.02	0.03 ± 0.00	0.03 ± 0.02	ns	0.02 ± 0.02	0.03 ± 0.03	0.03 ± 0.01	ns
20:4n6	0.16 ± 0.02 a	0.18 ± 0.02 ab	0.19 ± 0.03 b	*	0.15 ± 0.01 a	0.19 ± 0.03 b	0.18 ± 0.01 b	**

Table 1 (cont.)

20:5n3	0.09 ± 0.02	0.14 ± 0.11	0.07 ± 0.02	ns	0.09 ± 0.03	0.07 ± 0.04	0.06 ± 0.03	ns
22:5n3	0.17 ± 0.02 ab	0.16 ± 0.04 a	0.13 ± 0.04 b	**	0.19 ± 0.03 a	0.20 ± 0.05 a	0.12 ± 0.04 b	**
ΣPUFA	3.69 ± 0.61 a	4.28 ± 0.35 ab	4.62 ± 0.37 b	**	3.77 ± 0.40 a	4.44 ± 0.31 b	4.43 ± 0.25 b	**
Σn3	0.98 ± 0.14 a	1.10 ± 0.13 a	0.64 ± 0.12 b	**	1.06 ± 0.13 a	1.10 ± 0.18 a	0.61 ± 0.15 b	**
Σn6	2.72 ± 0.51 a	3.18 ± 0.40 a	3.98 ± 0.33 b	**	2.71 ± 0.36 a	3.34 ± 0.20 b	3.81 ± 0.15 c	**
n6/n3	2.79 ± 0.47 a	2.95 ± 0.63 a	6.38 ± 1.23 b	**	2.59 ± 0.42 a	3.09 ± 0.42 a	6.53 ± 1.39 b	**
ODD AND BRANCHED CHAIN FATTY ACIDS (OBCFA)								
11:0	0.12 ± 0.05	0.13 ± 0.07	0.10 ± 0.02	ns	0.12 ± 0.03 a	0.06 ± 0.04 b	0.10 ± 0.03 ab	*
13:0	0.12 ± 0.03	0.12 ± 0.04	0.10 ± 0.01	ns	0.12 ± 0.02	0.07 ± 0.04	0.10 ± 0.02	**ns**
*iso*14	0.15 ± 0.03 a	0.13 ± 0.03 a	0.10 ± 0.01 b	**	0.16 ± 0.01 a	0.14 ± 0.02 a	0.10 ± 0.01 b	**
*iso*15	0.23 ± 0.03	0.24 ± 0.05	0.23 ± 0.03	ns	0.24 ± 0.00	0.26 ± 0.04	0.24 ± 0.03	**ns**
*anteiso*15	0.65 ± 0.09 a	0.65 ± 0.05 a	0.52 ± 0.05 b	**	0.65 ± 0.06 a	0.57 ± 0.08 ab	0.54 ± 0.04 b	*
15:0	1.13 ± 0.10 a	1.10 ± 0.08 a	0.90 ± 0.05 b	**	1.15 ± 0.07 a	1.07 ± 0.06 a	0.94 ± 0.04 b	**
*iso*16	0.33 ± 0.06 a	0.27 ± 0.07 ab	0.25 ± 0.03 b	*	0.35 ± 0.04 a	0.32 ± 0.05 a	0.25 ± 0.03 b	**
*iso*17	0.35 ± 0.04	0.36 ± 0.06	0.40 ± 0.03	ns	0.36 ± 0.03	0.39 ± 0.02	0.40 ± 0.03	**ns**
*anteiso*17	0.82 ± 0.21	0.79 ± 0.10	0.72 ± 0.07	ns	0.79 ± 0.13	0.68 ± 0.04	0.72 ± 0.07	**ns**
17:0	0.72 ± 0.13	0.73 ± 0.19	0.60 ± 0.06	ns	0.74 ± 0.09 a	0.86 ± 0.07 a	0.60 ± 0.05 b	**
17:1	0.33 ± 0.03 a	0.33 ± 0.02 a	0.23 ± 0.02 b	**	0.33 ± 0.03 a	0.34 ± 0.03 a	0.23 ± 0.01 b	**
ΣOCFA	2.10 ± 0.16 a	2.07 ± 0.16 a	1.70 ± 0.06 b	**	2.54 ± 0.16 a	2.36 ± 0.23 ab	2.25 ± 0.10 b	*
ΣBCFA	2.52 ± 0.22 a	2.43 ± 0.13 a	2.21 ± 0.11 b	**	2.13 ± 0.05 a	2.07 ± 0.11 a	1.75 ± 0.05 b	**
ΣOBCFA	4.62 ± 0.26 a	4.50 ± 0.27 a	3.91 ± 0.14 b	**	4.66 ± 0.16 a	4.43 ± 0.27 a	4.00 ± 0.15 b	**

Table 1 (cont.)

HEALTH LIPID INDICES								
AI[3]	2.95 ± 0.84	2.64 ± 0.73	2.97 ± 0.2	ns	2.84 ± 0.48 a	2.25 ± 0.27 b	3.11 ± 0.30 a	**
TI [4]	2.92 ± 0.44 ab	2.59 ± 0.22 a	3.20 ± 0.23 b	**	2.84 ± 0.22 a	2.52 ± 0.20 b	3.32 ± 0.19 c	**
h/H [5]	0.65 ± 0.15	0.74 ± 0.18	0.60 ± 0.05	ns	0.64 ± 0.10 a	0.79 ± 0.09 b	0.57 ± 0.04 a	**

[a, b, c] = values within the same row associated with different letters are significantly different (* = $p < 0.05$; ** = $p < 0.01$), ANOVA and Tukey-HSD posthoc test.

[1] F/C = forage to concentrate ratio, calculated on the basis of average daily feed intake per goat, evaluated by mean of dry matter intake (DMI) prediction models for lactating goats reported by Pulina et al. (2013).

[2] = the 20:0 peak might include CLA c9t11, accounting for 0.6% of total FA in hay-fed goat milk (Bernard et al., 2005).

[3] AI (Atherogenic Index) = (12:0 + 4 × 14:0 + 16:0)/(n6 + n3 + MUFA).

[4] TI (Thrombogenic Index) = (14:0 + 16:0 + 18:0)/(0.5 × MUFA + 0.5 × n6 + 3 × n3 + n3/n6) (Ulbricht & Southgate, 1991).

[5] h/H (hypocholesterolemic/Hypercholesterolemic ratio) = (18:1n9 + 18:1n7 + 18:2n6 + 18:3n6 + 18:3n3 + 20:3n6 + 20:4n6 + 20:5n3 + 22:4n6 + 22:5n3 + 22:6n3)/(14:0 + 16:0) (Fernández et al., 2007).

PUFA are not synthesized in ruminants tissues, thus their concentration in milk and dairy products depend on the amount of PUFA provided to animals by the diet that partially escapes from the rumen microbial hydrogenation activity (Chilliard & Ferlay, 2004). It is known that fresh forage is richer in PUFA if compared with hay (Elgersma, 2015; Kalač & Samková, 2010; Mierlita et al., 2017). Although we would have expected to find higher amounts of PUFA in samples collected in the farms where goats were led to pasture, we detected a higher total amount of PUFA in farm M (4.28–4.44%) and C (4.43–4.62%) rather than in farm O (3.69–3.77%). As a matter of fact, the higher amount of PUFA in both fresh and semi-hard cheese samples collected in farm C can be mainly imputed to the significantly higher content of linoleic acid (c9c12, 18:2) found in such samples (3.17–3.28% in farm C vs. 2.13–2.14% in farm O and 2.47–2.71% in farm M). It is known that linoleic acid reaches significantly higher amounts in dairy products when the diet provided to goats contains a higher proportion of concentrates (Chilliard et al., 2007). Actually, the diet supplied to goats in farm C was characterized by the lowest F/C ratio and the higher amount of concentrates, naturally rich in linoleic acid (Elgersma, 2015). At the same time, alpha-linolenic acid (18:3 n3) followed the opposite trend, resulting in higher concentrations in samples collected in farm O (0.72–0.78%) and M (0.76–0.82%) than in farm C (0.43–0.45%). It is known that alpha-linoleic acid content is strongly influenced by the consumption of fresh grass and natural grassland hay, since they are natural sources of this FA, and a positive correlation between the content of alpha-linolenic acid in the diet and in dairy products exists (Chilliard et al., 2007; Chilliard & Ferlay, 2004). In agreement with this, we found the highest alpha-linoleic content in cheese samples collected in farms where goats' diet was represented by higher pasture and fresh grass intake and lower amounts of concentrates were supplied than in the conventional intensive farm (thus, a lower F/C ratio). These results are in accordance with the outcomes of other previous studies performed on organic versus conventional goat milk (Lopez et al., 2019; Tsiplakou et al., 2010). In addition to total PUFA and alpha-linoleic acid, also linoleic acid conjugated isomers (CLA), mainly represented by c9t11 18:2, are considered as markers of fibre intake in ruminants, showing higher percentages when goats are fed with fresh grass (Chilliard et al., 2007; Tudisco et al., 2014). Several studies investigated CLA content in goat milk and cheese. The outcomes of these studies suggested that goat products include CLA reaching amounts < 1% of total FA, depending on several factors (lactating period, genetics, dietary factors, etc.). The amount of CLA found in goat dairy products are comparable to those found in bovine dairy products and lower than those found in sheep dairy products (Cossignani et al., 2014; Prandini et al., 2011). Particularly in their papers, Tsiplakou et al. (2006; 2010) found that no differences were detectable when analyzing goat products obtained by organic vs. conventional production system or outdoor vs. indoor farming system, even suggesting that the ruminant species is the primary factor influencing CLA content in dairy products. Unfortunately, the analytical equipment used in the present study, provided with a 60 m length column, did not allow the separation of c9t11 18:2 that coeluted with 20:0 peak.

Linoleic acid and alpha-linoleic acid in milk and dairy products are the most representative FA for the n6- and n3- series, respectively. It is well known that n6- and n3- series FA are essential fatty acids (EFA) for humans, with peculiar and not inconvertible functional roles. n6- and n3- FA generally show opposite physiological functions toward cardiovascular diseases, cancer, inflammatory and autoimmune diseases and neural development, thus their balance is considered fundamental in order to assure their biochemical efficiency

(Simopoulos, 2010). It has been estimated that, nowadays, common diets can reach an n6/n3 ratio ranging from 10:1 to 20:1, whereas the target ratio, suitable to obtain a functional activity toward the prevention of the above mentioned diseases, should be from 1:1 to 2:1 maximum (Simopoulos, 2010). In the past, some international nutritional organizations have recommended an advisable n6/n3 ratio in human diet ranging between 5:1 and 10:1, as resumed by Ma et al. (2016) in a review. In our study, we found an n6/n3 ratio significantly higher in samples collected in farm C in both the typologies of products analyzed, reaching values > 6:1. On the contrary, in farm O and M, the n6/n3 ratio reached values ranging from 2.59:1 to 3.09:1, closer to the suggested optimum of 1:1. This result is highly significant, since samples coming from the conventional lowland production system (farm C) showed values for this ratio two times higher than the values recorded in samples collected in the two low-input system farms (O and M). This suggested that the organic and the mountain production systems led to the production of goat cheeses characterized by a more balanced EFA profile.

Many odd and branched chain fatty acids (OBCFA) were identified in fresh and semi-hard cheese samples in this study. OBCFA are characteristic FA of ruminants products, since they are components of rumen bacteria membranes that partially are absorbed and included in ruminants fats (Fievez et al., 2012; Vlaeminck et al., 2006). In our study, the sum of total OBCFA resulted higher in the two LIS farms (4.62–4.66% in farm O and 4.43–4.50% in farm M) than in the HIS farm (3.91–4.00% in farm C). Previously, other authors found a positive correlation between the F/C ratio in the diet supplied to goats and the OBCFA content in yielded milk (Lopez et al., 2019; Serment et al., 2011), particularly the iso forms of the branched chain FA (BCFA). It is known that rumen cellulolytic microbial populations are enriched in iso FA whereas amylolytic bacteria are enriched in the anteiso form and in linear odd-chain FA (OCFA) (Fievez et al., 2012; Vlaeminck et al., 2006). It has been demonstrated that many dietary factors, including the F/C ratio, can lead to modification of the rumen function and of the microbial populations, so that diets rich in starch reduce iso FA and, on the contrary, diets rich in forages increase iso FA in milk fat (Vlaeminck et al., 2006). According to this, we found iso 14:0 and iso 16:0 amounts significantly higher in samples coming from farm O and M than in samples from farm C, in both fresh and semi-hard cheese. These results supported the results of other authors (Cívico et al., 2017; Li et al., 2014) who found a positive correlation between the fiber content in the diet and iso 14:0 amount in goat milk, suggesting an enrichment of the cellulolytic populations in goats' rumen when fed with higher quantity of fiber. OBCFAs, particularly the branched-chained ones, are known to be important functional components in human nutrition since they showed (i) a positive activity on the gastrointestinal health, (ii) a cytotoxic activity comparable with the one of conjugated linoleic acid and (iii) positive effects against cardiovascular problems, such as atherosclerosis, thrombogenesis, coronary heart disease, blood cholesterol levels regulation and so on (Forouhi et al., 2014; Khaw et al., 2012; Ran-Ressler et al., 2014; Yang et al., 2000). Moreover, as evidenced by Haenlein (2004), goat milk generally contains a higher number of minor branched chain FA that, together with the lower content of trans 18:1 FA, represent a benefit for coronary heart disease risk.

Finally, we calculated the health lipid indices AI (atherogenic index), TI (thrombogenic index) and h/H (hypo/Hypercholesterolemic Index) that, together with n6/n3 ratio, are generally used to evaluate the nutritional value of dairy fat (Hanus et al., 2018). Our data for AI and TI were comparable to those previously obtained by other authors for goat cheese (Aguilar et al., 2014; Cossignani et al., 2014; Sant'Ana et al., 2019).

Rafiee-Yarandi et al. (2016) suggested that dairy products characterized by lower AI and TI values have a little incidence on the development of atherosclerosis and thrombosis in humans. In our study, we recorded the most interesting results in semi-hard cheese samples, where the AI and TI were significantly lower in farm M (2.25 and 2.52, respectively) than in farm O (2.84 and 2.84) and C (3.11 and 3.32). According to Hanus et al. (2018) and Sant'Ana et al. (2019) we found the highest values for AI and TI in samples collected in the farm managed with a high-input production system (more concentrates supplied, no pasture) and lower values for the farms managed by the low-input production systems (higher fibre content in the diet, access to the pasture). On the contrary, we found the highest h/H value in semi-hard cheese samples collected in farm M (0.79), followed by samples from farm O (0.64) and C (0.57), in a range comparable to the one previously detected in goat milk (Pamukova et al., 2018). It has been suggested that dairy products characterized by a high h/H ratio potentially have a protective effect against cardiovascular diseases (Rafiee-Yarandi et al., 2016).

In order to define if a discrimination among samples from the three farms was feasible by means of FA analysis, we applied a multivariate approach. A linear discriminant analysis (LDA) was performed with twenty-one variables as covariates, represented by OBCFA, n3- and n6- series PUFA, elaidic acid, 22:0, 20:1 n9, AI and TI. Such variables were selected in a first step, in which principal component analysis (PCA) was performed on data matrix to reduce dataset dimensionality, choosing factors associated with loadings > |0.5|. In Figure 1, the canonical plot obtained after the development of the LDA on our dataset, using the corresponding milk samples analyzed in a previous work (Lopez et al., 2019) as training set, is shown.

In the canonical plot, it is clear that almost all samples analyzed in this study were assigned to the group they belonged to, with 3 samples over the 17 of the validation set being misclassified (misclassification rate: 17.64%). Particularly, two samples collected on farm M were misclassified as samples of the O group and one sample collected on farm O was misclassified as a sample of the M group, whereas all samples from farm C were correctly classified, showing a clear differentiation from the samples collected in the other two farms.

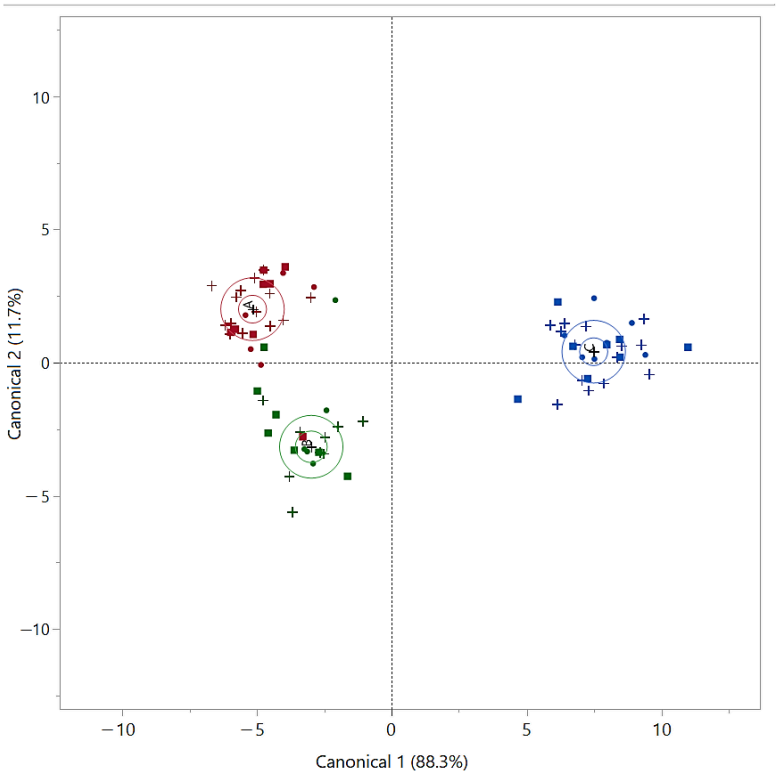

Figure 1. Canonical plot of the linear discriminant analysis performed on cheese samples, grouped by farm (O, M, C), using milk samples from Lopez et al. (2019) as training set. In the plot, each group is associated with a 95% confidence ellipse for the mean and a 50% prediction ellipsoid. Legend: red = Farm O (organic-certified farm); green = Farm M (mountain farm); blue = Farm C (conventional lowland farm); + = milk; ■ = fresh cheese; ● = semi-hard cheese.

In Figure 1, the similarity among O and M samples is clear. This phenomenon reflected the similarity observed in fatty acids percentages in O and M groups and it suggests that the difference in the production method (LIS versus HIS) was the main factor affecting the discrimination between O and M vs. C cheese samples. This result confirmed the assumption that farms where goats were fed with fresh grass or were allowed to graze (the organic-certified and the mountain farm) produced cheese represented by a FA composition clearly distinguishable from cheese coming from the conventional-lowland farm, where more concentrates were supplied to goats. Particularly, in the farms O and M, the higher F/C ratio (70:30 and 60:40, respectively) and the consumption of fresh grass and browsing plants, led to higher contents of health promoting fatty compounds, as previously remarked by other authors (Alonso et al., 1999; LeDoux et al., 2002; Sanz Sampelayo et al., 2002; Sepe & Argüello, 2019).

As to welfare outcomes deriving from the application of the AWIN welfare assessment protocol for goats, farm O presented the highest proportion of animals with no welfare problems and farm C also showed a good welfare level, whereas farm M showed the most critical situation, especially due to the high proportion of goats with poor hair coat conditions and presenting kneeling at the feeding rack, probably due to a poor management (Table 2).

Table 2. Result of the application of the AWIN (Animal Welfare Indicators) protocol, expressed as proportion (%) of animals with no welfare problems for each specific indicator.

Farm	PD*	AoA*	AoK*	FAaD*	FAaD*	GHCC*	AoO*	AoTS*	NG*
O	97.22	100.00	88.89	91.67	97.22	97.22	100.00	100.00	100.00
M	87.50	95.83	12.50	95.83	100.00	33.33	100.00	100.00	100.00
C	82.05	97.44	100.00	92.31	97.44	87.18	100.00	100.00	97.44

*PD= Proper Disbudding; AoA= Absence of Abscesses; AoK= Absence of Kneeling; FAaD= Free Access at Feeding; FAaD= Free Access at Drinking; GHCC= Good Hair Coat Condition; AoO= Absence of Oblivion; AoTS= Absence of Thermal Stress; NG= Normal Gait

In farm O, as well as in far-m M, we can also observe a very good human-animal relationship, as shown by the very low time of latency to the first contact test (2 s in farm O and 1 s in farm M), whereas in farm C goats approached the assessor only after 162 s. Bedding was dirt in all the three farms, and its quantity was also insufficient in farm M.

These welfare conditions are confirmed by QBA results, presented in Figure 2. PCA plot shows that farm M is positioned on the left side of PC1 (62.26% of explained variance), which carries information about the valence of the emotional state of goats. In fact, this farm was mainly characterized by descriptors such as irritated, frustrated and bored, indicative of a negative emotional state. On the contrary, farms O and C are on the right side of PC1, with higher loadings of positive descriptors such as content, lively, sociable, curious and relaxed. Some differences between farms O and C can be observed on PC2 (37.73% of explained variance), where C is characterized by higher loadings of aggressive, alert, suffering and fearful, and O is mainly described as agitated.

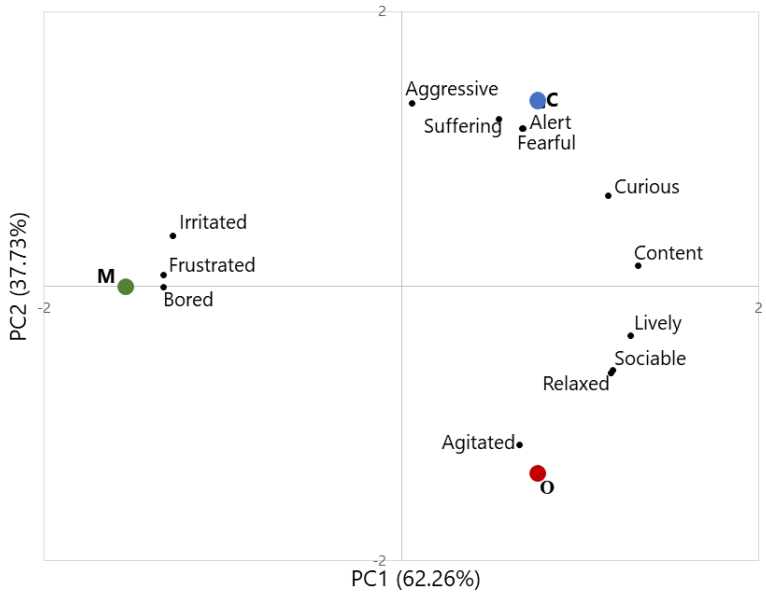

Figure 2. Scores plot of the principal component analysis performed on using QBA (qualitative behavior analysis) results. Legend: red = farm O (organic-certified farm); green = farm M (mountain farm); blue = farm C (conventional lowland farm); black = descriptors.

Overall, welfare outcomes showed a higher level of welfare in farm O, confirming the attention paid by organic producers to animal welfare, which is considered a priority in organic livestock farming by the current European legislation (EC, 2008). However, high levels of welfare can be achieved also in conventional farms, as highlighted by our results for farm C. This suggests that no clear relationship can be observed between the level of animal welfare and fatty acid composition of goat cheese.

4. Conclusions

Results obtained in this study confirmed the conclusions proposed in previous works on the fatty acid composition of goat milk. Generally, cheese from the LIS farms showed a more favourable fatty acid composition and better values for the health lipid indices, supporting the potential assessment of these products to the "functional food" market. Concerning origin discrimination among cheese from the three farms, a partial over imposition among samples coming from the two low-input management systems was evidenced, with a clear discrimination from cheese samples collected in the high-input system farm. However, we observed that, although LIS farms seem to provide cheese with better intrinsic qualities, they are not always able to guarantee high extrinsic qualities, for example in terms of animal welfare, as demonstrated by the contrasting results about lipid quality of cheese and goats' welfare in farms M and C.

As modern consumers are interested in both intrinsic and extrinsic quality of animal products our results suggest that assurance schemes cannot contain only information on the livestock system, because this information alone is not enough to provide a complete view of product quality; a more complete and detailed information on different quality traits should be provided in order to increase consumers' awareness and to allow them to make a more conscious food consumption.

Author Contributions: A.L., M.B.: investigation, formal and data analysis, writing. M.V.: main investigation and methodology. V.M.M.: co-supervision, validation and writing/editing. F.B., S.M.: conceptualization and supervision plus writing/editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the University of Milan [Piani di Sostegno alla Ricerca 2016–2017, Linea 2].

Acknowledgments: The authors acknowledge all the farmers for their availability in samples collection.

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Trial 3

Characterization of Fat Quality in Cow Milk From Alpine Farms as Influenced by Seasonal Variations of Diets

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Abstract. The production systems linked to mountain animal husbandry have had an environmental, social and cultural role in recent years. Zootechnical systems based on feeding strategies, such as pasture grazing and grass-fed strategies, contribute to a significant increase in the relative amounts of fa-vorable fatty acids (FAs) in animal products, indicating their ability to improve the long-term health of consumers. In this study, we compared different feeding strategies in two small moun-tain farms in the Piedmont Alpine region, Italy. Particularly, during the summer season, the two farms were distinguished by the exclusive employment of Alpine pasture (farm A), assumed as the best way to improve the quality of the FA profile in milk vs. the supply of daily fresh cut mountain grass plus a reduced implementation with hay and concentrates directly in the barn (farm B). The milk fatty acid profile was analyzed using gas chromatography. The results showed the high quality of alpine milk collected in the two farms. Even with some differences, particularly evidenced when comparing the summer diets, the milk FA profiles in farm A and farm B were favorable from a nutritional point of view in both seasons. Milk samples obtained using the exclu-sive employment of alpine grazing during summer were represented by an FA profile of higher quality (lower saturated FAs, higher branched FAs and monounsaturated FA, favorable n6/n3 ra-tio). However, milk obtained using the integrated strategy (fresh grass plus concentrates in the barn farm B) resulted in a more homogenous composition during the summer season, with a higher concentration of polyunsaturated FAs. These outcomes suggested that the integrated strategy, even if related to a lower ability in improving milk FA profile, could represent a valid and cost-effective alternative for mountain farmers to obtain an overall superior quality of milk, which was not strictly linked to the grazing practice. The multivariate analysis showed that in-formation contained in the milk FA profile may provide a valuable tool that can distinguish mountain-grass-based diet.

Results from this investigation have been published in the scientific journal *Animals*
Animals **2022**, 12(4), 515; doi.org/10.3390/ani12040515

1. Introduction

The mountain agricultural crisis in the Alpine region led to the loss of fundamental ecosystem services, including the loss of typical dairy products based on a pasture livestock system (MacDonald *et al.*, 2000; Hoffmann, From and Boerma, 2014; Montrasio *et al.*, 2020). However, in recent years, the production systems linked to mountain animal husbandry have been acquiring an environmental, social and cultural role, leading to policies aimed at supporting them and to the promotion of a generational change in rural areas activities that were abandoned. The economic sustainability of mountain animal husbandry, which is mainly family-centered, is most related to touristic multiservice approaches and its generating interest in consumers for mountain dairy products considered as top-quality products, both for specific organoleptic and superior nutritional properties (Mills *et al.*, 2011; Magan *et al.*, 2021).

Recently, the European Union has granted the "mountain product" label (European Union, 2014) as a strategy to sustain local products from mountain farming systems and local development politics of alpine areas, giving an obvious geographical connotation that can generate added value for mountain products (Santini, Guri and Gomez, 2013).

In dairy farming, low-input management systems, including feeding strategies based on pasture grazing, grass-fed and organic farming, are perceived by consumers as more sustainable, natural, healthy and respectful of animal welfare and biodiversity. Furthermore, dairy products from these rearing systems are known to possess an added value because of their favorable nutritional characterization and their high environmental sustainability, and are associated with authenticity features linked to their origin and traditional production processes (Butler *et al.*, 2008).

The milk fatty acid (FA) profile changes very quickly following changes in the feeding regime, affecting rumen microbial biohydrogenation (RBH) and mammary gland activity in cows. Modifications of the housing system (outdoor and indoor) and the feeding strategy (grazing and integration) in mountain and alpine farms are strictly linked to the seasonal changes in climatic conditions and feed availability, which are considered critical factors that affect the chemical composition of milk in ruminants (Serrapica *et al.*, 2020). Farming systems characterized by grazing and/or the supply of a reduced amount of conserved forage contributes to a significant increase in the relative amount of n3 FA in milk, mainly represented by α-linolenic acid (18:3n3), given that fresh grass contains about 1–3% FA/DM and 50–75% of these FAs are represented by α-linolenic acid (Collomb *et al.*, 2002). Moreover, together with the n3 FA series, fresh grass feeding and pasture grazing contribute to conveying a beneficial fatty acid composition to milk that involves a favorable composition in saturated/unsaturated FAs, *cis* and *trans* monounsaturated FAs (MUFAs), odd- and branched-chain FAs (OBCFAs) and polyunsaturated FAs (PUFAs), including conjugated linoleic acid (CLA) (Chilliard, Ferlay and Doreau, 2001; Chilliard *et al.*, 2007; Craninx *et al.*, 2008; Schwendel *et al.*, 2015). Particularly, the botanical composition of alpine pasture, which is mainly related to altitude, is known to induce significant modification in the milk FA profile, increasing favorable FAs and diminishing the unfavorable FA as a function of the elevation of the pasture (Collomb *et al.*, 2002; Povolo *et al.*, 2011, 2013).

Branched-chain fatty acids (BCFAs) in their *iso* and *anteiso* forms represent a lesser component of milk (about 2–3% of total fatty acids), but they are recognized as important bioactive components since their positive role

in gastrointestinal microbial ecology and their cytotoxicity might be compared with CLA. Increasing the forage:concentrate (F:C) ratio resulted in a higher proportion of *iso*14 and *iso*15 BCFAs in cow milk, which was related to the effect of diet on the *iso* form content in rumen bacteria (Bas *et al.*, 2003; Vlaeminck *et al.*, 2006). In addition, the odd-chain FAs (15:0, 17:0, 17:1) are generally higher in pasture cow milk and cheese and their concentration is influenced by different pasture vegetation types (Povolo *et al.*, 2011, 2013). Some of the abovementioned FAs could be interesting reliable chemical markers in chemometric approaches provided for the discrimination of the origin of milk fat, contributing to the characterization and protection of typical dairy products (Capuano *et al.*, 2014; Białek *et al.*, 2020).

The aim of the current study was to evaluate the variation in milk fat quality as affected by seasonal variation of the housing and feeding system in two Alpine farms in Piedmont (Italy), during summer and winter. The study was conducted under the usual conditions of farm management of herds in the Alpine region rather than under controlled experimental conditions.

2. Materials and Methods

2.1. Farming Conditions and Diets

The trial was carried out in the Northern Alpine area of Piedmont, a northwestern region of Italy, during two subsequent seasons, summer (August–September 2018, 51 days) and winter (January–March 2019, 77 days). Two alpine farms were involved in the trial, selected as representatives of the farming systems of interest that were typical of the alpine areas. Both farms processed raw milk in their dairy for cheese making; particularly, during summer, farm A transformed milk directly on mountain pasture.

The herd in farm A consisted of 40 multiparous dairy cows (Brown Swiss, Simmental and cross-bred cows). Farm A was set at 520 m above sea level and during winter (from October to late May), cows were housed indoors and fed a total mixed ration (TMR), consisting of alfalfa, mixed grass hays and concentrates. During summer, dairy cows were fed exclusively natural Alpine pasture, grazed at an altitude ranging from 1500 to 2000 m above sea level (100% of supplied DM).

Farm B was set in an alpine valley at 1300 m above sea level. The herd in farm B consisted of 43 multiparous Brown Swiss dairy cows housed indoors all year long. During winter (from October to late May) cows were fed a TMR consisting of meadow hay and concentrates. In summer, during the daytime, they were fed daily-cut fresh grass (31.7% of supplied DM), harvested in the grassland meadows of the surrounding Alpine valley (1250 to 1750 m above sea level), while in the evening, a TMR consisting of alfalfa hay and concentrates (58% of supplied DM) was furnished to the cows. Moreover, in this farm, cows were supplied with additional concentrates (10.2–10.3% of supplied DM) directly in the milking parlor in both summer and winter. The ingredient compositions of the summer and winter diets fed in the two dairy farms is reported in Table 1.

2.2. Feedstuffs and Milk Sampling

In both farms, dietary feedstuffs and bulk-tank milk samples were collected once every 7 days (8 sampling times for each season during the study). In farm A, summer pasture grass was harvested in six different randomly selected areas of the pastureland, cut to about 5 cm in height, in line with the feeding behavior of cows. In order to avoid the collection of grass discarded by animals, the sampling procedure was performed in pasture areas where cows had not grazed yet. In farm B, summer daily-cut fresh grass was sampled before being offered to cows. In both farms, the samples of tufts of pasture or daily-cut fresh grass were collected in triplicate and mixed in a pool in order to obtain a representative sample (500 g); they were then stored frozen under vacuum until analyses were performed. Bulk milk samples were collected directly from the farm in 50 mL plastic tubes after stirring the content of the tank. Hay and other feedstuffs and milk bulk samples were transported to university laboratories under refrigeration and stored at −20 °C until analysis.

Table 1. Compositions of the winter and summer diets in the two farms involved in the study. Each ingredient is expressed as kg/head per day on an as-fed basis. The components of the diets (fresh grass, concentrate, total mixed ration, TMR), in bold, are presented as a percentage of the total supplied dry matter (%SDM). AS—farm A summer, BS—farm B summer, AW—farm A winter, BW—farm B winter.

SUMMER				**WINTER**			
AS		**BS**		**AW**		**BW**	
Only pasture 100% SDM		**Fresh Forages** (31.7% SDM)		**TMR** (100% SDM)		**TMR** (89.8% SDM)	
	Daily-cut fresh grass		35 kg	-Alfalfa hay	10 kg	-Meadow hay	5.5 kg
	TMR (58% SDM)			-Mixed grass hay	4.5 kg	-Concentr. A *	12.5 kg
	-Alfalfa hay		6 kg	-Conc. (CP 18%)	4.5 kg	-Commercial concentrate **	3.5 kg
	-Concentr. A*		5 kg	-Flaked corn	2 kg	-Molasses	1 kg
	-Commercial concentrate**		3 kg	-Beet pulps	1 kg	Total TMR	22.5 kg
	-Molasses		0.5 kg	-Straw	1 kg	**Concentrate at Milking** (10.2% SDM)	
	Total TMR		14.5 kg	-Whey	12 kg	Concentr. B ***	2.5 kg
	Concentrate at Milking (10.3% SDM)			Total TMR	35 kg		
	Concentr. B ***		2.5 kg				

* Concentrate A: corn, barley and sodium bicarbonate. Analytical composition (% as fed): crude protein 8%, crude fibre 2.47%, crude fat 3.50%, ashes 1.87% and Na 0.05%. ** Commercial concentrate: soybean meal (dehulled), sunflower meal (decorticated), soybean (toasted), corn gluten feed, wheat bran, sunflower meal, calcium carbonate, sodium bicarbonate, dicalcium phosphate and sodium chloride. Analytical composition (% as fed): crude protein 33%, crude fibre 10.5%, crude fat 3.70%, ashes 9.8%, Na 0.57% plus trace elements and vitamin integration. *** Concentrate B: corn, wheat flour middlings, barley, soybean meal (dehulled), soybean (toasted), sunflower meal (decorticated), wheat bran, soybean hulls, dicalcium phosphate, sodium chloride, calcium carbonate, sodium bicarbonate and magnesium oxide. Analytical composition (% as fed): crude protein 14.2%, crude fibre 4.9%, crude fat 4.50%, ashes 6.5%, Na 0.3% plus trace elements and vitamin integration.

2.3. Composition and Fatty Acid Content of Dietary Feedstuffs

AOAC official methods (AOAC, 2002) were used to determine dry matter (DM), crude protein (CP), crude fat (CF) and ash according to the method of Van Soest et al. (Van Soest, Robertson and Lewis, 1991) to determine the neutral detergent fiber (NDF) and acid detergent fiber (ADF) of pasture, fresh daily-cut grass, concentrates and TMR samples. Lipids in feedstuff samples were extracted using ether extraction with petroleum ether. Fatty acid methyl esters (FAMEs) were prepared using base-catalyzed esterification with KOH/methanol and identified using gas chromatography and flame ionization detection using a TRACE™ 1300 chromatograph equipped with a TR-FAME column (Thermo Fisher Scientific Waltham, MA, USA). For the FA composition of fresh forage, we focused on the six main FAs that constituted the greatest majority of the total FA in grass: palmitic (16:0), palmitoleic (*cis9*-16:1), stearic (18:0), oleic (*cis9*-18:1), linoleic (*cis9cis12*-18:2) and α-linolenic (18:3n3) acids.

2.4. Fatty Acid Profile of Milk

Lipids of milk samples were extracted and quantified by means of liquid–liquid extraction with chloroform and methanol (2:1) according to the method of Folch et al. (Folch, Lees and Sloane Stanley, 1957). An aliquot of 40–50 mg of extracted lipids was employed for the fatty acid (FA) profile determination through the methyl-esterification in an alkalyne environment using sodium methoxide in methanol 1M, as described by Christie (Christie, 2003). This procedure allowed for the methyl-esterification of FAs linked to glycerol molecules in triacylglycerides (TAGs) of milk fat globules. FAMEs prepared in this way were identified using gas chromatography and flame ionization detection using a TRACE™ 1300 chromatograph equipped with a TR-FAME column (120 m, 0.250 mm id, 0.25 µm film thickness) from Thermo Fisher Scientific (Waltham, MA, USA). The programmed oven temperature started from 45 °C, was held for 8 min, increased at a rate of 10 °C/min up to 173 °C, and this temperature was kept for 47 min. Finally, a temperature gradient of 4 °C/min was set until reaching the final oven temperature of 220 °C, which was held for 30 min. FAMEs were identified by comparing peak retention times with FA standard mixtures and pure standard purchased from Sigma-Aldrich (St. Louis, MO, USA) and Larodan (Solna, Sweden) and analyzed under the same analytical conditions. Correction factors for the GC-FID response for FAMEs from C4 to C12 were applied (Ulberth, Gabernig and Schrammel, 1999); then, fatty acid amounts were expressed as g 100 g^{-1} of the total FA.

2.5. Data Analysis

Data analysis was performed using JMP Pro 15 from the SAS Institute (Cary, NC, USA). Milk FAs (dependent variables) were analyzed by means of a two-way ANOVA, considering the farm (F), the season (S) and their interaction (F × S) as factors influencing data variability. Tukey's HSD was used for post hoc comparisons, considering the observed differences as significant when $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). In order to reduce the dimensionality of the original data matrix and to observe the distribution of the samples, a principal component analysis (PCA) was performed as an unsupervised multivariate test, which was directed to detect the presence of eventual clusters or relationships between samples. Before developing the PCA, a low-level data

fusion (Smolinska *et al.*, 2019) was performed between the data on feedstuff gross compositions and FA profile and the milk FA profile, leading to a new data matrix consisting of 32 samples × 69 variables. Data were auto-scaled (van den Berg *et al.*, 2006) before performing the multivariate analysis in order to reduce the bias due to the influence of the parameters related to higher measure units. The score plot and the loading plot obtained by plotting PC1 vs. PC2 were used for data interpretation.

3. Results and Discussion

3.1. Diets Composition

The chemical composition and the FA profile of summer and winter diets supplied in the two Alpine mountain farms are reported in Table 2.

The outcomes obtained by the proximate analysis of alpine grass supplied to cows during the summer season (Table 2) were comparable to those previously obtained by other authors who studied the composition of meadows collected in different Italian alpine areas (Bovolenta *et al.*, 2002; Leiber *et al.*, 2005; Gorlier *et al.*, 2012; Povolo *et al.*, 2013; Peiretti *et al.*, 2017; Ravetto Enri *et al.*, 2017). Particularly, the alpine grass analyzed in this trial showed an average crude protein (CP) content ranging from 12.53% DM (farm A) to 16.12% DM (farm B) and an NDF content ranging from 60.21% DM (farm B) to 67.26% DM (farm A). Since the CP and NDF proportions are strongly related to the quality of forages (Peiretti *et al.*, 2017), the high values reported in this trial for these two parameters suggested an optimal quality of the meadows used as a forage source for dairy cows in the farms studied during the considered production seasons. Gorlier et al. (Gorlier *et al.*, 2012) detected negative and positive correlation coefficients between the phenological phases of the plants and the CP and NDF contents of the forages, respectively. The trend detected in this study for the grass collected in farm B (lower NDF and higher CP than in farm A) could suggest an average earlier phenological stage of the herbal essences present in the grassland meadows used in farm B.

Considering the total daily rations employed in summer, diets greatly differed between the two farms (Tables 1 and 2). While in farm A, cows were fed grass only on pasture with no integration, in farm B, the diet was based on daily cut fresh grass integrated with a TMR (alfalfa hay and concentrates) plus further concentrates furnished in the milking parlor. Consequently, as expected, the values of NDF and ADF were higher in the whole diet of farm A (respectively, 67.26 and 36.63% DM in farm A vs. 42.03 and 27.55% DM in farm B) due to the higher content of forages included in the total daily ration (100% DM in farm A vs. 56% DM in farm B).

The fatty acids (FAs) composition showed interesting differences between the pasture grass supplied in the two farms during summer. In particular, pasture grazed in farm A showed higher values of oleic (18:1n9) and linoleic (18:2n6) acid, while fresh grass collected in farm B showed higher values of α-linolenic acid (18:3n3).

Table 2. Chemical compositions and FA profiles of the summer and winter diets supplied in the two farms (mean values). The chemical composition (CP, EE, ash, NDF, ADF) is presented as the percentage of the dry matter (%DM), while the fatty acid composition is presented as a percentage of the total FA.

SUMMER DIETS					
	AS			**BS**	
	Pasture (Mixed Pools)	**Fresh Grass**	**TMR**	**Concentrate B**	**Total Daily Ration**
%DM	38.13 ± 6.71	18.79 ± 2.39	63.55	88.7	51.95 ± 0.76
CP %DM	12.53 ± 0.75	16.12 ± 1.54	15.78	16.22	15.93 ± 0.49
EE %DM	2.67 ± 0.21	2.88 ± 0.25	2.26	2.97	2.53 ± 0.08
Ash% DM	5.11 ± 0.86	12.65 ± 3.60	7.01	7.02	8.80 ± 0.56
NDF %DM	67.26 ± 6.96	60.21 ± 2.67	36.04	19.84	42.03 ± 0.85
ADF %DM	36.63 ± 1.79	40.97 ± 2.50	23.93	6.6	27.55 ± 0.85
F:C Ratio	**100**			**56:44**	
C16:0	11.75 ± 0.69	12.07 ± 2.09	13.94	22.51	14.23 ± 0.66
C16:1	1.58 ± 0.16	2.11 ± 0.42	0.37	0.31	0.91 ± 0.13
C18:0	1.97 ± 0.35	1.75 ± 0.43	3.33	5.19	3.02 ± 0.13
C18:1	12.91 ± 2.80	4.43 ± 0.98	26.55	36.67	20.58 ± 0.31
C18:2	21.46 ± 1.19	16.28 ± 2.69	37.86	23.07	29.50 ± 0.85
C18:3	27.80 ± 6.51	38.27 ± 5.67	3.91	1.87	14.59 ± 1.80
WINTER DIETS					
	AW			**BW**	
	Total Daily Ration		**TMR**	**Concentrate B**	**Total Daily Ration**
%DM	61.67		71.53	88.7	73.28
CP %DM	14.69		13.74	15.25	13.89
EE %DM	1.59		2.34	4.45	2.56
Ash% DM	9.05		7.73	7.17	7.67
NDF %DM	58.48		46.55	20.93	43.94
ADF %DM	34.42		29.79	7.72	27.54
F:C Ratio	**65:35**			**50:50**	
C16:0	13.51		13.26	23.14	14.27
C16:1	0.55		0.44	0.19	0.41
C18:0	2.34		3.28	5.78	3.54
C18:1	25.19		25.41	39.98	26.90
C18:2	47.75		44.76	20.38	42.27
C18:3	5.68		7.49	0.97	6.82

AS—farm A summer, BS—farm B summer, AW—farm A winter, BW—farm B winter. CP—crude protein, EE—ether extract, NDF—neutral detergent fiber, ADF—acid detergent fiber.

The differences observed in the FA compositions of the two summer grasslands could probably be related to several natural factors, such as the presence of different botanical families, different altitudes of pastures and distinct growth phases of the plants (Collomb *et al.*, 1999; Peiretti *et al.*, 2017). However, the amounts detected for oleic (4.43–12.91%), linoleic (16.28–21.46%) and α-linolenic (27.80–38.27%) acids in pasture grass from both farms were comparable to those detected by (Revello Chion *et al.*, 2010) in alpine pastures collected in the Piedmont region during summer, ranging from 6.01 to 13.5% for oleic acid, 18.1 to 23.9% for linoleic acid and 29.7 to 46.6% for α-linolenic acid.

Since fresh grass only represented one-third (30%) of the diet supplied to dairy cows in farm B during summer, the FA composition of the total daily ration furnished in this farm was found to be qualitatively different compared with farm A. Higher levels of saturated (palmitic acid 16:0 and stearic acid 18:0), monounsaturated (oleic acid) and n6 PUFA (linoleic acid) were observed for the total daily ration of farm B, where the FAs were highly representative of the concentrates furnished with the diet.

Winter diets adopted in the two mountain farms were comparable, with both consisting of TMR with hay and different concentrates, which were substantially different from the summer diets based on fresh grass (Table 1). Both the gross composition and the FA profile of the total daily rations furnished in farm A and farm B during winter showed smaller differences between them than the summer counterparts did (Table 2). When compared with the summer diets, the greatest differences were observed for the FA profile, with lower levels of α-linolenic acid (5.68 and 6.82% in farm A and farm B, respectively) and higher levels of oleic (25.1 and 26.90% in farm A and farm B, respectively) and linoleic acid (47.75 and 42.27% in farm A and farm B, respectively) detected in winter diets. The differences observed were due to the supply of concentrates at higher proportions during the winter season (reaching an F:C ratio of 65:35 in farm A and 50:50 in farm B). The greatest difference was observable between the summer and winter diets employed in farm A, where no integration with concentrates was performed during the summer season.

3.2. Milk Composition

Milk samples collected from the two farms showed average milk fat concentrations ranging from 3.7 ± 0.6% (AS) to 3.2 ± 0.2% (BS) during summer and from 3.0 ± 0.8% (AW) to 3.1 ± 0.8% (BW) during winter. Results obtained for the FA profile of milk fat are reported in Table 3.

Table 3. FA composition (g/100 g of FA) of milk samples collected from farms A and B during summer and winter. Data are presented as mean ± standard deviation. AS—farm A summer milk, BS—farm B summer milk, AW—farm A winter milk, BW—farm B winter milk.

Fatty acid	**AS**	**BS**	**AW**	**BW**	***sign***		
					F	**S**	**S × *F***
4:0	5.11 ± 0.67	4.91 ± 0.22	4.96 ± 0.17	4.89 ± 0.24	ns	ns	ns
6:0	2.46 ± 0.37 B	2.85 ± 0.36 A	2.57 ± 0.07 AB	2.70 ± 0.10 AB	ns	*	ns
8:0	1.35 ± 0.27 B	1.75 ± 0.19 A	1.53 ± 0.05 AB	1.68 ± 0.05 A	ns	***	*
10:0	2.44 ± 0.67 C	3.76 ± 0.30 A	3.03 ± 0.20 B	3.54 ± 0.12 AB	ns	***	**
12:0	2.70 ± 0.79 B	4.18 ± 0.23 A	3.35 ± 0.28 C	3.92 ± 0.14 AB	ns	***	**
14:0	9.16 ± 1.54 C	12.31 ± 0.15 A	10.65 ± 0.69 B	11.62 ± 0.30 AB	ns	***	**
16:0	27.42 ± 4.77	29.23 ± 0.91	26.40 ± 0.78	28.59 ± 0.41	ns	ns	ns
18:0	11.21 ± 2.63 A	8.20 ± 0.41 B	11.18 ± 0.77 A	10.31 ± 0.50 A	*	***	*
20:0	0.20 ± 0.05 A	0.16 ± 0.05 AB	0.19 ± 0.01 AB	0.15 ± 0.01 B	ns	**	ns
22:0	0.10 ± 0.04 A	0.06 ± 0.02 B	0.09 ± 0.01 AB	0.06 ± 0.00 B	ns	***	ns
24:0	0.07 ± 0.03 A	0.04 ± 0.02 B	0.06 ± 0.01 AB	0.04 ± 0.00 B	ns	**	ns
Total SFAs	62.21 ± 5.32 B	67.43 ± 0.85 A	64.01 ± 1.07 AB	67.51 ± 0.53 A	ns	***	ns
13:0	0.08 ± 0.02 B	0.11 ± 0.02 A	0.08 ± 0.01 B	0.10 ± 0.01 AB	ns	**	ns
15:0	0.78 ± 0.21 B	1.10 ± 0.06 A	1.14 ± 0.07 A	1.03 ± 0.03 A	**	*	***
17:0	0.74 ± 0.09 A	0.52 ± 0.01 B	0.66 ± 0.06 A	0.51 ± 0.02 B	ns	***	ns
17:1	0.09 ± 0.04 C	0.05 ± 0.01 D	0.26 ± 0.04 A	0.17 ± 0.01 B	***	***	*
21:0	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.06	0.03 ± 0.00	ns	ns	ns
Total OCFAs	1.73 ± 0.30 B	1.80 ± 0.06 B	2.19 ± 0.15 A	1.83 ± 0.04 B	***	*	**
*iso*13	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	ns	ns	ns
*iso*14	0.19 ± 0.04 A	0.13 ± 0.01 B	0.19 ± 0.02 A	0.17 ± 0.00 A	*	***	*
*iso*15	0.34 ± 0.07 A	0.24 ± 0.01 B	0.31 ± 0.02 A	0.23 ± 0.01 B	ns	***	ns
*anteiso*15	1.37 ± 0.27 A	0.53 ± 0.02 B	0.65 ± 0.04 B	0.52 ± 0.01 B	***	***	***
*iso*16	0.39 ± 0.08 A	0.27 ± 0.01 B	0.41 ± 0.04 A	0.30 ± 0.01 B	ns	***	ns
*iso*17	0.03 ± 0.06 B	0.02 ± 0.00 B	0.01 ± 0.00 B	0.23 ± 0.03 A	***	***	***
*anteiso*17	0.87 ± 0.11 A	0.26 ± 0.14 D	0.58 ± 0.06 B	0.39 ± 0.02 C	*	***	***
Total BCFAs	3.24 ± 0.50 A	1.50 ± 0.13 C	2.20 ± 0.16 B	1.88 ± 0.02 B	**	***	***
Total OBCFAs	4.97 ± 0.78 A	3.31 ± 0.11 C	4.39 ± 0.19 B	3.71 ± 0.04 C	ns	***	**
14:1	0.89 ± 0.36 AB	1.14 ± 0.09 A	0.87 ± 0.10 B	0.96 ± 0.05 AB	ns	*	ns
trans,16:1	0.10 ± 0.09	0.12 ± 0.01	0.08 ± 0.01	0.06 ± 0.00	ns	ns	ns
cis, 16:1	0.64 ± 0.50 B	1.55 ± 0.29 A	1.52 ± 0.18 A	1.24 ± 0.15 A	*	**	***
*trans*9, 18:1	0.31 ± 0.07	0.39 ± 0.15	0.37 ± 0.03	0.34 ± 0.03	ns	ns	ns
*trans*11, 18:1	1.97 ± 1.73	2.03 ± 0.33	1.26 ± 0.21	1.32 ± 0.011	ns	ns	ns
*cis*9, 18:1	24.64 ± 3.59 A	17.88 ± 0.57 B	22.22 ± 0.93 A	19.31 ± 0.45 B	ns	***	**
*cis*11, 18:1	0.56 ± 0.16 A	0.55 ± 0.05 A	0.50 ± 0.08 AB	0.40 ± 0.02 B	**	ns	ns
20:1 n9	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	ns	ns	ns
22:1 n9	0.03 ± 0.02	0.04 ± 0.03	0.03 ± 0.01	0.03 ± 0.00	ns	ns	ns
Total MUFAs	29.21 ± 4.34 A	23.75 ± 0.98 B	26.89 ± 0.88 A	23.71 ± 0.46 B	ns	***	ns

Table 3 (cont.)							
t9t12, 18:2 n6	0.01 ± 0.01 A	0.05 ± 0.08 A	0.23 ± 0.02 B	0.21 ± 0.02 B	***	ns	ns
c9t12, 18:2 n6	0.02 ± 0.01 B	0.03 ± 0.01 AB	0.04 ± 0.02 A	0.04 ± 0.02 AB	**	ns	ns
t9c12, 18:2 n6	0.04 ± 0.03	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	ns	ns	ns
c9c12, 18:2 n6	1.38 ± 0.49 C	2.73 ± 0.32 A	2.25 ± 0.15 B	2.80 ± 0.08 A	**	***	**
18:3 n6	0.02 ± 0.01 AB	0.03 ± 0.02 A	0.02 ± 0.01 B	0.0 ± 0.01 B	*	ns	ns
18:3 n3	0.62 ± 0.17	0.79 ± 0.19	0.73 ± 0.13	0.70 ± 0.03	ns	ns	ns
c9t11–18:2	1.12 ± 0.71 AB	1.31 ± 0.12 A	0.78 ± 0.12 B	0.73 ± 0.04 B	**	ns	ns
20:2	0.02 ± 0.01	0.05 ± 0.03	0.03 ± 0.00	0.03 ± 0.00	ns	ns	ns
20:3 n6	0.07 ± 0.02 B	0.13 ± 0.05 A	0.10 ± 0.01 AB	0.10 ± 0.00 AB	ns	**	**
20:4 n6	0.13 ± 0.02 B	0.16 ± 0.01 B	0.14 ± 0.01 B	0.14 ± 0.00 AB	ns	**	**
20:3 n3	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	ns	ns	ns
20:5 n3	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.05 ± 0.00	ns	ns	ns
22:5 n3	0.09 ± 0.06	0.10 ± 0.02	0.10 ± 0.01	0.08 ± 0.01	ns	ns	ns
Total PUFAs	3.60 ± 0.93 C	5.49 ± 0.29 A	4.51 ± 0.24 B	4.93 ± 0.12 AB	ns	***	**
Total n3	0.79 ± 0.21	0.96 ± 0.24	0.90 ± 0.14	0.84 ± 0.03	ns	ns	ns
Total n6	1.67 ± 0.50 C	3.17 ± 0.22 AB	2.80 ± 0.16 B	3.33 ± 0.11 A	***	***	***
PUFAs/SFAs	0.06 ± 0.02 B	0.08 ± 0.00 A	0.07 ± 0.00 AB	0.07 ± 0.00 AB	ns	**	*
n6/n3	2.20 ± 0.80 C	3.46 ± 0.69 AB	3.16 ± 0.48 B	3.98 ± 0.15 A	**	***	ns
DI[1]	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 00	ns	ns	ns

[1] DI—desaturase index, calculated as 14:1/(14:0 + 14:1), suggested as the best indicator for Δ9-desaturase activity (Lock and Garnsworthy, 2003). The significance obtained in the multifactorial analysis (S—season, F—farm, S × F—interaction) are presented (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Average values associated with different letters (A, B, C, D) on the same row are statistically different.

The correlation coefficients between the fiber-related parameters of the diets (NDF, ADF content and F:C ratio) and the concentrations of FAs detected in the milk are presented in Table 4.

Table 4. Pairwise correlation matrix between fiber-related parameters (NDF, ADF and F:C ratio) of the diet and the percentages of FAs detected in milk. Only significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) correlations are reported.

	Diet.NDF		**Diet.ADF**		**F:C Ratio**	
Fatty acid	Correlation Coefficient	*sign*	Correlation Coefficient	*sign*	Correlation Coefficient	*sign*
6:0	−0.4632	**	−0.5716	**	−0.4585	**
8:0	−0.699	***	−0.7464	***	−0.6903	***
10:0	−0.8094	***	−0.8296	***	−0.7797	***
12:0	−0.8076	***	−0.8107	***	−0.7515	***
14:0	−0.8101	***	−0.8306	***	−0.7626	***
16:0	−0.4064	*	−0.5001	**		ns
18:0	0.535	**	0.6184	**	0.3569	*
20:0	0.6408	**	0.7656	***	0.579	**
22:0	0.5596	**	0.7062	***	0.573	**
24:0	0.4687	**	0.6019	**	0.521	**
SFA	−0.686	***	−0.746	***	−0.5952	**
13:0	−0.5719	**	−0.521	**	−0.4094	*
15:0	−0.516	**	−0.3702	*	−0.6746	***
17:0	0.794	***	0.8788	***	0.7981	***
*iso*14	0.5696	**	0.6079	**	0.4239	*
*iso*15	0.7487	***	0.8459	***	0.7178	***
*anteiso*15	0.7539	***	0.7586	***	0.927	***
*iso*16	0.7349	***	0.7689	***	0.517	**
*iso*17	−0.478	**	−0.5356	**	−0.4515	*
*anteiso*17	0.8764	***	0.8825	***	0.8641	***
BCFAs	0.8184	***	0.8302	***	0.881	***
OBCFAs	0.7803	***	0.8489	***	0.755	***
14:1	−0.4955	**	−0.5035	**		ns
cis, 16:1	−0.6133	**	−0.4999	**	−0.6802	***
*cis*9, 18:1	0.8656	***	0.8404	***	0.7397	***
*cis*11, 18:1		ns		ns	0.376	*
MUFAs	0.761	***	0.7962	***	0.6888	***
t9t12, 18:2 n6		ns		ns	−0.5682	**
c9t12, 18:2 n6		ns		ns	−0.418	*
c9c12, 18:2 n6	−0.7971	***	−0.8484	***	−0.9023	***
20:0	−0.6655	***	−0.6302	**	−0.6721	***
20:3 n6	−0.6809	***	−0.6755	***	−0.7917	***
20:4 n6	−0.462	**	−0.5687	**	−0.4728	**
20:5 n3	0.5192	**	0.528	**	0.3586	*
PUFAs	−0.7067	***	−0.6561	***	−0.749	***
PUFAs/SFAs	−0.4921	**	−0.4132	*	−0.5477	**
n6	−0.7798	***	−0.8173	***	−0.9273	***
n6/n3	−0.7108	***	−0.7938	***	−0.8208	***

3.2.1. Saturated Fatty Acids (SFAs)

Overall, no statistical differences were detected for total SFAs between the two seasons for each farm. However, focusing on the summer samples, we detected significantly lower amounts of SFAs in farm A, where cows were fed just by grazing (62.21% SFAs in AS samples vs. 67.43% in BS samples). The differences were mainly related to higher amounts of short- and medium-chain FAs from 6:0 to 14:0 in farm B. In contrast, long-chain FAs from 18:0 to 24:0 were found higher in AS than in BS milk. According to this, we observed negative correlation coefficients (Table 4) between the F:C ratio, the NDF and the ADF amount of the diet and the amount of milk 6:0–14:0 FAs, with correlation coefficients reaching the highest values (near to −0.80) for 10:0, 12:0 and 14:0. In contrast, the correlation coefficients between the fiber-related parameters of the diet and the amount of long-chain SFAs (from 18:0 to 24:0) in milk were positive, even if represented by lower values (<0.80).

These results are in agreement with the scientific literature asserting that pasture practice and diets represented by high F:C values can increase the concentration of 18:0 and decrease the concentration of hypercholesteroleamic FAs (12:0, 14:0 and 16:0) in milk (Chilliard *et al.*, 2007; Hanus *et al.*, 2018). Revello Chion et al. (Revello Chion *et al.*, 2010) observed that cows fed with fresh grass produced milk characterized by a favorable FA composition compared with animals fed with dry diets, especially in the summer season, with reduced levels of saturated FAs. This outcome is very interesting for consumers since the medium-chain SFAs 12:0, 14:0 and 16:0 were shown to be associated with adverse effects on indicators of cardiovascular disease and their excessive consumption is related to an increased risk of atherosclerosis, hyperlipidemia, obesity and coronary heart diseases in humans (Hanus *et al.*, 2018).

3.2.2. Odd- and Branched-Chain Fatty Acids (OBCFAs)

Increasing the F:C ratio in the diet of dairy cows generally produces an increase of milk odd- and branched-chain fatty acids (OBCFAs), particularly the branched form (Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012). This modification is related to a shift in the proportion of the rumen bacterial population toward the growth of cellulosolytic bacteria, which was enhanced by increasing the proportion of forages in the diet. Several authors reported a positive correlation between the NDF content of the diet and milk OBCFA content (Kraft *et al.*, 2003; Vlaeminck *et al.*, 2006; Fievez *et al.*, 2012). Accordingly, in this investigation, we observed positive correlations between the dietary fiber content and the amounts of 17:0 and several branched-chain fatty acids (BCFAs), with the highest correlation coefficients (>0.80) observed between the F:C ratio of the diet and the BCFA content in milk (Table 4).

We found total OBCFAs to be significantly higher in the AS samples (4.97%) than in all the other groups. Particularly, the BCFAs that reached the higher concentration in AS samples were 17:0, *iso*14, *iso*15, *anteiso*15, *iso*16 and *anteiso*17. These FAs are associated with a particular significance, especially since

- They have been recognized as biomarkers of the rumen functionality, with their proportions strictly related to the ruminal microflora system (mainly, differences between cellulosolytic and amylolitic bacteria);
- They are known as bioactive food components since they display healthy functional properties that are comparable with that of greater debated CLAs isomers (Ran-Ressler *et al.*, 2014; Hanus *et al.*, 2018).

In this work, we observed and confirmed that the employment of pasture represents an effective strategy to maximize the presence of beneficial functional FAs in milk products. The highest values for the BCFAs (3.24%) were found in farm A during summer (AS) when cows were fed exclusively by pasture; this result was significantly different from all other milk samples. In contrast, no differences were detected for BCFAs between summer (BS) and winter (BW) samples in farm B; this can be noticeably related to the highest similarity between the feeding regime chosen in this farm, where the integration strategy with concentrates was practiced in both seasons (summer and winter). Similarly, the amount of total OBCFAs was found to be higher in farm A than in farm B in both seasons, recording the highest amount in AS samples.

3.2.3. Unsaturated Fatty Acids (UFAs)

Total monounsaturated fatty acids (MUFAs) content was higher in milk from farm A than in farm B in both seasons. The main MUFA accounting for this difference was oleic acid - *cis*9-18:1 - which reached the highest amount (24.64%) in AS samples. We observed a positive correlation index between the F:C ratio, the NDF and the ADF amount of the diet and the amount of oleic acid in milk (correlation coefficient around 0.80, Table 4). Chilliard et al. (Chilliard *et al.*, 2007) showed that oleic acid can be higher in pasture milk despite the low amount of this fatty acid in the pasture grass. Leiber et al. (Leiber *et al.*, 2005) suggested that this phenomenon could be related to the fact that pastures are usually enriched in α-linoleic acid - 18:3 n3 - which, after the rumen biohydrogenation processes, is converted to stearic acid and successively to its unsaturated counterpart oleic acid via mammary Δ9-desaturation. Moreover, it was suggested that the alpine pasture conditions could lead cow metabolism to a higher depletion of the adipose tissue (enriched in oleic acid) for milk FA synthesis, resulting in a higher amount of oleic acid in pasture milk (Chilliard *et al.*, 2007).

Palmitoleic acid - 16:1 - was found in significantly lower amounts in AS milk (0.64% vs. 1.24–1.55% in other samples). We hypothesize that the lower amounts of palmitic acid supplied with the diet in farm A during summer (11.75%, Table 2) led to the lower availability of this FA in the mammary gland for the desaturation operated by the Δ-9 desaturase, which adds a *cis*9-double bond in the carbon chain of saturated FA from C10 to C19, leading to the formation of palmitoleic from palmitic acid (Chilliard *et al.*, 2007). However, the range of amounts (0.64–1.55%) detected for this FA was comparable to that found in the literature for cows fed freshly cut grass indoors or at pasture (Capuano *et al.*, 2014; Benbrook *et al.*, 2018).

MUFAs with the double bond set in the *trans* configuration did not show statistically significant differences between the groups. It has been most often reported that pasture- and forage-based feeds increase the levels of *trans* FAs in milk, mainly *trans*18:1, simultaneously with increased levels of conjugated isomers of linoleic acid (CLAs) (Dewhurst *et al.*, 2006; Chilliard *et al.*, 2007; Collomb *et al.*, 2008). Indeed, vaccenic acid – *trans*11, 18:1 - is a common intermediate in the rumen biohydrogenation (RBH) of linoleic and α-linolenic acid in the rumen. The RBH averages 80% for linoleic acid and 92% for linolenic acid (Doreau and Ferlay, 1994). In the RBH pathway, vaccenic acid is reduced to stearic acid but, due to the low rate of this reaction, it accumulates in the rumen, becoming available for absorption and transport to the mammary gland, where it is transformed to rumenic acid—*c9t11,* 18:2 CLA—by the Δ9-desaturase. Since the hydrogenation of *trans*-18:1 constitutes the

limiting step for the full hydrogenation of unsaturated C18 FA, vaccenic acid frequently accumulates in the rumen. The comparable amount of vaccenic acid recorded in milk from the two farms and the two seasons in this study supported the consideration of this FA as a marker of high-forage diets (associated with a high F:C ratio), and not just linked to pasture practice. It can be observed that vaccenic acid amount showed a huge intra-group variability in AS samples, influencing the lack of significance when performing the statistical comparisons. Dewhurst *et al. (* 2006) had previously suggested that a higher variation between individual animals can be observed under grazing conditions than with a total mixed ration (TMR), a phenomenon that could potentially mask differences in experiments carried out on grazing systems in which we expected to find the highest level of vaccenic acid.

Similarly, no statistical difference between the amount of α-linolenic acid in milk delivered by the two farms in the two seasons was observed. The content of this fatty acid is known to vary according to botanical composition and maturation stage of the grassland, which would explain the higher proportion variability of α-linolenic acid, especially during the summer season, when cows were fed using pasture or freshly cut grass indoor (Capuano *et al.*, 2014).

One of the main intermediates of the rumen biohydrogenation (RBH) toward α-linolenic is rumenic acid (*c9t11*-CLA). During the RBH, a small proportion of the CLA is nonetheless absorbed in the intestine and secreted into milk through the mammary gland. The rumenic acid synthesis mainly occurs (probably more than 75%) in the udder, in proportion to the amount of vaccenic acid formed in the rumen (Chilliard *et al.*, 2007). We found *c9t11*-CLA in higher amounts in summer samples in both the farms (1.12–1.31%) than in the winter samples (0.73–0.78%). These outcomes agree with the knowledge that the consumption of fresh grass (both with grazing and furnishing it in the barn) is related to higher PUFA intake, higher RBH processes and a higher amount of the intermediates migrating to the mammary gland for the synthesis of milk FA (Chilliard *et al.*, 2007). However, the highest amount was detected in BS samples, even if the diet was associated with a higher proportion of concentrates compared with farm A. It is known that milk CLA composition in cows led to pasture are very variable due to the pasture composition (young grass contains higher 18:3n3) and the botanical origin of meadows. In this study, the α-linolenic acid content was higher in fresh cut grass from alpine meadows (35.74%, farm B) than in pasture grass (27.41 g/100 g FA, farm A), showing a trend with highest values in July (45.8%) and the lowest values when season advanced (late September, 22.97%) (seasonal data not reported). Furthermore, the dietary factors influencing the CLA concentrations in milk (18:2n6 and 18:3n3 precursors, forages and starchy sources) are interrelated and interactions between them can lead to wide variations (up to 4%) in CLA in pasture milk (Chilliard *et al.*, 2006).

Linoleic acid was found in higher amounts in BS (2.73%) than in AS (1.38%) milk. Negative correlation indexes were observed between the F:C ratio, NDF and ADF amount of the diet and the total n6 FA content, particularly linoleic acid, showing correlation coefficients ranging from −0.75 to −0.86 (Table 4). The difference observed in the linoleic acid amount was directly linked to the higher n6/n3 ratio found in BS (3.17) than in AS (1.67) samples. The n6/n3 ratio in milk essentially depends on concentrations of linoleic (n6) and α-linolenic (n3) acids (Elgersma, 2015). The n6/n3 ratio, well known as a healthy index to evaluate the quality of food fat, in dairy products can be improved (i.e., decreased) by shifts in cows diet, particularly enhancing the consumption of

fresh grass, such as it happens in the production of alpine milk (Leiber *et al.*, 2005; Elgersma, 2015). Accordingly, the n6/n3 ratio was already shown to reach values more than two times higher in conventional milk compared with extensive or pasture-based milk (Slots *et al.*, 2009; Davis *et al.*, 2020). All the values recorded for n6/n3 in this study can be counted as favorable. They ranged from 1.67 to 3.33, falling in the suggested range of 1–4, which is considered optimal from a functional perspective in order to prevent many cardiovascular diseases in consumers (Simopoulos, 2002). Nonetheless, it is notable that milk produced by feeding cows just through the grazing practice was represented by the best n6/n3 balance.

3.3. Multivariate Analysis

In Figure 1, the scores plot (A) and the loading plot (B) of a PCA that obtained the first (PC-1) and the second (PC-2) principal components (PCs) are reported. The two PCs accounted for 57.3% of the total variability in the 32 (samples) × 69 (analytical parameters) data matrix.

When observing the set of samples in the multivariate system (Figure 1a), we noticed that within the mountain dairy production systems evaluated, many qualitative differences were delivered to milk employing different livestock managing and feeding strategies. Both farms involved in this study can be considered as "low input farms", carrying a low farming pressure toward animals and the environment. However, in the PCA scores plot, samples were separated into three main groups, with BS and BW samples overlapping and clustering; these were separated from milk samples from farm A. AS milk was clearly separated from all the other samples; the main reason for this discrimination was due to by the total absence of any integration or concentrates included during the summer season in farm A. The PCA of average milk FA contents supported results previously obtained, which showed a clear separation between TMR-fed and pasture-based milk (Capuano *et al.*, 2014; O'Callaghan *et al.*, 2016). The factors with the highest negative loading scores for the PC-1 (in light blue in Figure 1b) were some SFAs (10:0, 14:0, 12:0, 8:0, 20:0 and 6:0) and n6 FAs (namely, c9c12-18:2n6, 20:3n6 and 20:4n6). Milk samples characterized by negative scores for PC-1 were the ones collected in farm B (Figure 1a), which were associated with the highest proportion of concentrates, and thus of linoleic acid in the dietary ration (Diet.18:2 in Figure 1b). This outcome suggested that, even if most of the linoleic acid introduced with the diet was transformed by the biohydrogenation processes occurring in the rumen, a certain amount of this FA escaped this pathway and was delivered to the mammary gland during milk fat synthesis, leading to a higher concentration in milk fat and, consequently, to a higher n6/n3 ratio in the milk. On the other hand, samples associated with the highest positive scores for PC-1 were AS samples (Figure 1a). The variables with the higher weight in the positive direction of PC-1 (in yellow in Figure 1b) were the fiber-related parameters of the diet (F:C, Diet.NDF and Diet.ADF) plus the milk content of many OBCFAs (namely, *anteiso*17, *anteiso*15 and 17:0) and oleic acid (*cis*9-18:1). BCFAs are known to be strictly related to a ruminal environment enriched in cellulosolytic bacteria, whose presence increase in the rumen when the diet is represented by a high amount of fiber, thus by a high F:C ratio. Hence, we suggest that the feeding strategy chosen during summer in farm A, represented by the exclusive employment of alpine grazing, involved a rumen functionality leading to a higher representation of beneficial functional FAs in milk.

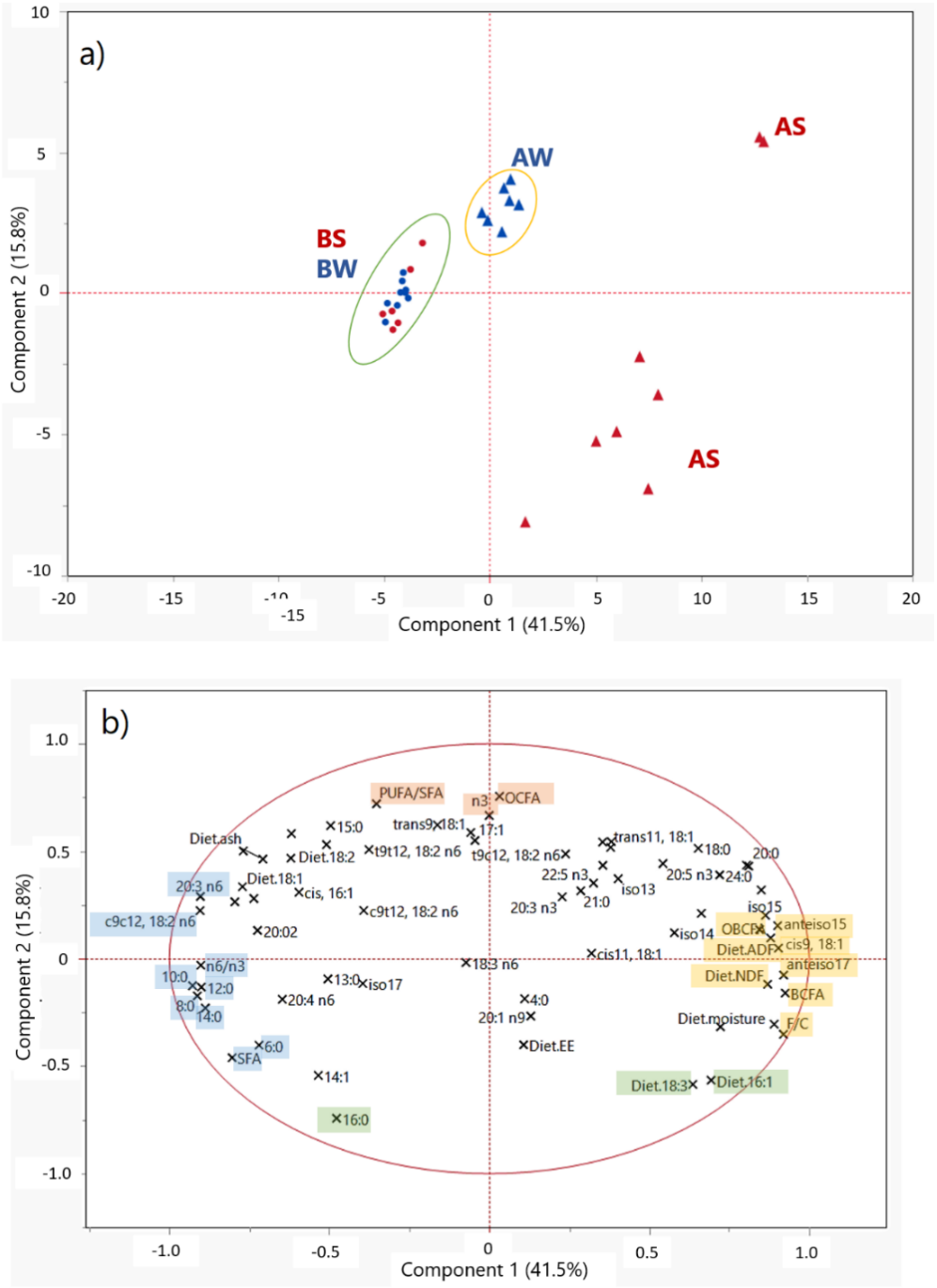

Figure 1. (**a**) PCA score plot. Legend: triangles—milk collected from farm A; points—milk collected from farm B; red—summer samples; blue—winter samples. (**b**) PCA loading plot.

AW samples represented a third separated cluster in the PC space, which was characterized by an intermediate position over PC-1 but clearly separated from AS samples over the direction of PC-2. Particularly, AS samples were characterized by negative PC-2 scores, while AW samples were characterized by positive PC-2 scores. The variables associated with positive loadings for PC-2 (orange in Figure 1b) were the OCFA, n3 and PUFA contents of milk; in contrast, the variables related to negative loadings for PC-2 (green in Figure 1b) were the milk content of palmitic acid (16:0) and the amount of α-linolenic acid and palmitoleic acid of feedstuffs (Diet.18:3 and

Diet.16:1, respectively). It was interesting to observe that the content of α-linolenic acid of the diet (Diet.18:3) and the proportion of this FA in milk (18:3n3 in Figure 1b) were negatively correlated following the direction of PC-2. This confirmed the knowledge that the rumen biohydrogenation processes affect most of the content of α-linolenic acid supplied to cows with the diet.

Nevertheless, the distribution of the scores in the PCA plot (Figure 1a) suggested that the exclusive use of grazing pasture in farm A during summer led to a lower homogeneity of milk quality during the sampling time in summer. The feeding strategy based only on pasture, even if related to an overall improvement of milk fat quality, was also associated with higher variability in the milk FA profile due to the less controlled conditions (higher seasonal variability of forages, possibility for cows to choose what to eat, etc.) that characterized the grazing practice.

4. Conclusions

Milk fat composition significantly contributes to the promotion of dairy products associated with an added value, especially when characterized by a favorable FA profile. This issue could be particularly important for mountain and alpine products since their high value, as generally perceived by consumers, can be supported by analytically assessed nutritional quality. The results obtained by this study supported the evaluation of alpine milk collected in the farms involved in the investigation as high-quality milk based on its fat composition. Even though there were some differences between the two farms, as particularly evidenced when comparing the summer diets, the milk FA profiles in farm A and farm B were favorable from a nutritional point of view (high amount of functional active FAs, such as BCFAs and CLAs, favorable n6/n3 ratio, etc.) in both the seasons. Particularly, milk samples obtained using the exclusive employment of alpine grazing (AS) were represented by an FA profile of greater quality, even if characterized by a higher variability due to the less controlled feeding conditions during grazing. On the other hand, milk obtained using the integrated strategy (fresh grass plus concentrates in the barn BS samples) resulted in a more homogenous composition during the summer season, with a higher concentration of PUFAs.

Reported results suggested how the supply of fresh grass directly in the barn might represent a valid alternative to alpine pasture, aiming to obtain increased quality of milk characterized by high homogeneity and being more feasible for farmers.

The limited number of farms involved did not allow for extending our conclusions to a broader context. However, our results supported the idea that information contained in the milk FA profile may be used to assess the fresh grass feeding. Further investigations extended to a higher number of farms with similar characteristics in the same geographical area could be taken into account in order to create a tool to support and enhance the consumers' perceptions toward alpine milk from the Piedmont alpine region.

Author Contributions: A.L.: investigation, formal and data analysis and writing. D.C.: conceptualization, supervision and writing/editing. G.S.: supervision, validation and writing/editing. V.M.M.: co-supervision, validation and writing/editing. F.B.: conceptualization, supervision and writing/editing. All authors read and agreed to the published version of the manuscript.

Funding: This research was founded by "Ager-Fondazioni in Rete per la Ricerca Agroalimentare" and the project "Integrated Alpine Livestock Systems: from ecosystem services to premium mountain products (IALS)".

Institutional Review Board Statement: Not applicable. The investigation presented in this manuscript only consisted in collecting raw bulk milk samples from milk tanks, considered as a routine practice in dairy farms.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors acknowledge all the farmers for their availability in samples collection.

Conflicts of Interest: The authors declare no conflict of interest.

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6.2 Discussions and conclusions of Chapter 6 – *Dairy products*

Analytical outcomes. The outcomes obtained in the trials performed on dairy products in this thesis mainly supported the knowledge reported in literature up to date. The GC-FID analytical methods developed allowed the identification of many FA as markers related to the feeding pattern and the livestock system employed in farms where samples were collected. In all the trials, FA were derivatized before performing the chromatographic analysis through a methyl-esterification step, obtaining the formation of the volatile equivalents as fatty acids methyl esters (FAMEs). A 1M sodium potassium methoxide in anhydrous methanol solution was selected as derivatizing agent in order to perform a base-catalyzed transterification, according to Christie (2003). The base-catalyzed transesterification was chosen as appropriate derivatization technique in the trials on dairy products since it was reported to avoid the isomerization of double bonds of polyunsaturated FA. According to the author, following this procedure, triacylglycerols are completely transesterified in 10 minutes and phosphoglycerides in 5 minutes. However, the drawback is represented by the fact that free fatty acids are not transesterified (Christie, 2003).

In both the studies, the chromatographic column selected was a TRACE™ TR-FAME (0.25 mm id, 0.25 μm film thickness), represented by a high polarity (70%) cyanopropylphenyl-based phase, specifically designed for the separation of FAMEs. In the case of goat products (**Trial 1** and **Trial 2**), the column was 60 m long. The programmed oven temperature started from 50°C and finished at 220°C, with an isotherm plateau of 30 min set at 170°C. The set of analytical condition chosen in this case allowed the separation and the identification (by means of analytical standards) of 38 FAMEs in a chromatographic run of 80.5 min; however, a bad separation performance was evidenced for many isomers, which peaks were not fully resolved. Such an example, with these analytical parameters, the two peaks for the *cis* and *trans* isomers of palmitoleic acid (16:1) coeluted. Moreover, it was not possible to separate the chromatographic peak for *cis*9,*trans*11-18:2 (c9t11-CLA, rumenic acid) and 20:0. However, the method allowed to identify several FAs already known as important biomarkers of the lipid fingerprint in dairy products (such as α-linolenic acid, linoleic acid, *trans* and *cis*-vaccenic acid, branched chain fatty acids, and so on). In order to avoid the same drawbacks, in **Trial 3** performed on the FA profile of cow mountain milk, a 120 m column was chosen, in order to optimize the separation of many isomers not separated with the previous chromatographic conditions. In this case, the programmed oven temperature started from 45°C and finished at 220°C, with an isotherm plateau of 47 min at 173°C. This analytical equipment effectively allowed the separation of the *cis* and *trans* isomers of palmitoleic acid, all the *cis* and *trans* isomers of linoleic acid (18:2n6) and also the separation of rumenic acid, further identified as a fundamental biomarker which amount highly varied related to the factors analysed (season and production method). The drawback in this case was the length of the chromatographic run that required a total of 109.5 min for the optimal separation of the target FAs. In figure 1, an extract of the chromatogram obtained in the region of 18:2 isomers in **Trial 3** is presented.

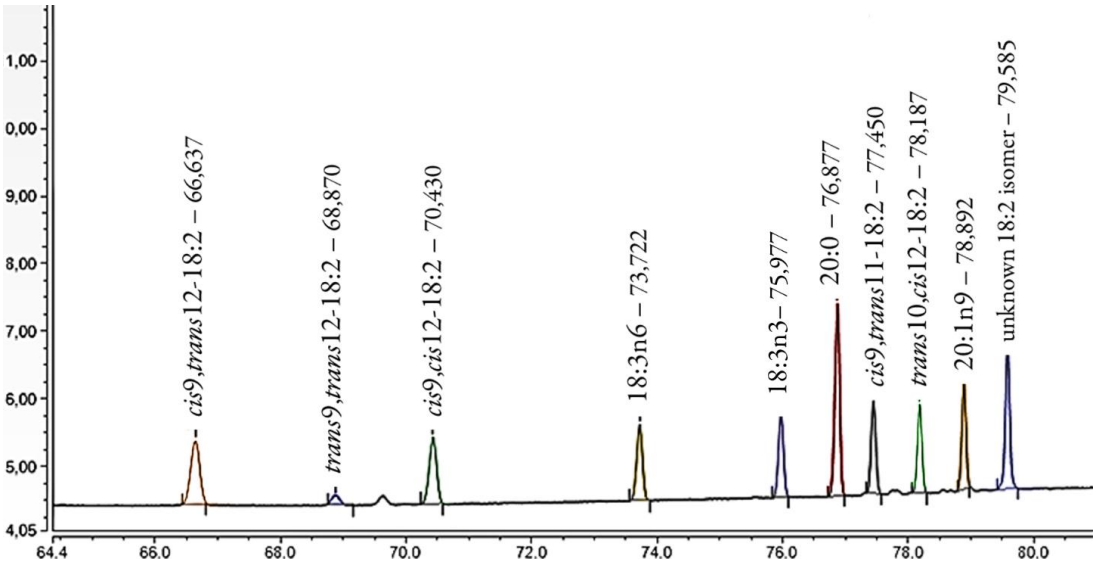

Figure 1. Exemplificative section of a chromatogram for the region of 18:2 isomers obtained under the analytical conditions set in **Trial 3** of this thesis (120 m length column, 109.5 min run).

Finally, because of the high volatility of short chain fatty acids methyl esters, highly represented in milk products, and the consequent carbon deficiency in the FID response, empirical response factor proposed by Ulberth et al. (1999) were applied for the determination of peak areas of FAMEs with <12 carbon atoms (4:0, 6:0, 8:0, 10:0 and 12:0).

Milk and cheese fatty acid profile. In all the trials, we confirmed many FA as indicators of the ruminal function inducted by the feeding strategy employed in the farms. Such an example, the group of odd and branched chain fatty acids (OBCFA) showed an interesting trend. Particularly, the branched chain FA (BCFA) were confirmed as indicators of a high consumption of fibre by the ruminants. BCFA are known as major components of the cellular membranes of cellulosolytic ruminal bacteria (Fievez et al., 2012; Vlaeminck et al., 2006), whose presence increase in the rumen when the diet is represented by high amount of fibre, related to a high forage to concentrates ratio (F/C) (Cívico et al., 2017; Serment et al., 2011). Actually, in the trials presented in this thesis, the group of BCFA showed higher percentages in the products collected in the farms where the dietary pattern was represented by higher F/C and animals were allowed to graze. Particularly, the pasture-based livestock system that characterized farm A during summer in **Trial 3**, showed the ability to maximize their amounts (especially for 17:0, *iso*14, *iso*15, *anteiso*15, *iso*16 and *anteiso*17), while no differences were detected in the winter and summer season in farm B, where diets were characterized by a high degree of similarity in both the seasons. Likewise, in goat products (**Trial 1** and **Trial 2**), BCFA were found at higher percentages in the products collected in the farms following production systems defined as "low-input", characterized by feeding strategies relate to high proportion of forages and/or the consumption of grasslands.

Similarly, oleic acid (cis9-18:1) was found in higher amounts in the products collected in the farms where the feed rations were characterized by the higher proportion of forages and by the access to the pasture (the organic and mountain farms in **Trial 1** and **Trial 2** and farm A in **Trial 3**). The concentration of oleic acid in milk is known to be inversely related to the amount of concentrates furnished to ruminants with the diet and to be increased by the pasture practice (Chilliard et al., 2007; Chilliard & Ferlay, 2004; Serment et al., 2011). Particularly, in **Trial 3**, the amount of oleic acid in cow milk showed positive correlation indexes with the F/C ratio and the NDF and ADF amount of the diets. These outcomes are in line to what expressed in the scientific literature available to date: the ingestion of higher amounts of α-linolenic acid present in pasture and fresh grass is followed by a higher biohydrogenation process toward this FA to stearic acid in the rumen (Leiber et al., 2005). Then, stearic acid is converted in its unsaturated counterpart, oleic acid, by the action of Δ9-desaturase in the mammary gland (Chilliard et al., 2001), as shown in figure 2. Moreover, the higher presence of oleic acid in milk from grazing cows has been related to a probable higher depletion of fat reserves, enriched in oleic acid due to higher energetic deficiency, and to the consequent mammary secretion (Chilliard et al., 2007).

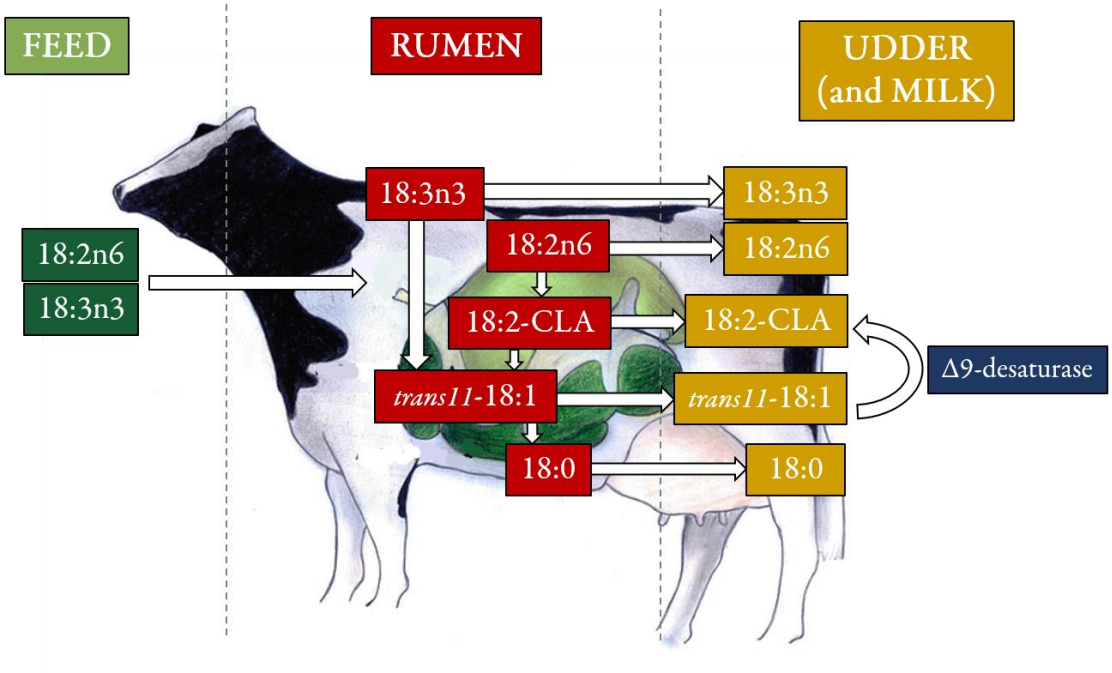

Figure 2. Adapted from Chinnadurai & Tyagi (2011) - Conjugated Linoleic Acid: A Milk Fatty Acid with Unique Health Benefit Properties

Rumen biohydrogenation (RBH) processes affect, on average, the 92% of α-linolenic acid (18:3n3) ingested with the diet, whereas the hydrogenation of linoleic acid (18:2n6) occurs at a rate around 80% of the ingestion (Doreau & Ferlay, 1994). α-linolenic and linoleic acid are considered the main FAs of the n6 and n3 series of the polyunsaturated fatty acids (PUFA) highly represented in dairy products. While α-linolenic acid mainly comes from the consumption of fresh forages, linoleic acid is highly represented in the concentrates furnished to

ruminants in the barn (Elgersma, 2015; Khiaosa-Ard et al., 2010). It has been demonstrated that an increase of the integration of concentrates, thus a decrease of the F/C ratio of the diets, leads to an increment of linoleic acid percentages in milk and dairy products (Chilliard et al., 2007). Actually, in the studies presented in this thesis, higher amounts of linoleic acid were found in the products collected in the farms characterized by the high-input system (**Trial 1** and **Trial 2**) and when feeding cows with higher proportion of concentrates in the total daily ration (**Trial 3**). Particularly, in **Trial 3**, the amount of linoleic acid in milk showed negative correlations with the F/C ratio and the percentages of NDF and ADF of the diets.

As explained before, the analytical equipment employed in **Trial 3** allowed even the separation and the identification of the principal conjugated isomer of linoleic acid, rumenic acid (c9t11-CLA). Rumenic acid is considered a fundamental marker of dairy products, since it is a specific key intermediate of the RBH processes. Moreover, a high proportion (up to 75%) of this CLA is formed by the unsaturation of *trans*-vaccenic acid (trans11-18:1) operated by Δ-9 desaturase in the mammary gland (Chilliard et al., 2007). Generally, rumenic acid content in dairy products increases with increasing values of F/C ratio (Chilliard et al., 2007; Tudisco et al., 2014). In **Trial 3** presented in this thesis, rumenic acid was found significantly higher in cow milk samples collected in the summer season, quite two times higher if compared to milk collected during the winter season. This phenomenon can be related to the higher PUFA intake occurred during the summer in both the farms, due to the grazing practice (farm A) and the consumption of fresh grass in the barn (farm B). The highest amount was detected in samples from farm B, even if the diet in this farm was associated to higher proportion of concentrates compared to farm A. Actually, it has to be specified that milk CLA composition in cows led to pasture can be very variable, due to inconstant pasture composition and to the influence of the different botanical origin of meadows. Furthermore, the dietary factors influencing the CLA concentration in milk (mainly, the availability of α -linolenic and linoleic acid as precursors, or the nature of forages and starchy sources of feedstuffs) are known to be interrelated and this interactions have been associated to variations (up to 4%) of CLA in pasture milk (Chilliard et al., 2006).

The results discussed until now assume an important role in the description *authenticity* properties of the dairy products analysed, since the FA introduced above have been proposed as specific markers of the livestock system employed in the dairy farms. Furthermore, most of these compounds are well-known indicators of the *nutritional quality* of milk and derived products. Particularly, BCFA and rumenic acid are considered important bioactive components which intake appears relevant for human health (Ran-Ressler et al., 2011) because of their positive role in the gastrointestinal microbial ecology and their cytotoxicity activity toward tumor cells (Hanus et al., 2018). Similarly, oleic acid was demonstrated to possess positive effects on consumers health, related to its anti-atherogenic properties, its positive effect on cholesterol level and anti-inflammatory properties, playing a pivotal role in the management of the oxidative stress (Piccinin et al., 2019). Moreover, the n6/n3 ratio, can be employed as lipid quality index. Actually, n6- and n3- series FA are essential fatty acids (EFA) for humans, with peculiar and not inconvertible functional roles, showing opposite physiological functions toward cardiovascular diseases, cancer, inflammatory and autoimmune diseases and neural development. Thus, their balance is considered fundamental in order to assure their biochemical efficiency in human health (Simopoulos, 2010). It

has been estimated that modern diets can reach n6/n3 ratios ranging from 10 to 20, whereas the target ratio, suitable to obtain a functional activity toward the prevention of the above mentioned diseases, should be from 1 to 2 maximum (Simopoulos, 2010). In all the trials introduced in this thesis, the n6/n3 ratio showed significant differences comparing samples collected in different farms related to the different production systems. Mountain milk analysed in **Trial 3** showed the fewer differences for the n6/n3 ratio among different seasons and different farms, reaching values in a range from 2.20 (in summer grass-based milk) to 3.98 (in winter milk from cows integrated with more concentrates). However, the values recorded for this ratio in trial 3 can be considered favourable independently by the season and the farm. Actually, international nutritional organizations have recommended an advisable n6/n3 ratio in human diet ranging between 5 and 10 (Ma et al., 2016) and all milk samples analysed in **Trial 3** were widely above this values. On the contrary, the highest and less favourable values (up to 6.53) were recorded in goat products (**Trial 1** and **Trial 2**) collected in the conventional low-land farm, where goats were fed with standardized ration all over the year, including the highest amounts of concentrates and, thus, the lowest F/C ratio. However, in these trials, the characteristic pattern of goat dairy products for the presence of caproic, caprilyc and capric acid (respectively, 6:0, 8:0 and 10:0) was evidenced. This outcome adds further knowledge about the nutritional quality of such products, being these FA related to unique metabolic activity for the consumers, providing direct energy instead of being deposited in the adipose tissue (Haenlein, 2004).

The discussion made upon the outcomes obtained by the chromatographic analysis were supported by the employment of multivariate statistical methods. Particularly, PCA was employed in all the trials as data-reduction technique, in order to identify the variables that affected more strongly the discrimination of the samples collected in different farms. The overall FA pattern analysed in the multivariate methods showed to be influenced by the production system in all the trials. Particularly, in **Trial 1** and **Trial 2**, it was possible to identify many class of FA as markers of origin related to the production system, namely 'low-input factors', such as OBCFA and n3 FA, and 'high-input factors', such as n6 FA and, particularly, linoleic acid.

Furthermore, in **Trial 2**, a combined PCA-LDA approach allowed the construction of a classification model able to discriminate cheese samples collected from different farms based on a set of covariates related to the FA profile of the milk previously analysed. Among the outcomes obtained by the application of the multivariate techniques in these trials, it is interesting to underline that few differences were observed between groups of samples related to slight differences in the production systems and in the feeding strategy employed. Moreover, it has been observed than samples collected in the farms employing less controlled or standardized feeding strategies (as the partial or total employment of the pasture) showed a higher variability in their FA composition, most likely due to the seasonal and compositional variations and quality of feed in pasture and grass-based feeding system. On the contrary, the more standardized conditions employed in the high-input farm (in **Trial 1** and **Trial 2**) and in the farm employing the total mixed ration as feeding supply (farm B in **Trial 3**), led to fewer variations of the FA pattern of dairy products and, thus, a more homogeneous lipid quality during the sampling time.

Figure 3. Illustrative picture of the organic-certified goat farm where milk and cheese analysed in **Trial 1** and T**rial 2** were collected. Photo by Annalaura Lopez.

The outcomes obtained in this section of this thesis provided interesting information about authenticity and quality aspects on dairy products collected in small-scale farms of northern Italy. Particularly, positive results obtained by the employment of grass-based and low-input livestock systems were evidenced, helping the scientific community to preserve the common positive perception of consumers toward such kind of foodstuffs. This can be considered of primary importance because the renewed interest of the community toward dairy products associated to an added value (mountain, grass-based, organic, etc.) requires a continuous support based on scientific evidence.

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Chapter 7

Authenticity and quality traits in fish products

Sturgeon meat and fish eggs

7.1 Authenticity and quality traits in fish products – *sturgeon meat and fish eggs*. General introduction

Scientific research in the field of aquaculture and fishery aims to develop and employ analytical methods that would allow to improve the identify preservation and the authentication of the products. Fish and seafood products are considered among the food product categories more susceptible to frauds, including species-substitution (high-value species with a cheaper alternative), mislabelling and others (Hassoun et al., 2020). Chromatographic techniques can be usefully employed in *food authentication* studies on fish and seafood products. Such an example, fatty acids (FAs) profiling emerged as a useful tool to discriminate among samples with different origin, in terms of species, production system, feeding strategies, in fish and fish roes products (Czesny et al., 2000; DePeters et al., 2013; Gessner et al., 2002; Gladyshev et al., 2018; Taşbozan et al., 2016; Vasconi et al., 2019).

FAs pattern on fish flash can provide information toward its origin directed to the investigation of the production methods, especially if coming from aquaculture or caught in the wild. Actually, it has been proved several times that the FA analysis provide information with a great discrimination power toward the production system in several fish species (Busetto et al., 2008; Gladyshev et al., 2018; Lenas et al., 2011; Molkentin et al., 2015; Strobel et al., 2012; Taşbozan et al., 2016; Vasconi et al., 2019). Particularly, linoleic acid, the main precursor of the n6 series FA, allowed the discrimination among wild and farmed specimens, being highly represented in vegetable raw materials included in commonly used aqua-feed formulations. Moreover, Strobel et al., (2012) evidenced a species-specific variation in the FA profile, allowing the identification of many species (oily sea fish) as associated to a higher nutritional value. The variation in the FA profile among different fish (species and individuals) is related to the feeding habits and the food availability in the natural environment, the season of catch and other factors such as the water temperature and salinity. Han et al. (2021) analysed the FA profile of the phospholipid fraction in salmonids by means of GC-FID and evidenced lower content of saturated FA and higher contents of monounsaturated FA in sea-water cultured fish compared to the freshwater cultured counterpart.

Authentication is a primary importance issues in the case of sturgeon products, which list among the foodstuffs more susceptible to food adulteration frauds. In 2015, Operation Opson V, a joint INTERPOL-EUROPOL initiative, led to the global seizure of more than 11,131 tonnes of potentially harmful products ranging from every day products to luxury good, such as caviar (INTERPOL - EUROPOL, 2016). Caviar is defined as the product made from fish eggs of the *Acipenseridae* family by treating with food grade salt (Codex Standard, 2013). Recent years have been characterized by the availability of caviar on the market from several species and from different sources. Subsequently to the sturgeon-preserving program of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), caviar aquaculture industry has enormously increased all over the world, with a special interest in those species that produce high quality caviar (Boscari et al., 2014). According to FAO data, current production of caviar in aquaculture largely exceeds production from wild populations (Bronzi et al., 2019).

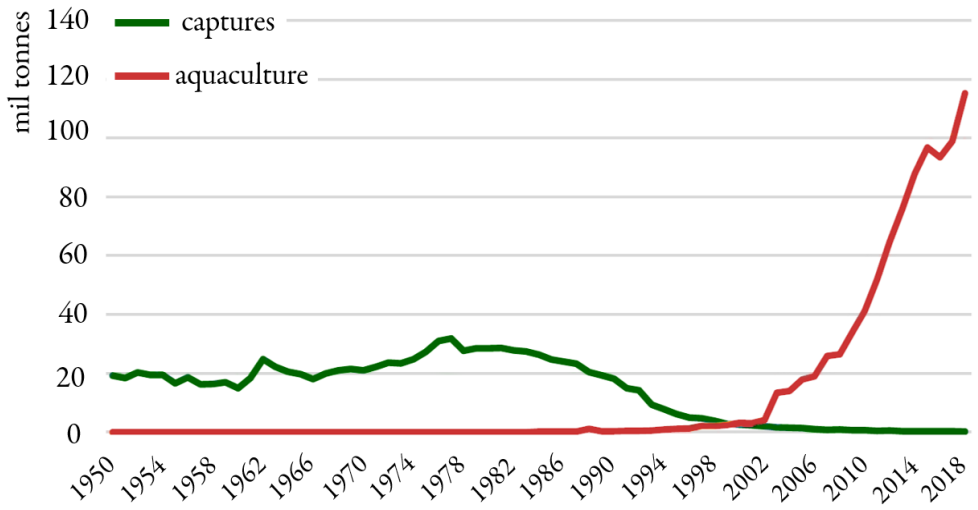

Figure 1. Capture and aquaculture of sturgeons from 1950 to 2018, adapted from EUMOFA report (2021).

In 2018, European sturgeon farmers produced about 164 tons of caviar, with Italy as production leader with 54 tons, followed by France, Poland and Germany (EUMOFA, 2021). At the same time, the import and the export exchanges among different countries have raised up and a black market has developed, due to the high profit linked to caviar trade (van Uhm & Siegel, 2016). The most representative types of illegal trade of caviar are, inter alia (FAO, 2018):

- the trade of caviar with label not in compliance with CITES requirement, or falsified CITES documents;
- caviar obtained by poaching from wild stock and sold off the books;
- wild sourced caviar from endangered sturgeon species traded after mislabelling as aquaculture derived species;
- aquaculture derived products deliberately declared as wild sourced and sold at higher price.

Processed fish roes are acquiring considerable importance in the modern food market, entering more and more often as an ingredient in food preparation. However, eggs obtained from different species of fish are known to have different market values, with caviar representing the most precious one. The success achieved in the production of caviar obtained from aquaculture has gradually promoted the study of its chemical characteristics, mainly aiming to compare the quality of caviar products with different origin (aquaculture versus fisheries, or caviar from different species) (Bledsoe et al., 2003; Czesny et al., 2000; DePeters et al., 2013; J. Gessner et al., 2008; Ovissipour & Rasco, 2011). Generally, caviar represents a food product characterized by a high nutritional profile, as it is rich in essential fatty acids (EFAs) and high biological value proteins (DePeters et al., 2013; Hamzeh et al., 2015; Mol & Turan, 2008). Despite the increasing importance of fish roe products alternative to caviar in the international market, there is a little technical information available about their chemical composition and quality attributes. In such a scenario, it comes forward how much important the development and the employment of reliable analytical techniques could be in order to develop a robust system of authenticity and integrity assurance within the caviar market. The qualitative evaluation of the product could represent a

very useful instrument for the protection of the market. The actual situation is characterized by an increase of the production of caviar incoming from aquaculture, with a reduction in producers profits (Bronzi et al., 2019). Moreover, the rapid growth in Chinese caviar production has increased the competition in the international caviar market. In the major importing markets, increased imports from China has led to a huge decrease in the average prices. For example, in the EU market the average import price decreased by almost 40% from 430 €/kg in 2014 to 264 €/kg in 2018 and the export price of 538 €/kg in 2014 decreased by 22% to 422 €/kg in 2018 (EUMOFA, 2021). Thus, market diversification, according to different products and prices, as well as different species and processing technology, appears primary in order to protect the business.

In the studies presented in chapter 7 of this thesis, the aim was to define quality and authenticity issues related to sturgeon meat and processed fish roe from different species by means of chromatographic analytical techniques. In a first attempt, FAs analysis by GC-FID method was employed to:

a) evaluate the lipid composition of meat and roes from three sturgeon species reared in an Italian production plant, in order to characterize the product and to support their appreciation on the market;
b) achieve a deeper knowledge of the chemical composition of several fish roe products from different species (sturgeon and not-sturgeon) collected on the Italian market; and
c) evaluate if a discrimination was achievable between different products (different species of origin) by means of the application of chemometric tools to the outcomes obtained by FA analysis.

Secondly, the research aim focused on the volatile and non-volatile metabolic profiling as a tool of quality and authenticity assurance in caviar from white sturgeon (*A. transmontanus*), representing the species most intensively reared for caviar production in Italian aquaculture plants. Chromatographic techniques coupled to mass spectrometry detection were employed in this step. Two lines of investigation were developed in order to characterize the volatolome and the metabolome of *A. transmontanus* caviar and particularly:

a) because of the primary importance of the sensory characteristic related to the flavour profile for consumers acceptance of caviar products, a GC-MS approach was firstly developed and employed to characterize the volatile profile of caviar treated with different preservative agents during the optimal and an extended ripening time;
b) secondly, a UHPLC-HRMS method was optimized and applied in a untargeted metabolomic approach, in order to characterize the non-volatile metabolome of caviar collected at different times of ripening.

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Trial 4

Sturgeon Meat and Caviar Quality from Different Cultured Species

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Abstract. Sturgeon raw eggs, caviar and meat obtained from different species reared in an Italian production plant were evaluated for their chemical composition, in order to improve their appreciation on the market and to detect any eventual distinctness related to the species. Mainly, fatty acid (FA) profile of eggs and caviar, determined by Gas-Chromatography coupled to Flame Ionization Detection, showed variability in the interspecific comparison, highlighted by chemometric methods (Linear Discriminant Analysis). Generally, all samples showed a prevalence of unsaturated fatty acids with respect to saturated ones, reaching a content of polyunsaturated fatty acids (PUFA) between the 40% and the 50% of total FA. A remarkable presence of n3 series PUFA was detected in all samples and a selective deposition of many FA into eggs' cellular membranes, yolk lipid and body fat reserves, imputable to the different biological role of single FA during sturgeon reproduction, was evidenced. Chemical composition of sturgeon flesh samples evidenced a high-protein and medium-fat content, characterized by a FA profile of high nutritional value. Moreover, color parameters (redness, yellowness, brightness, Chroma) were measured on sturgeon fillets, showing many species-specific characteristics of sturgeon meat.

Results from this investigation have been published in the scientific journal *Fishes*

Fishes **2020**, 5, 9; doi:10.3390/fishes5010009

1. Introduction

Sturgeons are fish species belonging to the *Acipenseridae* family originally distributed throughout the Northern hemisphere. In Europe, they inhabited mainly in the large river systems of the Ponto-Caspian region and the Black, Azov and the Caspian seas. The construction of barriers and dams on rivers during the years has largely contributed to prevent the migration of these species during the reproductive season, as they live in brackish waters and migrate to freshwater rivers for spawning. Moreover, the realization of quarries for the extraction of construction materials and water pollution have altered typical habitats dedicated to sturgeons' reproduction. Furthermore, the over-exploitation of the natural resources for caviar production has contributed to a worldwide decline of fish stocks. Thus, sturgeons species are now listed in Annex I and II of the Convention on International Trade in Endangered Species (CITES) and they are protected from the over-exploitation in all range states, with an exception for some Countries (mainly, Russia, Iran, Kazakhstan, Canada, USA) (Paolo Bronzi et al., 2019) where legal fisheries for limited quantities are still allowed. In 1869, the artificial reproduction of sturgeon used to support the natural populations began, with the pioneering work of Ovsyannikov on Sterlet (*A. ruthenus*) in USSR. The basic techniques and practices of artificial reproduction of sturgeon were then developed in the 1940's and 50's by Stroganov (Chebanov & Billard, 2001).

Only in recent decades specific farming techniques for sturgeons have been established, and under the strong pressure of market demand for caviar, the sturgeon farming industry has increased (P. Bronzi et al., 2011; P. Bronzi & Rosenthal, 2014; Paolo Bronzi et al., 2019), so that, since the end of the 20th century, it has been the fastest growing aquaculture sector. As recently reported by Bronzi et al. (Paolo Bronzi et al., 2019), a total of 2329 commercial sturgeon farms were recorded by 2017, with an increase of 7% compared to 2016. Nowadays, sturgeon farming exceeds fisheries, with China as the worldwide leader (79,638 tons (t) of sturgeon biomass production in 2017), followed by Russia (6800 t), Armenia (6000 t) and Iran (2514 t). Italy is the European leader in sturgeon farming, with a biomass production of 850 tons in 2017 (Paolo Bronzi et al., 2019). In Europe, sturgeon farming started as a meat-oriented production, but nowadays it has mainly shifted to caviar production, representing a more profitable source of income. On the contrary, in other countries such as China, sturgeon farming is still mainly oriented toward meat production. It has been estimated that global caviar production from aquaculture recorded amounts around 364 tons in 2017 (Paolo Bronzi et al., 2019). In this context, Italy is the first European producer, achieving a remarkable caviar production of 43 tons in 2017, being second just to China and Russia (Paolo Bronzi et al., 2019). CITES trade data (2018) reported that Italy exports both meat and caviar from many species (CITES, 2018) and the most commonly farmed sturgeon species in Italy are White sturgeon (*Acipenser transmontanus*), Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon (*Acipenser baerii*), Adriatic sturgeon (*Acipenser naccarii*), Beluga sturgeon (*Huso huso*) and Stellate sturgeon (*Acipenser stellatus*) (Parisi et al., 2014). Caviar and meat obtained from different species of sturgeons are known to have different market values. The most renowned and valuable caviars are named Beluga, from *H. huso*, Osetra, from *A. gueldenstaedtii*, and Sevruga, from *A. stellatus* and *A. persicus*. On the other hand, according to the producers' opinion, the highest quality for sturgeon meat is obtained from *A. transmontanus* sturgeons. However, sturgeon meat is still considered a secondary and/or by-product of caviar in Italy, where consumers

seem to avoid it, likely because they are not familiar with this product. Thus, at present, most of the sturgeon meat produced in Italy is frozen and sold abroad, especially in the Eastern European and Russian markets, where consumers actually appreciate this product, being more accustomed with it.

The success achieved in the production of caviar obtained from aquaculture has gradually promoted the study of its chemical characteristics, mainly aiming to compare the quality of caviar products with different origin (aquaculture versus fisheries, or caviar from different species). Several studies have been carried out on the determination of fatty acid (FA) profile, proximate composition, volatile compounds and amino acid profile of caviar (Bledsoe et al., 2003; Caprino et al., 2008). The chemical composition of caviar is influenced by various factors, such as the species, the age of the sturgeon, the stage of maturation of the eggs at the time of harvest, the type of diet and the origin of animals (Bledsoe et al., 2003; Czesny et al., 2000; DePeters et al., 2013; Gessner et al., 2008; Ovissipour & Rasco, 2011). In general, caviar is a food characterized by a high nutritional profile, as it is rich in essential fatty acids (EFAs) and high biological value proteins (DePeters et al., 2013; Hamzeh et al., 2015; Mol & Turan, 2008). The chemical quality of meat obtained from different sturgeon species (Anna Badiani et al., 1997) or hybrids (Vaccaro et al., 2005) of different ages and weights (Paleari et al., 1997) has been investigated during past years. All the authors found that the meat of farmed sturgeons contained variable amounts of lipid, depending on the species and the size of specimens. Protein content revealed a high biological value, depending on the amino acid composition. Fatty acid composition of sturgeon meat resulted in valuable omega-3 fatty acid contents, especially eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), mirroring the FA composition of the diet (commercial or experimental).

Thus, the aim of this study was to evaluate the composition (chemical and lipid composition, color parameters) of caviar and meat obtained from different species of sturgeon reared in an Italian production plant, in order to characterize these precious products and to promote them on the market.

2. Results

2.1. Proximate and Fatty Acid Composition of Eggs and Caviar

The proximate composition of raw eggs and caviar obtained after six months of can maturation, from three sturgeon species (*A. baerii*, *A. gueldenstaedtii* and *A. transmontanus*), is presented in Table 1. In Table 1, we can see that in egg samples, moisture content resulted higher in Siberian sturgeon eggs (59.5%), followed by White sturgeon (57.1%) and Russian sturgeon (53.9%) eggs. Protein level, indeed, was practically constant, ranging from 23.8% to 24.9%, without differences among species. Lipid content was higher in Russian (19.7%) and White (16.1%) sturgeon eggs than in Siberian sturgeon (14.9%) eggs. Ash did not show significant differences.

Table 1. Proximate composition (g/100 g) of fresh egg samples, before salting and canning, and of caviar samples, after six months of can maturation, from different sturgeon species. Data are expressed as g/100 g, mean ± standard deviation.

	EGGS			CAVIAR		
Species	Siberian *A. baerii*	Russian *A. gueldenstaedtii*	White *A. transmontanus*	Siberian *A. baerii*	Russian *A. gueldenstaedtii*	White *A. transmontanus*
N	5	5	5	8	12	5
Moisture	59.5 ± 0.9 ^{C}	53.9 ± 2.0 ^{A}	57.1 ± 1.3 ^{B}	57.3 ± 2.5 ^{B}	52.7 ± 2.2 ^{A}	54.6 ± 0.9 ^{A,B}
Protein	23.8 ± 0.8	24.1 ± 1.0	24.9 ± 0.6	23.9 ± 2.2	24.7 ± 1.2	24.0 ± 1.3
Lipid	14.9 ± 0.9 ^{A}	19.7 ± 1.8 ^{B}	16.1 ± 1.2 ^{A}	14.9 ± 0.7 ^{A}	19.1 ± 3.2 ^{B}	17.7 ± 1.2 ^{A}
Ash	1.9 ± 0.8	2.3 ± 0.6	1.9 ± 0.1	3.9 ± 0.3	3.5 ± 0.8	3.7 ± 0.3

^{A,B,C} = Different superscripts within a column indicate significant differences ($p < 0.05$).

Regarding caviar composition, our results were comparable with those previously reported in literature about caviar from farmed sturgeons (Caprino et al., 2008; Mol & Turan, 2008; Wirth et al., 2000). The protein content did not show significant differences between the examined species, ranging between 23.9% and 24.7%, according to the results of our previous studies (Caprino et al., 2008). Conversely, lipid content showed some significant differences, with *A. gueldenstaedtii* caviar significantly fatter (19.1%) than *A. baerii* (14.9%) and *A. transmontanus* (17.7%) caviar. Comparing raw eggs and caviar composition for each species, these two matrices differed for ash and moisture content. It can be suggested that a reasonable loss of moisture and an increase of ash occurred during caviar maturation, due to salt addition and to ripening processes.

Fatty acid composition of raw eggs and caviar in the three sturgeon species under investigation is shown in Table 2.

Table 2. Fatty acid profile (g/100 g of fatty acids) of fresh egg samples, before salting and canning, and caviar samples, after six months of can maturation, from three different sturgeon species. Data are expressed as g/100 g, mean ± standard deviation.

	EGGS				CAVIAR			
Species	Siberian *A. baerii*	Russian *A. gueldenstaedtii*	White *A. transomntanus*		Siberian *A. baerii*	Russian *A. gueldenstaedtii*	White *A. transomntanus*	
	N= 5	N= 5	*N=5*	*sign*	*N=8*	*N=11*	*N=12*	*sign*
14:0	0.9 ± 0.1 ^{B}	1.3 ± 0.1 ^{A}	1.4 ± 0.1 ^{A}	**	0.9 ± 0.1 ^{C}	1.2 ± 0.1 ^{B}	1.4 ± 0.2 ^{A}	**
16:0	18.7 ± 3.3	16.5 ± 0.6	17.4 ± 0.6	ns	17.5 ± 0.7	17.0 ± 1.2	16.6 ± 0.8	**ns**
18:0	3.3 ± 0.6	3.3 ± 0.3	3.1 ± 0.2	ns	3.2 ± 0.1 ^{A,B}	3.4 ± 0.4 ^{B}	3.0 ± 0.3 ^{A}	*
ΣSFA	22.9 ± 4.0	21.0 ± 0.6	21.9 ± 0.7	ns	21.5 ± 0.8	21.6 ± 1.0	21.1 ± 0.8	**ns**
16:1n7	3.6 ± 0.8	3.5 ± 0.2	3.3 ± 0.3	ns	3.5 ± 0.3 ^{A,B}	3.6 ± 0.4 ^{B}	3.3 ± 0.3 ^{A}	*
18:1n9	24.9 ± 11.6	24.6 ± 0.9	29.4 ± 1.4	ns	28.4 ± 2.5 ^{C}	25.4 ± 1.1 ^{B}	30.6 ± 1.3 ^{A}	**
18:1n7	2.3 ± 1.2	2.3 ± 1.3	3.0 ± 0.2	ns	3.0 ± 0.2 ^{B}	3.0 ± 0.1 ^{B}	3.2 ± 0.2 ^{A}	**
20:1n9	1.5 ± 0.4 ^{A}	0.8 ± 0.1 ^{B}	1.3 ± 0.1 ^{A}	**	1.3 ± 0.2 ^{A}	1.0 ± 0.2 ^{B}	1.4 ± 0.2 ^{A}	**
ΣMUFA	32.3 ± 11.7	31.3 ± 1.6	36.9 ± 1.1	ns	36.4 ± 2.4 ^{C}	33.2 ± 1.4 ^{B}	38.7 ± 1.3 ^{A}	**

Table 2 (cont)

18:2n6	13.4 ± 2.3 ^{A}	20.6 ± 0.6 ^{B}	14.2 ± 0.8 ^{A}	**	12.8 ± 1.0 ^{A}	16.8 ± 3.9 ^{B}	13.1 ± 1.4 ^{A}	**
18:3n6	1.5 ± 0.1 ^{B}	0.9 ± 0.2 ^{A}	0.8 ± 0.1 ^{A}	**	1.2 ± 0.2 ^{B}	0.6 ± 0.5 ^{A}	0.7 ± 0.6 ^{A,B}	*
18:3n3	2.0 ± 0.3	2.1 ± 0.1	1.8 ± 0.1	ns	1.8 ± 0.2	1.8 ± 0.3	1.8 ± 0.2	*ns*
20:2n6	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	ns	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	*ns*
20:3n6	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	ns	0.4 ± 0.1	0.2 ± 0.2	0.3 ± 0.2	*ns*
20:4n6	2.3 ± 0.3 ^{A}	1.7 ± 0.2 ^{B}	2.3 ± 0.2 ^{A}	**	2.1 ± 0.2 ^{C}	1.8 ± 0.2 ^{B}	2.4 ± 0.2 ^{A}	**
20:3n3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	ns	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	*ns*
20:5n3	5.6 ± 1.3 A ^{B}	6.5 ± 0.6 ^{B}	4.7 ± 0.7 ^{A}	*	5.4 ± 1.2 ^{A}	6.5 ± 0.6 ^{B}	5.0 ± 0.6 A	**
22:5n6	0.2 ± 0.0 ^{B}	0.2 ± 0.0 ^{B}	0.3 ± 0.0 ^{A}	*	0.2 ± 0.0 ^{B}	0.1 ± 0.1 ^{A}	0.1 ± 0.1 A,^{B}	*
22:5n3	1.4 ± 0.4 ^{B}	1.9 ± 0.1 ^{A}	1.6 ± 0.3 A ^{B}	*	1.5 ± 0.4 ^{A}	1.9 ± 0.2 ^{B}	1.6 ± 0.2 ^{A}	**
22:6n3	15.7 ± 3.2 ^{B}	11.2 ± 0.3 ^{A}	12.9 ± 1.0 A ^{B}	*	14.0 ± 0.7	13.0 ± 1.6	12.6 ± 1.0	*ns*
ΣPUFA	44.8 ± 7.8	47.7 ± 1.2	41.2 ± 1.6	ns	41.6 ± 2.0 ^{A}	44.9 ± 2.4 ^{B}	39.9 ± 1.2 ^{A}	**
Σn3	26.1 ± 5.2	23.0 ± 0.9	22.1 ± 1.4	ns	23.9 ± 1.9 ^{B}	24.4 ± 1.4 ^{B}	22.2 ± 1.0 ^{A}	**
Σn6	18.5 ± 2.8 ^{A}	24.4 ± 0.7 ^{B}	18.8 ± 0.4 ^{A}	*	17.4 ± 0.8 ^{A}	20.1 ± 3.2 ^{B}	17.2 ± 0.7 ^{A}	**
n3/n6	1.4 ± 0.1 ^{A}	0.9 ± 0.0 ^{B}	1.2 ± 0.1 ^{C}	*	1.4 ± 0.1	1.3 ± 0.3	1.3 ± 0.1	*ns*

^{A,B,C}= values in the same row for each product (eggs and caviar) with a different letter are significantly different, * = $p < 0.05$ ** = $p < 0.01$. SFA= Saturated Fatty Acids; MUFA= Monoinsaturated Fatty Acids; PUFA= Polyinsaturated Fatty Acids

The fatty acids profile detected in our samples matched with the general pattern reported in literature for fatty acid composition of sturgeon eggs and caviar (Caprino et al., 2008; Czesny et al., 2000; DePeters et al., 2013; Gussoni et al., 2006; Mol & Turan, 2008; Ovissipour et al., 2015; Wirth et al., 2000). Among the saturated fatty acids (SFA), palmitic acid (16:0) proved to be the most abundant in both eggs and caviar samples, regardless of the species considered, according to the results of several previous publications (Caprino et al., 2008; Czesny et al., 2000; DePeters et al., 2013; Ovissipour & Rasco, 2011; Shin et al., 2010). The main monounsaturated fatty acid (MUFA) was represented by oleic acid (18:1 n9) in all samples and many significant differences emerged among caviar samples, with White sturgeon caviar showing the highest value (30.6%), followed by Siberian sturgeon (28.4%) and then Russian sturgeon (25.4%) caviar. There is a general agreement in arguing that oleic acid is the most abundant fatty acid in sturgeon eggs, regardless of their origin and species, among the scientific literature available in the field (Caprino et al., 2008; Czesny et al., 2000; DePeters et al., 2013; Ovissipour & Rasco, 2011; Shin et al., 2010). This is not surprising, since it is well known that oleic acid represents the main source of energy during embryonic development in ova (Henderson, 1996), even if a recent study has shown how its content could decrease with the age of sturgeon (Shin et al., 2010). Considering the polyunsaturated fatty acids (PUFA) of the n6 series, the predominant one was linoleic acid (LA, 18:2 n6) in all samples, ranging from 13.4% to 20% in eggs and from 12.8% to 16.8% in caviar. In both cases, significant differences were observed among the different species under investigation. The high content of LA in sturgeon eggs and caviar is supported by literature data, which show a high percentage of linoleic acid (significantly greater than in wild samples) in caviar from cultured sturgeon, mainly due to the lipid composition of commercial diets (Bledsoe et al., 2003; Caprino et al., 2008; Chen et al., 1995; Ovissipour & Rasco, 2011; Wirth et al., 2002). It is well known that linoleic acid is widely represented in oils of vegetable origin (such as soy oil, sunflower oil, rapeseed oil, corn oil) commonly used in commercial feed as an alternative lipid source, to partial replacement of fish oils, more expensive and less eco-sustainable. For this reason, some authors previously reported that a high LA content can be used as an indicator of the origin of wild versus farmed caviar (Czesny et al., 2000). Arachidonic acid (ARA, 20:4 n6) was the second most abundant n6 series fatty acid, ranging from 1.7% to 2.3% in eggs and from 1.8% to 2.4% in caviar samples, with some differences among the species. As well as for LA, the level of ARA in caviar depends on the feed source supplied to sturgeons. Although ARA is the second most predominant among the n6 PUFA, our results showed significantly lower amounts than what is described in literature for wild caviar (Chen et al., 1995; Gessner et al., 2008; Ovissipour & Rasco, 2011; Wirth et al., 2002). This can be imputed to the fact that ARA is highly present inside the aquatic trophic chains, especially in some algae and in some crustaceans (Das, 2006) that represent the natural diet sources of wild sturgeons. Among the n3 series polyunsaturated fatty acids, the analyzed samples showed a clear prevalence of eicosapentaenoic acid (EPA, 20:5 n3) and docosahexaenoic acid (DHA, 22:6 n3) in all the species. In both the matrices, Russian sturgeon samples resulted the most enriched in EPA (6.5%), whereas Siberian sturgeon eggs and caviar showed the highest amount of DHA (15.7% and 14.0%, respectively). The selective deposition of EPA and DHA in caviar resulted to be high in any case, regardless of the type of diet supplied to fish. Caprino et al. (2008) reported similar concentrations of EPA and DHA in caviar from sturgeons fed with two different experimental diets, one containing squid oil and one soybean oil as the main lipid source. Similar values of EPA and DHA have also been demonstrated in caviar

of wild and farmed sturgeons, confirming the low influence of diet composition on the accumulation of these two fatty acids in fish eggs (DePeters et al., 2013; Wirth et al., 2002). A positive correlation between the EPA and DHA content in fish eggs and some reproductive parameters, such as the fertilization and hatching rate (Yanes-Roca et al., 2009), have been also highlighted and these FA are known to play an important role in the embryonic development of fish (Bell et al., 1996; Bell et al., 1995; Lee, 2001).

In order to evaluate if a discrimination of samples from different species based on their fatty acid composition was feasible, a multivariate statistical method was developed and employed. Since no great differences were found in fatty acids composition of raw eggs and caviar within the same species, the *Acipenser* species was considered as the only discriminant factor, and eggs and caviar from the same species were considered as a unique group. In Figure 1, the Canonical Plot of the Linear Discriminant Analysis (LDA) performed on fatty acids data is shown. In Table 3, the results of analysis of variance (ANOVA) performed on Canonical1 and Canonical2 scores as factors among groups are shown.

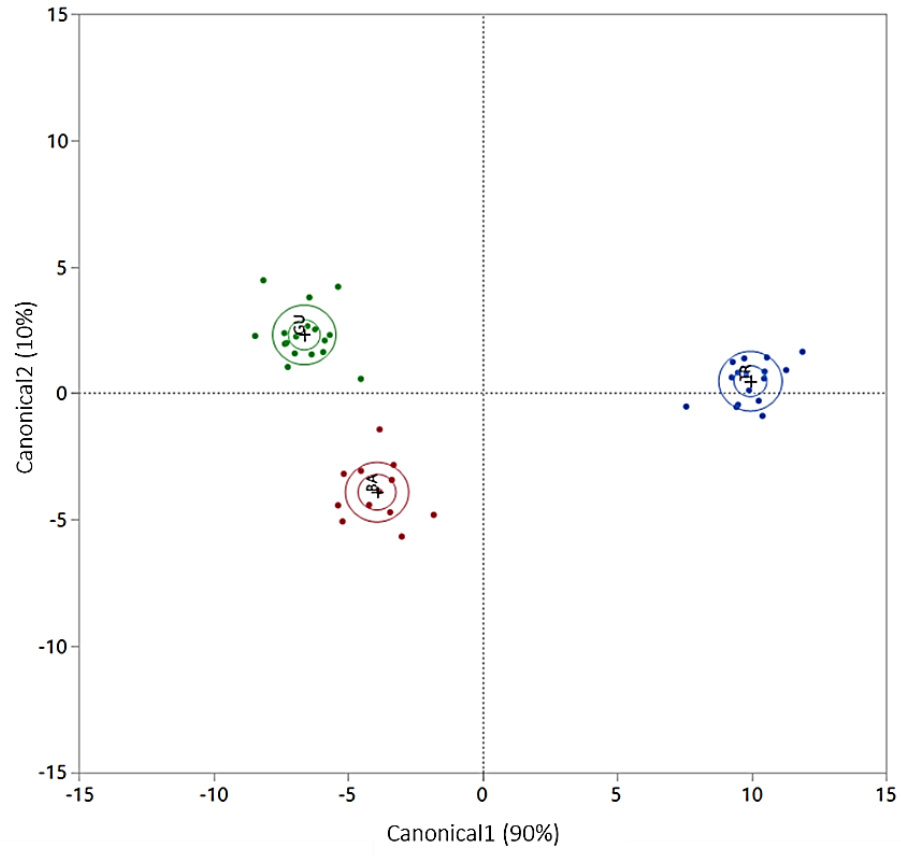

Figure 1. Canonical Plot of the Linear Discriminant Analysis (LDA) performed on eggs and caviar samples, using the sturgeon species as a discriminant factor. In the plot, each group is associated with a 95% confidence ellipse for the mean and a 50% prediction ellipsoid. Legend: green ● = *A. gueldenstaedtii* eggs and caviar, blue ● = *A. transmontanus* eggs and caviar, red ● = *A. baerii* eggs and caviar.

Table 3. Analysis of variance (ANOVA) by groups on first (C1) and second (C2) canonical functions obtained by LDA analysis performed on fatty acids data.

EGGS and CAVIAR				
Species	Siberian *A. baerii*	Russian *A. gueldenstaedtii*	White *A. transmontanus*	
N	13	16	17	*sign*
C1	−3.9 ± 1.0 ^{A}	−6.6 ± 0.1 ^{B}	10.0 ± 1.0 ^{C}	**
C2	−3.9 ± 1.2 ^{A}	2.3 ± 1.0 ^{B}	0.48 ± 0.8 ^{C}	**

^{A,B,C} = values in the same row with a different letter are significantly different, ** = $p < 0.01$.

The factors included in the LDA model were selected by a stepwise backward approach. Thus, we employed in the model such variables associated with a $p < 0.05$ by means of analysis of covariance among all the variables. Sixteen fatty acids were selected and included in the arrangement of the discriminant model, allowing 100% of cases in the training set to be correctly grouped. The variables that highly correlated with Canonical1 (correlation loading |>0.5|) were represented by EPA, PUFA, OA, MUFA, 14:0 and ARA, whereas the highest correlation loadings (|>0.5|) between the variables and Canonical2 were associated with 20:1 n9, DHA, LA, DPA and 14:0. As shown in Figure 1 and Table 3, a suitable group separation was obtained in the bi-plot determined by the two canonicals, C1 and C2 ($p < 0.01$). Particularly, Siberian sturgeon eggs and caviar samples (red dots) were characterized by negative values for both C1 and C2, Russian sturgeon eggs and caviar samples (green dots) were characterized by positive values for C1 and negative values for C2, and White sturgeon eggs and caviar samples (blue dots) were characterized by positive values for both C1 and C2.

2.2 Fatty acid composition of lipid fractions

It is well known that fatty acid composition of sturgeon eggs reflects the fatty acids composition of feed supplied to fish. In previous publications, Wirth et al. (2000, 2002) suggested that different factors, including the environment, can influence the lipid deposition in fish tissues. Particularly, the authors proposed that during the gonadogenesis, SFA and MUFA from female fish lipid reserves are mainly catabolized to provide the necessary energy for the pathways involved in eggs synthesis, whereas PUFA, especially long chain n3 fatty acids, are accumulated in eggs' lipids. Thus, they demonstrated that the fatty acid pattern in the different lipid fractions of eggs differed considerably, gathering that they should be estimated separately, even because of their different biological function (Wirth et al., 2000, 2002). For this reason, we analyzed the fatty acids pattern in the individual lipid fractions, phospholipids (PL) and neutral lipids (NL), of egg samples collected in our study. Results are shown in Table 4.

Table 4. Fatty acid (FA) composition (g/100 g of FA) of individual lipid fractions (phospholipids, PL and neutral lipids, NL) of egg samples from different sturgeon species. Data are expressed as mean ± standard deviation.

EGGS									
Species	Siberian *A. baerii*			Russian *A. gueldenstaedtii*			White *A. transmontanus*		
N	5			5			5		
Fraction	PL	*NL*	*sign*	PL	NL	*sign*	PL	NL	*sign*
14:0	2.1±0.5	1.3±0.1		2.3±0.5	1.7±0.2		2.1±0.2	1.9±0.3	
16:0	22.2±10.3	17.5±1.5		21.4±2.3	16.4±3.0		22.3±1.4	17.5±1.5	*
18:0	8.6±3.2	2.2±0.3	*	9.3±0.7	2.6±0.7	**	7.5±0.4	2.5±0.3	**
ΣSFA	32.9±15.1	21.1±2.0		33.0±5.3	20.7±3.9	*	31.9±3.1	21.8±2.0	**
16:1n7	1.8±0.8	4.1±0.3	**	1.9±0.3	4.1±0.8	**	2.0±0.2	3.8±0.4	**
18:1n9	15.8±8.9	32.4±3.5	*	14.8±1.7	25.7±4.8	*	18.4±1.6	31.8±2.6	**
18:1n7	2.2±1.1	2.9±0.3		2.4±0.2	2.9±0.6		2.2±0.3	2.9±0.4	
20:1n9	2.7±1.4	1.2±0.2		2.0±0.5	0.8±0.1	*	2.8±0.6	1.2±0.1	**
ΣMUFA	22.6±12.7	40.5±4.2		21.1±3.0	33.4±6.4	*	25.4±2.8	39.7±3.3	**
18:2n6	4.0±1.1	13.9±0.9	**	5.8±0.5	22.0±4.1	**	4.6±0.6	15.4±2.1	**
18:3n6	1.2±0.2	1.7±0.2	*	1.2±0.1	1.1±0.3		1.1±0.2	1.0±0.1	
18:3n3	1.3±0.2	2.2±0.2	**	1.3±0.1	2.4±0.5	*	1.2±0.2	2.1±0.3	*
20:2n6	1.7±0.4	0.6±0.1	**	1.9±0.1	0.7±0.1	**	1.7±0.2	0.7±0.1	**
20:3n6	1.6±0.4	0.6±0.1	*	1.5±0.1	0.5±0.1	**	1.6±0.4	0.6±0.1	**
20:4n6	3.5±1.2	1.6±0.3		3.0±0.5	1.4±0.5	*	3.9±0.5	1.8±0.2	**
20:3n3	1.0±0.1	0.3±0.0	**	1.1±0.1	0.3±0.0	**	1.0±0.2	0.3±0.0	**
20:5n3	8.4±3.0	4.4±0.8		10.8±1.0	5.9±1.8	*	6.8±0.8	4.0±0.8	*
22:5n6	0.7±0.4	0.2±0.0		0.8±0.4	0.2±0.1		0.8±0.1	0.2±0.1	**
22:5n3	1.4±0.8	1.4±0.2		2.0±0.4	2.2±0.4		1.2±0.3	1.7±0.4	
22:6n3	19.7±7.3	11.5±1.4		16.6±0.9	9.3±2.0	**	18.7±1.1	10.7±1.9	**
ΣPUFA	44.6±14.8	38.5±3.5		45.8± 2.2	45.9±10.1		42.6±3.3	38.5±5.9	
Σ n3	32.4±11.3	20.6±2.6		32.3±1.2	21.0±4.8	*	29.5±2.3	19.7±3.4	*
Σ n6	12.7±3.5	18.9±1.4	*	14.6±1.1	26.1±5.1	*	13.7±1.5	19.9±2.4	*
n3/n6	2.5±0.2	1.1±0.1	**	2.2±0.1	0.8±0.0	**	1.8±0.7	1.0±0.1	**

* = $p < 0.05$, ** = $p < 0.01$. SFA= Saturated Fatty Acids; MUFA= Monounsaturated Fatty Acids; PUFA= Polyunsaturated Fatty Acids

In Table 4, it is shown that the different lipid fractions (PL and NL) noticeably differed from each other if considering their fatty acids composition. In accordance with Gessner et al. (2008), we did not observe considerable interspecific variations in the FA profile of PL but greater variations of the NL fraction. The most interesting results are related to the differences in OA, LA, ALA, ARA, EPA and DHA content between the two fractions within the same species. In all three groups, we found OA, LA and ALA in higher amounts in NL (ranging from 25.7% to 32.4% for OA, from 13.9% to 22.0% for LA and from 2.1 to 2.2% for ALA,) in all cases reaching an amount two times, or more, higher than in the PL fraction. Conversely, we found ARA, EPA and DHA in higher amounts in the PL fraction (ranging from 3.0% to 3.9% for ARA, from 6.8% to 10.8% for EPA and from 16.6% to 19.7% for DHA).

The only divergence in testing significance emerged in the *A. baerii* eggs group, in which no significant differences were detected in EPA and DHA content. This phenomenon could be mainly due to the great variability registered in data distribution for EPA and DHA in the *A. baerii* group (higher standard deviations). However, the n3/n6 ratio resulted in all cases as significantly higher in the PL fractions, where it reached values between 1.8, in White sturgeon eggs, and 2.5, in Siberian sturgeon eggs. Our results matched with literature data (Mukhopadhyay & Ghosh, 2007; Wirth et al., 2000) and they may be explained by the known existing relationship between the sturgeon lipid consumption through the diet and the specific biological function of different fatty acids.

It can be suggested that OA, LA and ALA, highly representative in sturgeon feed formulations, are mainly stocked in the triacylglycerol fraction of oil droplets in yolk, as energy sources for the hatchlings. On the contrary, ARA, EPA and DHA may be particularly representative in the PL fraction because they are implicated in prostaglandine and eicosanoids formation in fish cell membranes, as previously suggested by other authors (Mukhopadhyay & Ghosh, 2007; Prabhakara Rao et al., 2013; Tocher & Sargent, 1984; Wirth et al., 2000). Particularly, it is known that PUFA of n3 series are incorporated into the phospholipid fraction of vitellogenin and transferred via the serum to the eggs during the gonadogenesis (Bransden et al., 2007). EPA and DHA are known to play a primary role in the embryonic development of fish, being fundamental for the development of nervous tissue and for maintaining the structural and functional integrity of cell membranes (Bell et al., 1996; Bell et al., 1995; Lee, 2001). EPA, in particular, would be involved in the modulation of steroidogenesis in the male fish gonad and, therefore, in the regulation of spermatogenesis, influencing the rate of egg fertilization (Ovissipour & Rasco, 2011). Moreover, it is known that the ARA is involved in the embryonic development of fish immune system, thus affecting the survival, development and growth of the fry (Tocher, 2010; Yanes-Roca et al., 2009). In the last decade, the role of the ARA in the diet has received more and more attention, since it has been recognized to lead to potential benefits on the reproductive performances. Bruce et al. (1999) and Mazorra et al. (2003) reported an improvement in the percentage of hatching and larval survival following ARA supplementation in the diet of sea bass (*Dicentrarchus labrax*) and halibut (*Hipoglossus hipoglossus*) breeders. Consequently, in order to optimize the aquaculture production, it would be desirable to decrease the level of linoleic acid and increase the level of arachidonic acid in sturgeon diets, getting as close as possible to the situation of wild fish, improving the growth and the reproduction performances of sturgeons. The FA profiles of eggs detected in this study, represented by a similar phospholipid content among different species, suggested a more conservative

composition of PL in all the involved species. This phenomenon indicated a selective FA inclusion in oocytes during the gonadogenesis and the formation of eggs' membranes.

2.3 Proximate composition and fatty acid composition of sturgeon fillets

Results obtained by proximate composition analysis on sturgeon meat fillets from different breeding species and size are presented in Table 5.

Table 5. Proximate composition (g/100 g) of fillet meat samples from different sturgeon species and size. Data are expressed as mean ± standard deviation.

	FLESH			
Species	Siberian *A. baerii*	White *A. transmontanus*	White *A. transmontanus*	
Farmed for	Caviar production	Caviar production	Meat production	
Gender	Female	Female	Male	
Average fish weight	5–8 kg	30–50 kg	6–10 kg	
N	5	5	5	*sign*
Moisture	75.5 ± 1.6	75.2 ± 3.3	77.7 ± 1.1	
Ash	1.3 ± 0.2	1.2 ± 0.3	1.1 ± 0.0	
Lipid	5.6 ± 1.7	3.9 ± 2.5	2.6 ± 0.8	
Protein	17.6 ± 0.5 ^{A}	19.6 ± 0.8 ^{B}	18.6 ± 0.5 ^{A,B}	**

^{A,B} = values in the same row with a different letter are significantly different, ** = $p < 0.01$, * = $p < 0.05$.

Results obtained by proximate composition analysis on sturgeon meat matched with those of previous studies conducted on *A. baerii* and *A. transmontanus* muscle by other authors (Badiani et al., 1996; Badiani et al., 1997). No great differences were detectable among the groups, with the exception of protein content, observing higher values in female White sturgeon meat (19.6%), followed by the male White sturgeon meat (18.6%) and then by the female Siberian sturgeon meat (17.6%). It is reasonable to suppose that genetic factors are at the basis of the differences observed in protein content, as suggested by Badiani et al. (1997). Lipid values ranged from 2.6% in White sturgeon and 5.6% in Siberian sturgeon. These outcomes confirmed the fact that sturgeon meat can be classified as a medium-fat high-protein product in the scale proposed by Stansby (1976), making it an appealing food for market, containing a restrained fat content with a high nutritional value (essential fatty acids). Moreover, it can be suggested that the medium-fat meat of Siberian sturgeon could be considered suitable for possible processing techniques, such as smoking and canning (Paleari et al., 1997), that usually lead to a greater appreciation of the product by consumers.

Fatty acids composition of sturgeon meat showed a prevalence of unsaturated fatty acids on saturated fatty acids in all samples. Interesting differences were found among different groups. Particularly, meat obtained by the males of White sturgeon seemed to be enriched in PUFA (44.2%) in comparison to meat obtained by female

sturgeons of the same species, designated to the production of caviar (33.9%), and the one obtained by the lightweight caviar-designated females of Siberian sturgeon (35.2%). This outcome allowed us to confirm, as previously mentioned above (Sections 2.1 and 2.2), that the metabolism of female sturgeons operates a selective deposition of fatty acids fundamental for reproduction (ARA, EPA, DHA) in eggs, leading to the stocking of the other FA (mainly represented by OA, LA and ALA) in fish fat deposits. Related to this, we found the highest level of EPA and DHA in male White sturgeon meat (8.6% and 12.3%, respectively), followed by female White sturgeon meat (5.7% and 9.7%) and female Siberian sturgeon meat (3.9% and 7.3%). The high levels of EPA and DHA in analyzed samples should be kept in great consideration, since these fatty acids are strictly related to the nutritional quality of the product, which appears to be characterized by a reduced lipid content of a high nutritional value. Moreover, this could be useful in order to promote sturgeon meat on the market, dismantling its actual consideration as a by-product of caviar and enforcing the activity of sturgeon aquaculture even for meat production.

The n3/n6 ratio ranged from 0.7 to 1.4 with no significant differences, resulting lower than values (around 4) previously reported in literature for *A. transmontanus* and *A. baerii* fillets (Badiani et al., 1996; Badiani et al., 1997; Paleari et al., 1997). It is worth to mention that this difference is highly influenced by the content of LA that, in our study, is four times higher than reported by the above-mentioned authors (11.9%–16.1%). This difference could be easily explained by the modifications which occurred in aquafeed formulations during the last years, in order to contain producers' costs and to increase aquaculture sustainability, substituting fish meals and oils with vegetable ones (Gatlin et al., 2007), enriched in LA. In Badiani et al. (1996; 1997), sturgeon diets, in fact, were only represented by fish meals and oils.

Comparing our data with those of Palmegiano et al. (2005) on Siberian sturgeon meat, it is possible to observe that LA and n3/n6 values obtained in our research perfectly agree with those referred to fish fed with recent commercial diets (control group). MUFA were detected in higher values in meat of Siberian sturgeons (45.5%) and female White sturgeons (42.7%) than in the males of white Sturgeon (31.5%). The most representative FA among MUFA was oleic acid in all samples, ranging from 25% and 37.5%, in accordance with results obtained by previous studies on fatty acids analysis in *A. baerii* and *A. transmontanus* meat (Badiani et al., 1996; Badiani et al., 1997; Paleari et al., 1997; Palmegiano et al., 2005).

Table 6. Fatty acid composition (g/100 g of FA) of fillets from different sturgeon species. Data are expressed as mean ± standard deviation.

	FLESH			
Species	Siberian *A. baerii*	White *A. transmontanus*	White *A. transmontanus*	
Farmed for	Caviar production	Caviar production	Meat production	
Gender	Female	Female	Male	
Average fish weight	5–8 kg	30–50 kg	6–10 kg	
N	5	5	5	*sign*
14:0	1.3 ± 0.1	1.9 ± 0.6	1.7 ± 1.3	ns
16:0	15.5 ± 0.7	17.6 ± 0.8	16.9 ± 1.9	ns
18:0	2.4 ± 0.5 ^{A}	3.9 ± 1.4 ^{A}	5.9 ± 1.2 ^{B}	**
ΣSFA	19.2 ± 0.6 ^{A}	23.4 ± 1.6 ^{B}	24.5 ± 3.3 ^{B}	**
16:1n7	3.3 ± 0.6	3.4 ± 1.1	2.9 ± 2.0	ns
18:1n9	37.5 ± 2.6 ^{A}	34.0 ± 2.3 ^{A}	25.0 ± 6.3 ^{B}	**
18:1n7	2.8 ± 0.1	3.1 ± 0.1	2.9 ± 0.3	ns
20:1n9	1.8 ± 0.1 ^{A}	2.2 ± 0.5 ^{A}	0.5 ± 1.1 ^{B}	**
ΣMUFA	45.5 ± 2.8 ^{A}	42.7 ± 3.5 ^{A}	31.3 ± 5.5 ^{B}	**
18:2n6	16.1 ± 0.3	11.9 ± 0.7	14.1 ± 5.1	ns
18:3n6	1.3 ± 0.4 ^{A}	0.4 ± 0.1 ^{B}	0.4 ± 0.2 ^{B}	**
18:3n3	2.7 ± 0.4 ^{A}	1.6 ± 0.4 ^{B}	1.9 ± 0.8 $^{A\,B}$	*
20:2n6	0.9 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	ns
20:3n6	0.5 ± 0.2 ^{A,B}	0.3 ± 0.0 ^{A}	0.6 ± 0.2 ^{B}	*
20:4n6	1.6 ± 0.4 ^{A}	2.0 ± 0.8 ^{A}	3.6 ± 0.9 ^{B}	**
20:3n3	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.2	ns
20:5n3	3.9 ± 0.8 ^{A}	5.7 ± 0.8 ^{A,B}	8.6 ± 4.5 ^{B}	*
22:5n3	0.9 ± 0.6	1.4 ± 0.3	1.8 ± 0.7	ns
22:6n3	7.3 ± 1.5 ^{A}	9.7 ± 1.9 ^{A,B}	12.3 ± 3.2 ^{B}	*
ΣPUFA	35.3 ± 2.7 ^{A}	33.9 ± 2.1 ^{A}	44.2 ± 2.9 ^{B}	**
Σ n3	15.0 ± 2.2 ^{A}	18.5 ± 1.5 ^{A,B}	24.7 ± 7.4 ^{B}	*
Σ n6	20.4 ± 1.0	15.4 ± 1.2	19.5 ± 5.0	ns
n3/n6	0.7 ± 0.1	1.2 ± 0.1	1.4 ± 0.9	ns

^{A,B} = values in the same row with a different letter are significantly different * = $p < 0.05$, ** = $p < 0.01$

2.4 Color Parameters of Sturgeon Fillets

We evaluated the colorimetric features of collected sturgeon fillets. Results of colorimetric analysis (redness, a*, yellowness, b*, lightness, L* and Chroma, C*) are shown in Table 7 and Figure 2.

Table 7. Color parameters (a*, redness, b*, yellowness, L*, brightness, C*, Chroma) of sturgeon fillets collected in our study. Average values were calculated as mean ± standard deviation of each group. a*, b*, L* values were obtained by two replicates in three different sites of each fillet.

	FLESH			
Species	Siberian *A. baerii*	White *A. transmontanus*	White *A. transmontanus*	
Farmed for	Caviar production	Caviar production	Meat production	
Gender	Female	Female	Male	
Average fish weight	5–8 kg	30–50 kg	6–10 kg	*sign*
a*	−0.86 ± 3.29	−1.98 ± 1.87	−3.27 ± 1.04	
b*	6.41 ± 1.91^{B}	2.99 ± 0.88^{A}	2.56 ± 1.59^{A}	*
L*	57.78 ± 7.85	53.76 ± 2.46	54.56 ± 2.70	
C*	7.15 ± 1.67^{B}	3.97 ± 0.79^{A}	4.36 ± 1.19^{A}	*

^{A,B} = values in the same row with a different letter are significantly different. * = $p < 0.05$, ** = $p < 0.01$.

Color parameters obtained in this study were different from those previously detected in literature in sturgeon meat from other species and hybrids (Ünal Şengör et al., 2010; Wedekind, 2002). Our data showed a negative value for the redness parameter, indicating a tendency to green in all samples analyzed, different to other authors' findings. This phenomenon could be obviously due to the species-specific characteristics of sturgeon meat. b* values were always positive, ranging between 2.56 in White sturgeon to 6.41 in Siberian sturgeon, showing a significantly different tendency to yellow in meat of the two species. L* value refers to the lightness of the meat surface. The detected values covered a range between 50 and 60, comparable to values previously found by Ünal Şengör et al. (2010) and Wedekind (2002), indicating an intermediate position between the dark and the bright appearance in all samples. In the end, we calculated C* value, referring to the Chroma, finding values in a range from 3.97 in White sturgeon, and 7.15 in Siberian sturgeon, with a significant difference between the two species. The bigger the C* parameter is, the more "definite/saturated" the colour is perceived. Our values indicated that all analyzed sturgeon species meat samples were characterized by almost achromatic hues, commonly recognized as different shades of grey.

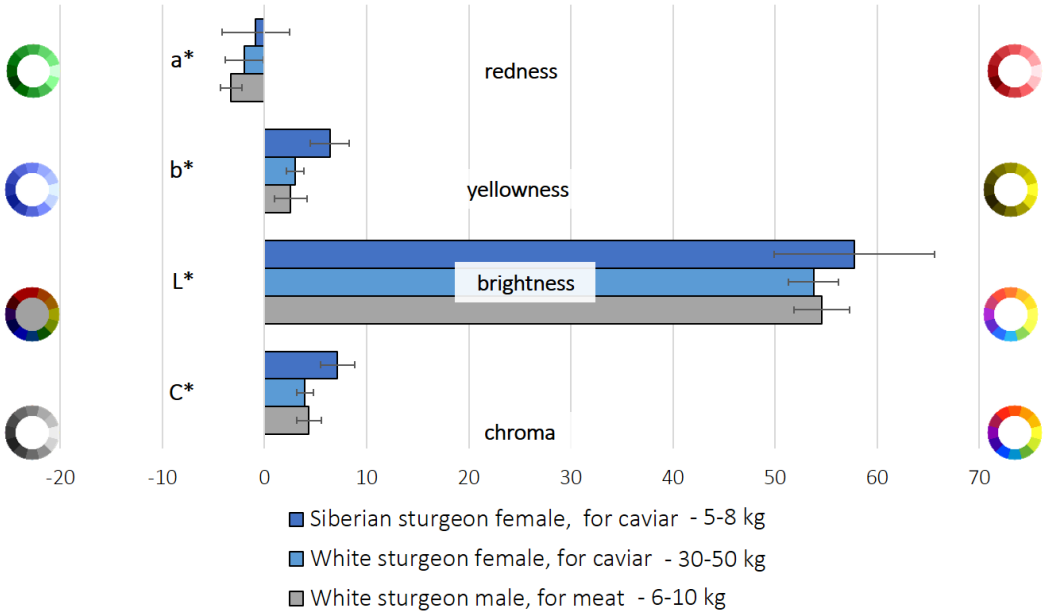

Figure 2. Colour parameters (redness, a*, yellowness, b*, lightness, L* and Chroma, C*) values (Minolta Chroma Meter II) in different sturgeon species fillets. Average values were calculated as mean ± standard deviation of each group. a*, b*, L* values were obtained by two replicates in three different sites of each fillet.

Results obtained by colorimetric analysis mainly showed a difference in meat from the two species (*A. baerii* and *A. transmontanus*), suggesting that the colour is a species-specific characteristic. No differences were found between male and female samples of *A. transmontanus*, suggesting that body composition and flesh appearance were not affected by a sex-effect. The characterization of sturgeon meat's typical features may be of primary importance in order to increase its importance in marketing of aquaculture products, being related to consumers' acceptance. As mentioned before, Italian consumers seem not to appreciate sturgeon meat, being not accustomed with it. The visual appearance of the product may be a crucial aspect to consider and to characterize in order to promote its acceptance by consumers, demonstrating and spreading the knowledge that the grey flesh feature is not related to a low quality of meat, but a specific characteristic of sturgeon meat.

3. Materials and methods

3.1 Samples collection

This study was carried out in cooperation with Agroittica Lombarda S.p.A. (BS), the most important Italian Company in sturgeon farming and caviar production. Fifteen sturgeon egg samples were collected during caviar preparation in the lab facilities of the production plant, belonging to the following species: *Acipenser baerii* (n = 5), *Acipenser gueldenstaedtii* (n = 5) and *Acipenser transmontanus* (n = 5). Roes were simply extracted from sturgeons after the separation from their connective tissue. After about six months of maturation in the storage room, 33 canned caviar samples were collected from the same species as above: *Acipenser baerii* (n = 8),

Acipenser gueldenstaedtii (n = 12) and *Acipenser transmontanus* (n = 11). Caviar was obtained through a simple and consolidated procedure, by light salting (3.6%–3.8%) of roes and freeze storage (−2 °C) in 500 g tin cans. Both the eggs and the caviar samples were transported to the laboratory and stored at −20 °C until the analyses were performed.

At the same time, 15 specimens of sturgeon meat from three different species were sampled: 5 samples from *A. baerii* females (fish weight 5–8 kg), 5 samples from *A. transmontanus* females (fish weight 30–50 Kg) and 5 samples from *A. transmontanus* males (fish weight 6–10 Kg). The meat slices, about 5 cm thick, were obtained by cross-cutting in a retro-cranial position of a freshly gutted fresh fish. They were then vacuum-packed and frozen at −20 °C until analysis. After adequate thawing they were portioned, to create the most representative samples possible of the whole slice. A portion equal to about 10 g of apical meat, a central one and a basal one was then taken. Subsequently, they were finely chopped with a knife and prepared for the chemical analysis.

3.2 Proximate composition and fatty acid analysis

Proximate composition was determined on eggs, caviar and sturgeon meat using standard methods (AOAC, 1996), Moisture content was determined by drying samples in an oven at 105 °C for 16–18 h (Association of Official Analytical Chemists (AOAC) Method 950.46). Total protein was determined by the Kjeldahl method (AOAC Method 940.25). For the analysis, an automated distillation unit (BÜCHI Labortechnik AG, Flawil, Switzerland) was used. Ash was determined by incineration of the sample in a muffle furnace at 550 °C for 18 h (AOAC Method 938.08).

The extraction and determination of total lipids was performed according to the Folch and Lees method (1957) with a chloroform:methanol mixture (2:1, v/v), using 0.7–1.0 g of sample, according to its lipid content. The preparation of fatty acid methyl esters was performed according to Christie (2003). Briefly, the lipid sample (20 mg) was dissolved in 10% methanolic hydrogen chloride (2 mL). A 1 mL solution of tricosanoic acid (1 mg mL^{-1}) in toluene was added as an internal standard. The sample was sealed and heated at 50 °C overnight. Then, 2 mL of a 1 M potassium carbonate solution and 5 mL of 5% NaCl were added to each sample. The fatty acids methyl esters (FAME) were extracted with 2 × 2 mL of hexane and the mixture was evaporated under nitrogen. The sample was dissolved in 1 mL hexane. Fatty acid analysis was carried out by capillary chromatography on an 6890 series gas-chromatograph (Agilent Technologies, Santa Clara, CA, USA) fitted with an automatic sampler model 7683 and Flame Ionization Detector (FID) by the same provider. 1 µL of sample was injected into the gas-chromatograph, in split mode (split ratio 1:100), with helium as a carrier gas, with a flow rate of 1.0 mL min-1 and an inlet pressure of 16.9. A HP-Innowax fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness, Agilent Technologies) was used to separate fatty acid methyl esters. The oven temperature program for separation was from 100 to 180 °C at 3 °C min−1, then from 180 to 250 °C at 2.5 °C min−1 and held for 10 min. Fatty acids were identified relative to known external standards and were expressed as percentage of total fatty acids.

3.3 Lipid fraction separation

Lipids extracted from egg samples were separated into fractions by a Solid Phase Extraction (SPE) method. Extraction of neutral lipids was performed using a DSC-NH2 disposable cartridge column (6 mL) (Supelco Inc., Bellefonte, PA, USA), according to the procedure of Kaluzny et al. (1985). The cartridge column was equilibrated by rinsing twice with 3 mL of hexane using a Visiprep™ SPE Vacuum Manifold (Supelco Inc., Bellefonte, PA, USA). 10 mg of lipids dissolved in 100 µl of chloroform were loaded onto the column and the chloroform was pulled through. Thereafter, the column was eluted with 4 mL of a chloroform-2-propanol 2:1 mixture, to obtain the neutral lipid fraction (NL). Then, 4 mL of methanol was used to elute phospholipids (PL). The flow rate was adjusted to approximately 3–5 mL/min. Each fraction collected was dried under a stream of nitrogen, dissolved in 2 mL of 10% methanolic hydrogen chloride, trans-esterified and analyzed as described in paragraph 4.2.

3.4 Color measurement on fillets

Color measurements of the flesh samples were performed in three different sites. Color was measured using a Minolta Chroma Meter II Reflectance instrument (Minolta Camera Co. Ltd., Osaka, Japan). Results were recorded as a*, b*, L* values, where a* describes redness, b* yellowness and L* lightness. Chroma values (brightness, C*) were calculated according to the following equation: $C^* = \sqrt{a^{*2} + b^{*2}}$. Two measurements were taken on each site and the measuring head was rotated 90° between each measurement.

3.5 Statistical Analysis

The statistical analysis was performed using JMP Pro 14 (SAS Institute Inc., Cary, NC, USA). Normality and homoscedasticity of data distribution for eggs, caviar and sturgeon meat were tested by the Shapiro–Wilk test and Levene test, respectively. When both the conditions were confirmed, the comparisons between means was performed by analysis of variance (ANOVA), setting the different Acipenser species as the discriminating factor between groups. The Tukey's Honest Significant Difference test was used as a post-hoc test between each pair of groups. When normality and/or homoscedasticity were not confirmed, the comparison between means was performed by the Wilcoxon Ranks test. Significance was declared at $p < 0.05$ (*) and $p < 0.01$ (**). Then, a multivariate statistical approach was employed on eggs and caviar fatty acids distribution. A Linear Discriminant Analysis (LDA) was performed involving fatty acids data in canonicals calculation, in order to generate a bi-plot where a clustering of different groups was perceptible. The LDA method was performed following a stepwise-backward approach, including variables in the model if p values that achieved testing for the group variable by analysis of covariance were < 0.05.

4. Conclusions

Results obtained in this work provided the characterization of food products obtained by sturgeons bred in Italy. Especially, caviar was characterized as a product with high nutritional value, even if its composition showed some variations from species to species. In comparison with the same products obtained by wild sturgeon stocks,

some relevant differences were detected, particularly in the content of fatty acids that derive from aquafeed (above all, linoleic acid from vegetable oils). In our opinion, it would be desirable to find a way to decrease the level of linoleic acid and increase that of arachidonic acid in the diet of sturgeons, getting as close as possible to the situation of wild caviar, in order to optimize production in aquaculture and to deliver products with a more favorable lipid composition. On the other hand, all the matrices analyzed showed remarkable values of essential fatty acids that cover a fundamental role in human nutrition. Particularly, data strengthened the hypothesis that the selective deposition of EPA and DHA in eggs during the gonadogenesis occurs regardless of the type of diet supplied to fish. The outcomes of our research might be fundamental to enhance the knowledge about this product, in order to promote its acceptance by Italian consumers, spreading fair information about its nutritional quality and helping to dismantle its actual evaluation as a caviar by-product. Results of this investigation, in fact, showed that sturgeon meat from aquaculture is a product of undeniable commercial interest, represented by a reduced lipid content of notable nutritional value.

Author Contributions: Conceptualization, M.V. and V.M.M.; Formal analysis, A.L. and M.V.; Investigation, A.L., M.V. and T.M.; Methodology, M.V.; Project administration, V.M.M.; Supervision, F.B.; Validation, M.V., F.B. and V.M.M.; Visualization, A.L. and V.M.M.; Writing—original draft, A.L., F.B. and V.M.M.; Writing—review and editing, M.V., F.B., T.M. and V.M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank Dr. Mario Pazzaglia, Head of Special Projects and Scientific External Relations in Agroittica Lombarda SpA, for his assistance with the collection and purchasing of sturgeon eggs, caviar and meat samples.

Conflicts of Interest: The authors declare no conflict of interest.

Ethical Approval: This article does not contain any experimental practice performed on animals by the authors. Only caviar and fillets' sampling was performed in order to develop the present research. No biological material was collected on alive animals. Authors guarantee that the farm involved in the study followed all the applicable guidelines for animal welfare established by harmonized EU rules. No approval by the institutional ethics committee was requested by University of Milan for this kind of research.

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Trial 5

Comparison of Chemical Composition and Safety Issues in Fish Roe Products: Application of Chemometrics to Chemical Data

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Abstract. Processed fish roes are acquiring considerable importance in the modern food market, entering more and more often as an ingredient in food preparation and as caviar substitutes. In this study, we defined quality, traceability and safety issues related to processed fish roe products from different species. The results obtained allowed to distinguish eggs originated from different fish species and to discriminate between fish roes and caviar samples obtained from four different sturgeons species. We observed that roes showed a trend of grouping according to ecological and reproductive habits of fish species. We highlighted the differences between eggs originated by farmed and freshwater fish, enriched in n6 polyunsaturated fatty acids (PUFAs), and all the others, in which n3 PUFAs were prevalent. In addition, we evaluated processed fish roes under a food safety point of view, combining microbiological analysis with the determination of organic acids, used in some products as authorized preservatives. Microbiological characterization has proved a general good hygienic level for these products. Organic acids determination showed values in compliance with European Union (EU) regulations in almost of samples; in some cases, we found a mismatch between the organic acids detected and what was reported in labels. Processed fish roes could be considered a safe product that can provide to human nutrition a valuable content of essential fatty acids.

Results from this investigation have been published in the scientific journal *Foods*

Foods **2020**, 9, 540; doi:10.3390/foods9040540

1. Introduction

Fish roe products has been historically present in many food cultures for a long time. The processing of mullet and tuna roes generates the Bottarga, a typical cured product of Mediterranean countries, while Ikura, Tarako and Tobiko are typical Japanese preparations that originate from salmon, pollock, and flying fish roes, respectively (Shirai et al., 2006). The most precious fish roe product is Caviar, obtained by light salting of roes extracted from sturgeons, separated from their connective tissue (Bledsoe et al., 2003). Caviar traditionally originated from the Russian and Persian empires and nowadays it is one of the most expensive luxury food products, appreciated all over the world. According to the *Codex Alimentarius* (2010), only the product coming from the processing of roes obtained from fish of the *Acipenseridae* family can be named as Caviar. Fish roes from other fish species than sturgeon can be defined as caviar substitutes. They are sold at a lower price, but they refer to caviar with the aim of exploiting its high appeal among consumers. This reference is evidenced by the habit that some producers have when they confer the black color to products that naturally are differently colored, in order to resemble as much as possible to sturgeon roe (P. Bronzi & Rosenthal, 2014). In some cases, with traditional products like lumpfish roes, fish processed roes have a specific niche market that is not in competition with caviar market, but, on the contrary, they can lead new consumers to approach the consumption of fish eggs, starting with the cheapest products and then moving towards the product of excellence represented by caviar (Paolo Bronzi et al., 2019). During last years, a great increase in the market of caviar substitutes occurred since (i) the availability of natural-sourced Caviar declined, due to the block of catch quotas granted in 2006 by the CITES (Convention on International Trade in Endangered Species) related to the steady decline of world sturgeon wild populations; (ii) the amount of Caviar produced by aquaculture was limited. In addition, the popularity of sushi led to the development of new products obtained by manufacturing roes from other fish species and to the expansion of their market. The estimated global market of processed fish roes covers 60,000 tons, while real caviar production does not reach 500 tons (P. Bronzi & Rosenthal, 2014; Sicuro, 2019). In 2000, European Union (EU) countries imported 5,000 tons of frozen roes and 1,000 tons of raw chilled roes from non-EU countries. At the same time, many EU countries (mainly in Denmark, Spain, and United Kingdom) also imported 7,500 tons of prepared fish roe, valued at 119 M€ (Monfort, 2002).

Egg processing techniques are different depending on the features of the raw material. The separation of eggs from the connective tissue could be achieved differently, according to egg dimensions. As an evidence, salmon and trout eggs are obtained by the manual separation, usually performed by a stainless steel grid (Shin & Rasco, 2007), while smaller fish roes, such as capelin, herring, and pollock roes, are automatically processed in drum filters separators. Enzyme separation, using collagenase and pepsine, has also been studied (Conde & Fernandes, 2016; Vilhelmsson, 1997). Eggs are then salted, generally by a saturated salt solution, then maintained in barrels at refrigerated temperatures in order to decrease the water activity, thus allowing their shelf stability. Other additives or ingredients, like sugar, could be added in these processing phases (Bledsoe et al., 2003). After a suitable maturation period, eggs are packaged in glass or aluminum cans for the retail. In addition, eggs may be pasteurized at mild temperatures, in order to avoid the denaturation of proteins, as happens with lumpfish roes (Johannesson, 2006). All fish roes are generally ready-to-eat products and they are sold under refrigerated

conditions. Nevertheless, organic acids, like citric acid or benzoic acid, are usually added in fish roe as antimicrobials (Bledsoe et al., 2003), although no studies aimed to investigate their presence are available in literature (Pereira da Costa & Conte-Junior, 2015).

Since usually fatty acid analysis performed by means of analytical chemistry leads to the yield of large amount of data, chemometrics methods appear to be necessary in order to investigate the structure of large datasets, especially when samples have different origins and can be divided into groups. This is the case of Principal Component Analysis (PCA), where the significant dimensionality of data matrices is strongly reduced, allowing the analyst to more easily understand the structure of chemical data, still retaining a good amount of the original information (Defernez & Kemsley, 1997). Moreover, qualitative chemometrics methods can be also very useful to create a classification model and to assign samples included in a dataset to a determinate group. In this scenario, multivariate methods appear to be very powerful, usually leading to a good fit of analytical data to a classification model. Particularly, the aim of Linear Discriminant Analysis (LDA) is to obtain an optimal separation among existing groups and, further, to maximize the prediction power in order to classify such samples whose membership is not known, on the basis of the distance of each observation from each group mean (Defernez & Kemsley, 1997). However, when the number of variables in the matrix is higher than the number of samples analysed, as often occurs with fatty acid analysis, it appears very advantageous to employ a PCA/LDA combined approach, in which firstly the dimensionality of raw data is reduced and then the discriminant model is built to a subset of the original variables (Defernez & Kemsley, 1997). Despite the importance of fish roe products in international market, there is a little technical information available about their chemical composition, food safety, and quality attributes. The aim of the present work was to achieve a deeper knowledge of the chemical composition, the fatty acid profile, and the organic acids content of several fish roe products collected on the Italian market. Moreover, we aimed to verify if a discrimination was achievable between caviar and fish roes by means of a chemometric approach. Finally, microbiological properties of the products and the assessment of the conformity with the label information have been investigated and discussed.

2. Materials and methods

2.1 Sampling

A total of 38 fish roe samples were purchased during the spring of 2016 (Table 1). The survey involved 12 samples of Chum salmon (*Oncorhynchus keta*) eggs coming from 4 different producers; 11 samples of lumpfish (*Cyclopterus lumpus*) roe purchased from 2 different producers; 3 samples of rainbow trout (*Oncorhynchus mykiss*) roe; 3 samples of pike (*Esox lucius*) roe; 3 samples of cod (*Gadus morhua*) roe, 3 samples of Alaska pollock (*Theragra chalcogramma*) roe, and 3 of capelin (*Mallotus villosus*) roe, one black colored, one orange colored, and one green colored. All samples were packaged in glass jars or metallic cans and sold refrigerated. Samples were transported to laboratory under ice in polystyrene boxes, and then they were opened for the microbiological analyses. The remaining portion of samples was stored at −20 °C until chemical analyses.

Table 1. Fish roes samples collected in the study with the additives listed according to what was reported in their labels.

		DIFFERENT PREPARATIONS						
SPECIES	***N* total**	***N* per producer**	**Nacl***	**Stabilizers**	**Preservatives**	**Antioxidants**	**Color additives**	**Others**
Salmon *O. keta*	12	3	2.5%	ND	ND	ND	ND	ND
		3	3.5%	ND	ND	ND	ND	ND
		3	2.5%	ND	ND	ND	ND	ND
		3	ND	ND	ND	ND	ND	ND
Lumpfish *C. lumpus*	8	2	ND	E413	E211	E330	E120, E160c	Chartamus
		3	ND	E413	E211	E330	E150d, E151	
		3	ND	E412, E422	E202, E211	E331	E141, E150d, E163, E151	E621
Capelin *M. villosus*	5	3	ND	E412, E422	E202, E212	E330	E120, E160c	E622
		1	ND		E202, E211	E330	E150d	Sugar
		1	ND	E413	E202, E211	E330	E160a, E120	Sugar, soy
Rainbow trout *O. mykiss*	3	3	<6%	ND	E200	ND	ND	ND
Pike *Esox lucius*	3	3	ND	E415	ND	ND	ND	ND
Cod *G. morhua*	3	3	ND	ND	E200	ND	ND	ND
Alaska pollock *T. chalcogramma*	3	3	ND	ND	E200	ND	ND	ND

*NaCl was present in all samples, table shows the amount as reported on the labels. Legend for the ingredients: ND: Not declared; E 412 Guar gum; E413: Tragacanth gum; E415 Xanthan gum; E422 Glycerol; E200: Sorbic acid; E202: Potassium sorbate: E211; Sodium benzoate: E212; Potassium benzoate; E330: Citric acid; E331: Sodium citrate; E102: Tartrazine; E120: Carminic acid; E133: brilliant blue FCF; E141: Chlorophylls and chlorophyllins, copper complexes; E150d: Sulfite Ammonia Caramel; E151: Brilliant Black BN; E160a: Carotenes; E160c: paprika oleoresin; E163: Anthocyanins; E621: Monosodium L-glutamate; E622: Monopotassium L-glutamate.

2.2 Chemical analysis

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). Before chemical analyses, three groups of eggs coming from each jar or can were weighed. Individual average weight of egg was calculated dividing the total weight with the number of eggs weighed. The pH was measured using five grams of roes, blended with 20 mL of distillate water with an Ultraturrax for 30 s and pH was registered by a digital pH-meter (Amel Instruments, Milan, I) standardized at pH 4 and 7. Proximate composition and NaCl content were determined using standard method (AOAC, 1996). The extraction and determination of total lipids were performed according to the Folch (1957) method with chloroform: methanol mixture (2:1, v/v), using 0.7–1.0 g of sample, depending on its lipid content. The preparation of fatty acid methyl esters was performed according to Christie (Christie, 2003). The sample was dissolved in 1 mL hexane and 1 µL sample was injected into the gas-chromatograph. Fatty acid analysis was carried out on an Agilent gas-chromatograph (Model 6890 Series GC) fitted with an automatic sampler (Model 7683) and FID detector (Agilent Technologies, Santa Clara, CA, USA) using helium as carrier gas. A HP-Innowax fused silica capillary column (30m × 0.25mm I.D., 0.25 µm film thickness, Agilent Technologies, Santa Clara, CA, USA) was used to separate fatty acid methyl esters. The oven temperature program for separation was from 100 to 180 °C at 3 °C min^{-1}, then from 180 to 250 °C at 2.5 °C min^{-1} and held for 10 min. Fatty acids were identified relative to known external standards (Supelco 37 FAME Mix, code CRM47885, Marine source, code 47,033 and Menhaden fish oil code 47085-U, Supelco, Bellafonte PA, USA) and were expressed as percentage of total fatty acids.

2.3 Microbiological analysis

For the microbiological determinations, 10 g of product were 10-fold diluted in chilled sterile diluent solution (0.85% NaCl and 0.1% peptone) and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). In some cases, due to the limited amount of material, pools of more than one sample were performed. Appropriate 10-fold dilutions of the homogenates were prepared in chilled saline solution. Total mesophilic viable count (TVC) was determined onto Plate Count Agar (ISO, 2013). Lactic acid bacteria were enumerated on De Man Rogosa Sharpe (MRS) agar (ISO), 2013), Enterobacteriaceae were enumerated on Violet Red Bile Glucose Agar (ISO, 2017), yeasts and moulds were counted on Sabouraud Agar (ISO, 2008), coagulase positive staphylococci were counted on Baird Parker Agar (ISO, 2018), presumptive Bacillus cereus was enumerated onto PEMBA, Enterococci were enumerated onto Slanetz agar, (Merck, Darmstadt, Germany). Spores of reducing sulphite Clostridia were counted after pasteurization of the samples at 80 °C for 10 min onto Tryptose Sulfite Cycloserine, then incubated in anaerobiosis at 37 °C for 48 h. Detection and enumeration of Listeria monocytogenes were performed according to the method AFNOR (1998). All the culture media were purchased from Biogenetics (Ponte San Nicolò, Italy).

2.4 Determination of organic acids

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). The amounts of citric, lactic, and acetic acids were determined by Reverse phase-High Performance Liquid Chromatography (HPLC) analysis with ion-exchange chromatography and UV detection (Tirloni et al., 2019). Briefly, 1 g of roe was added to 5.0 mL of water and vigorously shaken by a vortex for 20 s. After centrifugation, the supernatant was filtered through a 0.20 µm regenerated cellulose membrane filter. The HPLC system consisted of two pumps (510 by Waters S.p.A., Milano, Italy), an auto-sampler (717 plus by WatersS.p.A., Milano, Italy) and a UV-VIS detector (484 by Waters S.p.A., Milano, Italy) set at 210 nm. The separation was performed on a Rezex ROA column 300 mm × 7.8 mm, 8 µm (Phenomenex, Torrance, USA). The mobile phase (0.5 mL min^{-1} in isocratic mode) was 0.005 N sulphuric acid. External standards (acetic acid, code 1005706, Sigma Aldricht, lactic acid, code 252476, Sigma Aldricht and citric acid code 251275, Sigma Aldricht, Steinheim, Germany) were used for identification and quantification of acetic, citric, and lactic acid. The limit of detection (LOD: 0.076, 0.23, and 0.24 mM for acetic, citric, and lactic acid, respectively) and limit of quantification (LOQ: 0.39, 0.43, and 0.77 mM for acetic, lactic, and citric acid, respectively) were determined. LOD and LOQ of organic acids were calculated from the residual standard deviation of the regression line (SDrl) of the calibration curve and its slope (b) in accordance to the following equation: LOD = 3.3(SDrl/b) and LOQ = 10(SDrl/b) (EMA, 1995).

Concentration of benzoic and sorbic acids were determined by reverse phase HPLC method (Burana-osot et al., 2014). Briefly, 1 g of roes was homogenized with 10 mL of methanol:water (60:40 v/v) by an ultraturrax and filled up to 20 mL with the extraction solvent. The mixture solution was placed in an ultrasonic bath for 30 min to complete the extraction, after centrifugation 1 mL of supernatant was filtered through a 0.45 µm regenerated cellulose (RC) membrane filter and fill up to 5 mL with the extraction solvent. The chromatographic analysis was carried out in an Alliance 2695 HPLC system equipped with a PDA 996 (Waters S.p.A., Milano, Italy) diode array detector. The separation was performed at a flow rate of 1 mL/min throughout a C18 Chromolith column, 100 × 4.6 mm, 5 µm, (Merck Millipore, Burlington, Massachusetts, USA). The mobile phase consisted of 5mM ammonium acetate buffer pH 4.2 and methanol (70:30 v/v) and the eluent was monitored at 228 nm for benzoic acid and 260 nm for sorbic acid. External standards (sorbic acid code 47,845 Sigma Aldricht and benzoic acid, code 242,381 Sigma Aldricht, Steinheim, Germany) were used for identification and quantification of benzoic and sorbic acids. The limit of detection (LOD: 0.52, 0.50 mM for benzoic and sorbic acid, respectively) and limit of quantification (LOQ: 1.59 and 1.51 mM for benzoic and sorbic acid, respectively) were determined.

2.5 Statistical analysis

Since data distribution was characterized by unequal variances within the groups (Levene test), statistical analysis was performed by means of non-parametric tests (Welch test and Steel-Dwass post-Hoc test), declaring a significance when $p < 0.05$. A multivariate analysis was then performed by means of Principal Component Analysis (PCA) on a 38 × 28 matrix, including fatty acid data, in order to reduce the dimensionality of data matrix and to detect similarities among samples and correlations among variables, according to Scano et al.

(2010). Variables were selected when PC loadings score were > |0.5|. Then, a Linear Discriminant Analysis (LDA) was performed using the variables previously selected, in order to verify if they were satisfying in highlighting the differences among samples coming from different species. Since the distribution of the scores for Canonical-1 and Canonical-2 were confirmed for their normality and homoscedasticity, comparison among groups centroids scores were performed by means of ANOVA and the Tukey-HSD test. Furthermore, in a successive step, thirty-seven caviar samples (Borella et al., 2016; Lopez et al., 2020) were included in the dataset. The model was developed using roes from fish species analysed in this study as training set, including in the new matrix the variables selected in the previous steps, and caviar samples as validation set. Statistical analysis was performed using JMP Pro 14.0.0 (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1 Nutritional quality and authenticity of fish roes

Weights and proximate composition of eggs are presented in Table 2.

Table 2. Weight (mg), proximate composition (g/100g), and salt (g/100g) content of fish roes. Data are expressed as mean ± standard deviation.

FISH SPECIES	**SALMON** *N* = 12	**LUMPFISH** *N*= 11	**CAPELIN** *N*= 3	**TROUT** *N*= 3	**PIKE** *N* = 3	**COD** *N* = 3	**ALASKA POLLOCK** *N* = 3
Weight	217.3±31.33	6.0±1.07	0.7±0.09	54.7±7.36	4.7±0.50	0.2±0.04	0.2±0.02
Moisture	53.5±1.68	79.7±0.92	81.7±0.77	59.2±0.03	64.0±0.83	71.6±0.55	73.5±0.42
Protein	29.6±0.85	10.8±0.40	8.1±0.56	23.8±0.07	19.4±1.12	19.6±0.27	19.2±0.50
Lipid	12.8±0.85	4.3±0.58	4.5±0.23	12.5±0.08	12.7±0.57	3.2±0.17	2.8±0.10
Ash	4.2±0.79	5.2±0.89	5.7±0.02	4.6±0.05	3.9±0.04	5.5±0.11	4.4±0.05
NaCl	2.7±0.64	2.7±0.64	2.5±0.32	2.3±0.06	3.3±0.00	3.1±0.06	2.8±0.04

Egg size is influenced by the fish species of origin and by their reproductive behavior. Generally, fish with demersal spawning have bigger eggs if compared to species with planktonic eggs (Duarte & Alearaz, 1989). Biological features connected with the spawning site can also influence the chemical composition of roes that vary according to the maturation of fish from which they are obtained (Tocher & Sargent, 1984). The protein content of processed fish roes analyzed in this study ranged from 8.1% to 29.6%, with salmon and trout eggs reaching the highest values (29.6% and 23.8%, respectively), lumpfish and capelin eggs showing the lowest

values (10.8% and 8.1%, respectively), and pike, cod, and Alaska pollock eggs located in the middle (around 19%). According to their lipid content, samples could be divided in two groups. Salmon, trout, and pike roes recorded a lipid content > 12%, while all the other species showed a lipid content < 5%. An explanation for this phenomenon could be linked to the ecological habits of the various fish species. Fish that spawn in an environment that is poor of nourishment, like salmon and trout, produce eggs rich in lipid and protein, in order to guarantee an adequate nutrients supply to newborn generations for a prolonged period. Conversely, marine fish eggs (lumpfish, capelin, cod, Alaska pollock) contain minor reserves of nutrients, probably because the natural sites of spawning of these species are more able to easily provide food to newly hatched larvae. Ash content of roes ranged from 5.7%, recorded in capelin eggs, to 3.9%, recorded in pike eggs. The ash content is largely influenced by the amount of salt added to raw fish roes during their processing. Generally, our results agree with data previously reported by other authors, particularly regarding the species more commonly sold on the market, like salmon and lumpfish. Particularly, salmon roes analyzed in this work showed protein and lipid contents comparable to those reported by Bledsoe et al. in their review (2003), whereas the lipid content was slightly lower than the one found in 2006 by Shirai et al. (2006). Proximate composition of lumpfish roes in our samples showed a lower protein content and a similar lipid content when compared with those reported by several authors in previous studies (Basby et al., 1998; Johannesson, 2006; Kalogeropoulos et al., 2012). In salmon and lumpfish, the economic value of roes is higher than the one of meat (Bledsoe et al., 2003), thus fish from these species are specifically harvested for roes production and their caught is planned when the roes reach the optimal maturation. Conversely, capelin, pollock, and cod roes are considered a valuable by-product of fish meat industry, so their composition could vary according to the harvesting season that is not strictly controlled as occurs for salmon and lumpfish. In capelin roes, Tocher and Sargent (1984) reported a lipid content of 26.3%, corresponding to 7.89% on wet weight, which is superior to the value of 4.5% reported in the present paper. Regarding trout roes, we found a protein content slightly lower if compared with those reported by other authors (Coban, 2010; Mahmoud et al., 2008; Schubring, 2004; Vuorela et al., 1979) and a similar (Mahmoud et al., 2008; Schubring, 2004) or higher (Bledsoe et al., 2003; Kaitaranta & Ackman, 1981; Kalogeropoulos et al., 2012; Mahmoud et al., 2008; Vuorela et al., 1979) lipid content. For pike roes, Bledsoe et al. (2003) reported a protein content ranging from 14 to 27%, in agreement with the value of (19.4%) found in the present study; on the contrary they reported a lipid content between 1.5–2.4%, lower to the value (12.7%) of samples analyzed in the present survey. Proximate composition of cod roes characterized in our work was in agreement with data reported by Bledsoe et al. (2003) for protein content, whereas lipid content agreed with data presented by Tocher and Sargent (1984), recalculated as % of wet weight. Finally, Alaska pollock roes, used for the preparation of the typical Japanese fish preparation Tarako, showed a lipid content lower than the one reported by Shirai et al. (Shirai et al., 2006) and a protein and lipid content similar to the one reported by Chiou et al. (1989), recalculated on % wet weight.-

Furthermore, in a previous study, we analyzed the chemical composition of caviar obtained from four different sturgeon species (Borella et al., 2016). Caviar samples presented the mean moisture content of 54.71 ± 2.66 g/100g and a protein content of 24.31 ± 1.51 g/100g, while lipid content resulted 17.27 ± 2.81 g/100g and ash

content 3.70 ± 0.56 g/100g. An interesting difference between caviar and its substitutes can be observed in the ash content that in caviar was around 3.7 and 3.9%, while in roes from other fish species was higher, up to 5.7%. Since the ash content is linked to the amount of salt and additives added to raw material during processing, a lower ash content demonstrated the tendency of using as less salt as possible in caviar production, related to the aim to obtain a high quality product. Concerning protein content, salmon roes are the only analyzed roes that presented a higher amount of protein if compared with caviar, while trout roes showed a similar content.

Fatty acid (FA) composition in fish eggs is not directly related to the fatty acid composition of other fish tissues, as there is a certain selective fatty acid uptake in the ovarian tissues of female fish. As an evidence of this phenomenon, in 2017 Johnson et al. tested the ability of Coho salmon (*Oncorhynchus kisutch*) of storing essential fatty acids, especially arachidonic acid (ARA) and docosahexaenoic acid (DHA), in the ovary during the secondary oocyte growth. The authors demonstrated that salmons incorporated the essential fatty acids supplied by the diets into the ovarian tissue rather than into muscle tissue. At the same time, it seems that fish are able to reallocate the essential FA stored in muscles to the ovary, if necessary, in order to satisfy the FA requirements of larvae, as reported by Zhu et al. (2019). All samples of fish roes analysed in this study (Table 3) showed a prevalence of polyunsaturated fatty acids (PUFA), reaching amounts up to 52.4%, on monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). PUFA are present in fish eggs in large amounts since they represent an optimal nutritional supply for the growth of the embryo and the larva (Lopez et al., 2020). Particularly, we recorded a relevant proportion of n3 series FA on n6 series FA in all samples. This proportion, commonly represented by the n3/n6 ratio, is considered fundamental by a nutritional point of view, either for the reproductive performance of fishes, and consequential larval growth, or for the nutritional quality of fish roes as favourable food product. The results obtained on the n3/n6 ratio in roes analysed in this study largely varied among the species considered. The highest value was recorded in lumpfish eggs (30.63), followed by capelin (17.35), salmon (15.14), cod (13.20), and Alaska Pollack (12.78) eggs, while the lowest values were found in pike (4.26) and trout (2.55) eggs. The significant difference of the n3/n6 values among pike and trout roes and roes from the other species could be explained by the fact that trout and pike are freshwater species, living in an environment with lower n3 FA and higher n6 FA (Tocher, 2003). Moreover, trout roes originated from farmed fish; the use of plant derived oil in fish feed formulation involves an enrichment with n6 FA, mainly linolenic acid (LA, 18:2 n6), in farmed fish products (Turchini et al., 2009). As a consequence, trout roes showed the highest amount of LA, reaching 9.35% of total fatty acids. The trend found in n3 and n6 series FA in roes from these two species was similar to that found by Saliu et al. (2017), who analyzed the fatty acid content of roes extracted from European catfish (*Silurus glanis*), a freshwater predatory fish similar to pike for the habitat and the feeding habits. The authors found the prevalence of n3 FA on n6 FA but a lower n3/n6 ratio than in eggs of marine fish, recording a value of 3.4–3.8, comparable to the values we found in trout and pike roes. n3 series FA in fish roes are mainly represented by eicosapentaenoic acid (EPA, 20:5 n3) and docosahexaenoic acid (DHA, 22:6 n3), particularly in marine species, reaching higher amounts than in fish flesh because of the higher phospholipids content of eggs (Shirai et al., 2006). Actually, long chain FA of the n3 series are conserved in roes at the expense of other FA, since they are valuable essential components of the biological membranes to be

preserved during critical periods of larvae development (Turchini et al., 2009). According to this, we found the highest content of both EPA and DHA in roes from lumpfish (18.93% and 26.81%), Alaska pollack (18.18% and 28.05%), capelin (16.07% and 19.27%), salmon (15.41% and 22.04%), and cod (17.80% and 27.14%), usually obtained after the caught of wild fish in natural stocks. At the same time, cod and Alaska pollack roes showed also the highest amount of arachidonic acid (ARA, 20:4 n6), another FA considered fundamental during starvation of marine fish larvae (Izquierdo, 1996). Capelin eggs showed high levels of the monounsaturated FAs palmitoleic acid (16:1) and gondoic acid (20:1 n-9). Nevertheless, the sum of eicosenoic fatty acids did not reach the levels found by Cyprian et al. (2017), who analyzed the lipids from the whole capelin fish and found eicosanoic acids content near to 20% of total fatty acids. It can be suggested that these fatty acids do not have an essential role in larval metabolism and, for this reason, they are not stored in capelin roes. Pike eggs represented the only group in which MUFA were predominant (43.14%) on PUFA (36.02%). The prevalence of MUFA in pike eggs was mainly due to the higher content of 16:1 n7 (13.15%) and 18:1 n7 (7.67%) in samples if compared with eggs originating from other species. However, the most representative among MUFA in all analysed samples was oleic acid (OA, 18:1n9). This fatty acid, together with the SFA palmitic acid (16:0), is known to represent the primary energy source for fish larvae in many species (van der Meeren et al., 1993). We found the highest amount of oleic acid in trout roes (27.11%). As stated before, trout is a farmed species and, thus, its diet is strongly enriched in vegetable oils that represent a source of many FA, less representative in natural aquatic trophic chains, such as oleic acid. As for LA, an increase in the ingestion of OA could have led to the higher stocking of this fatty acid in trout organs and eggs, mainly in the storage fraction represented by triacylglycerols (Turchini et al., 2009).

Table 3. Fatty acid composition (g/100g of total fatty acids) of fish roe from different species purchased in the Italian market. Data are expressed as mean ± standard deviation.

Fish species	SALMON	LUMPFISH	CAPELIN	TROUT	PIKE	COD	A. POLLOCK
14:0	3.44 ± 0.51 b	1.37 ± 0.11 c	4.54 ± 0.01 a	1.58 ± 0.04 c	1.76 ± 0.03 c	1.47 ± 0.04 c	1.68 ± 0.19 c
15:0	0.54 ± 0.11a	0.27 ± 0.03 bc	0.36 ± 0.00 bc	0.19 ± 0.00 c	0.27 ± 0.00 bc	0.30 ± 0.01 bc	0.38 ± 0.11 b
16:0	10.63 ± 0.43 d	14.19 ± 0.37 c	17.07 ± 0.09 ab	10.85 ± 0.20 d	16.05 ± 0.12 b	17.59 ± 0.81 a	17.44 ± 0.56 ab
16:1n7	4.83 ± 0.53 c	1.82 ± 0.25 e	10.59 ± 0.10 b	2.93 ± 0.10 d	13.15 ± 0.30 a	4.89 ± 0.21 c	4.93 ± 0.14 c
16:2n4	0.14 ± 0.03 c	0.02 ± 0.06 d	0.36 ± 0.00 b	0.13 ± 0.02 c	0.70 ± 0.03 a	nd	nd
17:0	0.51 ± 0.10 a	0.41 ± 0.07 ab	0.08 ± 0.13 d	0.19 ± 0.00 bcd	0.40 ± 0.01 abc	0.25 ± 0.29 bcd	0.14 ± 0.24 cd
16:3n4	0.85 ± 0.33 a	0.02 ± 0.06 c	nd	0.05 ± 0.04 bc	0.53 ± 0.02 ab	0.08 ± 0.13 bc	nd
18:0	4.76 ± 0.23 a	4.37 ± 0.19 b	2.29 ± 0.01 b	3.90 ± 0.06 c	2.36 ± 0.04 de	2.83 ± 0.18 d	2.90 ± 0.28 d
18:1n9	18.25 ± 1.55 c	17.07 ± 1.81 c	11.21 ± 0.11 d	27.11 ± 0.22 a	21.65 ± 0.20 b	12.50 ± 0.60 d	12.82 ± 0.59 d
18:1n7	2.85 ± 0.40 b	4.02 ± 0.45 b	4.43 ± 0.04 ab	3.28 ± 0.06 b	7.67 ± 0.14 a	4.25 ± 3.68 b	2.09 ± 3.62 b
18:2n6	1.32 ± 0.11 d	1.04 ± 0.21 e	1.72 ± 0.01 c	9.35 ± 0.19 a	3.50 ± 0.04 b	0.53 ± 0.03 f	0.61 ± 0.04 f
18:3n3	1.22 ± 0.14 c	0.39 ± 0.06 e	0.78 ± 0.01 d	3.53 ± 0.04 a	2.20 ± 0.11 b	nd	nd
18:4n3	1.07 ± 0.11 c	0.88 ± 0.26 cd	3.08 ± 0.02 a	0.66 ± 0.02 de	1.73 ± 0.05 b	0.46 ± 0.03 e	0.47 ± 0.04 e
18:4n1	1.07 ± 0.11 c	nd	nd	0.19 ± 0.00 b	nd	nd	nd

Table 3 (cont.)

20:1n11	0.43 ± 0.15 c	nd	nd	nd	nd	1.69 ± 0.03 b	2.01 ± 0.23 a
20:1n9	0.85 ± 0.27 d	4.06 ± 0.92 ab	5.10 ± 0.08 a	2.85 ± 0.06 c	0.41 ± 0.02 d	2.79 ± 0.05 c	3.05 ± 0.17 bc
20:1n7	0.32 ± 0.03 b	0.54 ± 0.20 a	nd	0.03 ± 0.05 cd	0.26 ± 0.01 bcd	0.34 ± 0.02 abc	0.12 ± 0.21 bcd
20:2n6	0.32 ± 0.05 a	nd	nd	1.64 ± 0.03 a	0.36 ± 0.00 a	nd	0.16 ± 0.28 c
20:3n6	0.12 ± 0.05 c	nd	nd	0.75 ± 0.02 a	0.42 ± 0.00 b	nd	nd
20:4n6	1.42 ± 0.23 c	0.64 ± 0.16 d	0.68 ± 0.00 d	1.40 ± 0.04 c	2.34 ± 0.02 b	3.07 ± 0.16 a	3.07 ± 0.19 a
20:4n3	2.51 ± 0.19 a	0.97 ± 0.15 b	0.73 ± 0.00 bc	0.94 ± 0.02 bc	0.62 ± 0.01 c	0.12 ± 0.22 d	nd
20:5n3	15.41 ± 0.53 b	18.93 ± 0.77 a	16.07 ± 0.07 b	6.42 ± 0.02 d	9.03 ± 0.03 c	17.80 ± 1.07 a	18.18 ± 0.56 a
22:1n9	0.19 ± 0.12 b	0.49 ± 0.18 a	nd	nd	nd	nd	nd
22:5n3	5.56 ± 0.47 a	1.70 ± 0.16 c	1.64 ± 0.02 c	2.02 ± 0.01 bc	2.38 ± 0.01 b	1.90 ± 0.15 bc	1.90 ± 0.07 bc
22:6n3	22.04 ± 1.20 b	26.81 ± 1.53 a	19.27 ± 0.39 c	20.01 ± 0.37 bc	11.91 ± 0.29 c	27.14 ± 1.21 a	28.05 ± 1.09 a
SFA	19.88 ± 0.41 e	20.61 ± 0.63 d	24.33 ± 0.18 a	16.70 ± 0.22 f	20.84 ± 0.17 cd	22.44 ± 1.05 bc	22.54 ± 1.24 b
MUFA	27.72 ± 1.71 cd	28.00 ± 1.78 d	31.34 ± 0.23 c	36.21 ± 0.29 b	43.14 ± 0.24 a	26.47 ± 2.83 d	25.02 ± 2.82 d
PUFA	52.40 ± 1.66 a	51.39 ± 1.28 a	44.33 ± 0.36 b	47.09 ± 0.47 b	36.02 ± 0.40 c	51.09 ± 2.51 a	52.44 ± 1.79 a
n3	47.88 ± 1.43 a	49.68 ± 1.06 a	41.57 ± 0.37 b	33.57 ± 0.34 c	28.17 ± 0.41 d	47.43 ± 2.44 a	48.60 ± 1.71 a
n6	3.19 ± 0.31 d	1.67 ± 0.35 e	2.40 ± 0.01 d	13.15 ± 0.22 a	6.62 ± 0.03 b	3.59 ± 0.18 c	3.84 ± 0.47 c
n3/n6	15.14 ± 1.26 b	30.63 ± 5.19 a	17.35 ± 0.24 b	2.55 ± 0.05 c	4.26 ± 0.07 c	13.20 ± 0.18 b	12.78 ± 1.53 b

a, b, c, d = Value within the same raw not sharing a common letter are significantly different ($p < 0.05$); SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. nd= not detected

Salmon eggs were rich of 18:3 n3 and DHA, even if they presented values of n3 fatty acids lower than other marine species as lumpfish, cod, and Alaska pollock. This aspect could be linked to the anadromous behaviour of the species that partially lived in fresh waters during growing ages and during spawning season, but also to the feeding habits of the salmon species used to obtain roes. As a matter of fact, the Chum salmon (*O. keta*) feed mainly on copepods and euphausiids or gelatinous zooplankton, which have lower nutritional value than other prey (Qin & Kaeriyama, 2016).

In order to visualize the distribution of samples and eventual correlations between variables, a Principal Component Analysis was performed on the dataset including fatty acid data. The first two Principal Components (PC-1 and PC-2) introduced by means of this multivariate technique explained the 60% of the total variance. The PCA loading plot (Figure 1) showed that 16 variables over the original 28 were related to a loading > |0.5|, thus highly influencing the variability of data and their distribution in the new bi-plot delimited by PC-1 and PC-2. In the loading plot, we can observe that the first PC, which explained the 37.4% of the variance, was mainly described by fatty acids typical of the marine habitat (EPA, DHA, n3/n6) in the positive direction and by fatty acids coming from the farm feeding system (oleic acid, linoleic acid, linolenic acid) in the negative direction. PC-1 significantly separated three groups: (i) pike and trout roes, inversely corelated with the n3/n6 ratio and positively correlated to fatty acids typical of freshwater environment or vegetable oils, such as oleic, linoleic and linolenic acid; (ii) lumpfish, Alaska Pollock and cod roes, from pure marine species, positively correlated with EPA and DHA and, consequently, with the n3/n6 ratio; (iii) salmon and capelin roes, in an intermediate position. The second PC, which explained the 24.7% of the total variance in the dataset, was mainly described by palmitic and palmitoleic acid in the positive direction and by stearic acid, oleic acid, 20:4 n3, and docosapentaenoic acid (DPA) in the negative direction. PC-2 was able to separate four groups: (i) capelin roes, positively correlated to palmitic and palmitoleic acid and saturated fatty acids in general; (ii) salmon and trout roes, positively correlated to 20:4 n3, DPA and stearic acid; (iii) lumpfish roes and (iv)pike, cod, and Pollock roes, in an intermediate position. However, the combination of both PC-1 and PC-2 in a multivariate system allowed distinguishing groups that would have not been recognizable if considering just one over the two direction, as demonstrated also by another study based on processed fish roes (Caredda et al., 2018).

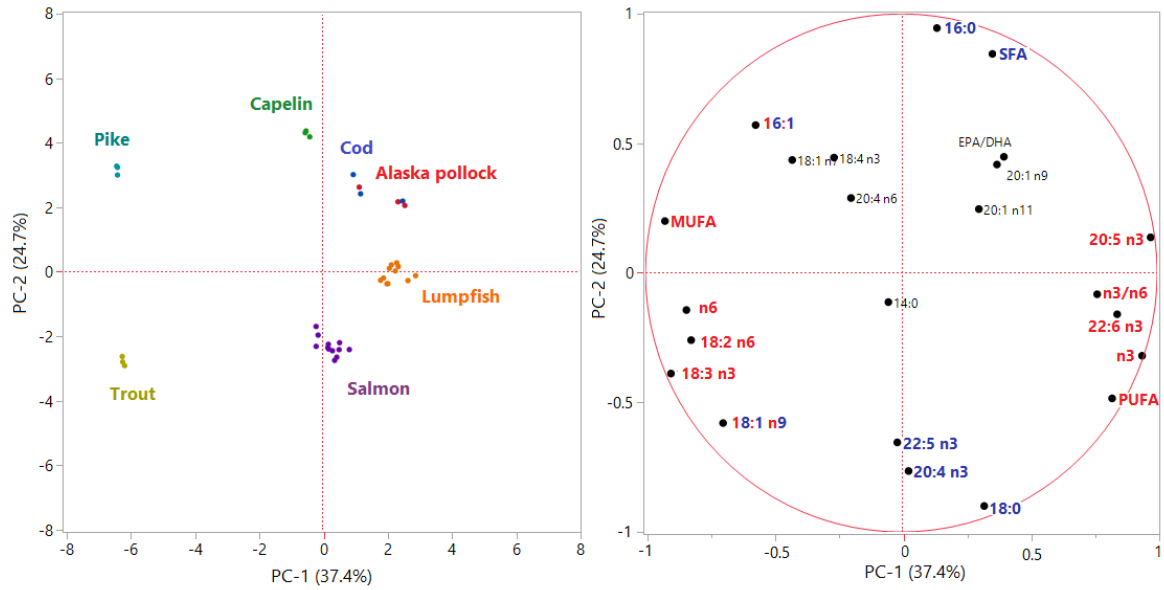

Figure 1. Principal Component Analysis score plot (left) and loading plot (right). In the loading plot, variables with loadings > |0.5| on PC-1 are in red, whereas variables with loadings > |0.5| on PC-2 are in blue.

The variables selected by means of PCA were then employed in the construction of a Linear Discriminant Analysis (LDA) model, performed in a new 38 × 16 matrix (38 samples and 16 fatty acids). The canonical plot obtained after the performance of the LDA is presented in Figure 2 whereas in Table 4, the Mahalanobis distances matrix is shown.

Table 4. Mahalanobis distances calculated as Euclidean distances among groups centroids in the canonical plot defined by Canonical-1 and Canonical-2 on the x- and y- axis, respectively.

	SALMON	LUMPFISH	CAPELIN	TROUT	PIKE	COD
LUMPFISH	52.3					
CAPELIN	70.6	23.7				
TROUT	144.9	149.4	138.3			
PIKE	74.7	57.4	44.9	93.6		
COD	71.0	19.3	22.7	160.1	66.6	
ALASKA POLLOCK	17.3	6.9	21.6	157.2	64.9	0.9*

*Comparison among group means on Canonical-1 and Canonical-2 scores did not show any statistical difference between Cod and Alaska Pollock centroids ($p > 0.05$).

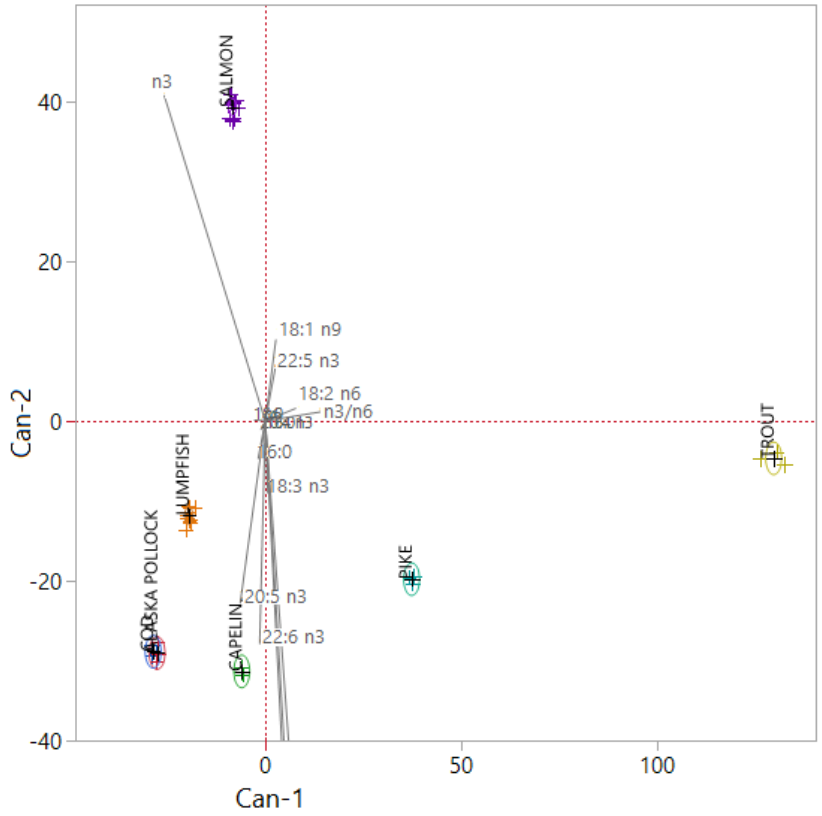

Figure 2. Linear Discriminant Analysis canonical plot. Each group is related with a 95% confident region density ellipse. In this graph, the scoring coefficients of the variables have been standardized and then multiplied by a scaling factor corresponding to 4.5, in order to better fit the canonical plot.

Sixteen variables were included in the model, in which Canonical 1 (Can-1) and Canonical 2 (Can-2) explained 83% of the total variance, so increasing the discriminant power if compared to the PCA previously performed. In order to investigate the influence of the variables on the construction of the discriminant model, their scoring coefficients were standardized and ordered by their weight on Can-1 and Can-2, respectively. In such LDA model, factors that most affected groups separation were 18:2 n6 and the n3/n6 ratio on Can-1; 18:1 n9, 22:5 n3, 22:6 n3 on Can-2; the total SFA, total MUFA, total PUFA, and total n3 FA, plus 20:5 n3, on both the canonicals. All roe samples coming from the different fish species analyzed in this study were clearly distinguishable by means of the model built on the basis of fatty acid data, with the exception of cod and Alaska pollack roes that showed a strong overlapping among groups. As a matter of fact, the LDA model allowed a proper classification of all samples even if one Cod sample was assigned to the proper group with an uncertainty corresponding to 24%. The superimposition between Cod and Alaska Pollock samples is also observable in Table 4, where the Mahalanobis distance of Cod and Alaska Pollock centroids revealed that they were not significantly different ($p > 0.05$) when testing group means. These outcomes reflected the similarity observed by means of univariate analysis on fatty acids composition. The two species, cod and Alaska Pollock, belong to the same family, the *Gadidae*. They share the same environment represented by the cold waters of the northern hemisphere, the

same reproductive habits and the same feed substrate; all these biological similarities resulted in a very close composition of their roes, not allowing our analyses to make a clear distinction between samples coming from their eggs. In the canonical bi-plot, samples belonging to groups cod, Alaskan pollock, lumpfish and capelin, pure marine species, were allocated in the same dial of the LDA chart related to n3 FA, particularly EPA and DHA, while pike and trout, freshwater and farmed species were in the opposite dial. Salmon showed a peculiar behavior, distancing itself from both marine and freshwater species. Salmon was the species that showed a higher value of n3 fatty acid, having the highest value of n3 fatty acid different from EPA and DHA, like eicosatetraenoic acid (ETA 20:4 n3) and docosapentaenoic acid (DPA 22:5 n3).

Finally, to ascertain whether the model would be able to distinguish among fish roes from these species, considered as caviar substitutes, and sturgeon caviar, we included in the dataset 37 samples of caviar previously analysed for fatty acid composition (Borella et al., 2016; Lopez et al., 2020). Then, we performed the same investigation, by means of LDA, on the new matrix (75 × 16); the canonical plot obtained is showed in Figure 3.

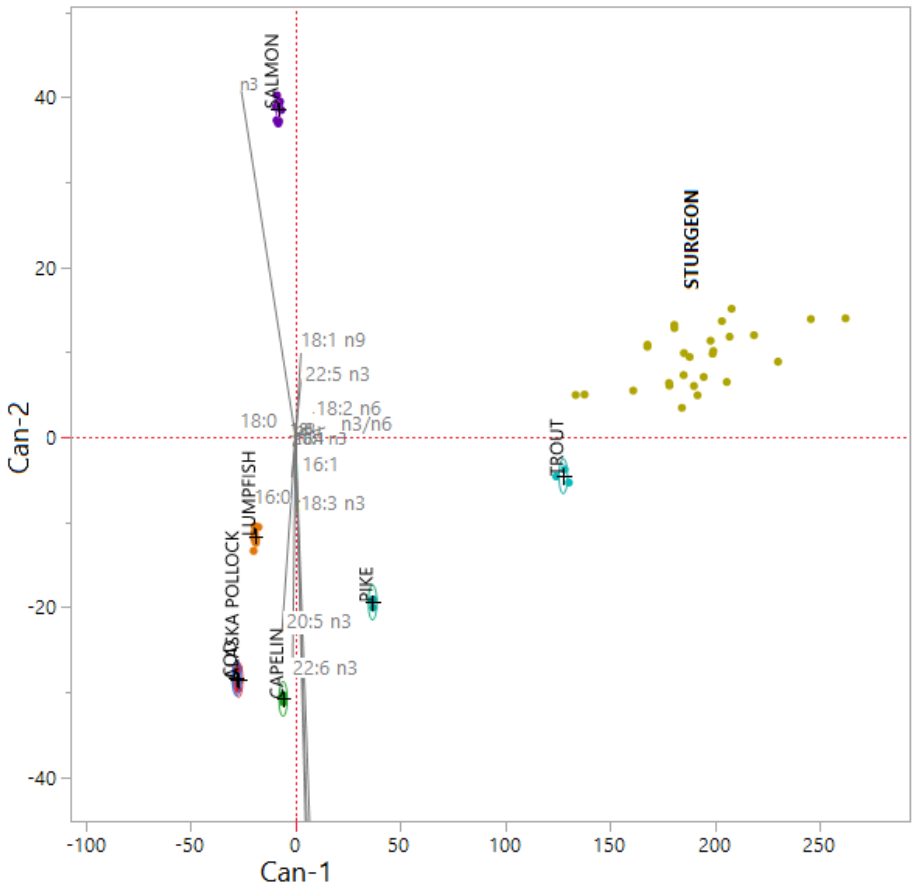

Figure 3. Canonical plot obtained after the employment of the linear discriminant analysis (LDA) model using fish roes from various species as training set and including 37 sturgeon caviar samples (Borella et al., 2016; Lopez et al., 2020) as validation set.

We can observe that all roe samples coming from the various fish species analyzed in this study were clearly distinguishable from caviar. Caviar samples showed a more intra-group spread if compared to other fish roes samples. This phenomenon is due to origin of caviar samples, that were obtained from four different sturgeon species, which presented significative differences in their fatty acid composition (Borella et al., 2016). Caviar showed a collocation in the plot close to trout roes samples. This phenomenon is linked to the origin of these samples; all of them came from farmed fish and their fatty acid signature is modified by the fatty acid of plan origin that characterize aquafeed, like linolenic acid (LA) and OA (Lopez et al., 2020).

3.2 Food safety of fish roes products

According to the information found on labels placed on packaging showed (Table 1), salmon roes was the only product where no additive were added during processing. Pike, capelin, and lumpfish roes have been treated with thickeners, probably to give a more consistent texture to eggs. Capelin and lumpfish eggs were also treated to confer red, black, and green colors to these products, which naturally have a pale yellow color, being unattractive for consumers. Preservatives are generally added to fish roes in order to lower the pH, to prevent lipid oxidation, and to make eggs an unfavorable substrate for the growth of microorganisms. The decrease of the pH value contributes to maintaining the quality of fish roes during processing and storage (Lapa-Guimarães et al., 2011). During the extraction, fish roes should be considered sterile; however, they rarely remain sterile as microorganisms present on the surface of fish could be transferred to the roes, as well as the screening stage of eggs could contribute to contamination (Bledsoe et al., 2003). Thus, good manufacturing practices and a correct refrigeration maintenance are crucial to avoid bacterial replication during storage (Lapa-Guimarães et al., 2011).

All the products analysed resulted safe as the presence of *L. monocytogenes* was never found. According to Reg. UE 2073/2005, for ready-to-eat food with more than 5 days of shelf-life and able to support the *L. monocytogenes* growth, a tolerance level of 100 CFU/g is indicated during their shelf-life. Although fish roes have a short shelf-life, they are considered to support the growth of this potential pathogenic microorganism. As reported by Miya et al. (2010), *L. monocytogenes* was found able to grow at 22 °C in 6 h; in the same study and differently from our findings, *L. monocytogenes* was found present with prevalence equal to 5.7% in salmon roes and 9.1% in cod roes, respectively. In a previous study, a prevalence *of L. monocytogenes* from 10 to 11.4% was found in fish roe products (Handa et al., 2005).

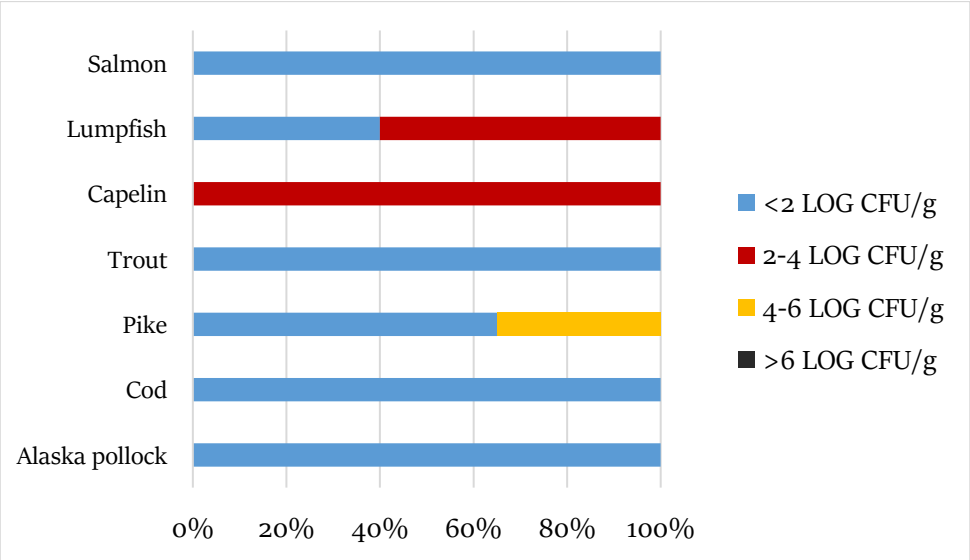

Figure 4. Total Viable Count of fish roe samples.

Total Viable Count (TVC) was generally very low, with 18 out of the 25 whole samples analysed (72%) showing values below the detection limit (2 Log CFU/g). Three out of the five samples of lumpfish roes analysed were countable with mean values equal to 2.26 Log CFU/g and all the capelin roes samples were countable with mean value equal to 3.14 Log CFU/g. The highest value of TVC detected was 5.00 and it was revealed in a sample of pike roes. The threshold limit of 6 Log CFU/g, frequently used for food products to designate the end of shelf-life of a fish product (Miya et al., 2010), was never exceeded. Moreover, values of total viable count above 7-8 Log CFU/g are often associated to sensory rejection. In agreement with our data, Oeleker et al. (2015) evidenced a good microbiological situation with 54% of fish roes analysed in which total viable count below 2 Log CFU/g, while 84% loads below 4 Log CFU/g. The loads obtained in our study were in general lower if compared to those obtained by Altug and Bayrak (2003) for caviar from Russia and Iran, where TVC was from 3 to 6.41 Log CFU/g, and those obtained by Hilmelbloom and Crapo (1998) in pink salmon ikura with loads from 3.48 to 6.48 Log CFU/g.

Lactic Acid Bacteria, Enterobacteriaceae, *B. cereus*, and Enterococci, resulted always below the detection limit (2 Log CFU/g). In all samples, Coagulase Positive Staphylococci resulted below the detection limit, except for a sample of lumpfish roes with a load of 3.88. Loads that are considered risky in terms of toxin production are generally set from 4 to 5 Log CFU/g, thus in our case, no particular concern resulted from their presence. Additionally, Oeleker et al. (2015) evidenced a single sample containing Coagulase Positive Staphylococci where the load resulted just above 3 Log CFU/g.

Clostridia were always below the detection limit in all the samples analysed: as in fish roe, sterilization could not be applied due to the protein irreversible denaturation process, particular attention should be used to avoid the germination of possible spores present in the product, especially those belonging to *C. botulinum*. The maintenance of low refrigeration temperatures according with salt content are key factors in order to produce a safe product, able to inhibit *C. botulinum* germination. FDA suggests a Water Phase Salt (WPS) above 3.5%; in

the products analysed, lumpfish roes, and capelin roes resulted to be characterized by a WPS below this limit (3.1 and 2.9%, respectively), thus the intrinsic characteristics of these two products could not guarantee the safety of the final product.

All pH values ranged from 5.12 to 6.28: at this pH range, benzoic acid may be effective against moulds and yeast in the range of 100–500 ppm (Jay, 2000). It is fundamental to maintain a certain pH range in food products in order to inhibit the metabolic activity of spoilage microorganisms. Salmon and pike roes were found to be permissive for moulds replication since they had low benzoic acid and sorbic acid concentrations (<LOD equal to 52 ppm and 41 ppm, respectively) (Table 5).

Table 5. pH and organic acids (mg/kg) content of fish roes. Data are expressed as mean ± standard deviation.

Fish Species	**SALMON** *N* = 12	**LUMPFISH** *N*= 11	**CAPELIN** *N* = 3	**TROUT** *N* = 3	**PIKE** *N* = 3	**COD** *N* = 3	**A. POLLOCK** *N* = 3
pH	6.28±0.08	5.77±0.09	6.19±0.06	5.95±0.03	5.88±0.05	5.96±0.06	5.95±0.03
Citric acid	nd	nd	1356±68	898±75	2695±192	<LOQ	<LOQ
Lactic acid	676±261	5343±2472	1546±172	10812± 746	232±7	11072±370	11752±2168
Acetic acid	263±92	nd	nd	713 ± 50	<LOD	875±75	1177±56
Sorbic acid	nd	nd	607 70	2021 ± 35	<LOD	2050±118	2573±491
Benzoic acid	<LOD	858±347	598±120	<LOD	<LOD	285±68	<LOD

LOD: Limits of Detection were 216 ppm for citric acid, 16 ppm for lactic acid, 47 ppm for acetic acid, 41 ppm for sorbic acid, and 52 ppm for benzoic acid. LOQ: Limits of Quantification were 722 ppm for citric acid, 52 ppm for lactic acid, 157 ppm for acetic acid, 151 ppm for sorbic acid, and 159 for benzoic acid.

Moulds were present in all the samples of pike roes in very limited concentration (values from 2.00 to 2.60 Log CFU/g). At the same time, trout, cod, and Alaska Pollock roes were found to be permissive for mould replications even if high levels of sorbic acid were found in these samples. Even if in limited concentrations, we found moulds in all the samples of trout roes (all values equal to 2.00 Log CFU/g), in one sample of cod roes and in two samples of Alaska Pollack roes (loads equal to 2.00 Log CFU/g). Despite the fact that in a pH range between 4.0 and 6.0 sorbic acid is more effective than benzoic acid, due to their different pK (pK of sorbate = 4.80 vs. pK of benzoate = 4.20), sorbic acid has higher undissociated ratio in products with low acidity if compared to benzoic acid. For this reason, we isolated moulds and yeasts also in products characterized by the presence of both sorbic and benzoic acid but a low acidity, like capelin roes. In two samples of capelin roes, we found yeasts with loads corresponding to 2.00 and 2.30 Log CFU/g: high values of yeasts >5 Log CFU/g are recognized to cause organoleptic spoilage that in our samples was never detected. Sorbic and benzoic acids maximum level permitted in EU (2000 ppm) is applicable to both of them, with the final levels expressed as free acid (EU Regulation 1129/2011). Cod and pollack roes were characterized by sorbic acid concentrations that exceed the limit, whereas

trout roes were found to have sorbic acid concentration close to the maximum. All the other products were in compliance to the EU regulation. Six out of eleven lumpfish products declared potassium sorbate (E202) in the list of ingredients, but sorbate salt was not detected in those samples. Cod roes did not declare benzoic acid (E211–E212) on the labels, however they all contained this acid, even if in low concentration. Other organic acids such as citric, lactic, and acetic acid and relative salts are permitted in fish roes with the principle of quantum satis. These organic acids are usually used to lower the pH in food. The order of their effectiveness towards pathogens is acetic > lactic > citric. As was the case for sorbic and benzoic acid, some of the labels did not match our results. Citric acid was found in trout and pike roes (898 ppm and 2695 pmm, respectively) but it was not reported on the labels. Citric and lactic acid (E270) was found in all the samples, ranging from 232 ppm (pike) to 11,752 ppm (Alaska pollock), but none of the products declared lactic acid on the label. Acetic acid (E260) was found in salmon, trout, cod, and Alaska pollock, but even in this case the preservative was not declared. The presence of these undeclared organic acid is not a safety concern but an unfair commercial practice. In Alaska pollock, cod, and trout, the organic acids mixture seem very effective against microbial growth. No data about organic acids in fish roes are available in literature; however, these organic acids were used to build predictive model for *L. monocytogenes* in lightly preserved and ready-to-eat seafood (Mejlholm & Dalgaard, 2009).

4. Conclusions

This study was undertaken to provide the seafood industry and the consumers with information on nutritional and safety properties of fish roe preparations on the market, using a chemometric approach. Proximate composition showed that these products are extremely variable and that their composition depends on the biology of fish, their reproductive environment, and their food habits. Furthermore, this study clearly indicates that the combination of fatty acid composition along with a chemometric approach can be successfully applied to give more information on the original fish species of roes. Generally, fish roes are very rich in essential fatty acid, especially EPA and DHA, and they could be considered a good source of these substances in human diets. Moreover, all the product analyzed were considerable safe for human consumption, since the low microbial loads detected, probably related to the presence of organic acids added to the products during manufacturing processes, were associated with a very limited replication of the present microorganisms.

Author Contributions: Data curation, M.V., E.T. and A.L.; Formal analysis, M.V., E.T., C.C. and C.B.; Funding acquisition, V.M.M.; Investigation, M.V. and E.T.; Methodology, M.V., C.P. and C.B.; Supervision, V.M.M.; Writing—original draft, M.V., E.T., C.C., A.L. and C.B.;Writing—review & editing, S.S., F.B. and V.M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflicts of interest.

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Trial 6

Volatile Organic Compounds Profile in White Sturgeon (Acipenser transmontanus) Caviar at Different Stages of Ripening by Multiple Headspace Solid Phase Microextraction

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Abstract. Caviar is considered a delicacy by luxury product consumers, but few data are available about its flavour chemistry to date. In this study, a multiple headspace-solid phase microextraction (MHS-SPME) followed by gas chromatography and mass spectrometry (GC-MS) approach was developed and employed to identify and quantitatively estimate key volatile organic compounds (VOCs) representative in white sturgeon (A. transmontanus) caviar at five different stages of ripening: raw eggs (to), after 60 days (t1), 120 days (t2), 180 days (t3), and 240 days (t4) of ripening. The method showed the ability to detect and estimate the quantity of 25 flavour compounds, without any severe alteration of the matrix before the analysis and in a short time. The VOCs detected as representative in caviar samples were primarily aldehydes and alcohols, already well known as responsible of fresh fish and seafood flavours, and mainly deriving from lipid peroxidation processes and microbial activity against lipids and amino acids. We found a significant ($p < 0.01$) increase in the amount of total aldehydes within to (29.64 ng/g) and t4 (121.96 ng/g); moreover, an interesting, great arise of 3-hydroxy-2-butanone at the final stage of storage (48.17 ng/g) was recorded. Alcohols were not detected in raw eggs (to) and then a decrease from t1 (17.77 ng/g) to t4 (10.18 ng/g) was recorded in their amount, with no statistical significance.

Results from this investigation have been published in the scientific journal *Molecules*

Molecules **2020**, 25, 1074; doi:10.3390/molecules25051074

1. Introduction

Caviar is defined as the product made from fish eggs of the *Acipenseridae* family by treating with food grade salt *(Codex Alimentarius* Standard, 2013). During the last 15 years, the presence on the market of caviar from aquaculture origin has increased and estimated to amount to 365 tons in 2017 (Bronzi et al., 2019), while the product coming from fisheries gradually disappeared on the legal market due to the global limit of capture of wild sturgeons. Sturgeons species are listed in Annex II and I of the Convention on International Trade in Endangered Species (CITES) and, starting from 2006, CITES has no longer issued any quota for the marketing of caviar from wild stocks, thus catches for caviar production are completely forbidden nowadays. European sturgeon farmers in 2017 produced about 140 tons of caviar, with Italy as production leader with 43 tons, followed by France, Poland and Germany (Bronzi et al., 2019). In such a scenario, the quality assurance and a solid characterisation of caviar as a precious product appear as fundamental issues for the safeguard of the Italian and European markets. Many analytical techniques have been developed during the years to assess caviar authenticity and quality factors. For instance, DNA testing, based on genetic interspecific divergences and variations, is a well-known technique used to verify the species source of the product to date (Pappalardo&Petraccioli, 2019; Fain et al., 2013; Rehbein et al., 2008), even if the presence of hybrid sturgeons could make it difficult to correctly attribute a caviar to a single species. Moreover, it has been demonstrated that the chemical composition of caviar can lead to discriminate, above all, between eggs obtained from farmed vs. wild sturgeons (Caprino et al., 2008; Fisheries et al., 2002; Gessner et al., 2008) or eggs coming from different species (Wirth et al., 2000).

Caviar producers are very careful about the sensory evaluation that is generally carried out in-factory by people purposely trained and in accordance with the Guidelines for the Sensory Evaluation of Fish and Shellfish in Laboratories (*CAC/GL 31-1999*, 1999). The Codex Alimentarius standard (*Codex Alimentarius* Standard, 2013) stands that caviar samples affected by odour and/or flavour indicative of decomposition, oxidation, taste of feed (supplied to farmed sturgeon) or contamination by xenobiotic substances must be considered defective and cannot be addressed to human consumption. In such a context, the interest in developing analytical techniques to determine the characteristic volatile organic compounds (VOCs) profile of this “luxury delicacy” becomes consistent. It is known that in fresh fish and seafood very fresh flavours and aromas are characterised by mild, green and planty notes. The chemical basis of the fresh fish flavour is centred on the polyunsaturated fatty acids very representative in fish lipids. The major flavour impact compounds are several 6-, 8- and 9-carbon aldehydes, ketones and alcohols, which are derived from the fatty acids via specific lipoxygenase activity (David B. Josephson & Lindsay, 1986). However, little information is available in the literature regarding caviar VOCs. Most data are obtained by extraction methods, such as simultaneous distillation–extraction (SDE), which is responsible for the formation of many artefacts, mainly due to the oxidation and thermal degradation of components during the extraction (Caprino et al., 2008). Such technique could be useful when the investigated food matrix consists of products that usually undergo cooking/heating processes, before human consumption. This is not the case of caviar, which is consumed raw, without any previous industrial process, just after the

addition of low concentrations of food grade salt and at the end of an optimal ripening time (generally, five or six months) under refrigeration (at −2 °C).

Solid phase microextraction (SPME) is an analytical technique that allows a solvent-free extraction of analytes of interest developed in 1990 by Arthur & Pawliszyn. SPME works by a partitioning process between the solid and the gaseous phase in the extraction chamber and then a partitioning process between the gaseous phase and a sorbent material, represented by a fused silica fibre coated with a thin layer of a selective coating. The fibre can be exposed to the sample matrix, in order to extract organic compounds of interest directly from the sample (direct immersion SPME) or from the sample headspace (HS). The extracted compounds are then desorbed and separated by HPLC or GC, often coupled with mass spectrometry (LC-MS and GC-MS) (Zhouyao Zhang & Pawliszyn, 1993) and the signal intensity provided by SPME and GC-MS is proportional to the free concentration of target compounds, defining the fraction of the analyte that is bioavailable (Ouyang & Pawliszyn, 2008). During last years, HS-SPME has been applied in many food analysis studies to detect the components responsible for the odour and aroma in a number of different food matrices (Bueno et al., 2019; Cordero et al., 2019; Costa et al., 2013; García-Vico et al., 2017). However, HS-SPME is a non-exhaustive extraction method, since a determinate amount of analyte is removed by the sample matrix until its concentration reaches the equilibrium between the solid and gaseous phases involved in the process. Several approaches have been developed to overcome this issue and to reach a reliable quantification of extracted compounds. One of these is the so-called multiple headspace (MHS) extraction method, a stepped procedure whose theory was introduced by Kolb in 1982. Briefly, an almost exhaustive extraction of analytes is performed exposing the fibre to the sample HS in several consecutive extractions. After this step, a logarithmic linear regression is performed, plotting the number of performed extraction versus the natural logarithm of the respective total ion current (TIC) area for each compound. The slope of the linear regression line obtained represents the natural logarithm of β, where β is an analyte-dependent constant that indicates the extent of the decay across successive extractions, as follows:

$$lnAi = ln\beta\ (i - 1) + lnA1$$

[Equation 1]

with i the number of extraction steps, β the exponential decay of the chromatographic peak area, and A1 the area detected after the first extraction.

The β factors obtained in this step allow the estimation of the total area (Atot) for each analyte or, in other words, the area of the TIC for the analyte if the SPME would not be an equilibrium but an exhaustive extraction technique. In fact, dividing the area obtained after the first extraction by 1-β, it is possible to estimate the total cumulative response area for each compound through a geometric regression function, as shown in Equation (2):

$$Atot = \frac{A1}{1 - \beta}$$

[Equation 2]

By means of Equations (1) and (2), the TIC area for each analyte after a single extraction on the sample can be used to estimate the total area. Consequently, a quantitation of the analytes can be carried out by the interpolation of the Atot in a calibration curve obtained by a typical linear regression model. In this way, the method based on the multiple extractions allows the analyst to quantify compounds in samples with a simplified procedure, since a single extraction is sufficient for the calculation of factors and curves required to estimate analytes amounts (Kolb & Ettre, 1991).

The aim of the present work was to optimise and employ a proper MHS-SPME-GC-MS method, with the final goal to identify and quantify key volatile compounds responsible for white sturgeon (*A. transmontanus*) caviar flavour and to detect expected changes in their amounts during the ripening time.

2. Results and discussion

2.1 MHS Extraction Method Development

The curves, equations and calculation factors obtained by the development of the MHS-SPME techniques are shown in Table 1 and Figure 1.

Table 1. Data obtained by the development of the multiple headspace-SPME (MHS-SPME) GC-MS method and by the external calibration curves. β factors were obtained by the logarithmic linear regression plot of the chromatographic areas recorded during the multiple extractions (shown in Figure 1a). The slope (m), the intercept (q) and the correlation coefficient (R^2) are referred to the external calibration curves obtained by the injection of four known concentration of standards (shown in Figure 1b).

TARGET FAMILY	TARGET COMPOUND	β	STUDIED RANGE (ng)	m	q	R^2	LOD (ng)
ALDEHYDES	nonanal	0.67463	1–50	286363	-1×10^6	0.9834	0.71
ALCOHOLS	oct-1-en-3-ol	0.74021	1–50	422066	-2×10^6	0.9773	0.91
ACIDS	nonanoic acid	0.44139	1–50	84741	572724	0.8744	1.92
TERPENES and HYDROCARBONS	α-Pinene	0.49902	1–50	910076	-2×10^6	0.9903	0.17
KETONES	heptan-2-one	0.67552	1–50	490916	-1×10^6	0.991	0.46
ESTERS	ethyl decanoate	0.3256	1–50	636175	-2×10^6	0.9843	1.02

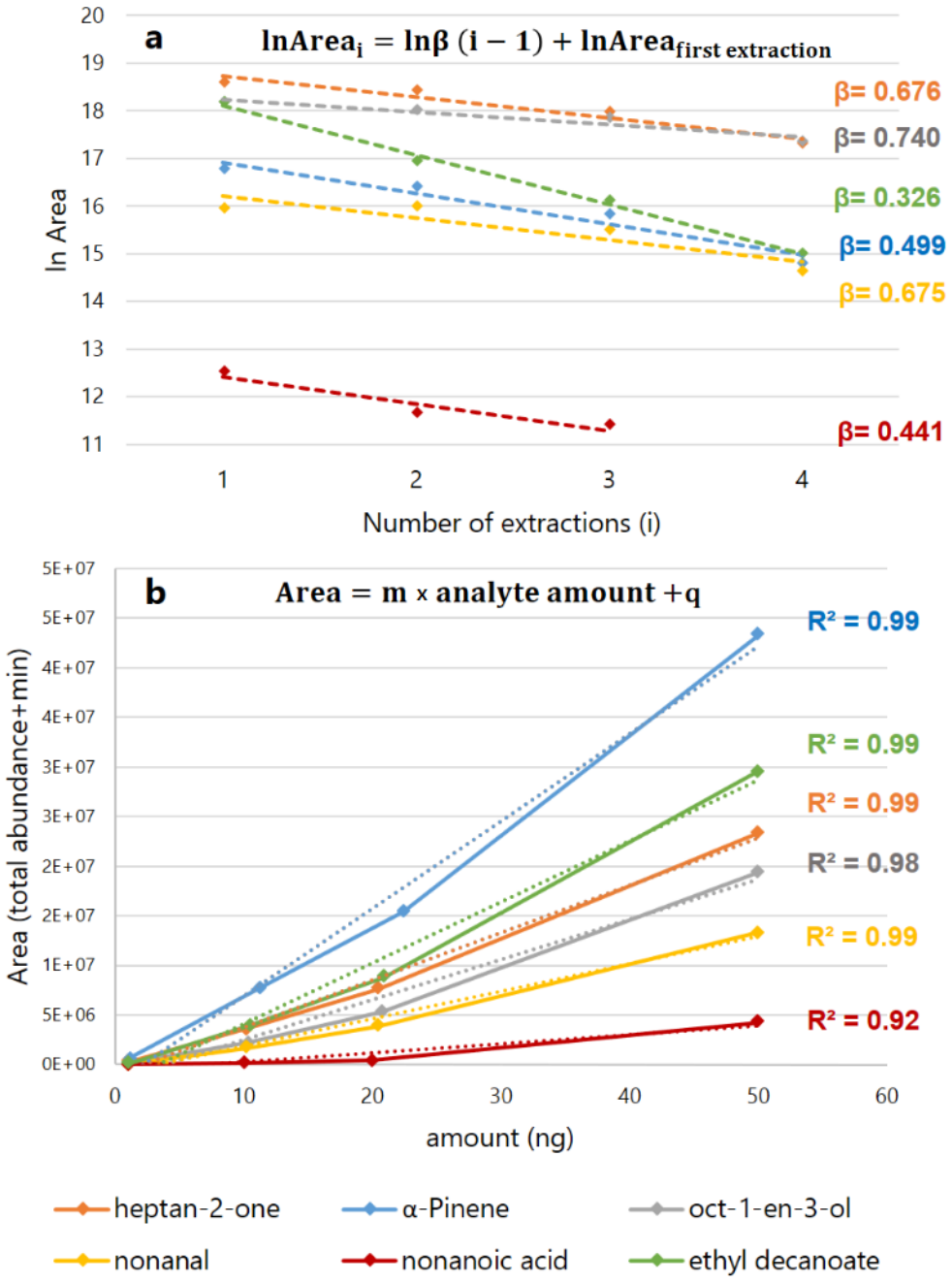

Figure 1. Linear regression plots employed in the determination of β values for each target compound, by means of the multiple extractions technique on calibration mixtures (**a**), and to the estimation of analytes' total areas in samples, by means of liquid injections of four different concentrations of calibration mixtures (**b**) (numerical data are shown in Table 1).

The development of the multiple extraction technique provided a good response when considering the exponential decay of target analytes during the consecutive extractions. With the only exception of ethyl decanoate ($\beta = 0.326$), the β factors obtained were included a range considered optimal for a correct estimation of the analytes' total areas ($0.4 < \beta < 0.95$). A β value higher than 0.95, in fact, would indicate that the chromatographic area of the analyte under investigation appears unchanged even after several extractions, meaning that the amount of analyte the fibre can extract is meagre if compared to its total amount. Conversely, a β value lower than 0.4 would mean that the reduction of the chromatographic area among successive extractions is very consistent and that the analyte could be exhaustively extracted even by means of a single extraction (Serrano et al., 2009; Tena & Carrillo, 2007).

We obtained $R^2 \geq 0.98$ for all the curves obtained after the external calibration of the instrument by means of liquid injection, with the exception of nonanoic acid that showed a $R^2 = 0.87$. This phenomenon can been imputed to the low solubility of highly polar carboxylic acids in the non-polar stationary phase of the column (DB-5MS) employed in the chromatographic separation of compounds, resulting in peak fronting and in a low system sensitivity for nonanoic (as well as a higher LOD).

In Figure 2, a TIC chromatogram of a representative sample analysed after 240 days of ripening (t4) is presented.

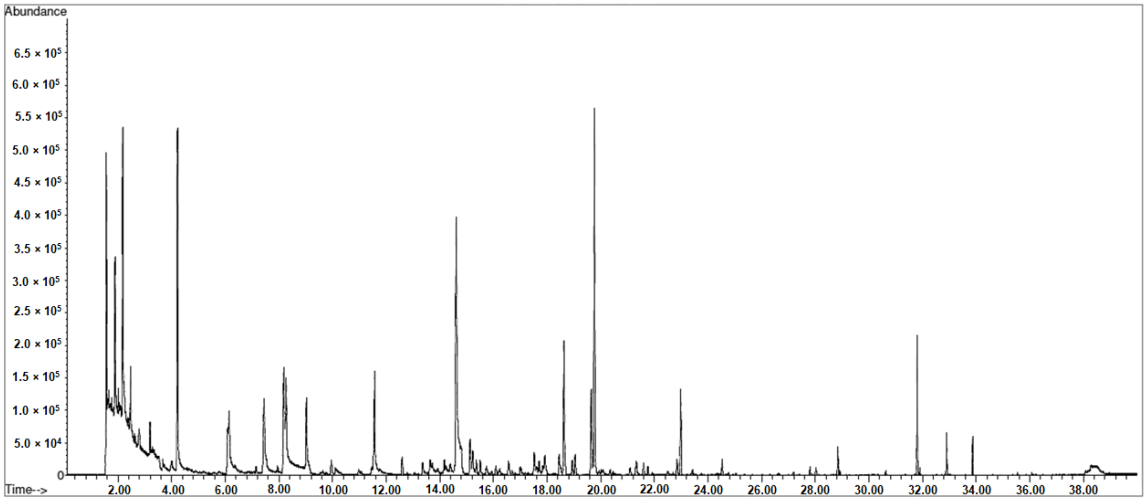

Figure 2. Illustrative TIC of volatile organic compounds in a caviar sample corresponding to a ripening time of 240 days (t4) by means of MHS-SPME-GC-MS.

2.2 Caviar VOCs identification and quantification

Twenty-five key volatile compounds were detected with a good degree of certainty in eggs and caviar samples, showing a significant variability among different ripening times. The estimative quantitation of compounds by means of the multiple extractions procedure provided reliable results. For many compounds, we found a great variability among samples collected at the same ripening time, reflecting considerable standard deviations within the same group (t0, t1, t2, t3, and t4). However, it has to be specified that such entity of data variability could be imputed to the fact that caviar analysed in this study was collected by the producer in cans of different

dimensions (500 g or 1800 g) and adding slightly different concentrations of NaCl (3.6% or 3.8%), which could have influenced the ripening process and led to the huge variability. To overcome this problem, during the construction of the statistical model, we considered cans dimension and NaCl percentage as within-subject factors, in order to evaluate only the significance of the ripening time.

VOCs detected in caviar samples were represented by aldehydes, alcohols, terpenes and non-terpenes hydrocarbons, one acid and one ketone, as shown in Table 2 and Figure 3.

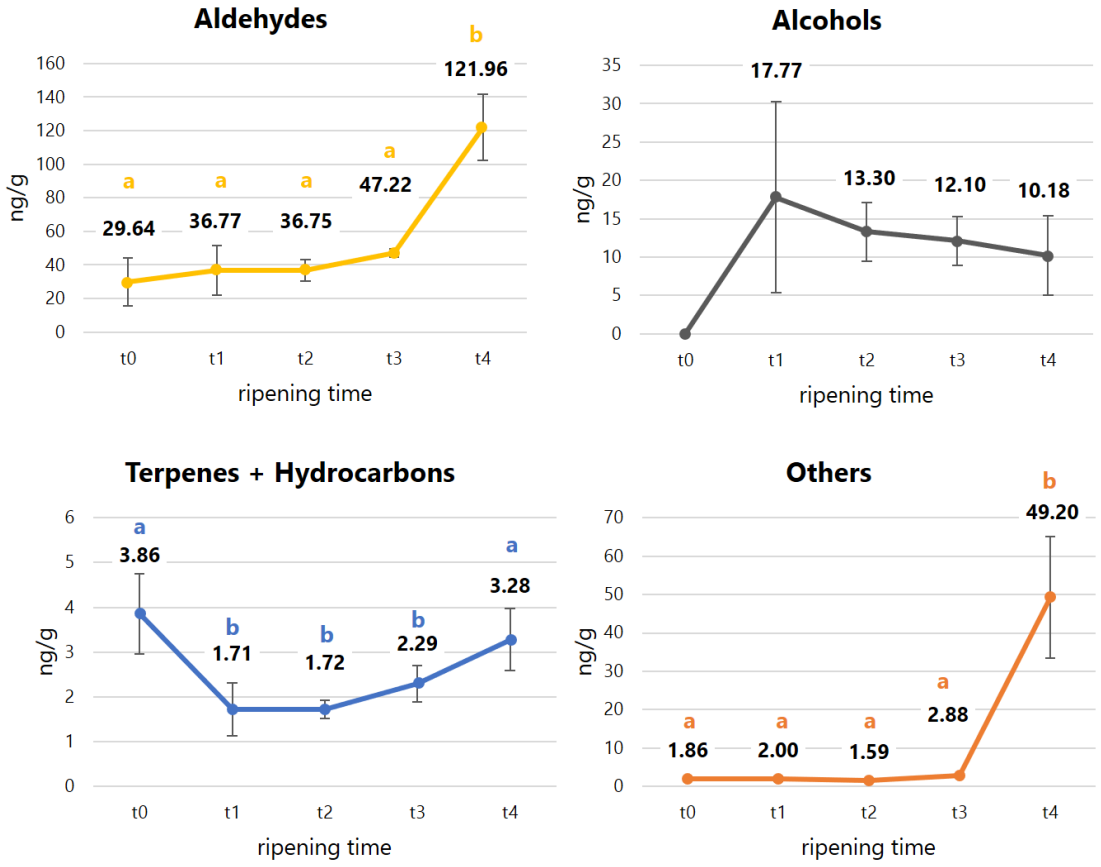

Figure 3. Development of the volatile compounds profile among to (raw eggs) and t4 (240 days) in caviar samples analysed by MHS-SPME-GC-MS.

Table 2. Volatile compounds profile of caviar analysed by MHS-SPME-GC-MS method.

VOLATILE COMPOUND	Rt (min)	MEAN OF IDENTIFICATION	LRI	To RAW EGGS	T1 CAVIAR 60 days	T2 CAVIAR 120 days	T3 CAVIAR 180 days	T4 CAVIAR 240 days	*sign*
3-methyl-1-butanal	3.196	MS, STD, LRI	655	3.48 ± 1.96 A	8.84 ± 5.88 A	9.07 ± 3.74 A	10.10 ± 1.73 A	29.66 ± 7.33 B	**
2-methyl butanal	3.345	MS, LRI	664	nd	2.58 ± 1.20 A	3.20 ± 1.46 A	3.48 ± 0.51 A	11.09 ± 2.76 B	**
Hexanal	7.449	MS, STD, LRI	801	6.70 ± 3.63 A	8.87 ± 2.86 A	7.77 ± 6.49 A	9.93 ± 1.46 A	19.31 ± 12.64 B	**
(E)-hex-2-enal	9.541	MS, STD, LRI	854	nd	nd	nd	0.59 ± 0.52 A	1.36 ± 0.76 B	**
3-methylsylfanylpropamal	11.611	MS, LRI	905	nd	1.49 ± 1.07 A	1.72 ± 0.62 A	2.29 ± 0.22 A	7.66 ± 3.25 B	**
Benzaldehyde	13.642	MS, STD, LRI	960	2.06 ± 0.82 A	1.60 ± 0.49 A	1.27 ± 0.24 A	2.19 ± 0.07 A	4.57 ± 0.97 B	**
Octanal	15.226	MS, STD, LRI	1003	2.48 ± 1.07 A	1.26 ± 0.31 BC	1.03 ± 0.21 B	1.11 ± 0.08 BC	1.64 ± 0.22 C	**
(2E,4E)-hepta-2,4-dienal	15.481	MS, STD, LRI	1010	nd	nd	nd	nd	0.75 ± 0.85	ns
2-phenylacetaldehyde	16.566	MS, LRI	1042	2.01 ± 0.97 A	7.92 ± 5.21 A	7.80 ± 3.33 A	11.46 ± 0.67 A	38.29 ± 14.96 B	**
(E)-oct-2-enal	17.082	MS, LRI	1058	nd	nd	nd	nd	0.97 ± 1.06	ns
Nonanal	18.630	MS, STD, LRI	1104	11.64 ± 7.00 A	3.24 ± 1.22 BC	3.88 ± 0.85 B	4.85 ± 0.67 BC	5.98 ± 1.22 B	**
Decanal	21.755	MS, STD, LRI	1205	1.28 ± 0.39 A	0.96 ± 0.22 AB	1.01 ± 0.12 AB	1.21 ± 0.22 AB	0.68 ± 0.54 B	*
∑ALDEHYDES				29.64 ± 14.31 A	36.77 ± 14.97 A	36.75 ± 6.54 A	47.22 ± 2.53 A	121.96 ± 19.80 B	**

Table 2 (cont.)

Pent-1-en-3-ol	3.675	MS, LRI	682	nd	2.00 ± 0.52	1.96 ± 0.51	2.23 ± 0.29	2.91 ± 2.56	ns
3-methylbutan-1-ol	5.126	MS, STD, LRI	734	nd	nd	nd	nd	4.95 ± 6.32	ns
Oct-1-en-3-ol	14.423	MS, STD, LRI	981	nd	2.07 ± 0.68	1.52 ± 0.70	1.46 ± 0.07	1.35 ± 1.50	ns
2-ethylhexan-1-ol	16.088	MS, LRI	1028	nd	13.70 ± 11.93	9.82 ± 3.84	8.41 ± 2.92	nd	ns
∑ALCOHOLS				nd	17.77 ± 12.45	13.30 ± 3.82	12.10 ± 3.21	9.22 ± 5.56	ns
α-pinene	12.606	MS, STD, LRI	932	0.95 ± 0.44	0.97 ± 0.50	0.73 ± 0.14	0.66 ± 0.04	1.11 ± 0.40	ns
3-carene	15.374	MS, STD, LRI	1007	nd	nd	0.44 ± 0.18	0.57 ± 0.03	0.36 ± 0.28	ns
1,2,3-trimethylbenzene	15.760	MS, STD, LRI	1018	0.66 ± 0.11 A	0.13 ± 0.23 B	nd	0.35 ± 0.31 A	nd	**
Limonene	16.103	MS, STD, LRI	1029	0.63 ± 0.05	nd	nd	nd	0.60 ± 0.03	ns
β-ocimene	16.700	MS, STD, LRI	1047	0.44 ± 0.30	nd	nd	nd	nd	ns
Caryophyllene	27.741	MS, STD, LRI	1423	0.14 ± 0.27	nd	nd	nd	nd	ns
Pristane	32.902	MS, LRI	1704	1.04 ± 0.43 AC	0.62 ± 0.06 B	0.56 ± 0.05 B	0.71 ± 0.03 AB	1.20 ± 0.22 C	**
∑TERPENES AND HYDROCARBONS				3.86 ± 0.90 A	1.71 ± 0.59 B	1.72 ± 0.20 B	2.29 ± 0.41 B	3.28 ± 0.69 A	**

Table 2 (cont.)

2-butanone, 3-hydroxy	4.797	MS, LRI	724	nd	nd	nd	nd	48.17 ± 16.87	**ns**
Nonanoic acid	23.424	MS, STD, LRI	1263	1.86 ± 0.33	2.00 ± 0.60	1.59 ± 0.13	2.88 ± 0.12	1.03 ± 1.65	**ns**
∑OTHER COMPOUNDS				1.86 ± 0.33 A	2.00 ± 0.60 A	1.59 ± 0.13 A	2.88 ± 0.12 A	49.20 ± 15.81 B	**

MS= Comparison with MS spectra obtained by NIST library

STD= comparison with retention time and spectra of authentic refer/ence compounds

LRI= comparison with Linear Retention Indices by van den Dool and Kratz (1963) for a DB-5MS capillary column, calculated by a n-alkanes series (Kovàts, 1958) found in the literature.

^{A,B,C}= values within the same row associated with different letters are significantly different (* $p < 0.05$; ** $p < 0.01$). Quantitative data are expressed as ng/g of sample (mean ± standard deviation)

Most of the volatile compounds found in fish products have been previously associated with the microbial and enzymatic activities occurring during the maturation of the products and with the lipoxygenases pathways acting against fatty acids (Duflos et al., 2006). The largest group of volatiles found in our work was represented by aldehydes. Several aldehydes have been previously found in different fresh and stored fish products (Aro et al., 2003; J. Iglesias et al., 2009; Jacobo Iglesias et al., 2010; D. B. Josephson et al., 1984; Milo & Grosch, 1993; Prost et al., 2004), including eggs and caviar (Caprino et al., 2008; Moretti et al., 2017), showing mostly an important increase during the storage time (Aro et al., 2003; J. Iglesias et al., 2009; Prost et al., 2004). Such aldehydes are considered aroma-active compounds in seafood since they contribute to the characteristic fish-like odour of fish products (D. B. Josephson et al., 1984), also during the cold storage (Guðrún Ólafsdóttir, 2005). According to this, we found a significant increase in the total amount of aldehydes in caviar between to (29.64 ng/g) and t4 (121.96 ng/g). Aldehydes are primarily recognised as secondary unsaturated fatty acids (UFA) peroxidation products (Frankel, 2005; Grosh, 1982; D. B. Josephson et al., 1984; David B. Josephson et al., 1983; Karahadian & Lindsay, 1989), formed by the action of several lipoxygenase systems on n3, n6 and n9 series UFA (D. B. Josephson et al., 1984; Kawai & Sakaguchi, 1996; Lindsay, 1990). For instance, 15-lipoxygenase acts on n3 or n6 polyunsaturated fatty acids (D. B. Josephson et al., 1984; Lindsay, 1990), mainly linoleic acid (Kawai & Sakaguchi, 1996); consequently, from the 13-hydroperoxide of linoleate, hexanal is produced. Octanal, nonanal and decanal are formed from autoxided n9 UFA, particularly oleic acid (Frankel, 2005; Jónsdóttir et al., 2008; Prost et al., 2004), while (E)-hex-2-enal and (2E,4E)-hepta-2,4-dienal originate from the oxidation of n3 PUFA (Prost et al., 2004), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Kawai & Sakaguchi, 1996). The presence of such aldehydes in sturgeon caviar at higher concentration at t3 is certainly related to the breakdown of the radicals of the most representative fatty acids in the matrix during the ripening time. As evidenced by many authors, in fact, oleic acid, linoleic acid, EPA and DHA represent more than 50% of total fatty acids in caviar from farmed sturgeon (Caprino et al., 2008; Gessner et al., 2008; Lopez et al., 2020; Wirth et al., 2000).

In addition, we found 3-methylbutanal and 2-methylbutanal, which are generally considered as key spoilage indicators derived by microbial activity (Balasubramanian & Panigrahi, 2011; Joffraud et al., 2001; Jørgensen et al., 2001), a consequence of amino acid degradation (Aro et al., 2003). The occurrence of many aldehydes, especially the branched and short chain ones, has been suggested to be associated with the breakdown of amino acids by several authors (Ardö, 2006; Giri et al., 2010). Particularly, 3-methylbutanal is thought to derive by the degradation of leucine (G. Ólafsdóttir & Jónsdóttir, 2010), while 2-methylbutanal from isoleucine (Ardö, 2006; Jónsdóttir et al., 2008; Milo & Grosch, 1995; Pripi-Nicolau et al., 2000). In the same way, 2-phenylacetaldehyde and 3-methylsulfanylpropanal derive are formed by the Strecker degradation breakdown of phenylalanine and methionine (Weenen & Van Der Ven, 2001). Generally, Strecker amino acids degradation is a process enhanced by high temperatures (Belitz et al., 2009), thus the presence of such compounds in a fresh product such as raw caviar, stored at −2 °C, might suggest that other degradation pathways could have occurred leading to the formation of such compounds. This hypothesis is supported by the fact that several pathways, other than Maillard reaction, are involved in Strecker aldehydes formation. For instance, the presence of mild oxidising

agents (such as metal catalysts) can lead to the oxidative decarboxylation of amino acids followed by hydrolysis of the imines also at ambient temperature (Yaylayan, 2003). Moreover, other authors previously detected Strecker aldehydes in fish tissues, even in cold storage conditions (Aro et al., 2002; Milo & Grosch, 1995; Selli et al., 2006; Selli & Cayhan, 2009), and, particularly in the case of 3-methylsulfanylpropanal, it showed a significant increase during the storage (Prost et al., 2004).

In the present work, we detected four alcohols, showing a variable trend within to and t4. Similar to aldehydes, alcohols in fish products are formed by the action of lipoxygenase on fatty acids (FA) (Duflos et al., 2006) and by the decomposition of the secondary hydroperoxides of FA (Tanchotikul & Hsieh, 1989, 1991). Particularly, it is known that pent-1-en-3-ol is formed by the action of 15-lypoxigenase on EPA and 12-lypoxygenase on arachidonic acid (ARA) while oct-1-en-3-ol derives by the enzymatic reaction of degradation of linoleic acid (LA) (Alasalvar et al., 2005; Hsieh & Kinsella, 1989; David B. Josephson et al., 1983; Kawai & Sakaguchi, 1996; Prost et al., 2004; Selli & Cayhan, 2009). Oct-1-en-3-ol has been identified as one of the principal volatile alcohols in several seafoods (Spurvey et al., 1998; Tanchotikul & Hsieh, 1991; Vejapham et al., 1988) and previously found also in caviar (Caprino et al., 2008). Pent-1-en-3-ol, indeed, other than lipid peroxidation product, is known to be related to the microbial spoilage activity (D. B. Josephson et al., 1984; Miller et al., 1973). The absence of pent-1-en-3-ol in to and its presence in t1, t2, t3 and t4 samples is in good agreement with results reported by other authors (Alasalvar et al., 2005; Aro et al., 2003; Jørgensen et al., 2001; Nordvi et al., 2007). In the same way, 3-methylbutan-1-ol and 2-ethylhexan-1-ol in fish products have been recognised as microbial spoilage compounds, deriving by the degradation of amino acids (mainly, valine) and lipids (Jørgensen et al., 2001; Miller et al., 1973; G. Ólafsdóttir & Jónsdóttir, 2010). Other authors have previously detected these alcohols in raw tissues of many species of fish and seafood products (Alasalvar et al., 2005; Aro et al., 2003; Duflos et al., 2006; Edirisinghe et al., 2007; Fall et al., 2010; Hsieh & Kinsella, 1989; J. Iglesias et al., 2009; D. B. Josephson et al., 1984; Laursen et al., 2006; Olafsdottir et al., 2005; Prost et al., 2004), with a trend of increase during the storage (Alasalvar et al., 2005; Aro et al., 2003; J. Iglesias et al., 2009; David B. Josephson & Lindsay, 1986; Laursen et al., 2006; Olafsdottir et al., 2005; Prost et al., 2004). For their characteristic marked production during the middle and later stages of fish products storage (Edirisinghe et al., 2007), volatile alcohols have been previously suggested as spoilage and oxidation indicators (Alasalvar et al., 2005; Jacobo Iglesias & Medina, 2008; Lindsay, 1990), also contributing to the off-odours in fish caused by the amino acids and lipid degradation (Miller et al., 1973).

Several odour-active terpene derivatives and two unsaturated hydrocarbons were identified in sturgeon eggs and caviar in this work. Several authors have previously found the same terpenes in fish products and suggested that this family of VOCs is most likely related to fish feed, deriving from algae or plants source (Alasalvar et al., 2005; Jacobo Iglesias & Medina, 2008; Laursen et al., 2006; Olafsdottir et al., 2005; Prost et al., 2004; Tanchotikul & Hsieh, 1991; Vejapham et al., 1988; Zhuomin Zhang et al., 2010). Even in our case, we can hypothesise that such compounds reached sturgeon eggs via the food chain and that their presence did not suggested any significant influence of the ripening pathways occurring in caviar during the storage time. On the contrary, the unsaturated hydrocarbon pristane (IUPAC name: 2,6,10,14-tetramethylpentadecane), a common hydrocarbon originating from fossil and biogenic sources, is known to be present in aquatic environments and

has been previously suggested to reach seafood products, included caviar, by means of the lipid autoxidation processes or from the decomposition of the carotenoids (Caprino et al., 2008; Turchini et al., 2004; Spurvey et al., 1998).

Finally, in our samples, we identified two compounds considered characteristic in fish (David B. Josephson & Lindsay, 1986; Olafsdottir et al., 2003). Meagre amounts of nonanoic acid, ranging from 1.03 to 2.88 ng/g, were found in caviar during the entire storage period, even if without any statistical significance. On the contrary, an interesting, great arise of 3-hydroxy-2-butanone was detected just at the final stage (t4) of the storage time, reaching an amount of 48.17 ng/g. Nonanoic acid is considered one of the major compounds in the original seaweed by-product, deriving from the degradation of polyunsaturated fatty acids either by auto-oxidation or by the action of enzymes, representing a precursor to seafood flavours (David B. Josephson & Lindsay, 1986; Le Pape et al., 2007). The presence of 3-hydrxoxy-2-butanone in fish products, indeed, has been related many times to the growth of microbial strains, e.g., by Ólafsdóttir et al. (2003, 2005). The significant increase of the amount of this compound in seafood products, even when cold storage, has led the authors to suggest this compound as an early indicator of spoilage, useful to monitor the loss of freshness.

The presence of several compounds derived from the lipid peroxidation processes leads us to suggest that, even if caviar were stored in controlled, strict conditions, the high amount unsaturated fatty acids could yield a relevant aptitude toward oxidation. The high unsaturation rate, in fact, could have balanced the reduction of lipid degradation due to the low storage temperature and operated by the antioxidant systems naturally active in the eggs.

3. Materials and methods

3.1 Samples

Four White sturgeon (*Acipenser transmontanus*) egg samples and twelve caviar samples were provided by an Italian caviar company (Agroittica Lombarda SpA, Calvisano, BS, Italy). Each set of samples was collected at different stages of production: raw eggs (t0, n = 4), 60 days (t1, n = 4), 120 days (t2, n = 4), 180 days (t3, n = 2) and 240 days (t4, n = 4) of ripening, for a total of eighteen samples. The caviar analysed was salted with 3.6% or 3.8% of NaCl and stored in 500 or 1800 g cans at −2 °C, with the exception of t3 samples that only include caviar ripened in 500 g cans. Other caviar samples, used as matrix to optimise the analytical procedure, were purchased from the same company. For each sample, an aliquot of 5 g of raw matrix (eggs or caviar) was employed in the analysis without any treatment before VOCs extraction; each sample was analysed in triplicate.

3.2 SPME, GC and MS Parameters

The extraction of volatile compounds was performed by HS-SPME, using a multipurpose sampler MPS2 XL (Gerstel GmbH, Mulheim and der Ruhr, Germany) equipped with the SPME option, followed by GC-MS analysis.

DVB/CAR/PDMS 1 cm SPME fibres were purchased by Supelco (Bellefonte, PA, USA) and used for the HS sampling. This fibre was chosen because of its capacity to extract a high number of VOCs, of different chemical species with different polarities and molecular weights. The bipolar compounds we expected to find in caviar samples, primarily aldehydes, ketones and alcohols, in fact, are known to be better extracted by fibres made of a combination of non-polar and polar materials (Balasubramanian & Panigrahi, 2011). Moreover, we expected to find VOCs in caviar samples at very low concentrations; the DVB/CAR/PDMS works by an adsorption mechanism that is strong and efficient, making this kind of device suitable for analysis on low concentrations compounds. The fibre was exposed to the calibration solutions or sample HS for 30 min at 60 °C. Extracted analytes were recovered by thermal desorption of the fibre into the injection port of the GC system at 250 °C for 1 min. The fibre was left in the injection port with the split valve open for 15 min for conditioning. The GC-MS system consisted of a 6890N Network GC system coupled to a 5973Network Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The column installed in the GC was a DB-5MS (30 m × 0.25 mm id, 0.25 ⌊m film thickness) from Agilent Technologies. During the SPME desorbing phase, the injection port of the GC system was set in splitless mode; during the liquid injection of standard solutions, it was set in split mode (split ratio 1:100). A purge flow of 50 mL/min was set at 2 min to avoid an oversaturation of the MS ion source. The carrier gas was helium with a flow 1.0 mL/min and a pressure of 6.71 psi. The oven temperature program was as follows: from 35 °C (5 min) to 150 °C at 5 °C/min, and then from 150 °C to 260 °C at 10 °C/min (2 min). The mass detector operated in electron ionisation (EI) mode at 70 eV. The scan range of the MS was set to m/z 35-300 with a scanning rate of 5.19 scans/s. Data were acquired by Enhanced ChemStation (Agilent Technologies, Inc., Santa Clara, CA, USA).

3.3 Identification of the volatiles

Key aroma compounds were experimentally selected by extractions performed on representative aliquots of eggs and caviar. Firstly, VOCs were tentatively identified by standard NIST MS library data, and then the identification of selected compound was performed by matching retention indices (RI) according to the theory by van den Dool and Kratz (1963). The LRI were calculated by retention times of a homologous series of n-alkane (Kovàts, 1958). The series of n-alkanes C7 to C30 (1 mg/mL) for determination of RI was purchased by Supelco (Bellefonte, PA, USA). Mass spectra of authentic standards purchased from Sigma Aldrich (Milan, Italy), when available, were collected for VOCs identity confirmation (STD in Table 2). Standard mixtures adopted in identity confirmation were prepared in hexane as solvent at a 10 mg/mL concentration and stored refrigerated. Before the injection, solutions were diluted to a final concentration of 1 mg/mL in hexane and a volume of 1 µL was injected.

3.4 Quantification by Multiple-Extractions and External Calibration Approach

A standard mixture was made selecting one control compound for each family of target compounds detected by the extractions performed on the representative aliquots of eggs and caviar, according to the method of Bueno et al. (2019). The peaks that better arranged in the chromatogram to avoid coelutions were chosen: heptan-2-

one for ketones, 1R-α-pinene for terpenes and unsaturated hydrocarbons, oct-1-en-3-ol for alcohols, nonanal for aldehydes, nonanoic acid for acids and ethyl decanoate for esters. All the analytical standards were purchased from Sigma Aldrich (Milan, Italy). The reference stock solution of target analytes was prepared in acetone as solvent at a 10 mg/mL concentration and stored in a vial under nitrogen at −18 °C for a maximum of four weeks. For the multiple-extraction method development, the reference stock solution was daily diluted and solutions were prepared fresh in 5 mL of HS-water (Sigma Aldrich, Milan, Italy), in order to cover a, for each analyte, the range of absolute amounts from 1 to 50 ng. Multiple extractions from the same calibration vial were performed setting the number of consecutive extractions at four, in order to achieve an almost-exhaustive extraction for all the analytes (a figure is provided in the Supplementary Materials). For the construction of the calibration equation, an external standard strategy was chosen to investigate the response of the instrumental equipment after known analyte amounts injections, as described by Serrano et al. (2009). Calibration solutions were prepared diluting the stock solution in hexane as solvent (Sigma Aldrich, Milan, Italy), covering four known concentration (100 μg/mL, 1 mg/mL, 2 mg/mL, and 5 mg/mL) in order to inject the corresponding total amount of 1, 10, 20, and 50 ng (injection volume 1 μL, split ratio 1:100). Each concentration was analysed in duplicate. The sensitivity of the detection system was measured by estimation of limit of detection (LOD) setting the signal to noise (S/N) ratio at 3 to the most diluted standard solution, according to other authors (Costa et al., 2013; Serrano et al., 2009).

3.5 Statistical Analysis

After data collection, the evaluation of the influence of storage time was performed by a univariate split-plot ANOVA for repeated measures. Significance was declared at $p < 0.05$ (*) and $p < 0.01$ (**). The statistical analysis was performed using JMP Pro 14 (SAS Institute Inc., Cary, NC, USA).

4. Conclusions

In this study, a method for the determination of caviar VOCs by means of MHS-SPME coupled to GC–MS was developed and employed, showing the ability to identify and quantify VOCs in samples without any severe alteration of the matrix before the analysis and in a relatively short time. This method allowed a reliable estimation of the analytes' quantities, solving the question of the non-exhaustive extraction due to the SPME working principle. The drawback in this kind of study remains the different analytes adsorption and partitioning behaviours that the authors think could have led to a competition among the components during the extraction phase and to interferences in the recovery rates. However, the results obtained predominantly show a trend in accordance to what is previously reported in the literature for the most of detected compounds. The relatively small number of compounds detected in caviar, if compared with the results obtained in previous studies on other fish products by headspace sampling techniques, may be because the storage conditions of analysed caviar samples were not so favourable for the microbial and enzymatic activities generally responsible for VOCs formation, as previously discussed. However, the results of this work mainly show the presence of several

compounds that have been identified as characteristic of fish products, with some significant variations along different ripening time. The identification and the quantitative analysis of compounds responsible for caviar flavour described within this research represent an innovation in the field, adding knowledge and providing data almost missing in the literature to date. This may represent a substantial contribution to the available literature, beneficial to acquire a deep knowledge about this outstanding Italian product and also to protect and enhance its market.

Author Contributions: Conceptualisation, A.L.; Data curation, A.L.; Formal analysis, A.L.; Investigation, A.L., M.V., T.M. and M.P.; Methodology, A.L.; Project Administration, V.M.M.; Supervision, V.M.M. and F.B.; Validation, V.M.M. and F.B.; Visualization, A.L.;Writing, A.L., F.B., M.V., T.M., M.P. and V.M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

Supplementary Materials: The following are available online at http://www.mdpi.com/1420-3049/25/5/1074/s1

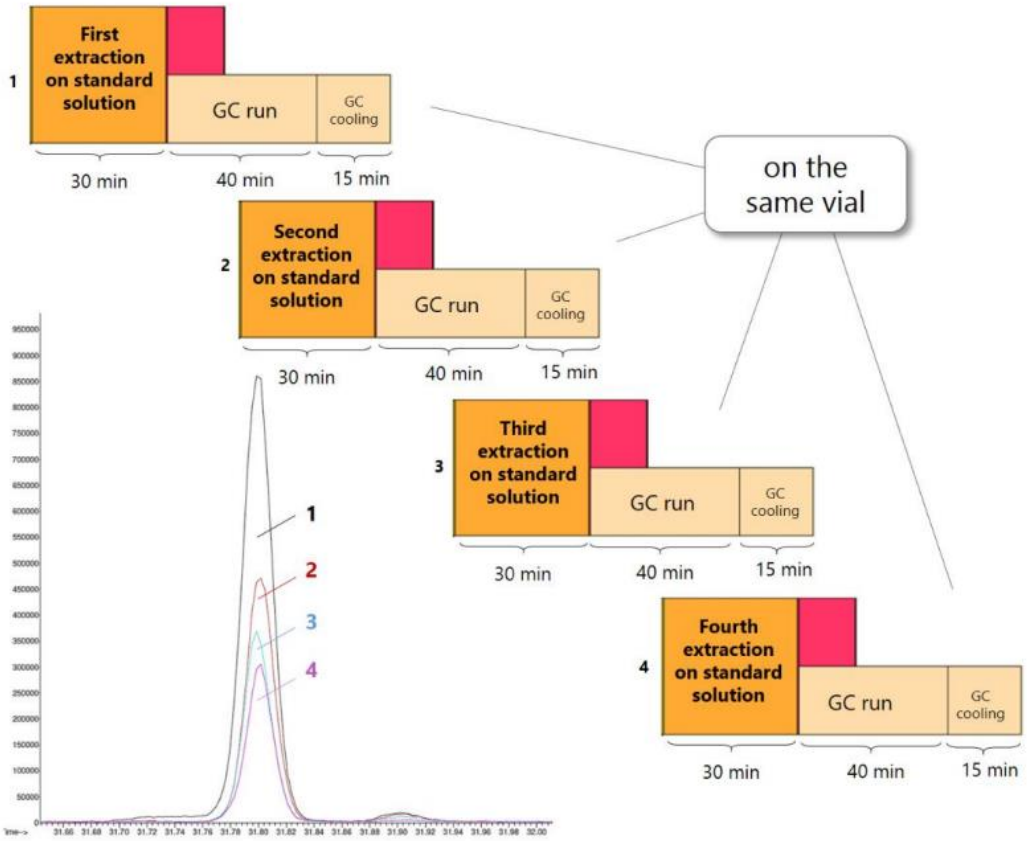

Figure S1. Illustrative multiple headspace solid phase microextraction protocol on a calibration mixture using an automated MPS multipurpose sampler (Gerstel Mullheim a/d Ruhr, Germany).

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Trial 7

Evolution of Food Safety Features and Volatile Profile in White Sturgeon Caviar Treated with Different Formulations of Salt and Preservatives during a Long-Term Storage Time

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Abstract. Caviar is a semi-preserved fish preparation in which cold storage (around 0 °C) and packaging under anaerobic conditions are fundamental to guarantee adequate safety parameters. Consumers seem to prefer caviar prepared with food salt only, but according to the needs of the different distribution channels, some preservatives are used in order to prolong its shelf life and to allow less restrictive storage conditions. Traditionally, the most common preservative was sodium tetra-borate (borax), a salt that contributes to the sensory profile of caviar. However, due to its toxicity, borax has been banned in many countries, and the current trend is to reduce or eliminate its use. In this study, we evaluated the evolution of food safety parameters (pH, water activity, microbiological parameters) and the volatile profile during 14 months of storage in caviar samples treated with three different preservatives: I. exclusively NaCl, II. a mixture of borax and NaCl, and III. a mixture of organic acids and salts. Microbial presence was studied by means of plate counts; vola-tile organic compounds were identified on the sample headspace by means of solid phase micro-extraction with gas-chromatography and mass spectrometry. Results showed relevant differences among the three treatments investigated, with salt samples characterized by the highest viable counts and the greatest presence of volatile products driven by oxidative and spoilage processes, mainly occurring toward lipid and amino acids. On the contrary, the mixture of organic acids and salts showed the best response during the entire storage period. Finally, the employment of a multiparametric statistic model allowed the identification of different clusters based on the time of ripening and the preservative treatments used.

Results from this investigation have been published in the scientific journal *Foods*

Foods **2021**, 10, 850; doi:10.3390/foods10040850

1. Introduction

Nowadays, caviar is a luxury food product distributed and appreciated world-wide. In the early 1700s, caviar was spread mainly in Caspian Countries, but during the 18th century, it started to be imported and cherished in Europe as well. Here it reached a "fashion status" among the wealthy class. During the last part of the 20th century, since the advantageous economical gain, caviar manufacturers started to practice overfishing toward wild sturgeon, and to massively produce caviar addressed to the export market (van Uhm & Siegel, 2016). This happened in any water basin representing the sturgeon's natural habitat, both in the Eurasian and American countries. Natural stocks of stur-geons started to be heavily threatened by both anthropic alteration of the natural en-vironment, pollution, and overfishing. Thus, in 2006 production of caviar coming from wild fisheries was banned by international law (CITES). Consequently, sturgeon aquaculture started to notice a re-markable growth for the first fifteen years of the 21st century. According to the latest estimates in 2017, Italy was the 7th producer worldwide in terms of sturgeon biomass production (850 tonnes), and the 3rd in terms of aquaculture caviar production (43 tonnes) (Bronzi et al., 2019)].

Caviar can be counted as a semi-preserved fish product (Huss, 1997). Following the guide-lines of the *Codex Alimentarius* Committee, within the European Union only the salt-treated eggs harvested from the *Acipenseridae* fish family specimens can be named as "caviar" (Codex Standard, 2013). This kind of product is characterized by values of NaCl in water phase > 6% (w/w) or a pH < 5.0. Chill storage (< 5 °C) for these products is essential, and, together with the employment of anaerobic packaging conditions, it represents the main preventive measure against the hazardous growth of pathogenic microorganisms, such as *C. botulinum*. Failing that, the mesophilic and salt-resistant pathogens would be able to grow, representing a great risk to the consumers, since semi-preserved products are eaten without cooking (Huss, 1997). Actually, even if caviar is produced under the highest hygiene conditions, many microbial contaminations may occur because of the high protein and lipid content and the lack of pasteurization steps (Heshmati et al., 2013). The caviar product more appreciated by consumers is represented by sturgeon eggs prepared with food salt only (Jubenot, 1992). Salt facilitates the preservation and brings out the exalted flavor. In 2006, Gussoni et al. (2006) showed evidence of a higher lipid mobility in salted sturgeon eggs compared to unsalted eggs by measuring the relaxation decay of lipid components through nuclear magnetic techniques. Since caviar that is only salted is not treated with any preservative, it is mandatory for it to be stored at −2 or −3 °C.

However, salt alone is generally considered insufficient to preserve a high quality of caviar for a long term (over 6–8 months, depending on the concentration) (Williot et al., 2001). Thus, according to the needs of the different distribution channels, in order to extend the shelf-life of caviar and to allow the mildest storage conditions, sturgeon eggs can be treated with some food preservatives. Particularly, the Commission Regulation (EU) No 1129/2011 requires that within the European Union (EU), sturgeon caviar is the only food product that can be treated with boron as a food additive, in the form of boric acid (E284) and sodium tetraborate (borax–E285). The treatment of sturgeon eggs with boric acid and borax allows the conservation of caviar even at higher temperatures (2 and 4 °C) (Williot et al., 2001). Boric acid and its salts are not considered suitable for use as food additives in many countries. However, in 2013, the European Food Safety Authority (EFSA) stated that boric acid and its salts are toxicologically acceptable for use as a preservative in genuine caviar. As a matter of fact,

the EFSA evaluated that it is un-likely that consumers' exposure to boron through caviar consumption occurs on a regular basis, and, thus, unlikely that a regular exceedance of the ADI, corresponding to 0.16 mg boron equivalent/kg bw/day, would occur (EFSA, 2013).

Organic acids and their salts are microbial preservatives, frequently employed to inhibit the growth of microorganisms in food. They are well known to inhibit the growth of both bacterial and fungal cells by means of several action mechanisms: membrane disruption, inhibition of essential metabolic activity, arousal of intracellular pH homeostasis stress, and so on. Particularly, sorbic acid (E200) and its salts, such as potassium sorbate (E202) are known to inhibit also the germination and outgrowth of bacterial spores (Brul & Coote, 1999). Furthermore, many organic acids, such as ascorbic and isoascorbic acid (E300 and E315) are known as powerful antioxidants. They work as oxygen radical scavengers, allowing the reduction of the peroxyl radicals that propagate lipid peroxidation, known as the main mechanism by which fats and fat-containing foods undergo spoilage (Parke & Lewis, 1992). Within the EU, the usage of sorbic acid (E200), isoascorbic acid (E315), and their salts as antimicrobial and antioxidant preservatives is approved in a wide range of foods, included many preserved and semi-preserved fish products, such as fish roes (Annex II, Reg. (EC) No 1129/2011).

Beyond the aspects of food safety, the concept of excellence for caviar is strictly linked to its flavor properties. Sensory evaluation is a fundamental step in the caviar production chain, and nowadays, the caviar market is defined by a wide diversification, linked to the availability of several products, each one characterized by different quality and different prices. High-quality caviar is undeniably characterized by the absence of off-flavors; samples that do not meet this requirement cannot be addressed to human consumption, as stated by the Codex Alimentarius standard (Codex Standard, 2013), and represent a waste, and consequently an economic loss, for producers. It is known that the presence of off-flavors in caviar is mainly related to microbial contamination of the product (Oeleker et al., 2015), to the presence of metabolites driven by enzymatic reactions occurring under both aerobic and anaerobic conditions (Heude et al., 2016), and to the presence of lipophilic compounds absorbed by fish eggs during the farming stage (Bonpunt, 2018). Incorrect procedures occurring during the product preparation could lead to unacceptable deficiencies. For example, incorrect handling, bad storage temperature control, or incorrect manipulation of cans during the canning and re-canning steps could lead to contamination of the matrix or to the undesired presence of oxygen in cans, leading to product oxidation and deterioration.

So far, the influence of additives on the stability of caviar and its volatile profile has not been described in any previous study. Therefore, the aims of this work were: (a) to evaluate the impact of the use of three different additives on the development of organic volatile compounds in white sturgeon (*Acipenser transmontanus*) caviar during an extended storage time; (b) to assess the overall safety properties of the product un-der these conditions; and (c) to establish, if possible, which is the ideal ripening period for the marketing of caviars with optimal organoleptic and safety properties.

2. Materials and methods

2.1 Sampling

Caviar samples were provided by an Italian caviar company (Agroittica Lombarda SpA, Calvisano, BS, Italy). Raw eggs were obtained from 12 sexually mature females of white sturgeon (*Acipenser transmontanus*). Roes were sieved and washed before the addition of the salt/preservative mixtures. Samples were treated following different formulas, added under aseptic conditions at the amount (% by weight) suggested by the good manufacturing practice rules which codify caviar production in the enter-prise that supported the investigation— always complying, however, with the limit levels established and defined in the Commission Regulation (EU) No 1129/2011 for fish roes and caviar (Section 09.3)— as follows:

I. NC series: sodium chloride (NaCl) was added at 3.8% by weight;

II. Experimental OAM (organic acids mixture) series: an experimental preservative, made of a mixture of sodium chloride, sorbic acid (E200), potassium sorbate (E202), and isoascorbic acid (E315), was added at 4% by weight;

III. BSM (borax and salt mixture) series: salt and sodium tetraborate (E285) mixture was added at 3.8% by weight.

Each sample was placed in a special metal can called the "original tin" containing 500 g or 1800 g of caviar. This type of tin helps the air and any excess liquid escape. Then, cans were kept at a storage temperature of −2 °C and handled according to the company's processing protocol. Samples were collected and analyzed starting from the day of extraction (t0), and then at three different time points during the ripening, corresponding to 4 months (t1), 8 months (t2), and 14 months (t3).

The samples collection design is briefly reported in Table 1.

Samples were sent to university laboratories under refrigeration conditions and kept at the storage temperature until the analysis. Unfortunately, an exception was represented by BSM samples at t0, which were stored in freezing conditions before the chromatographic analysis because of technical problems that unexpectedly occurred with the analytical equipment.

2.2 pH and a_w

The pH was measured at each sampling time using a pH meter (Amel Instruments, Milan, Italy), using 4.0 and 7.0 as calibration points. Water activity (aw) was determined using an Aw-DIO Hygromer humidity sensor (Rotronic, Bassersdorf, Switzerland), using 0.8 as the calibration point.

Table 1. Samples collection scheme. Legend: NC, sodium chloride (NaCl); OAM, organic acid mixture; BSM: borax and salt mixture.

			March 2019 Raw Eggs	July 2019 4 Months	November 2019 8 Months	May 2020 14 Months
ID	**Series**	**Can**	**to**	**t1**	**t2**	**t3**
1 **2** **3** **4**	NC 3.8%	500 g	*N* = 2	*N* = 2	*N* = 2	*N* = 2
		1800 g	*N* = 2	*N* = 2	*N* = 2	*N* = 2
1 **2** **3** **4**	OAM 4%	500 g	*N* = 2	*N* = 2	*N* = 1	*N* = 2
		1800 g	*N* = 2	*N* = 2	*N* = 1	*N* = 2
1 **2** **3** **4**	BSM 3.8%	500 g	*N* = 2	*N* = 2	*N* = 1	*N* = 2
		1800 g	*N* = 2	*N* = 2	*N* = 1	*N* = 2
		N tot=	12	12	8	12

2.3 Microbial counts

For microbial counts, 10 g of product were 10-fold diluted in chilled sterile diluent solution (0.85% NaCl and 0.1% peptone) and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Appropriate 10-fold dilutions of the homogenates were prepared in chilled saline solution. Total mesophilic viable count (TVC) was determined onto plate count agar (PCA) (ISO 4833-1:2013).

2.4 Volatile organic compounds profile

For each sample, an aliquot of 5 g of caviar was employed in the analysis without any treatment before volatile organic compounds (VOCs) extraction; each sample was analyzed in duplicate. VOCs were extracted by means of the solid phase microextraction (SPME) technique employed on the sample headspace (HS); then, analytes were separated and identified by gas chromatography coupled to mass spectrometry (GC-MS). The analytical conditions (SPME, GC, and MS parameters) were set according to the optimization used in Lopez et al. (2020). Briefly, DVB/CAR/PDMS 1 cm SPME fibers (Supelco) were used for the HS extraction, performed for 30 min at 60 °C. Then, VOCs were recovered by thermal desorption in the S/SL injection port of the GC sys-tem (6890 N

Network GC system by Agilent Technologies) set at 250 °C, in splitless mode. The chromatographic separation was performed by an optimized oven temperature program (from 35 °C to 150 °C at 5°C/min and then to 260 °C at 10 °C/min) employing a DB-5 MS column (30 m × 0.25 mm id, 0.25 um film thickness, Agilent Technologies). Helium was used as carrier gas, at a flow of 1 mL min^{-1} (pressure 6.71 psi). A 5973 Network Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA, USA) was employed for VOCs detection and identification, working in EI mode (70 eV, scan range m/z 35–300 amu, scanning rate 5.19 scans sec^{-1}). Data were acquired by Enhanced ChemStation software (Agilent Technologies, Inc., Santa Clara, CA, USA). A first tentative identification of key aroma compounds was performed by comparisons with mass spectra of compounds found in library data (NIST MS library). Then, the identification was performed by means of two approaches:

1. Identification by matching with van den Dool and Kratz (1963) retention indices, calculated on the basis of a homologous series of n-alkane injected in the same analytical conditions of samples (Kovàts, 1958);
2. Identification by mass spectra of authentic standards injected in the same analytical conditions, when available.

When analyzing the chemical standards, the injection port of the GC system was set in the split mode (split ratio 1:100) and 1 µL was injected. A purge flow of 50 mL min^{-1} was set at 2 min to avoid an oversaturation of the MS ion source.

2.5 Data analysis

Chromatographic signals were normalized for each sample weight, and noise was subtracted for each run. Unmatched chromatographic peaks were heightened to values close to zero. Both chromatographic peak areas and microbial counts were log-transformed before performing a data-fusion process. First, a multifactorial model was built in order to evaluate the effect of the factors (can volume, time of sampling, preservative treatment) and their interactions on the distributions of parameters analyzed by chemical, physical, and microbiological analysis. Since results of the full factorial model did not show a significant effect ($p > 0.05$) on composition for the factor “can volume”, results were presented pooling samples based on the preservative treatment (NC, OAM and BSM). When the significance of the effect due to the interaction between “time of sampling” and “preservative treatment” was observed ($p < 0.05$), multiple comparisons were performed over group least square means by the Tukey’s HSD test. Then, a multiparametric approach was developed. The whole dataset was submitted to power transformation, chosen as a nonlinear conversion pro-cess that allowed the reduction of data heteroscedasticity. Subsequently, Pareto scaling was used to adjust significance of variables represented by large and small fold changes (van den Berg et al., 2006). Finally, multilevel principal component analysis (multilevel-PCA) was applied as a multiparametric explorative tool, in order to reduce the dimensionality of the da-taset while retaining the maximum of the original information and preserving the structure of the dataset (time and treatment). The final dataset consisted of a 48 (volatile organic compounds + pH + water activity + microbial counts) × 44 (samples) matrix. Univariate statistical analysis was performed by the JMP® 15.2.0 tool of SAS (SAS Institute Inc., Cary, NC, USA). The multivariate analysis was performed by the mix-Omics package of R and the PLS Toolbox of MATLAB.

3. Results and discussion

3.1 pH, aw (Water Activity) and Microbial Counts

Caviar analyzed in this study was associated with pH values ranging from 5.78 to 6.46 and aw values ranging from 0.954 to 0.985, comparable to those reported by Bledsoe et al. (2003) for fish roes coming from various species, included sturgeon. Since these values are known to be sufficiently high to support the growth of some microbial species, an initial low microbial load and a strict control of hygiene and sanitation measures are considered to have a primary importance in managing eventual contamination during the entire process of caviar production (Altug & Bayrak, 2003). Actually, even when microbiologically sterile eggs are aseptically removed from fish, a secondary contamination during the production could occur, since this kind of product requires broad handling and multiple processing procedures (Bledsoe et al., 2003; Handa et al., 2005). Generally, the OAM and BSM series showed higher pH values for every sampling point when compared to salt samples. OAM and BSM samples ranged from 6.12 to 6.42, while the salt samples showed pH values always < 6, ranging from 5.84 to 5.97.

As reported in Table 2, the TVC recorded in this study at to showed very low values, always < 2 Log CFU/g in all the analyzed series, without any significant difference. Similar values recorded at to for the TVC in all three series suggested that, even if initial slightly different levels of microbial counts could be present because of individual fish characteristics, the processing conditions in the production plant were adequately standardized and effective in controlling the contamination. The microflora detected in this study was mainly composed of Bacilli and halophilic bacteria (data not reported), due to the natural microbial strains selection that occurred in the product related to the addition of salts.

Starting from t1, the TVC of the NC series showed a gradual increase, reaching average values up to 5.89, 5.90, and 6.89 Log CFU/g at t1, t2, and t3, respectively. The threshold limit of 6.0 Log CFU/g, frequently taken into consideration to designate the end of the shelf life of a fish product, was only recorded in the NC series at t2 and t3, corresponding to 8 and 14 months of ripening, respectively. In a previous study, Shin et al. (2010) found that white sturgeon caviar treated with 3–3.75% of salt and stored at refrigeration temperatures (3–7 °C) reached the total microbial count of 6.0 Log CFU/g in a time range of only 5–10 days. This outcome evidenced that the strictly low temperature (−2 °C) sustained in the production plant for caviar analyzed in this study had a fundamental role in maintaining the food safety requirement for the NC product even in a longer term.

Table 2. pH, a_w, and total viable count (TVC, expressed as Log CFU/g) detected in caviar under investigation during the ripening time. Data were expressed as mean ± SEM (standard error of the mean). For the TVC, the number of samples in which counts were detected is indicated in brackets. Legend: NC, sodium chloride (NaCl); OAM, organic acid mixture; BSM, borax and salt mixture. a_w: water activity, TVC: total viable counts.

	pH	aw	Total Viable Count (Log CFU/g) and Countable
t0= UNRIPENED ROES			
NC	5.86 ± 0.02	0.30 ± 0.00	1.24 ± 0.17 b (2/4)
OAM	6.12 ± 0.09	0.30 ± 0.00	1.08 ± 0.08 b (4/4)
BSM	6.15 ± 0.01	0.30 ± 0.00	1.48 ± 0.20 b (4/4)
t1= 4 MONTHS OF RIPENING			
NC	5.97 ± 0.01	0.29 ± 0.00	5.89 ± 1.29 a (4/4)
OAM	6.28 ± 0.07	0.29 ± 0.00	1.20 ± 0.17 b (4/4)
BSM	6.42 ± 0.02	0.29 ± 0.00	1.50 ± 0.17 b (3/4)
t2= 8 MONTHS OF RIPENING			
NC	5.90 ± 0.02	0.29 ± 0.00	5.90 ± 1.20 a (4/4)
OAM	6.26 ± 0.01	0.29 ± 0.00	nd
BSM	6.31 ± 0.04	0.29 ± 0.00	nd
t3= 14 MONTHS OF RIPENING			
NC	5.84 ± 0.02	0.29 ± 0.00	6.89 ± 0.09 a (3/4)
OAM	6.12 ± 0.08	0.29 ± 0.0	nd
BSM	6.30 ± 0.03	0.29 ± 0.00	1.30 (1/4)

a,b = values associated to a different letter within the same column were significantly different ($p < 0.05$) when testing for the interaction effect of ripening time and preservative treatment over group least squares means (Tukey HSD multiple comparisons) in a multifactorial model; nd = not detected.

The results obtained in this study about caviar's hygienic parameters can be considered positive, since the general picture regarding food safety of caviar and fish roes available in the literature is variable. As a matter of fact, a suitable hygienic level of processed fish roes from various species, including sturgeon, have been recently evidenced, with the limit of 6 Log CFU/g rarely exceeded in this kind of product (Oeleker et al., 2015; Vasconi et al., 2020). On the contrary, other authors had previously detected higher microbial counts in both caviar (Altug & Bayrak, 2003)and other fish roes products (Himelbloom & Crapo, 1998), in the range 3–6.5 Log CFU/g.

The fact that in this study we found the highest microbial counts in NC samples supports the theory that salting in low concentration alone cannot be a sufficient step to inhibit completely the microbial growth in fish roes products if the shelf life is extended beyond 8 months (Bledsoe et al., 2003). Actually, in the OAM and BSM series, the TVC detected, very low since to, did not show any relevant increase until the end of the study, showing a significant ($p < 0.05$) difference from the TVC detected in the NC at t1. At the end of the sampling time (t3), only one sample over the 4 in the BSM series showed the presence of microbial counts, with a value of 1.30 log CFU/g.

3.2 Volatile profile

Results of the VOCs profile detected by HS-SPME-GC-MS at different times of ripening for each treatment are presented in Tables 3 and 4.

Generally, we observed an enhancement of total VOCs amount during the sampling time in all the treatments tested. Aldehydes represented the first group for quantity, followed by alcohols (in all series), ketones (only in NC series), and acids (only OAM series). In all the series, sampling time matching 8 months of storage (t2) was detected as a "critical" time point, characterized by a massive enhancement of the signal provided by the VOCs detected, as a consequence of the development of volatile profile because of the ripening processes that occurred. This outcome is particularly interesting since the time corresponding to 6–8 months of ripening is considered by the producers as the optimal time range to obtain high-quality caviar for all the three treatments.

Many compounds were found in all the treatments tested. Some of them were detected earlier at to, then recorded in fluctuating amounts during the storage: 3-methylbutanal, benzaldehyde, octanal, nonanal, 2-phenylacetaldehyde, decanal, and nonanoic acid. Others were merely detected during the progression of the storage, as an outcome of the ripening processes: 2-methylbutanal, 3-methylsulfanylpropanal, heptanal, 2-hexenal, 2-nonenal, 4-methyl-1-heptanol, 6-methyl, 1-octanol, and 1-phenylethanone. Most of these compounds are known to belong to the typical volatile profile of fish products, as all of them had been previously found as characteristic volatile in fish products (Alasalvar et al., 2005; Aro et al., 2003; Duflos et al., 2006, 2010; Iglesias & Medina, 2008), and also in ripened fish roes (Caprino et al., 2008; Jonsdottir et al., 2004; Lopez et al., 2020). 2-methylbutanal, 3-methylbutanal, 2-phenylacetalhdehyde, and 3-methylsulfanylpropanal are Strecker aldehydes, known to be formed by the breakdown of amino acids: isoleucine for 2-methylbutanal, leucine for 3-methylbutanal and phenylalanine, and methionine for 2-phenyalcetaldehyde and 3-methylsulfanylpropanal (Ardö, 2006; Belitz et al., 2009; Pripi-Nicolau et al., 2000; Weenen & Van Der Ven, 2001). 2-metylbutanal and 3-methylbutanal were found by Parlapani et al. (2017) only in fish tissue models inoculated with bacterial strains, implying that these compounds were specific metabolites produced by the microbial spoilage. On the contrary, hexanal, heptanal, octanal, nonanal, and decanal were found by the authors also in non-inoculated samples, suggesting an enzyme-catabolic or oxidative origin. Actually, most of the saturated and unsaturated aldehydes ranging from 6 to 10 carbon atoms are secondary products of oxidation and enzyme-catabolism of polyunsaturated fatty acids (PUFA): heptanal, octanal, nonanal, decanal, 2-nonenal, and 2-hexenal, found in caviar in this study, are known to derive from the degradation of oleic, linoleic, linolenic, EPA, and DHA fatty acids peroxides (Belitz et al., 2009; Josephson & Lindsay, 1986). During the storage, carbohydrates, amino acids, and lipids act as substrate for degradation processes promoted by microbial or oxidation spoilage. The rate of lipid oxidation is affected by several factors, including fatty acid composition and the unsaturation degree (Belitz et al., 2009). White sturgeon caviar contains 40% of PUFA over the total of fatty acids, with half of them being represented by n3-series FA (Lopez et al, 2020). For this reason, it can be deduced that the high unsaturation degree, associated, in many samples, with the presence of microbial strains, could have promoted the formation of secondary oxidation products with volatile properties, and probably had an impact on caviar flavour (Belitz et al., 2009).

Table 3. Aldehydes detected in caviar during the ripening time for each treatment. Chromatographic areas are expressed in Log10. Data are expressed as Least Square Mean. Legend: NC, sodium chloride (NaCl); OAM, organic acid mixture; BSM: borax and salt mixture.

	t0= UNRIPENED ROES			**t1= 4 MONTHS OF RIPENING**			**t2= 8 MONTHS OF RIPENING**			**t3= 14 MONTHS OF RIPENING**		
	NC	OAM	BSM	NC	OAM	BSM	NC	OAM	BSM	NC	OAM	BSM
3-methyl-butanal	5.21d	5.38 cd	3.91e	5.51bcd	5.73cd	5.48bcd	6.36a	6.26a	6.03ab	6.36bcd	6.32a	5.96abc
2-methyl butanal	nd	nd	nd	4.98b	5.22b	4.99 b	5.92 a	nd	5.51ab	5.95 a	5.91a	5.54ab
2-methyl, 2-butenal	nd	5.46 b	nd	nd	4.89c	nd	nd	5.79 a	nd	5.84 a	5.53ab	nd
2-pentenal	nd	5.36ab	nd	nd	4.82c	nd	nd	5.49a	5.22ab	5.18bc	5.40ab	5.24ab
3-methyl, 2-butenal	nd	nd	nd	nd	nd	nd	nd	2.50ab	nd	nd	4.87a	nd
Hexanal	5.58def	5.78cdef	4.59g	5.43ef	5.37f	5.91cdef	6.14bcd	5.98 bcde	6.76 a	6.60ab	6.35abc	6.68ab
2-hexenal	nd	nd	nd	nd	nd	nd	4.99 bc	4.55c	5.44a	5.30ab	4.92bc	5.28ab
4-heptenal	nd	nd	nd	nd	nd	nd	nd	nd	5.23a	4.99b	nd	5.20ab
Heptanal	nd	nd	nd	nd	nd	nd	nd	nd	5.44 a	5.36ab	5.16 b	5.39ab
3-methyltio propanal	nd	nd	nd	4.88de	5.01cd	4.4e	5.71ab	5.87a	5.37bc	nd	nd	5.43bc
2-hetpenal	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.09 a	nd	5.13a

Table 3 (cont.)

Benzaldehyde	4.96 cde	5.53abc	nd	4.39e	5.07bcde	4.67de	5.55abc	5.84a	5.58abc	5.34abcd	5.74ab	5.44 abc
Octanal	5.04ab	5.18a	3.48d	4.16cd	4.58abc	4.58abc	4.99ab	4.97ab	5.20a	5.36a	5.27a	5.13ab
2,4-heptadienal	nd	nd	nd	nd	nd	nd	2.43b	nd	5.60a	5.30a	3.72ab	5.39a
Benzenacetaldehyde	4.88c	5.36bc	3.95d	5.45bc	5.6abc	5.46bc	6.47a	6.33a	6.14ab	6.24ab	6.23ab	5.96ab
2-octenal	nd	nd	nd	nd	nd	nd	2.54b	nd	5.43a	5.28a	5.10a	5.29a
Nonanal	5.83ab	*	4.5d	5.19c	*	5.37bc	5.72abc	*	6.00a	6.10a	*	6.05a
2,6-nonadienal	nd	nd	nd	nd	nd	nd	nd	nd	5.25a	nd	nd	nd
2-nonenal	nd	nd	nd	nd	nd	nd	nd	nd	4.87a	5.00a	4.93a	4.90a
Decanal	4.58	4.83	4.22	4.15	4.65	4.53	4.54	4.91	4.73	5.07	5.10	4.96
SUM OF ALDEHYDES	6.22c	6.35bc	5.03d	6.16c	6.28c	6.28c	6.99b	6.88 ab	7.09 a	7.12a	6.98ab	7.02a

*= the chromatographic peak for nonanal in the OAM series was not possible to integrate because a coeluition occurred with a huge peak of sorbic acid (present in the mixture); nd= not detected; a, b, c, d, e = values associated to a different letter within the same row were significantly different ($p < 0.05$) when testing for the interaction effect of ripening time and preservative treatment over group least squares means (Tukey HSD multiple comparisons) in a multifactorial model

Table 4. Alcohols, ketones and terpenes detected in caviar during the ripening time. Chromatographic areas are expressed in Log10. Data are presented as Least Square Means. Legend: NC, sodium chloride (NaCl); OAM, organic acid mixture; BSM: borax and salt mixture.

	t0= UNRIPENED ROES			**t1= 4 MONTHS OF RIPENING**			**t2= 8 MONTHS OF RIPENING**			**t3= 14 MONTHS OF RIPENING**		
	NC	OAM	BSM	NC	OAM	BSM	NC	OAM	BSM	NC	OAM	BSM
1-penten-3-ol	nd	nd	nd	4.75 d	nd	5.25 cd	5.53 bc	5.08 cd	6.10 a	5.65 bc	5.58 bc	6.06 ab
3-methyl, 1-butanol	nd	nd	nd	nd	nd	nd	2.98 b	nd	nd	6.26 a	nd	nd
2-penten-1-ol	nd	nd	nd	nd	nd	nd	nd	nd	5.57 a	nd	nd	5.49 b
1-octen-3-ol	nd	nd	nd	4.52 ab	nd	4.81 ab	5.32 bc	nd	5.98 a	5.97 a	5.62 a	6.08 a
2-ethyl, 1-hexanol	nd	nd	nd	5.70 ab	nd	5.87 a	4.85 c	5.31 ab	nd	5.18 bc	nd	nd
4-methyl, 1-heptanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.83 a	5.73 a	5.54 a
6-methyl, 1-octanol	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.41	5.54	5.35
SUM OF ALCOHOLS	nd	nd	nd	5.78 cd	nd	6.01 bcd	5.98 bcd	5.51 d	6.41 ab	6.65 a	6.25 abc	6.52 ab

Table 4 (cont.)

3-pentanone	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	5.06
3-hydroxy, 2-butanone	nd	nd	nd	nd	nd	nd	6.55	nd	nd	6.83	nd	nd
Acetophenone	nd	nd	nd	nd	nd	nd	nd	nd	nd	4.97 b	5.15 a	4.99 b
SUM OF KETONES	nd	nd	nd	nd	nd	nd	6.55 a	nd	nd	6.84 a	5.15 b	5.35 b
α-pinene	5.10 abc	5.65 a	4.71bc	4.63 c	5.24 abc	5.29 abc	5.38 abc	5.57 ab	5.66 a	5.35abc	5.59 ab	5.58 ab
3-carene	nd	nd	nd	3.72	4.43	4.27	4.64	4.73	4.76	5.13	5.13	5.06
Limonene	4.82 b	5.33 a	4.23 c	nd	4.93 abc	nd	nd	nd	5.20 a	nd	5.28 a	5.21 ab
β-ocimene	4.68 c	5.51 a	4.11 d	ndn	5.30 ab	4.88 bc	nd	5.42 a	5.29 ab	nd	5.41 a	5.32 ab
Caryophyllene	nd	5.05	nd	nd	4.47	3.79	nd	4.23	nd	nd	4.81	nd
SUM OF TERPENES	5.46 ab	6.03 a	4.92bc	4.68 c	5.75 a	5.47 ab	5.41 abc	5.86 a	5.94 a	5.57 ab	6.01 a	5.94 a

nd= not detected; a, b, c, d = values associated to a different letter within the same row were significantly different ($p < 0.05$) when testing for the interaction effect of ripening time and preservative treatment over group least squares means (Tukey HSD multiple comparisons) in a multifactorial model.

Many differences were detected among the three treatments tested. Samples treated with only sodium chloride (NC series) were those represented by the highest amounts of VOCs, associated to the highest increase until the final sampling time as well. Particularly, in the NC series we detected a substantial presence of alcohols and ketones (Table 4), distinctive specifically for this treatment. According to Olafsdottir et al. (2005) and Jonsdottir et al. (2008), who studied microbial metabolites in chilled fish, the formation of ketones in high levels, mainly represented by 3-hydroxy-2-butanone (also known as acetoin), can be associated to the active growth of specific spoilage organ-isms (SSO). Actually, acetoin is a microbial derived compound that can be formed from carbohydrate sources (via pyruvate and diacetyl), generally found in higher amounts if compared to lipid-derived ketones (Olafsdottir et al., 2005). In this study, 3-hydroxy-2-butanone was found exclusively in NC samples, starting from t2 (6.55 Log peak area). The same was observed for 3-methyl-1-butanol, detected only in NC samples and significantly increasing ($p < 0.01$) from t2 (2.98 Log peak area) to t3 (6.26 Log peak area). Even 3-methyl-1-butanol has been previously noticed to increase by authors who investigated the volatile profile of fish products and fish roes during cold storage (Alasalvar et al., 2005; Aro et al., 2003; Jonsdottir et al., 2004; Jørgensen et al., 2001). Particularly, Miller et al. (1973) identified the presence of 3-methyl-1- butanol in sterile fish muscle inoculated with spoilage bacteria strains and incubated for 7 days, thus advising the formation of this alcohol by microbial spoilage. For this reason, 3-hydroxy-2-butanone and 3-methyl-1-butanol have been suggested as early indicators of spoilage in fish products. According to this, we found that NC samples were associated with the highest values of TVC detected, even closer to the critical value of 6 Log UFC/g (Table 2). As a matter of fact, even during low temperature storage, both enzymatic and non-enzymatic changes in fish tissues persist, and, even if in slower rates, they can still support microbial growth and metabolism (Ghaly et al., 2010). Moreover, it is known that the use of no additive other than sodium chloride (in low concentrations) makes caviar suitable for consumption only in a relatively short time term (up to 6–8 months) (Williot et al., 2001) and with a storage temperature close to 0 °C. NaCl is known to have the power to reduce the autocatalytic activity in food products, but, when it is used as unique method of preservation without further processing, complete bacterial protection is not provided, because halophilic microorganisms can cause spoilage (Belitz et al., 2009). Thus, in such concentrations (< 4%), it seemed to be not enough to sustain a strong antimicrobial activity alone. The opposite condition was observed in the OAM series, which was represented by the lowest amount of VOCs, and showed a slower and more gradual increase throughout the ripening time. Actually, many compounds representative in NC and BSM samples were undetected in the OAM series. An example is represented by the unsaturated aldehydes 2-heptenal and 4-heptenal. 4-heptenal is an n3-series PUFA oxidation product previously found in other fish products (Aro et al., 2003; Jónsdóttir et al., 2008; McGill et al., 1974) and ripened fish roes (Caprino et al., 2008; Jonsdottir et al., 2004), well known to contribute to the expression of the overall cold stored and “fishy” flavor (Hardy et al., 1979; McGill et al., 1974). The absence of this compound in the OAM caviar samples suggests that oxidation phenomena occurred to a lesser degree in this series, probably because of the combination of the strongly controlled storage conditions and the efficiency of the preservative added, containing isoascorbic acid as an antioxidant. In a similar way, other compounds were found in the OAM series arising later during the storage time if compared to salt and BSM series. 1-penten-3-ol and 2-ethyl-1-hexanol were detected at t1 in NC and BSM while they appeared only at t2 in OAM samples. Similarly, 2,4-heptadienal, 2-octenal, and

1-octen-3-ol arose in NC and BSM samples at t2, but in OAM samples only at the end of the storage (t3) and in lower concentrations. These compounds are known to be related to the oxidation processes that occurs to-ward long-chain PUFA, and they have been found in the volatile profile of several fish products, also during cold storage (Alasalvar et al., 2005; Aro et al., 2003; Duflos et al., 2006, 2010; Iglesias & Medina, 2008; Josephson et al., 1984; Nordvi et al., 2007) and also in ripened fish roes (Caprino et al., 2008; Jonsdottir et al., 2004). 1-penten-3-ol and 2,4-heptadienal are listed among the main products of n3-series PUFA autoxidation (Josephson & Lindsay, 1986); similarly, 1-octen-3-ol is known to be a degradation product of n6-series PUFA hydro peroxides (mainly, linoleic and arachidonic acid) (Hsieh & Kinsella, 1989). More-over, 2,4-heptadienal and 1-penten-3-ol have been evidenced as markers for early oxidation in n3-PUFA-enriched fish products, contributing to the sensorial property of rancidity and representing important contributors to off-flavors. Since some authors suggested that 1-penten-3-ol can be produced by microorganisms arousing amino acids and lipids degradation bacteria (Joffraud et al., 2001; Miller et al., 1973), it has been pointed to as an indicator of microbial spoilage as well. Actually, in papers published in 2008 and 2009, Iglesias et al. (2009; 2008) showed that the presence of 1-penten-3-ol and 1-octen-3-ol in fish tissues was strongly correlated with the extent of fish oxidation phenomena occurring during the storage. Furthermore, in this study, the alcohol 2-ethyl-1-hexanol was found with a decreasing trend (between t1 and t2 in NC samples, $p < 0.01$) or completely disappearing (in BSM and OAM at t2 and t3, respectively) during the storage of caviar samples, according to what was previously found in literature (Alasalvar et al., 2005; Jørgensen et al., 2001). 2-ethyl-1-hexanol is known to be formed during the storage of fish products in vacuum conservation conditions and at low temperatures (Jørgensen et al., 2001; Olafsdottir et al., 2005). Parlapani et al. (2017) found this alcohol in both microbial-inoculated and non-inoculated fish tissues, suggesting that it could be a product of both non-microbial and microbial activity, but they detected significant higher amounts of this alcohol in microbial spoiled samples. According to this, we found the highest amount of 2-ethyl-1-hexanol in NC and BSM samples at t1 (5.87 and 5.70 Log peak area), while in the OAM samples, it was found only at t2 (5.31 Log peak area). The fact that 4-heptenal, 2,4-heptadienal, 1-penten-3-ol, 1-octen-3-ol, and 2-ethyl-1-hexanol were belatedly detected in the OAM series again agreed to the delayed process of oxidation hypothesized for caviar treated with this preservative.

In Figure 1a,b, the score plot (a) and the loading plot (b) obtained after the employment of a multilevel principal component analysis on the whole dataset (VOCs, pH, aw, microbial enumerable counts) are reported.

Changes in caviar chemical and microbiological profile occurred during the ripening time, and a tendency to form clusters among samples belonging to the same treatment can likely be observed in the multilevel-PCA score plot (Figure 1a). For all three series (NC, OAM, and BSM), an evolution from to and t3 was observed. Generally, the intensity of the signals recorded for volatile organic compounds peaks detected by chromatographic analysis showed an increase in all three series of samples from to to t3. This phenomenon could be linked to the proteolysis and hydrolysis phenomenon occurring toward polysaccharides and lipids during the first months of caviar aging, as previously indicated in the literature (Gussoni et al., 2006; Heude et al., 2016). However, the evolution of the whole profile of parameters measured in samples was related to the increase of different compounds for each group, and, thus, lead to the distribution of scores in different quarters of the PC space. Particularly, the NC samples clearly showed an opposite trend in comparison to the other two treatments. During

the sampling time, from to to t3, NC samples noticeably moved toward the area of the space related to the higher loadings of many compounds indicative of the occurrence of ripening and spoilage processes: 3-hydroxybutan-2-one, 3-methylbutan-1-ol, and 2-hethylhexan-1-ol. Similarly, the NC series was positively related on both PC-1 and PC-2 to the amount of viable counts detected (TVC, total viable count), showing a clear increase from to to t3. At t3, 3 samples out of 4 of the NC series showed the presence of microbial counts, reaching an average value of 6.89 log CFU/g. Thus, we evidenced that the NC samples at t3 showed a higher correlation with the values of all the parameters identified as markers of microbial spoilage. This outcome agreed with the fact that 14 months of storage are considered far and away the optimal ripening time for salted-only caviar from producers, usually indicated as 6–8 months maximum.

The microbial counts in this study were detected at different concentrations in the three series, according to the antimicrobial effectiveness of the preservatives employed. For the OAM series, no counts were evidenced at the end of the sampling time (t3). According to this, the OAM samples (the red diamonds in Figure 1b) showed the lowest dispersion from to and t3 and the highest tendency to form a condensed cluster characterized by scores inversely associated with the markers of spoilage processes. In the multilevel-PCA plot, this series was positively related on the PC-1 to the loadings for many compounds, such as terpenes and organic acids, which have not been associated with the spoilage of the product.

Finally, we observed an intermediate trend for the BSM series. A clear evolution was evidenced between t1 and t2 (4 and 8 months of ripening, respectively), with BSM moving toward the lower quarters of the PC space, positively related on the PC-2 with the loadings of many compounds, such as aldehydes, ketones, and alcohols, particularly 2-penten-1-ol. Then, the profile of the BSM series showed a smaller changes be-tween t2 and t3. Actually, the time range t1–t2 was the one in which the most interesting changes on the volatile and microbial pattern was evidenced in all samples analyzed. In this time range, enzymatic, oxidative, and microbial activities took place in samples from all the series, leading to the formation of several chemical compounds responsible for the fishy flavor of caviar (2-penten-1-ol, 3-pentanone, 4-heptenal), but at a different rate in the different treatments. These outcomes appear interesting since actually, according to the producers, the sampling time t2 (corresponding to 8 months of ripening) is the only storage time that sustains the consumption of high-quality caviar for all the treatments tested.

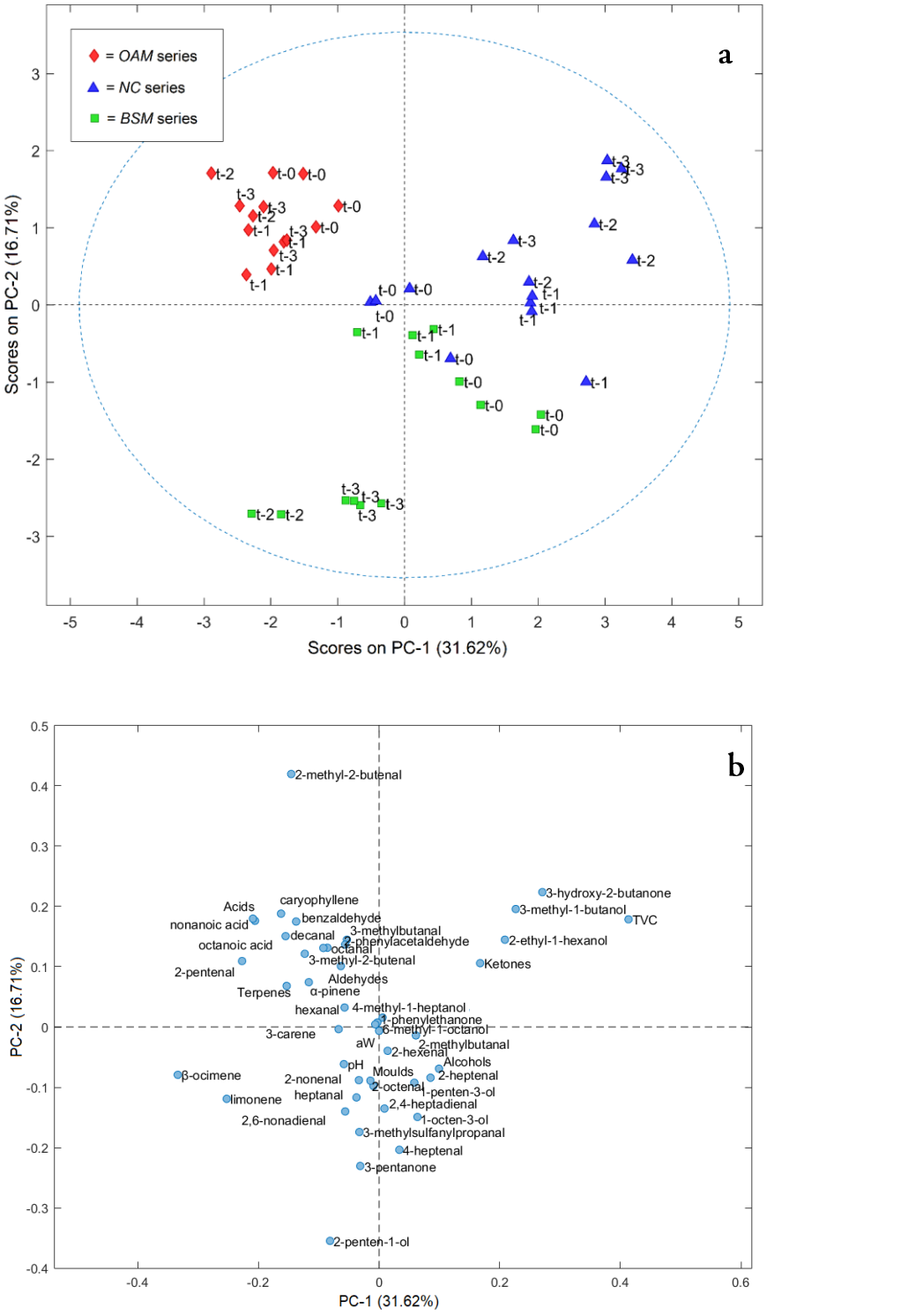

Figure 1. (**a**) Score plot of caviar samples analyzed in the study obtained by a multilevel principal component analysis (multilevel-PCA). t-0 = unripened roes, t-1 = 4 months of caviar ripening, t-2 = 8 months of caviar ripening, t-3 = 14 months of caviar ripening. (**b**) Loading plot of parameters measured in analyzed caviar samples obtained by multilevel-PCA. Variables associated with higher loadings on the 1st or the 2nd principal component (PC-1 and PC-2 on the x and the y axis, respectively) are related to a higher influence on variability recorded among samples in the multiple level data matrix.

4. Conclusions

The results obtained in this study evidenced interesting differences among caviar samples treated with different preservatives during a storage time of 14 months. The differences were particularly highlighted when combining all the parameters measured (physical, chemical, and microbiological) in a multivariate approach. The microbial counts (measured as total viable count, TVC) detected in samples analyzed in the present study generally evidenced an adequate management of samples processed in the production plant, leading to a good control of secondary contamination occurrence. However, the BSM (borax and salt mixture) and OAM (organic acid mixture) treatments were more effective in protecting the samples from the growth of microbial species in the long term (over 8 months) when compared to the addition of sodium chloride only (NC series). Similarly, all samples showed an increase for the chromatographic area of chemical compounds related to the ripening processes occurring to-ward sturgeon eggs during the sampling time. However, NC samples were associated to a volatile profile enriched in compounds driven by the spoilage occurring during the ripening processes (mainly, ketones). On the contrary, the OAM series showed the lowest amounts for all group of VOCs derived from the spoilage processes, while the BSM series showed an intermediate profile, although one characterized by the total absence of ketones. The outcomes obtained in this study showed that the BSM and, mainly, the OAM series displayed the highest stability of the product in the long term, suggesting the possibility of extending caviar shelf-life to a longer storage time (up to 14 months) when using such preservative mixtures.

Supplementary Materials: The following are available online at: https://www.mdpi.com/article/10.3390/foods10040850/s1, Table S1: Extended with replicates and additional data for Table 2 in main text.

Author Contributions: V.M.M. and M.P. conceived and planned the research. A.L. and M.V. carried out the chemical experiments. C.B. carried out the physical analysis. E.T. and S.S. carried out the microbiological analysis. F.B. and V.M.M. supervised and validated the research work. All the authors contributed to the interpretation of the results. A.L. took the lead in writing the manuscript. All authors provided critical feedback and revision for the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received financial support by Agroittica Lombarda S.p.A.–Calvisano, Brescia, Italy.

Conflicts of Interest: The funders contributed to conceive and to plan the design of the study, to the interpretation of the results and provided a feedback for the writing of the manuscript.

Supplementary materials.

Materials and methods of data not presented in the manuscript.

- Moulds were counted on Sabouraud Agar according to International Organization for Standardization (ISO) Microbiology of Food and Animal Feeding Stuffs—Horizontal Methods for the Enumeration of Yeasts and Moulds—Part 1: Colony Count Technique in Products with Water Activity Greater than 0, 95; ISO 21527-1:2008 2008.
- presumptive Bacillus cereus was enumerated onto PEMBA according to International Organization for Standardization (ISO) Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive Bacillus cereus — Colony-count technique at 30 degrees C. ISO 7932:2004 2004.
- halophilic bacteria were enumerated onto Tryptic Soy Agar supplemented with NaCl (30 g/L) then incubated at 30°C for 48h.

All the media were supplied by Scharlab (Barcelona, E).

In Table S1, microbiological data (Table 2 of the manuscript) extended with replicates and additional data are reported.

Table S1. Table 2 extended with replicates and additional data.

To= UNRIPENED ROES

	NC						OAM						BSM					
Sample no	1	2	3	4	Mean	C*	1	2	3	4	Mean	C*	1	2	3	4	Mean	C*
pH	5.84	5.88	5.84	5.88	5.86		6.13	6.23	6.3	5.83	6.12		6.17	6.12	6.16	6.14	6.12	
Aw	0.974	0.977	0.974	0.982	0.977		0.972	0.972	0.969	0.985	0.975		0.978	0.975	0.975	0.976	0.975	
TVC	<1	1	1.48	<1	1.24	2/4	1	1	1.30	1	1.08	4/4	1	1.30	1.78	1.85	1.48	4/4
Halophiles	<1	<1	<1	1	1	1/4	1.30	<1	1.30	<1	1.30	2/4	<1	<1	<1	<1	<1	4/4
Bacilli	1.30	1.30	1	<1	1.20	3/4	<1	1	<1	<1	1	1/4	1	<1	<1	1.6	1.30	2/4
Moulds	<1	<1	<1	<1	<1	4/4	<1	<1	1	<1	1	1/4	<1	<1	<1	<1	<1	4/4
T1= 4 MONTHS OF CAVIAR RIPENING																		
Sample no	1	2	3	4	Mean	C*	1	2	3	4	Mean	C*	1	2	3	4	Mean	C*
pH	5.96	5.96	6.01	5.95	5.97		6.33	6.35	6.42	6.03	6.28		6.44	6.41	6.37	6.46	6.42	
Aw	0.966	0.963	0.962	0.964	0.964		0.96	0.97	0.954	0.961	0.961		0.97	0.968	0.961	0.963	0.966	
TVC	5.84	6.02	5.21	6.51	5.89	4/4	1.60	1.00	<1	1.00	1.20	3/4	1.30	1.30	<1	1.90	1.50	3./4
Halophiles	4.48	4.30	4.48	4.60	4.46	4/4	1.00	<1	<1	<1	1	1/4	1	1	1.30	1.30	1.15	4/4
Bacilli	6.48	6.30	5.47	6.30	6.14	4/4	3.30	1.30	1.48	1.00	1.77	4/4	<1	<1	<1	2.08	2.08	1/4
Moulds	<1	<1	<1	<1	<1	4/4	<1	<1	<1	<1	<1	4/4	<1	<1	<1	<1	<1	4/4

T2= 8 MONTHS OF CAVIAR RIPENING																	
	NC						OAM						BSM				
Sample no	1	2	3	4	Mean	C*	1		3		Mean	C*	1		4	Mean	C*
pH	5.9	5.94	5.93	5.83	5.90		6.24		6.27		6.26		6.34		6.27	6.31	
Aw	0.959	0.961	0.96	0.963	0.961		0.962		0.963		0.963		0.97		0.966	0.968	
TVC	2	6.94	6.65	6.78	4.59	4/4	<1		<1		<1	2/2	<1		<1	<1	2/2
Halophiles	<1	6.72	<1	<1	6.72	1/4	<1		<1		<1	2/2	3.34		<1	3.34	1/2
Bacilli	2.30	<1	6.64	6.95	5.30	3/4	<1		<1		<1	2/2	<1		<1	<1	2/2
Moulds	<1	1.60	<1	<1	1.60	1/4	<1		<1		<1	2/2	<1		<1	<1	2/2
T3= 14 MONTHS OF CAVIAR RIPENING																	
Sample no	1	2	3	4	Mean	C*	1	2	3	4	Mean	C*	1	2	3	4	Mean	C*
pH	5.87	5.85	5.87	5.78	5.84		6.16	6.23	6.25	6.12			6.38	6.31	6.2	6.3	6.30	
Aw	0.96	0.955	0.956	0.957	0.957		0.957	0.956	0.954	0.955			0.966	0.959	0.963	0.958	0.962	
TVC	<1	7	6.68	7	6.89	3/4	<1	<1	<1	<1		4/4	<1	<1	1.3	<1	1.3	1/4
Halophiles	1.7	7	6.83	6.3	5.46	4/4	<1	1	<1	1		1/4	1	1	1	1.3	1.08	4/4
Bacilli	<1	<1	<1	<1	<1	4/4	<1	<1	1.7	1.59		2/4	1.3	<1	<1	<1	1.3	1/4
Moulds	1.3	<1	<1	1.48	1.39	2/4	<1	1	1.48	1.59		3/4	1.78	1.6	2.45	1	1.71	4/4

*C= countables

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Trial 8

Comprehensive untargeted metabolomics fingerprinting of white sturgeon caviar during the ripening time

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Abstract. In the present investigation, a ultra high performance liquid chromatography and high resolution mass spectrometry (UHPLC-HRMS) method was developed and optimized in order to characterize the non volatile metabolome of white sturgeon caviar during the ripening. Both the optimization of the method and data analysis were performed by chemometrics (mPLS-DA with variables selection). The results of the analytical optimization enabled to identify the best combination of extraction, chromatographic and mass spectrometry conditions to analyse the whole metabolome of white sturgeon caviar. Multivariate results showed a strong evolution of the non volatile compounds present in the aqueous phase of sturgeon eggs during the first 4 months of ripening, followed by a partial stabilization. Most of the compound tentatively identified were associated to a high nutritional value, comprising phospholipids species made of long chain and unsaturated fatty acids and vitamins. Moreover, the presence of chemical species related to have a fundamental impact on the characteristic *umami* taste in food, such as glutamic acid and nucleotides, were detected.

Unpublished data

1. Introduction

Metabolomic is an *omic* approach widely employed in systems biology and in food science, aiming to describe the metabolic profile in complex systems through the combination of high-throughput analytical techniques and multivariate data analysis (Naz et al., 2014). The 'metabolome' represents the complete set of metabolites synthetized in a biological system that, being the intermediate or end products of multiple enzymatic reactions occurring in an organism, constitute informative proxies of its biochemical activity (Alonso et al., 2015; Chatterjee et al., 2019). Metabolites present in a cell mainly include organic species characterized by a wide range of molecular weight and other physiochemical properties (polarity, solubility, volatility), such as amino and fatty acids, carbohydrates, vitamins, terpenoids, lipids, phenolic compounds, and so on (Dunn & Ellis, 2005) . The whole set of metabolites of a biological system varies depending on inner (genetic, metabolic) and outer (relation with the environment) features. In hypothesis-free metabolomics studies (called *untargeted*), metabolite profiling provides a picture of the whole range of compounds present in a biological system at one point in time (Chatterjee et al., 2019). The untargeted approach is characterized by the simultaneous measurement of a large number of metabolites, allowing the detection of novel biomarkers in the system under investigation, without mandatory *a priori* knowledge (Alonso et al., 2015; Chatterjee et al., 2019). Clearly, the metabolite fingerprint provided by the untargeted strategies is strictly dependent on the analytical methodology applied chosen (Gika et al., 2014).

To date, ultra-high performance liquid chromatograph (UHPLC) methods represent the gold standard for chromatographic methods in metabolomics investigations, thanks to their high peak capacity and sensitivity (Gika et al., 2014; Want et al., 2013). The chromatographic methods most commonly employed in metabolomics are reversed phase (RP) methods, allowing the separation of a large part of metabolome. However, the RP chromatographic columns allow the retaining and separation of medium-polar and nonpolar metabolites, but do not allow the separation and identification of polar or ionic species, due to the poor affinity of these molecules with the apolar stationary phase that characterize the RP chromatographic columns (Gika et al., 2014; Want et al., 2013). This means that many polar primary metabolites of high biological importance, such as amino acids, amines, organic acids, sugars and carbohydrates, involved in several biochemical pathways, cannot be effectively analysed in RPLC, eluting in or near the solvent front in the chromatographic separation step (Dunn & Ellis, 2005; Gika et al., 2014). To overcome this drawback, hydrophilic interaction chromatography (HILIC) strategies can be employed, allowing the separation and the detection of polar and ionic compounds, well retained by the HILIC column and, thus, providing complementary information to those obtained by RPLC (Dunn & Ellis, 2005; Gika et al., 2014). A combined RPLC and HILIC strategy can be suitable in order to cover the entire set of metabolites present in a biological system, characterized by different physiochemical properties (polar water-soluble and nonpolar hydrophobic compounds) (Theodoridis et al., 2011; Want et al., 2013).

High-resolution mass spectrometry (HRMS) is the detection method coupled to UHPLC most commonly employed in untargeted metabolomics research, leading to metabolite profiling with affordability, sensitivity and high resolution (Chatterjee et al., 2019; Dunn & Ellis, 2005; Gika et al., 2014). HESI is the preferred ionization mode in mass spectrometry when coupled with liquid chromatography. HESI is a soft ionization technique, which leads to minimal fragmentation and good sensitivity, enabling the detection of a wide range of metabolites

by addition of removal of a proton or by addition of other ionic species (Dunn & Ellis, 2005; Want et al., 2013). It can be performed in both positive (HESI+) or negative (HESI-) mode, influencing the metabolic profile obtained and revealed by the mass spectrometer. Actually, some molecules are ionized more efficiently in HESI+, such as basic species that forms protonated adducts $[M+H]^+$; on the contrary, other molecules are better ionized in HESI-, generally forming single or multiple protonated species $[M+zH]^{z+}$ or deprotonated species $[M-H]^-$ (Gika et al., 2014). In order to maximize the range of metabolites detected, a combined HESI+ and HESI- ionization approach can be employed, collecting separately the data obtained in the two runs (Dunn & Ellis, 2005; Want et al., 2013). The high-throughput matrix data obtained by UHPLC-HRMS analysis coupled with suitable chemometric techniques allow to investigate multiple authenticity issues within a single experiment, revealing the structure of the information and the relationship among the metabolomics features and the phenotypic features of the samples (Alonso et al., 2015; Chatterjee et al., 2019).

Since the nature of the metabolites present in a matrix can vary a lot (in terms of molecular weight, polarity, volatility, etc.), the metabolites extraction phase is a delicate process that needs to be optimized at best. The choice of the extraction protocol affects the molecular features detected in the analysis and, thus, the interpretation of the data obtained (Naz et al., 2014). In metabolomics studies, the main goal of the extraction step is the recovering the broadest possible range of metabolites at the higher concentration possible, thus the chosen strategy should be as nonselective as possible, reproducible, fast and easy (Cevallos-Cevallos et al., 2009; Naz et al., 2014). Combination of polar and nonpolar extraction methods are performed by the physical-chemical disruption of the cells of the tissue, removal of the pellet by centrifugation and the distribution of the metabolites between two solvent layers (polar and apolar) (Cevallos-Cevallos et al., 2009; Dunn & Ellis, 2005). The two-layer partition of the metabolites is highly employed in metabolomics studies performed in complex matrices, allowing the simultaneous extraction of hydrophilic and hydrophobic metabolites by the addition polar and nonpolar solvents (Naz et al., 2014). Polar and nonpolar metabolites have to be recovered in two different fractions (hydrophilic – HF, and lipophilic, LF) and analysed in different analytical runs, optimizing the UHPLC-MS conditions for each type of extract (Masson et al., 2011). Most of the extraction methods employed for metabolomics analysis of tissues containing high amounts of water (min 80%) are based on the (Blight & Dyer, 1959) protocol, carrying out the extraction and separation of solvent layers almost simultaneously, retaining the precipitate proteins between the two phases (García-Cañaveras et al., 2011; Le Belle et al., 2002; Masson et al., 2011; Naz et al., 2014; Wu et al., 2008). According to (Blight & Dyer, 1959)), it is imperative to keep the volumes of chloroform, methanol and water in the following proportion: $CHCl_3$:MeOH:H_2O 2:2:1.8 after the solvent addition. These ratios represent the total volumes present in the ternary system, including the water present in the sample. When the biological matrix does not contain 80% of water, the volumes of the solvents can be adjusted to give the correct proportions (Blight & Dyer, 1959). This method results in a biphasic separation where polar and nonpolar metabolites can be extracted simultaneously, representing an advantage most notably because lipids and phospholipids are excluded from the polar extract and because each fraction can be analysed separately.

Several studies have indicated that multistep extractions provide better results for the number of metabolites extracted and the concentrations recovered with the aim to analyse both the hydrophilic wand lipophilic

metabolites (Want et al., 2013). Many authors investigated the efficiency of different protocols of extraction based on the addition of $CHCl_3$, MeOH and H_2O, evaluating the efficiency in extracting both the polar and nonpolar metabolites in terms of number of metabolites extracted and repeatability, using multivariate approaches (Masson et al., 2010; Wu et al., 2008). The development of robust metabolomics approaches, built optimizing the conditions of sample treatment, metabolite separation, detection and data treatment, is fundamental in order to carry out reliable studies about food products in which authenticity represent a key issue for market protection and a fair trade warranty.

Fish and seafood products are among the foodstuffs more vulnerable to frauds (Hassoun et al., 2020), because of the high profit related to their economic value. During last years, the high availability and increasing presence on the market of caviar and substitutes, associated with different economic values, is considered to have led to a reduction in producers profits and to have made a way for the easy development of food frauds practices (Bronzi et al., 2019; van Uhm & Siegel, 2016). According to the definition of the Codex Alimentarius Committee, only the ripened roes harvested from fish of the *Acipenser* family can be defined as **caviar**. Caviar is the most valuable fish roes product (Tavakoli et al., 2021), associated to a floor price ranging between 300 €/kg to 500 €/kg in the European market (EUMOFA, 2021). Roes harvested from different sturgeon species can differ in quality and grade; consequently, the price can vary depending on several quality factors, such as size, texture, colour, fragrance, firmness, taste and maturity (EUMOFA, 2021). Traditionally, the quality of caviar is evaluated by means of sensorial parameters by trained panellists in the production plants before the placing on the market. The final quality of caviar can be affected both by factors arising before (habitat condition, diet, sexual maturity of the fish) and after (processing procedures, additives and preservatives added) the harvest of fish (Tavakoli et al., 2021). However, few information are available in literature about the biochemical modification occurring in sturgeon eggs during their processing to caviar of high quality.

During last years, several studies have been carried out on fish eggs and sturgeon caviar with the aim to define their composition and food safety parameters, covering proximate composition, amino and fatty acid profile, volatile organic compounds, colour and texture, microbial contamination, and so on (Altug & Bayrak, 2003; Bledsoe et al., 2003; Czesny et al., 2000; DePeters et al., 2013; Gessner et al., 2008; Gong et al., 2013; Hamzeh et al., 2015; Lopez et al., 2020; Mol & Turan, 2008; Oeleker et al., 2015; Ovissipour & Rasco, 2011; Park et al., 2015; Safari & Yousefi, 2010; Shin et al., 2010).

Contemporary, many studies were conducted combining different analytical approaches in order to characterize the lipophilic and hydrophilic metabolome of traditional fish roes products different from caviar. In 2013, Scano et al. investigated the lipid profile and the aqueous fraction containing low molecular weight metabolites of salt and dried tuna roes (*bottarga*) by means of combined GC, HPLC, ^{13}C NMR and ^{1}H-NMR analysis. The authors characterized the lipid composition of bottarga, in terms of presence of cholesterylesters, cholesterol, wax esters, triacylglycerols, phosphatidilycoline and free fatty acids. Moreover, they reported the presence of several nutrients dispersed in the aqueous phase of tuna roes, such as free amino acids, lactate, β-alanine, sugars, taurine, nucelosides and niacotinamide. The study of Scano et al. (2013) was conducted employing NMR as metabolomics analytical platform and HPLC as tool to identify cholesterol, unsaturated fatty acids and hydroperoxides in a targeted approach. No untargeted studies characterizing the whole metabolome of sturgeon

caviar by means of liquid chromatography and mass spectrometry are available in literature to date. Only two pertinent studies (Dal Bello et al., 2017; Porcari et al., 2014) were found, in which the authors employed HPLC-HRMS or shotgun lipidomics approaches in order to determine the lipidome of caviar and eventual modifications during the ripening.

Thus, the aim of the trial presented in this section was: a) the optimization and the employment of an UHPLC-HRMS method suitable to investigate the whole metabolome of white sturgeon (*Acipenser transmontanus*) caviar in an untargeted approach, and b) to determine if the outcomes obtained, combined with chemometrics, could provide useful information about the effect of the ripening process on the whole metabolic profile.

2. Materials and methods

2.1 Sampling

A total of 118 samples of caviar from white sturgeon (*Acipenser transmontanus*) analysed in this study were provided by Agroittica Lombarda SpA (Calvisano, BS, Italy). Sturgeon roes were collected by the egg sac removal from 12 sexually mature female specimens. According to the traditional production method, the egg sac were rolled by hand over a grate to separate the eggs from the surrounding membrane. Discoloured eggs and other impurities were removed and the eggs were washed. Clean egg samples were thus added with different preservatives formulas under aseptic conditions, according to the traditional preservative methods and component employed in caviar. Briefly, sodium chloride alone or in mixture with organic acids or sodium tetraborate was added at a percentages ranging 3.6-4.6%; the amount of sodium tetraborate was included in the latter preservative mixture complying with the limit levels established and defined in the Commission Regulation (EU) No 1129/2011 for fish roes and caviar (section 09.3). After few minutes, egg samples were put in metal tins, pressed in order to expel the air and any excess of water and then stored at -2°C in the refrigerated storage room of the company. The day of the harvest, one sample from each can was collected before (t0) and around ten minutes after (t01) the preservative addition, and transported to the laboratories of University of Milan in refrigerated conditions. Then, caviar was collected during the ripening time and analysed after 4 months (t4), 8 months (t8) and 14 months (t14) of ripening, according to the protocol described in Table 1.

At times t4, t8 and t14 caviar samples were collected by the company's collaborators and sent to the University of Milan laboratories under refrigerated conditions. Samples were stored at -80°C and sent in dry-ice to the laboratories of the Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA), were they were stored at -80°C until the metabolomics analysis.

Table 1. Sampling protocol of raw eggs and caviar analysed in the trial. To= raw eggs immediately after the cleaning phase; to1= raw eggs ten minutes after the addition of the mixtures; t4= caviar after 4 months of ripening; t8= caviar after 8 months of ripening; t14= caviar after 14 months of ripening.

	TREATMENT					
	NaCl ALONE		MIXTURE WITH ORGANIC ACIDS		MIXTURE WITH SODIUM TETRABORATE	
% of addition	3.6	3.8	4.00	4.65	3.8	4.2
Number of samples collected						
to	4	4	4	4	4	4
to1	4	4	4	4	4	4
t4	4	4	4	4	4	4
t8	4	4	2	2	2	2
t14	4	4	4	4	4	4

2.2 Method optimization workflow

The protocol developed for the optimization of the analytical method is resumed in Figure 1.

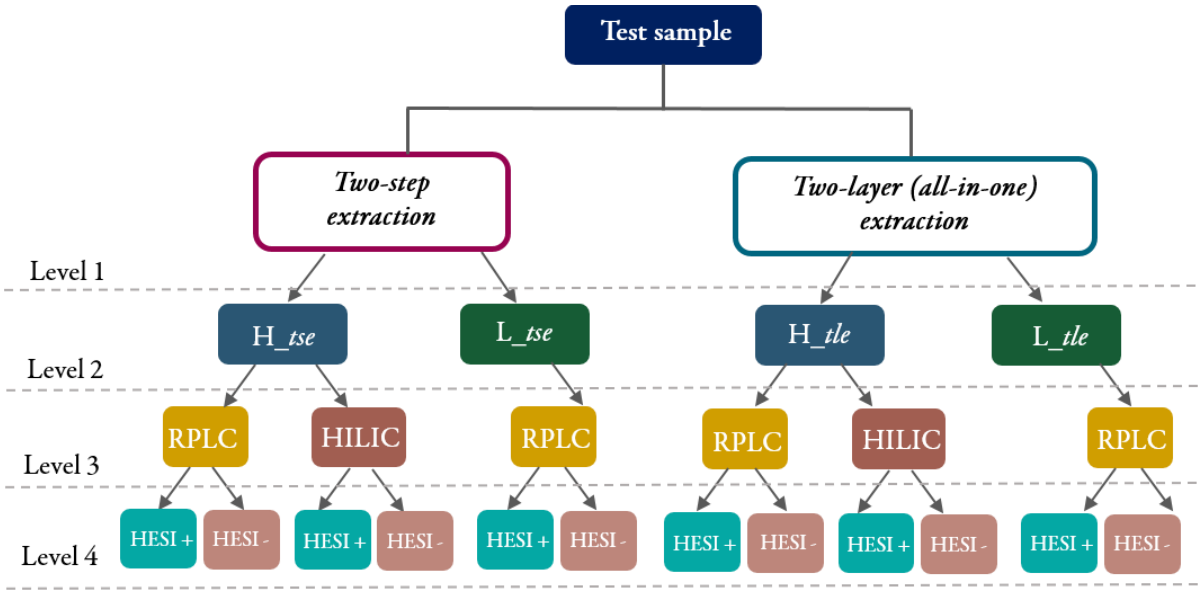

Figure1. Schematic representation of the workflow employed in the optimization of the method, with Level 1: Extraction method, Level 2: hydrophilic-lipophilic fraction, Level 3: chromatographic column, Level 4: ionization mode.

Briefly, in order to perform the optimization of the method, three samples were randomly selected in the sample set and from each one of them three aliquots were collected for the analysis. A quality control (QC) strategy was employed with the aim to test the efficiency and the repeatability of the analytical approach. Each sample was extracted by means of two different extraction protocols; then, the hydrophilic extracts were analysed by a combined RP and HILIC chromatographic approach, while the lipophilic extracts were analysed only by RP. The HRMS detection was performed testing both HESI+ and HESI- ionization mode in separated analytical runs.

2.3 Sample extraction protocols

In order to test and optimize the best extraction method for the metabolic fingerprinting of caviar, two extraction strategies were selected according to protocols suggested by Masson et al., (2010) and (Wu et al., 2008) with modifications, as follows.

> *Two-step extraction.* 150 mg of homogenized sample were added with 600 µL of a mixture of H_2O:MeOH 1:1 (v/v), vortexed for 1 minute, sonicated in a ultrasound bath for 10 minutes and centrifuged for 10 minutes at 15,000 rpm. The supernatant obtained in this step was collected as hydrophilic phase (H_tse). The remaining pellet was added with 900 µL of a mixture of $CHCl_3$:MeOH 2:1 (v/v), vortexed for 1 minute, sonicated in a ultrasound bath for 10 minutes and centrifuged for 10 minutes at 15,000 rpm. The supernatant obtained in this step was collected as lipophilic phase (L_tse).
>
> *Two-layer (all-in-one) extraction.* 150 mg of homogenized sample were added with 750 µL of a mixture of $CHCl_3$:MeOH:H_2O 2:2:1 (v/v), vortexed for 1 minute, sonicated in a ultrasound bath for 10 minutes and centrifuged for 10 minutes at 15,000 rpm. The supernatant obtained in this step was collected in a 200 µL plastic tube. On the remaining pellet, the extraction was repeated a second time with the same steps. The supernatant obtained by the second extraction was collected and united with the first one. The united extracts were centrifuged for 10 minutes at 15,000 rpm until obtaining a clear separation between two layers. The upper layer was collected as the hydrophilic phase (H_tle), while the lower layer was collected as the lipophilic phase (L_tle).

The combination of the mixture in the two extraction protocols were selected in order to maintain the same extraction volumes and proportions in the solvent used, namely $CHCl_3$:MeOH:H_2O 2:2:1, according to the ratios indicated by Bligh and Dyer (1959). In both the protocols, the H and L phases recovered were filtered by PTFE syringe filters (0.45 µm pore size, 13 mm diameter) and dried in a concentrator. Before the LC injection, the hydrophilic phase was suspended in 200 µL of H_2O:AcN 1:1 (v/v) whereas the lipophilic phase was suspended in 200 µL of IPA:AcN:H_2O 4:3:1 (v/v). The extraction workflows tested are graphically resumed in Figure 2.

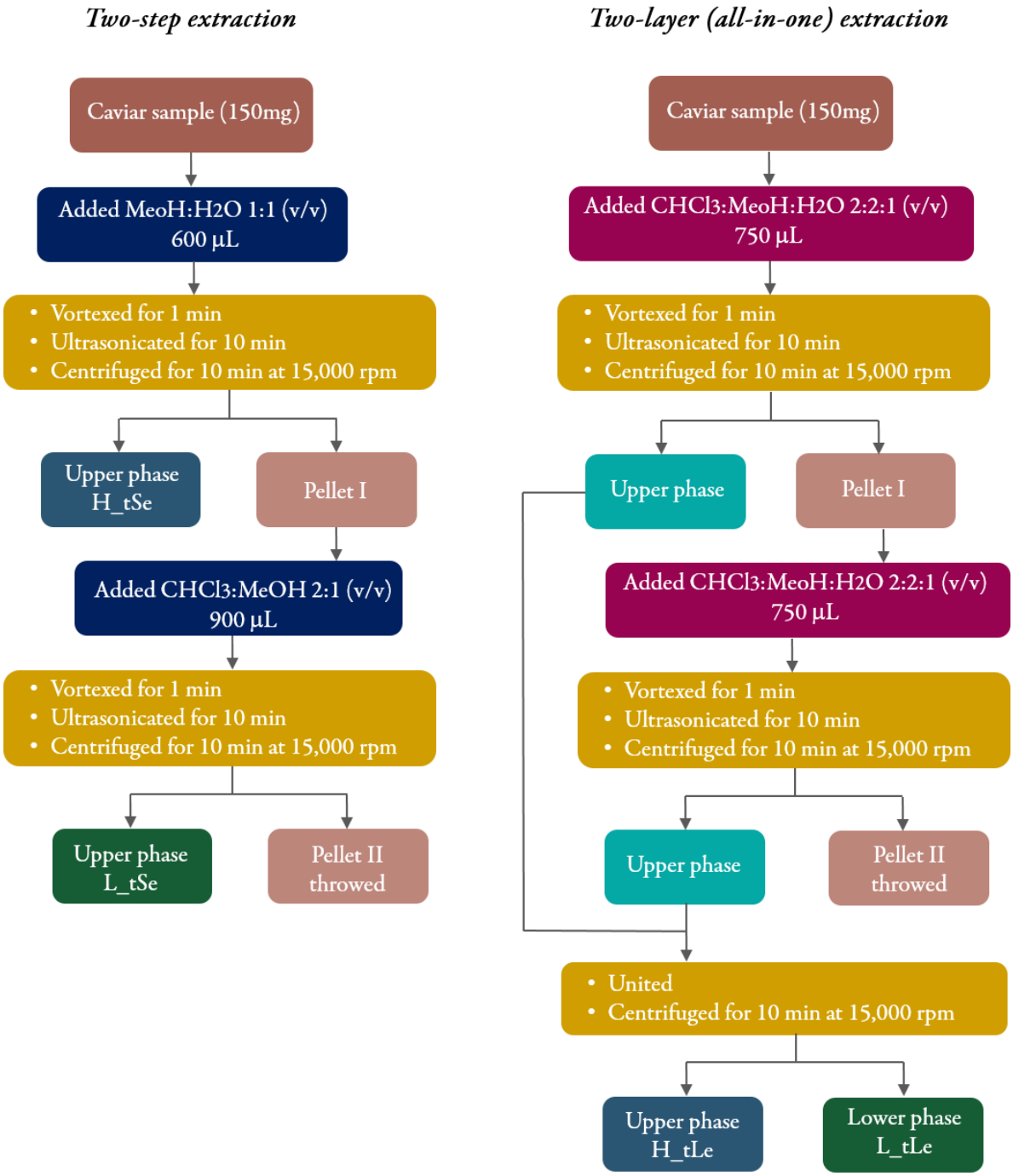

Figure 2. Workflow of the two extraction strategy employed for the recovering of both lipophilic and hydrophilic compounds of caviar.

2.4 Liquid Chromatography and Mass Spectrometry analysis

The metabolomics fingerprinting of caviar was determined by means of a ultra-high performance liquid chromatograph coupled to an Exactive Orbitrap mass spectrometer (UHPLC-HRMS platform by Thermo Scientific).

The combined RP and HILIC separation and HESI + and HESI – conditions were set as follows as follows:

- the lipophilic extract was analysed by in RP-LC mode combining the separation with both positive and negative HESI ionization. 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC, MW 621.83) was used in the optimization of the HESI for L compounds in both positive ($[M+H]^+$, m/z= 622) and negative ($[M-CH_3]^-$, m/z=606) modes.
- The hydrophilic extract was analysed in both RP-LC and HILIC mode, combining each one of the chromatographic condition with both positive and negative HESI ionization. Glutamine (Gln, MW 146) was used in the optimization of the ionization for H compounds in positive mode ($[M+H]^+$, m/z=147), while citric acid (MW=192) was used in the optimization of the negative HESI ($[M-H]^-$, m/z=191).

The column employed in the RP chromatographic method was a Zorbax SB-C18 RRHD (1.8 µm, 2.1 x 100 mm) purchased by Phenomenex. The RP chromatographic conditions were set as follows: mobile phase A= 0.1% FA in H2O (v/v), mobile phase B= 0.1% FA in AcN (v/v), injection volume= 5 µL, column temperature: 50°C, sample temperature: 10°C, flow: 0.200 mL min^{-1}. The chromatographic parameters employed in the separation were set according to García-Cañaveras et al. (2011), with modifications, as in Table 2.

Table 2. Gradient of elution employed with the Zorbax SB-C18 RRHD column.

LINEAR FLOW		
TIME (MIN)	**A (%)**	**B (%)**
0	99.9	0.1
2	99.9	0.1
6	75	25
10	20	80
12	10	90
21	0.1	99.9
23	0.1	99.9
24	99.9	0.1
30	99.9	0.1

The column employed in the HILIC chromatographic method was a ACQUITY UPLC 1.7 µm BEH amide (, 2.1 x 100 mm) purchased by Waters. The HILIC chromatographic conditions were set as follow: mobile phase A= mM ammonium formate in H2O:AcN 98:2, mobile phase B= AcN, injection volume 5 µL, column temperature: 40°C, sample temperature: 10°C. The gradient flow was set according to Muñoz-Redondo et al. (2021) with modifications, as described in Table 3.

Table 3. Gradient of elution employed with the ACQUITY UPLC 1.7 µm BEH amide column.

LINEAR GRADIENT			
TIME (min)	**A (%)**	**B (%)**	**FLOW (mL min^{-1})**
0	5	95	0.4
0	5	95	0.4
4	5	95	0.4
25	28	72	0.4
30	60	40	0.4
30.5	60	40	0.25
31.5	60	40	0.25
31.6	5	95	0.25
35.0	5	95	0.4
36.0	5	95	0.4

2.5 Data acquisition

Full scans were recorded in the 120-1200 m/z range with a resolution of 50,000 Hz and with a full AGC target of 100,000 charges, using 2 microscans. Analyses were also based on scans with in-source collision-induced dissociation (CID) at 25.0 eV. MS conditions for HESI positive mode were as follows: capillary temperature was 325°C, the heater temperature was 300°C, the sheath gas was 25 units, the auxiliary gas was 4 units, and the spray voltage was 4.0 kV. MS conditions for HESI negative mode were as follows: capillary temperature was 325°C, the heater temperature was 300°C, the sheath gas was 20 units, the auxiliary gas was 2 units, and the spray voltage was 4.0 kV.

Raw untargeted UHPLC-ESI-MS data were acquired in the Xcalibur file format (.raw) and converted to the international ANDI file format (.cdf) by means of the file converter tool implemented in the Thermo Xcalibur 3.0.63 software. Chromatographic full scan signals were integrated with the XCMS online Toolbox.

2.6 Analytical flow optimization performed on the test samples set

Raw signals were filtered and only the features with a RSD (relative standard deviation) ≤ 30% were employed for the optimization of the analytical condition. The selection of the chromatographic method (RP and HILIC) and the ionization mode (HESI+ and HESI-), was performed picking the combination of the parameters that led to the highest number of features identified. The selection of the extraction mode was operated by means of multivariate statistical tests. First, a PCA was performed on the raw data matrix. The main variation source in the samples was due to differences between subjects, and the whinit subject variation (i.e. the extraction mode effect) was not clearly observed. Thus ,a multilevel strategy was chosen as appropriate variability decomposition method, in order to highlight the systematic variation related to the factor 'extraction method' (Van Velzen et al., 2008). Subsequently, a multilevel PLS-DA (mPLS-DA) was performed in order to maximize and highlight the differences due to the extraction strategy employed. Then, a mPLS-DA was performed after the application of an iterative variable reduction procedure based on VIP (Muñoz-Redondo et al., 2021), employed to select the 'all relevant variables', with the aim to discover the maximum number of features associated to a significant discriminant power. Finally, in order to corroborate the results obtained by evaluation of the mPLS-DA, comparisons among the Least Square Means (LSM) were performed in a full-factorial model including the extraction method and all the other factors associated to the samples included in the test set. With this aim, peak intensity table containing the samples test set were expressed as fold-change compared to the highest signal recorded for each feature. Then, the fold-change signal for all the detected features was summed for each sample in the test set and the LSM comparisons were performed on this parameter (total fold-change signal), declaring a statistical significance when the factor 'extraction method' was associated to $p<0.05$.

2.7 Data treatment and statistical analysis on the real samples set

Before undergoing statistics, missing value imputation (MVI) was performed to complete the t8 matrix that was unbalanced (table 1), based on the values from the correspondent samples (same treatment) multiplied by a random number between 0.95 and 1.05. Then, following the outcomes obtained during the optimization of the method, a multilevel decomposition was applied to remove the contribution of the subjects on the total variability observed in the dataset and to highlight the contribution of the factor 'time of ripening'. Then, a random forest (RF) variable selection algorithm was used in order to reduce the number of original variables (features) but retaining the maximum of the variability in the data matrix. Finally, mPLS-DA was performed using the set of variables retained after the RF algorithm, chosen as an appropriate chemometrics tool in order to investigate for the most discriminative features and for classification tasks. The optimization and validation of the PLS-DA models were based on a double cross-validation (Szymańska et al., 2012) . All the statistical analyses were performed using the R v 4.1.1 software.

2.8 Putative identification of compounds

Tentative identification of caviar metabolites was performed based on the following criteria. Firstly, putative metabolite IDs were annotated based on m/z values searching in major compound databases available online (MONA, Metlin, HMDB, FoodB) by means of cloud-based applications, namely MetaboQuest (by Omicscraft) and the FoodB LC-MS search tool, and implemented using the MS-DIAL free software by Thermo Scientific. Then, the m/z values of features encountered were matched with m/z values of 604 metabolites present in a home-made database built using literature data covering metabolomes of fish and eggs products. When the identifications matched with an error < 10 ppm for the exact mass values, the targeted metabolites were manually searched in QC samples spectra, with the spectrum composition option of Xcalibur (Thermo). Briefly, the query m/z in the QC spectra at the correct retention time were used for the determination of the most likely chemical formulas associated to the m/z value of the experimental spectrum peaks. Target metabolites were retained for the putative identification when the chemical formula matched with the one of the metabolite suggested in the annotation step with an error < 10 ppm. Moreover, when available, the main fragment peaks were searched based on fragmentation LC-MS patterns obtained with analogous analytical conditions (same ionization mode, 10-20 eV CID fragmentation) available in online databases (PubChem, HMDB). Metabolites that satisfied the criteria described above were retained as putative identified compounds. Furthermore, amino acids were identified by comparisons of the retention times recorded injecting a mixture of reference analytical amino acid standards.

3. Results

3.1 Optimization of the extraction protocol and chromatographic-mass spectrometry conditions

The selection of chromatography and mass spectrometry parameters was performed picking the combination of the parameters that led to the highest number of features identified after the integration of the full signal. The results of this step are presented in table 4.

Table 4. Output of the features (raw signal) obtained by the combination of analytical approaches employed in the optimization step after the signal filtering (RSD ≤ 30%).

NUMBER OF FEATURES DETECTED			
		HYDROPHILIC FRACTION (HF)	**LIPOPHILIC FRACTION (LF)**
RP COLUMN	**HESI+**	**4,289**	**3,625**
	HESI-	1,150	1,159
HILIC COLUMN	**HESI+**	1,602	-
	HESI-	**12,873**	-

Due to the high amount of information obtained, a combined RP-HESI+ and HILIC-HESI- approach was selected as suitable for the analysis of the hydrophilic fraction of caviar. For the lipophilic fraction, the HESI+ was selected as the best ionization mode to apply after the RP chromatographic separation of compounds. The results obtained after the multivariate statistical analysis performed for evaluating the efficiency of the extraction technique are presented in Figure 3 and Figure 4.

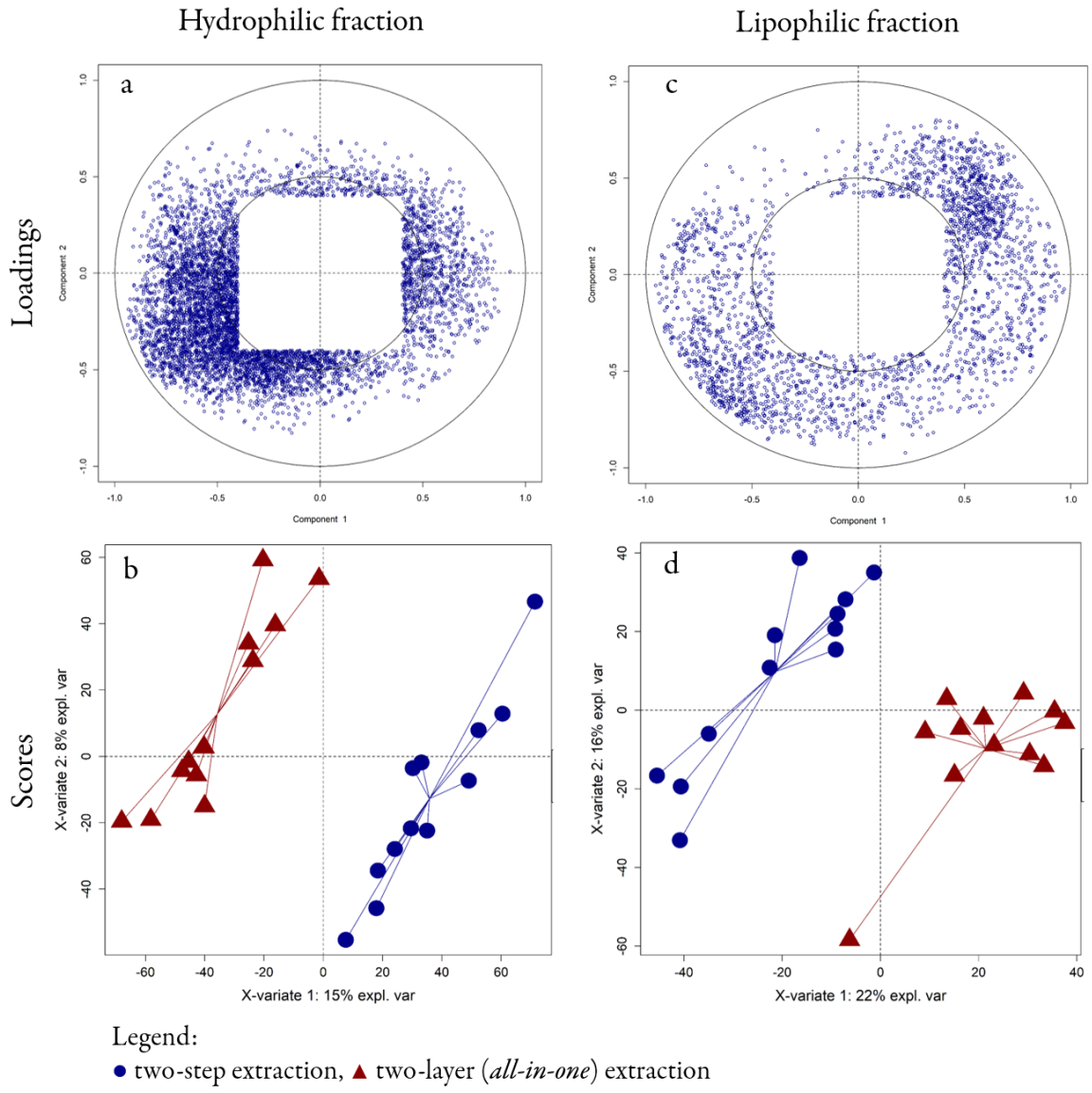

Figure 3. Scores and loadings plots from the mPLS-DA performed to discriminate the samples based on the extraction prodcedure by using the entire dataset. For an easier interpretation, only the features with a correlation value above 0.5 are shown.

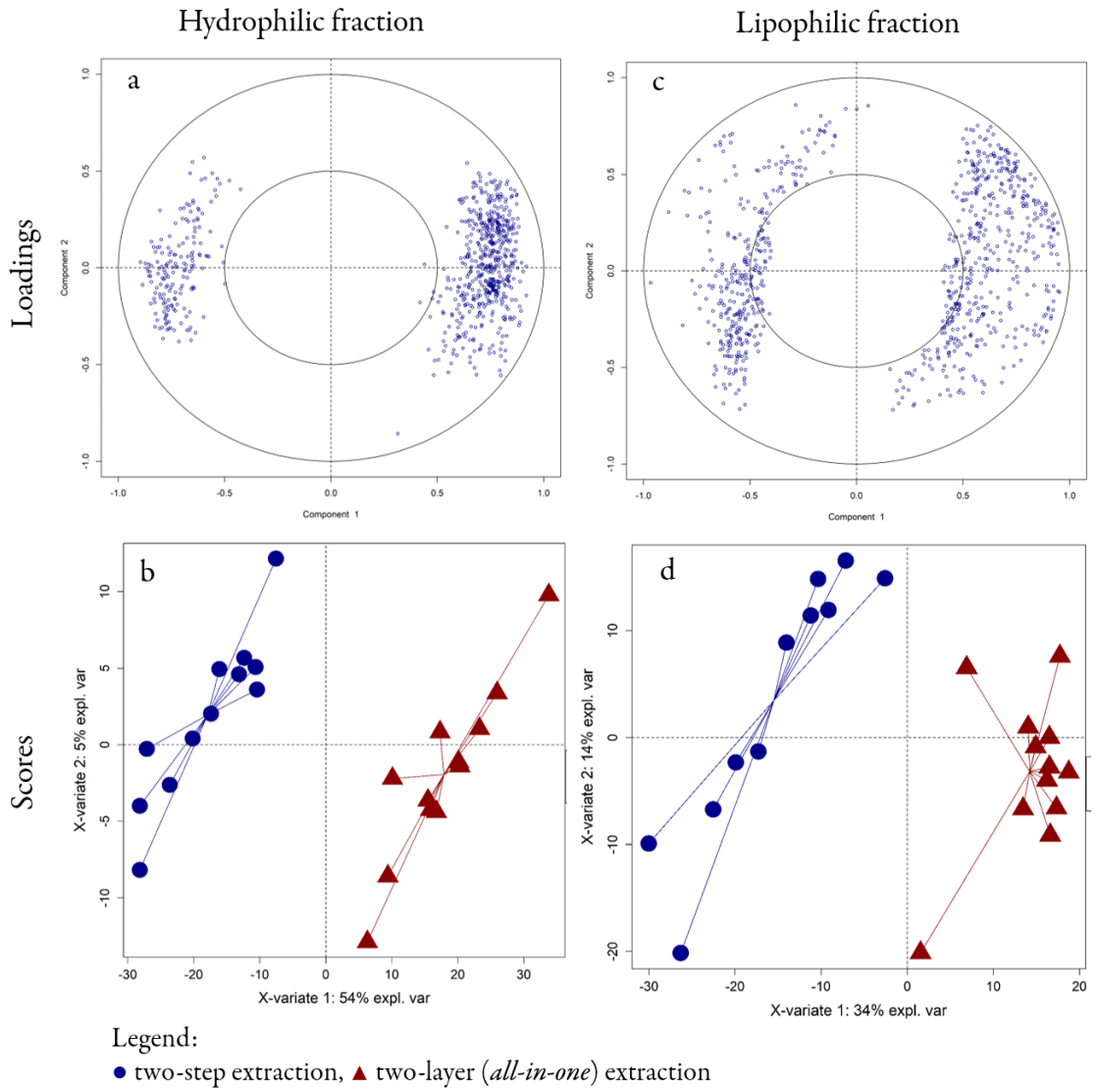

Figure 4. Scores and loadings plots from the mPLS-DA performed to discriminate the samples based on the extraction procedure by using the all-relevant variables from variable selection procedure.

All the mPLS-DA models before and after the slection of the most discriminatory variables (respectively, Figure 3 and Figure 4) showed an acceptable balanced error rate (BER) below 0.5 and low complexity (number of optimized components between 2-4). From the scores and loadings plots of these models, it was observed that the samples extracted by the two-layer (*all-in-one*) protocol (red triangles) were positively correlated with the highest number of features detected by UHPLC-HRMS, supporting a higher efficiency of this extraction protocol. Of note that this, multilevel pre-treatment allowed to evaluate the effect of the extraction approach avoiding interferences from the effect of the variability associated to the individual samples included in the test set.

LSM comparisons performed on the raw signal, expressed as fold change, corroborated the outcomes obtained by the multivariate tests, showing a significant increase ($p<0.05$, $p<0.01$, $p<0.001$) of the features detected in

the test samples for all the combination of chromatographic-mass spectrometry parameters when choosing the two-layer extraction protocol (Figure 5).

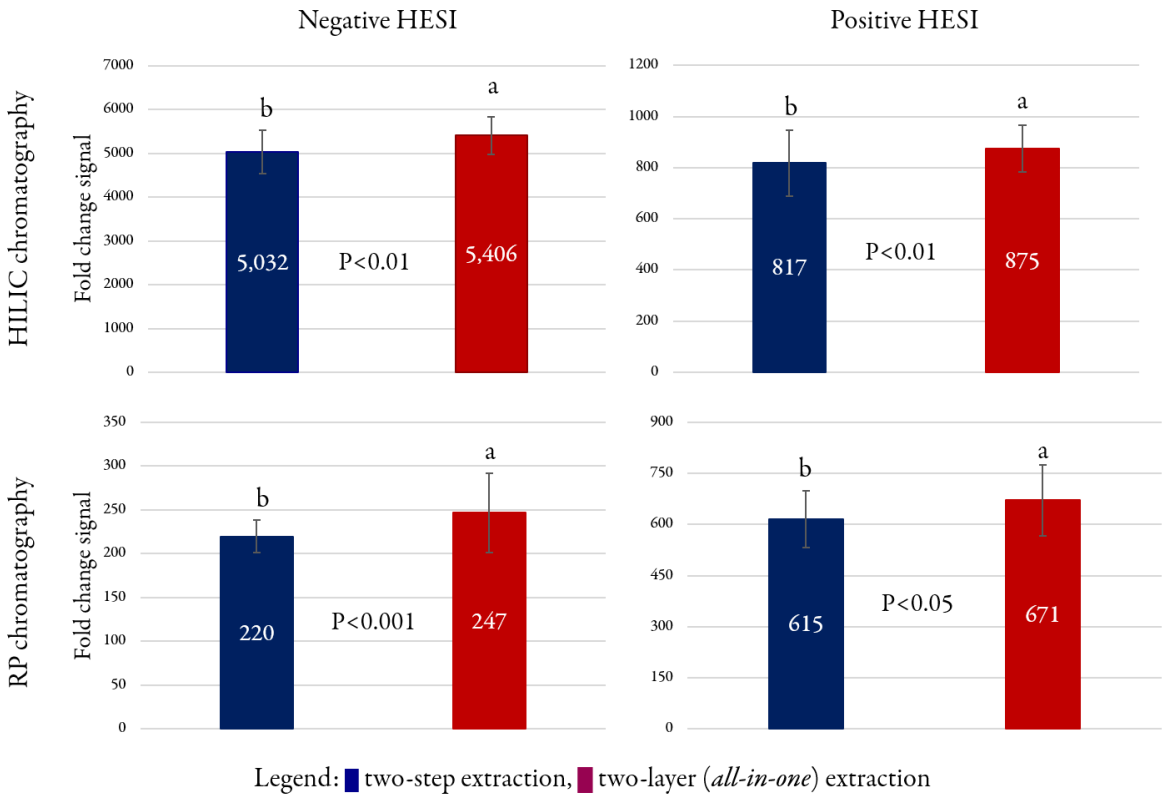

Figure 5. Results of the LSM comparisons between the two extraction methods tested (two-step *vs* two-layer, *all-in-one*) for each combination of chromatography (RP and HILIC) and mass spectrometry (HESI+ and HESI-) parameters employed. Different letters (a, b) associated to the histograms means that the LSM for the two groups (extraction methods) were statistically different (p<0.05, p<0.01, p<0.001).

Thus, in order to analyse the real caviar samples, the following conditions were selected:

- Extraction method: **two-layer (*all-in-one*) protocol**;
- UHPLC-HRMS parameters for the hydrophilic fraction: a combined **RP-HESI+** and **HILIC-HESI-** approach;
- UHPLC-HRMS parameters for the lipophilic fraction: **RP-HESI+**.

3.2 Chemometrics analysis on the evolution of the metabolomics profile of caviar

Multivariate statistics wetre employed in order to investigate the evolution occurring in the metabolome of sturgeon eggs during the ripening, retaining the whole information conveyed by the results of the untargeted analytical approach. PLS-DA was chosen for classification tasks; a multilevel decomposition was first applied to

remove the contribution of the individual subjects on the total variability in the dataset and to highlight the contribution of the factor 'time of ripening'. The results obtained by the mPLS-DA model are shown in Figure 6.

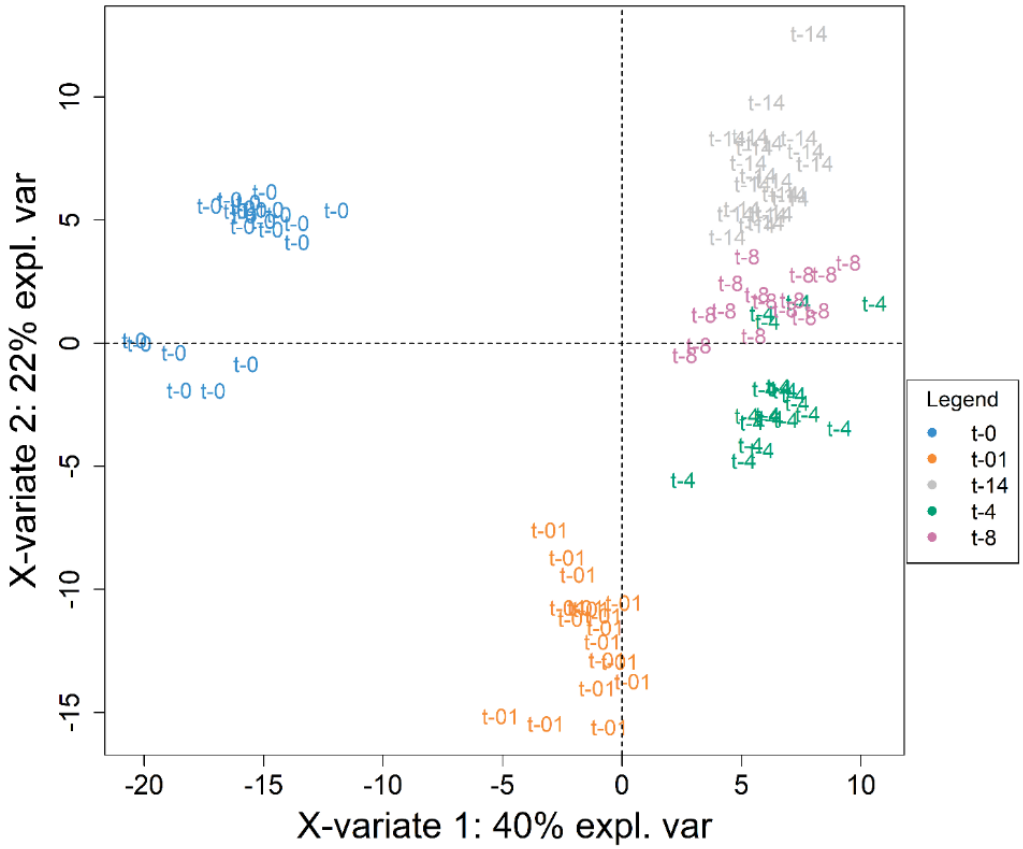

Figure 6. Score plot of mPLS-DA performed with the features selected from a random forest reduction procedure.

The mPLS-DA model was optimized for an average of 4 components and displayed a low BER of 0.06±0.02. The first three components explained the 76% of the variability in the metabolome profile of caviar induced by the ripening time. The Random Forest (RF) variable selection allowed to reduce the dimension of the original data matrix, reducing the number of features (originally, 9,329) to a smaller set of variables (208) accounting for the most of the variability. Interestingly, all the features selected by the Random Forest algorithm and included in the multivariate model were represented by m/z adducts obtained by the ionization of compounds present the hydrophilic fraction. This first outcome suggested that the evolution occurring during the processing of caviar and its storage affected to a larger extent the hydrophilic metabolic pattern of caviar than the lipophilic counterpart. This result was very interesting also from an analytical point of view. Actually, it suggested that, in order to build a classification model able to discriminate caviar samples based on the period of ripening (and, thus, the degree of maturation), it would be suitable to analyse only the hydrophilic extract, strongly reducing the analytical and data-managing effort and adjusting the time needed to obtain reliable results.

In the mPLS-DA scores plot, a significant difference was observable among the samples from to and to1 along the first component. Likewise, to1 and the successive groups (t4, t8 and t14) were separated on the first component direction, even if in a lesser extent. This fact suggested that original variables with the highest loadings for X-variate 1 were compounds influenced by the addition of preservative agents (sodium chloride and other organic acids and salts mixtures) during caviar manufacturing. It could be suggested that the addition of salts induced a partial dehydration of the egg cells, leading to a concentration of metabolites present in raw fish roes.

Furthermore, an interesting trend during the ripening time was observed over the X-variate 2 between to1 and the subsequent time points, reaching the highest scores at t14. This meant that the features associated with the higher loadings on component 2 were adducts of sturgeon roes metabolites evolving during the ripening, characterizing the transformation of raw roes into *caviar*. The highest increment was observed between to and t4 and then at a lesser and quite regular magnitude up to t14. This outcome allowed deducing that the strongest modifications in the non-volatile metabolome of sturgeon caviar occurs toward the hydrophilic compounds present in the eggs during the first 4 months of ripening and then continues during the successive months, but at a slower rate. This result is very interesting compared to the results previously obtained for the biochemical modifications occurring during caviar maturation. Actually, Gussoni et al. (2006) observed that the mechanism of lipid hydrolysis occurring in caviar during the storage time is not associated to significant oxidative process within 4 months. Actually, the authors also suggested that the lipoprotein hydrolysis occurring during this time frame in caviar might develop active components endowed with antimicrobial properties, responsible for the prevention of fast egg degradation. At the same time, previous research on the evolution of the volatilome of caviar during its ripening suggested the period between 6 and 8 months of storage as the critical time point in which volatile compounds indicative of spoilage are formed more intensively (Lopez et al., 2021).

In this study, the heaviest evolution of the non-volatile metabolome of sturgeon caviar was observed at 4 months of ripening, indicating a significant evolution of the hydrophilic metabolites already in this time range of maturation. This evolution could be considered responsible for the formation of chemical species in the aqueous phase, fundamental in determining the characteristic and unique caviar taste, such as free amino acids, particularly glutamic acid (Vilgis, 2020). Then, prolonging the ripening time above 8 months, degradation processes might occur more strongly toward compounds dispersed in the egg cell environment in the free form, thus more susceptible to oxidation, such as free fatty acids (FFA) and free amino acids (FAA), and leading to the formation of undesired compounds (smaller and volatile) responsible for the off-flavour. Actually, the volatile compounds formed during the prolonged storage of caviar, mainly represented by aldehydes and alcohols, have been mainly related to the degradation and oxidation of FFA and FAA (Lopez et al., 2021).

3.3 Metabolites identification in the hydrophilic extract of caviar

Since the Random Forest algorithm designated the hydrophilic compounds as the most affected during the ripening time of caviar, a tentative identification was performed on this set of variables detected by UHPLC-

HRMS, as explained in the section 2.6 of materials and methods. The results obtained by the tentative identification step are presented in Table 5, 6 and 7.

3.3.1 Amino acids, peptides and analogues

The first group of metabolites identified is represented by compounds belonging to the family amino acids, peptides and analogues (Table 5).

Table 5. Amino acids, peptides and analogues tentatively identified in caviar samples. Detailed information, such as the Δ ppm of the isotopic composition of the m/z adduct obtained with the spectrum composition option of Xcalibur (Thermo Scientifc) and CID fragmentation details are presented in Supplementary Materials Table S1.

m/z M+H	RT RP	m/z M-H	RT HILIC	Exact Mass	Proposed Formula	Proposed compound
175.1100	1.14	173.1034	32.79	174.1117	C6H14N4O2	Arginine
-	-	131.0454	19.011	132.0535	C4H8O3N2	Asparagine
-	-	132.0291	18.24	133.0375	C4H7NO4	Aspartate
132.0759	1.26	-	-	131.0695	C4H9N3O2	Creatine
161.0915	1.28	-	-	160.0848	C6H12N2O3	D-Ala-D-Ala
148.0597	1.16	146.0443	17.69	147.0531	C5H9NO4	Glutamic acid
147.0759	1.16	145.0606	18.62	146.0691	C5H10N2O3	Glutamine
156.0759	1.16	154.0625	32.79	155.0695	C6H9N3O2	Histidine
132.1015	1.53	130.8061	12.19	131.0946	C6H13NO2	Leucine + Isoleucine
147.1124	1.02	-	-	146.1055	C6H14N2O2	Lysine
150.057	1.54	-	-	149.0511	C5H11NO2S	Methionine
133.1007	1.26	-	-	132.0899	C5H12N2O2	Ornithine
220.1164	7.25	218.1022	2.89	219.1107	C9H17NO5	Panthotenic acid
166.0848	6.52	-	-	165.0790	C9H11NO2	Phenylalanine
-	-	104.0341	18.94	105.0426	C3H7NO3	Serine
-	-	124.0059	12.09	125.0147	C2H7NO3S	Taurine
120.0648	1.18	118.0496	17.18	119.0582	C4H9NO3	Threonine
205.0960	7.47	203.0815	12.72	204.0898	C11H12N2O2	Tryptophan
182.0803	1.94	180.065	15.10	181.0739	C9H11NO3	Tyrosine

The high presence of FAA, related to fundamental organoleptic properties, in fish roes products could be also related to their high biological importance in fish reproduction. Actually, FAA are associated to fundamental roles in larvae development stages and in their endogenous metabolic turnover, being the structural units for the

polymerization of embryos body proteins, hormones, neurotransmitters and cofactors and furnishing fuel in the energy aerobic catabolism (Rønnestad & Fyhn, 1993).

Moreover, some non-protein amino acids related to important biological functions were detected in caviar, among them creatine, previously found in high amounts in fish eggs from several species (Suzuki & Suyama, 1983), and in processed products such as mullet roes (Piras et al., 2014). Creatine plays a role in cellular energy metabolism in such tissues with high energetic requirements, functioning as an energy storage molecule related to the production of ATP by means of ADP dephosphorylation on demand (Reicher et al., 2020). Moreover, Fedorov et al. (2017) indicated the fundamental biological role of creatine in sterlet sturgeon spermatozoa, suggesting the biological importance of this α-amino acid in the reproductive organs of sturgeons.

Another non protein amino acids detected in caviar in this study was taurine (Tau), derived from the metabolism of sulphur containing amino acids in fish eggs, related to important physiological functions such as osmoregulation, energy storage, modulation of immune response, DNA repair and restoring disturbed expression of genes involved in fatty acids and amino acids metabolism (Guimarães et al., 2018). This amino acid has been previously found in fish roes from other species, indicated as one of the most abundant FAA in oocytes and ovary tissues of seawater and freshwater fish, and suggested to be incorporated in the FAA pool of eggs before spawning (Guimarães et al., 2018; Rønnestad & Fyhn, 1993).

Furthermore, pantothenic acid and ornithine have been detected in caviar. Pantothenic acid (vitamin B5) has been previously found in paddlefish roes (Herring & Mims, 2015). It is indicated in bibliography as important compound in fish oocytes, related to a minimum requirement ingestion by the adult fish for the fingerlings growth, prevention of deficiencies and optimal feed conversion (Murai & Andrews, 1979). This vitamin has also a fundamental nutritional function for consumers, being and essential compound for the proper functioning of metabolic processes that convert food in energy and for the production of hormones and cholesterol (Herring & Mims, 2015). Finally, ornithine was detected, confirming previously findings in fish roes from several species (Suzuki & Suyama, 1983), suggested as one of the main FAA in fish eggs, originated by the catabolism of arginine (Rønnestad & Fyhn, 1993).

3.3.2 Lipid species

The second group of compounds detected and tentatively identified was represented by lipid species characterized by a certain degree of polarity (Table 6) and, thus, present in the hydrophilic fraction of caviar extract. Actually, fish eggs vitellogenins are enriched in polar lipids and the yolk lipoprotein are mainly represented by phospholipids (PL) (Wiegand, 1996). Lipid compounds detected can be divided in the following classes: I. lysophosphatidylcholines (Lyso PC); II. lysophosphatidylethanolamines (Lyso PE); III. phosphatidylethanolamines (PE) and IV. free fatty acils (FFA). All the species detected in this study have been previously found in fish products, included fish roes, by other authors (Li et al., 2018; Porcari et al., 2014).

By means of the analytical equipment employed in this research, represented by a single quadrupole HRMS detector, it was not possible to suggest the composition for the PL species containing two FA chains, but only the total number of carbon and the number of unsaturated bonds present in the molecule (in the form No C atoms : No double bonds). However, previous research performed using MS/MS as detection method identified the exact composition of the FA chains, suggesting the following conformation of the PL detected: 16:0/20:4 for PE 36:4, 18:0/20:4 for PE 38:4, 18:1/22:6 for PE 40:7, 18:0/22:6 for PE 40:6 and p-16:0/10:4 for PE P-36:4 (Li et al., 2018). All the fatty acids (FAs) included in the PLs detected in this study, and also present in the free form, were already known as mayor FAs in caviar, highly represented in the sturgeon roes membrane.

Table 6. Lipid species tentatively identified in caviar samples. Detailed information, such as the Δ ppm of the isotopic composition of the m/z adduct obtained with the spectrum composition option of Xcalibur (Thermo Scientifc) and CID fragmentation details are presented in Supplementary Materials Table S2.

m/z M+H	RT RP	m/z M-H	RT HILIC	Exact Mass	Proposed Formula	Proposed compound
496.3383	14.06	-	-	495.331	C24H50NO7P	Lyso PC 16:0
508.3391	13.6	-	-	507.3317	C25H50NO7P	Lyso PC 17:1
522.3545	14.5	-	-	521.3473	C26H52NO7P	Lyso PC 18:1
520.3382	13.3	-	-	519.3309	C26H50NO7P	Lyso PC 18:2
546.3529	13.8	-	-	545.3457	C28H52NO7P	Lyso PC 20:3
542.323	12.7	-	-	541.3158	C28H48NO7P	Lyso PC 20:5
596.3679	14.0	-	-	595.3607	C32H55NO7P	Lyso PC 24:6
454.285	13.3	452.2773	8.23	453.2855	C21H44NO7P	Lyso PE 16:0
-	-	480.3104	8.49	481.3168	C23H48NO7P	Lyso PE 18:0
-	-	478.2938	8.06	479.3012	C23H46NO7P	Lyso PE 18:1
-	-	476.2792	7.92	477.2855	C23H44NO7P	Lyso PE 18:2
-	-	500.2787	7.73	501.2855	C25H44NO7P	Lyso PE 20:4
-	-	498.2663	7.89	499.2699	C25H42NO7P	Lyso PE 20:5
-	-	528.3119	6.72	529.3168	C27H48NO7P	Lyso PE 22:4
526.2934	12.8	524.2782	7.58	525.2855	C27H44NO7P	Lyso PE 22:6
-	-	738.5028	4.17	739.5152	C41H74NO8P	PE 36:4
-	-	766.5407	3.8	767.5465	C43H78NO8P	PE 38:4
		790.5412	1.04	791.5465	C45H78NO8P	PE 40:6
-	-	788.5222	3.94	789.5299	C45H76NO8P	PE 40:7
-	-	722.5114	3.7	723.5203	C41H74NO7P	PE P-36:4
-	-	281.2486	0.98	282.2599	C18H34O2	Oleic acid (18:1)
-	-	279.2323	0.80	280.2402	C18H30O2	Linoleic acid (18:2)
-	-	303.2313	0.34	304.2402	C20H32O2	Arachidonic acid (20:4)

Mostly, they were represented by long chain (>18 carbon atoms) and unsaturated (one or more double bonds) FA, being the most representative oleic acid (OA, 18:1), linoleic acid (LA, 18:2), arachidonic acid (ARA, 20:4), eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). Interestingly, DHA, well known as one of the mayor FAs in caviar (Lopez et al., 2020), was not detected in its free form. Accordingly, previous research performed by means of HPLC-HRMS (Dal Bello et al., 2017) on white sturgeon caviar detected DHA only at 2% among the FFA, while OA was the main FA in this fraction, accounting for the 71%. These outcomes suggested that DHA, which is a fundamental FA for the larvae development (Lee, 2001; Yanes-Roca et al., 2009), is mainly preserved in fish eggs in its bonded form, linked in the membrane phospholipids, where it unrolls its fundamental biological function. On the contrary, OA, which is considered the main lipid energy source for the larvae development (Henderson, 1996; van der Meeren et al., 1993), can be found in its free form, in which it can be easily catabolised to provide the required energy. These outcomes agreed with previous findings that indicated a significant higher distribution on DHA in the PL fraction of white sturgeon caviar compared to the neutral counterpart fraction and the opposite trend for OA, reaching the highest amount in neutral lipids (Lopez et al., 2020).

Even ARA, a FA highly represented in fish roes lipids as important precursor of intra- and extra- cellular massagers molecules such as eicosanoids (Wiegand, 1996), was detected in this study in both free and bonded form. Finally, among the n6 series FA even the presence of LA was observed. LA is a FA typical of the plant kingdom, and it presence in white sturgeon caviar from aquaculture has been related to its presence in the commercial farming diets, enriched in vegetable oils for sustainability purposes (Lopez et al., 2020). The high biological value of the lipid species detected in caviar in this study reflected also in an important nutritional significance, because in literature has been demonstrated that the uptake and the utilization of polyunsaturated fatty acids bonded to PL molecules are more efficient than those bound to neutral lipid species, such as triacylglycerols (Wiegand, 1996).

3.3.3 Other compounds

Finally, other classes of compounds were detected in the hydrophilic metabolome of caviar, particularly nucleosides, nucleotides and derivatives, carnitines, purines and derivatives and others (Table 7).

Table 7. Nucleosides, nucleotides (and derivatives), purines (and derivatives) and other compounds tentatively identified in caviar samples. Detailed information, such as the Δ ppm of the isotopic composition of the m/z adduct obtained with the spectrum composition option of Xcalibur (Thermo Scientifc) and CID fragmentation details are presented in Supplementary Materials Table S3.

m/z M+H	RT RP	m/z M-H	RT HILIC	Exact Mass	Proposed Formula	Proposed compound
Nucleosides, nucleotides (and derivatives)						
324.0581	1.36	322.0438	18.36	323.0519	C9H14N3O8P	CMP
284.0981	8.00	282.0837	10.566	283.0924	C10H13N5O5	Guanosine
364.0639	1.8	362.0494	18.86	363.0580	C10H14N5O8P	GMP
269.0873	4.4	267.0725	6.55	268.0808	C10H12N4O5	Inosine
123.0555	2.3	-	-	122.0476	C6H6N2O	Nicotinamide
-	-	662.1028	21.07	663.1091	C21H27N7O14P2	Beta-NAD
335.0613	4.91	-	-	334.0566	C11H15N2O8P	Beta-NAM
-	-	243.0609	3.35	244.0695	C9H12N2O6	Uridine
Purines (and derivatives)						
-	-	151.10602	5.10	152.0334	C5H4H4O2	Xanthine
137.0454	4.70	-	-	136.0382	C5H4N4O	Hypoxhantine
Miscellanous						
204.1225	1.6	-	-	203.115	C9H17NO4	Acetylcarnitine
-	-	103.0386	0.37	104.0473	C4H8O3	Hydroxybutyrate
184.0726	1.17	-	-	184.0739	C5H15NO4P	Phosphocholine
377.1439	8.54	375.1297	7.72	376.1383	C17H20N4O6	Riboflavin
-	-	117.0181	2.49	118.0266	C4H6O4	Succinic acid
516.2995	10.5	514.2848	0.98	515.2922	C26H45NO7S	Taurocholic acid
-	-	111.0181	1.63	112.026	C4H4N2O2	Uracil
222.0961	1.3	-	-	221.0889	C8H15NO6	N-acetyl-D-glucosamine

Among the last compound found in caviar in this study, it is worth to mention riboflavin. Riboflavin (vitamin B2) has been previously indicated as one of the main vitamins in roes from several species, among them salmon (Farag et al., 2021) and paddlefish (Herring & Mims, 2015). The latter, is a fish species biologically similar to sturgeon, being in the same taxonomy order of *Acipenseriformes*. Actually, paddlefish roes and sturgeon caviar have been indicated in literature as excellent sources of essential vitamins (Herring & Mims, 2015; Tavakoli et al., 2021). Particularly, riboflavin nutritionally fundamental being involved in the metabolic pathways that allow producing energy from food and maintaining the health of eyes and skin tissues (Herring & Mims, 2015). Furthermore, several nucleotides and nucleosides, and their derivatives, were found in caviar. Other authors

previously find these compounds in similar products, such as tuna roes. Particularly, nicotinamide was detected in both its free form or incorporated into nucleotides (Scano et al., 2013). This result is very interesting because nucleotides are compounds indicated among the main responsible for the *umami* taste of caviar (Vilgis, 2020).

The presence of nucleotides, nucleosides and their derivatives (such as inosine) in fish products have been linked to the same metabolic pathway of xanthine, hypoxhantine and uracil (Chang et al., 2020), also found in this study. Particularly, Chang et al. (2020) suggested that after catch of the fish, firstly ATP present in body tissues undergoes to breakdown, forming ADP and AMP. Thus, inosine is formed by the degradation of IMP (a deamination product of AMP) and then transformer to hypoxanthine. In a later stage, during the fish product storage even at refrigerated temperature, hypoxhantine is converted to xanthine and other ring cleavage products, such as uracil (Chang et al., 2020), a reaction enhanced by the developing of microflora on the surface of products. Similar processes were indicated to occur even in hen eggs during the storage (Cavanna et al., 2018) and thus could be hypothised to take place even in fish roes.

Similarly, phosphocholine (P-Cho), previously found in the aqueous phase of fish eggs, was suggested as lipolysis product induced by the addition of salt, and subsequent treatments, in tuna roes (Scano et al., 2013). Actually, P-Cho represents the hydrophilic polar head group of phospholipids (such as PC and Lyso PC), lacking the fatty acyl chain, thus it can be suggested that it is formed in caviar by early lipolityc processes, induced by the addition of salt, occurring toward PC and Lyso PC species, leading to the formation of free P-Cho and FFA. Moreover, this compound is structural part of the platelet-activating factor, a phospholipid with potent physiological functions, particularly as mediator of (patho)physiological events (Prescott et al., 2000).

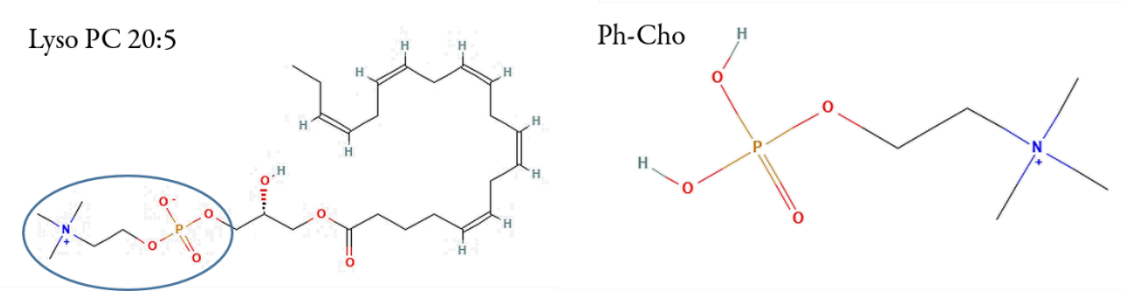

Figure 7. Illustrative representation of the 2D structure of Lyso PC 20:5 and Ph-Cho. The structures were provided on the online PubChem.

Finally, among the compounds detected in caviar it is worth to mention acetylcarnitine. This compound is an intermediate of the beta oxidation of very long chain fatty acids in mammals, involving both the perixosomial and the mitochondrial systems. L-Acetylcarnitine is an ester of carnitine that facilitates movement of acetyl-CoA into the matrices of mammalian mitochondria during the oxidation of fatty acids. In addition to his metabolic role, acetyl-L-carnitine posses unique neuroprotective, neuromodulatory, and neurotrophic properties this may play an important role in counteracting various disease processes. Chang et al. (2021) suggested that these

metabolic pathways are the responsible for the presence of this compound even in fish products, especially in the early moments after fish catch, when the metabolism of fatty acids is rapidly redirected form mitochondria to perixosomes. However, no evidences have been previously found for the presence of this compound in fish roes or eggs from other animal species, so further investigation is needed for eventual identification.

4. **Conclusions**

In this trial, an analytical method has been optimized for the first time in order to analyse the metabolome of sturgeon caviar in a comprehensive untargeted approach. The analytical workflow has been optimized in several steps, from the extraction method to the mass spectrometry detection, by means of robust multivariate statistics techniques. The application of the method developed in this way on caviar samples collected at different stages of ripening allowed to detect a significant evolution of the fingerprint of polar and medium-polar metabolites present in caviar. Actually, chemometrics revealed that the most interesting modification occurred toward the chemical species extracted from caviar in the hydrophilic fraction. Thus, tentative identification was performed focusing on this set of compounds, revealing the presence of several metabolites reliably present in sturgeon eggs and caviar, among them free amino acids, phospholipids, free fatty acids, nucleotides and derivatives, suggested to play a fundamental role in the development of unique caviar taste. The limitation of this kind of study was related to the analytical tool employed, since in absence of confirmation by means of analytical standard or tandem mass fragmentation, the identification of metabolites can only be considered as putative. However, the outcomes obtained by this study could lay the foundation to further investigation aimed to confirm the presence of important compounds undergoing to modifications linked to the development of the unique chemical and sensorial profile of sturgeon caviar during the ripening.

Supplementary materials

Table S1. Δ ppm of the isotopic composition of the m/z adduct obtained with the spectrum composition option of Xcalibur (Thermo Scientifc) and CID fragmentation details of amino acids, peptides and analogues.

Exact Mass	m/z M+H	RT RP	Δ ppm* ESI+	Fragm ESI +	m/z M-H	RT HILIC	Δ ppm* ESI-	Fragm ESI -	Formula and name
174.1117	175.1100	1.14	-6.409	158.0915 157.1075 130.0964	173.1034	32.79	-1.688	131.0815 154.0610 156.0766	C6H14N4O2 Arginine
132.0535	-	-	-	-	131.0454	19.011	-1.821	NA	C4H8O3N2 Asparagine
133.0375	-	-	-	-	132.0291	18.24	-0.032	115.0023 132.0291	C4H7NO4 Aspartate
131.0695	132.0759	1.26	-4.188	<120	-	-	-	-	C4H9N3O2 Creatine
160.0848	161.0915	1.28	-5.207	<120	-	-	-	-	C6H12N2O3 D-Ala-D-Ala
147.0531	148.0597	1.16	-5.095	130.0496	146.0443	17.69	-2.494	102.0547 128.0341 85.0281	C5H9NO4 Glutamic acid
146.0691	147.0759	1.16	-5.295	130.0500	145.0606	18.62	-1.439	127.0501 128.03406	C5H10N2O3 Glutamine
155.0695	156.0759	1.16	-4.505		154.0625	32.79	-1.318	93.0444 108.0554 137.0341	C6H9N3O2 Histidine
131.0946	132.1015	1.53	-4.809	<80	130.8061	12.19	-0.885	113.02304	C6H13NO2 Leucine + Isoleucine
146.1055	147.1124	1.02	-4.719	130.0859	-	-	-	-	C6H14N2O2 Lysine
149.0511	150.057	1.54	4.843	138.0905 121.0643 133.0313	-	-	-	-	C5H11NO2S Methionine
132.0899	133.1007	1.26	-4.765	<80	-	-	-	-	C5H12N2O2 Ornithine
219.1107	220.1164	7.25	-5.902	202.1085 184.0981	218.1022	2.89	-0.913	88.0389 146.0809 114.7000	C9H17NO5 Pantothenic acid
165.0790	166.0848	6.52	-8.220	120.0807 121.084 149.0591	-	-	-	-	C9H11NO2 Phenylalanine
105.0426	-	-	-	-	104.0341	18.94	-2.687	<120	C3H7NO3 Serine
125.0147	-	-	-	-	124.0059	12.09	0.804	106.9796 94.972 80.9637	C2H7NO3S Taurine
119.0582	120.0648	1.18	-3.662	<120	118.0496	17.18	0.003	118.0498	C4H9NO3 Threonine
204.0898	205.0960	7.47	-3.97	146.0548 495.0909 144.0805	203.0815	12.72	4.264	159.09178 116.0517 186.0551	C11H12N2O2 Tryptophan
181.0739	182.0803	1.94	-6.095	165.054 137.075 147.0433	180.065	15.10	-1.165	163.03883 119.0489 93.0332	C9H11NO3 Tyrosine

Table S2. Δ ppm of the isotopic composition of the m/z adduct obtained with the spectrum composition option of Xcalibur (Thermo Scientifc) and CID fragmentation details of lipid species.

Exact Mass	m/z M+H	RT RP	Δ ppm* ESI+	Fragm ESI +	m/z M-H	RT HILIC	Δ ppm* ESI-	Fragm ESI -	Formula and name
495.331	496.3383	14.06	-1.786	459.2463 313.2721	-	-	-	-	C24H50NO7P Lyso PC 16:0
507.3317	508.3391	13.6	-2.726	NF	-	-	-	-	C25H50NO7PL yso PC 17:1
521.3473	522.3545	14.5	-2.825	184.0725	-	-	-	-	C26H52NO7P Lyso PC 18:1
519.3309	520.3382	13.3	-2.202	467.2730 337.2718	-	-	-	-	C26H50NO7P Lyso PC 18:2
545.3457	546.3529	13.8	-2.482	277.2147	-	-	-	-	C28H52NO7P Lyso PC 20:3
541.3158	542.323	12.7	-2.057	184.0725	-	-	-	-	C28H48NO7PL yso PC 20:5
595.3607	596.3679	14.0	-4.973	ND	-	-	-	-	C32H55NO7P Lyso PC 24:6
453.2855	454.285	13.3	-2.962	239.2369 198.0526 436.2823	452.2773	8.23	0.187	140.0118 255.2326	C21H44NO7P LPE 16:0
481.3168	-	-	-	-	480.3104	8.49	2.362	283.2636 265.2537 140.0343	C23H48NO7P Lyso PE 18:0
479.3012	-	-	-	-	478.2938	8.06	0.762	281.2481 263.2380 140.0104	C23H46NO7P Lyso PE 18:1
477.2855	-	-	-	-	476.2792	7.92	0.177	279.2329 261.2224 140.0104	C23H44NO7P Lyso PE 18:2
501.2855	-	-	-	-	500.2787	7.73	1.448	303.2328 140.0105	C25H44NO7P Lyso PE 20:4
499.2699	-	-	-	-	498.2663	7.89	-0.162	NF	C25H42NO7P Lyso PE 20:5
529.3168	-	-	-	-	528.3119	6.72	0.595	NF	C27H48NO7P Lyso PE 22:4
525.2855	526.2934	12.8	-2.139	311.2354 385.2722	524.2782	7.58	-2.139	NF	C27H44NO7P Lyso PE 22:6
739.5152	-	-	-	-	738.5028	4.17	4.223	281.2481 277.2171	C41H74NO8P PE 36:4
767.5465	-	-	-	-	766.5407	3.8	3.051	303.2329 283.2637	C43H78NO8P PE 38:4
791.5465					790.5412	1.04	5.501	282.2516 327.2328 328.2358	C45H78NO8P PE 40:6
789.5299	-	-	-	-	788.5222	3.94	-3.537	329.2325	C45H76NO8P PE 40:7
723.5203	-	-	-	-	722.5114	3.7	3.949	NF	C41H74NO7P PE P-36:4
282.2599	-	-			281.2486	0.98	1.469	263.1277 209.1535	C18H34O2 18:1
280.2402	-	-			279.2323	0.80	1.301	261.18491 97.066 247.1695	C18H30O2 18:2
304.2402	-	-			303.2313	0.34	1.165	259.1679	C20H32O2 20:4

Table S3. Δ ppm of the isotopic composition of the m/z adduct obtained with the spectrum composition option of Xcalibur (Thermo Scientifc) and CID fragmentation details of nucleosides, nucleotides (and derivatives), purines (and derivatives) and other compounds.

Exact Mass	m/z M+H	RT RP	Δ ppm* ESI+	Fragm ESI +	m/z M-H	RT HILIC	Δ ppm* ESI-	Fragm ESI -	Formula and name
323.0519	324.0581	1.36	-4.096		322.0438	18.36	1.561	138.9790 211.0011 96.9684	C9H14N3O8P CMP
283.0924	284.0981	8.00	2.341	152.0565	282.0837	10.569	3.421	150.412 151.0444	C10H13N5O5 Guanosine
363.0580	364.0639	1.8	-2.487	152.0567	362.0494	18.86	-1.415	211.0002 96.9968 150.0549	C10H14N5O8P GMP
268.0808	269.0873	4.4	-0.609	137.0451 139.042 133.0484	267.0725	6.55	0.202	135.0303	C10H12N4O5 Inosine
122.0476	123.0555	2.3	-3.652	<80	-	-	-	-	C6H6N2O Nicotinamide
663.1091	-	-	-	-	662.1028	21.07	1.876	540.0549 407.8779 346.0555	C21H27N7O14P2 Beta-NAD
334.0566	335.0613	4.91	-2.114	123.0548	-	-	-	-	C11H15N2O8P Beta-NAM
244.0695	-	-	-	-	243.0609	3.35	3.034	200.0561 182.0448 110.0236 111.0188	C9H12N2O6 Uridine
136.0382	137.0454	4.70	-2.170	<120	-	-	-	-	C5H4N4O Hypoxhantine
203.1152	204.1225	1.3	-2.619	145.0521 144.045	-	-	-	-	Acetylcarnitine
104.0473	-	-	-	-	103.0386	0.37	-3.500	85.4000	C4H8O3 Hydroxybutyrate
184.0739	184.0726	1.17	-0.981	-	-	-	124.999	-	C5H15NO4P+ Phosphocholine
376.1383	377.1439	8.54	-0.453	243.0787	375.1297	7.72	1.544	255.0880 258.0555	C17H20N4O6 Riboflavin
118.0266	-	-	-	-	117.0181	2.49	-0.300	115.0036 112.9870	C4H6O4 Succinic acid
515.2922	516.2995	10.5	-2.479	462.2658 480.2764 463.2687 498.2879	514.2848	0.98	1.828	312.1323 317.1350 372.2146	C26H45NO7S7 Taurocholic acid
112.2922	-	-	-	-	111.026	1.63	-1.476	<80	C4H4N2O2 Uracil
221.0889	222.0961	1.3	-4.249	204.0851 138.0522 186.0752 114.0648	-	-	-	-	C8H15NO6 N-acetyl-D-glucosamine

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7.2 Discussion and conclusions of Chapter 7 – *Sturgeon and fish eggs products*

Analytical outcomes. In the trials performed on sturgeon meat and fish roes products (**Trial 4** and **Trial 5**), the GC-FID analytical method developed for the identification of the fatty acids (FA) profile was optimized for the type of product analysed. Particularly, since in fish products there is not the presence of as many geometrical isomers of FA as in dairy products, the derivatization step was performed through a methyl-esterification in acid environment. Acetyl chloride in methanol was selected as derivatizing agent in order to perform the acid-catalysed transterification, according to Christie, W. W. (2003). According to the author, by means of this derivatization technique, all fatty acids are esterified at the same rate, so there are unlikely to be differential losses of specific fatty acids during the esterification step, even if quantitative losses for the recovery of the short-chain ester can occur. However, fish products present a lipid profile starting from myristic acid (14:0), thus the short-chain FA problem can be easily avoided. The absence of the geometrical isomers for MUFA and PUFA highly representative in fish products also allowed the employment of shorter chromatographic column if compared to the ones selected in the trials on dairy products. Particularly, the FA profile of sturgeon meat and fish roes was determined by a 30 m x 0.25 mm i.d. x 0.25 μm film thickness HP-Innowax (polyethylene glycol stationary phase) column employing a temperature gradient without the needing to use isothermal plateau necessary for the separation of *cis* and *trans* isomers of 16:1, 18:1 and 18:2.

In **Trial 6**, a GC-MS analytical method was developed in order to perform a quantification of the volatile organic compounds (VOCs) of salted caviar during the ripening time by multiple headspace solid phase micro extraction (MHS-SPME), following the approach introduced for the first time by Bryan Kolb in 1982. This method was chosen in order to avoid the application of extraction techniques responsible for the formation of artefacts (such as simultaneous distillation-extraction) and, contemporary, to perform the quantification of the analytes extracted by SPME, which is a non-exhaustive extraction method. Briefly, in the multiple extraction approach developed:

I. An almost exhaustive extraction of many target analytes, selected by previous test extractions performed on a representative aliquots of caviar, was performed exposing the SPME fibre to the headspace of a vial in which standard mixture were prepared, for four consecutive times.
II. A logarithmic linear regression was performed, and the slope of the linear regression line obtained was identified as the natural logarithm of the β factor for each compound, indicating the extent of the exponential decay for the area of each compound across successive extractions. The β factor was thus employed in the estimation of the total area for each analyte or, in other words, the area of the analytes peak that we would have obtained if the SPME would have not been an equilibrium but an exhaustive extraction technique.
III. Finally, a quantitation of the analytes was carried out by the interpolation of the estimated total area for each analyte in a calibration curve obtained by a typical linear regression model built by injecting calibration solution in an external standard strategy, in order to measure the response of the instrumental equipment after known analyte amounts injections.

Results suggested that the MH-SPME approach developed allowed a quantification of several key VOCs of caviar avoiding severe alteration of the matrix before the analysis and in a relatively short time (30 minutes of fibre exposition plus 41 min of chromatographic run), obtaining scores for the β factors and the linear coefficient in an optimal range for quite all the classes of target compounds. An exception was represented by the class of organic acids (target compound: nonanoic acid) and this was imputed to the low affinity between these family of compounds and the stationary phase of the chromatographic column selected, a nonpolar phenyl arylene column, namely a DB-5MS (30 m × 0.25 mm id, 0.25 μm film thickness). The SPME tri-phasic fibre (DVB/CAR/PDMS) was chosen for the extraction of VOCs in sturgeon eggs and caviar, being represented by a combination of polar and nonpolar materials, and the results confirmed the suitability in extracting low concentration compounds represented by different polarities and molecular weights in complex matrices.

Finally, in **Trial 8**, an untargeted metabolomics method was developed by ultra high pressure liquid chromatography (UHPLC) and high resolution mass spectrometry (HRMS) for the first time, allowing the characterization of the non volatile fraction of caviar metabolome. The analytical method was developed and optimized by means of robust statistical tools. The optimization of the method covered three different steps: I. metabolite extraction protocol, II. chromatographic conditions (reverse phase or hydrophilic interaction chromatography), III. Ionization conditions (HESI + or HESI-). The results of the analytical optimization enabled to identify the *two-layer (all-in one)* extraction protocol as the best extraction method for the whole metabolome of caviar, being related to the highest number of metabolites detected; thus, it was chosen as standard procedure to extract the real samples. The principle was based on performing twice the extraction of the hydrophilic and lipophilic metabolites by means of a mixture of $CHCl_3$:MeOH:H_2O 2:2:1, the unification of the two extracts, the partition of the two layers by centrifugation and the drying of each fraction separately before the injection, as showed in Figure 1.

The chromatography and mass spectrometry optimal conditions were selected picking the combination of the parameters that led to the highest number of features identified after the integration of the full signal. Due to the high amount of information obtained, a combined RP-HESI+ and HILIC-HESI- approach was selected as suitable for the analysis of the hydrophilic fraction of caviar. For the lipophilic fraction, HESI+ was selected as the best ionization mode to apply after the RP chromatographic separation of compounds.

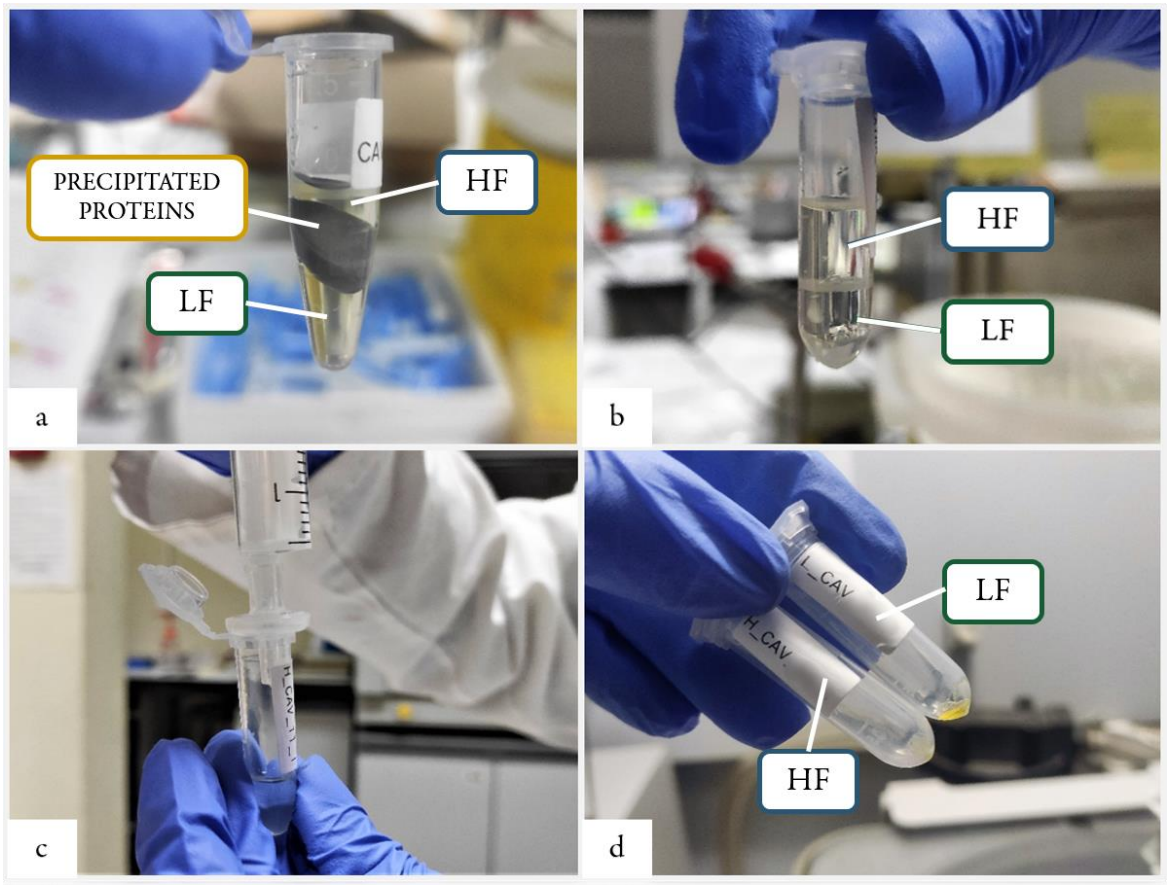

Figure 1. Illustrative representation of steps of the *two-layer (all-in-one)* extraction protocol. HF: hydrophilic fraction, LF: lipophilic fraction. In order: a) separation of the two solvent layers with the precipitate proteins between the two phases; b) separation of the two clean layers after centrifugation; c) filtration of the extract (each fraction separately) by means of PTFE syringe filters (0.45 µm pore size, 13 mm diameter); d) the two fractions after the drying step.

Fish roes and caviar fatty acid profile. The outcomes obtained in these trials provided appealing information about the chemical composition of fish roes related to fish biology, the production system and the processing method. Particularly, species-specific differences were evidenced in fish eggs and sturgeon caviar analysing the FA profile, and this outcome was considered particularly interesting in view of the importance of the ability to discriminate between caviar and its substitutes. Generally, in all the fish roes analysed, the FA profile showed the prevalence of palmitic acid (16:0), oleic acid (18:1n9), linoleic acid (18:2n6), arachidonic acid (20:4n6), EPA (20:5n3) and DHA (22:6n3). Oleic acid was evidenced as the most abundant FA regardless the origin and the species, representing the main source of energy accumulated in the oil droplets of yolk fish eggs during the embryonic development (Henderson, 1996; van der Meeren et al., 1993). In **Trial 4**, the role of this FA as primary lipid energy source in fish eggs was supported, being oleic acid detected at the highest proportion (25.7-32.4%, **Trial 4**, Table 4) in the neutral fraction of eggs lipids.

EPA and DHA showed a high deposition in fish roes regardless the species of origin. This result confirmed the recognition of these FA as primary biological important compounds in fish eggs, being highly represented in the phospholipid fraction of ovas as essential fatty acids for larvae development (Bell et al., 1996; Bell et al., 1995; Lee, 2001; Yanes-Roca et al., 2009). It is known that, during fish gonadogenesis, SFA and MUFA are mainly catabolized by the female fish to provide energy, while PUFA (above all, ARA and n3 PUFA) tend to accumulate in vitellogenin of ovas because of their fundamental biological functionality (Izquierdo, 1996; Tocher, 2010). In fact, the latter have a pivotal role in the embryonic development of fish, covering fundamental functions in the reproductive performances. In our studies, the highest proportion of long chain PUFA (such as ARA, EPA and DHA) was found in the phospholipid fraction of sturgeon eggs (T**rial 4**, Table 4), detected at similar percentages in roes harvested from different species. This outcome agreed with the reported thesis about the nonselective deposition of such FA in fish eggs, as fundamental precursors of prostaglandins and eicosanoids in fish eggs membranes, regardless the species of provenance (Izquierdo, 1996; Mukhopadhyay & Ghosh, 2007; Prabhakara Rao et al., 2013; Wirth et al., 2000). Furthermore, it has been suggested that keeping an ARA/LA ratio as higher as possible could promote the reproductive functionality of fish in aquaculture (Bell et al., 1996; Bell et al., 1995; Johnson et al., 2017; Ovissipour & Rasco, 2011; Tocher, 2010; Yanes-Roca et al., 2009; Zhu et al., 2019).

In **Trial 4** and **Trial 5**, linoleic acid was found in high amounts only in eggs of aquaculture origin. First, this observation allowed us to suggest it as indicator of the farming system compared to the wild origin of fish eggs. Secondly, the high values recorded for LA in roes from farmed fish, due to the high proportion of this FA in the commercial aquaculture diets enriched in vegetable oils, indicated that it would be suitable to increase the concentration decrease the ARA/LA ratio in modern aquaculture formulations, in order to improve the reproductive performances of fish (Turchini et al., 2009). Furthermore, it is reasonable to believe that this could have also a positive impact on the nutritional quality of fish eggs, since lowering the amount of linoleic acid would significantly increase the n3/n6 ratio.

Multivariate techniques applied to the FA data obtained in **Trials 4** and **Trial 5** allowed to distinguish eggs coming from the different fish species, with the combined PCA-LDA approach showing the highest discriminant power. FA associated to the highest selective power in both **Trial 4** and **Trial 5** were oleic acid, linoleic acid, linolenic acid, EPA and DHA. Particularly, the highest discrimination was determined by the opposite behaviour, in the multivariate bi-plots, among the typical FA of marine habitat (long chain n3) and the FA related to the common aquaculture feed ingredients (OA, LA, ALA). In the discriminant model built in **Trial 5.** all fish species were distinguishable and formed a separated cluster, with the exception of roes from cod and Alaska Pollock for which an overlap was observed. This outcome was imputed to the fact that the two fish species (*Gadus morhua* and *Theragra chalcogramma,* respectively) belonging to the same family (*Gadidae*) and sharing the environment of the cold waters of Northern Hemisphere, the reproductive habits and the feed substrate, produced eggs represented by a higher level of similarity. However, caviar was always assigned to a separate group respect to the eggs from the other fish species.

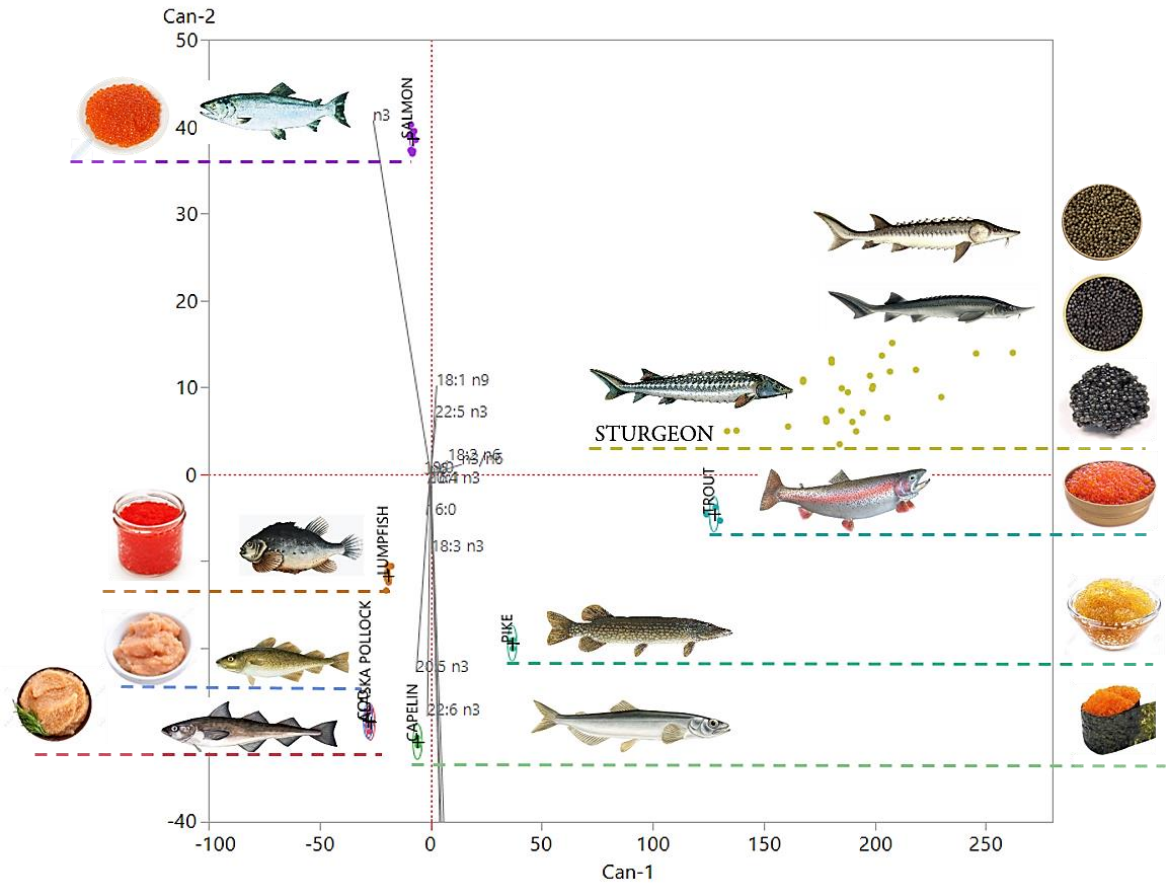

Figure 2. Canonical plot obtained in after the application of the combined PCA-LDA approach, thus selecting variables associated to PC loadings scores > |0.5|, and using roes from the different fish species analysed in trial 5 as training set and caviar samples from Borella et al. (2016) and Lopez et al. (2020) as validation set.

Sturgeon meat. The analysis performed on the biochemical composition of sturgeon meat (**Trial 4**, *Table 5*) revealed that sturgeon flesh could be classified as a medium-fat (2.6-5.6%) and high-protein (17.6-19.6%) fish product, according to the classification model of Stansby (1976). The FA analysis (**Trial 4**, *Table 6*) provided results comparable to what previously found in literature for meat of sturgeon fed with a common commercial diet (Palmegiano et al., 2005). Mainly, a prevalence of unsaturated (75-80%) FA over saturated FA was evidenced, with oleic acid as the most abundant, followed by linoleic acid, linolenic acid, EPA and DHA. It is interesting to note that the amount of linoleic acid detected in **Trial 4** (11.9-16.1%) was up to four time higher than the value reported by other authors for *A. transmontanus* and *A. baerii* fillets twenty years ago (Badiani et al., 1996; Badiani et al., 1997; Paleari et al., 1997). This factor reflected the huge modifications performed in the feed formulations commonly employed in aquaculture during the last years, made up in order to contain the costs and to increase aquaculture, substituting fish meals and oils with vegetable ones, enriched in linoleic acid (Gatlin et al., 2007). This was also notable in the reduced n3/n6 ratio, that in our study ranged from 0.7 to 1.4.

Many differences were evidenced above all comparing female and male sturgeon meat. It is reasonable to impute such differences both to genetic factors, as suggested by Badiani et al. (1997) and to the fact that, as introduced

before, the female fish FA metabolism tends to direct the FA essential for the reproduction (above all, ARA, EPA and DHA) in the gonads, while stores other FA (such as OA, LA and ALA) in fat deposits (Izquierdo, 1996; Tocher, 2010). On the overall, the high nutritional value for sturgeon meat evidenced in **Trial 4** represented an interesting outcome, since the characterization and the evaluation of sturgeon flash supported by scientific outcomes could help its promotion on the market and dismantle its actual role as by-product of caviar industry.

Caviar volatile profile. In **Trial 6** and **Trial 7**, the volatile profile of caviar treated with different preservative formulas and ripened for different time frames was investigated and characterized for the first time by solid phase micro exctration (SPME) coupled to gas chromatrography (GC) and mass spectrometry (MS). Mainly, the volatile organic compounds (VOCs) detected were indicated as markers of the development of the typical fish-flavour (mainly in the case of aldehydes) or spoilage and off-flavour (alcohols and ketones) (Hardy et al., 1979; McGill et al., 1974), with variable trends, but mostly in increasing amounts during the storage time. Particularly, in **Trial 7**, eight months of ripening was identified as critical time point in which the most of the modification in the VOCs profile occurred. Caviar treated with all the preservative tested (just salt, mixture of salt and organic acids, mixture of salt and sodium tetraborate) showed an increase of the VOCs during the storage, but each series was related to the increase of different compounds. Particularly, caviar just added with <4% of salt showed the highest increases of alcohols (3-methylbutanol, 2-ethylbutanol, 2-ethylhexanol) and ketones (acetoine) known as early indicators of spoilage. The other two series showed a different behaviour. Mostly, the mixture of organic acids was the treatment that led to the formation of the lowest concentrations and with a more gradual increase of the VOCs in caviar. Many VOCs related to the lipid degradation in fish products were undetected in this series and this outcome was related to the effectiveness of the presence of ascorbic acid as antioxidant agent in this series. The treatment represented by the mixture of salt and sodium tetraborate as preservative showed an intermediate behaviour, with a significant evolution of the VOCs profile from up to 8 months, due to the enzymatic, oxidative and microbial activity occurring, and then reaching an equilibrium.

The VOCs found in caviar in **Trial 6** and **Trial 7** were principally aldehydes, alcohols, ketones, acids and hydrocarbons already known as volatile compounds typical in seafood. Most of the aldehydes detected are known to derive by the peroxidation processes occurring toward the unsaturated FA highly represented in caviar and, particularly, 2,4-heptadienal from n3 FA, hexanal from n6 FA and octanal, nonanal and decanal from n9 FA. Similarly, short-chain alcohol, such as 1-octen-3-ol and 1-penten-3-ol, were detected and suggested as products of the peroxidation of LA and EPA and DHA, respectively (Aro et al., 2003; Belitz et al., 2009; Hsieh & Kinsella, 1989; Josephson & Lindsay, 1986; Nordvi et al., 2007). Since alcohols are generally formed during the middle and late storage of fish products, they have been indicated as spoilage and oxidation markers, associated to the development of the "off-flavour" in seafood (Alasalvar et al., 2005; Iglesias et al., 2009; Jonsdottir et al., 2004). Moreover, 1-penten-3-ol, together with other compounds such as 3-methylbutanal, 2-methylbutanal, 2-phenyalcetaldehdye, 3-methylsuflilpropanal and 3-hydroxy, 2-butanone, were found in caviar in increasing amount during the storage and were suggested as markers of microbial contamination and spoilage, being known derivative of the degradation activity of microorganisms toward amino acids and lipids (Ardö, 2006;

Belitz et al., 2009; Jónsdóttir et al., 2008; Olafsdottir et al., 2005; Parlapani et al., 2017; Pripi-Nicolau et al., 2000; Weenen & Van Der Ven, 2001). 3-hydroxy, 2-butanone and other ketones (3-pentanone and acetophenone) were detected after 8 months of ripening in caviar treated with only salt and after 14 months of ripening in caviar added with the organic acids and the salt and tetraborate mixture. 3-hydroxy, 2-butanone was thus indicated as an early indicator of caviar spoilage. Actually, caviar just added with salt, in the time frame between 8 and 14 months of ripening, was associated with a total viable count (TVC) near or higher than 6 Log CFU/g, considered as critical level in order to determine the acceptable shelf life of seafood products. On the contrary, caviar treated with the other preservative treatments never reached TVC values higher than 2 Log CFU/g. This outcome suggested that salting at low concentration alone, even if considered the gold practice to obtain caviar of the highest quality, is not sufficient if it is necessary to extend the shelf life of the product for a long time. However, the time needed to reach the limit for the shelf life acceptance in caviar analysed in **Trial 7** was quite long (8 months), more than what found in bibliography for caviar kept at refrigeration temperatures, that reached the total microbial count of 6.0 Log CFU/g in a time range of only 5–10 days (Shin et al., 2010). This suggested that the controlled storage temperature (-2°C) employed in the production plant that furnished caviar for this study effectively retarded the microbial proliferation, allowing to prolong the shelf life up to 6-8 months.

Caviar non-volatile metabolome. Results obtained by the application of chemometrics on metabolomics data in **Trial 8** allowed depicting the metabolites present in the aqueous phase of sturgeon eggs as the most affected during the ripening. Actually, 208 features detected in the hydrophilic fraction were retained by the classification model employed (mPLS-DA with Random Forest variables selection) over the 9,329 hydrophilic and lipophilic features included in the original data matrix. The mPLS-DA scores plot enabled detecting the time frame 0-4 months of ripening as critical time point in which the most of the modification in the non volatile profile occurred in the direction of both X-variate 1 and X-variate 2 (**Trial 8**, Figure 6). Particularly, a significant separation of raw egg samples analysed before and after the addition of the preservative treatment suggested that osmotic changes, due to the partial dehydration of the egg cells subsequent to the salt addition, led to a concentration of metabolites present in raw fish roes.

On the overall, these outcomes are very interesting if joined to the results obtained in **Trial 7** for the volatolome of caviar. Actually, whereas the critical time point for the formation of the VOCs responsible for the development of the 'off-flavour' was detected at 8 months of ripening, the most significant evolution of the non volatile metabolites in sturgeon eggs during the maturation to caviar was observed during the first 4 months, when raw roes become *caviar*. This early evolution occurring toward the hydrophilic species could be considered responsible for the formation of free compounds in the aqueous phase fundamental in determining the characteristic and unique caviar taste, such as free amino acids (particularly glutamic acid) and nucleotides, associated to the distinctive *umami* taste (Vilgis, 2020). Then, prolonging the ripening time up (and above) 8 months, degradation processes might occur toward the compounds generated during this phase, dispersed in the egg cell environment in the free form, thus more susceptible to oxidation and degradation, such as free fatty acids (FFA) and free amino acids (FAA). Actually, the VOCs characterized in**Trial 7** were mainly related to the degradation and oxidation of FFA and FAA.

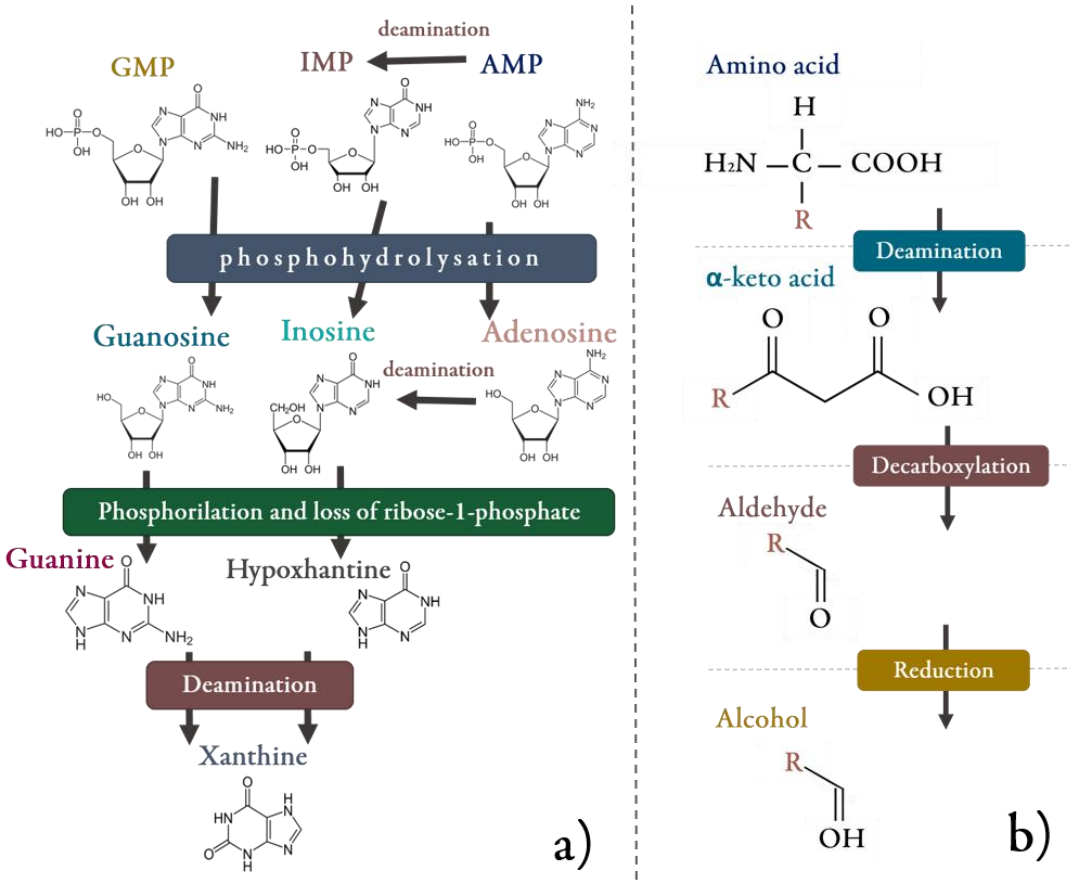

Figure 3. a) Metabolic pathways of purine nucleotides to xanthine; b) metabolic pathways of formation of volatiles from amino acids. Adapted from Sun et al. (2020).

The analytical platform employed for the detection (a single quadrupole MS) only allowed a putative identification of metabolites, based on the exact mass of the adducts detected by MS. However, features detected were only retained for biological interpretation when matching with a home-made database of metabolites found in literature for fish and eggs products and when the query m/z values in the QC spectra at the correct retention time generated the correspondent chemical formula with an error < 10 ppm. In addition, amino acids were identified by comparisons of the retention times recorded injecting a mixture of reference analytical amino acid standards.

Most of the compounds tentatively identified in caviar could be related to the biological requirement of fish eggs, in terms of energy substrate and active components necessary for larvae development stages. Among them, both protein and non protein amino acids were found (**Trial 8**, *Table 5*). The latter, represented by creatine, ornithine, taurine and pantothenic acid, were previously found in high amounts in fish eggs of several species and demonstrated to play fundamental role in metabolic processes occurring in the ova, including energy storage and modulation of immune response (Guimarães et al., 2018; J.L. Herring & Mims, 2015; Murai & Andrews, 1979; Reicher et al., 2020; Rønnestad & Fyhn, 1993; Suzuki & Suyama, 1983).

The second class of compound tentatively characterized was represented by phospholipids and free fatty acids (**Trial 8**, *Table 6*). The free form probably originated by lipid hydrolysis process, enhanced by the addition of salt, occurring in fish roes during production and storage, leading to the formation of FFA and free phosphocholine (Gussoni et al., 2006; Scano et al., 2013). On the overall, the FA composition was in line with findings of the previous trials, identifying palmitic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid, EPA and DHA as the most emblematic fatty species present in sturgeon caviar. It was particularly interesting to observe that the n3- series long chain PUFA were found exclusively in the bonded form with phosphocholine or phosphoethanolamine, in the class of phospholipids, while no free EPA or DHA were detected. This outcome interestingly matched with the results of **Trial 4**, where the n3- PUFA EPA and DHA were found at the higher proportions in the phospholipid fraction of sturgeon eggs. On the contrary, oleic acid was found in both the bonded and free form, the latter probably derived from the degradation of neutral lipids stored in the eggs as energy source. These outcomes further allowed supporting the selective metabolism of FA, and their deposition in different lipid species, in fish eggs, based on their biological function (Izquierdo, 1996; Lee, 2001; Mukhopadhyay & Ghosh, 2007; Prabhakara Rao et al., 2013; Tocher, 2010; Wirth et al., 2000; Yanes-Roca et al., 2009).

Furthermore, findings of this trial enabled promoting white sturgeon caviar as a food product characterized by a nutritionally favourable composition. Actually, the presence of beneficial lipid components, mainly represented by phospholipid of high nutritional value, and vitamins, such as vitamin B5 (pantothenic acid) and vitamin B2 (riboflavin), in food has been previously associated to positive consequences on the utilization and the uptake of PUFA and to beneficial functions on metabolic processes for consumers (Joshua L. Herring & Mims, 2015; Wiegand, 1996).

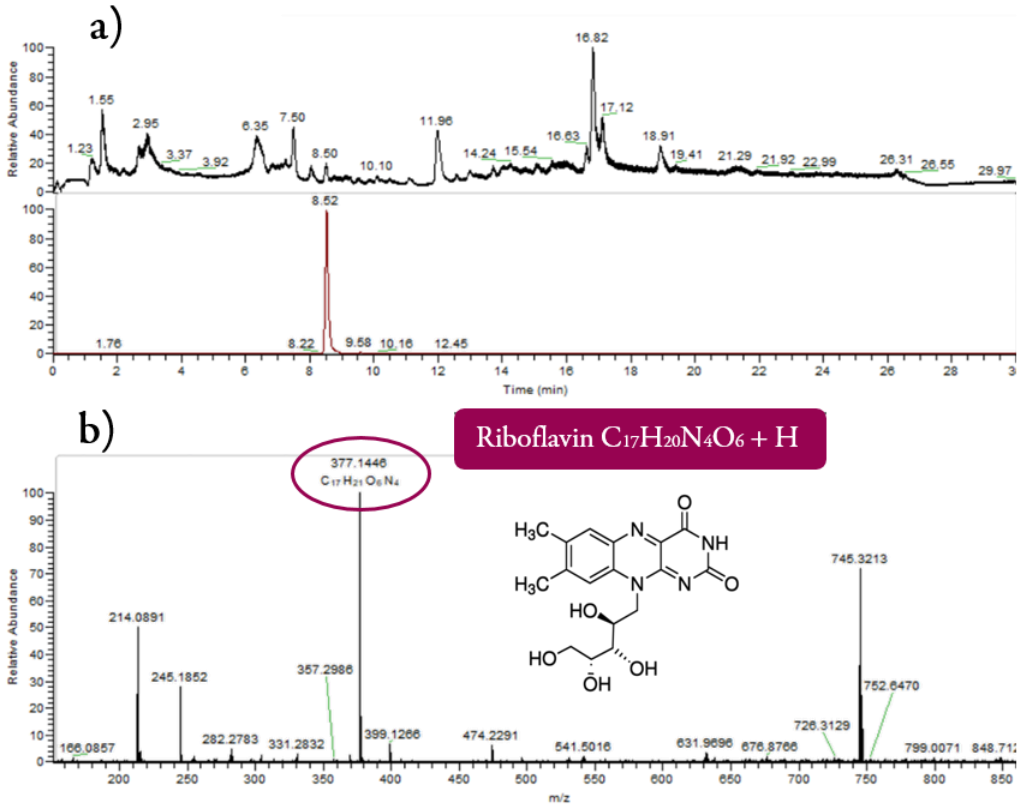

Figure 4. a) Illustrative full-scan chromatogram of a RP chromatographic run and focus on the mass range 377.1446 (± 10 ppm), corresponding to the $[M+H]^+$ adduct of riboflavin; b) spectrum of HESI+ ionization of riboflavin.

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Chapter 8

General Conclusions

The outcomes obtained in the experimental part of this thesis allowed to confirm chromatographic separation techniques coupled with different detection methods, such as flame ionization detection and mass spectrometry, as reliable analytical approaches able to characterize animal products in relation to the production systems and the processing techniques. The analytical methods developed and applied enabled the detection and/or the quantification of several kind of molecules characterized by different physical and chemical properties, such as molecular weight, polarity and volatility, as markers of quality and authenticity of the samples analysed. When coupled to proper data analysis techniques, the information collected by the analytical approaches chosen allowed the discrimination of samples with different origins, in terms of animal species, production system or manufacturing process.

In details:

Dairy products. The GC-FID analytical methods developed allowed the identification of many fatty acids (FA) as markers related to the feeding strategy and the livestock system employed in farms where samples were collected. Particularly, the GC-FID equipment arranged in **Trial 3**, employing a 120 mt long capillary column, allowed the separation of several *cis* and *trans* isomers of FA further identified as a fundamental markers (such as *c9t11*-18:2, CLA), unlikely detected with the analytical parameters set in **Trial 1** and **Trial 2**. On the overall, in all the trials, the analytical method developed allowed to identify and confirm many fatty acids (such as odd and branched chain fatty acids, oleic acid, linoleic acid, rumenic acid and PUFA of the n-3 series) as indicators of the production system and the feeding strategy practiced in the farm, interestingly linked to the nutritional quality of dairy products under investigation.

Fish products. The GC-FID analytical method developed for the identification of FA profile, optimized and standardized for these food matrices, enabled the detection of some differences in FA profile of products related to biological differences (**Trial 4** and **Trial 5**). Aditionally, the GC-MS analytical method developed in T**rial 6** allowed a reliable estimation of the amounts of volatile organic compounds (VOCs) detected in white sturgeon caviar, avoiding severe alteration of the matrix before the analysis and, contemporary, performing a quantification of the analytes extracted by SPME, that is a non-exhaustive extraction method. In both **Trial 6** and **Trial 7**, the SPME-GC-MS method allowed suggesting many VOCs as key markers of the development of the typical fish-flavour or, conversely, spoilage related off-flavour during the ripening time of caviar. Finally, the untargeted metabolomics method developed by UHPLC-HRMS in **Trial 8** allowed a characterization of the non-volatile fraction of caviar metabolome. The results of the analytical optimization performed by mean of multivariate statistic methods enabled to identify the best combination of extraction, chromatographic and mass spectrometry conditions to analyse the whole metabolome of white sturgeon caviar. Results obtained by metabolomics analysis allowed depicting the metabolites present in the aqueous phase of sturgeon eggs as the most affected during the key time-frame of ripening (0-4 months).

In all the trials, multivariate techniques applied to chromatographic data allowed to convert complex data matrix in bi-dimensional systems where significant differences among samples related to different origins (in terms of species of origin, livestock system or processing method employed) were more easily disclosable. Moreover, by means of chemometrics in most of the cases it was possible to select among the measured parameters the variables that most influenced the discrimination among different groups of samples, thus suggesting many compounds as possible markers of *authenticity* and *quality*.

To conclude, dairy products produced in small-scale farms of northern Italy were characterized for their nutritional quality, related to lipid composition. Particularly, milk and cheese collected in mountain and alpine farms, praticiting non-conventional (or *low input*) management systems, were characterized as remarkably valuable food products. This is particularly important because this outcome could help the promotion and appreaciation of such kind of products among consumers, supporting the generally perceived superior quality by mean of robust analytical method. Moreover, the outcomes obtained could also have an importance for the productive segment involved, since the results obtained suggested that the feeding system is the main factor affecting the nutritional quality of the final product and that, even in such cases in which the pasture practice is not feasible for farmes, supplying fresh grass directly in the barn might represent a valid alternative aiming to increase milk fat quality. These results seem promising and advising for future research in order to extend such kind of conlcusions to a broader framework and to support impactfully the characterization of dairy products from Italian small-scale farms by mean of scientific research, with positive feedbacks for both producers and consumers.

Regarding sturgeon eggs and meat, in many cases results obtained in this Thesis provided novel data, filling the lack of infomation available in literature and increasing the knowledge toward these food products that represent a valuable market segment for the Italian agri-food system. In the case of sturgeon meat (**Trial 4**), the high nutritional value depicted for the flesh of three sturgeon species typically farmed in Italy can be considered a significant outcome for producers, crucial in view of dismantling its actual role as by-product of caviar industry. In the case of sturgeon eggs, the ability to discriminate between caviar and its substitues was established by mean of analytical methods. These results surely represent a meaningful tool providing a robust support toward protection policies aimed to protect and to differentiate Italian caviar, that represent a fundamental outcome for the national aquaculture segment, **in a global market sector characterized by high levels of unfair competition and frauds**. Furthermore, the results obtained in **Trial 7** and **Trial 8** enabled identifying the pivotal time frames in which most of the modifications of the chemical profile of caviar occurred, providing interesting information and enhancing the knowledge toward the chemistry standing behind caviar flavour. Particularly, the period between 0 and 4 months of ripening was suggested as the most important for the early evolution of hydrophilic chemical species mainly responsible for the formation of the characteristic and appreaciated caviar taste. On the other hand, the period starting with 6-8 months of ripening, was identified as critical for the formation of volatile species, chemical markers of late degradation and oxidative process affecting caviar quality. These outcomes provided novelty in the dataframe available in literature and could represent a standpoint for

future remarkable research toward the nature and the evolution of caviar chemistry, particularly those achievable by targeted metabolomics approach.

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Section 7.2

Complete list of publications

Full papers

Scientific abstracts

Publications

Full papers

1. Vasconi, M., Lopez, A., Galimberti, C., Moreno-Rojas, J.M., Munoz Redondo, J.M., Bellagamba, F., Moretti., V.M. (2019) Authentication of farmed and wild european eel (*Anguilla anguilla*) by fatty acid profile and carbon and nitrogen isotopic analyses. In *Food Control*: 102, 112-121.

2. Lopez, A., Vasconi, M., Moretti, V.M., Bellagamba, F. (2019) Fatty acid profile in goat milk from high and low- input conventional and organic systems. In *Animals*, 9 (7), 452

3. Lopez, A., Vasconi, M., Bellagamba, T., Mentasti, T., Moretti, V.M. (2020) Sturgeon meat and caviar quality from different cultured species. In *Fishes*, 5 (9)

4. Lopez, A., Vasconi, M., Bellagamba, F., Mentasti, T., Pazzaglia, M. Moretti, V.M. (2020) Volatile organic compounds profile in white sturgeon (*Acipenser transmontanus)* caviar at different stages of ripening by multiple headspace solid phase microextraction. In *Molecules,* 25 (5)

5. Vasconi, M., Tirloni, E., Coppola, C., Lopez, A., Bellagamba, F., Bernardi, C. E., Moretti, V.M. (2020) Comparison of chemical composition and safety issues in fish roes products: application of chemometrics to chemical data. In *Foods,* 9 (5)

6. Lopez, A., Vasconi, M., Battini, M., Mattiello, S., Moretti, V.M., Bellagamba, F. (2020) Intrinsic and extrinsic quality attributes of fresh and semi-hard goat cheese from low- and high- input farming systems. In *Animals, 10 (9)*

7. Bongiorno, T., Foglio, L., Proietti, L., Vasconi, M., Lopez, A. Pizzera, A., Carminati, D., Tava, A. Vizcaíno, A. J., Alarcón, F.J., Ficara, E., Parati, K. (2020) Microalgae from biorefinery as potential protein source from siberian sturgeon (*A. baerii)* aquafeed. In *Sustainability*, 12, 8779

8. Lopez, A., Bellagamba, F., Tirloni, E., Vasconi, M., Stella, S., Bernardi, C., Pazzaglia, M., Moretti V.M. (2021) Evolution of food safety features and volatile profile in white sturgeon caviar treated with different formulations of salts and preservatives during a long term storage time. In *Foods*, 10 (4)

9. Bongiorno, T., Foglio, L., Proietti, L., Vasconi, M., Moretti, V.M., Lopez, A., Carminati, D., Galafat, A., Vizcaíno, A. J., Acién Fernández, F. G., Alarcón F.J., Parati, K. Hydrolyzed microalgae from biorefinery as a potential functional ingredient in Siberian sturgeon (*A. baerii*, Brandt) aquafeed. In *Algal Research, 6*

10. Lopez, A., Bellagamba, F., Savoini, G., Moretti, V.M., Cattaneo, D. (2022) Characterization of Fat Quality in Cow Milk from Alpine Farms as Influenced by Seasonal Variations of Diets In *Animals*, 12 (515)

Scientific abstracts

1. Vasconi et al. (2019) Fatty acid composition of ovarian fat and ovary of farmed Russian sturgeon (*Acipenser gueldenstaedtii)*. Conference poster at Conference and Exhibition on Aquaculture, Algaculture, Shellfish farming, fishing industry, new growning system, soilless and vertical farming – AQUAFARM, held in Pordenone (Italy)

2. Lopez et al (2019) Influence of different livestock production systems in caprine milk and cheese odd and branched fatty acids (OBCFA) composition. In *Italian Journal of Animal Science*, ISSN: 1825-021X vol 18, suppl 1

3. Lopez et al (2019) Development of a multiple-headspace SPME and GC-MS method for the determination of white sturgeon (*Acipenser transmontanus)* eggs and caviar volatile profile at different stages of ripening. Abstract in *International Symposium on recent advances in food analysis,* ISBN: 9788075920553

4. Bongiorno et al. (2019) Growth performance and quality traits of Siberian sturgeon (*A. baerii)* juveniles fed diets including *Nannochloropsis gaditana* and *Scenedesmus almeriensis* microalgae meal. Conference at *Aquaculture Europe, 2019,* held in Berlin

5. Fusi et al. (2021) Renal petfood for dogs and cats: fatty acids profile. In *Italian Journal of Animal Science,* ISSN 1594-4077, vol 20, suppl 1

6. Lopez et al. (2021) Characterization of mountain cheese from cows fed Alpine pasture grass by fatty acids composition and multivariate analysis. In *Italian Journal of Animal Science,* ISSN: 1828-051X vol 20, suppl 1

Other PhD activities

Scientific congresses

Courses and seminars

Courses attended

1. *The methodology of Life Cycle Assessment (LCA) in the food chain.* Course held by Prof. Riccardo Guidetti of the Doctoral School in Food Systems, University of Milan

2. *Multivariate statistics* held by Association for Animal Science and Production (ASPA) and the Department of Agronomy of University of Sassari

3. *Statistics for veterinary and animal science, pt. 1 & pt 2.* Held by Prof. Paola Crepaldi of the Doctoral School in Veterinary and Animal Science, University of Milan

4. *Scientific papers: from the first draft to proofs correction* held by Prof. Chiarella Sforza of the Doctoral School in Translation Medicine, University of Milan

5. *Advanced spectroscopic methods in food systems* held by Prof. Francesco Bonomi of the Doctoral School in Food Systems, University of Milan

6. *24th Course of Mass Spectrometry* held by Italian Chemistry Society (SCI) and University of Siena

7. *PH525.1x: Statistics and R* held by HarvardX, an online learning initiative of Harvard University

Scientific Congresses attended

1. AQUAFARM - International conference & trade show on aquaculture, algaculture, shellfish farming and fishing industry. February 13-14, 2019 – Pordenone (IT). Poster presentation.

2. *23rd National Congress of the Animal Science and Production Association* - "New challenges in Animal Science". June11-14, 2019 - Sorrento (IT). Oral presentation.

3. *9th International Symposium on RECENT ADVANCES IN FOOD ANALYS*IS. November 5-8, 2019, Prague (Czech Republic). Poster presentation.

4. AQUAFARM - International conference & trade show on aquaculture, algaculture, shellfish farming and fishing industry. February 19-20, 2020 – Pordenone (IT).