Metabolomic and volatilome profiling of milk to assess the application of Infrared radiation processing

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Abstract:

The use of Infrared (IR) in milk sanitation shows potential for reducing microorganisms while preserving quality. However, its use in milk and dairy products is still limited. Thermal processes are evaluated based on the thermal inactivation kinetics of the milk enzyme, alkaline phosphatase, which is not applicable to IR treatment because it remains active. The metabolomic and volatilome approach was used to compare the milk treated with IR radiation at 2 different energies with the starting raw milk, to identify possible treatment markers that can potentially be adopted at an inspection level. IR-treated milk samples showed by metabolomic only one up-regulated compound compared to raw: hypoxanthine. Moreover, we noted a statistically significant increase of some vitamins, amino acids and unsaturated fatty acids in IR-treated samples. From volatilome analysis, 2-propanone, 1,1-dichloro-propanone, dimethyl sulphide, 3-methyl-3-buten-1-ol and 2-ethoxy-2-methyl-propane, significantly increased after treatment, could serve as markers for the use of IR radiation.

Keywords: milk; infrared radiation; metabolomic; volatilome; food safety; sustainability

1. Introduction

Milk is a perishable food and its complex composition rich in nutrients in an aqueous matrix makes it a perfect medium for various microorganisms: *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* and *Listeria monocytogenes* are just some of the pathogenic organisms that can be ingested

through the consumption (Lucey, 2015). For this reason, according to the State-Region Agreement (SRA) of 25/01/2007, the sale of raw milk for human consumption in Italy is only allowed if it is sold directly from the farm or through dispensing machines located in the same province as the farm (Italian Republic, 2007). In all cases, as requested by the Health Ministry Decree of 12/12/2012, the words "to be consumed after boiling" must be indicated on the sales premises or dispensing machine as well as on the packaging (Italian Republic, 2012). The main heat treatments used today to guarantee safety are pasteurization (standard and ESL-Extended Shelf Life) and sterilization. In the food processing industry, the dairy sector is recognized as the most water-consuming segment in the agri-food system. Innovative technologies and strategies to reduce the environmental impact while maintaining high levels of safety and quality with efficient productivity are also mandatory according to the Sustainable Development Goals of the 2030 Agenda, to promote the challenge of a safe and environmentally friendly food system (Valentini, Sievenpiper, Antonelli, & Dembska, 2019). On the other hand, there is the need to support official control with analytical strategies capable of ensuring in terms of food safety and nutritional quality the adoption of the innovative treatment technologies and the recognition of specific markers to discriminate them from the raw matrix or from the conventional treatments. Innovative non-thermal technologies, as well as high-pressure processing (HPP), pulsed electric field (PEF), ultrasound, nonthermal cold plasma, membrane microfiltration, ultraviolet (UV) irradiations and infrared (IR) irradiation, have been developed to treat milk maintaining both safety criteria and milk nutritional quality (Shabbir et al., 2020).

IR is a portion of the electromagnetic spectrum between the visible and microwave ranges. Its wavelength ranges from 0.5 to 1000 μm and is divided into three categories: near-IR (NIR), mid-IR (MIR), and far-IR (FIR) (Jun et al., 2010). FIR (from 3 to 1000 μm) is the most commonly used in food, because most food components absorb this type of radiation (Aboud, Altemimi, RS Al-HiIphy, Yi-Chen, & Cacciola, 2019). The absorbed energy from IR radiation leads to intermolecular vibrations and friction between water molecules and other food components, resulting in heat generation.

IR heating inactivates the pathogen by damaging intracellular components such as DNA, RNA, ribosome, cell envelope and/or proteins within the cell (Sawai et al., 1995). Bacterial inactivation is influenced by the peak wavelength and surface temperature of the IR heating element, the physical state of the microorganism, penetration depth, composition, shape and surface characteristics of food, etc. The advantages of IR application showed high effectiveness in treating food against pathogens, energy efficiency, effective process control, reduction of process time, uniform product temperature, high heat transfer coefficient, and environmentally friendly traits (water saving, etc.) (Erdoğdu & Ekiz, 2011). The use of infrared radiation for milk and dairy products sanitisation is still limited (Aït-Kaddour et al., 2021). However, recently, a patented technology has been tested for its feasibility to trait milk according to safety criteria also investigating volatilome profile, according to Danesi et al. (2024), the application of IR for raw milk sanitisation appears as a promising technology in the dairy industry that can effectively reduce bacterial loads without significantly altering the aroma of the raw matrix.

Thermal processes such as pasteurization are evaluated during controls by monitoring the thermal inactivation of the endogenous milk enzyme, alkaline phosphatase (European Commission, 2005). Phosphatase determination is not applicable to IR treatment because it remains active despite being subjected to sufficient treatment to inactivate the pathogens (Danesi et al., 2024). Therefore, other analytical approaches are pivotal to support official controls to assess when milk is IR treated. Among comprehensive techniques, metabolomics could become a potential support to discover potential treatment markers as well as to investigate the changes that milk undergoes.

With the increasing demand for high standards in food quality guarantee and safety aspects of food, metabolomics has been developed to broadly assess these aspects providing valuable information on these topics. In fact, the growing interest in food-related topics, such as safety, quality, sensorial profile, traceability, and compliance with regulatory requirements, has accelerated the development and implementation of metabolomic approaches as well explained in the review on recent advances in the application of metabolomics for food safety control and food quality analyses by Li et al.

(2021). Metabolomics represents the golden standard approach to identify the chemical composition and quantify metabolites from products driven by various biochemical and treatment processes (Johnson, Ivanisevic & Siuzdak, 2016). It's also well known that milk metabolomics is influenced by several factors such as animal species (e.g., cow, goat, and sheep), thermal treatment, animal physiological conditions and farming systems (Suh, 2022). Knowledge of the chemical composition of milk and dairy products could provide clues to the nutritional quality and safety of dairy products, and some metabolites could be strategically adopted for both product safety and quality assurance (e.g., authentication). Metabolomics research in dairy products has dramatically increased in the last three years, as reported in the critical review by Suh (2022). Together with the metabolomic study, another very relevant aspect could emerge from a careful evaluation of the volatilome. Volatile organic compounds (VOCs) are responsible for the flavour of milk and are crucial for consumer evaluation as they are related to the sensory note of food (Starowicz, 2021). Heat treatments can significantly affect the flavour of the milk by degrading key components and the concentration of some volatile compounds thus resulting in cooked, scorched, and caramelized sensory notes (Cadwallader & Singh, 2009). In view of the above consideration and the the lack of literature on metabolomic characterization of IR-treated milk, the present work aimed to in deep characterize the milk treated with IR irradiation through a metabolomic and volatilome approach, to elucidate possible markers suitable to support the official controls.

2. Materials and Methods

2.1 Sample Collection and IR irradiation

Three different batches of raw bovine milk were provided by a northern Italy dairy industry producing different dairy products. The raw milk, consisting of 60 L for each batch, was sampled directly from the plant tank (100.000 L capacity, refrigerated at 4 °C) to minimize the variability related to the animal trait and feeding system. Raw milk samples were then transported to the laboratory (at 4 °C) to undergo IR treatment at two IR energies (80, and 85), which in order of effectiveness demonstrated the best results in terms of microbiological reduction and aroma preservation according to our recent

work (Danesi et al., 2024). The IR prototype instrument based on patented technology (N. 102020000007867) was used to treat the three batches of raw milk samples and configured to emit IR radiation with a wavelength in the 3–5 µm range (FIR) (Infrared S.r.l; Rho, Italy). The system, well described in the preliminary study of Danesi et al. (2024), consisted of an external pump that vehicles the milk (flow rate of 1.5 L/min) into 3 tubular quartz ducts (8 mm i.d. and 1250 mm length), a single emitter with a maximum power of 7000 W at 400 V radiates over 1100 mm (Infrared S.r.l; Rho, Italy) and an external panel to adjust the emitter power (Infrared S.r.l; Rho, Italy). The applied energies (80 and 85) indicate the percentage of installed power used from a total of 7000 W. The IR source, placed near the duct along the milk flow direction achieved a temperature of 800 °C. To perform the metabolomic analysis, the three different batches both raw milk and IR-treated samples were combined. This resulted in three final pools: one for raw milk, one for milk treated with IR80 and one for milk treated with IR85. This strategy was implemented to eliminate variables related to cow health, farming systems and animal diets, allowing us to focus solely on the changes associated with the treatment, consistent with an untargeted metabolomic approach. Conversely, for the analysis of volatile compounds, the samples were not pooled; instead, each sample was analysed individually after each test.

2.2 Metabolomic analysis

2.2.1 Extraction protocol

The 3 different pools (raw milk, IR80 and IR85) were extracted according to the protocol of Rocchetti Gallo, Nocetti, Lucini, & Masoero (2020) with some modifications. Briefly, milk samples were skimmed by centrifugation at 4500g for 10 min at 4 °C, and 2 mL of each pool were added of 4 mL acetonitrile LC-MS grade (Darmstadt, Germany) acidified with 3% formic acid, vortexed for 15 min and centrifuged at 5000g for 10 min at 4 °C to remove the precipitate. One mL of the supernatant was then filtered in a vial through a 0.45 µm cellulose syringe filter before HPLC-HRMS analysis, by injecting 5 µL of the extract. Ten replicates were collected and analysed for each pool. Three

procedural blank samples (without matrix) were extracted under the same conditions as the milk samples under investigation to obtain the exclusion list, while all the extracted samples were pooled by mixing equal aliquots to obtain Quality Control.

2.2.2 HPLC-HRMS analysis

Chromatographical separation was carried out on Vanquish HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) using an Accucore C18 column (100 × 2 mm i.d., 4 µm, (4 × 3 mm i.d.) (Thermo Fisher Scientific, San Jose, CA, USA). The column compartment was set at 35 °C and the autosampler was kept at 4 °C. The mobile phase consisted of water (A) and methanol (B) both acidified with 0.1% formic acid. The gradient with flow rate of 0.3 mL min^{-1}, started with 5% of eluent B increasing to 100% in 15 min and kept in this condition up to 20.5 min. The initial conditions restored at 20.7 min and maintained up to 26 min.

An Orbitrap Exploris 120 (Thermo Scientific, San Jose, CA, USA) acquiring separately data only in positive ESI mode and then only in the negative one was adopted as detector. The spray voltage was set at 3400 V for the acquisition in positive mode and 3200 V in the negative mode. Sheath gas was set at 40, auxiliary gas at 8 arbitrary units. The ion transfer tube temperature was 280 °C and the vaporizer temperature was 300 °C. Default charge state was 1 and the expected peak width was 8 s. two instrumental methods were employed: a Full scan acquisition (FS) method and a Full scan data-dependent acquisition (FS-dd-MS^2) one. The FS with resolving power of 120.000 (scan range of m/z 80–800), RF lens of 70%, normalized AGC target of 100% and an auto maximum injection time was used for the screening and statistical evaluation of the chromatographic profiles. The FS-dd-MS^2 was employed to obtain fragmentation of molecular ions detected in FS mode, which operated with resolving power of 30.000 (scan range of m/z 80–800), RF lens of 70%, normalized AGC target of 100% and an auto maximum injection time minimum, and intensity threshold of 9.00e+4, a dynamic exclusion duration of 2.5 s, dynamic exclusions and target mass tolerance of 5 ppm. dd-MS^2 operated with resolving power of 30.000, with an isolation window of 1.5 m/z, a stepped, normalized collision

energy (NCE) set at 20, 40 and 80 eV, with a normalized AGC target of 100% and a maximum injection time of 85 ms.

AcquireX software (Thermo Scientific, San Jose, CA, USA) with the Deep Scan workflow was used to create a background ion exclusion list from the procedural blank and an ion inclusion list from the sample pool (Quality Control) with dynamic modification of these inclusion and exclusion lists in between each replicate injection. Default Deep Scan settings were used with $[M+H]^{+1}$ and $[M-H]^{-1}$ as preferred ions, enabled automatic adding of isotopes and an exclusion and inclusion list minimum intensity of 9.00e+4. MS1 survey scans were performed on the procedural blank to create the ion exclusion list and on the QC to create the ion inclusion list, where MS^2 analysis was not performed at this step. Then, four injections of the QC were acquired by AcquireX by the FS-dd-MS^2 to generate MS^2 spectra (ID, Identification Only files). After each ID injection, the m/z for resolved ions were automatically appended to the exclusion list for the subsequent injection and continued until all inclusion list ions are fragmented. AcquireX Deep Scan workflow was performed separately in positive and negative ionization mode. After the AcquireX workflow the raw milk, IR80 and IR85 samples (10 replicates for each type) were analysed separately in the positive and negative ionization mode along with the quality controls which were analysed every five samples to monitor the stability and reproducibility, by the software Xcalibur™ 4.5 (Thermo Fisher Scientific, Waltham, United States).

2.2.3 Untargeted metabolomics approach with Compound Discoverer™ workflow

Thermo Fisher, MA, USA's Compound Discoverer (CD) 3.3 SP2 program elaborated the raw data (namely, procedural blank, ID, QC, and several milk samples) from Orbitrap Exploris 120 analysis for the purpose of chemical identification and statistical evaluation. Positive ion mode and negative ion mode were examined separately by this software. The entire procedure was based on several steps (the nodes) which constituted the workflow (Fig. S1): spectra selection, alignment of retention time and normalization of peak areas, further distinguishing background ions, searching and comparing

reference MS^2 spectral libraries for compound identification, and performing statistical analyses to assess relative differences in compound detection between samples (Cooper, & Yang, 2024). CD 3.3 employs the procedural blank files to identify other potential background ions that escaped the AcquireX exclusion operation, uses the QC files to align over time the chromatographic features of all samples, including the IDs and procedural blanks and performs refined normalizations based on QC features, also extracts MS^2 spectra from the ID files and associates them with corresponding FS ions of the sample files within retention time and parent m/z tolerances, and compares the resulting MS^2 spectra to references from the mzCloud and ChemSpider databases.

Criteria for putative identification of metabolites by the CD3.3 workflow were substantially a mzCloud best match score higher than 80% and the same identification being proposed by at least one external web database, i.e. Human Metabolome Platform HMDB (https://hmdb.ca/), Kyoto Encyclopedia of Genes and Genomes (KEGG), (https://www.genome.jp/kegg), Pubchem (www.pubchem.com) or Small Molecule Pathway Database (SMPDB) (http://smpdb.ca). If the mass fragmentation did not match any of the web databases, manual verification of the fragmentation pattern was performed using ChemDraw software. CD3.3 is also useful for pathway mapping and visualization using the Metabolika database.

2.3 Volatilome analysis

2.3.1 Extraction protocol

Headspace solid-phase microextraction (HS-SPME) was operated in this study, following the procedure by Panseri, Soncin, Chiesa, & Biondi (2011) to investigate the effect of different IR energies on the composition of the volatile compounds in milk samples. A glass vial with a capacity of 20 mL was used to hold 10 mL of milk. The vial was then sealed airtight with a cap equipped with a silicon-polytetrafluoroethylene septum (Supelco in Bellefonte, PA). Before sealing the vial, a solution of 4-methyl-2-pentanone, which served as an internal standard, was added to the milk. The solution was added in a quantity of 100 µL and had a concentration of 20 µl mL^{-1}. To prevent alterations to the matrix, a temperature of 10°C was selected for both the extraction and equilibration

phases. After allowing the sample to equilibrate for 1 hour, a conditioned StableFlex fiber made of 85 µm Carboxen/Polydimethylsiloxane (CAR/PDMS) was used for the headspace extraction (3h). The fiber (Supelco in Bellefonte, PA, USA), was conditioned for 1.5 hours at 280°C. The extraction was done using a CombiPAL system injector autosampler manufactured by CTC Analytics in Zwingen, Switzerland. To maintain a constant temperature, the vials were placed on a cooling plate during the analysis.

2.3.2 GC-MS analysis

A Trace GC Ultra (Thermo-Fisher Scientific; Waltham, MA, USA) Gas Chromatograph, coupled to a quadrupole Mass Spectrometer Trace DSQ (Thermo-Fisher Scientific; Waltham, MA, USA) and equipped with an Rtx-Wax column (30 m; 0.25 mm i.d.; 0.25 µm film thickness, Restek, USA), was used for HS-SPME analysis. The oven temperature program started from 35 °C and was maintained for 8 minutes, followed by a secondary increase of 4 °C per minute until the temperature reached 60 °C. From 60 °C to 160 °C, the temperature was raised at a rate of 6 °C per minute. For the last range, from 160 °C to 200 °C, the temperature increased at a rate of 20 °C per minute. After each analysis, the fiber was thermally desorbed in the GC injector at 250 °C for 5 minutes to avoid any carryover or contamination. The injections were carried out in splitless mode for 5 minutes, and helium was used as the carrier gas with a constant flow rate of 1 mL per minute. An *n*-Alkanes mixture (C_8-C_{22}, Sigma R 8769, Saint Louis, MO, USA) was run under the same chromatographic conditions as the samples to obtain the Kovats retention indices (KI) for each detected compound. During the experiment, the temperature of the transfer line leading to the mass spectrometer was maintained at 230 °C, while the ion source temperature was set at 250 °C. Mass spectra were obtained using an electron impact mass selective detector with a multiplication voltage of 1456 V and a data collection rate of 1 scan per second over the m/z range of 30-350, with an electron energy of 70 eV. Identification of the volatile compounds was carried out by comparing their retention times with those of authentic compounds tested under the same conditions, or by comparing Kovats retention indices with literature data when

authentic compounds were unavailable. The identification of MS fragmentation patterns was performed by comparing them with pure compounds or using the National Institute of Standards and Technology (NIST) MS spectral database. Quantitative evaluation was carried out using the internal standard procedure, with the assumption of a response factor of one. The quantity results (ng mL^{-1}) of each volatile compound were then calculated based on the intensity of the volatile compound peaks compared to the intensity of the internal standard added to the sample in a known amount and expressed as ng mL^{-1} internal standard (IS) equivalents (Panseri et al., 2011).

2.4 Statistical evaluation

Descriptive, univariate, and multivariate statistical analysis was carried out by the Differential Analysis node as an integral part of CD workflow allowing to obtain principal component analysis (PCA), Hierarchical Clustering Analysis (HCA) with the heat map, differential analysis with the Volcano Plot (VP) and the Box Whiskers Charts (BWC) with descriptive statistics to show the trend for the single identified compound. Multi-group comparison hypothesis test was performed by a one-way ANOVA model with Tukey as post-hoc test. P-values are adjusted by the Benjamini-Hochberg algorithm. For the VP, hypothesis test was performed by a multivariate paired t-test (assuming equal variance). VP based on Log2 (FC) and -log10 (P-value) is used to individuate metabolites of interest as differentiators between IR treated samples and raw matrix. HCA view help to individuate the correlation between detected metabolites in the investigated samples in a two-dimensional array of color-coded heat map where each rectangle represents the relative amount related to the area of a specific compound in each sample. HCA is based on an agglomerative bottom-up approach to find the similarities between sample groups. In fact, the analysis advances iteratively, at each stage grouping the two most similar clusters into a new cluster, continuing up to a single one cluster represented by a dendrogram. BWC presents median, first and third quartile, upper and bottom whiskers of each selected metabolite from each sample group. VOCs statistical analysis was performed using the GraphPad InStat software (version 3.10, GraphPad Software, Inc., La Jolla, CA,

USA). Raw milk samples and those treated with different energies were compared among each other. If the values passed the normality test, a paired t-test was applied for the comparison. Otherwise, the Wilcoxon matched pairs test was employed. A significant difference was considered when the p-value was less than 0.05. Considering the big differences in terms of concentrations among the compounds, they were normalized using the software RStudio (version 4.2.3, Integrated Development Environment for R. Posit Software, PBC, Boston, MA, USA) and subsequently reported as heatmap performed with GraphPad Prism (version 9.0.0 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results and discussions

3.1 Metabolomic analysis

To obtain a more reliable number of metabolites in a complex matrix, the metabolomic investigation in this research was performed in both positive and negative ionization mode in separate analytical sessions. Overall, 48 out of 3005 items were confirmed in the negative electrospray ionization mode and 49 out of 5240 in the positive one. Some compounds such as uric acid, hippuric acid and lactose were detectable in both modes, the positive acquisition denoted the identification of free amino acids, peptides, nucleosides, some vitamins, monosaccharides and disaccharides, (Table S1). The negative mode was more specific for organic acids and fatty acids (Table S2). For an first overall statistical visualization is reported the Fig. 1, which shows the PCA score plot, on the basis of the confirmed metabolites, classifying the raw milk, IR80 and IR85; the plots are based on the first 2 principal components (PC) for data acquired both in negative and positive electrospray ionization mode but did not reveal a substantial difference, explaining the of the 41 % and the 52.4% of the total variance in the dataset, respectively. From Fig. 1 it can be observed that IR80 milk is placed in an intermediate situation between the raw milk and IR85 and in particular, the raw milk samples and then the IR80 ones presented a greater uniformity of dispersion, compared to the IR85 samples which almost seem to present subgroups in the visualization.

The differentiation between the groups obtained by PCA, although not clearly substantial, finds confirmation in the representation of Fig. 2 relating to the differential analysis with the Volcano Plot, where only in the positive ionization mode only one up-regulated compound (P value<0.05, Log_2 Fold Change>2), corresponding to hypoxanthine, appeared in the comparison between both IR80 vs. raw milk and IR85 vs. raw milk. Moreover, in the positive ionization mode, 23 metabolites showed a statistically significant variation (P<0.05) in the comparison IR80 vs. raw milk and 14 metabolites in the comparison IR85 vs. raw milk. Fig. 2 also shows the Box Whisker Charts for hypoxanthine in the three different sample groups.

From a biochemical point of view, in the presence of oxygen, xanthine oxidase present in milk catalyses the oxidation of hypoxanthine to xanthine, further oxidized to uric acid, which is also formed during the catalysed oxidation of the purines and has been shown in the literature to have antioxidant activity in milk (Steffensen et al. 2002). The fact that hypoxanthine is higher in IR treated milk samples could suggest the deactivation of xanthine oxidase that might be an obvious candidate in the initiation of oxidative processes in both raw and pasteurized fresh milk, as it is not completely deactivated during milk pasteurization (Steffensen, Andersen, & Nielsen, 2002). On the other hand, the statistically significant variation of uric acid (P<0.05) in the IR samples indicates an increase in purine catabolism. However, in the volcano Plot related to the negative ionization mode, 30 and 28 confirmed metabolites (highlighted with red hatching in the heat maps, Fig. 3) showed a statistically significant difference in the group ratio IR80/raw milk and IR85/raw milk, respectively, while no compound exceeded the value of 2, which was set as the limit for the Log_2Fold Change to be able to define other potential markers. The evidence of PCA and VP together underlined the effectiveness and the sensitivity of both the extraction protocol and the potential of the metabolomic approach used through the combination of AcquireX and Compound Discoverer, which managed to detect and classify the samples based on minority differences.

Fig. 3 shows the Heat map of the compounds confirmed both in positive and negative ionization mode through their characteristic fragmentations which showed a high level of confidence. Here too is noticed a clear clusterization of the samples belonging to the 3 different groups of milk (those treated with IR80 in blue, with IR85 in orange, and raw milk in green). In particular, the colours of the figures ranging from green to red are correlated to the concentrations (from lower to higher) of the respective compounds reported on the right side of the heat maps and specified in Table S1 and S2. The red hatch next to the name indicates the statistically significant compounds obtained through the differential analysis, for a rapid visualization of the main differences. In particular, in the heat map of the negative ionization mode it is noticed that the milk treated with IR80 clusters primarily with the raw milk for greater similarity and then together with the milk treated with IR85; for compounds identified in positive mode, however, the opposite occurred.

In particular, since the IR irradiation is an ohmic treatment, the proposed potential marker was not detected in the studies or metabolomic reviews by Zhu et al. (2021), Qin et al. (2022), Suh (2022) and Sen, Ray, & Bhattacharyya (2021) on discriminating milk subjected to the different conventional heat treatments from raw milk. This to emphasize the fact, that since conventional heat treatments are different from IR one, the resulting metabolic processes are also different. Among the statistically significant compounds, for example, citric acid decreased in IR treated milk than in raw milk in our study, instead increased in the work of comparison between raw milk and UHT (Ajmal, Nadeem, Imran, & Junaid, 2018) and decreased linearly over time at a temperature-dependent rate in the comparison between raw and pasteurized milk (Edwards, Badiger, Heldman, & Klein, 2021). Mesaconic acid, which increased in the IR-treated samples in our study, is a carboxylic acid derived from citric acid, that is involved in the biosynthesis of vitamin B12. Oleic acid, a dominant unsaturated fatty acid in milk, usually decreases with thermal stress through oxidation because lipid catabolism is enhanced (Kumar, Devi, Sharma, & Somagond, 2020), but in our study it increases in IR-treated milk. Pyruvic acid, which can result from lactose degradation, as well as other organic

acids such as formic acid and acetic acid, is lower in IR-treated milk than in raw milk. These organic acids seem to be responsible for the increased acidity of UHT milk during the storage (Ajmal et al., 2018). The α-lactose is also lower in our IR-treated milk, as is the amount of glucose. Linoleic acid, higher in IR samples than in the raw milk in our study, has been reported to decrease with thermal treatments, possibly due to the partial inactivation of lipoprotein lipase by heat treatment (Suh, 2022). Linoleic acid, an essential fatty acid, is metabolized to dihomo-γ-linolenic acid, higher in the IR samples, and serves as an important component of neuronal membrane phospholipids and as a substrate for prostaglandin E, which appears to be important for maintaining nerve blood flow (Pop-Busui, Sima, & Stevens 2006). In general, free fatty acids, with the exception of some essential acids, were found in greater amounts in raw milk and it seems that the higher the free fatty acid content, the greater the likelihood of rancid off-flavors (Suh, 2022). Arachidonic acid, an endocannabinoid, essential for the brain and body development in infants and young children (Qin et al., 2022), was higher in IR samples, so particularly indicated for these categories. The nucleoside cytidine was higher in the IR-treated samples; it is a pyrimidine that, in addition to be incorporated into nucleic acids, can serve as a substrate for the salvage pathway of pyrimidine nucleotide synthesis. Cytidine, that was also higher in the IR-treated samples is converted to uridine; it is the major form of pyrimidine nucleosides taken up by the brain and it is phosphorylated to nucleotides, which are used for DNA and RNA synthesis as well as for the synthesis of membrane constituents and glycosylation. Riboflavin or Vitamin B2, also higher in IR samples and reported in literature as relatively stable during thermal and nonthermal food processing and storage (Choe, Huang, & Min, 2005), plays a fundamental role in the synthesis of all energy processes, in fact, its peculiarity is to release the right energy to the body to carry out regular daily activities. (±)12(13)-DiHOME and (±)9(10)-DiHOME, derivative of linoleic acid diol, are protoxins and in significantly lower quantities in IR-treated milk samples. Choline, acetylcholine, betaine, carnitine and creatine significantly higher in IR samples, play an important role in lipid metabolism. In mammals, different studies have documented the protective effects of betaine on antioxidant status (Ganesan, Buddhan, Anandan, Sivakumar, &

AnbinEzhilan, 2010). The health interest in betaine derives from its ability to give methyl groups to homocysteine, converting it to methionine (Day, & Kempson, et al., 2016). Alanine, proline, glutamic acid and tyrosine were also significantly higher in IR-treated samples.

3.2 Volatilome analysis

A comprehensive analysis of VOCs in milk identified 24 different compounds, including 6 ketones, 6 alcohols, 5 carboxylic acids, 3 sulphur compounds, 1 aldehyde, 1 ether, 1 hydrocarbon and acetamide (Table 1 and Fig. 4). The results show a general increase in the compounds evaluated as the energy of the treatment is raised. Ketones, sulphur compounds and alcohols seem more susceptible showing the most important differences. However, the main findings for each class of compounds, are detailed in the following subsections, focusing on the volatilome analysis of milk to assess the effects of IR treatment.

Table 1. VOCs profile of raw non homogenized milk before and after IR treatment with energies 80 and 85.

Compound	**KI[*]**	**Identification**	**Raw milk (*n*=3)**	**s.d. (±)**	**IR80 (*n*=3)**	**s.d. (±)**	**IR85 (*n*=3)**	**s.d. (±)**
			Aldehydes					
Hexanal	759	MS, LRI	0.08[a]	0.16	2.90[a,b]	1.39	4.21[b]	1.63
Total			**0.08[a]**		**2.90[a,b]**		**4.21[b]**	
			Ketones					
2-Propanone	527	MS, LRI	1212.95[a]	268.77	1553.42[b]	472.97	1613.35[b]	583.46
2-Butanone	591	MS, LRI	282.75	47.46	284.33	99.82	282.95	86.80
1,1-Dichloro-propanone	709	MS, LRI	0.19[a]	0.19	1.05[b]	0.80	1.77[b]	1.29
3-Hydroxy-2-butanone	728	MS, LRI	1.46	1.84	1.06	0.84	0.74	0.24
2-Heptanone	888	MS, LRI	0.49	0.33	0.33	0.12	0.37	0.22
6-Methyl-2-heptanone	963	MS, LRI	0.26[a]	0.29	2.93[a, b]	2.00	5.01[b]	3.61
Total			**1498.11[a]**		**1843.13[b]**		**1904.19[b]**	
			Sulphur compounds					
Dimethyl sulphide	565	MS, LRI	48.37[a]	15.09	57.78[b]	19.86	58.76[b]	20.86
Dimethyl disulphide	748	MS, LRI	n.d.	-	0.33	0.46	0.12	0.11

Dimethyl sulphone	912	MS, LRI	10.76^{a}	6.48	13.00^{a}	4.15	26.40^{b}	11.63
Total			**59.13^{a}**		**71.11^{b}**		**85.28^{c}**	
			Carboxylic acids					
Acetic acid	615	MS, LRI	1.13	0.77	0.83	0.37	1.10	0.40
Butanoic acid	779	MS, LRI	11.04	9.59	11.61	13.14	19.56	14.38
Hexanoic acid	980	MS, LRI	4.39	3.43	4.88	4.94	7.05	4.28
Octanoic acid	1166	MS, LRI	1.05	0.70	0.94	0.77	1.16	0.66
Decanoic acid	1356	MS, LRI	0.30	0.20	0.31	0.26	0.34	0.26
Total			**17.90**		**18.57**		**29.21**	
			Alcohols					
Ethanol	482	MS, LRI	6.43	1.16	5.71	1.94	5.77	1.46
2-Propanol	491	MS, LRI	2.70^{a}	1.66	$2.88^{a,b}$	1.41	4.06^{b}	2.35
2-Methyl-2-propanol	530	MS, LRI	66.82^{a}	79.10	$104.17^{a,b}$	66.32	101.93^{b}	49.94
3-Methyl-3-buten-1-ol	737	MS, LRI	0.42^{a}	0.37	1.08^{b}	0.80	1.08^{b}	0.59
1-Pentanol	771	MS, LRI	0.21^{a}	0.16	0.67^{b}	0.27	$0.47^{a,b}$	0.14
1-Hexanol	867	MS, LRI	5.09^{a}	2.85	$6.80^{a,b}$	5.06	9.49^{b}	6.96
Total			**81.67^{a}**		**$121.31^{a,b}$**		**122.79^{b}**	
			Ethers					
2-Ethoxy-2-methyl-propane	616	MS, LRI	10.17^{a}	8.93	39.18^{b}	29.22	71.23^{b}	48.06
Total			**10.17^{a}**	**8.93**	**39.18^{b}**	**29.22**	**71.23^{b}**	**48.06**
			Hydrocarbons					
Butyl-cyclopropane	712	MS, LRI	0.62	1.00	0.88	0.33	1.10	0.45
Total			**0.62**		**0.88**		**1.10**	
			Miscellaneous					
Acetamide	764	MS, LRI	0.03^{a}	0.04	$0.02^{a,b}$	0.04	0.09^{b}	0.06
Total			**0.03^{a}**		**$0.02^{a,b}$**		**0.09^{b}**	

*Kovats index calculated for Rtx-Wax column (30 m; 0.25 mm i.d.; 0.25 µm); MS: mass spectrum tentatively identified using NIST; LRI: linear retention index; different letters ^{a,b} and ^{c} mean statistically significant differences between samples ($P<0.05$). If two means share a letter, they are not significantly different from each other; n.d., not detected.

3.2.1 Aldehydes

Aldehydes are a class of compounds characterized by a significant impact on the flavour of milk, even at low concentrations (Vazquez-Landaverde, Velazquez, Torres, & Qian, 2005). Our findings confirmed that aldehydes are present in milk at low levels, with only hexanal being detected. Hexanal can occur naturally in raw milk, because of the breakdown of hydroperoxides and autoxidation of unsaturated fatty acids. However, the application of heat treatment can intensify these reactions, leading to an increase in the amount of this compound (Amador-Espejo, Gallardo-Chacón, Juan, & Trujillo, 2017). Our findings revealed a correlation between hexanal levels and the intensity of IR

radiation, with the highest energy level exhibiting a statistically significant increase compared to the raw sample. This suggests that IR radiation may promote the overcited oxidation process, potentially making hexanal a notable marker of this treatment. However, it's noteworthy that the mean amount detected (4.21 ng mL^{-1}) remained below the odour threshold reported in the literature for this compound (4.50 ng mL^{-1}) (Zabbia, Buys, & De Kock, 2012).

3.2.2 Ketones

This class of compounds is the most prevalent in milk (Contarini & Povolo, 2002). The results we obtained confirm this characteristic, with ketones identified as the compounds with the highest concentration. Ketones can originate from different sources. In milk, they can be formed from the feed composition or from metabolic reactions that occur in cows. These reactions mainly produce lower molecular weight methyl ketones such as 2-propanone and 2-butanone. However, when conventional heat treatments are applied, the levels of these ketones tend to increase (Amador-Espejo et al., 2017). In our study, 2-propanone was the most abundant compound detected. It showed a statistically significant increase after the IR radiation for each energy applied. Differently, 2-butanone maintains a constant value. The formation of higher molecular weight ketones, such as 2-pentanone, 2-hexanone and 2-heptanone, is instead attributable to the β-oxidation of saturated fatty acids followed by their decarboxylation and the decarboxylation of β-keto acids, both promoted by the heat (Hougaard, Vestergaard, Varming, Bredie, & Ipsen, 2011). Among these compounds, Contarini and Povolo (2002) suggested that 2-heptanone can be used as a marker to detect conventional heat treatment applications. However, our study did not find any alteration in this compound concentration, making it unsuitable for detecting IR treatments. Regarding the results obtained, only two other compounds belonging to the class of ketones showed a statistically significant variation as a result of IR treatment; 1,1-dichloro-propanone, which was seen to increase following treatment at both application energies, and 6-methyl-2-heptanone, which, on the other hand, showed a significant increase only following the application of energy 85.

3.2.3 Sulphur compounds

This class of compounds is mainly associated with the application of intense heat treatments, such as UHT. Sulphur compounds are often produced due to the release of sulphydryl groups when whey proteins are denatured (Coolbear, Janin, Traill, & Shingleton, 2022). An increase in sulphur compounds results in noticeable "cooked" notes, which are generally not appreciated by consumers. In our study, we identified three different molecules: dimethyl sulphide, dimethyl disulphide and dimethyl sulphone. Dimethyl sulphide was the most prevalent compound among the sulphurs. At low concentrations (5-10 ng mL^{-1}), it gives the milk a pleasant flavour, but its increase, caused by heat, could be disagreeable (Badings & Jong, 1984). Our results showed a significant increase in this compound after exposure to IR radiation. Even though the concentrations detected were well above the odour threshold found in the literature (20 ng mL^{-1}), raw milk samples also exceeded this value (48.37 ng mL^{-1}), which could be due to the animal's diet (Al-Attabi, D'arcy, & Deeth, 2008; Coolbear et al., 2022). Another notable compound in this class is dimethyl disulphide. Its significance is supported by Al-Attabi et al. (2008), who provided an odour threshold value for its concentration in milk, set at 20 ng mL^{-1}. Our results showed concentrations significantly lower, with an average of 0.33 ng mL^{-1} for the IR 80 samples and 0.12 ng mL^{-1} for those treated at energy level 85. Notably, despite its reduced concentrations, dimethyl disulphide was detected only in milk samples subjected to IR treatment and was not found in raw milk. This could be interesting as its appearance could be related to phenomena associated with IR application. Dimethyl sulphone exhibits a trend of rise after IR, with energy 85 characterized by a significant increase of this compound; forgetting that it has little impact on flavour, the statistical increase revealed could represent a potential marker for elevated energy application (Burbank & Qian, 2005).

3.2.4 Carboxylic acids

The production of carboxylic acids serves as an indicator of lipolysis and is a significant contributor to off-flavour formation in milk (Ziyaina, Rasco, Coffey, Mattinson, & Sablani, 2019). Although five different compounds were detected, none exhibited significant alterations, rendering them unsuitable as potential markers.

3.2.5 Alcohols

Alcohols are generally considered less relevant due to their odourless nature and often originate from the reduction of respective aldehydes (Zhang et al., 2011). In our study, we identified six different alcohols, with 3-methyl-3-buten-1-ol, being particularly noteworthy. While present in low concentrations in raw milk, its levels significantly increased after IR treatment using both energy levels. This suggests its potential as a marker for detecting IR applications in milk. Similarly, 2-methyl-2-propanol, 1-hexanol, and 2-propanol exhibited a statistically significant increase following the highest energy level application, indicating their candidacy as markers for detecting high levels of IR treatment.

3.2.6 Other compounds

Three additional compounds were detected, among which 2-ethoxy-2-methyl-propane exhibited interesting behaviour. Despite a lack of literature on its impact on milk aroma, this compound demonstrated a statistically significant increase following IR radiation treatment. Therefore, it could be regarded as an influential marker for evaluating the application of IR radiation in milk.

4. Conclusion

In this study the metabolomic approach was carried out in both positive and negative ionization mode in separate analytical sessions, to obtain a higher reliable number of metabolites in a complex matrix as well as raw milk, compared with IR treated samples at two different energies 80 and 85, trying to identify some identification markers useful for official inspection controls to discriminate IR treatment. In particular, only in the positive ionization mode one up-regulated compound, corresponding to hypoxanthine, emerged in the comparison between both IR80 and IR85 vs raw milk. Moreover, in the positive ionization mode, 23 metabolites showed a statistically significant variation in the IR80 vs raw milk comparison and 14 metabolites in the IR85 vs raw milk comparison. In the negative ionization mode, however, 30 and 28 confirmed metabolites showed a statistically

significant difference in the group ratio IR80/raw milk and IR85/raw milk, respectively, while no up- or down-regulated compound were evidenced as other potential markers. The metabolomic analysis provided also some important information regarding the nutritional profile of IR treated milk samples, which are richer in some vitamins, amino acids and unsaturated fatty acids. Currently, hypoxanthine could be a potential marker from an inspection point of view but further omic studies (e.g. lipidomic) will be conducted to discover other potential markers on this regard, deepening the topic through a more complete framework. The results showed also that the application of IR radiation could lead to a change in volatile compounds profile, as it happens with conventional heat treatments. Of particular interest are compounds such as 2-propanone, 1,1-dichloro-propanone, dimethyl sulphide, 3-methyl-3-buten-1-ol and 2-ethoxy-2-methyl-propane which demonstrated a significant increase after treatment with both energy levels. For that reason, these compounds could be deeply investigated as markers for the identification of the application of IR radiation on milk. On the other hand, compounds like hexanal, 6-methyl-2-heptanone, 1-hexanol and 2-methyl-2-propanol only showed a significant change after treatment at energy 85. Making them potentially considered markers of IR application at high energies. Further studies can be useful to clarify the mechanism associated with candidate marker formation in relation to the influence of milk processing and their stability in the treated matrix.

CRediT authorship contribution statement

Maria Nobile: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing-original draft and Writing-review & editing. **Luca Maria Chiesa:** Resources, Supervision and Project administration. **Luigi Danesi:** Formal analysis, Data curation, Investigation and Writing-original draft. **Mauro Fontana:** Conceptualization. **Sergio Ghidini:** Writing-review & editing. **Roberto Edoardo Villa:** Supervision. **Sara Panseri:** Conceptualization, Investigation, Resources and Supervision.

Declarations of conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Fig. S1. Workflow used during Compound Discoverer processing.

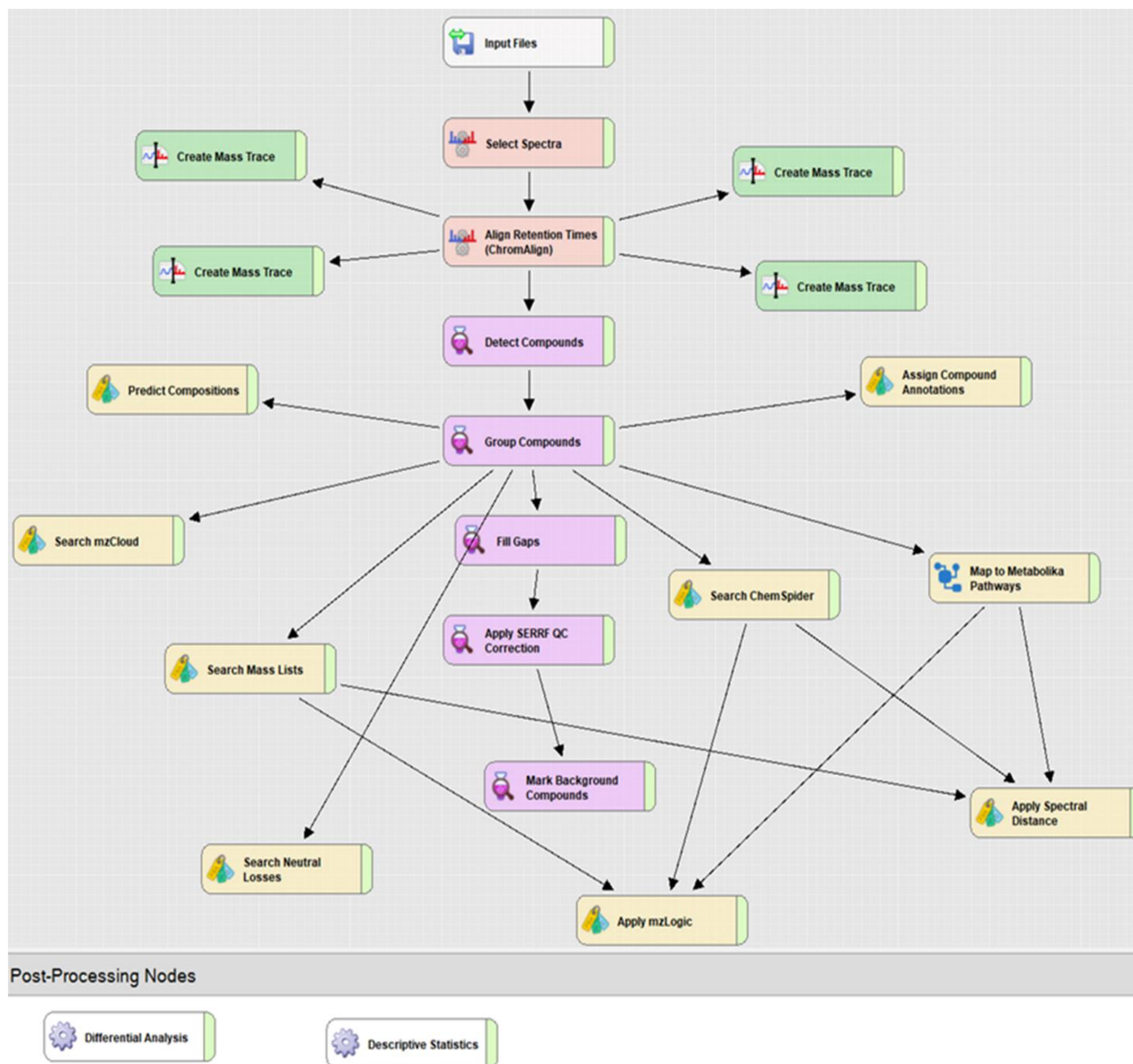


Fig. 1. Principal component analysis score plot representing 10 replicates from each of the 3 sample groups (Raw milk, IR80 and IR85).

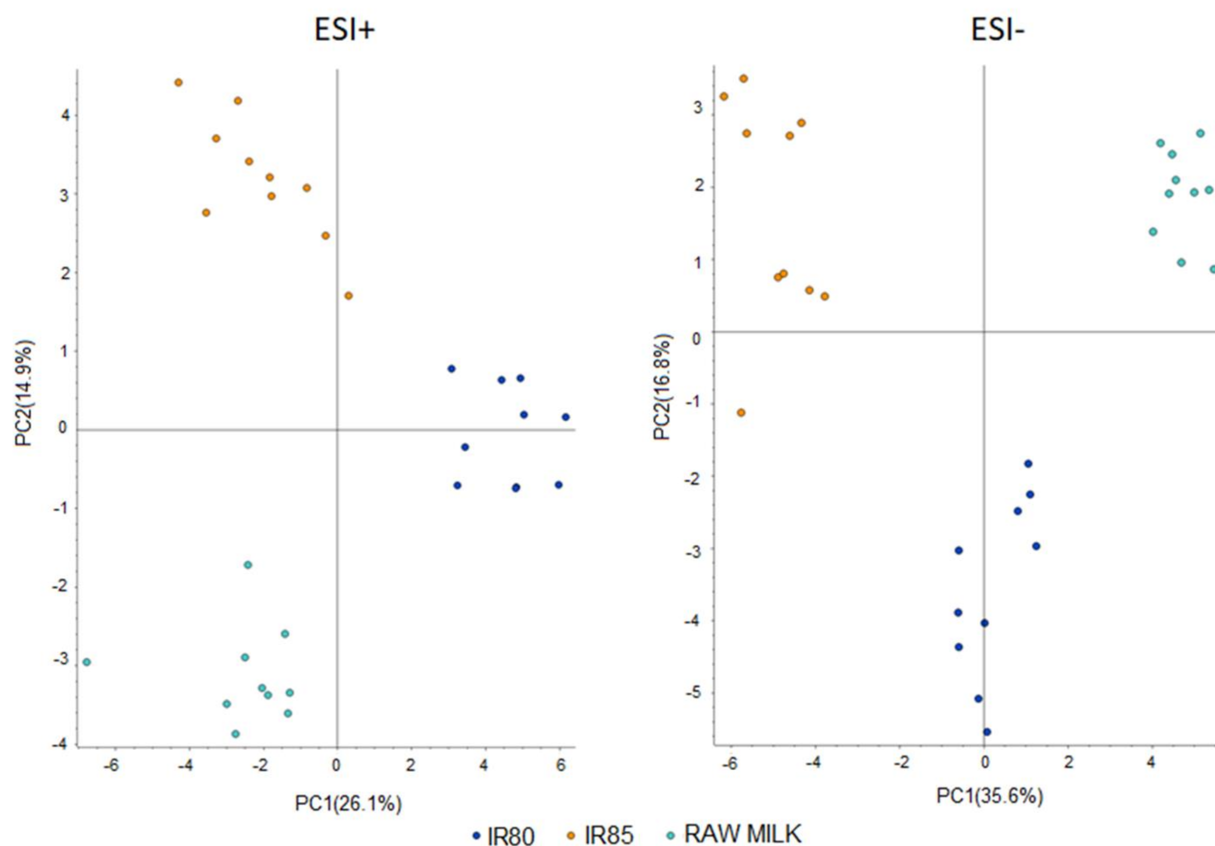


Fig. 2. Volcano Plot of metabolites detected in positive ionization mode for the group ratio IR80/raw milk (A) and IR85/raw milk (B); On the right sides the Box Whiskers Charts about hypoxanthine in the three different sample groups.

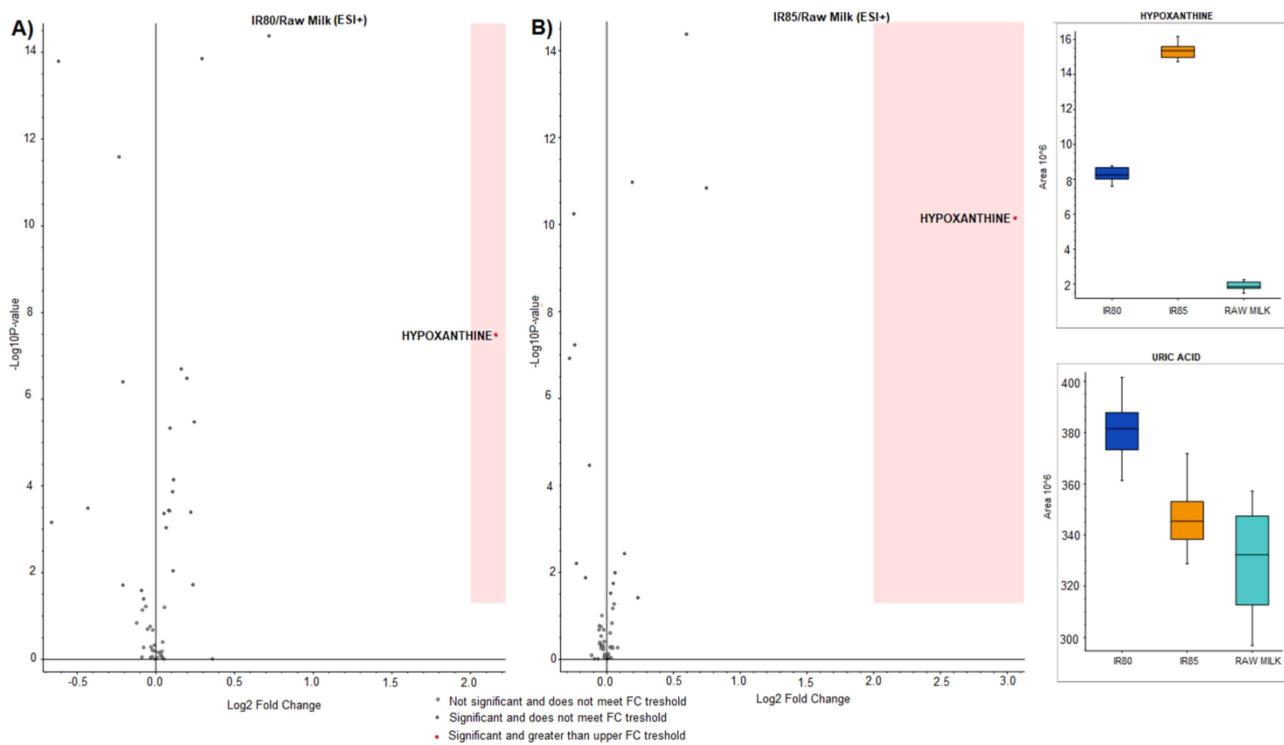
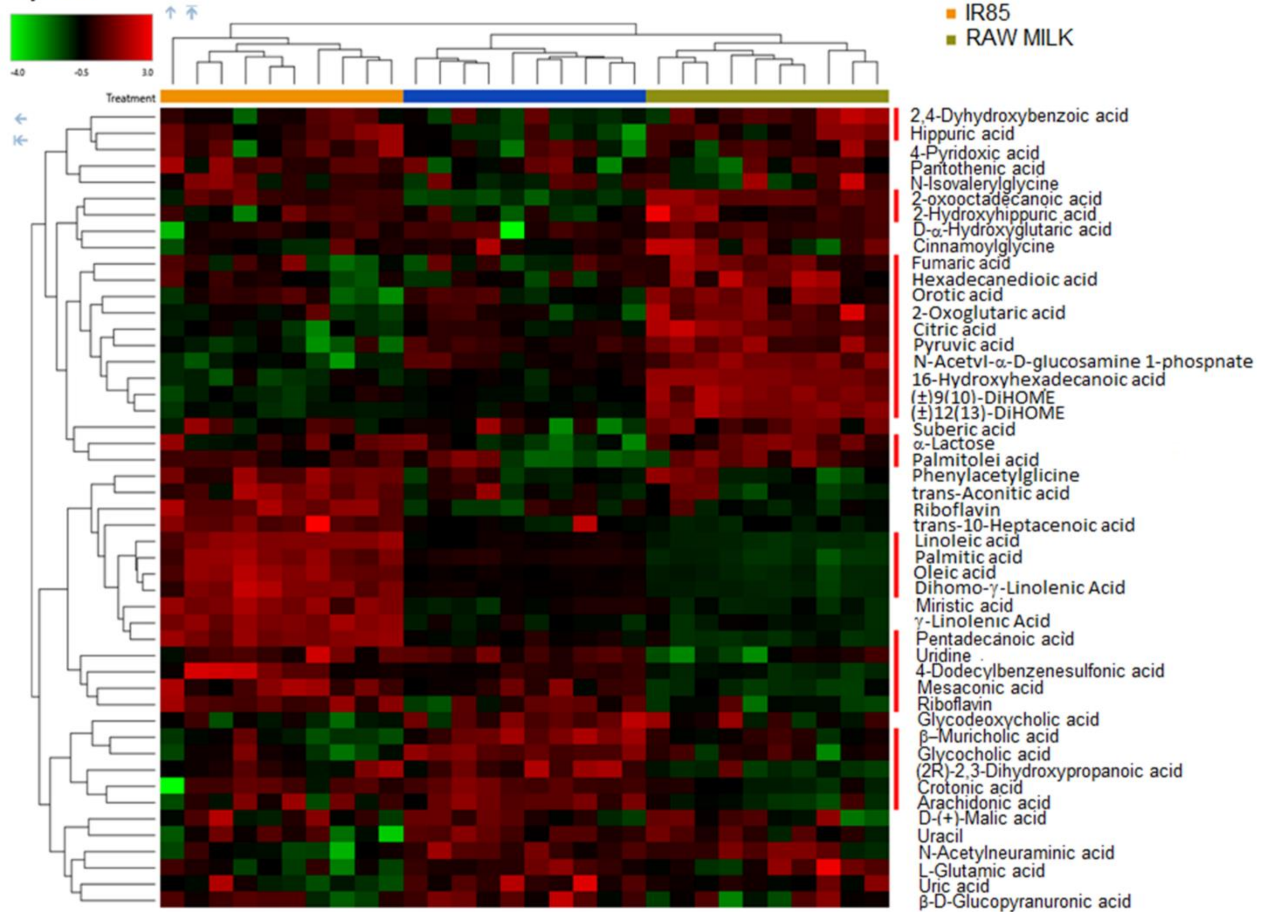


Fig. 3. Heat maps of A) ESI- and B) ESI+ mode related to the raw milk and IR treated milk samples (IR80 and IR85). Statistically significant compounds ($P < 0.05$) are highlighted with red hatching next to their respective names.

A) ESI-



B) ESI+

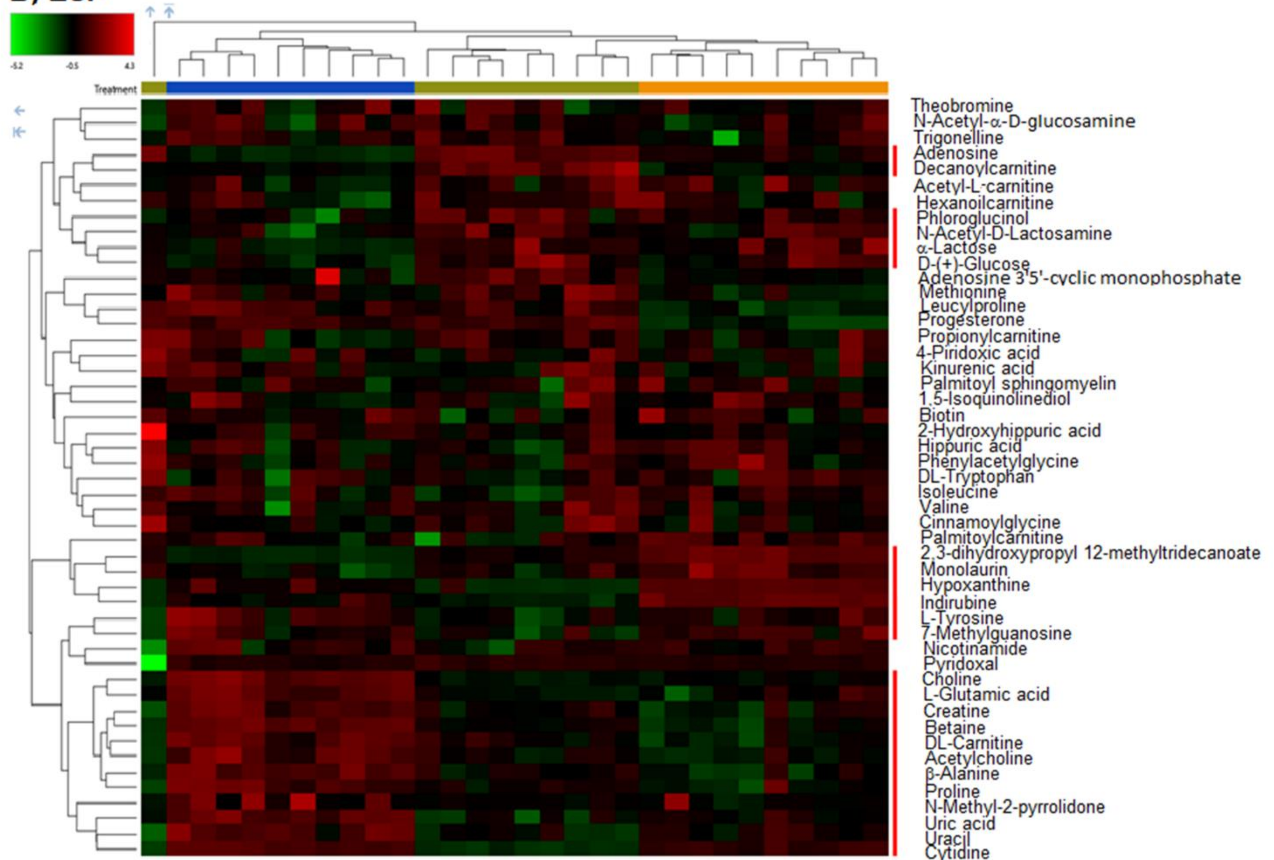


Fig. 4. Heat map of VOCs detected in Raw, IR80 and IR85 milk samples.