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Doctoral Thesis

ARTIFICIAL SENSES IN FEED ANALYSIS: BETWEEN RESEARCH AND LEGISLATION

(SSD AGR/18)

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When life gives you lemons,

make lemonade.

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Abstract

The ingredients used in animal feed are fundamentally important in terms of both the quality and safety of the resulting food products and for the potential human health impact associated with the animal-based food production chain (Sapkota et al., 2007). Feed analysis is an important topic in animal nutritional research and as a control: evaluation of feed quality and safety require a high number of analyses to be performed. Considering that feed science has progressively evolved, prompted by different factors such as the need to obtain validated and standardized methods of analysis, the analysis on feed should be a multi-analytical approach, in accordance with screening work conducted at different levels. For this reason new analytical laboratory instruments have been developed. Requirements for new analytical methods emphasize performance, sensitivity, reliability, speed, simplified use, low cost for high volume, and routine assays.

The starting point of my PhD was a thorough review of the literature to trace the state of the art in, *in vivo* and *in vitro* models, review analytical methods used, and to critically analyze the advantages and disadvantages of different models and methodological approaches, from "wet chemistry" to modern analytical techniques and to *in vitro* approaches.

This analysis showed clearly how the analytical approach is essential to evaluate feed and how farmers, researchers, industry and governments have been forced to give serious attention to animal feedstuff production process. New methods for evaluation of feed composition and safety have been developed.

My PhD project focused on feed analysis using new analytical methods (image analysis and electronic nose), to ensure quality and safety of feed and animal origin products for human consumption. Aspects of quantification of quality and safety in real time with the objective of an instrumental response were obtained using techniques based on use of the senses, such as vision and smell, which represent rapid methods for screening and quality control feed.

The research aim was to evaluate quality and safety of feed and pet food by the development and application of a multi analytical approach, as reported in the diagram below (Figure 1).

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Review

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State of the Art in Feedstuff Analysis: A Technique-Oriented Perspective

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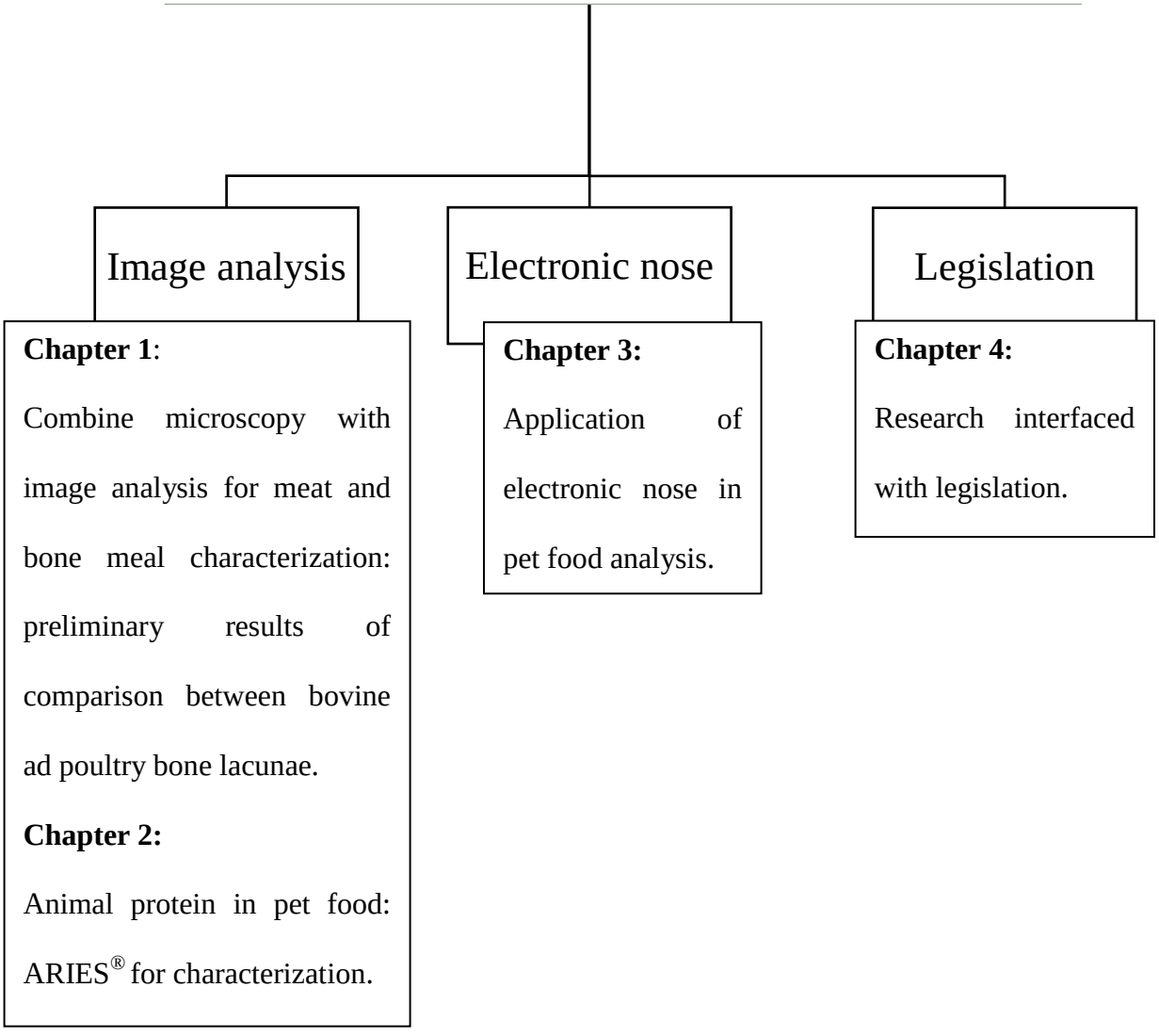

Figure 1: Multi analytical approaches for evaluating of quality and safety in feed and pet food.

In conclusion, the application of artificial senses in feed analysis can be considered an example of how science and engineering work effectively together. The image analysis and electronic nose represents new tools for rapid screening and quality control and as a support for decision making in the area of product quality. Chemometric tools are required for efficiently extracting qualitative or structural information from the wide volume of data collected. Image analysis allows, both in feed and pet food, the identification and characterization of products of animal origin (PAPs) so that morphometric descriptions of bone fragments can be used as possible markers in routine analysis. Electronic nose allows evaluation of the odour profile of pet foods, representing a promising and powerful tool able to provide immediate and satisfactory answers in complex matrices as pet food. Obviously, a legislative approach is an important issue to consider in a worldwide discussion regarding quality and safety of feed.

A study was carried out to address some aspects concerning feed and food-related issues, providing an update of the current EU Regulation and Directives. To give the reader a rapid first approach to the topic of his interest, a synoptic presentation of all law related to the above-mentioned topics is given, along with the main points of each law, cited in conjunction with its effect on previous laws (replacement, modification, amendments, and main related acts). Results were used to create a database to manage the consolidation and updating of the legislative texts.

Introduction

Animal nutrition in the 21st century aims to provide safe and good quality foodstuffs of animal origin so that they can better meet the requirements of human nutrition. The ingredients used in animal feed are fundamentally important in terms of both the quality of the resulting food products and the potential human health impacts associated with the animal-based food-production chain (Sapkota et al., 2007). In past years, feed science has progressively evolved, prompted by different factors such as the improved safety issues and relevant changes in the European Union agricultural policy (http://ec.europa.eu/agriculture/index_en.htm). Therefore farmers, researchers, industry and governments have been forced to pay serious attention to animal feedstuff production processes, thereby acknowledging that animal feed safety is an essential prerequisite for human food safety (Cheli et al., 2013). Feed sampling and analysis is an extremely important topic in animal nutrition research.

According to the European Feed Manufacturers' Federation (FEFAC, 2014) the production in 2013 of compound feed (complete and complementary) amounted to 155 million tonnes, while globally, according to data from Global Feed Tonnage Survey in 2013, there had been an increase of 1%, reaching 963 million tonnes. With globalization, the increase of feed production and global trade, new analytical methods for evaluation of feed quality, safety and functional features, were developed. Feed analysis in this context, with respect to animal nutritional requirements, health, reproduction and production, should be a multi-analytical approach, according to screening work conducted at different levels. Cheli, Battaglia, Pinotti and Baldi, (2012) report the state of the art on feedstuff analysis, considering advantages and

disadvantages of each method. This review attempts to bridge gaps within analytical methods in a multi-analytical approach to feed analysis, providing an overview of the most used and promising methods for feed composition, safety and functional properties evaluation. Although many classical methods are still widely used today and are officially recognized [European Commission, 2009. Commission Regulation (EC) No.152/2009], the first analytical techniques (Van Soest, 1963; Giger-Riverdin et al., 1994), have been eventually substituted by instrumental methods that provide lowered detection limits, increase analyte specificity, simplify use, reduce cost, and display higher sample throughput and automation capabilities. In past years, analytical methods have progressively evolved and the requirements for new analytical laboratory instruments emphasize performance, sensitivity, reliability, speed and simplified use, rapidity, and low cost for high-volume of routine analytical assays (Cheli et al., 2012).

In the early 20th century all feed analyses, that provided an exact description of the chemical composition of a feed, were performed using "wet chemistry". These methods do not give a complete estimate of feed nutritional value, which could be inferred by statistical association, and therefore different prediction equations based on Weende and Van Soest chemical analysis were proposed (Giger-Riverdin et al., 1994). Palatability, the impact of diet composition on feed intake and digestibility, or the feed functional properties, represent the building blocks for high quality of feed. Therefore, *in vivo* and *in vitro* feed evaluation techniques were developed. *In vivo* measurements may evaluate the animal response to a dietary treatment. The trials must be conducted under highly controlled experimental conditions and cannot be carried out for all possible feeding situations found in practice (Cheli et al., 2012). For example, for estimating digestibility and degradability of feedstuffs, taking into account the dynamic aspects of digestion

(such as the transit time and the digestibility kinetics of dietary constituents) several *in situ* and *in vitro* methods were developed (Huntington and Givens, 1995; Getachew et al., 1998; Ørskov, 2000 and Mold, 2003). Tilley and Terry, (1963) method is the original *in vitro* technique for the evaluation of ruminant. The results obtained with this method were extensively validated with *in vivo* results (Van Soest, 1994).

The techniques previously reported, are destructive, slow, relatively expensive, and time-consuming. Several noninvasive and nondestructive instrumental techniques have been developed, which represent, new analytical methods for the determination of feed composition, quality, and safety. New methods of analysis of feed have been developed from new analytical techniques married with chemometric tools (powerful analytical devices and data processing software) (Cheli et al., 2012). New analytical approaches are advantageous for many applications, as tools at-line and on-/in-line process control in processing and distribution of feed and feed products. With new analytical methods a large number of samples can be analyzed in short time, they are not nondestructive and can provide multiparametric measurements. The results obtained are multivariate data matrices and require the use of chemometric: this is the use of mathematical and statistical techniques for efficiently extracting quantitative, qualitative, or structural information from the data, through the analysis of data and the validation of the calibration curves. Cheli et al., (2012) reported that the selection of a training and a test dataset, although sometimes a third "tuning" set may be used, the discriminating variable selection, the use of classification and regression methods, and the validation of the models are the main steps for a qualitative and quantitative application in the field of feed analysis (Figure 1).

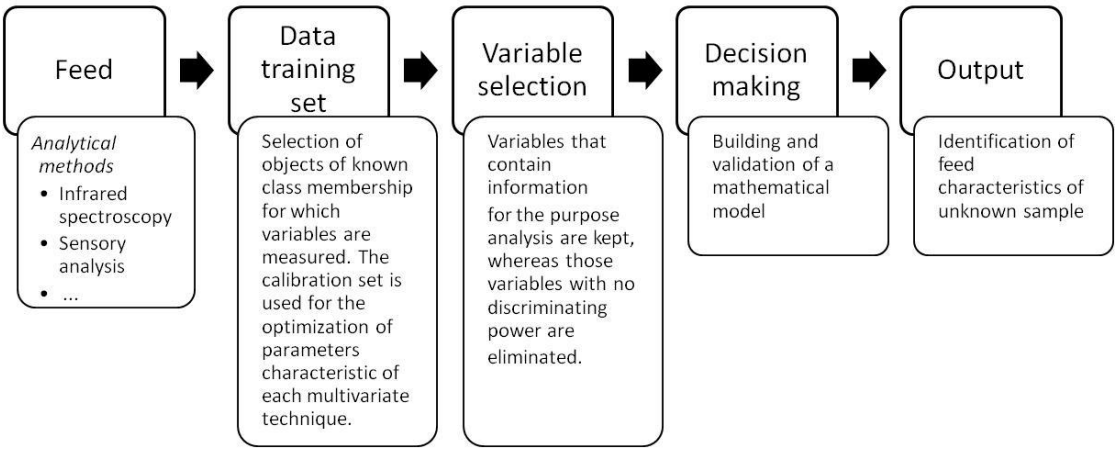

Figure 1: Analytical techniques coupled with chemometric tools: diagram of the procedure for feed analysis (Cheli et al., 2012).

Examples of new analytical methods coupled with chemometric tools are near-infrared (NIR) spectroscopy and sensor analysis.

Near-infrared (NIR) spectroscopy, is used for the analysis of feedstuffs to replace the "wet chemistry" techniques. It is the only technique that allows the analysis of large-scale samples and consistently makes decisions in real time (Roberts et al., 2004): it is the analytical technique, which most applies chemometrics (Cheli et al., 2012). It is routinely used in the feed industry as a quality assurance tool to determine feedstuff composition (De la Haba et al., 2007). NIR spectroscopy can give rapid answers to evaluate the composition of raw material and compound feedstuffs (Pérez-Marín et al. 2004), and nutritional value of compound feedstuffs (Verheggen et al., 1990; Valdes et al., 1992; De Boever et al., 1995; Aufrère et al., 1996; Berzaghi et al., 2000; Xiccato et al., 2003; Pérez-Marín et al., 2004). In addition to combined spectroscopy with microscopy technique (NIRM) as an alternative technology to detect and quantify banned ingredients in feedstuffs (De la Haba et al., 2007; de la Rosa-Delgado et al.,

2007; Pérez-Marín et al., 2009; Fernández-Ibáñez et al., 2010; Pavino et al., 2010) it can predict digestibility and voluntary intake of feedstuffs and forages. NIR calibrations were developed for real time prediction of the species composition of constituents of animal origin (De la Haba et al., 2009). For the evaluation of undesirable substances in feed and food, in screening control procedure, the development of fast, nondestructive and applicable methods is essential. Commission Regulation (EC) No. 1881/2006 (European Commission, 2006b) sets maximum limits, according to different foodstuffs: nitrate, mycotoxins, metals, dioxins and PCBs, polycyclic aromatic hydrocarbons, melamine and its structural analogues. For mycotoxins, DON, ZEA, OTA, fumonisins, guidance values were set in Commission Recommendation 2006/576/EC for maximum levels of undesirable substance content in feed and food. De Girolamo et al., (2009) report that FT-NIR (NIR and mid-infrared spectroscopy with attenuated total reflection) analysis may be suitable for the determination of dexoynivalenol (DON) in unprocessed wheat at levels far below the DON maximum permitted limits setfor feed and food, by the Commission Recommendation (EC) No. 2006/576/EC (European Commission, 2006a) and Commission Regulation (EC) No. 1881/2006 (European Commission, 2006b). Also Fernández-Ibáñez et al., (2009) highlighted the potential of NIRS methodology as a fast and nondestructive tool for the detection of AFB1. The authors found that NIR spectroscopy was successfully correlated with traditional quality methods commonly used to detect aflatoxin B1 (AFB1) in maize and barley. NIR spectroscopy is a powerful tool in the feed industry and on farms, regarding quality and safety control programs (Cheli et al., 2012). In the future remains the development of quantitative methods for ensuring compliance, with legal limits and indications of European Commission Regulation, improving the robustness of calibration curves.

Another new analytic method that can provide rapid, nondestructive, and particularly multi-parametric measurements is the sensory analysis. Currently the sensory technology for several applications is used in the feed and food industries. Several types of sensors are in commerce and all are composed of a sensing element "recognizing" the analyte and an analytical signal converter, which transforms a characteristic parameter of a chemical or biochemical reaction to a physical parameter (Figure 2).

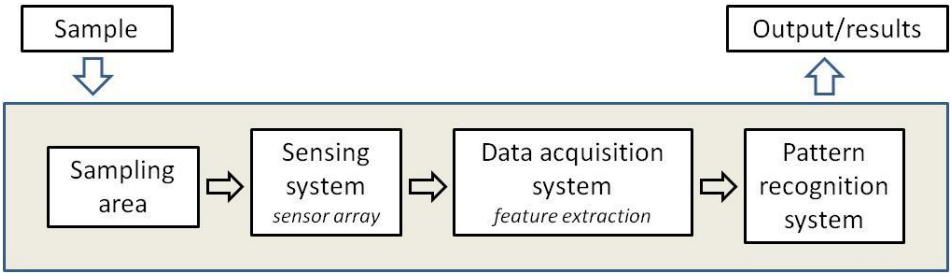

Figure 2: The general configuration of the sensor arrays technology (Cheli et al., 2012).

A huge variety of sensor devices were developed for food analysis (Table 1). Their characteristics, properties and specific use were reviewed by, Deisingh et al., (2004) and Van Dorst et al., (2010).

Table 1: Main sensor devices in feed/food analysis (Cheli et al., 2012)

Category	Sensing material	Examples of applications in feed/food analysis
Metal oxide semiconductors (MOS)	Metal oxide semiconducting film (metal coating may be zincoxide, tin dioxide, titanium dioxide, iron (III) oxide, nickel oxide or cobalt oxide)	Classification, authentication and recognition of feed/food VOC[a] based profiling for microbial and mould spoilage Feed/food quality control
Conducting polymer sensors	Polyaniline, polypyrrole and polythiophene	VOC[a] based profiling for feed/food spoilage Packaging smell Recognition of taste substances Feed/food quality control
Acoustic wave sensors	Chromatographic stationary phases and polymers $LiTaO_3$ substrate without chemical coating	VOC detection Recognition of taste substances Feed/food control
MOSFET/ISFET sensors[b]	Catalytic metal gate (covered with Pd, Pt, Rh)/gate covered by sensitive layer (plasticized polymers doped by ionophores)	Classification, authentication and recognition of feed/food VOC[a] based profiling for feed/food spoilage Food quality control
Optical	Fluorescent dyes, metalloporphyrines	VOC[a]-based metabolic profiling for feed/food spoilage

Potenziometric sensors	Plasticized organic polymers modified by ionophores Noble metals	Taste assessment Discrimination, classification and authentication of liquid food
Voltammetric sensors	Different type of metals for the working electrodes. Electrodes chemically modified with electroactive substances	Taste assessment Discrimination, classification and authentication of liquid food
Biosensors	Biological or biologically derived sensing element (such as an enzyme, antibody, microbe or DNA)	Detection of pathogens and toxic metabolites Routine analytical measurement of vitamins of drug residues

[a] Volatile Organic Compounds. [b] Metal Oxide Semiconductor Field-Effect Transistor/Ion Sensitive Field-Effect Transistor.

A subdivision of the sensor grouping is the biosensor. The biosensor has a biological sensing element positioned close to the transducer to give a reagent less sensing system for a target analyte (Hall et al., 1990). As NIRS, the sensor analysis uses advanced mathematical procedures for signal processing, based on pattern recognition and/or multivariate analysis, so as to approach a complex problem in a one-step analysis, with easy or no sample preparation. The tools for rapid and nondestructive analysis of feed for quality evaluation purposes, are increasingly used. The application of an array of non specific or low selective sensors in feed and food analysis is the base of the "multisensor system" electronic nose and tongue (ENT), used for the analysis of gases and liquids, respectively (Figure 3).

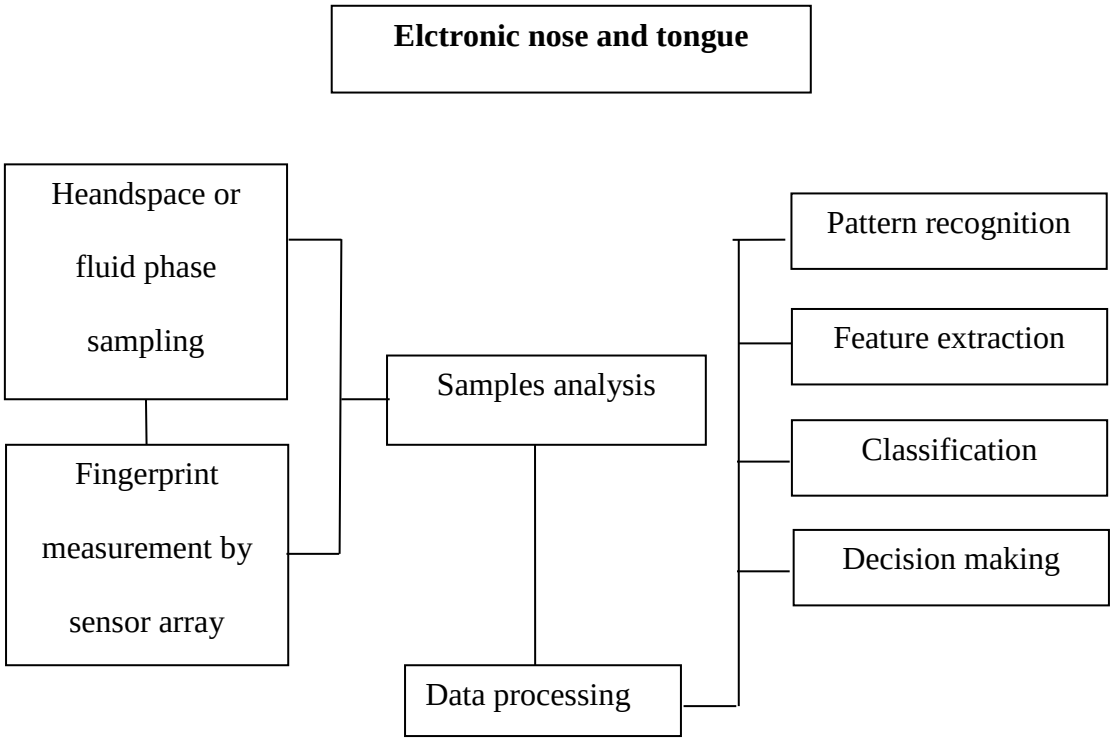

Figure 3: The general configuration of the Electronic nose and tongue (Cheli et al., 2007).

The ETN is capable of identifying simple or complex taste and aromatic profiles responsible for the quality of a given product (Gardner et al., 1994; Legin et al., 2002). As reported in Cheli et al., (2012) quality is a key factor for the modern feed industry because the high quality of a product is the basis for success in today's highly competitive market. EN is an excellent candidate for process monitoring, freshness evaluation, shelf life investigation, sensory and authenticity assessment, microbial contamination diagnosis in food and beverage industry, because these instruments replicate the human olfactory system (Deisingh et al., 2004; Peris et al., 2009; Berna, 2010; Concina et al., 2012). Recent studies indicate that EN technology can be used as a screening method for simple and rapid detection not only for fungal contamination, but also for mycotoxin presence in feed and food (Cheli et al., 2009; Campagnoli et al.,

2009 Campagnoli and Dell'Orto, 2013). A further application of electronic nose in the feed industry and an important issue in food safety comes from the identification of products of animal origin, generally known as processed animal proteins or PAP, in feedstuffs (Campagnoli et al., 2004). In addition, recent results suggest that the EN may have a potential application in the evaluation of fat quality in pet food. In conclusion, like the NIR, the electronic sense technology is also a powerful tool in the feed industry and possibly on farms with regard to quality and safety control programs. The literature reports a huge variety of sensor devices developed for food and feed analysis (Deisingh et al., 2004; Van Dorst et al., 2010).

Another technique that is based on the use of artificial senses is image analysis. Image analysis is a tool used for rapid screening and quality control in terms of composition and safety (Tognon et al., 2005; Cheli et al., 2007; Dell'Orto et al., 2007; Pinotti et al., 2013). EN and image analysis are the most successful and most advanced methods in the food industry. Today, image processing and image analysis are recognized as being at the core of computer vision (Figure 4).

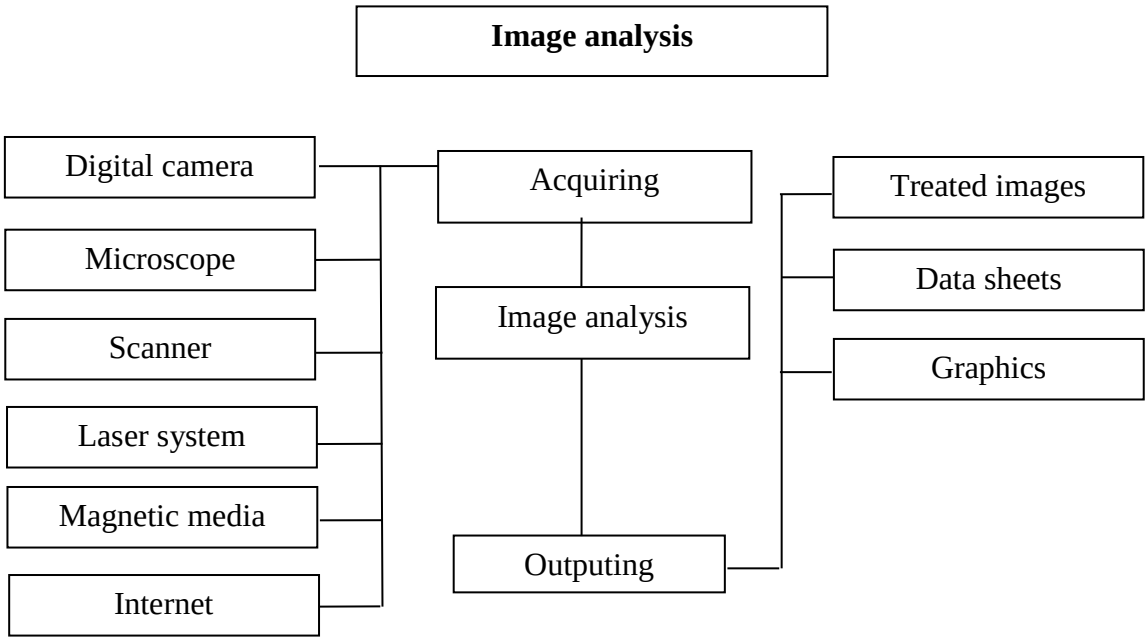

Figure 4: The general configuration of Image analysis (Cheli et al., 2007).

Image analysis requires the acquisition of an image of the object, which can be performed by different equipment such as cameras, scanners, microscopes and laser systems. The optical image is converted by digitization into a digital format (numerical form) and further processed in order to assess morphological, geometric and chromatic variables and produce quantitative information, which are used in the subsequent control system for decision-making. As the EN, chemometric tools carry out the analysis of image analysis data. Computer vision systems have been investigated for many applications involving grains and oilseeds and may have a wide application in the field of animal nutrition, feeding and health.

Future change for characterization of feed quality and safety will be a more collective use of sensors to obtain a multisensor data fusion. The literature already reports

examples of electronic nose/machine vision and electronic nose/ electronic tongue combinations, with improved prediction properties for both qualitative and quantitative analyses (Campagnoli et al., 2004, 2006; Oladipupu et al., 2011; Éles et al., 2013).

The "artificial senses" can be considered a great example of how science and engineering work together to produce something of real utility (Cheli et al., 2007). These "fit-to-purpose" analytical methods are rapid, user-friendly, adaptable, and coherent with the precision and accuracy level required for feed/food chain control and regulatory purposes, and useful for decision-making in the area of product quality.

In conclusion, the high-throughput analytical testing demands in the field of feed research, industry, and regulation indicate the need to move from the classical chemical compound approach to a multi-analytical and holistic approach (Cheli et al., 2012). If the global approach for feed evaluation is chosen, the future will see the increasing development of the analytical solutions marrying powerful analytical devices with data processing software.

Increasing interest in available feed and food supply worldwide has stimulated concerns over safety and quality issues, and given rise to legislation on traceability, control and labeling in order to prevent food crises in the future. Since the adoption of Commission Regulation (EC) No 178/2002 (European Commission, 2002), and establishing the EFSA, research must adhere to the legislation. As a result, researchers, farmers and industry, must consider the methods of sampling and new methods analysis for official control of feed (quality and safety in real time), reported in several EU Regulations.

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Chapter 1

Combine microscopy with image analysis for meat and bone meals characterization: preliminary results of comparison between bovine and poultry bone lacunae.

Introduction

The official term for meat and bone meals (MBM) and other animal by-products is processed animal proteins (PAPs): they are of animal origin and are produced mainly in the form of ground processed (rendered) slaughter by-products originating essentially from ruminants, pigs, poultry and fish (Hormisch, 2004). They are suitable for direct use as feedstuff or as an ingredient in feedstuff for animals (Gizzi et al., 2003). In the history of development of feeds with a high nutritional value, materials of animal origin were considered appropriate as ingredients in compound feeds (van Raamsdonk et al., 2007). As reported by van Raamsdonk et al., (2007), animal by products can be readily compared to soy bean hulls, but provide a higher amount of fat as energy source, higher levels of protein and minerals (Ca and P), and supply some essential vitamins. The meat and bone meals were the protein sources most used and least expensive, in the last decades of 1900s until the outbreak of bovine spongiform encephalopathy (BSE), commonly known as “mad cow” disease. The BSE disease belongs to the group of transmissible spongiform encephalopathies (TSE). It is a chronic, degenerative disorder affecting the central nervous system of cattle. Strong epidemiologic and laboratory evidence indicates that a new variant of Creutzfeld-Jakob disease (vCJD) caused by

BSE may be transmitted to humans by contaminated products with BSE agent (Bruce et al., 1997; Scott et al., 1999). The disease is fatal for humans and there is no known treatment or cure.

On 1 January 2001, through the Commission Regulation (EC) No. 999/2001 (European Commission, 2001), the use of PAPs in feed for any animals farmed for the production of food was suspended in the European Union, except for fish meal for non-ruminants. The European Union legislation does not make a distinction between different terrestrial animals and therefore only the presence of bone particles is currently sufficient to reach a positive conclusion (Liu et al., 2011). After 12 years, a new European Commission Regulation, the Commission Regulation (EU) No. 56/2013 (European Commission, 2013b), reformed the stringent rules on the use of PAPs from non-ruminants (fish, pigs and poultry) in feed or as feed ingredients in aquaculture, while also avoiding cannibalism. The specie used for pap productions, no may be used as feed in livestock if they are from same species, due to limitations in test methods that do not adequately differentiate between pig and poultry PAP. As a result, non-ruminant PAP cannot be used as feed for non-ruminants for fear that pig PAP are fed to pigs, or poultry PAP to chickens. This Regulation amends the Commission Regulation (EC) No. 999/2001 (European Commission, 2001) on transmissible spongiform encephalopathies (TSEs), TSE Regulation. Several analytical methods were described for the detection and identification of animal constituents in feed. The more important are: enzyme-linked immunosorbent assay (ELISA) (Hofmann, 1997), near infrared spectroscopy (NIRS) (Pérez-Marin et al., 2004), polymerase chain reaction (PCR) and Real-Time PCR (Myers et al., 2001; Gizzi et al., 2003; Mendoza-Romero et al., 2004) and microscopic method (Pinotti et al., 2004). The microscopic analysis is the official method for the

detection of PAPs in compound feeds or in their ingredients [Commission Directive 2003/126/EC, (European Commission, 2003)]. Briefly the MBM characterization with microscopy requires a sample processing to obtain a sediment. The methods are described in detail in Annex VI of the Commission Regulation No. 152/2009 (European Commission, 2009) and STRATFEED (van Raamsdonk et al., 2005; Liu et al., 2011) and in the literature (Makowski et al., 2011; Charoud-Go et al., 2012; Vermeulen et al., 2012; van Raamsdonk et al., 2012a, 2012b). These methods distinguish between constituents derived from terrestrial animals and those derived from fish, but are unable to quantify with sufficient accuracy the amount of animal constituents present in feed, and therefore should not be used for this purpose.

The implementation of the Commission Regulation (EU) No. 56/2013 (European Commission, 2013b), requires additional analytical methods for its enforcement. For this purpose, the methods of sampling and analysis for the official control of feed have been revised in Annex VI of the Commission Regulation (EU) No. 51/2013 (European Commission, 2013a) which amended Commission Regulation (EC) No. 152/2009 (European Commission, 2009). In this Regulation, besides the microscopy protocol, the polymerase chain reaction (PCR) was added as the official method for detecting the species origin of PAPs. The detection of PAPs using PCR has been extensively investigated (Prado et al., 2007; Fumière et al., 2009, 2012; Pegels et al., 2013). A combined approach of PCR and microscopy methods may be useful to obtain information on the origin of PAPs at species level. Neither of the methods (microscopy and PCR) independently meet all the requirements for the accurate identification of prohibited ingredients of animal origin (i.e. for the control of the correct implementation of the feeding prohibitions, differentiating between authorized and prohibited

ingredients). Therefore, a combined approach in which both methods can be used, implemented and eventually merged has been proposed (Veyes et al., 2012). Moreover, Pinotti et al., (2013), demonstrated that the use of microscopic methods in association with computer image analysis to identify the source of the PAPs may have promising application, particularly in class origin discrimination, which remains one of the main difficulties of the official methods. The computer image analysis procedure consists of a sequence of steps (Pinotti, 2009; Cheli et al., 2012) with the aim of capturing the important structural features of a digital image on which morphometric measurements can be performed (extract numeric descriptors) (Pinotti et al., 2013). Several authors as Gizzi et al., (2003) and Liu et al., (2011) have reported that the difference between poultry and mammalian meal is more difficult to detect and there are overlaps in the range of characteristics. In relation to this topic several studies, (Pinotti et al., 2004, 2007, 2013; Pinotti 2009) indicated the potential use of microscopic methods in association with computer image analysis for identifying and distinguishing between poultry and mammalian particles in feedstuff.

Aim

A study to evaluate the potential of image analysis measurements, in combination with the microscopic method, for the detection of constituents of animal origin in feedstuffs was carried out. The aim of the study was to identify morphometric descriptors of bone fragments as possible markers that can be used in routine analysis to distinguish between poultry and bovine lacunae.

Material and methods

Ten feed samples contained 0.5% pure bovine meal and twelve samples contained 0.5% pure poultry meal were processed to obtain sediments, according to Annex VI of Commission Regulation 152/2009 (European Commission, 2009) (Figure 1).

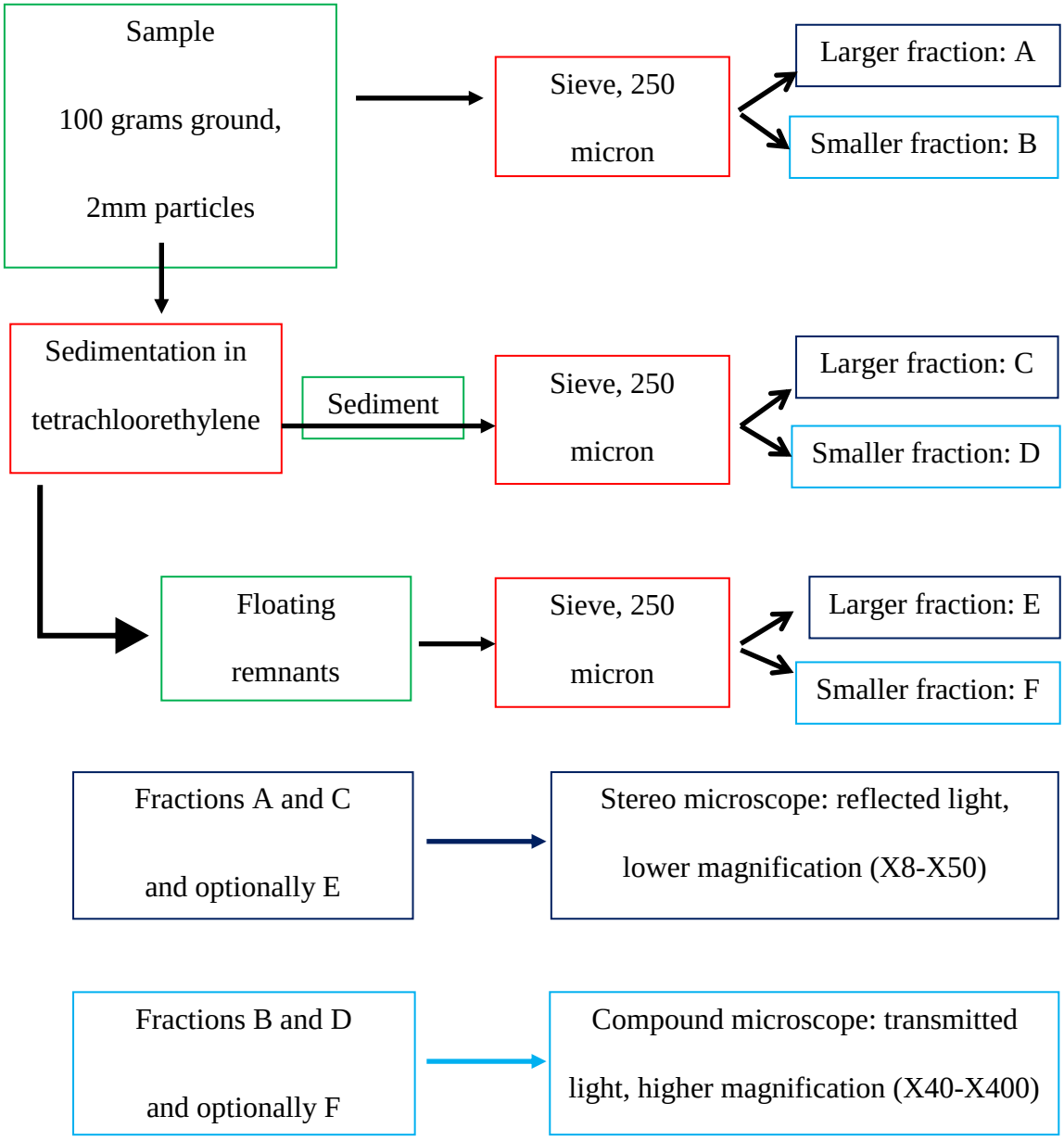

Figure 1: Main steps of the official microscope procedure.

In order to obtain several bone fragment lacunae images a X40 for each sample the sediment fractions of each were viewed under a compound microscope (Olympus BX41; Tokyo, Japan) at several magnifications.

Briefly, using a digital camera (Cool snap-Procf Roper Scientific Photometrics) and image analysis software (Image Pro Plus® 5.4.1, Media Cybernetics Inc. Rockville, MD,

USA) 430 lacunae (215 from bovine and 215 from poultry) were obtained, processed and elaborated at X40 (Figure 2; Figure 3; Figure 4).

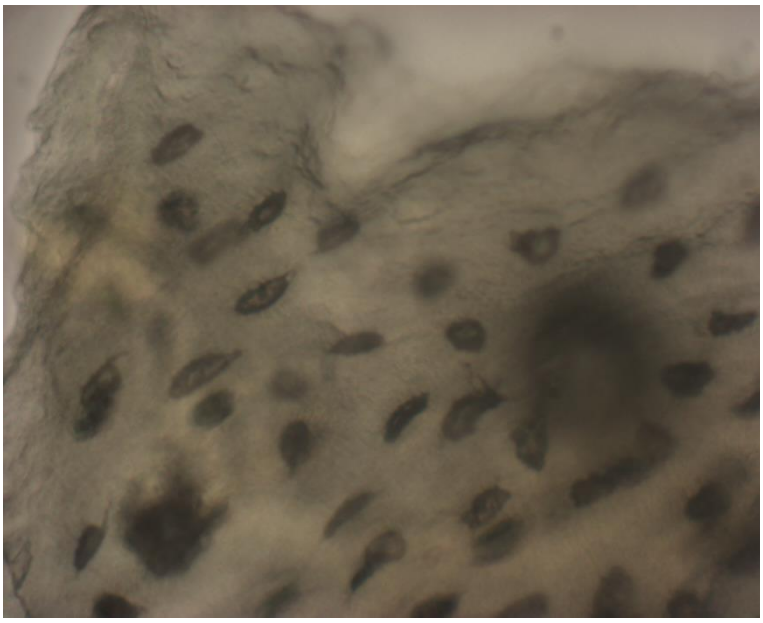

Figure 2: Example of poultry bone fragment at X40 magnification.

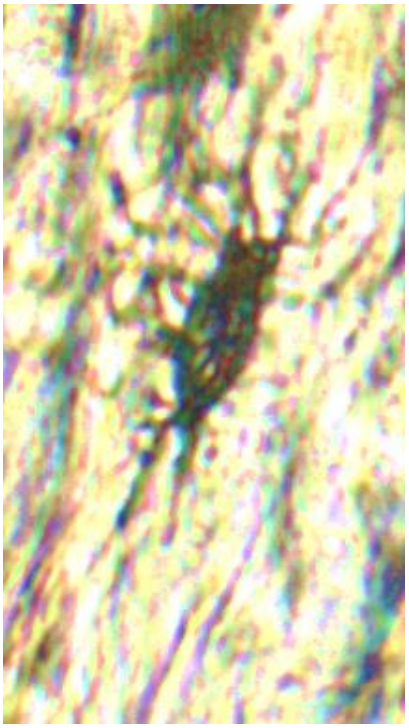

Figure 3: Example of detail of poultry bone fragment at X40 magnification.

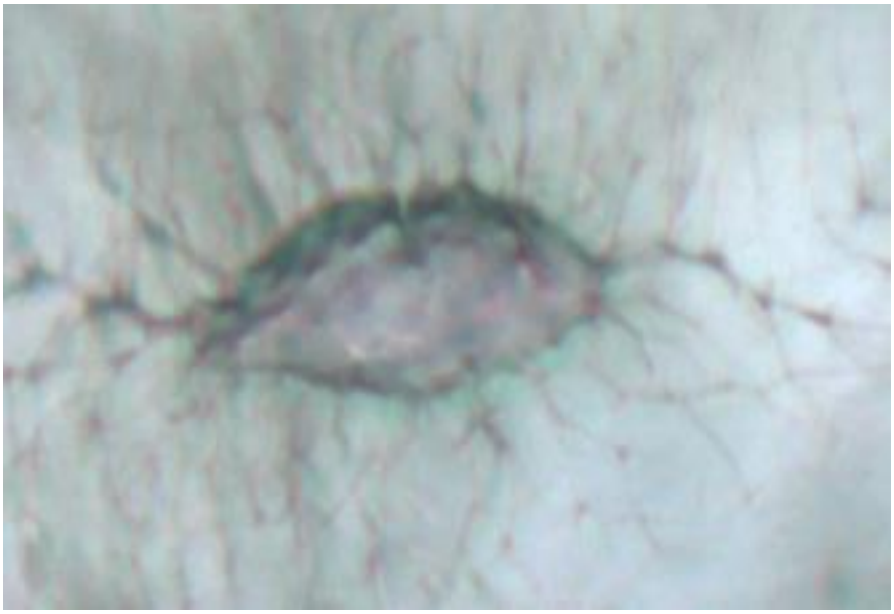

Figure 4: Example of detail of bovine bone fragment at X40 magnification.

Images were obtained according to Pinotti (2009) and Pinotti et al., (2013). General descriptions of lacunae obtained from bone of different mammalian, fish and avian origin, were reported in Gizzi et al., (2003); van Raamsdonk et al., (2005); Liu et al., (2011).

When an optimum image contrast was not available and could not be thresholded automatically, it was necessary to use a pen tablet (Intuos®3 Wacom, USA). The key step in the automatic and manual methods are illustrated in Figure 5.

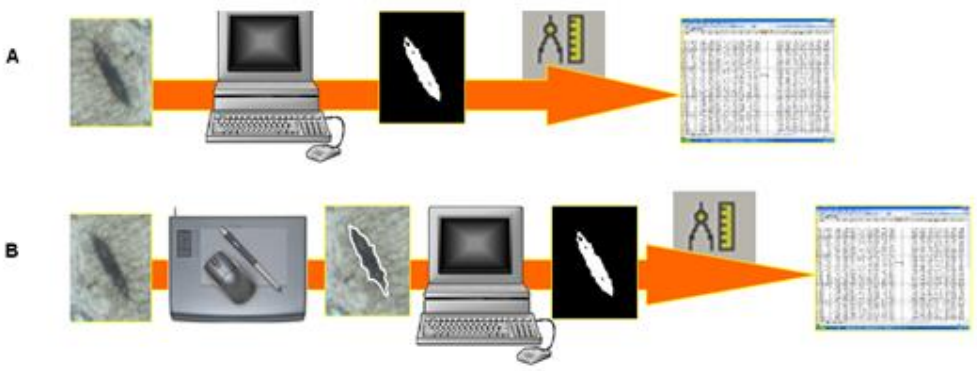

Figure 5: Key steps in (A) automatic image thresholding and (B) pen tablet definition of lacuna in the process of image analysis.

The images were processed in order to obtain, for each lacunae, a monochrome mask. On this 30 geometric variables were measured. The list and the description of all the 30 variable descriptors are reported in Figure 6.

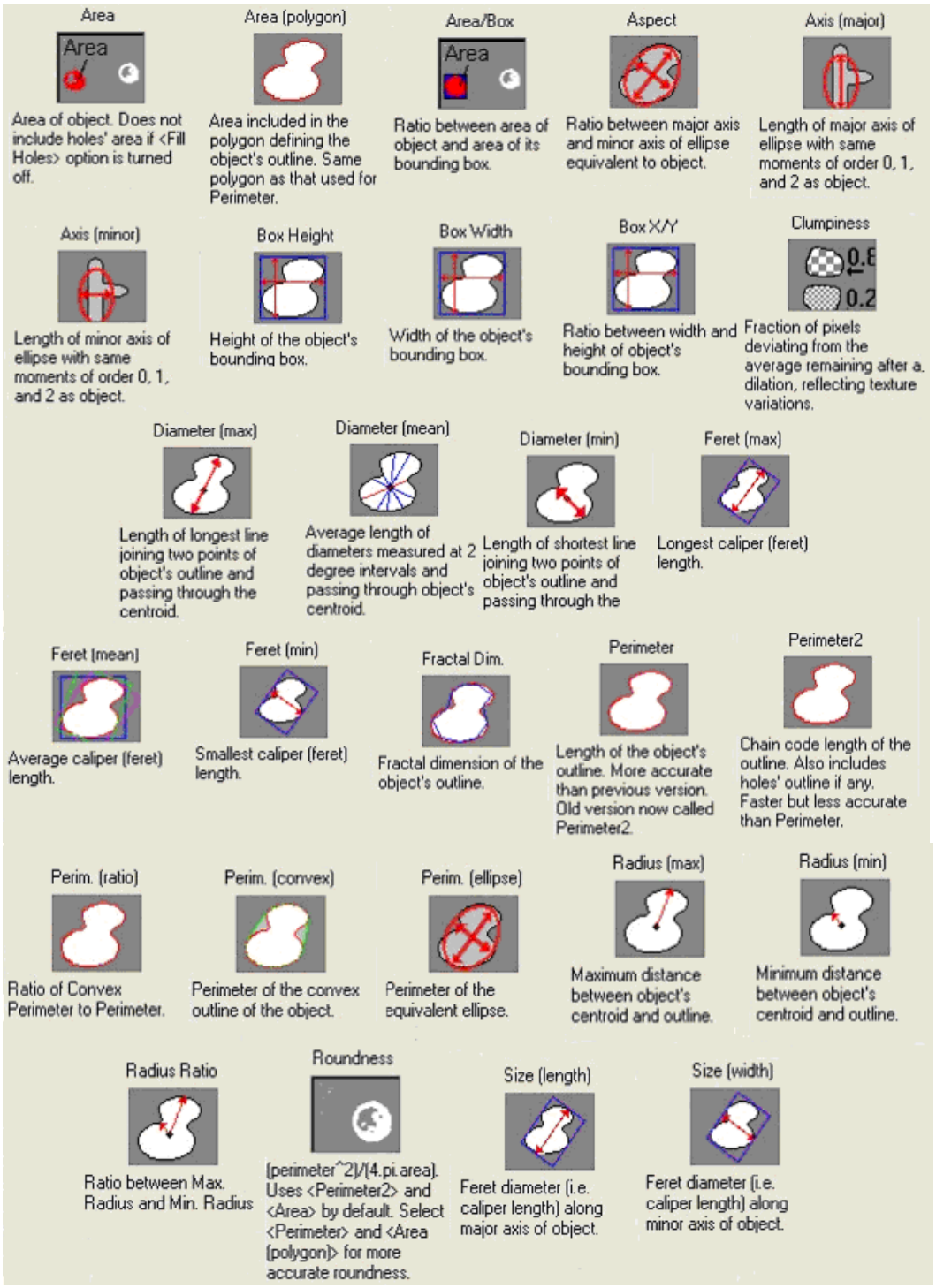

Figure 6: Informative descriptors as reported by Image-for Plus 4.5.1 version and 6.3 version (Media Cybernetics Inc., Silver Springs, USA).

In general, two main families of descriptors can be identified: size descriptors and derived shape descriptors (Table 1). The first represent direct measurements on bone lacunae, whereas derived shape descriptors are constructed by combining the various size variables so that dimension units cancel out (Russ, 2005). Derived shape descriptors are represented by V2, V3, V4, V20, V21, V34, V55, V56 and V58.

Table 1: ID code, nome, unit and description of size descriptors.

ID	Variable	Unit	Description
V1	Area	μm^2	Area of the object; includes a hole's areas if "Fill Holes" is turned on
V2	Aspect		Ratio between the major axis and the minor axis of the ellipse equivalent to the object
V3	Area/box		Ratio between the area of an object and the area of its bounding box
V4	Box X/Y		Ratio between the width and height of an object's bounding box
V11	Axis major	µm	Length of the major axis of the ellipse with the same moments of order 0,1 and 2 as the object
V12	Axis minor	µm	Length of the minor axis of the ellipse with the same moments of order 0,1 and 2 as the object
V13	Diameter max	µm	Length of the longest line joining two points of an object's outline and passing through the centroid
V14	Diameter min	µm	Length of the shortest line joining two points of

			an object's outline and passing through the centroid
V15	Diameter mean	µm	Average length of diameters measured at 2˚ intervals and passing through the object's centroid
V16	Radius max	µm	Maximum distance between an object's centroid and its outline
V17	Radius min	µm	Minimum distance between an object's centroid and its outline
V19	Perimeter	µm	Length of the object's outline. More accurate than previous version. Old version now called perimeter2
V20	Radius ratio		Ratio between the maximum radius and the minimum radius
V21	Roundness		$(\text{perimeter}^2)/(4\pi.\text{area})$. It uses "perimeter" and "area" by default. Select "perimeter" and "area" for more accurate roundness.
V28	Size (length)	µm	Feret diameter (i.e. calliper length) along the major axis of an object
V29	Size (width)	µm	Feret diameter (i.e. calliper length) along the minor axis of an object
V30	Perimeter2	µm	Chain code length of the outline. Also includes holes' outline if any. Faster but less accurate

			than perimeter
V32	Perimeter (convex)	µm	Perimeter of the convex outline of the object
V33	Perimeter (ellipse)	µm	Perimeter of the equivalent ellipse
V34	Perimeter ratio		Ratio of the convex perimeter and the perimeter
V35	Area polygon	μm^2	Area included in the polygon defining the object's outline. The same polygon as that used for the perimeter
V40	Box width	µm	Width of the object's bounding box
V41	Box height	µm	Height of the object's bounding box
V42	Feret min	µm	Smallest calliper (feret) length
V43	Feret max	µm	Longest calliper (feret) length
V44	Feret mean	µm	Average calliper (feret) length
V55	Form factor		$(4\pi \text{ area})/(\text{perimeter}^2)$. A form factor of 1.0 corresponds to a perfect circle
V56	Roundness 2		$(4\pi \text{ area})/(\pi.\text{major axis}^2)$
V57	Convex area	μm^2	Area of a polygon that has a major axis and a minimum axis for the sides
V58	Solidity		Ratio between are and convex area

From both families of descriptors 8 bidimensional morphometric measurements (size descriptors) for each lacuna (area, polygon area, axis minor, diameter min, radius min, size width, feat min and roundness) were of interest. The lacunae measurement data, of the 8 size descriptors, were collected in an Excel file and used for dataset assembly.

Bovine and poultry lacunae measurements were analyzed using one-way ANalysis Of VAriance (ANOVA) (GLM procedure of SAS statistical software 9.3). Mean, median and standard deviation values were calculated for each variable measured. Median for the same variables was always lower than mean value.

Results

Results obtained in this study indicated that all eight variables measured (Figure 7; Figure 8), except roundness variable, are higher in bovine than in poultry.

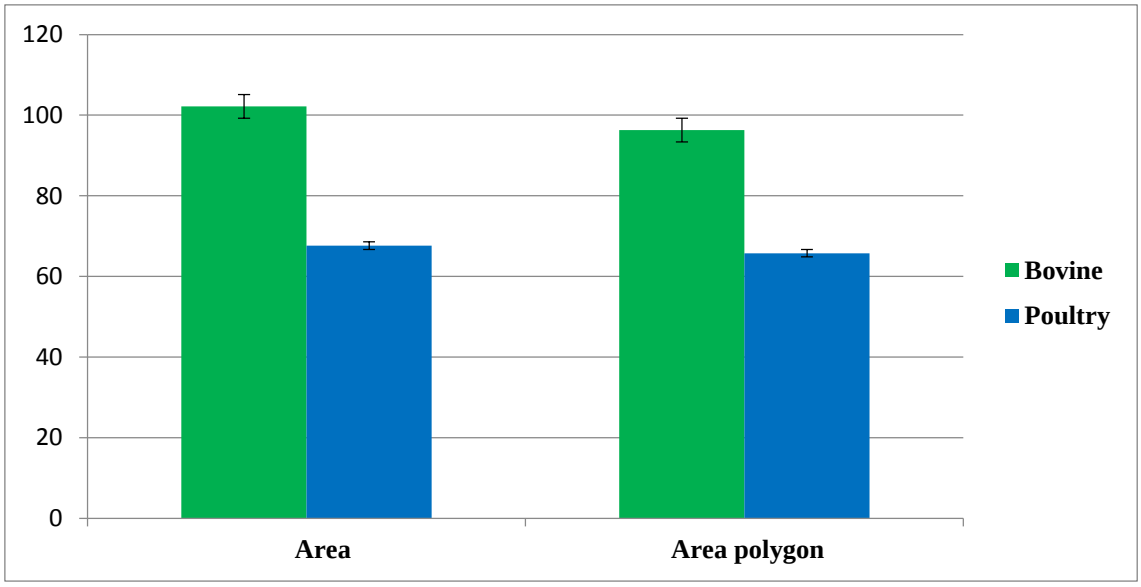

Figure 7: Means and standard deviation of area and area polygon variables measured in bovine and poultry lacunae.

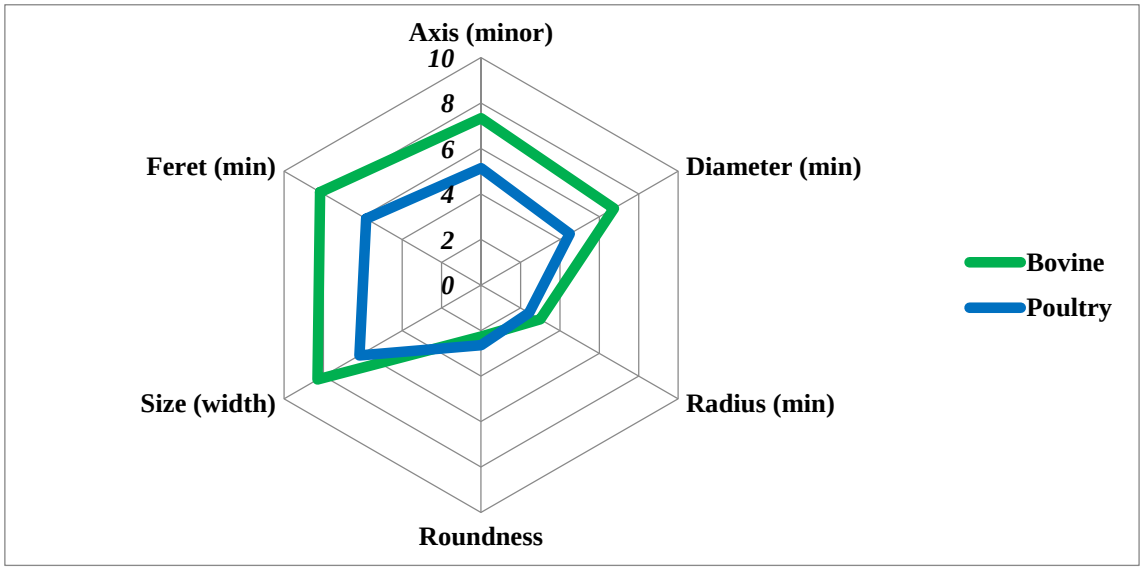

Figure 8: Other variables measured in bovine and poultry lacunae.

These eight variables were significantly ($P < 0,001$) different for discrimination among class (mammalian v. poultry). When other variables were considered, bovine and poultry bone lacunae overlapped. These eight variables are the most promising as potential markers in distinguishing between the two materials tested. These results are consistent with those obtained from a previous work (Figure 9, Figure 10, Figure 11) (Campagnoli et al., 2009).

Figure 9 shows the comparison of six descriptors for bovine and poultry lacunae obtained from Bovine 2013, Poultry 2013 and Campagnoli et al. 2009 (Bovine 2009, Poultry 2009).

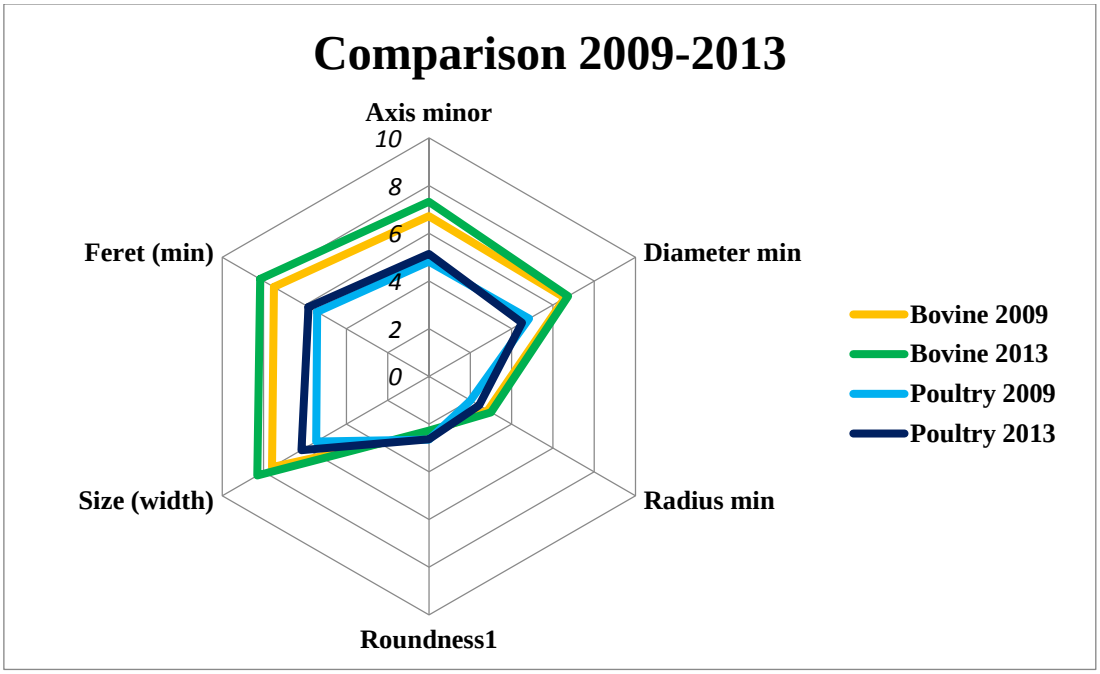

Figure 9: Comparison of six descriptors values measured in bovine and poultry (2009-2013).

In Figure 10 is shown the comparison of area and polygon area for bovine lacunae obtained between this work (Bovine 2013) and in Campagnoli et al. 2009 (Bovine 2009).

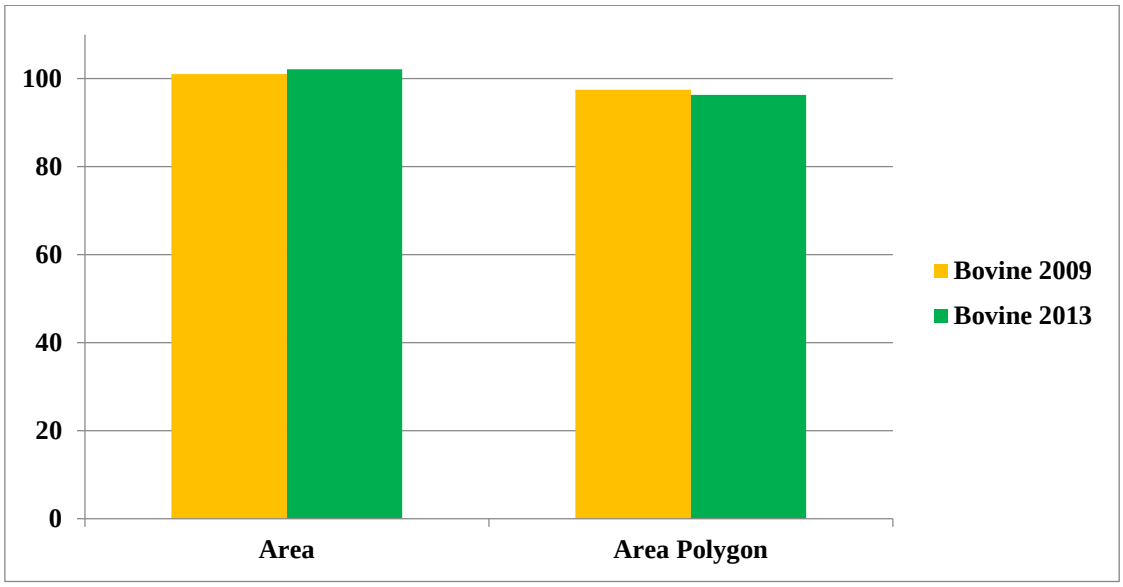

Figure 10: Comparison of other two descriptors values measured in bovine (2009-2013).

Figure 11 show the comparison of area and polygon area for poultry lacunae obtained from Poultry 2013 and Campagnoli et al. 2009 (Poultry 2009).

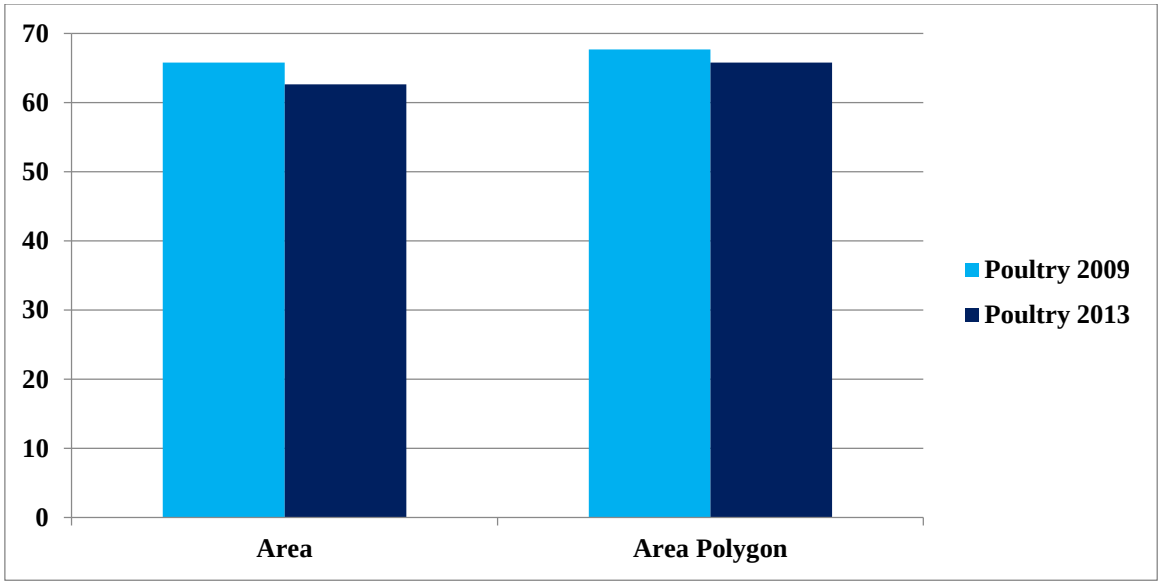

Figure 11: Comparison of other descriptors values measured in poultry (2009-2013).

Within the same class (poultry) or specie (bovine) no differences have been discussed in the comparison of differ dataset.

Discussion

Previously Pinotti el al., (2004) performed a preliminary study based on a limited number of poultry and mammalian lacunae. In this case, the 93.3% of lacunae were correctly classified using microscopy combined with image analysis. Only on two occasions (6.6%) the lacunae from poultry bone fragments were incorrectly classified as mammalian. The difference between poultry and mammalian meal is more difficult to detect because there are overlaps in measured variables (Gizzi et al., 2003; Liu et al., 2011). Combining microscopy with image analysis for MBM characterization allows, the addition of some objective information that can be used at least for class

identification in terrestrial animals and identify possible markers than could be used in routine analysis. In other works (Pinotti, 2009; van Raamdonsk et al., 2012b) the lacuna area has been considered a key marker in bone fragment identification. The value of this marker is confirmed by this work, and other measures (area polygon, axis minor, diameter min, radius min, size width, feat min) are worthy of consideration as good markers. However, the present study is based on a limited number of samples and additional observations/studies are needed. Successive studies were conducted in order to create a dataset representative of different animal bone material only of mammalian origin (pig and bovine in Ottoboni et al., 2014) and of both mammalian and avian origin (Pinotti et al., 2013).

To re-introduce the use of non-ruminant mammalian proteins to feed non-ruminant species, class discrimination is still not enough, but the availability of analytical methods to correctly differentiate PAPs at the level of species are necessary.

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Chapter 2

Animal protein in pet food:

ARIES® as decision support system for characterization.

Introduction

The compound feed industry consists of three distinct sections: farm animal feed, aqua feed and pet foods. The pet food market, particularly dog and cat food, is constantly growing, despite the gloomy economic situation. This industry offers a wide range of products to satisfy the pets' and owners' requirements (Di Donfrancesco et al., 2012). Ingredient composition, palatability, manufacturing processes, hygiene and appropriate storage, packaging, absence of undesirable components represents the building blocks for high quality pet foods.

The pet food labels must indicate either all ingredients present in the product or the categories to which the ingredients belong: they should be transparent, consistent, coherent and understandable [Commission Regulation 767/2009, (European Commission 2009b)]. In doing so, it prevents species substitution and the introduction of any food ingredient that might be harmful to human and/or animal health (Casazza et al., 2011; Pascoal et al., 2011). The development and use of new analytical methods were important for the identification of species (Rojas et al., 2011) and for quality and safety controls in pet food. The development and use of new analytical methods were important for the identification of species (Rojas et al., 2011) and for quality and safety controls in pet food.

Pet foods are formulated to be a balanced dietetic or complementary feed. There are three basic kinds: dry (maximum moisture of around 11%), semi-moist (around 25-35% of moisture) and moist (around 60-87% of moisture) (Zicker, 2008). Most commercially available pet foods are based on a mixture variety of animal- and plant-based ingredients and apply flavours in order to increase the palatability of their products.

As the feed, the pet foods contain mixtures of different ingredients and among these animal proteins and meat bone meal may be present. Usually, the pet food labels declare that products contain high biological value protein (e.g. proteins from avian species) but there is no guarantee that other questionable protein sources will not fraudulently substitute avian material , which causes economic damage and compromises the pet's health (Pegels et al., 2014). In addition to Commission Regulation (EU) No 294/2013 (European Commission, 2013b) laying down health rules as regards animal by-products and derived products not intended for human consumption, the processed animal proteins (PAPs) may be regarded as feed for pets if they are mixed in appropriate proportions with other normal feed consumed by those pet species.

Feeding of by-products to the same species as the source was prohibited (species-to-species ban) by the animal by product Commission Regulation 2002/1774/EC (European Commission, 2002). As a consequence adequate analytical methods to detect constituents of animal origin in feedstuffs were required. As reported in Commission Regulation 2009/152 (European Commission, 2009a) a support system such as ARIES® (Animal Remains Identification and Evaluation System) can be used and the reference samples can be documented (Figure 1). A first version was released in 2004 as a product of an EU funded project (STRATFEED).

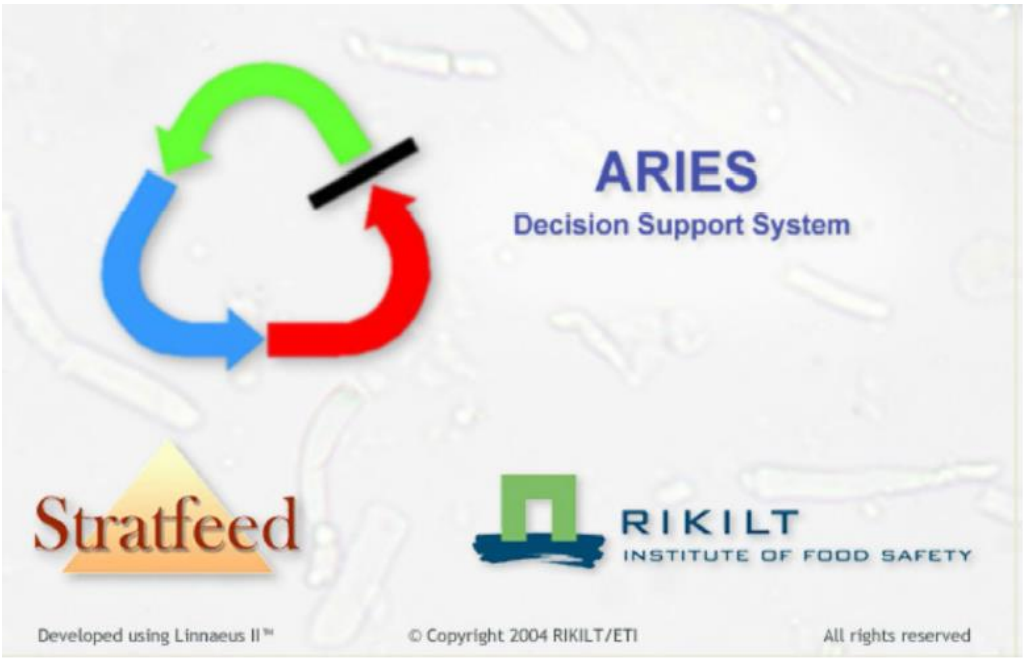

Figure 1: ARIES®, Decision Support System.

ARIES® is a stand-alone system developed by RIKILT (van Raamsdonk, 2002), based on the Linnaeus II software (ETI, 2003), in collaboration with partners NUTRECO, LAGC and ROLT (Vermeulen et al., 2003).

van Raamsdonk et al., 2004, 2007, 2010, demonstrated the potential power of decision support systems as well the possibility of identifying animal protein contamination in feed. ARIES® (Vermeulen et al., 2003) is a decision support system that provides a full range of animal meal descriptions, including eggshells, fish and a range of plant parts and minerals that can be confused with animal material. This software was designed to support the analyst in recognizing and classifying a single fragment at a time found in feed.

The software was divided into different modules (Figure 2), which in turn were divided into various sections.

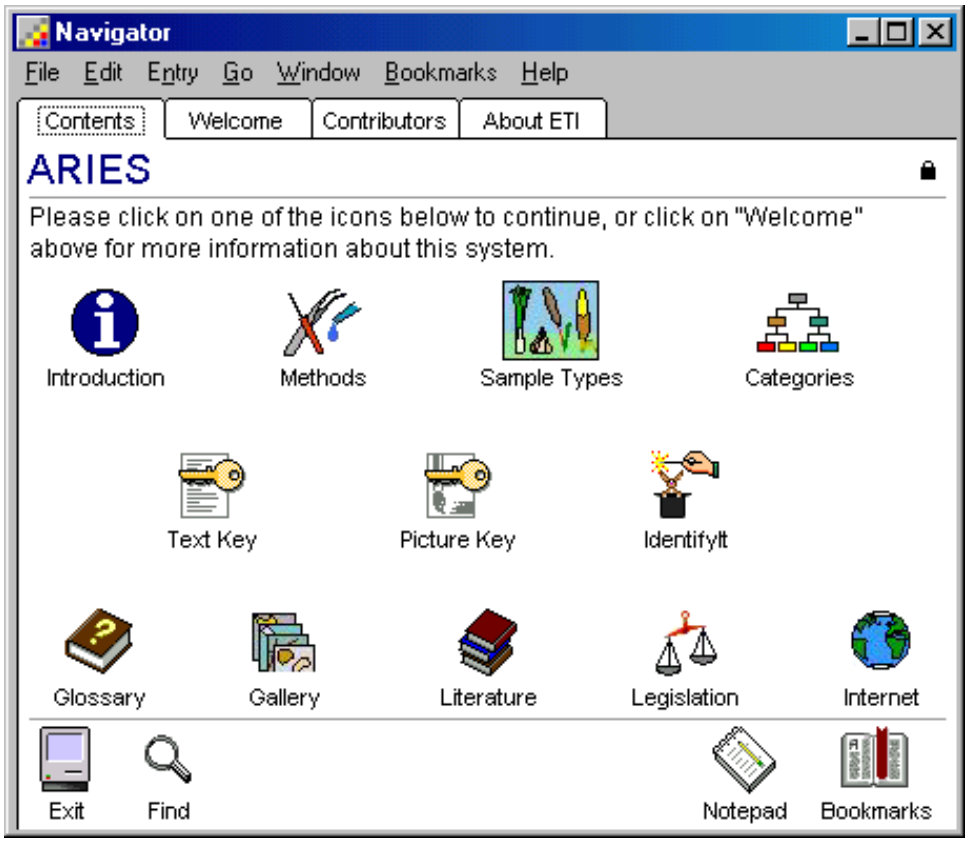

Figure 2: Menu of ARIES® presenting all the modules. (van Raamsdonk, 1999. www.stratfeed.cragx.fgov.be. Copyright by RIKILT, Wageningen, the Netherlands, 2002).

This system provides three identification modules: the first is based on text while the second is based on pictures, and both are based on decision trees. These two modules are based on decision trees. Every window shows two or three buttons with choices (texts or images); pressing a button, i.e. making a choice, leads to a next window with either new choices or a conclusion. Both modules can produce a report of the identification pathway. The third identification module is based on a multiple-entry key (matrix of taxa, characters and character states) (Vermeulen et al., 2003).

Some modules are descriptive: e.g. introducing the features of the software (introduction); indicate the current legislation (legislation), the general protocol for preparation of samples [categories, methods (Figure 3) and sample types)]; indicating the related bibliography and references on the Internet (literature and internet); one also is the gallery containing all the picture of the data set of the software (gallery) (Figure 4).

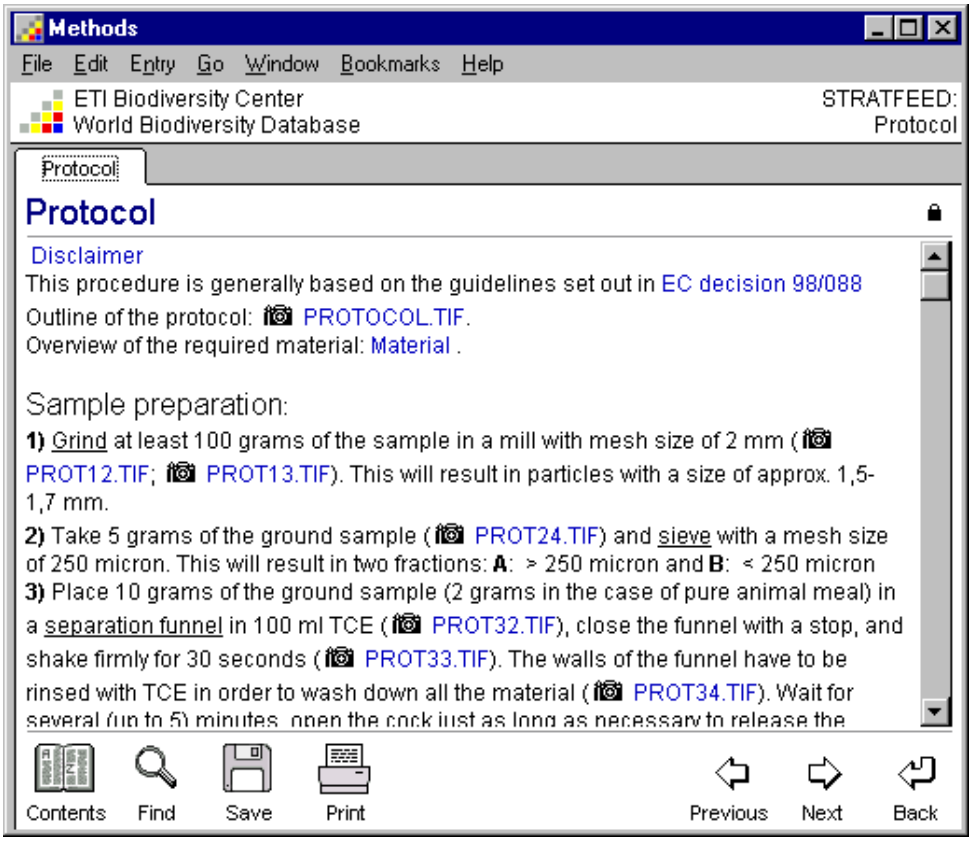

Figure 3: Example of method module. (van Raamsdonk, 1999. www.stratfeed.cragx.fgov.be. Copyright by RIKILT, Wageningen, the Netherlands, 2002).

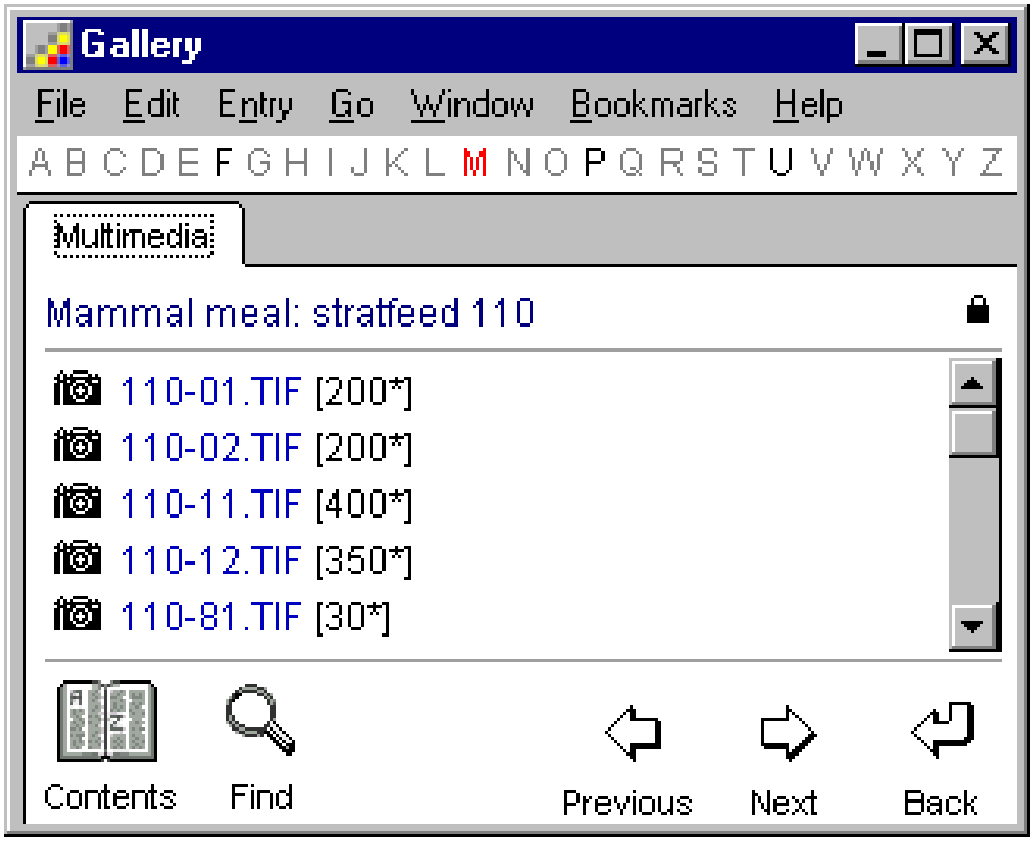

Figure 4: Example of gallery module. (van Raamsdonk, 1999. www.stratfeed.cragx.fgov.be. Copyright by RIKILT, Wageningen, the Netherlands, 2002).

Other three modules are the operational ones. The selection key allows to identify of the single particle in question: through the "text key" module (Figure 5) (it proceeds by exclusion through increasingly specific descriptions), the "picture key" module (the comparison with the images succession of the data set of the system), and finally through "identify it" module (according to information supplied by the user, the software calculates the probability that the fragment belongs to PAP of a specific zoological class).

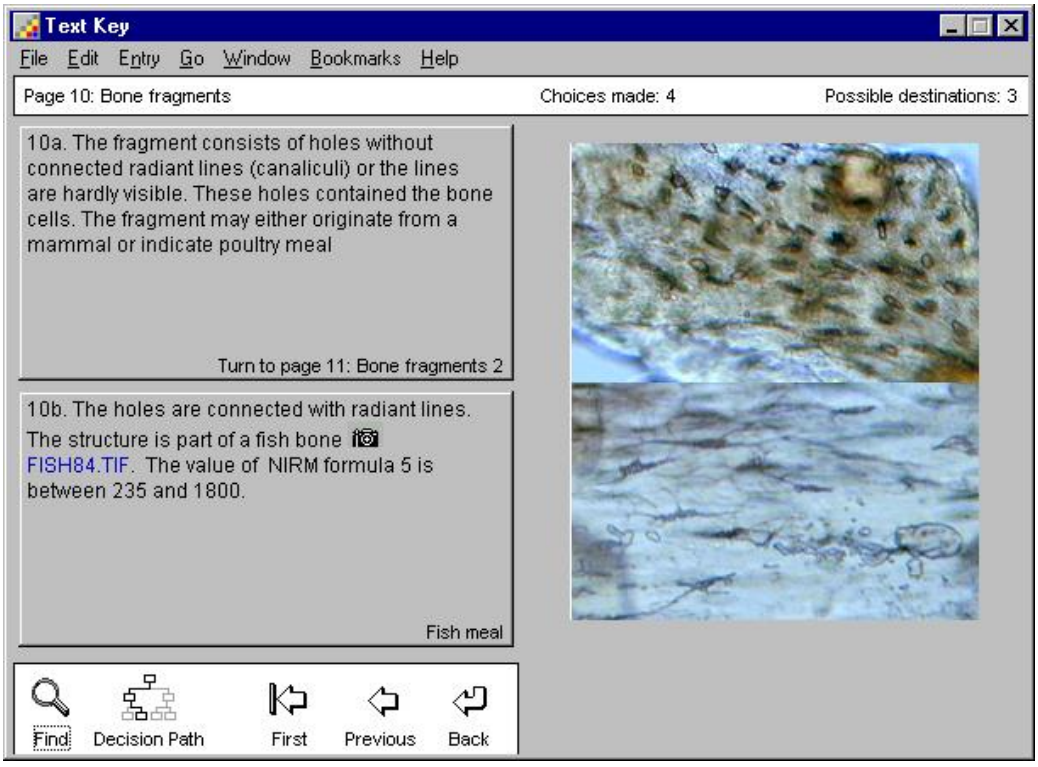

Figure 5: Example of text key module. (van Raamsdonk, 1999. www.stratfeed.cragx.fgov.be. Copyright by RIKILT, Wageningen, the Netherlands, 2002).

ARIES® was equipped with many forms of recognition. It is able to discriminate between the various kinds of subjects found in the surfactant (muscles, hair, feathers filaments) and also in the sediment (bones, egg shells, scales fish).

The availability of an informatic support such as ARIES® represents a fundamental tool to carry "crowding out", the analyst focusing on the morphological characteristics of the fragments under investigation. Thus the shape of the fragment in full (rounded or less) the dominant color, the shape of the gaps, the presence or absence of canals, as well as additional chemical testing if necessary, lead the analyst along a decision tree that should bring it to a fairly secure conclusion (Figure 6, Figure 7).

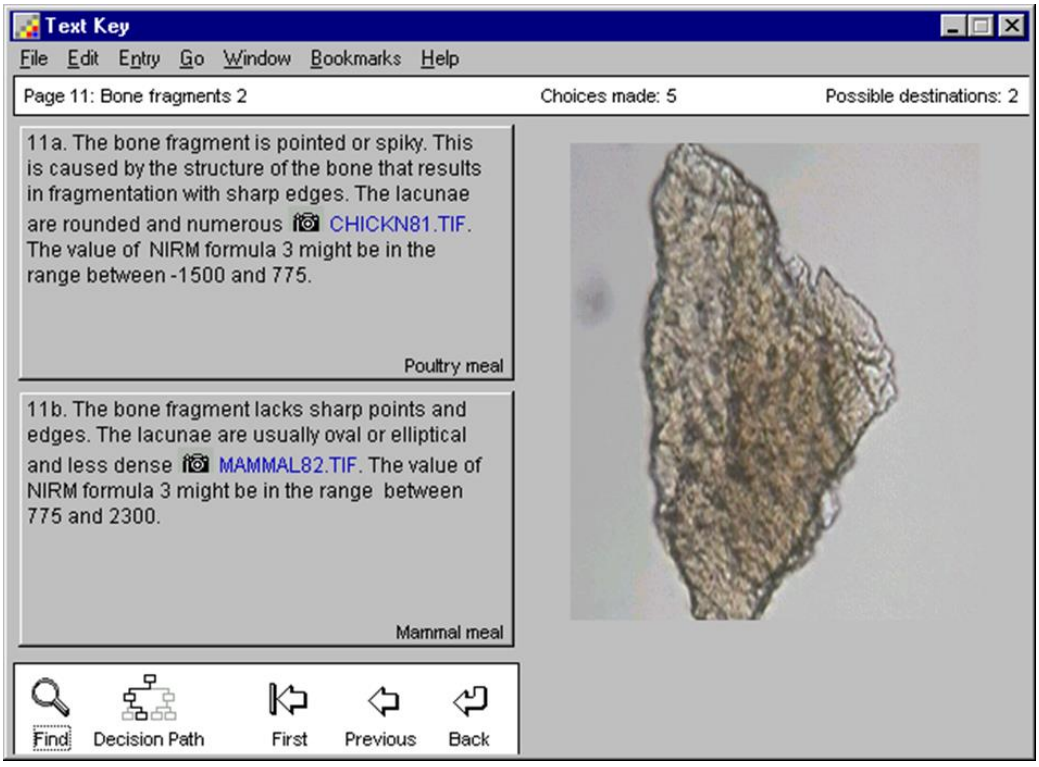

Figure 6: Example of chicken bone fragment analysis. (van Raamsdonk, 1999.

www.stratfeed.cragx.fgov.be Copyright by RIKILT, Wageningen, the Netherlands, 2002).

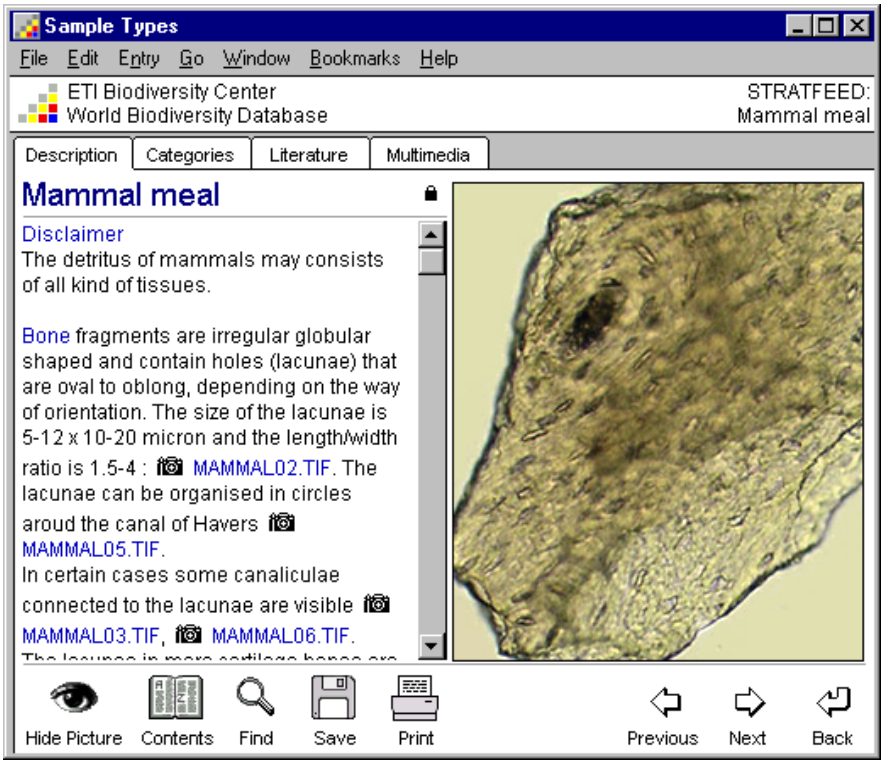

Figure 7: Example of database of mammalian bone fragment. (van Raamsdonk, 1999. www.stratfeed.cragx.fgov.be Copyright by RIKILT, Wageningen, the Netherlands, 2002).

Aim

Aim of the study was to evaluate the potential of decision support system (ARIES®), for characterization of fish material in pet food.

Material and methods

Two complete feed for adult dogs, containing 40% (sample 1) and 31% (sample 2) of fish and fish byproducts, were analyzed according to Commission Regulation (EC) No 51/2013 (Commission Regulation, 2013a), amending Regulation (EC) 152 /2009 (Commission Regulation, 2009a). The sediment fraction of each sample was observed with a compound microscope (Olympus BX41) at several magnifications in order to obtain several bone fragment lacunae images at X40 magnification.

Once we recognized and isolated the bone fragments and other constituents of animal origin, we proceeded with determining their origin and zoological classification using a "Decision Support System" called ARIES®DSS 0.7.

In the sediment fraction 30 bone fragments (10 bone fragments from samples num. 1 and 20 bone fragments from samples num. 2) were analyzed with ARIES®DSS 0.7. The ARIES® software is designed to individually identify these particles. Through ARIES® "Identify it" module, the software calculates the probability that the fragment belongs to specific specie.

Results

ARIES® was a good support for the identification of fish material in pet food. In Table 1 and Table 2 are reported the results of the analysis using "Identify it" module of ARIES® and data were expressed as a percentage of recognition. ARIES® is a tool to

carry "crowding out" the analyst focusing on the morphological characteristics of the fragments under investigation and precise focus. The full shape of fragment (rounded or not), the dominant color, the lacunae shapes and the presence of canaliculaes, with ARIES®, become accurate characteristics. This “decision support system” leads the analyst along several steps that should lead to a safe conclusion about the origin and zoological class of fragments analyzed.

Table 1: Results obtained using ARIES® (Decision Support System) software of sample number 1 of pet food.

	Fishmeal	Poultrymeal	Mammalianmeal	Cartilage	Calcium Carbonate	Bi-Calcium phosphate	Crystals of sand	Soy
Fragment1	44%	22%	55%	55%	66%	66%	77%	22%
Fragment2	60%	30%	60%	50%	80%	70%	70%	20%
Fragment3	33%	33%	55%	55%	77%	66%	66%	33%
Fragment4	75%	50%	37%	50%	87%	75%	75%	12%
Fragment5	66%	44%	66%	66%	66%	55%	55%	33%
Fragment6	77%	55%	33%	44%	77%	55%	44%	11%
Fragment7	66%	55%	33%	33%	77%	55%	44%	11%
Fragment8	66%	55%	33%	33%	77%	55%	44%	11%
Fragment9	62%	37%	37%	50%	75%	75%	62%	12%
Fragment10	54%	27%	45%	54%	63%	63%	54%	18%

Table 2: Results obtained using ARIES® (Decision Support System) software of sample number 2 of pet food.

	Fishmeal	Poultrymeal	Mammalianmeal	Cartilage	Calcium carbonate	Bi-Calcium phosphate	Crystals of sand	Soy
Fragment 1	66%	66%	66%	50%	33%	16%	16%	33%
Fragment 2	33%	66%	50%	83%	66%	50%	50%	50%
Fragment 3	50%	37%	87%	50%	62%	50%	37%	50%
Fragment 4	57%	14%	42%	42%	71%	57%	57%	28%
Fragment 5	20%	20%	60%	60%	80%	60%	60%	60%
Fragment 6	50%	50%	33%	33%	50%	50%	16%	16%
Fragment 7	71%	57%	28%	14%	42%	28%	0%	42%
Fragment 8	85%	42%	28%	14%	42%	28%	14%	14%
Fragment 9	71%	57%	14%	28%	85%	57%	42%	14%
Fragment10	75%	50%	50%	37%	50%	50%	25%	25%
Fragment11	71%	57%	14%	28%	85%	57%	42%	14%
Fragment12	55%	22%	44%	55%	77%	66%	55%	22%
Fragment13	25%	37%	62%	25%	37%	12%	12%	62%
Fragment14	62%	37%	37%	25%	62%	50%	25%	25%
Fragment15	50%	25%	25%	12%	62%	62%	25%	12%
Fragment16	57%	28%	14%	57%	71%	57%	57%	42%
Fragment17	57%	28%	28%	42%	42%	57%	28%	0%
Fragment18	77%	55%	55%	33%	66%	44%	22%	33%
Fragment19	42%	57%	71%	42%	57%	28%	28%	71%
Fragment20	33%	33%	55%	55%	66%	55%	55%	22%

In pet food samples results with high values, ranging between 60% and 85% were obtained using ARIES® for identifying fish fragments. Figure 8, shows a fish fragment with a 85% confirmation of identification.

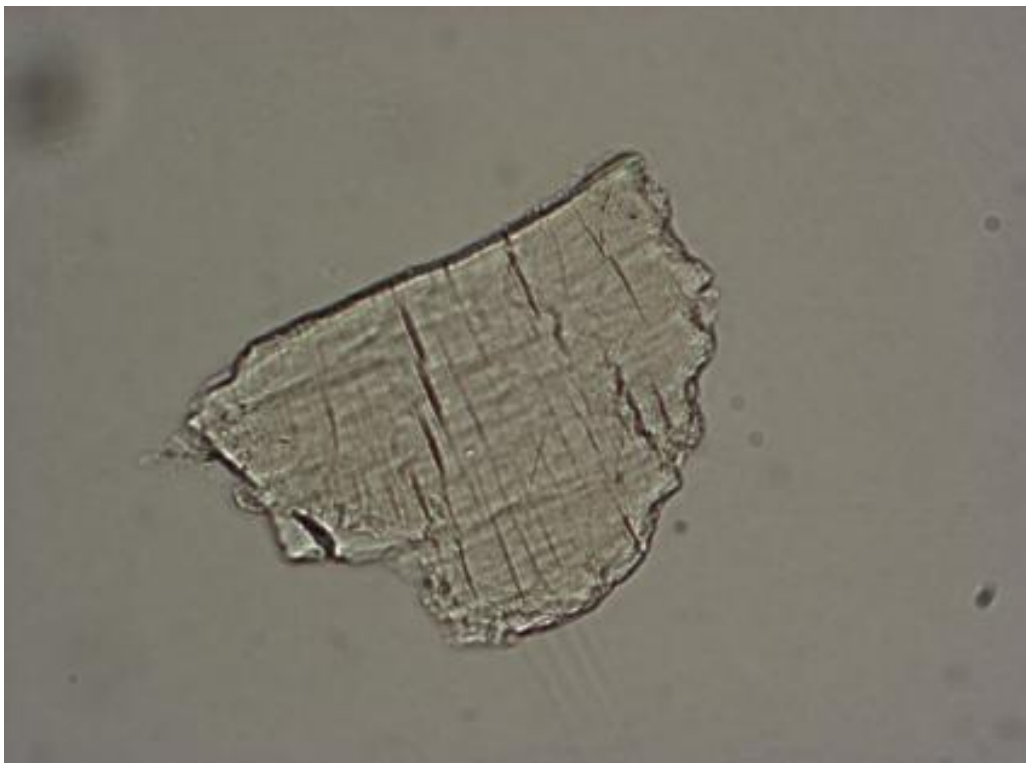

Figure 8: Example of fish lacunae at X40 magnification.

Several fragments (2, 4, 5, 6, 7, 8 and 9) obtained from sample number 1, with ARIES® were identified with a value of more than 60%, as fish fragments.

The fragment number 7 (Figure 9) and the fragment number 9 (Figure 10), are examples typical of the oyster shells.

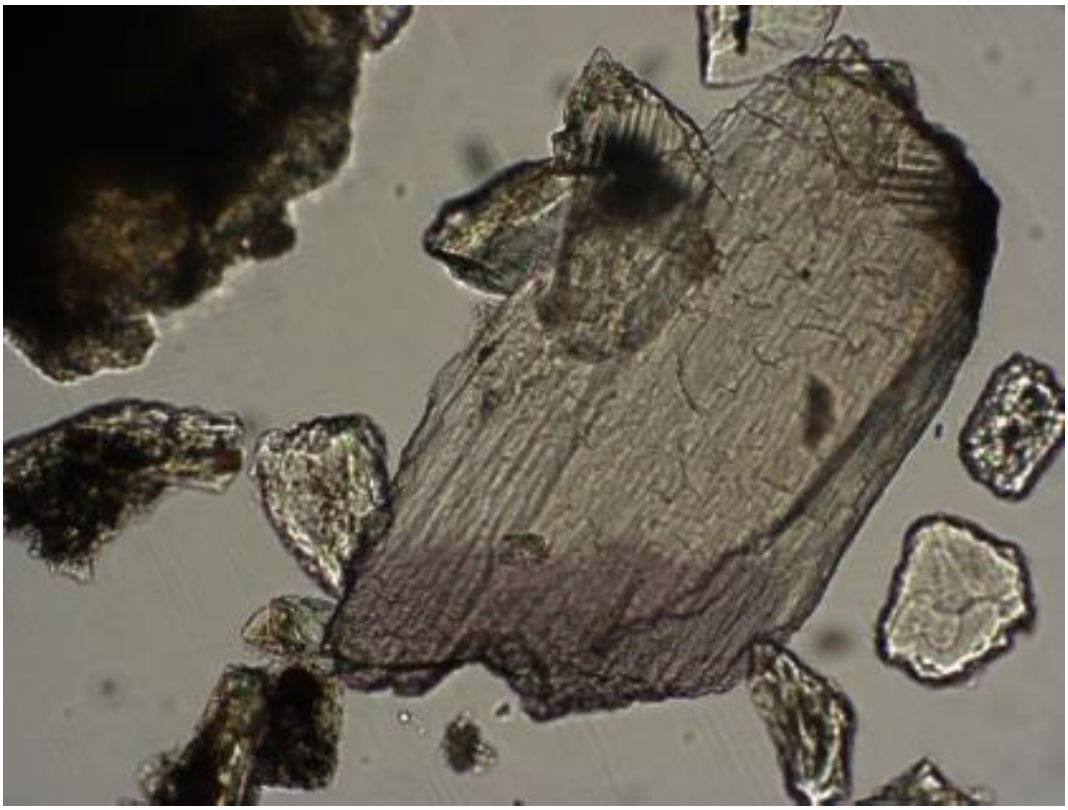

Figure 9: Fragment 7, sample 1; oyster shells at X40 magnification.

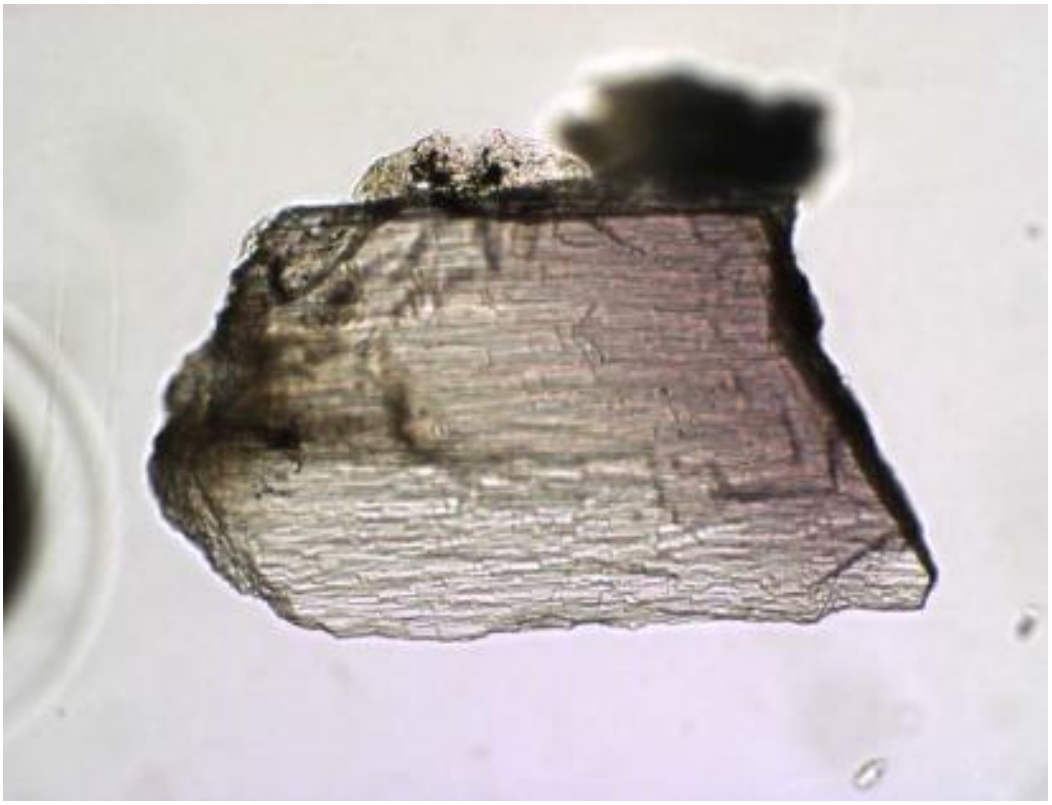

Figure 10: Fragment 9, simple 1; oyster shells at X40 magnification.

The pictures presented above were compared with the oyster shells picture shown in literature (Figure 11).

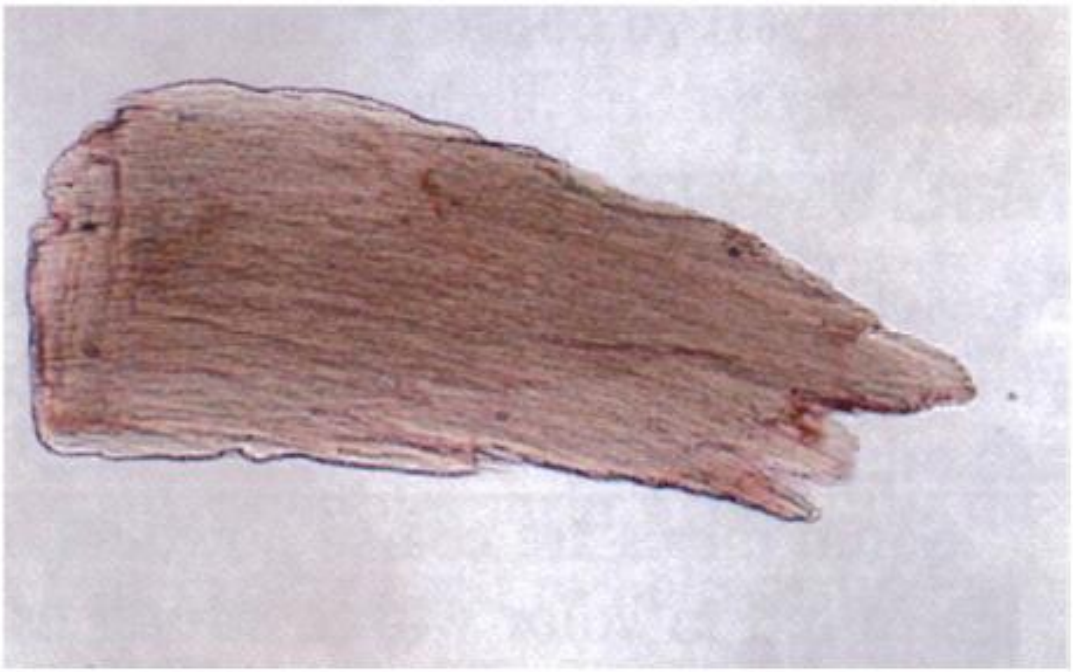

Figure 11: Oyster shells at X40 magnification (Makowski et al., 2011).

Makowski et al. (2011), reported that the fishmeal (FM) obtained from shells of oysters and mussels, showed a grayish-white color, while the FM obtained from squid, showed a scale of colors, from orange to red. The marine invertebrates, specially shellfish, are widely used as feed ingredients for its high calcium intake. As shown from Figures 9, 10 and 11, the oyster shell fragments are characterized by lacunae and canaliculae absence.

In sample number 2, several fragments (numbers: 1,4,6,7,8,9,10,11,12,14,15,17,18) showed results with high values (between 57% and 85%), obtained using ARIES®.

The fragment number 15 (Figure 12), represents remains of otoliths. An otolith is a minuscule concretion of calcium carbonate incorporated in gelatinous matrix in the human inner ear, and also reported to be found in fish.

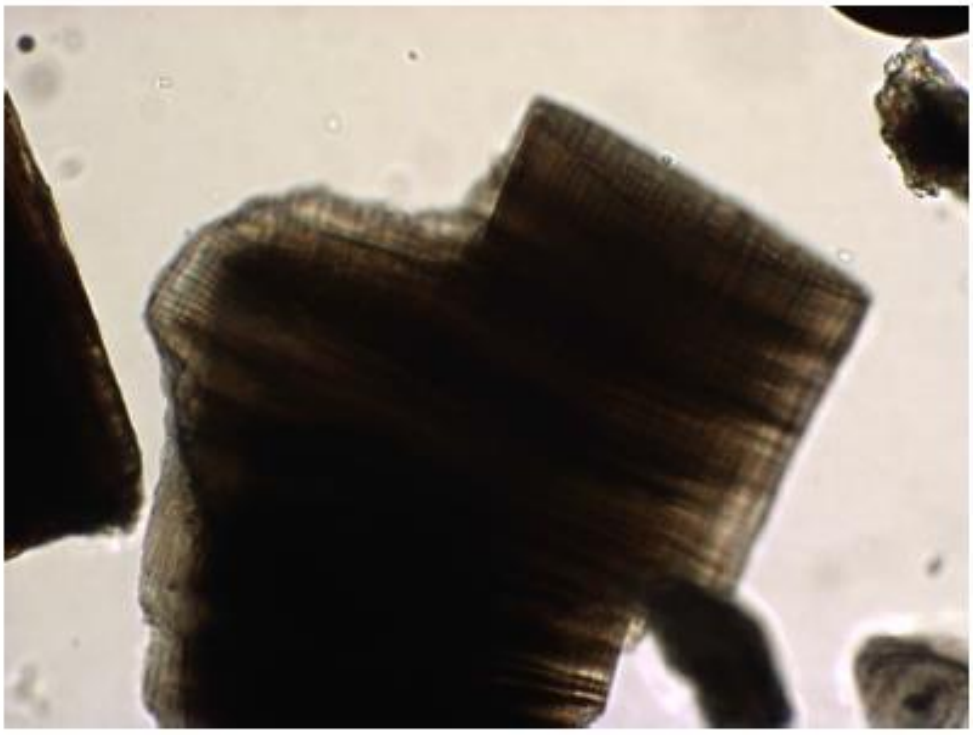

Figure 12: Fragment 15, sample 2; otoliths at X40 magnification.

The image in Figure 13 shows otoliths obtained from the ARIES® gallery.

Figure 13: Otoliths at X40 magnification. ARIES® gallery. (Copyright by RIKILT, Wageningen, the Netherlands, 2002.)

The fragment number 16 (Figure 14), represents shrimp/crab shells, that is crustacean.

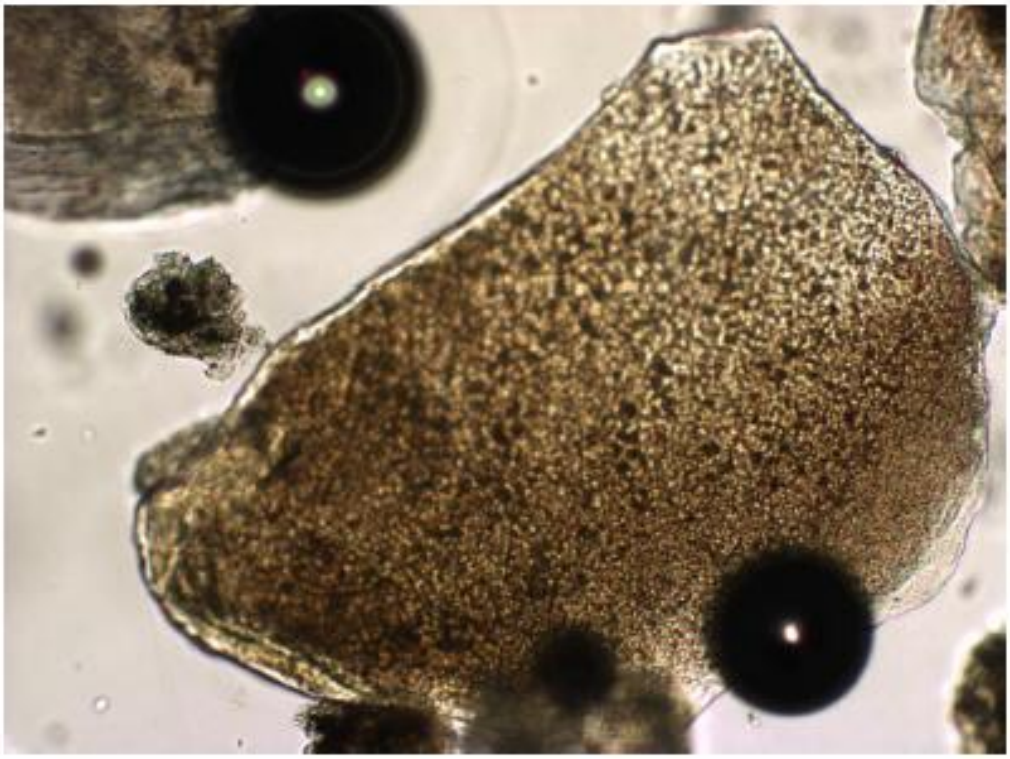

Figure 14: Fragment 16, sample 2; shrimp shell at X40 magnification.

In Figure 15 the image shows a shrimp shell obtained from ARIES® gallery.

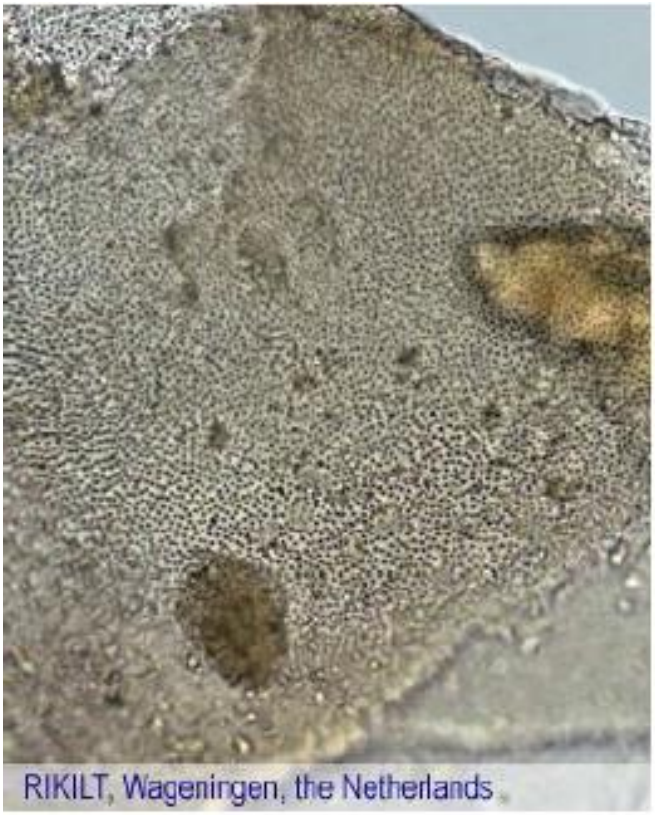

Figure 15: Shrimp shell at X40 magnification. ARIES® gallery. (Copyright by RIKILT, Wageningen, the Netherlands, 2002.)

The shrimp meal reported black dots that are part of the eye.

In this work, some fragments were not identified as fishmeal only. The fragment numbers 3 and 19, showed higher values when ARIES® recognized them as meat bone meal [87% in fragment number 3 (Figure 16) and 71% in fragment number 19 (Figure 17)] compared to fishmeal.

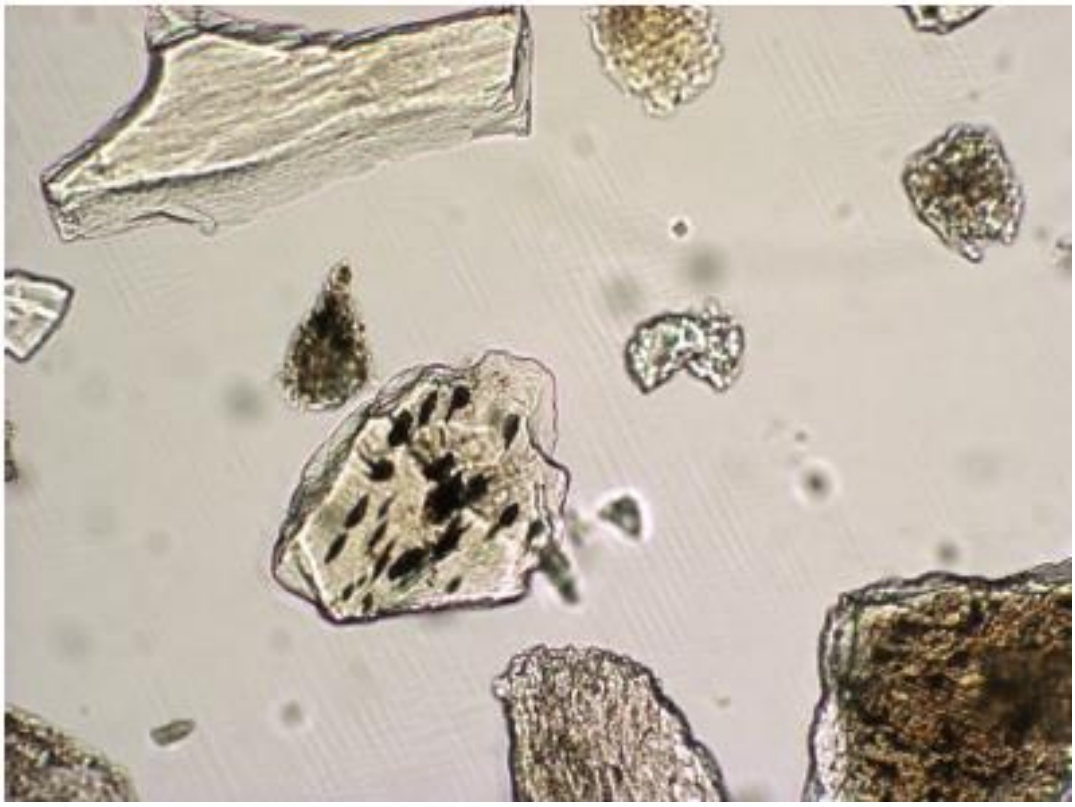

Figure 16: Fragment 3, sample 2; X40 magnification.

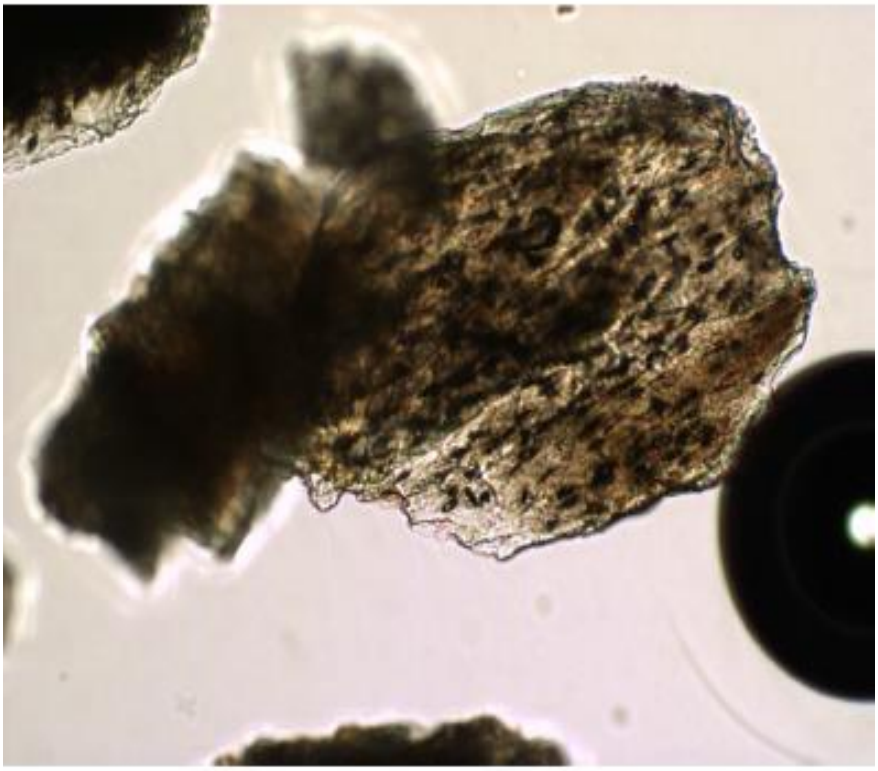

Figure 17: Fragment 19, sample 2; X40 magnification.

Figure 18 shows fragments and lacunae mammalian, obtained from ARIES® gallery**.**

Figure 18: Fragments and lacunae mammalian at X40 magnification. ARIES® gallery. (Copyright by RIKILT, Wageningen, the Netherlands, 2002).

Fish bones often show parallel sides. Lacunae in fish bones (Figure 19) are usually elongated with a clear fusiform net of canaliculae. However, there is a large diversity among fish species (Gizzi et al., 2003). Some fish lacunae, such as salmon are similar to mammalian lacunae (Jan Sten Jorgensen et al., 2012).

Discussion

Specific applications such as the "ARIES® Decision Support System" give responses to a specific request. In particular, this decision support system is the first known system applied to the detection of mammalian tissues in feedstuff, in support of current EU legislation (Vermeulen et al., 2003).

As reported by Pinotti (2009), the mammalian bone particles at high magnifications (from X10) are generally transparent, more or less rounded and contain elliptical to almost circular lacunae; canaliculae may be visible depending on the preservation and transparency of the particles. The bone particles from poultry, as reported by Gizzi et al., (2003) are dark, have a more splintered (sharp-edged) appearance, more rounded and denser lacunae, and canaliculae are rarely visible.

Thus it can be concluded that although, a considerable overlap between classes exists, fish materials have some specific features detectable using an *in silico* support system (i.e. ARIES®), moreover some of these characteristics can be confirmed by some measurements provided by an image analysis software.

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repealing Council Directive 79/373/EEC, Commission Directive 80/511/EEC, Council Directives 82/471/EEC, 83/228/EEC, 93/74/EEC, 93/113/EC and 96/25/EC and Commission Decision 2004/217/EC. In: Official Journal, L 229, 01/09/2009, pp 1-28.

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Chapter 3

Application of electronic nose in pet food analysis

Introduction

Despite the present economic situation the pet food market, particularly dog and cat food, is constantly growing with an increase of products on offer. The quality, safety and efficacy of foods intended for cats and dogs are important considerations for manufacturers.

Despite the present economic situation the pet food market, particularly dog and cat food, is constantly growing with an increase of products on offer. The quality, safety and efficacy of foods intended for cats and dogs are important considerations for manufacturers.

Dogs and cats use both taste and smell in the detection and selection of food (Di Donfrancesco et al., 2012). In pet food production, palatability plays an important role in food preference. Palatability is a composite function of a variety of factors including taste, aroma and mouthfeel (texture, shape, and particle size) (Thorn et al., 1992; Chaudhary et al., 2010). It is typically referred to as a measured value of food preference and ingestive behavior (Tran et al., 2008) and can be influenced by a number of factors, including diet nutrition composition, e.g. fat/carbohydrate ratio and processing (Hullár et al., 1998). Appearance, aroma, texture and flavor are sensory characteristics, important for determining pet food acceptability (Koppel, 2014). The dry food aroma comprises of animal (beef, poultry, pork or marine), vegetable (herbs or fruits and vegetables) or dairy (butter, milk or cheese) dry aromas while the pet food

palatability enhancer comprises of animal digest, animal fats or dairy products (Fournier et al., 2012). These aroma and enhancers have great impact on final product and pet food success. Accordingly the nutrients, used in pet foods, are measured depending on sensory properties and cannot be overlooked. Olfactory sense is important because "odor" needs to be paired with flavour in order to continuously accept the proposed food (Houp et al., 1978). The initial decision to eat is based on aroma (Houp et al., 1978) but according to Di Donfrancesco et al., (2012) the aroma attribute is not necessary to predict the characteristic flavor of the product.

The electronic nose (EN) is an instrument that comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern-recognition system that is capable of recognizing simple or complex odours (Gardner and Bartlett, 1994). The electronic nose, mimicking the sense of smell may represent a modern analytical approach in food and feed industry to monitor quality and safety of products and process (Deisingh et al., 2004; Peris and Escuder-Gilabert, 2009; Campagnoli and Dell'Orto, 2013).

For their complex formulation, pet foods are of interest for aromatic composition studies (Koppel et al., 2013). There is not much literature that discusses EN as unique tool used for pet food analysis. In a study on moist cat foods, several measurements types (such as EN, gas chromatography and texture parameters) were used to find correlation between aroma properties and appearance attributes (Denis et al., 1999). Electronic nose and tongue (ENT) technology were used for the classification of pet foods according to their aroma (Éles et al., 2013) and assessment of the quality of finished pet food flavours (Oladipupo et al. 2011).

Pickerinh (2009), conducted two studies on dry and wet cat foods using sensory analysis. These studies focused on both aromatic and flavor attributes. According to Pickerinh (2009) both wet and dry cat foods are highly complex in flavor characteristics. The dry dog food samples, were used to determine the influence of the aromatic compounds in the aroma perception, by descriptive analysis (Di Donfrancesco et al., 2012) and in Koppel at al., (2013) for to determine volatile compounds in same samples, solid-phase microextraction/gas chromatography/mass spectrometry were used.

The sensory analysis conducted on pet foods is often complicated and expensive (Koppel, 2014). Therefore, EN technology can be used for fast screening prior to conventional animal preference tests, saving time and money by speeding up the product development and also for quality control (Éles et al., 2013). Sensor array and pattern recognition (PR) system are capable of recognizing simple or complex odours, tend to predict the quality of a sample without providing hard data with respect to composition and concentration (Krantz-Rucker et al., 2001).

Aim

The aim of this study was to evaluate the application of EN to dog and cat pet food analysis and to distinguish pet food samples according to the species and/or the formula diets.

Material and methods

For this study, 12 commercial dry dog pet foods and 15 commercial dry cat pet foods were tested. The samples were classified according to: the species, the formula diets and the ingredient composition declared in label (presence or absence of fish and/or fish oil) (Table1).

Table 1. Ingredient composition of pet food samples.

Species	Formula Diet	Presence or absence of fish and/or fish oil	Number of samples
Dog	Complete and balance diet	Presence	6
	Dietetic diet	Presence	3
		Absence	3
Cat	Complete and balance diet	Presence	3
		Absence	3
	Dietetic diet	Presence	6
		Absence	3

The pet food samples (100g) were homogenized by ground using a mortar and pestle and samples were analyzed by the Electronic Nose Pen 2 (Airsense Analytics GmbH, Schwerin, Germany). Pet food samples (2g) were placed in airtight of 10-ml glass vials with a chlorobuty/PTFE magnetic cap (Chromacol Ltd., Welwyn Garden City, UK). The headspace of each sample was equilibrated for 24 hours at room temperature. The odour profile of each sample was determined by the 10 MOS (Metal Oxide Semiconductor) sensors of the EN. The MOS chemical sensors are made of a ceramic substrate heated by a wire resistor and coated by a metal oxide semiconducting film.

Four measurements were performed on each sample (n = 27 x 4). The sampling time was 2 min and the flush time between two sampling was 5 min. The flow rate was 400 ml/min.

During the measurement time, data from the raw sensor signals for each sample were singularly analyzed, and the mean value of the sensors' signals from each aliquot was calculated and recorded as a single odour profile. Thus, the ratio G/G0 (where G and G0 are the resistance of a sensor in a detecting gas and in clear air, respectively) was recorded by the EN dedicated software. Data were analyzed with chemometric tools such as Principal Component Analysis (PCA) and Cluster Analysis (Ward's Cluster Analysis) procedure of SPSS (IBM SPSS Statistics 21.0.). PCA and CA were performed to distinguish and form clusters among pet food samples based on their flavor fingerprints obtained by the EN.

Results

Figure 1a shows the PCA score plot on the whole data set (i.e. num=27). More than 80% of total data variability was explained by only two first principal components [corresponding to the two electronic nose sensors: sulphur-organic (W1A) and broadrange (W5B)] and the EN was not able to distinguish samples according to the species and the formula diets. One cat and one dog pet food sample (Figure 1a) were not correctly classified.

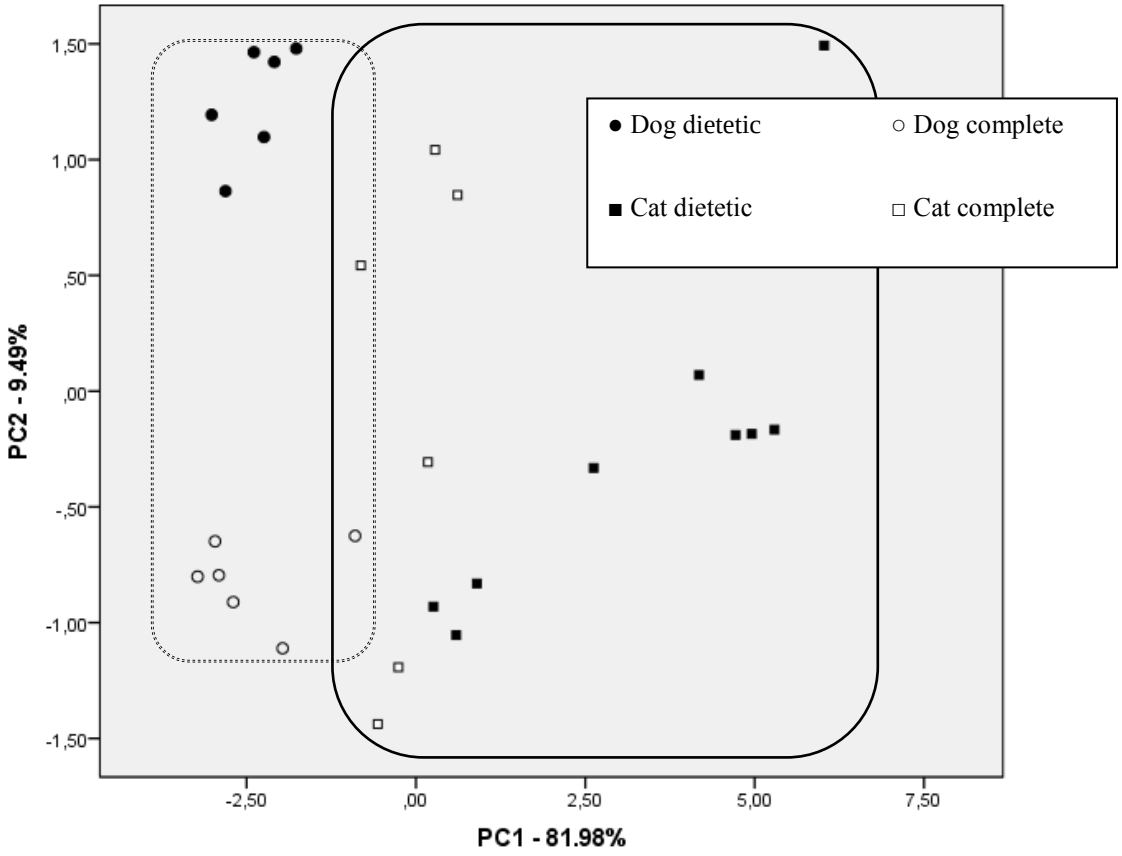

Figure 1a: Principal components analysis score plot of the whole dataset. The distribution of four pre-determinated classes (dog and cat, complete and dietetic) are indicated.

Data were analyzed by cluster analysis. The dendogram in Figure 1b shows that three clusters were identified, when CA was applied to the whole data set of pet food. Twelve samples of dog pet foods (six complete and six dietetic diets) and two samples of cat (complete diets) pet foods belong to one cluster. The second cluster constituted eight samples of cat pet foods (four dietetic and four complete) and a third cluster contained five samples of cat pet foods (dietetic diets).

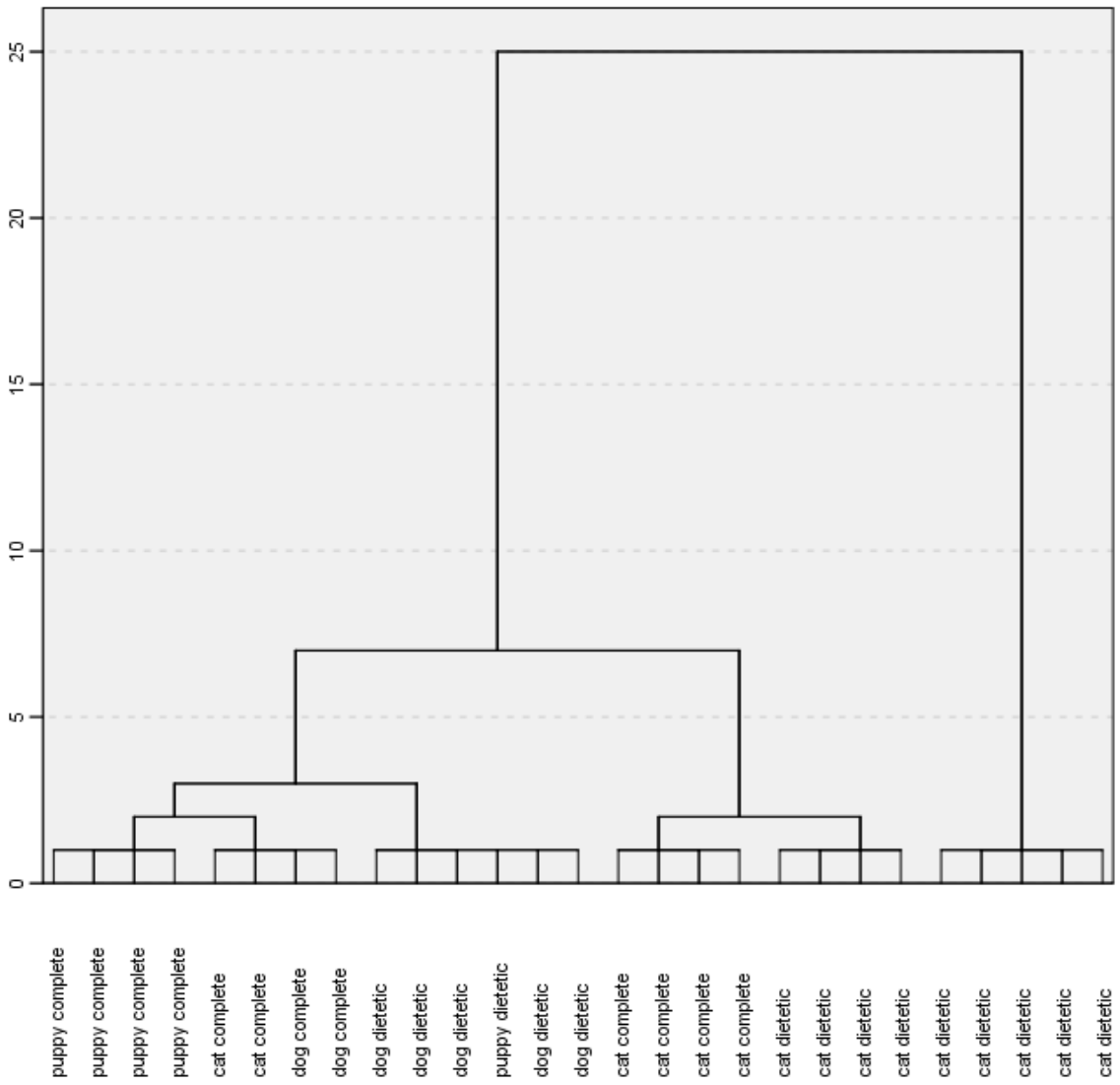

Figure 1b: Cluster analysis of the whole dataset.

Apparently, the sensors are not sensitive and/or specific enough to detect the volatile compounds that make it possible to distinguish between the different species, but EN was able (Figure 1b) to identify and isolate in the first cluster, puppy (four samples) and adult (2 samples) dog pet food, depending on complete and balance diets.

As a second step, the PCA was performed on the whole database according to presence or absence of fish and/or fish oil in pet foods (Figure 2a). The 81.89% of total data variability was explained by only the first two principal components (W1A and W5B).

EN was not able to distinguish samples according to these criteria. Few samples (with fish and/or fish oil) were located in an imaginary square, inside the group of pet food samples, which did not contain fish and/or fish oil.

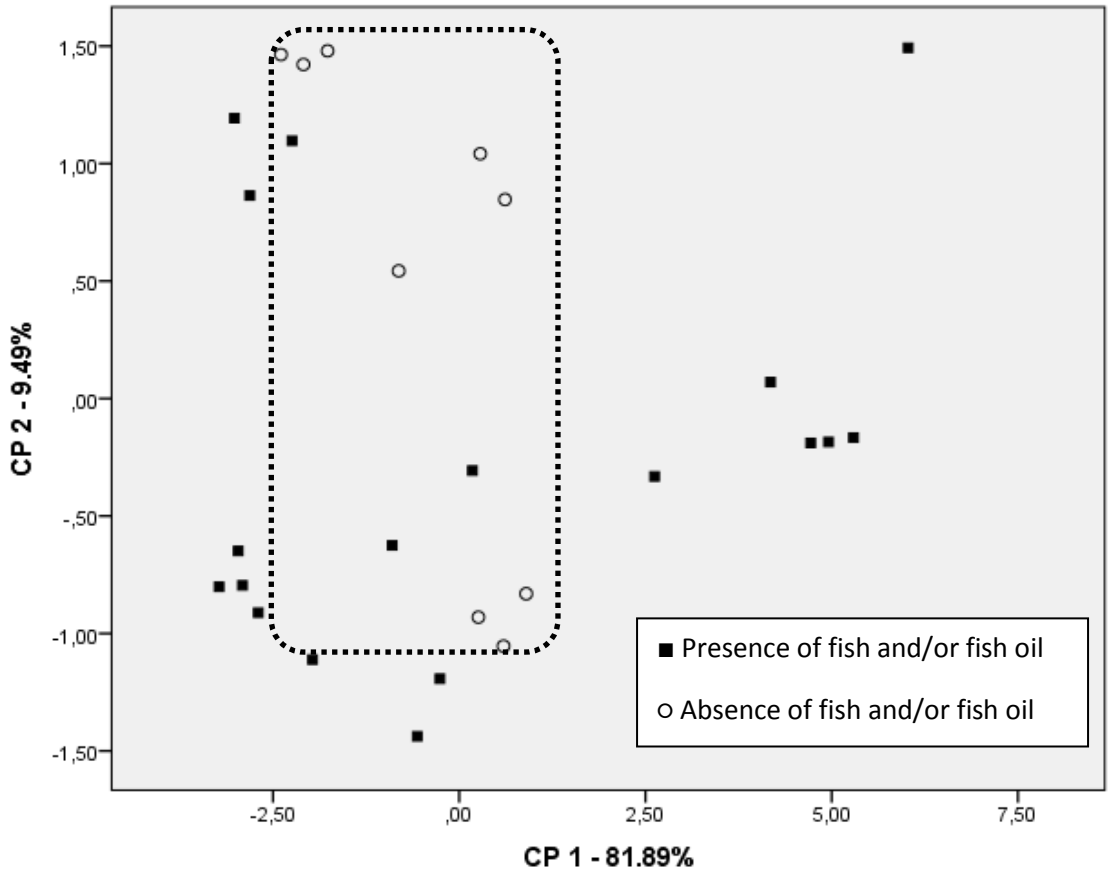

Figure 2a: Principal components analysis score plot of the whole data set. The distribution of two predetermined classes (presence or absence of fish and/ or fish oil in matrix) are indicated. An imaginary square reported the area of samples without fish and/or fish oil.

The dendrogram of CA formed according to presence or absence of fish and/or fish oil, is reported in Figure 2b. Three clusters were obtained. A first cluster constituted eleven samples with presence of fish or fish oil and three samples without, in dry pet foods. Six samples without and two samples with fish and/or fish oil belong to a second cluster.

Only samples (i.e. =5) of pet foods with presence of fish and/or fish oil belong to the last cluster. These results are in agreement with the results obtained with PCA shown in Figure 2a.

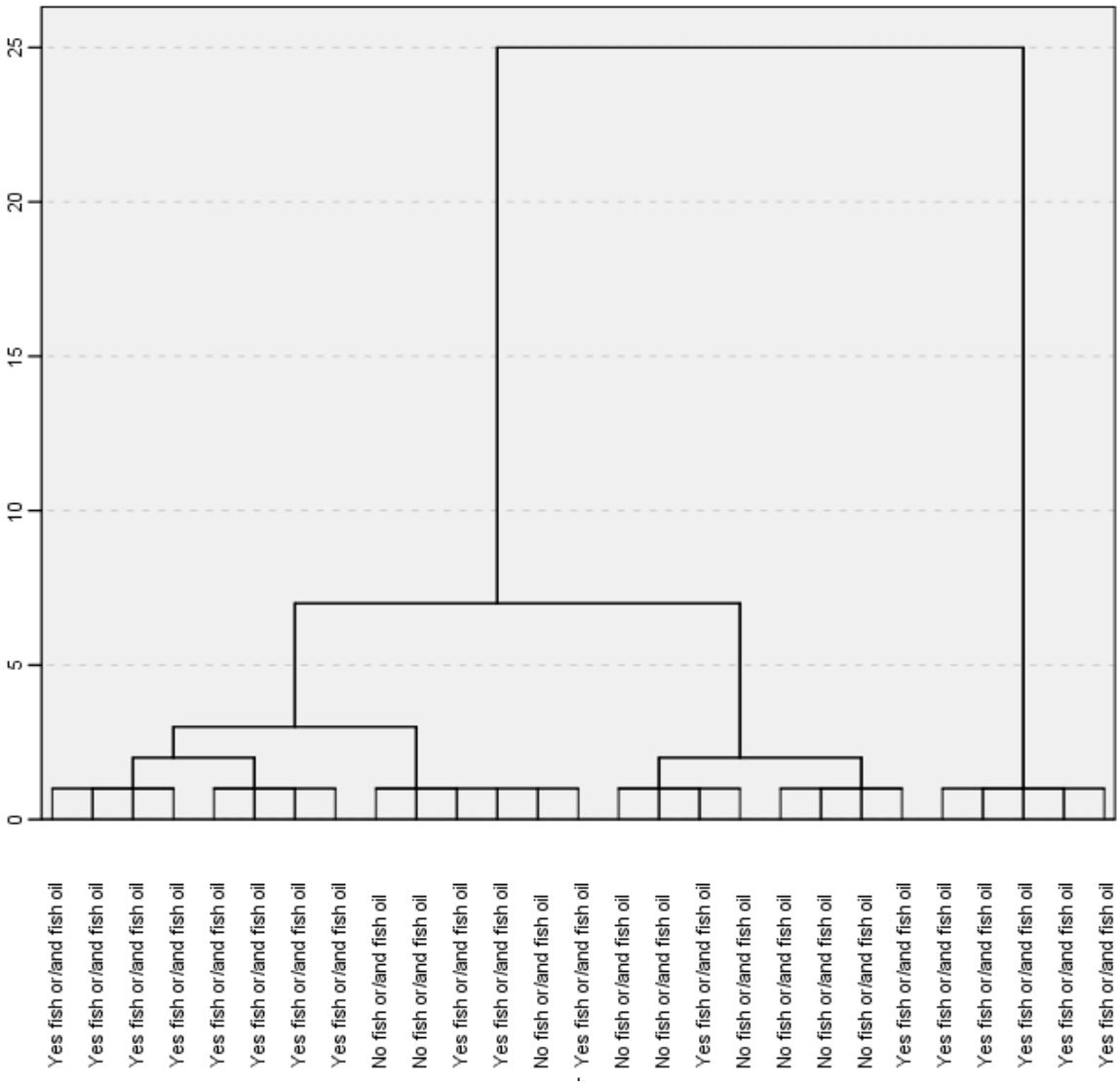

Figure 2b. Result Ward's Cluster Analysis of the whole data set among presence or absence of fish end/or fish oil in samples.

Finally, PC and cluster analysis were performed separately on a dataset of cat and dog sample. The EN was not able to discriminate cat samples according to the formula diets (Figure 3a). A single dietetic pet food sample was placed inside an imaginary square of complete and balance pet food samples. Figure 3a shows the principal component analysis score plot on the all cat samples (num=15) and that 87.50% of total data variability was explained by only two first principal components (W1A: 78.54% and W5B: 8.96%).

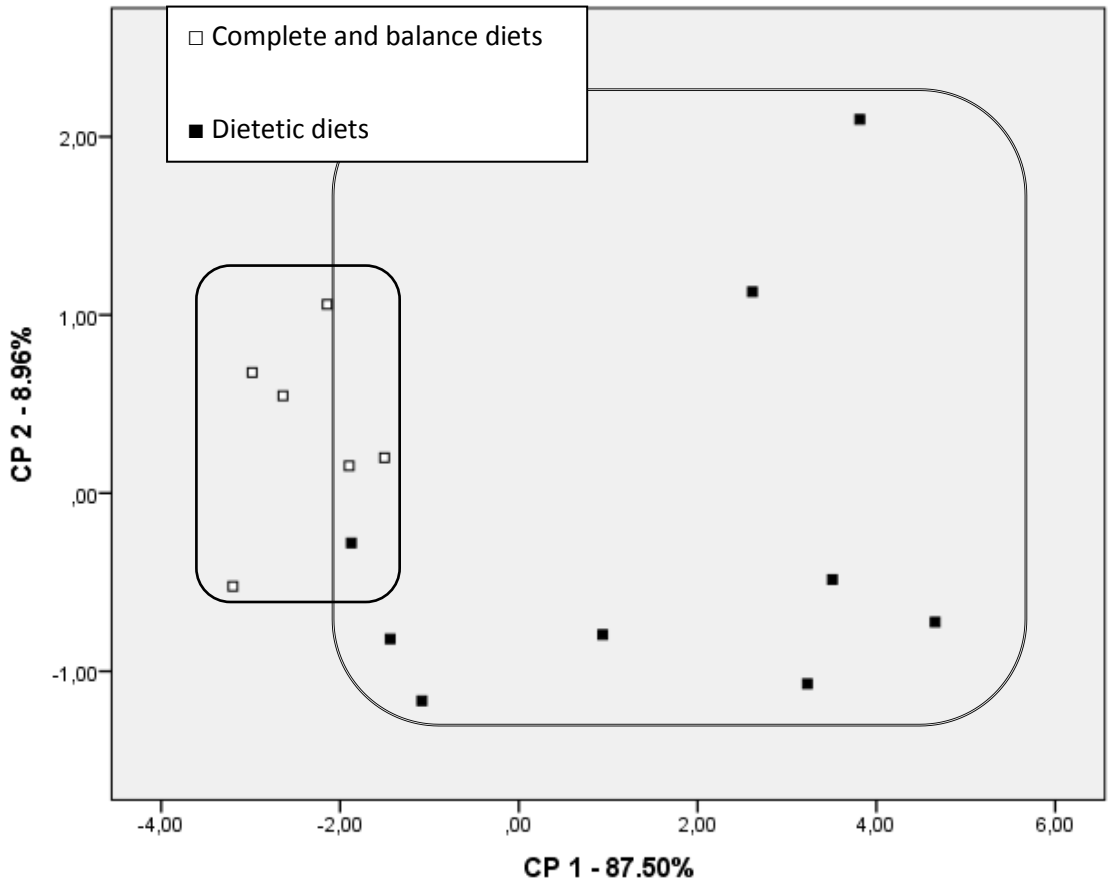

Figure 3a: Results of Principal components analysis of cat pet food samples. The distribution of two pre-determined classes (complete and dietetic diet) are indicated.

Clustering as shown in Figure 3b validated the PCA results. Three clusters were obtained. The six samples of complete and balanced diets, constituted a first cluster, while the nine samples of dietetic diets, constituted the second (four samples) and third (five samples) cluster.

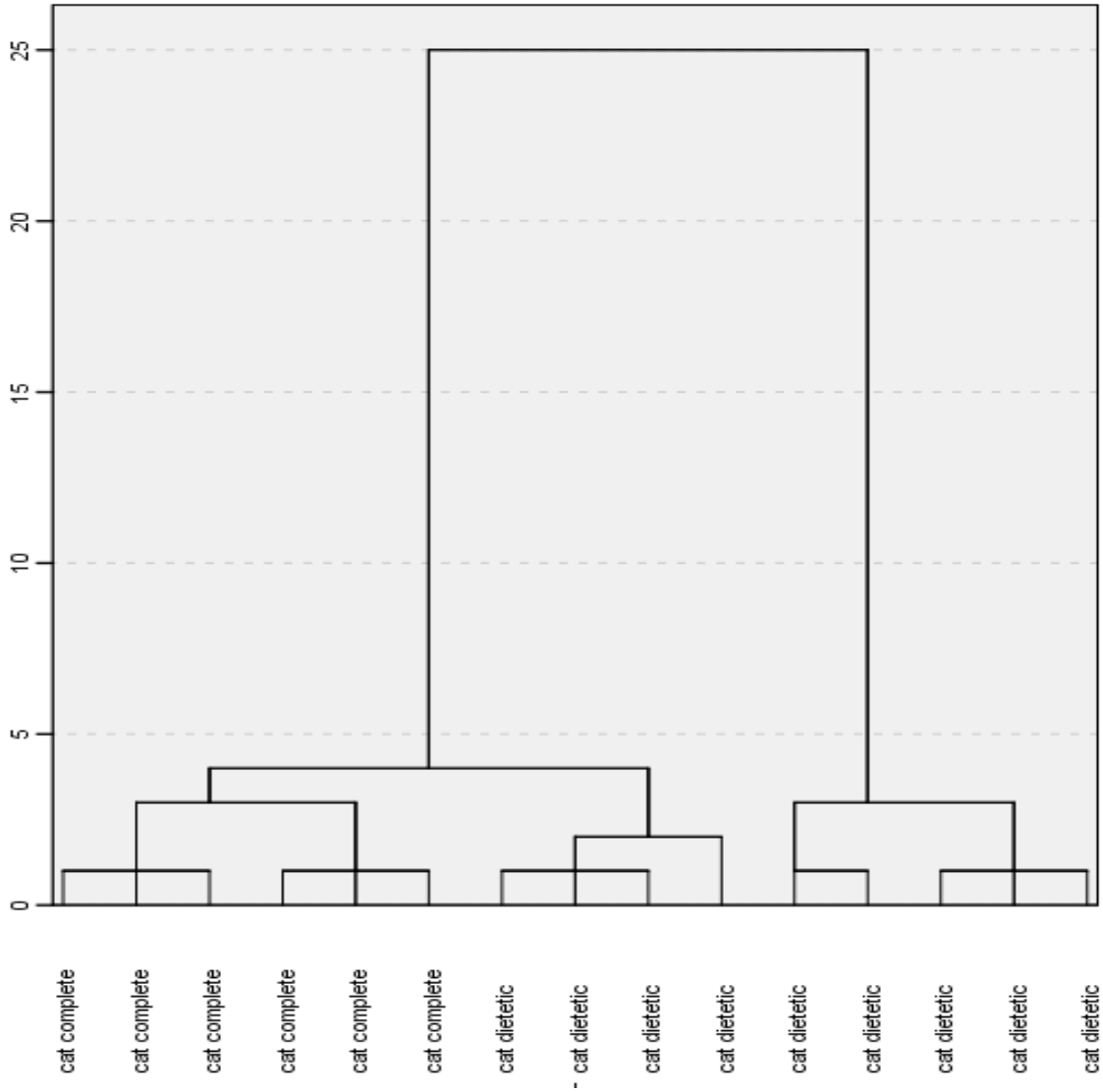

Figure 3b. Result Ward's Cluster Analysis of the cat samples data set among the formula diets.

The last PCA was performed on a dataset of dog pet food samples. Figure 4a represents the PCA score plot of dog pet food dataset (i.e. num= 12). PC1 (W1A) and PC2 (W5B) accounted for 56.10% and 26.13% of total variability, respectively. The EN was able to discriminate accurately the dog pet food samples, according to formula diets.

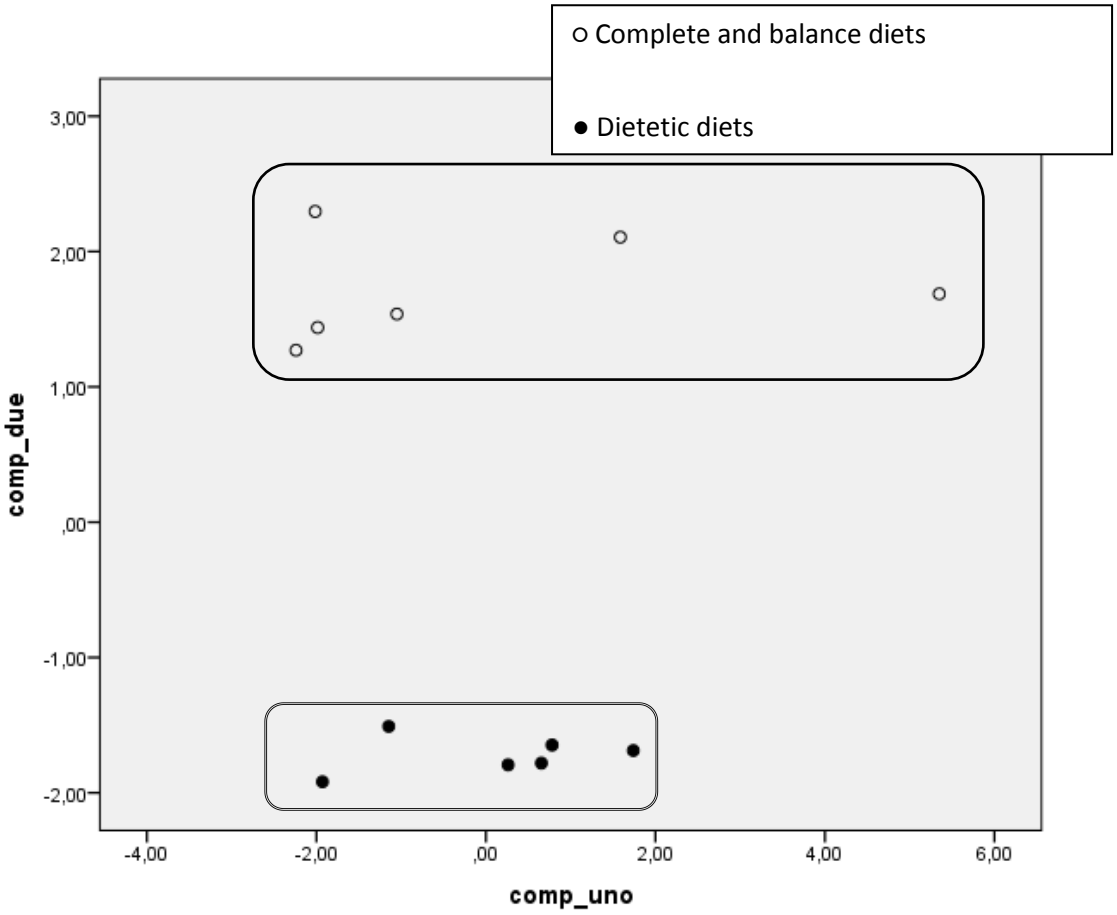

Figure 4a. Results of Principal components analysis of dog pet food samples. The distribution of two pre-determined classes (complete and dietetic diet) are indicated.

The dendrogram, reported in Figure 4b, shows the cluster obtained. The first cluster was composite of four complete pet foods (all puppy samples), six dietetic pet food samples belong to the second cluster and the last cluster showed two adult complete pet food samples. EN was able to recognize the different aroma between adult and puppy pet

food in complete and balance diet samples. The cluster analysis results are in agreement with the results obtained with PCA.

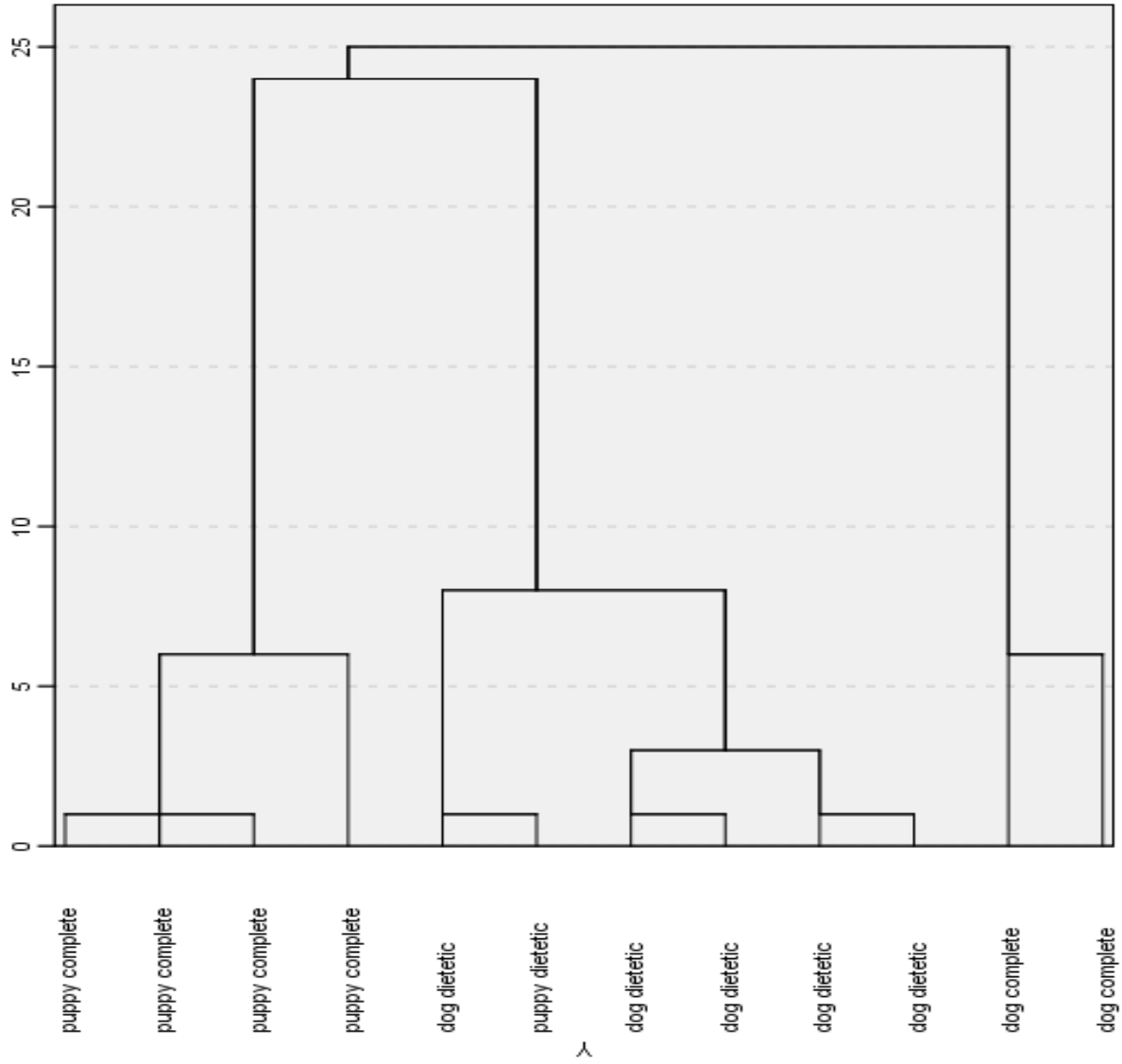

Figure 4b. Result Ward's Cluster Analysis of the dog samples data set among the formula diets.

Discussion

Sensory analysis has two main types of methods: analytical (descriptive and discrimination) and consumer (acceptance, preference, consumption and qualitative testing) (Meilgaard et al., 2006; Lawless and Heymann, 2010). The complexity of pet food aromas make them difficult to be characterized with conventional flavor analysis techniques. Typical methods for palatability measurement among dogs and cats include preference (using a two-bowl test) and acceptability testing (using a one-bowl test) (Griffin RW, 2003). The two-bowl test is based on: sniff or taste of sample, amount of food consumed and intake ratio. Sensory studies should aim at explaining acceptability and palatability based on sensory analysis and instrumental measurements. Koppel, (2014) reported the list of attributes (appearances, aroma and flavour and texture) used in descriptive sensory studies analysis of pet foods.

Denis et al. (1999) and Lin et al. (1998) looked at aroma properties of pet food. Liu et al. (1998) used descriptive sensory analysis to evaluate the sensory aromatic and appearance characteristics of extruded pet food. Dennis et al. (1999) determined the relationship among sensory aroma and appearance attributes, gas chromatography (GC), texture gas sensor measurements and palatability. These authors found that sensory analysis predicts palatability better than instrumental measurements, which may however provide a faster option for pet food industries.

Before the advent of EN the only possible instrumental analysis of "aroma" was the identification/quantification of individual chemical compounds after a separation step (e. g. GC-MS) (Koppel et al., 2013). The authors focused on the volatile compounds in dry dog foods (54 aromatic compounds were identified) and their possible influence on

sensory aromatic profile. They concluded that dry dog foods are products with complex odour characteristics.

The electronic nose does not distinguish each volatile substance, but express the global odour of a product (Gardner and Bartlett, 1994). Several studies were conducted on electronic nose, which was used for determining quality and safety of food, and feed processing.

PCA reduces the dimensionality in a data set, calculates a number of principal components having the greatest variance, eliminating the non-representative variables (*n* sensors) (Ampuero and Bosset, 2003) PCA have been used to explore and visualize e.nose (Ampuero and Bosset, 2003; Alasalvar et al., 2012; Di Donfrancesco et al., 2012; Papadopoulou et al., 2013). Clustering techniques have been widely used to explore and visualize e-nose data (Masoero et al., 2007; Alasalvar et al., 2012; Lin, et al., 2013; Torri et al., 2013). These algorithms are capable of generating a multi-level clustering using a tree structure known as dendrogram.

Results indicated that EN was not able to discriminate between samples according to species and formula diets. A misclassification of dog and cat pet food samples were obtained also in Éles et al., (2013). Although a non-complete correct classification was found, these authors concluded that EN and ET technology might be a promising tool to classify different commercial canned dog and cat food products.

Campagnoli et al. (2004, 2006) used the EN to detect the presence of PAP in feed samples. EN was able to discriminate different zoological classes in pure animal meal samples. In this case the electronic nose was able to distinguish among them the samples of different meals (poultry, feather, plasma and fish) (Campagnoli et al., 2006).

Otherwise, they found that EN was able to detect MBM at levels as low as 0.5% but not in samples with low MBM content (0.5%) e 5% of fishmeal. These samples were not discriminated from samples fortified with 5% of fishmeal solely. The author has supposed that fish flavor is able to mask MBM odour that was not discriminated by the electronic nose (Campagnoli et al., 2004). Cheli et al., (2007), carried out a study to evaluate electronic nose application in characterization of animal protein sources in dry dog pet food. The results demonstrated that EN was not able to discriminate the samples containing different meal (fish, poultry, mammalian and free meal) when all data set were analyzed with PCA. In previous study, Battaglia et al. (2014), the EN was able to discriminate the dog samples compared to those of cat when the two datasets were analyzed separately. Our results are in accordance with the previously reported data. The unambiguous identification of samples cannot be achieved using PCA in a whole dataset of pet food samples, because scatter diagrams were obtained. A discrimination of dataset by PCA was generated only in dog pet food samples.

All results confirm and highlight the blended nature of these products category and their variability. The pet food samples represent very heterogeneous and complex matrices to be analysed. Several authors conclude that pet food have a complex composition and a complex aroma (Di Donfrancesco et al., 2012, Éles et al., 2013; Koppel et al., 2013; Koppel, 2014).

Conclusion

This study demonstrated that electronic nose is a promising analytical approach to screening of the pet food. The unambiguous identification of samples cannot be achieved using PCA in a whole dataset of pet food samples, because scatter diagrams

were obtained. A discrimination of dataset by PCA was generated only in dog pet food samples. All results of cluster analysis are in agreement with all results obtained with PCA. We hypothesize that, the aroma profile of dog and cat pet foods are similar, in accordance with the results obtained in this preliminary work. Further study is also necessary to determine the real potential of the technique in this field. For instance, increasing the number of pet food samples and testing other independent samples, could be a way to test robustness of the models. This methodology could be applicable in evaluating aroma profile of different pet food when ingredients or palatability enhancers with low acceptability are incorporated.

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Chapter 4

Research interfaced with legislation

EU legislation update on feed and food related issues

Food and feed safety are issues that are of primary importance to producers and consumers. With the food and feed alerts of the 1990s (dioxin, BSE crisis, ecc.) the European Union (EU) has shown deficiencies in food regulation (Jensen and Sandøe, 2002). In order to ensure the safety of food, all aspects of the food production chain must be considered, from and including primary production and the production of animal feed up to and including sale or supply of food to the consumer (Cheli et al., 2013).

On 12 January 2000 the European Commission published the White Paper on Food Safety (COM 1999, 719 final). This Community measure sets out over 80 separate actions closely related to food safety with the scope to apply an integrated approach "from farm to table" covering all sectors of the food chain, including feed production, primary production, food processing, storage, transport and retail sale. As a result of the White Paper, the legislation has become more flexible, understandable and the degree of food safety has increased. Commission Regulation (EC) No. 178/2002 of the European Parliament and of the Council (European Commission, 2002) represents the operating phase of the White Paper. It is the milestone of the legislative structure for feed and food legislation. This regulation lays down the general principles and requirements of food law, establishes the European Food Safety Authority (EFSA), and lays down procedures of food safety with an integrated feed to food approach. The role of EFSA is

to assess the risk providing independent scientific advice on risks related to food and feed safety and support the EU risk managers to make their final decision. EFSA is not responsible for the laws regarding food safety or their application. The responsibility of the decision on food safety are: of the European Commission, European Parliament, Council of the European Union and competent national authorities of each Member State.

The current food safety policy is centred on a set of principles identified in the White Paper on Food Safety and in Commission Regulation (EC) No. 178/2002 (European Commission, 2002) to which a set of European Union Regulations, which represent "The hygiene package" followed: Commission Regulation (EC) No. 852/2004 (Commission Regulation, 2004a), Commission Regulation No. 853/2004 (Commission Regulation, 2004b) and Commission Regulation No. 854/2004(Commission Regulation, 2004c). Regulation No. 882/2004 (Commission Regulation, 2004d) is different in that it is concerned with official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.

After several food safety crises, which occurred within the EU, the public lost confidence in the quality of foods of animal origin. As a result the EU Commission, governments, industry, researchers, and farmers have been forced to pay serious attention to animal feedstuff production processes, thereby acknowledging that animal feed safety is an essential prerequisite for human food safety.

In this contest, two studies were conducted in order to provide a general frame of the EU feed and food legislation to give the reader a useful source of information.

First study: "EU legislation on feed related issues: an update"

The issue of food safety plays a role of primary importance for producers and consumers. As to market conditions, European Union (EU) countries, on average, import approximately 50% of their total food supply from other EU countries or from outside the EU. Food import shares vary strongly across EU members. Although food in Europe has probably never been safer, a number of issues have weakened the public's confidence on the quality and safety of foods of animal origin, and the methods of food production (Special Eurobarometer 354, 2010). Therefore, farmers, researchers, industry, and governments have been forced to pay serious attention to animal feedstuff production processes. In a review paper, Sapkota et al. (2007) emphasize that the ingredients used in animal feed are fundamentally important in terms of both the quality of the resulting food products and for the potential human health impacts associated with the animal-based food-production chain. Food legislation is vital to ensuring a fair act of authority, and is the guideline to address the food safety risks. The current food safety policy is centred on a set of principles identified in the White Paper on Food Safety (12 January 2000) and set out in Regulation (EC) 178/2002 (European Commission, 2002), which entered into force ten years ago. Decisions on food safety are the responsibility of the European Commission, the European Parliament, the Council of the European Union and the competent national authorities of each Member State. In this context, the European Food Safety Authority (EFSA) is not responsible for the laws regarding food safety or their application, but helps to ensure the safety of food and feed products. The role of EFSA is to assess the risks providing independent scientific advice on risks related to food and feed safety and to support the EU risk managers to make their final decisions. In recent years, besides health and consumer

protection issues, food legislation has acquired a fundamental role in the context of legal matters with a high economic and trade impact and implications. To meet the regulatory requirements, industry, food/feed official control professionals, and researchers in the field are increasingly faced with the continuous evolution of the regulatory aspects at EU and national level.

The aim of the paper (Cheli et al., 2013) is to address some aspects concerning feed-related issues, providing an update of the current EU Regulations and Directives. EU Regulations and Directives were classified into general food law, placing on the market and use of feed, official controls, sampling and analysis, hygiene, undesirable substances, additives, animal by-products, OGMs, feed intended for particular nutritional purposes, and organic production. To give the reader a rapid first approach to the topic of their interest, a synoptic presentation of all laws related to the above-mentioned topics is given, and the main points of each law, cited in conjunction with its effect on previous laws (replacement, modification, amendments, and main related acts), are reported in tables. In the reference list, the website of the main Regulations/Directives can be found where it is reported the consolidated versions of the main regulation.

Over the last 10–15 years several food safety crises occurred within the framework of the EU and weakened the public's confidence on the quality and wholesomeness of foods of animal origin. As a result, EU Commission, governments, industry, researchers, and farmers have been forced to pay serious attention to animal feedstuff production processes, thereby acknowledging that animal feed safety is an essential prerequisite for human food safety.

The target of the Community policies, in the development of food law, was to assure a high level of protection of human life and health. As a result a common EU framework basis for measures governing food and feed was developed in a non-discriminatory manner whether food or feed is traded in internal or international markets. The legislation in the field of feed/food chain is continuously evolving prompted by various factors, such as the dissemination of new scientific information, the activity of the EFSA's scientific committees, the changing of the epidemiological picture, and the availability of new analytical approaches. Globalization and the increased global trade associated with feed and food production pose the need for EU legislation to face with the different legislative framework of other countries. A lack of legislative harmonization is an important point to consider in a worldwide discussion regarding the managing risk and regulations in food security and safety governance.

Second study: "EU legislation on cereal safety: an update with a focus on mycotoxins".

Cereals are still by far the world's most important source of food, both for direct human consumption and, indirectly, as inputs to livestock production, although the use of cereals for bioethanol production is increasing (Cardona and Sànchez, 2007; Cassman and Liska, 2007; Lin and Tanaka, 2006; Luque et al., 2008; Pimentel et al., 2009). FAO's latest forecast for world cereal production in 2011 stands at nearly 2313 million tonnes, 3.3 per cent higher than in 2010 (FAO, 2011). Total EU-27 grain production forecast, including soft and durum wheat, barley, maize, rye, oats, triticale and other minor cereals, was 283 and 272 million tonnes for 2011 and 2012, respectively (from COCERAL, 2012/13-2011/12). The market share for cereals is approximately 51% for feed, 27% for food/human consumption, 19% for seed production and other uses, and 3% for bioethanol (Siegel and Babuscio, 2011). This ensures that such high volumes of products conform to adequate quality and safety standards and is a major undertaking of European Union legislation. Cereal contamination can be heterogeneous including biological, chemical and physical contaminants. The biological contamination comprising of microorganisms, natural occurring toxins (i.e. mycotoxins from fungi, phycotoxins from algae, toxins from cyanobacteria, histamine, vegetal alkaloids, etc.), and chemical contamination (i.e. agrochemicals as pesticides, plant growth regulators, and environmental contaminants as metals, dioxins, PCBs, etc.), get more concern for food and feed safety (Tang, Lu, Zhao, and Wang, 2009). In terms of food safety, among the most important risks associated to cereals' consumption are mycotoxins (Codex Alimentarius, 1991). The knowledge and control of the level and distribution of contaminants in cereals are a worldwide objective of producers, manufacturers, regulatory agencies and researchers due to the high economic and sanitary impact on

food and feed safety and human/animal health. Since it is impossible to fully eliminate the presence of undesirable substances and contaminants, maximum concentrations should be set at a strict level, which is reasonably achievable considering the risk related to food consumption. Consequently, an adequate surveillance and frequent checks are fundamental to assure quality and safety standards for raw materials destined for direct consumption or industrial processes. As a result, public authorities and regulatory agencies are pushing producers, manufacturers, and researchers to pay serious attention to food and feed production processes, and to develop comprehensive quality policies and management systems to improve food safety and try to improve consumer information so as to regain public confidence in food. To meet the regulatory requirements, industry, food/feed official control professionals, and researchers in the field have to face continuous change of the both EU and national regulatory functions .

This paper (Cheli et al., 2014) reviews the existing legislation associated with cereal safety, with a focus on mycotoxin contamination. Regulations and Directives were classified into the following topics: general food legislative framework, official controls (sampling and analysis), maximum levels for contaminants, prevention and reduction. To give the reader a rapid first approach to the topic of his interest, a synoptical presentation of all laws related to the above-mentioned topics is given, and the main points of each law, cited in conjunction with its effect on previous laws (repeal, modification, amendments, replacement, related acts), are reported in tables. Moreover, data regarding the worldwide occurrence of mycotoxins in cereals are reported.

In terms of food safety, this review focused on one of the most important risks associated with cereal consumption are mycotoxins. The legislation in the field of cereal and food safety is continuously changing prompted by different factors, such as the

availability of new scientific information, the activity of the EFSA's scientific committees, the results from the monitoring activity, and the availability of new analytical approaches. Globalisation and the increased global trade associated with cereal production pose the need for EU legislation to address the different legislative framework of other countries. A lack of a legislative harmonization is an important point to consider in a worldwide discussion regarding managing risk and regulations in cereal and food security and safety governance.

Database

To meet the regulatory requirements, industry, food/feed official control professionals and researchers in the field have to be increasingly faced with the continuous evolution of the regulatory aspects at EU and national level. Within the project "FoodCast - Forecasting and risk analysis in the markets of food commodities" a database, with the aim of providing a collection of references of normative documents as part of some issues related to food security, was developed.

Given the complexity of the problem, the database is not exhaustive, but is a platform and a starting point to provide an overview of European legislation and national laws: the aim of which is to guide readers in the vast and complex regulatory framework and to provide operational tools to help develop specific research. Through a system of links, the user will be directed to texts and sources making searching and selection of documents user friendly. This database is an update of the current European Union Regulation, Directives and related acts on feed related issues.

At present the database covers two main topics and indexes all related issues. The first topic "*Legislation on animal feed*" was divided into eleven sections: framework for the production of commercial feed; feed hygiene; dietetic feed; official controls / imports – sampling; additives / premixes in feed; animal waste and pathogens - framework to production of by-products animal and agri-food origin; food and feed genetically modified (OGM); food and feed from organic production; transmissible spongiform encephalopathies (TSEs) - Bans on feeding animal proteins to farmed; framework of medicated feed and intermediate products for production and marketing.

The second topic "*Legislation on contaminants in food*" was divided in six sections: community procedures for maximum levels contaminants in food; maximum levels of

contaminants, sampling and analysis for official control of the maximum levels of contaminants, recommendations for the prevention and reduction of contamination; recommendations on monitoring, safeguards.

For each issue is indicated the regulation at European Union and national level. A connection was created between our database and the EUR-Lex website (http://eurlex.europa.eu/en/index.htm) and at national leve with the Normattiva website (database of laws current in our Country) (http://www.normattiva.it/).

Also for the deepening sheets, our database was connected to the Europa website (summaries of European Union legislation) (http://europa.eu/legislation_summaries/index_en.htm).

Each European Union regulation is composed as follows: main regulation, the repeal, the amendment to, amended by and instruments cited. Clicking on the code law, you have direct access to European Union Law collected in EUR-Lex website.

The legislation on the feed and food chain is constantly changing. Using the database it is possible to manage the consolidation and updating of a legislative text. The database is available at: http://www.vespa.unimi.it/ecm/home/ricerca/gruppi-e-linee/alimentazione-e-nutrizione-animale/archivio-normativo.

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Conclusions

Over the last 10-15 years several food safety crises occurred within the EU and weakened the public's confidence in the quality of foods of animal origin. As a result, the European Union Commission, governments, industry, researchers and farmers have been forced focus their attention on animal feeding production processes, and thus acknowledge that animal feed safety is an essential prerequisite for human food safety (Cheli et al., 2012). As a consequence, the explicit and detailed formulation of the concept of feed and food safety and quality has given rise to worldwide legislation on traceability, control and labelling of both feed and food. The quality of a product is closely related to its physical and organoleptic properties. The use of the senses, such as smell and vision, are rapid field methods for feed quality control. Techniques based on the use of image analysis and electronic nose can provide quantization of quality and safety in real time.

The first two chapters were focused on the application of image analysis for the detection and characterization of constituents of animal origin in feedstuffs.

Microscopy combined with image analysis was used to identify morphometric descriptors of bone fragments as possible markers that can be used in routine analysis to distinguish between poultry and bovine lacunae.

The preliminary results described in the first chapter showed that bovine lacunae are much larger than poultry lacunae, which shows lacunae with an irregular shape. Values for all variable/descriptors measured in bovine have been reported to be higher than those in poultry, except for roundness (Campagnoli et al., 2009, Pinotti et al., 2009). In

previous studies (van Raamsdonk et al., 2012 and Pinotti et al., 2013) lacuna area (V1) and other primary descriptors have been considered keys markers in bone fragment identification.

In the second chapter, the decision support system ARIES (Animal Remains Identification Evaluation System) was used to characterize fish material in pet food. The "Identify it" module has proved to be a good support for the recognition of fish material, obtaining results with high values, ranging between 60% and 85% of identification confirmed.

The third chapter was focalized on the application of electronic nose technology for pet food analysis. The results obtained showed that the samples according to species, the formula diets and the presence or absence of fish and fish oil, were not always correctly classified. The unambiguous identification of samples cannot be achieved by principal component analysis in whole data set of pet food samples. A discrimination of data set by PCA was generated only in dog pet foods. All results of cluster analysis were in agreement with results obtained by PC analysis. In addition, the dataset of dog samples was divided into two main clusters according to adult, or puppy dog pet food samples. We can hypothesize that the aroma of puppy may be different from the aroma of adult pet food samples.

The legislation on the feed and food is constantly changing prompted by different factors: activity of the EFSA's scientific committees; the changing of the epidemiological picture; the availability of new analytical approaches. Using the

developed database, (chapter 4) you can manage the consolidation and updating of a legislative text.

In conclusion, the “artificial senses” can be considered a great example of how science and engineering can work together to produce something of real utility. Evidence suggests that these “fit to purpose” analytical methods are rapid, user-friendly, adaptable and coherent with the precision and accuracy level requested by the feed/food chain control and regulatory purpose, and useful for decision marking in the area of product quality. In the future, technological advances such as faster responding sensors, automated sampling system and unsupervised data analysis will make it possible to use these technologies on site for real monitoring and control of feedstuffs and industrial processes.

International and National Conferences

Abstract of poster or oral presentation

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MEDICAL MOLECULAR FARMING: RECOMBINANT PROTEINS FROM SALMONELLA TYPHIMURIUM EXPRESSED IN PLANT MODEL

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In livestock, plant-based vaccines could represent an innovative strategy for oral vaccination, especially to prevent infections by enteric pathogens; furthermore, edible vaccines could be an efficient way to reduce antibiotic treatments, according to EC Regulation 1831/2003. *Salmonella* spp. infections are responsible for serious human a nd animal diseases. In particular, *Salmonella typhimurium* strains are recognized as the most frequent cause of foodborne outbreak and the swine specie is the main reservoir. The first aim of this study was to isolate flgK flagellin gene, a principal component of bacterial flagella, recognized as virulence factor by the innate immune system. The gene encoding for flgK was isolated by a polymerase chain reaction (PCR) from genomic

DNA of a wild type *Salmonella typhimurium* strain, using specific oligonucleotides (including unique cloning sites for BamH I-5', Sac I-3'). The second aim of this study was to induce the seed-specific expression of flgK gene by the genomic transformation of tobacco plants. An overnight culture of EHA105 *Agrobacterium tumefaciens* strain, harboring the pBIpGLOB-flgK chimeric construct, was used for tobacco leaf disks infection. Regenerated plants and tobacco seeds were evaluated by PCR and immunoblotting techniques using established protocols. Obtained results showed that flgK gene can be incorporated into plant genome stably. Western blot analysis was carried out on all plants positive for flgK mRNA. flgK signals have been detected in all samples tested. By comparison with a positive control (flgK protein expressed by pET-system in BL21 *Escherichia coli* strain), the amount of flgK was estimated about 0.6 mg per gram of seeds. This amount corresponds to 0.3% of the total soluble proteins in tobacco seeds. In conclusion, flgK gene was specifically expressed, with the correct folding, in tobacco seeds. Tobacco plants represent an efficacious system for flgK flagellin expression and transformed tobacco seeds will be evaluated in vivo as a useful way for oral vaccination.

ANIMAL PROTEIN IN PET FOOD: MICROSCOPY, IMAGE ANALYSIS AND IN SILICO SUPPORT SYSTEM FOR CHARACTERISATION

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The aim of the present study was to evaluate the potential of microscopic analysis, image analysis and in silico decision support system for animal protein characterisation in pet food. Two complete feed for adult dogs, containing 40% and 31% of fish and by-products, were analysed according to Commission Regulation (EC) No 51/2013. In the sediment fraction 30 bone fragments were analysed with ARIES®DSS 0.7 (RIKILT, NL), whereas 180 bone lacunae were processed by image analysis software Image-Pro®Plus 7.0. A comparison with land animal literature data was also carried out. ARIES was a good support for the identification of fish material in pet food. Microscopic analysis, combined with image analysis, was used to integrate the qualitative analysis with bone material measurements. Both size and shape variables measured on fish bone lacunae were in line with reference values recorded in avian and mammalian materials. In spite of that, it has been also observed that fish measurements

were in a middle range when compared with land animal materials, i.e.: Avian<fish<mammalian. Thus it can be concluded that although, a considerable overlap between classes exists, fish material has some specific features detectable using an *in silico* support system (i.e. ARIES), moreover some of this characteristics can be confirmed by some measurements provided by an image analysis software**.**

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Electronic nose in commercial pet food evaluation

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The electronic nose sensor technology may represent a powerful tool in food and feed industry providing real time evaluation of quality and safety. The aim of this study was to evaluate the potential use of the electronic nose in pet food analysis. Twelve samples of commercial dry complete dog and cat pet food were used. In particular, the real potential of the electronic nose to discriminate 1) the concerning species (dog vs cat), 2) the pet food type (complete and balanced vs dietetic) and 3) the ingredient composition from label was evaluated. Each sample was tested in glass vials and the odour profile was determined by the 10 MOS (metal oxide semiconductor) sensors of the electronic nose PEN 2. Ten different descriptors, representing each sensor of the electronic nose, were used to characterise the odour of each sample. Data were analysed by Principal Component Analysis and Discriminant Analysis procedures using the Statgraphics Centurion XVI software. All analyses showed that the data variability was explained by

the two first principal components (corresponding to two electronic nose sensors: W1A-aromatic and W5B-broadrange) and was enough to explain more than 83.97% and 97.07% of total variability in odour pattern for PCA and DA, respectively. In the present study, the electronic nose did not correctly classified both categories dog and cat pet food and complete and balanced pet food, since two cat samples clustered close to dogs ones. By contrast, when dietetic pet food were considered, dog and cat samples were correctly classified. Pet food samples were not correctly classified according to the different ingredients reported in their label. Even though further studies using a wider set of samples are needed, results herein presented suggest that electronic nose can represent an effective tool in pet food industry in providing effective information about different formulated pet food and standardization of the aroma.

Publications

- Federica Cheli, **Debora Battaglia**, Luciano Pinotti and Antonella Baldi. State of the art in feedstuff analysis: a technique-oriented perspective. Journal of Agricultural and Food Chemistry.2012. 60(38), pp 9529-9542.

- Federica Cheli, Rossella Gallo, **Debora Battaglia**, Vittorio Dell'Orto. EU legislation on feed related issues: an update. Italian Journal of Animal Science. 2013. 12 (e48), pp 295-312.

- Federica Cheli, **Debora Battaglia**, Rossella Gallo, Vittorio Dell'Orto. EU legislation on cereal safety: an update with a focus on mycotoxins. Food Control. 2014. 37, pp 315-325. doi: 10.1016/j.foodcont.2013.09.059.

- **Debora Battaglia**, Federica Cheli. Metodi rapidi per l'analisi dei mangimi. Mangimi & Alimenti. N.5. Anno VI. Agosto-Settembre 2014. pp 16-19.

- **Debora Battaglia**, et al., **to be submitted** "Application of Electronic nose technology in pet food analysis" to Italian Journal of Animal Science.

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Review

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State of the Art in Feedstuff Analysis: A Technique-Oriented Perspective

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ABSTRACT: The need for global feed supply traceability, the high-throughput testing demands of feed industry, and regulatory enforcement drive the need for feed analysis and make extremely complex the issue of the control and evaluation of feed quality, safety, and functional properties, all of which contribute to the very high number of analyses that must be performed. Feed analysis, with respect to animal nutritional requirements, health, reproduction, and production, should be multianalytically approached. In addition to standard methods of chemical analysis, new methods for evaluation of feed composition and functional properties, authenticity, and safety have been developed. Requirements for new analytical methods emphasize performance, sensitivity, reliability, speed, simplified use, low cost for high volume, and routine assays. This review provides an overview of the most used and promising methods for feed analysis. The review is intentionally focused on the following techniques: classical chemical analysis; in situ and in vitro methods; analytical techniques coupled with chemometric tools (NIR and sensors); and cell-based bioassays. This review describes both the potential and limitations of each technique and discusses the challenges that need to be overcome to obtain validated and standardized methods of analysis for a complete and global feed evaluation and characterization.

KEYWORDS: *feed evaluation, chemical analysis, NIRS, sensors, cell-based bioassay*

INTRODUCTION

Feed analysis is an important topic in animal nutrition research. Once the nutrient requirements of the animal have been established, a diet that provides the correct balance of nutrients can be formulated if accurate information on the feedstuffs is available. Feed evaluation concerns the use of methods to describe animal feedstuffs with respect to their ability to sustain different types and levels of animal performance. Mainly in feed evaluation, emphasis is placed on determining specific chemical entities and the presence of contaminants and undesirable compounds, although other aspects such as digestibility, bioavailability, and functional properties of the feed are also of great importance. The need for global feed supply traceability, the high-throughput testing demand of the feed industry, and the regulatory enforcement drive the needs for feed analysis and make extremely complex the issue of the control and evaluation of feed quality, safety, and functional features and extremely high the number of analyses that must be performed.

Analytical methods are extremely important for the present and future of nutrition research. Without reliable and nutritionally significant methods, scientific advances are impeded. The early focus of feed analysis was to differentiate levels of feed components, assess purity, and exclude economic fraud. Later, through subsequent discoveries and further understandings of the roles of vitamins, minerals, proteins, lipids, and other essential nutrients, the need arose for the development of analytical methods that could link feed chemical composition and nutrition. In the past years, feed science has progressively evolved, prompted by different factors such as the improved safety issue and relevant changes in the European Union agricultural policy. Ensuring the safety of feed and food is an international mandate for processors and governmental agencies.[1] Therefore, requirements for new analytical laboratory instruments emphasize performance, sensitivity, reliability, speed and simplified use, rapidity, and low-cost for high-volume of routine analytical assays. More recently, European regulations have dealt with the topic of "nutritional and health claims". This means that, although analytical instruments have and will continue to have a fundamental role in the future of feed analysis, more biologically relevant analytical approaches are needed to evaluate feed functional properties. Therefore, feed analysis, with respect to animal nutritional requirements, health, reproduction, and production, should be multianalytically approached, according to a screening work conducted at different levels (Figure 1).

This review attempts to bridge gaps within analytical methods in a multianalytical approach to feed analysis, providing an overview of the most used and promising methods of analysis and their applications for feed composition, safety, and functional properties evaluation. Numerous techniques are used in this area and characterized mainly by two distinct approaches: instrumental and assays. The review is intentionally limited and focused on the following techniques: classical chemical analysis; in situ and in vitro methods; analytical techniques coupled with chemometric tools (NIR and sensors); and cell-based bioassays. The specific description of methodological approaches are outside the scope of this review, and readers are advised to consult other sources. This review describes both the potential and limitations of each

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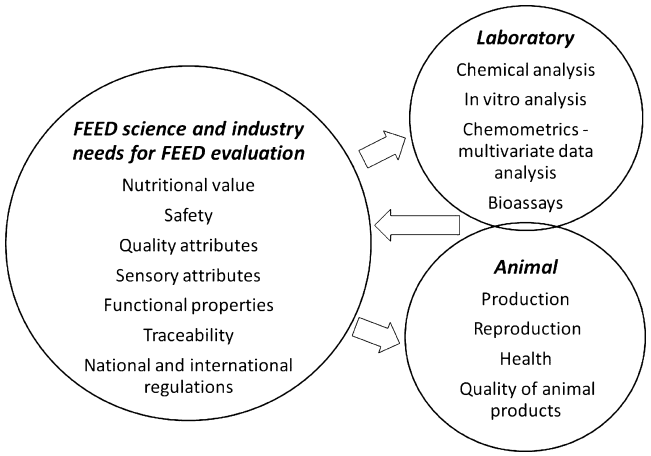

Figure 1. Feed evaluation: a multianalytical and multilevel approach.

method of analysis and discusses the challenges that need to be overcome to obtain validated and standardized methods of analysis for a complete and global feed evaluation and characterization.

"WET CHEMISTRY"

In the early 20th century, nearly all feed analyses were performed using "wet chemistry". In a typical chemical laboratory, analytical procedures, such as weighing, mixing, filtering, evaporation, distillation, or solvent extraction, for elemental analysis and isolation of organic substances, were developed. The main series of chemical analysis, which is performed by classical "wet chemistry" methods tailored for feedstuffs analysis, is called "proximate analysis" according to the Weende scheme: determination of dry matter (DM), organic matter, crude fiber (CF), crude protein (CP), crude fat, and ash content. The so-called Weende method for fiber estimation was not developed at Weende, but at Möglin after 1806. In the 1960s, the state of "wet chemistry" met a revolutionary approach with the research program of Peter Van Soest, which led to the detergent system of feed analysis. Over a number of years, within the scientific community, the Weende analysis system was replaced, at least for ruminants' feedstuffs, with the detergent system.[2] This replaces CF with neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin, and N-free extract with neutral detergent solubles (NDS). The detergent system was a cultural revolution as it made it possible to explain nutritional responses in terms of feed digestibility and intake. The nutritional rationale of the detergent system is based on the evaluation of feed factors with differences in digestibility. In this system, NDS constitutes the completely digestible fractions of carbohydrate and protein, as well as lipid and some ash, whereas NDF represents the structural fiber, which is only partially digestible, and lignin is the fraction of NDF that is totally indigestible. Milestones for the detergent system include the papers of Goering and Van Soest,[3] containing the first detailed description of the NDF method for laboratory use, Robertson and Van Soest,[4] in which a number of variants of NDF analysis were introduced, including the use of amylase, and Van Soest et al.,[5] presenting additional recommendations and changes, although no single method for all feed samples was recommended. Whereas NDF had largely replaced CF among scientists, CF is by no means an obsolete analysis, as it is still an approved method for legal trade use in many countries and must be reported in feedstuffs labels. The "wet chemistry" methods provide an exact description of the chemical composition of a feed. They do not give a complete estimate of feed nutritional value, which could be inferred by statistical association, and different prediction equations based on Weende and Van Soest chemical analysis were proposed.[6]

In the latter half of the 20th century, the use of "wet chemistry" analysis began to decline. Although many classical methods are still widely used today and they are officially recognized,[7] they are eventually substituted with instrumental methods that provide lowered detection limits, increase analyte specificity, simplify the use, reduce the cost, and display higher sample throughput and automation capabilities.

IN SITU AND IN VITRO METHODS

Despite the chemical analysis of feedstuffs, whatever methodology used is and will continue to be an invaluable tool for feed evaluation; it does not consider any animal–feed interactions such as palatability, the impact of diet composition on feed intake and digestibility, or the feed functional properties in a target animal. Knowledge of the gastrointestinal physiology, the dynamic processes of digestion and fermentation, and their influence on nutrient utilization oriented the research on feed evaluation techniques toward those that mimic the fate of feed nutrients in the gut. Therefore, in vivo and in vitro feed evaluation techniques were developed. In vivo measurements may provide the actual measure of digestibility as they evaluate the animal response to a dietary treatment. Traditionally, digestibility studies are conducted in sheep offered single feed at maintenance. Such trials must be conducted under highly controlled experimental conditions and cannot be carried out for all possible feeding situations found in practice. Therefore, a number of in situ and in vitro methods, which simulate the digestion process, were developed to estimate digestibility and degradability of feedstuffs, possibly taking into account the dynamic aspects of digestion, such as the transit time and the digestibility kinetics of dietary constituents. Specific reviews of the in vitro and in situ techniques are provided by Huntington and Givens,[8] Getachew et al.,[9] Ørskov,[10] and Mold.[11] Results indicate that these methodologies have several advantages and drawbacks to give an actual measure of feed digestibility and degradability. The in vitro technique of Tilley and Terry[12] is one of the milestones for the evaluation of ruminant feeds. The original methodology comprises two stages, representing the rumen and the lower digestive tract environment, respectively. The substrate is first fermented anaerobically in buffered rumen fluid and then subjected to an acid–pepsin incubation to digest undegraded plant cell and microbial protein. This method was extensively validated with in vivo results.[13] However, the main concern regarding this method is that it is an end-point measurement, not providing information on the kinetics of feed digestion. The use of enzymes, instead of rumen fluids, has appeared largely as a result of the increased availability of commercially produced enzymes.[14−18] This is an important step to standardize the methodology and for practical and ethical approaches, as enzymatic method does not require any fistulated animal. However, the enzymatic methods are used as end-point digestibility assays and therefore suffer from similar disadvantages as the original Tilley and Terry technique.

With regard to digestibility evaluation of feedstuffs, in vitro digestion methods have focused primarily on upper tract digestion. The need for accurate in vitro methods to study not only digestion but also fermentation in the hindgut has become increasingly more apparent and necessary, given the recently

recognized role of the hindgut in nutrition and gut health.[19,20] There are a number of detailed critical reviews of in vitro digestibility assays as applied to simple-stomached farm animals.[21−25] For monogastrics, models describing ileal or total tract digestion in pigs have been developed by Usry et al.,[26] Bastianelli et al.,[27] and Rivest et al.[28] A three-step multienzyme system, mimicking the digestion in the stomach, the small intestine, and the large intestine, was set up to predict organic matter digestibility in pigs.[29−31] This method isolates the hydrolysis process without taking into account specific processes of in vivo digestion such as endogenous secretions, absorption, and transit. Results indicate that this method could be an effective system to predict feed digestibility; however, as with the Tilley and Terry technique, this model obtains a single feed digestibility value and therefore suffers from similar disadvantages.

A dynamic methodological approach to obtain information regarding the extent and rate of digestion can be represented by different in situ techniques or the in vitro gas production technique. For an assessment of the impact of the rumen environment on degradation, the in situ technique based on the nylon bag technique represents an adequate and still valid methodology of analysis. The first description of the nylon bag technique was reported by Quins et al.[32] Thereafter, this technique was first standardized and provided with interpretative mathematical models that allowed protein ruminal degradation dynamics to be assessed.[33,34] With the use of this technique, degradation curves can be described for each feedstuff. Some concerns were raised regarding the equality of the bag environment with the rumen environment. The main problems concerning the use of the nylon bag techniques are related to a possible underestimation of feed degradation due to microbial contamination of the residues; overestimation of degradation due to excessive loss of particulate material; no possible application of this technique to finely ground feeds, entire or processed cereal grains and liquid feed; and interference with the presence of antinutritive factors in feed.[10] Moreover, the nylon bag technique requires the use of fistulated animals, with significant implications in terms of ethics and animal welfare, surgical skills, and facilities, availability of trained technicians, and high costs.

The close association between rumen fermentation and gas production has long been recognized, and the systems available for measuring gas production as a result of fermentation were reviewed by Getachew et al.[9] The history of the rumen fermentative gas-measuring technique started in the early 1940s.[35] The in vitro gas production technique (IVGPT) was originally developed as a means of obtaining information on the dynamics of rumen fermentation of feeds. Relationships between degradation and fermentative gas production can be used to evaluate the nutritional parameters of feedstuffs. A milestone of this technique is the paper of Menke et al.,[36] whose results indicate that there is a high correlation between in vitro gas production and in vivo apparent digestibility of feed. Since then, the in vitro gas production technique attracted the attention of researchers, and its role in feed evaluation research is still well recognized. An issue of *Animal Feed Science and Technology* was dedicated to this topic in 2005 (for a review, see Krishnamoorthy et al.[37] and Rymer et al.[38]). Kinetic estimates from gas production data can be transformed to inputs for mathematical models describing ruminant physiology. Results indicate that the gas production profiles are well related to in vivo measurements of rumen fermentation patterns, such as pH and the relative proportions of individual short-chain fatty acids.[39−41] Therefore, gas production technique may represent a powerful tool to run large batches simultaneously at low cost, to measure fermentation kinetics of soluble as well as insoluble fractions of feed, and to easily make relative comparisons among different feedstuffs, species, and interindividual variation and fermentation kinetics associated with these factors, using a minimum amount of sample.[42] Data from IVGPT may be useful when combined with other data, such as chemical composition of the substrate and/or its in vitro digestion, to act as inputs for more complex mathematical models that predict phenomena related to rumen function.[37] The concept at the base of the IVGPT is relatively simple; however, the related methodological issues are not trivial and include aspects related to different apparatus (e.g., syringes versus transducers) and the actual means of measuring gas production. Many factors may influence in vitro measured gas production profiles, such as the source and preparation of the inoculum and medium composition and preparation, as well as the preparation of the substrate. Therefore, from a practical perspective, there are a number of sources of variation in the evaluation of a gas production profile that must be considered to obtain a standardized procedure and comparable results. These include the apparatus, the species of inoculum donor, the animal diet, the rumen inoculum sampling site, and the preparation of both the rumen inoculum and the substrate. All of these methodological considerations were reviewed specifically by Rymer et al.,[38] to which the reader is directed. The gas production technique is capable of producing repeatable fermentation characteristics of a fermentation process with rumen microorganisms. However, for practical application in feed evaluation and for developing an extensive database of gas production profiles, comparable results must be obtained from different laboratories. van Gelder et al.[43] reported the results of ring tests to determine variation among laboratories of an automated gas production technique for measuring fermentation kinetics of feeds in the rumen. The authors concluded that, under standardized conditions (i.e., use of reference standards for variations due to atmospheric air pressure, different levels of calibration factors, or microbiological activity), acceptable repeatability can be obtained among laboratories using the same apparatus. Rymer et al.[44] calculated the variation among laboratories and between manual and automated techniques. The authors concluded that, although the methods of measuring pressure are sources of variation in the gas production profile estimation, the use of appropriate mathematical models, to account for differences in apparatus and laboratory, can permit standardization of data among laboratories so that gas production profiles of feeds may be comparable.

Although the IVGPT was primarily developed to evaluate ruminant feedstuffs, its application to hindgut fermentation of monogastric animals is gaining acceptance.[42,45,46] Like the rumen, the large intestine of simple-stomached animals is essentially a fermentation chamber where material is degraded by bacteria.[47] A cumulative gas production technique was used to test a range of different products to assess the end-products of in vitro fermentation in pigs.[19,46,48] The gas production technique was used after predigestion with pepsin and pancreatic enzymes to determine fermentation characteristics of organic matter and proteins in the large intestine of pigs.[45,49] An area of interest and potential application of IVGPT can be the evaluation of the health-promoting effects of feed

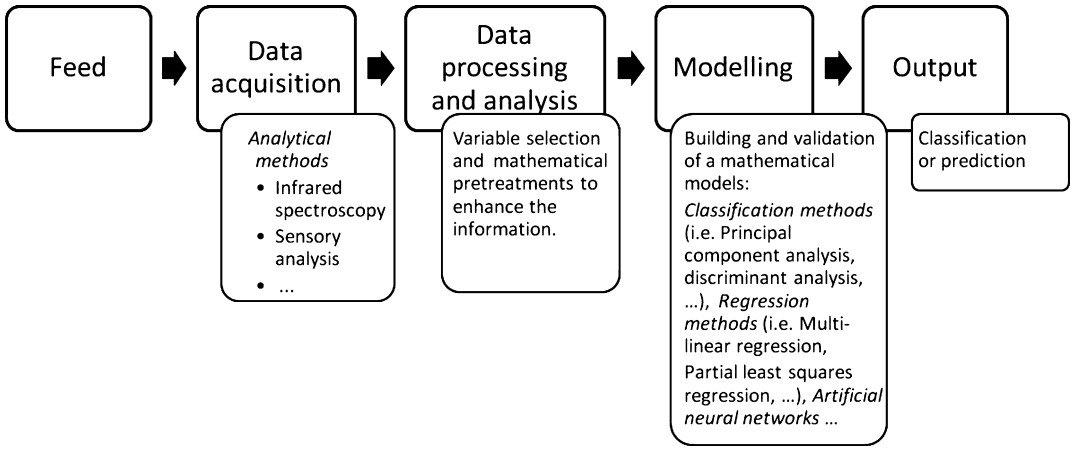

Figure 2. Analytical techniques coupled with chemometric tools: diagram of the procedure for feed analysis.

ingredients.[19] Particularly, great advantages may derive from the standardization of this technique in the identification and characterization of probiotics and prebiotics as potential feed additives.[50] With the aim to develop a standardized IVGPT procedure for single-stomached animals, to obtain comparable results, the most critical point is the choice of the best inoculum. The choice of the inoculum is based on the purpose of the evaluation. Given that microbial populations vary between areas of the gastrointestinal tract, from a theoretical point of view it would be better to choose a source of microorganisms from the gastrointestinal area under investigation and the appropriate animal. However, the use of fecal samples as inocula is most reported in the literature, as they are readily available and provide a source material for the major groups of intestinal bacteria. It was questioned whether the use of feces as an inoculum is truly representative of the intestinal microflora. In a detailed microbial study, Moore et al.[51] concluded that the composition of the bacterial flora of feces resembled that of the large intestine and that freshly passed feces collected under strictly anaerobic conditions could be considered as representative of the large-intestinal flora. In terms of VFA and cumulative gas production, some differences were found between inocula from the cecum, midcolon, and feces,[52] but it was concluded that feces did indeed give a reasonable estimate of the activity in the intestinal tract.

In conclusion, with a careful selection and correct application, the in situ and in vitro methods for feed analysis represent a relevant and powerful tool in feed research. However, they seem still far from a wide application as routine methods of analysis, but will play an increasingly important role in future animal production systems. These techniques may be used to answer many biological questions regarding feed impact on animal health and production and animal/feed environmental impacts.

■ ANALYTICAL TECHNIQUES COUPLED WITH CHEMOMETRIC TOOLS

The described methodological approaches are fundamental tools for feed evaluation. However, these techniques are destructive, slow, relatively expensive, and time-consuming, require highly skilled operators, and are not easily adapted to real-time feedstuff analysis and to an out-of-laboratory use or online monitoring. Therefore, they are not effective enough with respect to the increasing analytical demand of the feed industry. To meet these needs, a great number of noninvasive and nondestructive instrumental techniques have been developed for the determination of feed composition, quality, and safety. In this context, near-infrared (NIR) spectroscopy and sensor analysis are advantageous for many applications, because they can provide rapid, nondestructive, and particularly multiparametric measurements. A large number of samples can be analyzed in a relatively short time, and a great amount of information (variables or features) can be collected. This leads to the availability of multivariate data matrices, which require the use of chemometrics, that is, the use of mathematical and statistical techniques for efficiently extracting quantitative, qualitative, or structural information from the data.[53] This is a completely different analytical approach compared to classical chemical and in vitro analysis, as the analysis of data and the validation of the calibration curves are integral parts of the analysis. The selection of a training and a test data set, although sometimes a third "tuning" set may be used, the discriminating variable selection, the use of classification and regression methods, and the validation of the models are the main steps for a qualitative and quantitative application in the field of feed analysis (Figure 2). In feed analysis, where sampling uncertainty dominates in the final uncertainty of the result, the adoption of these rapid, low-cost, but high sample throughput analytical approaches, able to test a high number of samples, can represent a more efficient strategy than the choice of expensive, more specific, and complex analytical methods.[54] Moreover, these techniques are also suitable for at-line and on-/in-line process control, providing invaluable tools alleviating important problems in processing and distribution of feed and feed products.

NIR Spectroscopy. Nowadays, spectroscopic techniques are widely used for the analysis of feedstuffs to replace the "wet chemistry" techniques. NIR spectroscopy is routinely used in the feed industry as a quality assurance tool to determine feedstuff composition. The successful application of the NIR technology in the analytical field depends on a series of equally relevant factors. Most of the advantages of NIR spectroscopy come from the possibility of using intact samples with minimal or no sample preparation. Moreover, it provides rapid analysis and has the potential to run multiple tests on a single sample, with a low environmental impact, as no harmful chemicals are used. Coblentz, in 1900, was the first researcher to obtain absorbance spectra of pure substances and verified their usefulness for the identification of organic functional groups.[55] Since then, instrumental infrared spectroscopy analysis has

been continuously evolving, as can be deduced by comparing the old mid-IR equipment manufactured in the 1950s and based on dispersive monochromators with the present customized NIR instrumentation. The incorporation of the Fourier transform (FT) technique together with the interferometric spectrometers into the mid-IR instruments has increased the use of this technique in food analysis.[56] Almost all of the research and the use of NIR spectroscopy for feed analysis started with the work of Karl Norris on the determination of moisture in agricultural products by NIR in 1965.[57] The use of NIR spectroscopy to evaluate forage quality was first reported by the same author in 1976.[58] The 1980s represented the "boom" of this technique, with thousands of published papers dealing with NIR applications to different feeds and forages, attesting to the wide acceptance of this technique. In 1993, the first issue of the *Journal of Near Infrared Spectroscopy*, the only journal dedicated to NIR spectroscopy, was published. This journal, in 1996, republished, in a special issue honoring Karl Norris, the first results of his research. The applications of NIR spectroscopy in feedstuff analysis is huge in research, the feed industry, and field conditions. The use of NIR spectral information for analytical purposes relies on the multivariate approach for calibration. Currently, NIR spectroscopy is the analytical technique which most applies chemometrics. Due to these characteristics, NIR spectroscopy can give rapid answers to evaluate the composition of raw material and compound feedstuffs, to predict digestibility and voluntary intake of feedstuffs and forages, and, more recently, to evaluate the presence of prohibited and undesirable substances. For many years, NIR spectroscopy has been used for routine quality control in feed mills and nutritional feed analytical services as a rapid method in feed, forage, and food analysis for the determination of chemical constituents and other parameters of nutritional value with a precision comparable to that of the official methods of analysis, therefore enabling compliance with regulations concerning the production and circulation of raw materials in terms of the quantitative determination of chemical composition (for reviews, see refs 59−61). At present, NIR spectroscopy is the only technique that allows the analysis of large-scale samples and consistently taking decisions in real time.[62] A more limited number of publications concerning the use of NIR spectroscopy with compound feedstuffs was reported. This is because the considerable heterogeneity of these samples was supposed to require a great number of samples and fine milling to perform calibrations that would be robust in routine use. However, recently, several studies have demonstrated that NIR spectroscopy is a reliable method able to predict the chemical composition and nutritional value of compound feedstuffs, too.[63−69] The ability of NIR spectroscopy to predict the chemical and ingredient composition in compound feeds, not only at the end of the production process, but also at the mixing stage is of great interest for practical application in the feed industry.[69,70] This is a critical point in feed manufacture to ensure that a product meets the specifications for chemical and ingredient labeling. In the field of forage analysis, NIR spectroscopy has been used to evaluate chemical composition and chemical fermentative pattern and to predict in vivo digestibility and voluntary intake.[59,71−77]

A topic that still needs more studies is the possibility to avoid completely the sample preparation step (i.e., grinding or drying). This could further improve NIR spectroscopy potential to increase the speed of analysis and definitely promote NIR technology as a rapid analytical method for inspecting the huge volumes of the compound feedstuffs circulating across the world and ensuring compliance with regulations. Pérez-Marın et al.[69] presented the results of a study to obtain NIR calibrations for the instantaneous prediction of chemical composition of ground and unground commercial compound feedstuffs. They obtained accurate calibrations for moisture, CP, CF, fat, and ash, with excellent capacity for quality control of both ground and unground compound feedstuff samples. The possibility to avoid the sample preparation step in forage silage analysis by NIR spectroscopy is another important topic. Cozzolino et al.[78] concluded that NIR spectroscopy might be a suitable method to predict DM, CP, and ADF on wet whole maize silage samples. Park et al.[76] found that the freezing and thawing processes, in general, lower NIR spectroscopy prediction values of fresh silage. However, these differences with reference to the fresh silage predictions are within acceptable calibration errors for potential metabolic intake, pH, lactic acid, total acids, NDF, and ADF evaluation. All results confirm that the tedious and time-consuming step of feed milling or drying can be avoided, enabling a rapid turnaround in both the feed industry and farm advisory and quality control systems.

Recently, NIR spectroscopy applications for the detection of prohibited substances and contaminants were reported, suggesting that NIR can be a promising tool for feed safety and traceability evaluation, too. NIR calibrations were developed for the instantaneous and simultaneous prediction of the animal species composition of constituents of animal origin, confirming the potential of NIR technology in research and in routine quality control.[79] In this field, an analytical approach that combines spectroscopy techniques with the analytical advantages of microscopy (NIRM) was proposed as an alternative technology to detect and quantify banned ingredients in feedstuffs.[80−84] Results confirm that it is possible to reliably detect the presence of animal byproducts (terrestrial meals and fish meals) in complete feed. However, the authors conclude that further work is needed to develop an accurate quantitative method. With regard to feed safety issues, applications of NIR spectroscopy analysis for fungi and mycotoxin detection in cereals were reported, demonstrating that NIRS can be a workable screening tool.[85−87] NIR and mid-infrared spectroscopy with attenuated total reflection (IR/ATR and FT-IR/ATR) have been used to rapidly detect the presence of fungal infection and estimate the presence of fungal metabolites and mycotoxins in naturally and artificially contaminated products.[88−93] The development and establishment of fast, nondestructive, and actually applicable methods in a screening control procedure for the evaluation of undesirable substances content in feed and food must consider the maximum levels or guidance values established by the EU. De Girolamo et al.[92] reported evidence that FT-NIR analysis may be suitable for the determination of deoxynivalenol (DON) in unprocessed wheat at levels far below the DON maximum permitted limits set for feed and food by the European Commission.[94,95] Moreover, Petterson and Aberg[90] demonstrated that it may be possible to develop regression models for the prediction of DON in wheat kernels at levels just above the proposed EU maximal limits in wheat flour. Fernández-Ibáñez et al.[93] found that NIR spectroscopy is successfully correlated with traditional quality methods commonly used to detect aflatoxin B_1 (AFB_1) in maize and barley. The authors highlighted the potential of NIRS methodology as a fast and nondestructive tool for the detection

of AFB_1 at the 20 ppb level. This is an important result for feed analysis as the maximum AFB_1 level allowed in all feed materials and complete feedstuffs for cattle, sheep, and goats is 20 ppb with the exceptions of complete feedstuffs for dairy animals (5 ppb) and complete feedstuffs for calves and lambs (10 ppb).[96] Validation results showed no false negatives, minimizing the risk of including contaminated samples in the feed and food chain when the proposed method is applied. Improvements of the classification performance of FT-IR/ATR analysis can be achieved by optimizing sample preparation procedure and applying particle size analysis to samples.[97]

In conclusion, NIR spectroscopy is a powerful tool in the feed industry and on farms, regarding quality and safety control programs. Versatile NIR analyzers with different sample presentation attachments and large analysis windows, allowing the analysis of unground material, are now commercially available. In the field of feed safety, a number of challenges remain for the application of NIR methodology in terms of improving the robustness of calibration curves for the development of fully quantitative methods ensuring compliance with legal limits and indications.

Sensor Array Technology. Currently, sensor technology attracts increasing attention as an evolution of the conventional analytical techniques in the feed and food industries. Whatever the type of sensor, it is composed of a sensing element "recognizing" the analyte and an analytical signal converter, which transforms a characteristic parameter of a chemical or biochemical reaction to a physical parameter (Figure 3).

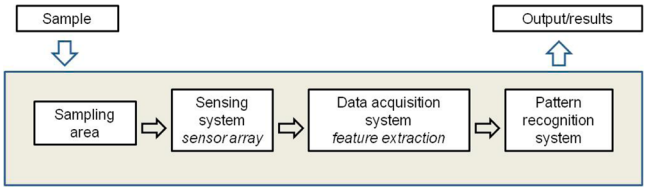

Figure 3. General configuration of the sensor array technology.

Integration of the sensing elements and the converter within a single analytical device represents a novel approach to analytical practice, rather than a formal procedure. A huge variety of sensor devices was developed for food analysis (Table 1), and their characteristics, properties, and use are specifically reviewed by Deisingh et al.[98] and Van Dorst et al.,[99] to which the reader is directed. A subdivision of the sensor grouping is the biosensor, which incorporates a biological sensing element positioned closed to the transducer to give a reagentless sensing system for a target analyte.[100]

For the very challenging field of feed analysis, the potential applications of low-selective sensors and the use of advanced mathematical procedures for signal processing, based on pattern recognition and/or multivariate analysis, are increasingly being employed and represent the most promising potential tools for rapid, nondestructive analysis of feed for quality evaluation purposes.[101] The application of an array of nonspecific or low-selective sensors in feed and food analysis is the base of the electronic nose and tongue, used for the analysis of gases and liquids, respectively. In 1982, Persaud and Dodd introduced the concept of artificial olfaction.[102] In 1994, Gardner and Bartlett introduced the term "electronic nose" for the first time.[103] At the end of the 1990s, the term electronic tongue was coined.[104,105] The development of the first commercial devices and research applications in the food industry began in the 1990s.

The rationale for application of electronic noses and tongues to the analysis of compounds responsible for taste and smell is based on an analogy to the biological organization of the olfactory and taste systems in mammals. Electronic noses and tongues are "multisensor systems" for gas and liquid analyses, based on chemical sensor arrays and patter recognition,[103,106] capable of identifying simple or complex taste and aromatic profiles responsible for the quality of a given product. Quality is a key factor for the modern feed industry because the high quality of a product is the basis for success in today's highly competitive market. The electronic nose and tongue instruments are mainly used in the food and pharmaceutical industries.[101,107−109] The majority of publications of foodstuff analysis by electronic nose instruments are related to meat and fish. The main areas of interest were the use of electronic noses to detect sensory quality, shelf-life spoilage, off-flavor, taints, and authenticity through the screening of volatile changes.[98,107] Electronic noses usually provide for the recognition and classification of the gas mixtures and in some cases for semiquantitative analysis, whereas electronic tongues are capable of performing both recognition of complex liquids and quantifications of the components.[110] The use of electronic noses to monitor dairy products in terms of quality and production processes, aging, or spoilage was reported.[111−114] However, the number of studies focused on dairy products is still limited, probably due to the complexity of their matrices.

Of great interest, for practical application in the feed and food industries, is the application of electronic noses for the detection of undesirable substances and contaminants. Results suggest that electronic noses can be a promising tool for feed and food safety and traceability evaluation.[115] An electronic nose was used for evaluating the presence of constituents of animal origin in animal feed.[116] Preliminary results confirm the potential of electronic nose technology to identify the presence of constituents of animal origin in feedstuffs, although there is still a need to implement the robustness of the models and expand the potential discrimination properties of the olfactometric analysis, especially when constituents of different animal origin co-occur in feedstuffs. In the field of feed safety, applications of electronic nose analysis for fungi and mycotoxin detection in cereals were reported, demonstrating that electronic noses can be a workable screening tool for the mycological quality of grains. Different species of molds, yeast, and bacteria can be discriminated with the electronic nose and tongue.[110] The ability of the electronic nose to differentiate grains and bakery products clean or contaminated (naturally or artificially infected) with different mold species was demonstrated.[117−121] Detection and differentiation between mycotoxigenic and nonmycotoxigenic strains of *Fusarium* spp. using volatile production profiles evaluated by electronic noses were also reported.[118,122−125] Further developments of studies carried out with the electronic nose technology were made to evaluate the possibility of using fungal volatile metabolites as indicators of mycotoxin contamination.[126] As for the NIR, the use of an electronic nose as a screening tool for the evaluation of the presence of undesirable substances in feed must consider the maximum levels or guidance values established by the EU. Results from a study carried out on naturally contaminated barley samples showed that it was possible to use volatile compounds to predict whether the OTA level in samples was below or above 5 μg/kg.[127] Electronic nose analysis enabled

Table 1. Main Sensor Devices in Feed/Food Analysis

category	sensing material	examples of applications in feed/food analysis
metal oxide semiconductors (MOS)	metal oxide semiconducting film (metal coating may be zinc oxide, tin dioxide, titanium dioxide, iron(III) oxide, nickel oxide, or cobalt oxide)	classification, authentication and recognition of feed/food VOC[a]-based profiling for microbial and mold spoilage feed/food quality control
conducting polymer sensors	polyaniline, polypyrrole, and polythiophene	VOC-based profiling for feed/food spoilage packaging smell recognition of taste substances feed/food quality control
acoustic wave sensors	chromatographic stationary phases and polymers $LiTaO_3$ substrate without chemical coating	VOC detection recognition of taste substances feed/food control
MOSFET/ISFET sensors[b]	catalytic metal gate (covered with Pd, Pt, Rh)/gate covered by sensitive layer (plasticized polymers doped by ionophores)	classification, authentication, and recognition of feed/food VOC-based profiling for feed/food spoilage food quality control
optical	fluorescent dyes, metalloporphyrins	VOC-based metabolic profiling for feed/food spoilage
potenziometric sensors	plasticized organic polymers modified by ionophore noble metals	taste assessment discrimination, classification, and authentication of liquid food
voltammetric sensors	different type of metals for the working electrodes electrodes chemically modified with electroactive substances	taste assessment discrimination, classification, and authentication of liquid food
biosensors	biological or biologically derived sensing element (such as an enzyme, antibody, microbe, or DNA)	detection of pathogens and toxic metabolites routine analytical measurement of vitamins of drug residues

[a]Volatile organic compounds. [b]Metal oxide semiconductor field-effect transistor/ion sensitive field-effect transistor.

correct classification of naturally contaminated maize and wheat with aflatoxins and DON, respectively.[128−132] Campagnoli et al.[133] reported that an electronic nose allowed the classification of naturally contaminated wheat samples on the basis of DON content according to the legislation limits.

In conclusion, one of the most important aspects of electronic senses is that there is the possibility of performing tasks traditionally entrusted to human senses with the objectivity and repeatability of calibrated instruments. In this sense, electronic sense technology is a powerful tool in the feed industry and possibly on farms with regard to quality and safety control programs. One of the main advantages is the possibility to approach a complex problem in a one-step analysis, with easy or no sample preparation. The future challenge of artificial senses will be the multisensor data fusion for characterization of feed quality and safety. Sensors can work collectively. In this direction there is already evidence indicating that combinations of electronic nose/machine vision and electronic nose/electronic tongue may enhance the prediction properties for both qualitative and quantitative analyses.[134]

CELL-BASED BIOASSAY

It is well-known that feeds may have biological activities that are beyond their nutritional value. Recently, this aspect has gained increasing attention mainly in the food industry but also in animal nutrition, and so-called nutraceuticals are offered for both food and feed applications. From a regulatory point of view, if foods and feeds are brought onto the market with "nutritional and health claims", these claims must be objective, scientifically supported, and verifiable by the competent authorities.[135,136] In this context, it is important to develop protocols and models to evaluate the bioaccessibility, bioavailability, and functional properties of feed bioactive components. For this purpose, neither the chemical analysis nor the supervised pattern recognition techniques, previously described, are "fit to purpose" methods of analysis. The transition to cell-based bioassays, to develop functional tests, may support the new need for feed analysis in terms of bioactivity and functional property evaluation. In vitro cell-based models have the advantage that they represent a possible alternative to animal experiments, thereby reducing the use of laboratory animals and costs for expensive animal experimentation. Although not reflecting fully in vivo conditions (all the effects of processes that occur in a living organism, such as

bioavailability, pharmacokinetics, metabolism, and distribution, cannot be considered), cell-based bioassays are an essential analytical support with a high information potential for preliminary studies before specific nutritional and clinical studies on animals are addressed. Up to now, cell-based bioassays are mainly used for food analysis and may represent a durable way to produce a valid documentation for claiming specific nutritional and health properties related to food, food components, and additives. The food industry is more interested in functional property evaluation of foods and dietary supplements for potential food applications and consumer acceptability. However, in the feed industry, research regarding the specific efficacy of additives and new functional feeds is an open issue and may take advantages from food research results to develop specific cell-based functional bioassays.

In the field of feed analysis, an area of research that has been developed in recent years is the use of cell-based bioassays for the evaluation of food/feed antioxidant components and food additives.[137,138] The concept of oxidative stress and the role that nutrition can play in preventing chronic inflammatory diseases are becoming very important topics in the field of medical and nutritional research.[139] There is evidence that dietary antioxidant components and antioxidant supplementation may have a protective role against oxidative stress induced diseases, although sometimes inconsistent results have been reported.[137,139−141] There are several chemical tests that are routinely used for the evaluation of antioxidant molecules. However, the chemical approach does not reflect the physiological conditions as it does not consider important factors related to the cellular uptake and metabolism of antioxidants.[142−146] When chemical assays were compared with cell-based methods for assessing antioxidants and antioxidant activity of foods and dietary supplements, different results have been found that are not always correlated with each other.[147,148] Primary cell culture and numerous cell lines have been used for the development of cell-based bioassays for food antioxidant activity analysis. This topic was reviewed specifically in a paper by Cheli and Baldi,[138] to which the reader is directed. Results indicate that cell-based bioassays may permit an evaluation of antioxidant capacity of different feed and feed components, in terms of real protection against damages from oxidation, as well as identify the mechanisms of actions (inhibition of oxidative processes, influence on the oxidant/antioxidant status, preservation of other antioxidant molecules). However, the data obtained by different researchers and laboratories are extremely difficult to compare and interpret. These results highlight the fact that the experimental models still cannot be transferred as such from the area of research to routine use, still needing standardization, optimization, automation, and, if possible, miniaturization. The validation of a cell-based bioassay is a complex process. For a cell-based bioassay as an "antioxidant test protocol", important topics regarding assay procedures, choice of cellular models, and the appropriate use and interpretation of nonlinear dose−responses still need to be defined to ensure more consistency in results.

In the field of feed safety evaluation, cell-based bioassay may be a methodology for assessing the presence of contaminants and/or undesirable substances. Notwithstanding the need for confirmatory instrumental methods, regulatory requirements in terms of maximum levels allowed for mycotoxins or other undesirable substances in animal feed, in a holistic approach to monitoring and surveillance of mycotoxin contamination of feed, cell-based bioassays allow an objective analysis and represent complementary analytical methods.[149] Results indicate that these cellular models, as well as providing a valuable tool to screen and assess mycotoxins, have an added value represented by the possibility of analyzing the effects and mechanisms of action of mycotoxins and assessing the ability of feed components to reduce their toxic effects.[150] Several cell lines have been shown to be very sensitive to a number of mycotoxins.[151−156] It is important to remember that, up to now, most of the reported studies worked with purified mycotoxins. Few studies have been carried out by testing naturally infected feed, where a copresence of different mycotoxins may occur.[157,158] In this context, the advantage of a cell-based bioassay is even more evident as it can evaluate the feed as "a unit" in which the copresence of mycotoxins, and therefore their synergistic effect, can be estimated and quantified or even the presence of a feed toxicity in the absence of a specific mycotoxin contamination. These results are extremely interesting in relation to the added value and the potential of cell-based bioassays as diagnostic tools for screening feeds in terms of safety assessment. This methodological approach, in fact, is able to detect a "safety problem" that can be connected to the presence and/or synergistic effects of mycotoxins or other undesirable substances, a problem that cannot be detectable by an analytical instrument.

The development of functional models of the intestinal ecosystem is one of the frontiers of research in the field of feed analysis, in relation to their broad potential application for the evaluation of nutritional and properties of functional feed. A good in vitro model would be beneficial in this area of study. It could be used to evaluate the bioavailability of nutrients from foods and feeds and offers a simple method to screen for factors that may affect intestinal absorption, such as the matrix, processing, and interactions with other foods.[137] Intestinal models are of great interest to the food and pharmaceutical industries, and they are principally used as toxicological and bioavailability tests of newly developed food ingredients and drugs before the products are put on the market.[159] Using mainly two cell lines, Caco-2 (intestinal cells isolated from human colon adenocarcinoma) and INT-407 cells (human embryonic intestine cells), both two-dimensional cell-based bioassays, where cells are grown in a monolayer on a plastic support, and three-dimensional models were developed. The latter, involving the cultivation of polarized cells on microporous membranes, are of particular interest because they reproduce the functional organization of the intestinal barrier. The microporous membrane (Figure 4) corresponds to the basolateral side of the intestine, whereas the compartment

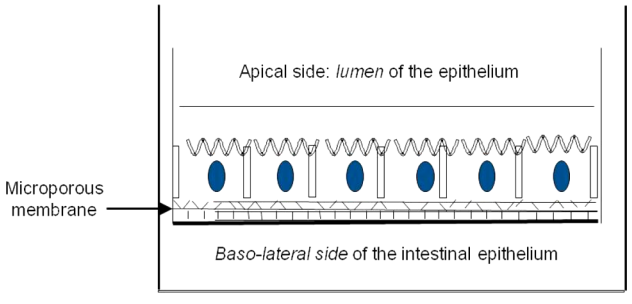

Figure 4. Schematic presentation of the functional (3D) model of the gut and functional polarity of intestinal epithelial cells growing on a microporous membrane.

above the microporous membrane represents the lumen side. These cellular models, used as part of a complex in vitro digestion model, were used as an analytical predictive tool for the evaluation of digestibility and absorption of food components in the diet. Particularly, in recent years, these models have been used in studies of human nutrition, with the goal, above all, to identify the transepithelial mechanisms of transport of amino acids and bioactive peptides and to assess iron bioavailability.[160,161] The good correspondence between the results obtained in vitro and in vivo[160] confirms that this model can represent a fast, realistic, and low-cost tool for the screening of feeds in terms of digestibility and bioavailability and for the analysis of the effects of structural changes due to technological treatments. Two-compartment models also provide the possibility of applying epithelial cells in combination with other cell types, such as immune cells such as macrophages or dendritic cells that attach on the bottom of the culture wells.[162] Cencič and Langerholc[159] have published an extensive review of the functional three-dimensional models of the intestinal ecosystem, emphasizing the potential for application to the study of the interactions between intestinal cells/nutrients/pathogens/pre and probiotics. The growing popularity of the use of probiotics in the diet and the lack of international consensus methodology for evaluating their effectiveness and safety have highlighted the need for guidelines, criteria, and methodologies for the evaluation of probiotics (FAO-WHO, 2002, ftp://ftp.fao.org/es/esn/food/wgreport2.pdf). Among the different methods identified and recommended, appropriate in vitro tests, which also include the use of cell cultures, have been suggested as a fit-to-purpose analytical approach. Two-dimensional cell-based bioassays using INT-407 and Caco-2 cells were used to study of adhesion of different strains of lactobacilli and bifidobacteria.[163−168] This parameter, associated with a large panel of other parameters, such as resistance to digestion in vitro, production organic acids, and the inhibition of bacteria potentially pathogens, is critical to the evaluation of the real potential of probiotic strains tested, which is of utmost importance to possible applications and uses in the feed industry to develop functional feeds.

A new frontier for the use of functional models of the intestinal ecosystem is the application for evaluating feed properties in terms of functionality and bioactivity. The gastrointestinal tract is an important target of dietary bioactive components that, by influencing the proliferation and activity of epithelial cells as well as the entire intestinal ecosystem, play an essential role for the proper development of the intestinal epithelium and for improving gut health. Particularly, milk bioactivities were given special attention, because of milk's important role in infant feeding in relation to its ability to modulate the intestinal development, the composition of the intestinal microflora, and to stimulate and modulate a local immune response.[169] Purup and Nielsen,[162] in a recent review, summarized some of the available cell-based models for testing milk-derived bioactives. The authors concluded that in vitro cell-based models for screening and testing of milk-derived bioactives represent a potential alternative to the use of a large number of experimental animals and that they have a high potential for the application for the study of bioactive compounds as functional foods or pharmaceutical products. They emphasized that there is still a need for validated in vitro models and that in vitro cell-based models have to be interpreted as such and need further studies in animal or human models.

Overall, results indicate that cell-based bioassays represent powerful tools for screening and testing feed properties, biological properties, and health claims. However, to develop the full potential of the cell bioassays and enable their effective transfer from research to routine analysis of feeds, there are mainly two fundamental requirements that must be met: the availability of a mobile platform that is easy to use and possibly automated and the ability to obtain reproducible results that are therefore comparable between laboratories. Moreover, the critical points that need to be defined and solved, in relation to specific analytical needs, are the correct choice of the cell type and model, the cell living environment, and the specific biomarkers that can be used to quantify the characteristics of quality, functionality, and also the safety of feed or of its components. All of these aspects are fundamental to ensure standardization of the model, uniformity, and sensitivity to evaluate specific feed properties. In vitro cell culture methods can be used in a two-tiered approach, one by which the simple effects on cell viability and proliferation are assessed, and the second by which more complex assays are made to elucidate the mechanism of action for the compound of interest. A screening system should achieve an optimal balance between high throughput, ease of performing experiments and analyses, adequate time, and expenses.

In conclusion, cell-based bioassays may represent a complementary approach to instrumental analysis of feed properties and improve our understanding and evaluation of the functional properties of feeds. Several cell-based assays were developed. Up to now, data from the literature indicate that there is a wide divergence of results, and for this reason, the data obtained by different researchers and laboratories are extremely difficult to compare and interpret. The transition of cell-based bioassays from research models to test models still needs optimization, standardization, and validation of the analytical protocols.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The high-throughput analytical testing demands in the field of feed research, industry, and regulation indicate the need to move from the classical chemical compound approach to a multianalytical and holistic approach. As the need for global food supply traceability grows, increasing numbers of feed products and ingredients will need to be routinely tested. Requirements for new analytical laboratory instruments will emphasize performance, sensitivity, reliability, fast and simplified use, and low cost for high volume routine assays. With the objective of feed evaluation, all of the techniques presented here are well suited and provide interesting information. Chemical analysis of feedstuffs is, and will continue to be, an indispensable part of feed evaluation, whether using traditional "wet chemistry" or analytical techniques coupled with chemometric tools. It is ideal, as much as possible, to arrange several techniques to gather a set of additional data allowing a better and global characterization of the feed. It is evident that an approach by chemical compounds is particularly well appropriated for the characterization of feeds from a chemical point of view. However, it is also evident that the characterization of products may not be attainable with a purely chemical vision as a global evaluation of feed quality and safety may be lost. If the global approach for

feed evaluation is chosen, the future seems to be linked to the increasing development of the analytical solutions marrying powerful analytical devices and data processing software. However, the lack of any animal interaction means that studies at higher hierarchical levels are required. Therefore, the use of in vitro feed evaluation systems and cell-based bioassays will continue to expand. Whatever the evaluation system used, it is fundamental to understand both the function and limitations of each methodology as well as be able to accurately interpret their findings to draw the appropriate conclusions.

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Notes

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ABBREVIATIONS USED

NIRS, near-infrared reflectance spectroscopy; DM, dry matter; CF, crude fiber; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NDS, nitrogen-free extract with neutral detergent soluble; IVGPT, in vitro gas production technique; GI, gastrointestinal; VFA, volatile fatty acid; NIR, near-infrared reflectance; FT, Fourier transform technique; mid-IR, mid-infrared spectroscopy; NIRM, near-infrared reflectance microscopy; IR/ATR, infrared spectroscopy in the attenuated total reflection mode; FT-IR/ATR, Fourier transform infrared spectroscopy in the attenuated total reflection mode; EU, European Union; DON, deoxynivalenol; AFB*1*, aflatoxin B*1*; ppb, parts per billion; OTA, ochratoxin A; Caco-2, intestinal cells isolated from human colon adenocarcinoma; INT-407, human embryonic intestine cells.

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REVIEW

EU legislation on feed related issues: an update

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Abstract

This review aims at providing an update of the current European Union (EU) legislation on feed-related issues. Regulations and Directives were classified into the following categories: general food law, placing on the market and use of feed, official controls, sampling and analysis, hygiene, undesirable substances, additives, animal by-products, OGMs, feed intended for particular nutritional purposes, and organic production. An overview of all laws related to the above-mentioned topics is given, and the main points of each law, cited in conjunction with its effect on previous laws (replacement, modification, amendments, and main related acts), are reported in tables.

Introduction

The issue of food safety plays a role of primary importance for producers and consumers. As to market conditions, European Union (EU) countries, on average, import approximately 50% of their total food supply from other EU countries or from outside the EU. Food import shares vary strongly across EU members. Although food in Europe has probably never been safer, a number of issues has weakened the public's confidence on the quality and safety of foods of animal origin, and the methods of food production (Report Special Eurobarometer 354 of 2010, http://www.efsa. europa.eu/en/factsheet/docs/reporten.pdf). Therefore, farmers, researchers, industry, and governments have been forced to pay serious attention to animal feedstuff production processes. In a review paper, Sapkota *et al.* (2007) emphasize that the ingredients used in animal feed are fundamentally important in terms of both the quality of the resulting food products and for the potential human health impacts associated with the animal-based food-production chain.

Food legislation is vital to ensuring a fair act of authority, and is the guideline to address the food safety risks. The current food safety policy is centred on a set of principles identified in the White Paper on Food Safety (12 January 2000, http://ec.europa.eu/dgs/health_consumer/library/pub/pub06_en.pdf) and set out in Regulation (EC) 178/2002 (European Commission, 2002d), entered into force 10 years ago. The responsibility of the decisions on food safety is of the European Commission, the European Parliament, the Council of the European Union, and the competent national authorities of each Member State. In this context, the European Food Safety Authority (EFSA) is not responsible for the laws regarding food safety or their application, but helps to ensure the safety of food and feed products. The role of EFSA is to assess the risks providing independent scientific advice on risks related to food and feed safety and to support the EU risk managers to make their final decisions. In recent years, beside health and consumer protection issues, food legislation has acquired a fundamental role in the context of legal matters with a high economic and trade impact and implications. To meet the regulatory requirements, industry, food/feed official control professionals, and researchers in the field have to be increasingly faced with the continuous evolution of the regulatory aspects at EU and national level.

The aim of the paper is to address some aspects concerning feed-related issues, providing an update of the current EU Regulations and Directives. EU Regulations and Directives were classified into general food law, placing on the market and use of feed, official controls, sampling and analysis, hygiene, undesirable substances, additives, animal by-products, OGMs, feed intended for particular nutritional purposes, and organic production. To give the reader a rapid first approach to the topic of his interest, a synoptic presentation of all laws related to the above-mentioned topics is given, and the main points of each law, cited in conjunction with its effect on previous laws (replacement, modification, amendments, and main related acts), are reported in tables. In the reference list, amendments to main Regulations/Directives are not reported, as they all can be found on the website of the main Regulations/Directives, where the consolidated versions are also reported.

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Food/Feed law: general principles and requirements

The integrated EU strategy aims to ensure a high level of food safety, animal health and welfare, and plant health within the EU, through coherent measures and adequate monitoring, while ensuring at the same time the effective functioning of the internal market. In order to ensure the safety of food, all aspects of the food production chain must be considered, from and including primary production and the production of animal feed up to and including sale or supply of food to the consumer. This approach involves the development of legislative and other actions in order to assure effective control systems, evaluate compliance with EU standards in the food chain within the EU and in third countries in relation to their exports to the EU, manage international relations with third countries and international organisations, manage relations with the EFSA, and ensure science-based risk management (Arvanitoyannis *et al.*, 2005). At EU level, on 28th of January 2002, the European Parliament and the Council adopted the Regulation (EC) 178/2002 (European Commission, 2002d) 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, with an integrated feed to food approach. The aim is to provide a framework to ensure a coherent approach in the development of food legislation. At the same time, the Regulation (EC) 178/2002 (European Commission, 2002d), that is the milestone of the legislative structure in the field of feed and

food legislation, provides the general framework for those areas not covered by specific harmonised rules but where the functioning of the Internal Market is ensured by mutual recognition. A summary of the EU Regulations and Decisions related to general principles and food law and of the related acts is given in Table 1.

Feed: placing on the market and use

Pinotti and Dell'Orto (2011) presented a synthetic description of the European feed sector sustaining the European livestock production, indicating that about 500 million tons of feedingstuffs are required each year within the EU-27. Approximately 50% of this volume is roughages produced on-farm, 10% are grains produced on-farm, 10% are purchased feed materials and 30% are industrial compound feeds. The EU-27 produces 152 million tons of compound feed per year, which is the second largest single share of the world compound feed market (Best, 2010; European Feed Manufacturers' Federation, 2011). Clearly, ensuring that such high volumes of traded products are conformed to adequate quality standards is a major undertaking of EU legislation in this area.

The placing on the market and the use of feed is regulated by Regulation (EC) 767/2009 (European Commission, 2009e). The objective of this Regulation, in accordance with the general principles laid down in Regulation (EC) 178/2002 (European Commission, 2002d), is to harmonise the conditions for the use of feed, in order to ensure a high level of feed safety and thus a high level of protection of public health. It lays down rules on the placing on the market and use of feed for both food-producing and non food-producing animals within the Community, including requirements for labelling, packaging and presentation. This Regulation shall apply without prejudice to other Community provisions applicable in the field of animal nutrition. The Regulation establishes the creation of a Community Catalogue of feed materials, to be used by feed business operators on a voluntary basis, as a tool to improve the labelling of feed materials and compound feed. The Catalogue shall facilitate the exchange of information on the product properties and list feed materials in a non-exhaustive manner. Regarding this topic, the Commission Regulation (EU) 68/2013 (European Commission, 2013) is of particular importance, as it updated the Community Catalogue of feed materials. In according to Commission Regulation (EU) 68/2013 (European Commission, 2013), all entries in the list of feed materials in Part C shall comply with the restrictions on the use of feed materials in accordance with the relevant legislation of the Union. Feed business operators using a feed material entered in the Catalogue shall ensure that it complies with Article 4 of Regulation (EC) 767/2009 (European Commission, 2009e). A register of feed materials is available at http://www.feedmaterialsregister.eu. Therefore, operators must regularly check that their feed materials comply with the requirements set for the Community Catalogue of feed Materials and the register.

A summary of the EU Regulations related to placing on the market and use of feed and of the related acts is given in Table 2.

Feed: official controls

The official controls of feeds are regulated by the Regulation (EC) 882/2004 (European Commission, 2004k). Official controls are defined as: any form of control performed by the competent authority or by the Community for the verification of compliance with feed and food law, as well as animal health and animal welfare rules. This Regulation describes in more details how the general principles, laid down in the Regulation (EC) 178/2002 (European Commission, 2002d), must be interpreted and implemented, and defines the European Union's duties as regards the organisation of these controls, as well as the rules which must be respected by the national authorities responsible for carrying out the official controls, including coercive measures adopted in the event of failure to comply with Community law. This Regulation re-organises official controls of food and feed so as to integrate controls at all stages of production and in all sectors, in the context of the review of food hygiene legislation (hygiene package). The main purposes of the Regulation (EC) 882/2004 (European Commission, 2004k) are to prevent or eliminate risks which may arise, either directly or via the environment, for human beings and animals, or to reduce these risks to an acceptable level, and to guarantee fair practices as regards trade in food and feed and the protection of consumers' interests, including labelling of food and feed and any other form of information intended for consumers. This Regulation does not apply to official controls for the verification of compliance with the rules on common market organisations agricultural products. The topic of the official control of feed is a critical point, therefore the legislative context is particularly complex. Therefore, there are several related acts to the Regulation (EC) 882/2004 (European Commission, 2004k) (Table 3).

Feed sampling and analysis

Sampling is the critical step in obtaining reliable results regarding feed composition, and evaluation of the presence of undesirable substances. A sampling plan may be defined as a test procedure combined with specific analytical procedures, and, in the case of undesirable substances, combined with a sample acceptance limit (Cheli *et al*., 2009). To plan a sampling procedure, the substance to be tested, the analytical method, the numbers of replicates samples, the numbers of replicate measurements per samples, and the sampling technique have to be selected.

Feed sampling and analysis topic is covered by the Commission Regulation (EC) 152/2009 (European Commission, 2009b) laying down the methods of sampling and analysis for the official control of feed. It is foreseen to update the sampling provisions in due time to take into account the recent developments in feed production, storing, transport, and marketing procedures. Methods of analysis for the official control of feed (control of the composition of feed materials and compound feed, control of the level of authorised additives, control of undesirable substances in feed, and determination of constituents of animal origin in feed) are described with specific references to the expression of the results. The specific topic regarding the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material is covered by existing regulations, such as Commission Recommendation 2004/787/EC (European Commission, 2004b) and Commission Regulation (EU) 619/2011 (European Commission, 2011d).

A summary of the EU Regulations related to feed sampling and analysis is given in Table 4.

Feed hygiene

Livestock production plays a very important role in the agricultural sector of the Community. Satisfactory results of this activity depend to a large extent on the use of safe and

Table 1. The basis of food/feed law: main Regulations and main related Acts.

Main documents	Main points	Relationship between documents
Regulation (EC) 178/2002 (European Commission, 2002d)	General principles and requirements of food law Common principles and responsibilities to provide a strong science base, efficient organizational arrangements and procedures to underpin decision-making in matters of food and feed safety General obligation of food trade Establishment of the European Food Safety Authority Establishment of a rapid alert system for the notification of a direct or indirect risk to human health deriving from food or feed (RASFF) Emergency measures for food and feed of Community origin or imported from a third country, and other emergency measures	Amendments Commission Regulation (EC) 1642/2003; 575/2006; 202/2008. Regulation (EC) 596/2009
Related Acts	**Main points**	**Relationship between documents**
Commission Decision 2004/478/CE (European Commission, 2004a)	Establishment of the general plan for food/feed crisis management Definition of practical procedures for the management of a crisis involving a serious direct or indirect risk to human	
Commission Regulation (EC) 2230/2004 (European Commission, 2004g)	Definition of detailed rules for the implementation of the Regulation (EC) 178/2002 (European Commission, 2002d) with regard to the network of organisations operating in the fields within the European Food Safety Authority's mission	
Regulation (EC) 854/2004 (European Commission, 2004j)	Establishment of specific rules for the organisation of official controls on products of animal origin	

Table 2. Main EU Regulations related to placing on the market and use of feed and main related acts.

Main documents	Main points	Relationship between documents
Regulation (EC) 767/2009 (European Commission, 2009e)	General rules on the placing on the market and use of feed for both food-producing and non food- producing animals within the Community General requirements in term of safety and marketing Responsibilities and obligations of feed businesses General and specific requirements for labelling, packaging and presentation Definition of a Community Catalogue of feed materials	Amending: Regulation (EC) 1831/2003 Repealing: Council Directives 79/373/EEC; 82/471/EEC; 83/228/EEC; 93/74/EEC; 93/113/EC; 96/25/EC. Commission Directive 80/511/EEC; 70/542/EEC. Commission Decision 2004/217/EC Amendments: Commission Regulation (EU) 454/2010; 568/2010; 939/2010.
Related Acts	**Main points**	**Relationship between documents**
Commission Regulation (EU) 892/2010 (European Commission, 2010b)	Definition of the status of certain products with regard to feed additives within the scope of Regulation (EC) 1831/2003 (European Commission, 2003e)	
Commission Recommendation 2011/25/EU (European Commission, 2011a)	Establishment of guidelines for the distinction between feed materials, feed additives, biocidal products and veterinary medicinal products	
Commission Regulation (EU) 68/2013 (European Commission, 2013)	Establishment of the Catalogue of feed materials Glossary of feed processes List of feed materials	

good quality feed.

Feed hygiene topic is covered by Regulation (EC) 183/2005 (European Commission, 2005d) laying down requirements for feed hygiene. The principal aim of this Regulation is to set out new hygiene rules in an integrated approach necessary to ensure: i) a higher level of consumer protection with regard to food and feed safety; ii) safety throughout the food chain, starting with primary production of feed up to and including the feeding of food-producing animals; iii) that all feed businesses, including aquaculture, operate in conformity with harmonised safety requirements. The general implementation of procedures is based on the principles of hazard analysis and critical control points (HACCP), that, together with the application of good hygiene practice, represents a valuable instrument to help feed business operators and should reinforce their responsibility. A complete application of a registration of feed business operators and approval system of establishments may guarantee full traceability.

The rules governing feed hygiene controls must consider certain zoonoses and zoonotic agents, for which specific requirements for controls have been laid down by the Regulation (EC) 2160/2003 (European Commission, 2003f) on the control of salmonella and other specified food-borne zoonotic agents, and the Directive 2003/99/EC (European Commission, 2003b) on the monitoring of zoonoses and zoonotic agents. The purpose of the Regulation (EC) No 2160/2003 (European Commission, 2003f) is to ensure that proper and effective measures are taken to detect and to control salmonella and other zoonotic agents at all relevant stages of production, processing and distribution, particularly at the level of primary production, including feed, in order to reduce their prevalence and the risk they pose to public health. The purpose of the Directive 2003/99/EC (European Commission, 2003b) is to ensure that zoonoses, zoonotic agents and related antimicrobial resistance are properly monitored, and that food-borne outbreaks receive proper epidemiological investigation, to enable the collection in the Community of the information necessary to evaluate relevant trends and sources. This Directive covers the monitoring of zoonoses and zoonotic agents, related antimicrobial resistance, the epidemiological investigation of food-borne outbreaks, and the exchange of information related to zoonoses and zoonotic agents.

A summary of the EU Regulation related to feed hygiene is given in Table 5.

Undesirable substances in animal feed

Livestock production is an important topic for the Community and satisfactory results in terms of public and animal health, animal welfare, environment and livestock producers' finances depend to a large extent on the use of appropriate good quality feedingstuffs. Rules on feedingstuffs are needed to ensure agricultural productivity and sustainability. Comprehensive regulations on undesirable substances have been set up in order to guarantee good quality and safety of feedingstuffs at farm level, if they are not commercially produced, or at commercial levels.

As it is impossible to fully eliminate the presence of undesirable substances, it is important to fix maximum limits, considering the substances' acute and chronic toxicity, bioaccumulation and degradability, in order to prevent undesirable and harmful effects. The undesirable substance presence in animal feed is covered by the Directive 2002/32/EC (European Commission, 2002c). In particular, this Directive, considering the continuous amending acts, fixes the maximum levels of undesirable substances in products intended for animal feed as regard to: inorganic contaminants and nitrogenous compounds, mycotoxins, inherent plant toxins, organochlorine compounds and dioxins and PCBs, harmful botanical impurities, authorised feed additives in non-target feed following unavoidable carry-over. This Directive must apply to products intended for animal feed as soon as they enter the Community. When a Member State has grounds, based on new information or a reassessment of existing information, suggesting that a maximum level might present a danger to animal or human health or to environment, that Member State may provisionally reduce the existing maximum level in its territory, fix a maximum level or prohibit the presence of that undesirable substance in products. In that case, it shall immediately notify the other Member States and the Commission of the measures taken with a statement of the reasons thereof. An immediate decision shall be taken, in accordance with the procedure laid down in the Directive for adapting the technical provisions in the Annexes to this Directive in the light of developments in scientific and technical knowledge. For a uniform approach in cases of increased levels it may be necessary to set action thresholds to trigger such investigations. These may be laid down in Annex II. So long as neither the Council nor the Commission has taken a decision, the Member State may maintain the measures it has implemented.

As a concrete result of European integration, in terms of ensuring the highest possible level of safety of the food chain and compliances with EU food and feed legislation, the Rapid Alert System for Food and Feed (RASFF) (http://ec.europa.eu/food/food/rapidalert/index_en.htm) was launched in 1979. The legal basis of the RASFF is Regulation (EC) 178/2002 (European Commission, 2002d) which established RASFF as a network involving the Member States, the Commission, as member and manager of the system, and EFSA. RASFF is a tool to exchange information between competent authorities on consignments of food and feed in cases where a risk to human and animal health has been identified and measures have been taken. In 2011, out of the 3730 original notifications transmitted in RASFF, 361 concerned feed, about 10 % of the total. Notifications concerning feed have been increasing for only a few specific categories. In decreasing order of importance these are: mycotoxins, non-pathogenic micro-organisms, industrial contaminants, heavy metals and fraud.

A summary of the EU Directives and Regulations related to undesirable substances in animal feed is given in Table 6. The specific topic regarding the methods of sampling and analysis for the official control of undesirable substances in feed has been previously presented (Table 4).

Additives for use in animal nutrition

Experience with the application of Council Directive 70/524/EEC (European Commission, 1970) concerning additives in feedingstuffs has shown that it is necessary to review all the rules on additives in order to take into account the need to ensure a greater degree of protection of animal and human health, and of the environment. It is also necessary to take into account the fact that the technological progress and the scientific developments have made available new types of additives, such as those to be used on silage or in water. The ban, from 1st January 2006, of the use of antibiotics as growth promoter feed additives within the European Union, resulted in a huge progress in the development of alternative and effective products. From a safety point of view, in order to protect human health, animal health and the environment, feed additives should undergo a

Table 3. EU regulations related to feed official controls and main related acts.

Main documents	Main points	Relationship between documents
Regulation (EC) 882/2004 (European Commission, 2004k)	Description of obligations relating to official controls Definition of the operational criteria that the competent authorities of each Member State for performing official controls must satisfy and of their obligations and requirements Definition of the criteria for the methods of sampling and analysis used within the context of official controls Indications for preparing an integrated multi-annual national control plan, and intervention plans in the event of an emergency Specific indications for the controls on products from Non-EU Member Countries (Commission experts may carry out controls in Non-EU Member Countries) Establishment of a number of Community Reference Laboratories assistance to the Commission. Definition of administrative measures	Repealing: Council Directive 70/373/ECC; 85/73/ECC; 85/591/ECC; 89/397/ECC; 93/383/ECC; 93/99/ECC; 95/53/EC. Council Decision 98/383/ECC; 98/728/EC; 1999/313/EC. Amending: Council Directive 96/23/EC; 97/78/EC; 2000/29/EC. Regulation (EC) No 854/2004. Amendments Commission Regulation (EC) 776/2006; 180/2008; 737/2008; 1029/2008; 1162/2009; 87/2011; 208/2011; 563/2012. Council Regulation (EC) 1791/2006; 301/2008. Regulation (EC) No 596/2009.

Related Acts	Main points	Relationship between documents
Commission Recommendation 2003/91/EC (European Commission, 2003a)	Recommendation on the coordinated inspection programme for the year 2003 in the field of animal nutrition	
Commission Regulation (EC) 136/2004 (European Commission, 2004e)	Definition of the procedures for veterinary checks at Community border inspection posts on products imported from third countries	
Regulation (EC) 852/2004 (European Commission, 2004h)	General rules for food business operators on the hygiene of foodstuffs	
Regulation (EC) 853/2004 (European Commission, 2004i)	General and specific hygiene rules for food of animal origin (unprocessed and processed)	
Regulation (EC) 854/2004 (European Commission, 2004j)	General and specific rules for the organisation of official controls on products of animal origin intended for human consumption	
Commission Recommendation 2005/925/EC (European Commission, 2005b)	Recommendation on the coordinated inspection programme for the year 2006 in the field of animal nutrition	
Regulation (EC) No 183/2005 (European Commission, 2005d)	Definition of requirements for feed hygiene See Table 5	
Commission Decision 2006/677/EC (European Commission, 2006a)	Guidelines for competent authorities' audit systems for the conduct of audits under Regulation (EC) 882/2004 (European Commission, 2004k) on official controls to verify compliance with feed and food law, animal health and animal welfare rules	
Commission Regulation (EC) 829/2007 (European Commission, 2007c)	Definition of rules regarding the placing on the market of certain animal by-products	Amending Regulation (EC) 1774/2002
Commission Decision 2008/798/EC (European Commission, 2008b)	Imposition of special conditions governing the import of products containing milk or milk products originating in or consigned from China	Repealing Commission Decision 2008/798/EC

Continued on next page.

Table 3. Continued from previous page.

Related Acts	Main points	Relationship between documents
Commission Decision 2009/821/EC (European Commission, 2009a)	Definition of a list of border inspection posts Definition of detailed rules for the inspections carried out by Commission veterinary experts at border inspection posts and at certain other points of entry into the Community Definition of a list of veterinary units in Traces	
Commission Regulation (EC) 669/2009 (European Commission, 2009c)	Definition of rules concerning the increased level of official controls on imports of certain feed and food of non-animal origin at the points of entry into the territories	
Commission Regulation (EC) 1162/2009 (European Commission, 2009d)	Definition of transitional measures for the implementation of Regulations, regarding hygiene rules for food of animal origin, organisation of official controls on products of animal origin intended for human consumption, and official controls for the verification of compliance with feed and food law, animal health	Implementation Regulation (EC) 882/2004 Amending Commission Decision 2006/504/EC
Commission Implementing Regulation (EU) 996/2012 (European Commission, 2012)	Imposition of special conditions governing the import of feed and food originating in or consigned from Japan following the accident at the Fukushima nuclear power station and repealing Implementing Regulation (EU) No 284/2012.	Repealing Commission Implementing Regulation (EU) 284/2012; 561/2012.

safety assessment through a Community procedure before being placed on the market, used or processed. This topic is covered by the Regulation (EC) 1831/2003 (European Commission, 2003e) on additives for use in animal nutrition. The purpose of this Regulation is to establish a Community procedure for authorising the placing on the market and use of feed additives and to lay down rules for the supervision and labelling of feed additives and premixtures in order to provide the basis for the assurance of a high level of protection of human health, animal health and welfare, environment and users' and consumers' interests in relation to feed additives, whilst ensuring the effective functioning of the internal market. In order to ensure a harmonised scientific assessment of feed additives, such assessment is carried out by the EFSA. Strict conditions for authorization are reported: no feed additive shall be authorised unless the applicant for such authorisation has adequately and sufficiently demonstrated that, when used in accordance with conditions set out in the Regulation authorising the use of the additive, it does not have an adverse effect on animal health, human health or the environment, and it is presented in a manner which may mislead the user. Moreover, each additive must be allocated within a specific category and one or more of the functional groups reported in the Regulation. An European Union Register of Feed Additives pursuant to Regulation (EC) 1831/2003 (European Commission, 2003e) (http://ec.europa.eu/food/food/animalnutrition/feedadditives/comm_register_feed_additives_1831-03.pdf) is available and it is updated every time, when a new authorization is given, obtained, modified, suspended, expired, revoked or extended. A summary of the EU Regulations related to additives in animal nutrition is given in Table 7.

Animal by-products: prevention, control, eradication of certain transmissible spongiform encephalopathies, and use in animal nutrition

Animal by-products not intended for human consumption may be a potential source of risks to public and animal health. Past crises related to outbreaks of foot-and-mouth disease, the spread of transmissible spongiform encephalopathies, such as bovine spongiform encephalopathy (BSE), and the occurrence of dioxins in feedingstuffs have shown the consequences of the improper use of certain animal by-products for the public and animal health, the safety of the food and feed chain, and the consumer confidence. In addition, such crises may also have a wider adverse impact on society with a high impact on the socioeconomic situation of the farmers and of the industrial sectors. Since 1990, the Community has adopted a series of measures to protect human and animal health from the risk of BSE. By now, this topic is covered by the Regulation (EC) 999/2001 (European Commission, 2001b) laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. This Regulation has been extensively amended by a huge number of Regulations, according to the new scientific information becoming available in the years, the changing of the epidemiological picture, and the availability of alternative and rapid tests. A complete list of the amending Regulations is available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001R0999:EN:NOT.

The topic of animal by-product use in animal nutrition is covered by the Regulation (EC) 1069/2009 (European Commission, 2009f), laying down health rules as regards animal by-products and derived products not intended for human consumption, and its corresponding implementing Commission Regulation (EC) 142/2011 (European Commission, 2011c), revoking and replacing the Regulation (EC) No 1774/2002 (European Commission, 2002e). It is the consequence of a long and comprehensive review carried out by the EU Commission to assess the operation of EU-wide controls on animal by-products. This Regulation defines community health rules for collection, transport, handling, treatment, transformation, processing, storage, placing on the market, distribution, use or disposal of animal by-products. The Regulations covers all animal products including meat, fish, milk and eggs when they are not intended for human consumption and other products of animal origin including hides, feathers, wool, bones, horns, and hoofs. It also covers carcasses of fallen stock on farms, pet animals, and wild animals where they are suspected of being diseased. It regulates the use of animal by-products for example as feed (including pet food), fertiliser or for technical products and lays down rules for their transformation through composting and biogas and their disposal via rendering and incineration.

A summary of the EU Regulations regarding animal by-product topic is given in Table 8. The

Table 4. EU regulations related to feed sampling and analysis and related acts.

Main documents	Main points	Relationship between documents
Commission Regulation (EC) 152/2009 (European Commission, 2009b)	General principles and definitions regarding sampling for the official control of feed, as regards the determination of constituents, additives and undesirable substances, with the exception of residues of pesticides and microorganisms Definition of the procedures for the preparation of samples for analysis and expression of results in accordance with the methods reported in this Regulation Methods of analysis for the official control of feed (composition of feed materials and compound feed; level of authorised additives; undesirable substances; determination of constituents of animal origin) Methods of analysis to control illegal presence of no longer authorised additives in feed	Repealing First Commission Directive 71/250/EEC Second Commission Directive 71/393/EEC Third Commission Directive 72/199/EEC Fourth Commission Directive 73/46/EEC First Commission Directive 76/371/EEC Seventh Commission Directive 76/372/EEC Eighth Commission Directive 78/633/EEC Ninth Commission Directive 81/715/EEC Tenth Commission Directive 84/425/EEC Commission Directive 86/174/EEC Eleventh Commission Directive 93/70/EEC Twelfth Commission Directive 93/117/EC Commission Directive 98/64/EC; 1999/27/EC; 1999/76/EC; 2000/45/EC; 2002/70/EC; 2003/126/EC. Amendments Commission Regulation (EU) 278/2012; 51/2013.

Related Acts	Main points
Commission Recommendation 2004/787/EC (European Commission, 2004b)	Technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) 1830/2003 (European Commission, 2003d). Definitions and general principles for sampling protocols Laboratory requirements Testing methods, expression and interpretation of the results of the analyses This guidance covers products that have received authorisations for their placing on the market
Commission Regulation (EC) 401/2006 (European Commission, 2006c)	General principles and definitions Description of the methods of sampling for official control of the levels of mycotoxins in foodstuffs, specifications for different commodities Criteria for sample preparation and for methods of analysis used for the official control of the levels of mycotoxins in foodstuffs Performance criteria for mycotoxin method of analysis to be used by the laboratory and laboratory control requirements Criteria for acceptance of a lot or sublot
Commission Regulation (EU) 619/2011 (European Commission, 2011d)	Definition of the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired

Table 5. EU regulations and Directives related to feed hygiene and main related acts.

Main documents	Main points	Relationship between documents
Regulation (EC) 183/2005 (European Commission, 2005d)	General principles and requirements for feed hygiene General and specific obligations for feed business operators Conditions and arrangements ensuring traceability of feed Rules for feed operators in order to put in place, implement and maintain a permanent written procedure or procedures based on the Hazard analysis and critical control points (HACCP) principles. Conditions and arrangements for registration and approval of establishments and record in a national list or lists Rules for import and export	Repealing Council Directive 95/69/EC Commission Directive 98/51/EC Amendments Regulation (EC) 219/2009 Commission Regulation (EC) 225/2012
Regulation (EC) 2160/2003 (European Commission, 2003f)	Definition of the Competent authorities Adoption of targets for the reduction of the prevalence of specified zoonoses in animal populations at the level of primary production, and where appropriate for the zoonosis or zoonotic agent concerned, at other stages of the food chain, including in food and feed Approval of specific control programmes established by Member States and food and feed business operators Adoption of specific rules concerning certain control methods applied in the reduction of the prevalence of zoonoses and zoonotic agents Adoption of rules concerning intra-Community trade and imports from third countries of certain animals and products Approval of laboratories, quality requirements and approved testing methods This Regulation shall not apply to primary production for private domestic use, or leading to the direct supply, by the producer, of small quantities of primary products	Amendments Commission Regulation (EC) 1237/2007; 199/2009; 213/2009; 517/2011; 1086/2011. Council Regulation (EC) 1791/2006 Regulation (EC) 596/2009
Directive 2003/99/EC (European Commission, 2003b)	General and specific rules for the monitoring of zoonoses and zoonotic agents, related antimicrobial resistance, the epidemiological investigation of food-borne outbreaks, and the exchange of information related to zoonoses and zoonotic agent Definition of a list of zoonoses and zoonotic agents to be included in monitoring, and to be monitored according to the epidemiological situation Rules for each Member State to designate a competent authority or competent authorities for the purposes of this Directive Rules for establishing coordinated monitoring programmes, especially when specific needs are identified Rules for exchange information Rules and procedures for the designation of Community and national reference laboratories	Repealing Council Directive 92/117/EEC Amending Council Decision 90/424/EEC Amendments Council Directive 2006/104/EC Council Decision 2009/470/EC Regulation (EC) 219/2009
Related Acts	**Main points**	**Relationship between documents**
Report from the Commission to the European Parliament and the Council (European Commission, 2007e)	On existing legal provisions, systems and practices in the Member States and at Community level relating to liability in the food and feed sectors and on feasible systems for financial guarantees in the feed sector at Community level in accordance with Article 8 of Regulation (EC) No 183/2005 laying down requirements for feed hygiene	
Commission Regulation (EC) 2073/2005 (European Commission, 2005c)	Definition of microbiological criteria for foodstuffs	Repealing Commission Decision 93/51/ECC Amendments Commission Regulation (EC) 1441/2007; 365/2010; 1086/2011.

Table 6. EU Directives and Regulations focused on undesirable substances in animal feed.

Main documents	Main points	Relationship between documents
Directive 2002/32/EC (European Commission, 2002c)	General principles and definitions Indications of relationships between Member States and the Commission in order to take immediate decision for adapting the technical provisions in the Annexes to this Directive, in the light of developments in scientific and technical knowledge. Setting of maximum admissible limits of undesirable substances in products intended for animal feed. Annex I: maximum levels of each undesirable substances are reported for different type of products intended for animal feed (inorganic contaminants and nitrogenous compounds; mycotoxins; inherent plant toxins; organochlorine compounds and dioxins and PCBs; harmful botanical impurities; authorised feed additives in non-target feed following unavoidable carry-over) Annex II: for dioxin and dioxin like PCB, action threshold relative to feedingstuffs, and comments and additional information (e.g. nature of investigations to be performed) are reported.	Repealing Council Directive 1999/29/EC Amendments Commission Directive 2003/57/EC; 2003/100/EC; 2005/8/EC; 2005/86/EC; 2005/87/EC; 2006/13/EC; 2006/77/EC; 2008/76/EC; 2009/8/EC; 2009/124/EC; 2009/141/EC; 2010/6/ECC. Regulation (EC) 219/2009 Commission Regulation (EU) 574/2011; 277/2012; 744/2012; 107/2013.

Related Acts	Main points	Relationship between documents
Commission Recommendation 2004/704/EC (European Commission, 2004c)	On the monitoring of background levels of dioxins and dioxin-like PCBs in feedingstuffs	
Regulation (EC) 882/2004 (European Commission, 2004k)	On official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare	
Regulation (EC) 396/2005 (European Commission, 2005e)	On maximum residue levels of pesticides in or on food and feed of plant and animal origin	
Commission Recommendation 2011/516/EU (European Commission, 2011b)	On the reduction of the presence of dioxins, furans and PCBs in feed and food	Amending Council Directive 91/414/EEC
Commission Recommendation 2006/576/EC (European Commission, 2006b)	On the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding	Repealing Commission Recommendation 2006/88/EC
Commission Regulation (EC) 1881/2006 (European Commission, 2006d)	Setting maximum levels for certain contaminants in foodstuffs	

specific topic regarding the methods of sampling and analysis for the official control of feed as regards presence of animal by-products has been previously presented (Table 4).

Genetically modified feed: placing on the market

The use of genetically modified (GM) plants in agriculture and their use as food and feed is a topic controversially discussed in academic, institutional and public debates. Many discussion forums, studies and publications have been devoted to evaluate if the release of GM crops is beneficial or harmful for the environment, and to assess the safety of GM food and feed. Other potential risks are: spread of pest resistance or herbicide tolerance to wild plants, inadvertent toxicity to benign wildlife, and increasing control of agriculture by biotechnology corporations. Perceptions of unnaturalness, ethical concerns, the failure to implement an efficacious traceability policy, and disparity between developing and developed countries (in terms of economics and sovereignty over decisions) have also been associated with a negative societal response and great differences in the perception of benefits and risks of GM Organisms (GMOs).

Since the 90', specific measures at the European level have been adopted with the aim to provide a legal framework for the control of GM crops and food: the Council Directive 90/219/EEC (European Commission, 1990a) on the contained use of genetically modified micro-organisms, and the Council Directive 90/220/EEC (European Commission, 1990b) on the deliberate release into the environment of genetically modified organisms. More recently, the European Union adopted a comprehensive and implemented legal framework regarding the authorization and the placing on the market of products consisting of or derived from GMOs. The authorisation procedure covers the use of GMOs and their derived products for food and feed, industrial processing and cultivation. The placing on the market of GMOs and foodstuffs containing GMOs, whether they are intended for consumption by humans or animals, is regulated by a specific authorisation procedure. Any GM food and feed intended for sale in the EU is subject to a rigorous safety assessment, which is the responsibility of EFSA. All these topics are covered by the Regulation (EC) 1829/2003 (European Commission, 2003c), and the Regulation (EC) No 1830/2003 (European Commission, 2003d).

Table 7. EU Regulations focused on additives for use in animal nutrition, and main related acts.

Main documents	Main points	Relationship between documents
Regulation (EC) 1831/2003 (European Commission, 2003f)	Definition and establishment of a Community procedure for authorising the placing on the market, use and placing on the market of feed additives Definition of a category list and additive functional groups within the categories General and specific rules for the supervision and labelling of feed additives and premixtures Definition of duties and tasks of the Community reference laboratory	Repealing Council Directive 70/524/EEC; 87/153/EEC. Amending Council Directive 82/471/EEC Amendments Commission Regulation (EC) 378/2005; 386/2009. Regulation (EC) 596/2009; 767/2009.

Related Acts	Main points
Commission Regulation (EC) 429/2008 (European Commission, 2008d)	On detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives
Commission Regulation (EU) 892/2010 (European Commission, 2010b)	On the status of certain products with regard to feed additives within the scope of Regulation (EC) No 1831/2003 of the European Parliament and of the Council (European Commission, 2003e)

These Regulations apply to three types of product: i) GMOs for food and feed use; ii) food and feed containing GMOs; iii) food and feed produced from or containing ingredients produced from GMOs. Food and feed consisting of, containing or produced from GMOs should undergo a safety assessment through a Community procedure before being placed on the market within the Community. Aspects and rules related to the authorisation procedures and supervision of GM food and feed are mainly covered by the Regulation (EC) 1829/2003 (European Commission, 2003c), while a framework for the traceability of OGMs at all stages of placing on the market, including the possibility of establishing thresholds, and labelling of GM products is covered by the Regulation (EC) 1830/2003 (European Commission, 2003d). A list of authorised GMOs is available at the website of the Directorate-General for Health and Consumers: http://ec.europa.eu/food/dyna/gm_register/index_en.cfm. This search covers the EU GMOs register, pursuant to Regulation (EC) 1829/2003 (European Commission, 2003c), and the products subject to EC decisions on withdrawal from the market.

A summary of the EU Regulations related to placing on the market to genetically modified food and feed is given in Table 9. The specific topic regarding the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material has been previously presented (Table 4).

Feed intended for particular nutritional purposes

It is well-known that feed may have biological activities that are beyond their nutritional value. Recently, this aspect has gained increasing attention mainly in animal nutrition and feed industry, and so-called nutraceuticals are offered for feed applications. From a regulatory point of view, if a feed is brought onto the market with nutritional and health claims, these claims must be objective, supported by scientific evidences, and verifiable by the competent authorities. Therefore, several information are required to bring evidence that these products have a specific composition, have been designed to meet particular nutritional needs of animal categories, have a beneficial effect on the animals, and/or are manufactured using special methods. Such feedingstuffs must be clearly distinguished in their characteristics and purpose from ordinary feedingstuffs. This

Table 8. EU Regulations focused on prevention, control, eradication of certain transmissible spongiform encephalopathies, and use of animal by-products in animal nutrition, and main related acts.

Main documents	Main points	Relationship between documents
Regulation (EC) 999/2001 (European Commission, 2001b)	General, specific rules and requirements for the production and placing on the market of live animals and products of animal origin (not for cosmetic or medicinal products or medical devices; products not intended for use in food, feed or fertilizers; products of animal origin for exhibition, teaching, scientific research) Criteria for the determination of BSE status of each Member state Definition and establishment of the measures for TSE prevention: monitoring system, rules for breeding programmes, prohibitions concerning animal feeding, education programmes Definition of rules and procedures for the control and eradication of TSEs: notification, measures with respect to suspect animals and confirmed presence of TSE, contingency plan Establishment of reference laboratories and sampling, testing and controls procedures	Amendments Complete list at http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001R0999:EN:NOT
Regulation (EC) 1069/2009 (European Commission, 2009f)	Establishment of community health rules for collection, transport, handling, treatment, transformation, processing, storage, placing on the market, distribution, use or disposal of animal by-products not intended for human consumption Definition of obligations from the starting point to end point in the manufacturing chain General animal health restrictions Categorisation and definition of three categories of animal by-products and derived products Establishment of general rules for collection, transport, disposal, placing on the market, and use of animal by-products and derived products Establishment of restrictions on use of the 3 category products General obligations, registration and approval procedures for operators as regard collection, transport and traceability Establishment of a list for each Member State of approved or registered establishments, plants and operators	Repealing Regulation (EC) 1774/2002 Amendments Council Directive 2010/63/EU

Related Acts	Main points	
Commission Decision 2002/248/EC (European Commission, 2002a)	Regard to transmissible spongiform encephalopathies and the feeding of animal proteins	Amending Council Decision 2000/766/EC Commission Decision 2001/9/EC
Commission Decision 2002/1003/EC (European Commission, 2002b)	Laying down minimum requirements for a survey of prion protein genotypes of sheep breeds	
Communication from the Commission (European Commission, 2005a)	TSE Road map	
Commission Decision 2007/453/EC (European Commission, 2007b)	Establishing the BSE status of Member States or third countries or regions thereof according to their BSE risk	
Communication from the Commission (European Commission, 2010c)	The TSE Road map 2: a strategy paper on Transmissible Spongiform Encephalopathies for 2010-2015	
Commission Regulation (EC) 142/2011 (European Commission, 2011c)	Laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive	

Table 9. EU Regulations focused on genetically modified food and feed: placing on the market.

Main documents	Main points	Relationship between documents
Regulation (EC) 1829/2003 (European Commission, 2003c)	Definition of Community procedures for the authorisation and supervision of genetically modified food and feed (GMOs for food use; food containing or consisting of GMOs; food produced from or containing ingredients produced from GMOs) Provisions and requirements for labelling of genetically modified food and feed. Rules and requirements for application of authorisation Rules for modification, suspension, revocation of authorizations, and renewal of authorizations Establishment of a Community register of genetically modified food and feed Duties and tasks of the Community reference laboratory Rules for establishment of National reference laboratories	Repealing Council Regulation (EC) 1139/98 Commission Regulation (EC) 49/2000; 50/2000. Amending Council Directive 68/193/EEC; 82/471/EEC Regulation (EC) 258/97 Council Directive 2002/53/EC; 2002/55/EC Amendments Commission Regulation (EC) 1981/2006 Regulation (EC) 298/2008
Regulation (EC) 1830/2003 (European Commission, 2003d)	Definition of a framework for the traceability of products consisting of or containing GMOs, and food and feed produced from GMOs Specific rules for the placing on the market of GMOs Traceability and labelling requirements for products consisting of or containing GMOs Traceability requirements for products for food and feed produced from GMOs Definition of inspection and control measures	Repealing Directive 2001/18/EC Amendments Regulation (EC) 1137/2008

Related Acts	Main points	Relationship between documents
Regulation (EC) 258/97 (European Commission, 1997)	Concerning novel food and novel food ingredients	
Regulation (EC) 1139/98 (European Commission, 1998)	Concerning the compulsory indication of the labelling of certain foodstuffs produced from genetically modified in Directive 79/112/EEC organisms of particulars other than those provided for	
Directive 2001/18/EC (European C ommission, 2001a)	On the deliberate release into the environment of genetically modified organisms	Repealing Council Directive 90/220/EEC
Commission Regulation (EC) 889/2008 (European Commission, 2008e)	Laying down detailed rules for the implementation of Council Regulation (EC) 834/2007 (European Commission, 2007) on organic production and labelling of organic products with regard to organic production, labelling and control	Amendments Complete list at: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32008R0889:EN:NOT
Commission Regulation (EC) 65/2004 (European Commission, 2004d)	Establishing a system for the development and assignment of unique identifiers for genetically modified organisms	
Commission Regulation (EC) 641/2004 (European Commission, 2004f)	On detailed rules for the implementation of Regulation (EC) 1829/2003 of the European Parliament and of the Council (European Commission, 2003) as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluations	
Commission Decision 2007/157/EC (European Commission, 2007a)	On emergency measures regarding the non-authorised genetically modified organism Bt10 in maize products	Repealing Commission Decision 2005/317/EC
Commission Decision 2010/315/EC (European Commission, 2010a)	On emergency measures regarding the non-authorised genetically modified organism "LL RICE 601" in rice products	Repealing Commission Decision 2006/601/EC
Commission Decision 2008/289/EC (European Commission, 2008a)	On emergency measures regarding the unauthorised genetically modified organism Bt 63 in rice products	
Commission Regulation (EU) 619/2011 (European Commission, 2011d)	Laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired	

Table 10. EU Directives and Regulations focused on feed for particular nutritional purposes.

Main documents	Main points	Relationship between documents
Commission Directive 2008/38/EC (European Commission, 2008c)	Provision of a list of intended uses of animal feedingstuffs for particular nutritional purposes ANNEX 1: list of particular nutritional purposes. For each purpose, requirements regarding the essential nutritional characteristics, species or category of animals, labelling declarations, recommended length of time, and other provisions are reported	Repealing Commission Directive 94/39/EC; 95/9/EC; 2002/1/EC; 2008/4/EC Amendments Commission Directive 2008/82/CE Commission Regulation (EU) 1070/2010
Regulation (EC) 767/2009 (European Commission, 2009e)	See Table 2	See Table 2

Table 11. EU Regulations focused on organic production.

Main documents	Main points	Relationship between documents
Council Regulation (EC) 834/2007 (European Commission, 2007d)	General and specific rules for the production and placing on the market of products originating from agriculture (live or unprocessed agricultural products, processed agricultural products and yeasts used use as food and feed, vegetative propagating material and seeds for cultivation), including aquaculture Provision of overall principles and general requirements of organic production (banned products, restriction of the use of external inputs) Establishment of general requirements and specific principles applicable to farming, processing of organic food and feed General requirements for farm, plant, seaweed, livestock, and aquaculture production General rules for production of processed feed, food, and organic yeast Criteria for the use and authorization of products and substances Definition of rules for the use of terms referring to organic production: labelling, compulsory indications, organic logo, specific labelling requirements Set up of a control system Set up of rules for trade with third countries	Repealing Council Regulation (EEC) 2092/91 Amendments Council Regulation (EC) 967/2008
Related Acts	**Main points**	**Relationship between documents**
Commission Regulation (EC) 889/2008 (European Commission, 2008e)	Laying down detailed rules for the implementation of Council Regulation (EC) 834/2007 (European Commission, 2007) on organic production and labelling of organic products with regard to organic production, labelling and control	Amendments Complete list at: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32008R0889:EN:NOT

topic is covered by the Commission Directive 2008/38/EC (European Commission, 2008c) establishing a list of intended uses of animal feedingstuffs for particular nutritional purposes. This is a positive list indicating, for each nutritional purpose, the essential nutritional characteristics, the labelling declarations and, where appropriate, the special labelling requirements of each feed. The established list may be modified and implemented, where appropriate, following developments in scientific and technical knowledge, and the availability of new Community methods of control. Feeds intended for particular nutritional purposes can be marketed only if their intended uses are included in the list, according to the general principles and requirements reported in Regulation (EC) 767/2009 (European Commission, 2009e), establishing the general principles and requirements regarding the placing on the market and use of feed (Table 2).

A summary of the EU Regulations related to animal feedstuffs intended for particular nutritional purposes is given in Table 10.

Organic production

Organic production is an overall system of farm management and feed 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 a production method in line with the preference of certain consumers for products produced using natural substances and processes. The share of the organic agricultural sector is increasing, providing products for a specific market and consumer, and delivering public goods contributing to the protection of the environment and animal welfare, as well as to rural development. An agricultural policy and a legislative framework on organic production are fundamental. This topic is covered by the Council Regulation (EC) 834/2007 (European Commission, 2007d), on organic production and labelling of organic products and repealing Regulation (EEC) 2092/91 (European Commission, 1991). This Regulation defines the objectives, principles and rules applicable to organic production, in order to contribute to transparency and consumer confidence as well as to a harmonised perception of the concept of organic production. Detailed rules for the implementation of Council Regulation (EC) 834/2007 (European Commission, 2007d), on organic production and labelling of organic products are reported in the Commission Regulation (EC) 889/2008 (European Commission, 2008e). The general Community framework provides a basis for the sustainable development of organic production, ensuring the effective functioning of the internal market and guaranteeing fair competition. The framework applies to both crop and animal production and all stages of production, preparation, and distribution of organic products.

A summary of the EU Regulations related to organic production is given in Table 11.

Conclusions

This review presented an update of the EU legislation regarding feed related topics in order to provide a general frame of the EU feed legislation and give the reader a useful source of information. Over the last 10-15 years several food safety crises occurred within the framework of the EU and weakened the public's confidence on the quality and wholesomeness of foods of animal origin. As a result, EU Commission, governments, industry, researchers, and farmers have been forced to pay serious attention to animal feedstuff production processes, thereby acknowledging that animal feed safety is an essential prerequisite for human food safety. The EU Commission enhanced the food safety level by either introducing new stricter Regulations/Directives or modifying the already existing ones. Since the adoption of Regulation (EC) 178/2002 (European Commission, 2002d) 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, the target of the Community policies, in the development of food law, was to assure a high level of protection of human life and health. So a common EU framework basis for measures governing food and feed was developed in a non-discriminatory manner whether food or feed is traded on the internal market or internationally. The legislation in the field of feed/food chain is continuously evolving prompted by different factors, such as the availability of new scientific information becoming available in the years, the activity of the EFSA's scientific committees, the changing of the epidemiological picture, and the availability of new analytical approaches. Globalisation and the increased global trade associated with feed and food production pose the need for EU legislation to face with the different legislative framework of other countries. A lack of legislative harmonization is an important point to consider in a worldwide discussion regarding the risk management and regulations in food security and safety governance.

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Review

EU legislation on cereal safety: An update with a focus on mycotoxins

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ABSTRACT

Cereals are still by far the world's most important source of food, both for direct human consumption and indirectly, as inputs to livestock production. FAO's latest forecast for world cereal production in 2011 stands at nearly 2313 million tonnes. Total EU-27 grain production forecast was 283 and 272 million tonnes for 2011 and 2012, respectively. Cereal contamination has an important impact on human and animal health. The European Union has established the most comprehensive regulations for food and cereal safety to facilitate world trade and protect consumer health. This paper reviews the existing legislation associated with cereal safety, with a focus on mycotoxin contamination. Regulations and Directives were classified into the following topics: general food legislative framework, official controls (sampling and analysis), maximum levels for contaminants, prevention and reduction. To give the reader a rapid first approach to the topic of his interest, a synoptical presentation of all laws related to the above-mentioned topics is given, and the main points of each law, cited in conjunction with its effect on previous laws (repeal, modification, amendments, replacement, related acts), are reported in tables. Moreover, data regarding the worldwide occurrence of mycotoxins in cereals were reported.

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1. Introduction

Cereals are still by far the world's most important source of food, both for direct human consumption and, indirectly, as inputs to livestock production, although the use of cereals for bioethanol production is increasing (Cardona & Sànchez, 2007; Cassman & Liska, 2007; Lin & Tanaka, 2006; Luque et al., 2008; Pimentel et al., 2009). FAO's latest forecast for world cereal production in 2011 stands at nearly 2313 million tonnes, 3.3 per cent higher than in 2010 (FAO, 2011). Total EU-27 grain production forecast, including soft and durum wheat, barley, maize, rye, oats, triticale and other minor cereals, was 283 and 272 million tonnes for 2011 and 2012, respectively (from COCERAL, 2012/13-2011/12). The market share for cereals is approximately 51% for feed, 27% for food/human consumption, 19% for seed production and other uses, and 3% for bioethanol (Siegel & Babuscio, 2011). Clearly, ensuring that such high volumes of products are conformed to adequate quality and safety standards is a major undertaking of European Union (EU)

Abbreviation: COCERAL, European association representing the trade in cereals, rice, feedstuffs, oilseeds, olive oil, oils and fats and agrosupply; DON, deoxynivalenol; EFSA, European Food Safety Authority; EU, European Union; FAO, Food and Agricultural Organization of the United Nations; GMOs, genetically modified organisms; OTA, Ochratoxin A; PCB, polychlorinated biphenyl; ZEA, zearalenone.

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legislation. Cereal contamination can be heterogeneous including biological, chemical and physical contaminants. The biological contamination, comprising microorganisms, natural occurring toxins (i.e. mycotoxins from fungi, phycotoxins from algae, toxins from cyanobacteria, histamine, vegetal alkaloids, etc.), and chemical contamination (i.e. agrochemicals as pesticides, plant growth regulators, and environmental contaminants as metals, dioxins, PCBs, etc.), get more concern for food and feed safety (Tang, Lu, Zhao, & Wang, 2009). In terms of food safety, among the most important risks associated to cereals' consumption are mycotoxins (Codex Alimentarius, 1991).

The knowledge and control of the level and distribution of contaminants in cereals are a worldwide objective of producers, manufacturers, regulatory agencies and researchers due to the high economic and sanitary impact on food and feed safety and human/animal health. Since it is impossible to fully eliminate the presence of undesirable substances and contaminants, maximum concentrations should be set at a strict level which is reasonably achievable considering the risk related to food consumption. Consequently, an adequate surveillance and frequent checks are fundamental to assure quality and safety standards for raw materials destined for direct consumption or industrial processes. As a result, public authorities and regulatory agencies are pushing producers, manufacturers, and researchers to pay serious attention to food and feed production processes and to develop comprehensive quality policies and management systems to improve food safety and try to enhance consumer information to regain consumers' trust in food. To meet the regulatory requirements, industry, food/feed official control professionals, and researchers in the field have to be increasingly faced with the continuous evolution of the regulatory aspects at EU and national level.

This paper reviews the existing legislation associated with cereal safety, with a focus on mycotoxin contamination. Regulations and Directives were classified into the following topics: general food legislative framework, official controls (sampling and analysis), maximum levels for contaminants, prevention and reduction. To give the reader a rapid first approach to the topic of his interest, a synoptical presentation of all laws related to the above-mentioned topics is given, and the main points of each law, cited in conjunction with its effect on previous laws (repeal, modification, amendments, replacement, related acts), are reported in tables. Moreover, data regarding the worldwide occurrence of mycotoxins in cereals were reported.

2. Food law: the legislative framework

The integrated EU strategy aims to ensure a high level of food safety, animal health and welfare, and plant health within the EU, through coherent measures and adequate monitoring, while ensuring at the same time the effective functioning of the internal market. In order to ensure the safety of food, all aspects of the food production chain must be considered, from and including primary production and the production of animal feed up to and including sale or supply of food to the consumer. This approach involves the development of legislative and other actions in order to assure effective control systems, evaluate compliance with EU standards in the food chain within the EU and in third countries in relation to their exports to the EU, manage international relations with third countries and international organisations, manage relations with the European Food Safety Authority (EFSA), and ensure science-based risk management (Arvanitoyannis, Choreftaki, & Tserkezou, 2005). At EU level, on the 28th of January 2002, the European Parliament and the Council adopted the 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, with an integrated feed to food approach (The European Parliament and the Council, 2002b). The aim of this Regulation is to provide a framework to ensure a coherent approach in the development of food legislation. At the same time, this Regulation, that is the milestone of the legislative structure in the field of feed and food legislation, provides the general framework for those areas not covered by specific harmonised rules but where the functioning of the Internal Market is ensured by mutual recognition.

A summary of the EU Regulations and Decisions related to general principles and food law is given in Table 1.

3. Cereal safety: official controls

The official controls of food and feed are regulated by the Regulation (EC) No 882/2004 (The European Parliament and the Council, 2004). Official controls are defined as *"any form of control that the competent authority or the Community performs for the verification of compliance with feed and food law, animal health and animal welfare rules"*. The rules contained in this Regulation underpin the integrated and horizontal approach necessary to implement a coherent control policy on the food chain: feed and

Table 1
The basis of food/feed law: main Regulation and related acts.

Main documents	Main points	Relationship between documents
Regulation (EC) No 178/2002 (The European Parliament and the Council, 2002b)	General principles and requirements of food law Common principles and responsibilities to provide a strong science base, efficient organisational arrangements and procedures to underpin decision-making in matters of food and feed safety General obligation of food trade Establishment of the European Food Safety Authority Establishment of a rapid alert system for the notification of a direct or indirect risk to human health deriving from food or feed (RASFF) Emergency measures for food and feed of Community origin or imported from a third country, and other emergency measures	Amendments Commission Regulation (EC) No 1642/2003; No 575/2006; No 202/2008; Regulation No 596/2009.
Related acts	**Main points**	**Relationship between documents**
Commission Decision 2004/478/EC (European Commission, 2004a)	Establishment of the general plan for food/feed crisis management Definition of practical procedures for the management of a crisis involving a serious direct or indirect risk to human	
Commission Regulation (EC) No 2230/2004 (European Commission, 2004c)	Definition of detailed rules for the implementation of the Regulation 178/2002 with regard to the network of organisations operating in the fields within the European Food Safety Authority's mission	

food safety, animal health and animal welfare. This Regulation lays down general rules for the performance of official controls, and defines the European Union's duties as regards the organisation of these controls, as well as the rules which must be respected by the national authorities responsible for carrying out the official controls, including coercive measures adopted in the event of failure to comply with Community law. This Regulation covers different areas such as animal nutrition including medicated feedingstuffs, feed and food hygiene, zoonoses, animal by-products, residues and contaminants, control and eradication of animal diseases with a public health impact, feed and food labelling, pesticides, feed and food additives, vitamins, mineral salts, trace elements and other additives, materials in contact with food, quality and compositional requirements, drinking water, ionisation, novel foods and genetically modified organisms (GMOs). The Regulation establishes a harmonised framework of general rules for the organisation of official controls of food and feed so as to integrate controls at all stages of production. Community legislation also provides quality parameters for laboratories involved in the analysis of official samples. Laboratories should work in accordance with internationally approved procedures or criteria-based performance standards and use methods of analysis that have been validated. A list of the Community and national reference laboratories is provided.

Rules concerning an increased level of official controls to be carried out on imports of certain feed and food of non-animal origin are laid down in Commission Regulation (EC) No 669/2009 (European Commission, 2009a). In this Regulation (Annex 1), a list of the feed and food of non-animal origin subject to the increased level of official controls, according to the intended use of the feed and food, the CN code, the country of origin, and the hazard, is reported. Moreover, the Commission Regulation (EC) No 1152/2009 (European Commission, 2009b) imposes special conditions governing the import of certain foodstuffs from certain third countries due to contamination risk by aflatoxins.

Tasks strictly related to official controls must be carried out using appropriate control methods and techniques such as monitoring, surveillance, verification, audit, inspection, sampling and analysis. Therefore, monitoring activity has a critical role in order to obtain an overview of the state of compliance with feed or food law, animal health and animal welfare rules. There are several Recommendations on this topic. With regard to those specific for cereals, the European Commission recently adopted the Commission Recommendation No 2013/165/EU on the presence of T-2 and HT-2 toxin in cereals and cereal products (European Commission, 2013). According to this Recommendation, Member States should perform monitoring for the presence of T-2 and HT-2 toxins in cereals and cereal products, and should encourage that samples are simultaneously analysed for the presence of T-2 and HT-2 and other *Fusarium*-toxins. Commission Recommendation No 2010/307/EU specifically applies to the monitoring of acrylamide levels in food (European Commission, 2010). Annual monitoring of acrylamide levels in food were carried out in the years 2007–2008–2009 according to Commission Recommendation No 2007/331/EC, and Commission Recommendation No 2012/154/EU on the monitoring of the presence of ergot alkaloids in feed and food (European Commission, 2007a, 2012). Monitoring investigations regarding contaminants and undesirable substances by the Member States and the Community, and the communication of the result to EFSA on a regular basis represent the basis for an evolution of the legislative framework to improve food safety and the risk related to the consumption of the food.

A summary of the EU Regulations and Recommendations related to cereal official controls is given in Table 2.

4. Cereal safety: maximum levels for contaminants

The general principles of the EU legislation on community procedures for contaminants in food were laid down in 1993 by the Council Regulation (EEC) No 315/93 (The Council of the European Communities, 1993). This legal act empowered the European Commission to take measures ensuring the protection of public health, including the introduction of maximum levels. It is essential, in order to protect public health, to keep contaminants at levels which are toxicologically acceptable. As it is impossible to fully eliminate food contamination, maximum levels should be set at a strict level which is reasonably achievable by following good agricultural and manufacturing practices and taking into account the risk related to food the consumption. Moreover, maximum levels have a direct impact on all European food/feed business operators and traders. Commission Regulation (EC) No 194/97 and Commission Regulation (EC) No 466/2001 established maximum limits for nitrates and mycotoxins (aflatoxins, ochratoxin A and patulin) in food, respectively (European Commission, 1997, 2001). These initial Regulations were updated several times and repealed in 2006 by Commission Regulation (EU) 1881/2006 setting maximum limits, according to different foodstuffs, for nitrate, mycotoxins, metals, dioxins and PCBs, polycyclic aromatic hydrocarbons, melamine and its structural analogues (European Commission, 2006d). As regards active substances and pesticide residues, the reference Regulations are Regulation (EC) No 396/2005 (The European Parliament and the Council, 2005) on maximum residue levels of pesticides on food and feed of plant and animal origin, and Regulation (EC) No 1107/2009 (The European Parliament and the Council, 2009) concerning the placing of plant protection products on the market. This Regulation and the subsequent implementing ones introduced new procedures for the evaluation and authorisation of active substances and plant protection products. These procedures require the involvement of the Member States of a specific agro-climatic characteristics, in strict collaboration with the Member States of other area and the relevant bodies of the European Commission.

An EU pesticide database, where active substances according to the before mentioned Regulations are reported, is available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm.

As stated before, in terms of food safety, among the most important risks associated to cereal contaminants are mycotoxins (Codex Alimentarius, 1991). Mycotoxins are metabolites of fungi capable of having acute toxic, carcinogenic, mutagenic, teratogenic, immunotoxic, and oestrogenic effects in man and animals (D'Mello, Placinta, & Macdonald, 1999; Wild & Gong, 2010). Since the discovery of aflatoxins in 1960 and subsequent recognition that mycotoxins are of significant health concern to both humans and animals mycotoxins have received considerable attention as biotoxins in the food chain. Extensive mycotoxin contamination has been reported to occur in both developing and developed countries. It has been estimated that up to 25% of the world's crops grown for feed and food may be contaminated with mycotoxins (Fink-Gremmels, 1999; Hussein & Brasel, 2001). This means that, if the estimated world production is about 2300 million tonnes (2011), there are potentially about 500 million tonnes of mycotoxin contaminated grains entering the food and feed supply chain. Furthermore, according to the possible carry-over of each toxin, feed contamination can represent also a hazard for the safety of food of animal origin and can contribute to mycotoxin intake in human population (Jorgensen, 2005; Monaci & Palmisano, 2004). Although it is known that mycotoxins are ubiquitous and not just limited to humid and hot countries, where the climate is more favourable to microbial and fungal contamination, it has been reported that some toxins can occur more frequently than other according to the producing area of the food/feed material. Thus

Table 2
Main EU Regulations and Recommendations related to cereal official controls.

Main Documents	Main points	Relationship between documents
Regulation (EC) No 882/2004 (The European Parliament and the Council, 2004)	General rules for the performance of official controls to verify compliance with rules aiming, in particular, at preventing, eliminating or reducing to acceptable levels risks to humans and animals, either directly or through the environment, and guaranteeing fair practices in feed and food trade and protecting consumer interests, including feed and food labelling and other forms of consumer information Description of obligations relating to official controls Definition of the operational criteria that the designed competent authorities of each Member State for performing official controls must satisfy and of their obligations and requirements Definition of the criteria for the methods of sampling and analysis used within the context of official controls Indications for preparing an integrated multi-annual national control plan, and intervention plans in the event of an emergency Specific indications for the controls on products from Non-EU Member Countries (Commission experts may carry out controls in Non-EU Member Countries) Establishment of a list of EU reference laboratories for feed and food Definition of administrative measures General principles and guidelines for the preparation of multi-annual national control plans General and specific import conditions National and Community enforcement measures	Repealing Council Directive 70/373/ECC; 85/73/ECC; 85/591/ECC; 89/397/ECC; 93/99/ECC; 95/53/EC. Council Decision 93/383/ECC; 98/728/EC; 1999/313/EC. Amending Council Directive 96/23/EC; 97/78/EC; 2000/29/EC Regulation (EC) No 854/2004 Amendments Commission Decision 2006/677/EC Council Regulation (EC) 1791/2006; 301/2008. Commission Regulation (EC) No 776/2006; No 180/2008; No 737/2008; No 1029/2008; No 596/2009; No 1162/2009; No 87/2011; No 208/2011; No 563/2012.
Commission Recommendation 2006/576/EC (European Commission, 2006a)	On the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding.	
Commission Recommendation No 2010/307/EU (European Commission, 2010)	On the monitoring of acrylamide levels in food Member States should perform the monitoring of acrylamide levels in the foodstuffs and provide to the EFSA the data Description of sampling points and procedure Specific information for products, sample numbers and frequencies, analytical requirements, minimum additional information for each product	
Commission Recommendation No 2012/154/EU (European Commission, 2012)	On the monitoring of the presence of ergot alkaloids in feed and food Member States should perform with the active involvement of the feed and food business operators monitoring on the presence of ergot alkaloids in cereals and cereal products intended for human consumption or intended for animal feeding, in pasture/forage grasses for animal feeding and in compound feed and food The analytical results should be provided on a regular basis to EFSA	
Commission Regulation (EC) No 669/2009 (European Commission, 2009a)	Regarding increased level of official controls on imports of certain feed and food of non-animal origin (cereal are not listed)	Repealing Commission Decision 2005/402/EC Amending Commission Decision 2006/504/EC Implementing Commission Regulation (EC) No 882/2004 Amendments Commission Regulation (EU) No 212/2010; No 878/2010; No 1099/2010; No 187/2011; Commission Implementing Regulation (EU) No 433/2011; No 799/2011; No 1277/2011; No 294/2012; No 514/2012; No 889/2012; No 1235/2012; No 91/2013; No 270/213.

zeralenone, fumonisin and aflatoxin were the most widespread toxins found in Asian commodities. By contrast, zeralenone and deoxynivalenol were the most prevalent toxins in continental Europe samples, even after adjusting for the seasonality of contamination for these different toxins (Binder, Tan, Chin, Handl, & Richard, 2007; Rodrigues & Naehrer, 2012; Taylor-Pickard, 2009). Examples regarding the worldwide occurrence of mycotoxins in cereals are reported in Figs. 1 and 2. It is well documented that milling and thermal processing are treatments that may affect redistribution, stability, change and removal of mycotoxins in the processed food (Berthiller et al., 2013; Brera et al., 2006; Bullerman & Bianchini, 2007; Castells, Marin, Sanchis, & Ramos, 2008; Cheli et al., 2010; Cheli, Pinotti, Rossi, & Dell'Orto, 2013; Scudamore, 2008). Cereal by-products, commonly used as animal feed (such as bran, screenings, middling and short middling) typically contain higher levels of mycotoxins' contamination compared to whole grains. Therefore, from a safety perspective, controls are needed at all stages of cereal production and processing in order to guarantee the quality and safety of the production. Different factors play a role in the decision-making process of setting limits for mycotoxins, including the availability of toxicological and occurrence data, detailed knowledge about possibilities for sampling and analysis, and socio-economic issues. All the factors affecting the promulgation of mycotoxin regulations and the setting of limits have been discussed in a detailed reviewed by van Egmond, Schothorst, and Jonker (2007). Aflatoxins, ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEA), fumonisins, and T-2 and HT-2 toxin maximum limits are reported for cereals and by-products according to the type and the technological process (European Commission, 2006d). For mycotoxin contamination in cereals intended for feed use, only aflatoxin B_1 is regulated with fixed maximum limits by Commission Directive 2002/32/EC (The European Parliament and the Council, 2002a). For other mycotoxins, DON, ZEA, OTA, fumonisins, guidance values were set in

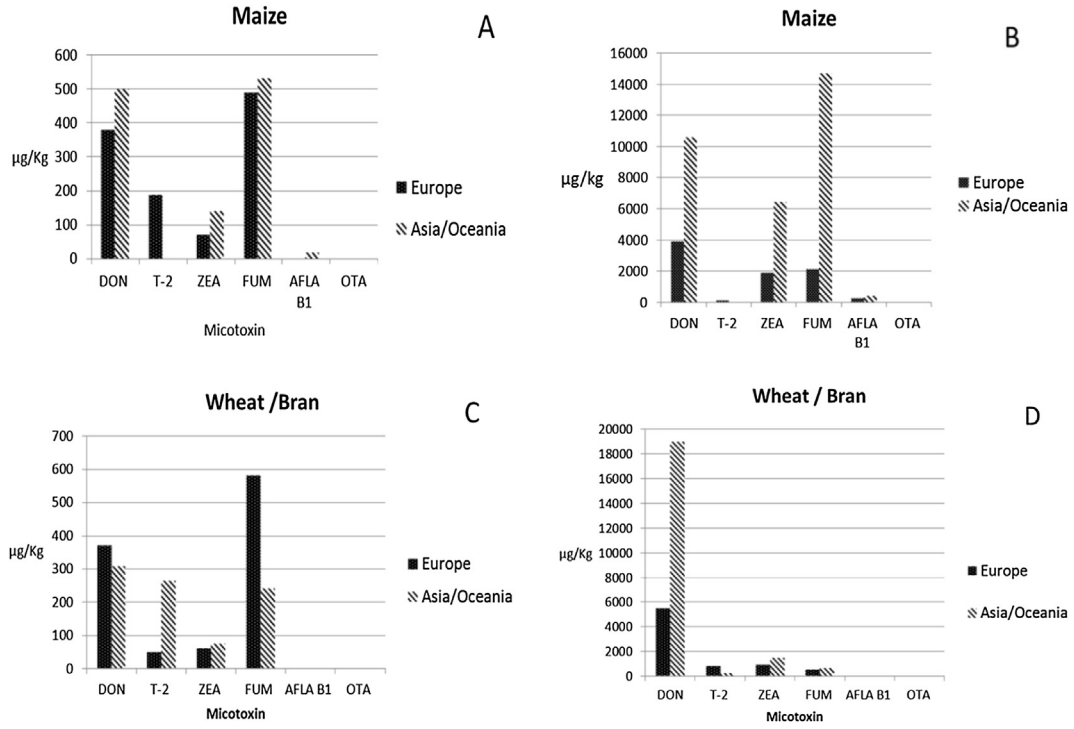

Fig. 1. Worldwide mycotoxin occurrence (µg/kg) in maize and wheat/bran samples (A,C: median of positive samples; B,D: maximum levels) (modified from Binder et al. (2007)).

Commission Recommendation 2006/576/EC (European Commission, 2006a). For T-2 and HT-2 toxin, indicative levels for cereals and cereals products were set in Commission Recommendation 2013/165/EU (European Commission, 2013). In the case of lots intended for industrial purposes (e.g. bioethanol or biopolymer production) neither maximum limits nor guidance levels have been established. The maximum levels, guidance values and indicative levels for mycotoxins in cereals and cereals by-products intended for human and animal consumption are reported in Table 3.

As a concrete result of the European integration, in terms of ensuring the highest possible level of safety of the food chain and compliances with EU food and feed legislation, The Rapid Alert System for Food and Feed (RASFF) (http://ec.europa.eu/food/food/rapidalert/index_en.htm) was launched in 1979. The legal basis of

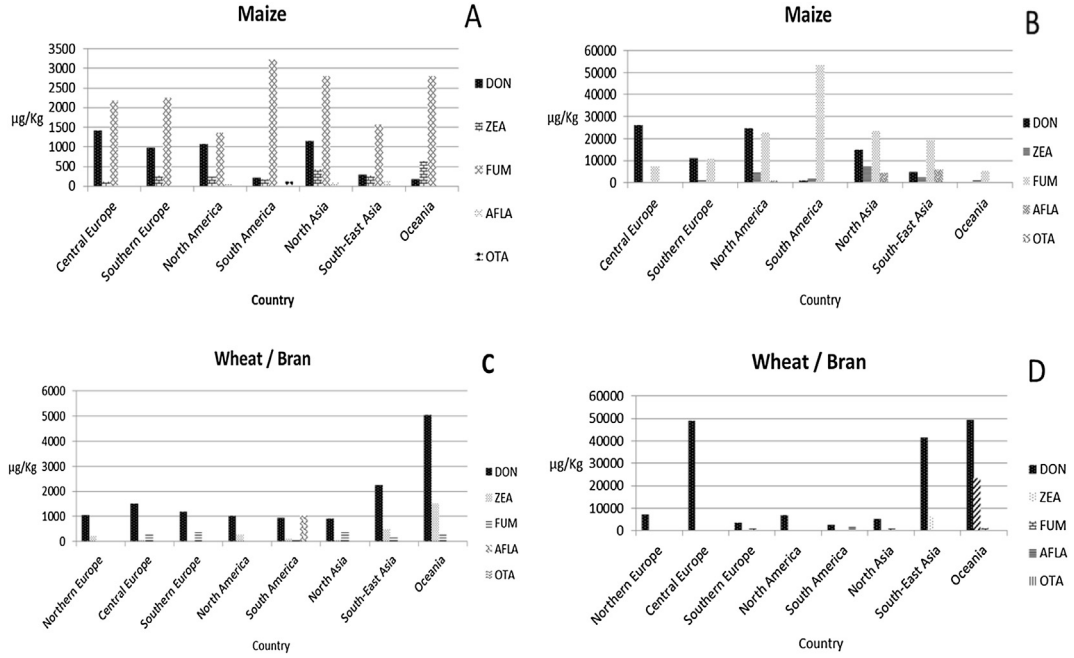

Fig. 2. Worldwide mycotoxin occurrence (µg/kg) in maize and wheat/bran samples (A,C: median of positive samples; B,D: maximum levels) (modified from Rodrigues and Naehrer (2012)).

Table 3
Maximum levels for mycotoxins in cereals and cereal-based products for human (European Commission, 2006d) and animal consumption (The European Parliament and the Council, 2002a). Mycotoxins indicative levels for cereals and cereals products (European Commission, 2013) (*) and guidance levels for products intended for animal feed (European Commission, 2006a) (**).

Mycotoxin	Cereal and cereal products	Food	Feed	Maximum levels, μg/kg
Aflatoxin B_1	All cereals and all products derived from cereals	+		2.0
	Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	+		5.0
	Processed cereal-based foods and baby foods for infants and young children	+		
	Feed materials		+	20
Aflatoxins, sum of B_1, B_2, G_1 and G_2	All cereals and all products derived from cereals	+		4.0
	Maize and rice to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	+		10.0
Deoxynivalenol	Unprocessed cereals other than durum wheat, oats and maize	+		1250
	Unprocessed durum wheat and oats	+		1750
	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	+		1750
	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	+		750
	Pasta (dry)	+		750
	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	+		500
	Processed cereal-based foods and baby foods for infants and young children	+		200
	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	+		750
	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	+		1250
	Cereals and cereal products (**)		+	8000
	Maize by-products (**)		+	12,000
Zearalenone	Unprocessed cereals other than maize	+		100
	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	+		350
	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	+		75
	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	+		50
	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	+		100
	Processed cereal-based foods and baby foods for infants and young children	+		20
	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	+		200
	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	+		300
	Cereals and cereal products (**)		+	2000
	Maize by-products (**)		+	3000
Ochratoxin A	Unprocessed cereals	+		5.0
	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption	+		3.0
	Processed cereal-based foods and baby foods for infants and young children	+		0.05
	Wheat gluten not sold directly to the consumer	+		8
	Cereals and cereal products (**)		+	250
Fumonisin $B_1 + B_2$	Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	+		4000
	Maize intended for direct human consumption, maize-based foods for direct human consumption	+		1000
	Maize-based breakfast cereals and maize-based snacks	+		800
	Processed maize-based foods and baby foods for infants and young children	+		200
	Milling fractions of maize with particle size > 500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size > 500 micron not used for direct human consumption falling within CN code 1904 10 10	+		1400
	Milling fractions of maize with particle size ≤ 500 micron falling within CN code 1102 20 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption falling within CN code 1904 10 10	+		2000
	Maize and maize products (**)		+	60.000
Sum T-2 and HT-2 toxin	*Unprocessed cereals* (*)			
	Barley and maize	+		200
	Oats	+		1000
	Wheat, rye and other cereals	+		100
	Cereals grains for direct human consumption (*)			
	Oats	+		200
	Maize	+		100
	Other cereals	+		50
	Cereal products for human consumption (*)			
	Other cereal milling products	+		50
	Cereal based foods for infants and young children	+		15
	Cereal products for feed and compound feed (*)			
	Oat milling product		+	2000
	Other cereal products		+	500

the RASFF is Regulation (EC) 178/2002 (The European Parliament and the Council, 2002b) which established RASFF as a network involving the Member States, the Commission as member and manager of the system and EFSA. RASFF is a tool to exchange information between competent authorities on consignments of food and feed in cases where a risk to human and animal health has been identified and measures have been taken. In 2011, out of the 3730 original notifications transmitted in RASFF, 635 concerned mycotoxin in food, of which 33 was related to aflatoxins in cereals and bakery products, and 361 concerned in general feed, about 10% of the total (RASFF, 2011). Notifications concerning feed have been increasing for only a few specific categories with mycotoxin as the most important.

A summary of the EU Regulations and Directives related to cereal contaminants is given in Table 4.

5. Cereal contaminants: prevention and reduction

Cereal production plays a very important part in the agricultural sector of the Community, and several measures can be adopted as parts of an overall strategy in order to stimulate a pro-active approach to reduce the presence of contaminants and undesirable substances. Proper and effective measures can be taken at all relevant stages of production, processing and distribution, particularly at the level of primary production, in order to reduce their prevalence and the risk they pose to public health. There are several Recommendations on this topic. With regard to those for cereals, Commission Recommendation 2006/583/EC (European Commission, 2006b), and Commission Recommendation 2011/516/EU (European Commission, 2011a) must be cited. Commission Recommendation 2006/583/EC identifies risk factors, sets out the

Table 4
Main EU Regulations and Directives related to cereal contaminants.

Main documents	Main points	Relationship between documents
Council Regulation (EEC) No 315/93 (The Council of the European Communities, 1993)	General community procedures for contaminants in food Definition of contaminant as any substance not intentionally added to food which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food, or as a result of environmental contamination This Regulation shall not apply to contaminants which are the subject of more specific Community rules	Amendments Regulation (EC) No 1882/2003; No 596/2009
Commission Regulation (EC) No 1881/2006 (European Commission, 2006d)	Setting maximum levels for certain contaminants in foodstuffs taking into account new information and developments in Codex Alimentarius General rules regarding prohibitions on use, mixing and detoxification Specific provisions for cereals Reference Regulations for sampling and the analysis for the official control of the maximum levels Rules for monitoring and reporting According to different foodstuffs, maximum levels for Nitrate, Mycotoxins (aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins, T-2 and HT-2 toxins), Metals, Dioxins and PCBs, Polycyclic aromatic hydrocarbons, Melamine and its structural analogues are reported The foodstuffs listed in the Annex of the Regulation cannot be placed on the market where they contain a contaminant at a level exceeding the maximum level set out	Repealing Commission Regulation (EC) No 466/2001 Amendments Commission Regulation (EC) No 1126/2007; No 565/2008; No 629/2008; No 105/2010; No 165/2010; No 420/2011; No 835/2011; No 1258/2011; No 1259/2011; No 219/2012; No 594/2012; No 1058/2012.
Regulation (EC) No 396/2005 (The European Parliament and the Council, 2005)	This Regulation shall apply to products of plant and animal origin or parts to be used as fresh, processed and/or composite food or feed in or on which pesticide residues may be present MRLs are set at Community levels Establishment of a list of groups of products for which harmonised MRLs shall apply Extensive list of commodities covered by the Regulation (plant and animal origin) (Annex I) Annex II contains the list of definitive MRLs Annex III Establishment of temporary MRLs Establishment of a list of active substances for which no MRLs are required (Annex IV) Procedure for applications for MRLs Rules for national and community controls	Repealing Council Directive 76/895/EEC; 86/362/EEC; 86/363/EEC; 90/642/EEC. Amending Council Directive 91/414/EEC Amendments Available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32005R0396:en:NOT
Directive 2002/32/EC (The European Parliament and the Council, 2002a).	General principles and definitions Indications of relationships between Member States and the Commission in order to take immediate decision for adapting the technical provisions in the Annexes to this Directive, in the light of developments in scientific and technical knowledge. Setting of maximum admissible limits of undesirable substances in products intended for animal feed. Annex I: maximum levels of each undesirable substances are reported for different type of products intended for animal feed (inorganic contaminants and nitrogenous compounds; mycotoxins; inherent plant toxins; organochlorine compounds and dioxins and PCBs; harmful botanical impurities; authorised feed additives in non-target feed following unavoidable carry-over) Annex II: for dioxin and dioxin like PCB, action threshold relative to feedingstuffs, and comments and additional information (e.g. nature of investigations to be performed) are reported.	Repealing Council Directive1999/29/EC Amendments Commission Directive 2003/57/EC; 2003/100/EC; 2005/8/EC; 2005/86/EC; 2005/87/EC; 2006/13/EC; 2006/77/EC; 2008/76/EC; 2009/8/EC; 2009/141/EC. Regulation (EC) No 219/2009 Commission Directive 2010/6/ECC Commission Regulation (EU) No 574/2011; No 277/2012; No 744/2012; No 107/2013.

Related acts	Main points	Relationship between documents
Regulation (EC) No 1107/2009 (The European Parliament and the Council, 2009)	Rules for the authorisation of plant protection products in commercial form and for their placing on the market, use and control within the Community Rules for placing on the market of treated seeds	Repealing Council Directives 79/117/EEC; 91/414/EEC

principles for the prevention and reduction of *Fusarium* toxin contamination in cereals and cereal products, adopting measures, directed at operators in the cereal chain, to prevent, reduce, control, and manage *Fusarium* toxin contamination of cereals for human food and animal feed (European Commission, 2006b). Commission Recommendation 2011/516/EU is the relevant test on the reduction of the presence of dioxins, furans and PCBs in feed and food (European Commission, 2011a). In any case a conclusive statement on the quality of a cereal lot can only be made when its mycotoxin content is known to a sufficient degree of accuracy (Siegel & Babuscio, 2011).

A summary of the EU Recommendations related to prevention and reduction of cereal contamination is given in Table 5.

6. Cereal safety: sampling and analysis for official controls

Sampling is the critical step to obtain reliable results regarding food composition, and evaluation of the presence of undesirable substances and contaminants. A sampling plan may be defined as a test procedure combined with specific analytical procedures, and in the case of undesirable substances, combined with a sample acceptance limit (Cheli, Campagnoli, Pinotti, Fusi, & Dell'Orto, 2009). To plan a sampling procedure, the substance to be tested, the analytical method, the numbers of replicates samples, the numbers of replicate measurements per samples, and the sampling technique have to be selected. Adequate sampling is necessary to make justified management decision about what to do with lots that may be contaminated with mycotoxins (van Egmond et al., 2007).

The topic of sampling and analysis for official controls of the presence of contaminants and undesirable substances in cereal and food is particularly complex. Their distribution, within a lot can be very different due to the characteristics of both food matrix and undesirable molecules themselves. Usually contaminants are divided into two groups, substances uniformly distributed (pesticides, additives, heavy metals, PCBs, dioxins, etc) and non-uniformly distributed (natural toxins, GMOs, etc.). The type of distribution of contaminants in food has major implications for attempts to precisely and accurately measure the level of contamination in a commodity bulk that is fundamental for products intended for food/feed uses in order to respect the final purposes, i.e. fixed maximum tolerable levels or other operational targets for food/feed industry. A good example is provided by mould and mycotoxin distribution in food commodities. It well known that, when sampling for mycotoxin is considered, cereals bulk moisture usually facilitates the development of localised clumps particularly rich in moulded kernels. These small percentages of extremely contaminated portions ("hot spots") are randomly distributed in a lot (average value usually registered about 0.1%) (Johansson et al., 2000). Therefore, the plan of an effective sampling procedure for cereal mycotoxin detection or quantification represents a complex challenge for operators.

There are several Regulations covering the topic of food sampling and analysis for official controls: Commission Regulation (EC) No 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (European Commission, 2006c); Commission Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs (European Commission, 2007b); Commission Regulation (EC) No 1882/2006 laying down methods of sampling and analysis for the official control of the levels of nitrates in certain foodstuffs (European Commission, 2006e); Commission Directive 2002/63/EC establishing Community methods of sampling for the official control of pesticide residues in and on products of plant and animal origin (European Commission, 2002). The specific topic regarding the methods of sampling and analysis for the official control of food as regards presence of genetically modified material is covered by Commission Recommendation 2004/787/EC and Commission Regulation (EU) No 619/2011 (European Commission, 2004b, 2011b). Each Regulation/Directive/Recommendation gives precise details regarding the methods of sampling for each food, acceptance parameters, the criteria for sample preparation, the analytical performance criteria of the methods of analysis used for the official controls, and the criteria for reporting and interpretation of the results.

A summary of the EU legal acts related to cereal sampling and analysis for the official control is given in Table 6.

7. Conclusions

This review presented an update of the EU legislation regarding cereal contaminants in order to provide a general frame of the EU food legislation and give the reader a useful source of information. As in terms of food safety, among the most important risks associated to cereals' consumption are mycotoxins, this review is focused on this topic.

The EU Commission enhanced the food safety level by either introducing new stricter Regulations/Directives or modifying the already existing ones. Since the adoption of 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, the pursuit of Community policies, in the development of food law, was to assure a high level of protection of human life and health (The European Parliament and the Council, 2002b). The legislation in the field of cereal and food safety is continuously evolving prompted by different factors, such as the availability of new scientific information becoming available in the years, the activity of the EFSA's

Table 5
Main EU Recommendations related to cereal prevention and reduction measures.

Main documents	Main points	Relationship between documents
Commission Recommendation 2006/583/EC (European Commission, 2006b)	Principles for the prevention and reduction of *Fusarium* toxin contamination in cereals Identification of risk factors to be taken into account in good agricultural practices (GAP): crop rotation, choice of variety/hybrid, crop planning, soil and crop management, harvesting, drying, storage, transport from storage	
Commission Recommendation No 2011/516/EU (European Commission, 2011a)	On the reduction of the presence of dioxins, furans and PCBs in feed and food In cases of non-compliance Member States should, in cooperation with operators, initiate investigations to identify the source of contamination, and take measures to reduce or eliminate the source of contamination	Repealing Commission Recommendation 2006/88/EC

Table 6
Main EU Regulations and Directives related to cereal sampling and analysis for the official controls.

Main documents	Main points	Relationship between documents
Commission Regulation (EC) No 401/2006 (European Commission, 2006c)	Methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs Definitions of 'lot', 'sublot', 'incremental sample', 'aggregate sample', and 'laboratory sample' General provisions for personnel and sample preparation Precise description of methods of sampling according to the different type of foodstuffs (cereals, dried fruits, …) Criteria for acceptance of a lot or sublot Criteria for sample preparation and for methods of analysis used for the official control of the levels of mycotoxins in foodstuffs General and specific requirements of the methods of analysis: performance criteria for each mycotoxin Quality standards for laboratories involved in official controls	Repealing Commission Directive 98/53/EC; 2002/26/EC; 2003/78/EC; 2005/38/EC. Amendments Decision of the EEA Joint Committee No 100/2010 Commission Regulation (EU) No 178/2010
Commission Regulation (EC) No 1882/2006 (European Commission, 2006e)	Methods of sampling and analysis for the official control of the levels of nitrates in certain foodstuffs Definitions of 'lot', 'sublot', 'incremental sample', 'aggregate sample', and 'laboratory sample' General provisions for personnel and sample preparation Precise description of methods of sampling according to the different type of foodstuffs Criteria for acceptance of a lot or sublot Criteria for sample preparation and for methods of analysis used for the official control of the levels of nitrates in foodstuffs General, specific requirements, and performance criteria of the methods of analysis Quality standards for laboratories involved in official controls	
Commission Regulation (EC) No 333/2007 (European Commission, 2007b)	Methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs Definitions of 'lot', 'sublot', 'incremental sample', 'aggregate sample', and 'laboratory sample' General provisions for personnel and sample preparation Precise description of sampling plans Specific sample preparation procedures according to the type of undesirable substance General, specific requirements, and performance criteria of the methods of analysis Criteria for acceptance of a lot or sublot Quality standards for laboratories involved in official controls	Repealing Commission Directive 2001/22/EC; 2004/16/EC; 2005/10/EC. Amendments Commission Regulation (EU) No 836/2011
Commission Directive 2002/63/EC (European Commission, 2002)	Methods of sampling for the official control of pesticide residues in and on products of plant and animal origin and repealing Definitions of 'analytical portion', 'analytical sample', 'bulk sample/ aggregate sample', 'lot', … Precise description of sampling procedures Criteria for determining compliance	Repealing Commission Directive 79/700/EEC
Commission Recommendation 2004/787/EC (European Commission, 2004b)	Technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) 1830/2003 (European Commission, 2006e). Definitions and general principles for sampling protocols Laboratory requirements Testing methods, expression and interpretation of the results of the analyses This guidance covers products that have received authorisations for their placing on the market	
Commission Regulation (EU) No 619/2011. (European Commission, 2011b)	Laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired	

scientific committees, the results from the monitoring activity, and the availability of new analytical approaches. Globalisation and the increased global trade associated with cereal production pose the need for EU legislation to face with the different legislative framework of other countries. A lack of a legislative harmonisation is an important point to consider in a worldwide discussion regarding the managing risk and regulations in cereal and food security and safety governance.

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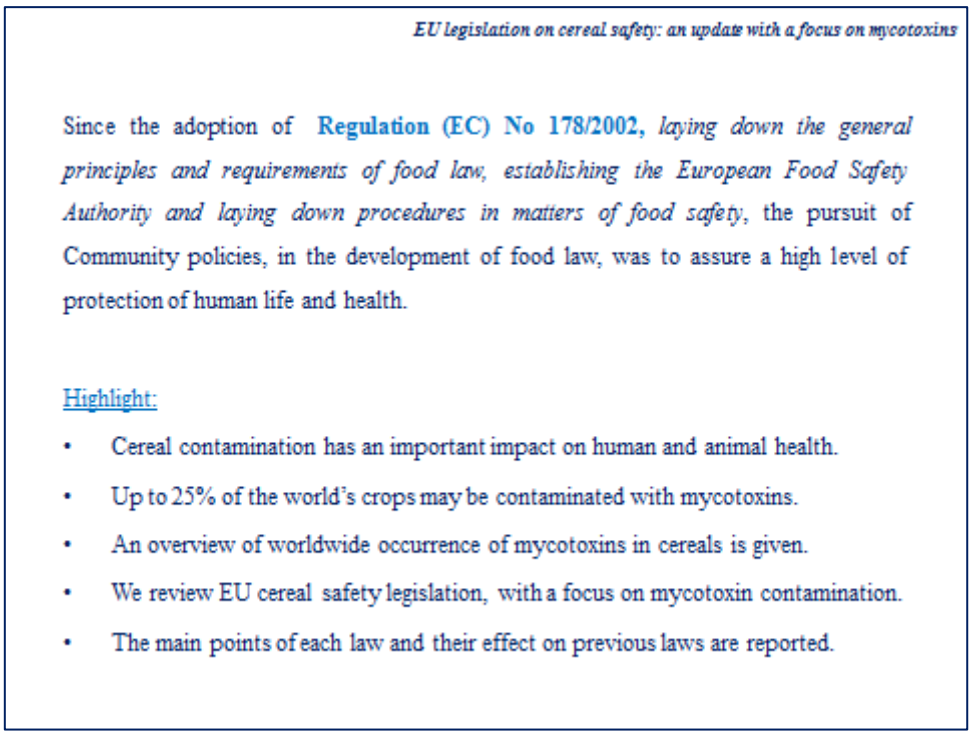
Slide 1:

Slide 2:



Slide 3:

General frame of the EU food legislation:

Regulations, Recommendations & Directives

Topics:

- Food law: the legislative framework
- Cereal safety: official controls (sampling and analysis)
- Cereal safety: maximum levels for contaminants
- Cereals contamints: prevention and reduction
- Cereal safety: sampling and analysis for official controls

Slide 4:

For each topic:

- a synoptical presentation of all laws is given;
- the main points of each law, cited in conjunction with its effect on previous laws (repeal, modification, amendments, replacement, related acts), are reported in tables.

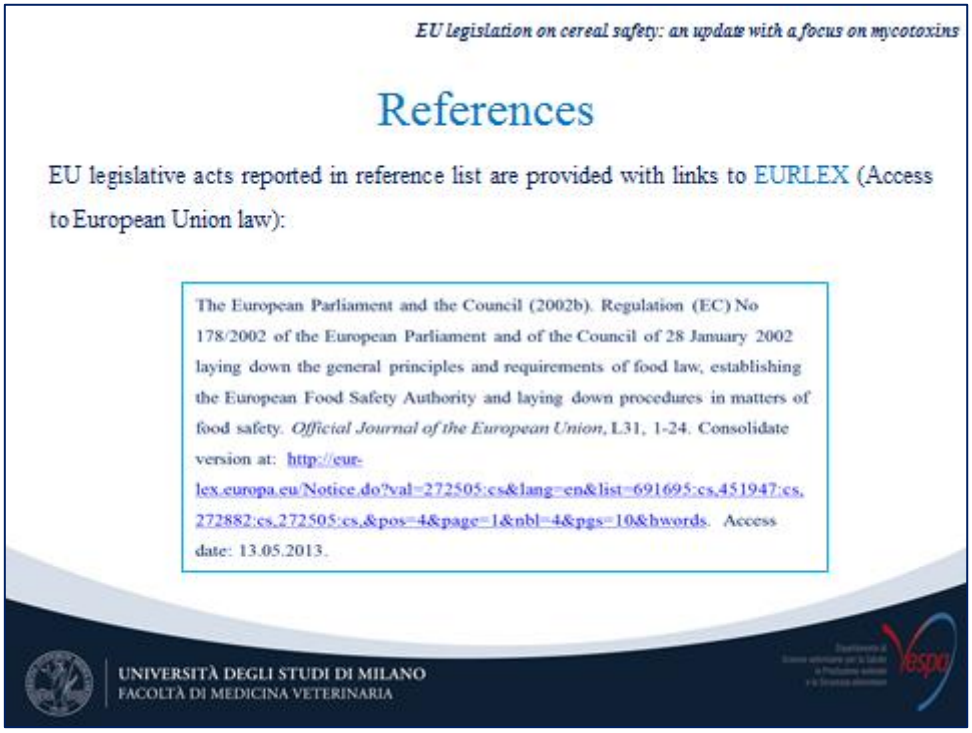
Example: Table 1.

Table 1
The basis of food/feed law: main Regulation and related acts.

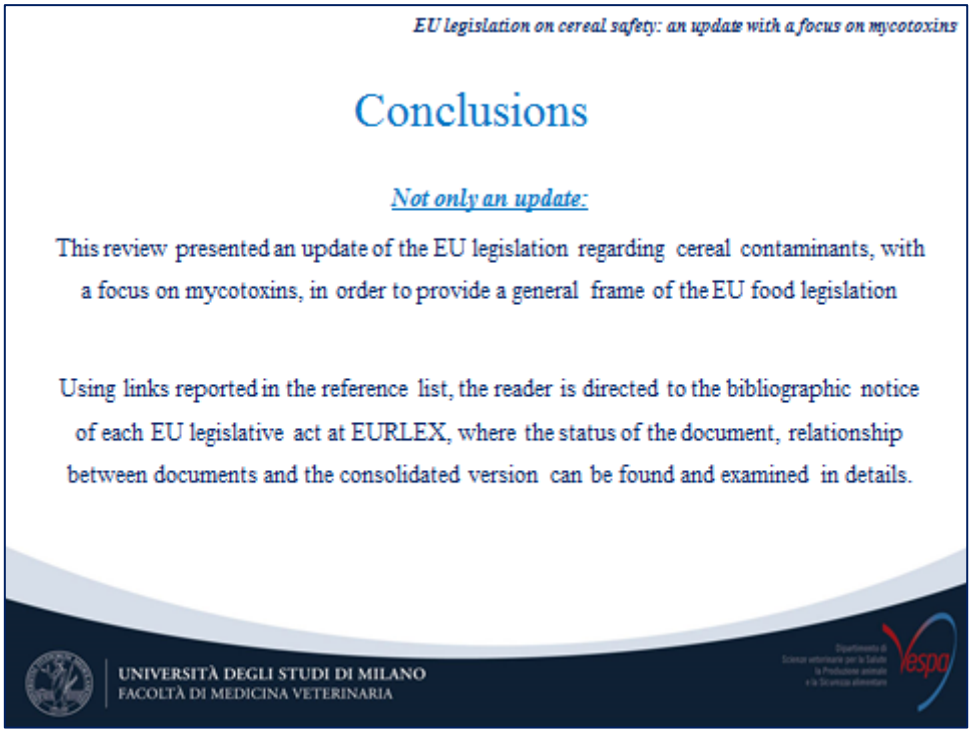
Main documents	Main points	Relationship between documents
Regulation (EC) No 178/2002 (The European Parliament and the Council, 2002b)	General principles and requirements of food law Common principles and responsibilities to provide a strong science base, efficient organisational arrangements and procedures to underpin decision-making in matters of food and feed safety General obligation of food trade Establishment of the European Food Safety Authority Establishment of a rapid alert system for the notification of a direct or indirect risk to human health deriving from food or feed (RASFF) Emergency measures for food and feed of Community origin or imported from a third country, and other emergency measures	Amendments Commission Regulation (EC) No 1642/2003; No 575/2006; No 202/2008; Regulation No 596/2009.

Related acts	Main points	Relationship between documents
Commission Decision 2004/478/EC (European Commission, 2004a)	Establishment of the general plan for food/feed crisis management Definition of practical procedures for the management of a crisis involving a serious direct or indirect risk to human	
Commission Regulation (EC) No 2230/2004 (European Commission, 2004c)	Definition of detailed rules for the implementation of the Regulation 178/2002 with regard to the network of organisations operating in the fields within the European Food Safety Authority's mission	

Slide 5:



Slide 6:



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(Enzo ♥*, my mother, my father)*

to you*...*

and

to MYSELF!