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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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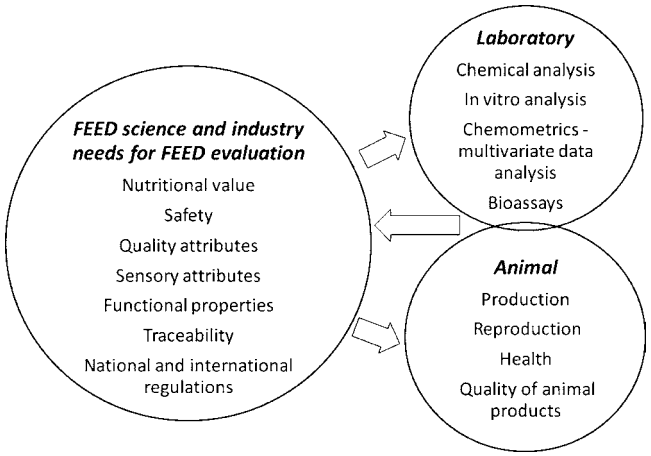

Figure 1. Feed evaluation: a multianalytical and multilevel approach.

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

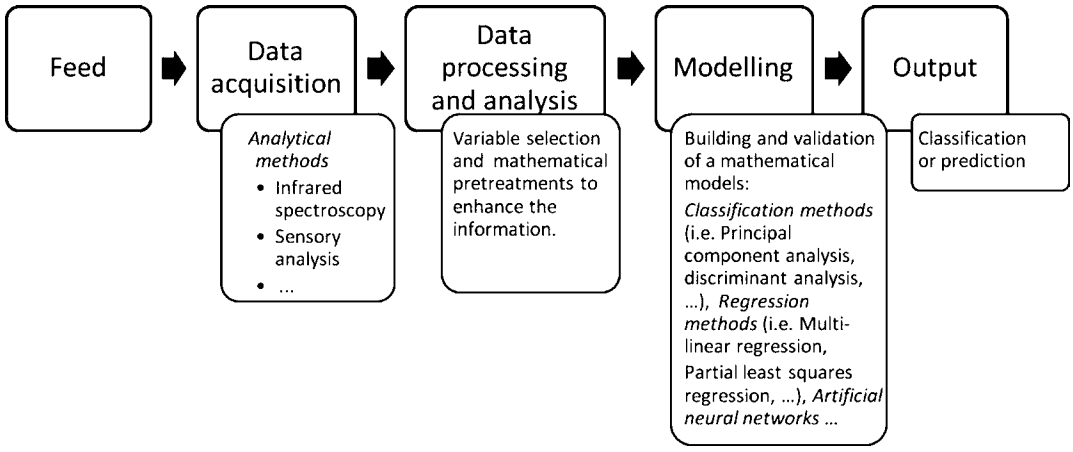

Figure 2. Analytical techniques coupled with chemometric tools: diagram of the procedure for feed analysis.

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).

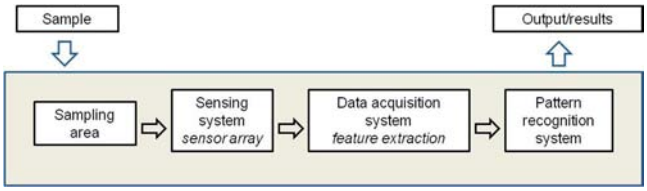

Figure 3. General configuration of the sensor array technology.

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

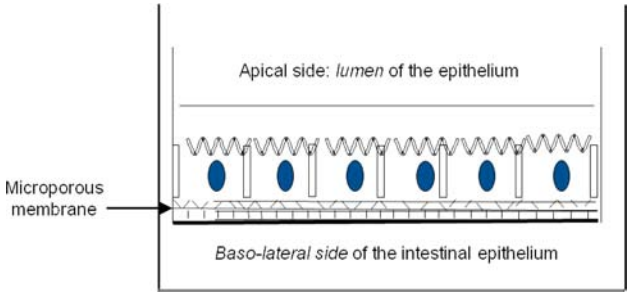

Figure 4. Schematic presentation of the functional (3D) model of the gut and functional polarity of intestinal epithelial cells growing on a microporous membrane.

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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