RESEARCH PAPER

Origin authentication of distillers' dried grains and solubles (DDGS)—application and comparison of different analytical strategies

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Abstract In the context of products from certain regions or countries being banned because of an identified or non-identified hazard, proof of geographical origin is essential with regard to feed and food safety issues. Usually, the product labeling of an affected feed lot shows origin, and the paper documentation shows traceability. Incorrect product labeling is common in embargo situations, however, and alternative analytical strategies for controlling feed authenticity are therefore needed. In this study, distillers' dried grains and solubles (DDGS) were chosen as the product on which to base a comparison of analytical strategies aimed at identifying the most appropriate one. Various analytical techniques were investigated for their ability to authenticate DDGS, including spectroscopic and spectrometric techniques combined with multivariate data analysis, as well as proven techniques for authenticating food, such as DNA analysis and stable isotope ratio analysis. An external validation procedure (called the system challenge) was used to analyze sample sets blind and to compare analytical techniques. All the techniques were adapted so as to be applicable to the DDGS matrix. They produced positive results in determining the botanical origin of DDGS (corn vs. wheat), and several of them were able to determine the geographical origin of the DDGS in the sample set. The maintenance and extension of the databanks generated in this study through the analysis of new authentic samples from a single location are essential in order to monitor developments and processing that could affect authentication.

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Introduction

With the recent and dramatic increase in bio-ethanol production, distillers' dried grains and solubles (DDGS) have rapidly become a global commodity playing an important role in the animal feed industry. The origin of feed is not as closely linked economically to a high quality background as in the case of food, where such terms as "protected designation of origin" (PDO) and "protected geographical indication" (PGI) have a major impact on the market price of the relevant goods. There is a trend, however, towards an environmentally sustainable approach with regard to producing animal feed with minimal environmental impact, such as producing feed on a regional

basis and producing feed products on the farm [1]. In the case of banned products from certain regions or countries because of an identified or non-identified hazard, and because of differing legislation, proof of geographical origin is essential in terms of feed and food safety issues. In July 2014, China's General Administration of Quality Supervision, Inspection and Quarantine (AQSIQ) notified the US Department of Agriculture's Foreign Agricultural Service (FAS) that it had found MIR 162, a banned biotech trait, in 963 batches of US DDGS imports, totaling 425,600 t. China decided to reject all DDGS shipments that did not meet the certification requiring that products did not contain the MIR 162 GMO corn strain [2]. Apart from GM-related issues, imports also need to be screened for antibiotics because these products can be used in the fermentation processing to fight lactic acid bacteria that compete with yeast for the sugars to make ethanol. Any producer hoping to export DDGS to Europe therefore has to ensure that they are free of antibiotics [3].

Usually, the product labeling of a feed lot shows its origin, and the paper documentation shows its traceability. Incorrect product labeling is common in embargo situations, and alternative analytical strategies for controlling feed authenticity are therefore needed. In this study, DDGS were chosen as the product on which to base a comparison of analytical strategies aimed at identifying the most appropriate one. DDGS are obtained through the production of alcoholic beverages or industrial bio-ethanol and are used as feed material that contains valuable amounts of protein, fat, and fiber. They are produced worldwide, and their market volume is increasing.

Various analytical techniques were considered for this study and were assessed for their ability to authenticate DDGS. The techniques included rapid and economic ones, such as near-infrared spectroscopy (NIRS) and microscopy (NIRM), mid-infrared (MIR), and Raman spectroscopy as well as more sophisticated approaches such as proton transfer reaction mass spectrometry (PTR-MS), direct analysis in real time coupled with Orbitrap mass spectrometry (DART-OrbitrapMS), and liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOFMS). In addition, proven techniques for authenticating food, such as stable isotope ratio analysis (via isotope ratio mass spectrometry, IRMS) and DNA analysis (via polymerase chain reaction, PCR), were applied to determine the DDGS origin and check the botanical origin labeling.

Methods based on the untargeted detection of contaminants and foreign bodies in food/feed, as well as on untargeted analysis of food components for product authentication, have already shown promise [4, 5]. This study sought to assess the applicability of untargeted analyses for feed materials, particularly with regard to proof of origin. The so-called fingerprinting techniques used for this untargeted approach benefit from the ever-increasing possibilities for data acquisition and processing. A major challenge with fingerprinting techniques, however, is to extract the information of interest (e.g., geographical origin) from the large amount of other information contained in the data (e.g., botanical and processing origin).

This study sought to develop and improve methods for assessing the traceability and authenticity of feed materials, using DDGS as the feed product. The paper outlines the analytical techniques selected for the study and evaluates them in relation to a sample set externally validated by a procedure known as the system challenge. The techniques are then compared based on their complementarities, and a global strategy for tracing and confirming the origin of DDGS is proposed.

Material: sample set, characterization, and validation approaches

DDGS samples

A total of 191 DDGS samples were collected from reliable sources between July 2011 and September 2013. DDGS were defined according to the numbering in the EU feed catalogue [6]: either (a) number 1.12.10 (distillers' dried grains) or (b) number 1.12.11 (distillers' dried grains and solubles/distillers' dark grains). The sample set included DDGS from Canada, China, Europe, and the USA produced from corn (*Zea mays*) and wheat (*Triticum aestivum* L.) and obtained during the industrial production of bio-ethanol or alcoholic beverages. The DDGS samples were stored at 4 °C in the dark, and each sample was then ground using a centrifugal mill (ZM 200, Retsch, Germany, mesh size 0.5 mm). The ground samples were homogenized in plastic containers (filling level ~2/3) for 6 h using a drum hoop mixer (RRM 100, Engelsmann, Germany). Portions of the ground samples, again stored at 4 °C in the dark, were then distributed in six batches to laboratories in the EU's Quality and Safety of Feeds and Food for Europe (QSAFFE) project [7] for analysis.

Control of botanical origin using IRMS and PCR

IRMS methods to determine the stable isotope ratio of DDGS powder

Stable isotope ratios of carbon ($^{13}C/^{12}C$) were determined using the pre-ground DDGS samples. Analytical procedures were performed as previously reported [8]. The botanical origin of DDGS samples was assessed according to the $\delta^{13}C$ values, with pure C_4 plant origin (corn) indicated by $\delta^{13}C$ values higher than −14.5‰ and pure C_3 plant origin (wheat) indicated by $\delta^{13}C$ values lower than −25.5‰.

One subset of samples which was indicated by the provider to be corn DDGS samples showed unexpected $^{13}C/^{12}C$ ratios and were thereafter excluded from the model development work. These samples were assessed separately as the system

challenge 3 set in order to evaluate the ability of the analytical methods to determine the proportions of corn and wheat in DDGS (see Table 5).

PCR methods to determine DNA from DDGS powder

In addition to the IRMS method, a molecular biological method based on PCR was used to assess the botanical origin of the mixed samples. DNA from these samples was extracted and purified following the CTAB-based method described in Annex A.3.1 of the ISO 21571:2005 international standard [9]. DNA concentration was estimated using a Nanodrop ND-1000 spectrophotometer. The ability of plant DNA to be amplified and detected by PCR was assessed using a universal real-time PCR assay with RbCL-F and RbCL-R primers developed by Debode et al. [10].

Six of the 14 mixed DDGS were analyzed using PCR based on this protocol. The DDGS amount of the other samples was too low to perform the analysis. The results showed the same trends as those that emerged using the IRMS method (for more detail, see Table 5).

Nutrient content assessment of DDGS samples (NIRS)

In order to study the composition and variability of the DDGS samples, all of them were initially analyzed using a FOSS XDS NIR spectrometer active in the 400–2,500-nm range. Quality parameters such as moisture, protein, fat, fiber, and ash were estimated using equations constructed with historical NIRS databases [11]. These calibration equations were characterized by a coefficient of determination (R^2) and a standard error of cross-validation (SECV) defined in Table 1. Corn DDGS differed from wheat DDGS by having higher fat ($P<0.001$) and moisture ($P=0.0011$) content. Wheat DDGS differed from corn DDGS by having higher protein ($P<0.001$) and ash ($P=0.023$) content. The greater variability in the corn DDGS sample set was linked to its fat content (standard deviation (SD)=3.01). No difference in fiber content was noted between the corn and wheat DDGS ($P=0.98$). These results accord with results reported by Pedersen et al. [12]. With regard to geographical origin, the corn DDGS from China differed from those from Europe and the USA by having a lower fat ($P<0.001$) content and greater variability in fat content (SD=2.52). The corn DDGS from Europe differed from those from the USA by having higher protein ($P<0.001$) and fiber ($P<0.001$) content and lower ash ($P<0.001$) content. The greater variability in the European corn DDGS sample set was linked mainly to the fiber (SD=1.12) and ash (SD=0.88) content. With regard to processing origin, the USA corn DDGS obtained via alcoholic beverage production differed from those obtained via bio-ethanol production by having a lower moisture ($P=0.009$) content. No difference was noted for the other parameters between those two processes.

In order to characterize the variability of the DDGS samples according to the production origin, principal component analysis (PCA) was performed, using the five quality parameters, with normalization and autoscale pre-processing being applied to the data. Based on the compositional profiles of the DDGS, PCA allowed corn DDGS from three bio-ethanol plants (two in China, origins 1 and 2, and one in the Czech Republic) to be visually distinguished from corn DDGS from bio-ethanol and alcoholic beverage plants in the USA, indicating the potential of each ethanol plant to produce DDGS with consistent compositional characteristics (data not shown). The DDGS from China (origin 1) were characterized by very low fat content, which could be explained by fat extraction in the production process. The DDGS from the Czech Republic were characterized by very high fiber content and very low ash content. The DDGS from the USA were characterized by very high fat content and very low protein and fiber content. The DDGS from China (origin 2) were characterized by intermediate protein, fat, and fiber content levels. Table 1 presents a summary of the samples used in this study and their predicted moisture, protein, fat, fiber, and ash content, using NIRS calibration equations.

Datasets and the concept of classification and external validation

The most significant aspect of the experimental design was that all the study laboratories received and analyzed the same sample batch, which enabled the various analytical approaches for DDGS authentication to be compared.

For the first analysis on sample clustering, PCA was conducted on the full dataset (191 samples). This constructs a small number of new variables which explain most of the variability in the measured variables, enabling any clustering to be visualized in low dimensions. The results showed the samples clustered according to their botanical origin, as well as some trends in geographical origin among the corn DDGS samples, confirming the results obtained from the PCA on the quality parameters of the DDGS.

For better classification, various approaches for determining botanical, geographical, and processing origin were applied. The classification techniques used depended on the expertise of the laboratories; they included canonical discriminant analysis (CDA), soft independent modeling of class analogy (SIMCA), principal component analysis and linear discriminant analysis (PCA-LDA), partial least squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA). Models for predicting botanical origin were developed for corn and wheat DDGS. Models for predicting geographical origin used only corn DDGS from China, the EU, and the USA. In some cases, models were also developed to discriminate corn DDGS from particular bio-ethanol plants: two in China and one in the Czech Republic. A two-step procedure

Table 1 Characteristics of the NIRS calibration equations and the samples for five quality parameters

		Moisture (%)		Protein (%)		Fat (%)		Fiber (%)		Ash (%)	
	N	901		1,153		741		439		945	
	R^2	0.91		0.98		0.98		0.83		0.87	
	SECV	0.75		0.60		0.34		0.69		0.27	
Group	N	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Wheat	29	8.5	1.08	33.1	2.07	4.9	0.66	6.6	1.05	4.7	0.38
Corn	148	9.3	1.20	28.7	2.08	8.3	3.01	6.7	0.94	4.4	0.73
China	48	9.0	0.86	30.5	1.56	5.0	2.52	7.2	0.70	4.7	0.50
Origin 1 (bio-ethanol)	30	9.3	0.78	31.1	1.36	3.2	0.65	7.1	0.72	4.6	0.39
Origin 2 (bio-ethanol)	18	8.6	0.78	29.4	1.25	8.0	1.09	7.3	0.66	4.7	0.65
USA	51	9.0	1.15	27.1	1.49	10.0	1.37	6.1	0.61	4.6	0.42
USA (alcoholic beverage)	34	8.7	1.03	26.7	1.48	10.1	1.34	6.2	0.55	4.6	0.39
USA (bio-ethanol)	10	9.8	1.23	27.3	1.45	9.8	1.63	6.0	0.86	4.7	0.57
Other	7	9.2	1.18	28.2	1.12	10.1	1.27	6.0	0.49	4.4	0.28
EU	33	9.6	1.36	29.3	1.32	9.6	1.66	7.2	1.12	3.6	0.88
Czech Republic (bio-ethanol)	21	10.0	1.20	29.7	0.90	8.4	0.60	7.9	0.26	3.0	0.24
Other	12	9.0	1.41	28.7	1.74	11.6	0.61	5.9	0.84	4.7	0.56
Other	16	9.8	1.55	27.3	1.19	10.5	1.67	6.1	0.70	4.9	0.62
Mix wheat corn	14	10.0	1.38	31.3	1.23	8.8	1.93	7.2	0.44	4.7	0.53

N number of samples, R^2 coefficient of determination, SECV standard error of cross-validation

was applied to the data acquired: (1) the statistical models were validated initially by cross-validation using the leave-one-out method, where successively each sample was left out of the model calculation and subsequently predicted once (internal validation), and (2) the developed models were then validated by predicting the origin of samples initially isolated from the calibration dataset. In spite of recommendations for data evaluation, the single groups of samples subjected to statistical data analysis to determine botanical, geographical, and processing origin were not always exactly the same size and sometimes, for various reasons, specific samples were removed from the datasets (e.g., statistical outliers, conspicuous samples, or a limited amount of samples). Nevertheless, a general comparability of the results was achieved.

In order to compare the analytical techniques used to authenticate the botanical, geographical, and processing origin of the DDGS, an external validation procedure known as the system challenge was used. In this procedure, the botanical/geographical/processing origins of "unlabeled" DDGS samples were predicted using the analytical techniques in combination with the statistical models developed. The procedure served as an independent method of external validation for the developed models because the relevant information about the samples used for the system challenge was not available until the prediction results were evaluated by the person coordinating the study. This procedure, which so far as we know is unique in validation and proof of authenticity studies, consisted of system challenge 1 and 2 sets, summarized below. The two-step character was necessary because DDGS sampling was carried out consecutively, and the statistical models were updated after the first step.

System challenge 1 set of unlabeled samples The botanical and geographical origin of 16 samples (SC1-01–SC1-16) was predicted using the statistical models developed on the basis of 132 DDGS samples: for botanical origin, DDGS produced from corn (n=106) and wheat (n=26); for geographical origin, corn DDGS from China (n=45), the EU (n=14), and the USA (n=31). It should be noted that only nine of the 16 system challenge sets were new samples that had not yet been included in the models. Seven of the 16 system challenge samples (SC1-01 to SC1-07) had been included in the models but were nevertheless considered useful for the system challenge approach.

System challenge 2 set of unlabeled samples Prior to the system challenge 2 set, the statistical models were updated with 12 further DDGS samples including new geographical origin and the system challenge 1 set (only samples SC1-08 to SC1-16 used). The updated models were therefore based on 153 DDGS samples; for botanical origin, DDGS produced from corn (n=125) and wheat (n=28); for geographical origin, corn DDGS from China (n=47), the EU (n=22), and the USA (n=39). Consequently, the predictions for the system challenge 2 set (botanical origin, 24 DDGS samples; geographical origin, 23 corn DDGS samples; processing origin, 18 corn DDGS samples obtained via bio-ethanol or alcoholic beverage production) were done using the updated models.

System challenge 3 set of mixed samples A third step focused on predicting the botanical origin of 14 mixed DDGS samples using the final models for botanical origin that had been developed after the inclusion of the system challenge 1 and 2 sets. As noted earlier, despite the care taken in sample collection, it emerged that the information on botanical origin of 14 DDGS provided was incorrect. This sample set was therefore used as a third system challenge set, and predictions using the models were made on 177 DDGS samples from corn ($n=148$) and wheat ($n=29$). This third step was an extension of the system challenge approach and demonstrated the applicability of the models in determining DDGS botanical origin of samples produced from mixed raw materials. Figure 1 gives an overview of the analytical approaches as well as the calibration and system challenge sets used in the study to develop and validate the discrimination models, respectively.
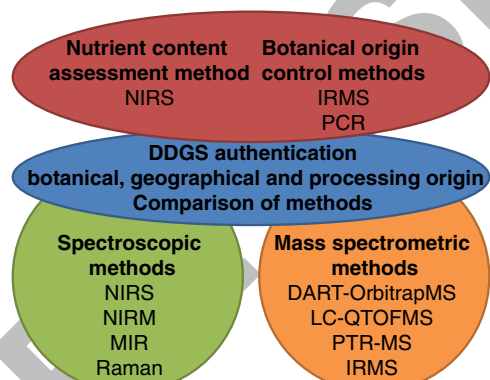
Analytical and chemometric methods for DDGS authentication

Various analytical techniques for DDGS authentication were assessed. The determination of the botanical and geographical origin of DDGS was of particular interest, but it emerged that other issues (e.g., processing origin, basic parameters on matrix composition) could also be studied using the selected techniques. The analytical techniques applied in this study were NIRS, NIRM, MIR, and Raman spectroscopic methods as well as MS-based approaches such as PTR-MS, DART-OrbitrapMS, and LC-QTOFMS. IRMS was also used, so as

Fig. 1 Scheme of the study showing the analytical approaches and the DDGS sets used at each step to develop and validate the discrimination models

to include a proven technique for assessing food authenticity and one potentially able to determine geographical origin.

Regarding the rapid screening spectroscopic methods, two Fourier transform (FT) NIR spectrometers (PerkinElmer and Thermo Fisher) combined with different chemometric packages for PLS-DA and OPLS-DA were used in two separate laboratories to assess the feasibility of classifying the origin of DDGS powders [13]. The FT-NIRM method applied on DDGS powder involved using an FT-NIR spectrometer from PerkinElmer (PerkinElmer Spectrum One NTS system) equipped with a PerkinElmer Spotlight microscope [14–16]. The ATR-FT-MIR method involved using attenuated total reflection (ATR) in conjunction with a diamond and a FT-MIR spectrometer. Three methods were developed: one based on DDGS powder analysis [17] and two on the oil extracted chemically [17] or in situ by filter [18]. In the latter case, the authentication was based on analyzing only the composition of the DDGS oil fraction; spectral signals of protein and fiber constituents were therefore not acquired. Raman analysis was performed with an Advantage 1064 Raman Spectrometer (DeltaNu Inc., Laramie, Wyoming, USA) on oil from DDGS extracted using accelerated solvent extraction (ASE) [17, 19].

Regarding the more complex screening mass spectrometric methods, the LC-QTOFMS analyses were performed using an Acquity Ultra-Performance LC system coupled with a Synapt G2 HD spectrometer (Waters, USA). The method used was that reported by [20], adapted for DDGS. A system consisting of a DART ion source (DART-SVP, IonSense, Saugus, MA, USA) coupled with an Exactive benchtop mass spectrometer with an Orbitrap analyzer (Thermo Fisher Scientific, San Jose, CA, USA) was also used on polar and non-polar extracts of DDGS [21]. The volatile fingerprint of DDGS samples was measured using a high sensitivity PTR-MS instrument (Ionicon Analytik, Innsbruck, Austria) applying the analytical procedures previously reported [22]. Finally, stable isotope ratios of hydrogen ($^2H/^1H$), carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), and oxygen ($^{18}O/^{16}O$) were determined in the pre-ground DDGS samples [8].

System challenge sets: results and discussion

DDGS system challenge 1 and 2 sets of unlabeled samples

The analytical approaches and statistical models developed for DDGS authentication were tested in a "real-life" situation that resembled conditions under which the techniques would be applied in laboratories in the future. The system challenge procedure was performed blindly. Tables 2, 3, and 4 show the classification of results obtained for the system challenge 1 and 2 sets by applying the best models developed using varying techniques to determine botanical, geographical, and processing origin. The actual information on sample origin is

Table 2 Botanical origin of DDGS in the system challenge 1 and 2 sets

Sample code	Botanical origin	NIRS Powder PLS-DA	NIRS Powder OPLS-DA	NIRM Powder PLS-DA	MIR Powder PCA-LDA	MIR ASE oil PCA-LDA	MIR Oil in situ PLS-DA	RAMAN ASE oil OPLS-DA	LC-QTOFMS Extract PLS-DA	DART-OrbitrapMS Non-polar extract PLS-DA	PTR-MS Extract KNN	IRMS Powder c3/c4
SC1-01 [a]	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-02 [a]	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-03 [a]	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-04 [a]	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
SC1-05 [a]	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
SC1-06 [a]	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-07 [a]	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-08	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-09	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-10	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-11	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-12	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-13	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-14	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC1-15	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
SC1-16	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat
SC2-01	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-02	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-03	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-04	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-05	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-06	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-07	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-08	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-09	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-10	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-11	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-12	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-13	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-14	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-15	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-16	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-17	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-18	Wheat	Wheat	Wheat	Wheat	Wheat	Wheat	**Corn**	Wheat	Wheat	Wheat	**Corn**	Wheat
SC2-19	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-20	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-21	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-22	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-23	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC2-24	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
Classification (summarized) Correct		100 %	100 %	100 %	100 %	100 %	98 %	100 %	100 %	100 %	98 %	100 %
Ambiguous		–	–	–	–	–	–	–	–	–	–	–
False		–	–	–	–	–	2 %	–	–	–	2 %	–

Legend: Correct — Ambiguous — False — Not analyzed

[a] Samples included in the calibration set to build the models

given and compared with the results of the single analytical techniques.

For botanical origin (Table 2), all techniques showed excellent results, although it should be noted that most of the samples were produced from corn because this matrix was to be used for predicting geographical origin. One sample (SC2-18) was misclassified by two techniques. A more in-depth analysis of the results showed that it was close to the discrimination limit between both species.

For geographical origin (Table 3), the correct classification rates ranged from 34 to 94 % and were judged as very good. The results demonstrate that, in addition to highly sophisticated techniques (IRMS, PTR-MS, and LC-QTOFMS), some spectroscopic techniques (NIRS, NIRM, MIR analysis of DDGS powders) can be used for DDGS authentication with a correct classification rate above 80 %. Several analytical techniques gave ambiguous geographical origins for some samples, indicating that the exact origin cannot be classified precisely with the models developed. This was the case with MIR analysis using in situ oil extraction, where a binary classification tree was built to identify geographical origin. The China vs. US–EU corn DDGS models were applied first to the

samples. The origin 1 (O1) vs. origin 2 (O2) China corn DDGS or EU–Czech Republic vs. other EU–USA corn DDGS were then applied in order to identify the specific bio-ethanol plants. Of the 12 ambiguous results, 11 were DDGS produced in the USA or Europe but not in the Czech Republic. With the Raman and DART-OrbitrapMS techniques, there was quite an overlap between EU and US samples, and the latter were therefore were more closely assigned to the EU cluster. The DART-OrbitrapMS technique showed quite poor results for the three geographical origins (EU, USA, and China).

Processing origin was predicted by some laboratories in order to check if the developed strategies could also be used for this purpose (Table 4). The models developed for processing origin, however, were based only on a sample set consisting of DDGS obtained via alcoholic beverage production in only a few geographical places. There is therefore more to be done in this area, as is the case with the results of the system challenge sets, and models need to be continuously validated before an appropriate strategy can be put into practice. Two techniques (NIRS and LC-QTOFMS) showed a correct classification rate higher than 90 %.

Table 3 Geographical origin of DDGS in the system challenge 1 and 2 sets

Sample code	Geographical origin	NIRS Powder PLS-DA	NIRS Powder OPLS-DA	NIRM Powder PLS-DA	MIR Powder PCA-LDA	MIR ASE oil PCA-LDA	MIR Oil in situ PLS-DA	RAMAN ASE oil OPLS-DA	LC-QTOFMS Extract PLS-DA	DART-OrbitrapMS Non-polar extract PLS-DA	PTR-MS Extract KNN	IRMS Powder c3/c4
SC1-01[a]	USA	USA	USA	China (O1)	USA	USA	USA	USA	EU	China	China	USA
SC1-02[a]	USA	USA	USA	USA	USA	EU	USA	USA	EU/USA	EU/USA	USA/China	USA
SC1-03[a]	USA	USA	USA	China (O1)	USA	USA	USA	USA	USA	China	USA	USA
SC1-06[a]	USA	USA	USA	China (O1)	USA	EU	USA	USA	USA	China	USA	USA
SC1-07[a]	EU-Poland	USA	EU-Poland	USA	USA	USA	East-EU	Unclass.	EU	USA	EU	USA
SC1-08	EU-Cz	EU	EU-Cz	EU	EU	EU	USA	EU-Cz	EU	USA	Unclass.	EU
SC1-09	USA	USA	USA	China (O1)	USA	USA	USA	USA	USA	China	USA	USA
SC1-10	EU-Cz	EU	EU-Cz	EU	EU	EU	East-EU	EU-Cz	USA	EU	EU	EU
SC1-11	China (O1)	China	China (O1)	China	China	China	China	China (O1)	China	China	China	China
SC1-12	China (O2)	China	China (O2)	China	China	China	China	China (O2)	China	China	China	China
SC1-13	USA	USA	USA	USA	USA	USA	USA	EU-Cz	EU/USA	EU/USA	USA	USA
SC1-14	USA	USA	USA	USA	USA	USA	USA	USA	USA	China	USA	USA
SC2-01	USA	USA	USA	USA	USA	EU	USA/EU	USA	USA	USA/EU	USA	USA
SC2-02	USA	USA/EU	USA	USA	USA	EU	USA/EU	EU	USA	USA/EU	USA	USA
SC2-03	USA	USA	USA	USA	USA	USA	USA/EU	EU	USA	USA/EU	USA	USA
SC2-04	USA	USA	USA	USA	USA	EU	USA/EU	EU	USA	USA/EU	USA	USA
SC2-05	USA	USA	USA	USA	USA	USA	USA/EU	EU	USA	EU	USA	USA
SC2-06	USA	USA/EU	USA	USA	USA	EU	USA/EU	USA/EU	USA	USA/EU	USA	USA
SC2-07	EU-Cz	EU	EU	EU	EU	EU	EU-Cz	EU	EU	USA/EU	EU	EU
SC2-08	EU-Cz	EU	EU	EU	EU	USA	EU-Cz	EU	EU	EU	EU	EU
SC2-09	EU-Cz	EU	EU	EU	EU	EU	EU-Cz	EU	EU	USA/EU	EU	EU
SC2-10	EU-Cz	EU	EU	EU	EU	EU	EU-Cz	EU	EU	China/EU	EU	EU
SC2-11	EU-Cz	EU	EU	EU	EU	EU	USA/EU	EU	EU	EU	EU	USA
SC2-12	EU-Cz	EU	EU	EU	EU	EU	EU-Cz	EU	EU	EU	EU	EU
SC2-13	EU-Cz	EU	EU	EU	EU	EU	EU-Cz	EU	EU	un-classified	EU	EU
SC2-14	China (O1)	China	China (O1)	China	China	China	China (O1)		China	China	USA	China
SC2-15	EU-Poland	USA	USA	Unclass.	EU	EU	USA/EU	EU	EU	EU	EU	USA
SC2-16	EU-Netherlands	EU	EU	Unclass.	EU	EU	USA/EU	EU	EU	USA/EU	EU	EU
SC2-17	EU-Netherlands	EU	EU	EU	USA	EU	USA/EU	EU	EU	USA	EU	EU
SC2-19	USA	USA/EU	USA	USA	USA	USA	China (O2)	USA	USA	USA	USA	USA
SC2-20	USA	USA	USA	USA	USA	USA	EU-Cz	USA	USA	USA	USA	USA
SC2-21	USA	USA/EU	USA	USA	USA	USA	EU-Cz		USA	USA	USA	EU
SC2-22	USA	USA/EU	USA	USA	USA	USA	EU-Cz	USA/EU	USA	USA/EU	USA	EU
SC2-23	USA	USA/EU	USA	USA	USA	USA	USA/EU	EU	USA	EU	EU	EU
SC2-24	EU-Hungary	EU	China/EU	EU	EU	EU	USA/EU		EU	EU	EU	EU
Classification (summarized) Correct	77 %	94 %	80 %	94 %	77 %	51 %	72 %	88 %	34 %	86 %	83 %
Ambiguous	17 %	3 %	–	–	–	34 %	6 %	6 %	34 %	3 %	–
False	6 %	3 %	20 %	6 %	23 %	14 %	22 %	6 %	31 %	11 %	17 %

Legend: Correct | Ambiguous | False | Not analyzed

Unclass unclassified

[a] Samples included in the calibration set to build the models

DDGS system challenge 3 set results for mixed samples

After receiving the prediction results for the system challenge 1 and 2 sets, the laboratories were given full information about the samples and amended their final models accordingly. These optimized models were then applied to the system challenge 3 set, which included 14 DDGS samples of mixed botanical origin, to confirm the mislabeling indicated by the IRMS technique. Table 5 shows the botanical origin prediction made using IRMS and PCR, as well as the final classification models using NIRS, MIR, Raman spectroscopy, and LC-QTOFMS. No other methods were investigated for this dataset.

Contrary to the information provided, the IRMS method showed that 10 of the 14 DDGS from this set were not of pure botanical origin (C_3/wheat from ~22 to ~78 %; C_4/corn from ~22 to ~78 %), two corn/wheat mixture DDGS (SC3-13 and SC3-14) were actually pure corn DDGS (C_4/corn >85), and one pure corn DDGS (SC3-02) was actually pure wheat DDGS. Only one sample was actually pure corn (SC3-05). Only six of the 14 DDGS were analyzed by PCR; the DDGS amount in the other samples was too low for PCR to be performed. Assuming that DNA content indicated botanical origin, real-time PCR results for samples SC3-03, SC3-10, and

SC3-11 accorded with the findings for mixed corn and wheat feed, whereas SC3-14 seemed to have been produced from corn but contained traces of wheat. Even if corn DNA was detected in sample SC3-13, low DNA content in samples SC3-06 and SC3-13 did not allow conclusions to be drawn regarding botanical origin. The samples were then classified as pure corn, pure wheat, or a mixture (corn > wheat, corn = wheat, wheat > corn), depending on the rough content of C_3 and C_4 material or the haploid genome equivalents.

Applying the NIRS method to DDGS powder confirmed these results, except for sample SC3-11. The MIR method applied to DDGS powders classified the full set as corn DDGS, using the PCA-LDA model; the SIMCA model, however, classified the samples correctly in compliance with the IRMS/PCR approach. An interesting result related to approach used for extracted ASE oils, based on MIR and the SIMCA model. Here, the botanical origin of seven DDGS samples (SC3-02/04/05/07/08/13/14) was predicted correctly, if the IRMS results are considered as true, and only one DDGS sample (SC3-01) was not assigned to one of the groups, although it contained 71 % C_3 material (probably wheat). No DDGS samples with a ratio of C_3/C_4 between 70:30 and 30:70, however, were assigned to the corn or wheat DDGS groups. Therefore, apart from the PCA-LDA models, the

Table 4 Processing origin of DDGS in the system challenge 1 and 2 sets

Sample code	Processing origin	NIRS Powder PLS-DA	NIRS Powder OPLS-DA	NIRM Powder PLS-DA	MIR Powder PCA-LDA	MIR ASE oil PCA-LDA	MIR Oil in situ PLS-DA	RAMAN ASE oil OPLS-DA	LC-QTOFMS Extract PLS-DA	DART-OrbitrapMS Non-polar extract PLS-DA	PTR-MS Extract KNN	IRMS Powder c3/c4
SC1-01 [a]	Bioeth.	Bioeth.					Bioeth.		Bioeth.			
SC1-02 [a]	Alc. bev.	Alc. bev.					Alc. bev.		Alc Bev/Bioeth.			
SC1-03 [a]	Bioeth.	Bioeth.					Alc. bev.		Alc Bev/Bioeth.			
SC1-04 [a]	Bioeth.	Bioeth.										
SC1-05 [a]	Bioeth.	Bioeth.										
SC1-06 [a]	Alc. bev.	Alc. bev.					Alc. bev.		Alc. bev.			
SC1-07 [a]	Bioeth.	Alc. bev.					Bioeth.		Bioeth.			
SC1-08	Bioeth.	Bioeth.					Bioeth.		Bioeth.			
SC1-09	Bioeth.	Bioeth.					Bioeth.		Bioeth.			
SC1-10	Bioeth.	Bioeth.					Bioeth.		Bioeth.			
SC1-11	Bioeth.	Bioeth.							Bioeth.			
SC1-12	Bioeth.	Bioeth.							Alc Bev/Bioeth.			
SC1-13	Alc. bev.	Alc. bev.					Beverage		Alc Bev/Bioeth.			
SC1-14	Bioeth.	Bioeth.					Alc. bev.		Bioeth.			
SC1-15	Bioeth.	Bioeth.										
SC1-16	Bioeth.	Bioeth.										
SC2-01	Alc. bev.		Alc. bev.						Alc. bev.	Bioeth.		
SC2-02	Alc. bev.		Alc. bev.						Alc. bev.	Un-classified		
SC2-03	Alc. bev.		Alc. bev.						Alc. bev.	Bioeth.		
SC2-04	Alc. bev.		Alc. bev.						Alc. bev.	Un-classified		
SC2-05	Alc. bev.		Alc. bev.						Alc. bev.	Bioeth.		
SC2-06	Alc. bev.		Alc. bev.						Alc. bev.	Bioeth.		
SC2-07	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-08	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-09	Bioeth.		Bioeth.						Bioeth.	Un-classified		
SC2-10	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-11	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-12	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-13	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-15	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
SC2-16	Bioeth.		Bioeth.						Bioeth.	Un-classified		
SC2-17	Bioeth.		Bioeth.						Bioeth.	Un-classified		
SC2-23	Alc. bev.		Alc. bev.						Alc. bev.	Bioeth.		
SC2-24	Bioeth.		Bioeth.						Bioeth.	Bioeth.		
Classification (summarized) Correct		94 %	100 %	–	–	–	80 %	–	90 %	56 %	–	–
Ambigous		–	–	–	–	–	–	–	10 %	–	–	–
False		6 %	–	–	–	–	20 %	–		44 %	–	–

Legend: Correct | Ambiguous | False | Not analyzed

Bioeth. bio-ethanol, *Alc. bev.* alcohol beverage

[a] Samples included in the calibration set to build the models

SIMCA model for the botanical origin of the oils could be useful for DDGS composed of mixed raw material. The results achieved using MIR (based on in situ extracted oil) are in line with the IRMS/PCR results. For data obtained using the Raman method, the IRMS/PCR results were confirmed for 11 of the 14 samples. A more precise classification approach was used by labeling samples as pure wheat or corn when the predicted score was higher than 0.8. A similar classification approach was used for the LC-QTOFMS method, and the IRMS/PCR results were confirmed.

This approach illustrates the applicability of botanical origin models for samples produced from mixed raw material.

Comparison and complementarity of the methods and guidelines and a strategy for identifying DDGS origin

All the analytical techniques were evaluated for applicability and future use by the laboratories on the basis of detailed knowledge of these techniques. Crucial criteria in the future use of these techniques for authenticating feed, particularly DDGS, are summarized in Table 6, under these key features: (1) applicability, (2) technical limitations, (3) DDGS authentication issues, (4) costs/laboratory effort, and (5) transferability.

Applicability relates to several parameters in the application of the method. These parameters are state and fraction of the DDGS sample being analyzed (ground powder, oil, extract; Table 6, 1.1); quantity of material needed for analysis (Table 6, 1.2); use of an organic solvent or other reagents needed for sample preparation (Table 6, 1.3); destructive character of the method (Table 6, 1.4); working speed of the method (rapid > fast > slow; Table 6, 1.5); possible use on site (industry, laboratory; Table 6, 1.6) and in-line (Table 6, 1.7); and need for skilled and trained personnel (i.e., expertise; Table 6, 1.8).

The main limitations of individual methods from a technical point of view are also described (Table 6, 2).

The DDGS authentication issues investigated, based on the system challenge set results, are summarized in terms of botanical origin (i.e., discrimination between corn/wheat; Table 6, 3.1); geographical origin (Table 6, 3.2), particularly continent (i.e., China/Europe/US discrimination) and/or bio-ethanol plant origin (China origin 1/China origin 2/Czech Republic, companies); and processing origin (i.e., discrimination between bio-ethanol and alcoholic beverage production; Table 6, 3.3).

Table 5 Botanical origin of DDGS in the system challenge 3 set

Sample code	Botanical origin	IRMS rough content of C3/wheat [%]	IRMS C4/corn [%]	IRMS Concl.	PCR Haploid genome equivalents Wheat	PCR Corn	PCR Concl.	NIRS DDGS powders PLS-DA	NIRS OPLS-DA	MIR DDGS powders PCA-LDA	MIR DDGS powders SIMCA	MIR Oils extracted chemically (ASE) PCA-LDA	MIR Oils extracted chemically (ASE) SIMCA	MIR Oils extracted in situ PLS-DA	RAMAN Oils extracted chemically (ASE) OPLS-DA	LC-QTOFMS Polar extracts PLS-DA
SC3-01	Corn	71	29	**Wheat>Corn**	/	/	/	Wheat	Wheat	Corn	Wheat=Corn	Wheat	not assigned	Wheat	Wheat	Wheat=Corn
SC3-02	Corn	85	15	**Wheat**	/	/	/	Wheat	Wheat	Corn	Wheat	Wheat	Wheat	Wheat	Wheat>Corn	Wheat>Corn
SC3-03	Corn	64	36	**Wheat>Corn**	70	36	**Wheat>Corn**	Wheat	Wheat	Corn	Wheat	Wheat	not assigned	Wheat	Corn	Wheat=Corn
SC3-04	Corn	22	78	**Corn>Wheat**	/	/	/	Corn	Corn	Corn	not assigned	Corn	Corn	Corn	Corn	Wheat=Corn
SC3-05	Corn	8	92	**Corn**	ND	ND	not assigned	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC3-06	Wheat	50	50	**Wheat=Corn**	/	/	/	Wheat	Wheat	Corn	not assigned	Wheat	not assigned	Wheat	Corn>Wheat	Wheat=Corn
SC3-07	Wheat	71	29	**Wheat>Corn**	/	/	/	Wheat	Wheat	Corn	Wheat	Wheat	Wheat	Wheat	Wheat>Corn	Wheat=Corn
SC3-08	Wheat	78	22	**Wheat>Corn**	/	/	/	Wheat	Wheat	Corn	Wheat	Wheat	not assigned	Wheat	Wheat>Corn	Wheat>Corn
SC3-09	Wheat	64	36	**Wheat>Corn**	/	/	/	Wheat	Wheat	Corn	Wheat	Wheat	Wheat	Wheat	Corn>Wheat	Wheat=Corn
SC3-10	Wheat	64	36	**Wheat>Corn**	174	148	**Wheat>Corn**	Wheat	Wheat	Corn	Wheat	Wheat	not assigned	Wheat	Corn>Wheat	Wheat=Corn
SC3-11	Wheat	43	57	**Corn>Wheat**	41	58	**Corn>Wheat**	Wheat	Wheat>Corn	Corn	Corn	Wheat	not assigned	Corn	Corn	Corn>Wheat
SC3-12	Wheat	50	50	**Wheat=Corn**	/	/	/	Wheat	Wheat>Corn	Corn	Corn	Wheat	not assigned	Wheat	Corn	Corn>Wheat
SC3-13	Corn/Wheat	15	85	**Corn**	ND	7	not assigned	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn
SC3-14	Corn/Wheat	15	85	**Corn**	2	90	**Corn**	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn	Corn

– no data, *ND* not detected (not enough DNA), *Wheat > corn* slightly more wheat, *Corn > wheat* slightly more corn, *Wheat = corn* ratio 50/50

The results in bold show the classifications achieved using new analytical method which are in line with the IRMS/PCR results

The costs/laboratory effort are assessed and quantified by cost of the instrument (Table 6, 4.1); sample preparation (Table 6, 4.2); time needed to perform the analysis of one sample (Table 6, 4.3); and the number of samples analyzed by one analyst per day (Table 6, 4.4).

Assessment of the transferability of the method is based on analytical approach (Table 6, 5.1) and data compatibility (Table 6, 5.2).

Stable IRMS is a well-established technique in food authenticity testing. For many food commodities (e.g., wine, olive oil, honey, and meat), the stable isotope ratios of the bioelements H, C, N, O, and S are valuable factors for authentication. In a study by Nietner et al. [8], IRMS was used for the first time in the analysis of δ^2H, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ values in the animal feed DDGS matrix. Both this study and the present study showed that IRMS could be used to authenticate the botanical and geographical origin of DDGS on the basis of the δ^2H, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values, but not the $\delta^{34}S$ value, which is influenced by other factors (e.g., sulfuric acid used for production process). To perform the analyses, samples must be well homogenized and consist of fine particles.

Stable isotope ratios of DDGS feed such as corn or wheat, however, can be altered by various factors (e.g., climate), as Nietner et al. [8] reported and as confirmed by the present study (data not shown). In general, it can be assumed that certain deviations from the observed mean values for single origins need to be considered in the application and interpretation of stable isotope ratios for DDGS authentication. Before the isotope ratios of the elements of interest can be used routinely to authenticate geographical origin, the models need to be tested over several years.

Since the IRMS protocols can be transferred easily, this technique could be used in the analysis of other animal feed materials. This would require a fully equipped laboratory with expert knowledge in stable isotope mass spectrometry to guarantee valid results. Alternatively, the analysis could be outsourced to external laboratories. Since the analytical method in isotope ratio analysis is based on reference scales and the use of internationally accepted reference materials, the results obtained from different laboratories would be comparable.

Spectroscopic methods, based mainly on *NIR* techniques, are often described as rapid (no need for time-consuming wet chemical analysis), non-destructive, clean (no harmful chemical reagents), cost-effective, and easily applicable (requiring no or limited expertise), and they are seen as the most suitable approaches for at-line, on-line, and in-line control of food product quality. NIRS can record the spectrum of a sample in a few seconds and provide the prediction results immediately once the calibration model has been developed. The most important limitation of spectroscopic methods lies in the development of a calibration model in advance. The samples for building this model should be highly representative and in sufficient numbers to represent normal production

Table 6 Main features of the NIRS, NIRM, MIR, Raman, LC-QTOFMS, DART-OrbitrapMS, PTR-MS, and IRMS methods in DDGS

	NIRS	NIRM	MIR	MIR	MIR	Raman	LC-QTOF-MS	DART-Orbitrap-MS	PTR-MS	IRMS
1 Applicability										
1.1 State/fraction of the DDGS sample	Solid ground (0.5 mm)	Solid ground/ sieved (<0.25 mm)	Solid finely ground (~0.1 mm)	Oil (solvent extraction)	Oil (in situ extraction)	Oil (solvent extraction)	Extract (polar/ non-polar)	Extract (polar/ non-polar)	Extract (volatile organic compounds)	Solid ground (0.5 mm)
1.2 Quantity of material	10 g	30 g	<1 g	~20 g	<1 g	~20 g	<1 g	<1 g	25 g	<1 g
1.3 Solvents	No	No	No	Yes	No	Yes	Yes	Yes	No	No
1.4 Destructive	No	No	No	Yes	(Yes)	Yes	Yes	Yes	No	Yes
1.5 Working speed	Rapid	Fast	Rapid	Fast	Rapid	Fast	Slow	Fast	Fast	Slow
1.6 On site	Industry	Laboratory	Laboratory industry	Laboratory	Laboratory industry	Laboratory	Laboratory	Laboratory	Laboratory industry	Laboratory
1.7 In-line	Yes	No	No	No	No	No	No	No	Yes	No
1.8 Expertise	No	No	Limited	Limited	Limited	Limited	Yes	Yes	Yes	Yes
2 Technical limitations	Variations due to environment have to be considered (e.g., temperature)	Not specified	Close contact to ATR crystal; samples must be grindable	Requires sufficient oil content	Close contact to ATR crystal (fine particles, press bridge)	Self-fluorescence of samples must be small	Sample extract must be compatible with mobile phase	Not suitable for powders or inhomogeneous samples	Ambiguous mass-to-charge ratios; separate room needed	Sample must be fine/homogeneous; δ34S values not always promising
3 DDGS authentication issues										
3.1 Botanical origin	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3.2 Geographical origin										
Continent origin	Yes	Yes	Yes	Yes	Partly yes	Yes	Yes	Yes	Yes	Yes
Plant origin	Yes	Partly yes	Not assessed	Not assessed	Yes	Partly yes	Not assessed	No	Yes	Yes
3.3 Processing origin bio-ethanol/ alcoholic beverage	Yes	No	Not assessed	Not assessed	No	Not assessed	Yes	No	Not assessed	Not assessed
4 Costs/laboratory effort										
4.1 Instrument	€ 50,000	€ 150,000	€ 40,000	€ 40,000	€ 30,000	€ 20,000–€ 150.000	€ 500,000	€ 195,000	€ 200,000	€ 200.000–€ 300.000
4.2 Sample preparation	Milling	Milling	Milling	Milling/ extraction	Milling	Milling/ extraction	Milling	Milling	(Milling)	Milling
4.3 Instrument time/sample	10 min	75 min	15 min	15 min	15 min	5–10 min	Depending on number of samples/ batch	Depending on number of samples/ batch	Few minutes	100 min (C/N) 180 min (O)
4.4 Samples/day	40	10	20	20	20	40	Depending on number of samples/ batch	Depending on number of samples/ batch	>50	14 (C/N) 8 (O)

Table 6 (continued)

	NIRS	NIRM	MIR	MIR	MIR	Raman	LC-QTOF-MS	DART-Orbitrap-MS	PTR-MS	IRMS
5 Transferability										
5.1 Analytical approach	Possible	Not investigated	Possible	Possible	Not investigated but possible	Possible	Possible	Possible	Yes	Yes
5.2 Data compatibility	Possible	Not investigated	Not investigated but possible	Not investigated but possible	Not investigated but possible	Not investigated	Not investigated	Not investigated	Not investigated	Yes

variation. In order to ensure the robustness of the calibration model, other variations should be considered during calibration, such as environmental conditions (e.g., temperature and humidity), instrument status, and reflectance standard. These techniques are already routinely used in the industry to control raw materials and finished products in line with specific production standards [23]. Several studies have also shown the potential of combining NIRS with chemometric methods for determining the geographical origin of food and feed [24–26]. This work has shown that the NIR method can be used successfully in DDGS authentication for determining botanical, geographical, and processing origin. NIRS could be considered as one of the best rapid screening methods for this purpose. It can be used for in-line analysis in industry and is easily transferred from one instrument to another, as shown by several studies over the past 25 years [11, 27–31]. A comparative study showed that, with the same dataset, two laboratories using different spectrometers combined with different chemometric software packages achieved similar results [13].

The *NIRM* method was also investigated. It is a new method that adds a spatial dimension to the spectral data and enables low levels of contaminants to be detected and quantified. It combines the analytical advantages of microscopy and spectroscopy (i.e., non-destructive, clean, low level of expertise needed, unlike NIRS) [32, 33]. In the present study, 625 spectra per sample were recorded instead of one spectrum/sample using classical NIRS. Since all the spectra collected per sample were averaged for DDGS authentication, the NIRM instrument was used as a classical NIR spectrometer. The NIRM results confirmed the NIRS results. Using NIRM, analysis costs are higher and analysis time is longer. In addition to using NIRM for DDGS authentication, it could also be used in the analysis of contaminants. The transfer of NIRM analytical procedures from one laboratory to another is feasible [15, 34]. A study by Fernández et al. [16] described, for the first time, the application of NIRM instrument standardization using a measurement cell in an inter-laboratory study on the qualitative determination of animal proteins in compound feeds, based on spectra obtained using eight instruments.

As with other infrared spectroscopic methods, the *FT-MIR* technique is rapid, non-destructive, and environmentally friendly, and it needs little or no sample preparation. The use of FT-MIR spectroscopy in feed and food analyses has been increasing [35]. Recently, more flexible presentation techniques have been proposed as ATR accessories. With these accessories, the sample is put in close contact with a crystal that has a high refraction index, made mainly of ZnSe, Ge, ZnS, Si, or diamond. In the present study, analyses were performed on powder and extracted oil fractions of DDGS. In order to analyze DDGS in the solid state, the samples were finely ground to small particles (less than 100 μm) to achieve close contact with the crystal, as described by Nietner et al. [17]. In comparison with other vibrational spectroscopic

methods performed on DDGS, the ATR-FT-MIR technique enabled the ratio between free fatty acids and triglycerides, the presence of short/long chain fatty acids, and the ratio between saturated and unsaturated fatty acids to be determined. This information was used to identify the botanical and geographical origin in relation to the processing origin of wheat/corn DDGS [18]. The use of the in situ oil extraction method, developed in this study, reduced sample preparation time. In comparison with the ATR-FT-MIR method applied to oil extracted by solvent (petroleum benzine, ASE), where triacylglycerides might also be hydrolyzed and converted into free fatty acid (FFA), the ATR-FT-MIR method applied to oil extracted in situ enabled the free fatty acid content to be determined, reflecting the constraints in ethanol production processing. The significant improvements in vibrational spectroscopy technology have led to less sophisticated and less expensive ATR-FT-MIR instruments that could be used in routine analysis in any control laboratory or small laboratories in the food/feed industry.

Raman spectroscopy, which provides a unique fingerprint of the molecular structure of samples, was also evaluated for DDGS authentication. The Raman spectra of DDGS powder are affected to a large degree by fluorescence, which masks the signals from Raman scattering [36]. In this study, only the chemically extracted oil fraction was analyzed using this technology. Raman data used in conjunction with chemometric analysis enabled botanical and geographical origin to be determined. Recent advances in optoelectronics have reduced both the size and cost of the instrumentation required. In addition, the new generation of compact, fully automated instruments requires limited expertise and shorter analysis time. These advances have changed Raman spectroscopy from a method of last resort to a first-choice technique for a range of analytical problems. More experienced operators might be needed, however, to perform the oil extraction during sample preparation.

Advanced technologies employing *LC-QTOF*, *DART-Orbitrap*, and *PTR* combined with mass spectrometric detection (*MS*) are powerful, but more expensive (higher investment and cost per analysis) and complex (greater expertise), techniques. They can be used in a laboratory for full scan analyses of feed materials. Until now, this full scan information has been used mainly for library-based targeted multi-class screening and confirmation of known contaminants [37–39]. In combination with suitable chemometric approaches for normalized fingerprinting of the samples, these techniques can be used to authenticate a range of matrices, including DDGS [40–44]. In the present study, all the MS techniques proved reliable for botanical origin authentication. In addition, MS techniques offer the possibility of detecting deviations from "normal" patterns, thus flagging up mixed feed materials. The techniques could be used for the routine control of the batch-to-batch quality and stability of raw materials used for feed production and of the final products. Transferring these analytical procedures among laboratories is feasible. Issues relating to instrumentation, however, need to be considered: conditions for analyses might need to be modified, resulting in re-validation, and obtained data might vary, depending on the specific instruments used.

Apart from botanical origin determination, the *LC-QTOFMS* method can be used for authenticating the geographical and possibly also the processing origin of feed. One technical limitation of this method is that the sample extract has to be compatible with the LC mobile phase and must be pure, without solid particles or turbidity.

The *DART-OrbitrapMS* method is not suitable for the analysis of powdered or inhomogeneous samples and is unable to detect some polar compounds with a large molecule (e.g., phospholipids). Data processing is more time-consuming compared with LC-MS because analysis and data mining are not fully automated. An advantage, however, is that because DART-OrbitrapMS does not use chromatographic separation prior to detection, analysis time can be significantly reduced. If a comprehensive database of a wide range of (normalized) fingerprints/profiles was established and continuously updated over time, at a certain level, the authentication of feed would be feasible using this method.

The *PTR-MS* technique is a fast method. The sample preparation is limited to heating the samples to 37 °C. The volatile organic compounds can be monitored in-line with real-time measurements. Due to well-defined constant conditions within the system, absolute concentrations of compounds can be determined without the use of a reference gas. Characterization of compounds can raise difficulties as concentrations of mass-to-charge ratio protonated VOCs are monitored, and more than one compound can have the same mass. PTR-MS method can be also used for authentication of the geographical origin. It can be applied in other laboratories if that the instrument can be installed in a separate room in order to prevent measurements from being influenced by external scents.

Conclusions

This study demonstrated that all the analytical techniques investigated, once adapted for applicability to the DDGS matrix and coupled with univariate or multivariate data analysis, could clearly determine botanical origin (corn DDGS vs wheat DDGS). Some techniques showed potential in determining the geographical origin (China, EU, USA) of the DDGS in the sample set. The use of stable isotope ratios as a viable tool for identifying geographical origin (already established in the food industry) was also confirmed by the study results for DDGS. Other analytical techniques used to authenticate DDGS gave acceptable results for initial proof of geographical origin, especially NIRS and MIR, which proved

to be relatively fast and cost-efficient. In the context of using a decision tree, the study showed that botanical origin should be identified before geographical origin. Clearly, the overlap between geographical and processing origin has to be considered during data evaluation. Only a few techniques, however, were able to classify processing origin correctly. Most of them were unable to distinguish between bio-ethanol and alcoholic beverage production (whiskey, vodka) correctly.

The developed approaches should be applicable for authenticating other materials in the animal feed sector in the same way that has been shown for DDGS. Strategies using analytical techniques (spectroscopic and spectrometric) combined with dedicated statistical data analysis tools could be implemented in both specialized and routine laboratories. Although many of the techniques are not currently used routinely in the industry and therefore might be difficult to implement, NIRS is already widely accepted in the animal feed sector. The high throughput of this method, its capacity to determine in one analysis a large variety of parameters (from major constituents to criteria such as digestibility), and the possibility of building a network of spectrometers make this technique very attractive for the feed sector [45]. It can be also used on-line in feed production plants, thus adding to its attraction as a screening tool [46]. NIRS would therefore be the first technique of choice to apply in an industrial context, especially in feedmills, which routinely use the NIR spectra of raw materials to help authenticate the botanical and geographical origin of these materials. The introduction of high-resolution MS also enables non-target screening to be done, which means that, unlike spectroscopic methods, blind signal(s) can be identified as a potential source of difference from a reference. In order to increase the confidence in the results of both spectroscopic and spectrometric methods, an extensive database of spectral data would need to be built up and maintained, using the most appropriate data processing tools from the available chemometric software packages.

This study has shown that established analytical approaches in food analysis can be applied to feed materials. With the increase in feed exchange in the world, more complex processes used in plant feed companies, and the trend to promote not only regional feed production but also organic feed production, the authentication of feed material will become more and more important in efforts to improve the safety of animal feed.

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