Characterization of Distillers Dried Grains and Solubles using FT-IR Spectroscopy and Isotope Ratio Mass Spectrometry

Dissertation

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The following publications and presentations were derived from work undertaken during the studies for this dissertation.

Journal Articles

Nietner, T., Pfister, M., Glomb, M.A., Fauhl-Hassek, C. (2013). Authentication of the botanical and geographical origin of distillers dried grains and solubles (DDGS) by FT-IR spectroscopy. *Journal of Agricultural and Food Chemistry, 61(30),* 7225-7233. **(Publication A)**

Nietner, T., Haughey, S., Ogle, N., Fauhl-Hassek, C., Elliott, C.T. (2014). Determination of geographical origin of distillers dried grains and solubles using isotope ratio mass spectrometry. *Food Research International, 60,* 146-153. **(Publication B)**

Nietner, T., Pfister, M., Brakowiecka-Sassy, B., Glomb, M.A., Fauhl-Hassek, C. (2015). Screening for sulfate in distillers dried grains and solubles by FT-IR spectroscopy. *Journal of Agricultural and Food Chemistry, 63(2),* 476-484. **(Publication C)**

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Oral Presentations

Nietner, T., Pfister, M., Lahrssen-Wiederholt, M., Fauhl-Hassek, C. (2012). Determination of the geographical origin of DDGS with FT-IR spectroscopy. 4th International Feed Safety Conference - "Methods and Challenges", 11-13 September 2012, Beijing, China.

Nietner, T. (2013a). Authentizitätsprüfung von Trockenschlempen - Distillers Dried Grains and Solubles. Jahrestagung der Futtermittelüberwachungsbehörden der Länder und des Bundes, 16-18 April 2013, Magdeburg, Germany.

Nietner, T., Fauhl-Hassek, C., Lahrssen-Wiederholt, M. (2013). Bestimmung der botanischen und geographischen Herkunft von DDGS mittels FTIR-Spektroskopie und Stabilisotopen-Massenspektrometrie. 125. VDLUFA-Kongress, 17-20 September 2013, Berlin, Germany.

Nietner, T. (2013b). Application of FT-IR spectroscopy for authentication of Distillers Dried Grains and Solubles (DDGS) – Part of Workshop "Infrared spectroscopy, Raman spectroscopy and chemometrics for monitoring of food and feed products, lab-to-the-sample". 6th International Symposium on Recent Advances in Food Analysis, 5-8 November 2013, Prague, Czech Republic.

Nietner, T., Haughey, S., Ogle, N., Fauhl-Hassek, C., Elliott, C.T. (2013). Traceability of Distillers Dried Grains and Solubles by stable isotope ratio mass spectrometry. 6th International Symposium on Recent Advances in Food Analysis, 5-8 November 2013, Prague, Czech Republic.

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Poster Presentations

Nietner, T., Esslinger, S., Lahrssen-Wiederholt, M., Fauhl-Hassek, C. (2011). Traceability and authenticity of feed materials – report on QSAFFE Work Package 2 activities. Poster presented at 5th International Symposium on Recent Advances in Food Analysis, 1-4 November 2011, Prague, Czech Republic.

Nietner, T., Pfister, M., Fauhl-Hassek, C. (2014). Determination of sulphate levels in DDGS by FT-IR spectroscopy. Poster presented at 2nd Food Integrity and Traceability Conference, 8-10 April 2014, Belfast, United Kingdom.

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Abbreviations

ADF	acid detergent fiber
AOAC	association of official analytical chemists
ATR	attenuated total reflexion
CDA	canonical discriminant analysis
CF	crude fiber
CP	crude protein
DDGS	distillers dried grains and solubles
DG	distillers grains
DM	dry matter
DWG	distillers wet grains
DWGS	distillers wet grains and solubles
FT-IR	Fourier transform infrared
HPLC	high performance liquid chromatography
IQR	interquartile range
IR	infrared
IRMS	isotope ratio mass spectrometry
LDA	linear discriminant analysis
MIR	mid infrared
MRL	maximun residue limit
MS	mass spectrometry
NDF	neutral detergent fiber
NIR	near infrared
NIRS	near infrared spectroscopy
PC	principal component
PCA	principal component analysis
PCB	polychlorinated biphenyl
PLS	partial least squares
QSAFFE	quality and safety of feeds and food for Europe
SIMCA	soft independent modeling of class analogy
SNV	standard normal variate transformation

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1 Introduction and Objectives

Distillers Dried Grains and Solubles (DDGS) have become an important product for the animal feed industry, particularly due to the recent increase in bioethanol production (Liu, 2011; OECD-FAO Agricultural Outlook, 2014). With regards to global trade, nutrient specifications need to be guaranteed and, therefore, quality standards for DDGS are tested by methods of chemical analysis (Thiex, 2012). In addition, paper documentation and electronic traceability systems list further information on DDGS, such as place of production, date of production and storage stability, or the designation of particular methods of production applied (e.g., heat treatment) or raw materials used (e.g., genetically modified corn). However, most of the information specified in electronic/paper information systems cannot be proven by analytical methodology yet. Also with regards to the high variability in DDGS composition, the various production technologies and sometimes long supply chains (Spiehs, Whitney and Shurson, 2002; Belyea *et al.*, 2010; Buckner *et al.*, 2011), reliable analytical approaches to verify the information provided could be of valuable use.

In this respect, analytical authentication approaches have demonstrated suitability in the food sector and a magnitude of applications already exists here (Lees, 2003; Esslinger, Riedl and Fauhl-Hassek, 2014). In particular, multivariate analysis of spectroscopic and spectrometric data showed successful strategies to the authentication of food matrices (Leardi, 2003). In order to develop strategies for authentication of DDGS, attenuated total reflection Fourier Transform Infrared spectroscopy (FT-IR spectroscopy) and Isotope Ratio Mass Spectrometry (IRMS) have been applied in this dissertation. These two techniques have commonly been used in authentication of agricultural and food products (Karoui *et al.*, 2008; Vermeulen *et al.*, 2010; Kelly, 2003) and, therefore, should be promising to the authentication of DDGS.

Authentication of DDGS in this thesis concentrated on the botanical and geographical origin of DDGS, as well as the method of production, i.e., bioethanol production vs. alcoholic beverage production. For this purpose, DDGS were collected from various sources and countries of origin and analytical data need to be evaluated in univariate and multivariate statistical approaches. Since statistical models developed in authentication studies always should be updated and carefully validated (Downey, 1996; Riedl, Esslinger and Fauhl-Hassek, 2015), authentication approaches were continuously compared on different sample sets and different validation methods were considered. In this way, the authentication strategies developed in this thesis could be assessed for final and future use in the field of chemical analysis of DDGS.

Moreover, FT-IR spectroscopy was employed together with multivariate data analysis for non-targeted detection of DDGS ingredients. Multivariate pattern recognition techniques already showed potential for detection and determination of ingredients or compound classes in other matrices (Murray, 1996; Honigs, 2009; Abbas *et al.*, 2013). Therefore, in the course of this work, FT-IR spectroscopic data should be assessed in a similar way, ideally, in order to find quantitative information on ingredients present in DDGS.

2 Conventional Chemical Analysis of DDGS

2.1 Chemical Analysis in the Animal Feed Sector

Methods of chemical analysis have traditionally been used for determination of the nutritive value of animal feeds. Besides contamination of feeds with bacteria and fungi (microbiological concerns) or toxic ingredients, general composition of the diets has always been a significant factor with regard to animal health and animal production. With the beginning of 'scientific agriculture' in the 19th century, animal nutrition has started to be based on knowledge of single nutrients present in animal feed. The effects of nutrients on the rearing of different animal species and the advantages of additives (supplements) for special purposes have become increasingly important since that time. Chemical analysis of animal feed helped to gain a deeper understanding of animal nutrition and information on the composition of animal feed became the basis of scientific feeding and contributed to optimized animal performance. Thus, long time ago the development of analytical procedures for the determination of the nutritive values, or rather for the chemical composition of animal feeds was initiated. (Midkiff, 1984; Mueller-Harvey, 2004) Besides the analysis of nutrients, the determination of single additives (e.g., minerals, vitamins, drugs) and undesirable substances (e.g., mycotoxins, heavy metals) have become more important over the years. Nowadays, methods of chemical analysis also are applied to ensure the conformity of animal feed with legislation and quality assurance systems of feed business operators. In general, many different aspects have to be considered and play a role in the chemical analysis of animal feed (Figure 1).

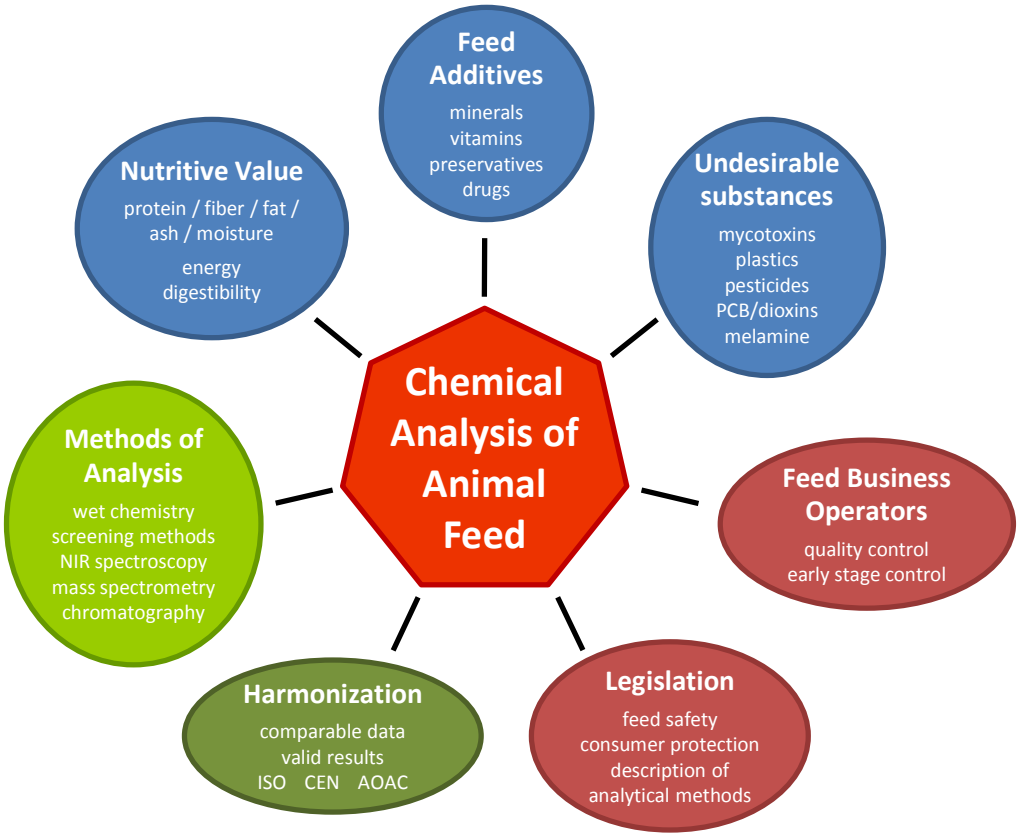

Figure 1: Important factors for chemical analysis of animal feed.

One of the most well-known organizations in the field, the Association of Official Analytical Chemists (AOAC), originally named Association of Official Agricultural Chemists, began to pay attention to the analysis of animal feed already in the year 1886, two years after its foundation. (Midkiff, 1984) From the very beginning, the interest of AOAC in crops and animal feed went along with a discussion about the agreement on methods of chemical analysis to obtain comparable data and valid results. Later on, this resulted in a collection of analytical procedures for different kinds of commodities and different analytes, the Official Methods of Analysis of AOAC INTERNATIONAL. Besides AOAC, other organizations, such as the Association of German Agricultural Analytic and Research Institutes (VDLUFA), set standards in the field, decades before international harmonization was faced. Nowadays, methods of analysis for animal feed are ideally standardized on an international level by the International Organization for Standardization (ISO) or the European Committee for Standardization (CEN). Furthermore, the European Union regulated the methods of sampling and analysis for the official control of feed in Commission Regulation No 152/2009, which is in many cases consistent with the respective ISO/CEN standards.

Scientific milestones with regard to the chemical analysis of animal feed have been the results of Henneberg and Stohmann in 1860 (Henneberg and Stohmann, 1860), later referred to as 'Weende scheme', and the findings of van Soest in 1963/1967 (van Soest, 1963; van Soest, 1967), later referred to as 'van Soest scheme'. The Weende scheme introduced the determination of the principal constituents crude protein (CP), crude fiber (CF), crude fat, crude ash and nitrogen-free extractives besides the moisture content respectively the dry matter (DM) of animal feed by the conventional, so-called 'wet chemistry' methods (Table 1). Already in the year 1860, Henneberg and Stohmann highlighted the importance of the "chemical composition" of animal feed when considering "economic or supposed progress" (Henneberg and Stohmann, 1860). One century later, van Soest developed methods able to distinguish between neutral detergent fiber (NDF) and acid detergent fiber (ADF), thus leading to an extended differentiation of structural carbohydrates (Table 1). Besides cellulose and lignin, recognized as crude fiber before, NDF additionally includes hemicelluloses, which are branched polymers of different sugars and are causing big differences with regard to digestibility of animal feed also. (Balthrop *et al.*, 2011) Several other constituents listed in Table 1, such as starch, lignin or organic acids had been included in the analysis scheme together with the respective methods over the following decades. With regard to the compositional analysis of animal feed, however, the basic parameters of the Weende and van Soest schemes are still of importance in practice.

Apart from the parameters mentioned, evaluation of animal feed with regard to gross/net energy, general digestibility, degradable, undegradable and metabolizable protein have always been related to methods of chemical analysis, although these values were not/could not necessarily be analyzed by the respective methods directly. In contrast, estimations or calculations on the basis of single nutrients often stand behind these parameters in terms of 'feed evaluation'. Nutrients for these calculations have been determined mostly by the wet chemistry methods, of course, in accordance with the 'Weende' and 'van Soest' schemes. Besides the multitude of wet chemistry methods for the determination of constituents, near infrared reflectance spectroscopy (NIRS) has also been used

for the respective analyses. NIRS had been applied firstly for the analysis of nutrients and digestibility related parameters in forages by Norris in 1976 (Norris, Barnes, Moore and Shenk, 1976; Norris, 1992) and has been used extensively for more than 20 years for the compositional analysis of animal feed. Moreover, NIRS has shown potential for the prediction of the more complex parameters, such as energy values, amino acids or specific minerals (Givens and Deaville, 1999).

Table 1: Weende and van Soest schemes for analysis of animal feed constituents, adapted from Balthrop *et al.* (2011).

<table>
<tr><th>Constituents</th><th>Weende scheme</th><th>Van Soest scheme</th></tr>
<tr><td>water</td><td>moisture</td><td>-</td></tr>
<tr><td>protein
non-protein N</td><td>crude protein</td><td rowspan="3">neutral detergent soluble</td></tr>
<tr><td>lipids
pigments</td><td>crude fat</td></tr>
<tr><td>starch
sugars
organic acids
pectin</td><td rowspan="2">nitrogen-free extractives</td></tr>
<tr><td>hemicellulose</td><td rowspan="2">neutral detergent fiber (NDF)</td></tr>
<tr><td>cellulose
lignin
fiber-bound N</td><td>crude fiber</td><td>acid detergent fiber (ADF)</td></tr>
<tr><td>insoluble ash
soluble ash</td><td>crude ash</td><td>silica</td></tr>
</table>

In addition to the parameters of ‘chemical composition’ and ‘feed evaluation’, there had been a rising demand to develop analytical methods for the determination of minerals and additives, which occur as minor components in animal feed, but nonetheless have a high impact on the nutrition of the different animal species. As the focus of interest in the animal feed sector was always driven by the question how animal performance could be forced up and production could be made more efficient (Henneberg and Stohmann, 1860; Balthrop *et al.*, 2011), the beneficial use of both minerals and additives in animal feed had been practiced. Although the occurrence of different minerals had already been tackled by researchers in the early days of scientific agriculture (Henneberg and Stohmann, 1860), research on major minerals (i.e., Ca, P, Mg, K, Na, Cl, S) and trace elements (i.e., Fe, Cu, Zn, Mn, Co, I, Se) deepened over the decades (Midkiff, 1984). The respective methods of analysis have continuously developed since the 1920s and have adapted to technical and scientific progress from titration to highly-sophisticated techniques based on spectroscopic or mass spectrometric detection. On the other hand, the use of ‘non-mineral’ additives in animal nutrition has been and still is going to be a fundamental issue (e.g., vitamins, preservatives, antibiotics, veterinary drugs, herbs and spices). As it is for minerals, methods of chemical analysis for different compounds added to animal feed have been continuously developed,

also due to legislative regulation (e.g., Regulation 152/2009). Since the publication of the very first methods for the analysis of vitamins in 1940 and drugs in 1950 (Midkiff, 1984), chemical analysis of feed additives has expanded and in the meantime covers the full range of modern analytical techniques, such as diverse chromatographic and mass spectrometric technologies.

However, modern feed analysis not only pays attention to compositional parameters, the nutritive value and minor components of animal feed. In recent years, undesirable substances have been targeted, "[…] which are present in and/or on the product intended for animal feed and which present a potential danger to animal or human health or to the environment or could adversely affect livestock production." (Balthrop *et al.*, 2011, p. 82) Such undesirable substances derive either from the cultivation of plants, e.g., pesticides, heavy metals, mycotoxins, or from the processes after harvest, e.g. plastics, mycotoxins produced during storage. Also, contamination of animal feed with dioxins or polychlorinated biphenyls (PCBs) are of current interest (Hoogenboom, 2012). These compound classes can occur in animal feed firstly due to contamination of crops by factory fumes (incineration of waste) or smoke during fire accidents but secondly due to contamination of feed ingredients by (un)intentional addition of waste oils. A prominent example for contamination of animal feed with PCBs/dioxins happened in Belgium in 1999, when a feed business operator added oil, most likely from discarded transformers, containing PCBs and dioxins to melted animal fat from slaughterhouses (Bernard *et al.*, 1999; van Larebeke *et al.*, 2001). Another issue that has to be addressed, not only because of recent incidents, is the intentional adulteration of animal feed (ingredients) with chemicals such as melamine. In 2007, melamine has been found in pet food from the USA (protein isolates delivered from China) and in 2008 in organic soya expeller originating from China delivered to France, Germany and the United Kingdom (Hilts and Pelletier, 2009; Food Standards Agency, 2014).

It is obvious that chemical analysis of animal feed these days does not only involve the basic parameters related to animal nutrition. Moreover, methods of chemical analysis are related to feed safety and feed security, for example, in terms of crises due to undesirable substances, contaminants or fraud. Last but not least, the effect of animal feed in the whole food chain, the so-called 'from-farm-to-fork-principle', is considered of great importance. Official organizations have recognized current and future risks that might be associated with animal feed due to the consumption of food of animal origin, and tighten rules for single substances in animal feed together with requirements for the respective analytical procedures. Besides general requirements on animal feed with regard to feed safety (e.g., European Regulation 178/2002) and maximum levels of additives in animal feed (e.g., European Regulation 1831/2003, Directive 70/524/EEC), maximum levels for contaminants and residues (MRLs) have been regulated in many countries (e.g., European Directive 2002/32/EC, European Recommendation 2006/576/EC). The respective methods of analysis are recommended or often compulsory for official control by means of specific regulations (e.g., Commission Regulation No 152/2009). Apart from legislation, feed business operators have realized that methods of chemical analysis could not only be used for pricing but even more for improved monitoring of both feed quality and feed safety. Also, early stage control of animal feed in harbors or feed mills, in view of the increasing globalization of trade, is a crucial

point. In conclusion, methods of chemical analysis in the animal feed sector have improved significantly, but at the same time the historic wet chemistry methods for determination of nutrients are still in place and are often used as 'reference'.

2.2 Chemical Analysis of DDGS

Utilization of DDGS in Livestock Feeding

Distillers Dried Grains and Solubles (DDGS) are by-products of the alcoholic beverage production (e.g., whiskey, vodka) or the production of fuel ethanol (Figure 2). DDGS are obtained by drying the solid residues of fermented grains (e.g., corn, wheat, barley), which remain in the distillation pot, and to which pot ale syrup or evaporated spent wash, the so-called Solubles, have been added (Commission Regulation 68/2013). Distillers Wet Grains (DWG) and Distillers Wet Grains and Solubles (DWGS) result from the same process when the drying step is omitted, and show much higher moisture contents than DDGS (DWGS/DWG: 50-70 %, DDGS: 10-15 %; Dicostanzo and Wright, 2012). If DWG are dried but the Solubles are not added, this leads to Distillers Dried Grains (DDG). All these by-products of the alcohol production are subsumed under the term 'Distillers Grains' (DG).

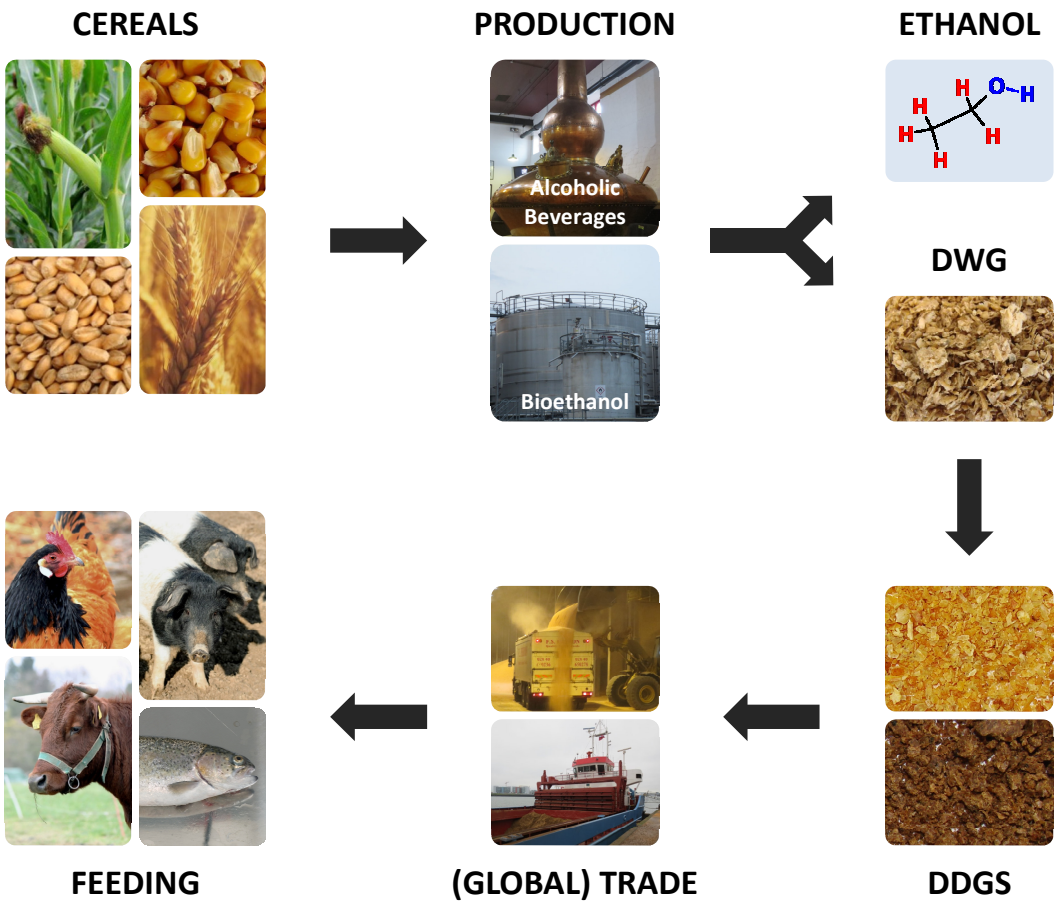

Figure 2: Production and utilization of DDGS.

Distillers Grains have traditionally been used in the rearing of animals in regions where distilleries produced alcoholic beverages and have been fed at feedlots next to production plants, especially in the wet forms (DWG/DWGS). More recently, Distillers Grains are also fed close to places of fuel ethanol (= bioethanol) production, which has rapidly grown in the last years and accounts for the clear majority (98 %) of the production volume in North America (Shurson *et al.*, 2014/unknown). However, production volume increased in such a way, that especially fuel ethanol producers also dry the by-products and ship them to regions far from the ethanol plants (Figure 2), for both domestic and international use (Saunders & Rosentrater, 2009). The share of DDGS in the total amount of DG varies by plants, regions and countries, but the dried forms of DG benefit from an extended shelf-life (US Grains Council, 2012a).

In 2012, production of DDG/DDGS has reached nearly 37 million tons in the USA, 3.5 million tons in the European Union and 1.2 million tons in Canada and China respectively (OECD-FAO Agricultural Outlook, 2014), representing the most important producers of Distillers Grains. The US production is not only limited to domestic use but also takes advantage of export to China and several other countries, with a recent volume of more than 9 million tons (US Grains Council, 2014). Forecasts until 2020 expect the total production volume of DDG/DDGS to increase globally to 61 million tons, with 47 million tons in the US, 7.7 million tons in the EU, 1 million tons in Canada (unchanged to 2012) and up to 2.5 million tons in China and the rest of the world (OECD-FAO Agricultural Outlook, 2014; Jensen, Björnsson and Lind, 2012). Of course, the production volume of DDG/DDGS in 2012 only represents a limited share in the total amount of 658 million tons of cereals (including DDG/DDGS) used for animal nutrition (OECD-FAO Agricultural Outlook, 2014). But the percentage of 5 % at the moment might possibly increase in the next decades, due to political efforts to push the bioenergy sector (Jensen, Björnsson and Lind, 2012).

Distillers Grains are used mainly in the rearing of ruminants (beef and dairy cattle), poultry, pigs and fish in aquaculture (Liu, 2011). Furthermore, DDGS are ingredients for compound feed as they feature a high content of protein and oil, but a negligible content of starch (Table 2), being of general economic interest in the current struggle for protein sources in the feed sector (Liu, 2011). However, DDGS show a high variation in their chemical composition (cf. Table 2), also with regards to the main components protein, fiber and oil. This is due to the fact that production of DDGS is dependent on various factors, such as the composition and the quality of the grains (e.g., corn, wheat, barley), which can for example vary between production years, but also on the different production technologies applied (batch-wise fermentation vs. continuous fermentation, liquefaction, drying), as reported before (Spiehs, Whitney and Shurson, 2002; Balyea *et al.*, 2010; US Grains Council, 2012a). Variation in the nutrient content of DDGS (cf. Table 2) exists within and also among ethanol plants, even within plants using the same fermentation and production technology (Spiehs, Whitney and Shurson, 2002; Belyea *et al.*, 2010; Buckner *et al.*, 2011). As such, variability of DDGS nutrient composition directly affects the usage of DDGS in animal nutrition and makes the inclusion of DDGS into diets more complex than for comparable cereals like corn or wheat.

Table 2: Composition of DDGS. Mean values and ranges extracted or calculated from Monceaux and Kuehner (2009), Belyea, Rausch and Tumbleson (2004), Belyea *et al.* (2010), Spiehs, Whitney and Shurson (2002), Liu (2009), Chrenková *et al.* (2012), Westreicher-Kristen, Steingass and Rodehutscord (2012), Nuez Ortín and Yu (2009), McKeown *et al.* (2010), Cozannet *et al.* (2010), Noblet, Cozannet and Skiba (2012).

	Corn DDGS		**Wheat DDGS**	
	Mean	Range	Mean	Range
DRY MATTER (DM)	89.6	87.2 - 91.4	92.7	89.9 - 95.0
BASIC NUTRIENTS (% of DM)				
Protein	30.4	25.8 - 33.8	37.2	30.2 - 43.0
Crude Fiber	8.7	7.2 - 10.6	8.1	5.9 - 10.9
Fat	11.5	5.7 - 16.5	4.9	3.6 - 6.6
FURTHER COMPOUNDS (% of DM)				
Ash	4.8	3.5 - 6.7	5.1	3.9 - 6.7
NDF	43.7	32.9 - 62.1	36.5	25.1 - 48.1
ADF	16.4	13.2 - 25.2	15.4	7.5 - 24.2
ADL	4.0	2.8 - 5.0	5.5	2.1 - 13.0
Starch	4.6	3.2 - 5.9	5.7	2.4 - 18.5
Na	0.26	0.12 - 0.51	0.23	0.08 - 0.63
K	0.98	0.69 - 1.06	1.11	0.91 - 1.65
Mg	0.31	0.25 - 0.37	0.27	0.21 - 0.31
Ca	0.12	0.03 - 0.40	0.17	0.07 - 0.39
P	0.79	0.70 - 0.99	0.86	0.75 - 0.97
S	0.62	0.33 - 0.74	0.52	0.39 - 0.66
Cl	0.16	0.09 - 0.19	0.15	0.12 - 0.17
Arginine	1.29	1.11 - 2.17	1.36	0.81 - 1.67
Histidine	0.79	0.72 - 0.82	0.70	0.60 - 0.79
Leucine	3.51	3.43 - 3.81	2.31	2.10 - 2.44
Isoleucine	1.14	1.05 - 1.17	1.23	1.18 - 1.28
Lysine	0.88	0.72 - 1.02	0.69	0.30 - 1.09
Methionine	0.57	0.49 - 0.69	0.52	0.47 - 0.55
Phenylalanine	1.49	1.41 - 1.57	1.60	1.52 - 1.68
Threonine	1.13	1.07 - 1.21	1.06	0.97 - 1.13
Tryptophane	0.25	0.21 - 0.27	0.38	0.31 - 0.44
Valine	1.53	1.43 - 1.57	1.52	1.43 - 1.58

For example, the protein content of DDGS can be highly variable among different sources but specifications on the protein content are often necessary for the formulation of diets containing DDGS (Belyea *et al.*, 2010). Actual protein contents could differ clearly from average values given for DDGS produced from different grains (corn DDGS, wheat DDGS) causing protein deficiency or wastage and resulting in reduced animal productivity (Belyea *et al.*, 2010). In particular, large amounts of high-fat Distillers Grains might have a negative impact on ruminants, as the intakes may decrease and ruminal fermentation might be disrupted (Buckner *et al.*, 2011; Dicostanzo and Wright, 2012). In addition, pigs fed with DDGS of high fat content showed soft fat that also tends to be highly unsaturated, where it could be necessary to reduce the amounts of DDGS in diets

during the late finishing period (Stein, 2012). Further, Batal and Bregendahl (2012) reported that DDGS could generally be used for the rearing of poultry species but care should be taken of the high variation in nutrient content and digestibility, and thus recommended the consistent monitoring of the nutrient composition of DDGS.

Fields of Chemical Analysis

All the facts mentioned in the previous section illustrate the need for chemical analysis of DDGS. The different parameters that are typically analyzed and the usual ranges for those in corn DDGS and wheat DDGS are summarized in Table 2. Generally speaking, there are three main reasons for the analysis, as mentioned by Thiex (2012, p. 194): "[…] (1) quality standards for trading purposes; (2) to determine nutrients for inclusion in livestock feeds; and (3) to determine contaminants that may limit inclusion in livestock feeds." These reasons lead to methods of chemical analysis for the determination of (1) basic nutrients, (2) further compounds and (3) undesirable substances, and more details of those are highlighted in the following paragraphs.

(1) Basic Nutrients

Information on the nutrient profile is imperative for trading purposes, and chemical analysis of the respective parameters guarantees quality standards with regards to the whole supply chain (Thiex, 2012). However, as DDGS show high variation in the chemical composition, it becomes necessary to analyze nutrients in DDGS more frequently than in comparable feed materials (e.g., coarse grains, soybeans) in order to ensure guaranteed specifications of the product. The parameters analyzed for this purpose are more or less the same parameters that are analyzed in animal feed as a whole (cf. previous chapter). Dry matter, crude protein, crude fiber and crude fat, either analyzed by wet chemistry methods or using NIRS, are the basic parameters for determination of the nutritive value (Thiex, 2012). A number of analytical methods are in common use and have been evaluated by intra-/ interlaboratory studies, initiated by the American Feed Industry Association (AFIA), the Renewable Fuels Association (RFA) and the National Corn Grain Association (NCGA), and reported by Thiex (2009; 2012). Based on the results of the intra-/interlaboratory studies, reference methods for use in commercial trade of DDGS have been recommended and often are representing standardized AOAC methods of analysis (Thiex, 2009). Besides these reference methods, NIRS prediction models have been developed for determination of DDGS basic nutrients in research (X. Zhou *et al.*, 2012) and routine (NIRPerformance, 2013). In view of the great variability of DDGS, it has been recommended to use NIRS for quality check of DDGS as frequently as possible, since it offers convenient handling and could enable routine delivery checks (Gady, 2012).

(2) Further Compounds

The second major reason for analyzing DDGS is the inclusion of DDGS in livestock feeds. In addition to the determination of basic nutrients, the analysis of further nutrients and other compounds is the basis for feeding DDGS in accordance with the requirements of different species. It enables the formulation of complex compound feeds regarding main components (e.g., protein, fiber, oil) and minor constituents (e.g., major minerals, trace elements) at the same time. Thiex (2012) reported that besides the basic nutrients, the analysis of detergent fibers (NDF, ADF and lignin), ash, major elements and trace elements, amino acids and starch are of general interest for inclusion of DDGS in livestock feeds. The corresponding methods of analysis were discussed by Thiex (2012) and reference methods have been recommended also on the basis of standardized AOAC protocols. In fact, both wet chemistry methods, closely related to the methods developed by van Soest (1963; 1967), and more sophisticated methods applying modern techniques of analysis are involved. The latter ones include atomic absorption spectroscopy or inductively coupled plasma atomic emission spectroscopy for the analysis of major and minor elements, ion exchange chromatography and high performance liquid chromatography (pre- and postcolumn derivatisation) or mass spectrometry for the determination of amino acids, and specific methods involving fluorometry (e.g., selenium), potentiometry (e.g., chlorine) or spectrophotometry (e.g., phosphorus). For some of the parameters and compounds mentioned above, also NIRS prediction models can be developed by using values determined by the reference methods for calibration. NIRS is routinely used for measurement of DM, protein, fat, fiber (CF, ADF, NDF), ash and starch in DDGS, and the determination of DDGS fat content by NIRS was readily accepted by the feedlot industry (NIRPerformance, 2013). Further, NIRS showed potential for the rapid determination of amino acids in corn DDGS or the determination of phosphorus (AllAboutFeed, 2012; Tahir *et al.*, 2012).

Moreover, chemical analysis of DDGS incorporates the quantification of the sulfur content as one of the most important parameters with regard to the use of DDGS in animal nutrition. Large amounts of sulfur can be found in DDGS, because (1) sulfuric acid is often used in the production process and (2) the concentration of sulfur-containing amino acids present in the grains is concentrated approximately by a factor of three during production (Liu, 2011). Especially fuel ethanol plants use sulfuric acid to control the pH of the cereal mash during fermentation and/or use sulfuric acid as cleaning agent. In this way, a considerable amount of sulfur gets into the cereal mash in the form of sulfate, and finally into the animal feed DDGS. Shurson, Tilstra and Kerr (2012) reported that sulfur levels in DDGS can range from 0.31 up to 1.93 % on a dry matter basis and are much higher than the respective sulfur levels of 0.12 % in corn (Watson, 2003) and 0.14 % in wheat (Zhao, Hawkesford and McGrath, 1999). However, ruminants are susceptible to diets high in sulfur as high sulfur levels in the rumen can cause H_2S intoxication and polioencephalomalacia (PEM), in severe cases leading to the death (Schoonmaker and Beitz, 2012). Also, it has been reported that both ruminants and non-ruminants show decreased feed intake and gain when feeding a high amount of sulfur in the rations (Erickson, Klopfenstein and Watson, 2012; NRC, 2005). Thus, quantitative information on sulfur in DDGS is of great interest to parties involved. DDGS producers using sulfuric acid in the production particularly aim at the analysis of sulfur in order to

establish maximum sulfur levels in their products and finally to guarantee specifications. Quantification of sulfur is most conveniently carried out by ICP-AES (ISO 27085:2009) or is based on determination of SO_2 after combustion of DDGS at high temperatures, but also the traditional gravimetric approach can be used: oxidation of the organic sulfur compounds to sulfate, subsequent ashing and precipitation of barium sulfate by adding barium chloride solution (AOAC 923.01), as reported by Thiex (2012).

Another nutrient that is of great importance for the inclusion of DDGS in livestock diets is lysine. This is due to the fact, that the available lysine content in DDGS is highly variable but at the same time lysine is the first limiting amino acid when feeding dairy cattle and represents the most important amino acid for monogastric species (pigs: first limiting, poultry: second limiting) (Cromwell, 2010; Roush, 2010; Kalscheur *et al.*, 2012). During the drying of Distillers Grains, a major part of the lysine present reacts with reducing sugars in the Maillard reaction (Cromwell *et al.*, 1993; Stein *et al.*, 2006), supported by the high drying temperatures. Air temperatures in the dryer can be over 500 °C at the dryer inlet and over 100 °C at the dryer discharge (Rosentrater *et al.*, 2012). In the course of the reaction, the lysine's ε-amino group is bound by reducing sugars, Amadori compounds (e.g., furosine) are produced and finally high molecular weight polymeric compounds (melanoidins) are generated; the available amount of lysine in DDGS is reduced accordingly (Pahm *et al.*, 2008; US Grains Council, 2012a). Methods of analysis for lysine are typically based on hydrolysis of the protein, followed by separation of lysine using ion exchange chromatography and detection by ninhydrin. Alternatively, HPLC methods in combination with pre- or postcolumn derivatisation or detection by mass spectrometry could be applied (Thiex, 2012). Another important parameter is the amount of lysine that is biologically available, also known as 'lysine digestibility' that could be predicted most easily by colorimetric analyses of DDGS (US Grains Council, 2012b). Besides the color measurement, prediction equations for the ileal digestible lysine in DDGS have been developed on the basis of furosine determination by HPLC (Pahm *et al.*, 2008b), using the lysine content and crude protein determined by methods of chemical analyses (US Grains Council, 2012b) or prediction equations on the basis of NIRS (Gady, 2012).

(3) Undesirable Substances

The third reason for analyzing DDGS is the occurrence of undesirable substances or contaminants that can limit the inclusion in livestock feeds. With regard to DDGS, the most important compound groups are mycotoxins and antibiotic residues.

Mycotoxins are naturally occurring, secondary metabolites of molds and fungi and can be produced during the cultivation of raw materials (i.e., corn, wheat) or if DDGS are stored under conditions that favor the growth of molds, for example high humidity (Thiex, 2012). They can cause adverse effects on animal and human health and regulatory levels have therefore been established for animal feed materials (Liu, 2011; Liu, 2012; Caupert *et al.*, 2012). The most important mycotoxins with regard to DDGS are aflatoxins (B_1, B_2, G_1 and G_2) fumonisins (B_1, B_2, B_3), trichothecenes

(DON, T-2), zearalenone, ochratoxin A (Thiex, 2012); and their levels in DDGS could be higher than in raw materials due to concentration during production (Liu, 2011; Caupert *et al.*, 2012). Respective methods of analysis aiming at the specific detection of single mycotoxin analytes (e.g., for the detection of fumonisins) are in use, and recently also multi-analyte methods, mainly on the basis of HPLC and detection by fluorescence or mass spectrometry, have been developed (Zhang *et al.*, 2009; Caupert *et al.*, 2012; Thiex, 2012; Oplatowska-Stachowiak, 2015). In contrast to bioanalytical methods that are applied for rapid screening (i.e., immunosensor-based methods, ELISA), methods of (physico-) chemical analysis are used as confirmatory and reference methods (Liu, 2011).

Apart from the natural occurrence of mycotoxins, another important point is the exogenous addition of antibiotics during DDGS production. Because bacterial contamination (e.g., lactic acid bacteria) can reduce ethanol yield and can also result in lower quality DDGS, some countries (e.g., USA, Canada) have permitted the use of antibiotics, such as virginiamycin, penicillin, erythromycin or tylosin, to control the fermentation process (US Grains Council, 2012c; Granby *et al.*, 2012). However, concerns are that bacteria could possibly evolve resistances to single substances and that antibiotic residues remaining in DDGS could have a negative impact on animal health or finally could be carried over to humans (Ziggers, 2011). Of course, DDGS have to comply with regulatory levels for antibiotic residues or pharmaceutical drugs in animal feed (e.g., Regulation (EC) No 1831/2003), and antagonistic effects or negative interactions with other drugs (e.g., combination of erythromycin with penicillin or monensin) have to be considered (US Grains Council, 2012c). The Food and Drug Administration of the United States (FDA) developed a multi-method for the detection of 13 different antibiotic residues in Distillers Grains, based on two-step extraction of analytes (aqueous EDTA-trichloroacetic acid; methanol), cleanup on SPE columns, separation of analytes by HPLC and detection by ion trap tandem mass spectrometry (De Alwis and Heller, 2010). Furthermore, Kaklamanos *et al.* (2013) developed a method for the detection of up to 96 veterinary drugs in DDGS, including antibiotic residues, using extraction by acetonitrile/methanol/water/formic acid followed by HPLC coupled to high resolution mass spectrometry (Orbitrap MS). Although some antibiotics, such as virginiamycin, can be inactivated or destroyed during the distillation or drying steps in DDGS production, studies have been reporting the presence of antibiotic residues in Distillers Grains with detectable levels for virginiamycin, erythromycin, tetracycline, narasin and penicillin (US Grains Council, 2012c; Kaklamanos *et al.*, 2013).

Further Fields and Future Developments

The analytical parameters mentioned in the sections above are representing the most important ones with regards to the chemical analysis of DDGS. Of course, there has been a large number of articles dealing with the determination of other substances and compound groups in DDGS, such as the determination of vitamins (Brown *et al.*, 2012), the consideration of different forms of phosphor (Liu and Han, 2011) or the occurrence of free fatty acids (Moreau *et al.*, 2010). However, the aim of this chapter was to give an overview on the most important parameters concerning

DDGS analysis and to highlight the most conventional parameters and methods of chemical analysis that are in routine use for DDGS investigation.

In addition to the detection and quantification of single compounds, methods of (physico-)chemical analysis have been used in the investigation of specific protein classes, e.g., diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy (Yu & Nuez-Ortin, 2010; Liu *et al.*, 2012). NIRS has also been applied to the prediction of minor constituents in DDGS and thus showed promise for future routine applications (L.J. Zhou *et al.*, 2012; Pekel *et al.*, 2013). Of course, the development of methods of analysis for the determination of undesirable compounds or nutrients that will show correlation to reduced animal performance is steadily in progress. Also, methods of analysis that are already used for other feed materials will be adapted to DDGS (and probably are adapted at the moment), because the matrix DDGS is relatively new in the animal feed sector and production volume has increased in the last few years.

Moreover, the combination of laboratory analysis with multivariate statistical data evaluation, enabled by chemometric software tools, could provide great opportunities for the analysis of DDGS. Especially spectroscopic techniques (e.g., NIR or MIR spectroscopy) but also highly sophisticated analytical procedures (e.g., ultra-high separation techniques, high resolution mass spectrometry) that acquire large amounts of data, could not only be applied by means of classical compound-oriented analysis, but also by means of non-targeted authenticity-oriented approaches, similar to the study of de Jong *et al.* (2016) on soybean meal and vegetable oils. Such approaches could lead to the detection of abnormalities, and thus could offer the chance for detection of compounds that have never been detected in DDGS before; either naturally present or intentionally added (i.e., adulterants like melamine) (cf. Murray, 1996; NIRPerformance, 2014). Most notably, rapid screening techniques (e.g., NIR or MIR spectroscopy) that can be applied on-site, and the availability of micro-spectroscopes (i.e., handheld devices) in particular, could lead to the development of comprehensive strategies for analysis of DDGS that meet with future challenges in the face of feed safety.

3 New Methods for the Chemical Analysis of DDGS

Conventional chemical analysis of feed materials is typically aimed at the quantification of compounds that are known to be present in the matrix, either constituents of nutritive value like protein and fiber, or substances of anti-nutritive character like contaminants. However, most of the methods applied in the field fail to identify feed materials, because qualitative information on the raw material is not collected (Murray, 1996). What these methods have in common is that they are testing for the obvious, and consequently there is a danger that unexpected analytes or more serious flaws in a raw material remain undetected (Murray, 1996). On the contrary, methods that enable qualitative product identification, or generally speaking, analytical approaches that target the products authenticity, could prove whether a feed material tested is typical of its kind (Murray, 1996). If it is not, further analytical testing or traceability procedures could then be initiated, in order to clarify the reason.

The situation is similar with regards to DDGS: Methods of chemical analysis focus on the determination of nutrients and undesirable substances, but up to now, no methods directly address the question whether a DDGS batch is what it is stated to be. However, the last point is of great importance to feed business operators, because in general, qualitative analysis as the first step in feed evaluation ensures the identity of a feed material offered for sale (Murray, 1996). With regard to DDGS, simple information on the identity of a certain batch, for example whether it has been produced from corn or wheat, or mixtures thereof, could be worth for parties involved and could be the starting point for specific quantitative analysis. Also, properties that cannot be detected by conventional chemical analysis, but represent further factors of diversity, can be of particular interest to feed business operators, for example, place of origin, method of production or date of production. Although such information typically is available by paper documentation and electronic traceability systems, assurance on the information provided could become relevant for new suppliers or if supply chains have been modified, especially in a globalized market situation.

Moreover, feed business operators might acknowledge analytical strategies that enable the detection of abnormalities in general. The main reasons for this are: great variability in DDGS composition, globalization of the feed market, supply of DDGS by different and thereby possibly unknown manufacturers, as well as potential hazards that may arise in the future (Spiehs, Whitney and Shurson, 2002; Balyea *et al.*, 2010; US Grains Council, 2012; publication A). In this respect, screening for abnormalities, or inversely, confirmation of the materials authenticity, could introduce an extended system of quality assurance by analytical methods. Such systems could initiate the search for compounds that might have been added to DDGS, for example by means of adulteration (similar to melamine in feed), or simply indicate a quality concern, for example due to differences in the content of single nutrients, which has been reported similarly for food and feed applications (NIRPerformance, 2014; Baeten *et al.*, 2014; Fernández Pierna *et al.*, 2015).

The starting point for these kinds of analyses is the construction of databases by collecting analytical data of DDGS from various sources, similar to the development of spectral libraries of

ingredients in animal feed described by Fernández-Ibanez *et al.* (2009; 2010). Together with the itemization of all relevant properties of the samples (e.g., composition, origin), the so-called meta data, the spectral libraries could then in a second step provide the basis for a 'conformity check' of newly analyzed DDGS batches. By collection of analytical data from a large number of authentic DDGS samples, provided these derive from various origins and suppliers, libraries could be built that represent the natural variability of DDGS and that could help to authenticate new DDGS samples. In other words, systems that enable authentication of DDGS could lead to the information whether a DDGS batch looks similar to DDGS batches analyzed before (e.g., same provider, origin), or whether this DDGS batch seems to be different to previous batches. This information could be beneficial for early-stage control of DDGS (i.e., at feedmills or feed manufacturers) in a way that (1) further laboratory analysis of basic nutrients or specific compounds could be requested for conspicuous batches, (2) specific proof of documents could be initiated and credibility of provided information (composition, origin, etc.) could be confirmed and (3) DDGS delivered could be authenticated with regard to chemical fingerprints and labeling information at the same time. But the spectral libraries could also be used together with advanced pattern recognition software to flag-up hazards not detected at all by conventional methods of analysis, as it has been discussed by Murray (1996) for the application of NIRS in the animal feed sector.

3.1 Authentication using FT-IR Spectroscopy and IRMS

Authentication means to prove that something is 'authentic', i.e., it is "real and genuine and not a copy […] true and accurate […] made to be exactly the same as the original" (Oxford Learner's Dictionaries, 2014), or in other words, that it is "of undisputed origin and not a copy; genuine" (Oxford Dictionaries, 2014). Following Downey's definition of authentic food (Downey, 1996), authentic animal feed could be defined as animal feed that is what it purports to be, and conforms to the description provided by the producer or processor. From a more practical point of view, authentication comprises the comparison of a product's properties with the labeling, and vice versa, the verification whether the properties and characteristics specified by the labeling can be confirmed for the product. The fundamental question that has to be answered is, whether the product meets the criteria for membership in the class of genuine products. Besides sensory analysis and verification of paper documentation, this can be tackled by chemical analysis, since products in question can be compared to a set of values established as typical for the genuine products (cf. Downey, 1996). In food control, authentication also describes "[…] the confirmation of all requirements regarding the legal product description or the detection of fraudulent statements […]" (Esslinger, Riedl and Fauhl-Hassek, 2014, p. 189).

In the most basic sense, analytical authentication of agricultural and food products is realized by evaluation of key properties or analytical parameters that are characteristic for the product in question. For example, the quantitative analysis of different sugars (e.g., fructose, glucose, sucrose) could confirm the identity of juice produced from a certain fruit. Also, more complex questions on

the products provenance (i.e., botanical origin, geographical origin) could be investigated by single analytical parameters, for example applying stable isotope ratio analysis of single elements. On the other hand, single analytical parameters could also detect the non-conformity of a product, such as the detection of starch, if added to meat products (e.g., chicken meat patties). Generally speaking, single parameters can be used as 'identifiers' or 'markers' by means of verification of the products authenticity.

However, it is easy to understand that sometimes single properties or parameters do not suffice for describing a products authenticity, but the combination of parameters is more valuable in this respect. For example, the consideration of isotope ratios of several elements enhances the dimensionality of the authenticity matrix that describes the product, and often a reasonable evaluation with regards to the products provenance only then can be made. In other words, multivariate analysis of analytical data is often superior to the commonly used univariate approach, especially when addressing food and feed authenticity (Leardi, 2003). For this purpose, mathematical and statistical methods are applied to the analysis of complex analytical data, subsumed under the expression 'chemometrics'.

In addition, one has to distinguish between techniques that are based on the detection of marker compounds and techniques that acquire a large amount of data, both with the aim to verify the authenticity of a certain product. For example, the detection of single deoxyribonucleic acid (DNA) sequences that are characteristic for animal species could enable the authentication of meat (Lees and Popping, 2003), whereas the collection of infrared spectra from meat would usually require further processing of the data to draw a conclusion on meat authenticity (Karoui *et al.*, 2008). The latter includes the use of multivariate data analysis to reveal variables, or in other words identifiers, for the verification of the products authenticity.

3.1.1 Techniques and Strategies

Authentication has mainly been an issue in the food sector and only few articles have been published on authentication of feed materials, such as Pinotti *et al.* (2005), Bauermann *et al.* (2008), Fernández-Ibánez *et al.* (2009; 2010), van Ruth *et al.* (2010), Tres and van Ruth (2011), Tres *et al.* (2012). However, as feed materials are agricultural products, it can be assumed that the same analytical techniques as those applied to food authentication, in principle, can be used for authentication of feed materials. A magnitude of analytical techniques is utilized in food authentication, but the most important groups are: (1) conventional (wet) chemistry methods, often aimed at the specific determination of single substances (e.g., color reactions, titrimetric methods); (2) spectroscopic techniques, in particular infrared spectroscopy (NIR, MIR), nuclear magnetic resonance spectroscopy (NMR) and UV-Vis (ultra violet visible) spectrophotometry; (3) chromatographic/spectrometric techniques, mainly gas chromatography (GC), high performance liquid chromatography (HPLC) and/or combinations with mass spectrometry (MS); (4) bioanalytical techniques, especially polymerase chain reaction (PCR), enzyme-based methods like enzyme-linked immunosorbent assays (ELISA) or metabolome and proteome analyses; (5) stable

isotope analysis, realized by isotope ratio mass spectrometry (IRMS) or site-specific natural isotope fractionation nuclear magnetic resonance spectroscopy (SNIF-NMR); and (6) multi-elemental analysis, mainly by atomic absorption/emission spectroscopy (AAS/AES) and inductively coupled plasma atomic emission spectroscopy or mass spectrometry (ICP-OES or ICP-MS) (Schieber, 2008; Lees, 2003).

Two techniques have been applied to the authentication of DDGS in this thesis: Fourier transform infrared (FT-IR) spectroscopy and stable isotope ratio mass spectrometry (IRMS). The suitability of both techniques for authentication of agricultural and food products has already been demonstrated (Karoui *et al.*, 2008; Vermeulen *et al.*, 2010; Kelly, 2003) and therefore, the application of these analytical techniques seemed to be promising also for authentication of DDGS. Special attention was paid to authentication of the botanical and geographical origin, as well as the method of production, because these three facts are generally of interest and the respective information was available through DDGS sample suppliers.

Fourier Transform Infrared Spectroscopy (FT-IR Spectroscopy)

FT-IR spectroscopy gives information on the presence of different functional groups in molecules, like O-H, C=O and CH_3, or generally speaking, it gives information on the molecular structure (Shurvell, 2002). The principle behind this analytical technique is the absorption of electromagnetic radiation between 4000 cm^{-1} and 400 cm^{-1} (2.5-25 μm), which is due to transitions between the quantized vibrational energy states of molecules (Griffiths, 2010). The absorption of IR radiation at characteristic frequencies may be related to single functional groups but absorptions usually occur at several frequencies for the same group because of the different vibrational modes (Karoui *et al.*, 2008). Besides these fundamental modes of vibration also the possibility of overtones and combinations of the fundamentals exists, which increases the total number of absorbed frequencies in the IR spectrum, especially in the case of complex molecules (Shurvell, 2002). The data acquired by FT-IR spectroscopy usually show the absorbance ($A = \log_{10} 1/T$, with T = transmittance) over the wavelength/wavenumber range (Shurvell, 2002). The proportional correlation of absorbance and concentration of the absorbing molecules results in quantitative information about single molecular species present in the samples (Shurvell, 2002). In addition, unique information about chemical composition, protein structure, protein-protein and protein-lipid interactions can be obtained (Vermeulen *et al.*, 2010; Karoui *et al.*, 2008). As FT-IR spectroscopy considers the effects of all the different functional groups of the molecules in the sample, the resulting spectrum can be regarded as a unique molecular fingerprint of the sample analyzed (Karoui *et al.*, 2008). Depending on the complexity of the matrix present, FT-IR spectroscopy is mainly used for achieving information on the purity and identity of samples (e.g., plastic materials, food additives) or in order to quantify compounds or compound classes (e.g., fat and trans fatty acids, peroxide value) in samples of known matrices (van de Voort *et al.*, 1994; Chalmers, Hannah and Mayo, 2002; Mossoba *et al.*, 2004; Vermeulen *et al.*, 2010).

FT-IR spectroscopy is often carried out by using attenuated total reflexion (ATR) devices, which have the advantage that for analysis, the sample only needs to be pressed against a crystal (e.g., diamond, ZnS, Ge). At the crystal, a part of the IR beam interacts with the sample (evanescent waves), specific wavelengths are absorbed and finally the non-absorbed wavelengths are reflected back to the detector (Karoui *et al.*, 2008). Thus, the measurement is non-destructive and only little sample preparation is needed for ATR/FT-IR spectroscopy, which makes it more comfortable compared to the traditional analysis with KBr pellets. Recently, portable and handheld FT-IR instruments using the ATR technique and on-the-instrument data analysis have been developed for out-of-lab applications (Agilent Technologies, 2014). The capability of such instruments for composition verification and counterfeit detection has been demonstrated for polymeric materials (Seelenbinder and Rein, 2014) and possibly similar applications for animal feed could be realized in the future.

For authentication of agricultural products, FT-IR spectroscopy is often combined with chemometrics, a discipline that uses mathematical and statistical methods for the multivariate analysis of analytical data (Schieber, 2008). One advantage of multivariate data analysis lies in the consideration of all variables available, the complete spectral data, at the same time. By doing so, also smallest but statistically significant differences in absorbance values between the samples can be detected. Such differences are not always readily apparent in the spectra themselves (not visible by eye), but could be revealed by multivariate data analysis. As mentioned before, IR spectra can be regarded as molecular fingerprints of the samples analyzed, and it is easy to understand that respective differences in the composition of samples are represented also in the spectra. In combination with multivariate data analysis, FT-IR spectroscopy has shown suitability for authentication of many different food matrices, like meat, wine, olive oil or honey (Vermeulen *et al.*, 2010; Rodriguez-Saona and Allendorf, 2011; Riovanto *et al.*, 2011; De Luca *et al.*, 2011; Ruoff *et al.*, 2006). Also studies on authentication of cereals, most similar to the matrix DDGS, have been published: For example, Cocchi *et al.* (2004) classified different cereal flours (wheat, oat and buckwheat) by using chemometric analysis of MIR spectra, and Fernández Pierna *et al.* (2005) compared different statistical methods for the classification of modified and unmodified starches based on IR data. Moreover, vibrational spectroscopy in general is especially useful for authenticating the geographical origin, the botanical origin (species/variety), and the production process of food and feed products (Vermeulen *et al.*, 2010). It could already be shown that discrimination of botanical and geographical origin of cereals is realizable by combining NIR spectroscopy and multivariate data analysis (Bertrand *et al.*, 1985; Osborne *et al.*, 1993; Kim *et al.*, 2003). However, only few studies dealing with the authentication of animal feed have been published and therefore it was the aim of this work to apply FT-IR spectroscopy together with subsequent multivariate data analysis to the authentication of DDGS.

Stable Isotope Ratio Mass Spectrometry (IRMS)

IRMS is applied to determine the stable isotope ratios of several light (bio-)elements: hydrogen ($^{2}H/^{1}H$), carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$), oxygen ($^{18}O/^{16}O$) and sulfur ($^{34}S/^{32}S$) are the elements

of interest in food authentication (Kelly, 2003). The measurement principle is based on the difference in mass of the atoms in question and special mass spectrometers which are used for the appropriate analysis. Sample material has first to be converted into measurement gases like H_2, CO_2, N_2, CO and SO_2, which is usually carried out by elemental analyzers or high temperature pyrolysis units. The gases produced are then separated by gas chromatography (packed columns) and ionized by bombardment with electrons, the so called electron impact ionization (EI), forming positively charged ions, like CO_2^+ (Gremaud & Hilkert, 2008). Subsequently, ions are accelerated into a magnetic field, which deflects the ions vertical to the flight direction and vertical to the magnetic field, so that an ion of low mass (e.g., $^{12}C^{16}O^{16}O^+$) describes a smaller radius than an ion of high mass (e.g., $^{13}C^{16}O^{16}O^+$) (Gremaud & Hilkert, 2008). Finally, the intensity of the ion beams (e.g., $^{14}N^{14}N$ and $^{15}N^{14}N$) is measured simultaneously by dedicated Faraday cups with individual amplifier electronics, positioned according to the deflection of the ion beams (Gremaud & Hilkert, 2008). This results in the collection of the single elements isotope ratios, measured against a reference and typically given in the so called delta notation in per mil, which expresses the isotopic abundance of a sample relative to the reference, multiplied by a factor of 1000 (Kelly, 2003).

The distribution of stable isotopes in plant raw materials is the result of isotopic fractionation effects, either due to physico-chemical processes such as evaporation and diffusion, or caused by chemical and biochemical reactions (Kelly, 2003; Gremaud & Hilkert, 2008). Isotope ratios of bio-elements can reflect the enrichment of single isotopes by plant metabolism and could also be representative for geochemical phenomena or environmental and climatic factors (Gremaud & Hilkert, 2008; Benson *et al.*, 2006). For example, differences in the heavy isotopes of water, i.e. ^2H and ^{18}O, are correlated with factors like latitude, distance from the coast and altitude, which tend to result in depletion of the heavy isotopes at continental areas of higher altitude (Gremaud & Hilkert, 2008). At the same time, plant metabolism and evapo-transpiration account for the enrichment of ^{18}O in plant water compared to ground water (Förstel, 2007). The ^{15}N enrichment is primarily affected by regional agricultural practices and soil types (fertilizers, bacterial nitrification/denitrification processes), whereas the $^{13}C/^{12}C$ ratio is resulting from plant metabolism (C_4, C_3 and CAM plants) (Gremaud & Hilkert, 2008). However, $\delta^{13}C$ of plants can also be different according to geographical growing regions, since the dryness of climate often is correlated with the geographical region and "[…] C_3 plants predominate at higher latitudes and C_4 plants are more common in warmer climates at lower latitudes […]" (Kelly *et al.*, 2005, p. 557). Indications on the geographical origin of a plant can also be drawn by the $^{34}S/^{32}S$ ratio, which is influenced by soil types (geological structures) and by sulfate-rich sea sprays in coastal areas (Gremaud & Hilkert, 2008). On the other hand, the sulfur isotope ratio can also be connected with sulfur-containing fertilizers and the atmospheric deposition of sulfur (Gremaud & Hilkert, 2008); thus drawing final conclusions on the geographical origin of a plant by the $^{34}S/^{32}S$ ratio exclusively is not appropriate but could confirm the origin of products indicated by $\delta\,^2H$ and $\delta\,^{18}O$ values. In brief, the analysis of stable isotope ratios of bio-elements can provide information on botanical origin (plant type) and geographical origin (growing region) of plant material, as well as further characteristics associated with primary production, such as fertilization.

In contrast to analytical techniques that acquire large amounts of data, e.g., FT-IR spectroscopy, IRMS reports single values on which the final conclusions about the products' provenance or authenticity are drawn. For this purpose, the isotope values of the samples are compared with isotope values that are expected for the respective commodity. However, seasonal and regional variability of the isotopic data exist and therefore meaningful results could only be obtained after a large number of authentic samples has been analyzed and databases have been established (Rossmann, 2001). Besides the comparison of single isotope ratios, authentication strategies on the basis of IRMS involve the simultaneous consideration of several isotope ratios analyzed. Such multi-isotope approaches are usually carried out by scatter plots with up to three dimensions, e.g. $\delta^2H/\delta^{18}O/\delta^{13}C$, or by multivariate data analysis for more than 3 isotope ratios. Sometimes, stable isotope ratios of the light elements are combined with the ratio of strontium isotopes ^{87}Sr and ^{86}Sr or with multi-element composition data, which can be analyzed by ICP-MS, in order to better describe the provenance of samples (Kelly *et al.*, 2005).

As mentioned before, IRMS showed to be a powerful analytical tool for food authentication and can provide information on the botanical and geographical origin of agricultural products (Rossmann, 2001; Kelly, 2003). In addition, stable isotope ratios proved to be useful in the detection of food adulteration, as they allow the differentiation of chemically identical materials, but of different origin, by the use of a physical parameter, the isotope ratio (Rossmann, 2001). Various applications of IRMS exist in the food sector, e.g., for olive oil and wine (Camin *et al.*, 2010; Wachter *et al.*, 2009), and also cereals have been analyzed with regards to authentication of provenance (Asfaha *et al.*, 2011). Brescia *et al.* (2002) reported on differentiation of the geographical origin of durum wheat semolina on the basis of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ values, and Branch *et al.* (2002) conducted a preliminary study on wheat samples, where $\delta^{13}C$ values alone enabled the determination of geographical origin of the samples analyzed in the study. However, studies dealing with authentication of feed materials on the basis of IRMS are few in number, and up to now have mainly focused on the effects of different diets (e.g., C_3/C_4 plants, animal protein vs. plant protein) on animal products like milk or meat (Bahar *et al.*, 2005; Camin *et al.*, 2008; Knobbe *et al.*, 2006; Moreno-Rojas, 2008). Therefore it was the aim of this work to determine stable isotope ratios of light elements by IRMS in order to authenticate DDGS, especially with regards to botanical and geographical origin.

Chemometrics - Multivariate Data Analysis

Chemometrics has been defined as "[…] the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods." by the International Chemometrics Society (IUPAC, 2008). The aim of chemometrics with regards to food and feed authentication is to enable the evaluation of a huge set of variables acquired by methods of chemical or physicochemical analysis. Different chemometrical techniques for multivariate analysis can be used to reveal variables out of the data set in order to define and describe the products authenticity. In the following section these techniques are described and some of them were used in this dissertation in order to develop authentication strategies for DDGS.

(1) Pre-Processing

The first step in multivariate data analysis is pre-processing of the analytical data. Although this step is not compulsory, it is often necessary for a successful evaluation of the analytical data. This has also been the case for evaluation of analytical data in the present work. With regard to spectroscopic techniques, like FT-IR spectroscopy, the need for data pre-processing results from instrumental issues, experimental conditions or physical characteristics of the samples, such as scattering, interferences or baseline shifts (Engel *et al.*, 2013; Oliveri and Downey, 2013). Sometimes, spectroscopic data are pre-processed in order to improve the linear relationship between absorbance and the analytes' concentration, if quantitative correlations according to Lambert Beer's Law are investigated (Rinnan *et al.*, 2009). On the other hand, it could also be necessary to eliminate systematic differences between heterogeneous variables prior to multivariate analysis (Oliveri and Downey, 2013), as it was the case in this thesis for isotope ratios, which differed in the range of absolute values and their standard deviation.

Generally, two groups of pre-processing techniques can be distinguished: reference-dependent techniques and reference-independent techniques (Rinnan *et al.*, 2009). The main difference between the two techniques is that reference-dependent techniques always use a meta variable, the reference value, in addition to the analytical variables itself, e.g., the crude fat content of the samples that have been measured. A prominent example of a reference-dependent technique is orthogonal signal correction (OSC) with the general aim to remove variability which does not correlate to the reference values (Rinnan *et al.*, 2009). However, those techniques are of interest especially if quantitative regression techniques are carried out, in contrary to reference-independent techniques, which are applied mainly in food and feed authentication. In order to find the best authentication approach for DDGS, reference-independent techniques were applied also in this dissertation. Reference-independent techniques are based on mathematical corrections, either sample-based (all variables in one sample) or variable-based (one variable in all samples). Commonly accepted sample-based techniques are (1) row centering, where each variable of the analytical data from one sample is corrected by subtracting a specified value (e.g., mean, median of the sample data); (2) standard normal variate transformation (SNV), where each variable of a sample is first corrected by the mean of the variables of this specific sample and then divided by the standard deviation of all variables of this specific sample; and (3) first- and second-order derivation after smoothing, applied to correlated (spectroscopic) data, according to the Savitzky-Golay routine (Barnes *et al.*, 1989; Oliveri and Downey, 2013; Rinnan *et al.*, 2009). On the other hand, accepted variable-based techniques involve (1) column centering, where the same variable of all the samples analyzed is corrected by subtracting a specified value (e.g., mean, median of the sample data); (2) column autoscaling, where the same variable of all samples is first corrected (individually) by the mean of this variable in all samples and then divided by the standard deviation of this variable in all samples (Oliveri and Downey, 2013).

(2) Techniques used in Chemometrics

Certainly, the use of pre-processing techniques is specific to the analytical data that is evaluated, but usually improves the subsequent multivariate data analysis. On the basis of well pre-processed data, the actual chemometric tools can be employed. In general, statistical and mathematical methods that are applied in chemometrics can be categorized as: (A) 'unsupervised' techniques for exploratory data analysis and (B) 'supervised' techniques for the modeling of quantitative or qualitative responses. The main difference between the two categories is that the latter one makes use of additional information, which is not part of the analytical data itself but is incorporated into data analysis. Such information could be the membership to a class, e.g., the membership of DDGS samples to a certain geographical origin, or the content of a compound analyzed by a reference method, e.g., the crude fat content of DDGS samples.

(A) 'Unsupervised' Techniques

'Unsupervised' techniques include 'unsupervised' pattern recognition methods, such as principal component analysis (PCA) or cluster analysis, and are applied in order to get an overview of the data (Leardi, 2003). When having measured n variables on k samples, PCA projects the data in a reduced hyperspace (cf. Figure 3), defined by the so called principal components (PCs), which are m (with $m \leq n$) linear combinations ($y_1, y_2, \ldots y_m$) of the original variables (Leardi, 2003; Ballabio & Todeschini, 2009). It is a variable compression method that reduces the data matrix **X** (**K x N**) to a smaller one, while deleting/hiding useless information (Blanco Romia & Alcala Bernardez, 2009). The corresponding mathematical transformation can be expressed by the equation **X = TP + E**, where the matrix **T** (**K x M**) contains m scores for the k samples, the matrix **P** (**M x N**) contains m loadings for the n variables and the matrix **E** (**K x N**) represents the residuals/the error made by the product **TP** (Brereton, 2009).

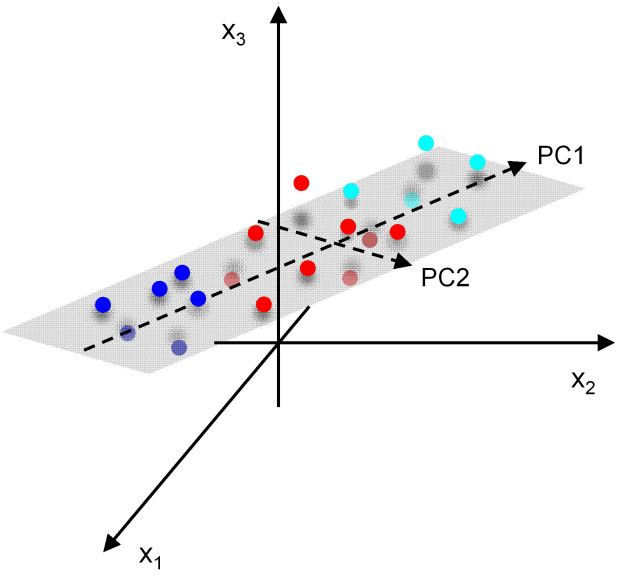

Figure 3: Principal Component Analysis - Geometrical visualization of the principle.

From a geometrical point of view, PCA determines the direction of greatest variance in the space of the original variables, which leads to the first PC, then it determines the direction explaining the greatest part of the residual variance, which leads to the second PC, that is orthogonal to the first one, and so on (Leardi, 2003). The advantage of PCA is that it reduces the dimensionality of the data and extracts the most relevant part of the information contained in the data to the first PCs, but non-structured information, i.e., the noise, gets hidden in the last PCs (Leardi, 2003).

By using PCA, structured information contained in very complex data matrices, such as spectroscopic data from FT-IR spectroscopy (~1.800 variables/sample) or NMR spectroscopy (up to ~130.000 variables/sample), can be visualized in just one or a few plots. On the one hand, these plots show the samples' scores values for the different PCs (i.e., scores plots) and thus enable detection of similarities between different samples, but on the other hand PCA could also be used to detect outliers or to verify the impact of single variables on the separation of samples (i.e., loadings plots). In this way, one could find out more about the chemical reasons for a separation, e.g., the separation according to absorption values in wavenumber regions that are characteristic for water would probably mean a separation due to the water content. With regards to DDGS authentication, PCA could therefore also reveal variables in a set of analytical data that are specific to single groups of DDGS samples.

In contrast to PCA, cluster analysis identifies concentrated groups of samples without any efforts required by the users, i.e., clusters are directly displayed (Varmuza and Filzmoser, 2009). It is based on the detection of similarities between samples and finds groups in the data by calculating distances (Ballabio & Todeschini, 2009). However, cluster analysis does not necessarily assign samples to only one group, but could lead to the result that a sample belongs to two or more groups (Varmuza and Filzmoser, 2009). The most commonly known cluster analysis method is called hierarchical cluster analysis, which arranges samples in a hierarchy and results in a graphical representation, a tree-like dendrogram (Varmuza and Filzmoser, 2009).

In conclusion, PCA and cluster analysis are chemometrical techniques that are applied in order to discover similarities and differences between samples, or in other words to detect different groups of samples, commonly called clusters. Finally, they could be used to find parameters in the huge pool of analytical data that are relevant to the authentication of agricultural and food products. Therefore, exploratory analysis in the form of PCA was carried out on DDGS analytical data in this dissertation. Cluster analysis was also evaluated for potential use but finally not applied, because it turned out that no superior statements could be made in this way.

(B) 'Supervised' Techniques

If new batches of agricultural products shall be authenticated, for example if a new batch of DDGS should be authenticated with regard to its geographical origin, unsupervised techniques only show limited possibilities. Usually, PCA allows only for visual statements whether new samples that are evaluated show similar scores than some of the old samples. In order to do so, the new batch of DDGS could be projected to the PCA that has been calculated before on the basis of the old

samples. The best thing one could then make use of is optimizing the PCA with regards to group building, e.g., by pre-selection of variables that will improve the separation of groups and projection of the new samples accordingly. However, such procedures do not lead to numerical values (e.g., probability) or assignments of a sample to a certain group of samples. For this purpose, chemometrical techniques are required that say with a reasonable certainty whether the new samples belongs to group A or group B. From a more practical point of view, the question is, whether the new samples (e.g., DDGS labeled US origin) belong to the authentic group of samples that is of interest (e.g., DDGS from the United States) or if those do not show the characteristics and are deemed 'non-authentic' (i.e., DDGS from another geographical origin). Part of the solution lies in the application of so-called 'supervised' techniques for the modeling of quantitative or qualitative responses, and thus those were applied in this work accordingly.

Such 'supervised' techniques, or 'supervised' pattern recognition methods, require *a priori* information on the set of samples (= objects) that is used for classification purposes (Ballabio & Todeschini, 2009). This information is used together with the analytical data in order to find mathematical models that are able to recognize the membership of each object to its proper class (Ballabio & Todeschini, 2009). Usually, classification methods define decision boundaries for class discrimination by delimiting regions of the hyperspace into which the objects are predicted, or they define classification rules by finding mathematical relationships between the classes (Leardi, 2003; Ballabio & Todeschini, 2009). The calculation of these decision boundaries and classification rules is based on a limited set of samples that are belonging to different groups and for each sample the group must be known before (*a priori*). Once the model has been built, new samples can be predicted with a reasonable certainty to one of the modeled classes using the analytical data of the sample. While regression methods like multiple linear regression or partial least squares regression model quantitative responses on the basis of a set of explanatory variables, classification techniques are methods for the modeling of qualitative responses (Ballabio & Todeschini, 2009).

The simplest classification methods are the nearest mean classifier (NMC) and the K-nearest neighbor (KNN) rule. The NMC method considers the centroid of each class (i.e., the mean of the parameter values of the respective samples) and predicts new samples to the class of the nearest class centroid, thus being based on a measure of distance (Ballabio & Todeschini, 2009). KNN is classifying samples according to the majority of its K-nearest neighbors, where, for example, the Euclidean distance of each object from all other objects in the data space is calculated. Thus, the assignment to a class is also depending on a measure of distance, the smallest distance to K objects, but the (optimal) K value can be selected (Ballabio & Todeschini, 2009). In this dissertation, classification methods for DDGS on the basis of KNN have not been applied, since model performance was highly dependent on the optimal K value and showed an extremely wide difference.

The most commonly used classification techniques are linear discriminant analysis (LDA), quadratic discriminant analysis (QDA) and partial least squares discriminant analysis (PLS-DA). The first two methods are based on the classical discriminant analysis (DA) introduced by Fisher

(1936) and include the calculation of discriminant functions that separate objects into classes by minimizing the within-class variance and maximizing the between-class variance (Ballabio & Todeschini, 2009). Here, discriminant functions divide the hyperspace of objects in as many subspaces as the number of classes, each point of the hyperspace belongs to one and only one subspace, and new objects (samples) are predicted according to these discriminant functions (Leardi, 2003). However, for LDA, the number of objects must be significantly greater than the number of variables (Ballabio & Todeschini, 2009). Therefore, huge sets of parameters/variables, like the ones acquired by FT-IR spectroscopy in the present dissertation, require the calculation of PCs (by PCA) that are used instead of the original variables for applying LDA. More recently, PLS-DA has been applied to classification purposes. It produces essentially the same results as LDA but with the noise reduction and variable selection advantages of PLS (Ballabio & Todeschini, 2009). PLS-DA is based on an algorithm that searches for latent variables featuring a maximum covariance with the so called Y variables, i.e., the depending variables that describe the class membership of the objects. Finally, new objects can be predicted by the PLS models constructed, resulting in Y vectors that indicate the assignment of the objects to the single classes. The advantage of PLS-DA is that the membership to a class is not described by single number values that have been given to the classes (e.g., 0 and 1) but is given by predicted values that result from the calculations. For example, a value of 0.781 would indicate that a new object is more related to group '1' than to group '0'. Therefore, threshold values between zero and one can be determined for each class and decision rules can be applied in order to describe the model more properly (Ballabio & Todeschini, 2009).

The classification methods described above all share the same principle: classification by discrimination. However, this means that new samples that are predicted by the respective methods must belong to one of the classes that have been used for model calculation. Such 'hard' classification techniques are not able to handle completely different samples, which do not belong to any of the groups modeled, as they will assign these samples to one of the classes used before (Leardi, 2003). For example, DDGS samples from Europe that are predicted to a model developed on the basis of DDGS from China and USA will be assigned either to China or to USA, but indeed, both assignments are wrong. In contrast to that, class-modeling methods such as unequal class modeling (UNEQ) or soft independent modeling of class analogy (SIMCA) model analogies between the objects of a class and separate classes by defining boundaries in the hyperspace (Ballabio & Todeschini, 2009). In this way, single class models can overlap in the hyperspace and also some regions of the hyperspace are left unassigned (Leardi, 2003). For example, SIMCA is based on the calculation of N PCA models, one for each of the N classes, and finally prediction is carried out by projecting the new objects to the different class subspaces and assessing the distances to the different classes (Ballabio & Todeschini, 2009). Generally, three 'types' of classification of new objects are possible with such methods: (1) unique – object is assigned to one single class; (2) ambiguous – object is assigned to 2 or more classes; and (3) outlier – object is assigned to none of the classes. Thus, for example, DDGS samples from Europe that are predicted to a SIMCA model for DDGS from China and USA could be predicted as belonging to none of

these classes, which would be correct. In contrast to discrimination methods like LDA, SIMCA verifies whether or not a sample is really compatible with one of the class models derived from the authentic pool of samples (Oliveri and Downey, 2013). Therefore, class-modeling methods can be very useful for authentication purposes if not each of the possible categories of a product has been sampled, and different groups could be expected to occur in the future. For this purpose, class-modeling using SIMCA was also applied for authentication of DDGS in this work.

Besides the supervised pattern recognition methods described above, some other methods exist that feature even more possibilities for data evaluation but have not been used in this dissertation. Examples are artificial neural networks (ANNs) that mimic the action of a biological network of neurons by means of interconnected processing algorithms working in parallel, and support vector machines (SVMs) which support classification on hyperplanes that are calculated to separate objects in the hyperspace (Ballabio & Todeschini, 2009; Gonzálvez *et al.*, 2009).

(3) Validation

Statistical models developed for authentication of food and feedstuffs always need to be carefully validated (Downey, 1996; Riedl, Esslinger and Fauhl-Hassek, 2015). Generally, the mathematical models are constructed on the basis of a specific sample set and the question is whether these models will show similar results if the sample set is extended. In order to achieve models that are eligible for future use, the sample sets examined should be most representative, with a large number of authentic samples that are collected from various sources (Downey, 1996). Also, models should be tested by means of validation and in a way that conclusions can be drawn how the model will perform in the future and if it is already robust enough. Thus, several validation strategies can be applied during model development: (1) cross-validation approaches (internal validation), (2) validation by prediction of test sample sets, either split from the original (training) sample set or collected separately (external validation), (3) blind-testing by prediction of unknown and independent samples ('system challenge') (Downey, 1996; Kelly, 2005; Riedl, Esslinger and Fauhl-Hassek, 2015). In this dissertation, different strategies for model validation have therefore been applied and models for authentication of the botanical and geographical origin of DDGS have been constructed on different sample sets during the course of the work.

It is also important that statistical models are optimized before test sets are predicted or blind-testing is carried out, for example, by selection of pre-processing or variables, but that the statistical data analysis procedure is remained unchanged during and after the validation itself. Of course, changes need to be implemented if the model shows worse validation results, but then, first internal validation should be optimized on the basis of the training set (i.e., cross-validation) before external validation is targeted by means of test sets, as it has been similarly explained in the field of metabolomics and food authenticity by Broadhurst & Kell (2006) and Oliveri & Downey (2012), respectively. Also, it could become necessary that samples of the test set must be included into the training set, which has been used for model construction, especially if unique samples are contained in the test set but are not included in the training set. In this thesis, for example, this was

the case for European corn DDGS samples, which need to be included into the authentication model for the botanical origin of DDGS during the course of the work (cf. section botanical origin). Generally, training sample sets with a large number of authentic samples that are representative of various sources are the best way in order to achieve robust authenticity models with long-term stability (Downey, 1996; Gonzálvez & de la Guardia, 2013; Vermeulen *et al.*, 2015b). However, if those are not available (yet), sample selection strategies could also be used for the selection of representative training sample sets (always on the basis of the samples collected). For example, this could be realized by CADEX or DUPLEX algorithms, developed by Kennard and Stone (1969) and Snee (1977) respectively. Therefore, also in this work, the DUPLEX algorithm has been used for the selection of training and test sets for DDGS authentication models.

Besides validation of the models constructed for authentication questions, the goodness of the analytical data itself must be guaranteed. Although analytical method validation parameters, for example, estimation of repeatability, are only reported in a few authentication studies, the quality of the analytical data is imperative for reliable and feasible multivariate data analysis (Riedl, Esslinger and Fauhl-Hassek, 2015). Furthermore, quality control samples could be used to 'monitor' the multivariate data analysis itself, as has been discussed by Esslinger, Riedl and Fauhl-Hassek (2014). Thus, noise sources could be better addressed and biased influence of noise on the discrimination of different populations (cf. Norris, 2009) could possibly be checked and avoided. With regards to DDGS authentication in this dissertation, this was partly approached at PCA level through the repetitive instrumental and subsequent data analysis of selected matrix-like and matrix-similar samples (cf. following sections and publication A).

3.1.2 Botanical Origin

In this work, the botanical origin has been defined as the plant material used for DDGS production, which usually is corn or wheat. In the sample set used, DDGS derived mainly from corn and wheat, but single samples have been produced from grain mixtures (i.e., corn, wheat, barley) or rice.

IRMS

The analysis of DDGS bulk material by IRMS revealed clear differences in $\delta^{13}C$ values of DDGS produced from corn and wheat, as expected for C_3 and C_4 plants (publication B). It turned out that some samples, claimed to be produced exclusively from corn or wheat, showed significantly lower/higher $\delta^{13}C$ values than the respective group of samples with the same botanical origin. Although no reference method for detection of species composition (e.g., DNA analysis) was carried out, this indicated that these samples were not produced from pure corn or wheat and information provided by the producer/supplier on the botanical origin was considered as questionable. With regards to publication B, the results shown were based on a limited sample set. As a follow-up to this, an extended sample set of 196 DDGS produced from corn (n = 151), wheat (n = 29), mixed raw materials (n = 15) and rice (n = 1) has been evaluated in view of $\delta^{13}C$ values (Figure 4). These results confirmed the findings described in publication B and thus also the suitability of IRMS for authentication of the botanical origin.

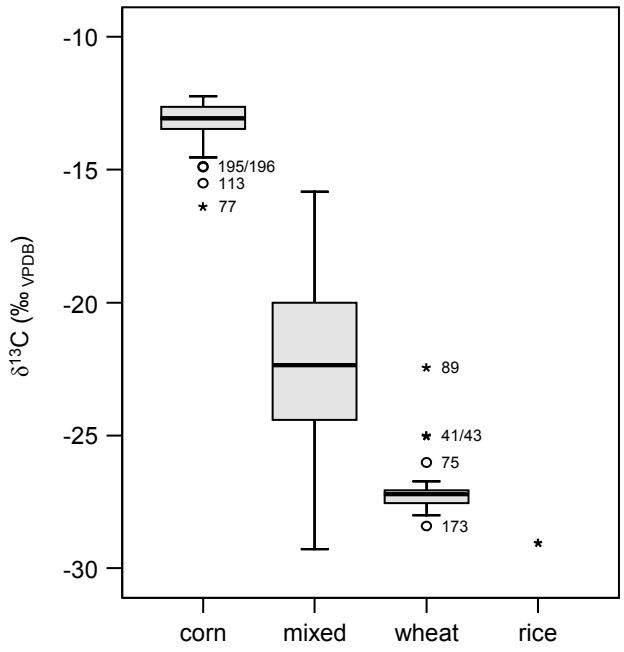

Figure 4: $\delta^{13}C$ values of DDGS bulk material produced from corn (n = 151), wheat (n = 29), mixed raw materials (n = 15) and rice (n = 1), according to the providers information. Sample numbers according to Appendix 4. Box length represents the data between first and third quartile (IQR); bold line shows the respective median value; whiskers represent minimum and maximum values which are not considered as outliers (within 1.5x IQR calculated from first/third quartile); outliers are indicated by circles (data between 1.5x and 3.0x IQR calculated from first/third quartile); extreme outliers are indicated by asterisks (data after 3.0x IQR calculated from first/third quartile), shown in the style of publication B.

FT-IR Spectroscopy

In contrast to the univariate approach undertaken after IRMS analysis, authentication of the botanical origin by FT-IR spectroscopy required multivariate data analysis. With publication A of this dissertation it was shown that in this way differentiation between corn DDGS and wheat DDGS is also achievable. Indeed, the most dominant factor after PCA of FT-IR spectra was the botanical origin of DDGS (publication A). Further, this was confirmed by FT-IR spectroscopy of an extended sample set of 194 DDGS produced from corn (n = 150), wheat (n = 28), mixed raw materials (n = 15) and rice (n = 1), and subsequent PCA (Figure 5).

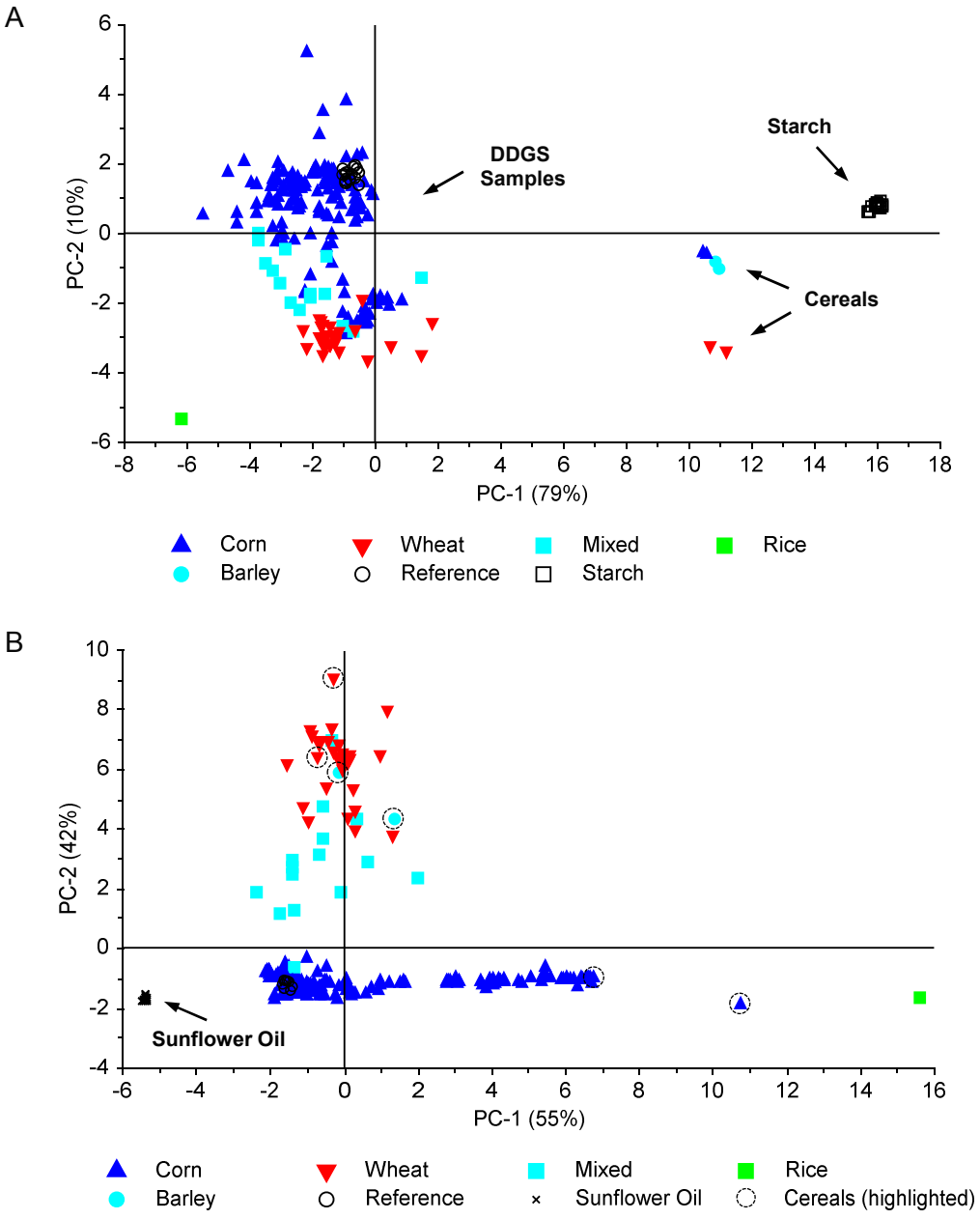

Figure 5: Authentication of botanical origin after PCA of FT-IR spectra from (A) solid DDGS and (B) oils extracted from DDGS. Results based on extended sample set (194 DDGS) produced from corn (n = 150), wheat (n = 28), mixed

raw materials (n = 15) and rice (n = 1); data analysis and style according to publication A, except for additional SNV pre-processing of oil spectra.

Moreover, it was shown that the botanical origin of DDGS could be verified by the use of supervised classification methods: PCA-LDA and SIMCA models have been established for differentiation of corn DDGS and wheat DDGS with good classification rates (publication A), also with regards to the follow-up sample set (Table 3). In view of the classification approaches by PCA-LDA and SIMCA, the concept of model validation was slightly modified for the follow-up sample set: DUPLEX algorithm (Snee, 1977) was used for splitting sample sets of corn DDGS (n = 150) and wheat DDGS (n = 28) into training sets for model calculation (100 corn DDGS; 19 wheat DDGS) and test sets for model validation (50 corn DDGS; 9 wheat DDGS). Data splitting was realized using a script (provided by courtesy of Dr. J.A. Fernández Pierna, CRA-W Gembloux, Belgium) in Matlab version 7.11.0584 [R2010b] (The Mathworks Inc., Natick, MA, USA), but data analysis and construction procedures for classification models were carried out in the same way as described in publication A.

Table 3: Classification results of DDGS samples according to the botanical origin. Results for extended sample set of corn DDGS (n = 150) and wheat DDGS (n = 28), either based on 100% prediction procedure or DUPLEX validation procedure. #PCs represents the number of principal components selected, for SIMCA first number represents the class model 'corn', second number the class model 'wheat'.

	PCA-LDA			**SIMCA**					
	# PCs	classification		# PCs	α	classification			
		correct	incorrect			correct	incorrect	outlier	ambiguous
SELF-PREDICTION									
solids	7	100 %	0 %	5;3	0.10	80 %	0 %	4 %	16 %
oils	2	100 %	0 %	1;2	0.01	98 %	0 %	2 %	0 %
TRAINING SET									
solids	5	100 %	0 %	5;4	0.10	86 %	0 %	3 %	11 %
oils	2	100 %	0 %	1;2	0.01	97 %	0 %	3 %	0 %
TEST SET									
solids	5	93 %	7 %	5;4	0.10	80 %	0 %	8 %	12 %
oils	2	100 %	0 %	1;2	0.01	95 %	0 %	5 %	0 %

Generally, it can be assumed that the models constructed for the botanical origin are robust and results are likely to remain steady if sample sets will be further extended, because (1) both original sample set (publication A) and extended sample set featured similar classification rates, (2) number of latent variables used for the models remained comparable for the extended sample set, and (3) both model validation strategies, random splitting (publication A) and DUPLEX splitting worked well. In conclusion, classification results confirmed the suitability of FT-IR spectroscopy, combined with multivariate statistics, for verification of the botanical origin of DDGS.

However, the models developed up to now were considering primarily the two raw materials mainly used for DDGS production, i.e., corn and wheat. The tentative separation of DDGS

produced from mixed raw materials on the PCA level (publication A and Figure 5) indicated that, possibly, classification models could be applied for this question as well. Although this approach has not been carried out due to the small number of samples for mixed DDGS, it could be an interesting option for DDGS suppliers in the future. Especially the use of SIMCA models and the construction of models that predict the percentage of corn/wheat/barley that was used for DDGS production could reveal valuable information for animal feeding and could initiate the analysis of more specific parameters.

Besides the plant species, which has been defined as the botanical origin in this work, also different plant varieties or cultivars could be subject to the statistical models developed. Similar to the results of Bertrand *et al.* (1985) on the feasibility of NIRS combined with multivariate data analysis for wheat variety identification, the grouping of DDGS according to the botanical origin after FT-IR spectroscopy (publication A) could, for example, feature further sub-groups of DDGS derived from single varieties. Thus, future work on authentication of DDGS should include DDGS produced from different 'sub-types' of raw materials, provided that the respective meta-data can be obtained.

Techniques described in Literature

Authentication of DDGS with regards to the raw material used for production has also been realized by other analytical techniques. As part of the EU research project Quality and Safety of Feeds and Food for Europe (QSAFFE) several analytical techniques, besides those employed in this thesis, were applied to authentication of DDGS (Nietner *et al.*, 2011; QSAFFE, 2014; www.qsaffe.eu). In this context, DDGS samples were collected for the whole QSAFFE project consortium, which led to the fact that several other studies (Vermeulen *et al.*, 2015a; Tres *et al.*, 2014; Zhou *et al.*, 2015; Tena *et al.*, 2015; Vermeulen *et al.*, 2015b) utilized the same DDGS samples as in this thesis.

Vermeulen *et al.* (2015a) also applied FT-IR spectroscopy to authentication of oils extracted from DDGS, resulting in 100 % correct classification of the botanical origin (i.e., corn and wheat) after PLS-DA and nearly 100 % correct classification based on three variables selected by the Fisher coefficient (1699, 1716 and 2923 cm^{-1}). Similar to the findings of this work (cf. Table 3), sensitivity slightly decreased to 96 % when the external validation set was predicted (Vermeulen *et al.*, 2015a), but here, according to the authors, probably because the calibration set did not include DDGS of the respective geographical origin (Austria). Although Vermeulen *et al.* (2015a) mentioned that compared with the ASE oil extraction method described in publication A, their in situ extraction method prevents triglycerides from hydrolysis to free fatty acids, they have reported strong correlations with the band around 1712 cm^{-1} (Vermeulen *et al.*, 2015a), which just represents the free fatty acid content. Therefore, a final statement whether the ASE extraction method boosted the free fatty acid content that is already present in native DDGS could only be made by future analysis of the total acid number (acid value).

Further studies on authentication of the botanical origin of DDGS have been based on fatty acid fingerprinting by gas chromatography mass spectrometry (GC-MS) and volatile fingerprinting by proton transfer reaction mass spectrometry (PTR-MS), combined to multivariate data analysis (Tres *et al.*, 2014). As well, several other analytical techniques showed to be capable tools for differentiation of DDGS produced from corn and wheat, using multivariate models and challenged by an independent test set of DDGS samples (Vermeulen *et al.*, 2015b). In the study of Vermeulen *et al.* (2015b), the botanical origin of DDGS samples was evaluated also by analysis of DNA sequences specific for corn and wheat, based on real-time polymerase chain reaction (PCR), and these results were compared to the results achieved by other analytical techniques (Vermeulen *et al.*, 2015b). Except for the application reported by Vermeulen *et al.* (2015b), PCR and DNA-based techniques are not known to be widely-used for the determination of the botanical origin of DDGS, although these techniques are capable approaches in food authentication as highlighted by Pereira, Amorim and van Asch (2013), for example. As reported for food and feed matrices in scientific literature, in most cases the DNA assays allow for quantitative or semi-quantitative statements on the botanical origin of cereal species or even of varieties (Terzi *et al.*, 2005; Casazza *et al.*, 2011; Pegels *et al.*, 2015). In addition to that, one may speculate that microscopic investigation of DDGS samples could offer further possibilities for differentiating the botanical origin, since corn and wheat show characteristic starch granules and tissue structures (van Raamsdonk, personal communication, September 2012). However, in DDGS production starch content is reduced to a minimum and starch granules are damaged in order to increase ethanol yield (Liu, 2012), which could reduce the amount of intact starch granules for determination of the botanical species.

Moreover, Pedersen *et al.* (2014) analyzed compositional data of DDGS produced from corn, wheat and mixed raw materials in a multivariate way. They demonstrated that PCA of ten common constituents predicted by NIRS (e.g., moisture, CP, NDF), ideally in combination with non-starch polysaccharides profiles (e.g., cellulose, xylose, arabinose) analyzed by gas chromatography, enabled differentiation of DDGS from these three feedstock origins. In this context, Pedersen *et al.* (2014) have drawn conclusions on the constituents which are the most responsible ones for the observed separations, and logically, more precise statements on the chemical differences can be made, compared to the strategy based on FT-IR spectroscopy in this thesis. However, no classification models or external validation have been carried out in their study (Pedersen *et al.*, 2014), which can be regarded as a strong point in this dissertation.

In addition, a group of researchers from the University of Saskatchewan reported on the suitability of ATR/FT-IR to detect effects of blend DDGS inclusion level to hulless barley-based feed (Zhang & Yu, 2012), and applied DRIFT (diffuse reflectance infrared Fourier transform spectroscopy) to investigate the spectral profile of carbohydrates and lipids with regards to co-products and the original feedstocks (Yu *et al.*, 2011; Yu, 2011). Multivariate data analysis of DRIFT spectra revealed structural differences between corn and corn DDGS, between wheat and wheat DDGS (Yu *et al.*, 2011; Yu, 2011), and, particularly with regards to the botanical origin of DDGS, structural differences between blend DDGS (wheat/corn) and wheat DDGS are described in the

work of Yu (2011). The selection of specific wavenumber regions for cluster analysis and PCA conducted in these studies, for example the consideration of amide I and II regions (Zhang & Yu, 2012), could be another option how to improve the FT-IR statistical models of this work in the future.

3.1.3 Geographical Origin

In this work, the geographical origin has been defined as the place of DDGS production, since (1) information on the place of cultivation (of cereal grains) was not available for all samples, and (2) information on the place of production is of great importance with regard to potential queries related to DDGS. First and foremost, DDGS were sampled from the major producing countries, and finally, the sample set comprised DDGS from Canada, China, India, USA, Austria, the Czech Republic, the Netherlands, and several other countries in the European Union.

IRMS

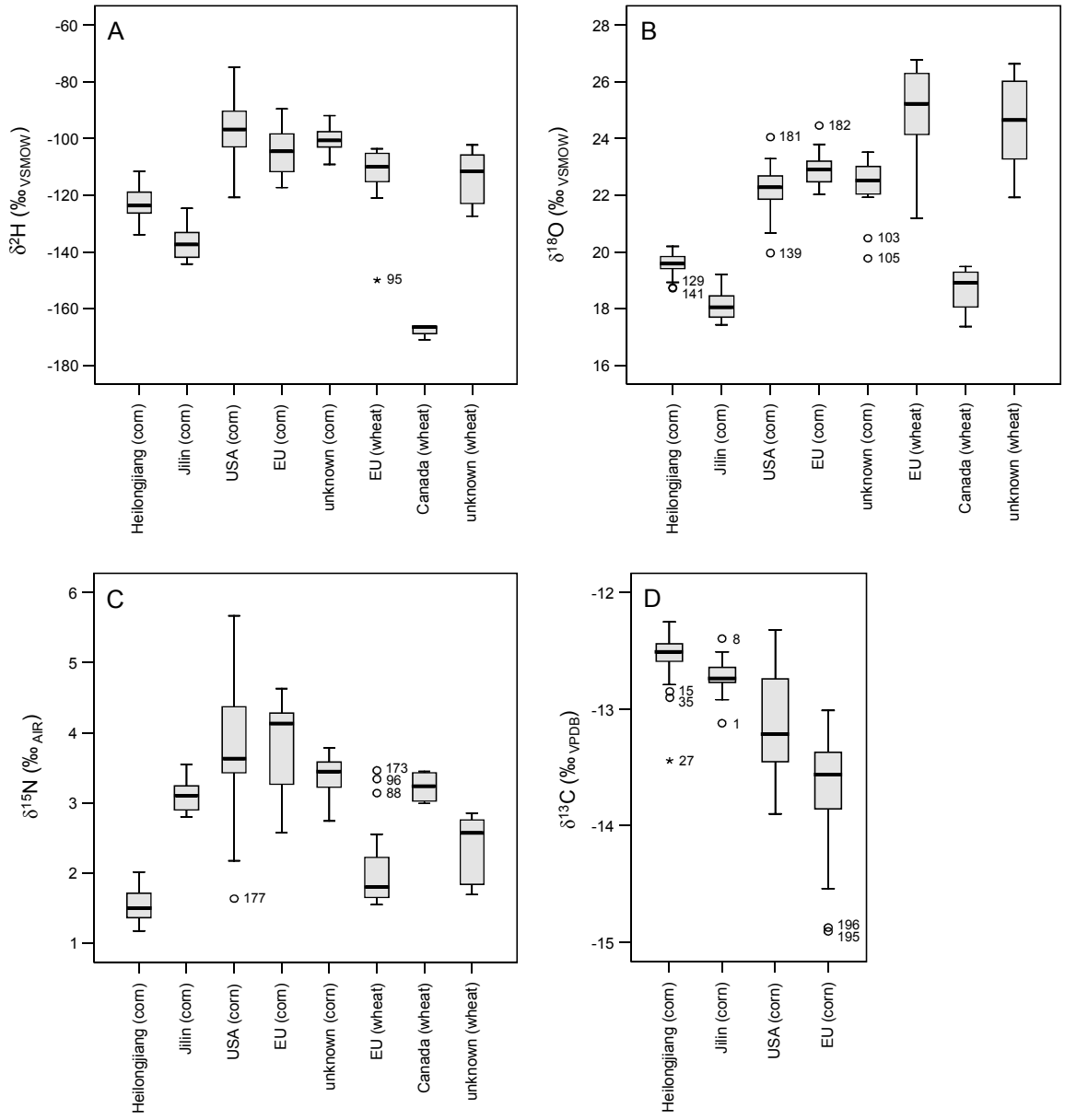

Figure 6: Boxplot of (A) δ^2H, (B) $\delta^{18}O$, (C) $\delta^{15}N$ of corn DDGS (n = 149) from Heilongjiang (n = 30), Jilin (n = 18), USA (n = 50), EU (n = 35) or unknown origin (n = 16) and wheat DDGS (n = 26) from EU (n = 16), Canada (n = 4) or unknown origin (n = 8); (D) $\delta^{13}C$ values only shown for corn DDGS from Heilongjiang (n = 30), Jilin (n = 18), USA

(n = 50) and EU (n = 35). Sample numbers according to Appendix 4. Box length represents the data between first and third quartile (IQR); bold line shows the respective median value; whiskers represent minimum and maximum values which are not considered as outliers (within 1.5x IQR calculated from first/third quartile); outliers are indicated by circles (data between 1.5x and 3.0x IQR calculated from first/third quartile); extreme outliers are indicated by asterisks (data after 3.0x IQR calculated from first/third quartile), shown in the style of publication B. $\delta^{2}H$ and $\delta^{18}O$ values have been provided by courtesy of Dr. S.A. Haughey, Dr. N. Ogle and Prof. Dr. C.T. Elliott (Queens University Belfast).

The analysis of stable isotope ratios in DDGS bulk material showed that DDGS from different locations could be differentiated mainly by the respective delta values of hydrogen ($^{2}H/^{1}H$) and oxygen ($^{18}O/^{16}O$), but also with regards to carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) (publication B).

Possible reasons for this differentiation have been discussed in publication B and include isotopic fractionation in the water cycle and during evapo-transpiration of plants, local agricultural practices and the use of production chemicals, or climatic conditions during plant growth. The fact that DDGS of different geographical origins could not be completely distinguished by consideration of only one element (e.g., $\delta^{2}H$) was confirmed by the analysis of an extended sample set of 196 DDGS produced from corn (n = 151), wheat (n = 29), mixed raw materials (n = 15) and rice (n = 1), with regards to $\delta^{2}H$, $\delta^{15}N$ and $\delta^{18}O$ (Figure 6). However, DDGS could not be differentiated according to the geographical origin by the analysis of stable sulfur isotopes ($^{34}S/^{32}S$), as reported in publication B, and thus, analysis of $\delta^{34}S$ was omitted for the extended sample set. Instead, $\delta^{13}C$ values indicated slight differences between corn DDGS from the two Chinese provinces, EU and USA in the extended sample set (Figure 6D), more clearly than it has been mentioned in publication B for the original sample set.

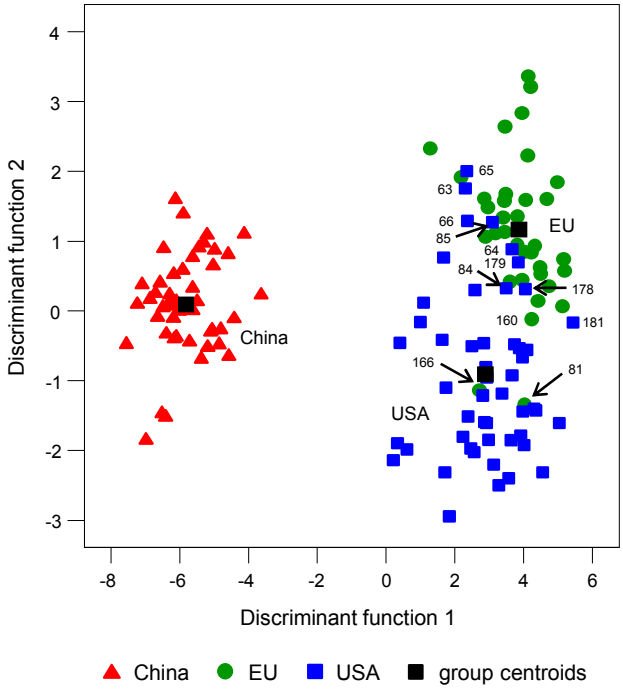

Figure 7: Authentication of geographical origin of DDGS samples derived from China (n = 48), EU (n = 35), and USA (n = 50) after canonical discriminant analysis of pre-processed isotope data, carried out according to the procedures described in publication B, but without DUPLEX data splitting. Sample numbers according to Appendix 4.

Differentiation of DDGS provenance, i.e., geographical origin, was improved when δ^2H and $\delta^{18}O$ were considered together (publication B), but was certainly superior when δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values were regarded simultaneously, enabled by multivariate data analysis. With publication B, it was shown that corn DDGS from three continents (China, EU, USA) could be differentiated after canonical discriminant analysis (CDA), which was confirmed by evaluation of the extended DDGS sample set (Figure 7). Although tentative separations of wheat DDGS collected in the EU have also been observed (publication B), the respective sample numbers were only appropriate, in terms of statistical analysis, for corn DDGS derived from China, EU and USA. The classification results after CDA with regards to self-prediction/cross-validation and prediction of test sets selected by DUPLEX splitting were satisfactory, for both the original sample set (publication B) and the extended sample set (Table 4).

Table 4: Classification results of DDGS samples from different geographical origins based on CDA of pre-processed isotope data. Results for extended sample set, data splitting according to publication B (DUPLEX procedure).

		PREDICTION			**PERFORMANCE**	
Procedure	Origin	China	EU	USA	sensitivity	specificity
SELF-PREDICTION (training set)	China	32	0	0	100 %	100 %
	EU	0	21	2	91 %	91 %
	USA	0	6	27	82 %	96 %
CROSS-VALIDATION (training set)	China	32	0	0	100 %	100 %
	EU	0	21	2	91 %	91 %
	USA	0	6	27	82 %	96 %
PREDICTION (test set)	China	16	0	0	100 %	100 %
	EU	0	11	1	92 %	94 %
	USA	0	2	15	88 %	96 %

Generally speaking, multivariate evaluations of stable isotope ratios in DDGS bulk material can be regarded as a capable tool for authenticating the geographical origin of corn DDGS. It can be assumed that the models constructed could be of valuable use in the future in order to verify the geographical origin of corn DDGS. This becomes clear when comparing the classification rates of both the original and the extended sample set: The classification rates of more than 80 % remained almost unchanged after the sample set was extended, which underlines the models` quality. Although it is apparent that the continuous model updating by further DDGS samples is necessary, especially in order to allow for altering of stable isotope composition (by climatic, seasonal or annual fluctuations or changes in production agents), the models and strategies developed in publication B could be applied for DDGS provenance authentication in the future. This may also become relevant for authenticating the geographical origin of wheat DDGS, as tentative discrimination was already observed for wheat DDGS from Canada and the EU (publication B).

Besides implementation of IRMS for DDGS provenance authentication, the present work demonstrated that multivariate analysis of δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values could be useful in identification of conspicuous DDGS batches. Indeed, after analysis of the extended DDGS sample

set, it turned out that two DDGS samples derived from a production plant in Poland showed similar discrimination values (after CDA) than most of the DDGS derived from USA (cf. Figure 7, samples #81 and #166). As such, this could be associated to characteristic multi-isotope patterns of the Polish samples; however, as the sample provider stated the geographical origin as 'mainly Poland', it could also be possible that the corn used for production of these DDGS batches originated (partly) from USA. A scenario like this is realistic, as additional purchases of corn originating from other continents (e.g., North America) by European bio-ethanol producers can occur in situations when local feedstocks are scarce (Schenkel, H., personal communication, 19 September 2013). Then, DDGS provenance could be falsely identified by the respective models, namely as the geographical origin of the raw material used for production, i.e., the corn from USA, and not as the place of production, i.e., the production plant in Poland. However, if DDGS batches are delivered from a certain producer and the respective analysis/statistical models indicate another origin than the producers' origin, specific proof of traceability documents could be initiated and the reason of deviation could be clarified.

In addition to the use of $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values for authentication of DDGS provenance, future work on the analysis of strontium isotopes ($^{87}Sr/^{86}Sr$) by inductively coupled plasma mass spectrometry (ICP-MS) or multi-element profiling could possibly improve the approaches undertaken in this work. For example, the results of Asfaha *et al.* (2011) on provenance authentication of European cereal samples, a matrix similar to DDGS, based on the combination of isotopic signatures (C, N, O, S, Sr) and contents of five elements (Na, K, Ca, Cu, Rb) indicate that such a procedure could be valuable. Moreover, future work on the development of isotope maps that combine isotope ratios with geo-climatic factors (e.g., geographical maps, annual mean temperature) could be of interest, since their potential has already been shown for food commodities (Kelly *et al.*, 2011).

FT-IR Spectroscopy

Similar to the procedure reported for authentication of the botanical origin (cf. 3.1.2), data acquired by FT-IR spectroscopy needed investigation by multivariate analysis in order to authenticate the geographical origin of DDGS. In view of the published data, essential findings were the separation of corn DDGS samples from China and USA, and the separation of wheat DDGS samples from Canada and Europe, after PCA had been conducted (publication A). Signals associated with the spectral absorption of free fatty acids, but also other spectral variables were found to contribute to the separation of DDGS according to the geographical origin (publication A). As a follow-up to this, an extended sample set of 194 DDGS produced from corn (n = 150), wheat (n = 28), mixed raw materials (n = 15) and rice (n = 1) has been evaluated (Figure 8). These results confirmed the findings described in publication A, and further, a separation of DDGS samples produced in the European Union was observed (Figure 9), as it has been indicated already in publication A. Moreover, it was shown that the geographical origin of DDGS could be verified by the use of supervised classification methods: PCA-LDA and SIMCA models have been established for differentiation of corn DDGS derived from China (n = 31) and USA (n = 23) with good

classification rates (publication A), and with regards to the follow-up sample set, even discrimination of corn DDGS from three geographical origins, i.e., China (n = 48), EU (n = 36) and USA (n = 50), was achieved by PCA-LDA (Table 5).

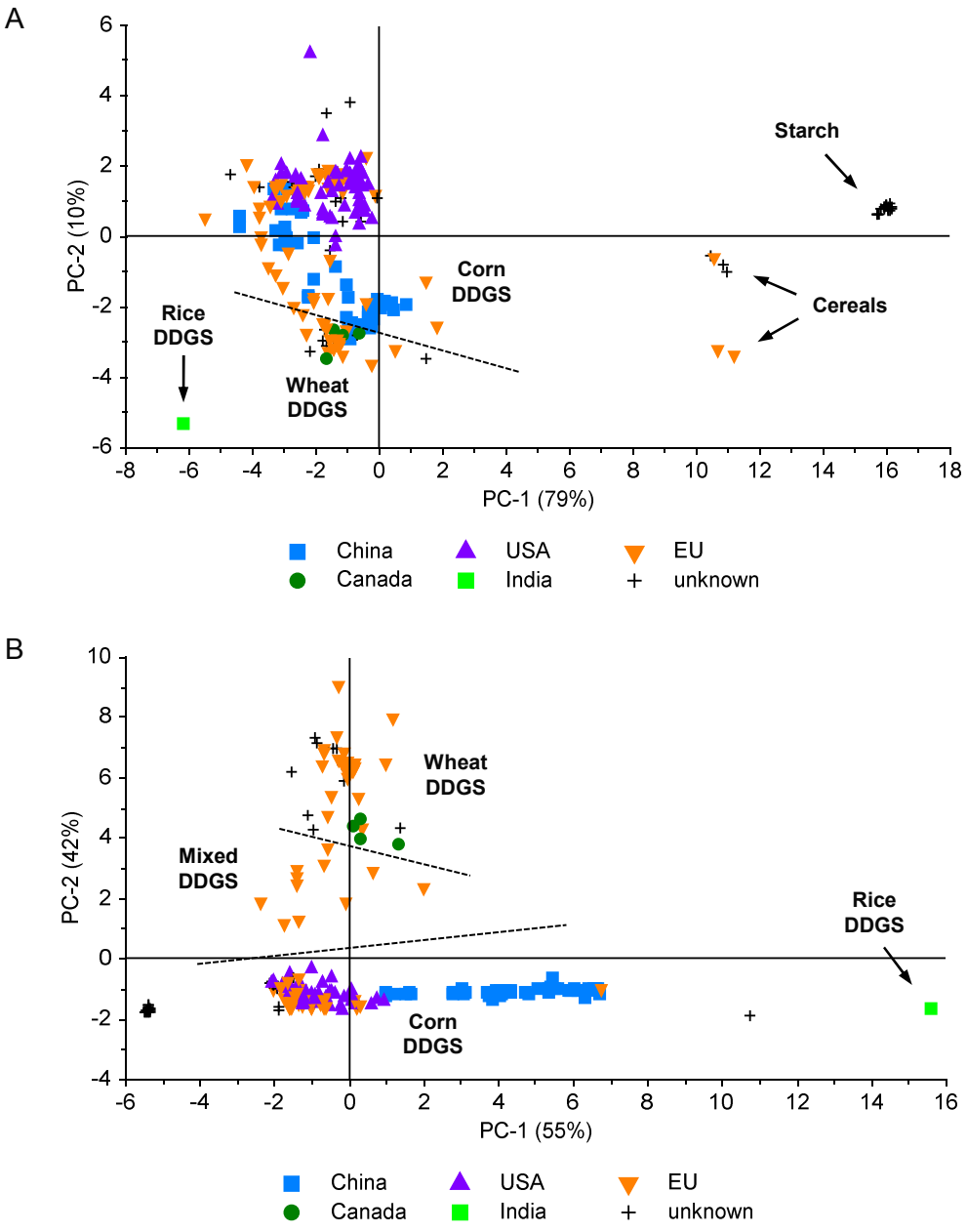

Figure 8: Authentication of geographical origin after PCA of FT-IR spectra from (A) solid DDGS and (B) oils extracted from DDGS. Results based on extended sample set (194 DDGS). Corn DDGS derived from China (n = 48), EU (n = 36), USA (n = 50) and unknown geographical origin (n = 16); wheat DDGS derived from Canada (n = 4), EU (n = 17) and unknown geographical origin (n = 7), mixed DDGS derived from EU (n = 14) and unknown geographical origin (n = 1), rice DDGS derived from India (n = 1); data analysis and style according to publication A, except for additional SNV pre-processing of oil spectra.

In view of the classification approaches by PCA-LDA and SIMCA, the concept of model validation was slightly modified for the follow-up sample set: DUPLEX algorithm (Snee, 1977) was used for splitting sample sets of corn DDGS from China, EU and USA into training sets for model calculation (China: n = 32; EU: n = 24; USA: n = 33) and test sets for model validation (China: n = 16; EU: n = 12; USA: n = 17). Data splitting was realized using a script (provided by courtesy of Dr. J.A. Fernández Pierna, CRA-W Gembloux, Belgium) in Matlab version 7.11.0584 [R2010b] (The Mathworks Inc., Natick, MA, USA), but data analysis and construction procedures for classification models were carried out in the same way as described in publication A.

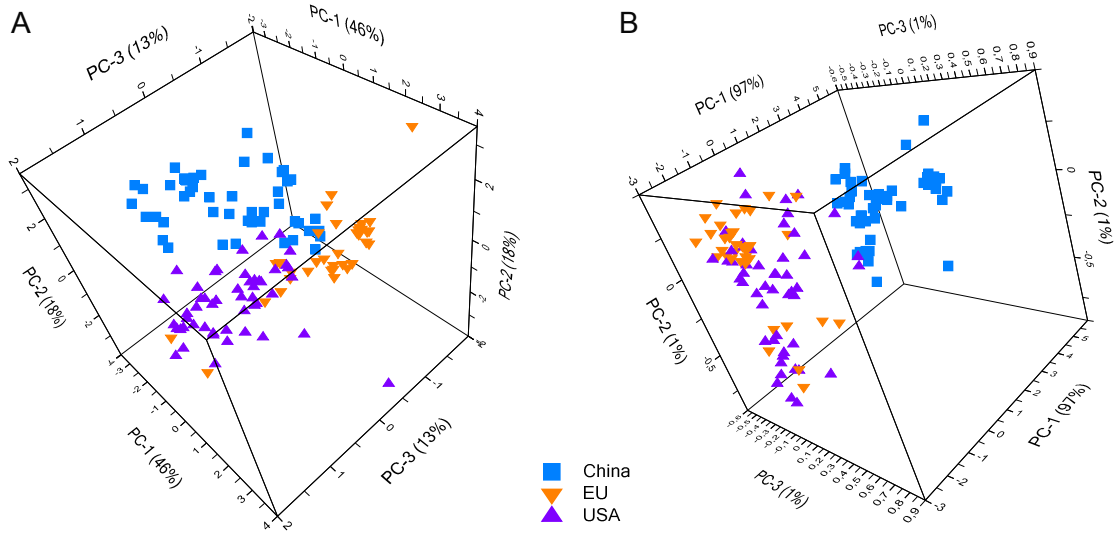

Figure 9: Authentication of geographical origin after PCA of FT-IR spectra from (A) solid DDGS and (B) oils extracted from DDGS. PCA performed only with corn DDGS samples (n = 134) derived from China (n = 48), EU (n = 36) and USA (n = 50); data analysis and style according to publication A, except for additional SNV pre-processing of oil spectra.

Classification results of the extended PCA-LDA models for authentication of the geographical origin were, generally, in agreement with the results of publication A. Only the percentage for correct classification of the DUPLEX test set was slightly lowered, compared to the findings of publication A, which indicates that for future applications, the respective models still need to be extended by further samples in order to reach consistent models. In contrast to PCA-LDA models, SIMCA models of the extended sample set were only of use for the differentiation of corn DDGS samples from China vs. EU/USA, since most of the DDGS samples from EU/USA were predicted ambiguously: In fact, for those, a class-membership to both EU and USA, was predicted (Table 5). However, the developed models for authentication of geographical origin, with regards to corn DDGS from China, EU and USA, could be of future use whenever DDGS provenance is questionable. In conclusion, classification results confirmed the suitability of FT-IR spectroscopy, combined with multivariate statistics, for verification of the geographical origin of DDGS.

Table 5: Classification results of DDGS samples according to the geographical origin. Results for extended sample set of corn DDGS derived from China (n = 48), EU (n = 36) and USA (n = 50), either based on 100 % prediction procedure or DUPLEX validation procedure. #PCs represents the number of principal components selected, for SIMCA first number represents the class model 'China', second number the class model 'EU', third number the class model 'USA'.

	PCA-LDA			SIMCA					
	# PCs	classification		# PCs	α	classification			
		correct	incorrect			correct	incorrect	outlier	ambiguous
SELF-PREDICTION									
solids	6	96 %	4 %	2;5;3	0.01	43 %	5 %	10 %	42 %
oils	6	93 %	7 %	1;3;2	0.05	39 %	0 %	1 %	60 %
TRAINING SET									
solids	6	96 %	4 %	2;4;3	0.05	58 %	0 %	1 %	41 %
oils	6	94 %	6 %	1;4;1	0.05	38 %	1 %	1 %	60 %
TEST SET									
solids	6	87 %	13 %	2;4;3	0.05	55 %	7 %	0 %	38 %
oils	6	89 %	11 %	1;4;1	0.05	40 %	5 %	2 %	53 %

For future use, strategies and models developed in this work could be applied and modified according to special demands of feed business operators involved in the DDGS supply chain. From a statistical point of view, operators should recognize that, so far, PCA-LDA models presented in this thesis (publication A and follow-up results) are limited to certain classes of geographical origin that have been modeled. This means that only DDGS of the origins included in model building can be predicted correctly, or in other words, the prediction principle of discrimination will result in a certain prediction for any DDGS sample, whether or not the sample belongs to one of the modeled classes. In this respect, SIMCA models are superior since they are not limited to the modeled classes (cf. chapter 3.1.1). Although they resulted in a high number of ambiguous predictions in the present work, such models could be of valuable use if larger DDGS sample sets will be processed in future authentication approaches.

Moreover, it is recommended to pre-select DDGS produced from defined raw materials (i.e., corn, wheat, mixtures, etc.) before authentication of DDGS provenance is carried out. In view of the published data (publication A and follow-up results), FT-IR spectroscopy and multivariate data analysis could enable the selection of DDGS with defined botanical origin (e.g., corn DDGS), and thereafter the specific authentication of geographical origin. By doing so, differentiation of DDGS according to the geographical origin would not interfere with variation in spectra due to different botanical origins. Hence, FT-IR spectroscopy and multivariate data analysis might be used to verify DDGS provenance also with regards to geographical origins not considered up to now, particularly if DDGS sample sets will be further extended.

Techniques described in Literature

Authentication of the geographical origin of DDGS has also been carried out by other analytical techniques, as a part of the EU research project QSAFFE (www.qsaffe.eu), and partly on the basis of the same DDGS samples that have been analyzed in this thesis.

The study published by Vermeulen *et al.* (2015a) highlights an alternative approach for DDGS provenance authentication, in particular in view of publication A, since both studies employed FT-IR spectroscopy in combination with chemometrics. In agreement with the results of Vermeulen *et al.* (2015a), it could be distinguished between DDGS from China and USA in the present work (publication A). Superior to the results reported by Vermeulen *et al.* (2015a), and with regards to the extended sample set used in this dissertation (cf. Table 5), classification of DDGS samples from EU against DDGS from China and USA was achieved. However, the study by Vermeulen *et al.* (2015a) was based on only 113 corn DDGS samples, which might be one reason for the improved classification ability for the question USA-EU in the present work (in total 134 corn DDGS samples).

Another study has been published by Zhou *et al.* (2015): Two independent laboratories applied near infrared reflectance spectroscopy and developed statistical models for the classification of geographical origin, based on 137 corn DDGS samples (Zhou *et al.*, 2015). On the basis of PLS-DA and OPLS-DA, they distinguished between DDGS from China (Jilin and Heilongjiang province), the USA and Europe with correct classification rates of 100 % (Zhou *et al.*, 2015). For PLS-DA models, Zhou *et al.* applied a variable selection algorithm, which led to improved sensitivity and specificity in terms of cross-validation. In the future, it would be interesting to evaluate whether such a procedure, if applied in an appropriate way, could result in better classification models for the geographical origin of DDGS based on FT-IR spectroscopy. Furthermore, transfer of the two-step discrimination strategy used by Zhou *et al.* (2015) for DDGS from (1) China (Heilongjiang + Jilin) and Europe/USA, (2a) Jilin province and Heilongjiang province, (2b) Europe and USA, could improve the classification models developed in this work (publication A and publication B). Classification tree methods, in general, could be a good way to further improve the models developed in this dissertation, since this methodology could explain and predict differences between similar samples (Gonzálvez and de la Guardia, 2013). Another study using NIR spectroscopy was conducted by Tena *et al.* (2015): They applied near infrared microscopy and PLS-DA to the identification of geographical origin of DDGS, and concluded that their results are in line with the results reported in publication A. Their concept of estimating the probability of wrong classification using t-statistics (Tena *et al.*, 2015) is interesting for future work in food and feed authentication, and also for the methods described in this thesis. Together with this concept, the target value of 5 % for false classifications defined by Tena *et al.* could, generally, be worth for assessment of model suitability in feed authentication studies.

Moreover, Tres *et al.* (2014) reported that fatty acid and volatile profiling, by GC-MS and PTR-MS respectively, showed promising results for classification of the geographical origin of DDGS. Corn DDGS from the USA and Chinese provinces (Heilongjiang and Jilin) could be discriminated using

PLS-DA (Tres *et al.*, 2014). However, much higher sample numbers have been analyzed in this dissertation, and therefore results of both studies are not fully comparable, although partly the same DDGS samples were included in the respective models. Such challenges were faced in a study by Vermeulen *et al.* (2015b), where different analytical strategies involved in the EU project QSAFFE (www.qsaffe.eu) were compared using the same sets of DDGS samples. The models conducted in this thesis were also part of the study by Vermeulen *et al.* (2015b). Based on the completely external validation, the approaches used in the present work showed very satisfactory results, also in comparison to different analytical strategies (Vermeulen *et al.*, 2015b). Therefore, the suitability of IRMS and FT-IR spectroscopy (together with multivariate data analysis) for authentication of DDGS geographical origin was substantiated.

In agreement with this work (cf. previous sections), Tres *et al.* (2014), Zhou *et al.* (2015) and Vermeulen *et al.* (2015b) mentioned that further DDGS samples should be collected in order to update the respective models for authentication of geographical origin. Also, DDGS of further geographical locations, for both corn DDGS and wheat DDGS, should be included in the analytical provenance authentication strategies developed, as it has been discussed for the models developed with IRMS and FT-IR data (cf. publication A + B, and upper sections) and by other authors (Tres *et al.*, 2014; Zhou *et al.*, 2015). Generally, robust classification models can be achieved if high sample numbers are included for modeling and all possible sources of variation are included (Capuano *et al.*, 2012; Gonzálvez and de la Guardia, 2013). Especially spectroscopic techniques require permanent updating of the respective spectral libraries (Downey, 1996; Downey, 1998), first and foremost, with regards to dynamic matrices such as most food and feedstuffs. Therefore, the authentication approaches for the geographical origin of DDGS developed in this work should be maintained and enhanced in an appropriate way in the future. The continuous collection and analysis of high sample numbers could then result in statistical models for the geographical origin that are even ready-to-use in routine control.

3.1.4 Method of Production

In this work, authentication of DDGS with regards to botanical and geographical origin was based on DDGS samples that derived from bioethanol production or alcoholic beverage production (i.e., whiskey, vodka). In principle, differentiation of DDGS from different geographical origins could be closely related to different production processes used in the plants, besides differences according to climatic, soil-related or isotopic occurrences. Therefore, great attention must be paid whether the corresponding separations are possibly due to production processes. However, almost no information on the detailed production processes was obtained from the DDGS suppliers and producers, since usually this was proprietary information. The only information available for a large part of the samples was whether the DDGS had been obtained as by-products from alcoholic beverage production or fuel ethanol production, and thus, the 'method of production' was defined accordingly.

With regards to FT-IR spectroscopy, it turned out that after PCA no satisfying separation according to the method of production (named 'production process' in publication A) could be achieved (publication A). However, the respective sample set mainly comprised DDGS produced in the fuel ethanol industry. As a follow-up to these results, an extended sample set of 159 DDGS derived from bioethanol production (n = 125) and alcoholic beverage production (n = 34) was evaluated with regards to the method of production. Applying PCA still revealed no significant separation when the whole sample set was considered (Figure 10), and with regards to corn DDGS, only indicative separation according to the method of production was achieved (Figure 11).

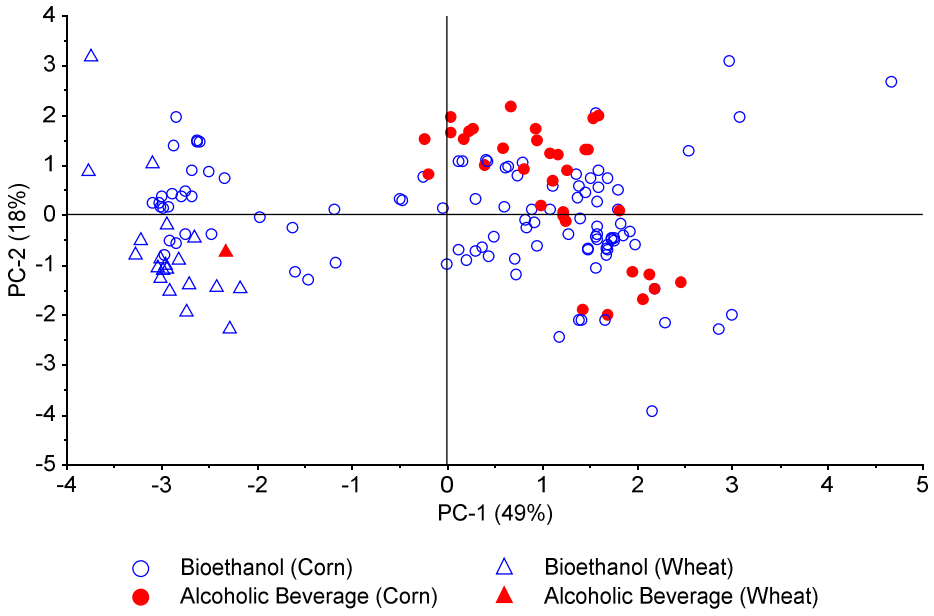

Figure 10: Authentication of DDGS produced in bioethanol production from corn (n = 105) or wheat (n = 20) and produced in alcoholic beverage production from corn (n = 33) or wheat (n = 1), applying SNV pre-processing and PCA on FT-IR spectra of solid DDGS.

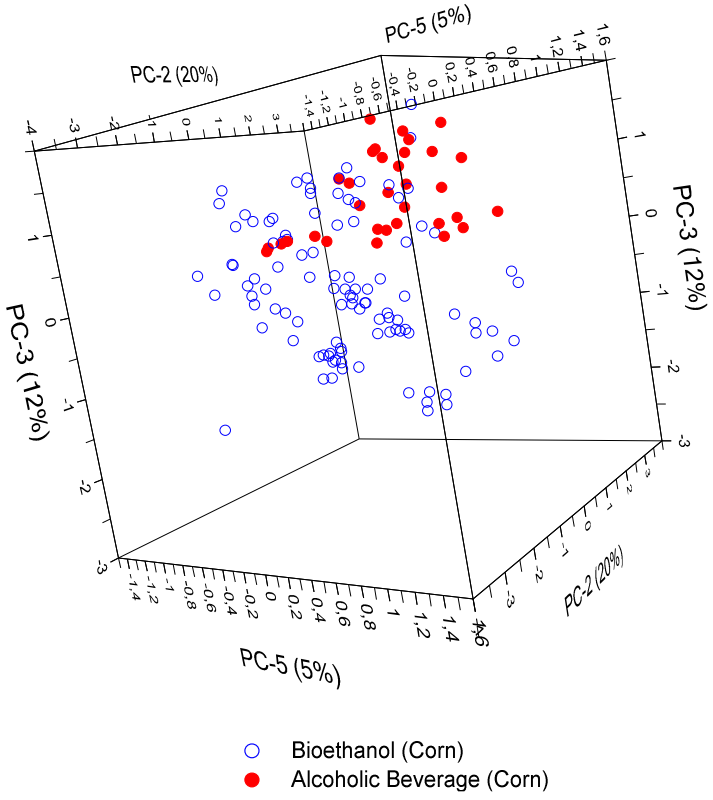

Figure 11: Authentication of corn DDGS produced in bioethanol production (n = 105) and alcoholic beverage production (n = 33), applying SNV pre-processing and PCA on FT-IR spectra of solid DDGS.

Discrimination of corn DDGS by PCA-LDA using up to 10 PCs also did not show satisfactory results (data not shown), and generally, the situation was comparable for both solids and oils. However, the sample set used for the calculations only comprised DDGS from US alcoholic beverage production, whereas the DDGS from bioethanol production originated from various countries and continents. Therefore, for future applications, it is essential to extend the DDGS sample sets accordingly, in order to evaluate whether FT-IR spectroscopy and multivariate data analysis might be used to verify the method of production.

With regards to IRMS, no new findings have been achieved on the basis of the extended sample set, which indicate the differentiation of DDGS according to the method of production. As it has been mentioned in publication B, the only fact in this context was the potential influence of production chemicals on $\delta^{34}S$ values (publication B).

Techniques described in Literature

Several studies reported evidence that DDGS could be authenticated with regard to the method of production. Tres *et al.* (2014) analyzed volatile fingerprints of 31 corn DDGS samples by PTR-MS and could clearly discriminate DDGS from ethanol beverage production and DDGS from biofuel ethanol based production. They reported that the volatile profiles could be tentatively assigned to several compounds, such as acetic acid and diacetyl, which are associated with fermentation by-products (Tres *et al.*, 2014). In contrast, fatty acid fingerprinting did not show consistent results,

according to the authors, possibly because the geographical origin had a higher influence on the fatty acid profile than the method of production (Tres *et al.*, 2014). One could speculate that this could also be a reason for the limited separation of DDGS derived from alcoholic beverage and fuel ethanol production in the present work (publication A), since FT-IR spectra are not showing separate signals for volatiles that are, with regards to DDGS, usually present in much lower concentrations than fatty acids. In addition, it remains unclear, why the volatile profile of DDGS is that different in relation to the method of production.

In another study, Pedersen *et al.* (2014) observed an individual grouping of corn DDGS samples from 5 different ethanol plants in the US. In their study, separation of DDGS was obtained after PCA on combined data of 10 common constituents (e.g., moisture, CP, NDF) and non-starch polysaccharides profiles (e.g., cellulose, xylose, arabinose), which indicated a consistent compositional profile for each ethanol plant (Pedersen *et al.*, 2014). However, their findings were not based on comparison of DDGS from alcoholic beverage and fuel ethanol production, and in fact they reported that "[…] the nutrient composition of the DDGS in part reflected the composition in the parent grain […]" (Pedersen *et al.*, 2014, p. 138). On the other hand, the compositional profile can also be influenced by the production technology: For example, starch content is influenced by the fermentation process, moisture content and Klason lignin are influenced by the drying process, according to Pedersen *et al.* (2014).

Furthermore, Vermeulen *et al.* (2015a) reported that DDGS samples from certain process origins (Heilongjiang province) could be differentiated due to the low fat content, which has also been indicated by gravimetric fat content analysis in publication A. The reason for low fat DDGS usually is the extraction of oil before (front-end) or after (back-end) the fermentation process, in order to use the oil fraction in food manufacturing or biodiesel production (Watkins, 2007). Thus, models for authentication of DDGS with regards to the method of production are obviously highly dependent on the meta-data provided, such as details on the single production steps. In this respect, information whether the raw material used for production was harvested in the area around the plant is also important and should ideally be considered for future authentication approaches of DDGS.

3.2 The Principal Capability of Non-Targeted Screening

Classification of Analytical Procedures

Chemical analysis of animal feed involves analytical procedures that are primarily used to determine the presence of compounds (i.e., qualitative analysis) or the content of compounds (i.e., quantitative analysis), as it has been described in the first sections of this thesis. The respective methods of analysis can be classified according to many different features, e.g., purpose of use, length, costs, practicability, degree of mechanization, or susceptibility of instruments. Moreover, different methods of analysis often lead to differing results, either because of varying limits of detection (qualitative aspects), or in terms of accuracy (quantitative aspects). Of course, each analytical procedure could be distinguished according to a magnitude of further features, advantages and disadvantages, but overall, the following characteristics need to be considered.

An important fact is that, basically, specific values and signals are evaluated in order to analyze specific compounds, but further data or signals due to the presence of 'unknown' analytes are usually not regarded in routines. Furthermore, most routine procedures are optimized for the detection of compounds of interest, e.g., the selective separation of compounds using specific columns and settings of chromatography, or the focus of mass spectrometric detection on specific fragmentation patterns and selected ion monitoring. Thus, the potential for detection of new compounds is limited.

However, approaches to the detection of 'unknowns' could be realized by utilization of analytical techniques in terms of a non-targeted way of analysis. Techniques that in principle can collect data apart from the selected analytes, e.g., FT-IR spectroscopy or mass spectrometry, could be also of use for the detection of 'unknowns', particularly if combined with multivariate statistical data analysis. This is the principle of non-targeted screening procedures, where anything that can be detected as abnormal compared to a pool of normal samples is searched for, in contrast to targeted screening procedures, where known substances are focused on (NIRPerformance, 2014).

From Authentication to Identification

In light of the melamine crises in 2007 and 2008 it quickly became clear that analytical techniques are strongly needed for the 'detection of the unknown', not only in the food sector but particularly in the animal feed sector. With regards to DDGS, strategies to reveal the 'unknowns' could be valuable, because of several facts, such as (1) great variability of DDGS composition, (2) supply of DDGS by different and thereby possibly unknown manufacturers, as well as (3) various production processes existing.

By using pattern recognition software, e.g., on spectroscopic data collected from DDGS, the matrix in question could be assessed by means of authentication approaches (cf. above sections of this thesis). Beyond the targeted authentication of single properties, e.g., botanical or geographical

origin of DDGS, multivariate analysis of analytical data and the reflected inspection of such data could be the starting point for detection of important signals or identification of new compounds.

For example, in a first step similarity and dissimilarity analysis could be carried out in order to detect abnormalities or adulterants (Honigs, 2009). Provided that a large number of DDGS samples from various origins and suppliers are included, databases could be the basis for detection of abnormalities. This could be achieved by simple statistical evaluation of the analytical data using PCA, cluster analysis, or approaches based on outlier detection, such as spectral residuals, Mahalanobis distance, or H statistics, as described in a similar way in research studies (Abbas *et al.*, 2013; Fernández Pierna *et al.*, 2015) and in several contributions to the USP Food Protein Workshop (USP, 2009). If suspicious samples or abnormalities have been noted, in a second step, one could look behind the analytical data related to the findings (e.g., initiated by high loadings of single variables/signals in PCA) in order to find the reason for the abnormality and to further elucidate the chemistry behind the unknown signal. Finally, this could end up in the detection of new compounds in the matrix DDGS, that might be relevant to product quality or safety, as it has been the case in this thesis (cf. following sections). Of course, the possibilities for each technique are dependent on the analytical data itself, for example, it is more likely that mass spectrometric data can be associated to specific structures and molecules than this would be the case for FT-IR spectroscopic data. However, also on the basis of spectroscopic data or combined datasets of single compounds (e.g., multi-element profiles), further analytical testing could be initiated in order to find the reason for an ascertained deviation.

For example, strategies for different matrices have been suggested by several research studies (Fernández-Ibánez *et al.*, 2009; Fernández-Ibánez *et al.*, 2010; Graham *et al.*, 2012; Abbas *et al.*, 2013; Baeten *et al.*, 2014) or have been reviewed (Cozzolino *et al.*, 2015, Ellis *et al.*, 2015) and their use has been successfully demonstrated for the animal feed industry (Fernández Pierna *et al.*, 2015; de Jong *et al.*, 2016). It is interesting to note that all of these studies applied spectroscopic techniques, which are generally regarded as a valuable tool to face adulterant detection (USP, 2009; NIRPerformance, 2014) and qualitative analysis of feed materials (Murray, 1996).

Also in this thesis, the analysis of non-targeted spectroscopic data in combination with inspection and further investigation allowed for two findings with regard to the feed material DDGS:

(1) Quantitative statement on the fat content using FT-IR spectroscopy (publication A), and

(2) Screening for sulfate using FT-IR spectroscopy (publication C).

In the following sections, the respective methods are highlighted and particularly the usefulness of multivariate data analysis in the detection of the respective analytes is explained. However, already here, it should be mentioned that the potential of this application was not fully investigated up to the final stage. Possibly, the concepts for non-targeted screening could deliver further findings for the analysis of DDGS that have not been regarded in this thesis so far.

3.2.1 Example A: Prediction of Crude Fat by FT-IR Spectroscopy

In publication A of this thesis a correlation between the crude fat content and FT-IR spectroscopic data of DDGS was reported (publication A). Although infrared spectroscopy (MIR and NIR) is generally known as a suitable tool for analysis of crude fat and fatty compounds (e.g., van de Voort *et al.*, 1994; Lefier, Grappin and Pochet, 1996; Mossoba *et al.*, 2004; Vermeulen *et al.*, 2010), the concept of non-targeted screening was the real approach for the reported findings in this work. After applying PCA and coloring the DDGS samples according to three fat content classes, it was indicated that FT-IR spectroscopy could be a valuable tool for the quantitative analysis of the crude fat content of DDGS (publication A, Figure 3). The idea to investigate the PCA scores plot with regards to the fat content, however, was initiated by inspection of the respective loading plots (publication A, Figure 4), since high loadings for the absorption bands associated to C-H stretching vibrations between 3000 and 2850 cm^{-1} have been found. Although it was not explicitly mentioned in publication A, high loadings on PC-1 for the absorption band associated to C=O ester stretching vibrations (1745 cm^{-1}) substantiated this indication as well (publication A, Figure 4). Therefore, a quantitative relation between crude fat content and the spectroscopic data was expected and the data was evaluated accordingly.

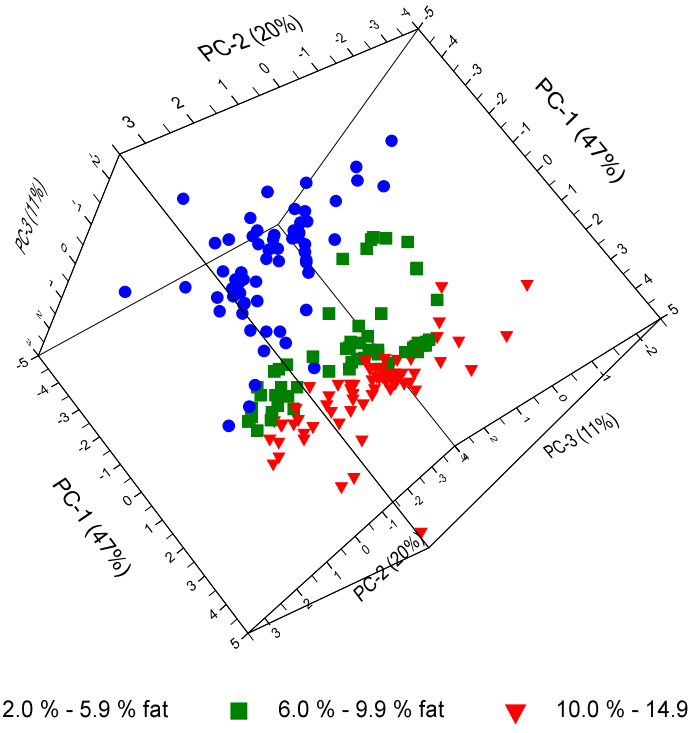

Figure 12: PCA of 193 DDGS samples from corn (n = 150), wheat (n = 28) and mixed origin (n = 15) colored according to different fat content classes. DDGS samples # 77, #87, #89 and #113 (cf. Appendix 4) were excluded for this PCA.

As a follow-up to this, an extended sample set of 193 DDGS was evaluated in the same manner, which supported the findings of publication A (Figure 12). Furthermore, a truly quantitative calibration (Figure 13) could be achieved by applying PLS (partial least squares) regression to the FT-IR spectroscopic data of 178 DDGS samples produced from corn (n = 150) and wheat (n = 28),

in a similar procedure as it was reported for PLS regression in publication C. But here, reference data for the crude fat content were obtained from gravimetric determination as described in publication A and listed in Appendix 4. Finally, PLS regression was based on 4 latent variables (explaining 96.4 % of the variance after calibration), a total number of 980 spectral variables and 10-fold cross-validation was applied in such a way that the data set was split into 10 parts instead of (but similar to) leave-one-out cross-validation (explaining 96.0 % of the variance after validation). With an error of 0.66 % (cf. Figure 13), crude fat content of DDGS could be predicted on the basis of the investigated sample set. In the future, it would be beneficial to include mixed DDGS that were produced from more than one botanical raw material, which might increase the error, but would probably make the calibration more robust.

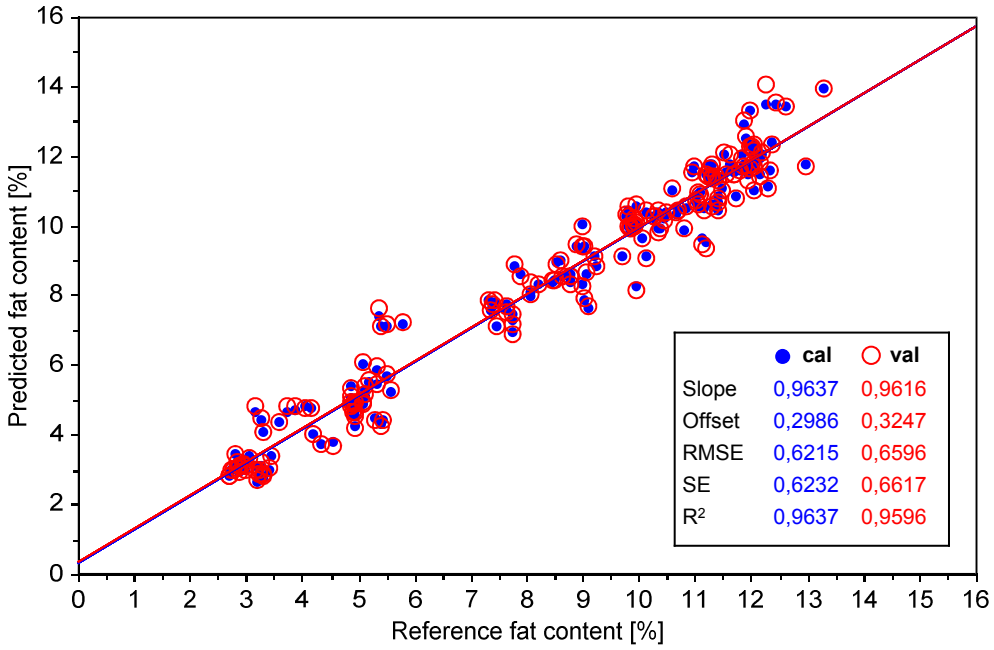

Figure 13: PLS regression of crude fat content (reference values from gravimetric determination) using FT-IR spectra of 178 solid DDGS samples; RMSE: root mean squared error; SE: standard error.

In conclusion, and according to the multivariate calibration shown, FT-IR spectroscopy can be used for the prediction of the fat content of DDGS. Although this approach was not fully validated under consideration of the criteria listed in Annex III of Regulation No 882/2004 and no certified reference material was included, it could still be used as a screening method in order to estimate the crude fat content. It could be of future use to feed business operators, since the demonstrated calibration assists the use of FT-IR spectroscopy for analysis of DDGS. To the best knowledge at the time this thesis was written, quantitative calibrations for prediction of crude fat using FT-IR spectroscopy combined with PLS regression have not been reported so far for DDGS. However, it should be mentioned that for different matrices several quantitative methods based on FT-IR spectroscopy and multivariate data analysis have already been implemented, as surveyed by Firestone & Mossoba (1997) and Fagan & O'Donnell (2008), for example.

3.2.2 Example B: Screening for Sulfate by FT-IR Spectroscopy

Another screening method for analysis of DDGS was reported in publication C: It was demonstrated that FT-IR spectroscopy could be used for the determination of sulfate levels in the matrix DDGS (publication C). In the first instance, this was proven by PLS regression on the basis of reference values from capillary electrophoretic analysis of sulfate levels in various DDGS samples, showing a solid calibration with only small errors (publication C). Application of the method could be of use for DDGS suppliers, since the sulfate and/or sulfur content is known to be a crucial factor (Shurson, Tilstra & Kerr, 2012; Schoonmaker & Beitz, 2012) and maximum tolerable dietary sulfur levels have been defined with regards to animal health (NRC, 2000). In view of the published data, FT-IR spectroscopy could be recommended as a prescreening method for the selection of DDGS batches with high sulfate levels (publication C). The respective reference methods, quantification of sulfate by gravimetric analysis ($BaSO_4$) or ICP-MS (AOAC 923.01 and ISO 27085:2009, respectively), could then be carried out in a second step. In conclusion, the main advantages of the FT-IR spectroscopic method developed (publication C) are rapid analysis and relatively low expenses.

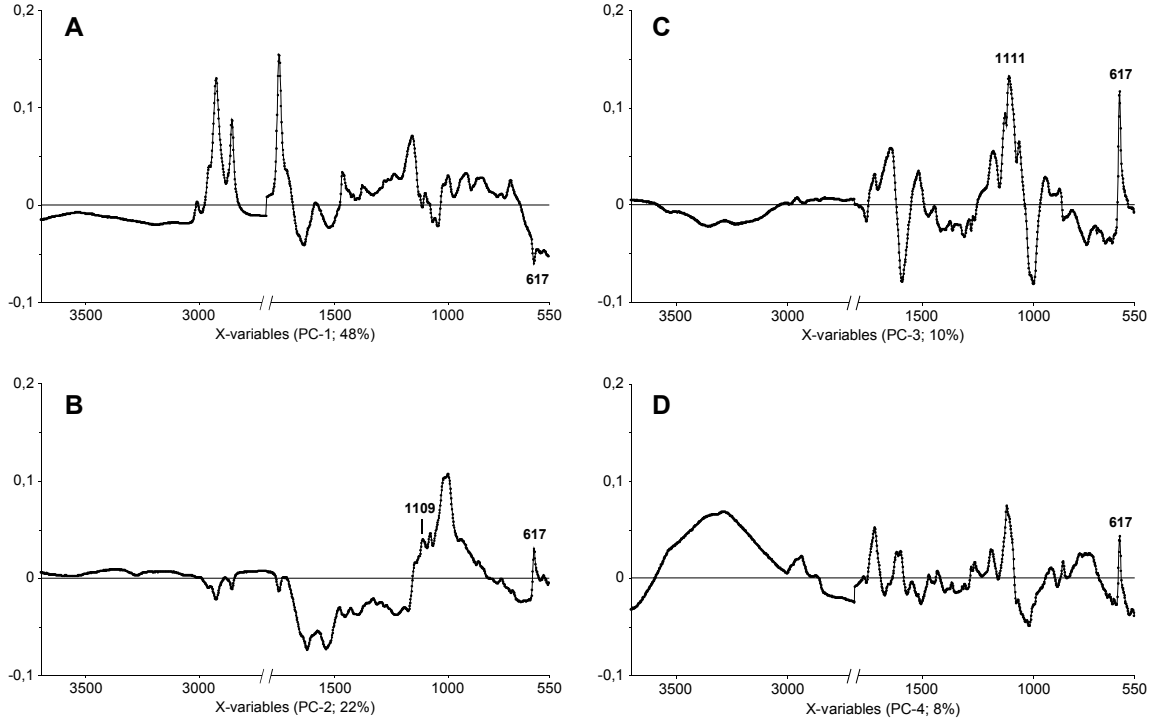

Figure 14: PCA loading plots of FT-IR spectra of solid DDGS samples. (A) and (B) taken from publication A, (C) and (D) show higher PCs.

The starting point for the reported findings (publication C), however, was not an educated guess that sulfates show specific signals in the FT-IR spectrum. By contrast, the search for the quantitative relation of sulfate and single absorption bands turned out during the evaluation of analytical data for authentication approaches, which are described in the previous sections of this thesis. First of all, it was noted that separation of DDGS samples after chemometric data analysis

(PCA) was not only dependent on spectral regions that are characteristic to C-H stretching vibrations or signals related to the fat content (publication A). In fact, PCA loading plots of the solid DDGS spectra (publication A, Figure 4) showed signals that were later on found to be related to the fundamental vibrations of the sulfate anion (publication C, Figure 1). For example, loading plots for PC-1 and PC-2 of solid DDGS samples showed the influence of wavenumbers at about 615 cm^{-1} (cf. publication A and Figure 14). This effect was even more obvious when loading plots for PC-3 and PC-4 were considered (Figure 14, data not shown in publication A). However, as the chemical structure responsible for these signals, i.e., the sulfate anion, was not identified yet, conclusions could not be drawn in publication A.

Nevertheless, the wavenumber regions indicated by PCA loading plots in publication A were subjected to further investigations. First, it turned out that the raw spectra of some DDGS samples also showed narrow absorption bands at about 615 cm^{-1}, which has simply been overlooked in the quantity of spectra (cf. publication A, Figure 1) or was misjudged as spectral noise in the lower wavenumber region. Second, FT-IR spectral libraries (Socrates, 2001) and scientific literature (e.g., Hunt *et al.*, 1950; Nyquist *et al.*, 1997; Nyquist & Kagel 1997; Nakamoto, 2009) were surveyed for compounds that could be causing absorption in the spectral ranges in question, and could possibly occur in the matrix DDGS. Third, several inorganic salts of anions that were supposed to be causing the explicit signals were prepared for FT-IR spectroscopy, in the same way as the spiked DDGS samples in publication C. In order to verify whether the bands result from seal abrasion of the ball mill cylinders, PTFE (Polytetrafluoroethylene) was included additionally. In summary, the resulting spectra indicated that most probably sulfates might be the reason for the absorption at about 615 cm^{-1} (Figure 15). To investigate this assumption, DDGS had been spiked with different inorganic salts and the research conducted in publication C has finally been initiated, also to obtain the relevant ‘meta data’ on the sulfate levels of DDGS samples.

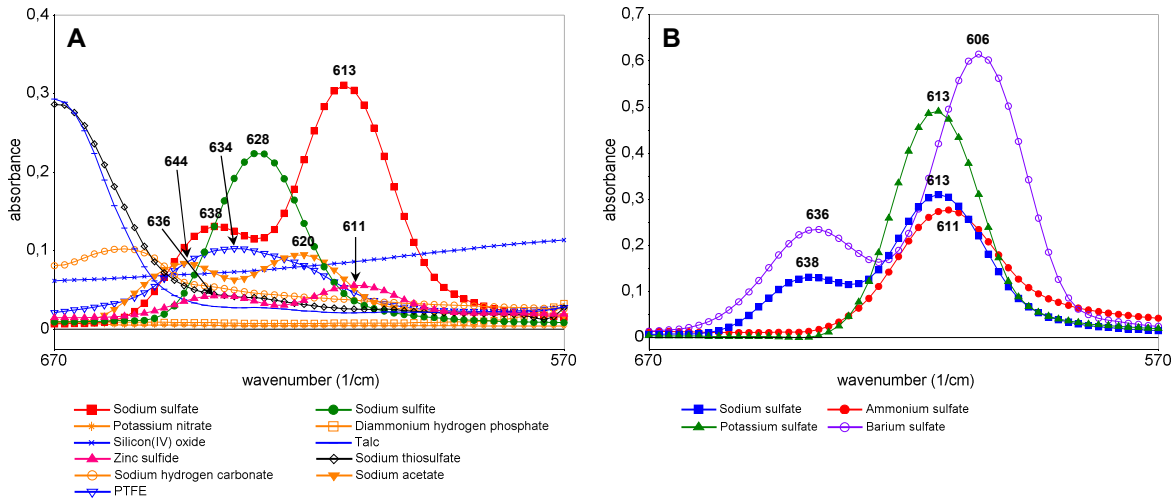

Figure 15: FT-IR absorbance spectra of inorganic compounds and PTFE from 670 to 570 cm^{-1} (SG smoothed, analysis similar to publication C). (A) Several inorganic salts, (B) Different inorganic sulfates.

As this example shows, applying statistical data analysis could uncover important information that is contained in spectroscopic data but may not be regarded in routine workflows yet. Of course, expert knowledge of analytical procedures and particular feed matrices is always the best way of analyzing compounds and detecting specific characteristics. Multivariate data analysis could, however, be used additionally to detect smallest but statistically significant differences in spectral data of feed samples, which could be overseen, for example, by visual observation. Also, qualitative or quantitative information on compounds that are not prevalent in DDGS or are not established yet could be found.

Indeed, multivariate data analysis has, in general, been described as a useful tool in the detection of abnormalities, adulteration or quality concerns (cf. above chapters). With this work, it could be shown that multivariate data analysis could broaden the capability of FT-IR spectroscopy in the analysis of DDGS. Thus, screening approaches have been established for the (semi-)quantitative analysis of DDGS (fat content and sulfate levels). In particular with regards to the potential for DDGS authentication (cf. previous chapters) and at the same time the applicability for analyzing the levels of different compounds, FT-IR spectroscopy could not only offer one way of analysis. In addition, the correlation of further analytical parameters obtained by classical analysis with FT-IR data could certainly result in a broader spectrum of analytical screening approaches (quantitative or semi-quantitative) using FT-IR spectroscopy in the future.

Moreover, further applications for FT-IR spectroscopy and the matrix DDGS could possibly be obtained, first and foremost, if the potential of FT-IR spectroscopy as screening tool is regarded and non-targeted approaches are carried out. Manufacturers have noticed the need for multivariate data analysis already years ago, and FT-IR instruments have been introduced, for example, WineScan™ and MilkoScan™ for the analysis of multiple parameters in wine and milk using multivariate regression models (FOSS, 2016a; FOSS, 2016b). Besides the analysis of homogenous liquid samples, FT-IR instruments have been optimized for viscous products, such as yogurt and cream, (FOSS, 2016c) or are applied for different matrices (e.g., material identification, detection of explosives or narcotics) in terms of handheld and portable ATR/FT-IR devices together with integrated software tools (Agilent, 2016a; Agilent 2016b; Agilent 2016c; Thermo, 2016a; Thermo 2016b; Thermo 2016c), which has recently also been reviewed for on-site food fraud analysis by Ellis *et al.* (2015). The software tools available today focus mainly on comparison of spectra against a library or on quantitative determination of single parameters using pre-calibrated methods (Thermo, 2016a; Agilent, 2016d). However, together with portable devices software tools could be implemented that similarly allow for detection of abnormalities or that point out adulteration, which might be a strong perspective also for the animal feed sector.

4 Summary

In this thesis Distillers Dried Grains and Solubles (DDGS) were analyzed by attenuated total reflection Fourier Transform Infrared spectroscopy (FT-IR spectroscopy) and Isotope Ratio Mass Spectrometry (IRMS). The aim was to develop authentication strategies for DDGS in terms of botanical origin, geographical origin, and method of production. For this purpose, more than 190 DDGS samples were collected in the major producing countries (Canada, China, USA, several countries in the European Union), derived from different cereals (mainly corn or wheat) in the bioethanol or the alcoholic beverage industry. In order to develop authentication strategies, the analytical data was evaluated by univariate and multivariate statistical analysis. Besides authentication, FT-IR spectroscopy was considered as screening technique for determination of crude fat and sulfates in the matrix DDGS, since quantitative correlation was indicated by multivariate analysis of the spectroscopic data in the course of the work.

In summary, FT-IR spectroscopy and IRMS showed to be suitable analytical techniques for authentication of DDGS with regards to their botanical and geographical origin. Suitable analytical strategies were demonstrated together with the use of statistical data analysis, based on the DDGS sample sets evaluated in this thesis. With regards to FT-IR spectroscopy, principal component analysis (PCA) of spectroscopic data allowed for differentiation of corn DDGS and wheat DDGS, which was clearly confirmed by PCA-LDA and SIMCA models with good validation results. Furthermore, corn DDGS from China, Europe and USA were distinguished when FT-IR spectroscopic data was evaluated by PCA and correspondent classification models based on PCA-LDA showed good results. With regards to IRMS, clear differences in $\delta^{13}C$ values of DDGS produced from wheat and corn were observed, as expected for C_3 and C_4 plants. By multivariate analysis of $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values using CDA, geographical origin of corn DDGS from China, Europe and USA was authenticated.

In contrast, no appropriate strategy for authentication of DDGS with regards to the method of production was found, based on FT-IR spectroscopy or IRMS, although positive results have been reported for different techniques in literature. It was concluded that it would be crucial to extend the sample set analyzed in this thesis in order to evaluate the final suitability, particularly in terms of FT-IR spectroscopy. Generally, this is also relevant if the analytical strategies and statistical models developed for authentication of botanical and geographical origin should be transferred into routine procedures in the future. Since most of the models presented in this thesis were limited to certain classes of botanical and geographical origin, it will be necessary to include DDGS of other origins (e.g., different provenances, botanical varieties) to meet with the demands of feed business operators involved in the DDGS supply chain. On the other hand, it should be stressed that the developed authentication approaches showed continuous validity throughout the analysis of different sample sets. Therefore, the strategies should be appropriate if continuously updated with further samples and could be of valuable use in verification of the botanical and geographical origin of DDGS.

Moreover, with the present thesis it was demonstrated that FT-IR spectroscopy could be used as screening technique for the quantitative analysis of crude fat and sulfates in DDGS. These findings were achieved by the use of multivariate analysis of spectroscopic data in a non-targeted manner. Relevant variables were detected in the PCA loading plots of DDGS spectra and were finally attributed to absorption bands of fatty compounds and sulfates. In addition, PLS regression was successfully implemented on the basis of reference values from gravimetric analysis (crude fat) and capillary electrophoretic analysis (sulfate). The resulting calibrations for estimation of crude fat and sulfate levels featured only small errors in comparison to the reference values underlying the PLS calculation. In conclusion, FT-IR spectroscopy could serve as screening technique for these compounds, but should be cross-validated with reference methods before taken into routine analysis. Application of FT-IR spectroscopy for screening sulfate levels could be particularly valuable, because ruminants are susceptible to high sulfur diets, and thus, selecting DDGS batches with low sulfate/sulfur levels is relevant.

In the future, the analytical procedures and authentication strategies developed in this thesis could be transferred to authentication of other animal feed ingredients. The approaches for authentication of botanical and geographical origin could be valuable with regards to different animal feed commodities and also with regards to different analytical techniques. In addition, features that were not regarded in this work, such as genetically modified ingredients, specific production technologies or factors associated with shelf-life, could possibly be verified if the developed strategies were applied accordingly in the future. This could become relevant for both DDGS and other animal feed commodities. Furthermore, the methods investigated in this thesis could be useful to determine whether DDGS are genuine or adulterated. Besides applications for detection of single compounds or compound classes, as it has been demonstrated for crude fat and sulfate, the analytical procedures used in this thesis could be evaluated also by concepts of data analysis that allow to screen for abnormalities. In the ideal scenario, potential adulterants but also further natural compounds could be identified by non-targeted screening using FT-IR spectroscopy and other analytical techniques. In conclusion, the benefit of multivariate data analysis in non-targeted approaches, as demonstrated in this work, could assist in the way from detection to prevention and could possibly broaden the field of chemical analysis in the animal feed sector.

5 Zusammenfassung

In dieser Arbeit wurden Distillers Dried Grains and Solubles (DDGS) mit Fourier Transformation Infrarotspektroskopie (FT-IR-Spektroskopie) im Modus der abgeschwächten Totalreflexion und mit Isotopenverhältnis-Massenspektrometrie (IRMS) analysiert. Ziel war es, Strategien zur Authentizitätsprüfung von DDGS hinsichtlich der botanischen Herkunft, der geographischen Herkunft und dem Produktionsverfahren zu entwickeln. Zu diesem Zweck wurden mehr als 190 DDGS-Proben aus den Haupterzeugerländern (Kanada, China, USA, mehrere Länder der Europäischen Union) gesammelt, die aus verschiedenen Getreidesorten (hauptsächlich Mais oder Weizen) bei der Herstellung von Bioethanol bzw. alkoholischen Getränken gewonnen wurden. Zur Entwicklung von Strategien zur Authentizitätsprüfung wurden die analytischen Daten univariat und multivariat ausgewertet. Zusätzlich zur Authentizitätsprüfung wurde die FT-IR-Spektroskopie auch als Screening-Verfahren zur Bestimmung des Gehaltes an Rohfett und Sulfat in der Matrix DDGS eingesetzt, da sich im Laufe der Arbeit quantitative Korrelationen für diese Parameter über die multivariate Datenanalyse der spektroskopischen Daten ergaben.

Zusammenfassend lässt sich festhalten, dass sowohl die FT-IR-Spektroskopie als auch die IRMS geeignete Analysenverfahren sind, um DDGS hinsichtlich der botanischen und geographischen Herkunft zu authentifizieren. Auf Grundlage des in dieser Dissertation untersuchten Probensatzes wurden geeignete Analysenstrategien unter Verwendung der multivariaten Datenanalyse aufgezeigt. Mit der FT-IR-Spektroskopie konnten Mais-DDGS und Weizen-DDGS über eine Hauptkomponentenanalyse (PCA) der spektroskopischen Daten unterschieden werden, was auch die guten Validierungsergebnisse der Klassifizierungsmodelle mittels PCA-LDA und SIMCA bestätigten. Über eine PCA der Daten aus der FT-IR-Spektroskopie konnten außerdem Mais-DDGS aus China, Europa und den USA unterschieden werden und die entsprechenden Klassifizierungsmodelle mittels PCA-LDA zeigten gute Ergebnisse. Hinsichtlich der IRMS wurden, wie für C_3 und C_4-Pflanzen erwartet, deutliche Unterschiede in den $\delta^{13}C$-Werten von Weizen-DDGS und Mais-DDGS festgestellt. Die multivariate Analyse der $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$ und $\delta^{18}O$-Werte mittels CDA ermöglichte die Authentifizierung der geographischen Herkunft von Mais-DDGS aus China, Europa und den USA.

Im Gegensatz dazu wurde zur Authentizitätsprüfung von DDGS hinsichtlich des Produktionsverfahrens keine geeignete Strategie basierend auf FT-IR-Spektroskopie oder IRMS gefunden, obwohl in der Literatur bereits positive Ergebnisse für andere Analysentechniken beschrieben wurden. Es wurde festgestellt, dass zur endgütigen Beurteilung der in dieser Arbeit untersuchte Probensatz erweitert werden müsste, vor allem in Hinblick auf die FT-IR-Spektroskopie. Dies gilt auch für eine zukünftige Übertragung der Analysenstrategien und der statistischen Modelle zur Authentifizierung der botanischen und geographischen Herkunft in die Routine. Da die meisten Modelle in dieser Arbeit auf bestimmte botanische bzw. geographische Herkünfte begrenzt waren, wird es nötig sein, DDGS anderer Herkünfte (z.B. verschiedene Herkunftsgebiete, botanische Arten) in die Modelle aufzunehmen um den Anforderungen von Futtermittelunternehmen in der DDGS-Lieferkette zu erfüllen. Allerdings sollte betont werden,

dass die entwickelten Strategien zur Authentizitätsprüfung während der Analyse verschiedener Probensätze stets Validität zeigten. Wenn eine kontinuierliche Erweiterung mit weiteren Proben erfolgt, sollten die Strategien daher geeignet sein die botanische und geographische Herkunft von DDGS zu verifizieren.

Zudem wurde mit der vorliegenden Arbeit gezeigt, dass die FT-IR-Spektroskopie als Screening-Verfahren zur quantitativen Analyse von Rohfett und Sulfat in DDGS eingesetzt werden kann. Diese Erkenntnisse wurden durch den Einsatz der multivariaten Analyse der spektroskopischen Daten in einem nicht-zielgerichteten Ansatz gewonnen. So wurden relevante Variablen über die loading plots der PCA von DDGS-Spektren erkannt und schließlich auf Absorptionsbanden von Fettverbindungen und Sulfaten zurückgeführt. Auf der Basis der Referenzwerte aus der Gravimetrie (Rohfett) und Kapillarelektrohorese (Sulfat) wurde darüber hinaus eine PLS-Regression erfolgreich eingesetzt. Die resultierenden Kalibrierungen zur Abschätzung der Gehalte an Rohfett und Sulfat wiesen nur kleine Fehler im Vergleich zu den Referenzwerten auf, die der Berechnung der PLS zugrunde lagen. Letztendlich könnte die FT-IR-Spektroskopie als Screening-Verfahren für diese Verbindungen genutzt werden, sollte allerdings vor dem Einsatz in der Routineanalytik mit Referenzverfahren kreuzvalidiert werden. Die Anwendung der FT-IR-Spektroskopie für das Screening von Sulfatgehalten könnte besonders nützlich sein, da Wiederkäuer empfindlich auf hohe Schwefelgaben reagieren und somit eine Vorauswahl von DDGS-Chargen mit niedrigen Sulfat-/Schwefel-Gehalten relevant ist.

Zukünftig könnten die Analysenverfahren und Strategien zur Authentizitätsprüfung, die in dieser Arbeit entwickelt wurden, auch auf die Authentizitätsprüfung anderer Futtermittel(-bestandteile) übertragen werden. Die Ansätze zur Authentifizierung der botanischen und geographischen Herkunft könnten für andere Futtermittel und andere Analysetechniken nützlich sein. Außerdem könnten auch Eigenschaften, die in dieser Arbeit nicht betrachtet wurden – wie z.B. genetisch modifizierte Bestandteile, bestimmte Herstellungsverfahren oder Faktoren die mit der Haltbarkeit verbunden sind – möglicherweise überprüft werden, wenn die entwickelten Strategien zukünftig entsprechend angewendet werden. Dies könnte sowohl für DDGS als auch für andere Futtermittelprodukte relevant werden. Weiterhin könnten die in dieser Arbeit untersuchten Methoden nützlich sein, um herauszufinden, ob DDGS wirklich echt sind oder verfälscht wurden. Neben der Anwendung für den Nachweis einzelner Stoffe oder Stoffklassen, wie es für die Parameter Rohfett und Sulfat gezeigt wurde, könnten die analytischen Verfahren dieser Arbeit genauso mittels Datenanalysenstrategien ausgewertet werden, die es ermöglichen Abweichungen von der Norm zu erkennen. Im Idealfall könnten somit Verbindungen, die zur Verfälschung verwendet wurden, aber auch weitere natürliche Verbindungen durch die nicht-zielgerichtete Analytik mittels FT-IR-Spektroskopie oder anderen Analysetechniken identifiziert werden. Schließlich könnten die Vorteile der multivariaten Datenanalyse für nicht-zielgereichtete Ansätze, wie in der vorliegenden Arbeit gezeigt wurde, auf dem Weg von der Detektion zur Prävention genutzt werden und möglicherweise das Gebiet der chemischen Analytik im Futtermittelbereich erweitern.

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A Appendices

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Authentication of the Botanical and Geographical Origin of Distillers Dried Grains and Solubles (DDGS) by FT-IR Spectroscopy

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Supporting Information

ABSTRACT: Distillers dried grains and solubles (DDGS) were investigated with attenuated total reflection FT-IR spectroscopy both directly in their solid state and as the isolated oils (fat fractions). The collected spectra were evaluated in a first step with principal component analysis (PCA) according to the botanical origin (corn, rice, wheat) and the geographical origin (Canada, China, European Union, India, United States) of the DDGS. In a second step, statistical models were constructed for the characterization of the botanical and geographical origin using linear discriminant analysis (LDA) and soft independent modeling of class analogy (SIMCA). For this purpose, the botanical origin was investigated more deeply for corn and wheat as the most important raw materials used for DDGS production. Also, the geographical origin was investigated exemplary for corn DDGS, derived from China and the United States. Models were validated by a randomized batchwise procedure and showed satisfactory classification rates, in most cases better than 80% correct classification.

KEYWORDS: *distillers dried grains and solubles, Fourier transform infrared spectroscopy, traceability, authenticity, geographical origin, botanical origin, feed material, multivariate data analysis*

INTRODUCTION

The complexity of food and feed production systems is steadily increasing, and in particular the trade in animal feed has become global. In addition, feed materials are obtained from new sources or are produced by new technologies. These facts may contribute to new and unforeseen risks for animal and human health. In a crisis situation due to contamination or adulteration of feed materials, the identification of origin can be essential in regard to feed safety as well as food safety for the following reasons: (a) A risk (e.g., reduced animal welfare) has been associated with a particular product but the reason is not identified. (b) A particular contamination was already identified but the analysis of the contaminant is difficult/impossible and/or expensive. In addition, the level of an assigned risk might be linked to certain areas of origin and a differentiated level of control might be appropriate, which requires that the origin must be traceable and verifiable, not only on the basis of documentation systems provided by the producer or distributor but also by analysis in the laboratory. Thus, "place of origin" will be increasingly linked to the quality of feed materials in a globalized market and will become more and more important. These changing situations underpin the necessity of analytical authentication systems particularly suited to "proof of origin".

Within work package 2 of the European Union (EU) research project Quality and Safety of Feeds and Food for Europe (QSAFFE), distillers dried grains and solubles (DDGS) were chosen to be analyzed—exemplary for feed ingredients—targeting the proof of origin. DDGS are a coproduct of the alcohol-distilling process obtained by drying solid residues of fermented grains (e.g., corn, wheat, barley) to which pot ale syrup or evaporated spent wash was added[1] and are used mainly in feeding of ruminants, poultry, pigs, and fish in aquaculture. As a result of the upgrowth of the fuel−ethanol industry, DDGS became a global commodity and play an even more important role in the feed market due to their high nutrient content (proteins, fat) in relation to the price.[2] Particularly, DDGS were chosen to be analyzed because of the increasing trade of DDGS among different countries and the fact that in the production of DDGS, especially from the fuel−ethanol industry, factors such as the yield of ethanol could possibly become more relevant than quality issues of DDGS.

In addition to these points, DDGS are a feed material showing a relatively high variability. As bioethanol is produced from different botanical raw materials, the quality of the resulting coproduct DDGS depends on the characteristic composition of the cereals. For example, DDGS produced from wheat are usually lower in fat than DDGS obtained from corn[3,4] because the harvested grains show different fat contents themselves. The quality and the nutrient profile of DDGS could even be associated with its geographical origin, as climate conditions for plant growth or soil parameters can be related to the geographical region. Also, variation in the nutrient content of DDGS exists within and among ethanol plants, even within plants using the same fermentation and processing technology.[5,6] In addition, the composition of DDGS can vary in relation to the method of production, too.[2,6] Production factors such as fermentation efficiency, usage of chemicals and enzymes, drying time and temperature, the ratio of distillers wet grains (DWG) to condensed distiller solubles (CDS) in the drying process, and fractionation of raw materials (e.g., removal of germ or pericarp) or fractionation of the coproducts (e.g.,

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extraction of oil or carotenoids) have an impact on the final feed material DDGS. Furthermore, DDGS are produced from many different companies and in different countries worldwide, and therefore production processes are manifold. In addition, ethanol production and production of DDGS might be regulated by national legislation.

However, the variability and particularly the ignorance of the different factors in the product chain could also lead to a situation in which DDGS from particular origins cause further inquiries. Thus, a scenario appears to be reasonable that feed materials from particular regions or countries could be banned for importation, for example. In the case of such an embargo, the proof of the geographical origin is known to be essential in terms of feed and food authenticity issues. In the present study, ATR/FT-IR spectroscopy was therefore used in combination with multivariate data analysis to construct models for the evaluation of the botanical and geographical origin of DDGS. Authentication and traceability approaches of agricultural and food products with this technique already have shown great potential,[7,8] and FT-IR spectroscopy as a fast and robust analytical technique was applied together with chemometrics, for example, in the authentication of olive oil, honey, or wine.[9−13] In principle, an identification of the botanical origin of DDGS could also be possible with other techniques such as microscopy, identifying particles of glumes, kernels, or whole grains of single botanical species. However, the main objective of the present study was to find a procedure that reveals also differences with regard to the geographical origin or other factors such as the production process—besides the botanical origin—of the DDGS samples. The goal was therefore to develop a strategy that could be applied for the proof of origin of feed materials like DDGS. Within work package 2 of the project QSAFFE, ATR/FT-IR spectroscopy was one of the analytical techniques selected for this purpose, and the results of this approach are reported in this study.

MATERIALS AND METHODS

DDGS Samples and Chemicals. Eighty-eight samples of DDGS were collected in the dry state in the context of the EU project QSAFFE. DDGS samples in this study are defined as feed materials according to the numbering of the EU feed catalogue:[1] either (a) number 1.12.10 (distillers' dried grains) or (b) number 1.12.11 (distillers' dried grains and solubles/distillers' dark grains). The DDGS samples used for the study derived from different botanical raw materials and were collected from different geographical origins as stated by the producer and summarized in Table 1. Detailed information on the botanical and geographical origin of the DDGS samples as well as on the production process is provided in the Supporting Information. All DDGS samples were stored at 4 °C in the dark until analysis.

Table 1. Botanical and Geographical Origin of the DDGS Samples

	Canada	China	EU	India	USA	unknown
corn		31	3		23	12
wheat	2		6			7
mixed			2			1
rice				1		

Petroleum benzine for fat extraction was of p.a. grade with boiling range of 40−60 °C (AnalR Normapur, VWR Prolabo, Fontenay-sous-bois, France); hexane and methanol for cleaning of the ATR diamond surface were of p.a. grade (AnalR Normapur, VWR Prolabo); glass fiber filters for accelerated solvent extraction (ASE) were 30 mm in diameter and obtained from Dionex (Thermo Fisher Group, Waltham, MA, USA); 2 mL glass vials and Teflon/silicone/Teflon septa were used from WICOM (Heppenheim, Germany).

Sample Preparation. The DDGS samples were investigated both directly in their solid state and as their isolated fat fractions (subsequently called oils). For the solid DDGS approach, samples were preground with a centrifugal mill (ZM 200, Retsch, Germany; mesh size = 0.5 mm) in a first step and subsequently homogenized in plastic containers (filling level approximately two-thirds) for 6 h using a drum hoop mixer (RRM 100, Engelsmann, Germany). Finally, 0.5 g of the homogenized preground sample was finely ground with a ball mill (MM 301, Retsch; 5 mL steel cylinders and 2 steel balls with 7 mm diameter, 30 Hz) for 4 min. The particle size of the finely ground DDGS was determined via microscopy with 150× magnification using a Continuμm IR microscope in visible light mode in combination with a Cassegrain lense and digital camera (Thermo Fisher Scientific). More than 99% of the particles ranged from 10 to 200 μm, and only single particles of a size up to 500 μm were detected.

The oils (fat fractions) were isolated from the preground (homogenized) DDGS samples with an ASE 300 accelerated solvent extractor (Dionex Corp., Thermo Fisher Group) using petroleum benzine as solvent. Extraction conditions were chosen according to their applicability under consideration of methods of previous studies dealing with oil extraction from DDGS.[14−17] The samples were extracted in 33 mL ASE cells, equipped with two glass fiber filters (one on the top and one on the bottom of the cell), at 80 °C and 150 bar without a preheat step, a 5 min heating period, three 5 min static extraction cycles, a 50% flush volume, and a 60 s purge time. ASE cells were disassembled and visually checked for leakage after extraction. The solvent was removed directly from the extracts in 250 mL ASE glass bottles (30 min at 300 mbar followed by 30 min at 50 mbar) with a Multivapor P-6 (Büchi, Flawil, Switzerland) at 40 °C, and the remaining oil was dried directly in the ASE glass bottles for 60 min at 103 ± 2 °C. The gravimetric fat content was determined (d = 0.1 mg, analytical balance Sartorius ME254S, Goettingen, Germany) after 60 min of storage in the desiccator, and oil samples were subsequently transferred in 2 mL glass vials and sealed with Teflon/silicone/Teflon septa for further storage at −20 °C in the dark. Oil samples (in the glass vials) were defrosted for 10 min at 50 °C, homogenized with an overhead shaker for 10 min, and centrifuged for 10 min (3112g) prior to FT-IR analyses.

FT-IR Spectroscopy. FT-IR spectra were collected with a Nicolet 6700 series spectroscope (Thermo Fisher Scientific) equipped with a Smart Performer Accessory (single ATR with diamond crystal), a KBr beamsplitter, and a deuterated triglycine sulfate (DTGS) detector. The optics were continuously flushed with dried nitrogen gas (purity 5.0). Spectra were recorded within the wavenumber range of 4000 and 525 cm^{-1} for each sample in triplicates with a spectral resolution of 4 cm^{-1} (data spacing of 1.928 cm^{-1}, Happ−Genzel apodization). The finely ground solid DDGS samples were placed on the diamond using a micrometric pressure device (Thermo Fisher Scientific) with a standardized pressing force (unit 4 of 20 on the scale of the micrometric pressure device). The oils were measured at 25 ± 0.1 °C, maintained with a DC-50-K10 temperature control unit (Thermo Fisher Scientific), by pipetting 1 μL of the oil on the ATR crystal. Prior to acquisition of each sample, a background spectrum (laboratory air) was collected, and each sample was subsequently measured against its particular background spectrum. The number of scans per spectrum (n = 64 for solid DDGS and n = 32 for oils) was selected with regard to a sufficient signal-to-noise ratio (of the main signals in comparison to the baseline) and an acceptable acquisition time. After each measurement, the diamond was cleaned thoroughly with hexane and methanol in a two-step procedure and dried with a soft tissue afterward. Background spectra were controlled visually for remaining solvent or sample residues. The spectrometer was checked regularly for spectral resolution, signal-to-noise-ratio, and wavenumber accuracy (with a polystyrene film) to ensure the stability of the instrument during the measurements.

Quality Assurance Samples. One additional sample of DDGS was used as quality control sample (described as reference in all

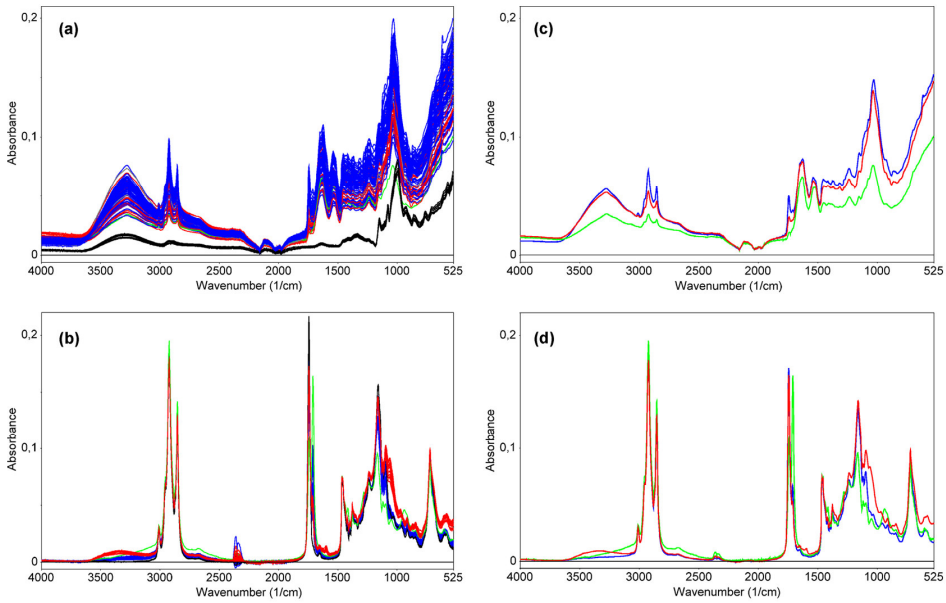

Figure 1. Raw spectra of (a) the solid DDGS samples and (b) the oil extracts of the DDGS samples; mean (averaged) spectra of (c) the solid DDGS samples and (d) the oil extracts of the DDGS samples: blue lines, corn DDGS; red lines, wheat DDGS; green lines, rice DDGS; black lines in (a), starch as quality assurance sample for solids; black lines in (b), sunflower oil as quality assurance sample for oils. All spectra were analyzed with FT-IR spectroscopy between 4000 and 525 cm^{-1}.

figures). As well, starch (starch, soluble extra pure; MerckKGaA, Darmstadt, Germany) was used as quality assurance sample for the solid DDGS measurements, and one sample of sunflower oil (blend of three different sunflower oil samples, produced in-house) was used as quality assurance sample for the oil DDGS measurements. Quality assurance samples were included in FT-IR spectroscopy for each measurement day and were subsequently evaluated in data analysis together with the DDGS samples. Also, six samples of cereals ground from the whole grains (two samples of corn, wheat, and barley, respectively) were included in the study to monitor the quality of the data analysis procedure.

Chemometric Data Analysis. Multivariate data analysis was carried out using The Unscrambler X 10.2 software (CAMO Software, Oslo, Norway). For all multivariate approaches, the triplicate spectra of each sample were averaged prior to data analysis. Wavenumber regions that did not provide relevant spectral information (baseline areas) or which were assigned to disturbing signals (e.g., CO_2 absorption) were cut off prior to the data analysis. Thus, spectra of the solid DDGS samples were evaluated in the regions 3700−2700 and 1800−550 cm^{-1}; spectra of the oils extracted from the DDGS were analyzed in the regions 3700−2600 and 1850−525 cm^{-1}.

Principal component analysis (PCA) was performed for explorative data analysis to recognize potential clustering (similarities and differences) of the DDGS samples. For this purpose raw spectra as well as spectra that had been preprocessed by standard normal variate (SNV) transformation, mean normalization, first or second Savitzky−Golay derivative, Savitzky−Golay smoothing, and also combinations thereof were evaluated. SNV transformation was finally applied to the spectra of the solid DDGS samples and describes a method that calculates the mean and standard deviation of a single spectrum (row-wise). Each absorbance value x_{ij} (at a wavenumber j) is corrected according to eq 1 with $\bar{x}_i$ being the mean of all absorbances x_{ij} in spectrum i.

$$x_{ij}(\mathrm{SNV}) = \frac{(x_{ij} - \bar{x}_i)}{\sqrt{\frac{\sum_{j=1}^{k}(x_{ij} - \bar{x}_i)^2}{k-1}}} \quad (1)$$

PCA was based on seven components, applying the nonlinear iterative partial least-squares algorithm (NIPALS). For detection of possible outliers, Hotelling's T^2 95% confidence ellipse was included in the respective score plots (enabled by The Unscrambler software) during data analysis. Hotelling's T^2 probability distribution is a generalized Student's t distribution, which was described first by Harold Hotelling[18] and is frequently used for outlier detection in multivariate statistics.

According to the grouping of the DDGS samples at the PCA level, classification approaches were performed to analyze the indicated geographical and botanical origin of the DDGS samples. Classification was performed with linear discriminant analysis based on PCA and the Mahalanobis distance (PCA-LDA) and with soft independent modeling of class analogy (SIMCA) based on a 5% significance level. Classification of DDGS samples was evaluated according to their botanical origin of corn (n = 69) or wheat (n = 15) and their geographical origin, exemplary conducted with corn DDGS from China (n = 31) and the United States (n = 23).

For both questions (botanical and geographical origin), models were constructed in the first instance with all data (100% of the samples were used as training set), and all samples were predicted in the generated model (100% prediction procedure). In a second step, the models were validated in a randomized batchwise procedure. For this purpose, the data sets of the botanical and geographical origin, respectively, were randomized (with the software The Unscrambler) in a training set, which consisted of two-thirds of the samples and a test set (internal validation set), which consisted of the remaining one-third of the samples, separately for each modeled class. This randomization was performed three times, and the modeling was repeated afterward. Therefore, validation results are based on average classification results concerning three different randomly selected test sample sets. Depending on the randomization of the data sets, PCA-LDA models were based on three to six principal components (PCs) for the solid DDGS and two or three PCs for the oils. Accordingly, for class modeling within SIMCA models, three to six PCs have been used for the solid DDGS and two to six PCs for the oils. To ensure a direct comparability between the solid DDGS and the oils, the randomized training and test sets consisted of the same samples (in each of the

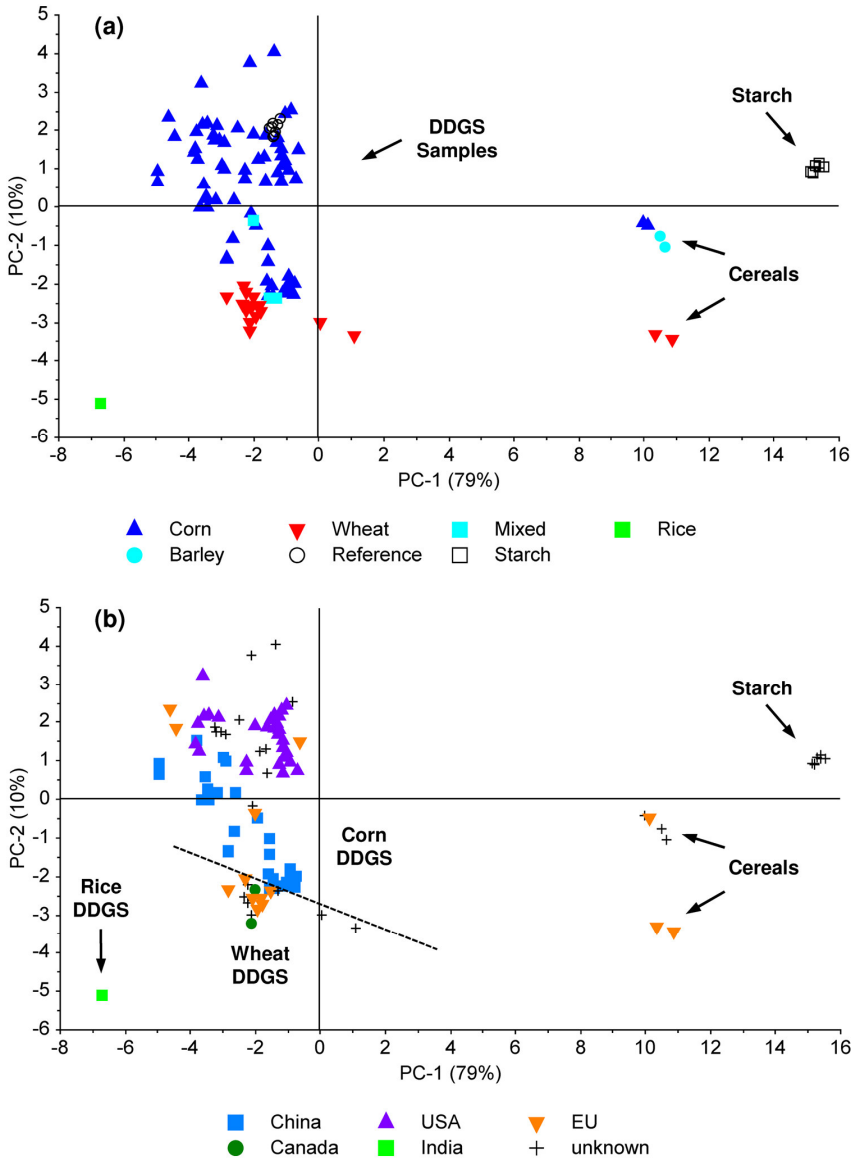

Figure 2. PCA scores plot of FT-IR spectra of solid DDGS samples colored according to (a) botanical origin and (b) geographical origin including quality control samples (starch samples and whole grain samples of corn, wheat, and barley). Standard normal variate (SNV) preprocessing of the solid DDGS spectra was performed. The broken line illustrates the two clusters of corn DDGS and wheat DDGS and does not represent statistical results. PCA was based on seven components. Explained variance for each component is given in parentheses.

three repetitions) for the evaluation of the respective solid DDGS and oils approach.

■ RESULTS AND DISCUSSION

ATR/FT-IR Spectra of Solid DDGS and Oils Extracted from DDGS. The mid-infrared spectra of solid DDGS samples and starch as the respective quality assurance sample are shown in Figure 1a, and the mid-infrared spectra of oils extracted from the DDGS and sunflower oil as the respective quality assurance sample are shown in Figure 1b. In addition, mean (averaged) spectra for corn DDGS, wheat DDGS, and rice DDGS are shown for the solid DDGS (Figure 1c) and the oils (Figure 1d).

For the solid DDGS a clear difference of the DDGS spectra in comparison to the starch spectra is observed (Figure 1a), but the visual differences in the original infrared spectra between the botanical species rice, wheat, and corn DDGS are quite small and obvious only after the spectra of each botanical species have been averaged (Figure 1c). Overall, the spectra of the solid DDGS are similar to previously published mid-infrared (ATR) spectra of DDGS,[19,20] except for the wavenumber regions between 2300 and 1900 cm^{-1} (disturbing signals), which were excluded for data analysis. Within the spectra of the oils extracted from the DDGS (Figure 1b) differences of the respective botanical species can already be

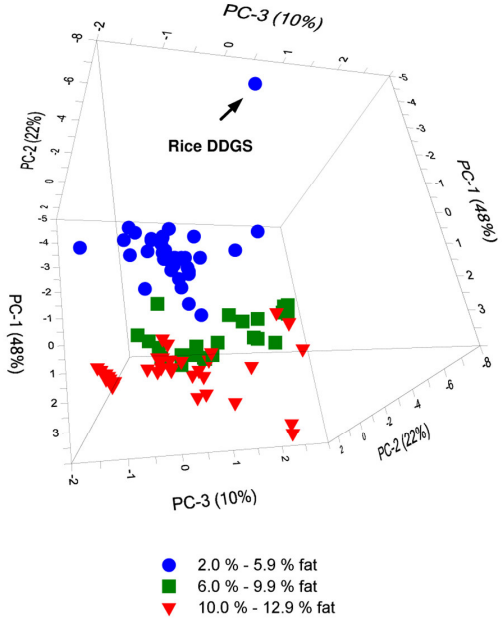

Figure 3. 3D-PCA scores plot of FT-IR spectra of solid DDGS samples colored according to different fat content classes. Explained variance for each component is given in parentheses.

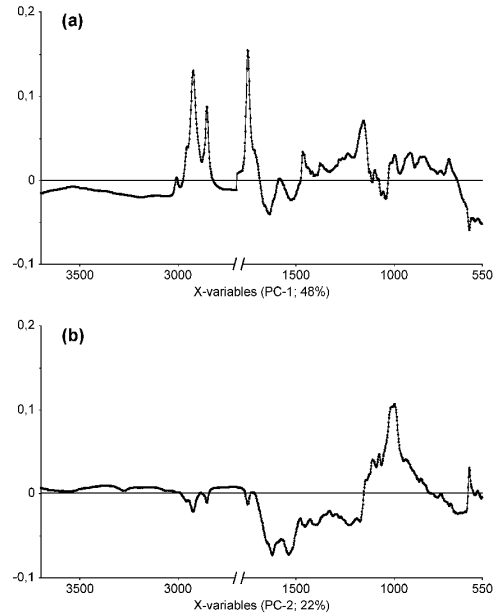

Figure 4. PCA loadings plot of FT-IR spectra of solid DDGS samples for (a) PC-1 and (b) PC-2 indicating the influence of single-wavenumber regions on the variance explained by the respective PC.

observed by visual inspection, especially in the region between 2000 and 800 cm^{-1}. Here, considerable differences for the stretching vibrations of the C═O carboxylic group of esters at 1743 cm^{-1} and the C═O carboxylic group of the free fatty acids at 1711 cm^{-1}, stated as 1746 and 1711 cm^{-1}, respectively, by Guillén and Cabo[21] for edible oils and fats, can be observed. The presence of free fatty acids in DDGS oil is a known fact, reported especially for corn DDGS produced in the dry-grind process in levels up to 22% free fatty acids in the oil.[22−24] Besides differences in the C═O bands, differences in the fingerprint region, especially in the wavenumber range of 1000−800 cm^{-1}, are noticeable at the level of visual spectrum inspection. Furthermore, the oils extracted from corn DDGS were consistently of orange color, the oils extracted from wheat DDGS of brown color, and the oils extracted from the rice DDGS of ocher color. Hence, a first indication for the determination of the botanical origin of the DDGS samples could be obtained by visual inspection of the oils extracted from the DDGS.

Principal Component Analysis. PCA was performed for explorative data analysis to recognize potential clustering (similarities and differences) of the DDGS samples. A removal of samples from the data set was omitted, as all samples that had been indicated as possible outliers by statistics (Hotelling's T^2 statistics) were found as plausible due to certain characteristics of the respective samples. The FT-IR spectra of the solid DDGS samples were preprocessed by SNV transformation to minimize effects due to light scattering or shifted spectra. The SNV preprocessed spectra of the solid DDGS samples showed superior PCA results compared to data analysis of the original spectra and also compared to differently preprocessed spectra (such as mean normalization, first and second derivative, or Savitzky−Golay smoothing). In contrast, preprocessing of the spectral data of the oils did not improve results after PCA, whereupon raw data were used here.

The PCA results (scores plot) of the solid DDGS samples are shown in Figure 2 with symbols colored according to their botanical origin (Figure 2a) and their geographical origin (Figure 2b), respectively. As expected, DDGS samples are separated from starch samples and whole grain samples of corn, wheat, and barley (cereals), additionally included in the analysis, at the first principal component (PC-1). Furthermore, the DDGS derived from corn and wheat cluster clearly at the second principal component (PC-2), in the same direction as observed for the cereal samples of corn and wheat (Figure 2a), indicating that information of the botanical origin can be associated with PC-2. Also, the rice DDGS sample is clearly separated from other DDGS samples, and the samples of mixed botanical origin are located between the wheat and corn DDGS samples, which emphasizes the separation along PC-2 according to the plant raw material used for production.

With regard to the geographical origin of the solid DDGS samples (Figure 2b), within the corn DDGS samples a separation of samples from China toward samples from United States and Europe is observed in the direction of PC-2. Samples from the EU belonging to the corn DDGS group are not clearly separated yet; possibly separation will be achieved if more samples from Europe will be analyzed in the future. Concerning the geographical origin of the wheat DDGS samples, a first separation of wheat DDGS samples from Europe and wheat DDGS samples from Canada is observed in the direction of PC-3 (data not shown). Furthermore, it appears that the separation of solid DDGS samples in the PCA can be correlated to the crude fat content of the samples (Figure 3). For demonstration purposes, three fat content classes (based on gravimetric results, cf. the Supporting Information) were defined, and samples clustered along PC-1 according to these classes (Figure 3). The corresponding loading plots of PC-1 and also PC-2 (Figure 4) do not indicate single-wavenumber

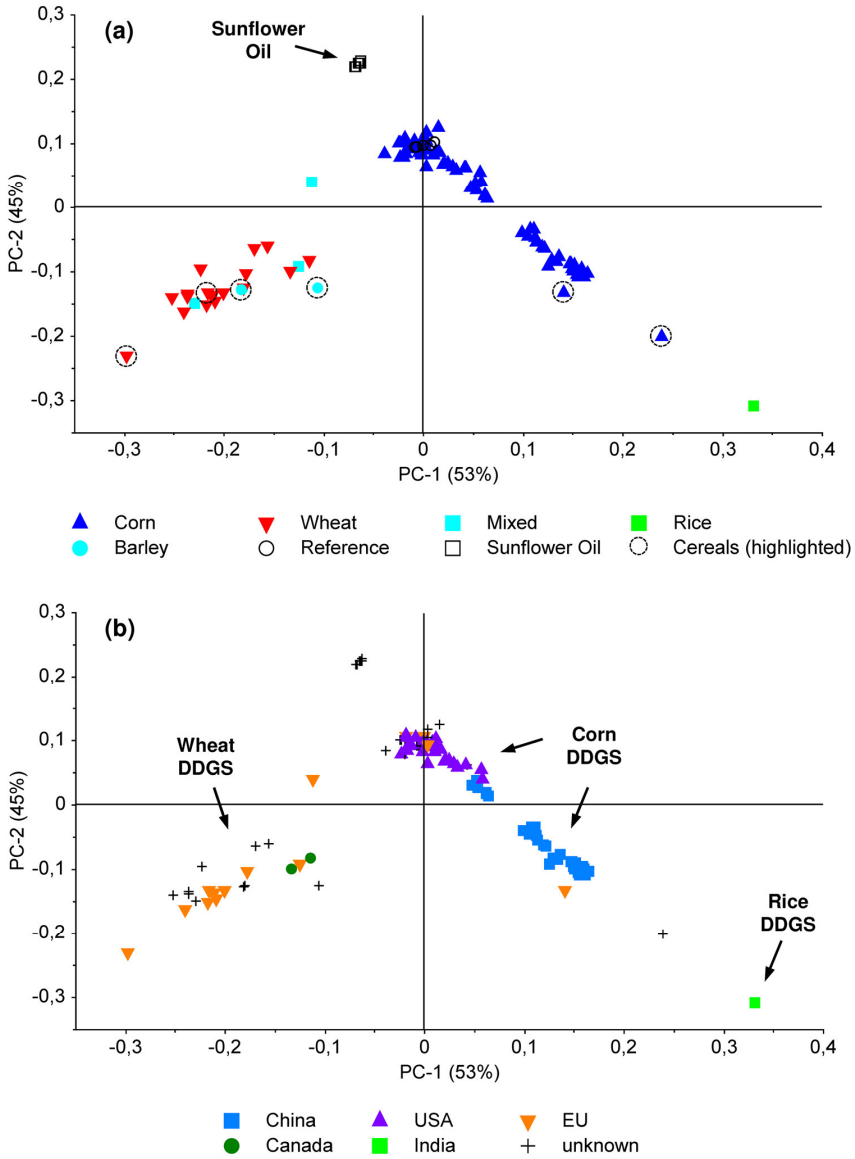

Figure 5. PCA scores plot of FT-IR spectra of oils extracted from DDGS samples colored according to (a) botanical origin and (b) geographical origin including quality control samples (sunflower oil samples and extracted oils of whole grain samples of corn, wheat, and barley are highlighted). PCA was based on seven components. Explained variance for each component is given in parentheses.

regions with outstanding influence on the explained variance of the respective PCs, except for the absorption bands of the CH stretching vibrations between 3000 and 2850 cm^{-1} (Figure 4), indicating the influence of the fat content. As corn and wheat grains have typically different fat contents, 3.2−4.3% for corn and 1.6−2.1% for wheat,[25] a separation of the respective botanical species of DDGS samples according to the fat content could be expected. However, the clear separation of the rice DDGS sample from the corn/wheat DDGS samples (Figure 3) cannot be explained by the fat content alone, as the rice DDGS sample consisted of 3.3% fat and several corn DDGS samples showed fat contents around 3.0% (data not shown). In fact, separation of DDGS samples after chemometric data analysis seems to be dependent on other spectral regions as well (Figure 4) but cannot be explained by specific signals.

The oils extracted from the DDGS samples (Figure 5) reveal a slightly different picture compared to the solid DDGS approach. Corn DDGS samples are clearly separated from wheat DDGS samples at PC-1 (Figure 5a). Again, the rice DDGS sample is well separated from the DDGS samples with different botanical origins, but the oils extracted from the whole grains (quality control samples) fall into the cluster of the DDGS samples. With regard to the geographical origin of the samples, again within the corn DDGS group, samples from China and from the United States are separated into two groups (Figure 5b). This separation becomes even more

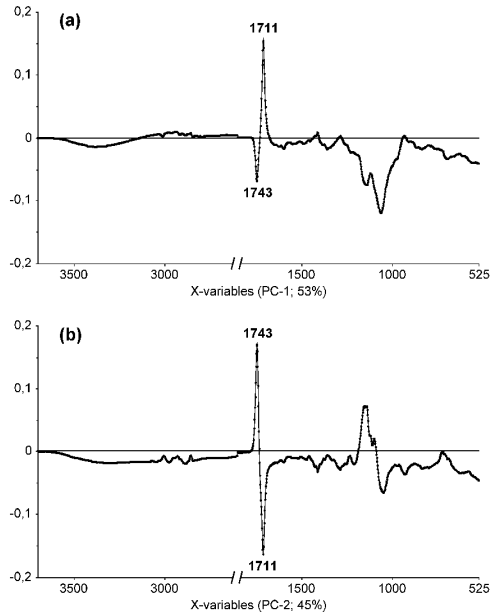

Figure 6. PCA loadings plot of FT-IR spectra of oils extracted from DDGS samples for (a) PC-1 and (b) PC-2 indicating the influence of single-wavenumber regions on the variance explained by the respective PC.

obvious when only these two groups are considered in a separate PCA model (data not shown). Within the corn group the three European corn DDGS samples appear close to the corn DDGS samples from the United States. Here, a separation was not achieved at the PCA level, possibly due to the low number of samples. In general, the separation in both PC-1 and PC-2 is correlated mainly to the absorption of the stretching vibrations of the C═O carboxylic group of esters at 1743 cm^{-1} and the C═O carboxylic group of the free fatty acids at 1711 cm^{-1}, as can be seen in the high loadings on PC-1 and PC-2, respectively (Figure 6). On the one hand, the content of free fatty acids—and therefore also the IR signal for free fatty acids at 1711 cm^{-1}—could depend on the storage of the DDGS samples and continuous hydrolysis of triglycerides by lipases or the analytical method chosen for extraction of the oil from the DDGS.[26] On the other hand, the content of free fatty acids in DDGS was reported to be higher after a dry-grind ethanol process compared to processes preventing germ breakage such as coarse wet-grinding ones.[24] According to Wang et al.,[24] the hydrolysis of the corn oil, resulting in free fatty acids, is presumably caused by the endogenous lipase or the exogenous enzymes secreted by yeast, or both. Therefore, it remains unclear whether the content of free fatty acids can be explained by one of the above-mentioned factors or if various reasons have to be taken into consideration. However, the separation of the oils in PC-1 also depends on spectral absorption in the fingerprinting region, as can be seen especially in the loadings for the wavenumber range of 1200−800 cm^{-1}. Here, characteristic absorption signals such as the asymmetric stretching vibrations of the CO−O−C group[27] can be observed, which influence the clustering of the oil samples accordingly. However, due to the overlapping signals (combination bands) in this region, absorptions at single wavenumbers cannot be assigned to single chemical compounds. In summary, it seems that the different content of free fatty acids is one reason for the separation of the oils, but also other chemical compounds appear to be important in this respect and should be taken into consideration in further research investigations.

PCA results were also evaluated with regard to the production process from which the DDGS samples originated (bioethanol production vs alcoholic beverage production). Neither for the solid DDGS samples nor for the oils could a separation according to the production process be achieved. In addition, data analysis revealed no significant impact of the production process with regard to the two questions of botanical or geographical origin.

Reference DDGS Sample for Monitoring of Stability. To monitor the stability of the approach over the time of the FT-IR measurements, one DDGS sample (the reference DDGS sample) was stored over the sampling period and sequentially measured several times. PCA reveals that the scores of this DDGS sample (black circles in Figures 2a and 5a) were very similar, indicating that the FT-IR measurements were fairly comparable over the entire study.

Classification Approaches and Validation of Models. According to the grouping of the DDGS samples at the PCA level, classification approaches were performed to investigate the botanical and geographical origin of the DDGS samples. Classification of DDGS samples was evaluated with both PCA-LDA and SIMCA modeling according to their botanical origin of corn (n = 69) or wheat (n = 15) and their geographical origin, exemplary conducted with corn DDGS from China (n = 31) and the United States (n = 23). Classification results are shown in Tables 2 and 3.

For both questions (botanical and geographical origin), the models were constructed in the first instance with all data (100% of the samples were used as training set) and resulted in a very good classification performance, except for the question of the geographical origin in the analysis of the oils after SIMCA modeling (Table 2). However, it has to be mentioned that a 100% prediction procedure is typically very optimistic, and validation results after a randomized batchwise procedure are judged to be more reliable.[28]

Table 2. Classification Results (Percent) of DDGS Samples According to the Botanical and Geographical Origin Based on 100% Prediction Procedure

		PCA-LDA		SIMCA		
question	sample state	correct classification	misclassification	correct classification	outlier	ambigous
botanical origin (corn vs wheat)	solids	100	0	95	1	4
	oils	100	0	97	3	0
geographical origin (China vs USA)	solids	100	0	98	2	0
	oils	100	0	83	2	15

Table 3. Validation Results (Percent) after Randomized Batchwise Validation Procedure for the Botanical and Geographical Origin of DDGS

		PCA-LDA		SIMCA		
question	sample state	correct classification	misclassification	correct classification	outlier	ambigous
botanical origin (corn vs wheat)	solids	95	5	81	4[a]	14
	oils	100	0	98	2	0
geographical origin (China vs USA)	solids	94	6	68	32	0
	oils	94	6	81	4	15

[a] + misclassifier (1%).

Therefore, in a second step, the models were validated in a randomized batchwise procedure with a training set consisting of two-thirds of the samples and a test set (internal validation set) including the remaining one-third of the samples, for both questions (botanical and geographical origin), respectively. After the randomized batchwise procedure, best classification results of the unknown internal test samples were obtained after PCA-LDA with both the solid DDGS and the oils (Table 3). In summary, the classification results after SIMCA modeling are the best for the oils with correct classification rates of 98% for the botanical origin and 81% for the geographical origin (concerning corn DDGS from China versus corn DDGS from United States). At this point, it should be mentioned that SIMCA modeling must be considered as more suitable for an extension of the models with further classes, that is, botanical raw materials such as barley and sorghum or geographical origins such as the EU, which are not yet included in the model. In fact, the advantage of class modeling (e.g., SIMCA) is that "class spaces defined for different classes may overlap ... and a portion of the global domain may be covered by no class space: samples found in such a region are not therefore compatible with any class studied".[29] In SIMCA models, such samples can also build up new classes outside the present class spaces in contrast to discriminant classification approaches such as PCA-LDA. In conclusion, the extraction of the oils from the DDGS led to slightly superior validation results for the determination of the botanical and geographical origin of DDGS samples, compared to the solid DDGS.

Authentication of the Botanical and Geographical Origin of DDGS. The analytical proof of origin of DDGS—exemplary for feed materials—was the objective of the present study. For this purpose, FT-IR analysis was successfully adapted for the matrix of solid DDGS and coupled with multivariate data analysis of the resulting spectra. Although the most dominant factor after data analysis was the botanical origin of the DDGS, the discrimination of the geographical origin of DDGS was indicated for the example of corn DDGS from China and corn DDGS from the United States. However, the influence of factors such as the production process (e.g., beverage production vs bioethanol production) has to be considered in the data analysis. It is not clarified yet at which step in the development of the statistical models these factors have to be included. For example, if it turns out that another factor, such as the production process, has a stronger influence on the separation of DDGS samples than the geographical origin, models should first be evaluated for this factor (e.g., different production processes) before the geographical origin can be assessed. In the future, it has therefore to be verified with samples from different production systems if the identification of the geographical origin is linked to the production process. Furthermore, it would be of interest to analyze more samples derived from corn to improve the constructed models for the geographical origin and to validate the generated models with iterative bootstrap procedures. However, results obtained so far underpin the suitability of the proposed analytical strategy (ATR/FT-IR spectroscopy combined with multivariate data analysis) for the proof of origin of DDGS. This strategy could be used to verify feed authenticity by the initiation of traceability procedures whenever discrepancies from paper documentation are observed.

■ ASSOCIATED CONTENT

Supporting Information

Detailed information of investigated DDGS samples with their botanical and geographical origin, the production process, and the gravimetric fat contents. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Determination of geographical origin of distillers dried grains and solubles using isotope ratio mass spectrometry

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ABSTRACT

In recent years distillers dried grains and solubles (DDGS), co-products of the bio-ethanol and beverages industries, have become globally traded commodity for the animal feed sector. As such it is becoming increasingly important to be able to trace the geographical origin of commodities in case of a contamination incident or authenticity issue arise. In this study, 137 DDGS samples from a range of different geographical origins (China, USA, Canada and European Union) were collected and analyzed. Isotope ratio mass spectrometry (IRMS) was used to analyze the DDGS for $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{34}S/^{32}S$ isotope ratios which can vary depending on geographical origin and processing. Univariate and multivariate statistical techniques were employed to investigate the feasibility of using the IRMS data to determine botanical and geographical origin of the DDGS. The results indicated that this commodity could be differentiated according to their place of origin by the analysis of stable isotopes of hydrogen, carbon, nitrogen and oxygen but not with sulfur. By adding data to the models produced in this study, potentially an isotope databank could be set up for traceability procedures for DDGS, similar to the one established already for wine which will help in feed and food security issues arising worldwide.

1. Introduction

In recent decades the animal feed trade has increasingly been subject to globalization and subsequently feed materials are traded worldwide. Agricultural products are often sold via traders and shipped to other regions or even continents. Besides foodstuffs this trend has also been extended to animal feed commodities. Due to population growth, industrial development, urbanization and an increasing demand for meat production, resources for animal nutrition are much more difficult to source locally and global trade with animal feed materials is consequently of growing importance. For example, world trade in coarse grains used for animal nutrition has grown from ~24 million tonnes in 1960/61 to more than 130 million tonnes in 2011/12 (USDA statistics, 2013). Currently, the origin of feed materials is less linked to the quality of the commodity as compared to the food sector, where for example the terms "Protected Designation of Origin" (PDO) and "Protected Geographical Indication" (PGI), defined in Council Regulation No 510/2006(CR 510/2006), have a major impact on the market price of the relevant products. However, a trend towards the use of regionally sourced feeds in animal production is already noticeable. Recently Wägeli and Hamm (2012) reported that consumers of organic food products expect organic feed to be produced regionally also. European legislation regulates production systems for organic food and specifies the amount of organic feed which has to be used for production of organic food (Council Regulation No 834/2007; Commission Regulation No 889/2008). Furthermore, private organizations for organic food production and farmers associations (e.g. Demeter, Bioland, Bio Suisse, The Soil Association) are imposing their own standards on their members e.g. by using regionally produced animal feed exclusively, resulting in special logos on food products of animal origin (Bioland, 2012a, 2012b; Janssen & Hamm, 2012). Hence, the geographical origin of animal feed and feed materials is already partially designated for special use (organic foods of animal origin) and will become more important in the future as a measure to improve consumer confidence.

Moreover, animal feed no longer only comprises the classical feed materials like grains, soya and forages, but also co-products from industrial food production, animal feed materials from new sources or new technologies. New sources for animal feed materials have to be carefully considered with regard to their potential impact on the whole feed and food supply chain. It should be noted that, particularly in a crisis situation due to contamination or adulteration of a feed material, the identification of origin is essential with regard to feed and food safety. Such situations arise where a risk (e.g. reduced animal performance, health or welfare) has been associated with a particular product but the reason cannot be identified, or where the analysis of a causal contaminant

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cannot be undertaken quickly or at all due to the complexity or cost of the analysis. Although traceability systems are already available on the basis of document based systems (e.g. paper documentation, labeling, electronic databanks etc.), such systems can be exploited in terms of relabeling and are far from being considered as forgery-proof. A reliable analytical approach to prove the origin in the laboratory, however, could substantially improve the traceability of feed materials.

Authentication approaches for agricultural products, which are also targeting the application of analytical based traceability, have been performed using different techniques, e.g. stable isotope analysis (IRMS and SNIF-NMR), multi-element analysis or spectroscopic techniques such as NIR, MIR, Raman spectroscopy (Gonzalvez, Armenta, & de la Guardia, 2009; Garrigues & de la Guardia, 2013). So far, stable isotope ratio analysis is well established and recognized in authenticity testing of food as it is cited in nearly 50% of the studies in this field (Gremaud & Hilkert, 2008). This methodology benefits from so-called isotope effects observed in nature, which lead to global differences in the isotopic patterns reflected in plants, feed materials and food (Benson, Lennard, Maynard, & Roux, 2006; Gremaud & Hilkert, 2008; Schmidt, 2003). The stable isotope ratios of the bio-elements C, O, H, N and S are valuable factors for verification of the botanical and geographical origin of food or the synthetic origin of food ingredients and have been used extensively for the proof of origin in food research (Anderson & Smith, 2006; Boner & Förstel, 2004; Kelly, Heaton, & Hoogewerff, 2005; Martinelli et al., 2011; Meier-Augenstein, Kemp, & Hardie, 2012; Rossmann, 2001; Rossmann et al., 2000; Schellenberg et al., 2010).

Throughout the growth of plants, e.g. during the ripening of fruits, and also during the rearing of animals, environmental factors, such as climate or geographical origin or nutrition, determine the ratios of these indicative isotopes. The reasons for these differences are linked to the isotopic content of the respective sources (e.g. water with certain isotope ratios) and to the isotopic fractionation that occurs during physical, chemical or biochemical reactions (Benson et al., 2006; Schmidt, 2003). For example, in the water cycle a well-known isotopic fractionation occurs in evaporation of the water from oceans where a depletion in heavy isotopes ^{2}H and ^{18}O is observed in the vapor with respect to that of the liquid state and furthermore in clouds moving inland from the coast or from lower altitude to higher altitude (Gremaud & Hilkert, 2008). Another isotopic fractionation occurs in the evapotranspiration of water from plants. Higher $^{18}O/^{16}O$ isotope ratios are therefore observed in plant water with respect to those of the ground water (Förstel, 2007). The ^{15}N enrichment is primarily affected by regional agricultural practices and soil types (fertilizer, bacterial nitrification/denitrification) whereas the $^{13}C/^{12}C$ ratio reflects mainly the plant metabolism (C_4, C_3 and CAM plants) but can also be used as a semi-geographic marker, since C_3 plants "predominate at higher latitudes and C_4 plants are more common in warmer climates at lower latitudes" (Kelly et al., 2005, p.557). Finally the ratio of $^{34}S/^{32}S$ is influenced by soil types (geological structures) which can be connected with geographic regions, but also by the use of sulfur-containing fertilizers (Gremaud & Hilkert, 2008). Hence, the isotope ratio analysis of the elements C, O, H, N and S enables the authentication of an agricultural product with regard to its botanical and geographical origin.

However IRMS studies investigating the geographical origin feed and feed materials are few in number, although feed and feed materials are based on plant material, for which various applications exist in the food sector, e.g. olive oil, wine, juice, cereals and potatoes (Asfaha et al., 2011; Camin, Larcher, Nicolini, et al., 2010; Gonzalvez et al., 2009; Wachter, Christoph, & Seifert, 2009). Until now stable isotope analysis of feed materials has focused mainly on effects of different diets (e.g. animal protein/plant protein, C_3/C_4 plants) on animal products like milk or meat (Bahar et al., 2005; Camin, Perini, Colombari, Bontempo, & Versini, 2008; Knobbe et al., 2006; Moreno-Rojas, Tulli, Messina, Tibaldi, & Guillou, 2008). Therefore the present study considers the determination of the geographical origin of distillers dried grains and solubles (DDGS) as an example of feed ingredients. This study was part of the workplan of the EU project, Quality and Safety of Feeds and Food for Europe (QSAFFE), with one of the major objectives being to improve systems of traceability and authenticity of feed materials. The chosen commodity DDGS is the co-product of the alcohol distilling process by drying the solid residues of fermented grains (e.g. corn, wheat, barley), to which pot ale syrup or evaporated spent wash was added (Commission Regulation No 68/2013). DDGS are mainly used as feedstock for ruminants, poultry, pigs and fish in aquaculture offering a high nutrient content (proteins, fat) in relation to the price and became a global commodity as a result of the booming fuel-ethanol industry (Liu, 2011). DDGS were chosen for analysis because they are increasingly traded amongst different countries and in the production of DDGS (especially from the fuel-ethanol industry) strategies to increase the yield of ethanol could possibly become more relevant than the quality issues of DDGS.

2. Material and methods

2.1. DDGS samples and sample preparation

A total of 137 DDGS samples were collected in the frame of the EU project QSAFFE from reliable sources. DDGS samples in this study are defined as feed materials according to the numbering of the EU feed catalog (Commission Regulation No 68/2013): either (a) number 1.12.10 (distillers' dried grains) or (b) number 1.12.11 (distillers' dried grains and solubles/distillers' dark grains). The samples were produced from different botanical raw materials (corn, rice, wheat) either in the production of bio-ethanol or alcoholic beverages (whiskey, vodka) and were obtained from different geographical origins as stated by the manufacturer. Origins of the DDGS samples are summarized in Table 1 and details, including the sample numbers used in the study, are provided in the supplementary material (Table A.1). All samples were ground with a centrifugal mill (ZM 200, Retsch, Germany, mesh size 0.5 mm) and subsequently homogenized in plastic containers (filling level ~2/3) for 6 h using a drum hoop mixer (RRM 100, Engelsmann, Germany). The DDGS samples were stored at 4 °C in the dark until analysis.

2.2. Stable isotope ratio analysis

Stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) were analyzed by EA-IRMS using an elemental analyzer (Europa EA-GSL Sample Preparation System, Sercon Ltd, Crewe, UK) coupled to a continuous flow isotope ratio mass spectrometer (20–22 Stable Isotope Ratio Mass Spectrometer, Sercon Ltd, Crewe, UK). For analysis, 1.5 ± 0.2 mg of the ground sample material were weighed into tin capsules and crimped tight. Combustion of the samples was performed at 1000 °C in a combustion tube (packed with quartz wool, quartz chips and reduced copper) together with a quartz liner to collect ash and capsule residues; the sample gases then passed through a magnesium perchlorate water trap and reduction of gases occurred at 890 °C in a reduction tube (packed with quartz wool, quartz chips and reduced copper), similar to the method described by Fry (2007). Isotope ratio analyses of measurement gases N_2 and CO_2 was carried out consecutively in one run utilizing the separation of measurement gases in a packed GC column (0.25 inch i.d., 1 m length, filled with HayeSep Q 60/80 mesh) at 60 °C followed by a second packed GC column

Table 1
Botanical and geographical origin of the DDGS samples.

	Canada	China	EU	India	USA	Unknown
Corn	–	45	14	–	31	16
Wheat	4	–	15	–	–	7
Mixed	–	–	3	–	–	1
Rice	–	–	–	1	–	–

(0.25 inch i.d., 2 m length, filled with HayeSep Q 60/80 mesh) at room temperature before the open split inlet into the mass spectrometer. Flow of helium carrier gas was set to 60 mL/min (grade 5.0) and signals of N_2 (m/z 28/29/30) and CO_2 (m/z 44/45/46) were within the linear intensity range of the system. The mass spectrometer itself was tuned every measurement day for high tension setting of the source and, if needed, for different source settings (ion repeller, beam focus, etc.) with regard to sensitivity and stability using laboratory tank gases (CO_2 and N_2) directly injected into the source.

Stable isotope ratios of hydrogen ($^2H/^1H$) and oxygen ($^{18}O/^{16}O$) were analyzed by TC/EA-IRMS on a TC/EA (Thermo Fisher Scientific, Bremen, Germany) coupled to a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). 0.15 ± 0.05 mg and 0.4 ± 0.05 mg of the ground sample material was weighed into silver capsules and crimped for hydrogen and oxygen isotope analysis respectively. Pyrolysis of the sample was performed in a combustion reactor at 1447 °C (made up of glassy carbon chips and glassy carbon tube within a ceramic outer tube). The samples were combusted in a graphite crucible positioned in the hot zone of the reactor and the combusted gases (H_2 and CO) passed into the mass spectrometer.

For sulfur isotope ratio analysis ($^{34}S/^{32}S$) a Flash EA 1112 elemental analyzer (Thermo Fisher Scientific, Bremen, Germany) was coupled to the Delta V mass spectrometer. 10 ± 0.5 mg of the ground sample were weighed into a tin capsule. Approximately 4 mg of vanadium pentoxide was added to improve reproducibility and the capsule was crimped and placed into a second tin capsule which was subsequently crimped. Samples were combusted in a combustion column filled with tungstic oxide and copper, any NO_x compounds were removed by the copper. The eluted gas (SO_2) passed over a magnesium perchlorate water trap and subsequently entered the mass spectrometer.

Isotope ratios of the samples were expressed in delta notation (δ) in per mil relative to the internationally accepted standards VPDB (Vienna Pee Dee Belemnite) for $\delta^{13}C$, AIR (atmospheric air) for $\delta^{15}N$, VSMOW (Vienna Standard Mean Ocean Water) for δ^2H and $\delta^{18}O$, and CDT (Cañon Diablo Troilite) for $\delta^{34}S$ according to formula 1:

$$\delta[‰] = \frac{R_{sample} - R_{standard}}{R_{standard}} \cdot 1000 \quad (1)$$

where R_{sample} is the ratio of the heavy and light isotope of the sample (e.g. $^{13}C/^{12}C$) and $R_{standard}$ is the ratio of the heavy and light isotope of the international standard.

The ratios of $^{13}C/^{12}C$ and $^{15}N/^{14}N$ of the samples were measured relative to an in-house L-cysteine standard (≥98%, Sigma-Aldrich Co., St. Louis, MO, USA), which was calibrated with reference materials from the International Atomic Energy Agency (IAEA): IAEA-CH-7 Polyethylene ($\delta^{13}C = -32.15‰_{VPDB}$) for carbon; IAEA-NO3 Potassium Nitrate ($\delta^{15}N = +4.7‰_{AIR}$) for nitrogen. Calibration resulted in values of $-29.90‰_{VPDB}$ for $\delta^{13}C$ and $-6.36‰_{AIR}$ for $\delta^{15}N$ for the L-cysteine standard. Within one batch run of DDGS samples the L-cysteine standard was run twice (one standard for calibration, one standard for verification) every 12–15 batch samples and results for $\delta^{13}C$ and $\delta^{15}N$ of the batch samples between two respective L-cysteine standards (used for calibration) were drift corrected using the Callisto CF-IRMS software version 10.0.48 (Sercon Ltd, Crewe, UK). In every batch run, laboratory standards (fructose and one particular DDGS sample for quality assurance) and different IAEA standards (IAEA-CH-6 Sucrose with $\delta^{13}C = -10.45‰_{VPDB}$, IAEA-C-8 Oxalic Acid with $\delta^{13}C = -18.30‰_{VPDB}$, IAEA-N-1 Ammonium Sulfate with $\delta^{15}N = +0.4‰_{AIR}$) were included spread over the whole batch to monitor the stability of the instrument and the accuracy of different isotope ratios closer to the results of the samples (e.g. IAEA-CH-6). The amounts of all standards weighed into the capsules were well adapted for peak intensities within the linear range of the mass spectrometer.

For analysis of $\delta^{18}O$ and $\delta^{34}S$, IAEA reference materials IAEA-601 Benzoic Acid ($\delta^{18}O = +23.3‰_{VSMOW}$) and NBS-127 Barium Sulfate ($\delta^{34}S = +20.3‰_{VCDT}$) respectively were ran in duplicate at the beginning, the middle and the end of a sample batch. All results were subsequently calibrated against these references. An internal standard check of "off the shelf" barium sulfate (99%, Sigma-Aldrich Co., St. Louis, MO, USA) was also measured at the same time as the IAEA reference material during sulfur analysis. For hydrogen analysis two standards previously calibrated against international standards (by Environment Canada, Gatineau, Canada), Kudu (KHS) keratin ($\delta^2H = +54.1‰_{VSMOW}$) and Caribou (CBS) keratin ($\delta^2H = -197‰_{VSMOW}$), were used to calibrate the samples. Within one batch run, four samples of KHS were measured at the start and four samples of CBS at the end.

Each sample was measured in triplicate for analyses of hydrogen, carbon and nitrogen isotopes and in duplicate for analyses of oxygen and sulfur; results are presented as the mean. Analyses of samples were repeated according to the following criteria: (a) δ^2H: if the largest difference between the replicates was >1.5‰; (b) $\delta^{13}C$: if the standard deviation was >0.2‰; (c) $\delta^{15}N$: if the standard deviation was >0.2‰; (d) $\delta^{18}O$: if the difference between samples was >1.0‰ (not exceeded during the study); and (e) $\delta^{34}S$: if the difference between replicates was >1.0‰. Analytical precision was calculated as standard deviation of the repeated analyses of standards (based on different batch runs) and resulted in ±1.0‰ for δ^2H (based on KHS and CBS standards), ±0.10‰ for $\delta^{13}C$ (based on the DDGS sample measured in each batch run for quality assurance), ±0.17‰ for $\delta^{15}N$ (based on the DDGS sample measured in each batch run for quality assurance), ±0.15‰ for $\delta^{18}O$ (based on IAEA-601) and ±0.4‰ for $\delta^{34}S$ (based on IAEA-NBS-127).

2.3. Data analysis

Univariate data analysis included descriptive statistics (calculation of mean and median values, boxplot evaluation) and was carried out with the Software SPSS 12.0.2G for windows (Lead Technologies Inc., Charlotte, NC, USA). Multivariate data analysis was performed using PLS toolbox version 7.0.3 (Eigenvector Research, Wenatchee, WA, USA) together with Matlab version 7.11.0584 [R2010b] (The MathWorks Inc., Natick, MA, USA) and also SPSS (used for canonical discriminant analysis, CDA). Different data preprocessing methods (mean centering, division by standard deviation, absolute value/$\log_{10}$, and combinations thereof) were applied to overcome difficulties due to the different ranges of the respective isotope ratios (e.g. low range of $\delta^{15}N$ compared to great range of δ^2H). Based on the corn DDGS group, different class groups according to the geographical origin of China (n = 45), USA (n = 30) and the EU (n = 13) were split into training sets (2/3 of the respective samples) and test sets (1/3 of the respective samples) using the DUPLEX algorithm (Snee, 1977) in Matlab. Data splitting was carried out for the differently preprocessed data and resulted in 59 samples belonging to the training set (China: 30 samples; USA: 20 samples; EU: 9 samples) and 29 samples belonging to the test set (China: 15 samples; USA: 10 samples; EU: 4 samples). Subsequently, principal component analysis (PCA) was applied to check for well distribution of the training and test sets over the first 3 principal components (PCs). Then, classification models for the indicated geographical origin were built applying stepwise canonical discriminant analysis (using SPSS) and partial least squares discriminant analysis (PLS-DA) with a 5% significance level (using the PLS-toolbox) on the basis of the δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values. In a first step, models were constructed using the samples from the training set together with cross validation based on leave-one-out cross validation for CDA and on leave-multiple-out cross validation (59 samples were divided in 7 random subsets) for PLS-DA. CDA was calculated stepwise on the assumption that all group covariance matrices are equal and on a priori probabilities from the individual group sizes, resulting in two discriminant functions. PLS-DA models were calculated with the NIPALS algorithm and 4 latent variables, the number of latent variables for constructing the model was selected considering the minimum root mean squared error of cross validation (RMSECV), the eigenvalues and the percent of variance captured

by the model. In this way, classification/prediction results for samples used as the training set (self-prediction) and subsets of the training set (cross validation procedure) were obtained. In a second step the external test data set (29 samples) was predicted using the established canonical discriminant functions and the PLS-DA models respectively, and results were expressed by means of sensitivity and specificity according to the definitions given by Oliveri and Downey (2012).

3. Results and discussion

3.1. Univariate results

3.1.1. Botanical origin of DDGS

As expected for C_4 and C_3 plants, clear differences for the $\delta^{13}C$ values of DDGS samples produced from corn ($\delta^{13}C_{median} = -12.8‰$) and wheat ($\delta^{13}C_{median} = -27.2‰$) were observed. Some samples that were claimed to be produced from corn (#4, #71) were detected as outliers after statistical boxplot evaluation (Fig. 1), which was the same for samples reported to be produced from wheat (#113, #119, #121) showing $\delta^{13}C$ values much higher than the median value. As well, one sample produced from 92% wheat and 8% barley (#108) differed significantly from the main wheat DDGS group and one sample produced from 50% corn and 50% wheat (#110) consequently showed a $\delta^{13}C$ value of −20.3‰. With −29.0‰ the rice DDGS sample (#111) presented an expected $\delta^{13}C$ value, i.e. C_3 plant origin, and similar to previously reported results for long grain rice coming also from India (Kelly et al., 2002). For further evaluation with respect to the geographical origin of the DDGS samples, the detected outliers were excluded from data analysis. Also, two samples that were claimed to be of mixed botanical origin (#107, #109), with $\delta^{13}C$ values of −26.3‰ and −27.3‰ (most probably mixtures of wheat and barley) were excluded.

3.1.2. Geographical origin of DDGS

Regarding the geographical origin of the DDGS samples, differences were mainly evident when $\delta^{2}H$ and $\delta^{18}O$ values were considered (Fig. 2a, b). Firstly, considering the $\delta^{18}O$ values, corn DDGS samples from the two Chinese provinces Jilin ($\delta^{18}O_{median} = +18.1‰$) and Heilongjiang ($\delta^{18}O_{median} = +19.6‰$) could be well distinguished from corn DDGS samples produced in the EU ($\delta^{18}O_{median} = +22.8‰$) and USA ($\delta^{18}O_{median} = +22.2‰$). Relatively low $\delta^{18}O$ values could therefore be an indicator that a corn DDGS sample has been produced in China. Also, Canadian wheat DDGS showed different $\delta^{18}O$ values ($\delta^{18}O_{median} = +18.9‰$) compared to the wheat DDGS from the EU ($\delta^{18}O_{median} = +25.7‰$). The $\delta^{2}H$ values showed a comparable pattern for the various geographical sources, however this was not as pronounced as in case of the $\delta^{18}O$ values. A possible explanation could be that water added during the production of the DDGS (for the mash), could have different $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios compared to the water present in the plant material, and influences the respective isotope ratios in the final feed material. To find out whether the results are more dependent on water added during the production, it would, in the future, be valuable to analyze either the water fraction of the DDGS or, more feasibly, their fat fraction. Indeed, more meaningful results could possibly be obtained by analysis of $\delta^{18}O$ or $\delta^{2}H$ of the fat, as isotope ratios of hydrogen and oxygen of the lipid molecules should basically represent the isotope ratios which were present in the plant material.

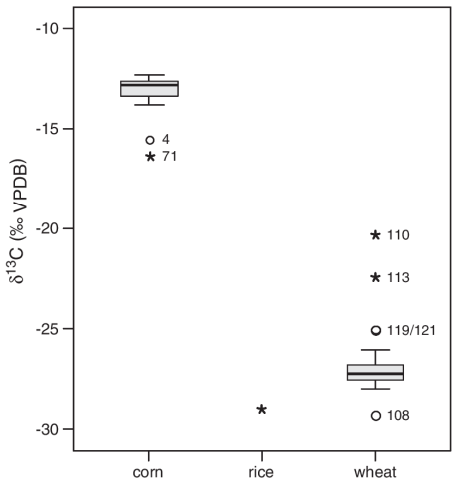

Fig. 1. $\delta^{13}C$ values of DDGS produced from corn (n = 106), rice (n = 1) and wheat/mixed raw materials (n = 30). The group of wheat DDGS contains 4 samples claimed to be produced from mixed botanical raw materials. Box length represents the data between first and third quartile (IQR); bold line shows the respective median value; whiskers represent minimum and maximum values which are not considered as outliers (within 1.5× IQR calculated from first/third quartile); outliers are indicated by circles (data between 1.5× and 3.0× IQR calculated from first/third quartile); extreme outliers are indicated by asterisks (data after 3.0× IQR calculated from first/third quartile).

$\delta^{15}N$ values also differentiate the samples originating from the two Chinese provinces compared to the EU and USA (Fig. 2c). Indeed, differences in the $\delta^{15}N$ values of agricultural products are known to be dependant on regional agricultural practices and soil types (Kelly et al., 2005). For example, the use of organic fertilizers (e.g. manure) in general can lead to higher $\delta^{15}N$ values compared to mineral fertilizers, such as diammonium hydrogen phosphate or potassium nitrate (Förstel, 2007). However, bacterial nitrification/denitrification processes can also affect the isotopic ratio of nitrogen isotopes towards a depletion in ^{15}N (Gremaud & Hilkert, 2008), whereas the use of legumes causes an enrichment of ^{15}N in the soil. Furthermore, the $\delta^{15}N$ values of DDGS samples could be influenced by addition of nutrient salts for yeasts (e.g. urea or ammonium sulfate) during fermentation, similarly discussed on IRMS analysis of wine (Rossmann, 2001). Nevertheless, the differences in the nitrogen isotopes could be correlated to single production sites and/or regions of origin in the current study. Also, due to the fact that DDGS production in some countries is based on only a few production plants (according to information obtained during sampling), consideration of $\delta^{15}N$ values for determination of the place of origin of DDGS is reasonable.

Differences in the $\delta^{34}S$ values were mainly present for samples from China (slightly higher values) against samples from all other regions (Fig. 2d). Surprisingly, six DDGS from one Chinese province sampled in 2012 showed high values ($\delta^{34}S_{median} = +11.7‰$) compared to DDGS from this province sampled in 2011 ($\delta^{34}S_{median} = +3.2‰$). Also, one corn DDGS from the USA sampled in 2013 showed a $\delta^{34}S$ value of +10.6‰ whereas similar samples were in the range of −0.6‰ to +5.6‰. Therefore, the correlation of $\delta^{34}S$ values with the geographical origin of the DDGS samples was limited. Possibly, chemicals used for production with considerable amounts of sulfur and different $^{34}S/^{32}S$ ratios, like sulfuric acid for pH adjustment and/or cleaning purposes or ammonium sulfate used as nutrient salt, which were carried over to the DDGS, could be the reason for this. For example, barium sulfate even from the same manufacturer and catalog number but purchased in different years varied in the sulfur isotope ratio, as Coplen et al. (2002) described.

With regards to the $\delta^{13}C$ values within the corn DDGS group, small differences were also observed according to the geographical origin. While Chinese corn DDGS samples presented a mean (= median) $\delta^{13}C$ value of −12.6‰ (range: −12.3‰ to −13.4‰), samples from the US and EU resulted in a mean (= median) $\delta^{13}C$ value of −12.9‰ (range: −12.3‰ to −13.7‰) and −13.4‰ (range: −13.0‰ to −13.8‰) respectively. Camin, Larcher, Perini, et al. (2010) stated that increasing $\delta^{13}C$ values of olive oil are probably correlated with the dryness of the climate, which could also be a possible explanation for the results of the current study. However, the production year of the raw

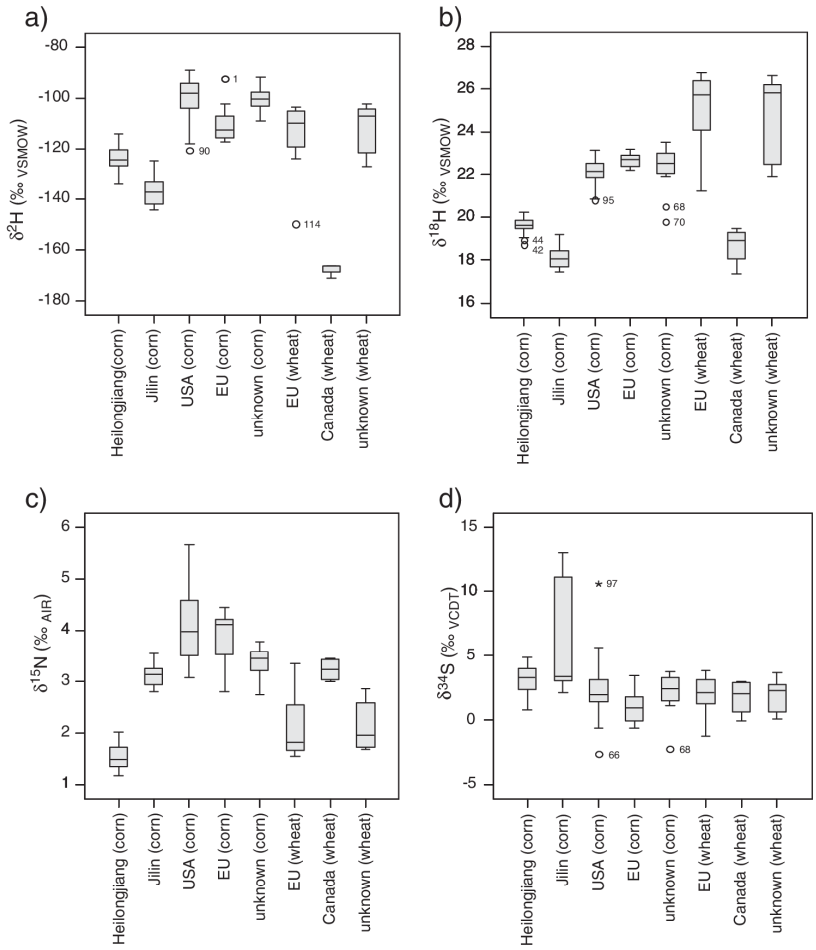

Fig. 2. Boxplot of (a) δ^2H, (b) $\delta^{18}O$, (c) $\delta^{15}N$ and (d) $\delta^{34}S$ values of DDGS produced from corn (n = 88) and wheat (n = 23) according to geographical origin. Box length represents the data between first and third quartile (IQR); bold line shows the respective median value; whiskers represent minimum and maximum values which are not considered as outliers (within 1.5× IQR calculated from first/third quartile); outliers are indicated by circles (data between 1.5× and 3.0× IQR calculated from first/third quartile); extreme outliers are indicated by asterisks (data after 3.0× IQR calculated from first/third quartile).

material used for production of DDGS is not clear in every case and therefore final statements cannot be made in this respect.

3.2. Multivariate results

As univariate data analysis showed, discrimination of samples according to their place of origin was best for δ^2H and $\delta^{18}O$ values. Combination of these two parameters leads to an even better result (Fig. 3) with samples from Asia (China and the single sample from India) being well separated from the EU/USA samples, which themselves are approximately clustered in two groups. Most of the wheat DDGS samples derived from the EU showed higher $\delta^{18}O$ values than the European corn DDGS samples (see also Fig. 2b), possibly connected with the climatic conditions in the respective production areas of the raw materials and/or influenced by the water used for production of the DDGS in the same way. Only one wheat DDGS sample known to be produced in the EU (#115) and two samples with unknown origin (#117, #118) end up close to the European/American corn DDGS samples, perhaps indicating that climatic conditions within Europe could be the reason for cultivation of corn or wheat used for DDGS production. The exceptional $\delta^2H/\delta^{18}O$ values of the wheat DDGS sample (#114) appearing between the wheat DDGS from the EU and the wheat DDGS from Canada, could possibly be explained by the use of special water (potentially depleted in ^2H and ^{18}O) during its production as alcoholic beverage. The 16 corn DDGS samples of unknown origin are showing similar δ^2H and $\delta^{18}O$ values to the corn DDGS produced in the USA.

By also taking the nitrogen isotopes into account, a three dimensional plot of δ^2H, $\delta^{15}N$ and $\delta^{18}O$ values leads to well distinguished groups of samples from the two Chinese provinces (Heilongjiang and Jilin) and the Canadian samples, whereas the overlap of the European and American DDGS samples still remains (data not shown). Concentrating on the DDGS produced from corn only, which could in practice be separated from wheat DDGS using the $\delta^{13}C$ values in a first step, a similar picture is apparent (Fig. 4). The differentiation of Chinese and European/American DDGS samples is clearly visible, and also the $\delta^{15}N$ values contribute to the enhanced separation of the DDGS from the two Chinese provinces.

However, discrimination of corn DDGS samples produced in the EU to corn DDGS samples from the USA is not clearly presented on the three dimensional scatter plot. Therefore multivariate evaluation of the δ^2H, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values was tested to see if discrimination could be achieved. Classification models for the discrimination of corn

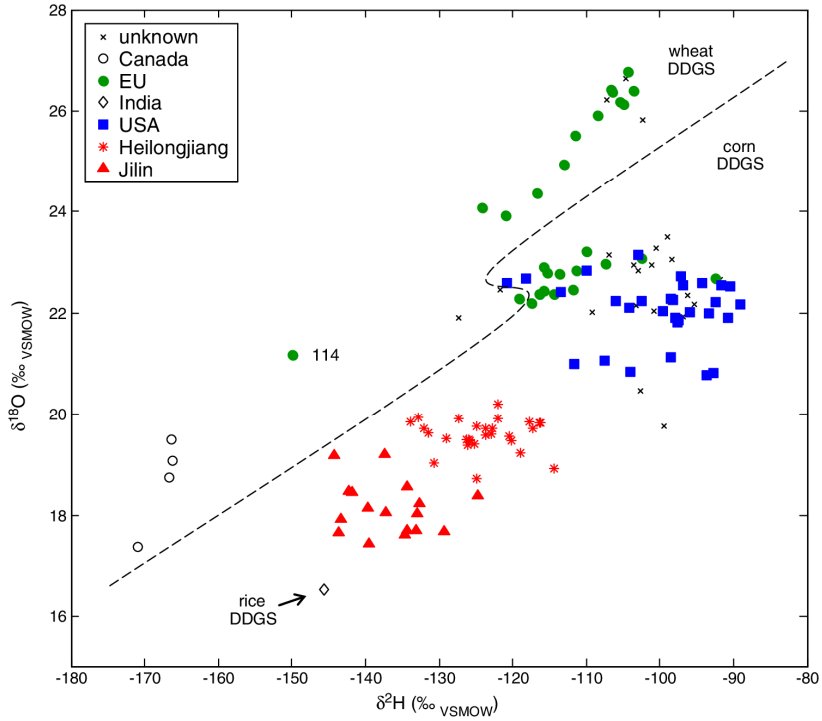

Fig. 3. Differentiation of 127 DDGS samples according to their geographical origin, based on δ^2H and $\delta^{18}O$ values. Heilongjiang and Jilin are provinces in China; information on the geographical origin was not available for samples labeled 'unknown'. The broken line illustrates the botanical raw material used for production of DDGS (corn and wheat) and is not representative of statistical results.

DDGS samples from China, the EU and USA resulted in a statistically more proven result, for example observed in the scatter plot displaying the discriminant functions after CDA (Fig. 5). Classification results after CDA and PLS-DA with regard to self-prediction (based on 2/3 of the

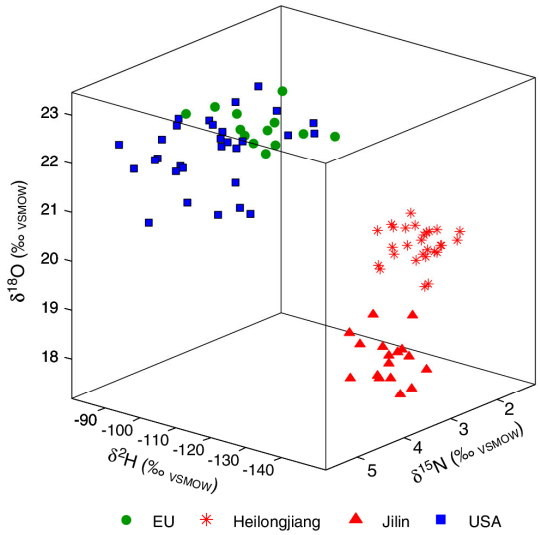

Fig. 4. Differentiation of 88 DDGS samples produced from corn according to their geographical origin, based on δ^2H, $\delta^{15}N$ and $\delta^{18}O$ values. Heilongjiang and Jilin are provinces in China.

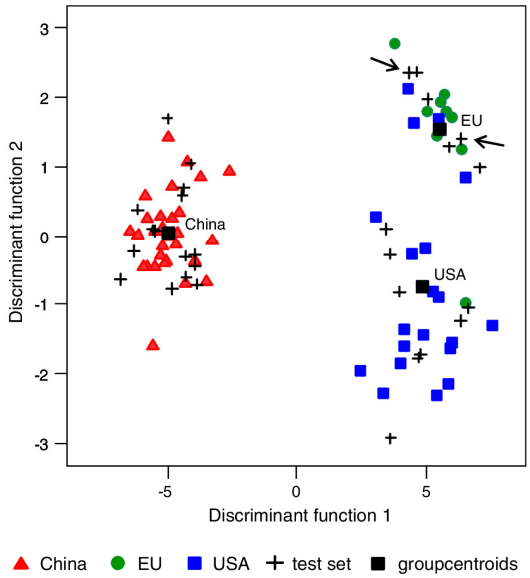

Fig. 5. Discrimination with respect to the geographical origin of DDGS samples derived from China (n = 45), EU (n = 13) and USA (n = 30) after canonical discriminant analysis of preprocessed isotope data ($\log_{10}$ of the absolute value, followed by mean centering and division by standard deviation). Arrows are highlighting two US samples of the test set which were misclassified to the EU group.

Table 2
Classification results of DDGS samples from different geographical origins based on CDA and PLS-DA (results in parentheses).

Procedure	Origin	MeanSD[a]		AbsLogMeanSD[b]	
		Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Self-prediction of training set	China	100 (100)	100 (100)	100 (100)	100 (100)
	EU	89 (89)	92 (90)	89 (89)	92 (92)
	USA	80 (75)	97 (97)	80 (80)	97 (97)
Cross validation[c]	China	100 (97)	100 (100)	100 (97)	100 (100)
	EU	89 (89)	92 (88)	89 (89)	92 (90)
	USA	80 (75)	97 (97)	80 (80)	97 (97)
Prediction of test set	China	100 (100)	100 (100)	100 (100)	100 (100)
	EU	100 (100)	92 (92)	100 (100)	92 (100)
	USA	80 (80)	100 (100)	80 (70)	100 (100)

[a] Preprocessing of isotope data based on mean centring and division by standard deviation, PLS-DA with two latent variables.
[b] Preprocessing of isotope data based on $\log_{10}$ of the absolute value followed by mean centring and division by standard deviation, PLS-DA with three latent variables.
[c] Cross validation was based on leave-one-out cross validation for CDA and on random subsets (leave-multiple-out) for PLS-DA.

respective samples of one group) and prediction (based on 1/3 of the respective samples of one group, not used for model building) were satisfactory (Table 2). As multivariate isotope data analysis is strongly influenced by the different ranges of the respective isotope ratios (e.g. low range of $\delta^{15}N$ compared to great range of $\delta^{2}H$), different data preprocessing methods were applied to overcome different weights of the variables (δ values). Logically, data preprocessing based on MeanSD (mean centering and division by standard deviation) and AbsLogMeanSD ($\log_{10}$ of the absolute value, followed by mean centering and division by standard deviation) resulted in the best classification models. Although DDGS samples from the EU could be well discriminated from DDGS samples from the USA in the current approach, it should be noted, that such models have to be continuously updated, as already the bi-variate and tri-variate results are showing only small differences for the respective isotope ratios. However, models could be used to evaluate the origin of a corn DDGS sample, for which a geographical origin of either EU or USA is already indicated by bi- or tri-variate results, more thoroughly.

4. Conclusion

In the current study DDGS were analyzed with the aim of discriminating geographic origins of this globally traded animal feed commodity. Stable Isotope Ratio Analysis of carbon ($^{13}C/^{12}C$) showed clear differences for DDGS samples produced from corn and wheat. Combination of stable isotope ratios for hydrogen ($^{2}H/^{1}H$), oxygen ($^{18}O/^{16}O$) and nitrogen ($^{15}N/^{14}N$) enabled a differentiation of DDGS produced in China from DDGS produced in the EU and USA. In addition, tendencies for discrimination of corn DDGS samples produced within the EU and corn DDGS samples produced in the USA were noted based on differences according to the isotope patterns of hydrogen and oxygen mainly, but also due to small differences in the $\delta^{13}C$ values of carbon. Multivariate data analysis of the $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$ values based on CDA and PLS-DA showed that corn DDGS samples from the EU and USA could be discriminated by classification models. In conclusion, the results indicated that DDGS could be differentiated according to their place of origin by the analysis of stable isotopes of hydrogen, carbon, nitrogen and oxygen. However, stable isotope composition of raw materials used for production of DDGS can be altered by climate, seasonal or annual fluctuations or simply by differences in the isotope ratios of ingredients and agents used for production (e.g. local water). Therefore, it will always be necessary to continuously up-date the results by analysis of new authentic samples from the single locations. In this way, an isotope databank could be set up for traceability procedures of DDGS, similar to the one established already for wine.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.11.002.

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Article

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Screening for Sulfate in Distillers Dried Grains and Solubles by FT−IR Spectroscopy

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Supporting Information

ABSTRACT: Distillers Dried Grains and Solubles (DDGS) are an animal feed containing varying levels of sulfur. As ruminants are susceptible to high sulfur diets, sulfur content is of major interest to the parties involved. The variation in levels of sulfur in DDGS is mainly due to sulfate derived from the addition of sulfuric acid during the production. ATR/FT−IR spectroscopy was used to determine sulfate levels in 90 DDGS samples from various origins (Canada, China, EU, and U.S.A.). Specific absorption bands for sulfate at 615 and 1107 cm^{-1} enabled the analysis of sulfate in the DDGS matrix. Besides direct quantification (using band at 615 cm^{-1}), PLS regression was applied for the prediction of sulfate using FT−IR spectra and calibration with reference values analyzed by capillary electrophoresis. An extended calculation on the total sulfur estimated that 11% of the DDGS samples analyzed in this study featured sulfur contents higher than 0.80%.

KEYWORDS: *FTIR, capillary electrophoresis, bioethanol, DDGS, sulphate, feed safety, QSAFFE*

INTRODUCTION

The animal feed sector is affected more than ever by the rising lack of protein sources. The involved business has forced efforts to find alternative sources for animal nutrition, and novel feed materials are considered for entry into the market. Also, by- or coproducts from food production or industrial processes based on natural raw materials are considered and already used in the rearing of livestock. For example, Distillers Dried Grains and Solubles (DDGS) as byproducts of the bioethanol industry recently have gained importance in the animal feed sector. As such, the ethanol industry is primarily interested in the production of ethanol by fermentation of cereals (i.e., corn, wheat, barley, and sorghum), while at the same time obtaining a byproduct of high nutritional value (rich in proteins, raw fiber, fat, and minerals). However, it is of the utmost importance that animal feed materials derived from industrial processes or novel sources comply with legal requirements and that feed safety as well as food safety can be guaranteed in terms of the farm-to-fork principle.

With regard to DDGS, consistency in the nutritional value and quality of the feedstuff is particularly relevant, as sources of production are manifold and variation in the products composition is not only present among but also within production plants.[1,2] Besides the nutritional composition, one of the most important aspects regarding DDGS is its content of sulfur (S), which is strongly affected by the process of ethanol production. First, the concentration of sulfur-containing amino acids is increased by a factor of approximately three compared to the grain during the production process,[3] resulting in a content of S about 0.30 to 0.40 mass percent of endogenous origin depending on the raw material (corn or wheat). Second, sulfuric acid is used in many plants to control the pH of the cereal mash during fermentation and/or as a cleaning agent and can lead to high sulfate/sulfur levels in DDGS.[4] Many studies have already paid attention to the presence of S in DDGS and as Shurson, Tilstra, and Kerr (2012)[5] indicated, sulfur levels in DDGS can range from 0.31 to 1.93% (average 0.69%) on a dry matter (DM) basis. In addition, the sulfur content of Distillers Grains was reported to vary typically, showing a within-plant coefficient of variation from 6.4 to 40.8%[1] and 2.2 to 12.8%[6] respectively.

The particular interest in the sulfur content of DDGS is linked to the fact that ruminants are susceptible to high sulfur-containing diets. Although microbes present in the rumen require sulfur for their normal growth and metabolism, high S intake can decrease the bioavailability of trace minerals (e.g., copper, iron, zinc) and also lead to the production of H_2S by sulfate reducing bacteria (e.g., *Desulfovibrio* spp. and *Desulfotomaculum* spp.).[7] In ruminants, the major part of H_2S from the rumen will be eructated and inhaled during rumination and after entering the pulmonary vein in the lung, the H_2S consequently reaches the brain of the animal. As Schoonmaker and Beitz (2012) summarized, high concentrations of H_2S decrease cellular respiration and limit the rate of ATP generation causing necrosis of the cerebral cortex and softening of the brain tissue.[7] Severe cases of H_2S intoxication can result in polioencephalomalacia (PEM) and if not treated with thiamine, in the death of the animal.[7]

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Risk for steers contracting PEM was reported to increase when the dietary S levels and the rumen available S levels in the diet increased; this was observed most clearly at dietary S levels above 0.6% and low levels of forage NDF in the diets.[8] However, Schoonmaker and Beitz (2012) mentioned that PEM incidence correlates not only with dietary S contents, but also with variation in the S content from day to day.[7] Therefore, consistency of the S content of DDGS but also of the whole diet must be regarded as a crucial factor in rearing of ruminants. According to the U.S. National Research Council,[9] the maximum tolerable dietary S level is 0.4% (DM basis) for beef cattle. For example, this dietary level could be exceeded if DDGS with sulfur content greater than 0.80% (DM basis) were fed to ruminants at high inclusion rates (DDGS: 40% of DM intake) in addition to corn and corn silage, assuming low sulfate levels in drinking water.[5,10]

In addition, inorganic forms of sulfur (i.e., sulfate) are described to be better available for ruminal bacteria.[7,8,11] In this context, Schoonmaker and Beitz (2012) mentioned that DDGS could show high sulfate levels of 0.5 to 1.7%, which can cause a dramatic increase in H_2S production in the rumen.[7] Moreover, Sarturi et al. (2013) observed greater ruminal H_2S concentrations for steers fed with Wet Distillers Grains with Solubles (WDGS) and sulfate-enriched diets compared with conventional or organic sulfur-enriched diets.[11] Therefore, great attention has to be devoted not only to sulfur levels, but also especially to sulfate levels in DDGS preventing ruminants of H_2S intoxication. Besides the risk of PEM, there is also a general interest in reducing sulfate levels (and sulfur levels) in DDGS because both ruminants and nonruminants show decreased feed intake and gain at high dietary S levels.[12,13] Sulfate levels in DDGS could be reduced most easily, if the use of sulfuric acid would be avoided and phosphoric acid or hydrochloric acid would be used instead for pH control in the production process.[14] Alternatively, the analysis of sulfate and/or sulfur levels in DDGS could enable the selection of feeds and batches of feed with acceptable S levels, and diets could also be kept to a more consistent S level in this way.[7]

Thus, the quantitative information on sulfur and/or sulfate in DDGS is of major interest to parties involved and certainly improves the quality and safety of DDGS. Traditionally, quantitative analysis of sulfur in animal feed has been performed by gravimetric analysis ($BaSO_4$) after oxidation of S-containing compounds to sulfate (e.g., AOAC Method 923.01).[15] Nowadays, X-ray fluorescence spectroscopy or inductively coupled plasma mass spectrometry (ICP-MS) are commonly used for this purpose. In the current study, the application of ATR/FT−IR spectroscopy for the determination of sulfate levels in DDGS as a rapid screening method is highlighted. The application targets the screening of sulfate levels in different batches of DDGS and was developed to easily preselect batches of DDGS with prominent sulfate levels. The technique itself could be used routinely in the field as laboratory expenses are limited and it could also serve as a process-oriented solution to control the sulfate content in DDGS on-site.

■ MATERIALS AND METHODS

DDGS Samples and Sample Preparation. 90 DDGS samples were collected in the frame of the EU project QSAFFE from reliable sources in the years 2011−2013. In this study, DDGS samples are defined as feed materials according to the numbering of the EU feed catalogue (Commission Regulation No. 68/2013):[16] either (a) number 1.12.10 (Distillers' dried grains) or (b) number 1.12.11 (Distillers' dried grains and solubles/Distillers' dark grains). The samples were produced from the bioethanol industry or the alcoholic beverage industry (whiskey, vodka) and were obtained from different geographical origins as stated by the provider. They were produced mainly from corn or wheat; details of the samples are provided as supplementary table (Supporting Information, SI). All samples were ground with a centrifugal mill (ZM 200, Retsch, Germany, mesh size 0.5 mm) and subsequently homogenized in plastic containers (filling level ∼2/3) for 6 h using a drum hoop mixer (RRM 100, Engelsmann, Germany). The DDGS samples were stored at 4 °C in the dark until analysis.

One preground DDGS sample without substantial sulfate level [#40, cf. SI], named sample A in this study, was spiked with different amounts of anhydrous sodium sulfate (analytical grade; ≥ 99.0%; Merck KGaA, Darmstadt, Germany) to levels of 1% to 5% sodium sulfate, in triplicates (each of 0.500 g) for FT−IR spectroscopy and in triplicates (each of 4.000 g) for capillary electrophoresis (CE). Also, one preground DDGS sample with medium sulfate content (1.44%) [#29, cf. SI], named sample B in this study, was analyzed to estimate precision of (1) FT−IR spectroscopy in the spectral range of interest, and (2) CE. In order to demonstrate the effect of Na_2SO_4-spiking to sulfate-containing DDGS, one further preground DDGS sample with medium sulfate content [#33, cf. SI], named sample C in this study, was spiked with 3% sodium sulfate.

FT−IR Spectroscopy. Prior to FT−IR spectroscopy, the preground DDGS samples were finely ground using a ball mill (MM 301, Retsch, Germany; 5 mL steel cylinders and 2 steel balls with 7 mm diameter, 30 Hz, 4 min) to achieve small-sized particles (more than 99% between 10 to 200 μm), as described before.[17] FT−IR spectra were collected with a Nicolet 6700 series spectroscope (Thermo Fisher Scientific, Waltham, MA, U.S.A.) equipped with a Smart Performer Accessory (single-bounce-ATR with diamond crystal), a micrometric pressure device enabling a standardized pressing force, a KBr beamsplitter, and a deuterated triglycine sulfate (DTGS) detector. In general, FT−IR spectroscopy of the DDGS samples was carried out as described in a previous study.[17] The spiked mixtures of sample A and sample C were ground with a ball mill (instrument setup specified above) for 4 × 1 min and mixed manually using a spatula after each grinding to improve homogeneous distribution of the sodium sulfate in the sample, which led to equal particle sizes as mentioned above. For estimation of precision, 10 replicates of sample B underwent fine grinding, triplicate FT−IR spectra of each replicate were collected, peak areas and sulfate contents (cf. following sections) were calculated. Then relative standard deviation (coefficient of variation) of the averaged sulfate contents of the triplicates (n = 10) was calculated.

Extraction of Sulfate and Capillary Electrophoresis. The extractable sulfate content of the DDGS samples was analyzed by capillary electrophoresis (CE). Sulfate was extracted out of the DDGS by weighing exactly 4.000 g of the preground sample material into a 50 mL Polypropylene Conical tube (30 × 115 mm; BD Biosciences, San Jose, U.S.A.) and adding 40.00 mL deionized water (18.2 MΩ; purified by Milli-Q Reference A+ System; Millipore, Schwalbach, Germany). The tube was capped and vigorously shaken until the sample material was dispersed in the water; then homogenized with an overhead shaker for 10 min, followed by 5 min supersonic wave treatment (35 kHz) and centrifugation for 10 min at an average of 7020g (8000 rpm, J2-HS Centrifuge with rotor JA-10, Beckman Coulter, U.S.A.). The supernatant was first filtered through an ashless filter paper (589/3, Whatman, Dassel, Germany) and subsequently through a 0.2 μm syringe filter (Perfect-flow Nylon membrane, WICOM, Heppenheim, Germany).

For capillary electrophoresis, 1000 μL of the filtered sample extract and 1000 μL of an internal standard solution of 2000 mg/L sodium chlorate (ACS reagent, ≥ 99.0%; Sigma-Aldrich, St. Louis, MO, U.S.A.) were diluted with deionized water to a final volume of 50 mL (using 50 mL glass flasks). Finally, 500 μL of this solution was pipetted to a 1 mL polypropylene vial and used for electrophoresis. Organic Acids Buffer (Agilent Technologies, Waldbronn, Germany) was used for electrophoretic separations. Three independent sulfate stock

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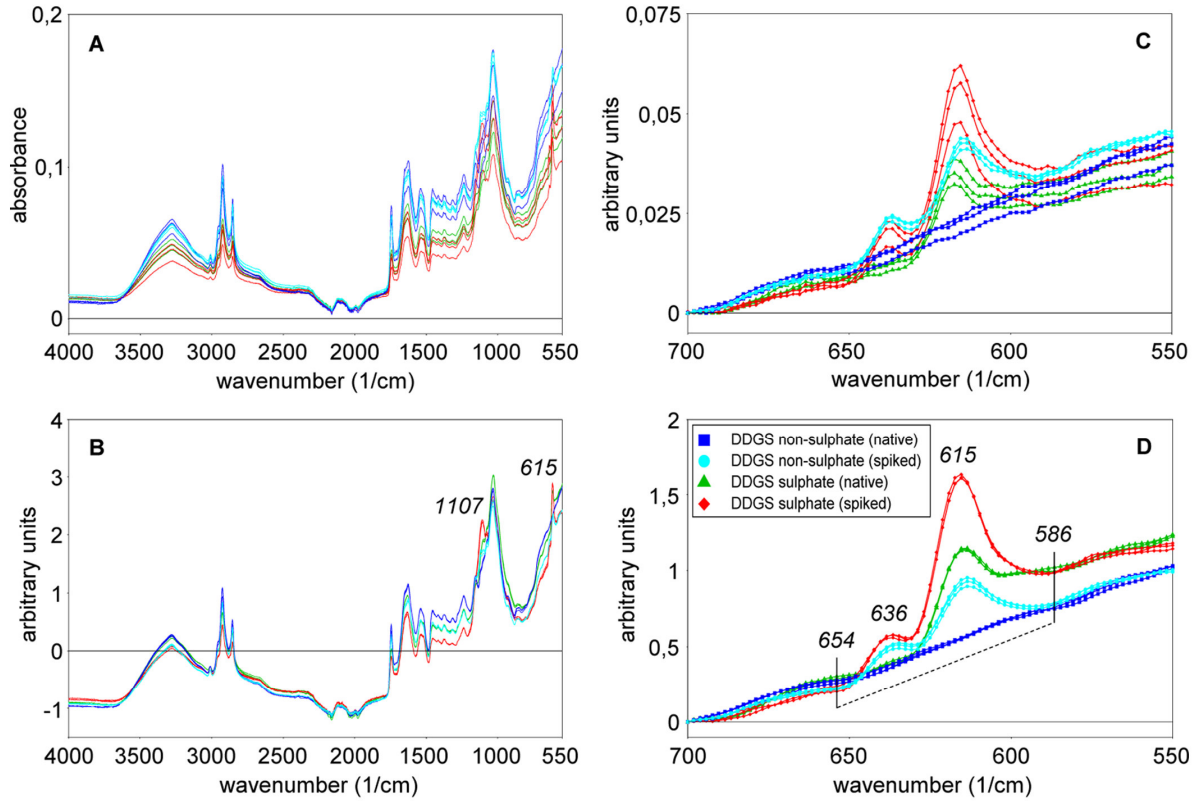

Figure 1. (A)+(C): FT−IR triplicate spectra of DDGS; (B)+(D): FT−IR spectra after preprocessing (SG smoothing and SNV transformation); exemplary shown for DDGS without natural sulfate content (sample A) and DDGS with natural sulfate content (sample C), both native and Na_2SO_4-spiked. (C)+(D): Spectra were zeroed to signal at 700 cm^{-1} for demonstration purposes (not conducted in actual data processing). (D): dashed line indicates wavenumber region for integration of bands; legend is representative for all single figures.

solutions of 500 mg/L were prepared by dissolving appropriate amounts of anhydrous sodium sulfate (analytical grade; ≥ 99.0%; Merck KGaA, Darmstadt, Germany) in deionized water to a volume of 1000 mL. Sulfate standard solutions (5, 20, 40, 60, and 80 mg/L) were prepared from these stock solutions for calibration and were run daily in the beginning, the middle and the end of each sample sequence. Calibration curves were calculated on the average results of the three independent sets of standard solutions and used for determination of the sulfate concentrations in the sample extracts. Also, sulfate concentrations in sample extracts (and in standard solutions) were corrected using the chlorate peak as internal standard in the respective electropherogram. Finally, sulfate contents in the solid DDGS samples were calculated from the sulfate concentrations of the sample extracts.

The CE system employed for the study was an Agilent 7100 system (Agilent Technologies, Waldbronn, Germany) equipped with a fused-silica capillary of 72 cm length and 75 μm I.D. and UV−vis detector (190−600 nm) for indirect detection of analytes. The capillary was initially activated with 0.1 mol/L $NaOH_{aq}$ and water, rinsed with buffer daily before analyses and for preconditioning (240 s) before each injection. Sample solutions were injected in the hydrodynamic mode for 2 s at 50 mbar and separations were performed at 30.0 °C and a constant voltage of −25 kV. Total run time (without preconditioning) was 10 min and detection was based on a wavelength of 254 nm using a reference wavelength of 200 nm. Fresh buffer solutions were used after every 6 samples, and the detector was autobalanced after 60 s.

The spiked mixtures of sample A were homogenized using a spatula and extracted as described above; extracts were analyzed by CE, and the results served for estimation of recovery. Sample B was extracted ten times and analyzed by CE accordingly for estimation of precision. Extraction of sample B was carried out consecutively during the extraction of the samples and CE analysis of the respective replicates went along with samples on the single days of measurement by means of quality control.

Data Analysis. *Univariate Calibration Using Corrected Peak Area.* FT−IR raw spectra were investigated using the OMNIC 7.4 software (Thermo Fisher Scientific). Raw spectra (triplicates) were imported into The Unscrambler X 10.2 software (CAMO Software, Oslo, Norway) and preprocessed by Savitzky-Golay smoothing (centered spectral window 11 points, third polynomial for fitting) followed by Standard Normal Variate (SNV) transformation. Preprocessed spectra were then exported to ASCII text files using semicolons as item delimiter, transcribed to csv-format using KNIME version 2.9.1 (KNIME, Zurich, Switzerland) and finally imported into the OMNIC software again. Here, a macro (written in the OMNIC Macros Basic 7.4) was used for the calculation of the corrected peak height and corrected peak area in the range of 653.750 and 586.246 cm^{-1} for all spectra and results were automatically saved as text file. Peak location for the calculation of the peak height was defined at 617 cm^{-1} (seek closest peak enabled) and a new linear baseline (in the range of 653.750 and 586.246 cm^{-1}) was used automatically to calculate the corrected values of height and area, respectively. The text file was finally imported into Excel 2010 (Microsoft, Redmond, WA, U.S.A.) to draw graphs and calculate regression equations.

Regression analysis was performed by fitting a linear model (least-squares approach) to the obtained data of DDGS sample A spiked with different amounts of sodium sulfate. The double sided confidence interval bands for prediction were calculated according to eq 1:

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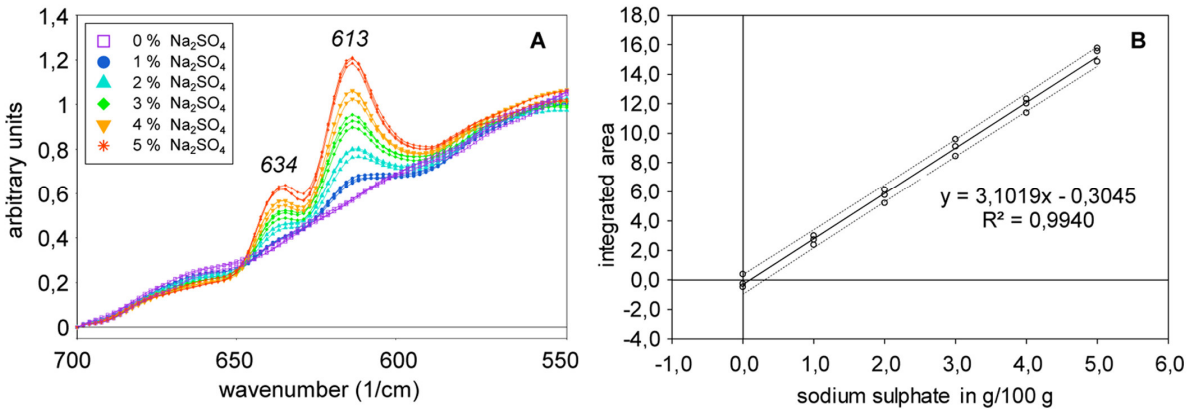

Figure 2. FT−IR triplicate spectra of DDGS sample A (SG smoothed, SNV transformed) spiked with different amounts of sulfate (A) and resulting calibration line with confidence intervals drawn by dashed lines (B). Spectra were zeroed to signal at 700 cm^{-1} in (A) for demonstration purposes (not conducted in actual data processing). Calibration was based on integration of bands between 654 and 586 cm^{-1}.

$$\hat{y} = (\hat{a} + \hat{b}x_i) \pm s_y t_{(p,df)} \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(x_i - \bar{x})^2}{Q_{xx}}} \quad (1)$$

where $\hat{a}$, $\hat{b}$, and x denote the intercept and slope estimated from calibration data and sulfate content respectively and s_y is the standard deviation of the residuals, calculated according to eq 2:

$$s_y = \sqrt{\frac{\sum (y_i - \hat{y}_i)^2}{n - 2}} \quad (2)$$

In eq 1, t is the student's t factor with p = 95% and degrees of freedom $df = n - 2$ are considering the number of samples (n) used for calibration. The number of replicates is indicated by m and Q_{xx} (sum of squared differences of contents) is calculated according to eq 3:

$$Q_{xx} = \sum (x_i - \bar{x})^2 \quad (3)$$

First, a calibration line was calculated for the sample spiked with different levels of sulfate and the respective corrected peak areas from the FT−IR spectra (matrix calibration). Then, this calibration was used to calculate the sulfate contents in all other samples analyzed in the study. Finally, these calculated sulfate contents were plotted against the sulfate contents analyzed by CE. The process coefficient of variation V_{x0}, based on the calibration using DDGS sample A, was calculated according to eq 4:

$$V_{x0} = \frac{s_y}{b \cdot \bar{x}} \quad (4)$$

Multivariate Calibration Using CE Reference Values (PLS Regression). For multivariate regression, FT−IR spectra were imported into The Unscrambler X 10.2 software, all sample triplicates were preprocessed by Savitzky-Golay smoothing (centered spectral window 11 points, third polynomial for fitting), then the triplicates were averaged, and finally SNV transformation was conducted. Partial least-squares regression (PLS regression) was calculated using the results from CE as reference values, based on NIPALS (Nonlinear Iterative Partial Least Squares) algorithm, mean centring and 7 requested latent variables (factors). Most important spectral variables of the calculated PLS models were consecutively selected three times (through the respective regression coefficients plot) and calculation of the new models with reduced number of variables was repeated accordingly. The PLS regression model was based on 2 latent variables (explaining 95.04% of the variance), a total number of 188 spectral variables (originally 1803, second step 568, third step 318, fourth step 188) and leave-one-out cross-validation was applied.

RESULTS AND DISCUSSION

Determining Sulfate by FT−IR Spectroscopy. FT−IR spectra of DDGS containing sulfate showed a unique band with local absorption maxima around 615 cm^{-1} (range: 640 cm^{-1} to 590 cm^{-1}) which were not present in DDGS without prominent sulfate levels (Figure 1). Also, a specific band around 1107 cm^{-1} was detectable in DDGS with high sulfate content (spiked with sodium sulfate). The band with the maximum around 615 cm^{-1} could be clearly attributed to one of the two fundamental IR active vibrations of the sulfate anion (SO_4^{2-}). In general, inorganic tetrahedral XY_4 molecules such as SO_4^{2-} show four normal modes of vibration; all are Raman-active, but only ν_3 and ν_4 are infrared-active.[18] For the sulfate anion in the free state, these vibrations appear at 1105 cm^{-1} (ν_3) and 611 cm^{-1} (ν_4) as it was described by Nakamoto.[18]

Addition of sodium sulfate to DDGS samples clearly increased the signal at 615 cm^{-1}, whereas the signal at 1107 cm^{-1} could only be detected at very high sulfate levels (Figure 1, red spectra: ∼4.2% sulfate) due to overlaying bands of compounds naturally present in the DDGS (e.g., C—O stretching vibrations of cellulose). In contrast to the maximum absorption signals reported in literature for pure sulfate salts,[18−20] the absorption maxima of ν_3 and ν_4 for sulfate in the matrix DDGS were sometimes found to be slightly shifted to lower or higher frequencies. The reason could possibly be the difference between the ATR technique and the transmission mode, as ATR generally introduces a shift to lower frequencies, or the DDGS matrix accounting for shifts in both directions of the frequency scale. Furthermore, the addition of sodium sulfate lead to an additional band at 636 cm^{-1} (Figure 1D) caused by the lowering of symmetry of the sulfate anion through coordination of the sodium cation. Possibly, coordination exists in the form of a bridged bidentate complex (C_{2v} instead of T_d), as it was previously reported for Na_2SO_4 and sulphato complexes.[21,22]

Recently, Zarrinbakhsh, Mohanty, and Misra (2013) reported on a water-soluble portion of DDGS which exhibits peaks at 1105 and 615 cm^{-1} only in the spectra of solid DDGS, but not in washed DDGS.[23] In consideration of the literature and our current study, their findings clearly indicate the presence of sulfate and their ascription of the major peak at

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1105 cm^{-1} to the respective C—O vibrations of organic compounds should therefore be adjusted.

Quantification of Sulfate in DDGS by FT−IR Spectroscopy. In terms of quantification of sulfate, it was crucial to include both the signal of the normal sulfate anion at 615 cm^{-1} and the signal of the distorted sulfate anion at 636 cm^{-1}. Although for DDGS samples naturally containing sulfate simply the absorption at 615 cm^{-1} was noted, for all spiked DDGS samples linear relationship between band area and spiking level was only reached when integrating over both signals. This indicates that all distorted ions absorbing energy around the maximum of 636 cm^{-1} did not contribute to the absorption signal at 615 cm^{-1} anymore (due to band splitting). Hence, quantification of sulfate was based on the integration of the preprocessed FT−IR spectra of both spiked and nonspiked (native) DDGS samples between 654 and 586 cm^{-1} (Figure 1D). For calibration, DDGS sample A, derived from corn and without substantial sulfate level, was spiked with different amounts of sodium sulfate in levels of 1% to 5%, equal to 0.6% to 3.3% sulfate (Figure 2A). Integration of the respective preprocessed FT−IR spectra in the range of 654 cm^{-1} to 586 cm^{-1} resulted in a linear correlation of integrated area and sulfate content with superior confidence limits (Figure 2B). This matrix calibration was therefore used to calculate the sulfate contents in all other DDGS samples analyzed in the study. Generally, precision of FT−IR spectroscopic measurement in the spectral range of interest was good. Coefficient of variation of calculated sulfate contents (n = 10), based on DDGS sample B, was 2.4%, and the process coefficient of variation, based on the matrix calibration of sample A, was 5.6%.

It is also notable that the absorption maxima of the spiked DDGS (sample A) used for calibration (Figure 2A) compared to those of a spiked DDGS (sample C) with native sulfate content (cf. Figure 1D) were shifted two wavenumbers to lower frequency for both bands of the sodium sulfate vibrations (ν_3 and ν_4). However, this did not affect the integration, as the integration window was chosen broad enough to compensate for such shifts in the wavenumber scale. Furthermore, a quantification procedure aiming at the maximum height of the band around 615 cm^{-1} (using corrected absorbance values, data not shown), as it can generally be applied for quantitative IR spectroscopy,[24] was not as good as the approach by band/peak area demonstrated here. The procedure applied for calculation of the band/peak area included linear baseline correction of the spectra in the range of 654 to 586 cm^{-1}, before integration, and was carried out in the OMNIC Macros Basic 7.4 (Thermo Fisher Scientific). Although for the sample spectra, this resulted in slightly negative amplitudes around 640 cm^{-1} (Figure 3), the procedure was necessary in order to apply the calibration after addition of sodium sulfate using DDGS sample A (Figure 2) to the calculation of sulfate levels in all other samples. The main reason for choosing 654 cm^{-1} as the upper wavenumber for the baseline correction (Figure 3) was the additional signal of the distorted sulfate anion.

Comparison of FT−IR Spectroscopy and Capillary Electrophoresis. *Capillary Electrophoresis.* Sulfate content of DDGS samples was not only analyzed by the previously mentioned FT−IR approach, but also by the use of capillary electrophoresis (CE). The results served as reference values for comparison with FT−IR spectroscopy and also for multivariate calibration.

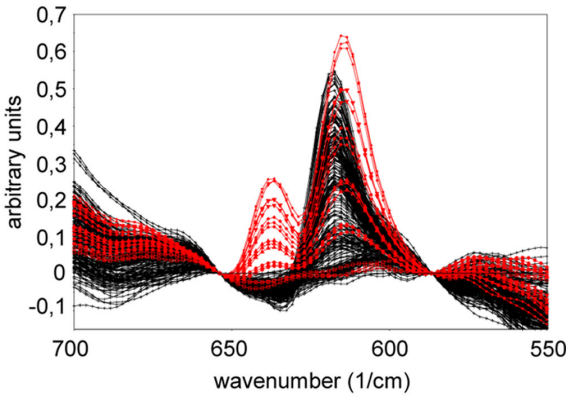

Figure 3. Linear baseline correction of preprocessed FT−IR spectra between 654 and 586 cm^{-1}. Shown are spectra of DDGS sample A, spiked with different amounts of sulfate (red) and spectra of all other DDGS analyzed in the study (black).

The vast majority of the sulfate present in DDGS is derived from sulfuric acid added in the production process and is probably not bound or located in the plant tissue, and therefore a preferably simple method for the extraction of sulfate out of the DDGS samples should work. In addition, several extraction methods for inorganic ions in plant materials had been reported before,[25−27] with sufficient extraction recoveries of sulfate and results indicating even quantitative extraction in comparison to reference methods.[28] Thus, extraction conditions and sample preparation procedure for CE were chosen in consideration of the methods for extraction of sulfate in these previous studies. Accordingly, the extractable sulfate contents analyzed in this study were considered to be capable to serve as reference values for comparison with the results achieved by FT−IR spectroscopy.

To support this assumption, DDGS sample A, without substantial sulfate content, was spiked with different amounts of sodium sulfate (1% to 5%) and analyzed for estimation of recovery. Also, DDGS sample B, with medium sulfate content (1.44%), was extracted ten times and accordingly analyzed by CE to underpin the precision of CE and its suitability as a reference method. Recovery ranged from 94% to 97% dependent on the amount of sulfate added to the sample, relative standard deviation of repetitive measurements (interday precision, n = 10) reached 1.7%. Sulfate was eluting about 3.5 min after the injection of the samples (Figure 4) and was identified by a separate standard addition procedure as well as all the identified peaks in the electropherogram (data not shown). Quantification of sulfate in all DDGS samples was based on correction of the peak area by an internal standard (chlorate) and calculation of the respective sulfate contents in the sample extracts using averaged calibration curves of the single working days.

Comparison of FT−IR and CE Based on Univariate Data. Regression of sulfate contents analyzed by FT−IR spectroscopy against the reference values determined by CE resulted in a linear relationship showing a slight overestimation of the sulfate content by FT−IR (Figure 5A). Although the results for DDGS samples which did not contain sulfate according to CE were leading to both positive and negative sulfate levels by FT−IR (e.g., due to integration of a negligible negative band), the offset in total was nearly zero indicating a good strategy for the

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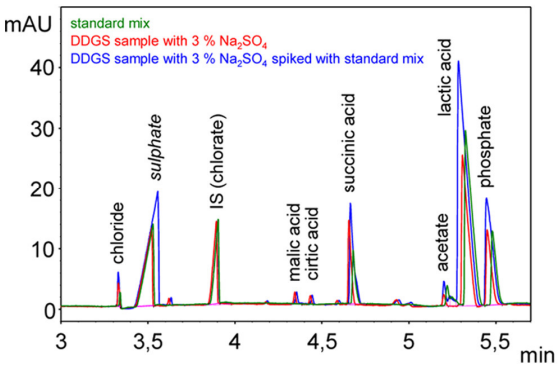

Figure 4. Analysis of sulfate in aqueous extracts of DDGS and in pure standard mix (containing different anions and organic acids) by capillary electrophoresis.

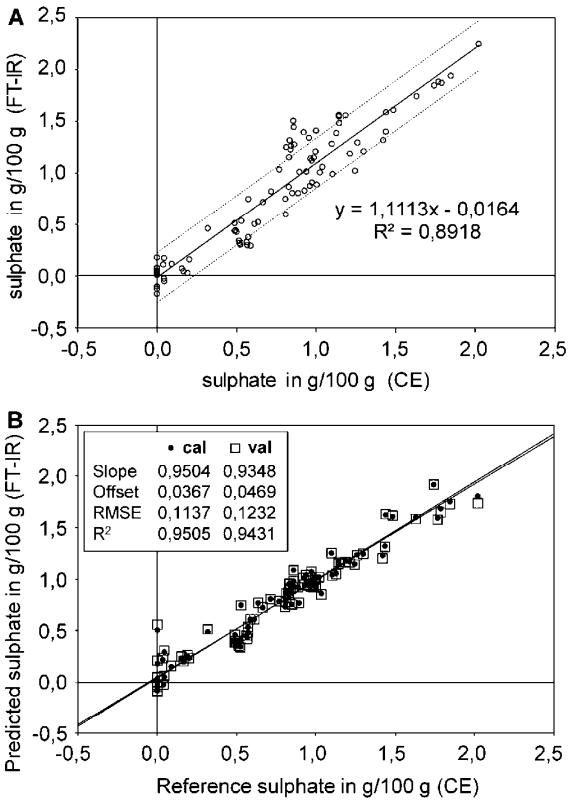

Figure 5. Capability of FT−IR spectroscopy for analysis of sulfate in DDGS. (A) based on integration of bands between 654 and 586 cm^{-1} with confidence intervals drawn by dashed lines; (B) based on PLS regression and 2 latent variables using CE results as reference values; RMSE stands for root mean squared error.

determination of sulfate by FT−IR. However, it was necessary to reintegrate 4 of the 90 samples that had been analyzed by FT−IR spectroscopy (by adjusting the right spectral wavenumber regions manually) as they were slightly shifted outside the region that had been selected for the automated calculation of the peak area (cf. above). In general, it was decided to include all DDGS samples that had been measured for the method comparison (elimination of outliers was omitted) to demonstrate the capability of the proposed strategy on a sample set of DDGS from various origins derived from corn and wheat (cf. SI).

Multivariate Calibration Using PLS Regression. The regression of the univariate results showed a rather high uncertainty for sulfate contents by FT−IR, especially for levels around 0.9% according to CE (cf. Figure 5A). In order to improve the significance of the approach and to overcome difficulties due to spectral window selection for peak area calculation, multivariate calibration was performed applying PLS regression. For this purpose, CE results were used as reference values and after subsequent selection of the most important spectral variables, PLS regression based on 2 latent variables (explained Y-variance: 95.0%) showed a reduced uncertainty with regards to the predictability of sulfate levels of DDGS using FT−IR spectra (Figure 5B). In this case, sulfate content was slightly underestimated by FT−IR spectroscopy, but was nearly unaffected when tested by leave-one-out cross-validation (explained Y-variance: 94.3%) indicating a robust calibration (Figure 5B). The most important finding with regard to the chemical information behind the data was, that the spectral variables used for the final PLS regression were consequentially correlated with the two fundamental IR active vibrations of the sulfate anion. The highest regression coefficients were found for the spectral regions from 1109 to 1088 cm^{-1} and from 623 to 599 cm^{-1} representing the S—O vibrations ν_3 (1105 cm^{-1}) and ν_4 (611 cm^{-1}) of the sulfate anion (Figure 6).

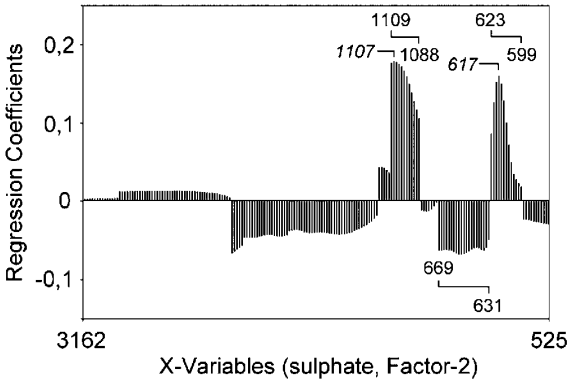

Figure 6. Most important spectral variables used for PLS regression and consistency with the fundamental S—O vibrations of the sulfate anion ν_3 (1105 cm^{-1}) and ν_4 (611 cm^{-1}). Scale of *X*-axis is not continuous.

Without a doubt, there is still a need to prove the robustness and validity of the calibration with an independent external sample set in the future. But in consideration of the sample set with a huge number of different origins included (cf. SI) and the strong correlation to the fundamental vibrations of the sulfate anion, the presented calibration is regarded as already solid at this stage. In general, improved specificity and the nonexisting influence of spectral window selection make the multivariate calibration advantageous, compared with the univariate procedure based on peak area calculation. First and foremost, the approach by FT−IR spectroscopy and subsequent multivariate data analysis could be used for the rapid

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analysis of sulfate levels in DDGS (in terms of screening). By this, single batches of DDGS with high levels of sulfate could be identified and selected for further investigations. If the results indicate high sulfate levels, then verification of the results and analysis of the total sulfur levels (including sulfate) should consequently be carried out by reference methods, such as ICP-MS or X-ray fluorescence spectroscopy.

Estimation: Theoretical Calculation of Total Sulfur Contents. In general, quantitative information on sulfur and/or sulfate in DDGS is of interest to DDGS manufacturers, animal feed producers and farmers. Regarding DDGS, the total amount of sulfur (S) can be classified into S derived from organic compounds and S derived from inorganic compounds: S-rich organic compounds are mainly composed of amino acids containing S atoms or enzymes with S in the prosthetic group; inorganic compounds that contain S are mainly due to sulfate, derived mainly from sulfuric acid used in the production process of DDGS.

In the current study, it was shown that FT−IR spectroscopy could be used for estimation of sulfate levels in DDGS. However, organic forms of S, like S-containing amino acids and proteins, cannot be identified and thus will not be quantified by FT−IR spectroscopy. Nevertheless, the presented approach could be used for routine purposes, as the variability in the total S content of DDGS mainly depends on the inorganic form of S (i.e., sulfate) linked to the amount of sulfuric acid used in the production. In contrast, S-containing organic compounds mainly derive from the plant material (natural origin) and generally only a very low amount is added in the form of yeasts or nutrient salts during the production. Additionally, S derived from the plant material gives a relatively constant input to the production of DDGS, and the major part of it is organically bound with only about 10% of the S from natural origin being present in the form of sulfate.[29,30]

Bearing this in mind, the total S content of DDGS could be estimated, on the basis of a theoretical calculation, as the sum of: (1) the amount of S derived from sulfate (which could be determined by FT−IR spectroscopy) and (2) the amount of S derived from S-containing compounds which were already present in the natural plant material (and would in the main part be due to organic S-containing compounds). The amount of S naturally present in the plant material is dependent on the plant species but is well examined: based on the DM, corn contains an average of 0.12% S,[31] whereas for wheat, cultivated in the 1990s, a mean S content of 1.35 mg/g (0.14%) was reported.[30]

In the final product DDGS, all constituents are concentrated by a factor of approximately three compared to the grain and this would in theory, with regards to the above-mentioned mean S contents of the cereals, result in levels of 0.36% S for corn DDGS and 0.42% for wheat DDGS on a DM basis. However, for a final estimation of the total S contents in DDGS, one would have to consider the percentage of sulfate derived from the plant material, which is approximately 10% of the total S of plant-origin[29,30] and is already covered by the sulfate levels analyzed in this study. Therefore, the total S content of the DDGS samples was theoretically estimated as the sum of the sulfate content (based on the DM) and the "sulfate-corrected" mean S contents from the plant material (based on the DM) of 0.32% for corn DDGS and 0.38% for wheat DDGS (and DDGS of mixed origin). Finally, this resulted in estimated total S contents for the DDGS samples between 0.29% and 1.06% (median 0.63%) based on FT−IR spectroscopy using PLS regression, and consistently, in 0.32% to 1.08% (median 0.63%) when calculating with the reference values which have been analyzed by CE (Figure 7).

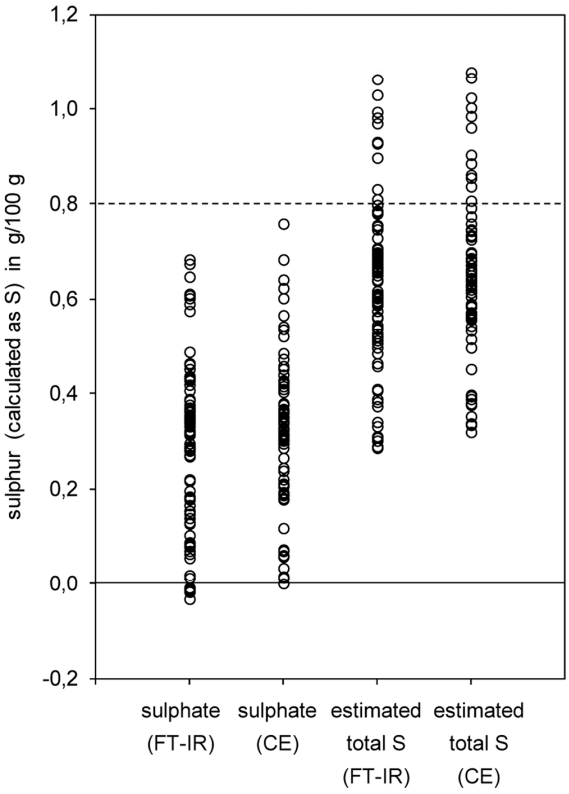

Figure 7. Distribution of sulfur contents of DDGS samples based on sulfate (from chemical analysis) and on estimation of total S contents (based on theoretical calculation). Sulfate (FT−IR) and sulfate (CE) are representing the sulfate levels (calculated as S) based on the DM of the samples; negative values are due to the multivariate calibration approach and should be regarded as zero/not detectable. Total S (FT−IR) and total S (CE) are estimated results of total S contents (calculated as S) additionally considering the theoretical input of plant-derived sulfur. The dashed line illustrates the maximum level of 0.8% S in DDGS discussed in literature.

With regards to the maximum tolerable dietary S level for beef cattle of 0.4% on a DM basis,[9] one has to consider that this dietary concentration could be exceeded if DDGS with a sulfur content greater than 0.80% (DM basis) were fed to ruminants at high inclusion rates (DDGS: 40% of DM intake), in addition to corn and corn silage assuming low sulfate levels in drinking water.[5,10] In such a scenario, 11% (based on FT−IR prediction), respectively 13% (based on CE results) of the DDGS samples analyzed in this study showed excessive sulfur levels on the basis of the theoretical calculation (Figure 7). However, such DDGS could still be fed at a lower inclusion rate or even mixed with batches of DDGS containing lower sulfur levels. This would not only protect ruminants of S toxicity and PEM, but could also show general advantages, because it has been reported that both ruminants and nonruminants show decreased feed intake and gain when feeding a high dietary S level.[12,13]

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General Aspects and Future Perspectives. The study demonstrated that ATR/FT−IR spectroscopy can be applied to the determination of sulfate levels in the matrix DDGS. With regard to the maximum tolerable dietary S level for beef cattle of 0.4%, FT−IR spectroscopy could evaluate whether sulfate levels are low enough to use DDGS in high inclusion rates of the diet. It could serve as a prescreening method to select batches of DDGS with high sulfate levels before using techniques such as X-ray fluorescence spectroscopy or ICP-MS, especially if those are not affordable or FT−IR spectroscopy is already in use for other purposes. However, the final evaluation of sulfur levels in the study was based on an theoretical estimation combining the analyzed sulfate levels with the theoretical percentage of plant-derived sulfur in the form of organic S (as mean levels). Further investigation whether such an approach can be used in routine analysis should therefore be cross-validated with a complementary method (e.g., X-ray fluorescence spectroscopy) in the future. In conclusion, FT−IR spectroscopy can be applied for the comparison of DDGS batches regarding the sulfate content, which could be especially useful for blending of animal feed or adaption of process parameters in the production of DDGS.

■ ASSOCIATED CONTENT

Supporting Information

Detailed information on investigated DDGS samples (botanical origin, geographical origin, and method of production). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Table A1: Detailed information on investigated DDGS samples (extended sample set)

Sample #	Sample code	Botanical origin	Geographical origin of DDGS (country)	Method of production	Crude fat (%) [1]
1	CORN-CAU-01	corn	China, Jilin province	bio-ethanol	7,33
2	CORN-CAU-02	corn	China, Jilin province	bio-ethanol	7,35
3	CORN-CAU-03	corn	China, Jilin province	bio-ethanol	7,40
4	CORN-CAU-04	corn	China, Jilin province	bio-ethanol	7,42
5	CORN-CAU-05	corn	China, Jilin province	bio-ethanol	8,06
6	CORN-CAU-07	corn	China, Jilin province	bio-ethanol	11,13
7	CORN-CAU-08	corn	China, Jilin province	bio-ethanol	11,19
8	CORN-CAU-09	corn	China, Jilin province	bio-ethanol	8,80
9	CORN-CAU-10	corn	China, Jilin province	bio-ethanol	8,53
10	CORN-CAU-11	corn	China, Jilin province	bio-ethanol	8,63
11	CORN-CAU-12	corn	China, Heilongjiang province	bio-ethanol	2,70
12	CORN-CAU-13	corn	China, Heilongjiang province	bio-ethanol	2,88
13	CORN-CAU-14	corn	China, Heilongjiang province	bio-ethanol	2,98
14	CORN-CAU-16	corn	China, Heilongjiang province	bio-ethanol	3,21
15	CORN-CAU-17	corn	China, Heilongjiang province	bio-ethanol	3,60
16	CORN-CAU-18	corn	China, Heilongjiang province	bio-ethanol	2,84
17	CORN-CAU-19	corn	China, Heilongjiang province	bio-ethanol	3,32
18	CORN-CAU-20	corn	China, Heilongjiang province	bio-ethanol	3,74
19	CORN-CAU-21	corn	China, Heilongjiang province	bio-ethanol	3,29
20	CORN-CAU-22	corn	China, Heilongjiang province	bio-ethanol	4,16
21	CORN-CAU-24	corn	China, Heilongjiang province	bio-ethanol	5,41
22	CORN-CAU-25	corn	China, Heilongjiang province	bio-ethanol	2,70
23	CORN-CAU-26	corn	China, Heilongjiang province	bio-ethanol	2,94
24	CORN-CAU-27	corn	China, Heilongjiang province	bio-ethanol	3,07
25	CORN-CAU-28	corn	China, Heilongjiang province	bio-ethanol	2,99
26	CORN-CAU-29	corn	China, Heilongjiang province	bio-ethanol	2,82
27	CORN-CAU-30	corn	China, Heilongjiang province	bio-ethanol	3,16
28	CORN-CAU-31	corn	China, Heilongjiang province	bio-ethanol	2,85
29	CORN-THOM-03	corn	USA	alc. beverage	8,78
30	CORN-THOM-04	corn	USA	alc. beverage	9,11
31	CORN-THOM-05	corn	USA	alc. beverage	8,47
32	CORN-THOM-06	corn	USA	alc. beverage	8,79
33	CORN-THOM-07	corn	USA	alc. beverage	8,50
34	CORN-CAU-06	corn	China, Jilin province	bio-ethanol	10,67
35	CORN-CAU-15	corn	China, Heilongjiang province	bio-ethanol	4,06
36	CORN-CAU-23	corn	China, Heilongjiang province	bio-ethanol	2,87
37	CORN-THOM-08	corn	USA	alc. beverage	9,01
38	CORN-THOM-09	corn	USA	alc. beverage	11,03
39	CORN-THOM-10	corn	USA	alc. beverage	10,81
40	MIXED-BfR-01	wheat, corn	Germany	bio-ethanol	5,19

Table A1 (continued): Detailed information on investigated DDGS samples (extended sample set)

Sample #	Sample code	Botanical origin	Geographical origin of DDGS (country)	Method of production	Crude fat (%) [1]
41	WHEAT-PROV-01	wheat	unknown	unknown	5,32
42	WHEAT-PROV-02	wheat	unknown	unknown	3,89
43	WHEAT-PROV-03	wheat	unknown	unknown	4,87
44	WHEAT-PROV-04	wheat	unknown	unknown	4,93
45	WHEAT-PROV-05	wheat	unknown	unknown	3,28
46	WHEAT-PROV-06	wheat	France	bio-ethanol	5,12
47	WHEAT-PROV-07	wheat	France	bio-ethanol	4,19
48	WHEAT-CRAW-01	wheat	unknown	bio-ethanol	5,40
49	CORN-PROV-01	corn	unknown	unknown	11,42
50	CORN-PROV-02	corn	unknown	unknown	11,40
51	CORN-PROV-03	corn	unknown	unknown	12,34
52	CORN-PROV-05	corn	unknown	unknown	11,22
53	CORN-PROV-06	corn	unknown	bio-ethanol	9,84
54	CORN-PROV-07	corn	unknown	bio-ethanol	9,20
55	CORN-PROV-08	corn	unknown	bio-ethanol	9,04
56	CORN-PROV-09	corn	unknown	bio-ethanol	10,12
57	CORN-PROV-10	corn	unknown	bio-ethanol	11,93
58	CORN-PROV-11	corn	unknown	bio-ethanol	11,61
59	CORN-THOM-02	corn	USA	alc. beverage	9,08
60	CORN-THOM-11	corn	USA	alc. beverage	9,23
61	CORN-THOM-12	corn	USA	alc. beverage	11,05
62	CORN-THOM-13	corn	USA	alc. beverage	10,13
63	CORN-THOM-14	corn	USA	alc. beverage	11,88
64	CORN-THOM-15	corn	USA	alc. beverage	11,54
65	CORN-THOM-16	corn	USA	alc. beverage	11,99
66	CORN-THOM-17	corn	USA	alc. beverage	12,31
67	CORN-RIKILT-01	corn	Netherlands	bio-ethanol	12,39
68	MIXED-PROV-01	mixed	unknown	unknown	4,17
69	MIXED-RIKILT-01	50% wheat and 50% corn	Belgium	bio-ethanol	7,15
70	WHEAT-QUB-01	wheat	Canada, Saskatchewan	bio-ethanol	4,96
71	WHEAT-QUB-02	wheat	Canada, Saskatchewan	bio-ethanol	5,12
72	WHEAT-THOM-01	wheat	France	bio-ethanol	5,07
73	WHEAT-THOM-02	wheat	France	bio-ethanol	4,87
74	WHEAT-THOM-03	wheat	France	bio-ethanol	4,90
75	WHEAT-THOM-04	wheat	France	bio-ethanol	5,50
76	WHEAT-THOM-06	wheat	France	bio-ethanol	4,95
77	CORN-QUB-01	corn [2]	USA, SE Indiana	alc. beverage	11,02
78	CORN-QUB-02	corn	USA, SE Illinois	bio-ethanol	9,00
79	CORN-QUB-04	corn	USA, Wisconsin	bio-ethanol	9,02
80	CORN-QUB-05	corn	USA, Wisconsin	bio-ethanol	11,41

Table A1 (continued): Detailed information on investigated DDGS samples (extended sample set)

Sample #	Sample code	Botanical origin	Geographical origin of DDGS (country)	Method of production	Crude fat (%) [1]
81	CORN-BfR-01	corn	Poland, Goswinowice	bio-ethanol	10,33
82	CORN-BfR-02	corn	USA, Illinois	bio-ethanol	8,20
83	CORN-PROV-04	corn	unknown	unknown	12,07
84	CORN-THOM-18	corn	USA	alc. beverage	11,85
85	CORN-THOM-19	corn	USA	alc. beverage	12,02
86	CORN-JRC-01	corn	Spain	bio-ethanol	12,43
87	RICE-QUB-01	rice	India	alc. beverage	3,30
88	WHEAT-BfR-01	wheat	EU (probably Sweden)	unknown	4,55
89	WHEAT-BfR-02	wheat [2]	Belgium	unknown	7,45
90	WHEAT-QUB-03	wheat	Canada, Saskatchewan	bio-ethanol	4,35
91	WHEAT-QUB-04	wheat	Canada, Saskatchewan	bio-ethanol	5,30
92	WHEAT-THOM-08	wheat	France	bio-ethanol	4,92
93	WHEAT-THOM-09	wheat	France	bio-ethanol	4,87
94	WHEAT-THOM-10	wheat	France	bio-ethanol	4,95
95	WHEAT-BfR-03	wheat	Sweden	alc. beverage	5,20
96	WHEAT-BfR-04	wheat	Sweden	bio-ethanol	5,32
97	WHEAT-CRAW-02	wheat	unknown	bio-ethanol	5,45
98	WHEAT-CRAW-03	wheat	unknown	bio-ethanol	5,59
99	CORN-PROV-13	corn	USA	bio-ethanol	8,61
100	CORN-PROV-14	corn	USA	bio-ethanol	12,26
101	CORN-PROV-15	corn	USA	bio-ethanol	9,01
102	CORN-PROV-16	corn	USA	bio-ethanol	12,16
103	CORN-PROV-17	corn	unknown	unknown	5,51
104	CORN-PROV-18	corn	unknown	unknown	11,52
105	CORN-PROV-19	corn	unknown	unknown	5,37
106	CORN-THOM-21	corn	USA	alc. beverage	8,88
107	CORN-THOM-22	corn	USA	alc. beverage	5,81
108	CORN-THOM-23	corn	USA	alc. beverage	10,69
109	CORN-THOM-24	corn	USA	alc. beverage	11,74
110	CORN-CRAW-01	corn	unknown	bio-ethanol	12,61
111	CORN-CRAW-02	corn	unknown	bio-ethanol	13,30
112	CORN-BfR-03	corn	Hungary, Dunaföldvar	bio-ethanol	12,07
113	CORN-BfR-04	corn [2]	Poland, Goswinowice	bio-ethanol	14,16
114	CORN-VSCHT-02	corn	Czech Republic	bio-ethanol	10,83
115	CORN-VSCHT-03	corn	Czech Republic	bio-ethanol	10,48
116	CORN-VSCHT-04	corn	Czech Republic	bio-ethanol	10,37
117	CORN-VSCHT-05	corn	Czech Republic	bio-ethanol	10,45
118	CORN-VSCHT-06	corn	Czech Republic	bio-ethanol	9,71
119	CORN-VSCHT-07	corn	Czech Republic	bio-ethanol	10,29
120	CORN-VSCHT-08	corn	Czech Republic	bio-ethanol	10,06

Table A1 (continued): Detailed information on investigated DDGS samples (extended sample set)

Sample #	Sample code	Botanical origin	Geographical origin of DDGS (country)	Method of production	Crude fat (%) [1]
121	CORN-VSCHT-09	corn	Czech Republic	bio-ethanol	10,58
122	CORN-VSCHT-10	corn	Czech Republic	bio-ethanol	10,34
123	CORN-CAU-32	corn	China, Heilongjiang province	bio-ethanol	3,42
124	CORN-CAU-33	corn	China, Heilongjiang province	bio-ethanol	3,32
125	CORN-CAU-34	corn	China, Heilongjiang province	bio-ethanol	3,45
126	CORN-CAU-35	corn	China, Heilongjiang province	bio-ethanol	3,22
127	CORN-CAU-36	corn	China, Heilongjiang province	bio-ethanol	3,22
128	CORN-CAU-37	corn	China, Heilongjiang province	bio-ethanol	3,16
129	CORN-CAU-38	corn	China, Heilongjiang province	bio-ethanol	3,28
130	CORN-CAU-39	corn	China, Heilongjiang province	bio-ethanol	2,77
131	CORN-CAU-40	corn	China, Jilin province	bio-ethanol	7,74
132	CORN-CAU-41	corn	China, Jilin province	bio-ethanol	7,66
133	CORN-CAU-42	corn	China, Jilin province	bio-ethanol	8,06
134	CORN-CAU-43	corn	China, Jilin province	bio-ethanol	7,48
135	CORN-CAU-44	corn	China, Jilin province	bio-ethanol	7,76
136	CORN-CAU-45	corn	China, Jilin province	bio-ethanol	7,63
137	MIXED-BfR-02	92% wheat, 8% barley	Scotland, North East	alc. beverage	4,82
138	CORN-VSCHT-01	corn	Czech Republic	bio-ethanol	9,84
139	CORN-PROV-20	corn	USA	bio-ethanol	9,94
140	CORN-VSCHT-11	corn	Czech Republic	bio-ethanol	10,28
141	CORN-CAU-46	corn	China, Heilongjiang province	bio-ethanol	2,74
142	CORN-CAU-47	corn	China, Jilin province	bio-ethanol	7,74
143	CORN-THOM-20	corn	USA	alc. beverage	11,89
144	CORN-QUB-03	corn	USA, NW Illinois	bio-ethanol	12,97
145	WHEAT-THOM-05	wheat	France	bio-ethanol	4,88
146	WHEAT-THOM-07	wheat	France	bio-ethanol	5,10
147	CORN-THOM-26	corn	USA	alc. beverage	11,81
148	CORN-THOM-27	corn	USA	alc. beverage	11,50
149	CORN-THOM-28	corn	USA	alc. beverage	12,04
150	CORN-THOM-29	corn	USA	alc. beverage	10,94
151	CORN-THOM-30	corn	USA	alc. beverage	11,08
152	CORN-THOM-31	corn	USA	alc. beverage	10,98
153	CORN-THOM-32	corn	USA	alc. beverage	11,71
154	CORN-THOM-33	corn	USA	alc. beverage	11,38
155	CORN-VSCHT-12	corn	Czech Republic	bio-ethanol	9,86
156	CORN-VSCHT-13	corn	Czech Republic	bio-ethanol	9,92
157	CORN-VSCHT-14	corn	Czech Republic	bio-ethanol	9,95
158	CORN-VSCHT-15	corn	Czech Republic	bio-ethanol	9,83
159	CORN-VSCHT-16	corn	Czech Republic	bio-ethanol	9,82
160	CORN-VSCHT-17	corn	Czech Republic	bio-ethanol	9,92

Table A1 (continued): Detailed information on investigated DDGS samples (extended sample set)

Sample #	Sample code	Botanical origin	Geographical origin of DDGS (country)	Method of production	Crude fat (%) [1]
161	CORN-VSCHT-18	corn	Czech Republic	bio-ethanol	9,93
162	CORN-VSCHT-19	corn	Czech Republic	bio-ethanol	9,78
163	CORN-VSCHT-20	corn	Czech Republic	bio-ethanol	9,82
164	CORN-VSCHT-21	corn	Czech Republic	bio-ethanol	9,92
165	CORN-BfR-06	probably corn	China, Heilongjiang	probably bio-ethanol	2,89
166	CORN-BfR-05	corn	Poland, Goswinowice	bio-ethanol	11,68
167	CORN-THOM-34	corn	Netherlands	bio-ethanol	12,02
168	CORN-THOM-35	corn	Netherlands	bio-ethanol	12,19
169	CORN-THOM-36	corn	USA	alc. beverage	11,30
170	CORN-THOM-37	corn	Netherlands	bio-ethanol	12,17
171	CORN-THOM-38	corn	Netherlands	bio-ethanol	11,92
172	CORN-THOM-39	corn	Netherlands	bio-ethanol	12,05
173	WHEAT-QUB-05	wheat	Sweden	unknown	5,07
174	CORN-QUB-06	corn	USA	probably bio-ethanol	7,79
175	CORN-QUB-07	corn	USA	probably bio-ethanol	7,91
176	CORN-QUB-08	corn	USA	probably bio-ethanol	11,31
177	CORN-QUB-09	corn	USA	unknown	9,96
178	CORN-QUB-10	corn	USA	unknown	11,32
179	CORN-QUB-11	corn	USA	unknown	11,42
180	CORN-QUB-12	corn	USA	unknown	11,06
181	CORN-QUB-13	corn	USA	alc. beverage	11,30
182	CORN-QUB-14	corn	Hungary	bio-ethanol	11,97
183	CORN-QUB-15	corn (mixed*)	Austria	bio-ethanol	7,55
184	CORN-QUB-16	corn (mixed*)	Austria	bio-ethanol	6,83
185	CORN-QUB-17	corn (mixed*)	Austria	bio-ethanol	7,73
186	CORN-QUB-18	corn (mixed*)	Austria	bio-ethanol	11,54
187	CORN-QUB-19	corn (corn*)	Austria	bio-ethanol	11,98
188	WHEAT-QUB-06	wheat (mixed*)	Austria	bio-ethanol	7,42
189	WHEAT-QUB-07	wheat (mixed*)	Austria	bio-ethanol	7,97
190	WHEAT-QUB-08	wheat (mixed*)	Austria	bio-ethanol	6,08
191	WHEAT-QUB-09	wheat (mixed*)	Austria	bio-ethanol	7,60
192	WHEAT-QUB-10	wheat (mixed*)	Austria	bio-ethanol	7,47
193	WHEAT-QUB-11	wheat (mixed*)	Austria	bio-ethanol	9,31
194	WHEAT-QUB-12	wheat (mixed*)	Austria	bio-ethanol	8,41
195	MIXED-QUB-01	mixed (corn*)	Austria	bio-ethanol	11,19
196	MIXED-QUB-02	mixed (corn*)	Austria	bio-ethanol	11,28
197	CORN-PROV-12	corn [3]	EU	bio-ethanol	11,16

[1] crude fat content obtained from gravimetric determination, analytical procedure described in publication A

[2] $\delta^{13}C$ values analyzed by IRMS, not used for PCA after FT-IR spectroscopy; CORN-QUB-01 and CORN-BfR-04 not included in CDA, PCA-LDA, and SIMCA models for geographical origin

[3] analyzed by FT-IR spectroscopy (not by IRMS) and included in respective models for geographical origin

* botanical origin indicated after IRMS analysis; equals the class membership used for statistical data analysis (of both FT-IR spectroscopy and IRMS); only corn* samples included in CDA, PCA-LDA, and SIMCA models for geographical origin

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Weiterhin versichere ich, dass ich keine vergeblichen Promotionsversuche unternommen habe und die Dissertation in der gegenwärtigen bzw. in einer anderen Fassung keiner anderen Fakultät vorgelegen hat.

Remshalden, den 28. Dezember 2016

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