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Analytical strategies for the early quality and safety assurance in the global feed chain
Approaches for nitrogen adulterants in soybean meal and mineral and transformer oils in vegetable oils

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ABSTRACT

In the past decade, several major food safety crises originated from problems with feed. Consequently, there is an urgent need for early detection of fraudulent adulteration and contamination in the feed chain. Strategies are presented for two specific cases, viz. adulterations of (i) soybean meal with melamine and other types of adulterants/contaminants and (ii) vegetable oils with mineral oil, transformer oil or other oils. These strategies comprise screening at the feed mill or port of entry with non-destructive spectroscopic methods (NIRS and Raman), followed by post-screening and confirmation in the laboratory with MS-based methods. The spectroscopic techniques are suitable for on-site and on-line applications. Currently they are suited to detect fraudulent adulteration at relatively high levels but not to detect low level contamination. The potential use of the strategies for non-targeted analysis is demonstrated.

Contents

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1. Introduction

Controls performed to ensure the quality and safety within the feed chain are mainly carried out at the laboratory level. The analytical methods applied are usually focused on a single adulterant or contaminant or on a group of adulterants or contaminants in the case of multi-residue methods. The limitation of such targeted approaches is their incapacity to detect unforeseen problems. Two recent examples where official control failed with strong impact on the feed and food chain were fraudulent adulterations of feed materials used as protein sources with melamine [1,2] and oils with technical fats containing dioxins [3].

The dioxin and melamine crises were only discovered at a late stage due to the fact that only a small percentage of the products entering the feed chain is tested for contaminants and, in the case of melamine, also because this chemical compound was not included in the regular monitoring schemes.

Feed chain controls have been adapted in order to tackle these specific problems, but little has been done in relation to the early detection of new adulterants and contaminants and consequently for prevention of the next crises. The questions that are now being posed are “What will be the next problems with feed adulterants / contaminants?” and “How to be able to detect them before they contaminate the feed chain and the feed business?”

There is an urgent need to investigate ways to combine existing test methods and emerging technologies into comprehensive analytical strategies for the early quality and safety assurance in the feed chain. It should be emphasized that, although adulterations do not necessarily lead to contamination at high levels, in many instances this was the case, e.g. in the Belgian dioxin crisis where fraudulent adulteration with transformer oil led to high dioxin and PCB levels in fat used as feed material [4]. Therefore there is a need for methodology allowing the detection of possible problems at different strategic points in the chain, viz. at the entrance of feed materials into the European Union (i.e. ports of entry) or at the entrance of feed materials into feed production plants. It is crucial to develop tools that flag suspicious lots at an early stage and, after appropriate confirmation of the adulterant/contaminant, remove these products from the feed (food) chain.

For initial screening, several types of methods could be applied, viz. immunochemical methods, such as dipsticks, effect assays and spectroscopic methods. Important requirements for strategies for early quality and safety assurance in the feed chain are that methods can be applied on-site, on-line and in-line and moreover that the methods are suitable for non-targeted screening. While all types of methods can be used on-site, only the spectroscopic techniques lend themselves for on-line and in-line applications and non-targeted approaches. For this reason spectroscopic methods, viz. Near Infrared (NIR)-based techniques and Raman spectroscopy were selected as candidate initial screening methods. These techniques are already routinely used in the industry to control both raw materials and finished products for specific production standards, flagging deviations from quality standards that occur during production. Near infrared spectroscopy (NIRS) is the most widely used non-destructive method in the feed industry to determine quality parameters of feed materials. The high throughput of the method, the capacity to determine in one single analysis a large variety of parameters (from major constituents to criteria such as digestibility) and the possibility to build a network of spectrometers made this technique very attractive for the feed sector [5–7]. Raman spectroscopy is now well established as a non-destructive, non-contact analysis method, which provides vibrational spectra of molecular samples within seconds, i.e. molecular fingerprints. Advances in optoelectronics have driven down both the size and the cost of the instrumentation required. In addition, the new generation of compact, fully automated instruments can be used by non-specialist operators. These advances have upgraded Raman spectroscopy to a highly regarded technique across a range of analytical problems in agricultural and food analysis which has recently been reviewed [8]. Since the major components of feeds are fats, proteins and carbohydrates, Raman offers itself as a potential technology for feed analysis. It can be directed towards the determination of components of interest such as fatty acids [9,10], but also the spectral patterns can be used as fingerprints to detect adulteration within samples, e.g. fats or olive oil [7,8,11–13].

New technologies based on (ultra) high performance liquid chromatography combined with high resolution mass spectrometric detection (e.g. UPLC-TOF-MS, UPLC-Orbitrap-MS), comprehensive gas chromatography combined with mass spectroscopy (e.g. GCxGC-TOF-MS) and ambient mass spectrometry (DART)) can be applied at laboratory level for fast full scan analysis of feed materials. Until now, this full scan information has mainly been utilized for library-based targeted multi-class screening and confirmation of known contaminants [14–19]. However, these techniques also have high potential for non-targeted or semi-targeted fingerprinting [20,21], with possibilities to detect deviations from normal patterns, flagging suspicious feed materials for further analysis and confirmation with the same type of techniques.

In this paper, strategies will be presented to apply existing methods and new approaches, including fingerprinting techniques, and to combine them into an integrated approach that can be applied to check (at ports, in feedmills and in the laboratory) the quality and safety of feed materials, to detect non-conformity and subsequently to identify adulterants and contaminants. The methods and procedures described in this paper have mainly been developed in the framework of the European Research project Quality and Safety of Feed and Food for Europe (QSAFFE).

Strategies have been elaborated for two specific cases, viz.

(i) Adulteration of feed materials that are included in animal feed diets as protein sources with melamine and its by-products (cyanuric acid, ammelide and ammeline) and with other types of adulterants/contaminants. In QSAFFE soybean meal has been selected as a model matrix.
(ii) Adulteration of vegetable oils and fats with mineral oil or transformer oil or with alternative oil/fat sources with a lower economic value.

2. Materials and methods

2.1. NIR spectroscopy to detect N-adulterants in feed at laboratory, feed mill or port of entry level

Two different near Infrared (NIR) spectrometers, which are active in the 400–2500 nm VIS-NIR scanning were used. The spectral data generated, at laboratory level, by samples of pure soybean meal were used to develop chemometric tools to authenticate it [22]. Soybean meal samples contaminated with different percentages of melamine, cyanuric acid or whey milk serum were used to validate the chemometric protocol, which was later transferred to the feed mill and to the port of entry [23].

2.2. Raman spectroscopy to detect N-adulterants in soybean meal

Soybean meal was ground and the fraction smaller than 250 µm was used for the analysis. The spectra were acquired in a Fourier Transformed Raman module coupled to a Fourier Transformed Infrared Spectrometer equipped with two excitation lasers at 785 nm and 1064 nm and a Germanium detector. Dedicated software was used for instrument control and spectral acquisition. The data processing and treatment is described elsewhere [24].

2.3. NIR hyperspectral imaging spectroscopy to determine N-adulterants in feed

Hyperspectral images were collected using an NIR hyperspectral line scan or push-broom imaging system combined with a conveyor belt that includes a spectrograph with a cooled, temperature-stabilized Mercury-Cadmium-Telluride (MCT) detector. Prior to analysis, the spectral imaging system was calibrated with a dark image and a white image. The spectra were then automatically corrected accordingly. The images obtained with this instrument for this work consist of 2000 frames of 320 pixels acquired at 209 wavelengths (1100–2400 nm).

Based on the images of pure products (soybean meal, melamine and cyanuric acid), a complete database was built, allowing a complete exploratory analysis of the data using Principal Component Analysis (PCA) and the construction of chemometric models using Partial Least Squares Discriminant Analysis (PLS-DA) to discriminate between the species, and therefore to characterize the soybean meal [6,25].

2.4. NIR microscopy to detect melamine in soybean meal

A detailed description of the methodology used to detect melamine by NIR microscopy in soybean meal can be found elsewhere [26,27]. Briefly, samples were analyzed on a line imaging system whereby 16 spectra are acquired simultaneously. The wavenumber range is 7800–4000 cm^{-1}. Furthermore, a calibration set that included pixels of melamine and soybean meal was also used to construct models based on Partial Least Squares Discriminant Analysis (PLS-DA) and Support Vector Machine Discriminant Analysis (SVM-DA) to discriminate melamine and cyanuric acid in soybean meal.

2.5. LC-HRMS to analyze N-adulterants in soybean meal

An amount of homogenized sample was extracted with acetonitrile/water. The extract was centrifuged, filtered, diluted and injected into the LC-HRMS system. The chromatography was performed on a weak anion exchange column. A gradient containing acetonitrile/isopropanol/water with 50 mM ammonium acetate (85/10/5, v/v) (A) and water/acetonitrile (90/10, v/v) (B) was applied. The LC system was coupled to a high-resolution mass spectrometer. Full-scan data were acquired from 123 to 185 m/z. A high resolving power of 50,000 FWHM (full width at half maximum) was applied.

2.6. LC-QTOF-MS and LC-Orbitrap-MS to analyze pesticides residues, mycotoxins and plant toxins in soya-based matrices

The method to simultaneously determine 306 pesticides, namely organophosphorus, carbamates, triazines, urea-derivates, pyrethroids, triazoles, strobilurins, neonicotinoids, imidazoles, imidazolinones, chloroacetamides, etc., 51 mycotoxins (regulated and emerging ones) and 11 pyrrolizidine alkaloids (PAs) was presented elsewhere [28].

The homogenised samples were extracted with acidified water with formic acid and acetonitrile. The extract was centrifuged and an aliquot of the extract filtered and transferred into an autosampler vial.

The LC-QTOF-MS analyses were performed using an Ultra-Performance LC system equipped with a C18 column compatible with 100% aqueous mobile phase. 5 mM ammonium formate and 0.2% formic acid in both Milli-Q water and methanol were used in ESI(+). In ESI(−) 5 mM ammonium acetate in Milli-Q water and pure methanol were used. This UHPLC system was connected to an orthogonal accelerated high-resolution time-of-flight mass spectrometer, which operated in both ESI(+) and ESI(-). Raw mass spectra were acquired in the m/z range 50–1200. A high resolving power greater than 25,000 FWHM was applied.

The UHPLC-Orbitrap-MS analyses were performed using a quaternary-pump LC system equipped with a new generation C18 column. Mobile phase composition was (A) 5 mM ammonium formate and 0.1% formic acid in Milli-Q water and (B) methanol and 0.1% formic acid. This system was connected to a high-resolution Orbitrap mass spectrometer operated in polarity switching ESI(+) and ESI(−) modes. The data were acquired over a mass range of m/z 50–1000 at a mass resolving power of 50,000 FWHM.

For both systems, specific software was used for data acquisition and data processing.

2.7. UHPLC-HRMS/MS to determine mycotoxins in feed and DDGs

The details of the extraction and analysis procedure to analyse 57 mycotoxins, regulated and emerging ones, are described elsewhere [29,30]. Briefly, a representative and homogeneous sample was extracted using a QuEChERS procedure.

Analyses were carried out by an Ultra-Performance LC system equipped with a C18 column compatible with 100% aqueous mobile phase. The mobile phases were different for ESI(+) and ESI(−) analysis. The 5 mM ammonium formate and 0.2% formic acid in both Milli-Q water and methanol was used in ESI(+). In ESI(−) 5 mM ammonium acetate in Milli-Q water and pure methanol were used. The LC system was coupled to a high-resolution MS/MS that is able to operate in both positive and negative mode.

2.8. LC-MS/MS for the screening of mycotoxins

This method was capable of detecting up to 72 mycotoxins. Mycotoxins included in the method were aflatoxins, fumonisins, trichothecenes, *Alternaria* toxins, ergot alkaloids, zearalenone and derivatives, enniatins, beauvericin as well as many other *Fusarium* and *Penicillium* mycotoxins, as well masked forms of some of the mycotoxins.

The sample was weighed, extracted using a mixture of acetonitrile/water/acetic acid and centrifuged. An aliquot of the supernatant was diluted and filtered by syringe filter. The final extract was analysed by UPLC-MS/MS. Two analytical runs, one using neutral

mobile phase conditions and one using acidic conditions, were required.

2.9. LC-HRMS to analyze veterinary drugs in dried distiller grains with solubles (DDGs)

A detailed description of the method to determine veterinary drugs, namely ionophore and non-ionophore authorized coccidiostats, banned coccidiostats, macrolides, tetracyclines, quinolones, sulphonamides, nitroimidazoles, benzoimidazoles, non-steroidal anti-inflammatory drugs, tranquillizers and amphenicols in DDGs can be found elsewhere [31]. Briefly, approximately 3 g of DGGs were extracted with a mixture of acetonitrile/methanol/water in 1% formic acid. Then, the sample was centrifuged, filtered and the extracts were directly injected in the LC-HRMS system set in full scan mode. The separation of the analytes was carried out in reverse phase using a C18 analytical column. The mobile phase consisted of 0.5% (v/v) formic acid in water (eluent A) and 0.5% (v/v) formic acid in methanol (eluent B). The LC system was coupled to a high-resolution mass spectrometer. The mass resolving power was set at 50,000 FWHM.

2.10. Raman spectroscopy to detect adulterations of vegetable oil with transformer or mineral oil

The method developed to detect adulteration of oil by Raman spectroscopy at laboratory level is described elsewhere [32]. Briefly, 300 µL of an oil sample were pipetted into a glass vial and analysed using a Raman Spectrometer. All the samples were computed at 10 cm^{-1} resolution across the spectral range 200–2200 cm^{-1}. Instrument control was performed using specific software and the library development software, which was provided with the instrument. The Raman spectroscopic data were analysed by multivariate analysis.

This methodology has been successfully installed and implemented in a feed mill and applied to samples directly taken from a ship at the port entry.

2.11. GCxGC-ToFMS to screen environmental contaminants in fats and oils

The screening method to screen for the presence of environmental contaminants was presented elsewhere [33]. The oil samples were diluted with hexane and cleaned up with silica deactivated at 2% (w/w) with water. The eluate was evaporated with solvent exchange (*iso*-octane) under a gentle nitrogen stream and the final extract transferred to GC-vials. The extracts were injected on the GC × GC–ToFMS. The configuration of the GC × GC system was as follows: a semi-polar column was used for the first dimension, while a non-polar stationary phase for the second dimension. The modulation time was set 3 s. The data acquisition rate was 200 scans/s, covering a mass range of 50–750 m/z. For automated spectrum-based detection of contaminants MetAlignID, a non-proprietary software (freely downloadable), was used [34]. For detection by spectral features, a scripting software module, embedded in the LECO ChromaTOF software, was used [35].

2.12. DART-Orbitrap-MS analysis to screen oil adulteration

The profile of the oil samples was screened using DART-Orbitrap-MS technology. A few grams of the oil were dissolved in hexane and directly analysed. The DART–Orbitrap-MS instrument was operated in positive ionisation mode and the settings of the system parameters were optimized for the specific application [36]. An aqueous solution of ammonia was used to enhance the ionization of the triacylglycerides (TG). The acquisition rate was set at 2 spectra/s, under this setting the mass resolving power was 50,000 FWHM. The mass spectral data were background-subtracted. Principal component analysis (PCA) and (orthogonal) partial-least squares discriminant analysis (O)PLSDA of the data was performed.

3. Results and discussion

3.1. Strategies to detect adulteration and/or contamination

3.1.1. Nitrogen-adulterants and other adulterants/contaminants in soybean meal

Several techniques have been developed in the framework of QSAFFE (see Table 1) to screen for the presence of melamine and its by-products, urea, whey, DDGs, mycotoxins, pesticides and veterinary drugs.

The proposed strategy for the early quality and safety assurance in the feed chain is depicted in Fig. 1. NIRS or Raman are the methods of choice for fast screening. Both techniques do not require any sample pre-treatment, except grinding for Raman, and can be applied at the port of entry (or even the port of loading) or in feed plants. Both methods require a database of unsuspected samples for calibration. However, NIRS is already used in many feed companies for the determination of nutritional values (crude protein, fat, etc.) and databases of feed materials and compound feeds are already existing and available in the market. For these reasons NIRS has an advantage compared to Raman.

Studies developed in the context of QSAFFE have permitted the construction of an effective and complete procedure based on NIR spectra and chemometric data treatment to provide early evidence of non-conformity in feed mills and to check the presence of possible adulterants. This methodology was validated at the laboratory level and adapted to be applied at feed mill level. In those studies pure soybean meal samples were tested in order to create an early control system for the detection and quantification of melamine, cyanuric acid, whey and DDGs. Multiple samples from one sea vessel, boat or truck can be analysed, which facilitates the detection of hot spots of adulteration. The techniques are also potentially suitable for in-line applications [8]. Within the QSAFFE-project, an on-line NIR sensor was successfully installed in a feed mill [23]. This study indicated that the use of NIR, combined with some simple chemometric tools based on distances and regression equations, is appropriate for the authentication of soybean meal and for the detection of the presence of abnormal samples, viz. soybean meal contaminated with whey. Applying an on-line NIR system, a larger portion of the lots being loaded into the feed mill can be measured [23].

It should be emphasized that the screening methods developed within QSAFFE are meant to detect adulterations with melamine and not to check for compliance with the maximum permitted concentration for melamine in feed set by the European Commission at 2.5 mg/kg as unavoidable background presence [37]. Therefore the detection limits, approx. 0.5% (=5000 mg/kg) for NIRS and 0.1% (=1000 mg/kg) for Raman, are considered fit for the purpose of screening since fraudulent adulteration levels will normally be higher.

NIRS and Raman are also available as portable instruments [38–40]. Portable NIRS instruments can be purchased starting from 5000 €. Handheld Raman instruments cost between 12,000–60,000 € depending on configuration and branding.

NIR Hyperspectral Imaging could also be applied as a primary screening technique in feedmills. It has been tested in the QSAFFE-project in a line scan system for the detection of melamine and cyanuric acid [41]. It is also potentially suitable for in-line applications [42,43]. Taking into account the current state of the art, NIR Hyperspectral Imaging has certain disadvantages compared to NIRS and RAMAN. These limitations are the requirements of a laborato-

Table 1
Characteristics of the methods developed in QSAFFE for detection of N-adulterants and other types of adulteration/contamination in soybean matrices

[a]Technique	Matrix	Analytes tested	[b]T	[c]S/C	[d]Q/q	[e]Level	LOD/LOQs	Time per sample	#sample per day	Destructive	On-line	Cost (€) instrument
NIR-Spectroscopy	Soybean meal	Melamine, cyanuric acid, whey, DDGs	T/NT	S	q	L, F, P*	0.50% (LOD)	5 min	100	No	Yes	80,000
Raman spectroscopy	Soybean meal, pig meal, poultry meal, cattle meal	Melamine, cyanuric acid, ammelide, ammeline	ST	S	q	L, F*, P*	0.10% (LOD)	2–5 min	50–100	No	Yes	120,000
NIR-HSI	Soybean meal	Melamine, cyanuric acid	T/NT	S	q	L, F*	0.1% (LOD)	5 min	200	No	Yes	100,000
NIR-Microscopy	Soybean meal	Melamine, cyanuric acid	T/NT	S	q	L	50 mg/kg (LOD)	<4 h	2	No	No	150,000
LC-HROrbitrapMS	Soybean meal	Melamine, cyanuric acid	ST	S/C	Q	L	0.1 mg/kg (LOQ)	4 h	25	Yes	No	300,000
LC-HROrbitrapMS	DDGs/Soybean meal	Veterinary drugs	ST	S/C	Q	L	0.002–0.2 mg/kg (LOQ)	2 h	25	Yes	No	300,000
LC-HROrbitrapMS	Soya-based matrices, incl. soybean meal	Pesticides residues, mycotoxins and pyrrolizidine alkaloids	T	S	Q	L	[f]0.016 mg/kg (LOQ)	2.4 h	36	Yes	No	300,000
LC-QTOFMS	Soya-based matrices, incl. soybean meal	Pesticides residues, mycotoxins and pyrrolizidine alkaloids	T	S	Q	L	[f]0.016 mg/kg (LOQ)	2.4 h	18	Yes	No	300,000
LC-HRMS/MS	Wide range of feeds, incl. soybean meal	Mycotoxins	T	S/C	Q	L	0.0005–0.050 mg/kg (LOQ)	2.5 h	12	Yes	No	450.000
LC-MS/MS	Wide range of feeds, incl. soybean meal	Mycotoxins	T	S/C	Q/q	L	0.0025–0.50 mg/kg	4 h	30	Yes	No	300,000

[a] NIR-Spectroscopy: near infrared spectroscopy; NIR-HSI: near infrared hyperspectral imaging; NIR-Microscopy: near infrared microscopy; LC-HROrbitrapMS: liquid chromatography-high resolution Orbitrap mass spectrometry; LC-QTOF: quadropole time of flight; HRMS/MS: high resolution tandem mass spectrometry.
[b] T: Targeted; NT: non-targeted; ST: semi-targeted.
[c] S: screening; C: confirmation.
[d] Q: Quantitative; q: qualitative.
[e] L: laboratory; F: feed plant; P: port of entry; * not tested in QSAFFE.[f] Lowest calibration level for most of the analytes.

Fig. 1. Strategy for soybean meal samples.

ry environment and staff with specific expertise and training to perform the analysis, the need to analyse the sample in a monolayer to be able to detect the contaminant and the need to use fast computers with large storage capacity to analyse the high number of data acquired by imaging. False positives and false negatives can be reduced by applying an image analysis based on the object identification rules on these groups of pixels [44].

Representative spectra proving the ability of NIRS and Raman to detect the presence of melamine and its by-products in soybean meal are shown in Figs. 2 and 3. For NIRS specific bands appear in the region of 1460–1540 nm and 2200–2400 nm. The bands in the Raman spectra used to identify the presence of melamine and its structural by-products are: (1) out of plane blending vibration mode (693 cm^{-1}) used to identify ammeline; (2) in plane deformation vibration mode (678 cm^{-1}) used to identify melamine; (3) out of plane blending vibration mode (706 cm^{-1}) used to identify cyanuric acid; (4) asymmetric vibration mode of NH group (561 cm^{-1}) used to identify ammelide (5) stretching vibration mode of C = O group (1728 cm^{-1}) used to identify cyanuric acid (see Fig. 3).

NIRS, Raman and NIR Hyperspectral Imaging also have the potential to be used for detection of other adulterations through non-targeted fingerprinting. In these cases, the spectra of a lot are compared with reference spectra from the database and if, based on the evaluation of the parameters described below, deviating fingerprints are found that cannot be attributed to the presence of

Fig. 2. First derivative NIRS spectra (SG 9: 2: 1) of a pure soybean meal (black line), soybean meal contaminated with 5% of melamine (dark grey) and soybean meal contaminated with 5% of whey (light grey). For melamine specific bands appear around 1460–1540 nm and 2200–2400 nm, for whey bands appear in the vicinity of 2200–2400 nm.

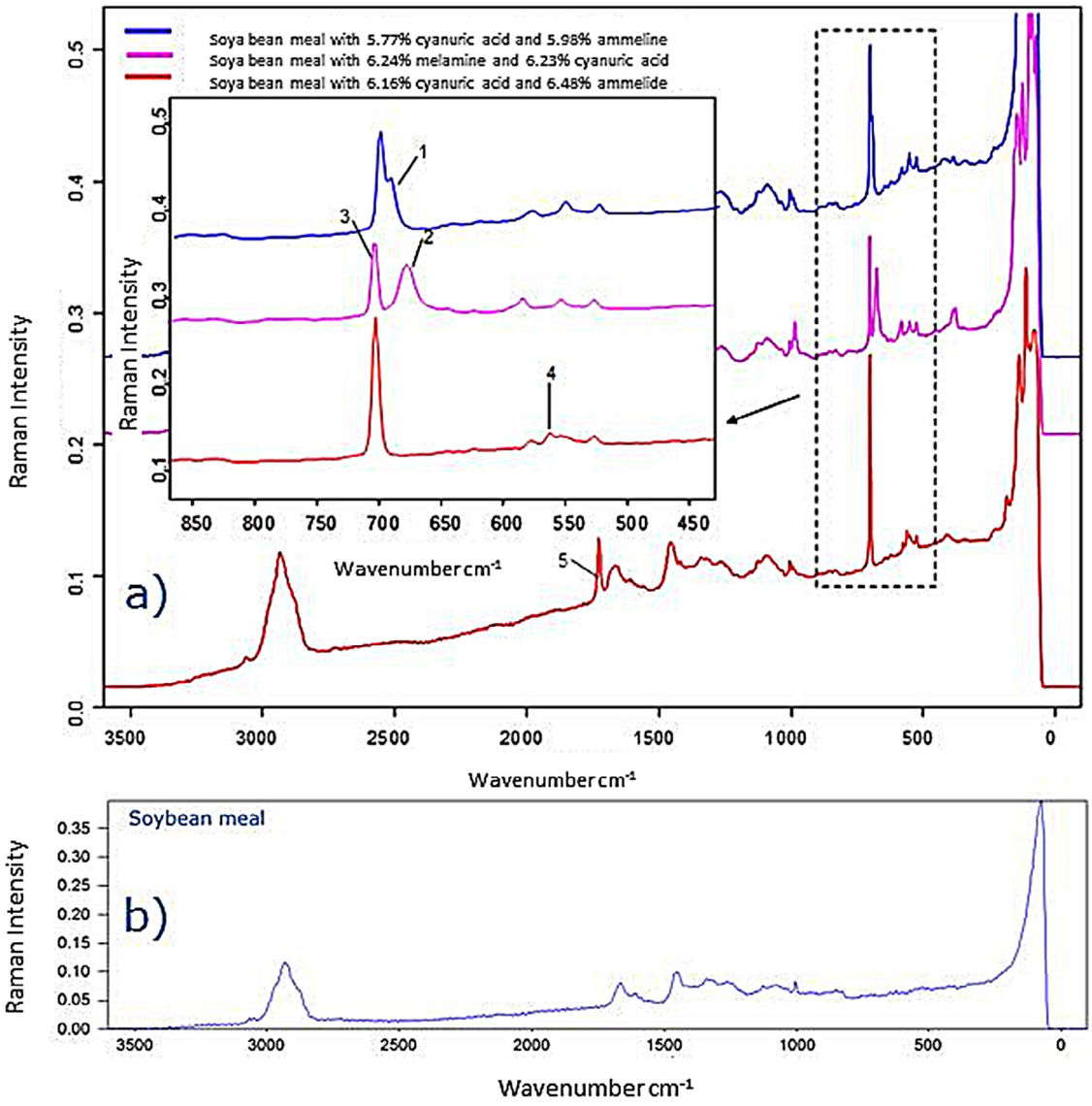

Fig. 3. (a) Raman spectra (100–3500 cm^{-1}) of different concentrations (w/w) of melamine, ammeline, ammelide and cyanuric acid in soybean meal. Spectral region enlarged (300–900 cm^{-1}) to locate the peaks responsible for the identification of each of the target analytes (1) ammeline, (2) melamine, (3 & 5) cyanuric acid, (4) ammelide.; (b) Raman spectra (100–3500 cm^{-1}) of soybean meal.

melamine, these samples should be further tested with other methods (see Fig. 1). It has been shown within QSAFFE that NIRS is also suitable to detect adulteration of soybean meal with whey powder (see Fig. 2) and DDGs [23,45].

Soybean meal has a typical NIR fingerprint spectrum. Deviant behaviour (spectral shifts or/and apparition of spectral bands) means that a band which is not characteristic for soybean meal is present in the spectrum, thus indicating a possible contamination. The criteria for concluding that a sample is suspect are based on the chemometric tools applied. In the case of NIR, three criteria were used to characterize the soybean meal and detect the presence of contaminants [23]: the global H (GH) criterion and two regression equations. The GH criterion is a modification of the Mahalanobis distance (H) of each sample from the average spectrum in which H2 is divided by the number of dimensions used to derive H. This provides information about the distances between each sample and the average sample in the principal components space. Two PLS regression models (one for protein and one for fat) were determined based on historical datasets and then used to predict and characterize new (unknown) samples. Where the prediction fell within the limits defined by the PLS models, the samples were in compliance with the specifications and therefore considered as authentic soybean meal. Where this was not the case, the samples were considered as suspect. In the case of hyperspectral imaging, the criteria are based on the chemometric model PLS-DA as described elsewhere [22].

If the spectra of NIRS, Raman or NIR Hyperspectral Imaging do not show any deviant behaviour, the lots can be accepted by the importer or feed producer. If deviations are observed, this may be an indication of adulteration and/or contamination of the material and other more specific techniques should be applied to identify and quantify the adulterant/contaminant.

If the soybean meal contains melamine and/or its by-products, this will be indicated by the spectra of NIRS, Raman or NIR Hyperspectral Imaging. In this case, the presence of melamine and/or its by-products can be confirmed by means of LC-MS methods (see Fig. 1). Nowadays, for confirmation MS-based techniques are generally applied because of their excellent and unrivalled confirmatory power. While screening for adulteration with spectroscopic techniques is performed at relatively high levels (above

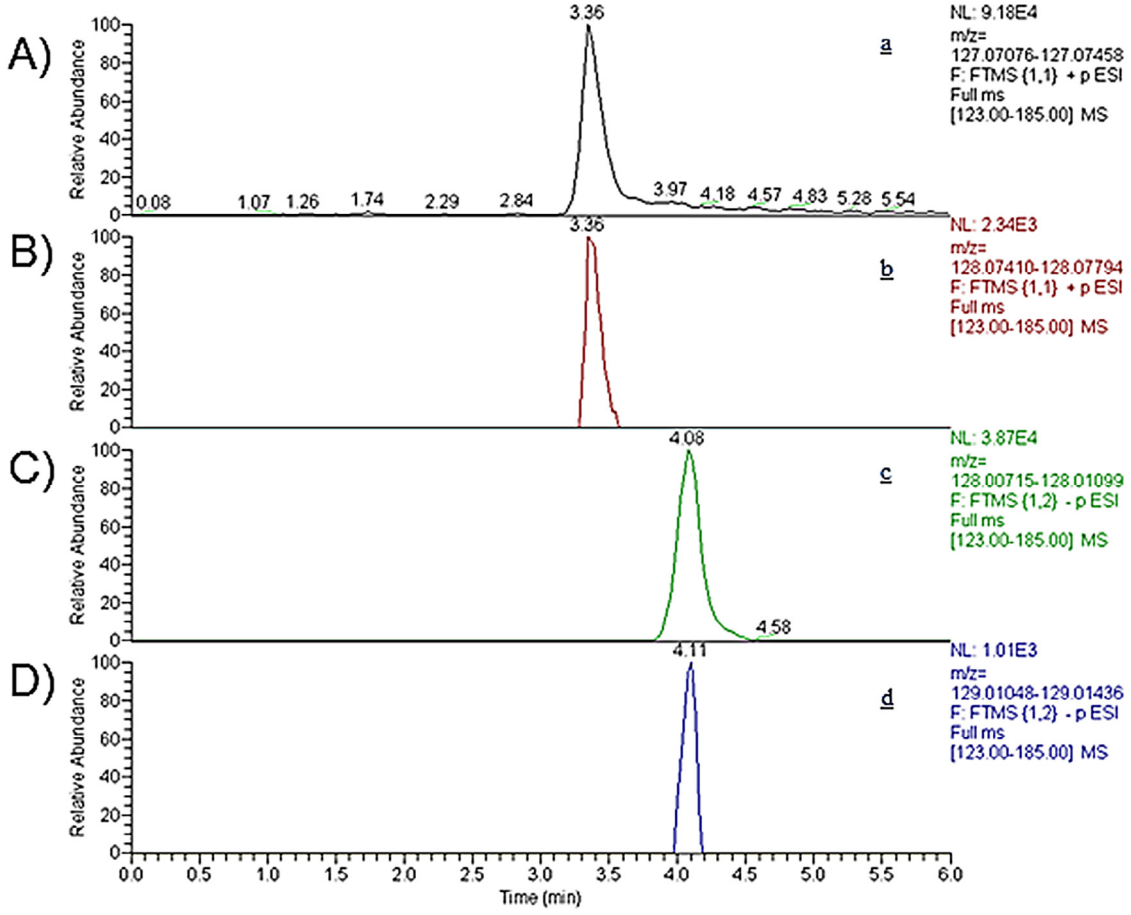

Fig. 4. LC-HRMS Chromatogram of a soybean meal sample spiked with melamine and cyanuric acid at 2.5 mg kg^{-1}. (A) melamine (RT: 3.36); (B) $^{13}C_3$-melamine (RT: 3.36); (C) Cyanuric acid (RT: 4.08); (D) $^{13}C_3$-cyanuric acid (RT: 4.08).

1000–5000 mg/kg; see above), it is not really necessary to use these techniques from the sensitivity point of view. However, from the confirmation point of view they are the best choice for suspect samples. Several LC-MS/MS methods have been described, which can be applied directly as quantitative confirmatory methods [46,47] or first as post-screening methods [48]. Within QSAFFE, an LC-full scan-HRMS method was developed [49], representative chromatograms are shown in Fig. 4. The benefits of HRMS instruments are the greater insight into the composition of a sample through the collection of full-scan spectra and the possibility of retrospective data analysis [50].

The NIR Microscopy method developed in QSAFFE (see Table 1) can be applied as post-screening method [27]. Melamine is a triazine heterocyclic organic compound, only composed of a nitrogen heterocyclic ring and $-NH_2$. Melamine has three strong characteristic peaks in the range of 6900–6450 cm^{-1}. Among them, 6805 cm^{-1} is a very sensitive wavelength (See Fig. 5), which corresponds to the N–H combination band ((νN–H asymmetric and νN–H symmetric combination) from primary amides. Soybean meal is a natural mixture, which has a broader absorption band in the range of 6900–6450 cm^{-1} without a strong absorption peak at 6805 cm^{-1}. Spectra of other feed raw materials are similar to soybean meal in the range

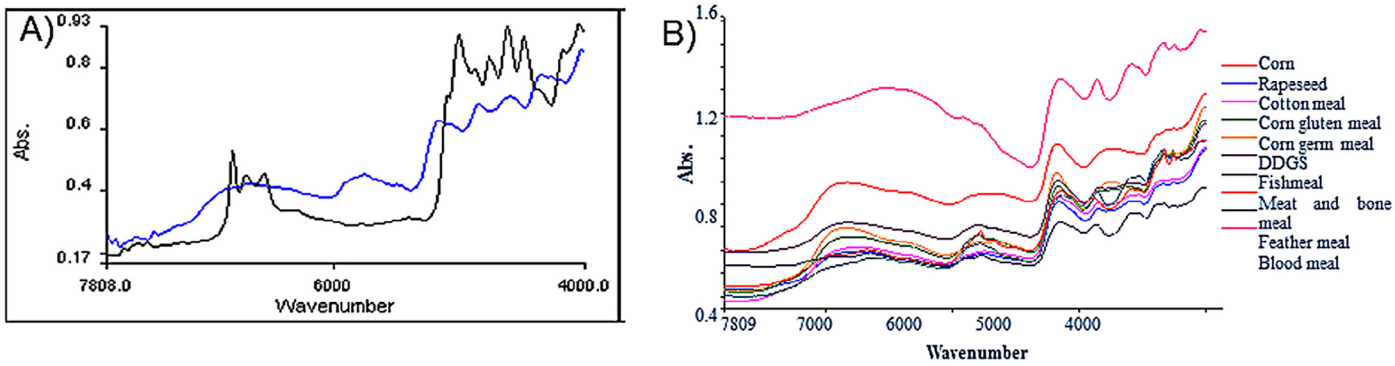

Fig. 5. NIR microscopy to detect melamine in soybean meal. (A) spectra of melamine and soybean meal. (B) comparison of the spectra of different feed raw material.

Table 2
Characteristics of the methods developed in QSAFFE to detect adulterations of vegetable oils with mineral oil, transformer oil or other oils

[a]Technique	Matrix	Analytes tested	[b]T	[c]S/C	[d]Q/q	[e]Level	LOD/LOQs	Time/ sample	#sample per day[f]	Destructive	On-line	Cost (€) instrument
Raman spectroscopy	Soya oil and basic vegetable blend oil	Mineral oil/ Transformer oil	T	S	q	L, F, P	0.25%	<5 min	>50	Non-destructive	Yes	20,000–150,000
GCxGC-ToFMS	Vegetable and fish oil, animal fats	[g]PCBs, PBDEs, PAHs and nBFR	ST/T	S	Q	L	5.0 µg/kg ΣPCB6, 1.0 µg/kg BaP, 4.0 µg/kg ΣPAH4, 17.5 µg/kg ΣPBDE7, 52.5 µg/kg ΣnBFR6	6 h	10–12	Destructive	No	350,000
DART-HRMS	Fats, oils	[h]TG, DG, MG, oxidized forms of TG/DG/MG, cholesterol	ST	S/C	q	L	Not applicable	3 min	50	Destructive	No	300,000

[a] GCxGC-ToFMS Comprehensive gas chromatography-time of flight mass spectrometry; DART-HRMS: Direct analysis in real time ionization high resolution mass spectrometry.
[b] T: Targeted; NT: non-targeted; ST: semi-targeted.
[c] S: screening; C: confirmation.
[d] Q: Quantitative; q: qualitative.
[e] L: laboratory; F: feed plant; P: port of entry.
[f] Based on duplicate analysis.
[g] PCBs: polychlorinated biphenyls; BaP: Benzo [*a*] pyrene; PAH: polycyclic aromatic hydrocarbons; PBDEs: polybrominated diphenyl ethers; nBFRs emerging brominated flame retardants: 2,3,4,5,6-pentabromoethylbenzene, tetrabromo-*p*-xylene, 2,3,4,5,6-pentabromotoluene, hexabromobenzene, tetrabromo-*o*-chlorotoluene and 1,2-bis (2,4,6-tribromophenoxy) ethane.
[h] TG: triacylgycerols; DG: diacyglycerols; MG: monoacylglycerols.

of 6900–6450 cm^{-1} (see Fig. 5). So this technique is useful for detecting melamine in feed raw materials, based on this wavelength pre-processed by baseline offset correction.

If deviating fingerprints are found that cannot be attributed to the presence of melamine or its by-products, these samples should be further tested and investigated with other methods. From the NIRS spectra of a suspect soybean meal, indications could be obtained about the presence of high levels of contaminants. NIR spectroscopy is suitable to detect fungal contamination of agricultural commodities, particularly cereals [51], potentially leading to the production of mycotoxins. Moreover, NIR is a promising technique for the detection of mycotoxins [51,52], pesticides [52,53] and antibiotics [52] in food, although in these reviews it was generally concluded that the sensitivity needs further improvement. Besides indications from NIRS screening, there may be indications from investigations about the origin, production or fate of the soybean meal that the lot may contain elevated levels of mycotoxins, pesticides or veterinary drugs. E.g., if the soybean meal would be adulterated with DDGs this could lead to the presence of antibiotics.

For further testing and confirmation of suspect samples, other than those suspect for the presence of melamine, MS-based methods are available. For mycotoxins a number of confirmatory methods have been published based on LC-MS/MS with various extraction/clean up procedures, i.e. extraction with SPE cleanup, without further cleanup, or with liquid-liquid partitioning [54–58]. Confirmatory methods for pesticide residue analysis nowadays are often based on acetonitrile extraction followed by salt-induced liquid-liquid partitioning ('QuEChERS' or modifications thereof [59]) followed by LC-MS/MS and GC-MS/MS analysis [14,60–64]. For veterinary drugs LC-MS/MS and LC-HRMS methods have been described [65,66].

LC-MS-based methods, mainly LC-HRMS methods have also been developed in QSAFFE for (a) mycotoxins, pesticides and pyrrolizidine alkaloids [28] (b) mycotoxins [29,30] and (c) veterinary drugs (see Table 1). The method for veterinary drugs was validated for DDGs [31] and shown to be suitable also for soybean meal [49]. These methods can be applied as screening methods. For the mycotoxins (referred to under (b)) and the veterinary drugs, the same methods can be used for confirmation.

3.1.2. Adulterations of vegetable oils with mineral oil, transformer oil or other oils

Several techniques have been developed in the framework of QSAFFE (see Table 2) to screen for the presence of these adulterations.

For primary broad screening in the port of entry or the feed plant Raman is the method of choice (see Fig. 6). As shown in Fig. 7, Raman spectra differ between basic vegetable blend oil (BVB), soya oil, transformer oil and mineral oil. Applying chemometric analysis, this allows to discriminate between adulterated and non-adulterated vegetable oils [32]. As shown in Fig. 8, 1% mineral oil can be detected in soya oil but it is not possible to fully discriminate between pure BVB and 1% mineral oil in BVB [32].

Similar to what has been described before for the use of Raman in soybean meal, the technique has advantages with regards to high sample throughput, potential for in-line applications [8] and portable instruments [40] that can be used in ports of entry. Again a database of unsuspected samples is required for calibration.

It should be emphasized that the Raman screening method developed within QSAFFE is meant to detect fraudulent adulterations, which normally will mean that the contents will be much higher

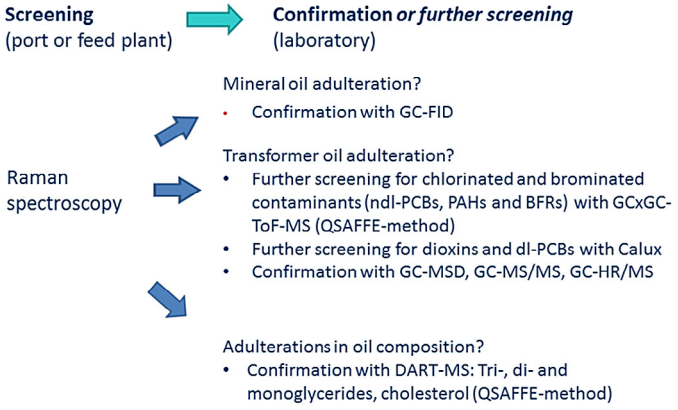

Fig. 6. Strategy for vegetable oil samples.

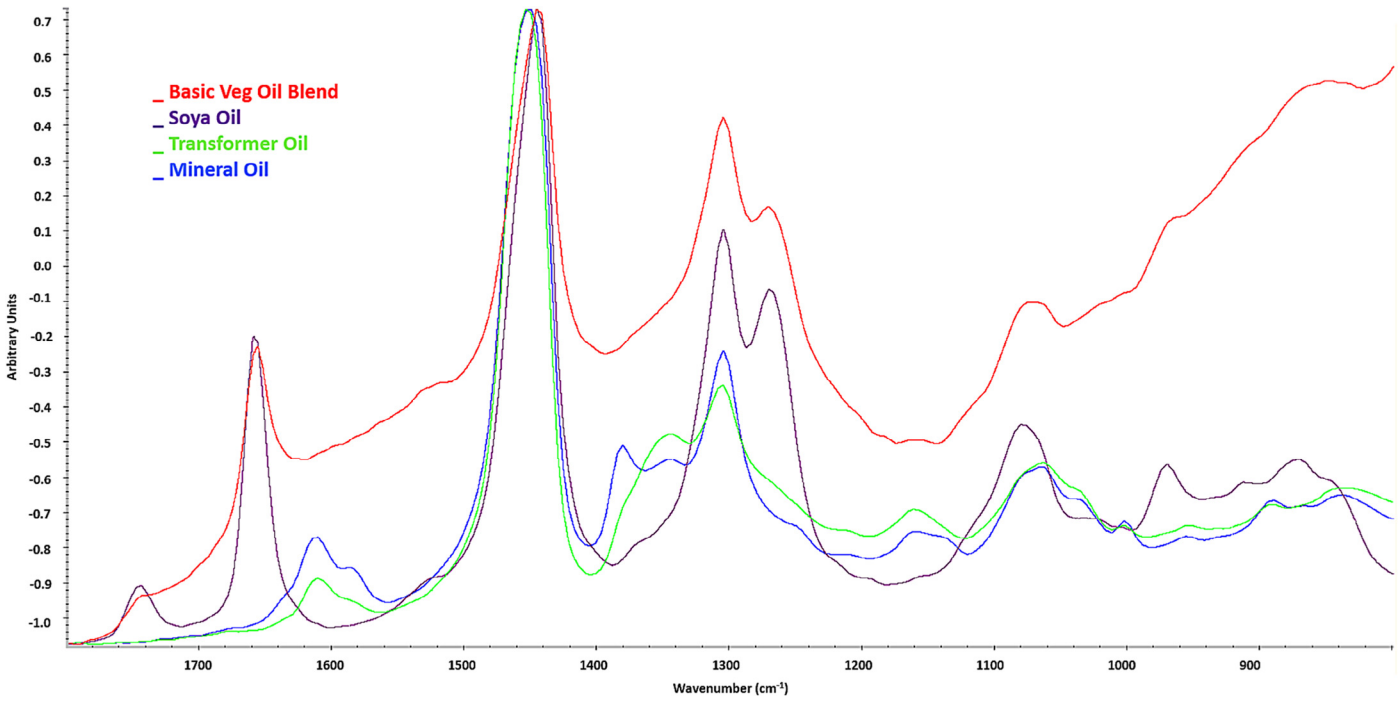

Fig. 7. Raman spectra for basic vegetable blend oil (BVB), soya oil, transformer oil and mineral oil.

than the maximum limit of 50 mg/kg that applied in the European Union until August 2014 for mineral oil in sunflower oil originating from Ukraine [67]. The detection limit is approx. 0.25% (=2500 mg/kg).

If the Raman spectra do not show any deviant behaviour, based on the results from the chemometric model [32], the lots can be accepted by the importer or feed producer.

If the Raman spectra observed are deemed out of specification, based on the results from the chemometric model, this can be an indication that the vegetable oil is adulterated with mineral oil, transformer oil or other, e.g. lower priced or inferior quality oils/fats. For these suspect samples further analysis is required (Fig. 6).

The presence of mineral oil can be checked and confirmed by means of Gas Chromatography-Flame Ionization Detection (GC-FID), a technique that is commonly applied as reference method [68–71].

The potential presence of transformer oil implies a risk that the lot contains contaminants such as PCBs, PBDEs, PAHs, emerging flame retardants and/or dioxins. Due to this wide variety of potential contaminants, the best approach is post-screening by means of GCxGC-ToF-MS, developed within QSAFFE (see Fig. 6). In this way, the presence of PCBs, PBDEs, PAHs and emerging flame retardants at the regulated maximum levels in food and feed can be detected. A representative 2D-chromatogram is shown in Fig. 9. GCxGC-ToF-MS can both be used in the semi-targeted mode and in the targeted mode. In the semi-targeted mode chlorine (Cl) and bromine (Br)-containing compounds with more than 2 Cl/Br atoms can be detected through their isotopical pattern [35]. Afterwards, the identity of these compounds can be confirmed with established quantitative GC-MSD, GC-MS/MS and GC-HRMS confirmatory methods for PCBs, PBDEs and other flame retardants and PAHs, which apply more time-consuming and thorough sample clean up procedures [72–74]. Additionally, samples can also be tested for dioxins. For this purpose the Calux screening assay could be applied [75], followed by GC-HRMS or GC-MS/MS confirmation [76].

Adulteration with other, e.g. lower priced, or inferior quality oils/fats can be checked by means of DART-MS. DART-MS is suitable for differentiation of fats and oils through the analysis of triacylglycerols (TG), diacylglycerols (DG), monoacylglycerols (MG), oxidized forms of TG/DG/MG and cholesterol and so to get information about the type and quality of oils/fats and the composition of mixtures [77,78]. A score plot that shows the possibilities to discriminate between different types of oil is shown in Fig. 10. DART-MS has the advantage of a high sample throughput. If deviations in the fat/oil composition are found, these samples can be further investigated with specific confirmatory techniques for organic contaminants as described above.

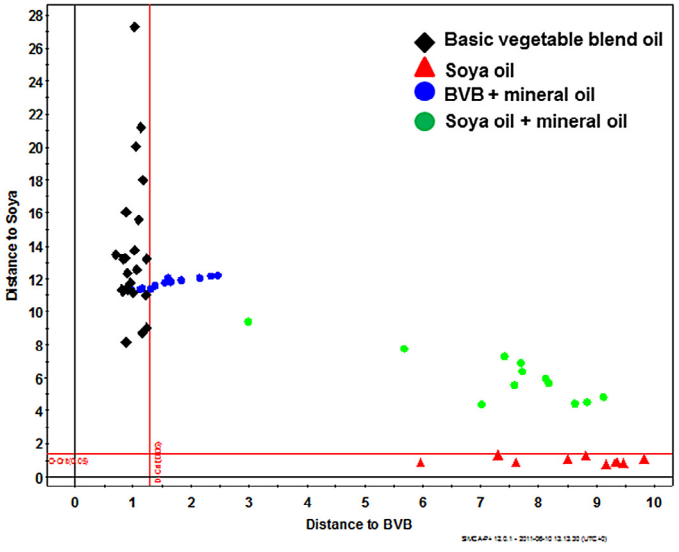

Fig. 8. Adulteration of basic vegetable blend oil and soya oil with 1% mineral oil. Cooman's plot from the data generated by Raman.

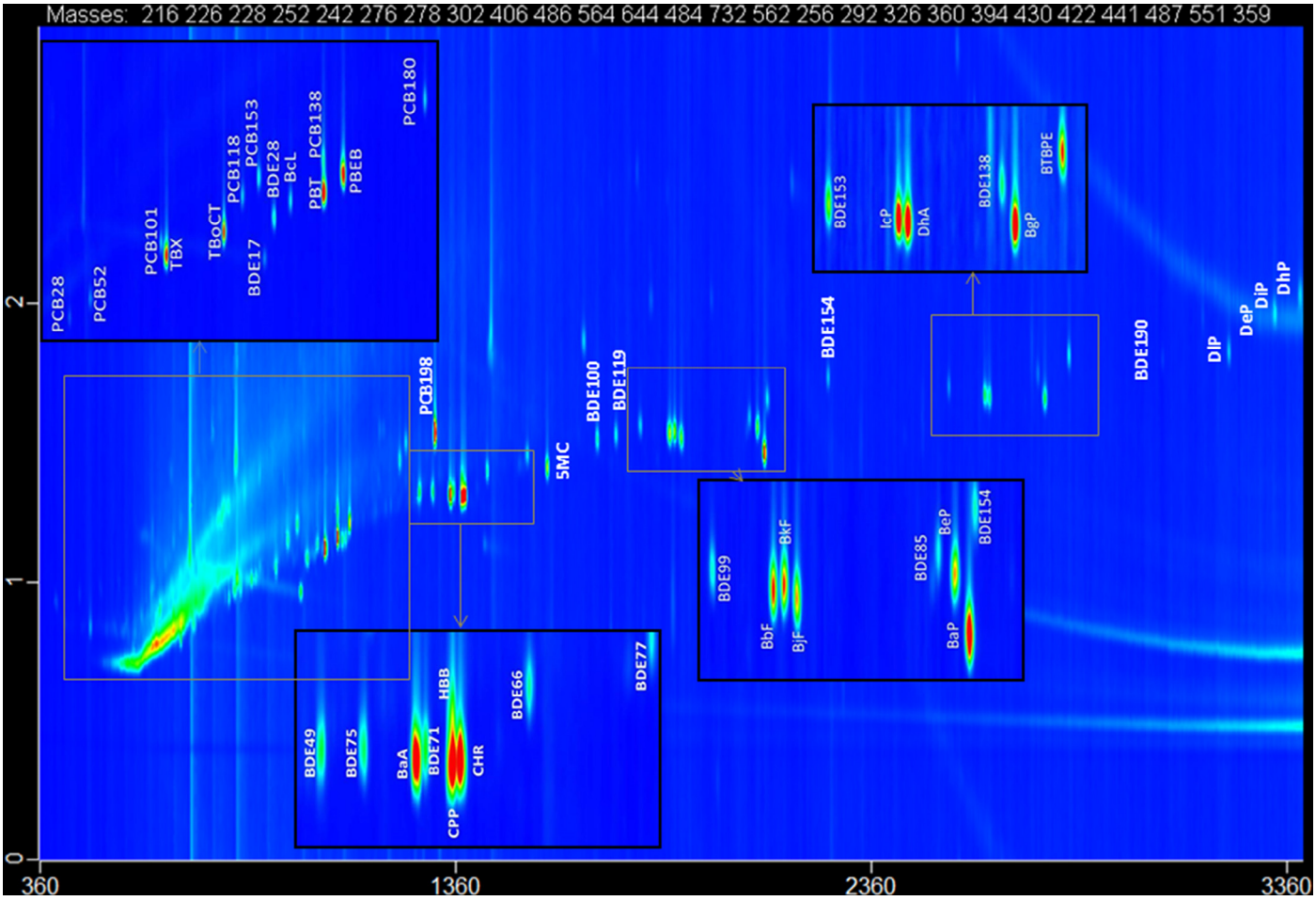

Fig. 9. GCxGC-ToF-MS chromatogram of standards showing the separation of PAHs, PBDEs and non-dioxin-like PCBs.

When applying GCxGC-ToF-MS or DART-MS, acquisition of MS-data is done in full-scan mode, which entails the possibility of conducting retrospective analyses.

GC-FID, GCxGC-ToF-MS, DART-MS, Calux, GC-HRMS and GC-MS/MS can only be run in well-equipped laboratories and for this reason they are not suitable for primary screening by feed business operators. It means that samples showing deviant Raman spectra have to be sent to a laboratory for further investigations.

4. Conclusions

Early quality and safety assurance in the feed chain is feasible by means of strategies based on screening at the feed mill or port of entry with non-destructive spectroscopic methods (NIRS and Raman), followed by post-screening and confirmation in the laboratory with MS-based methods. Strategies have been elaborated for adulterations of (i) soybean meal with melamine and other types of adulterants/contaminants and (ii) vegetable oils with mineral oil, transformer oil or other oils. For the detection of adulterations in soybean meal, NIRS or Raman were shown to be the most suitable methods for primary screening. NIRS has an advantage compared to Raman because it is already used in many feed plants. Within the QSAFFE-project, an on-line NIR sensor was successfully installed in a feed mill. For primary screening of adulteration of vegetable oils, Raman is the method of choice. Within the QSAFFE project, a Raman instrument was successfully tested in a feed mill for off-line screening of vegetable oils as they arrived on-site. High-resolution LC-MS and GC-MS methods, developed in QSAFFE, are suitable for unambiguous confirmation, give greater insight into the composition of a sample through the collection of full-scan spectra and yield possibilities for retrospective data analysis of suspect samples, adding extra value to the control strategies with respect to non-targeted approaches.

In principle, similar strategies could also be applied to other feed and food products, e.g. milk powder, provided that NIR and/or Raman databases containing spectra of sufficient numbers of unsuspected samples are available.

The major limitation of the proposed strategies is that, through the use of spectroscopic methods for initial screening, these strategies can only be used for the detection of adulteration at relatively high levels and not for low level contamination (ppb/ppm level). However, spectroscopy is the only technology suitable for on-line and in-line control in feed mills and ports, allowing detection at an early stage when the adulterant content is high. Later detection will be more complicated due to dilution of the adulterant. On-line and in-line detection also allows a larger portion of the lots to be measured, which facilitates the detection of hot spots of adulteration. Finally, NIRS and Raman spectroscopy both have the potential to be applied as non-targeted techniques, as was demonstrated through the detection with NIRS of whey and DDGs in soybean meal and the Raman spectral differences between transformer oil and vegetable oil. GCxGC-ToF-MS, included as a post-screening method in the strategy for vegetable oils, can also be used in the semi-targeted mode to detect compounds containing more than 2 Cl/Br-atoms.

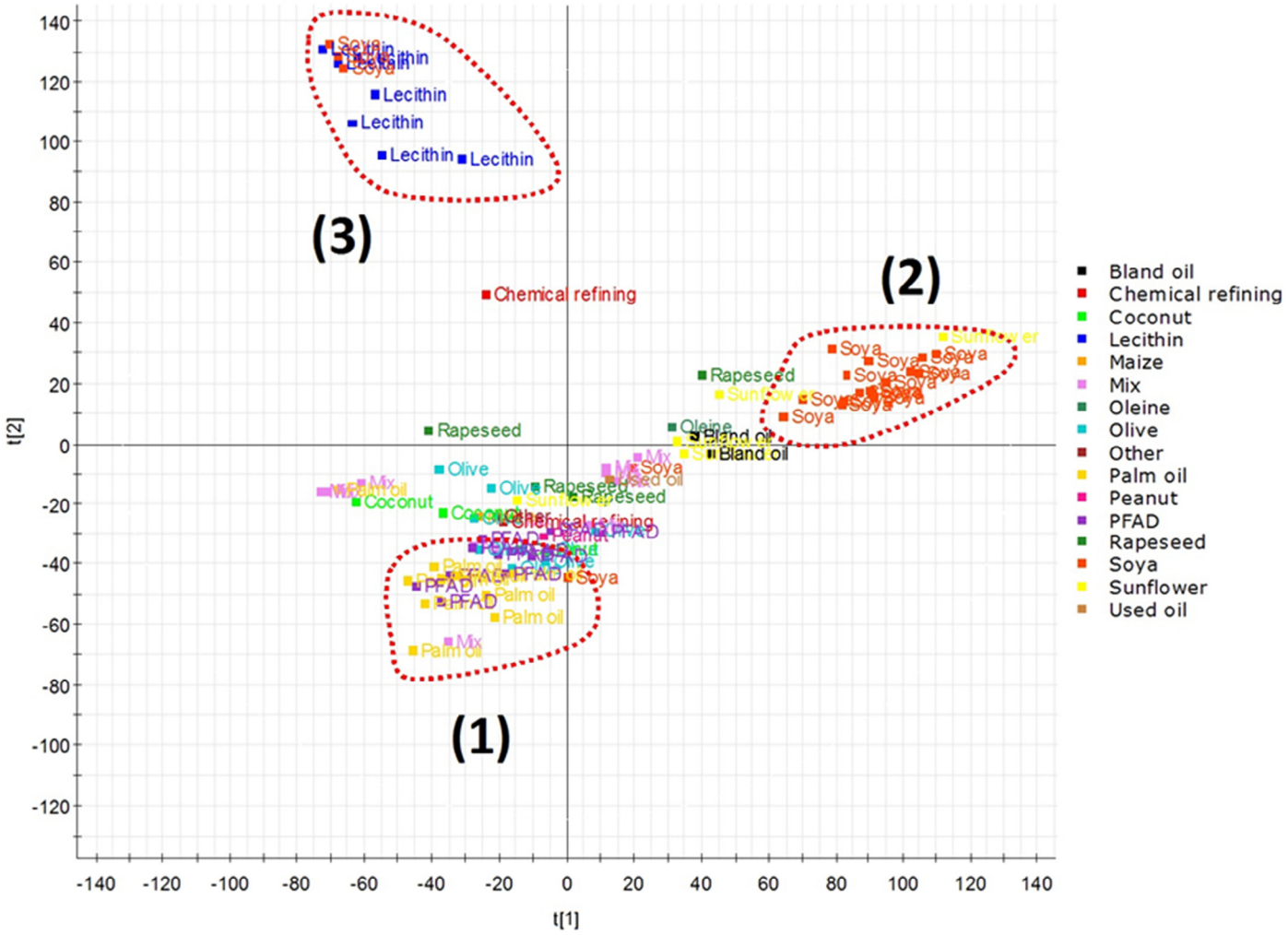

Fig. 10. Score plot (PCA) for different types of oils obtained using DART–Orbitrap-MS.

Future research should focus on improvements of the sensitivity of the spectroscopic techniques, application of the strategies to other feed and food products, elaboration of on-line and in-line applications and the use of portable instruments in the feed mill and the port of entry. Finally further studies are needed to yield further evidence that NIRS and Raman can be used for non-targeted screening.

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