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ORIGINAL PAPER

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Discriminating animal fats and their origins: assessing the potentials of Fourier transform infrared spectroscopy, gas chromatography, immunoassay and polymerase chain reaction techniques

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Abstract The objective of the reported study was to assess the abilities of various methods to differentiate the sources of fats used in feedstuff formulations. The main target was the identification of tallow (ruminant fat) and its differentiation from non-ruminant fats. Four different techniques were compared in terms of their suitability for enforcing existing and upcoming legislation on animal by-products: (1) Fourier transform infrared spectroscopy (FT-IR) applied to fat samples, (2) gas chromatography coupled with mass spectrometry (GC–MS) to determine fatty acid profiles, (3) immunoassays focusing on the protein fraction included in the fat, and (4) polymerase chain reaction (PCR) for the detection of bovine-specific DNA. Samples of the different fats and oils as well as mixtures of these fats were probed using these analytical methods. FT-IR and GC–MS differentiated pure fat samples quite well but showed limited ability to identify the animal species or even the animal class the fat(s) belonged to; no single compound or spectral signal that could permit species identification could be found. However, immunoassays and PCR were both able to identify the species or groups of species that the fats originated from, and they were the only techniques able to identify low concentrations of tallow in a mixture of fats prepared by the rendering industry, even when the samples had been sterilised at temperatures >133 °C. Fats used in animal nutrition come mainly from the rendering industry, thereby confirming the suitability of PCR and immunoassays for their identification. However, neither of these latter techniques was able to detect ''premier jus'' tallow, representing the highest quality standard of fat with extremely low protein concentration.

Keywords Animal fats · Species identification · FT-IR · GC–MS · Immunoassay technique · PCR · TSE

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Introduction

Animal fat is an important animal by-product also used as an ingredient in ruminant feedstuffs. Since the first verified case of bovine spongiform encephalopathy (BSE) in 1986 [1], the ingredients of feed have been continuously evaluated regarding their BSE risk. Therefore, the feeding of ruminant animals with meat and bone meal (MBM) from mammals was prohibited in 1994 by European legislation [2]. Currently this ban also prohibits the feeding of processed proteins derived from terrestrial animals and fishes to farmed animals [3], which also reflects the lack of validated analytical methods that allow for species-specific identification of MBM in feed. Other important measures are pressure-cooking (133 °C, 3 bar, 20 min) of certain mammalian animal by-products [4] and the removal of cattle-, sheep- and goat-specified risk material (SRM) from human and animal food chains [5]. In 2001 the Scientific Steering Committee (SSC) [6] of the European Commission gave its opinion on the safety of fat from certain ruminant animal by-products, summarising the different techniques involved in the production of animal fat: fat melting and rendering processes. The risk from transmissible spongiform encephalopathy (TSE) infectivity

associated with fat was considered to be low when various criteria were fulfilled. Taking into account that the TSE risk from fat is mainly due to protein residues in the end- product, it was recommended that the weight of insoluble impurities should be below 0.15% of the total weight. Tallow from the fat melting industry, obtained using discrete adipose tissue from parts of the animals that were intended for human consumption, was considered to be safe, whereas fat from the rendering industry needs to fulfil additional criteria, such as having undergone an appropriate sterilisation process. The reason for the inclusion of this criterion was that in the rendering industry the probability is somewhat higher that infectious material is included in the final product. On the other hand, Appel et al. [7] reported that pressure sterilisation becomes less effective in terms of inactivation of prions when they are suspended in lipids. Regarding the use of tallow in animal nutrition, the SSC recommended that ruminant fat produced by the rendering industry should not be used as an ingredient in ruminant feed [6].

Criteria for the safe use of ruminant fat in animal nutrition in Europe are defined by Regulation 1774/2002 [4], which requires that the material belongs to category 3 (animal by-products fit for human consumption) and the maximum concentration of residual insoluble impurities (RII) after purification does not exceed 0.15%. These residues mainly consist of proteinaceous impurities (up to 85%) [6]. In contrast, ruminant fat is excluded from the food and feed chains when the material belongs to category 1 (such as specified risk material) or category 2 (such as fallen stock), whatever fat treatment is applied. In general, the feeding of rendered ruminant fat to ruminants is prohibited within the European Union in countries and regions with a high incidence of BSE [8]. These criteria could be adjusted according to the results from a quantitative risk assessment.

The objective of this study was to establish whether the identification of tallow (ruminant fat) and its differentiation from lard (porcine fat) is possible using an analytical methodology that can be easily applied by enforcement laboratories. The tests were evaluated at two levels: (1) the correct identification of pure fat samples, and (2) the identification of tallow when mixed with fat from other species. The identification of tallow could become important when, for instance, the practical implementation of the animal by-products regulation [4] shows that the separation of ruminant material according to their BSE risk is not effective, or in cases in which the fraction of insoluble impurities is above 0.15%.

Tallow and lard contain esters from palmitic, oleic and stearic acids and over 50 triglycerides in concentrations higher than 0.1%, but there isn't a predominant triglyceride specific to tallow which is therefore suitable as an easily detectable target analyte for differentiation from lard or other fats [9]. Ruminant fatty acids contain [10] specific fatty acids such as elaidic acid (t9-C-18:1) and t-vaccenic acid (t11-C-18:1), which are both *trans*-fatty acids, whereas fat from non-ruminants contains mainly *cis*-fatty acids. However, *trans*-isomers can also be formed artificially during fat processing, thereby increasing the risk of false positive results when the evaluation is only based on the presence of these fatty acids. In this study, we analysed fatty acids after transesterfication, employing multivariate statistical tools for the data evaluation. Multivariate statistics were also applied to FT-IR-spectral data from the fat samples. Chromatographic and spectroscopic methods have already been shown to be promising techniques for the authentication of various oils and fats [11–14]. However, we also tested some alternative approaches—commercially-available immunoassay and polymerase chain reaction (PCR) techniques, that can be used to analyse insoluble proteins and the residual DNA in fat, respectively.

Materials

Appropriate samples were selected, considering the specific characteristics of the analytical methods investigated. Both FT-IR and GC–MS focus on differences in *fat* composition, and so it was important to include various fat samples of different origins in the study in order to investigate the selectivities of these methods. We also measured industrially-processed samples. In contrast to FT-IR and GC–MS, the PCR and immunoassay techniques analyse insoluble *residues* in the fat, thereby requiring that samples from different industrial

Table 1 Samples used in the MIR and GC analyses. Mixtures are described in terms of their respective mass fractions. Lard and tallow were produced by melting fat (from the melting industry)

Sample type	Number of samples	
	MIR	GC
Cocoa butter	1	1
Flaxseed oil	1	1
Sunflower + palm	1	1
Olive oil	1	–
Soyabean oil	3	1
Sunflower oil	4	4
Corn oil	4	1
Peanut oil	4	1
Butter	2	2
Chicken fat	5	4
Fish oil	2	2
Horse fat	2	2
Lard	12	13
Lamb fat	1	–
Rabbit fat	3	3
Tallow	3	3
Bovine	9	10
Mix 1: Lard/bovine fat (10/90, 25/75, 50/50, 25/75, 90/10)	7	7
Mix 2: Bovine fat/lard/fish oil (49/49/2)	5	–
Mix 3: Bovine fat/lard/chicken fat (33/33/33, 45/45/10)	–	2

processes are included. Aspects of these processes that are important in the context of this study include the concentration of residues that remains after the purification of the raw fat, and the damage caused to proteins [15] and the DNA segment to which the PCR is targeted [16] by the heat treatment, especially when the fat is subjected to steam pressure sterilisation.

Test samples for FT-IR and GC analysis

Table 1 describes the different samples analysed. The different sample types were obtained from various sources, local and industrial retailers and producers, in order to represent a realistic variation within each sample type. In addition to the pure samples, several mixes were prepared in order to investigate the ability of each method to identify tallow when present in a mixture of animal fats.

Test samples for immunoassay and PCR analysis

To evaluate the immunoassay and PCR, different samples were selected reflecting the various industrial processes applied to the production of tallow and lard. In principal, tallow used for animal nutrition is produced in two separate production lines, which are (1) the rendering of animal by-products, which are frequently mixtures of fat, meat and bones, and (2) the melting of almost pure slaughterhouse fat [6]. Tallow of the highest quality (“premier jus”), characterised by an extremely low content (below 0.02%) of residual insoluble impurities, is only produced by the melting industry, by applying gentle process conditions. When applying a wet melting process, the fat is heated up to 95 °C by direct steam injection followed by purification steps such as centrifugation and filtration. In contrast, tallow produced in the rendering industry contains higher amounts of RII (up to 0.15%). Depending on which rendering technology is applied, the tallow is subjected to severe heat treatment (such as steam pressure sterilisation above 133 °C), possibly leading to a change in protein structure or damaging the DNA present in the impurities. In order to examine the latter aspect, MBM samples containing about 50% bovine material and corresponding fat samples were analysed. Both the MBM and the fat samples were from a batch-type commercial rendering plant, processing carcasses and animal by-products as described elsewhere [15]. The heat treatment varied from 126 to 138 °C. In addition, we have analysed tallow and lard from the melting industry and mixtures of these fats. The samples used are described in Tables 2 and 3. For the purpose of this study it is important to note that tallow used in animal nutrition derives mainly from the rendering industry, and it contains up to 0.15% of RII.

Table 2 Description of the samples containing tallow used to test the discriminating abilities of PCR and immunoassay, and the results of analysis. The fraction of tallow is given as a mass ratio

Description	PCR result	Immunoassay
Meat and bone meal (MBM)		
Rendering: 138 °C	+	+ +
Rendering: 136 °C	+	+ +
Rendering: 133 °C	+	+ +
Rendering: 129 °C	+	+ +
Rendering: 126 °C	+	+ +
Samples with tallow (50%), RII (%) > 0.15		
Fat extracted from MBM 138 °C	+	+
Fat extracted from MBM 136 °C	+	+ +
Fat extracted from MBM 133 °C	+	+ +
Fat extracted from MBM 129 °C	+	+
Fat extracted from MBM 126 °C	+	+ +
Samples with tallow, RII (%) < 0.15		
Wet rendering: tallow (A)	n.a.	+ +
Wet rendering: tallow (B)	n.a.	+
Wet rendering: tallow (C)	+	+
Rendering: Bovine fat (H)	+	+ +
Lard (A)/tallow (B) 95:5	n.a.	+
Lard (A)/tallow (C) 95:5	n.a.	+
Lard (C)/tallow (C) 90:10	+	+
Lard (C)/tallow (C) 95:5	+	+
Lard (C)/tallow (C) 98:2	+	–
Lard (A)/bovine fat (H) 50:50	+	+ +
Lard (A)/bovine fat (H) 90:10	+	±
Lard (A)/bovine fat (H) 95:5	+	+
Lard (A)/bovine fat (H) 98:2	+	–
Samples with tallow, RII (%) < 0.02		
Melting: bovine fat (premier jus) (D)	–	–
Melting: bovine fat (premier jus) (E)	–	–
Melting: bovine fat (F)	–	–
Melting: bovine fat (G)	+	±
Lard (C)/bovine fat (D) 90:10	+	–
Lard (C)/bovine fat (D) 95:5	+	–
Lard (C)/bovine fat (D) 98:2	+	–
Lard (G)/bovine fat (G) 50:50	+	±
Lard (G)/bovine fat (G) 75:25	+	±
Lard (G)/bovine fat (G) 90:10	– (±)	–
Lard (G)/bovine fat (G) 95:5	–	–
Lard (G)/bovine fat (G) 98:2	–	–

RII = Residual insoluble impurities in tallow; n.a. = not analysed, since there was not enough sample amount available. Codes for the lard samples are taken from Table 3. Visibility of the immunoassay line: – = no line; ± = inconclusive result; + = sufficient; + + = very intensive. Interpretation of the PCR analysis: + = proof of the presence of tallow; – = no proof of the presence of tallow; ± = inconclusive result

Table 3 Description of the samples without tallow used to test PCR and immunoassay, and the results of analysis

Description	PCR result	Immunoassay
Melting: lard (A)	–	–
Melting: lard (B)	–	–
Rendering: lard (C)	+	–
Melting: lard (D)	–	–
Melting: lard (E)	–	–
Melting: lard (F)	+	–
Rendering: lard (G)	–	–
Rendering: poultry fat (A)	–	–
Rendering: poultry fat (B)	–	–
Melting: poultry fat (C)	–	–

Methods

Fourier transform infrared spectroscopy

The study was carried out on a Perkin Elmer FT-IR Spectrum 2000 (PerkinElmer, Inc., Boston, MA, USA) coupled with an autoimage microscope. The measurement set-up comprised a single beam Michelson interferometer with beam splitters covering a wavenumber range of 15,000–30 cm^{-1}. Analyses were performed in two regions of the infrared spectrum, near-infrared (NIR) and mid-infrared (MIR). For the NIR measurements, a mercury cadmium telluride (MCT) detector cooled with liquid nitrogen was used; in the MIR region, spectra were acquired using a deuterated triglycerine sulphate (DTGS) detector with KBr window. The spectra were collected with the instrument's software (Spectrum software, Perkin Elmer).

NIR spectra were collected from 9,000 to 4,000 cm^{-1} or 1,100 to 2,500 nm with a resolution of 16 cm^{-1}, 16 scans were co-added, interval 8 cm^{-1}, optical path difference (OPD) 0.5 cm^{-1}, and a gain of 1 was used. The spectra were obtained by placing the fat directly onto a steel tablet and then analysing it with the FT-IR microscope.

The MIR spectra were collected from 4,000 to 600 cm^{-1} with a resolution of 4 cm^{-1}, 32 scans were co-added, interval 2 cm^{-1}, OPD 1 cm^{-1}, and using a gain of 1. Before each scan, a new reference air background spectrum was taken. The spectra were taken with an attenuated total reflectance mode (ATR) sample presentation instrument. The ATR crystal used was a heated (50 °C) ZnSe crystal.

Fatty acid analysis with gas chromatography and gas chromatography–mass spectrometry

Samples were made ready for analysis by preparing methyl esters from the fats using a procedure based on a work of Christopherson and Glass [17]. This procedure was modified to render the obtained methyl esters more stable. The procedure used for methylation by means of transesterification was modified as follows: Approximately 2 g of anhydrous sodium sulphate were placed along with 5 ml of hexane in a 10 ml vial. Then we added 300 µl of fat and 350 µl of the solution for methylation (KOH/methanol 2 N) to the vial. The vial was closed and mixed for 30 s. It was placed in a bath at 40 °C for 15 min. The sample was removed from the bath, stirred using a vortex for 30 s, and cooled using ice. After 5 min, 300 µl of a solution of saturated citric acid was added in order to stop the reaction, and the solution was mixed for another 30 s by vortex stirring. The sample was kept in ice for another 5 min. The success of the reaction was indicated by the presence of an upper limpid solution and the appearance of the glycerine in the zone of the anhydrous sodium sulphate. For analysis with the on-column injection system, an ulterior dilution was prepared. The analysis revealed that optimal dilution occurred with a factor of ~160 (We therefore placed 0.8 ml of hexane and 5 µl of the obtained methyl esters in a 1 ml vial with a PTFE stopper).

The study was carried out on a MEGA 2 series gas chromatograph (Fisons Instruments Inc. Beverly, MA, USA), with an on-column injector and a FID detector from Carlo Erba (Carlo Erba/Fisons Instruments Valencia, CA, USA). The software used for the evaluation was the instrumental software from Unipoint Gilson. The method applied only integrated the peaks between 10 and 50 min retention time. Several measurements were repeated with a Hewlett Packard (HP 5890) gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, USA), with a split/splitless injector and a mass selective detector HP 5971 (Electronic Impact Mode, Full Scan) for constituent identification. The GC–MS spectra were also evaluated with the instrument's software (ChemStation G1701BA). The capillary column used for the GC and GC–MS measurements was a 50 m DB 5, 5% phenyldimethylpolysiloxane 0.5 µm column (J&W Scientific Inc., Folsom, CA, USA), 0.32 mm i.d. The sample solutions were analysed using helium as carrier gas, flow 1.5 ml/min, 1 µl injection volume, with an oven program of 60 °C (3 min), heating 20 °C/min to 120 °C (1 min), and second ramp of 3.5 °C/min to 280 °C (10 min).

Multivariate statistics

The results from the investigation with FT-IR and GC were partially evaluated using multivariate statistical tools. The software package Unscrambler (Camo Process AS, Oslo, Norway) was used. Spectral data and data extracted from chromatograms were evaluated using principal component analysis (PCA) and partial least-square analysis (PLS). These multivariate statistical tools permit the identification and qualitative analysis of the constituents of a complex mixture [18, 19]. PCA is used to depict data in a graphical representation that describes the majority of the variation in a data set. PLS was used in this study to build discriminant models that are able to discriminate between groups using all of the information included in the spectra. These so-called chemometric methods are described in several textbooks and publications [20–22].

Immunoassay dipsticks

The investigation was performed using a commercially-available immunoassay ruminant test kit, actually developed for the detection of MBM in feed: "Agri-Screen for ruminant in feed strip test". This kit is marketed by Neogen (Agri-Screen, Neogen Corporation, Lansing, MI, USA) and consists of a single-step immunochromatographic lateral flow assay used for the detection of processed proteins from ruminants in feed.

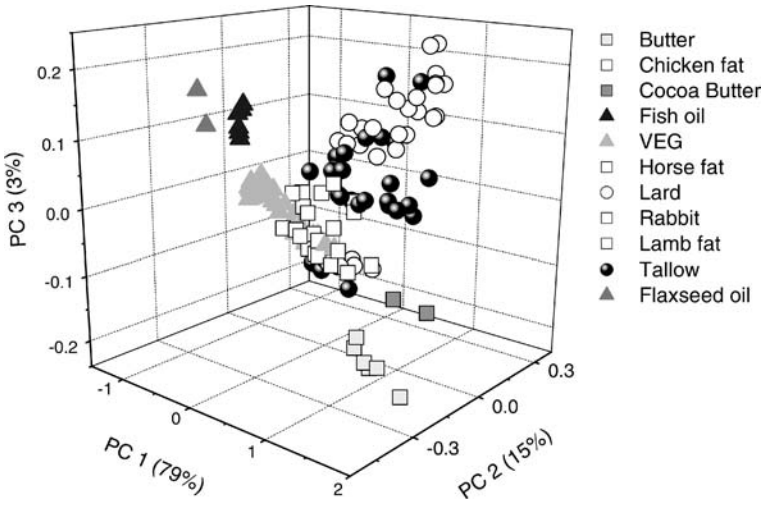

Fig. 1 Principal component analysis (PCA) visualisation of the results obtained for the MIR spectra

The extract is brought into contact with antibodies specific to heat stable ruminant muscle protein, conjugated to coloured particles. If ruminant by-product protein is present, it will be captured by the conjugated antibodies and it forms a visible line with immobilised antibodies specific to the ruminant muscle protein [23]. The reaction result was interpreted according to the manufacturer's recommendations: if two lines were present, the sample was positive. If one line was present (in the control zone), the sample was negative. If no line develops at all after 10 min, the test is invalid and should be repeated with another test strip.

Two methods for the extraction of proteins from the fat were evaluated: centrifugation and filtration of the liquid fat in an oven using a paper funnel. The extraction of the proteins using a funnel proved less effective and more time-consuming. Therefore, the extraction was performed using three cycles of centrifugation. For this, about 30 g of the fat was placed in an oven at 65 °C until it was molten, and then it was transferred to a centrifuge tube (50 ml) for 10 min centrifugation at 40 °C, 3,500 rpm. After this first centrifugation, the protein impurities present in the fat were clearly separated from the lipid fraction. In order to eliminate the fat completely, we removed most of the upper fat-fraction from the centrifuge tube and added hexane. Then we centrifuged the sample a second time for 10 min at 30 °C. After this step, we repeated the previous operation but we removed the mixture of fat/hexane left without also removing the fraction containing the protein. The sample then was centrifuged a third time under the same conditions. The hexane was removed. Then 5 ml of the extraction solvent together with a small amount of extraction additive, both provided with the kit, were placed directly into the centrifuge tube containing the proteins. The amount of extraction solvent added was measured to be ten times the weight of the residue, but no less than 0.5 ml, in accordance with the test strip dimensions. The tube was heated for 10 min in a bath with boiling water. The test strip was then dipped into the hot liquid sample and checked after 10 min; the results appeared as coloured bands on the dipstick.

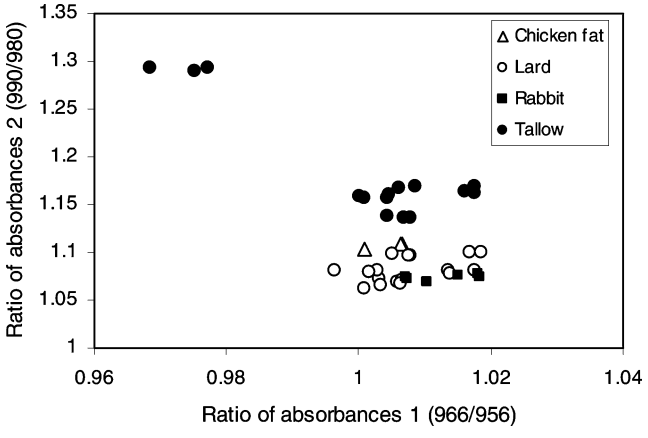

Fig. 2 Discrimination of tallow from other terrestrial animal fat, based on two absorbance ratios from the MIR spectra

Polymerase chain reaction

Before performing a genetic amplification by means of PCR, the fat samples were first treated in order to extract their contents in nucleic acid. Therefore, each sample was extracted twice on 2.00 g using the protocol advised by Promega for vegetable oil using their kit "Wizard Magnetic DNA purification for food". The protocol was slightly adapted as follows: (1) the test portion was of 2.00 g instead of 40 g; (2) the volumes of buffers used were all divided by two; (3) at the final step the DNA was recovered in 100 µl of water.

The obtained DNA was submitted to a real-time PCR on a GeneAmp 5700 real-time PCR device (Applied Biosystems, USA). Each reaction was performed in a total volume of 35 µl with 5 µl of template DNA (at one- and tenfold dilutions of the extract). Other components of the reaction were 17.5 µl of qPCR Mastermix

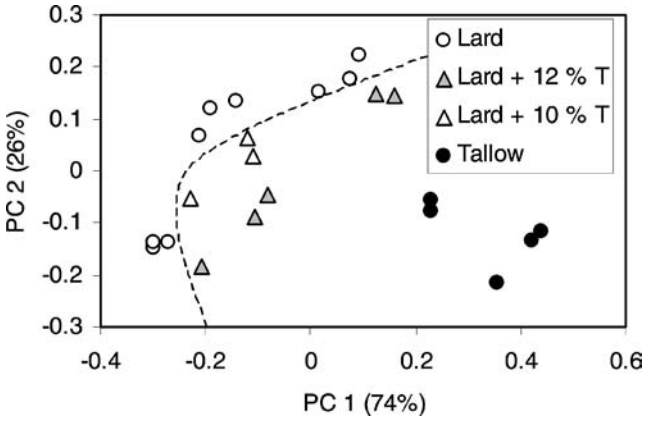

Fig. 3 Discrimination of tallow, lard, and mixtures of lard with 10 and 12% tallow using PCA. The dotted line indicates the border between pure and contaminated lard

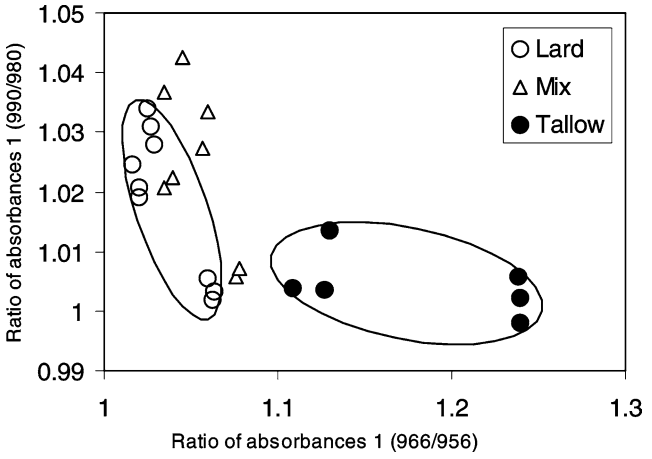

Fig. 4 Discrimination of tallow, lard, and mixtures of lard with 10 and 12% tallow using the a plot of absorbance ratio 1 (966/956) against absorbance ratio 2 (990/980)

(Eurogentec, Belgium), 0.75 µl of each "bovine" primer at 5 µM (Invitrogen, The Netherlands) and 2.5 µl of a bovine *Taq*Man-like probe (Eurogentec, Belgium), and 12 µl of PCR-grade water. The bovine primers and probe as well as the PCR conditions used over 50 cycles were identical to those initially developed for the detection of animal DNA targets in MBM samples [24, 25]. All results were analysed with the GeneAmp SDS software. The critical fluorescence used to calculate the Ct value (cycle threshold) of each sample was determined in such a way that the Ct value of a pure bovine DNA reached 19 cycles.

The following controls were used on each plate: negative extraction control, positive amplification control, and no template control. Moreover, on those samples considered to be negative, the PCR was performed again in the presence of cattle DNA to check whether the absence of signal on the original sample was due to PCR inhibition, but this was never the case.

Results

Potential of near-infrared and mid-infrared techniques

The NIR spectra (1,100–2,500 nm or 9,000–4,000 cm^{-1}) of fats and oils showed various overlapping bands that were the result of the first and the second overtones of fundamental bond vibrations as well as the combinations of two or more fundamental vibrations that occur in the MIR region. From low to high wavelengths, we encountered the regions corresponding to the second C–H overtone (9,500–8,000 cm^{-1}), a C–H combination band (7,700–6,900 cm^{-1}), the first C=H overtone (6,250–5,550 cm^{-1}), the OH combination band (5,320–5,180 cm^{-1}), and a CH combination band (4,770–4,170 cm^{-1}) [26]. Visual investigation of the NIR spectra did not reveal any specific indicators allowing the direct identification of the species the fats originated from. Similarly, analysis of data with PCA did not allow us to cluster the spectra according to the species. The NIR region, although allowing relatively fast analysis cycle times and a convenient measurement set-up, did not prove to be suitable for the task of animal identification, even for pure fat samples.

The MIR spectrum (4,000–400 cm^{-1}) of fats and oils contains well-separated peaks, mainly in the region 3,100–1,700 cm^{-1}, and overlapping peaks in the fingerprint region from 1,500 to 700 cm^{-1}. The MIR region of the electromagnetic spectrum contains mainly fundamental vibration bands where the vibration results in a change in the dipole moment of the molecule under consideration. From high to low wavenumbers, we have, at around 3,000 cm^{-1} a group of bands from C–H stretching vibrations; near 1,745 cm^{-1} a single peak from the C=O stretching vibration of carbonyl groups; in the 1,400–1,200 cm^{-1} region a bending vibration band from CH_2 and CH_3 groups; and, in the range 1,125–1,095 cm^{-1}, bands characteristic of the stretching vibrations of C–O and C–C groups appear [26]. As for the NIR spectra, the absorbances measured at one specific frequency did not allow the identification of a single species.

However, a PCA using the full spectra obtained with the MIR ATR infrared technique allowed the separation of various fats from tallow and lard. Figure 1 displays the samples according to the first three principal components (PC), obtained when PCA was applied to unpreprocessed full MIR spectra. These three PCs explained 79, 15 and 3% of the variance of the data set respectively. The region 1,000–900 cm^{-1}, located in the fingerprint region, is the most important when discriminating tallow and lard samples. Distinguishing fish oil, butter and cocoa butter from any other fat proved to be easy. The clusters of these groups were significantly separated from any other sample groups. Classification of the mammalian species the fats originated from was possible after removing the well-separated data for vegetable fats, oils and butter during the iterative pro-

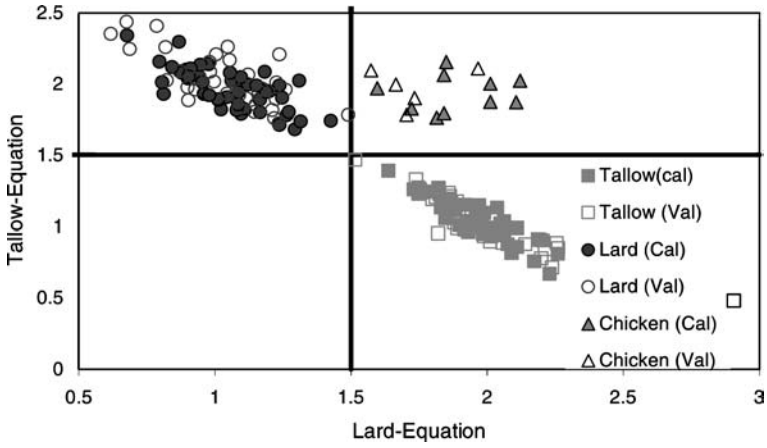

Fig. 5 PLS prediction of tallow, lard and chicken validation data using tallow and lard spectra for calibration

cess of building the statistical model. The spectra of tallow and lard then formed two separate clusters, allowing us to identify the origins of the pure products.

Noting a previous study on the potential of MIR for the discrimination of fat and oils [12], the discrimination power of two ratios of absorbances at defined wavenumbers was also studied. Figure 2 shows the discrimination of tallow from other terrestrial animal fats. The discrimination of tallow from any other mammalian fat is possible for the pure compound, and this is robust against variations in origins. The ratios use absorbances at frequencies characteristic of *trans*-isomers and that are positively correlated to the degree of unsaturation of the samples [14].

However, the discrimination performance of MIR data did not appear to be sufficient for the reliable detection of tallow contamination in lard. A content of 10% tallow in lard could be discriminated only after the exclusion of any other fat or oil samples based on multivariate statistics (a new statistical model was built in an iterative process after the removal of fat or oil samples from the sample base). Figure 3 shows the PCA for selected normalised spectral data from pure and mixed samples of tallow and lard. The figures showing the simple ratios of selected spectral wavenumbers show the good performance attained when distinguishing between pure samples (Fig. 3), but also the difficulties found when attempting to identify tallow contamination in a mixture of fats (Fig. 4).

The identification of tallow contamination would be even more difficult for mixtures containing fats from various sources. Several statistical approaches have been tested for the data but it was not possible to find a method for detecting tallow in a mixture of fats that is robust against variations in the matrix. However, for the pure compounds, a model could be built that allowed the identification of the species despite variations in origin and treatment.

We generated three PLS2 regression equations based on lard, tallow and chicken calibration spectra (Fig. 5). Here, the axes of the plot depict the prediction and reference values for the respective equations, where "1" corresponds to the correct identification of the lard or tallow calibration sample. We could discriminate samples by dividing the graph area into four zones used for prediction (see Fig. 5). The samples from the same species cluster in the same zone, and samples from other species cluster in other zones. This analysis showed that it may be possible to classify mixtures of tallow and lard, but an identification of a sample containing a mixture of 10% tallow in lard could not be verified. Analysing a larger number of samples could improve the classification.

Potential of gas chromatography

A total of 58 fat and oil samples and 21 fatty acid isomers were used to evaluate the potential applicability of GC to the identification of the species that the fat originates from. No single fatty acid could be identified that could be used to classify the fats. Upon building a PCA model with seven fatty acids, 99.98% of the variance in the data could be explained. Table 4 shows the relative areas of the chromatographic peaks for these selected fatty acids identified in chicken fat, lard and

Table 4 Gas chromatography investigation: relative chromatographic areas of the fatty acids (%, RT)

Sample	Tetra-decenoic methyl ester C14:1	Myristic acid methyl ester C14:0	Palmitoleic acid methyl ester C16:1	Linoleic acid methyl ester C18:2	Stearic acid methyl ester C18:0	Gadoleic acid methyl ester C20:1	Arachidic acid methyl ester C20:0
Chicken fat	0.20	1.10	4.14	2.09	0.13	0.42	0.02
Lard	0.02	1.55	2.41	3.18	0.15	1.04	0.07
Tallow	0.66	4.48	3.07	2.38	0.49	0.42	0.05

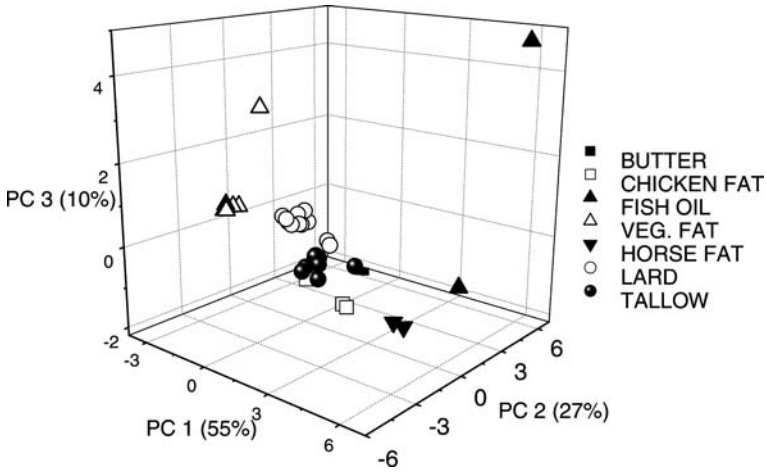

Fig. 6 Principal component analysis (PCA) visualisation of the GC results for different fats based on the chromatographic areas determined for fatty acids

tallow. Although the levels of single constituents can give a hint as to the origin of a sample—for example the relative abundance of C14 in tallow—this is not sufficient for clear and indisputable identification.

The use of multivariate statistical tools allowed the classification of fat samples according to their origin. Figure 6 displays the samples according to the three first principal components derived from the GC data (the relative peak areas). The three PC explain 55, 27 and 10% of the total variance respectively.

Vegetable fats and oils and fish oils are very well distinguished from any other fat. It is possible to derive the origin of the fat for pure fats. Tallow and lard form two separate clusters, allowing us to identify their respective origins. Table 5 shows the abundances of selected fatty acids measured in fat mixtures.

As discussed previously, no single fatty acid can be used to detect the contamination of fat with tallow. Therefore, it is only possible to discriminate between tallow and lard for the pure materials. Using multivariate analysis to exploit the GC data, it is only possible to detect tallow adulteration levels higher than 10% (Fig. 7). Indeed, a lard sample contaminated with 10% of tallow is included in a group of pure lard samples (highlighted with a circle in Fig. 7).

The results for the immunoassay and PCR

The results from PCR and immunoassay analyses are shown in Table 2 for the samples with tallow and in Table 3 for the samples without tallow. The samples and the corresponding results in Table 2 were further subdivided into four groups, which were (1) pure MBM samples, (2) fat with a residual insoluble impurity (RII) content above 0.15%, extracted from the MBM samples, (3) tallow and fat mixtures with a RII content below 0.15%, and (4) tallow and fat mixtures with a RII content below 0.02%.

Potential of the immunoassay dipstick technique

Analyses of the MBM samples treated at different temperatures and analyses of the corresponding fat samples revealed that the immunoassay was capable of detecting the presence of ruminant proteins, even in material that was heat treated up to 138 °C. This is a remarkable result, since former studies showed that the immunoassay response depend largely on the heat treatment of the test material [15]. The analysis of the fat samples demonstrated that detection of processed ruminant proteins

Table 5 Gas chromatography investigation: relative chromatographic areas (%) of selected fatty acids, measured for mixtures of tallow and lard

Sample	Myristic acid methyl ester C14:0	Palmitoleic acid methyl ester C16:1	Linoleic acid methyl ester C18:2	Stearic acid methyl ester C18:0	Gadoleic acid methyl ester C20:1
MIX 1 a Lard + 10% tallow	1.776	1.911	2.482	0.197	0.848
MIX 1 b Lard + 10% tallow	1.653	1.703	2.278	0.137	0.867
MIX 1 c Lard + 10% tallow	2.191	3.219	3.863	0.32	1.067
MIX 1 d Lard + 22.8% tallow	2.093	2.074	2.694	0.332	0.805
MIX 1 e Lard + 50.3% tallow	2.502	2.222	2.285	0.473	0.593
MIX 1 f Lard + 72.4% tallow	2.805	2.329	1.956	0.352	0.443
MIX 1 g Chicken + 10% tallow	1.417	5.072	2.164	0.22	0.468
MIX 3 a T/L/C (33/33/33)	2.06	3.274	2.286	0.379	0.559
MIX 3 b T/L/C (45/45/10)	1.653	0.324	2.452	0.139	0.69

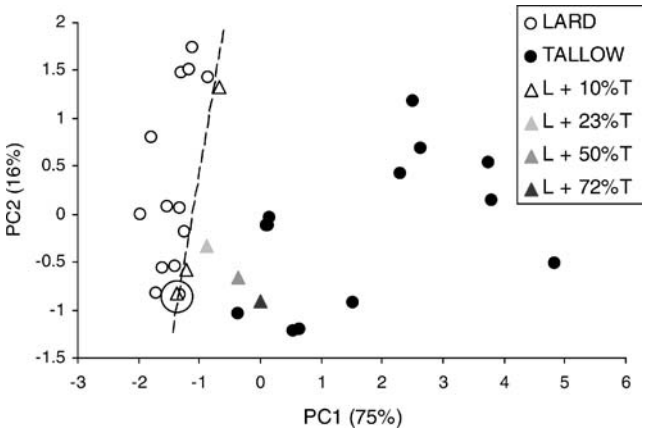

Fig. 7 Principal component analysis (PCA) visualisation of the GC data (chromatographic area, normalised data) from tallow, lard and mixtures. The dotted line indicates the border between pure and contaminated lard

was also possible when it is present at low concentrations in fat.

The third group comprised samples derived from tallow but containing up to 0.15% of RII. Pure samples and mixtures of tallow with lard produced positive responses at a tallow concentration of 5%. However, when the tallow portion was 2%, the test failed to detect the presence of tallow. It can be concluded that at tallow concentrations below 5%, the amount of ruminant protein is too low for a positive reaction to occur during the immunoassay.

The results from the fourth group containing the samples with very a low content of RII (0.02%) demonstrated that no detection of tallow was possible.

Therefore, the immunoassay technique demonstrated sufficient specificity, since the results from the analyses of all of the samples that did not contain tallow were negative, as shown in Table 3.

Potential of the PCR technique

The results from the real time PCR were interpreted visually, by checking to see whether any amplification curve appeared before 40 cycles. Indeed, based on the experience gained on the MBM samples, we found that signals that occur after 40 cycles may be not significant. Each sample provided at least four results: two test portions each at two dilutions of the extracts. Each test portion was considered as positive if it gave a significant signal from at least one dilution. Samples that were at the threshold (Ct of 40 cycles) or that were ambiguous for both tested portions (positive result for one portion, negative or not significant for the other one) were retried to reach a conclusion. Based upon these rules, the results obtained are listed in Tables 2 and 3. All MBM samples that contained processed proteins from cattle (first group of samples in Table 2) also contained PCR-detectable amounts of cattle DNA, whatever the temperature of the process (up to 138 °C). It is remarkable to observe that the fat samples still contained a considerable amount of mitochondrial DNA (second group of samples in Table 2).

Both tallow samples from the third group in Table 2 (samples with tallow, RII (%) <0.15) were tested positive, whereas only one out of the four samples belonging to the fourth group (samples with tallow, RII (%) <0.02) gave a positive result for the detection of cattle DNA. This is most likely due to the fact that there was no more DNA present in the samples. It is important to note that the results from the PCR analysis corresponded quite well with the results from the immunoassay analysis, because the latter method was also not able to detect tallow samples from the fourth group in Table 2.

Evaluating the mixtures from the third group (samples with tallow, RII (%) <0.15) revealed that the detecting the presence of tallow was even possible at 2% levels, indicating that the PCR technique was even more sensitive than the immunoassay method.

Two lard samples out of the seven tested gave a positive signal (Table 3). However, it is very difficult to determine whether this discrepancy was an artefact of the PCR or whether it was due to the fact that the test materials were real world samples that might contain some amount of cattle DNA. The specificity of the targets used is generally quite good, which was also confirmed by the chicken samples, which did not react to the PCR test.

Table 6 PCR results for specific samples from Table 2. Results derive from the same run and were performed on undiluted extracts in four replicates. Ct indicates the cycle threshold. Sample codes are taken from Tables 2 and 3

Mix	Composition	Ct				Mean Ct	Result PCR
Mix 1, Samples with tallow, RII (%) <0.15	Bovine fat (H)	25.9	25.4	30.7	31.1	28.3	+
	Lard (A)/Bovine fat (H) 50:50	28.2	28	27.9	27.8	28	+
	Lard (A)/Bovine fat (H) 90:10	32.7	33	33.5	32.9	33	+
	Lard (A)/Bovine fat (H) 95:5	32.7	32.1	35.3	35.5	33.9	+
	Lard (A)/Bovine fat (H) 98:2	36.9	35.4	33.7	34.4	35.1	+
Mix 2, Samples with tallow, RII (%) < 0.02	Bovine fat (G)	37.4	36.3	39.9	40.1	38.4	+
	Lard (G)/Bovine fat (G) 50:50	36.7	36.8	35.2	37.1	36.5	+
	Lard (G)/Bovine fat (G) 75:25	37.9	38.9	37.8	37.7	38.1	+
	Lard (G)/Bovine fat (G) 90:10	39.1	50	50	47.5	46.7	− (±)
	Lard (G)/Bovine fat (G) 95:5	45.7	50	50	50	48.9	−
	Lard (G)/Bovine fat (G) 98:2	50	50	50	50	50	−

The analysis of the mixtures from the fourth group in Table 2 (samples with tallow, RII (%) <0.02) gave six out of eight correctly positive results. However, for the samples containing lard (C), the analytical significance of these results is limited because the lard gave a positive PCR result (Table 3), while conversely when bovine fat (D) was used in the mixtures, it was undetectable by PCR (Table 2). The analyses of mixtures based on bovine fat (G) only gave a positive PCR result above a bovine content of 25%. These results show that mixtures of fat containing tallow of the highest quality are very difficult to detect, thereby confirming the results of the immunoassay.

In order to investigate the limit of detection for this PCR technique, the results from the mixtures (mix 1) based on bovine fat H (samples with tallow, RII (%) <0.15) and the results from the mixtures (mix 2) based on bovine G (samples with tallow, RII (%) <0.02) were subjected to close scrutiny. Table 6 gives more detailed information on the results obtained for these two mixtures. As can be seen from the data collected, both mixtures reacted in very different ways. In mix 2, rather high levels of tallow are required in order to be able to detect the bovine target using PCR, as the detection limits lies somewhere between 10 and 25%. The 10% sample has to be considered as giving a negative with the various analyses performed, although for one of the replicates on this sample (see Table 6), the Ct was smaller than 40, indicating that there is the beginning of detection at this level. The results clearly indicate that the performance of PCR in the detection of tallow is highly dependent on the type of treatment it has undergone. Also, the level of purity of the tallow turned out to be more important than the degree of heat treatment of the material; if sufficient DNA is left then detection might still be possible. Finally, it should be stated that a ruminant target would have been more appropriate, but as the aim here was just to assess the potential of PCR, we preferred to use a target known to be very sensitive.

Conclusion

We investigated four methods that could potentially be used to analyse fats used in the formulation of feedstuffs: FT-IR, GC, immunoassay-based detection kits and PCR. GC and FT-IR allowed us to classify pure samples depending on their species origin using multivariate statistical data evaluation, but it was not possible to reliably use any single compound for species identification. Also, neither of the methods proved to be suitable for detecting a specific fat, namely tallow, at concentrations of less than 10% in a mixture of fats. Of the four methods tested, only immunoassay dipsticks and PCR analyses were able to detect a contamination level as low as 5% of tallow in a matrix of other fats. PCR even reached the 2% detection level in one of the mixtures, but this performance level is in fact highly dependent on the type of tallow to be analysed and the heat process to which it was submitted. In addition, the immunoassay and PCR techniques were robust against variations in heat treatment. However, when analysing tallow of the highest quality (''premier jus''), the immunoassay and PCR techniques are not applicable, since the amount of residual protein or DNA in the sample is too low. However, this limitation does not pose a problem when using the immunoassay technique or PCR for this specific purpose, since ''premier jus'' tallow is not considered to be relevant in terms of TSE infectivity. Therefore, we can conclude that these methods could potentially be applied to the analysis of fat.

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