

**ASSAY FOR IDENTIFYING ANIMAL SPECIES IN RAW AND THERMALLY PROCESSED  
CONDITIONS**

**5 FIELD OF THE INVENTION**

The present invention relates to a method for identifying animal species in raw and thermally processed conditions based on animal fats detection.

**10 BACKGROUND OF THE INVENTION**

Mixing undeclared species in meat products is illegal under Food Labeling Regulations. Apart from regulatory reasons, the prevention of adulteration of meat products with less desirable meat species is important to the consumers for economic, health, food safety and  
15 religious reasons.

A conventional PCR technology for halal authentication by extracting DNA from animal species has been well known. However, this thermal processing limits the use of DNA technologies as heat treatment degraded the protein due to heat denaturation.

20

Several methods have been developed to identify meat species including electrophoresis, chromatography, DNA hybridization, and immunoassays. Immunological techniques, including agar-gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) are most commonly applied for meat species identification.

25

There are several disadvantages to the official method AGID. Concentrated antiserum preparations are required to obtain visible precipitin lines in AGID. Obtaining the antiserum is expensive in large-scale testing. Furthermore, the sensitivity of AGID is variable. Usually ten percent or more contamination must be present to detect adulteration with this method.

30

Lastly, AGID cannot be used for species identification in cooked meat because of the shortage of commercial antiserum specific to cooked meats.

The ELISA method has emerged as a sensitive, rapid, and specific method for meat speciation. This method can detect levels as low as one percent or less of meat  
35 contamination. Furthermore, it requires only simple sample preparation and no expensive

equipment or highly skilled operator. In addition, the ELISA technique can be used for both qualitative and quantitative analysis of meat proteins. Specific antibodies, either polyclonal antibodies or monoclonal antibodies (MAbs), are required as capture reagents in ELISA.

- 5 Monoclonal antibodies as disclosed in **WO02065126** and **US2003022248** documents which bind to Troponin I, a thermostable muscle protein for raw and cooked meat species identification, however, adulteration with fat and oil of different/other animal species limit the use of monoclonal antibodies (the antibodies are only binded to muscle protein).
- 10 Most of the ELISA methods currently employed for meat species identification use polyclonal antibodies as a capture reagent. However, polyclonal antibodies have disadvantages such as limited production, variable affinity and a requirement for further purification to eliminate cross reaction.
- 15 All the technologies mentioned above involves protein as an analyte or medium for detection and the adulteration with pure fat and oil of different/other animal species definitely limits the use of the technology. Further, when protein is exposed to heat, denaturation occurs during thermal processing of food. This is a constraint to the conventional technologies as food consumed in the developed countries has been subjected to varying degrees of heat
- 20 treatment at one stage or another, either through cooking, baking, broiling, toasting, roasting, canning, concentrating, pasteurizing, drying or frying to modify its flavour or texture and to improve the storage ability.

The present invention overcomes these and other deficiencies of the above-mentioned

25 drawbacks by providing a method for meat species identification by analysing the animal fat triacylglycerol (TAG) regioisomers in raw and thermally processed conditions. The present invention provides a greater efficiency and economically during operation.

## 30 SUMMARY OF THE INVENTION

Separation of six groups of animal fat TAG regioisomers on a single stage reversed phase high performance liquid chromatography (RP HPLC) coupling with atmospheric pressure chemical ionization tandem mass spectrometry (APCI) and ion trap mass spectrometer has

been developed for a potential method for discriminating different animal species (beef, pork, chicken and lamb) in raw and thermally processed food. The TAG profiles were analysed by the application of discriminant analysis (DA) to explore the extent of variation occurring with different animal species and different types of thermal processing.

- 5 The present invention provides a method for analysis of triacylglycerol regioisomers from animal fats comprising providing a preparation of a sample comprising triacylglycerol regioisomers; applying the preparation of sample as determined by chromatography having a separation material; separating and detecting the triacylglycerol regioisomers content of said preparation of the sample; collating the detection data into a data analysable form; and  
10 analysing the data using a discriminant analysis (DA) for identifying and determining animal fats belonged to different animal species.

In one of the preferred embodiment, the discriminant analysis (DA) is based on distribution of different animal species according to triacylglycerol (TAG) isomers.

15

In another preferred embodiment, the discriminant analysis (DA) provides a discriminant analysis plot with discrimination of meat from different animal species for raw and processed meat products.

- 20 In yet another embodiment, the animal species are cow, pig, chicken and lamb in raw condition and thermally processed condition.

- A method for identifying animal species in raw and thermally processed conditions comprising providing a preparation of a sample comprising triacylglycerol regioisomers; applying the preparation of sample as determined by chromatography having a separation material ;separating and detecting the triacylglycerol regioisomers content of said preparation of the sample; collating the detection data into a data analysable form; and characterized in that analysing the data using a discriminant analysis (DA) for identifying and determining animal fats belonged to different animal species.

30

**BRIEF DESCRIPTION OF THE DRAWINGS**

The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate embodiments of the invention and, together with the description,  
5 serve to explain the principles of the invention.

**Figure 1A** illustrates a RP HPLC-APCI MS Chromatograms of beef fat labelled with six groups of separated regioisomers of TAGs eluted with the mobile phase in accordance of the present invention.

10

**Figure 1B** illustrates a RP HPLC-APCI MS Chromatograms of TAG isomers from beef, lamb, chicken and pork eluted with the mobile phase in accordance of the present invention.

**Figure 2A** illustrates a RP HPLC-APCI MS Chromatograms of POM\*/PMO\* isomers in beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention.

15

**Figure 2B** illustrates a RP HPLC-APCI MS Chromatograms of OOP\*/OPO isomers from beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention.

20

**Figure 2C** illustrates a RP HPLC-APCI MS Chromatograms of POP/PPO\* isomers in beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention.

25

**Figure 2D** illustrates a RP HPLC-APCI MS Chromatograms of OOS\*/OSO isomers in beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention.

30

**Figure 2E** illustrates a RP HPLC-APCI MS Chromatograms of SOP\*/SPO\* isomers from beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention.

**Figure 2F** illustrates a RP HPLC-APCI MS Chromatograms of SOS/SSO\* isomers from beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention.

- 5 **Figure 3A** illustrates a Discriminant Analysis (DA) plot for raw meat of different animal species in accordance of an embodiment of the present invention.

**Figure 3B** illustrates a Discriminant Analysis (DA) plot for raw and processed meat products of different animal species in accordance of an embodiment of the present invention.

10

## DETAILED DESCRIPTIONS OF THE INVENTION

- 15 The present invention will now be described in detail in connection with specific embodiments with reference to the accompanying drawings. Unless the context requires otherwise, throughout the specification and claims which follow, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open, inclusive sense that is as "including, but not limited to". Reference throughout this
- 20 specification to "one embodiment" or "an embodiment" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics
- 25 may be combined in any suitable manner in one or more embodiments. With respect to the use of substantially any plural and/or singular terms herein, those having skill in the art can translate from the plural to the singular and/or from the singular to the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for sake of clarity. It will be further understood by those within
- 30 the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description, claims, or drawings, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or "B" or "A and B."

35

HPLC is a very well-known method for the separation and analysis of solute molecules. The separation process is effected through liquid chromatography and relies on the fact that a number of component solute molecules in a sample stream of fluid (mobile phase) flowing through a packed bed of particles (stationary phase) can be efficiently separated from one another with a high degree of resolution. This is based on the fact that individual components in the mobile phase have a different affinity for the stationary phase and therefore a different rate of migration and exit through the column. The efficiency of separation is determined by the amount of spreading through the column which is determined by the column composition.

Triacylglycerol (TAG) or triglyceride are the main components of fats and oils and are comprised of three fatty acids esterified at the stereo-specific number (sn) positions of sn-1, sn-2 and sn-3 of a glycerol molecule. Triacylglycerols in fats and oils have many properties which depend upon their fatty acid composition and especially the stereo-specific distribution of the fatty acids on the glycerol moiety. The three letter code of TAGs in the present invention includes the suffix "\*" where more than one regioisomer can exist in a form that can, potentially, be separated chromatographically".

By meat type or class is intended a broader class of meats including several meat species.

Thus, type or class refers to mammalian, avian, poultry, and the like. By meat or meat species is generally intended meats which are commonly used for consumption, including poultry such as chicken and mammalian meats, such as pork, beef and lamb. Thus, particular meat species include but are not limited to chicken, pork, beef, lamb and the like.

The present invention relates to a separation of animal fats, TAG regioisomers on a single stage reversed phase high performance liquid chromatography (RP HPLC) coupling with atmospheric pressure chemical ionization tandem mass spectrometry (APCI) and ion trap mass spectrometer has been developed for a potential method for discriminating different animal species (beef, pork, chicken and lamb) cooked by microwave, roasting, hotpot and currying. The TAG regioisomer profiles are analysed by application of discriminant analysis, (DA).

Analysis of TAG regioisomers by mass spectrometer (MS) has been achieved by analyzing linear calibration plots of fractional DAG fragment intensities against the fractional composition of binary mixtures of standards (Malone and Evans, 2004; Li and Evans., 2005

and Fauconnot et al., 2004). However, as the regioisomers were not separated, the MS/MS spectra across the chromatographic peaks of co-eluting regioisomers give composite spectra. Thus, it is difficult to retrieve the relative abundance data from the composite spectra. Regiospecific characterisation of the TAGs in animal fats has been performed using HPLC-APCI-MS by Mottram et al. (2001), who determined the identification of FAs in the sn-2 position by lipase degradation and validated the HPLC-APCI-MS method for determining the sn-2 FAs from the relative intensities of DAG fragment ions in lamb and pork fat. The compositions of the TAG fraction of lard, tallow and a mixture of both were determined using a multidimensional HPLC RP HPLC-UV and silver ion HPLC-APCI MS and the technique was shown to allow discrimination of tallow in lard via the sn-POP/sn-PPO ratio (Dugo et al., 2006). The combination of RP HPLC and Ag HPLC allowed the resolution of critical pairs and isomers in rice oil due to the different retention mechanism of TAGs under these two distinct HPLC modes (Dugo et al., 2004). Separation of the regioisomers of the synthetic monosaturated and disaturated TAGs POP-PPO and PLP-PPL was demonstrated for the first time on a single RP HPLC column (Momchilova et al., 2004).

However, to date, there is no report for complete separation of regioisomers in animal fat. In the present invention, a method is developed for complete separation of six groups of TAG regioisomers from animal fats. A developed mobile phase gradients (combination of acetonitrile, dichloromethane and 10 mM ammonium acetate in methanol) are in Table 1.

**Table 1.** The mobile phase gradient for separation of TAG regioisomers.

| Time (min) | Flow rate<br>(mL/min) | % acetonitrile | %<br>dichloromethane | % ammonium<br>acetate (10 mM) |
|------------|-----------------------|----------------|----------------------|-------------------------------|
| 0          | 1                     | 76             | 20                   | 4                             |
| 82         | 1                     | 75             | 21                   | 4                             |
| 145        | 1                     | 61             | 35                   | 4                             |

In APCI, TAGs are identified from the ammoniated molecules and their diacylglycerols (DAG) ions in MS/MS or tandem mass spectrometry (MS<sup>2</sup>). The fatty acid (FA) positions are identified from the presence and relative intensities of the DAG product ions in MS<sup>2</sup>. The relative intensities of DAG product ions in the MS<sup>2</sup> spectra of the ammoniated TAG ions

depend on FA position along the glycerol backbone (Byrdwell, 2005). The TAGs exhibiting no co-elution give clean spectra, which is important in obtaining the correct DAG ion ratios for compound identification.

- 5 The TAG isomers of the present invention produce identical ammoniated and protonated molecules and the dissociation of ammoniated TAG isomers produce the same DAGs product ion in MS<sup>2</sup> but in differing relative intensities. The DAG product ion abundances are very important for distinguishing between isomers. For example, OOP\* and OPO isomers produce a protonated molecule at m/z 859.5; in MS<sup>2</sup>, DAG product ions occur in the ratio of [OP]<sup>+</sup>: [OO]<sup>+</sup> about 3:1 for OPO and about 1:1 for OOP\*.
- 10

The TAG isomer separation for the four different animal species examined shows that TAG isomers are prevalent in beef and lamb but not in pork and chicken in which only the most abundant isomers are observed. The prevalence of regioisomers in the beef and lamb fat is obvious due to one isomer being more abundant than the other. The peaks observed eluting after PMO\*, OOP\*, POP, OOS\*, SOP\* and SOS in beef and lamb fat are identified in MS<sup>2</sup> to their regioisomers. Thus, six groups of TAG regioisomers are resolved from beef and lamb fat: POM\*/PMO\*, OOP\*/OPO, POP/PPO\*, OOS\*/OSO, SOP\*/SPO\*, SOS/SSO\*. The most abundant isomers in chicken are OOP\*, POP, OOS\*, SOP\* and SSO\*. In pork, isomers of OPO, PPO\*, OOS\*, SPO\* and SSO\* are the most abundant. TAG of POM\*/PMO\* is not observed in chicken and pork.

15

20

The most important influence on the elution order of the regioisomers of the present invention is the position of the unsaturated FA in the TAG molecules. In all cases, the isomer with the unsaturated FA in either the sn-1 or sn-3 position is retained more strongly than the isomer with the unsaturated FA in the sn-2 position. This is due to the polarity of the stationary phase, which can interact more strongly with the more polar unsaturated FA when it is located in the more accessible position of the TAG molecule (Momchilova et al., 2004). In the case of TAGs containing both saturated and unsaturated FAs, the regioisomer with the unsaturated FA in the sn-2 position is always the most abundant in animal fats.

25

30

The individual TAG isomer peak areas of all identified compounds for all animal species are expressed relative to the total peak area of six TAG isomer groups. To overcome the difficulties in attempting to detect adulterants that have approximately the same chemical composition as the original food product, the instrumental techniques of RP HPLC APCI MS

35

used are associated with the use of chemometrics which is discriminant analysis (DA) in this present invention. All of six TAG isomer groups are included in the discriminant analysis and plot.

- 5 The discrimination between different species is very clear in the discriminant analysis plot. All animal species of beef, pork, chicken and lamb are well separated in the discriminant analysis plot of TAG isomer profiles. The meat products processed or cooked by microwave, roasting, hotpot and currying are also well separated in the discriminant analysis scores plot. The present invention confirms that the discrimination of meat from different animal species  
10 is achievable for both raw and processed meat products, and reveals that the differences produced by the various processing methods are less than the variations observed between species.

- In one preferred method for analysis of triacylglycerol regioisomers from animal fats of the present invention comprising by providing a preparation of a sample comprising triacylglycerol regioisomers at the beginning. The preparation sample is determined by chromatography having a separation material. A process of separation and detection of triacylglycerol regioisomers content of the preparation of the sample is occurred. The detection data collected is then collated into a data analysable form and finally discriminant  
15 analysis is used to analyse the data to identify and determine the animal fats belonged to different animal species. This method is further applied to identify animal species in raw and thermally processed conditions. The method for analysis of triacylglycerol regioisomers from animal fats of the present invention is further provided with examples as illustrated below:

25

### **Examples**

#### ***Materials and Methods***

##### **A. Sampling**

###### **Different anatomical locations of adipose tissue from pigs**

- A total of 24 samples of adipose tissue from pork fat were taken from four different locations  
35 from three different pigs. The four locations were; ham, shoulder, loin and belly. The adipose tissue samples (about 5 g) were taken from subcutaneous and intermuscular sections at each of the locations, following Timon et al., 2001. All samples were extracted immediately.

### Raw/unprocessed meat samples

Meat samples from beef (labelled fresh British beef sirloin steak), chicken (labelled fresh British chicken) and lamb (fresh British lamb chops) were purchased from a local supermarket. Chicken samples were deboned (skin and muscle were used) whereas beef and lamb were used as purchased. All samples (about 5 g) were homogenised in the laboratory and extracted on the day of purchase.

### Processed meat products

Three different methods of processing were studied to determine the effect of each on the TAG profiles in the meat products:

**Microwave open cooking:** Different homogenised samples ( $\approx 5$  g) of beef, lamb and chicken as specified in raw/uncooked meat samples, and pork ham were cooked in a microwave oven (700 W, full power, 4 min) and extracted immediately.

**Cooked and roasted cooking:** Samples of cooked and roasted pork, beef and chicken were purchased from a local supermarket, labeled as; 'cooked and roasted pork formed from cuts of pork', 'sliced cooked and roasted beef' and 'reformed, cooked and roasted chicken with added pea fibre' on their packaging. The samples (about 5 g) were homogenised and extracted on the day of purchase.

**Curry and hotpot:** Samples of beef and chicken curry (labeled as 'beef curry: pieces of beef in a spicy curry sauce' and 'chicken curry: chicken pieces in a mild curry sauce') and lamb hotpot (labeled as 'Cumbrian lamb hotpot slow-cooked traditional kitchen recipes') were purchased from a local supermarket. The samples (about 5 g) were homogenised and extracted on the day of purchase.

### **B. Extraction procedure - Accelerated Solvent Extraction (ASE)**

The samples of raw and processed meat products were extracted according to Dionex Application Note 334 i.e. Rapid determination of fat in meat using accelerated solvent extraction (ASE).

### C. RP HPLC APCI MS separation and detection of TAG profiles

5 The procedure for separation of TAG regioisomer profiles and detection by reversed phase high performance liquid chromatography (RP HPLC) coupling with atmospheric pressure chemical ionization tandem mass spectrometry (APCI) and ion trap mass spectrometer. A developed mobile phase gradients (combination of acetonitrile, dichloromethane and 10 mM ammonium acetate in methanol) are shown in Table 1. Two Waters Spherisorb ODS2 (150  
10 mm x 4.6 mm, 3  $\mu$ m) columns connected in series were used for the elution of the TAG regioisomers and the injection volume was 25  $\mu$ L and the flow rate 1 mL/min in the present invention. An Ultimate 3000 (Dionex) HPLC was used in combination with a Bruker HCT ultra ETD II ion trap. The APCI source was operated in the positive ion mode. The vaporiser temperature was set to 450°C and drying temperature to 150°C. Nitrogen was used as a dry  
15 gas at a flow rate of 3 L/min and nebulizer gas at 50 psi. Scans were obtained from  $m/z$  330 – 1000. The smart tune and auto MS<sup>n</sup> to MS<sup>3</sup> were selected. The TAG regioisomer profiles were then analysed by application of discriminant analysis (DA).

## 20 RESULTS

Figure 1A illustrates a RP HPLC-APCI MS Chromatograms of beef fat labelled with six groups of separated regioisomers of TAGs eluted with the mobile phase in Table 1 above in accordance of the present invention. Figure 1B illustrates a RP HPLC-APCI MS  
25 Chromatograms of TAG isomers from beef, lamb, chicken and pork eluted with the mobile phase in Table 1 above in accordance of the present invention.

Figure 2A illustrates a RP HPLC-APCI MS Chromatograms of POM\*/PMO\* isomers in beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention. The first TAG isomer pair eluted in beef fat corresponds to POM\* and  
30 PMO\*. Both isomers produced identical ammoniated and protonated molecules at different retention times, and the dissociation of these ammoniated POM\* and PMO\* isomers produce the same DAGs product ions: [PM]<sup>+</sup>, [PO]<sup>+</sup> and [MO]<sup>+</sup> in MS<sup>2</sup> but in differing relative intensities. The POM\* isomer eluted first with the unsaturated FA being located in the sn-2  
35 position. The MS<sup>2</sup> spectrum showed the least abundant DAG ion [PM]<sup>+</sup>, formed by loss of oleic acid from the sn-2 position, at  $m/z$  523. The later eluting and minor isomer, PMO\*,

showed loss of margaric acid from the sn-2 position to produce the least abundant [PO]<sup>+</sup> ion at *m/z* 577. A peak corresponding to MPO was not detected, though it was not possible to rule out its presence as a minor constituent. The separation of this isomer pair was completed by 79 min for this particular run and the resolution of the peaks, *R<sub>s</sub>* was about 1.2.

5

**Figure 2B** illustrates a RP HPLC-APCI MS Chromatograms of OOP\*/OPO isomers from beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention. Within the OOP\* group the isomer could either be symmetric or asymmetric. The OOP\* isomer eluted first due to one of the unsaturated oleic acids being located in the sn-1/3 position. The OPO isomer in which oleic acid occupied, both the sn-1 and sn-3 positions exhibited greater retention. OPO and OOP\* had the same ammoniated and protonated molecules at *m/z* 876 and 859, respectively, and provided the same DAG product ions in MS<sup>2</sup>: [OP]<sup>+</sup> (*m/z* 577) and [OO]<sup>+</sup> (*m/z* 603) but in differing relative intensities. The ratio of [OP]<sup>+</sup>: [OO]<sup>+</sup> was higher for OPO (about 3:1) than for OOP\* where it was 1:1.

10

15

**Figure 2C** illustrates a RP HPLC-APCI MS Chromatograms of POP/PPO\* isomers in beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention. For the POP/PPO\* isomer pair, POP was the most abundant isomer in the samples and was the first to elute. The unsaturated FA, being located in the sn-2 position, provided the shorter *t<sub>R</sub>* and the MS<sup>2</sup> spectrum showed a DAG product ion ratio of [OP]<sup>+</sup> to [PP]<sup>+</sup> of about 3:1 for POP and about 1:1 for PPO\*. The POP/PPO\* isomers were fully separated within 93 to 96 min in this particular separation of beef fat.

20

25

**Figure 2D** illustrates a RP HPLC-APCI MS Chromatograms of OOS\*/OSO isomers in beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention. For the OOS\* and OSO isomer pair, OOS\* eluted first: the unsaturated oleic acid occupied the sn-1 and sn-3 positions in OSO, hence the isomer was retained preferentially. OOS\* was the most abundant isomer in beef fat. The structure was confirmed from the MS<sup>2</sup> spectrum by consideration of the DAG product ions: the ratio of [OS]<sup>+</sup> to [OO]<sup>+</sup> was about 2:1 for OOS\* and 3:1 for the OSO isomer. The separation of this isomer pair was completed within 106.1 min for this particular run.

30

35

**Figure 2E** illustrates a RP HPLC-APCI MS Chromatograms of SOP\*/SPO\* isomers from beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention. Within the SOP\*/SPO\* isomer group, SOP\* was first eluted as the

unsaturated FA was located in the sn-2 position. The DAG ions in the MS<sup>2</sup> spectra were [PO]<sup>+</sup> (m/z 577), [PS]<sup>+</sup> (m/z 579) and [SO]<sup>+</sup> (m/z 605). The SOP\* isomer MS<sup>2</sup> spectrum showed the least abundant DAG ion to be [PS]<sup>+</sup> which resulted from loss of oleic acid from the sn-2 position illustrated that the compound was SOP\*. The later eluting isomer showed [SO]<sup>+</sup> as the least abundant DAG ion due to loss of palmitic acid from sn-2 position. This dissociation confirmed that the compound was SPO\*. The PSO isomer was not detected; this could be connected to a very low concentration or co-elution with SPO\*. The separation of this isomer pair was completed within 109.8 min for this particular run.

10 **Figure 2F** illustrates a RP HPLC-APCI MS Chromatograms of SOS/SSO\* isomers from beef fat and insert MS<sup>2</sup> spectra (PI = precursor ion) in accordance of an embodiment of the present invention. For the isomer pair SOS/SSO\*, SOS was first eluted as the unsaturated FA was located in the sn-2 position and SSO\*, which had the unsaturated FA in the sn-1/3 position, was retained preferentially. Both isomers undergone dissociation to produce two DAG ions at m/z 605 ([OS]<sup>+</sup>) and m/z 607 ([SS]<sup>+</sup>) in MS<sup>2</sup> due to loss of stearic and oleic acids, respectively. The spectrum of the first eluting isomer showed a ratio for [OS]<sup>+</sup> to [SS]<sup>+</sup> of about 4:1, confirmed that it was SOS. The later eluting isomer dissociated to produce the same product ions in a 1:1 ratio, corresponding to the SSO\* isomer. The elution of the SOS and SSO\* isomer pair occurred by 121 min for this particular run, and with a resolution of about 1.36.

**Figure 3A** illustrates a Discriminant Analysis (DA) plot for raw meat of different animal species in accordance of an embodiment of the present invention. The first two factors correlations in the DA scores plot showed the distribution of different animal species according to their TAG isomers and with 99.2% of the variance being explained; 84.1% from the first factors (F1) and 15.1% from the second one (F2). All animal species were separated well and no overlap was observed between them. The discrimination between different species was very clear.

30 **Figure 3B** illustrates a Discriminant Analysis (DA) plot for raw and processed meat products of different animal species in accordance of an embodiment of the present invention. The discrimination between different species of thermal processed product was very clear. The first two factors correlations in the DA scores plot showed the distribution of raw and processed meat according to their TAG isomers and with 97% of the variance being explained; 89% from the first factors (F1) and 8% from the second one (F2). All of the raw

and processed meat products grouped well by species and were well separated between species with no overlapped between them. It was particularly noteworthy that the processed meats plotted close or overlap to the raw meat for each species. This indicated similarity in their TAG isomer profiles and showed that the discrimination of different species was achievable for both raw and processed meat products.

From the above results, it is concluded that the meat products of different species (beef, pork, chicken and lamb) cooked by microwave, roasting, hotpot and currying are separated well in the Discriminant Analysis (DA) scores plot. The present invention confirms that the discrimination of meat from different animal species is possible for both raw and processed meat products, and reveals that the differences produced by the various processing methods are less than the variations observed between species.

The techniques developed in the present invention have made important advances in the analysis of TAG regioisomers demonstrating clear improvements in their chromatographic resolution and, by utilizing APCI in combination with ammonium acetate to identify regioisomers, enabling their distributions to be characterised more accurately.

The foregoing detailed description and examples are merely illustrative of the preferred embodiments. They are by no means meant to be the exclusive description of the inventive concept hereby disclosed. It will be recognized by one of ordinary skill in the art that certain aspects of the practice of the invention are readily susceptible to modification or practice by alternative, known means.

**REFERENCES****1. Fauconnot et al., 2004;**

Fauconnot L., Hau J., Aeschlimann JM., Fay L.B., Dionisi F., Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 2004; **18**: 218-224.

**2. Dugo et al., 2004;**

Dugo P., Favoino O., Tranchida P.Q., Dugo G., and Mondello L., Off-line coupling of non-aqueous reversed-phase and silver ion high-performance liquid chromatography-mass spectrometry for the characterization of rice oil triacylglycerol positional isomer. *Journal of Chromatography A.* 2004; **1041**: 135-142.

**3. Dugo et al., 2006;**

Dugo F., Kumm T., Fazio A., Dugo G., Mondello L., Determination of beef tallow in lard through a multidimensional off-line non-aqueous reversed phase-argentation LC method coupled to mass spectrometry. *J. Sep. Sci.* 2006; **29**: 567-575.

**4. Li and Evans, 2005;**

Li X., Evans J.J., Examining the collision-induced decomposition spectra of ammoniated triglycerides as a function of fatty acid chain length and degree of unsaturation. I. The OXO/YOY series. *Rapid Commun. Mass Spectrom.* 2005; **19**: 2528-2538.

**5. Mottram et al., 2001**

Mottram H.R., Crossman Z.M., Evershed R.P., Regiospecific characterisation of the triacylglycerols in animal fats using high-performance liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry. *Analyst.* 2001; **126**: 1018-1024.

**6. Malone and Evans., 2004**

Malone M., Evans J.J., Determining the relative amounts of positional isomers in complex mixtures of triglycerides using reversed-phase high-performance liquid chromatography-tandem mass spectrometry. *Lipids.* 2004; **39**(3):273-284.

**7. Momchilova et al., 2004**

Momchilova S., Tsuji K., Itabashi Y., Damyanova B.N., Kuksis A., Resolution of triacylglycerol positional isomers by reversed-phase high-performance liquid chromatography. *J. Sep. Sci.* 2004;27 : 1033-1036.

**8. Byrdwell, 2005**

Byrdwell W.C., Atmospheric pressure ionization techniques in modern lipid analysis. *Modern Methods for Lipid Analysis by Liquid Chromatography/Mass Spectrometry and Related Techniques*. 2005; Chapter 1: 1-18, Chapter 7: 299-412.

**9. Timon et al., 2001**

Timon M.L., Ventanas J., Carrapiso A.I., Garcia J.C. , Subcutaneous and intermuscular fat characterization of dry-cured Iberian hams. *Meat Science*. 2001; 58:85-91.

**10. Dionex Application Note 334**

Rapid determination of fat in meat using accelerated solvent extraction (ASE).

<http://www.dionex.com/en-us/documents/application-notes-updates/lp-84398.html>

**CLAIMS**

1. A method for analysis of triacylglycerol regioisomers from animal fats comprising
  - 5 providing a preparation of a sample comprising triacylglycerol regioisomers;  
applying the preparation of sample as determined by chromatography having a  
separation material;  
separating and detecting the triacylglycerol regioisomers content of said  
preparation of the sample;
  - 10 collating the detection data into a data analysable form; and  
analysing the data using a discriminant analysis (DA) for identifying and  
determining animal fats belonged to different animal species.
2. The method as claimed in Claim 1 wherein the discriminant analysis (DA) is
  - 15 based on distribution of different animal species according to triacylglycerol  
(TAG) isomers.
3. The method as claimed in Claim 2 wherein the discriminant analysis (DA)
  - 20 provides a discriminant analysis plot with discrimination of meat from different  
animal species for raw and processed meat products.
4. The method as claimed in Claim 1 wherein the animal species are cow, pig,  
chicken and lamb in raw condition and thermally processed condition.
5. A method for identifying animal species in raw and thermally processed
  - 25 conditions comprising  
  
providing a preparation of a sample comprising triacylglycerol regioisomers;  
applying the preparation of sample as determined by chromatography having a  
30 separation material;  
separating and detecting the triacylglycerol regioisomers content of said  
preparation of the sample;  
collating the detection data into a data analysable form; and  
analysing the data using a discriminant analysis (DA) for identifying and  
35 determining animal fats belonged to different animal species.

6. The method as claimed in Claim 5 wherein the discriminant analysis (DA) is based on distribution of different animal species according to triacylglycerol (TAG) isomers.

5

7. The method as claimed in Claim 6 wherein the discriminant analysis (DA) provides a discriminant analysis plot with discrimination of meat from different animal species for raw and processed meat products.

10

8. The method as claimed in Claim 5 wherein the animal species are cow, pig, chicken and lamb in raw condition and thermally processed condition.

15

20

25

30

35