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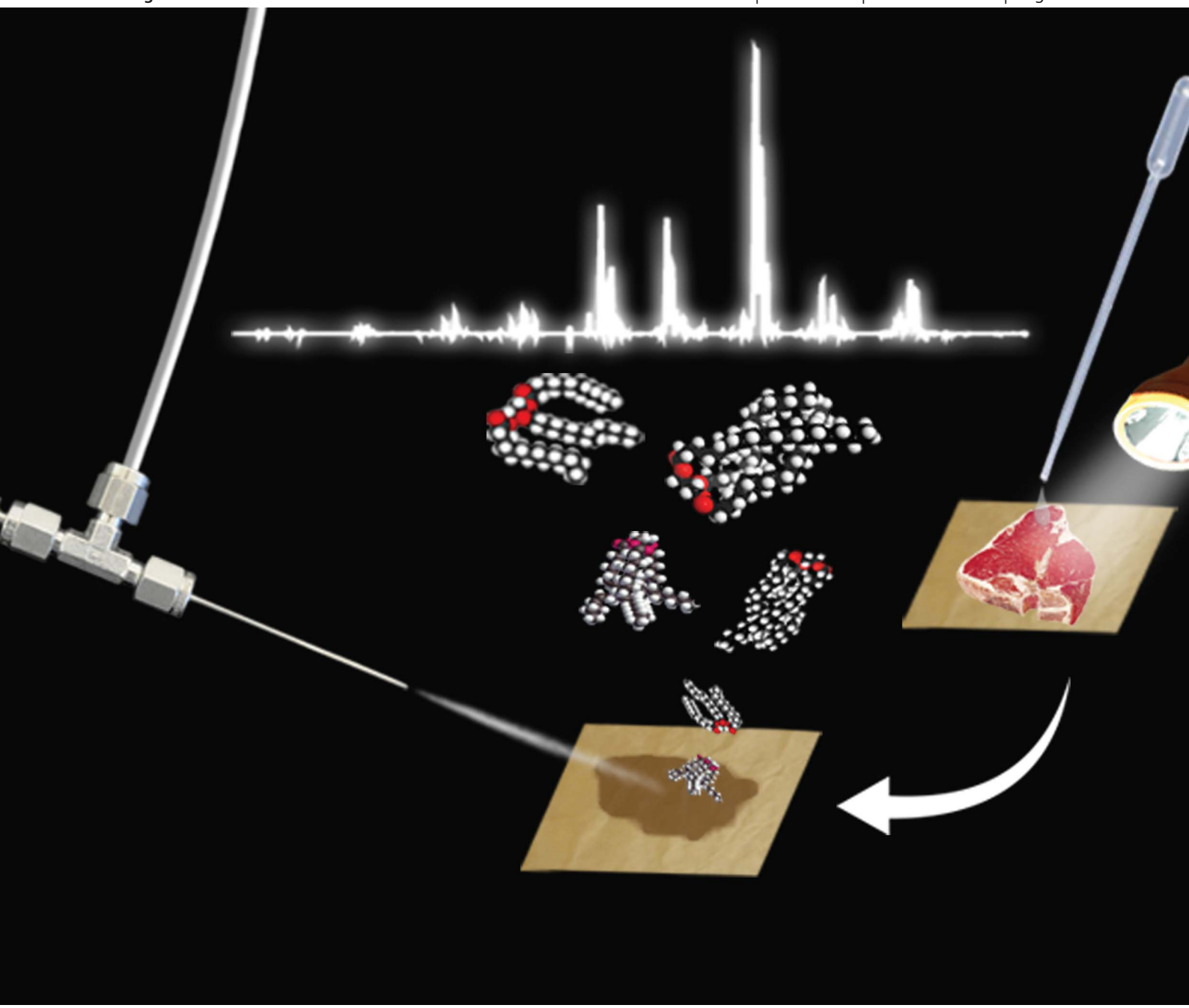

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PAPER

Intact triacylglycerol profiles of fats and meats *via* thermal imprinting easy ambient sonic-spray ionization mass spectrometry

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Thermal imprinting (TI) on a paper surface, using minimal solvent amounts, followed by direct analysis of the triacylglycerols (TAG) content *via* easy ambient sonic-spray ionization mass spectrometry (EASI-MS) is shown to provide a fast protocol to analyze TAG in meats and fats. The technique is simple, fast and eco-friendly requiring no hydrolysis, derivatization or chromatographic separation. The entire TI-EASI-MS protocol is performed in a few minutes and with minimal sample handling and solvent consumption. The TAG profiles obtained *via* TI-EASI-MS are shown to be quite similar to those obtained using GC and MALDI-MS analyses, and the imprinting and mailing of the imprinted paper in a sealed plastic bag is proposed for remote TI-EASI-MS analysis of meat and fat samples.

Introduction

Triacylglycerols (TAG) are the major constituents of oils and fats, and are responsible for energy storage in animals and plants, acting also as solvents for liposoluble vitamins. TAG have also great nutritional value which varies according to the level of unsaturation in their fatty acyl chains.[1] These key lipids also affect the structure, stability, taste, aroma, storage quality and sensory and visual characteristics of foods.[2]

TAG composition or its variation as a function of age, diet, maturation or degradation is therefore a major parameter of animal meat and fat quality. The determination of TAG composition represents normally a highly demanding and complex task due to the variety of natural fatty acids (FA) and their specific location on the glycerol backbone of triacylglycerols. Gas chromatography (GC) has been the most widely used technique for "indirect" TAG analysis,[3,4] but it requires hydrolysis and derivatization of the free FA to more volatile derivatives. High-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) has also been employed to quantify and characterize intact TAG with no need for derivatization.[5–8] Direct MS techniques with no previous chromatographic separation, such as matrix-assisted laser desorption ionization MS (MALDI-MS)[9–13] and electrospray ionization MS (ESI-MS),[14,15] have also been used for intact TAG profiling. TAG analysis in biological matrices such as tissues, meats and fats is even more demanding since many of the TAG contents are encapsulated by cellular membranes; hence extraction procedures (usually involving solvent extraction, centrifugation and filtration) are required. There are many consolidated liquid extraction methods for TAG and total lipids in such matrices, but their application is time-consuming and they also demand relatively high quantities of high quality solvents or gases, as it is also the case for chromatographic separation techniques.[16,17]

In a continuous trend towards ease and simplicity in MS analysis, a set of ambient desorption/ionization MS techniques has been recently introduced.[18–22] These techniques eliminated or greatly simplified sample preparation protocols therefore allowing direct analysis of molecules placed on inert or selective surfaces or on their natural matrices. Among these techniques, easy ambient sonic-spray ionization mass spectrometry (EASI-MS)[23–25] has been shown to be one of the simplest and easiest to assemble. EASI provides ultra soft ionization without the need for voltages, UV lights, laser beams, corona or glow discharges, or heating. EASI is therefore inherently free of electrical, thermal and discharge interferences. Based on sonic-spray ionization[26] which promotes unbalanced charge distribution, the EASI spray is composed of very minute bipolar droplets. These bipolar droplets desorb and ionize the analyte molecules from surfaces. EASI-MS has already been applied to instantaneously characterize different vegetable oils *via* TAG and free fatty acids (FFA) profiles using a tiny droplet of the oil placed on an inert paper surface under ambient conditions.[27,28] EASI-MS has also been used for TAG analysis in liver of hypertriglyceridemic mice[29] and for the monitoring of TAG oxidation in oils and fats in vegetable oils.[30]

Herein we describe the use of EASI-MS, assisted by thermal imprinting and minimal solvent extraction, to obtain characteristic TAG profiles directly from raw meat and fat samples. The approach couples the immediacy and simplicity of EASI-MS

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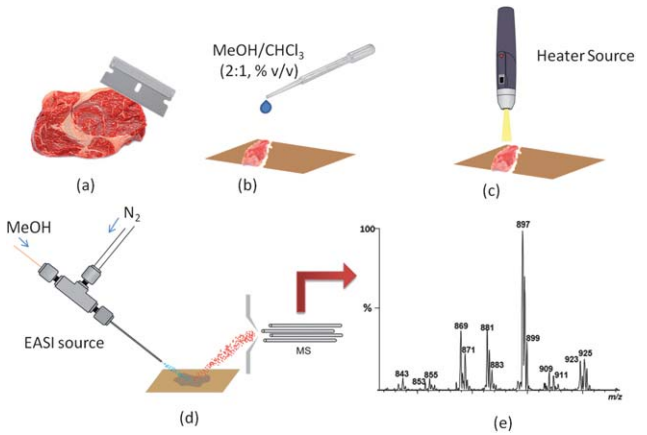

Fig. 1 Workflow of TI-EASI analysis: (a) a piece (*ca.* 1 cm^2) of meat or fat is manually sliced into ~10 mm thick sections; (b) it is placed on a brown Kraft paper surface and a few drops (*ca.* 3) of a MeOH–$CHCl_3$ solution (2 : 1 v/v) are dripped on the sample surface; (c) the sample surface is heated for 20 s (for fats) or 90 s (for meat); (d) the TAG content imprinted on the paper surface is submitted to sonic-spray and (e) mass spectrum obtained by EASI-MS.

analysis with the benefits of a very simple fast extraction step performed *via* thermal imprinting directly onto a paper surface, with minimal solvent extraction.

Experimental

a. Chemicals and samples

HPLC-grade methanol, chloroform and *n*-heptane were purchased from Burdick & Jackson (Muskegon, MI, USA) and used without further purification. Fatty acid methyl esters (FAMEs) mix containing butyrate (4 : 0), caproic (6 : 0), caprylic (8 : 0), capric (10 : 0), undecanoic (11 : 0), lauric (12 : 0), tridecanoic (13 : 0), myristic (14 : 0), myristoleic (14 : 1), pentadecanoate (15 : 0), *cis*-10-pentadecenoate (15 : 1), palmitic (16 : 0), palmitoleic (16 : 1), heptadecanoic (17 : 0), *cis*-10-heptadecenoic (17 : 1), stearic (18 : 0), elaidic (18 : 1n-9t), oleic (18 : 1n-9c), linolelaidic (18 : 2n-6t), linoleic (18 : 2n-6c), arachidic (20 : 0), γ-linolenic (18 : 3n-6), *cis*-11-eicosenoic (20 : 1), α-linolenic (18 : 3n-3), heneicosanoic (21 : 0), *cis*-11,14-eicosadienoic (20 : 2), behenic (22 : 0), *cis*-8,11,14-eicosatrienoic (20 : 3n-6), erucic (22 : 1n-9), *cis*-11,14,17-eicosatrienoic (20 : 3n-3), arachidonic (20 : 4n-6), tricosanoic (23 : 0), *cis*-13,16-docosadienoic (22 : 2), lignoceric (24 : 0), *cis*-5,8,11,14,17-eicosapentaenoic (20 : 5n-3), nervonic (24 : 1), and *cis*-4,7,10,13,16,19-docosahexaenoic (22 : 6n-3) was obtained from Sigma Aldrich (St Louis, MO, USA) and used as standard. Beef, chicken, pork, mutton, sardine, trout and salmon were obtained from a local food store. Samples were refrigerated immediately and stored at −18 °C.

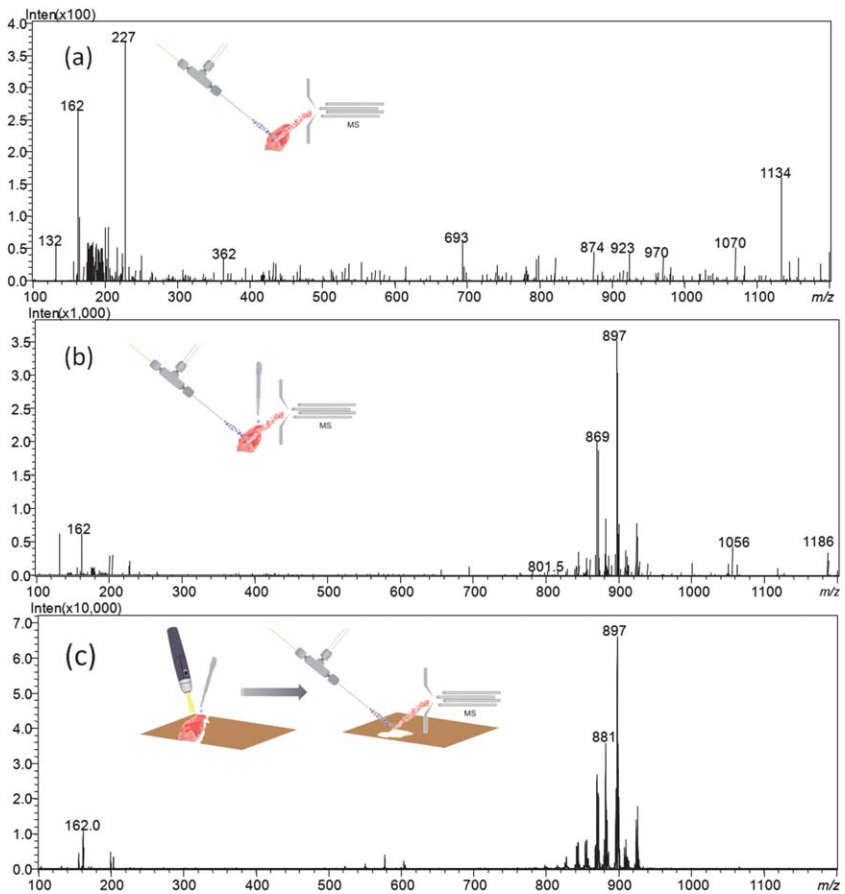

Fig. 2 EASI(+)-MS of beef *via* spraying of (a) the untreated meat, (b) the meat surface after *in situ* solvent extraction and (c) the imprinted paper surface *via* heating (fat) or solvent plus heating (meat) imprinting.

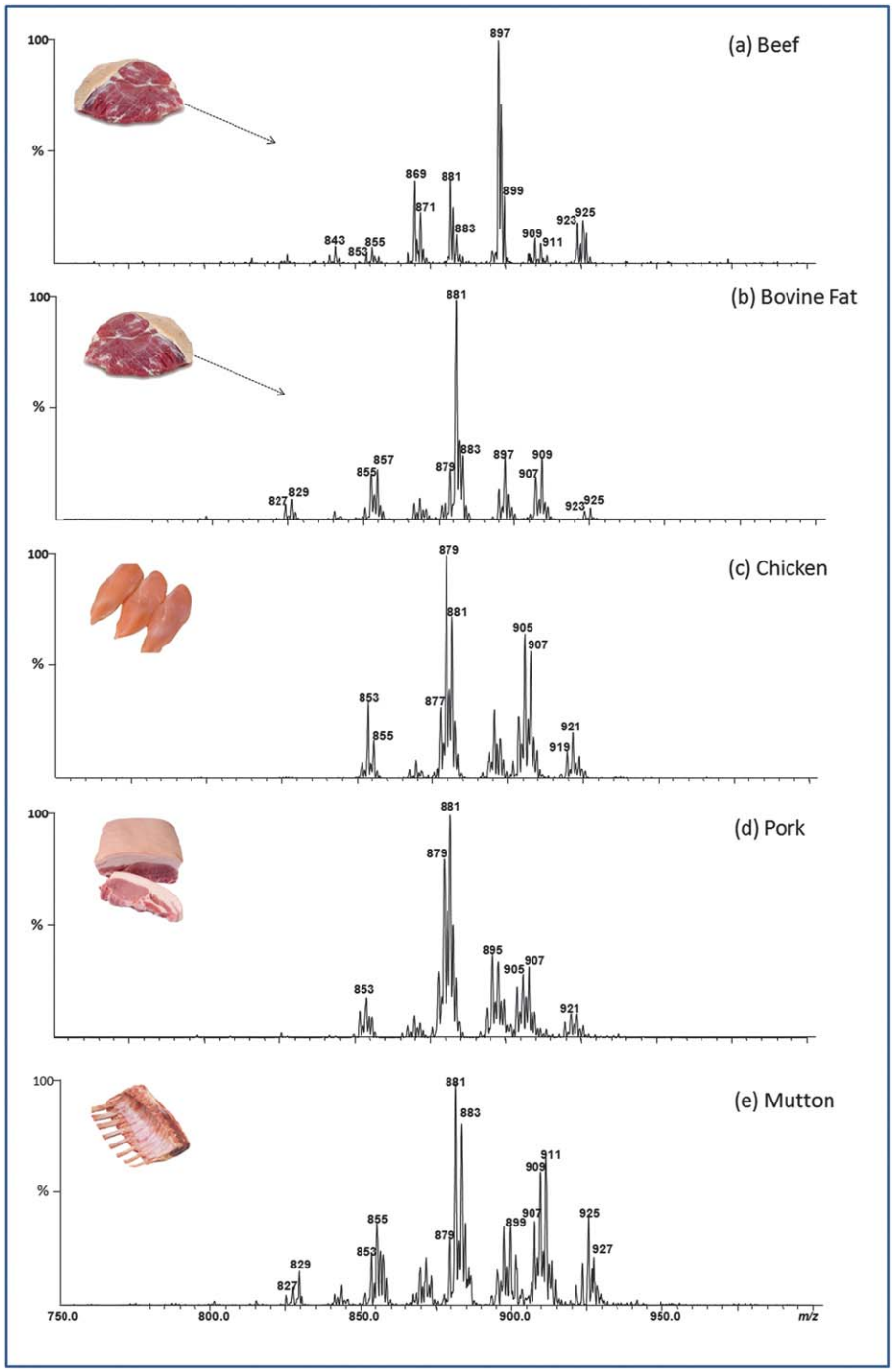

Fig. 3 TI-EASI(+)-MS of (a) beef; (b) bovine fat; (c) chicken; (d) pork and (e) mutton. Note that $[TAG + K]^+$ adducts, $[POO + K]^+$ of *m/z* 897 for instance (see Table 1), are predominant for beef whereas $[TAG + Na]^+$ adducts, $[POO + Na]^+$ of *m/z* 881 for instance, are predominant for the other meat samples.

b. Gas chromatography analysis

The meat and fat lipids were extracted using the Bligh and Dyer protocol.[17] For the esterification step, a mass of *ca.* 1.0 g of the extracted lipids was vortexed with 10.0 mL of *n*-heptane. After that, 0.5 mL of a NaOH solution (2 mol L^{-1} in MeOH) was added and the content was stirred for 20 s and the upper layer was collected for gas chromatography analysis according to the ISO (1978) procedure.[31]

Chromatographic analysis was carried out on a Thermo Scientific GC equipped with a flame ionization detector (FID), split/splitless injector and a fused silica capillary column CP-7420 (Select FAME, 100 m, 0.25 mm i.d. and 0.25 μm cyanopropyl). The operation parameters were: column temperature of 165 °C for 18 min and 235 °C (4 °C min^{-1}) for 20 min. The injector and detector temperatures were kept at 230 and 250 °C, respectively. The gas flow rates used were 1.2 mL min^{-1} for the

Table 1 FA composition for beef, bovine fat, chicken, pork, mutton, trout, salmon and sardine determined by GC-FID

		% composition (GC-FID)							
CN/DB[a]	FA[b]	Beef	Bovine fat	Chicken	Pork	Mutton	Trout	Salmon	Sardine
14 : 0	Myristic acid (M)	3.5	3.5	—	1.3	3.5	1.5	3.0	6.1
16 : 0	Palmitic acid (P)	25.9	26.0	23.9	22.8	24.6	21.0	13.9	12.1
16 : 1n-7	Palmitoleic acid (Po)	4.2	4.3	5.5	2.1	—	6.4	4.4	4.3
18 : 0	Stearic acid (S)	16.3	16.2	6.8	11.8	31.9	5.5	3.8	1.2
18 : 1n-9	Oleic acid (O)	37.4	38.9	43.4	42.0	6.6	35.9	32.6	8.0
18 : 1n-7	*Cis*-vaccenic acid (V)[c]	1.1	—	2.2	2.6	24.0	2.7	3.2	1.4
18 : 2n-6	Linoleic acid (Ln)	2.4	—	14.6	13.3	—	16.8	15.5	1.9
20 : 1n-9	Gondoic acid (G)[c]	—	—	—	—	—	2.1	2.4	3.7
20 : 1n-7	Paulinic acid (Pa)							—	15.3
20 : 4n-6	Arachidonic acid (AA)	—	—	—	—	—	—	0.7	22.2
20 : 5n-3	Timnodonic acid (EPA)	—	—	—	—	—	1.2	6.7	8.3
22 : 6n-3	Cervonic acid (DHA)	—	—	—	—	—	1.5	4.5	8.3
Others		9.2	11.1	3.6	4.1	9.4	5.4	8.9	7.3

[a] CN/DB: carbon number/double bond. [b] Usual nomenclature. [c] Suggested abbreviation.

carrier gas (H_2), 30 mL min^{-1} for the make-up gas (N_2) and 30 and 300 mL min^{-1} for the flame gas H_2 and synthetic air, respectively. The sample split mode was 1/80. Both the lipid extraction and the posterior FAME injection were carried out in triplicate and the injection volume was 1 μL. FAMEs were identified by comparison of retention time of the sample constituents with Sigma FAME and results were expressed as relative percent of total fatty acids according to Visentainer (2012).[32]

c. Thermal imprinting easy ambient sonic-spray ionization mass spectrometry (TI-EASI-MS) analysis

For TI-EASI-MS analysis, a suitable analysis overflow (Fig. 1) was established: a piece (*ca.* 1 cm^2) of fat or meat (beef, chicken, pork, mutton, sardine, trout, and salmon) was manually sliced into ~10 mm thick sections and placed on a brown Kraft paper surface. Three to four drops of a MeOH–$CHCl_3$ solution (2 : 1 v/v) were dripped on the meat surface, and a homemade heater containing a 150 W halogen bulb was directed to the sample for

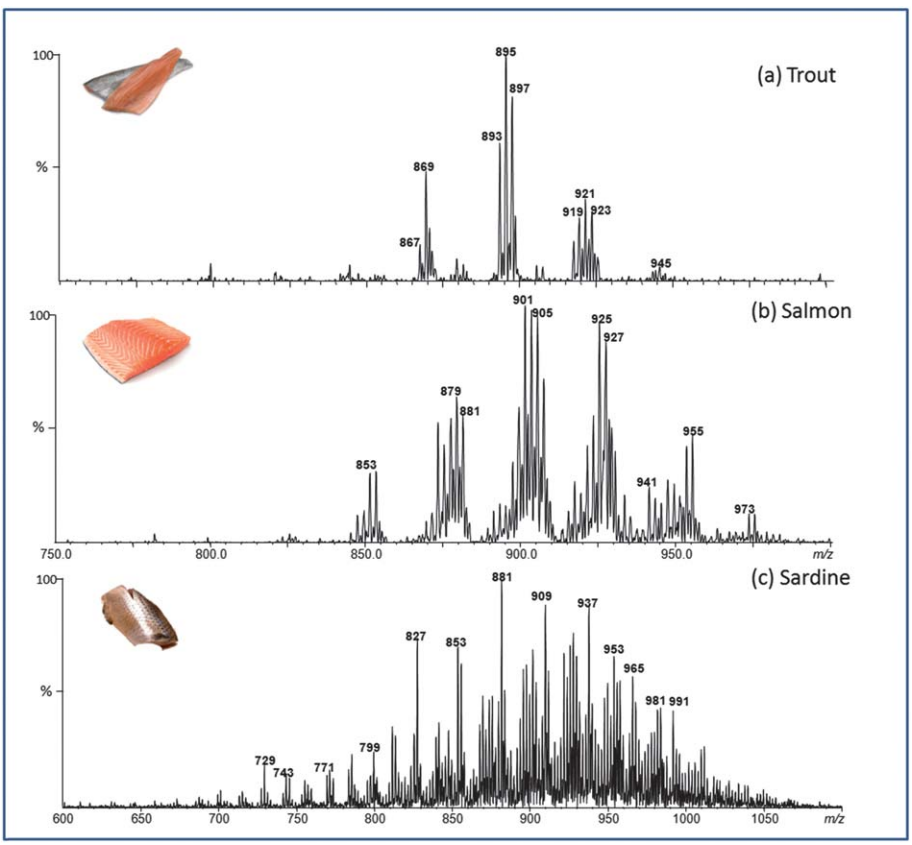

Fig. 4 TI-EASI(+)-MS for the fish samples: (a) trout; (b) salmon and (c) sardine.

Table 2 Possible assignment of TAG ions detected by TI-EASI-MS and their sodium and potassium adducts

TAG[a]	CN/DB[b]	$[M + Na]^+$	$[M + K]^+$
MMPo	44 : 1	771	787
MPPo or MMO	46 : 1	799	815
PPoPo	48 : 2	825	841
PPPo or MPO	48 : 1	827	843
MPS	48 : 0	829	845
PPoL or PoPoO or MOL	50 : 3	851	867
MOO or PPL	50 : 2	853	869
PPO	50 : 1	855	871
PPS	50 : 0	857	873
PLL	52 : 4	877	893
POL	52 : 3	879	895
POO or MOPa	52 : 2	881	897
PSO	52 : 1	883	899
PSS	52 : 0	885	901
P-L-EPA or P-Po-DHA or LLLn or OLnLn	54 : 7	899	915
LLL or OLLn	54 : 6	901	917
OLL	54 : 5	903	919
OOL or SLL	54 : 4	905	921
OOO	54 : 3	907	923
SOO or O-L-AA or Po-Pa-EPA or P-O-DHA	54 : 2	909	925
SSO	54 : 1	911	927
O-L-EPA	56 : 8	925	941
S-L-EPA or P-O-DHA or M-Pa-DHA	56 : 7	927	943
S-O-EPA or P-S-DHA or O-O-AA	56 : 6	929	945
Ln-EPA-EPA	58 : 12	943	959
O-AA-AA	58 : 9	951	967
O-O-DHA or S-L-DHA or Po-Pa-DHA	58 : 8	953	969
S-O-DHA	58 : 7	955	971
L-AA-DHA or AA-AA-AA	60 : 12	973	989
S-EPA-DHA	60 : 11	975	991
Pa-AA-AA	60 : 9	979	995
O-Pa-DHA	60 : 8	981	997
Pa-Pa-Pa	60 : 3	991	1007

[a] FA abbreviations: M, myristic acid; Po, palmitoleic acid; P, palmitic acid; Ln, linolenic acid; L, linoleic acid; O, oleic acid; S, stearic acid; Pa, paulinic acid; AA, arachidonic acid, EPA, timnodonic acid; DHA, cervonic acid. [b] CN/DB is the carbon number/number of double bonds of the three fatty acid moieties.

20 s (for fats) or 90 s (for meat samples). An infra-red (IR) thermometer was used to estimate the temperature. Afterwards, the sample was removed and its TAG content imprinted on the paper surface was analyzed by EASI-MS.

The TI-EASI-MS experiments were performed in the positive ion mode on a single quadrupole mass spectrometer (LCMS-2010EV-Shimadzu-Japan) equipped with a homemade EASI source described in detail elsewhere.[23–25] To produce the sonic-spray, pure methanol at 30 μL min^{-1} and N_2 nebulizing gas flow of 3 L min^{-1} were used. The paper-entrance angle of ~30° and the distance from the paper to the cone of 2 mm were used. Mass spectra were accumulated over 60 s and scanned over the 400–1100 *m/z* range.

Results and discussion

Fully direct TAG analysis of meats and fats by EASI-MS (Fig. 2a) was performed for all samples. At first, the sample surface was sprayed with the EASI spray but, by this way, TAG ions escaped detection or were detected at very low abundances with unacceptable *S/N* ratios. To improve the sensitivity, "*in situ*" solvent extraction of the TAG was performed by adding three or four droplets of a MeOH–$CHCl_3$ solution (2 : 1 v/v) directly to the meat surface. TAG ions were detected (Fig. 2b), but still with low signal intensity. The best results were obtained *via* thermal imprinting directly onto a paper surface using a slice of the sample leading to very abundant TAG ions (Fig. 2c). For fats, only thermal assistance was used. For meats, the addition of a few (3–4) droplets of the extraction solution (MeOH–$CHCl_3$ 2 : 1 v/v) on the top of the meat slice followed by heating was found to considerably increase the sensitivity.

Simple heating for fats or heating plus extraction using some droplets of the MeOH–$CHCl_3$ solution for meat samples prior to TI-EASI-MS analysis facilitates therefore the transfer of TAG from the sample to the paper surface. The solvents of the binary mixture chloroform–methanol used have the capacity to extract neutral and polar lipids efficiently.[33] The melted or extracted TAG flows through the sample and are imprinted on the paper under the sample. This efficient accumulation provides enough TAG material to generate a stable and intense signal when EASI-MS analysis is performed. When no thermal or solvent assistance is used, and the meats or fats are simply pressed onto the paper surface, insufficient ion signal was also observed. Even though thermal assistance (*ca.* 70 °C) is used, no signs of thermal degradation or oxidation products due to heating could be detected. By using the TI process just described, TAG profiles could be obtained by EASI-MS without the use of hydrolysis or derivatization in a few seconds and with minimal sample handling and solvent consumption (4–5 droplets), leading to fast characterization of meats and fats *via* intact TAG profiles.

Fig. 3 shows representative TAG profiles obtained by TI-EASI(+)-MS from different fat or meat samples. TAG were detected mainly as $[TAG + Na]^+$ ions with minor $[TAG + K]^+$ ions, except for beef samples where $[TAG + K]^+$ ions were predominant. The detection of TAG as $[TAG + K]^+$ and $[TAG + Na]^+$ ions for meat is believed to be due to the natural relatively high content of salts in meat matrices, which is related to the muscular tissue physiology, in which these elements are highly needed for the muscular contraction process. The most abundant TAG observed in TI-EASI spectra for the analyzed samples was found to be composed of the major FA determined after hydrolysis and derivatization by gas chromatography analysis (Table 1).

The TAG profiles from beef, chicken and pork were quite diverse and dominated by TAG containing mainly palmitic acid (P) and oleic acid (O). For beef meat (Fig. 3a), the most abundant $[TAG + K]^+$ ion was that of *m/z* 897 corresponding to POO. Except for the predominance of $[TAG + Na]^+$ ions instead of $[TAG + K]^+$ ions, the TAG profile of bovine fat (Fig. 3b) is almost the same as that of beef meat (Fig. 3a). The most abundant TAG ion for bovine fat corresponds to $[POO + Na]^+$ of *m/z* 881. Chicken (Fig. 3c) and pork (Fig. 3d) displayed TAG with relatively higher amounts of linoleic acid (L) than those from beef and their $[TAG + Na]^+$ profiles were similar. The *m/z* 879/881 (PLO/POO) ratios for chicken and pork meats were however quite distinct. TAG from mutton are known to be rich in stearic acid (S) (*ca.* 32%, Table 1), hence its TAG profile (Fig. 3e) is characterized by an abundant ion of *m/z* 883 (PSO).

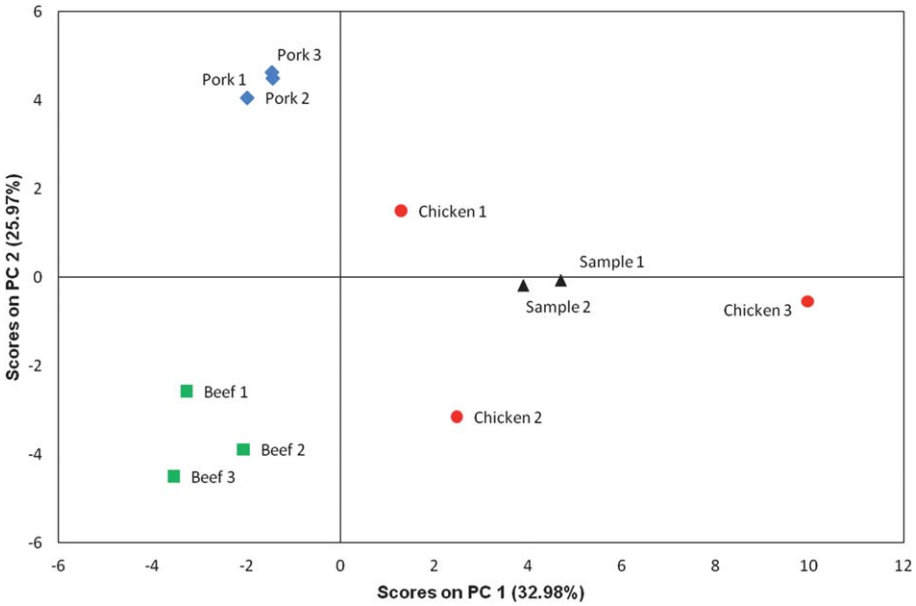

Fig. 5 PCA analysis of beef ($N = 3$) and chicken ($N = 3$) spectra obtained by TI-EASI(+)-MS.

The TI-EASI(+)-MS data for fish samples (Fig. 4) provided very characteristic profiles due to the detection of TAG containing arachidonic (AA), timnodonic (EPA) and cervonic (DHA) acids. Trout (Fig. 4a) showed a TAG profile quite similar to beef meat (Fig. 3a) in which the more abundant $[TAG + K]^+$ ions are those of *m/z* 895 (POL) and 897 (POO). Salmon (Fig. 4b) is rich in EPA and DHA; hence its TAG profile displayed mainly $[TAG + Na]^+$ of *m/z* 925 (O-L-EPA) and 927 (S-O-DHA). Sardine, in addition to EPA and DHA, also contains high quantities of AA (*ca.* 22%, Table 1) hence it displayed a very rich and unique TAG profile (Fig. 4c).

The differences mainly in the fatty acids composition (EPA, DHA, AA) for these fish can be directly related to species feeding and diet supplementation.[34] Feeding for some species can significantly interfere in their TAG expression; hence TI-EASI-MS seems to be useful to evaluate nutritional alteration in meat samples related to exposure of the animals to different diets. Table 2 summarizes intact TAG ions attribution and their respective adducts.

To investigate the ability of TI-EASI-MS to provide a tool for the quality control and to test statistically the performance of this technique for meats evaluation, three pork, beef and chicken samples were analyzed and Principal Component Analysis (PCA) was performed. Calculations of the PCA model were implemented from the PLS Toolbox 2.0 (Eigenvector Research Inc.), for use with Matlab 6.0 (Mathworks, Inc). PCA was conducted over the full variable range and auto scaling of the data was used for data pretreatment in order to minimize the effects of ionization differences between TAG species and to make the analysis restrict to the differentiation based on the absence/presence of marker TAG. Clear differentiation was achieved between these three meats (Fig. 5). Two chicken samples (1 and 2) were then used as "unknown", for external validation of the model, and their imprinted TAG content was properly classified as from chicken.

a. TI-EASI-MS *versus* MALDI-MS

The TAG profiles of bovine fat obtained *via* TI-EASI-MS (Fig. 1b) were compared to those from GC analysis (estimated from FFA, Table 1) as well as to those previously reported from matrix-assisted laser desorption ionization (MALDI).[11] TAG profiles obtained by TI-EASI-MS were in excellent agreement with these techniques, but note that TI-EASI-MS analysis is performed with very simple sample preparation, with little solvent consumption and in a few minutes of total analysis time.

b. Remote TI-EASI-MS

To access the stability of the extracted oil content imprinted on the paper, three chicken samples were extracted and one of them was immediately analyzed by TI-EASI-MS. The other two papers were stored in a sealed plastic bag and kept in a dark envelope at room temperature for three and seven days before EASI-MS analysis. No substantial changes were observed in the TAG profiles (data not shown) even after seven days, thus suggesting that TAG extracts by thermal imprinting onto a paper surface, using minimal amounts of solvent, could be mailed long distances for remote TI-EASI-MS analysis.

Conclusions

A simple, fast and more eco-friendly method to analyze TAG in meats and fats has been demonstrated. It requires no hydrolysis or derivatization, and the whole TI-EASI-MS protocol is performed in a few minutes and with minimal sample handling and solvent consumption, leading to proper characterization and quality control of meats and fats. The TAG profiles obtained *via* TI-EASI-MS showed to be quite similar to those obtained using other well established techniques such as GC and MALDI-MS analyses. As shown for the fish samples, most particularly for trout, such TAG profiles could be used to monitor feeding and

diet supplementations. The oil extracts stored on paper surfaces *via* the fast and simple thermal imprinting method were unaltered for several days in a plastic bag showing that remote TI-EASI-MS analysis of meat and fat samples is feasible.

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