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Article

TAG, DAG and FFA Profiles of Dry-Cured Ham by Easy Ambient Sonic-Spray Ionization Mass Spectrometry After Thermal Imprinting

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Espectrometria de massas ambiente com ionização por *sonic-spray* (EASI-MS), uma técnica de espectrometria de massas ambiente recém desenvolvida, foi diretamente utilizada para a obtenção do perfil de triacilgliceróis (TAG), diacilgliceróis (DAG) e ácidos graxos livres (FFA) em diferentes tipos de presuntos crus curados, após impressão térmica. A técnica é simples, rápida e confiável, além de não requerer nenhum tipo de hidrólise, derivatização ou separação cromatográfica, o que representa vantagens sobre outros procedimentos analíticos normalmente utilizados para este propósito.

Easy ambient sonic-spray ionization mass spectrometry (EASI-MS), a recently developed ambient mass spectrometry technique, was used for the direct analysis of triacylglycerols (TAG), diacylglycerols (DAG) and free fatty acids (FFA) in different types of dry-cured ham, after thermal imprinting. The technique is simple, fast and reliable, not requiring hydrolysis, derivatization or chromatographic separation, which represents advantages over other analytical procedures usually used for this purpose.

Keywords: dry-cured ham, thermal imprinting, ambient mass spectrometry, triacylglycerols, diacylglycerols, free fatty acids

Introduction

Dry-cured hams are very appreciated by consumers due their superior sensory properties and their reputation as traditional products. At present, traditional technologies are used to produce high quality dry-meat products with attractive sensory characteristics, such as color, aroma and texture, and high nutritional value due to very high protein content. The drying and long ripening periods are processes that can produce not only typical sensory characteristics and microbiological stability, but also changes in the composition and nutritional value of proteins and lipids.[1]

The quality of dry-cured hams is closely related to the lipids of raw material and the complex biochemical reactions that take place during the curing process. Lipids, mainly triacylglycerols (TAG) located in muscle and adipose tissues and phospholipids (PL) located in membrane of muscular cells, are largely involved in sensory attributes of the final dry products, since during processing they undergo lipolysis and oxidation, which generates numerous volatile compounds.[2,3] In southern European countries, the production processes include the standard steps of salting, drying and ripening. There are, however, large differences in the time, temperature and humidity of the different steps, according to the processes used for each product in each country. These large variations in processing conditions can affect the kinetics of lipolysis and oxidation reactions.[4]

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Several analytical approaches have been proposed based on the monitoring of characteristic properties of the fats, such as melting and slip point, iodine value and fatty acids profiles. More selective parameters, such as the composition of TAG are also being increasingly used for ham authentication based on the breeding and feeding conditions using either gas chromatography (GC) with flame ionization detection or high performance liquid chromatography (HPLC) coupled to diode-array, which require laborious and time consuming procedures.[5,6]

Recently, we have introduced thermal imprinting easy ambient sonic-spray ionization mass spectrometry (TI-EASI-MS) for direct analysis of TAG profiles in meats and fats in such way that no previous sample preparation, derivatization or chromatographic separation are required.[7] EASI-MS approach put together the immediacy and simplicity for the analysis.[8] The technique belongs to a new group of desorption/ionization techniques known collectively as ambient mass spectrometry,[9,10] with the benefits of a very simple fast extraction step performed via thermal imprinting directly onto a paper surface, with minimal use of solvent. The EASI-MS technique has shown great efficiency in the analysis of TAG present in vegetables oils;[11-15] PL and TAG in commercial lecithins;[16] in the ratio PL/TAG in liver tissue[17,18] as well as in the monitoring of TAG oxidation in oils.[19]

In this work a set of three different types of Iberian pig ham (a high value dry-cured product) known as Cebo, Recebo and Bellota and two different types of white pig ham Serrano and Parma, whose pigs greatly differ with respect to the breed and diet, were selected as a case of study and analyzed by both EASI(+)-MS and EASI(–)-MS after a simple process of thermal imprinting onto a paper surface. TAG, diacylglycerol (DAG) and free fatty acid (FFA) profiles in dry-cured hams were easily and quickly obtained by TI-EASI-MS.

Experimental

Chemicals and samples

HPLC-grade methanol and chloroform were purchased from Merck SA (Rio de Janeiro, Brazil) and used without further purification.

Five dry-cured ham samples were used for lipid profile evaluation, one sample of each ham type. Cebo, Recebo, Bellota and Serrano (Table 1; names associated with designation of origin, D. O.) hams were obtained from a local market in Spain, whereas Parma (name associated with D. O.) ham was from Italy. All samples were sliced (± 1.5 mm thickness), vacuum-packed (transparent packing) and were then stored at 4 ºC until analysis.

For analysis only the three slices in the middle were used (the first and last slices were discarded since they were susceptible to lipid light oxidation). From the muscular fraction of each slice we took three standard pieces (1 cm × 1 cm × 1.5 mm) for TI-EASI-MS analysis. The remaining muscular fraction was utilized for lipid extraction.

Gas chromatography

To characterize the fatty acid (FA) profile, which helps the TAG assignment, the lipids were extracted from 2 g of muscular ham sample, according to Bligh and Dyer,[20] and esterified by the method of Hartman and Lago.[21] The fatty acid methyl esters (FAME) were separated according to AOCS standard method Ce 2-66, in a capillary gas chromatograph (CGC Agilent 6850 Series GC System, Santa Clara, CA) with a DB-23 Agilent capillary column (50% cyanopropyl-methylpolysiloxane, 60 m × 0.25 mm × 0.25 mm film). Oven temperature was 110 ºC for 5 min, 110-215 ºC (5 ºC min^{-1}), 215 ºC for 24 min; flame ionization detector (FID) detector temperature: 280 ºC; injector temperature 250 ºC; carrier gas: helium; split ratio 1:50; injection volume: 1 µL.[22] The fatty acid (FA) identification was determined by comparing peak retention times with the respective standards using Supelco 37 Component FAME Mix from Supelco (Bellefonte, USA). All analyses were done in triplicate.

Thermal imprinting

A piece (1 cm × 1 cm × 1.5 mm, as already described) of ham was placed on a brown Kraft paper surface. Four drops of a MeOH-$CHCl_3$ solution (2:1 v/v) were dripped on the

Table 1. Dry-cured ham characteristics

Dry-cured ham	Pig	Pig breed	Pig feed	Ripening time / month
Parma	White	Landrace, Large-White, or Duroc	Grain, nut and whey	13 to18
Serrano	White	Landrace, Large-White or Duroc	Grain	12 to 20
Cebo	Iberian	Black Iberian	Grain	ca. 24
Recebo	Iberian	Black Iberian	Grain and bellota	ca. 24
Bellota	Iberian	Black Iberian	bellota	ca. 36

meat surface, and a homemade heater containing a 150 W halogen bulb was directed to the sample for 90 s.[7] The sample was then removed and its TAG content imprinted on the paper surface was analyzed by EASI-MS.

Easy ambient sonic-spray ionization mass spectrometry

The TI-EASI-MS data was collected both in the positive and negative ion modes using a single quadrupole mass spectrometer LCMS-2010EV (Shimadzu, Japan) equipped with a homemade EASI source described in detail elsewhere.[8] To produce the sonic-spray, pure methanol at 20 μL min^{-1} and N_2 nebulizing gas flow of 3 L min^{-1} were used. Methanol containing 1% of ammonium hydroxide was used in the negative ion mode. The paper-entrance angle of ca. 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 *m/z* 500-1000 range or *m/z* 100-500 range in the positive or negative ion modes, respectively.

Statistical analysis

A statistical analysis of the experiment results was performed on the Microsoft Office Excel program. The methodology for EASI-MS analyses was carried out using three different pieces of each ham, in which one of them was thermally imprinted on paper in triplicate, and analyzed by EASI(+/–)-MS in triplicate, resulting in a total of twenty seven measurements for each ham in both positive and negative ion modes. The respective extracts were also analyzed by GC in triplicate resulting in nine measurements for each ham; the results were expressed as means ± standard deviations (SD).

Results and Discussion

The lipid characteristics in the muscle and adipose tissues are strongly related to the rearing systems of the pigs and can vary, both quantitatively and qualitatively, according to a range of factors, including the breed, age, sex and diet.[1,2,23] For example, Bellota D. O. (Iberian acorn-fed crude ham) is from pigs fattened in outdoor-based systems with grass, legumes and acorns, Cebo D. O. (Iberian cereal-fed crude ham) is from pigs fattened in indoor-based systems with compound feed and Recebo D. O. (Iberian mix-fed crude ham) is from pigs fattened using both systems; Serrano D. O. (Spanish dry-cured ham) is from white pigs, raised on compound feed farms, produced on the mountains and Parma D. O. (Italian dry-cured ham) is from white pigs, with a diet based on a blend of grains, produced by a regional consortium (Table 1). These differences in the breed and diet produce different sensorial and physicochemical characteristics in the hams.[24-26]

Table 2 shows the FA composition of five different types of ham samples determined by GC. The FA composition obtained was characteristic of pork meats with palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) as the main components. Regarding the FA composition, Bellota ham sample is shown to contain much higher amounts of oleic acid (ca. 60%) than the other hams (44-48%), as well as lower amounts of palmitic, stearic and

Table 2. FA composition of dry-cured hams determined by GC-FID

Fatty Acid	Parma / %	Serrano / %	Cebo / %	Recebo / %	Bellota / %
C10:0	0.17 ± 0.01	n.d.	0.42 ± 0.08	n.d.	n.d.
C12:0	0.45 ± 0.11	n.d.	1.20 ± 0.16	1.22 ± 0.32	n.d.
C14:0	1.74 ± 0.02	1.41 ± 0.10	1.29 ± 0.36	2.62 ± 0.76	1.43 ± 0.13
C16:0	21.05 ± 2.30	22.65 ± 0.89	22.28 ± 0.85	24.10 ± 0.06	18.99 ± 1.91
C16:1	2.52 ± 0.14	2.01 ± 0.15	2.23 ± 0.17	3.07 ± 0.31	2.77 ± 0.48
C17:0	0.26 ± 0.03	0.34 ± 0.08	0.39 ± 0.07	0.60 ± 0.03	0.40 ± 0.00
C17:1	0.24 ± 0.02	0.30 ± 0.01	0.36 ± 0.06	0.60 ± 0.07	0.53 ± 0.04
C18:0	10.02 ± 1.81	11.83 ± 0.76	10.98 ± 0.44	10.66 ± 1.31	6.63 ± 0.76
C18:1	48.18 ± 3.36	44.45 ± 2.76	48.01 ± 1.33	46.91 ± 1.36	59.91 ± 3.07
C18:2	13.38 ± 0.08	13.83 ± 1.67	10.63 ± 1.29	8.40 ± 1.00	7.05 ± 0.12
C18:3	0.72 ± 0.02	0.89 ± 0.04	0.65 ± 0.26	1.02 ± 0.01	0.66 ± 0.03
C20:0	0.08 ± 0.00	0.47 ± 0.06	0.26 ± 0.05	0.21 ± 0.21	n.d.
C20:1	0.91 ± 0.14	0.89 ± 0.06	0.69 ± 0.07	0.58 ± 0.05	1.09 ± 0.11
C20:4	0.29 ± 0.07	0.94 ± 0.13	0.61 ± 0.01	n.d.	0.54 ± 0.05

Data are expressed as mean percentage ± SD; n.d. = not detected.

linoleic acids. As previously mentioned, these differences are strongly related to the rearing systems of the pigs.

Figure 1 shows representative TI-EASI(–)-MS profile from Cebo and Bellota hams. The dominant free fatty acids (FFA) detected in the form of $[FFA - H]^-$ were oleic (C18:1, *m/z* 281) and linoleic (C18:2, *m/z* 279) acids, as well as palmitic (C16:0, *m/z* 255) and palmitoleic (C16:1, *m/z* 253) acids. When Cebo ham (Figure 1a) is compared to Bellota ham (Figure 1b), the *m/z* 281/279 and *m/z* 281/255 ratio increase, thereby confirming that the content of oleic acid increases while the content of palmitic and linoleic acids consequently decreases. This trend is also the same as that predicted by gas chromatography, in the analysis of the total FA (Table 2).

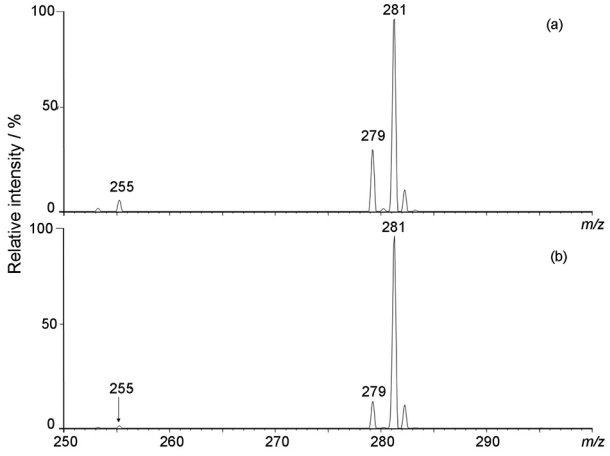

Figure 1. TI-EASI(–)-MS of (a) Cebo and (b) Bellota hams.

More recently, the attention has been focused on TAG composition because it represents the chemical form in which the fatty acids are in tissues. The main TAG in pig fat are palmitoyl-dioleoyl-glycerol (POO, 32-36%), palmitoyl-dioleoyl-*rac*-glycerol (POO, 32-36%), palmitoyl-oleoyl-stearoyl-*rac*-glycerol (POS, 19-28%), palmitoyl-oleoyl-linoleoyl-*rac*-glycerol (POL, 5-8%), trioleoyl-glycerol (OOO, 4-10%) and dipalmitoyl-oleoyl-*rac*-glycerol (PPO, 3-4%). TAG composition is more markedly influenced by the feeding and production system than the FA composition. So TAG composition is more efficient for distinguishing hams according to the rearing conditions.[27] Figure 2 shows representative TI-EASI(+)-MS profiles from five different types of ham samples. The spectra of all the samples show similar profile, with two clusters of peaks in the *m/z* range of 600-650 and 850-950. The ions observed in the *m/z* 850-950 range correspond to TAG, which were detected mainly in the form of sodium adducts $[TAG + Na]^+$. Potassium adducts $[TAG + K]^+$ were also detected with minor abundances. The most abundant TAG was found to be composed of the major FA determined by GC (Table 2). The main $[TAG + Na]^+$ ions are assigned as follows: *m/z* 853 [dipalmitoyl-linoleoyl-*rac*-glycerol, PPL]; *m/z* 855 [PPO]; *m/z* 877 [palmitoyl-dilinoleoyl-*rac*-glycerol, PLL]; *m/z* 879 [POL]; *m/z* 881 [POO]; *m/z* 883 [POS]; *m/z* 905 [dioleoyl-linoleoyl-*rac*-glycerol, OOL or dilinoleoyl-stearoyl-*rac*-glycerol, LLS]; *m/z* 907 [OOO or oleoyl-linoleoyl-stearoyl-*rac*-glycerol, OLS]; and *m/z* 909 [dioleoyl-stearoyl-*rac*-glycerol, OOS or distearoyl-linoleoyl-*rac*-glycerol, SSL]. Although the TAG profiles obtained for different types of hams are quite similar by visual inspection, the comparison of mass spectra from Bellota (Figure 2e) with the other types of ham (Figures 2a-d) reveals higher relative abundance for the *m/z* 907 ion [OOO]. This trend has also been observed using direct infusion electrospray mass spectrometry.[6] When the role profiles of TAG are sorted out by composition (Table 3), the major presence of oleic acid for the Bellota ham is also clear.

During ham ripening, lipolysis is one of the most common reactions and the formation of DAG molecules

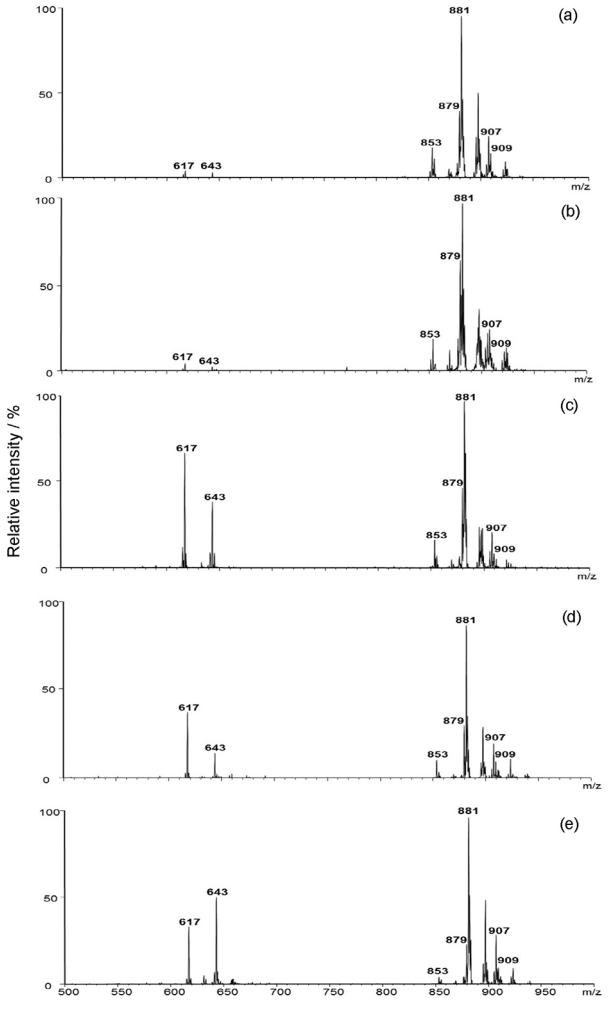

Figure 2. TI-EASI(+)-MS of (a) Parma; (b) Serrano; (c) Cebo; (d) Recebo and (e) Bellota hams.

Table 3. Main TAG of dry-cured hams obtained by TI-EASI(+)-MS

TAG[a]	ACN/DBN[b]	[TAG + Na]$^+$ *m/z*	Parma / %	Serrano / %	Cebo / %	Recebo / %	Bellota / %
PPL	50:2	853	4.61 ± 0.75	2.73 ± 1.29	3.13 ± 1.53	2.76 ± 1.86	1.44 ± 1.46
PPO	50:1	855	3.02 ± 0.83	2.52 ± 1.05	2.02 ± 1.50	2.14 ± 1.63	0.97 ± 0.43
PLL/POLn	52:4	877	2.82 ± 0.62	4.57 ± 1.85	3.17 ± 0.99	1.58 ± 0.99	0.78 ± 0.81
PLO/PSLn	52:3	879	17.05 ± 2.08	21.51 ± 4.23	18.00 ± 3.53	13.71 ± 1.84	11.09 ± 2.51
PSL/POO	52:2	881	47.83 ± 3.75	41.97 ± 8.03	51.97 ± 3.99	55.37 ± 6.12	54.44 ± 3.94
PSO	52:1	883	11.03 ± 2.08	12.66 ± 3.76	9.05 ± 3.05	11.00 ± 3.12	8.31 ± 1.95
LOO/SLL/SOLn	54:4	905	2.66 ± 0.65	5.55 ± 2.92	2.57 ± 0.97	2.21 ± 1.11	3.69 ± 1.17
OOO/SOL/SSLn	54:3	907	7.46 ± 1.04	6.84 ± 2.04	6.99 ± 2.49	7.34 ± 2.17	15.14 ± 1.83
SOO/SSL	54:2	909	3.50 ± 0.98	1.66 ± 1.30	3.11 ± 1.53	3.88 ± 1.38	4.15 ± 1.24

[a]FA abbreviations: P = palmitic acid, O = oleic acid, L = linoleic acid, Ln = linolenic acid, and S = stearic acid; [b]acyl carbon number/double bond number of the three FA moieties. Data are expressed as mean percentage ± SD.

is very important to release FFA, which are essential to formation of ham flavor. In this way the presence of higher amounts of DAG ions can be related with high ripening time of ham. The ions observed in the *m/z* 600-650 range correspond to DAG, which were detected mainly in the form of sodium adducts [DAG + Na]$^+$ as ions of *m/z* 617 [palmitoyl-oleoyl-*rac*-glycerol PO, C34:1] and of *m/z* 643 [dioleoyl-*rac*-glycerol, OO or stearoyl-oleoyl-*rac*-glycerol SL, C36:2]. Parma and Serrano hams have shorter ripening times (Table 1) hence exhibit ions of *m/z* 617 and 643 with much lower abundances (Figures 2a and 2b). As for ripening, therefore, Cebo, Recebo and Bellota hams display similar levels of overall relative abundances of the DAG ions of *m/z* 643 and 617 when compared to the TAG ions in the *m/z* 850-950 range but again, due to the very oleic acid-rich unique composition of the Bellota ham, its TI-EASI-MS shows a characteristic signature (Figure 2e) due to the higher abundance of the ion of *m/z* 643 (OO) as compared to the ion of *m/z* 617 (PO).

In a promising way, the TAG, DAG and FFA profiles can give us information from both the diet of pigs, by the TAG composition, as well as the curing time, through the presence of DAG provided from the TAG hydrolysis. This method could therefore be used to monitor the lipid hydrolysis during ripening, as well as be directly used as a quality control parameter related to lipid markers of ham characterization and ripening period.

Conclusions

The application of TI-EASI-MS from dry-cured ham intramuscular fat allows simple, fast and reliable TAG, DAG and FFA fingerprinting. With the proposed method, sample handling is minimal and chromatographic separation is not necessary, which represents advantages over other analytical procedures usually used for this purpose. The technique seems therefore quite promising as an effective tool for the analysis of ham and similar products providing characterization, origin, process and quality control.

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