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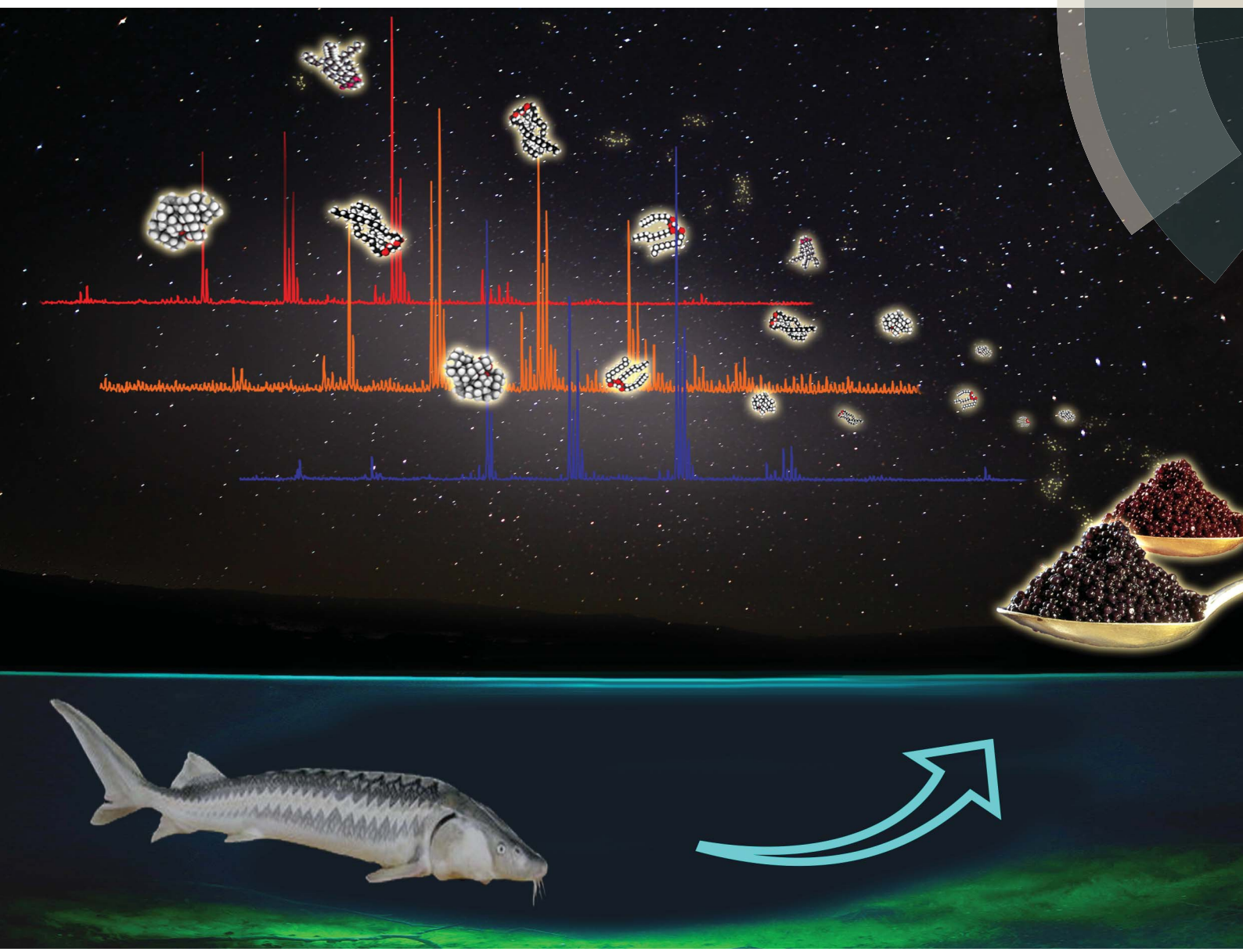

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The lipid profile of *Sturgeon* roe (caviar) was monitored by matrix assisted laser desorption/ionization mass spectrometry (MALDI(+)-MS), thermal imprinting easy ambient sonic-spray ionization mass spectrometry (TI-EASI(+)-MS) and electrospray mass spectrometry (ESI(+)-MS). Freshly salted and commercially salted pasteurized caviar samples of Atlantic sturgeon (*Acipenser sturio*) were stored either at +4 °C or at room temperature for 4 weeks. The different types of chemical information achieved by these MS techniques were compared: MALDI(+)-MS detects mainly phospholipids (PL) whereas TI-EASI(+)-MS allows monitoring of both triacylglycerols (TAG) and PL. ESI(+) coupled to Fourier transform ion cyclotron resonance high resolution mass spectrometer (FT-ICR-MS) and MS/MS experiments were used to fully characterize the detected lipids, ensure the absence of oxidation products in the degradation process and confirm the high efficiency of the thermal imprinting extraction. TI-EASI(+)-MS, *via* a more comprehensive profiling and easier operation, has therefore been demonstrated to provide caviar lipidomic profiles and discriminate its changes as a function of storage time and temperature. The data have also confirmed that hydrolysis is the main process of lipid degradation in caviar.

1. Introduction

Quality control and validation of origin are hot issues in the production, distribution and storage of food products, and they are of primary concern to food and agriculture organizations. Modern mass spectrometry (MS) provides unique, reliable and affordable methodologies to approach analytical tasks,[1] with a high degree of scientificity, for example the characterization and degradation of complex mixtures of organic compounds, such as lipid fractions in food products.[2,3] Lipids play an important role when dealing with the quality control of food products, since their composition is closely related to sensorial aspects, including attributes of texture, structure, mouth feel, flavor and color.[4] Lipids are however one of the most chemically unstable food components and readily undergo degradation which can speed up the spoilage.[5,6] Therefore, food industries and regulatory agencies need high-throughput MS techniques that are able to provide exact data on a large scale. For this purpose, shotgun lipidomics have been demonstrated to be a very suitable way to fully achieve lipid characterization.[7] Such an approach uses the direct infusion of a lipid extract into an atmospheric pressure ionization (API) source followed by the identification and/or quantification of the lipid species by tandem MS.[8] Indeed, shotgun lipidomics performed by electrospray ionization mass spectrometry (ESI-MS) has become a popular and powerful technology for both identification and quantitation of lipid species in food.[9,10] Information on detailed molecular composition of diverse edible oils and fats has been obtained by ESI-MS identifying more than 200 elemental compositions attributable to diacylglycerols, triacylglycerols (TAG), and their oxidation products.[11] Interestingly, ESI-MS was applied to identify and quantitatively determine 146 polar lipid species in wheat.[12]

Another widespread MS technique employed in lipidomics is matrix-assisted laser desorption/ionization (MALDI). MALDI-MS has been shown to be fast, requiring relatively simple sample preparation protocols, and high tolerance to salts and impurities. For instance, MALDI-MS has been used to investigate the adulteration and monitor food quality by analyzing the phospholipids profile,[13,14] characterizing Amazonian vegetable oils[15] and evaluating lipidic stability by monitoring oxidation and hydrolysis products.[16,17]

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Recently, the speed and simplicity of ambient MS techniques have been applied to study lipids in food products.[18] One of the simplest ambient ionization techniques, easy ambient sonic spray ionization (EASI-MS), was developed in 2006 by Eberlin and co-workers[19] showing high efficiency in lipid analysis in several matrices such as vegetables oils, commercial lecithin and tissues.[5,20,21] Therefore, by engaging the efficiency of EASI-MS with the benefits of a very simple fast extraction step by thermal imprinting (TI) directly onto a paper surface with minimal solvent extraction, we have developed thermal imprinting easy ambient sonic-spray ionization mass spectrometry (TI-EASI-MS) for direct analysis of TAG profiles in meats and fats in a way that requires no previous sample preparation, derivatization or chromatographic separation.[10,12,19] TI-EASI-MS has never been compared to other analytical techniques, except to gas chromatography coupled to a flame ionization detector (GC-FID).[20] Therefore, we decided to compare TI-EASI-MS to MALDI and ESI-MS using the analysis of caviar as a case study.

Hard-roe (caviar) of sturgeon[22] is a masterpiece created by nature. However, due to its delicacy and high commercial value, caviar requires the most delicate handling during collection, canning and storage so as to keep the product quality and its remarkable taste. Because of its fast natural degradation, conservation protocols, such as salting and pasteurization, must be applied to protect industrialized caviar from spoilage.[23] Pasteurization is however believed to reduce the culinary and the economic value of caviar. Distinguishing between fresh salted and pasteurized caviar[24,25] is an analytical challenge since no standardized methods have been set up yet to detect compositional differences in these samples. Moreover, the storage effect has never been studied by a high throughput analytical technique.

For instance, the effects of storage at +4 °C on the quantity and quality of chemical constituents in the caviar roes obtained from farmed *Acipenser transmontanus* have been analyzed by scanning electron microscopy (SEM), wet chemical methods, nuclear magnetic resonance (NMR) and magnetic resonance imaging (MRI) techniques.[26] This monitoring indicated hydrolysis as the main process of caviar degradation with no detectable oxidation even after 4 months at +4 °C. The present study aimed at comparing the performance of three MS techniques applied to caviar lipidomics. More specifically, the MS techniques of MALDI-MS,[27] the recently developed TI-EASI-MS[12] and the ESI-FT-ICR-MS were used to characterize the lipid fraction of salted and pasteurized sturgeon caviar at different times and storage conditions.

2. Materials and methods

a. Chemicals and samples

HPLC-grade methanol and chloroform were purchased from Burdick & Jackson (Muskegon, MI, USA) and used without further purification. Ultrapure water was obtained by a Direct-Q water system (Millipore, Bedford, MA, USA). The MALDI matrix 2,5-dihydroxybenzoic acid (DHB) was purchased from Sigma Aldrich. In our study, weighed salt granular caviar (S) and canned salted pasteurized granular caviar (P) of Atlantic sturgeon (*Acipenser sturio*) were selected and analyzed at different storage times and conditions (+4 °C and room temperature *ca.* 25 °C) up to 4 weeks. S and P caviar samples were analyzed by MALDI(+)-MS, TI-EASI(+)-MS and ESI(+)-MS at ThoMSon Lab (UNICAMP, Campinas, SP, Brazil).

b. Lipid extraction

The lipid profiles of S and P caviar were investigated by MALDI(+)-MS at day 0 (d_0), after seven days (d_7) and twenty-one days (d_{21}) of storage both at room temperature (RT, +25 °C) and in a refrigerator (+4 °C). The total lipids were extracted by Bligh Dyer liquid extraction (BD protocol).[15] Therefore, for each analyzed sample, three fish eggs were submitted to the extraction of the lipids by adding 150 μL of water, 190 μL of methanol and 370 μL of chloroform. After prolonged vortexing (2 min), each sample was centrifuged (5 min at 13 000 × *g*). The apolar fraction (down layer) was separated and dried for 40 min in a vacuum concentrator (Concentrator plus, Eppendorf) with the subsequent evaporation of the solvents. The dried samples have been resuspended with 50 μL of a MeOH–$CHCl_3$ solution (1 : 1, v/v). A volume of 1 μL of the resuspended sample was spotted on the MALDI plate, allowed to dry and then covered with 1 μL of DHB matrix (30 mg mL^{-1} in MeOH).

c. MALDI-MS analysis

The experiments were run by using a MALDI-TOF/TOF Autoflex III (Bruker Daltonics, Germany) instrument equipped with a λ 337 nm laser. Each spectrum was acquired from 300 laser shots on a single spot. The *m*/*z* range over which ions were detected was *m*/*z* 700 to 1000. The laser power range was adjusted to 30–50% and measurements were performed under operating conditions as follows: ion source 1 = 19.00 kV, ion source 2 = 16.72 kV, lens voltage = 8.30 kV, reflector voltage = 21.00 kV, reflector voltage 2 = 9.70 kV, pulsed ion extraction time = 10 ns, and suppression = 500 Da. Samples (N = 15) were analyzed in triplicate.

d. TI-EASI-MS analysis

The lipid profiles of the S and P caviar samples were investigated by TI-EASI(+)-MS over a period of four weeks (d_{28}) of storage at both RT and in a refrigerator (+4 °C). A few units of the caviar sample (*ca.* 500 mg) were placed on an envelope paper surface. Four drops of a MeOH : $CHCl_3$ (2 : 1 v/v) solution were dripped on the sample surface, and a homemade heater with a 150 W halogen bulb was placed close to the sample for 2 min. Afterwards, the sample was removed and the lipid fraction, which had been imprinted on the paper surface, was analyzed by EASI-MS.[12] The TI-EASI(+)-MS experiments were performed in the positive ion mode using a single quadrupole mass spectrometer (LCMS-2010EV-Shimadzu-Japan) equipped with a homemade EASI source.[19] To produce the sonic-spray, pure methanol at 20 μL min^{-1} and a 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 *m*/*z*

700–1000 range. Additional analyses for the confirmation of hydrolysis products were run in the *m/z* 100–1000 range with the same source parameters. Samples were analyzed in triplicate.

e. ESI-MS analysis

ESI-MS analysis was performed using a 7.2T LTQ FT Ultra mass spectrometer (Thermo Scientific, Bremen, Germany). For that, thermal imprinting of the samples was performed by placing a few caviar units (*ca.* 500 mg) on a glass plate which was then heated for 2 min, as previously described (Section 2d). After heating, the oily content on the glass plate was washed with 2 mL of MeOH (0.1% formic acid) and collected in a vial and analyzed by ESI(+)-FT-ICR-MS, without further dilution. Samples were analyzed in triplicate. The BD extracts (previously analyzed by MALDI(+)-MS) were ten-fold diluted in a MeOH : H_2O (1 : 1, 0.1% formic acid) solution and again analyzed by ESI(+)-FT-ICR-MS. MS and MS/MS (not shown) experiments were run for both BD and thermally imprinted extracts, allowing the identification (elemental composition) of the ions. Accurate experimental *m/z* values were compared to the exact mass using a database search (http://www.lipidmaps.org). It was considered a match between the experimental *m/z* value and the theoretical *m/z* value from the library when the mass error was <10 ppm. The isotope distribution pattern of the ions identified was also considered with the proposed chemical formula. Table 1 reports the accurate mass measurements.

f. Data analysis

MS fingerprints were obtained for each caviar sample (S and P) at different times and storage conditions by using the three different ionization sources. The spectra obtained by MALDI-MS, TI-EASI-MS and ESI-FT-ICR-MS were compared. The

Table 1 PL and TAG detected in the S and P caviars by MALDI(+)-MS or TI-EASI(+)-MS. The exact assignment is based on MS/MS data and high resolution mass spectrometry measurements coupled to database search

Experimental[a,b] *m/z*	Assignments (CN:DB)[c]	Molecular formula	Accurate[d] *m/z*	Theoretical *m/z*	Error (ppm)
715.5[a]	$[PG\ 34:1 + Na]^+$	$C_{38}H_{68}O_{10}P$	715.45610	715.4545	2.2
732.6[a]	$[PC\ 32:1 + H]^+$	$C_{40}H_{79}NO_8P$	732.55432	732.5538	0.7
760.6[a]	$[PC\ 34:1 + H]^+$	$C_{42}H_{83}NO_8P$	760.58719	760.5851	2.7
780.6[a]	$[PC\ 36:5 + H]^+$	$C_{44}H_{79}NO_8P$	780.55599	780.5538	2.8
	$[PC\ 34:2 + Na]^+$	$C_{42}H_{80}NO_8PNa$		780.5514	−5.9
782.6[a]	$[PC\ 36:4 + H]^+$	$C_{44}H_{81}NO_8P$	782.57218	782.5694	3.6
	$[PC\ 34:1 + Na]^+$	$C_{42}H_{82}NO_8PNa$		782.5670	−6.6
782[b]	$[PC\ 34:1 + Na]^+$	$C_{42}H_{82}NO_8PNa$	782.56827	782.5670	1.62
786.6[a]	$[PC\ 36:2 + H]^+$	$C_{44}H_{85}NO_8P$	786.60361	786.6007	3.7
802.5[a]	$[PC\ 40:8 + H]^+$	$C_{46}H_{77}NO_8P$	802.53845	802.5381	0.4
	$[PC\ 36:5 + Na]^+$	$C_{44}H_{78}NO_8PNa$		802.5357	−3.4
802[b]	$[PC\ 36:5 + Na]^+$	$C_{44}H_{78}NO_8PNa$	802.53721	802.5357	1.88
806.6[a]	$[PC\ 38:6 + H]^+$	$C_{46}H_{81}NO_8NP$	806.57169	806.5694	2.8
	$[PC\ 36:3 + Na]^+$	$C_{44}H_{82}NO_8PNa$		806.5670	−5.8
828.6[a]	$[PC\ 40:9 + H]^+$	$C_{48}H_{79}NO_8P$	828.55428	828.5538	0.6
	$[PC\ 38:6 + Na]^+$	$C_{46}H_{80}NO_8PNa$		828.5514	−3.5
828[b]	$[PC\ 38:6 + Na]^+$	$C_{46}H_{80}NO_8PNa$	828.55284	828.5514	1.74
830.6[a]	$[PC\ 40:8 + H]^+$	$C_{48}H_{81}NO_8P$	830.56944	830.5694	0.0
	$[PC\ 38:5 + Na]^+$	$C_{46}H_{82}NO_8PNa$		830.5670	−2.9
832.6[a]	$[PC\ 40:7 + H]^+$	$C_{48}H_{83}NO_8P$	832.58809	832.5851	3.6
	$[PC\ 38:4 + Na]^+$	$C_{46}H_{84}NO_8PNa$		832.5827	−6.5
834.6[a]	$[PC\ 40:6 + H]^+$	$C_{48}H_{85}NO_8P$	834.60371	834.6007	3.6
	$[PC\ 38:3 + Na]^+$	$C_{46}H_{86}NO_8PNa$		834.5983	−6.5
844.6[a]	$[PE\ 44:8 + H]^+$	$C_{49}H_{83}NO_8P$	844.58567	844.5851	0.7
	$[PC\ 39:5 + Na]^+$	$C_{47}H_{84}NO_8PNa$		844.5827	−3.5
853[b]	$[TAG\ 50:2 + Na]^+$	$C_{53}H_{98}O_6Na$	853.71841	853.7256	−8.42
854.6[a]	$[PC\ 42:10 + H]^+$	$C_{50}H_{81}NO_8P$	854.57062	854.5694	1.4
	$[PC\ 40:7 + Na]^+$	$C_{48}H_{82}NO_8PNa$		854.5670	−4.2
856.6[a]	$[PC\ 42:9 + H]^+$	$C_{50}H_{83}NO_8P$	856.58485	856.5851	−0.3
	$[PC\ 40:6 + Na]^+$	$C_{48}H_{84}NO_8PNa$		856.5827	−2.5
860.6[a]	$[PC\ 42:7 + H]^+$	$C_{48}H_{79}NO_{10}P$	860.61978	860.6164	3.9
	$[PC\ 40:4 + Na]^+$	$C_{48}H_{88}NO_8PNa$		860.6140	−5.9
875[b]	$[TAG\ 52:5 + Na]^+$	$C_{55}H_{96}O_6Na$	875.71152	875.7099	1.85
877[b]	$[TAG\ 52:4 + Na]^+$	$C_{55}H_{98}O_6Na$	877.71816	877.7256	−8.48
879[b]	$[TAG\ 52:3 + Na]^+$	$C_{55}H_{100}O_6Na$	879.73392	879.7412	−8.28
881.8[a]	$[TAG\ 52:2 + Na]^+$	$C_{55}H_{102}O_6Na$	881.75975	881.7593	0.5
881[b]	$[TAG\ 52:2 + Na]^+$	$C_{55}H_{102}O_6Na$	881.75831	881.7569	1.60
901[b]	$[TAG\ 54:6 + Na]^+$	$C_{57}H_{98}O_6Na$	901.72715	901.7256	1.72
927[b]	$[TAG\ 56:7 + Na]^+$	$C_{59}H_{100}O_6Na$	927.74282	927.7412	1.75

[a] Data acquired by MALDI(+)-MS. [b] Data acquired by TI-EASI(+)-MS. [c] CN:DB: carbon number/double bound; PC: phosphatidylcholines; TAG: triacylglycerols; PG: phosphoglycerols; PE: phosphoethanolamines. [d] Data acquired by ESI(+)-FT-ICR-MS.

comparison was based only on the MS-profile with no quantitative purposes.

3. Results and discussion

Three MS techniques were used to properly characterize caviar samples. MALDI-MS and TI-EASI-MS could both distinguish the two types of samples. Changes in the lipid profile which could be related to the time and conditions of storage were monitored by using the three techniques. MALDI-MS offers more sensitive PL detection whereas TI-EASI-MS offers richer information regarding the lipid composition of the sample since it detects both PL and TAG species. ESI-MS confirmed both MALDI-MS and TI-EASI-MS data and provided a full characterization of the samples, through it coupling to an ultra high resolution mass analyzer.

a. MALDI(+)-MS

By analyzing the lipid extracts (BD protocol) of S and P caviars by MALDI(+)-MS using a DHB matrix, it was possible to mainly detect a high number of phospholipids, more specifically phosphatidylcholines (PC). The preference in MALDI-MS to produce sodiated molecules $[M + Na]^+$ as compared to protonated molecules $[M + H]^+$ is related to the salt concentration of each type of sample, which can be different according to the conservation process.

The lipid extracts of S and P caviar samples were analyzed as a function of the time of storage. The first comparison of lipid extracts of S and P caviar samples was done at day 0 (d_0). Fig. 1a and d show the observed profiles, characterized by the PL ions of *m/z* 715.5, 760.6, 780.6, 782.6 and 806.6, identified as PC and PG (Table 1).

Considering the theoretical fatty acid (FA) composition of the sample, it is possible to estimate the most probable attribution of a lipid ion.[28] Since the major FA in caviar are known to be oleic (18:1, *ca.* 30%), palmitic (16:0, *ca.* 23%), docosahexaenoic (DHA, 22:6, *ca.* 17%), eicosapentaenoic (EPA, 20:5, *ca.* 5%), linoleic (18:2, *ca.* 4%) and myristic acids (14:0, *ca.* 2%),[26] the most likely constitutions of PL ions detected by MALDI(+)-MS are $[PG\ 34:1\ (18:1/14:0)+Na]^+$ of *m/z* 715.5, $[PC\ 34:1\ (16:0/18:1)+H]^+$of *m/z* 760.6 and its corresponding sodiated adduct of *m/z* 782.6, $[PC\ 36:5\ (16:0/20:5)+H]^+$of *m/z* 780.6, $[PC\ 38:6\ (16:0/22:6\ or\ 18:1/20:5)+H]^+$of *m/z* 806.6 and $[PC\ 38:6\ (16:0/22:6\ or\ 18:1/20:5)+Na]^+$ of *m/z* 828.6.

Although basically the same pattern of PL ions was detected in both types of caviar, their distinction was achieved by the significant and reproducible differences in the relative ion abundances (Fig. 1a and d). An opposite ratio of the ions of *m/z* 760.6 and 780.6 can be observed comparing the spectra of S and P at d_0. This difference is probably due to the contrasting lipid composition of the two kinds of samples.

The capability of MALDI(+)-MS to monitor the changes in the PL profile due to spoilage has been verified. The S and P caviar samples were kept at RT in open flasks. These conditions were meant to simulate and speed up the spoilage of caviar due to inappropriate storage and resulted in high dehydration and lipid hydrolysis. The marine lipids are known to be highly stable, and because of this steady property, they are largely used in cosmetics. Fig. 1b and e show the MALDI(+)-MS of S and P caviars at d_7, respectively, whereas Fig. 1c and f show their respective spectra at d_{21}. When comparing the MALDI(+)-MS acquired at d_0 (Fig. 1a and d) with the spectra of both types of caviar at d_7 (Fig. 1b and e), several new or much more abundant ions of *m/z* 828.6, 830.6, 832.6, 834.6, 844.5, 854.6, 858.6, 860.6 and 881.8 were observed at d_{21} at RT (Fig. 1c and f) for both P and S caviars, with higher abundance for the S samples.

The changes in the caviar lipid profiles as a function of time also resulted in a linear increase of sodium adducts, as exemplified by the higher relative abundances of those of *m/z* 782.6,

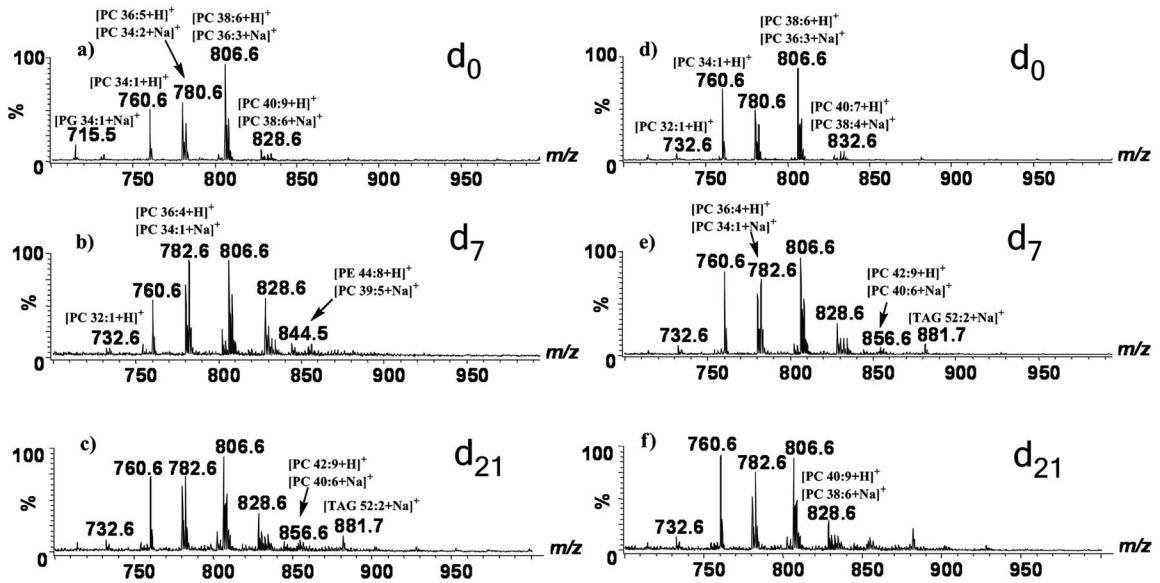

Fig. 1 MALDI(+)-MS after storage at RT for S caviar at (a) d_0, (b) d_7 and (c) d_{21}; and for P caviar at (d) d_0, (e) d_7 and (f) d_{21}.

828.6, and 802.6. This increase is likely related to the decreasing water content of samples exposed to RT in an open vessel, therefore favoring the decrease of protonated molecules.

Note that, probably due to the consumption of the short chain phospholipid ions *via* hydrolysis,[26] the less abundant ions, such as the long chain phospholipids of *m*/*z* 844.5, 854.6, 858.6, and 860.6 (characterized by a tight inter-molecular packing conformation and less capability to hydrolyze[21]) and the TAG ion at *m*/*z* 881.8, which were probably suppressed at d_0, could be instead detected at d_7 and d_{21} (compare Fig. 1a and b with Fig. 1d and f). This observation was posteriorly confirmed by TI-EASI-MS and ESI-MS (data presented below) and verified in previous reports.[8,20] Therefore, at d_{21}, with the decrease of the intensity of easy-to-ionize short chain PL ions mainly by hydrolysis, ionic suppression became less severe and it was possible to detect the long chain phospholipids and the TAG ions.

S and P caviar samples stored for d_{21} at 4 °C have also been extracted and analyzed by MALDI(+)-MS. As Fig. 2a and b show, the spectra acquired from caviar samples under these conditions are very similar to those at d_0 (Fig. 1a and d), thus indicating that no or little degradation occurs. Abundances of $[M + Na]^+$ ions remained nearly the same as a function of storage time, likely because refrigeration at 4 °C results in a minor loss of water.

The same profiles were also revealed by TI-EASI-MS, indicating that, when stored at +4 °C, caviar degradation is prevented by both pasteurization and salt addition. As Table 1 summarizes (and as mentioned before), the most significant lipids detected by MALDI(+)-MS are long chain PC with a high degree of unsaturation. Although marine phospholipids (MPL) contain a high percentage of long-chain polyunsaturated FA (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), their tight intermolecular packing conformation at the *sn*-2 position and their synergism with alpha-tocopherol present in fish extracts are known to inhibit oxidation.[1]

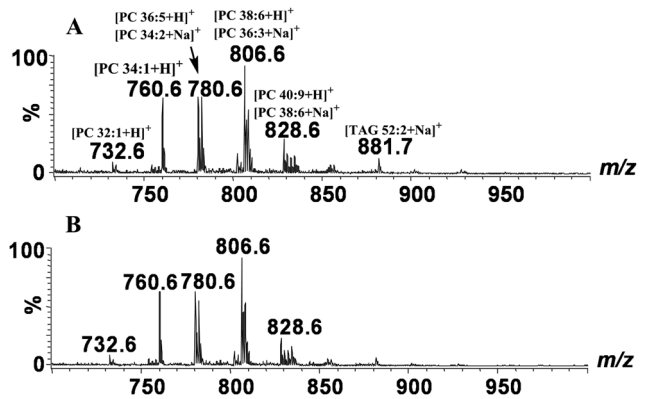

Fig. 2 MALDI(+)-MS of lipid extracts of (a) S and (b) P caviar acquired at d_{21} and kept at +4 °C.

b. TI-EASI(+)-MS

As already demonstrated for EASI(+)-MS lipid analysis,[21] TI-EASI(+)-MS detects both PL (mainly PC) and TAG. Fig. 3a and c show TI-EASI(+)-MS of S and P caviars after d_{28} at +4 °C, whose spectra are very similar to those observed for d_0 samples by MALDI(+)-MS (Fig. 1a and d). This similarity is mainly observed when considering the opposite ratio between PC 34:1 and PC 38:6, detected as sodium adducts of *m*/*z* 782 and 828, respectively. An interesting feature of the TI-EASI(+)-MS (Fig. 3), as compared to MALDI(+)-MS (Fig. 1), is therefore its more comprehensiveness due to the detection of a combined TAG and PC profile, with the exclusive detection of sodium adducts in detriment of protonated molecules.

All TI-EASI(+)-MS show predominance of $[M + Na]^+$ ions, which is a very common feature for TAG analysis using EASI.[29] The most abundant ion of *m*/*z* 881 is assigned to $[TAG\ 52:2 + Na]^+$. Other TAG species of *m*/*z* 927, 901, 879, 877 and 853 were also observed. Note that the same set of PC ions of *m*/*z*

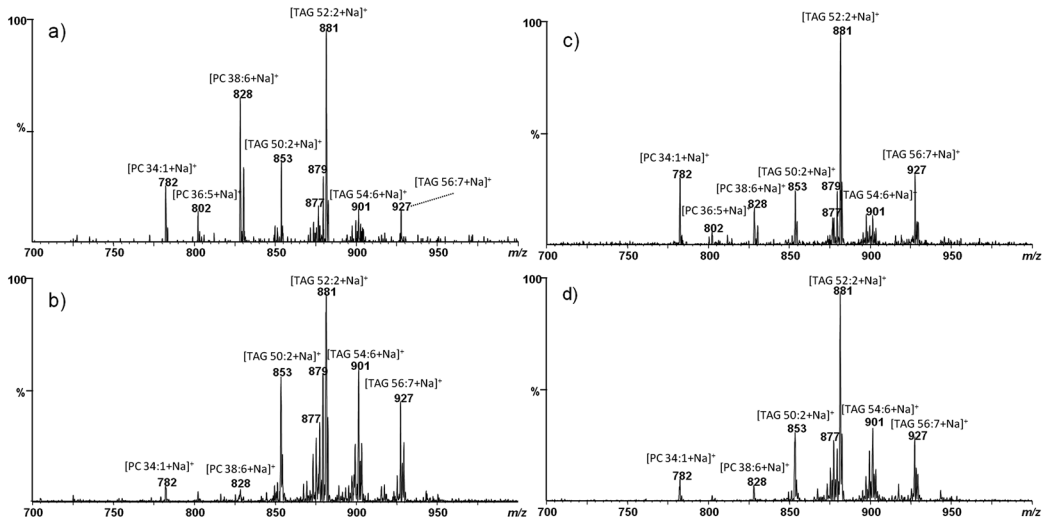

Fig. 3 TI-EASI(+)-MS of S caviar at (a) d_{28} at 4 °C and (b) d_{28} at RT and P caviar at (c) d_{28} at 4 °C and (d) d_{28} at RT.

782, 802, and 828 detected by MALDI(+)-MS (Fig. 1) was also detected by TI-EASI(+)-MS. As previously discussed in Section 3a, oleic acid, palmitic acid, DHA and EPA are the major FA in caviar. Therefore, the probable constitutions of TAG detected by TI(+)-EASI-MS are [TAG 52:2 (16:0/18:1/18:1)+Na]$^+$ of *m*/*z* 881, [TAG 56:7 (16:0/18:1/22:6 or 18:1/18:1/20:5)+Na]$^+$ of *m*/*z* 927, and [TAG 54:6 (16:0/16:0/22:6 or 16:0/18:1/20:5)+Na]$^+$ of *m*/*z* 901. Table 1 summarizes the assignments for the other major ions.

The major differences between the TAG + PL profiles of both types of caviars at d_{28} at 4 °C (Fig. 3a and c) are clearly due to the relative abundances of the ions of *m*/*z* 828 [PC(16:0/22:6 or 18:1/20:5)+Na]$^+$ and 927 [TAG 56:7+Na]$^+$. The P caviars show a less abundant ion of *m*/*z* 828, and a significantly more abundant ion of *m*/*z* 927, indicating that P caviar has a much lower content of DHA (22:6) or EPA (20:5) as phospholipids and a higher content of these acids as TAG than S caviars. These changes could be related to the pasteurization process.

When comparing S caviars at d_{28} at 4 °C and d_{28} at RT, a great difference in TAG profiles was noted (Fig. 3a and b). For RT samples, the relative abundances of the PC ions of *m*/*z* 782, 802 and 828 notably decreased as compared to those at d_0 at +4 °C.

Comparing P caviars at d_{28} at 4 °C with those at d_{28} at RT, although more similar spectra were observed (Fig. 3c and d), the relative abundances of the same PC ions of *m*/*z* 782, 802 and 828 also decreased, as for the S caviar samples. As it will be discussed in the next topic, these changes may be due to extensive hydrolysis of PC species and slight hydrolysis of TAG species to DAG and MAG.

Therefore, an important advantage of the TI-EASI(+)-MS analysis is the simultaneous detection of both PC and TAG ions leading to a more comprehensive lipid profile. Actually, the almost unchanged TAG profile facilitated the perception of the decrease in the intensity of the PL ions. In other words, the constant TAG profile can therefore be considered a natural internal standard for comparative tasks.

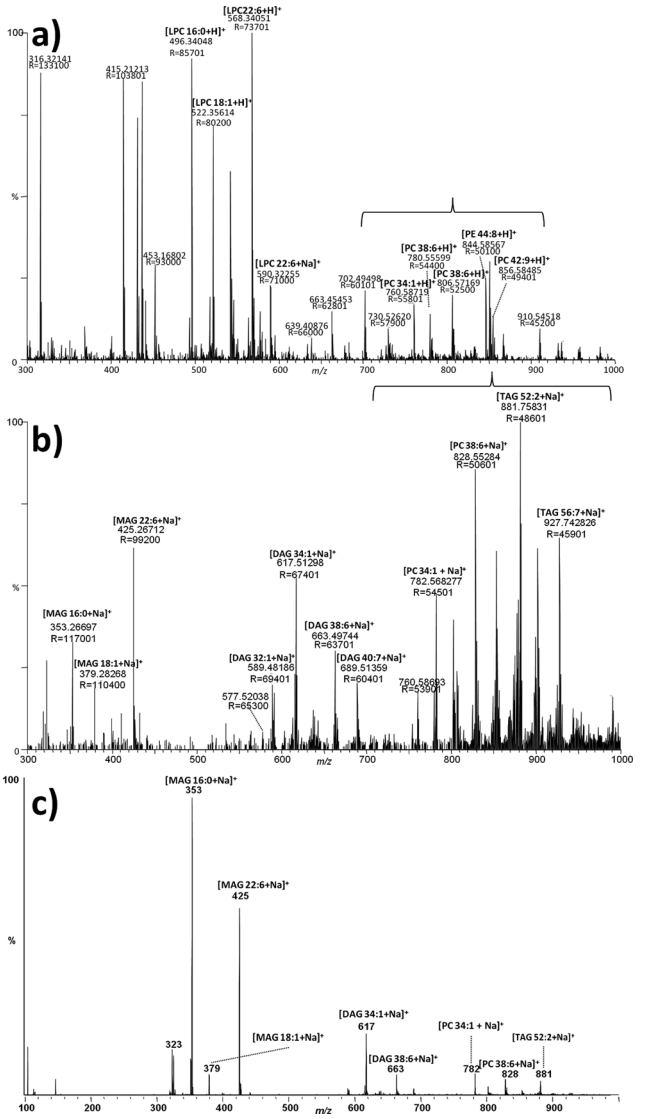

Fig. 4 ESI(+)-FT-ICR-MS of S caviar at d_{21} at RT for lipids extracted (a) *via* the Bligh & Dyer protocol or (b) *via* thermal imprinting into a glass surface and (c) full TI-EASI(+)-MS for S caviar at d_{28} at RT.

c. ESI(+)-MS

ESI(+)-FT-ICR-MS allowed characterizing the lipidic content of the caviar samples after storage at RT whose data confirmed the occurrence of the hydrolysis process. Analyses were performed for extracts (BD protocol) of both kinds of caviar samples after d_{21} at RT, but now scanning within a broader *m*/*z* range (from 100 to 1000) (Fig. 4a), which is not possible by MALDI-MS because of the interference of the matrix in the low *m*/*z* range. Table 1 summarizes the ESI(+)-FT-ICR-MS plus online database search assignments. Ions were assigned either as protonated or sodiated molecules with errors lower than 10 ppm. As for MALDI-MS, mainly PL ions were observed (Fig. 4) in the BD extracts. Note that for ESI(+)-FT-ICR-MS analysis, the caviar extracts were acidified in order to favor [M + H]$^+$ ions. The high resolution and accuracy of ESI(+)-FT-ICR-MS again confirmed that no oxidized lipids are formed at d_{21} for samples kept at RT. Instead, hydrolysis products, such as lysophosphatidylcholines (LPC), were observed and assigned by accurate *m*/*z* measurements and database search. As an example, Fig. 4 shows the ESI(+)-FT-ICR-MS of S caviar at d_{21} at RT with abundant ions of *m*/*z* 496.34, 522.36 and 568.34.

The present results are therefore in accordance with those reported by Gussoni *et al.*[26] who performed NMR on salted and unsalted caviar stored for 4 months at 4 °C and found that the total fat content remained practically unchanged with no signs of oxidation. Their data also indicated that lipid degradation (faster for unsalted eggs) for caviar kept at +4 °C was mainly due to hydrolysis.

The samples kept at RT for 4 weeks (d_{28}) were also thermally imprinted on a glass slide and the obtained extract was analyzed by FT-ICR-MS. Both PL plus TAG were detected, suggesting that the thermal imprinting favors the extraction of TAG. Again, no oxidized lipids were detected *via* thermally imprinted extracts of d_{28} at RT samples which have been analyzed by TI-ESI-FT-ICR-MS (Fig. 4b). Hydrolysis products such as DAG and MAG are however clearly detected *via* the ions of *m*/*z* 425.26672,

617.51250 and 663.49695 which could be assigned respectively to $[MAG\ 22:6 + Na]^+$, $[DAG\ 34:1 + Na]^+$ and $[DAG\ 38:6 + Na]^+$. These data allow us to confirm the hypothesis of lipid hydrolysis since the hydrolysis products of all lipids classes were observed.

d. Comparison between BD and TI extraction protocols

The use of the same MS technique (*i.e.* ESI) for analyzing both BD and TI extracts allowed the comparison between these two extraction protocols. BD is undoubtedly an exhaustive extraction process, which enables the use of small amounts of sample (3 eggs in this case). It can be readily used for quantitative tasks and provides information regarding all the lipid classes.

The TI protocol is however fast and easy to perform, avoiding the use of centrifugation and evaporation steps. Moreover, almost no solvent is employed, leading to an eco-friendly sample treatment when analyzing lipids. It can also be easily performed together with other MS techniques, as exemplified here with ESI.

As it has been previously stated, the use of TI-ESI(+)-FT-ICR-MS ensured the absence of oxidation products and properly characterized the lipid content on the thermally imprinted extract. This result demonstrated that the TI extraction process does not cause degradation of the sample due to the heat. Heating the sample for a few minutes failed therefore to modify the lipid profile of the sample analyzed here, making the TI protocol a convenient tool for lipid extraction.

e. Comparison between MS techniques

Comparing the MALDI(+)-MS and TI-EASI(+)-MS data, it can be noted that the most abundant PC identified by MALDI(+)-MS as $[M + H]^+$ ions of *m*/*z* 760.6, 780.6 and 806.6 were also detected by TI-EASI(+)-MS as $[M + Na]^+$ ions of *m*/*z* 782, 802 and 828. However, TI-EASI(+)-MS can also monitor TAG species. Although MALDI-MS has a unique sensitivity for PL detection the greater coverage of ion species provided by EASI-MS leads to more comprehensive chemical information. The observed favoring of TAG detection may be related to the extraction obtained by thermal imprinting and the ionization process of EASI-MS. In fact, comparing the TI-ESI-FT-ICR-MS (Fig. 4b) with the ESI-FT-ICR-MS of the BD extract (Fig. 4a), it seems that TI is decisive for the TAG extraction since in this process no water was used either in the extraction procedure or in the ionization step. The ESI(+)-FT-ICR-MS provides complementary lipid information allowing exact assignment and confirming the hydrolysis hypothesis of caviar spoilage. This technique can obtain results which are comparable to both MALDI and EASI results, depending on the applied conditions. Although FT-ICR-MS is an expensive technique to be commercially applied to caviar screening, it was very useful here to confirm the structural assignments. Similar results could be obtained using other lower resolution MS equipment such as triple quadrupole, linear traps and the new generation of orbitraps.

Note also that not only ESI-MS allows the detection of hydrolysis products. In fact, among the mentioned techniques EASI-MS could also be used for this task, as Fig. 4c shows, which shows the EASI(+) spectrum of a caviar extract in a broader *m*/*z* range. By comparing Fig. 4b and c we can observe that the hydrolysis products are even easier to detect by EASI-MS than the TAG and PL ions and the abundances of MAG and DAG ions are much higher than PL and TAG abundances as Fig. 4c shows. This trend is related to the EASI process which favors the desorption of lighter/more soluble molecules from the analyzed surface.

The detection of hydrolysis products was performed along all the time of storage for EASI-MS and a trend was observed as an increase in the relative abundance of such ions (data not shown). Nevertheless we decided to focus on the comparison among the techniques in a higher *m*/*z* range, since the interference of the matrix in MALDI-MS could disable the proper detection of these lighter ions.

4. Conclusions

Lipid profiles of S and P caviars can be rapidly obtained by the use of three fast and direct MS techniques: MALDI(+)-MS, ESI(+)-MS and TI-EASI(+)-MS. MALDI-MS, after BD extraction, allows the observation of mainly PL species whereas TI-EASI-MS detects both PL and TAG species thus providing a more comprehensive profile. Both PL and TAG ions were detected and characterized by TI-EASI-MS as lipids containing a relatively high degree of unsaturation as expected from the known high content of PUFA in caviar. Analyses of both the BD and TI extracts of caviars by ESI-FT-ICR-MS have demonstrated that the comprehensiveness of TI-EASI is also related to the extraction method. Therefore, as compared to other techniques, TI-EASI(+)-MS analysis of caviar is simpler and faster, and more comprehensive, providing both TAG + PL profiles and a more comprehensive caviar characterization and quality control. TI-EASI(+)-MS seems indeed promising not only for caviar analysis but also for other similar food products providing proper characterization as well as origin, process and quality control. The main degradation process of caviar in regard to its lipid composition was confirmed to be hydrolysis, whereas no traces of oxidation were detected. Hydrolysis rapidly consumes PL species whereas TAG species are slowly hydrolyzed to DAG and MAG.

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