Food Research International 116 (2019) 1258–1265

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Assessing fish authenticity by direct analysis in real time-high resolution mass spectrometry and multivariate analysis: discrimination between wild-type and farmed salmon

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ARTICLE INFO

Keywords:
Direct Analysis in Real Time-High Resolution Mass Spectrometry (DART-HRMS)
Food authenticity
Salmon
Multivariate analysis
Validation

ABSTRACT

The constant increase in seafood consumption worldwide has led to a parallel growth of the incidence of products obtained by aquaculture on the market, but also of the fraudulent commercialization of farmed products as wild-type ones. A careful characterization of the lipid component of seafood products based on chromatography-mass spectrometry techniques has been reported as a promising approach to reliably differentiate farmed from wild-type products. In this context, a fast method based on Direct Analysis in Real Time (DART) coupled to High Resolution Mass Spectrometry (HRMS) based on a single stage Orbitrap mass analyzer, integrated by Principal Component Analysis (PCA), was developed in the present study and applied to scout for spectral features useful to discriminate wild-type from farmed salmon of *Salmo salar* species. In particular, normalized intensities obtained for the 30 most intense signals (all referred to fatty acids, FA) detected in negative ion DART-HRMS spectra of the lipid extracts of salmon fillets [26 wild-type from Canada, 74 farmed from Canada (25), Norway (25) and Chile (24)] were considered as the variables for PCA. The *scatterplot* referred to the first two principal components showed a clear distinction between wild-type and farmed salmon, which gathered as a unique cluster, despite the remarkable differences in their geographical origin. In accordance with previous studies based on more complex and time-demanding analytical approaches, three saturated (14:0, 16:0 and 18:0) FA, along with unsaturated ones having 20 or 22 carbon atoms, were found as the main discriminating variables for wild-type salmons, whereas FA with compositions 18:1, 18:2, 18:3 and several oxidized forms arising from them were found to have a significantly higher incidence in farmed salmon. The method was further validated by Discriminant Analysis (DA) performed on the same dataset used for PCA integrated by data obtained from 6 commercial samples, putatively referred to farmed Norwegian salmon. Results showed that 100% of the latter were correctly classified as farmed type. Relative abundances of DART-HRMS signals related to specific FA appear then very promising for the differentiation of wild-type salmon from farmed ones, a very relevant issue in the context of consumers' protection from seafood frauds.

1. Introduction

Increasing interest has been devoted in recent years to fishery products and in particular to salmon, due to the health benefits offered by the consumption of this fish. This is related to its high content in polyunsaturated fatty acids (PUFA), like docosahexaenoic (DHA, chain composition 22:6, according to the conventional nomenclature), eicosapentaenoic (EPA, 20:5), linolenic (LA, 18:2) and alpha-linolenic(ALA, 18:3) ones (Blanchet et al., 2005; Hamilton et al., 2005; Sprague et al., 2016). Although human body is able to synthesize most of the fats it needs starting from nutrients introduced through a normal diet, linoleic and alpha-linolenic acids are essential fatty acids, i.e., they cannot be naturally synthesized and must be assumed directly from the food. Indeed, they represent the starting point for the biosynthesis of the so-called omega-3 and omega-6 fatty acids (Jones & Rideout, 2014), particularly abundant in plants, including flaxseeds, walnuts and soybeans, and in fish like salmons (Baker et al., 2016).Omega-3 fatty acids play important roles in the body as components of the phospholipids

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https://doi.org/10.1016/j.foodres.2018.10.013
Received 15 June 2018; Received in revised form 20 September 2018; Accepted 2 October 2018
Available online 04 October 2018

representing the building blocks of cell membranes (Institute of Medicine, Food and Nutrition Board, 2005). In addition to their structural role in cell membranes, omega-3 and omega-6 FA provide energy for the body and are used to synthesize compounds known as *eicosanoids*, that in turn have wide-ranging functions in the cardiovascular, pulmonary, immune, and endocrine systems (Jones & Rideout, 2014; Jones & Papamandjaris, 2012). In particular, ALA can be converted into EPA and then to DHA, but the conversion (which occurs primarily in the liver) is very limited, with reported rates of less than 15% (Harris, 2010). Therefore, consuming EPA and DHA directly from foods and/or dietary supplements is the only practical way to increase the levels of these fatty acids in the body.

The European Food Safety Authority (EFSA) has recently issued a recommendation for an adult population intake of 250 mg of EPA + DHA/day (EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA), n.d.) and a consumption of fish twice a week (one of which to be oily fish) for individuals considered to be at risk of cardiovascular disease (Perk et al. 2012).

Salmon is certainly a source of valuable polyunsaturated fatty acids, yet the actual amount and integrity of the latter into its tissues can be significantly influenced by its origin, i.e., if it is obtained by capture or aquaculture, and, in the case of aquaculture, by the type of feeding and the conditions of farming (Bell et al., 2003; Cahu et al., 2004; Sprague et al., 2015).In particular, it is well known that wild-type salmon is richer in the more valuable omega-3 fatty acids, compared to omega-6 (Blanchet et al., 2005; Lundebye et al., 2017). In the last years this has caused a growing demand for wild-type salmon,often with a specified geographical origin, yet the actual availability of this type of salmon is increasingly limited. As a result, wild-type salmon prices have been increasing significantly and, accordingly, food frauds aiming at classifying and selling farmed salmon as wild-type one have become a serious concern (Kappel & Schröder, 2016; Cline, 2012; Thomas et al., 2008). In order to protect consumers from such frauds and, at the same time, to guarantee their safety, a labeling regulation for fishery and aquaculture products has been issued in 2000 in the European Union (EU) (Council Regulation (EEC) No. 104/2000 on the common organization of the markets in fishery and aquaculture products; Grundy et al., 2012). This regulation, emphasizing the commercial and scientific name, the modality of production (capture or aquaculture) and the geographical origin of a seafood product, represents a helpful set of information for the consumers who can be aware of the qualitative, geographic and productive features of fishery items. A system for the traceability of food (including fishery and aquaculture products) and feed has been also set up by the European Food Safety Authority (EFSA) to assure food safety at all stages (Charlebois et al., 2014). EU Regulations 1169/2011 (Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers), dealing with Food Information to Consumers (FIC), and 1379/2013 (Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organization of the markets in fishery and aquaculture products, n.d.), concerning the Common Market Organization (CMO) of fishery and aquaculture products, provided a more extensive legislation framework about identification and labelling of fishery and aquaculture products. In particular, Regulation 1379/2013 contributed to the implementation of traceability protocols for fishery products, encouraging competent national authorities to make use of the forefront technology to deter operators from the false labeling of products (Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organization of the markets in fishery and aquaculture products).

The context discussed so far has generated the urgent need to develop sensitive and fast analytical methods able to deliver, in the shortest time possible, a useful result in terms of fish authenticity. Several methods and techniques, mainly based on the measurement of sensory and physical characteristics, and the analysis of volatile compounds, microorganisms, proteins and lipids, have been developed in recent years to assess fish freshness or geographic origin (Cheng et al., 2015; Farag El Sheikha & Montet, 2016; De Battisti et al., 2013; Liu et al., 2013; Fiorino et al., 2018a). These methods can be in general categorized into two types, based on biological or physico-chemical techniques (Farag El Sheikha & Montet, 2016; Downey, 2016). Among the latter, mass spectrometry based on different ionization sources has been successfully exploited to assess fish (including salmon) quality and geographic origin (Thomas et al., 2008; Cubero-Leon et al., 2014; Mazzeo & Siciliano, 2016; Arvanitoyannis, 2016). A further MS-related promising approach to the assessment of food authenticity, described for the first time by Vaclavik et al. (Vaclavik et al., 2009), is based on the use of the so-called Direct Analysis in Real Time (DART) ionization source. Many papers recently published on this regard proved the applicability of DART-MS in detecting food adulteration or in discriminating authentic from non-authentic food through the monitoring of specific markers and appropriate statistical analysis (Gross, 2014; Vaclavik et al., 2011). As for fishery products, DART-HRMS was applied to study fish metabolomics (Cajka et al., 2013), and, recently, it was exploited in our laboratory to evaluate salmon freshness (Fiorino et al., 2018b). The latter study was targeted on fatty acids released in salmon muscles as the result of hydrolysis occurring on main lipids (phospholipids, triacylglycerols) during prolonged storage. Actually, due to its rapidity and high throughput potential, DART-HRMS may represent a powerful approach also to the assessment of seafood authenticity, yet, to the best of our knowledge, no study based on this technique has been performed so far in this context. Starting from this consideration, the development of an untargeted method based on DART-HRMS, to be applied for a fast and high throughput analysis of salmons of *Salmo salar* species, aiming at the discrimination between farmed and wild-type individuals, has been undertaken in our laboratories and will be described in detail in the present paper.

2. Materials and methods

2.1. Chemicals and salmon samples

Liquid chromatography-grade methanol and 2-propanol were purchased from VWR International (Fontenay-sous-Bois, France), while chloroform (same grade) was provided by Merck (Darmstadt, Germany).

The salmon samples analyzed during the present work were obtained in the framework of an international, EU-funded, project called *Food Integrity* and, in particular, within Working Package 18, whose aim is to harmonize and develop fast and untargeted analytical methods to assess the integrity of foods. More specifically, 26 wild-type (WT) salmon captured in Canada and a total of 74 farmed salmon, arising from aquaculture plants of Canada (25), Norway (25) and Chile (24), all of *Salmo salar* species, were considered in this investigation. The bones and skin were removed from each individual salmon and fillets were grinded under refrigeration from the provider (Meriex Nutriscience, Chicago, IL, USA) by following the same sample preparation protocol. The homogenate of each sample was weighed and a 200 g aliquot was placed into a glass jar and shipped to our laboratory under freezing conditions (−20 °C). Once arrived at the laboratory the samples were stored at −20 °C before lipid extraction and DART-MS analysis. In order to validate the approach developed during the present study, 6 additional salmon samples, purchased from local retailers and claimed as farmed in Norway, were considered for lipid extraction and subsequent DART-HRMS analysis.

2.2. Lipid extraction from salmon samples

All homogenized samples were extracted according to a slightly modified version of the Bligh & Dyer protocol (Bligh & Dyer, 1959). Briefly, 2.5 g of homogenized sample were withdrawn, transferred into

a glass tube and combined with 5 mL of refrigerated chloroform and 5 mL of refrigerated methanol. The resulting mixture was manually stirred for a few seconds using a metallic spatula and left under shaking for 15 min at room temperature, then was subjected to a decanting phase for 2 h (although specific tests showed that even 30 min could enable an efficient decantation) at 4 °C. After this step, the chloroform layer was collected using a Pasteur pipette and stored at −20 °C before being analyzed. Subsequently, 200 μL of the chloroform-based extract were transferred into glass vials, evaporated under a gentle stream of nitrogen and then re-dissolved in 200 μL of 2-propanol before proceeding to DART-HRMS analysis. The last experimental procedure was adopted to minimize the sample volume variability related to chloroform volatility.

2.3. DART-HRMS analysis: instrumentation and operating conditions

DART-HRMS analyses were performed using a DART ionization source SI-140-GIST (DART Thermo Ion Max Vapur Interface, Ion Sense Inc., Saugus, MA, USA) mounted on an Exactive™ monostage Orbitrap™ High Resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A 2 μL aliquot of the lipid extract obtained from a salmon sample was placed on the metallic grid of one of the open spot sample cards provided by the DART source manufacturer and left to evaporate at 60 °C for 5 min before introducing the card into the appropriate holder on the DART source. Once the card was introduced, the sample was exposed for 1 min to a 3.2 L/min heated helium stream subjected to a discharge generated on a needle kept at −6 kV. The operating conditions of the DART source were the following: negative ion mode; helium flow heated at 150 °C; grid electrode voltage set to −350 V. The distance between the DART exit and the MS inlet was set at 5 mm. The major settings for the Exactive™ mass spectrometer were as follows: mass scan range, 100–900 *m/z*; resolution, 50,000 (FWHM at *m/z* 200); microscan number, 2; Automatic Gain Control (AGC) Target, $3*10^6$; maximum injection time (IT), 250 ms; capillary voltage, 30 V; tube lens voltage, 65 V; capillary temperature, 250 °C. The spectrometer was periodically calibrated by direct infusion-ESI-MS analysis of the negative ion calibrating solution provided by the manufacturer and a mass accuracy usually better than 5 ppm was achieved. A DART-SVP controller (v. 4.0.x) was used to set operating source parameters, whereas the software Xcalibur™ v. 2.1 (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the Exactive™ spectrometer and for a first elaboration of DART-HRMS data.

It is worth noting that, to get the spectral background for subsequent subtraction, a blank card was analyzed before each sample analysis and the relevant DART-HRMS spectrum was acquired for 30 s. Two replicated analyses were performed for each sample.

2.4. Data processing and chemometric analysis

The Xcalibur™ software was used in the first step of data processing, i.e. to average DART-HRMS spectra obtained in the time range (1 min) corresponding to the exposure of each sample to the DART gas beam. Afterwards, the spectral background obtained from a 30 s acquisition performed on a blank card before each sample analysis (see section 2.3) was subtracted, using the same software.

The background-subtracted DART-HRMS spectra referred to the two replicates corresponding to each of the 100 analyzed lipid extracts were averaged and then carefully processed to find the most abundant and reproducible signals. In particular, the *mMass 5.5.0* software was used for data mining, setting a relative abundance threshold equal to 0.5%, since this level corresponded approximately to ionic noise. The resulting list of accurate *m/z* ratios and intensities was then processed to select only *m/z* ratios corresponding to the first isotopologues of the detected isotopic clusters. A comparison between the lists of such ratios obtained for the different samples enabled the selection of the top 30 signals, in order of abundance, that were systematically detected in all the acquired spectra, both for wild-type and for farmed salmon samples, although some relevant differences in the distribution of their abundances were observed between the two sample types. The corresponding *m/z* values were subsequently searched in the *LipidMaps* database (freely accessible at the Internet address *www.lipidmaps.org*), setting a tolerance on *m/z* values matching of 0.005 units, consistent with the accuracy typically obtained on the Exactive spectrometer. For each sample the intensity of each selected signal was normalized to the sum of intensities referred to the 30 signals set, thus providing values not affected by response fluctuations eventually occurring between different analyses and/or due to variations in the lipid extraction yield. Indeed, when the sums of intensities observed for the 30 selected signals were averaged, respectively, for the two types of salmon samples and the standard deviations were calculated, the following values were obtained: for wild-type $(1.4 \pm 0.8) \times 10^6$ counts/s and for farmed $(2.0 \pm 1.4) \times 10^6$ counts/s. The remarkable variations observed for absolute intensities made the choice of internally-normalized intensities inevitable, if a reliable comparison between the two salmon sample types was pursued.

Normalized intensity values obtained for the 30 selected signals from all the 100 samples were finally used as input values for a Principal Component Analysis based on the Nipals algorithm and performed using the *Statistica* v.7 software (StatSoft Inc., now TIBCO Software Inc., Palo Alto, CA, USA). The same dataset, integrated by data obtained for 6 farmed Norwegian salmon samples purchased from local retailers, was also employed for Linear Discriminant Analysis (LDA), performed by *Statistica* v.7 software.

3. Results and discussion

3.1. Comparison of DART-HRMS spectra obtained from farmed and wild type salmon lipid extracts

As shown in Fig. 1, DART-HRMS spectra obtained for the lipid extracts of both wild-type and farmed salmon evidenced that the most intense signals (intended as those related to the major isotopologues of the detected isotopic clusters) were all odd-numbered and located in the *m/z* range 150–330. These features suggested that fatty acids, detected as $[M\text{-}H]^-$ ions, were dominating the spectra, as already observed during the analysis, based on the same instrumentation and operating conditions, of lipid extracts of salmons stored under refrigeration (Fiorino et al., 2018b). In particular, as emphasized in Fig. 1, signals typically detected at *m/z* 281.2479 and 255.2323 (note that variations occurred on the fourth decimal figure of *m/z* ratios obtained from different analyses) were always the base peaks in DART-HRMS spectra referred to farmed and wild-type salmons, respectively. The subsequent search on the *LipidMaps* database clearly showed the two signals to be related to fatty acids with respective compositions 18:1 and 16:0. When the search was extended to the first 30 signals, in terms of relative abundance, common to all samples and then selected for the subsequent chemometric analysis, only FAs were found to correspond to their *m/z* values within a reasonable tolerance, as shown in Table 1. The compositions of saturated (14:0, 16:0 and 18:0) and unsaturated (16:1, 18:1, 18:2, 18:3, 20:5) fatty acids, whose presence in salmon muscles (either as free fatty acids or embedded into major lipids like phospholipids or triacylglycerols) is well known in the literature (Refsgaard et al., 2000), were easily retrieved from the database search. It is worth noting that an investigation on the positions of C=C bonds and on the presence of eventual chain branching for the detected FA, eventually retrievable using tandem mass spectrometry or more complex analytical approaches, was beyond the goal of the present study. In accordance to that, only the overall chain compositions were reported in Table 1. As emphasized in the table, only half of the 30 species selected for chemometric analysis corresponded to unmodified FA. The remaining ones exhibited *m/z* values that could be explained only by considering different types of chain oxidation, namely hydroxylation,

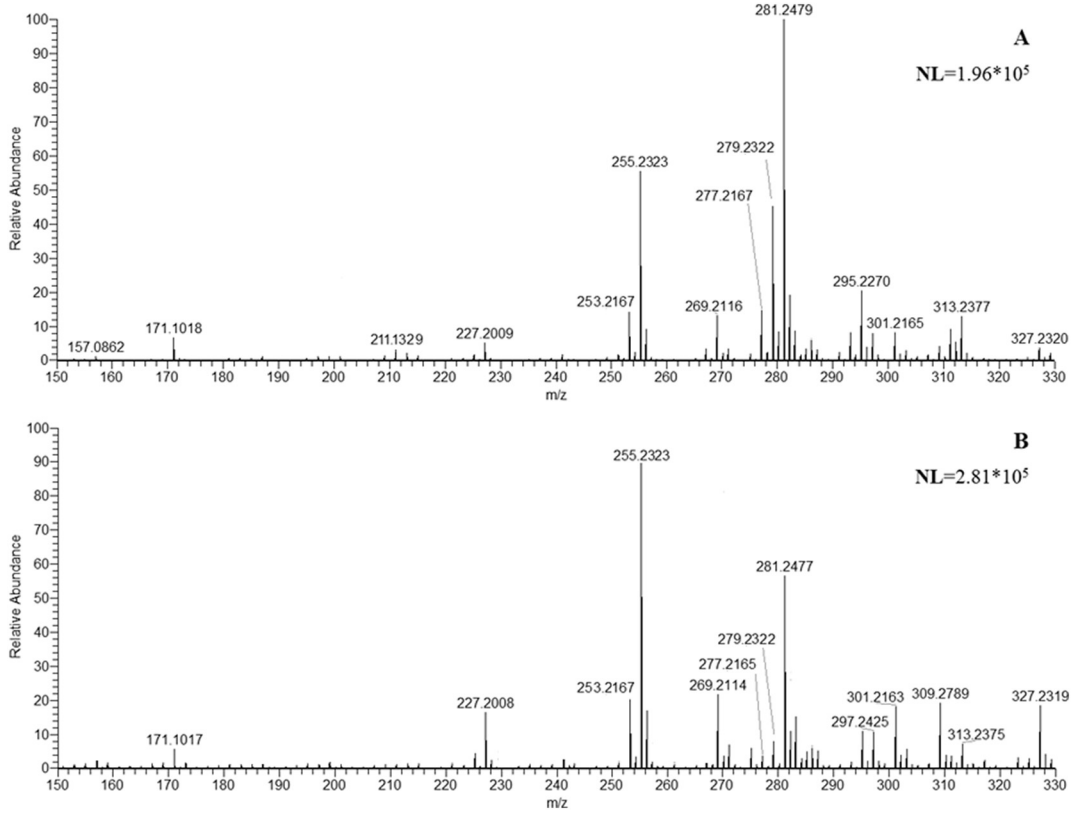

Fig. 1. Representative DART-HRMS negative ion spectra obtained for the fillet lipid extracts of farmed (A) and wild-type (B) salmon. See Table 1 for the compositional assignments given to the 30 most abundant signals generated from FA after a search on the *LipidMaps* database.

epoxylation and/or carbonylation. In many cases, due to the lack of further measurements, like MS/MS ones, more than a single assignment could be made, yet the detection of so many oxidized species was a very interesting result. Subsequent chemometric analysis showed that these species played a remarkable role in determining the discrimination between wild-type and farmed salmons.

3.2. Chemometric elaboration of the generated data

3.2.1. Principal Component Analysis (PCA)

Principal Component Analysis (PCA) represents one of the most widespread exploratory chemometric tool used in the field of multivariate statistics, typically utilized to simplify and gain better knowledge of a dataset, by removing redundancies and noise while retaining meaningful information. PCA has been largely exploited, in conjunction with DART-MS analysis, for the assessment of food quality and authenticity (Vaclavik et al., 2009; Guo et al., 2017; Hrbek et al., 2014). In the present case, PCA was based on the normalized intensity values of the 30 most abundant signals selected from the DART-HRMS spectra obtained from the 100 salmon lipid extract samples, all corresponding to fatty acids, unmodified or oxidized (see Table 1). As first step, PCA was performed only on Canadian salmon, since they represented the only sub-set of samples in which farmed and wild-type salmon were obtained from the same geographical area. As shown in Fig. 2, the *scatterplot* (*score plot*) referred to the two first principal components, accounting for ca. 77% of the total variance, showed a clear separation between Canadian farmed and wild-type salmon along the first principal component.

Further interesting indications were obtained when PCA based on the 30 selected FA was extended to the entire set of samples available, i.e., including also salmon farmed in Norway and Chile. As shown by the scatterplot reported in Fig. 3, still referred to the first two principal components (in this case accounting for ca.74% of the total variance), the separation between farmed and wild-type salmon along the first principal component persisted. Interestingly, the plot clearly indicated that farmed salmon were not distinguishable, at least using the selected set of FA as variables, despite that their geographical origin was very different.

The method based on DART-HRMS integrated by PCA proved then extremely powerful in the discrimination between farmed and wild-type salmon, enabling its achievement through a much faster approach, at least for the data accumulation stage, compared to other methods already described in the literature (Thomas et al., 2008). Indeed, one of the most common methods among the latter is based on the combination between multielement isotopic analysis and determination of fatty acid composition, with the latter obtained through transesterification and subsequent GC–MS analysis of lipid extracts, which is certainly more complex and time-consuming than DART-HRMS. In other words, although all the approaches reported so far, including the one described in the present paper, require a relatively long time for the extraction of lipids from salmon samples, DART-HRMS is able to reduce significantly the time required for analysis without loss of information.

Further useful insights could be obtained in the present case by considering the loading plots related to the described PCA elaborations. Indeed, as shown in Fig. 4, in which the *loading plot* correlated to the *scatterplot* of Fig. 3 is reported, 14 out of the 30 FA selected as variables had a clearly negative loading (lower than −0.6) on the first principal component, thus they were expected to be more abundant in wild-type salmons. On the other hand, 12 FA exhibited a clearly positive loading (higher than +0.5) on the first principal component, thus their amount was expected to be higher in farmed salmons.

In particular, as suggested by a comparison between Table 1 and Fig. 4, saturated FA with compositions 14:0, 16:0, 16:1, 18:0, along with mono- or bi-oxidized FA related to compositions 16:0, 16:1 and 18:0, and polyunsaturated FA with compositions 18:4, 20:4, 20:5, 22:5 and 22:6, thus including also the important omega-3 FA EPA and DHA,

Table 1
Summary of MS-related data, chemical formulas and possible chain compositions inferred for the 30 fatty acids that were considered for the discrimination between wild-type and farmed salmons during the present study. In the last two columns average values and standard deviations referred to normalized abundances observed for each fatty acid in the 26 wild-type and the 74 farmed salmons are reported.

#	Average Experimental m/z	Theoretical m/z	Chemical formula	Mass accuracy (ppm)	Chain composition(s)	Wild type	Farmed
1	157.0862	157.0870	$C_8H_{13}O_3$	-5.09	Hydroxy-8:1 Oxo-8:0	0.45 ± 0.16	0.35 ± 0.11
2	171.1018	171.1027	$C_9H_{15}O_3$	-5.26	Hydroxy-9:1 Oxo-9:0	1.10 ± 0.30	1.67 ± 0.37
3	181.0860	181.0870	$C_{10}H_{13}O_3$	-5.52	Oxo-10:2	0.50 ± 0.09	0.21 ± 0.07
4	211.1329	211.1340	$C_{12}H_{19}O_3$	-5.21	Hydroxy-12:2 Oxo-12:1	0.17 ± 0.03	0.44 ± 0.07
5	227.2008	227.2017	$C_{14}H_{27}O_2$	-3.96	14:0	4.09 ± 1.61	1.80 ± 0.91
6	253.2167	253.2173	$C_{16}H_{29}O_2$	-2.36	16:1	5.40 ± 0.82	3.97 ± 1.09
7	255.2323	255.2330	$C_{16}H_{31}O_2$	-2.74	16:0	25.37 ± 3.13	15.04 ± 2.67
8	269.2116	269.2122	$C_{16}H_{29}O_3$	-2.23	Hydroxy-16:1 Oxo-16:0	5.22 ± 0.88	3.04 ± 0.77
9	271.2271	271.2279	$C_{16}H_{31}O_3$	-2.95	Hydroxy-16:0	1.59 ± 0.32	0.75 ± 0.15
10	275.2006	275.2017	$C_{18}H_{27}O_2$	-3.99	18:4	1.86 ± 0.57	0.57 ± 0.13
11	277.2167	277.2173	$C_{18}H_{29}O_2$	-2.16	18:3	1.13 ± 0.17	3.76 ± 0.68
12	279.2322	279.2330	$C_{18}H_{31}O_2$	-2.86	18:2	2.08 ± 0.22	11.82 ± 1.39
13	281.2479	281.2486	$C_{18}H_{33}O_2$	-2.49	18:1	15.64 ± 2.10	27.85 ± 2.52
14	283.2633	283.2643	$C_{18}H_{35}O_2$	-3.18	18:0	3.86 ± 0.36	2.63 ± 0.39
15	285.2066	285.2071	$C_{16}H_{29}O_4$	-1.75	Hydroxy, oxo -16:0	1.17 ± 0.23	0.81 ± 0.30
16	287.2222	287.2228	$C_{16}H_{31}O_4$	-2.09	Dihydroxy-16:0	1.27 ± 0.15	1.87 ± 5.18
17	293.2114	293.2122	$C_{18}H_{29}O_3$	-2.73	Hydroxy-18:3 Epoxy-18:2 Oxo-18:2	0.46 ± 0.04	1.85 ± 0.30
18	295.2270	295.2279	$C_{18}H_{31}O_3$	-3.04	Hydroxy-18:2 Epoxy-18:1 Oxo-18:1	2.64 ± 0.34	4.82 ± 0.70
19	297.2425	297.2435	$C_{18}H_{33}O_3$	-3.36	Hydroxy- 18:1 Epoxy-18:0 Oxo-18:0	2.17 ± 0.40	2.07 ± 0.47
20	301.2165	301.2173	$C_{20}H_{29}O_2$	-2.66	20:5	5.87 ± 1.56	3.18 ± 0.68
21	303.2315	303.2330	$C_{20}H_{31}O_2$	-4.95	20:4	1.67 ± 0.37	1.08 ± 0.23
22	305.2483	305.2486	$C_{20}H_{33}O_2$	-0.98	20:3	0.24 ± 0.06	0.40 ± 0.08
23	307.2636	307.2643	$C_{20}H_{35}O_2$	-2.28	20:2	0.43 ± 0.10	0.52 ± 0.10
24	309.2063	309.2071	$C_{18}H_{29}O_4$	-2.59	Oxo,epoxy-18:1 Hydroxy, epoxy-18:2 Hydroperoxy-18:3	0.32 ± 0.03	0.88 ± 0.19
25	309.2789	309.2799	$C_{20}H_{37}O_2$	-3.23	20:1	4.97 ± 1.29	1.16 ± 0.70
26	311.2219	311.2228	$C_{18}H_{31}O_4$	-2.89	Hydroxy,epoxy-18:1 Hydroxy,oxo-18:1 Dihydroxy-18:2 Hydroperoxy-18:2	0.75 ± 0.13	2.05 ± 0.37
27	313.2375	313.2384	$C_{18}H_{33}O_4$	-2.87	Hydroxy,epoxy-18:0 Dihydroxy-18:1 Hydroperoxy-18:1	1.72 ± 0.31	3.07 ± 0.44
28	327.2320	327.2330	$C_{22}H_{31}O_2$	-3.06	22:6	5.27 ± 1.90	1.51 ± 0.34
29	329.2317	329.2334	$C_{18}H_{33}O_5$	-5.16	Trihydroxy-18:1	1.86 ± 2.68	0.52 ± 0.10
30	329.2477	329.2486	$C_{22}H_{33}O_2$	-2.73	22:5	0.74 ± 0.22	0.33 ± 0.08

were found to be most relevant in DART-HRMS spectra referred to wild-type samples. On the other hand, FA with compositions 18:1, 18:2 and 18:3, accompanied by several of their possible oxidized forms, and those with compositions 20:2 and 20:3 were more relevant in farmed samples. Average values and standard deviations obtained for normalized intensities observed for FA species in the two types of salmon samples, reported in the two last columns of Table 1, clearly confirm the indications arising from the PCA loading plot.

These findings, at least with respect to non-oxidized FA (since, to the best of our knowledge, no data have been reported so far for oxidized FA in salmons), are in excellent agreement with the information already reported in the literature. Indeed, the higher incidence of saturated FA in the muscles of wild-type salmon is well known (Cahu et al., 2004). On the other hand, 18:2 and 18:3 FA have been reported to be more abundant in farmed salmon, as the result of the increasing use of oils obtained from the seeds of soy, flax and rape, rich in FA like 18:1, 18:2 and 18:3, in farmed salmon feeds (Blanchet et al., 2005; Bell et al., 2003; Cahu et al., 2004; Thomas et al., 2008). Interestingly, while oxidized derivatives of unsaturated FA with 18 carbon atoms on the side chain were found in the lipid extracts of farmed salmon, this was not the case for polyunsaturated FA with 18 (18:4), 20 (20:4) and 22 (22:5 and 22:6) carbon atoms, found to be more relevant in wild-type salmons, although such species are potentially susceptible of oxidation. This finding seems to confirm the correlation between oxidative stress and specific aspects of fish aquaculture, which has been often proposed in the literature (see Ref. (Secci & Parisi, 2016) for a recent review on the topic). In particular, pre-slaughter starvation, aimed at reducing the amount of feces in the intestine of fishes a few days before collection, crowding, occurring just at the moment of collection, and several procedures adopted for killing have been found to increase oxidative stress in farmed fishes.

As already discussed, the scatterplot based on the first two principal components (see Fig. 3) was unable to distinguish farmed salmons in terms of their geographical origin. A tri-dimensional scatterplot, including also the third principal component, was then tentatively reproduced to search for a distinction between farmed salmons based on that parameter. As shown in Fig. 5, even considering the third principal component, accounting for an additional 7.49% of variance, farmed

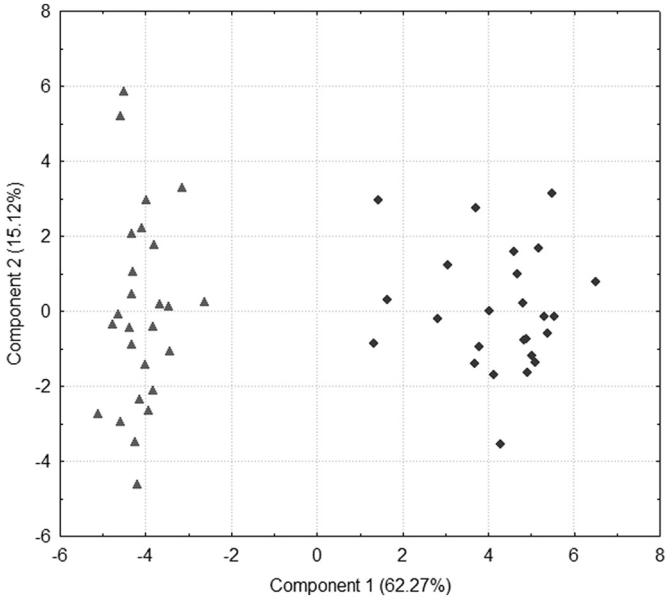

Fig. 2. *Scatterplot* obtained for the first two principal components after applying PCA to normalized intensities retrieved for the 30 most abundant signals generated from FA after the DART-HRMS analysis of fillet lipid extracts of 26 wild-type (triangles) and 25 farmed (diamonds) Canadian salmons. See Table 1 for a list of the selected fatty acids.

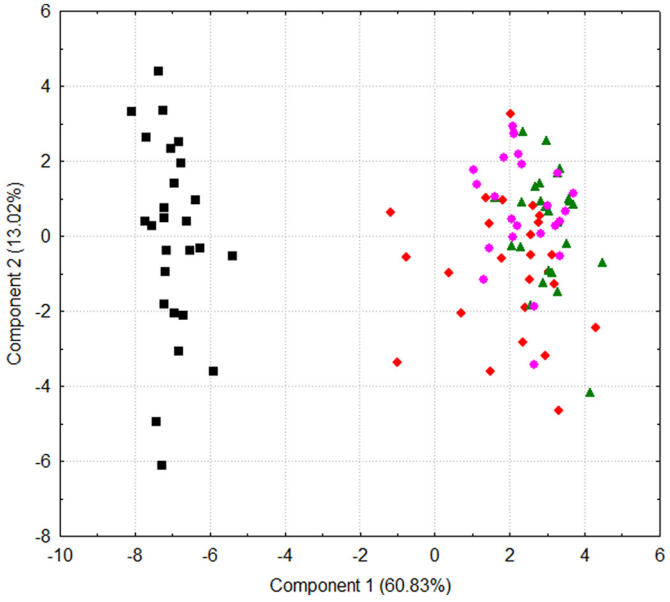

Fig. 3. *Scatterplot* obtained for the first two principal components after applying PCA to normalized intensities retrieved for the 30 most abundant signals generated from FA after the DART-HRMS analysis of fillet lipid extracts of: 26 Canadian wild-type (black squares) and 25 Canadian (red diamonds), 25 Norwegian (green triangles) and 24 Chilean (purple circles) farmed salmons. See Table 1 for a list of the selected fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

salmon samples arising from different countries could not be distinguished. This result, confirming those reported both for farmed and for wild-type salmons in a previous study based on the combination of multielement isotopic analysis and FA profiling (Thomas et al., 2008), suggest that the international standardization of aquaculture practises adopted for salmon may level off the eventual differences, at the FA level, due to the geographic location.

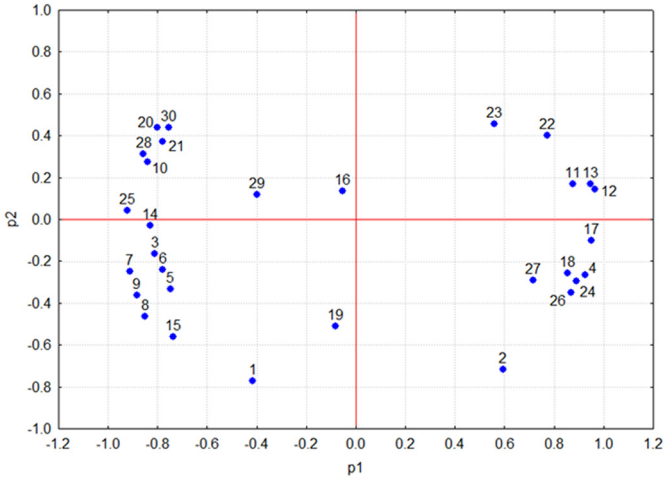

Fig. 4. *Loading plot* referred to the first two principal components obtained after applying PCA to normalized intensities retrieved for the 30 most abundant signals generated from FA after the DART-HRMS analysis of fillet lipid extracts of: 26 Canadian wild-type and 25 Canadian, 25 Norwegian and 24 Chilean farmed salmon. Numeric labels indicated for each point correspond to the numbers adopted to distinguish fatty acid compositions in Table 1.

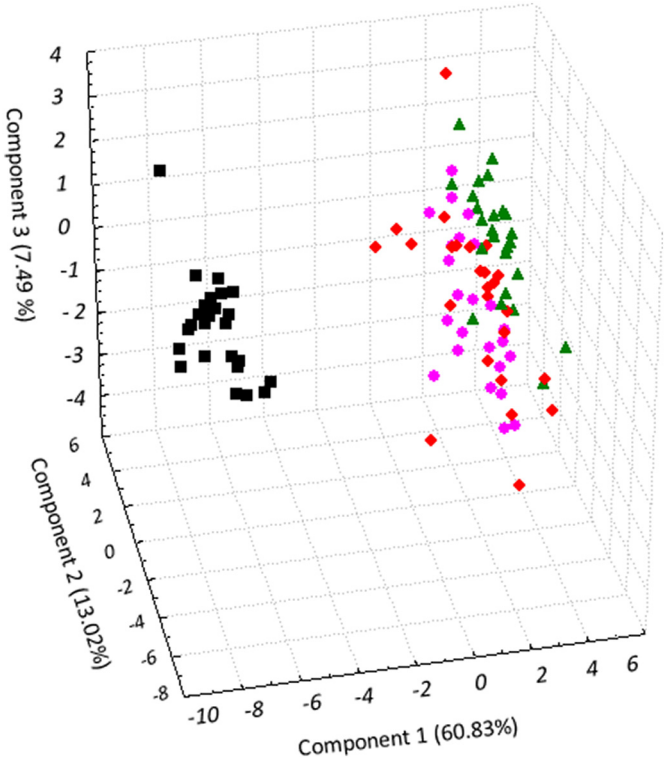

Fig. 5. *Scatterplot* obtained for the first three principal components after applying PCA to normalized intensities retrieved for the 30 most abundant signals generated from FA after the DART-HRMS analysis of fillet lipid extracts of: 26 Canadian wild-type (black squares) and 25 Canadian (red diamonds), 25 Norwegian (green triangles) and 24 Chilean (purple circles) farmed salmon. See Table 1 for a list of the selected fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is worth noting that only wild-type salmon captured in Canada could be taken into consideration for DART-HRMS analysis in the present investigation. At least in principle, salmons captured in other areas, like Norway, Chile, etc., could exhibit peculiar spectral features, different from those shown by Canadian wild-type salmon and

Table 2
Average values and standard deviations referred to normalized DART-HRMS intensities obtained for the 6 fatty acids exhibiting the most relevant differences in terms of average values when comparing Canadian wild-type salmons with farmed salmons collected in Canada, Chile and Norway. In the last six columns average values and standard deviations (n = 3) referred to normalized intensities obtained for the same fatty acids in the lipid extracts of six salmon samples (NRW F 1X-NRW F 6X) purchased in local markets and claimed as farmed in Norway are reported.

Average Experimental m/z	Chain composition(s)	Wild type	Farmed	NRW F 1X	NRW F 2X	NRW F 3X	NRW F 4X	NRW F 5X	NRW F 6X
227.2008	14:0	4.09 ± 1.61	1.80 ± 0.91	0.82 ± 0.17	0.90 ± 0.24	0.84 ± 0.19	0.97 ± 0.25	0.96 ± 0.15	1.10 ± 0.21
255.2323	16:0	25.37 ± 3.13	15.04 ± 2.67	12.69 ± 1.09	13.57 ± 2.31	13.27 ± 1.56	11.43 ± 2.03	11.37 ± 1.74	15.98 ± 1.92
277.2167	18:3	1.13 ± 0.17	3.76 ± 0.68	3.00 ± 0.11	3.00 ± 0.29	3.06 ± 0.18	2.87 ± 0.24	3.26 ± 0.16	2.68 ± 0.22
279.2322	18:2	2.08 ± 0.22	11.82 ± 1.39	7.56 ± 0.23	7.96 ± 0.55	7.68 ± 0.34	7.28 ± 0.41	8.04 ± 0.52	6.96 ± 0.26
281.2479	18:1	15.64 ± 2.10	27.65 ± 2.52	25.35 ± 0.56	25.71 ± 2.35	25.49 ± 1.83	24.73 ± 0.98	28.00 ± 2.27	23.30 ± 1.39
327.2320	22:6	5.27 ± 1.90	1.51 ± 0.34	2.96 ± 0.23	2.70 ± 0.72	2.90 ± 0.65	3.21 ± 0.43	3.38 ± 0.52	1.94 ± 0.55

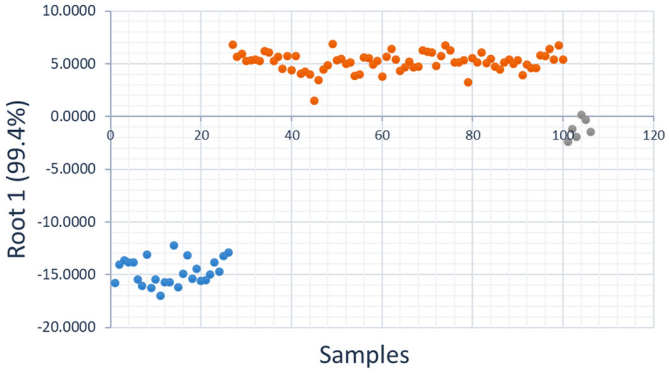

Fig. 6. Graphical representation of the values of *Root 1* obtained after applying Linear Discriminant Analysis to DART-HRMS normalized intensity data referred to wild-type (blu circles) and farmed salmons (orange circles) previously adopted for PCA elaboration. The analysis was further enlarged to include additional 6 commercial salmons claimed as farmed in Norway (grey circles).

potentially impairing a neat discrimination from farmed salmon. The accordance discussed before between the present study and previous ones, that considered wild-type salmon of various origins, suggests this eventuality to be unlikely, yet a specific investigation is ongoing in our laboratory to clarify this aspect.

In any case, due to the remarkable differences observed between farmed and wild-type salmon, as displayed in the last two columns of Table 1, some markers can be suggested for a fast screening of unknown salmon samples, namely to attribute them to one or the other class depending on the specific normalized ion intensities recorded from the DART-HRMS spectrum. In particular, after analysing values reported in Table 1, a sub-list of six ions, showing the most remarkable intensity differences between the two salmon types and corresponding to compositions (in order of *m/z* ratio) 14:0, 16:0, 18:3, 18:2, 18:1 and 22:6, could be proposed for discrimination purposes. In order to validate the approach, six salmon samples purchased from local retailers and claimed farmed of Norway origin, were subjected to lipid extraction and DART-HRMS analysis in triplicate. In Table 2 average values and standard deviations of normalized DART-HRMS intensities retrieved, for each of the six selected fatty acids, from such samples were compared with the corresponding values obtained from the populations of wild-type and farmed salmon used for the PCA elaboration. As a result, normalized intensities related to FA 14:0, 16:0, 18:3 and 18:1 in commercial salmon were quite close to those typical of farmed salmon. Values obtained for FA 18:2 and 22:6 appeared to be intermediate between those obtained for the two types of salmon, although they were closer to values typical of farmed salmon. It is worth noting that these findings might have been influenced, at least partially, by the effect of thermal history on the profile of FA detected by DART-HRMS. Indeed, the six commercial salmon samples were reasonably subjected to a longer refrigeration (during transportation and then storage before purchase) than those provided in the context of the *Food Integrity* project. On the other hand, they were not subjected to freezing and storage at −20 °C, i.e., the treatment systematically adopted for the latter samples. Nonetheless, the comparative evaluation described in Table 2 appeared to confirm the recognition of the six commercial samples as farmed salmons. This issue was further investigated using Discriminant Analysis.

4. Discriminant Analysis (DA)

DA is a frequently used supervised pattern recognition method and it has been applied to food-related data for a long time (Berrueta et al., 2007). In the present case, Linear Discriminant Analysis (LDA) was exploited to verify the classification of wild-type and farmed salmon samples, with the latter including also the six commercial samples described in the previous section. The elaboration was performed using the *Statistica* v. 7 software, with normalized DART-HRMS intensities referred to the 30 ions previously selected as features for PCA adopted as variables. As additional input features, the *General Discriminant Analysis* option was chosen, with the *all effects DA* selected as classification method. As a result, one discriminant function obtained from LDA (*Root 1*), whose values for each of the samples in the dataset are represented graphically in Fig. 6, led to a clear classification of salmons as wild-type or farmed ones, accounting for 99,38% of variance explained (Wilk's lambda: 0.0127; χ^2: 362.52; df: 30). Interestingly, the 6 commercial salmon samples (represented by grey circles in Fig. 6) were also classified among the latter (prediction ability = 100%). Nonetheless, in accordance with the results reported in Table 2, the corresponding values of the discriminant function were slightly different from those obtained from non-commercial farmed samples (see Fig. 6).

5. Conclusions

The profiling of the 30 major fatty acids in salmon lipid extracts, rapidly provided by DART-HRMS and then integrated by data elaboration based on Principal Component Analysis (PCA), enabled a faster discrimination, compared to previously reported approaches, between Canadian wild-type salmons and farmed salmons from Canada, Chile and Norway. In agreement with the results already reported in the literature, but arising from more complex analytical approaches, saturated FA and polyunsaturated FA with 20 or 22 carbon atoms on their side chain, including the omega-3 species EPA and DHA, were found to be more abundant in wild-type salmons, whereas unsaturated FA like 18:1, 18:2 and 18:3 and several oxidized derivatives of the latter appeared to be more relevant in farmed salmons. A careful evaluation of the normalized spectral abundance of these FA by DART-HRMS was then suggested as an easy and targeted MS-based approach to be used for discriminating the two types of salmons (wild type versus farmed). Finally, Discriminant Analysis (DA) was exploited to validate the approach and six commercial samples, added to the original set of samples, were correctly classified as farmed salmons with a rate of 100%.

Interestingly, in spite of the presence of several potentially

oxidizable polyunsaturated FA in wild-type salmons, no evidence was obtained for the occurrence of the corresponding oxidized derivatives in the lipid extracts of this type of sample. This result corroborates, on a molecular basis, the hypothesis, made in several previous studies, that aquaculture may induce a relevant oxidative stress in fishes. Lastly, the farming-related features described so far were comparable for salmons farmed in very distant countries, thus suggesting that internationally consolidated aquaculture practices can influence the profile of major FA in salmons much more than geographical origin.

Acknowledgement

The present research has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration, under grant agreement No. 613688 2 “Food Integrity”. The equipment used in this work was funded by project BioNet – PTP – “BIODIVERSITA’ PER LA VALORIZZAZIONE E SICUREZZA DELLE PRODUZIONI ALIMENTARI TIPICHE PUGLIESI (codice n. 73, PO Regione PugliaFESR 377 2000-2006)”.

Dr. Rosa Pilolli is kindly acknowledged for her support in the statistical analysis. The authors are thankful to Dr. Emiliano De Dominicis from Mérieux-Nutrisciences for the helpful discussion and for providing the salmon samples analysed in the present project.

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