





## Article

# Aqueous Extracts of Fish Roe as a Source of Several Bioactive Compounds

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**Abstract:** Regular consumption of seafood and, in particular, fish has been associated with important health benefits. A fish product that has been increasingly included in the human nutrition is roe. Despite its nutritional value has been established (fatty acid profile and protein content), the knowledge of the composition of its aqueous extracts is still limited. This work describes the bioactive compounds profile in the roe-derived aqueous extracts of three different marine species (sardine, horse mackerel and sea bass) using a method based on liquid chromatography coupled to high-resolution mass spectrometry with an electrospray ionisation source (LC-ESI/HRMS). The presence of substances with well-known nutritional and functional properties (e.g., antioxidant and anti-inflammatory properties) was demonstrated, namely essential amino acids (e.g., taurine), peptides (e.g., anserine and carnosine), B-group vitamins (e.g., nicotinamide) and gadusol. Therefore, roe-derived aqueous extracts are excellent sources of bioactive compounds and may be used as a font of functional components for several medical and veterinary applications.

**Keywords:** fish roe; liquid chromatography; high-resolution mass spectrometry; electrospray ionization; aqueous extracts; bioactive compounds



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## 1. Introduction

Fish as well as seafood in general is common in the world population diet. Importantly, the regular consumption of fish has been associated with several beneficial effects [1]. Indeed, the association between the intake of fish and positive health benefits, such as prevention of cardiovascular diseases and cancer, decreased incidence of inflammatory diseases and normal brain development and function, is well documented [2]. These benefits have been mainly related to the high content of  $\omega$ 3 polyunsaturated fatty acids (PUFA; mainly eicosapentaenoic acid—EPA, C20:5 $\omega$ 3—and docosahexaenoic acid—DHA, C22:6 $\omega$ 3), proteins, vitamins and minerals as well as the low amount in cholesterol [1].

As fish, its eggs, commonly referred to as roe, are nowadays considered a high-quality food, but they were a waste of the fish industry in the past [3]. The consumption of this fish product is auspiciously increasing worldwide since they are rich in essential nutrients and bioactive molecules, including essential amino acids,  $\omega$ 3 PUFA, water and fat-soluble vitamins and other antioxidants (e.g., glutathione and gadusol) as well as minerals [4–6].

Considering their composition, it is not surprising that important biological functions of fish roe have been scientifically evidenced. Indeed, diverse biological activities have been reported for fish roe, namely anti-cancer [7], anti-inflammatory, immunostimulating [8], antioxidant and antihypertensive activities [9,10]. Additionally, fish roe has been shown to have a valuable role in lipid and glucose metabolism [11], in the prevention of cardiovascular diseases [12] and in the improvement of the visual function and learning ability [13]. Consequently, this fish product may be further explored to discover and/ or develop new nutraceuticals or drugs for biomedical applications.

Bioactive and hydrophilic compounds in fish tissues may comprise, for instance, ascorbic acid, B vitamins, glutathione and gadusol. These compounds regulate several physiological processes, and they are well-known mainly for their antioxidant activity. These important antioxidants protect cellular components against oxidative damage caused by reactive oxygen or nitrogen species that are involved in the development of various life-threatening diseases [14]. However, the knowledge of the composition of the fish roe-derived aqueous extracts is limited and most of the data available for the individual components describes the proximate composition, which includes the protein, lipid, moisture, ash and carbohydrate contents [3,15].

In this study, the fish roe-derived aqueous extracts of three different species, namely sardine (*Sardina pilchardus*), horse mackerel (*Trachurus trachurus*) and sea bass (*Dicentrarchus labrax*) were analysed. These marine species are found in the Northeast Atlantic and Mediterranean Sea and present great economic importance [16]. For instance, in 2016, sardine and horse mackerel were included in the group of the most important species consumed globally. Moreover, sardine was the main species caught in Africa in that year. Sea bass is the third most important farmed species consumed in Europe. All species studied in this work also present an elevated nutritional value, since, for instance, they are rich in  $\omega$ 3 PUFA and protein, and present a low content in cholesterol [17–19].

The profile of polar compounds with biological activity present in the roe-derived aqueous extracts samples of the three selected species was evaluated by liquid chromatography with an orbitrap high-resolution mass spectrometer with an electrospray ionisation (ESI) source (LC-ESI/HRMS). To the best of our knowledge, there are no previous published results on the assessment of the composition of the sardine, horse mackerel or sea bass roe-derived aqueous extracts.

## 2. Materials and Methods

### 2.1. Chemicals

Standards of nicotinamide (vitamin B3), riboflavin (vitamin B2), thiamine hydrochloride (vitamin B1), L-tryptophan, L-histidine, L-phenylalanine, L-isoleucine, taurine and hypoxanthine (Table S1 [20]) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Standards of L-anserine and L-carnosine (Table S1) were acquired from Acros Organics (Geel, Belgium) and abcr GmbH (Karlsruhe, Germany), respectively. Acetonitrile HPLC grade was obtained from Fisher Chemical (Leicestershire, UK). Ammonium acetate and tert-butyl methyl ether were supplied by Sigma-Aldrich (Saint Louis, MO, USA). Methanol was purchased from Fisher Chemical (Loughborough, Leicestershire, UK). Ultrapure water was obtained from a Milli-Q<sup>®</sup> Direct Water Purification System (Millipore, Darmstadt, Germany).

### 2.2. Standard Solutions

Three B-group vitamins (thiamine—B1, riboflavin—B2 and nicotinamide—B3), five amino acids (tryptophan, histidine, phenylalanine, isoleucine and taurine), two peptides (anserine and carnosine) and a purine derivative (hypoxanthine) were the selected target compounds. Standard stock solutions of peptides and amino acids were accurately prepared in a mixture of acetonitrile/ultrapure water (1:1, v/v), except for tryptophan, which was dissolved in methanol, at 1 mg/mL and stored at  $-20$  °C. Nicotinamide, riboflavin and thiamine hydrochloride were precisely prepared in ultrapure water, at 1 mg/mL and

stored at  $-80\text{ }^{\circ}\text{C}$  until further analyses. All working solutions (including mixed standard solutions) were prepared daily by diluting the stock solutions to appropriate concentrations with acetonitrile/ultrapure water (1:1, *v/v*).

### 2.3. Aqueous Extracts of Fish Roe

Fresh roes of sea bass (*Dicentrarchus labrax*), horse mackerel (*Trachurus trachurus*) and sardine (*Sardina pilchardus*), caught at the Portuguese mainland coast, were kindly donated by a local fisherman. Fish roe samples were weighed, freeze-dried (LyoQuest Plus ECO, Telstar) and stored at  $-20\text{ }^{\circ}\text{C}$  until processing.

The extraction of hydrophilic compounds was carried out as depicted elsewhere [5]. In brief, 3 g of a freeze-dried roe sample was homogenized with 120 mL of ice-cold 75% methanol using an Ultra Turrax (T18 Basic, IKA, Darmstadt, Germany), for 2 min. Then, 300 mL of tert-butyl methyl ether was introduced, and the samples were shaken for 1 h. After 1 h of incubation at room temperature (RT), 75 mL of ultrapure water was added and 10 min later, the samples were centrifuged for 15 min at  $12,000\times g$ , at  $4\text{ }^{\circ}\text{C}$ . The methanol/ultrapure water-rich phase was collected, and the organic solvent was evaporated under vacuum using a rotary evaporator (RE300, Stuart, Staffordshire, United Kingdom), at  $25\text{ }^{\circ}\text{C}$ , and the ultrapure water was removed through freeze-drying (LyoQuest Plus ECO, Telstar, Barcelona, Spain). The freeze-dried extracts were stored protected from light, at  $-20\text{ }^{\circ}\text{C}$ . Before LC-ESI/HRMS analyses, 100 mg of freeze-dried extracts were dissolved in 1 mL acetonitrile/water (1:1, *v/v*) and filtered through a  $0.2\text{ }\mu\text{m}$  PTFE membrane.

### 2.4. Chromatographic Analysis

#### 2.4.1. Equipment

The optimization of chromatographic conditions was first performed in a Shimadzu UFLC Prominence System (Shimadzu Corporation, Tokyo, Japan), consisting of a system controller (CBM-20A), three pumps (LC-20AD), an autosampler (SIL-20AC), a column oven (CTO-20AC) and a UV/VIS detector (SPD-20A). A HPLC Accela system (Thermo Fischer Scientific) with an Accela PDA detector, Accela Autosampler and Accela 600 Pump, coupled to a LTQ Orbitrap<sup>TM</sup> XL hybrid mass spectrometer (Thermo Fischer Scientific), and equipped with an electrospray ionisation (ESI) source was employed for method validation and sample analysis.

#### 2.4.2. Chromatographic Conditions and Analyses

Different chromatographic columns with several chromatographic conditions were tested. The selected chromatographic columns were LiChroCART<sup>®</sup> LiChrospher<sup>®</sup> 100 RP-18 ( $250\times 4.6\text{ mm}$ ,  $5\text{ }\mu\text{m}$ , Merck, Darmstadt, Germany), Luna<sup>®</sup> PFP (2)  $100\text{ \AA}$  ( $150\times 4.6\text{ mm}$ ,  $3\text{ }\mu\text{m}$ , Phenomenex) and Luna HILIC ( $150\times 4.6\text{ mm}$ ,  $3\text{ }\mu\text{m}$ , Phenomenex, Alcobendas, Spain). Several mobile phase compositions were investigated, in reversed phase and in hydrophilic interaction liquid chromatography (HILIC), in isocratic and gradient elution modes. Mobile phases composed of methanol, acetonitrile, water, different buffers at different concentrations and pH values were evaluated. Different injection volumes (between  $10\text{ }\mu\text{L}$  and  $40\text{ }\mu\text{L}$ ) and flow rates (between  $0.5\text{ mL/min}$  and  $1.2\text{ mL/min}$ ) were also tested as well as the column oven temperature (between  $23\text{ }^{\circ}\text{C}$  and  $40\text{ }^{\circ}\text{C}$ ) and the autosampler temperature ( $4$ ,  $10$  and  $23\text{ }^{\circ}\text{C}$ ).

The optimized conditions were achieved with a Luna HILIC ( $150\times 4.6\text{ mm}$ ,  $3\text{ }\mu\text{m}$ , Phenomenex) column in gradient mode. Acetonitrile (solvent A), ultrapure water (solvent B) and  $100\text{ mM}$  ammonium acetate (solvent C) were used as the mobile phase, in a gradient mode as follows:  $0\text{--}4\text{ min}$ ,  $90\%$  A,  $5\%$  B and  $5\%$  C;  $4\text{--}20\text{ min}$ ,  $90\text{--}50\%$  A,  $5\text{--}45\%$  B,  $5\%$  C; at  $20.50\text{ min}$  the system returns to the initial conditions that were held for  $9.5\text{ min}$  (until  $30\text{ min}$ ) as a re-equilibration step. The flow rate was set at  $0.5\text{ mL/min}$  and the injection volume was  $10\text{ }\mu\text{L}$ . The autosampler tray and column oven temperatures were maintained at  $4\text{ }^{\circ}\text{C}$  and  $23\text{ }^{\circ}\text{C}$ , respectively. Elution was monitored at  $268\text{ nm}$ .

To identify and quantify the different compounds, the positive and negative-ion modes were used, with MS and MS<sup>2</sup> spectra acquired over a mass range from 120 to 2000 *m/z*. The system was controlled by LTQ Tune Plus 2.5.5. and Xcalibur 2.1.0 software (Thermo Fisher Scientific, Bremen, Germany). The remaining mass spectrometric parameters were the following: capillary voltage of the ESI, 3.1 kV; capillary voltage, 15 V; tube lens voltage, 65 V; capillary temperature, 275 °C; sheath gas and auxiliary gas flow rate, 40 and 10 (arbitrary unit as provided by the software settings), respectively.

The MS data handling software (Xcalibur QualBrowser software version 2.2, Thermo Fisher Scientific, Bremen, Germany) was employed to search other target compounds in the fish roe samples, which were not previously included in the standards list, using the *m/z* of the precursor ions and the respective MS<sup>2</sup> spectra. These parameters were further compared with public databases, namely the European MassBank, ReSpec and MassBank of North America, and data present in the literature.

Aqueous extracts of roe from three different species of fish were obtained as described in the Section 2.3 and analysed (each one in triplicate, *n* = 3). The identification of the target compounds was performed by comparison of the retention times, the *m/z* of the precursor ions and respective MS<sup>2</sup> of the standard spectra.

#### 2.4.3. Method Validation

The developed method was validated following the International Conference on Harmonization (ICH) guidelines [20] in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision.

Linearity was determined by measuring the peak area of the standard solutions in a range of known concentrations. Peak areas were plotted against the concentrations of each standard for the construction of calibration curves, and the correlation coefficient (*r*<sup>2</sup>) and slope of the regression line were determined.

Equations (1) and (2), respectively, were used to calculate LOD and LOQ:

$$\text{LOD} = 3.3 \times \frac{s}{S} \quad (1)$$

$$\text{LOQ} = 10 \times \frac{s}{S} \quad (2)$$

where *s* is the standard deviation of the *y*-intercept of the regression line and *S* is the slope of the calibration curve.

Accuracy and precision were determined using quality controls (QCs) standard solutions. These QC standard solutions were prepared in triplicate by dilution of the mixed standard stock solution to selected concentrations with acetonitrile/ultrapure water (1:1, *v/v*). Accuracy was expressed as the percentage of agreement between measured and real concentration of the standard. Precision was determined as the degree of the scatter of a series of measurements and expressed as relative standard deviation (% RSD). At least three samples of each species were analysed in triplicate. Three mixed standard solutions, with the respective concentration, were prepared and analysed for the method validation. Data are expressed as the arithmetic mean ± standard deviation of at least three independent measurements.

### 3. Results and Discussion

#### 3.1. Analytical Method for Screening and Quantification

A LC-ESI/HRMS method was developed for the screening and quantification of eleven target compounds in the aqueous extracts of roe samples of three different fish species, namely sardine, horse mackerel and sea bass. Due to the complexity of the sample matrices and the different physicochemical properties of the target compounds, the optimization of the chromatographic conditions was a challenge. Consequently, three different chromatographic columns in many different conditions were tested (data not shown). The LiChroCART<sup>®</sup> LiChrospher<sup>®</sup> 100 RP-18 (250 × 4.6 mm, 5 μm, Merck) and

Luna<sup>®</sup> PFP (2) 100 Å (150 × 4.6 mm, 3 µm, Phenomenex) columns were initially tested, but with poor retention, selectivity and sensitivity. The Luna HILIC column demonstrated to be the best option for the analyses of the target polar compounds. HILIC elution mode has been widely employed as an alternative approach to efficiently separate small polar compounds in complex matrices [21]. The polarity of the stationary phase allows the use of a relatively hydrophobic mobile phase, offering enhanced retention to the highly polar target compounds. Moreover, as mobile phases present high volatility, they are suitable for MS with electrospray ionisation. HILIC has also the advantage to improve method sensitivity in MS analysers.

The first approach to analyse the roe-derived aqueous extracts composition was to separate and identify some water-soluble vitamins and other antioxidants (namely glutathione, carnosine, anserine, putrescine, spermidine and spermine), potentially present in fish roe, as previously reported [22–24]. The best chromatographic conditions were first explored in the LC-UV/Vis. However, as samples present high complexity, the conditions were adapted to LC-ESI/HRMS for compounds screening and search. As expected, the full scan LC-ESI/HRMS analyses of the aqueous extracts revealed the presence of other compounds, which were identified by the *m/z* of their precursor ions and their respective MS and MS<sup>2</sup> spectra by association with public databases as well as data for the literature [25–27]. Thus, the standards were acquired (taurine, L-phenylalanine, L-tryptophan, L-iso-leucine, L-histidine and hypoxanthine) and included in the analytical method for further analyses. Table 1 summarizes the chromatographic and MS parameters used for the identification and further quantification of the target compounds. The MS and MS<sup>2</sup> spectra of all target compounds are presented in Figure 1.

**Table 1.** Chromatographic and MS parameters for the mixed standard solution. Retention time and *m/z* values are shown as arithmetic means ± SD (n = 3).

Compound	Retention Time (min)	Selected Ion Monitoring (SIM)	[M-H] <sup>+</sup> ( <i>m/z</i> )	Error (ppm)	MS <sup>2</sup> Fragment Ions
Riboflavin	7.81 ± 0.09	367–387	377.1451 ± 0.0003	−1.19	243.1 (100), 359.2 (14)
Tryptophan	13.20 ± 0.08	195–215	205.0965 ± 0.0002	−1.12	188.0 (100)
Thiamine	13.63 ± 0.30	256–276	265.1114 ± 0.0004 *	−0.59	121.9 (100), 144.0 (51), 155.9 (5)
Histidine	17.65 ± 0.38	146–166	156.0762 ± 0.0001	−3.33	109.9 (100), 94.9 (3)
Anserine	17.92 ± 0.06	231–251	241.1290 ± 0.0003	−2.28	170.0 (100), 224.1 (76), 197.1 (36), 108.9 (27)
Nicotinamide	5.05 ± 0.06	113–133	123.0549 ± 0.0001	−2.84	122.9 (100), 79.8 (48), 106.0 (22), 95.8 (17), 95.2 (3)
Hypoxanthine	8.06 ± 0.04	127–147	137.0455 ± 0.0001	−1.97	137.0 (100), 109.9 (58), 93.8 (37), 119.0 (36)
Phenylalanine	12.93 ± 0.04	156–176	166.0859 ± 0.0002	−2.19	119.9 (100), 148.9 (4)
Isoleucine	13.53 ± 0.03	122–142	132.1016 ± 0.0002	−2.02	85.9 (100)
Taurine	13.96 ± 0.04	116–136	126.0215 ± 0.0001	−3.13	107.8 (100)
Carnosine	18.12 ± 0.02	217–237	227.1134 ± 0.0002	−2.04	210.1 (100), 209.3 (55), 109.9 (14), 179.9 (11), 156.0 (10)

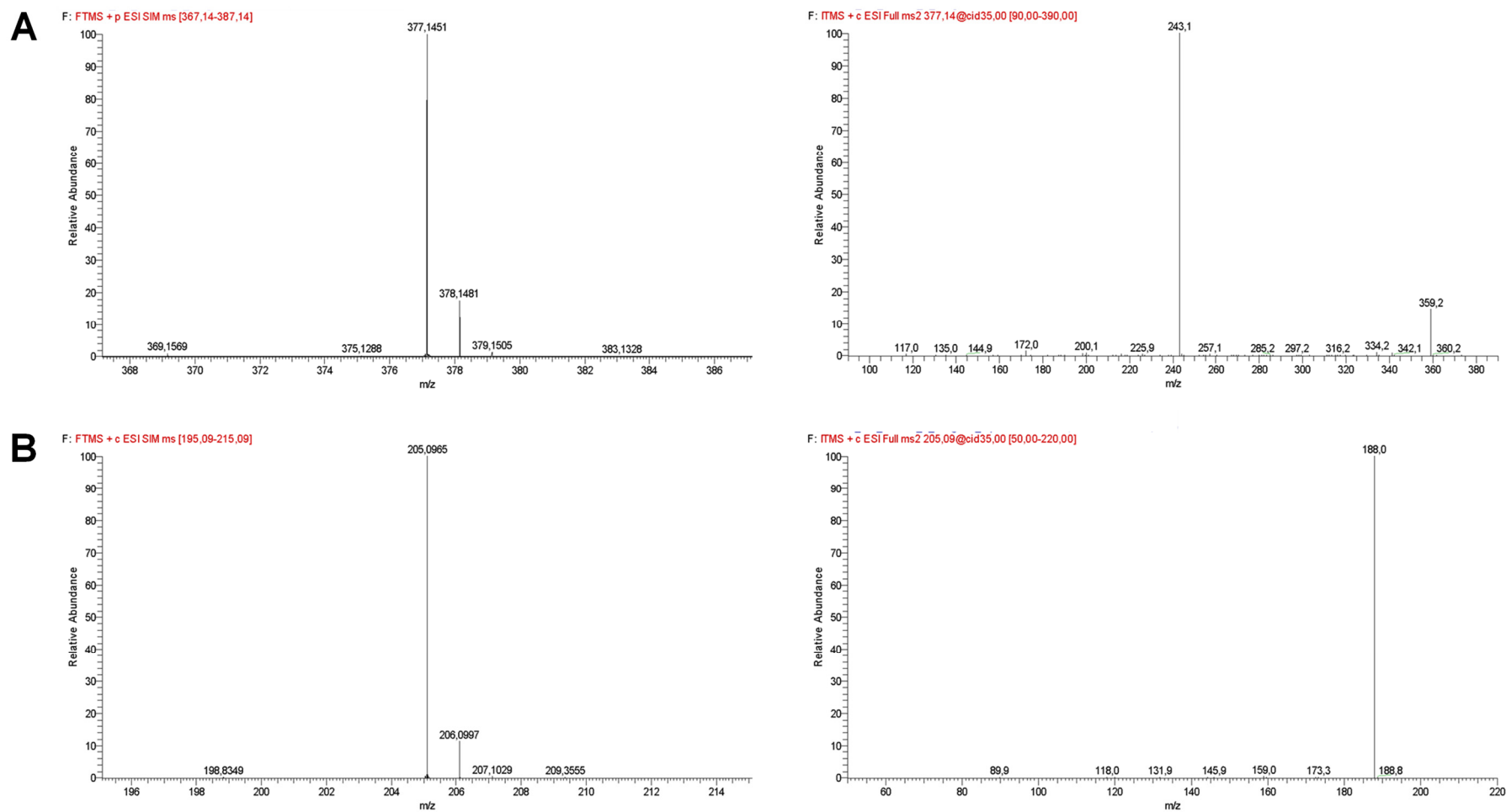


Figure 1. Cont.

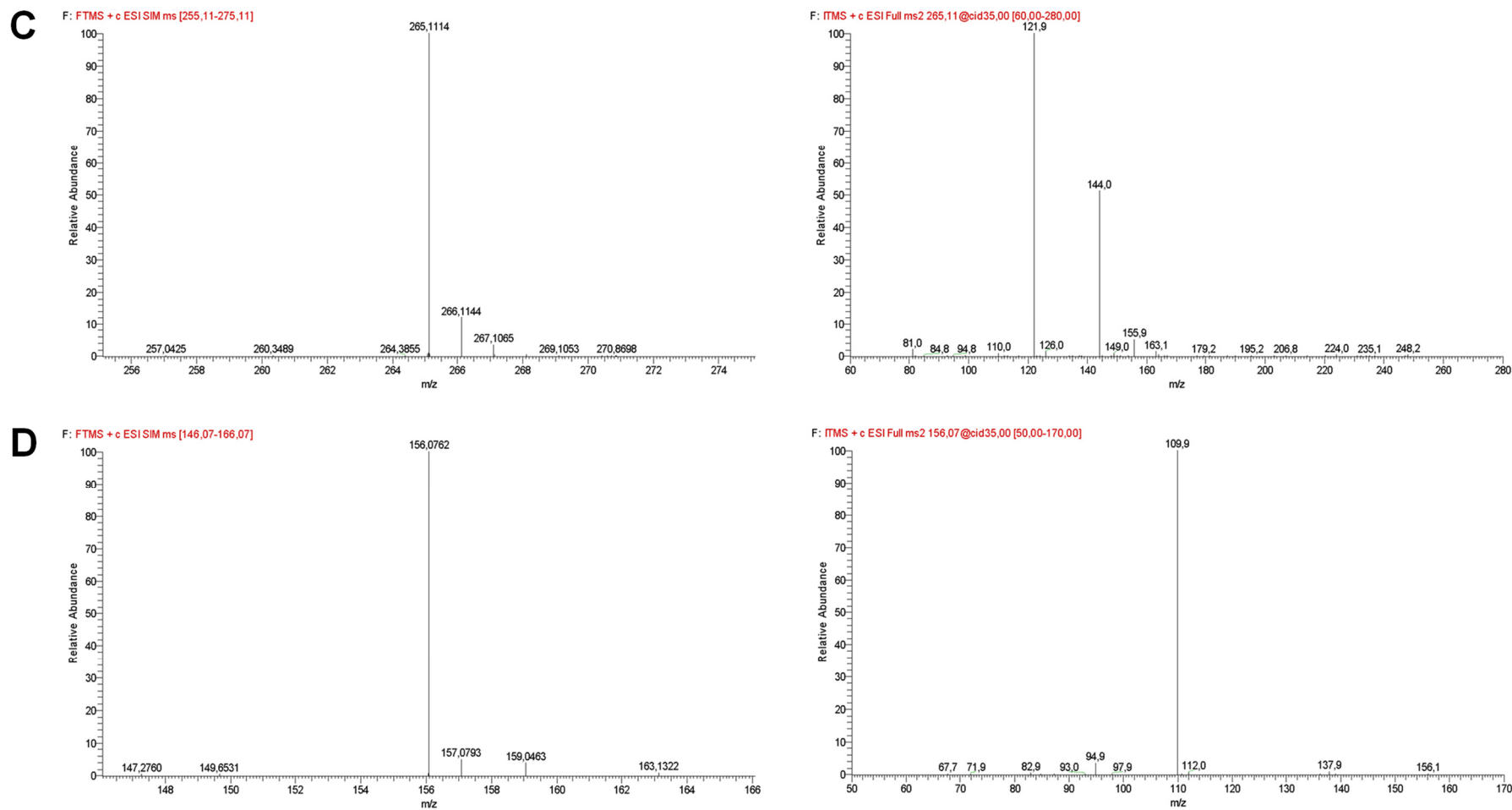


Figure 1. Cont.

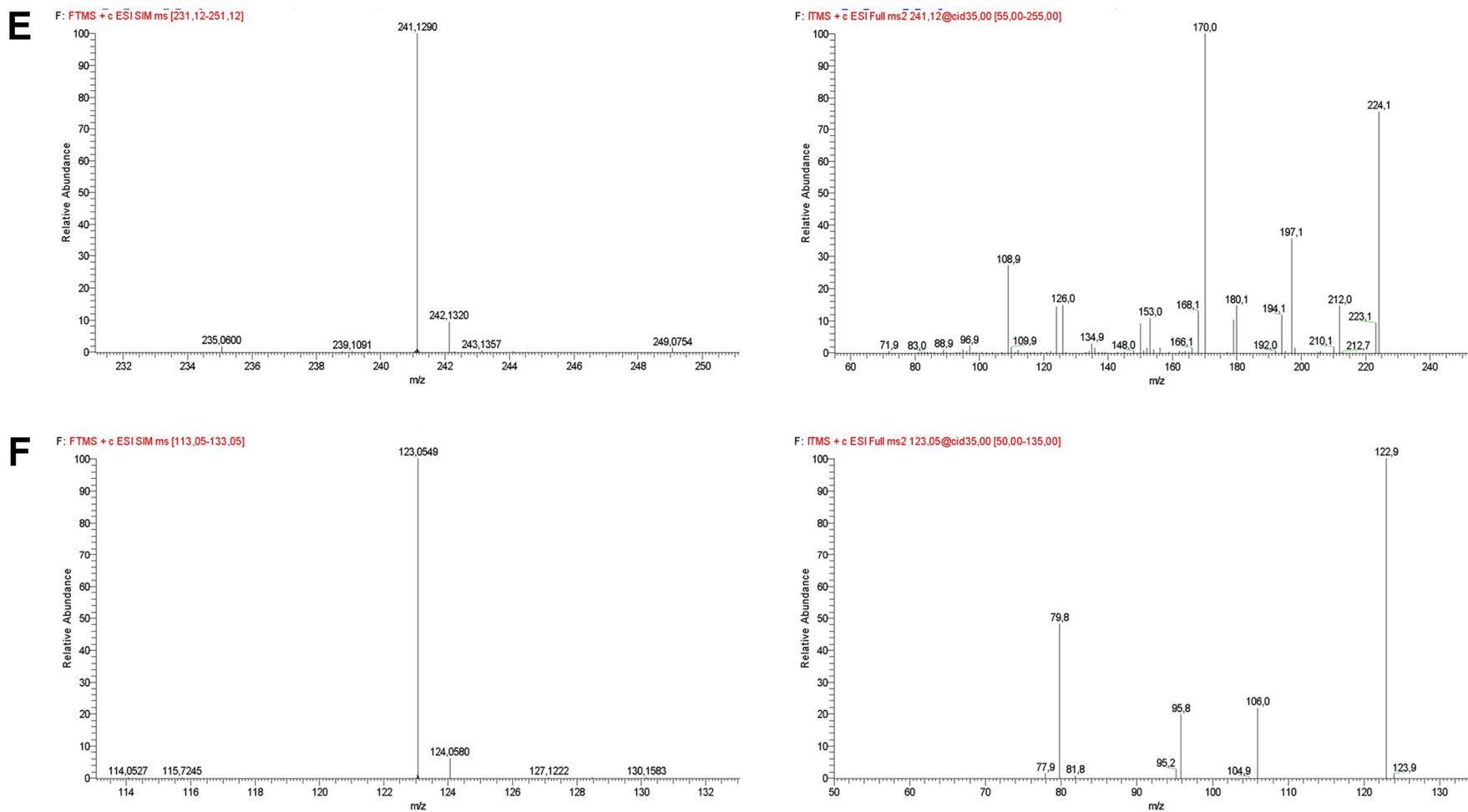


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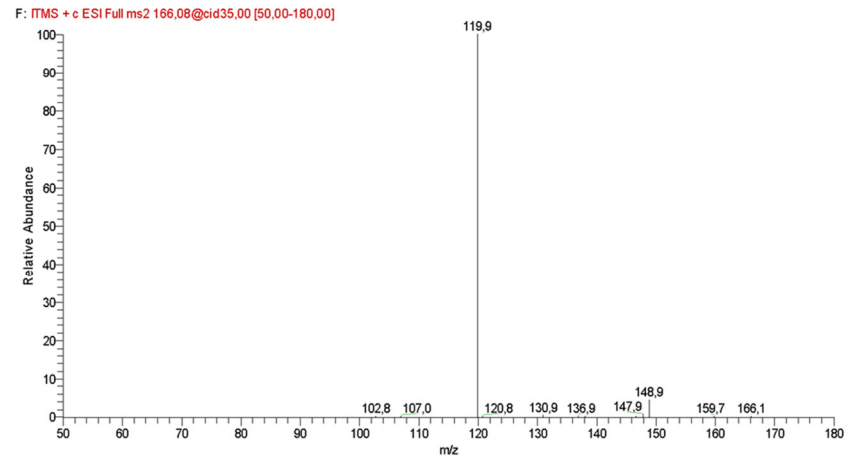
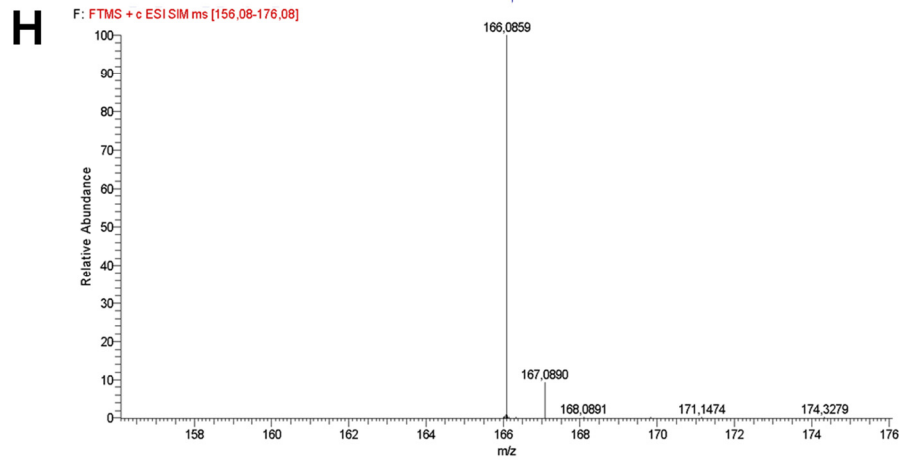
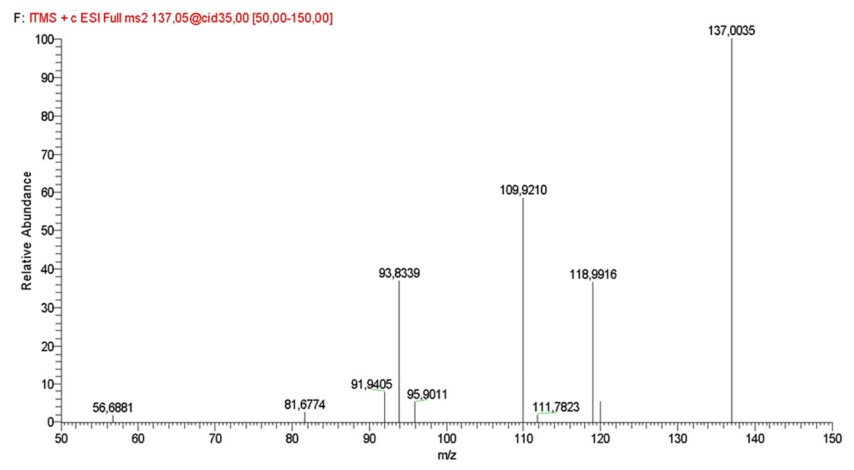
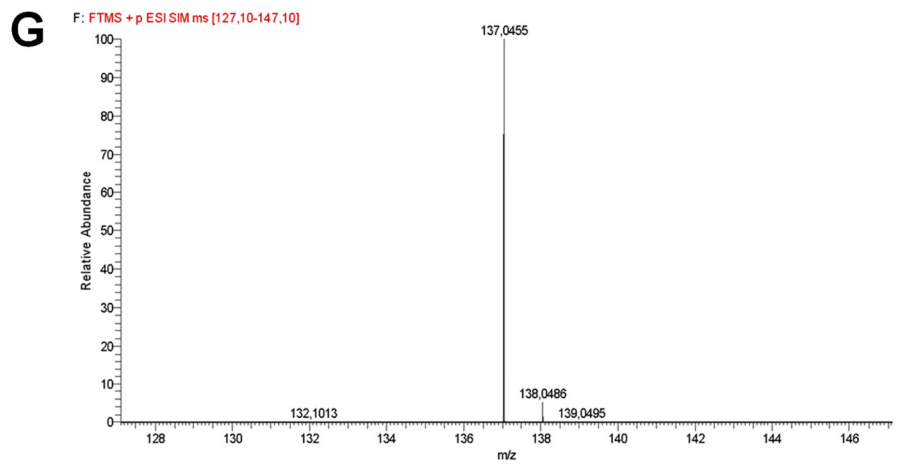


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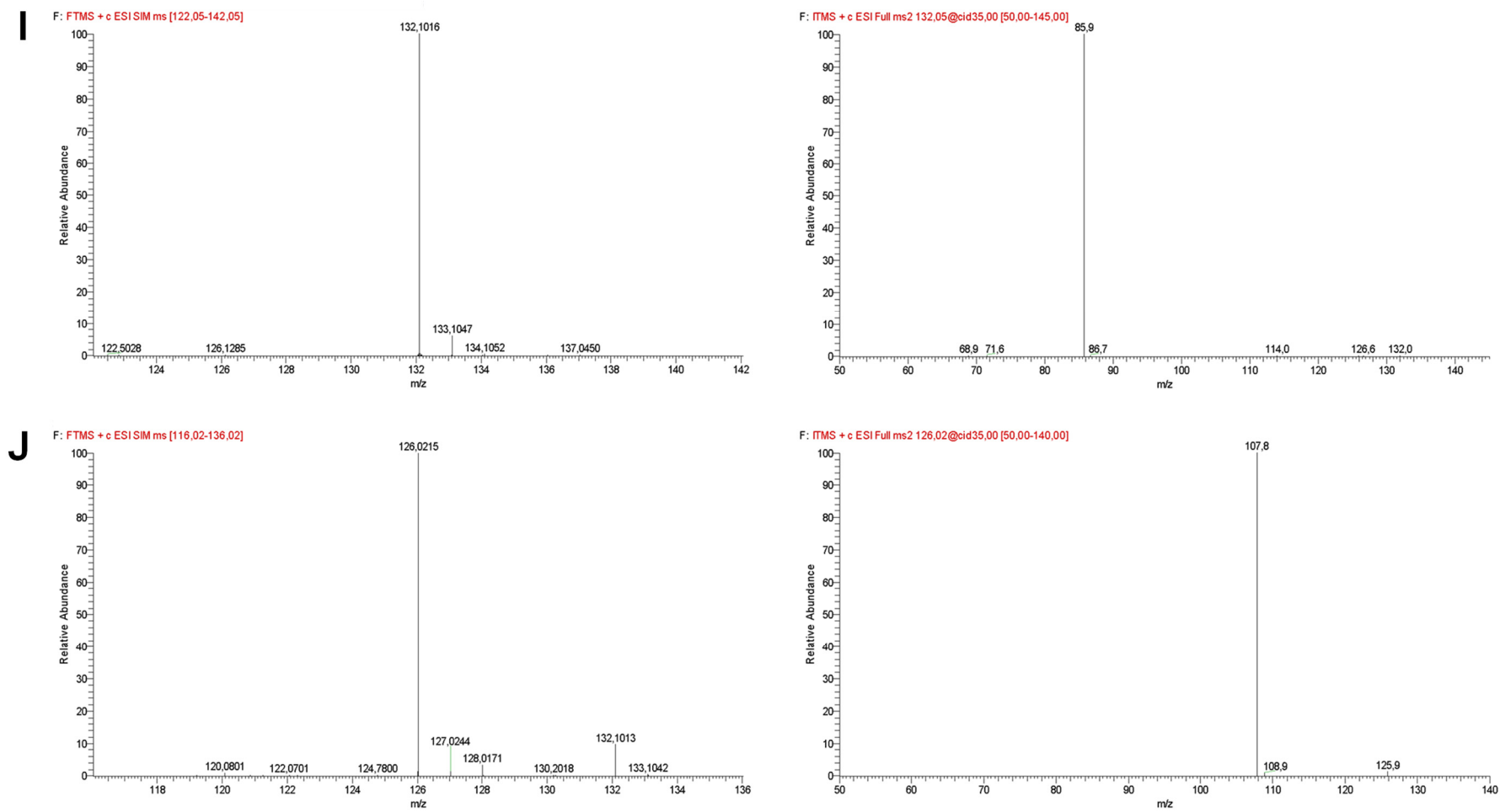
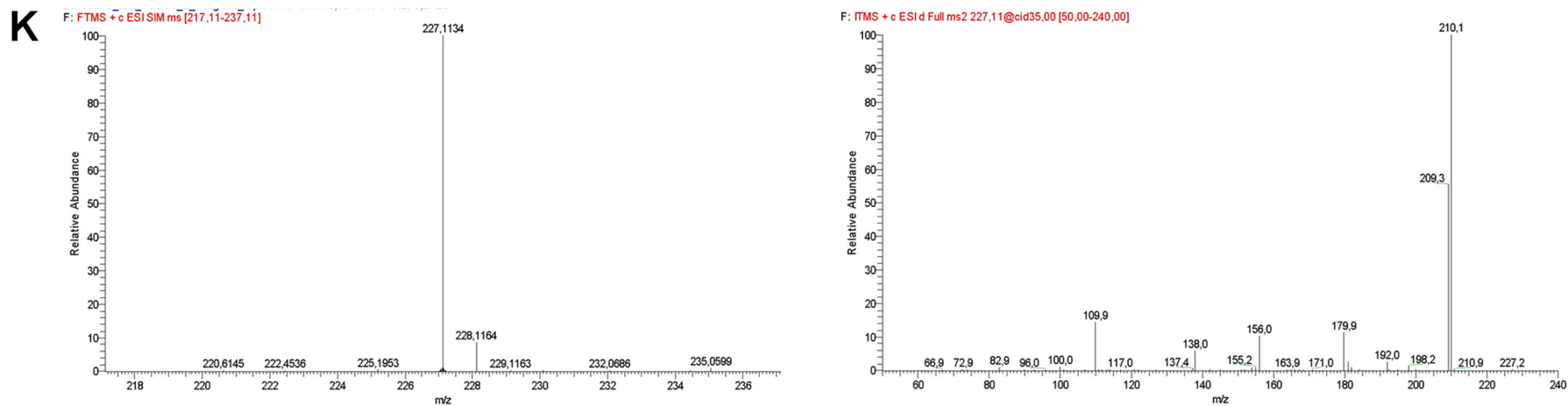


Figure 1. Cont.



**Figure 1.** MS and MS<sup>2</sup> spectra of riboflavin (A), tryptophan (B), thiamine (C), histidine (D), anserine (E), nicotinamide (F), hypoxanthine (G), phenylalanine (H), isoleucine (I), taurine (J) and carnosine (K) in the mixed standard solution.

For method validation, its linearity, LOD LOQ, accuracy and precision were considered. Regarding linearity, calibration curves of all target compounds were built, and their respective linear regression presented correlation coefficients higher than 0.99, within the studied range (Table 2). As can be observed in Table 2, LOD ranged from 0.33 to 4.39 µg/mL, whereas LOQ ranged from 1.0 to 13.3 µg/mL. The accuracy ranged between 90.58 and 118.52%, values that are within the range for quantitative determinations in complex matrices. Precision presented RSD% values lower than 20%, except for riboflavin and tryptophan.

Table 2. Method validation parameters.

Compound	Range (µg/mL)	Calibration Curve Equation	r <sup>2</sup>	LOD (µg/mL)	LOQ (µg/mL)
Riboflavin	5–50	$y = 2,597,970.9083x + 22,883,398.7348$	0.9987	1.65	5.00
Tryptophan	6.7–100	$y = 1,419,395.4086x + 21,252,564.7864$	0.9934	2.21	6.70
Thiamine	1.3–33.3	$y = 4,397,787.5153x - 270,937.1986$	0.9941	0.43	1.30
Histidine	20–150	$y = 1,975,845.0061x + 2,836,204.2581$	0.9998	3.14	9.50
Anserine	5–75	$y = 955,314.8167x + 9,065,014.7500$	0.9987	1.65	5.00
Nicotinamide	10–75	$y = 2,817,664.2995x + 7,778,762.8222$	0.9995	2.19	6.63
Hypoxanthine	6.7–46.7	$y = 3,640,970.0000x + 46,010,702.0000$	0.9920	2.21	6.70
Phenylalanine	6.7–46.7	$y = 2,861,270.2246x + 16,386,226.9065$	0.9970	2.21	6.70
Isoleucine	13.3–100	$y = 1,525,464.4712x + 40,827,232.0979$	0.9955	4.39	13.30
Taurine	13.3–80	$y = 629,499.1431x + 9,232,958.8045$	0.9941	4.39	13.30
Carnosine	1–25	$y = 1,594,248.8705x + 1,402,639.7461$	0.9990	0.33	1.00

### 3.2. Analysis of the Roe-Derived Aqueous Extracts Samples from the Three Fish Species

The antioxidant and anti-inflammatory activity of the roe-derived aqueous extracts obtained from different fish roe samples were already investigated by our team and they revealed to be an auspicious font of antioxidant and anti-inflammatory compounds (manuscript under review in the Innovative Food Science and Emerging Technologies). Indeed, the aqueous extracts of these roes presented antioxidant and anti-inflammatory activities, at non-cytotoxic levels, with a similar efficacy to the positive controls tested. Moreover, these properties were potentiated after their combination with liposomes obtained from sardine roe-derived lipidic extracts [28]. Therefore, fish roe-derived aqueous extracts may potentiate the intrinsic bioactivity of these or other drug delivery devices.

The MS spectra of all compounds detected in the aqueous extracts of roe samples of sardine, horse mackerel and sea bass are presented in Figure S1.

Table 3 summarizes the content of the target compounds, expressed as µg/100 mg of freeze-dried aqueous extract of fish roe. Most of the analysed B-group vitamins, amino acids, peptides and purine derivative were identified in the studied samples. Different contents of the compounds were found among the three species. Sardine and horse mackerel roes were the richest samples in these compounds, being the amino acids levels much higher than those of the B vitamins and peptides. Amino acid taurine was detected in higher quantity in all the studied samples, followed by isoleucine, histidine, phenylalanine and tryptophan. Histidine was at levels lower than LOQ in sea bass roe-derived aqueous extracts. Tryptophan, histidine and phenylamine were observed in sardine roe at higher concentrations when compared with those of other species. However, isoleucine and taurine levels were higher in horse mackerel samples. Amino acids combine to form

proteins and are key precursors of many types of biologically crucial biomolecules. They can regulate key metabolic pathways and are also important sources of energy [29]. Consequently, these molecules have important roles in both health and nutrition. The selected amino acids detected in the present work are essential, playing specific physiological roles, such as antioxidant and anti-inflammatory activities. Tryptophan has immune functions and is the precursor of serotonin (neurotransmitter), *N*-acetylserotonin, melatonin, anthranilic acid and niacin. These products have also antioxidant and anti-inflammatory properties. Histidine participates in protein and DNA methylation, haemoglobin structure and function, and it is the precursor of antioxidative dipeptides (e.g., anserine and carnosine), histamine and urocanic acid. Phenylalanine is involved in the activation of tetrahydrobiopterin (a cofactor for nitric oxide synthase) synthesis, in the synthesis of tyrosine, catecholamines (e.g., epinephrine and norepinephrine), dopamine and thyroid hormones, and in the neurological development and function. Isoleucine is a substrate for glutamine and alanine synthesis in animal tissues, and it contributes to the balance among branched-chain amino acids. Other studies using roe from different marine species also reported the presence of the amino acids detected in the present work [3,15]. Considering the species used in this work, Bandarrra and co-authors reported that tryptophan was detected in sardine (3.5 mg/100 g wet weight), horse mackerel (3.7 mg/100 g wet weight) and sea bass (3.5 mg/100 g wet weight) although in the raw edible parts [30]. In raw meat of wild and cultured sea bass, Baki et al. [31] reported high concentrations for isoleucine (590–685 mg/100 g wet weight), phenylalanine (738–752 mg/100 g wet weight) and histidine (622–563 mg/100 g wet weight). Taurine was not evaluated in these studies. Taurine is considered a conditionally essential nutrient since humans present a limited capability for its biosynthesis. It may have beneficial effects on neuronal development, and inflammatory and cardiovascular diseases. All fish roe derived-aqueous extracts were rich in taurine. Variable levels of this amino acid are often present in marine organisms [32]. For instance, the taurine concentration reported for *Trachurus japonicus* muscle, a species of the same genus as the horse mackerel species used in the present work, was 75 mg/ 100 g wet weight [32].

**Table 3.** Concentrations of the water-soluble compounds ( $\mu\text{g}/100\text{ mg}$  of freeze-dried extracts) identified in sardine, horse mackerel and sea bass roe-derived aqueous extracts. Data are shown as arithmetic means  $\pm$  SD ( $n = 3$ ).

	Compound	Concentration ( $\mu\text{g}/100\text{ mg}$ Dry Extracts)		
		Sardine	Horse Mackerel	Sea Bass
Vitamins	Nicotinamide	18.32 $\pm$ 0.61	16.66 $\pm$ 2.69	<LOQ
	Riboflavin	<LOQ	<LOQ	<LOQ
	Thiamine	<LOQ	1.72 $\pm$ 0.32	<LOQ
Amino acids	Tryptophan	100.96 $\pm$ 7.83	49.72 $\pm$ 8.63	27.41 $\pm$ 5.69
	Histidine	130.77 $\pm$ 6.72	118.92 $\pm$ 5.00	<LOQ
	Phenylalanine	275.49 $\pm$ 12.28	70.22 $\pm$ 6.59	47.89 $\pm$ 7.75
	Isoleucine	427.01 $\pm$ 19.92	643.48 $\pm$ 97.29	380.11 $\pm$ 2.64
	Taurine	1131.08 $\pm$ 150.16	1306.43 $\pm$ 124.52	596.54 $\pm$ 54.43
Peptides	Anserine	not detected	<LOQ	<LOQ
	Carnosine	not detected	10.86 $\pm$ 1.91	<LOQ
Purine derivative	Hypoxanthine	200.97 $\pm$ 9.47	158.28 $\pm$ 25.55	54.48 $\pm$ 5.94

Three vitamins of B-group were also detected. Among them, nicotinamide was the most abundant, but this vitamin was only quantified in sardine and horse mackerel roe samples. Conversely, thiamine was only quantified in horse mackerel roe samples. Finally, riboflavin was detected at a concentration lower than LOQ in all the evaluated samples. High concentration of nicotinamide was also reported by Bandarrra and co-authors, however in edible parts [30]. Chatterjee et al. also observed similar results in samples of sardine and

mackerel flesh [33]. Vitamins are a group of essential dietary components necessary to retain normal cellular, metabolic and physiologic functions [34]. Their absence or excess can cause specific diseases. Despite these biomolecules belong to the same group, they widely vary in their chemical and physiological roles and nutritional significance [35]. The B vitamins operate mainly as coenzymes in the catabolism of food to generate energy [34]. Thiamine, as thiamine diphosphate, serves as a coenzyme for enzymes which are engaged in the metabolism of carbohydrates and amino acids. Riboflavin plays its biological roles through two flavin coenzymes, termed flavin adenine dinucleotide and flavin mononucleotide. These coenzymes are required in oxidation-reduction reactions in several metabolic routes as well as other specific reactions (e.g., in the interconversion of vitamin B6 vitamers and B9 vitamers, and in the transformation of kynurenine into 3-hydroxykynurenine in the production of nicotinamide adenine dinucleotide from tryptophan). Nicotinamide is the reactive moiety of the coenzymes nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. They function as proton and electron donors in a wide variety of oxidation-reduction reactions (e.g., the energy released from carbohydrates, fatty acids, and amino acids degradation, and the synthesis of amino acids, fatty acids and pentose sugars).

Anserine was detected at lower levels than LOQ in horse mackerel and sea bass roes, whereas carnosine was only quantified in horse mackerel roe samples. These peptides were not detected in sardine roe-derived aqueous extracts. Carnosine and anserine are histidine containing dipeptides of similar structure. They possess several beneficial characteristics, including the maintenance of the pH balance as well as antioxidant, neuroprotective and chelating activities. Mori et al., for instance, determined carnosine amount in bonito (0.17 mg/g wet tissue) and eel (4.20 mg/g wet tissue) muscle, but not anserine [24].

Hypoxanthine was present in all the samples examined. High amounts of hypoxanthine were reported by Li and co-authors in edible parts and internal organs of the marine fishes studied by them (ranged from 51 to 1016 mg/kg) [36]. This compound is the principal purine nucleobase which participates in the salvage synthesis of purines and subsequent synthesis of nucleic acids [37]. In the brain, the basal ganglia are mainly dependent on the salvage pathway to retain the normal levels of purine. Moreover, hypoxanthine is an endogenous inhibitor of the nuclear enzyme poly (ADP-ribose) polymerase, protecting against oxidant-induced cell injury [38].

In addition to the compounds analysed and described in Table 3, it was also possible to identify the presence of gadusol in two of the studied samples (sardine and horse mackerel). Due to the lack of an appropriate standard, it was not possible to validate the method for this compound. The identification of gadusol was made by comparison of the MS and MS<sup>2</sup> spectra (obtained in a negative ESI-MS analysis) with public databases and literature. The precursor ions  $[M-H]^- = 203.0555$  (sardine roe) and 203.0558 (horse mackerel roe) as well as the fragment ions at  $m/z$  185.0, 116.0 and 143.9 were observed, indicating the presence of gadusol in both samples (Figure S2). This compound has been detected in roe of some marine teleost fishes, including the species of horse mackerel [39], and other marine organisms [40]. In the work performed by Plack and collaborators, gadusol was estimated at a concentration several times greater than that of ascorbic acid (1.21–4.34 mg/g dry weight) [41]. In roe of three species from Argentine Sea, Arbeloa et al. reported gadusol concentrations ranging from 0 to 245 mg/kg of wet tissue [4]. A functional role of gadusol in embryonic development has been suggested and this feature is associated with its antioxidant properties.

Many studies reported the quantification of the target compounds in fish, but to the best of our knowledge, the composition of the roe-derived aqueous extracts of sardine, horse mackerel and sea bass was never reported before.

#### 4. Conclusions

Despite the significance of fish roe products, the data available in the literature about their chemical and nutritional composition is limited. Therefore, this work presents a

method based on LC-ESI/HRMS with HILIC elution mode to quantify target compounds in the aqueous extracts of fish roe samples of three different species, namely sardine, horse mackerel and sea bass. The B vitamins (nicotinamide, riboflavin and thiamine), essential amino acids (tryptophan, histidine, phenylalanine, isoleucine and taurine), peptides (anserine and carnosine), a purine derivative (hypoxanthine) and gadusol were identified in the aqueous extracts, in different amounts. Amino acids presented the highest concentrations for all the studied samples, and taurine was the most abundant amino acid. Other antioxidants were also detected, including carnosine and anserine in horse mackerel and sea bass roe-derived aqueous extracts, and gadusol in sardine and horse mackerel roe samples. Thus, roe-derived aqueous extracts from the three fish species studied may be used as a font of natural bioactive compounds for nutraceutical, veterinary, pharmaceutical and medical applications.

To the best of our knowledge, this work is the first study that analyses the composition of the roe-derived aqueous extracts of sardine, horse mackerel and sea bass.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/separations9080210/s1>. Table S1: IUPAC name, chemical structure, CAS number, molecular formula, mass exact and pKa values of the water-soluble compounds identified in sardine, horse mackerel and sea bass roe-derived aqueous extracts. Figure S1: MS spectra of nicotinamide (A), riboflavin (B), thiamine (C), tryptophan (D), histidine (E), phenylalanine (F), isoleucine (G), taurine (H), anserine (I), carnosine (J), hypoxanthine (K) and gadusol (L) in the sardine (1), horse mackerel (2) and sea bass (3) roe-derived aqueous extracts. Figure S2: MS2 spectra of gadusol in the sardine (A) and horse mackerel (B) roe-derived aqueous extracts.

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