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Detection of nitrate and nitrite in different seafood

Running title: Detection of nitrate and nitrite in different seafood

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Abstract

High-performance ion-exchange chromatography with suppressed conductivity (HPIEC-SCD) was validated for the determination of nitrite (NO_2^-) and nitrate (NO_3^-) in the edible part of diverse seafood species. Samples analyzed by HPIEC-SCD that were devoid of nitrite and nitrate were subjected to HRMS using a Q-exactive Orbitrap platform. As NO_2^- is not detectable in Q-Exactive Orbitrap, it was necessary to perform the oxidation of NO_2^- to NO_3^-. Accordingly, suitability of NO_2^- oxidation by H_2O_2 as a part of sample preparation for HPIEC-HRMS was elaborated. The difference in the concentration of NO_3^- before and after H_2O_2 treatment was used for the evaluation of eventual NO_2^- presence. The edible part of 53 fish, shrimp and bivalve species presented significant differences in NO_3^- levels especially between farmed (median=44µg/g) and wild species (median=16µg/g). The highest concentration of NO_3^- was found in smoked salmon samples (median=60µg/g) while NO_2^- was not revealed in any of the samples studied.

1. Introduction

The nitrogen cycle, involving atmosphere, terrestrial and marine ecosystems with appurtenant flora and fauna, exhibits a strong influence on the food chain and consequently on the properties of deriving food products. Nitrogen is incorporated into nitrogenous compounds through several pathways including microorganisms, plants and industrial agro/aquacultural resources (Rose et al., 2014). Nitrite (NO_2^-) and nitrate (NO_3^-), that are involved in these pathways, are natural constituents of plants and vegetables, which represent the most important source of these two anions to which humans are exposed by nutrition (Mensinga, Speijers & Meulenbelt, 2003; Iammarino, Di Taranto, Cristino, 2013). Several other foodstuffs, such as cereals and dairy and meat products may contain levels of these two anions which are not negligible (Bahadoran, Mirmiran, Jeddi, Azizic, Ghasemi, Hadaegh, 2016).

NO_2^- and NO_3^- are also well-known food additives recorded as official preservatives (European Commission, 2011) and are exploited as important antimicrobial agents in meat. Indeed, NO_2^- prevents the development of extremely dangerous bacteria, such as *clostridium botulinum,* which generates the botulin toxin, responsible for muscular paralysis and neuronal complications (Cammack, Joannou, Cui, Martinez, Maraj, Hughes,1999). Furthermore, NO_2^- is extensively used in food production because of its ability to react with meat myoglobin attributing a red colour to the treated meat (Wang, Yan, Su, Zhao, Xia, Chen, 2018). Despite several applications in the meat industry, NO_2^- is reported to be one of the contaminants which can react with biogenic amines giving carcinogenic N-nitroso compounds (EFSA 2017a). Moreover, the level of NO_3^- also has to be controlled, especially in meat products, mainly because of the fact that in the favourable milieu within the gastrointestinal tract, NO_3^- might be converted into NO_2^- (EFSA 2017b). Nevertheless, there is no specific limitation for seafood, apart from that for marinated herrings and sprats: the

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maximum allowed level for NO_3^- is fixed at 500 μg/g (European Commission, 2011). Consequently, the application of NO_3^- and NO_2^- as additives for the preservation/conservation of aquatic food products is not allowed in general. However, it remains to be established what the "natural", endogenous NO_3^- and NO_2^- levels that may appear in edible seafood matrices would be. It has already been estimated for ~~the~~ meat: Iammarino & Di Taranto (2012) verified a constant presence of NO_3^- at low concentrations in ~~the~~ fresh meat from different species which were not subject to any NO_3^- and/or NO_2^- treatment.

In the past decades, the massive introduction of nitrogen fertilisers has markedly increased the residual amounts of NO_3^- and NO_2^- not only in primary agricultural products but also influencing ~~the~~ aquatic nitrogen expanse and balance as well. At the same time, aquaculture has increasingly become an important producer of aquatic food in coastal areas. Well-managed aquaculture can contribute positively to coastal environmental integrity, and particularly nitrogen pathways (FAO, 2016). Additionally, the equilibrium of the nitrogen cycle (especially NO_3^- and NO_2^- amount and ratio) has to be considered in recently introduced recirculation aquaculture systems (RAS). Such systems allow fish and plants to be raised together in one integrated system along with the nitrifying microorganisms that convert ammonia from the fish waste firstly into NO_2^-, and then into NO_3^-. By combining those basic metabolic pathways, modern aquaculture practice capitalizes on their benefits, where constant control of NO_3^- and NO_2^- appears to be mandatory (Wongkiew, Park, Chandran & Khanal, 2018).

Fish and seafood in general are not usually considered as a source of NO_3^- and NO_2^- although for live fish they are a well-known toxicant. The level for intoxication varies considerably and depends on numerous external and internal factors (Kroupova, Machova & Svobodova, 2005). The number of recent investigations for NO_3^- and NO_2^- in eatable fish parts and in ~~the~~ fish products is rather sporadic (Karimzadeh, Koohdani, Mahmodi, Safari & Babaee, 2010; Iammarino & Di Taranto, 2012;

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Bahadoran, Mirmiran, Jeddi, Azizic F, Ghasemi A, Hadaegh F, 2016). An extensive study that included different marine seafood species was performed two decades ago (Karl 1998) where the majority of the samples contained low levels of NO_3^- (in the range of few µg/g), with no NO_2^- revealed. The question that arises is whether ~~the~~ natural concentrations have been altered, particularly concerning the possible accumulation of those anions in the emerging aquaculture sector.

Therefore, the primary aim of this study ~~was~~ focused on the evaluation of NO_3^- and NO_2^- levels in fish, shrimps and bivalves that were selected randomly from the market. The species included in this study originated in different environmental surroundings (open sea or aquaculture) and were subdivided into three classes: smoked, fresh, and frozen on arrival. Preliminary analysis was performed by means of high-performance ion chromatography with suppressed conductivity detection (HPIEC-SCD) which has frequently been used for NO_3^- and NO_2^- determination in different food samples (Iammarino & Di Taranto, 2012; Lopez-Moreno, Viera Perez & Urbano, 2016). During our initial experimentation and method set-up it was not possible to detect NO_3^- and/or NO_2^- in some of the samples analysed. Thus, to reach ~~the~~ ultra-trace levels, those samples were additionally analyzed by means of high-resolution mass spectrometry (HRMS) detection. Although triple-quadrupole mass spectrometry techniques have already been used for NO_3^- determination in meat samples (Siddiqui Wabaidur, AL Othman, Rafiquee, 2015), to the best of our knowledge, HRMS has not yet been exploited for this purpose. Therefore, this paper reports the results of our work regarding the validation of both analytical approaches, HPIEC-SCD alone and coupled with HRMS.

2. Materials and methods

2.1 Sample collection

A total of 38 fish from different species, 8 shrimps and 7 bivalves were collected from the fish market in Milan and investigated for the presence of NO_3^- and NO_2^-. On the declarations that accompanied

samples there were no indications of any NO_3^-/NO_2^- treatment. Details regarding the species, their geographical origin, method of preservation (if any), and whether they were farmed or caught in open sea, are summarised in **Table 1.**

2.2 Chemicals and working standard solutions

Certified reference material (CRM) of NO_3^- and NO_2^- standard solutions for ion chromatography (1000 mg/L) were purchased from Sigma–Aldrich (Stenheim, Germany). Ultrapure water supplied by a Milli-Q RG unit from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 MΩ cm^{-1}, was used to prepare all standard solutions, mobile phases and for sample preparation. Working solutions were prepared by dilution CMR in ultrapure Milli-Q water to get the required concentrations.

2.3 Sample preparation

A 5g portion of the test sample was homogenised by blade homogenizer, 0.5g was then taken and mixed for 1 min with 5 mL of ultrapure Milli-Q water. The resultant suspension was then placed in an ultrasound bath for 20 min at 60 °C. The mixture was then centrifuged for 10 min at 5000 rpm on 4^0C (Centrifuge SORVALL™ ST 8 SERIES, Thermo Fisher). The supernatant was then treated with 5mL of hexane, followed by centrifugation at 5000 rpm on 4^0C for 5 min. After removal of the organic phase, the sample was divided into two 1mL-aliquots. Both aliquots were brought to pH 3.6 by adding 50 µL of the $HCOONH_4$/$HCOOH$ buffer 20mM. In one aliquot 10 µL of ultrapure Milli-Q water was added and in the second one 10 µL of 3% H_2O_2. After 10 min at room temperature, a few MnO_2 particles were added in order to neutralize excess H_2O_2. After brief centrifugation at 14000rpm for 2min (Eppendorf Minispin microcentrifuge), 10 µL of each sample was injected into the ion chromatographic instrumental system.

2.4 NO_2^- oxidation to NO_3^- by H_2O_2

The reaction conditions that lead to the quantitative conversion of NO_2^- to NO_3^- by H_2O_2 were adjusted by performing oxidation under different pH conditions. Once the best pH needed for oxidation had been established, the optimum concentration of the selected buffer was explored as well. Three different concentrations of $HCOONH_4/HCOOH$ buffer were evaluated for this purpose: 20, 50 and 100mM. All experiments were performed on ~~the~~ routine blank samples that were separately fortified with CRM of NO_2^- and NO_3^- at two concentration levels each: 5 µg/g for HPIEC-SCD and 0.05 µg/g for HPIEC-HRMS. These samples were used ~~in quality of~~ as Fortified Reference Samples (FRS). The reaction yield was calculated comparing the response of the pure NO_3^- with that obtained by NO_2^- oxidation at the same concentration level.

2.5 Instrumental methods

2.4.1 Analyses of nitrate and nitrite by high-performance ion-exchange chromatography with suppressed conductivity (HPIEC-SCD)

The analyses were accomplished by an Ionic Chromatography (IC) Dionex ICS-5000+ system (Sunnyvale, CA, USA) made up of a Dual Pump (DP), a Conductivity Detector (EG), a Detector/Chromatography Module (DC) and an Autosampler (AS-AP). The ion chromatography separation column was a Thermo Scientific Dionex IonPac AS19-4 µm (2 × 250 mm, 4 µm particle size) with a guard column Dionex IonPac AG19-4 µm (2 × 50 mm) maintained at 30 °C. The eluent flow rate was 0.30 mL/min with a gradient from 15 mM KOH (aq), held for 8 min, increased to 55 mM KOH (aq) at 20 min, held in these conditions for 4 min and back to 15 mM KOH (aq) at 24.1 min, with a cycle time of 30 min. The KOH eluent was neutralized using Dionex anion self-regenerating suppressor set to 50 mA (ASRS II, 4 mm). Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system and to elaborate the data obtained.

2.4.2 Ultra trace analyses of nitrate by high-performance ion-exchange chromatography coupled to Q-Exactive Orbitrap mass spectrometry

All ''negative'' samples were furthered analyzed by using the same anion-exchange chromatographic method but with Q-Exactive mass spectrometry detection as the final confirmation tool. The detector was a Thermo Q-Exactive Orbitrap™ (Thermo Scientific, San Jose, CA, USA), equipped with heated electrospray ionization (HESI) source. Capillary temperature and vaporizer temperature were set at 330°C and 280°C, while the electrospray voltage was set at 3.50 kV operating in negative mode. Sheath and auxiliary gas were set at 35 and 15 arbitrary units, with S lens RF level of 60. Instrument calibration was performed for every analytical session with a direct infusion of a LTQ Velos ESI Negative Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). Full Scan acquisition (FS) with resolving power set at 70000 Full Width at Half Maximum (FWHM) was used. Detection of NO_3^- was based on its retention time and exact mass (61.98834) accompanied ~~with~~ by characteristic isotopic pattern. Chromeleon™ software (Thermo Fisher Scientific, Waltham, MA) was used to control the IC system while Xcalibur™ 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA) was used to control the HRMS system and the exact mass of the compounds and to record and elaborate data.

2.6 Method validation

Validation of methods was performed taking into consideration the following parameters: specificity, linearity, precision, recovery, limit of detection (LOD) and limit of quantification (LOQ). Linearity was established using squared correlation coefficients (r^2) on calibration curve points prepared in the fish matrix in the range 5-250 µg/g for HPIEC-SCD and 0.05-5 µg/g for HPIEC-HRMS. Two fortified levels of NO_3^- and NO_2^- added to the blank fish samples as FRS were used for the evaluation of method accuracy (precision and recovery). Precision was estimated as the coefficient

of variability (CV) for the intra-day and inter-day repeatability, while recovery (expressed as %) was determined by comparing the response obtained for the same blank sample spiked before and after the extraction. Finally, limits of detection (LODs) and quantification (LOQs) were determined by fortifying blank matrix samples at low concentration levels. Detection (LOD) and quantification (LOQ) limits were calculated according to the following equations (Miller & Miller, 1993): LOD = 3.3SD∕b and LOQ = 10SD∕b, where SD is the standard deviation of the intercept for the low concentration levels and b is the slope of the regression line obtained from the principal calibration curve.

2.7 Statistical analysis

Preliminary statistical evaluation (Shapiro-Wilk Test) revealed that data were not normally distributed. Therefore, non-parametric statistical estimation was applied. Non-parametric Mann-Whitney Rank Sum Test was used to check the differences between the median values of two datasets while Kruskal-Wallis One Way analysis followed by all pairwise multiple comparison processes (Dunn's method) were used to check the differences between the medians of the three datasets. Statistical analyses were performed using Sigma Stat (Statistical Analysis System, version 12.5) software (Jandel Scientific GmbH, Erkrath, Germany). A P-value of 0.05 was set as statistically significant.

3. Results and discussion

3.1 Method validation

Fish/crustaceous/bivalve edible parts are challenging matrices to analyse for the presence of NO_2^- and NO_3^- by HPIEC mainly because of their heterogeneous and variable protein and fat quantities. Protein can consume column capacity and interfere with SCD detection particularly when it comes to near-to-trace levels of those anions. Fat can damage the column by a number of mechanisms

including the generation of excessive backpressure. Therefore, removal of the protein and fat from the sample is required for a successful application of HPIEC, but for NO_2^- and NO_3^- it is quite hazardous as those anions might be lost during sample treatment. The variability in the lipid percentage in fish samples, especially high in farmed fishes, required a defatting step through hexane washing after the extraction procedure. In addition, it is noteworthy that application of ultrasounds to the samples in a water bath helped the movement of the analytes from the matrix to the extraction solvent. Therefore, the preparation procedure described herein involved only defatting the sample previously suspended in water, while the removal of residual protein interferences was supported by $HCOONH_4/HCOOH$ buffer addition. Bringing the pH to 3.6 is also favourable if eventual oxidation of NO_2^- to NO_3^- by H_2O_2 has to be performed, as a part of sample preparation for HRMS detection. Concretely, when the HPIEC-SCD technique did not reveal any presence of NO_2^- and NO_3^- nor reach the concentrations near to method LODs, those samples were additionally analysed by Q-exactive Orbitrap mass spectrometer, which is much more sensitive. Q-Exactive Orbitrap does not detect m/z ratios below 50, thus it is not possible to follow the nitrites directly which is why a simple oxidation step was introduced. This procedure was validated in terms of pH adjustment and subsequently buffer concentration (20, 50 and 100mM). The buffer that was taken into consideration was $HCOONH_4/HCOOH$ that is volatile and thus compatible with HRMS detection. Furthermore, water solution of formic acid (0.1%, pH-2.7) and pure MiliQ water (pH-7) were studied as a medium for NO_2^- oxidation and were found to be unsuitable. As is clearly shown in **Figure 1** the best results were obtained for pH 3.6 for both HPIEC-SCD and HPIEC-HRMS methods. There were no significant differences in NO_3^- yield regarding the buffer concentration, and that is ~~reason~~ why the lowest concentration (20mM) was chosen ~~for~~ as final.

The validation parameters for both methods are summarised in **Table 2**. It is evident that the two methods are complementary: HPIEC-SCD showed excellent accuracy, while for HPIEC-HRMS the CVs

for intra- and inter-day precision were acceptable, although higher than those for SCD acquisition mode. Acceptable linearity was verified for both methods within corresponding concentration ranges, with correlation coefficients (r^2) higher than 0.988 for both anions, demonstrating a good correlation between the increasing concentrations in the sample and the responses obtained. NO_2^- determination by oxidation with H_2O_2 gave satisfactory results, as far as recovery is concerned, bearing in mind that the lower fortification level was near to the LOD method. LOD reached by Q-exactive method is in line with results obtained for gas chromatography–mass spectrometry method (Akyuz & Ata, 2009) and ultra performance liquid chromatography–single quadrupole mass spectrometry technique (Siddiqui, Wabaidur, AL Othman, Rafiquee, 2015).

The HPIEC-SCD analysis performed following our method showed LOD and LOQ values (**Table 2**) equivalent or better than those already reported in literature. For example, Iammarino & Di Taranto (2012) reported 4,5 and 9,7 µg/g for nitrite and nitrate, respectively. For the evaluation of method specificity, different types of seafood products were analysed in order to verify the absence of matrix interfering peaks in the intervals where the nitrites and nitrates appear. Actually, chromatograms obtained for the real sample (**Figure 2A**) and fortified blank sample (**Figure 2B**) show that the peaks relative to the aforementioned ions were well separated and that the matrix does not interfere with analysis. This is very important as many ion exchanged chromatographic methods faced problems with the chloride ion, which, when present in extremely high concentration may co-eluate with nitrite and thus interfere with its accurate determination (Lopez-Moreno, Viera Perez & Urbano, 2016). The HPIEC-HRMS method, apart from being specific, showed extremely high selectivity as can be seen from the ion-extracted chromatogram obtained for a real sample **(Figure 3).** Selectivity showed a good compliance with relative retention times, which, in our case, were within 2.5% tolerance for nitrate related to its standard. Moreover, isotopic ion ratio was within the recommended tolerances when compared with theoretical data.

3.2 Nitrate and nitrite content of fish, crustaceous and bivalve species

In order to confirm feasibility, the proposed methods were applied to fish, crustaceous and bivalve samples, as a primary aim of this project was to evaluate NO_3^- and NO_2^- incidence in different commercially available marine foodstuffs. Presence of NO_2^- was not discovered in any of the samples analysed. The results for the NO_3^- level in the analysed species are presented in **Figure 4.** It is worth mentioning that enormous differences occurred between mussel specimens (2.8 µg/g) and clam samples (89.3 µg/g). The data collected herein are in contrast to those reported in the study conducted by Iammarino, Taranto & Cristino (2013) who did not find NO_3^- in any of the clams examined, while a value of 42.4µg/g was the maximum NO_2^- level measured in mussels. It is reasonable to assume that such a disparity is a direct consequence of the origin of the bivalves studied. Indeed, the clams analyzed in our study were fished, while the mussels were cultivated. Having no detailed information on the conditions of their cultivation, it can be supposed that the mussels were raised in quite elevated densities in a way that could compromise the aquatic cycle of nitrogen and consequently reduce the presence of NO_3^- in their tissues. It is well known that bivalves are filtration organisms that contribute to the maintenance of the nitrogen cycle in aquatic systems. In fact, filtering mollusks assimilate nitrogen, especially through phytoplankton, and, at the same time, deposit excreted nitrogen in organic form in marine sediment. Organic nitrogen is converted into ammonium and subsequently into NO_3^- through nitrification processes promoted by aerobic sediment bacteria (Rice 2008). However, excessive density of mollusk farming can lead to an inefficiency in nitrification processes. This overload of the nitrification system is due to the excessive storage of organic nitrogen in the sediment by a high number of bivalves (Rose, Bricker, Tedesco, Wikfors, 2014). The reason for relatively high concentrations of NO_3^- might be that the clam samples that are caught in the open sea are in an equilibrated nitrification process in the open ecosystem,

which would maintain a constant, stable NO_3^- concentration. This, most probably, would influence NO_3^- accumulation in the clam's edible parts.

The analyzes carried out on two types of frozen shrimp samples show a median nitrate content of 22 and 35.3 µg/g. These data can be considered preliminary as in the literature there is no evidence for nitrate content in the marine shrimp. Comparing these values with those obtained from the only study that deals with the shrimp species (Karl, 1998), lower amounts of nitrates were found, specifically below 2 µg/g, with the exception of only one freshwater shrimp sample in which the level of nitrates reaches 100 µg/g.

The overall results of the fish samples are presented in **Figure 4**, that indicates the differences in NO_3^- content between the samples analyzed. The results obtained were additionally divided according to two criteria: on the basis of breeding (farmed or wild) and according to the state of preservation (smoked, fresh, and frozen on arrival).

By careful observing the graphs it can be deduced that the amount of NO_3^- present in the farmed fish is higher than that in the caught fish, as was confirmed by statistical non-parametric analysis (**Figure 5**). Since detailed information was not available regarding the origin and breeding systems adopted, it can be only speculated that this discrepancy between the two categories is due to the fact that intensive aquaculture systems do not always adopt an effective recycling and purification system for wastewater, hence resulting in nitrate accumulation in the micro-environment where fish are cultivated. For example, modern RAS save considerable amounts of water for fish farming by means of special recirculation systems, resulting in an important impact on the quality of the water itself. Although this system is very effective and generally safe, the level of NO_3^- and NO_2^- must be constantly controlled as there is a risk of excessive accumulation. This would subsequently lead to increased absorption of NO_3^- and NO_2^- by the fish through the gills (or through food ingestion), and hence their accumulation in the edible tissues. Therefore, our results for farmed fish

(median 44.4 µg/g) are in line with those of Manthey-Karl and Schimdt, (2018) that found up to 52.2 µg/g. The same authors found, in particular, the lowest concentrations in the fish grown in the tanks equipped with denitrification systems, which is comparable with our results for the wild fish specimens.

The content of NO_3^- in fresh, frozen and smoked fish presented statistically significant differences, in particular between smoked samples compared with fresh and then with frozen ones (**Figure 6**). Indeed, it ~~has~~ emerged that smoked fish products contain higher quantities of NO_3^- probably due to cross-contamination during the processing of the raw material through the water and / or the salts used in the preliminary phases that precede the actual conservation process. Actually, nitrogen species are ubiquitous in the environment, especially in water and air, so brine treatment and subsequent smoking may have concentrated such contaminants in the final finished product. However, the concentration of NO_3^- and NO_2^- in smoked fish depends on storage conditions, progressively decreasing over time, and strongly depends on temperature settings (Karimzadeh, Koohdani, Mahmodi, Safari & Babaee, 2010).

For the fresh and frozen samples, there is no significant difference regarding NO_3^- content: median values of 18 µg/g and 12 µg/g for fresh and frozen samples, respectively. It is reasonable to consider obtained values as endogenous, natural levels that should be taken into account for the evaluation of eventual treatment with additives.

4. Conclusions

Results obtained in this study demonstrate that the consumption of fishery products does not pose a significant health risk ~~in relation to~~ as regards the content of NO_3^- and NO_2^-, since in the samples analyzed nitrate quantity is limited and nitrite is completely absent. The HPIEC-SCD (with alternative HRMS detection) method used in this study can be applied to check real fish and fish products, as safeguarding of aquaculture products becomes an imperative. Being able to analyse seafood

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samples before putting them on the market is becoming an essential issue in terms of food safety. Furthermore, the content of nitrates and nitrites in fishery products must be monitored constantly in order to provide a more detailed scenario for the role of biogenic amines in the formation of nitrosamines (EFSA Journal, 2017a). In fact, fish matrices that are subject to the formation of different biogenic amines (Chiesa, Panseri, Pavlovic & Arioli, 2018), under favorable post-harvest conditions, could react with nitrogenous compounds leading to nitrosomanine production, an issue that has emerged as important for food in general (Lu et al 2017).

Conflicts of interest

All authors declare that there is no conflict of interest.

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Figure captions

Figure 1. Effect of pH on the efficiency of NO_2^- oxidation to NO_3^- by H_2O_2

Figure 2. HPIEC-SCD chromatograms comparison: (A) Fish sample with measured nitrates (12.7 µg/g) (B) Blank sample fortified with nitrites (50 µg/g) and nitrates (50 µg/g)

Figure 3. HPIEC–q-Exactive Orbitrap ion extracted chromatogram of real fish samples with nitrate measured at concentration (2.2 µg/g).

Figure 4. Median concentration of nitrates in the fish, crustaceous and bivalve species in the study

Figure 5. Distribution of nitrites according to breeding method.

Data are reported as median with 5th–95th percentile range. Comparison was done using Mann-Whitney Rank Sum Test (* statistical significance of P=0.005); wild n=15, farmedn=9.

Figure 6. Distribution of nitrites according to the preservation method.

Data are reported as median with 5th–95th percentile range. Comparison was done using Kruskal-Wallis One Way Analysis of Variance on Ranks with all Pairwise Multiple Comparison Procedures (Dunn's Method). Following subscripts refer to statistically significant pairwise difference: * smoked vs fresh; ** smoked vs frozen; smoked n=8, fresh n=12, frozen=8.

Table 1. Specific data related to the fish/bivalve/shrimp samples analysed

Common name	Scientific name	FM/WD[a]	FR/FZ/SM	FAO Catch Area	Samples number
FISH					
Atlantic salmon	*Salmo salar*	FM	SM	42	4
Gilt-head (sea) bream	*Sparus aurata*	FM	FR	37	4
Humpback salmon	*Oncorhyncus gorbuscha*	WD	FZ	67	4
Red salmon	Oncorhynchus nerka	WF	SM	67	4
Sea bass	*Dicentrarchus labrax*	FM	FR	37	4
Shortfin mako shark	*Isurus oxyrhincus*	WD	FZ	47	2
Spiny dogfish	*Squalus acanthias*	WD	FR	21	2
Swordfish	*Xiphias gladius*	WD	FR	87	4
Yellowfin tuna	*Thunnus albacares*	WD	FR	51	5
BIVALVES					
Mediterranean mussel	*Mytilus galloprovincialis*	FM	FR	37	4
Saltwater clam	*Chamelea gallina*	WD	FR	37	4
SHRIMP					
Caridean shrimp	*Pandalus borealis*	FM	FZ	21	3
Argentine red shrimp	*Pleoticus muelleri*	WD	FZ	41	4

[a] **FM**-farmed; **WD**-wild; **SM**-smoked

[b] **FR**-fresh; **FZ**-frozen

Table 2. Analytical performance and validation parameters of the proposed HPIEC-SCD and HPIEC-Q-Exactive methods

	Calibration curve	R^2	LOD (µg/g)	LOQ (µg/g)	Spiked level (µg/g)	Recovery	Precision (CV; *n*=5)	
							intra-day	inter-day
HPIC-SCD method (range 5-250 µg/g)								
NO_3^-	0.0404x + 0.1219	0.998	0.31	0.95	5	79	5.6	7.4
					100	89	8.7	9.0
NO_2^-	0.0612x + 0.299	0.994	0.20	0.59	5	72	7.3	6.9
					100	108	2.9	5.2
HPIC-Q-Exactive method (range 0.05-5 µg/g)								
NO_3^-	3042453x + 450223	0.9965	0.029	0.087	0.1	79	12.8	18
					1	110	13.8	17.6
*NO_2^-	11981980x - 120183	0.9888	0.036	0.108	0.1	90	15.5	18.7
					1	87	19.2	20.2

*NO_2^- concentration was determined after oxidation to nitrites

Figure 1.
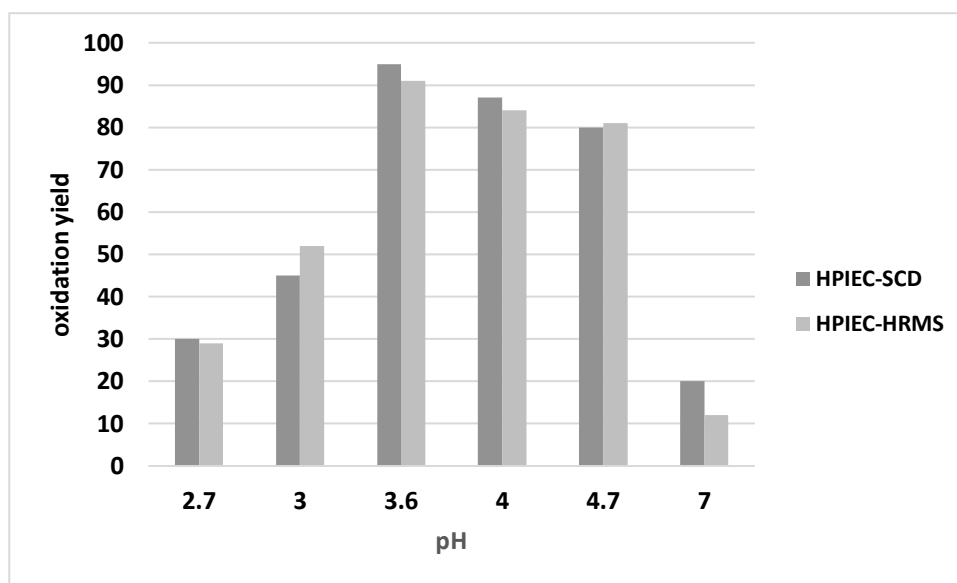
Figure 2.
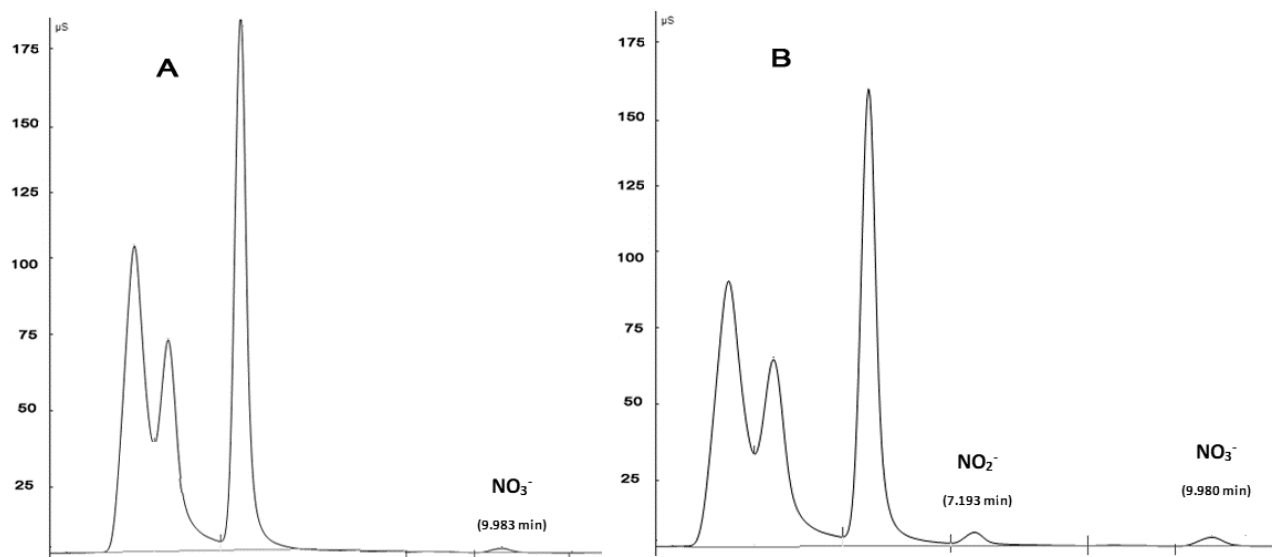
Figure 3.
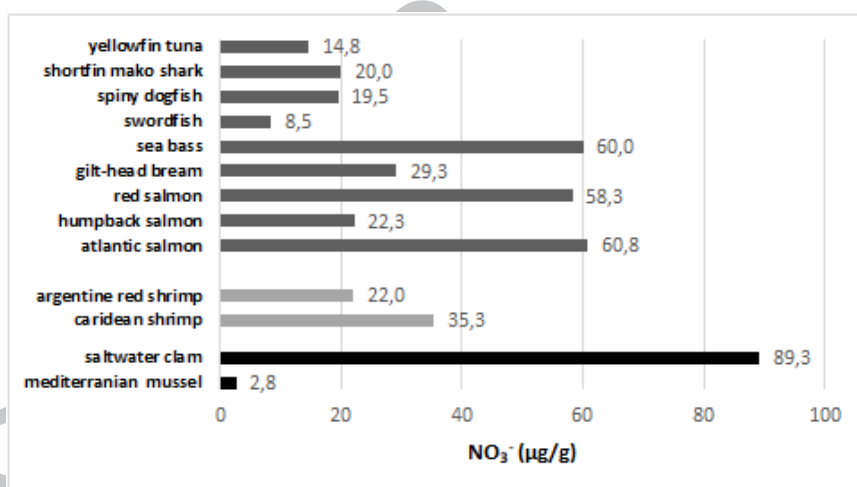
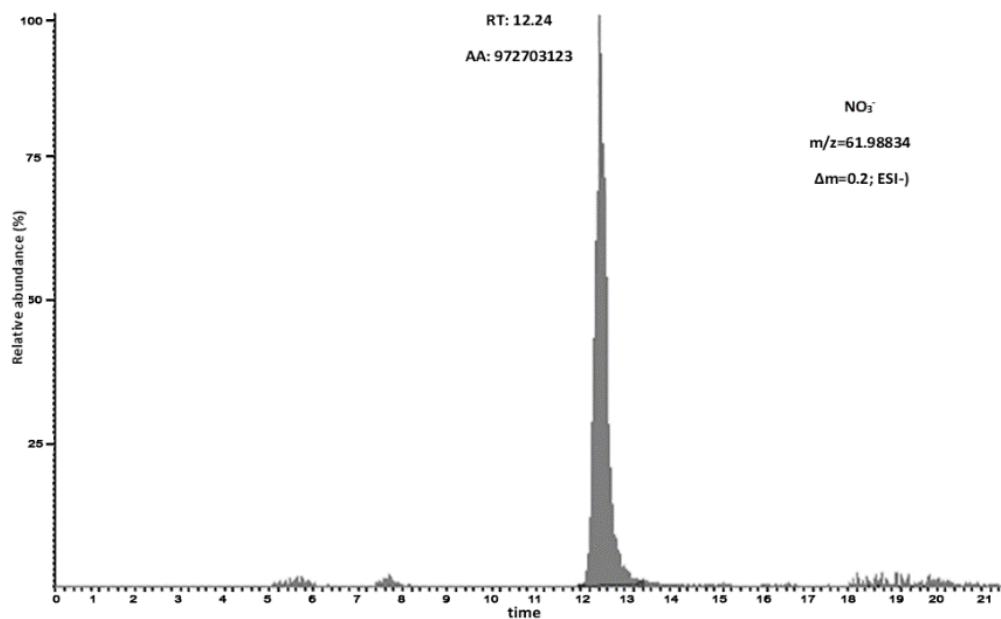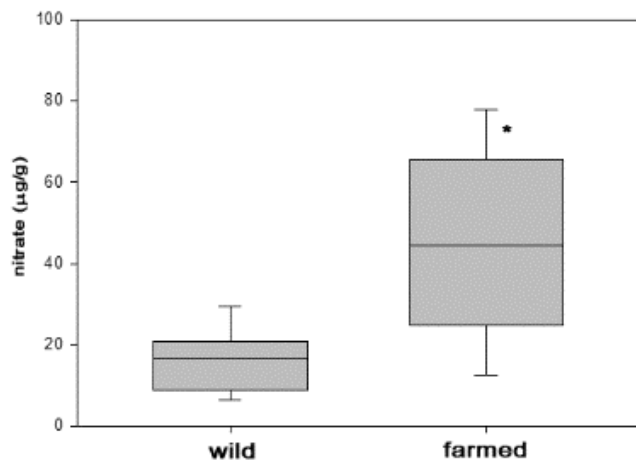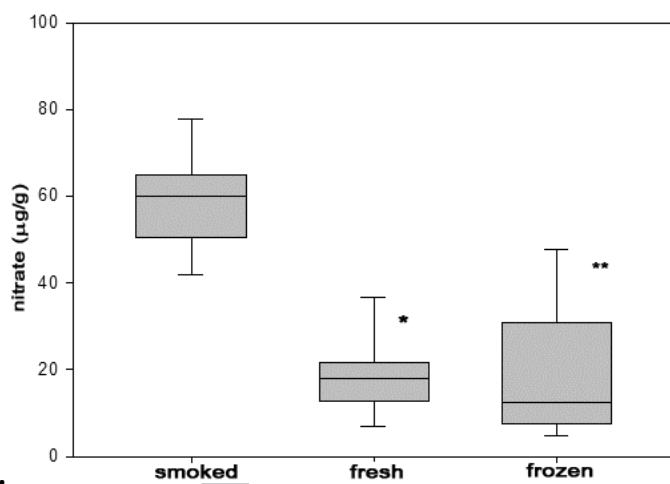**Figure 4.**

Figure 5.

Figure 6.

Highlights

- A method for NO_2^- and NO_3^- determination in seafood based on ion chromatography with suppressed conductivity ~~for~~ was set-up

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- High resolution mass spectrometry is used as a confirmatory detection tool for revealing ~~the~~ NO_2^- and NO_3^- ultra-trace levels

- Significant differences in NO_3^- levels between ~~the~~ farmed and wild seafood species ~~was~~ were found

- The highest ~~concertation~~ concentration of NO_3^- was ~~find~~ found in smoked salmon samples

- NO_2^- was not detected in any sample studied