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Analytical Methods

Selective post-column derivatization coupled to cation exchange chromatography for the determination of histamine and its precursor histidine in fish and Oriental sauce samples

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ABSTRACT

Histamine is a biogenic amine that is formed from histidine by action of the enzyme histidine decarboxylase and can be toxic at high intakes. Thus, the quantification of these analytes in foods constitutes a significant axis of food safety. In this study we present the development, validation and application of a new method for the determination of histamine and its precursor histidine in fish products and oriental sauces. The analytes were separated rapidly through a cation exchange column using an acidic mobile phase (7 mmol L^{-1} nitric acid) and reacted downstream with *o*-phthalaldehyde in post-column mode in the absence of nucleophilic reagents. The derivatives were detected spectrofluorimetrically at $\lambda_{ex}/\lambda_{em.}$ = 360/440 nm. Following investigation of the chromatographic and post-column conditions, the method was validated as for its intended applications. The limits of detection were 0.16 and 0.17 μmol L^{-1} for histidine and histamine respectively (ca. 0.1 mg kg^{-1}) and the precision was better than 5%. Various food samples were successfully analyzed without matrix interferences following minimal pretreatment. The percent recoveries ranged between 91.3 and 117.9%.

1. Introduction

Histamine (HIM) is a heterocyclic amine formed by the decarboxylation of the amino acid L-histidine (HIS) and belongs to the biogenic amines (Taylor & Eitenmiller, 1986). HIM can be gained either from L-histidine or food intake (Ai et al., 2006). High levels of HIM cannot be metabolized by the body, thus it binds to specific receptors and causes a number of food poisoning cases (Taylor et al., 1989). It is found especially in fish and fish products, in meat, fruits and vegetables and in fermented products, such as soy and alcoholic beverages (Papageorgiou et al., 2018; Shalaby, 1996). The poisoning associated with HIM in fish is called scombroid fish poisoning and is produced by the following bacteria Enterobacteriaceae, Clostridium, Lactobacillus, Vibrio, Pseudomonas and Photobacterium (Guillier et al., 2011).

The World Health Organization (WHO) associates HIM with many food poisoning outbreaks. The Food and Agriculture Organization of the United Nations (FAO) and the WHO addressed the public health risks of HIM from fish and fishery products at an expert meeting, conducted in July 2012. The appropriate hazard level of HIM was defined as a dose of 50 mg/kg, which is the no-observed-adverse-effect level (NOAEL) (FAO & WHO, 2013). According the US Food and Drug Administration (FDA), an edible fish should contain less than 50 mg kg^{-1} of HIM and a level of 500 mg kg^{-1} can be toxic (U.S. Food and Drug Administration, 2019).

The official AOAC method for the determination of HIM in fish samples is based on the off-line reaction of the analyte with *o*-phthalaldehyde under strictly controlled conditions (4 min) (AOAC, 2012). Selectivity is achieved by ion-exchange solid-phase extraction of the methanolic extracts through a suitable resin and this laborious step could not be ignored even in the modified and simpler alternative approach proposed recently by Bjornsdottir-Butler et al. (2015). A pre-treatment step based on solid-phase extraction seems to be necessary even in the case of combining the classic generic OPA/2-mercaptoethanol method for the derivatization of HIM with HPLC in a pre-column configuration (Kounnoun & Maadoudi, 2020; Peng et al., 2008). Direct derivatization of HIM in fish samples without prior clean-up would be problematic due to the competitive side-reactions with co-existing primary amines and amino acids. A typical example is HIS, the precursor of HIM, that may be present in large excess. Application to complex samples with extreme differences in concentration levels of active compounds is a well-known generic disadvantage of pre-column derivatization (Zacharis & Tzanavaras, 2013; Zacharis, Tzanavaras, & Themelis, 2009).

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Non-separation methods that offer adequate specificify for the determination of HIM in food matrices after simple pretreatment are based on the use of enzymes (enzymatic methods) and indirect competitive ELISA, with both approaches being also available as commercial kits (PerkinElmer MaxSignal Histamine Enzymatic Assay; Rocky Mountain Diagnostics HistaSure ELISA Fast Track). In both cases quantification is based on well-plate readings using dedicated detectors, while strict following of the reactions timing is mandatory for accurate results. Additionally, enzymes from natural sources (horse radish peroxidase, alkaline phosphatase, glucose oxidase etc) have many disadvantages, including instability, complex preparation and high cost (Li et al., 2021).

A viable alternative for the analysis of fish samples for HIM content could be liquid chromatography coupled to on-line post column derivatization (PCD) (Zacharis, Tzanavaras, & Zotou, 2011). Compared to the procedures described above, PCD involves a post-separation reaction and if separated efficiently from the matrix, HIM would ideally react with the derivatization reagent "in isolation", this way avoiding competition from excess of co-existing endogenous primary amines. Efficient HPLC separation of the polar underivatized HIM cannot be expected using reversed-phase stationary phases. Viable alternatives seem to be ion-pair, cation exchange and hydrophilic interaction LC (HILIC).

Due to the high content of organic solvents in the mobile phase, HILIC is "happily married" with mass spectrometric detection. In fact, a HILIC–MS/MS method has recently been reported for the analysis of HIM in Japanese sauces (Todoroki et al., 2014). On the other hand, the cation-exchange and ion-pair mechanisms have been combined with post column derivatization schemes. Cation-exchange LC has mainly been reported with the generic OPA-thiol chemistry (Triki et al., 2012). Despite the advantages of the cation-exchange separation mechanism for the analysis of amines, the generic character of the PCD reaction requires baseline separation of all active compounds and therefore a prolonged 30-min analysis cycle. An alternative PCD chemistry for both primary and secondary amines using NBD-Cl (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) has the disadvantage of the necessity of post reaction acidification for the reduction of the background signal of the excess of the reagent (Zacharis et al., 2011). The main advantage of ion-pair chromatography is the employment of routinely used reversed-phase columns for the separation of polar compounds. On the other hand, extremely slow equilibration and permanent modification of the columns are important and well-known disadvantages (Yerneni, 2017). In recently reported PCD methods for the determination of HIM by ion-pair LC the generic OPA-thiol chemistry has been applied (Evangelista et al., 2016; Rosinská & Lehotay, 2014). In both cases, due to the non-specific character of the PCD reaction a separation cylce of 30–60 min was required for complete separation.

The goal of the present study is to propose a rapid, selective and robust alternative for the determination of HIM in fisheries and Oriental sauces. To achieve this task we combined the efficient and rapid separation of HIM through a generic cation exchange column with an alternative highly specific PCD OPA-based reaction (Yoshimura et al., 1990). The absence of nucleophilic compounds excluded most primary amines from fluorimetric detection, enabling fast elution of HIM. Additionally, due to the kinetic flow-based character of the PCD reaction, stabilization of the OPA-HIM derivative by acidification can be omitted. The latter feature enables the simultaneous detection of the fast-eluting HIS, the precursor of HIM, as well. Compared to the pre-column and non-specific post-column methods reported in the literature, the developed scheme is capable of rapidly determining HIM in the presence of large excess of its precursor HIS with minimal sample preparation and sufficient sensitivity at the low mg kg^{-1} level.

2. Experimental

2.1. Instrumentation

The HPLC instrumentation consisted of an AS3000 autosampler (Thermo Scientific); a LC-9A binary pump (Shimadzu, Kyoto, Japan); an RF-551 spectrofluorimetric detector operated at high sensitivity (Shimadzu), an Elite^{TM} vacuum degasser (Alltech, U.S.). A Minipuls^{TM} 3 peristaltic pump (Gilson, Middleton, WI) was used for delivering the reagents. Two column ovens (Jones Chromatography and HiChrom Limited) were utilized in order to maintain the HPLC column and the reaction coil at the required temperatures. Chromatographic separations were performed by using a MetroSep C4 column (150 × 4.0 mm, i.d., 5 μm) (Metrohm AG, Herisau, Switzerland). The PCD reaction coil (200 cm) and connections were made of PTFE tubing (i.d. = 0.5 mm). Data acquisition was carried out using Clarity® software (version 4.0.3; DataApex, Prague, Czech Republic).

Off-line spectra were recorded using an RF-5301PC batch spectrofluorophotometer (Shimadzu). Other typical laboratory equipment included: ultrasonic bath (Elma Transonic), pH-meter (Orion), centrifuge (Hermle) and PTFE syringe filters (0.45 μm, Rigas Labs).

2.2. Reagents and solutions

The following analytical grade reagents were used throughout the study; histidine (HIS) and histamine (HIM) both from Sigma-Aldrich, *o*-phthalaldehyde (OPA, Fluka), HNO_3 (Fluka), KH_2PO_4 (Merck) and NaOH (Merck). Doubly de-ionized water was produced by a Milli-Q system (Millipore).

The standard stock solutions of HIS and HIM were prepared at a concentration level of 1000 μmol L^{-1} by dissolving accurately weighed amounts in HNO_3 (7 mmol L^{-1}). Working standard solutions were prepared daily by gradual dilutions in the same solvent. The post-column derivatization reagent solution (OPA) was prepared at a concentration level of 10 mmol L^{-1} by dissolution in 5 mL methanol following by dilution to 100 mL with doubly de-ionized water. The OPA solution proved stable for 5 working days when stored at 4 °C in an aluminum foil-wrapped container, although it was typically consumed within 1–2 working days. Phosphate buffer was prepared at 100 mmol L^{-1} and was adjusted to the pH value of 9 by dropwise addition of 2.0 mol L^{-1} NaOH solution. The mobile phase consisted of 7 mmol L^{-1} HNO_3 and was prepared daily, including ultrasonic degassing and filtration under vacuum through 0.45-μm membrane filters (Whatman®).

2.3. HPLC–PCD procedure

Standard solutions and samples (20 μL injection volume) were separated with isocratic elution (7 mmol L^{-1} nitric acid) on the cation exchange column at a flow rate of 1.0 mL min^{-1}. The column was thermostated at 60 °C. The eluted compounds were mixed downstream with the reagents (OPA and buffer, 0.25 mL min^{-1} each stream) forming the OPA-derivatives, on passage through the thermostated reaction coil (200 cm / 60 °C). The products were delivered to the fluorescence detector and were monitored at 360/440 nm.

2.4. Preparation of samples

2.4.1. Fish samples

Three types of commercially available fish products, namely tuna (canned), mackerel (canned) and sardine (frozen) were randomly collected from the Greek market. A representative amount of 10.0 g of each fish was exactly weighed and was blended/homogenized with 10 mL of HNO_3 0.1 mol L^{-1} and 40 mL ice-cold acetonitrile to facilitate proteins precipitation. Extraction was further promoted by ultrasonication for 15 min. A fraction of the resulting suspension was transferred into a 15-mL plastic centrifuge tube and was centrifuged at

5,000 rpm for 10 min. The supernatant was filtered through a syringe filter and – if necessary – was diluted in mobile phase prior to HPLC–PCD analysis (up to a maximum of 500:1 depending on the sample). It should be noted that due to the extreme differences of the concentrations of HIS and HIM in the samples, in several cases two different dilution levels were required for quantitative analysis.

2.4.2. Storage/freshness study

Each fish product was unpacked to allow contact with the environment and each sample was independently incubated for 0, 6 and 24 h at room temperature. Subsequent sample preparation was carried out as mentioned above. All samples were extracted and analyzed in triplicate unless mentioned otherwise.

2.4.3. Oriental sauces

Various types of commercially available Oriental sauce samples were collected, including soy sauce, dark soy sauce, two brands of light soy sauce, organic soy sauce, mushroom flavored soy sauce, tamari and fish sauce. All the above products were purchased from local markets and following opening, were kept refrigerated. In brief, 1.0 g of each sauce was accurately weighed into 15-mL plastic centrifuge tubes and dispersed in 1 mL HNO_3 0.1 mol L^{-1} and 4 mL ice-cold acetonitrile. The mixture was vortexed for 3 min and centrifuged at 5,000 rpm for 10 min. The supernatant was filtered through a syringe filter and was diluted in mobile phase prior to the analysis. All samples were extracted and analyzed in triplicate unless mentioned otherwise.

Analogous sample preparation was followed in pooled samples of fish products and Oriental sauces to evaluate the matrix effect. The analytical procedure is referred in Section 3.5.

3. Results and discussion

3.1. Preliminary studies

HIS is an amino acid with an imidazole ring connected to a two-carbon-atom chain. The chemical structure of HIM is similar to HIS, but the carboxyl group is absent. Besides the primary amino group of both compounds (pKa ≅ 9), the imidazole ring contains a secondary amine with a $pK_a \cong 6.0$. Therefore, HIS/HIM are protonated in acidic media and they could be separated through cation-exchange chromatography (Haddad et al., 2008). For this reason, a cation-exchange MetroSep C4 column (150 cm × 4.0 mm i.d., 7 μm) was used; this is a universal standard column in cation-exchange analysis for accomplishing high separating efficiency in a short time. It contains carboxyl functional groups to interact with the positively charged groups of the analytes and it is compatible with acidic mobile phases containing 0–100% organic modifiers such as acetone and acetonitrile. Preliminary comparative experiments demonstrated the advantages of the C4 column *versus* the analogous C2 Metrosep in terms of peak shape and sample throughput.

Off-line spectra confirmed the formation of the derivatives at alkaline pH (Fig. 1S in Supplementary Information). These experiments also confirmed the reported instability of the HIM-OPA derivative under the basic reaction pH (Yoshimura et al., 1987). In fact, >50% of the FL intensity was lost within a few minutes from the first scanning. Published off-line analytical protocols require a post-derivatization acidification step to stabilize the fluorescent derivative until analysis (Yoshimura et al., 1990). In our case however, this step can be overcome due to the flow based kinetic character of the PCD procedure (Douabale, 2003). Additionally, the absence of post-reaction acidification enables the simultaneous determination of both HIM and HIS, since the OPA-HIS derivative is non-fluorescent at low pH values.

The HPLC-PCD setup depicted graphically in Fig. 1 was employed in all cases. The starting values of the main HPLC and PCD variables were c (OPA) = 10 mmol L^{-1}, 50 mmol L^{-1} phosphate buffer (pH = 9.0), T (column) = T(RC) reaction coil = 30 °C, l(RC) = 200 cm and Q_V = 0.25 mL min^{-1} for each PCD stream. The concentration of HIS and HIM was 10 μmol L^{-1}.

3.2. Study of the HPLC conditions

The optimization of the chromatographic conditions was mainly focused on the effective separation of both analytes at a reasonable time. Other HPLC evaluation parameters such as number of theoretical plates (N), peak width and asymmetry were of course taken into consideration.

The concentration of HNO_3 proved to be critical in terms of HIM retention, while HIS was less affected due to the presence of the carboxylic group. For example, when using 3 mmol L^{-1} HNO_3 as mobile phase HIM is strongly retained, requiring an analysis time of > 25 min (data not shown). Late elution of HIM is of course accompanied by increased peak width, lower S/N ratios and higher consumption of the PCD reagents. As can be seen in Fig. 2S (Supplementary Information) both 5 and 7 mmol L^{-1} HNO_3 speeded-up the analysis without compromising the resolution between the analytes. Higher concentrations of the acid were not examined to avoid (i) pH-related fouling of the column and (ii) rapid elution of HIS at the void volume. HNO_3 at 7 mmol L^{-1} was finally selected for subsequent experiments as it offered acceptable chromatographic figures of merit ($R_s > 3$, $N > 15000$ m^{-1}) and analysis cycle of less than 10 min.

In ion-exchange separation mechanisms elevated temperature leads to the significant improvement of column efficiency, due to increase of the kinetics of the LC equilibrium (Dybczyński & Kulisa, 2005; Kulisa, 2004). The column temperature was set at three different levels (30, 45 and 60 °C, Fig. 3S in Supplementary Information). Temperature increase improved the efficiency and peak shape for both analytes and reduced the separation time. For example, the peak asymmetry for HIM was improved from 1.98 to 1.70 and the peak width was reduced from 0.27 to 0.20. For the above reasons, 60 °C was selected for further experiments, being compatible with the specifications of the column.

Under the optimum chromatographic conditions, the retention times of HIM and HIS were 2.4 min and 5.3 min respectively, the resolution factor was 9.15, the number of theoretical plates per column meter was 15,180 for HIS and 27,900 for HIM and the asymmetry factors were 1.39 and 1.70, respectively.

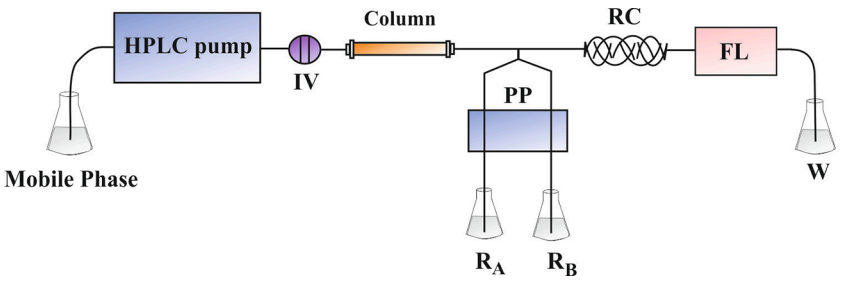

Fig. 1. Schematic depiction of the HPLC–PCD setup: IV = autosampler (V = 20 μL); PP = peristaltic pump; R_A = OPA (10 mmol L^{-1}, Q = 0.25 mL min^{-1}); R_B = phosphate buffer (100 mmol L^{-1} / pH = 9, 0.25 mL min^{-1}); RC = reaction coil (200 cm / 60 °C); FL = fluorimetric detector (340/460 nm); W = waste.

3.3. Study of the PCD conditions

Under the above LC conditions, the reaction efficiency was examined by varying the following parameters: the pH and the amount concentration of phosphate buffer, the amount concentration of the derivatization reagent, the flow rate of the reagents and the temperature of the reaction coil. All the above factors were individually investigated, apart from the flow rate and the temperature, which were examined simultaneously by co-variation.

Both analytes react with OPA under alkaline conditions with the HIM derivative being unstable (see Section 3.1). Flow experiments in the pH range of 8–12 showed that the effect of pH on the HIS-OPA reaction was negligible at all levels (Fig. 4S, Supplementary Information). HIM behaved similarly with the exception of sensitivity decrease at pH > 10. An intermediate value of pH = 9 was therefore selected offering adequate sensitivity and robustness against accidental pH variations.

On the other hand, the concentration of the derivatization reagent proved to have a significant effect on the sensitivity for both compounds (Fig. 5S, Supplementary Information). Increase of the concentration of OPA over the range of 2.5–15.0 mmol L^{-1} resulted in a 4-fold peak area increase for HIS and 3-fold for HIM. Taking into account the reagent consumption under the continuous flow PCD mode, 10 mmol L^{-1} was selected as a compromise. The concentration of phosphate ions in the buffer also improved the sensitivity in the range of 50–100 mmol L^{-1}, with the phenomenon being more pronounced in the case of HIS (ca 60% increase in the peak area of HIS). The value of 100 mmol L^{-1} was selected since higher concentrations (i.e., 150 mmol L^{-1}) had less impact (less than10% variation).

The flow rate of the PCD reagents is a significant factor because the interaction among the reagents and analytes and therefore the reaction time are regulated through the flow rate. In post-column systems, the stopped-flow approach is not feasible and therefore adequate length of the reaction coil (200 cm) was used to promote the reaction. Temperature is another important factor, affecting the kinetics of the derivatization. For this reason, the influence of the flow rate and the temperature of the reaction coil were examined simultaneously. Three flow rate levels of the PCD reagents (Q_V(OPA) + Q_V(buffer) = 0.3, 0.5, 0.7 mL min^{-1}) were combined with four temperature levels (30, 45, 60, 75 °C) in a total of 12 experiments. The experimental results are depicted in Fig. 2A and B, for HIS and HIM, respectively. The influence of the flow rate and temperature caused negligible variations on the HIM-OPA derivatization in the range of 0.3–0.7 mL min^{-1} and 30–60 °C. On the other hand, as can be seen in Fig. 2A, temperature rising sufficiently improved the rate of the HIS-OPA reaction in an almost linear way, offering a >2-fold increase of the peak areas in the examined range. The combination of 60 °C and 0.5 mL min^{-1} flow rate were selected as the optimum conditions due to reasonable reagents consumption and risk of bubble formation at higher temperatures.

3.4. Method validation

The proposed analytical method was validated in terms of linearity, limits of detection (LOD) and quantification (LOQ), precision, robustness, matrix effect and accuracy.

In order to evaluate the linearity of the method, numerous independent analyses of aqueous standards were obtained under the optimal conditions at different working days throughout the validation studies and were combined to one cumulative curve. The calibration curves include the results from >100 injections for HIS and HIM, with the aim of minimizing the effects of between-days variations. The regression equations for HIS and HIM were (1) and (2) respectively;

$$A = 373.78(\pm 3.85) \times c(HIS) - -5.89(\pm 20.49) \quad (1)$$

$$A = 264.17(\pm 1.99) \times c(HIM) - -4.97(\pm 15.09) \quad (2)$$

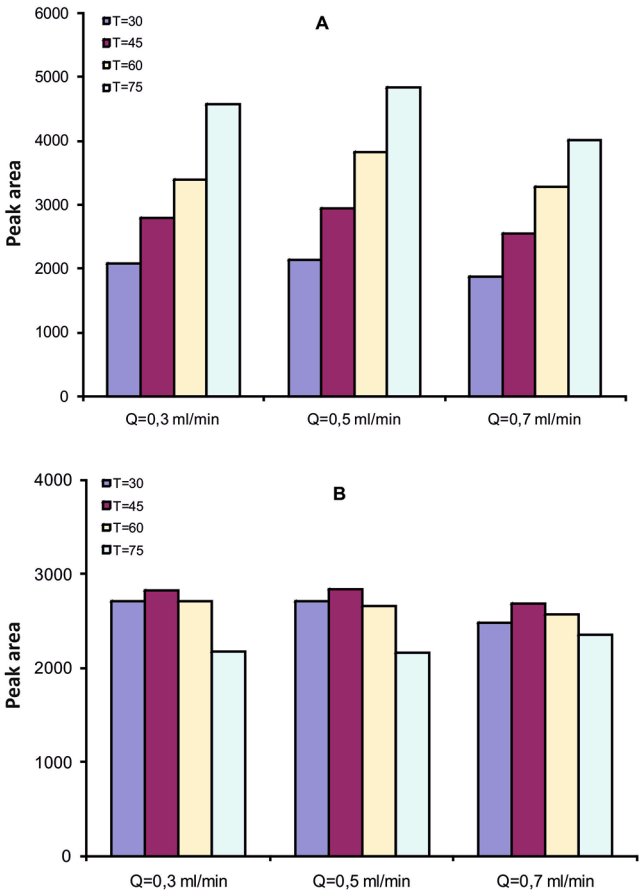

Fig. 2. Effect of the temperature of the reaction coil and the flow rate of the PCD reagents on the sensitivity; (A) histidine and (B) histamine.

Where A = is the peak area and c the concentration of the analytes (in μmol L^{-1}). Despite the large number of measurements at different days, the regression coefficients were satisfactory; $r^2 > 0.992$ for HIS and $r^2 > 0.994$ for HIM. The linearity of the method was evaluated in the range of 0.5–20.0 μmol L^{-1} (corresponding to ca. 0.4–15.5 mg kg^{-1}) and 0.5–15.0 μmol L^{-1} (corresponding to ca. 0.3–8.5 mg kg^{1}) for HIS and HIM, respectively. The percent residuals ranged between –19.5% (one data point at the LOQ level) and + 10.6% for HIS and –12.2 and + 12.9% for HIM. The calculation of the LODs was based on the standard deviations of the intercepts. They were found to be 0.16 μmol L^{-1} for HIS and 0.17 μmol L^{-1} for HIM (corresponding to 120 and 90 μg kg^{-1}, respectively). The lowest concentration level of the calibration curves, namely 0.5 μmol L^{-1} was set as the LOQ for both analytes.

Successive injections (n = 8) at 10.0 μmol L^{-1} of HIS and HIM were conducted to evaluate the within-day precision. The RSD values of fluorescence (peak area) and the retention times were calculated to be 2.2 and 0.1% for HIS, and 1.6 and 0.2% for HIM, respectively. The between-day precision was validated by ten independent aqueous calibration curves at non-consecutive working days and the RSDs of the regression slopes were found to be 3.3% for HIS and 4.6% for HIM. The experimental results are shown in Table 1S (Supplementary Information), proving the reproducibility of the proposed analytical method.

The robustness of the method was evaluated in terms of slight intentional variations of the following parameters: column temperature, HNO_3 concentration, flow rate of PCD reagents, temperature of the reaction coil, OPA concentration and pH of the buffer solution. All the experiments were conducted individually, modifying one parameter at a time at an analyte concentration level of 10 μmol L^{-1}. As can be observed in Table 2S (Supplementary Information), the effects of the above experiments caused acceptable fluctuations in the peak areas, in the range

of 95.3–102.3%. These results confirm the robustness of the proposed method.

3.5. Selectivity and matrix effect

The potential matrix effect was studied by spiking elevated concentrations levels of standards (at final concentrations corresponding to 2.5, 5.0, 10.0, 15.0 μmol L^{-1}) in three series of pooled samples of fish and Oriental sauces. The experimental results were evaluated by comparison of the slopes of the matrix matched curves to the aqueous ones. As can be seen in Table 3S, the absence of significant matrix effect was verified, being in the range of –2.8 to + 7.2%. These results confirm the direct applicability of the method in a complex matrix with minimal sample preparation.

Due to the combination of the cation-exchange separation mechanism and especially the selective post-column reaction chemistry (absence of nucleophilic reagent), no interfering peaks from endogenous components were recorded in all cases of the pooled food samples. To further demonstrate the contribution of the PCD "section" to the selectivity of the analytical method, we have carried out an additional series of experiments keeping the same HPLC conditions and using instead a non-specific PCD reaction. We have selected the OPA-NAC chemistry that enables derivatization of ammonium ion, amino acids and biogenic amines in alkaline medium (for experimental details see figures' captions and Tzanavaras et al. (2013)). Representative chromatograms from tuna, fish sauce and sardine analysis can be seen in Fig. 3 and Figs. 6S and 7S (Supplementary Information), respectively. The results were quite impressive, revealing a plethora of co-existing compounds containing primary amino-groups in the food samples that were practically undetectable by the proposed method. Especially in the case of tuna and fish sauce HIM and HIS respectively could not be resolved from co-eluting primary amines and therefore could not be quantified.

3.6. Analysis of fish products and oriental sauces

Fish and Oriental sauce products were collected, prepared as described in the experimental section and analyzed.

To confirm the reproducibility of the sample preparation, each fish product and each Oriental sauce was independently processed in triplicate. Fish analysis included storage studies as well; in brief, each fish sample was analyzed at three different time intervals, i.e., immediately after package opening and after storage at room temperature for 6 and 24 h. The experimental results are shown in Table 1. HIS was detected in all fish samples at high levels ranging between 784 and 3445 mg kg^{-1}. On the contrary, HIM was detected at levels < LOQ in mackerel during

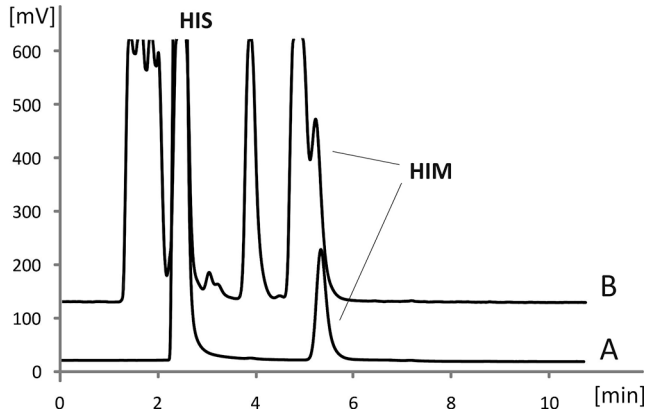

Fig. 3. Representative chromatogram from a tuna sample by (A) the present method and (B) using a non-specific PCD reaction (R_A = 2 mmol L^{-1} OPA and R_B = 5 mmol L^{-1} *N*-acetylcysteine in 100 mmol L^{-1} borate buffer (pH = 10), $\lambda_{ex}/\lambda_{em}$ = 340/460 nm).

Table 1
Analysis of fish samples.

Fish samples (storage time)	Histamine (mg kg^{-1}) (S.D.)	Histidine (mg kg^{-1})[a] (S.D.)
Sardine (0 h)	<LOQ[b]	1720 (±115)
Sardine (6 h)	<LOQ	1555 (±120)
Sardine (24 h)	50 (±3.1)	1495 (±90)
Mackerel (0 h)	<LOQ	784 (±45)
Mackerel (6 h)	<LOQ	850 (±70)
Mackerel (24 h)	<LOQ	840 (±65)
Tuna (0 h)	N.D.	3445 (±135)
Tuna (6 h)	68[c] (±2.9)	3331 (±250)
Tuna (24 h)	1370[a] (±180)	2013 (±315)

[a] 500-fold dilution.
[b] LOQ(HIM) = 0.3 mg kg^{-1}.
[c] 10-fold dilution.

all storage conditions. In sardine and tuna samples, the levels of HIM increased only after storage. For example, HIM in sardines and canned tuna reached concentrations of 50 and 68 mg kg^{-1} after 24 and 6 h, respectively. These values are lower than the EU recommended level of 200 mg kg^{-1} (EC, 2013) but according to FDA guidelines require further investigation since there is the possibility of significantly higher amounts of HIM in different parts of the fish. Canned tuna samples developed drastically higher amounts of HIM after 24 h of storage at room temperature reaching toxic levels of > 1000 mg kg^{-1} (FDA, 2011) being unfit for human consumption according to the FDA (U.S. Food and Drug Administration, 2019). It was observed that during storage of tuna samples, increase of HIM is accompanied by proportional decrease of HIS (see tuna analysis). This phenomenon could be more or less expected, due to the fact that HIM is formed by decarboxylation of HIS (Ai et al., 2006). On the other hand, in the absence of HIM, as in the case of canned mackerel, no significant changes in the levels of HIS were observed over the 24-h frame (Ai et al., 2006). Representative chromatograms from the effect of storage time on tuna and sardine samples can be seen in Figs. 4 and 8S (Supplementary Information).

In Oriental sauces, HIS was detected in measurable levels in 6 out of 8 samples, ranging between 14 and 42 mg kg^{-1}. HIM was detected in 3 samples with concentration levels between 11 and 34 mg kg^{-1}, values within the permitted limits (Table 2).

An interesting feature of the HPLC–PCD method compared to analogous pre-column derivatization procedures (Nakano et al., 2015; Tahmouzi et al., 2011; Zacharis & Tzanavaras, 2013; Zacharis, Tzanavaras, & Zotou, 2011) is the efficient and direct analysis of HIM in the presence of 30 to 50-fold excess of HIS. Representative examples are the results for sardine and tuna during storage at room temperature (Table 1). Taking also into account the complexity of the food matrix and especially the presence of numerous other primary amines in excess (see chromatograms in Figs. 3 and 6S and discussion in Section 3.5) the

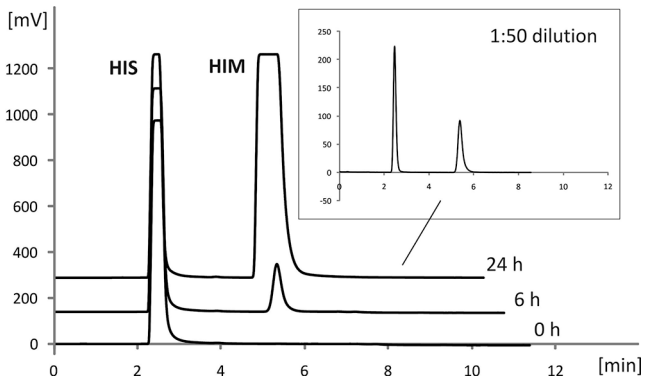

Fig. 4. Representative chromatograms from the effect of storage time on a tuna sample.

Table 2
Analysis of oriental sauces.

Oriental sauces	Histamine (mg kg^{-1}) (S. D.)	Histidine (mg kg^{-1}) (S. D.)
Soy sauce	11[b] (±1)	14[b] (±1)
Dark soy sauce	N.D.	24[b] (±3)
Light soy sauce (brand 1)	N.D.	19[b] (±2)
Light soy sauce (brand 2)	13[b] (±2)	17[b] (±3)
Organic soy sauce	34[b] (±3)	<LOQ
Mushroom flavored soy sauce	<LOQ[a]	<LOQ
Tamari	N.D.	42[b] (±5)
Fish sauce	<LOQ	14[b] (±3)

[a] LOQ(HIM) = 0.3 mg kg^{-1} and LOQ(HIS) = 0.4 mg kg^{-1}.
[b] 10-fold dilution.

development of a generic robust and reliable pre-column scheme would be a real analytical challenge. For example two methods that employ the OPA-based chemistry for the determination of HIM in similar samples require extensive sample clean-up by solid-phase extraction and extremely precise handling of the reaction time either off-line (210 s Kounnoun & Maadoudi, 2020) or using the capabilities of the auto-sampler of the HPLC setup (Peng et al., 2008).

The applicability of the proposed method was further evaluated by spiking experiments at final concentration levels of 5 and 10 μmol L^{-1}. As can be seen in Table 4S, in fish samples the percent recoveries ranged between 98.5 and 116.4% for HIS and 91.3 and 117.9% for HIM. The corresponding recoveries in Oriental sauces ranged between 97.6 and 118.7% for HIS and 93.1 and 116.4% for HIM, respectively (Table 5S, Supplementary Information). These results prove the applicability of the method in real samples and its effectiveness in complicated matrices. Representative chromatograms from the analysis of non-spiked and spiked tuna and a soy sauce sample are depicted in Figs. 9S and 10S in the Supplementary Information.

4. Conclusions

In the present work we have developed a method for the determination of histamine and its precursor histidine in fish using cation-exchange chromatography coupled to highly selective post-column derivatization with OPA in the absence of nucleophilic reagents. The combination of the advantages of cation-exchange chromatography with the selective PCD reaction offers some interesting features: (i) fish samples can be processed following simple treatment that is restricted only to protein precipitation and dilution; (ii) due to the selective post column derivatization of the separated analytes, reliable results can be obtained even in large excess of histidine and other amines; (iii) the critical solid-phase extraction pretreatment step that seems to be necessary in pre-column derivatization HPLC methods can be avoided; (iv) due to the on-line kinetic character of the PCD reaction, the instability of the OPA-HIM derivative is eliminated avoiding the extra acidification step; (v) the analytical figures of merit of the proposed analytical scheme are perfectly compatible with FDA, WHO and EU regulations; (vi) in our opinion the proposed method could be applied as a reliable and widely available tool for the monitoring of histamine levels in fish.

CRediT authorship contribution statement

Apostolia Tsiasioti: Methodology, Investigation, Validation, Writing - original draft. **Paraskevas D. Tzanavaras:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129351.

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