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Development and Validation of an RP-HPLC-FLD Method for the Determination of Biogenic Amines after Pre-column Derivatization with a New Pyrazoline Based reagent, 3- Naphthyl-1-(4-trifluoromethyl)-5-(4-carboxy phenyl)-2-pyrazoline

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ABSTRACT: Application of 3-Naphthyl-1-(4-trifluoromethyl)-5-(4-carboxy phenyl)-2-pyrazoline (NFCP) as a novel fluorigenic label for the derivatization of biogenic amines (BAs) and separation by liquid chromatography (LC) was investigated. Optimum conditions for the pre-column derivatization and chromatographic separation were investigated. The resulting derivatives are fluorescent at λex/λem: 380/460 nm. For the sake of evaluating the efficacy of the proposed analysis of BAs of different nature, the formation of nine NFCP-BAs derivatives was tested individually. Simultaneous derivatization and separation of four BAs (histamine, tyramine, tryptamine, and phenylethylamine) were achieved under the isocratic elution mode. Furthermore, the derivatives were identified by the characteristic product ion obtained during LC-MS/MS analysis. The viability of the method was established by measuring levels of histamine in fresh and spoiled fish samples. Histamine concentrations were found to be 0.4 $\mu g g^{-1}$ and 4.1 $\mu g g^{-1}$ in fresh and spoiled tuna fish samples respectively. As a simple, reliable, and sensitive pre-column derivatization method, this work provides for future research to develop a novel HPLC method for the analysis of BAs from food samples such as fish and various other matrices by utilizing the new pyrazoline compound.

Keywords: Pyrazoline, Biogenic amines, HPLC, LC-MS/MS, Sample analysis

تطوير والتحقق من فاعلية التحليل بطريقة RP-HPLC-FLD لتحديد الأمينات الحيوية التي يتم اشتقاقها قبل العمود باستخدام كاشف جديد قائم على البيرازولين، 3-نفثايل-1-(4-ثلاثي فلوروميثايل)-5-4-كربوكسي فينايل)-2- بيرازولين

أمل الصبحي، صالح البوصافي، بينا فرجيز ،سلمى الكندي و فخرالدين عثمان سليمان

الملخـص: اسـتخدم 3-نافثيـل-1-(4-ثلاثـى فلـورو ميثيـل)-5-(4-فينيـل الكاربوكسـيل)-2-البـايرازولين كمركـب فلوروسـينى جديـد لاشـتقاق الأمينـات الحيويـة ومـن ثـم فصـلها عـن طريـق الكروماتوجرافيـا السـائلة (LC)). تـم دراسـة الظـروف المثلـى لاشـتقاق مـا قبـل الفصـل الكروماتوجرافي. المشـتقات الناتجـة مشـعة عنـد 380/460 :λ_{ex} / $_{em}$ نـانومتر. مـن أجـل تقيـيم فاعليـة طريقـة التحليـل المقترحـة لـلأمينات الحيويـة ذات الطبيعـة المختلفـة ، تـم اختبـار تكـوين تسـعة مشـتقات بشـكل فـردي مـع المركـب الجديـد. تـم التحقـق مـن الاشـتقاق والفصـل المتـزامن لأربعـة مركبـات (هيسـتامين ، وتيـرامين ، وتريبتـامين ، وفينيـل إيثيـل أمـين). عـلاوة علـى ذلـك ، تـم تحديـد المشـتقات بواسـطة أيـون المنـتج الأساسـى الـذي تـم الحصـول عليـه بواسـطة تحليـل MS / LC-MS. تـم إثبـات جـدوى الطريقـة مـن خـلال قيـاس مسـتويات الهيسـتامين فـي عينـات الأسـماك الطازجـة والفاسـدة كـل علـى حـده. وجـد أن تركيـز الهيسـتامين كـان (0.4 ميكروجرام/جـرام) و (4.1 ميكروجرام/جـرام) فـي عينـات أسـماك التونـة الطازجـة والفاسـدة علـى التـوالي. كطريقـة اشـتقاق بسـيطة وموثوقـة وحساسـة قبـل الفصـل ، يـوفر هـذا العمـل بحثًـا مسـتقبليًا لتطـوير طريقـة HPLC جديـدة لتحليـل درجـات الأمينـات الحيويـة فـى عينـات الطعـام مثـل الأسـماك ومـواد أخـرى متنوعـة مـن خـلال اسـتخدام مركبـات البيرازولين الجديدة.

الكلمات المفتاحية: بيرازولين، الأمينات الحيوية، الكروماتوغرافيا السائلة مرتفعة الأداء HPLC ، الكروماتوغرافيا السائلة-طيف الكتلة/طيف الكتلة LC-MS/MS ، تحليل العينات.

1. Introduction

An area of research that is very active in separation science is the development of new fluorescent probes for tagging and labeling organic functional groups, before trace analysis using sensitive analytical instruments. Among the more promising compounds that offer versatility, the heterocycles have generated an increasing interest for application in many areas. However, their application in fluorescent labeling is still limited. Sufficient flexibility in synthetic strategies, derivatization procedures, and efficiency in modulating the charge transfer process throughout the system are the key aspects to consider while designing a labeling reagent. In this regard, one group of electron-rich nitrogen heterocycles, pyrazolines, has received our attention as a useful candidate for tagging the analytes of interest.

There is a huge amount of research on the exploitation of the blue light emission efficiency of this organic skeleton in multi-disciplinary areas such as dye-sensitized solar cells, organic light-emitting diodes (OLEDs), organic field effect transistors (OFETs), sensors, nonlinear optical (NLO) devices, the textile industry and electrophotography [1-9]. In the area of biomedical applications, these compounds are widely used as anti-microbial, anti-amoebic, anti-nociceptive, anti-cancer, anti-depressant, anti-inflammatory, anti-convulsant, anti-fungal, anti-hyperglycemic, anti-epileptic, anti-malarial, antipyretic, and so on [10-17]. It is known that the optical behaviors of pyrazoline derivatives are medium sensitive and they are being adapted to method development in bioanalytical applications. For this to be widely adopted, in this work we have evaluated the reactivity of a recently introduced pyrazoline dye, 3- Naphthyl-1-(4-trifluoromethyl)-5-(4-carboxy phenyl)-2-pyrazoline (NFCP) for tagging biogenic amines (BAs) under optimized derivatization conditions.

Biogenic amines are basic nitrogenous compounds with a small mass. They are present in large quantities in food and beverages. The main BAs occurring in foods are histamine, tyramine, tryptamine, phenylethylamine, putrescine, cadaverine, spermine, and spermidine. They are formed as a result of enzymatic action on poor-quality raw materials, contamination, or are generated under unhygienic conditions during aging and storage, because of microbial decarboxylation of food amino acids [18-21].

When present in food samples in low concentration, BAs do not pose a serious risk to humans. However, high levels of BAs are notoriously worrisome in terms of the detrimental effects they can have on our health. For instance, cadaverine, histamine, and putrescine may cause hypotension. In severe cases, the presence of tyramine may bring about death while mild cases result in hypertension, headache, dizziness, cardiac palpitation, nausea, intracerebral hemorrhage, and anaphylactic shock syndrome [22-24]. The presence of BAs in food samples indicates food spoilage; hence, the determination of BAs is important for the quality control of food commodities. To this end, chromatographic techniques have found wide applications due to their high sensitivity and adaptability for simultaneous quantification of most BAs [24]. Since BAs are either weakly chromophoric or non- fluorogenic; the tagging of these molecules to the chromophores or fluorophores is essential to improve the detection of derivatized BAs using UV and fluorescence techniques [25]. It can be noted that pre / post-column derivatization technique with UV-vis /fluorigenic labels in conjunction with high-performance liquid chromatography (HPLC), has successfully overcome the challenges in detection due to lack of volatility and chromophores.

In addition, pre-column derivatizations of polar BAs are often needed, to reduce their polarity and to improve the resolution in reversed-phase HPLC columns [25]. Various fluorescent reagents such as *o*-phthaldialdehyde (OPA), 5-dimethylaminonaphthalene-1-sulfonyl chloride (DNS-Cl), benzoyl chloride, 9- fluorenyl methyl chloroformate (FMOC-Cl), 4-fluoro-3- dinitrofluoro-methylbenzene (FNBT), 2-chloro-1,3-dinitro-5-(trifluoromethyl)benzene, 4-chloro-3,5-dinitrobenzotrifluoride (CNBF), 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DBS-Cl), fluorescamine, mercaptopropionic acid (3-MPA), 2-mercaptoethanol, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), para-nitrobenzyloxycarbonyl chloride (PNZ-Cl), and 3,5-dinitro benzoyl chloride (DNBZ-Cl) have proved their efficiency in BAs labeling [26-38].

Fish and fish products constitute an important source of proteins, minerals, and vitamins. Fish foods that are widely consumed all over the world. They form an integral part of the regular diets of many due to their high nutritional content, low caloric value, ease of digestibility, and moderate cost. However, if not properly preserved, fish is easily perishable and is susceptible to the formation of BAS, which leads to the loss of quality, and it can undergo deterioration and spoilage. The presence of BAs in fish is an indicator of the quality of the freshness or spoilage of food and gives information about the edibility of fish [18]. Hence, to this end, several studies on the determination of BAs in fish have been reported in the literature [25, 27, 39-43]. These methods include liquid chromatography-tandem mass spectrometry [39, 40], ion chromatography [41], the HPLC method with fluorescence detection [25, 27, 42], and the colorimetric and fluorescence method [43], to mention a few. To the best of our knowledge, the methods reported in the literature for the determination of BAs are devoid of any compounds based on the pyrazoline scaffold. To

address this omission, this work has focused on (i) testing the suitability of NFCP for the pre-column derivatization of BAs, (ii) optimizing the derivatization as well as chromatographic parameters to maximize the yield and fluorescence response, (iii) confirming the structure of NFCP-BAs using LC/MS/MS, (iv) demonstrating the practical application of the validated method on fish matrix.

2. Experimental

2.1 Reagents and Chemicals

Analytical grade reagents purchased from Sigma-Aldrich, USA, and Ultrapure water from a Milli-Q water purification system (Millipore, Billerica, MA, USA) were used in this study.
The fish samples (goatfish, white tuna fish, and cobia fish) were collected from the local market. Synthesis of NFCP has been published elsewhere [**44**]. The biogenic amines used in this study are 2-phenylethylamine (PEA), tyramine (TYR), dopamine (DA), octopamine (OCT), synephrine (SYN), ephedrine (EPH), histamine (HIS), tryptamine (TRY), and serotonin (SER).

A 3 mmol L^{-1} stock solution of NFCP was prepared in acetonitrile. A standard stock solution of each of the studied BAs (10 mmol L^{-1}) was individually prepared in a phosphate buffer of pH 8.5. Working solutions were produced daily by dilution with the same buffer.
The activation reagents N-(3-dimethyl aminopropyl)-N'-ethyl carbodiimide hydrochloride, EDC.HCl (0.1 mol L^{-1}) and dimethylamino pyridine, DMAP (0.4 mol L^{-1}) were also prepared in acetonitrile. All standards were stored in a refrigerator at 4 °C. All solutions were filtered through a 0.45 µm nylon membrane filter (Thomas Scientific, USA).

2.2 Apparatus

Derivatization was carried out after mixing the contents on a Stuart vortex mixer SA8 (UK) at 1400 RPM and heating on a B490 Butchi heating bath (Switzerland). A Cole Parmer ultrasonic instrument (Cole Parmer, USA) was used for degassing of the solvents, while Clinspin horizon 642E -Woodley equipment (Fischer Scientific, USA), was used for all centrifugation activities. Monitoring of pH was carried out on a Thermo pH meter (Russell RL060P, Singapore).

2.3 High-performance liquid chromatography (HPLC)

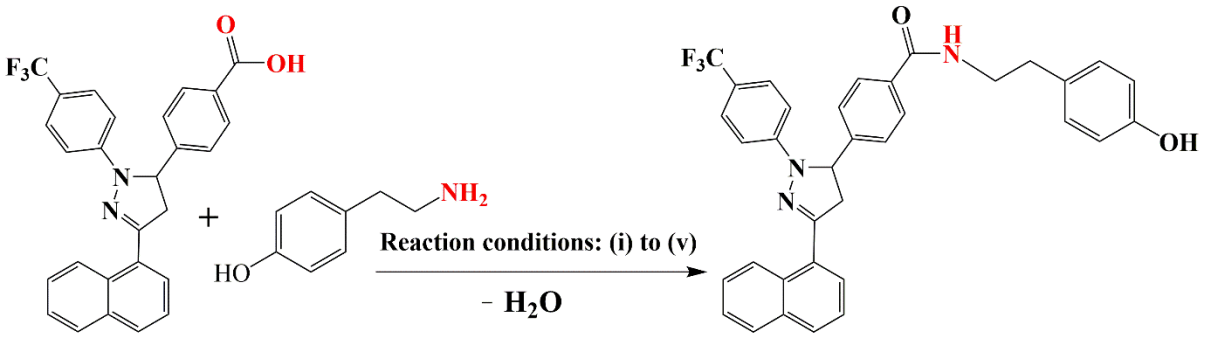
The analysis of NFCP-BAs derivatives was performed on a HPLC Agilent 1260 Infinity capillary LC, USA unit equipped with a G1376A binary capillary pump, G1377A autosampler, and coupled to a G1315C UV diode array detector and a G13218 fluorescence detector. The column compartment (G1316A) was maintained at a constant temperature by a thermostat. Open Lab ChemStation (version C.01.04) software was used to control the chromatographic system. NFCP derivatized BAs were separated on a reversed-phase poroshell EC-C18 column (100 mm× 3.0 mm, particle size 2.7 µm) and were eluted under isocratic conditions using 80:20, v/v acetonitrile and water mobile phase at a flow rate of 0.2 ml min^{-1}. The column temperature was maintained at 40°C throughout the separation process. Before the analysis, the column was pre-equilibrated with the mobile phase for 30 min. 1 µL samples volumes were injected onto the column. Detection in the UV mode was carried out at λmax of 360 nm. On the other hand, fluorescence was monitored at 460 nm with the excitation wavelength set up at 380 nm. All the sample analysis was performed in triplicate and the mean result was reported.

2.4 Liquid chromatography-Mass spectrometry (LC-MS/MS)

For the mass-spectrometric analysis, a Triple Quadrupole mass spectrometer (Shimadzu 8040, Japan) was used. Ionization was carried out with an electrospray ionization source (ESI). The operating conditions were set as follows: ESI temperature 400 °C, the desolvation line (***DL***) 250 °C, 3 L min^{-1} flow rate of argon (nebulizing gas), and 15 L min^{-1} flow rate of drying gas. The LC-MS was fitted with a poroshell EC-C18 column (100 mm, 3.0 mm, 2.7 µm) and controlled by Lab-solutions software. Separation of NFCP-BAs was achieved using a mobile phase composed of A (acetonitrile), B (0.1% formic acid and water) at 80-20 % (v/v). The pump was operated under a low-pressure gradient at a flow rate of 0.2 ml min^{-1}. The oven temperature was set at 40 °C for all of the separation processes. The mass analyzers Q1 and Q3 were operated at a unit mass resolution.

2.5 Derivatization procedure

The derivatization reaction is shown in **scheme 1**. To an Eppendorf vial (2 ml), 100 µl of NFCP, EDC. HCl, and DMAP were added consecutively and the contents were vortexed for half a minute. Then 100 µl of BAs mixture was added dropwise with vortex mixing for at least 30 seconds, after which the mixture was made up to 1 ml with acetonitrile. The reaction mixture was maintained at 65 °C for 40 min and cooled to ambient temperature before injecting a 1-µL sample into the HPLC column.



Scheme 1. Derivatization reaction of NFCP with a representative amine, Tyramine. Reaction conditions: (i) Acetonitrile, (ii) [NFCP/BA] 0.3/0.025 mmol L^{-1}, (iii) [EDC/DMAP] 0.1 / 0.4 mmol L^{-1}, (iv) Temperature 40 ^{0}C and (v) Time 60 minutes.

2.6 Pre-treatment of the fish samples

The three fish samples (goatfish, white tuna fish, and cobia fish) were first divided into two portions. The first part was immediately stored in the freezer at -10 °C and the latter which was kept at room temperature for 48 hrs. All fish samples (5 g of each) were homogenized separately before extraction using a mortar and dissolved in 10 mL of 5% Trichloroacetic acid (TCA). After being sonicated, the mixture was centrifuged at 6000 rpm for 30 minutes three times, to ensure complete extraction of biogenic amines. When not in use, the collected supernatant was stored at 4 °C after filtration through a 0.45 µm filter paper. Working solutions were prepared as follows: into a 5 ml volumetric flask were added 1 mL of the biogenic amine extract followed by 0.4 mL of 1 M NaOH. The contents were made up to the mark with a pH 8.5 phosphate buffer solution.

2.7 Analytical performance of the method

The proposed method was validated in terms of the analytical parameters for quality assurance by the ICH guidelines (International Conference on Harmonization Tripartite Guideline: Validation of Analytical Procedures) **[45]**.

Linearity was assessed by plotting calibration curves of four BAs' standard concentrations in the range between 0.5 µmol L^{-1} to 100 µmol L^{-1}. Each standard was injected three times. Correlation coefficients (R^2) were calculated using least-squares regression; the limits of detection (LD) and the limits of quantification (LQ) were evaluated using the equation 3σ/m and 10σ/m respectively where σ is the standard deviation of the y-intercept of the regression analysis and m is the slope of the calibration curve. Accuracy was established by injecting standard solutions of BAs at three different concentrations (4, 10, and 40) µmol L^{-1}, with six replicates.

The intra-day and inter-day analysis, which is taken to be the relative standard deviations (RSD) was determined from the peak areas of the four derivatives' six replicates (n=6) for three different concentrations above on the same day and four consecutive days, respectively. Recovery and matrix effect was estimated by fortification of fresh and spoiled tuna extract with BAs standard ranging from 0 to 25 µmol L^{-1}. Standard additions plots were constructed and the slopes were matched with standard solutions prepared in water. The matrix effect is given by the equation = (Sm/Ss) × 100%, where Sm and Ss represent the slope of the calibration curve of each analyte in matrix solutions and standard working solutions, respectively. The recovery of histamine in this method was estimated by the standard addition method; by adding four concentration levels of histamine (2.5-25µmol L^{-1}) to two types of fish samples (fresh and spoiled white tuna).

3. Results and Discussion

3.1 HPLC and LC-MS/MS analysis

Conventionally, the reaction map for the condensation of amines and carboxylic acids involves thionyl chloride ($SOCl_2$) or the combination of various activation reagents with some base catalysts. Based on introductory experiences, it seemed likely that the conversion of –COOH to –COCl and later replacement by an amino group would be viable for the synthesis of crude derivatives; but it leads to unknown interference in the chromatogram. Acetonitrile is appropriate for derivatization as well as chromatographic separation involving pyrazoline-based probes since most of the reagents used are soluble in acetonitrile and are favorably compatible with the HPLC system. Much of the literature features examples of different activation agents for an efficient coupling reaction to trigger a favorable

derivatization reaction with the desired chromatographic separations, [46-47] The most prominent example is the combination of 2, 2'-dipyridyl disulfide, (DPDS) with triphenylphosphine (TPP) [46]. The high moisture sensitivity, storage under inert conditions to avoid inactivation and the relatively weaker derivative peaks reduce our interest in these reagents. On the other hand, 4-ethyl dimethylamino propyl carbodiimide with 4-Dimethylaminopyridine (EDC/DMAP) couple was found to be best for the derivatization of BAs at very low concentrations. In addition, it features longer stability and faster reaction, and more importantly, there is no need for product extraction from the reaction system before sample analysis in the HPLC.

For the derivatization of aromatic BAs with EDC, it was reported that the coupling efficiency is six times greater than its counterpart DCC (dicyclohexyl carbodiimide) when an equal concentration of both coupling reagents was used and its . superiority to four other coupling reagents was emphasized [47]. The EDC.HCl (0.1 mol L^{-1})/DMAP (0.4 mol L^{-1}) coupling system was selected as undoubtedly being the optimum for the reaction of biogenic amines with NFCP.

As shown in figure 1, as the concentration of the labeling reagent increases, the fluorescence intensity of the derivatives and hence yield was observed to increase. A rapid increase in the intensity was observed when the concentration of NPCP rises from 0.2 to 0.3 mmol L^{-1}. No further increase in the intensity was observed beyond 0.3 mmol L^{-1}. These results indicate that the maximum yield of the derivatives was obtained using an NFCP concentration of 0.3 mmol L^{-1}, which is equal to tenfold the concentration of the biogenic amine mixture (HIS, TYR, TRY, and PEA) in the reaction vial. To drive the equilibrium towards the formation of the derivative a high excess of the reagent is required. Similar results were previously obtained for the derivatization reaction of alcohols and NFCP label whereby an optimum concentration of NFCP to alcohol was 5:1 [48].

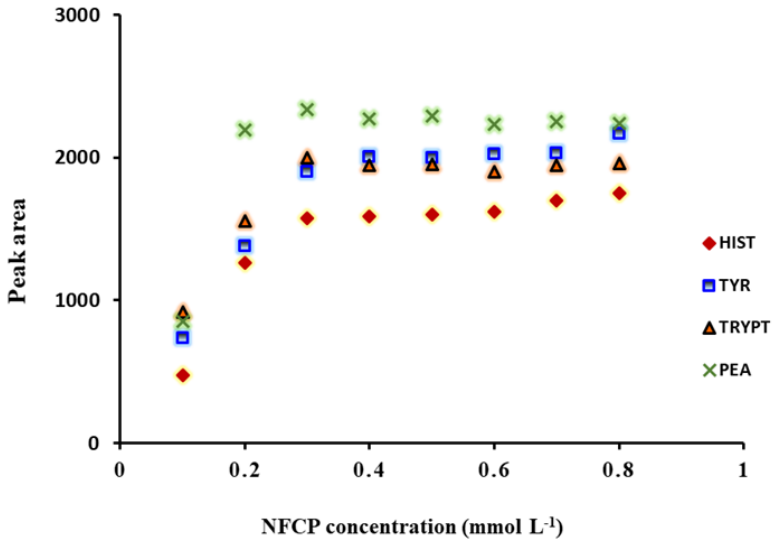


Figure 1. Effect of NFCP concentration on the labeling reaction of four biogenic amines. Chromatographic conditions: Poroshell EC- C18 column (100 mm, 3.0 mm, 2.7 µm), ACN-water (80 %v/v), flow rate 200 µL min^{-1}; λex/λem: 380/460 nm, column temperature 40 °C; Reaction conditions: [BAs] 0.025 mmol L^{-1}, [EDC/DMAP] 0.1/0.4 mmolL^{-1}, Acetonitrile, 65 °C, 40 min.

Time and temperature are considered to be significant factors influencing reaction kinetics. Therefore, to optimize these factors, the derivatization reaction was carried out at 20, 40, 60, 80, and 100 mins. Figure 2 shows that a reasonable yield of the NFCP-BAs derivatives was achieved at a reaction time of 20 minutes; however, the highest peak areas were attained at 40 minutes. Reaction yields of the derivatives obtained at different temperatures were compared and it was found that HIS and TYR produced maximum efficiency at 65 °C, whereas the peak area of TRY and PEA derivatives at 50 °C was slightly higher than those at 65 and 80 °C (figure 3). Accordingly, 40 minutes and 65 °C were selected to be the optimal values for time and temperature, respectively.

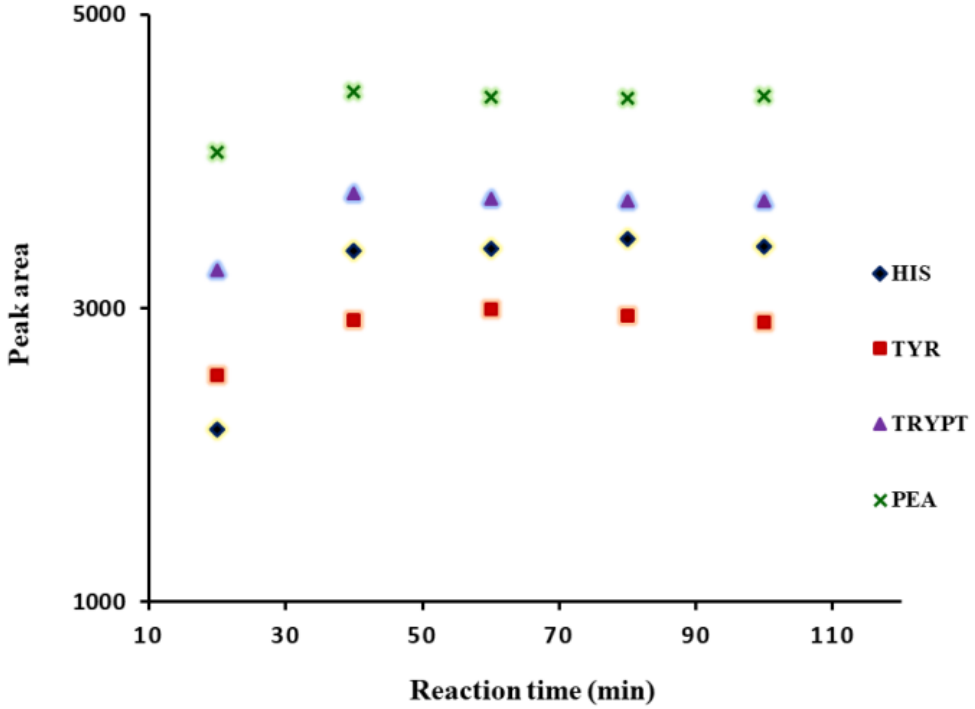


Figure 2. Effect of derivatization time on the labeling reaction of four biogenic amines. Chromatographic conditions: Poroshell EC- C18 column (100 mm, 3.0 mm, 2.7 µm), ACN-water (80 %v/v), flow rate 200 µL min^{-1}; λex/λem: 380/460 nm, column temperature 40 °C; Reaction conditions: [NFCP] 0.3 $mmolL^{-1}$, [BAs] 0.025 mmol L^{-1}, [EDC/DMAP] 0.1/0.4 $mmolL^{-1}$, Acetonitrile, 65 °C.

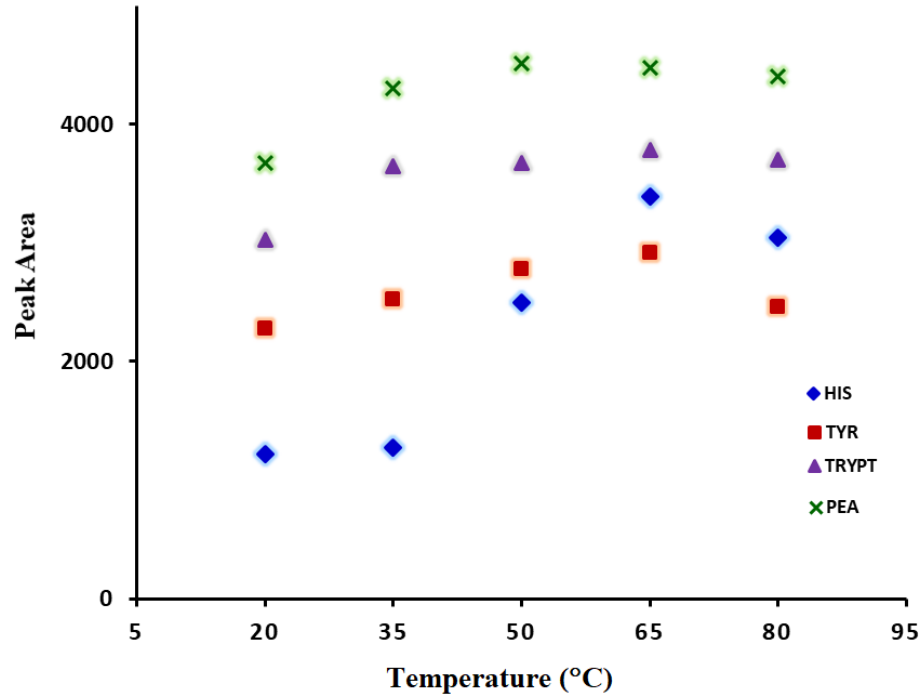


Figure 3. Effect of derivatization temperature on the labeling reaction of four biogenic amines. Chromatographic conditions: Poroshell EC- C18 column (100 mm, 3.0 mm, 2.7 µm), ACN-water (80 %v/v), flow rate 200 µL min^{-1}; λex/λem: 380/460 nm, column temperature 40 °C; Reaction conditions: [NFCP] 0.3 $mmolL^{-1}$, [BAs] 0.025 mmol L^{-1}, [EDC/DMAP] 0.1/0.4 $mmolL^{-1}$, Acetonitrile, 65 °C.

It could be expected that the derivatization reaction involving –COOH and $-NH_2$ is catalyzed in a basic media and that the pH of the solution has to be above the pKa of amines. To switch on a nucleophilic

acyl substitution reaction, the phosphate, acetate, and formate buffers of pH 8.5 were employed and it was found that NFCP – BAs derivatives were not formed with acetate and formate buffers. Then, the optimum pH value of the phosphate buffer was examined (figure 4). Since the maximum response was revealed as between pH 8 and 9.5, pH 8.5 was chosen for subsequent work. The optimized derivatization method was applied to nine BAs of different nature: PEA and TYR (primary), SYN and EPH (secondary), HIS and SER (highly polar), PEA (less polar), DA and OCT. The formation of all derivatives was tested individually and finally, four of the BAs (HIS, TYR, TRYP, PEA) were selected to perform the quality control procedure to validate our newly developed method. A graphical representation of the overall study is shown in scheme 2.

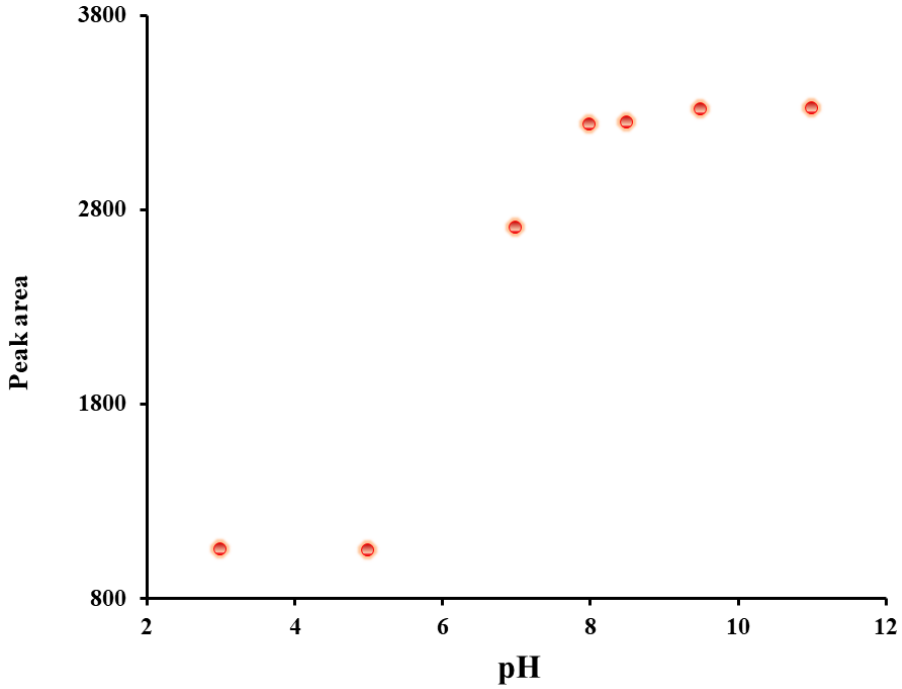
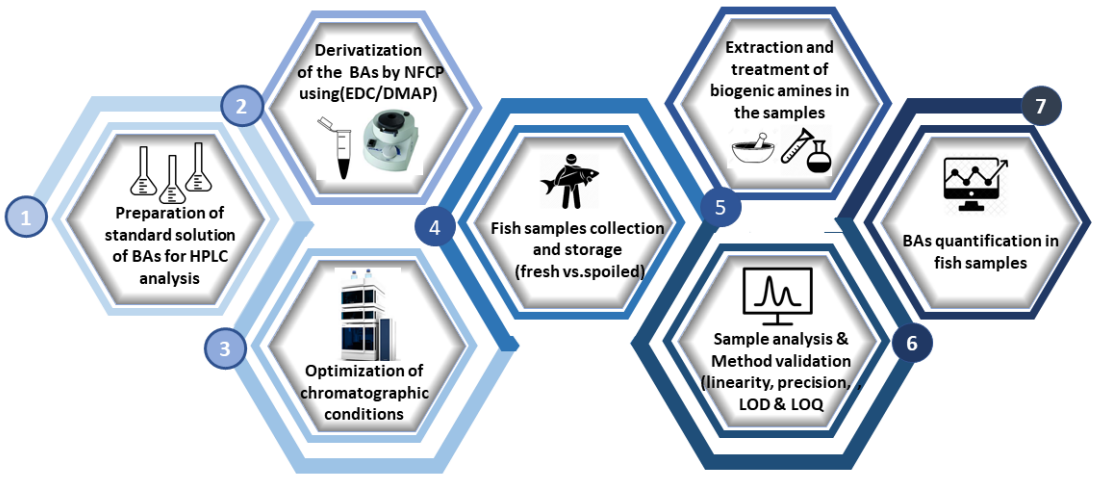


Figure 4. The effect of pH on the labeling reaction of NFCP-BAs. Chromatographic conditions: Poroshell EC- C18 column (100 mm, 3.0 mm, 2.7 µm), ACN-water (80 %v/v), flow rate 200 µL min-1; λex/λem: 380/460 nm, column temperature 40 o C; Reaction conditions: [NFCP] 0.3 mmolL -1 , [BAs] 0.025 mmol L -1, [EDC/DMAP] 0.1/0.4 mmolL -1 , Acetonitrile, 65 o C, 40 min.



Scheme 2: A graphical illustration of the overall study.

Among the analytical columns of varying particle and dimensions, a poroshell C18 column (100 mm × 3.0 mm × 2.7 µm) was found to be most efficient to elute the derivatives in good resolution and shortened analysis time. The best mobile phase was tested with acetonitrile-water composition, along with

phosphate, formate, and acetate buffers at pH 8.5, and no noticeable changes were observed. However, later in the method development, we chose pure water to overcome the risk of the accumulation of salts in the column, which would affect its lifetime. The proportion of water was limited to 20% to avoid the quenching of peaks. The optimum excitation wavelength was selected after scanning the analytical signals for the derivatives at different excitation wavelengths. A wavelength of 380 nm was found to be the best for the detection of all of the derivatives studied. Moreover, the optimum emission wavelength was obtained by conducting multi-emission wavelengths at 380 nm using the fluorescence detector in the instrument. As a result, 460 nm was taken to be the optimum fluorescence wavelength for all derivatives throughout the run.

Besides the evident success of derivatization and HPLC separation, it was noted that a sole peak appeared along with the main derivative peak of BAs used for HPLC analysis, but that it was only 1/10 of the size of the main peak height (Figure 5a and 5b). Interestingly, the resolution of the peaks of interest is not affected by these peaks. A change of column and acetonitrile gradient to separate the daughter peak from the parent was unsuccessful. Finally, the four biogenic amines mixture was separated. The resultant chromatogram is shown in Figure 6.

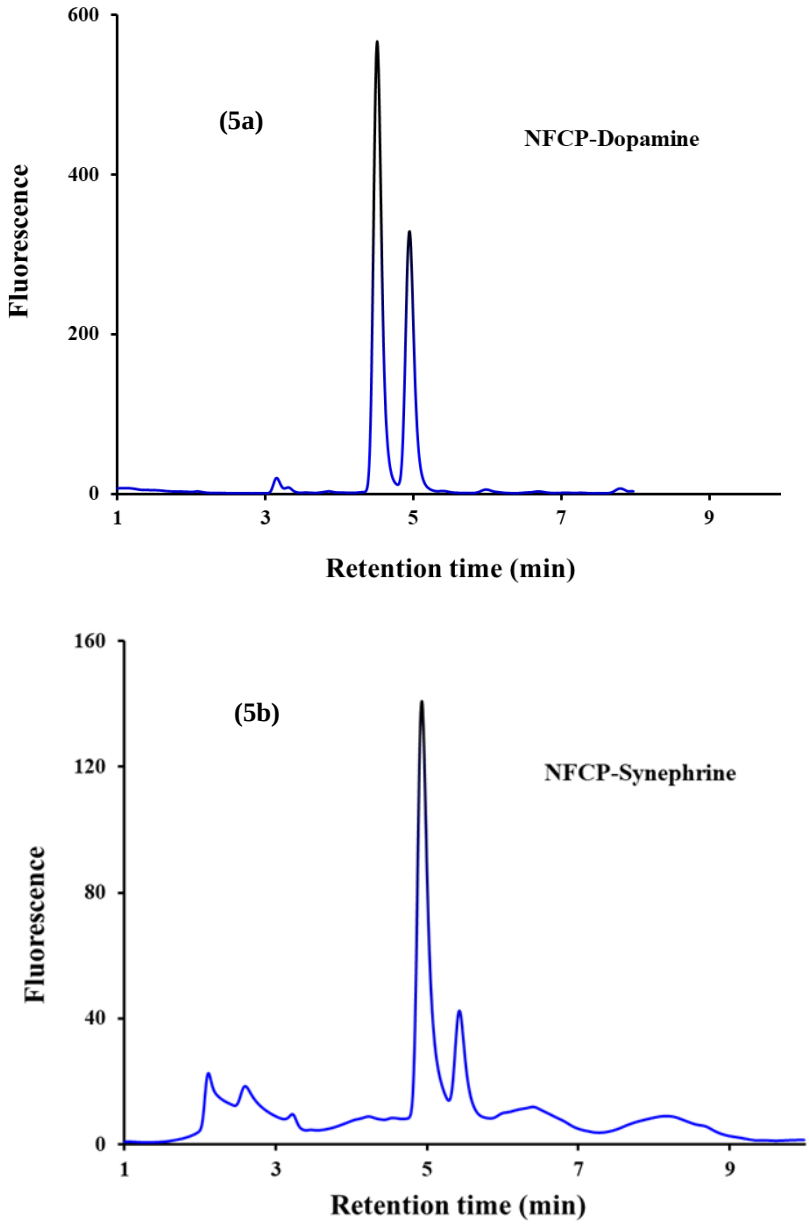


Figure 5a and 5b. Chromatograms of Dopamine and Synephrine derivatives. Chromatographic conditions: Chromatographic conditions: poroshell EC- C18 column (100 mm, 3.0 mm, 2.7 µm), ACN-water (80 %v/v), flow rate 200 µL min^{-1}, λex/λem: 380/460 nm, Column temperature 40 °C; Reaction conditions: [NFCP] 0.3 $mmolL^{-1}$, [BAs] 0.025 mmol L^{-1}, [EDC/DMAP] 0.1/0.4 $mmolL^{-1}$, Acetonitrile, 65 °C, 40 min.

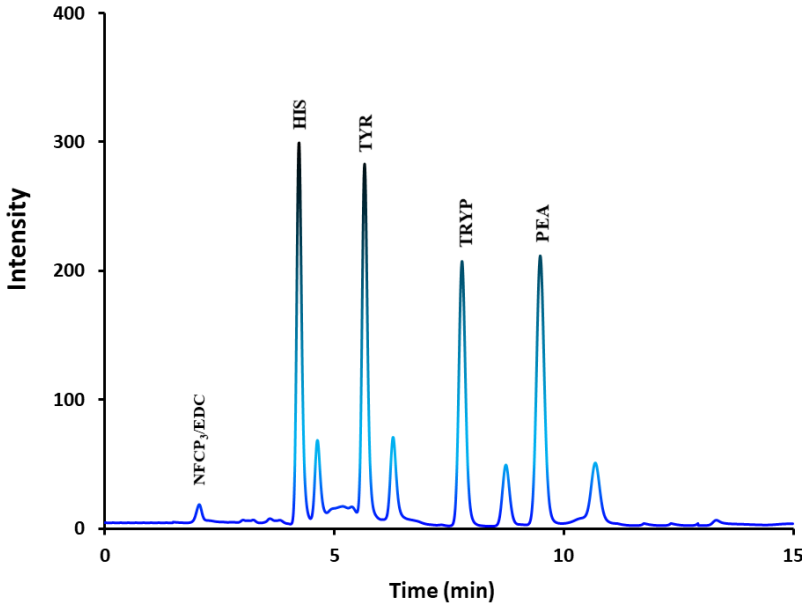


Figure 6. Chromatogram of four biogenic amines derivatives with NFCP. Chromatographic conditions: poroshell EC- C18 column (100 mm, 3.0 mm, 2.7 µm), ACN-water (80 %v/v), flow rate 200 µL min^{-1}; λex/λem: 380/460 nm; Reaction conditions: [NFCP] 0.3 mmol L^{-1}, [BAs] 0.025 mmol L^{-1}, [EDC/DMAP] 0.1/0.4 $mmolL^{-1}$, Acetonitrile, 65 °C, 40 min. NFCP peak at 2.1 min, histamine at 4.2 min, tyramine at 5.6 min, tryptamine at 7.7 min, phenylethylamine at 9.4 min.

The four derivatives were characterized by MS/MS and the fragmentation patterns identified. Characterization was brought about by the direct infusion of the standard solution derivative of each analyte at their retention times when passed through the HPLC column with the same mobile phase and flow rate. In this study, the molecular ions and diagnostic fragments of all the NFCP – BAs derivatives (except of histamine. because of their low abundance), were generated by negative ionization mode, $[M-H]^-$. Running the Q1 scan for the peaks that appeared at specified retention times revealed that the molecular mass of smaller peaks matched well with the abundant production. In response to this, it is suggested that NFCP-BAs derivatives exhibit cis-trans isomerism and eluted as two closer peaks since they have close polarity. It can be presumed that the dominant peak belongs to the trans-form and the cis-isomer migrates slowly and is then eluted as a baby peak. This trend was observed in the product ion spectra of all derivatives in which the parent peaks are at the m/z 562.3, 578.3, and 601.3, corresponding to the $[M-H]^-$ of the derivatives in trans-form (Figure 7). On the other hand, the small intensity peaks registered at m/z 563.62, 579.3, and 602.3 reflect the cis-form. The loss of CF_3 and C_4H_8O fragments were shown in the spectra of TYR-derivative at m/z 509.1 and 541.3, but seem to be negligible. The $[M-H]^-$ spectrum of HIS derivative shows the parent peak at m/z 577.5.

3.2 Analytical parameters and sample analysis

Under the optimized derivatization, separation, and detection conditions, calibration curves for the four derivatives studied were linear, with the calibration equation of 42.35±(0.48)x+59.00±(15.22), 72.40±(1.00)x-106.65±(25.46), 74.50±(0.97)x+536.41±0.74) , 85.47± (0.86)x+220.93±(27.31) with r^2 values of 0.997, 0.996, 0.998 and 0.996 for HIS, TYR, TRYP and PEA, respectively. Since the volume of the injected sample was 1µL, the lowest detection limits (LOD) for PEA, HIS, TYR, and TRY are 0.9, 1.1, 1.1, and 1.2 µmol L^{-1} and quantification (LOQ) limits are 3.2, 3.6, 3.5 and 4.1 µmol L^{-1}, respectively. The comparison of initial response with the peak areas of the BAs derivatives stored in the instrument at room temperature for one week showed only minor variations of 1.1 to 9.9 %, highlighting its considerable stability. For assessing the stability of derivatives in the fish matrix, fresh tuna solution was spiked with BAs and the differences in peak areas were analyzed for three consecutive days. The RSD values within 2.87 seem to be satisfactory for the first two days but a decrease in the absolute peak area on the third day emphasizes the derivative degradation after two days at room temperature.

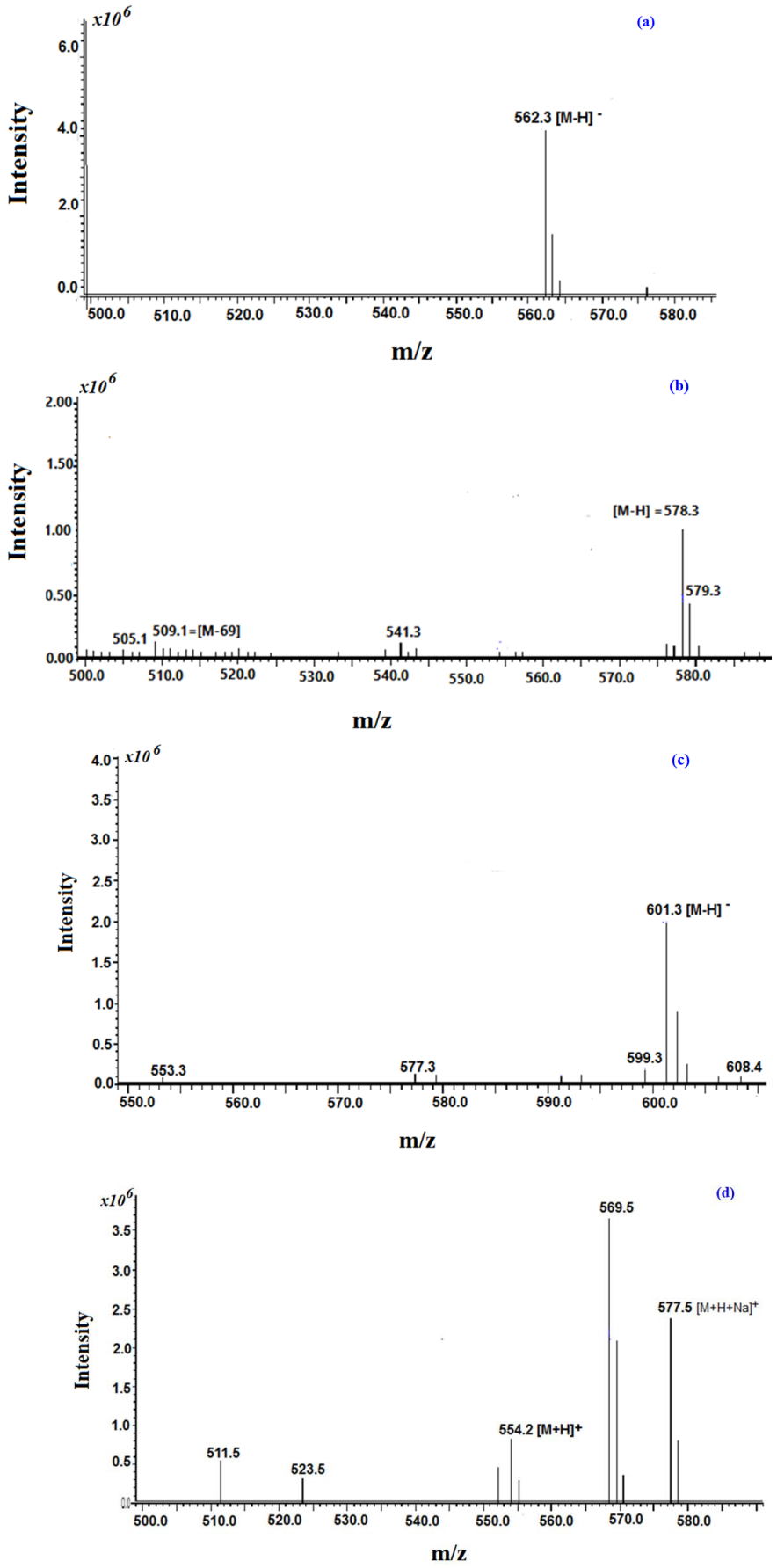


Figure 7. The LC-MS/MS spectra of NFCP- (a) PEA (b) TYR (c) TRY and (d) HIS derivatives.

The values obtained for intra-day precision range from 0.18-1.54, 0.32-1.41, 0.22- 1.06, and 0.18-1.37 for HIS, TYR, TRYP, and PEA, verifying the excellent repeatability and reproducibility of the entire analytical procedure (Table 1).

Table 1. Intraday precision, stability of derivatives in the matrix, and the matrix effect.

Biogenic amine	Precision (%RSD)			Stability (%RSD)[b]			Matrix effect ±SD (Sm/Ss)×100%
	4[a]	10[a]	40[a]	2.5	5	10	
HIS	1.55	1.30	0.18	1.05	0	1.05	76.9 ± 3.7
TYR	1.41	0.93	0.32	1.19	-	1.04	81.1 ± 12.0
TRY	1.06	0.75	0.22	2.89	1.07	2.87	87.7 ± 1.7
PEA	1.37	1.20	0.18	0.50	0.50	1.25	NM

The superscripts 'a' denote concentrations in µmol L-1, 'b' stability measured in fresh tuna matrix, NM=not measured. HIS=histamine, TYR=tyramine, TRY=tryptamine, PEA=phenylethylamine. RSD=rrelative standard deviation, SD= standard deviation. Sm= slope of calibration curve of the analyte in matrix solutions, Ss= slope of calibration curve of the analyte in standard working solutions.

To assess the reliability and selectivity of the HPLC method, the matrix effect was tested by adding 0 to 25 µmol L^{-1} standards to the fish sample solution, HIS, TYR, and TRYP (PEA, not measured). The slopes of the linear calibration curves for the studied biogenic amines in the solvent were compared with the slopes of the same analyte in the fresh or spoiled fish matrix. The matrix effect values obtained were 76.9, 81.1, and 87.7 % of the fish solution, respectively, which inferred the strong effect of the matrix solution on the complete derivatization of the mentioned BAs. The fish extract is expected to also contain various amounts of biogenic amines other than the ones studied in this work and this broad matrix contribution may consume some amounts of the derivatization reagents leading to variable chances of the studied analytes to be derivatized. The selectivity of the assay was evaluated by analyzing extracts from three types of fish samples, and no interference from the matrix was observed with the evaluated BAs in any of the samples studied as shown in Figure 8. However, the presence of additional peaks, other than the studied BAs, was observed in the chromatogram (Figure 8).

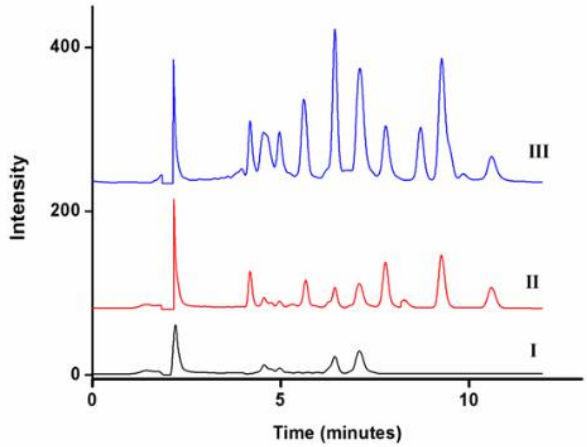


Figure 8. Chromatograms of biogenic amines (HIS, TYR, TRYP, PEA) spiked in the fish matrix solution at concentration, (I) 0, (II) 5, and (III) 10 µmol L^{-1}, after derivatization with NFCP at optimum conditions.

3.3 Method application

Contemplated as major indices of fish spoilage and poisoning, the maximum acceptable limit of histamine in fish was adopted by European Union and The U.S. Food and Drug Administration as 200 mg Kg^{-1} and 50 mg Kg^{-1}, respectively **[49-50].** The proposed method was evaluated in the determination of histamine in two types of fish samples (fresh and spoiled white tuna). The result indicates 0.4 µg g^{-1} of histamine in fresh goatfish as well as tuna samples and as expected, higher content (4.1 µg g^{-1}) in spoiled tuna. On the other hand, no traces of histamine were found in the fresh cobia sample (**Table 2**).

Table 2. Quantification of histamine in different fish samples.

Sample	Found	
	µmol L^{-1} ±SD	µg/ g sample
Fresh tuna	0.80±0.23	0.4
Spoiled tuna	7.38±0.66	4.1
Fresh goatfish	0.79±0.23	0.4
Fresh cobia	ND	ND

ND-not detected

3.4 Comparison with other methods

To reveal the uniqueness of NFCP over other frequently used reagents for amine labeling, a comparison was done with the performance parameters of some existing methods. It is inferred from Table 3 that the method developed has an advantage over other existing methods for some substances. Among the reported reagents from references [51-61], the amine derivatives were detected by fluorescence (FL) response, whereas CNBF, DNBZ-Cl derivatives were by their absorption at 254 and 260 nm, respectively, and DEEMM by photodiode array (PD) detector at 280 nm. Regarding the detection wavelength, except in OPA and DNS-Cl (523 nm), all other derivatives were reported in the wavelength range between 315 and 440 nm. The typical intra-molecular charge transfer (ICT) characteristics of pyrazoline derivatives are reflected in the optimal emission wavelengths of NFCP- BAs at 460 nm. Moreover, the derivatization reaction is simple, being a single step of adding and mixing the reagents in less than one minute, and has reasonable reaction completion at 20 minutes. It does not require a lengthy procedure in extracting the derivative from the reaction media or derivative cleanup before injection. This is outstanding when revisiting reagents (FMOC-Cl, OPA, OPA/2-ME, BC, DNBZ-Cl, and DNS-Cl) which involve complications in the formation of multiple derivatives and significant interference if the excess reagent is not extracted or carefully controlled before chromatographic separation.

Table 3. Comparison of the derivatization conditions and detection limit of some common reagents reported for amines.

Reagent	**Matrix**	**Derivatization Conditions**				**LOD**	**Ref.**
		Time	**Temp**	**Alkaline Medium**	**pH**	**µmol L^{-1}**	
Fmoc-Cl	Fish	30	RT	Borate buffer.	10	1.97×10^{-7}- 3.12×10^{-6}	50
OPA	Fish	>5	RT	Potassium borate buf	12	13.49*	51
OPA-2ME	Milk, Beer	>1	RT	Borate buffer.	10	9×10^{-5}-3.1×10^{-4}	52
DNS-Cl	Wine	25	NR	NaOH	11	0.02-0.31	53
Dbs-Cl	Wine	116	70	HCl–TDPA	8.2	0.08-0.69	54
BC	Wine	10	25	NaOH	NR	6.44-18.22*	55
BCEC-Cl	Shrimp catsup	3	RT	Borate buffer.	9	1.77×10^{-12}-1.44×10^{-11}	56
EAC	Food sample[1]	6	55	Borate buffer.	9.7	2.2×10^{-3}-3.3×10^{-3}	57
AQUA	Musts, Wines	NR	RT	Borate buffer.	8.8	4.53×10^{-3}-0.20	58
DNBZ-Cl	Food sample[2]	3	RT	NaOH	NR	0.95-6.29	59
DEEMM	Wine	150	80	Borate buffer.	9	0.20-0.39	60
CNBF	Beer	30	60	Borate buffer.	9.5	0.056-0.87	61
NFCP	Fish	40	65	Phosphate buffer.	8.5	0.9 - 1.2	current work

Reagent: Fmoc-Cl-Fluorenylmethyloxycarbonyl chloride, OPA- *o*-phthaldialdehyde, OPA-2ME- *o* - Phthaldialdehyde/ 2-mercaptoethanol, Dns-Cl- Dansyl Chloride, Dbs-Cl- Dabsyl Chloride, BC-Benzoyl chloride, BCEC-Cl- 2-(11H-benzo[a]carbazol-11-yl) ethyl chloroformate, EAC-ethyl-acridine-sulfonyl

chloride, CNBF- 2-Chloro-1,3-dinitro-5-(trifluoromethyl)benzene (4-chloro-3,5-dinitrobenzotrifluoride), DNBZ-Cl-3,5-dinitrobenzene chloride, DEEMM- diethyl ethoxymethylenemalonate, AQC- 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; Food sample[1]- Beer, cheese, fish, sausage and shrimp, Food sample[2]- Fermented cabbage juices, soy sauces, misos (soy pastes), fermented fish sauces, and anchovy paste. Time-min, Temperature-°C, RT-Room temperature, NR-Not reported, The superscript '*' denotes LOD in samples.

One key feature that stands out is the stability of NFCP- BAs derivatives on exposure to daylight when compared to OPA and Dns-Cl derivatives [**40, 61-62**], which are light sensitive. The ability of NFCP to react with various types of BAs, including primary and secondary, makes it superior as a derivatization reagent over awards its transcendent specialty over OPA and its analogs, which react only with primary amino groups. While comparing the LODs reported for NFCP (0.9-1.2 µmol L^{-1}) with other reagents, the proposed method can provide a potential alternative in the determination of amines. Above all, NFCP, itself is highly fluorescent, can be synthesized easily in two steps from available, less expensive starting materials, and possesses a long shelf life. However, despite all the positive outcomes of NFCP derivatization and HPLC analysis, the non-separable co-eluted peaks in the chromatogram need to be addressed by rescheduling the method development with chiral columns and other chromatographic parameters.

4. Conclusion

This work presents a pyrazoline-based compound, NFCP as an appealing pre-column derivatization reagent, which facilitates the rapid assay of BAs of different nature. In contrast to the more labour-intensive procedure involving hazardous organic solvents, NFCP seems to be highly reactive towards the selected BAs under mild reaction conditions. The method developed showed linearity for standard BAs quantification in the range of 0.5 to 100 µmol L^{-1} and detection limits of 0.9 - 1.2 µmol L^{-1}. The nuisance of less intense isomeric peaks that appears in the chromatogram is compensated for by the short elution time, feasibility, sensitivity, and reproducibility of this method. The difference in the histamine level estimated in fresh (0.4 µg g^{-1}) and spoiled white tuna (4.1 µg g^{-1}), proved the feasibility of this method to assess histamine toxicity hazards of seafood. With minor modification, there is ample scope with this method to broaden the application of NFCP in food industries and biomedical analysis.

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Conflict of interest

The authors declare no conflict of interest.

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