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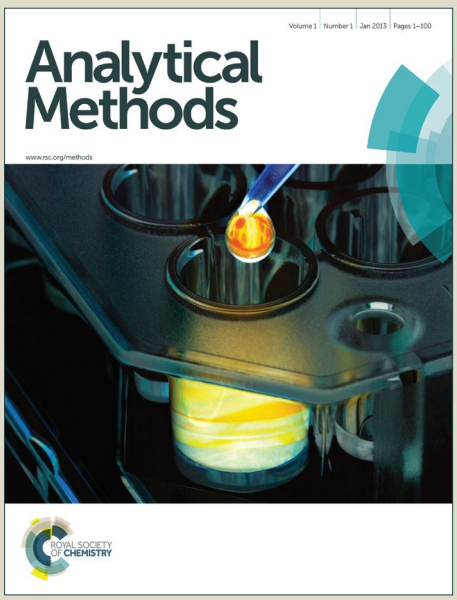

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ARTICLE

3-naphthyl-1-phenyl-5-(4-carboxyphenyl)-2-pyrazoline – A pyrazoline based heterocyclic dye as a fluorescent label for biomolecules containing amino group and its evaluation using HPLC

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Beena Varghese[a], Saleh N. Al-Busafi[a], FakhrEldin O. Suliman[a] and Salma Al-Kindy[a]

The use of 3-naphthyl-1-phenyl-5-(4-carboxyphenyl)-2-pyrazoline as a pre-column derivatization agent for the HPLC analysis of biomolecules containing $–NH_2$ group is proposed in this article. The derivatization conditions to obtain quantitative reaction were optimized by considering different parameters (time, temperature, pH and reagent concentration) using seven amino acids as model analytes. The resulting derivatives were well separated on a reversed-phase column with isocratic elution profile and detected fluorimetrically at excitation and emission wavelength of 370 and 480 nm respectively. The proposed method was validated for accuracy, precision, sample stability, linearity, detection and quantification limits and were all found to be satisfactory. Through this study, we are forecasting the emergence of pyrazoline based heterocyclic dyes as organic fluorescent markers for the labelling of diagnostically important compounds, for their determination and quantification of practical interest.

Introduction

Recently, fluorescent heterocyclic dyes are gaining more attraction in bioanalytical chemistry due to its favourable photophysical properties compatible with different separation techniques. An important aspect of incorporating heterocycle moiety while designing fluorescent dyes includes its efficient photophysical properties and its crucial role in charge transfer process throughout the system. Pyrazolines, a group of electron–rich nitrogen containing heterocycles constitute a class of fluorescent compounds and a large number of pyrazoline core molecules have been prepared and evaluated for its optical and electronics as well as pharmacological activities [1-3]. Though variously fashioned chromophoric systems like coumarins, phenylisothiocyanate (PITC), dansyl chloride (Dns-Cl), ortho-phthaldialdehyde (OPA), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), 9-fluorenylmethyl chloroformate (FMOC-Cl), 4,7-phenanthroline-5,6-dione (phanquinone), naphthalene- 2, 3- dicarboxaldehyde (NDA), 4-chloro-7-nitrobenzooxadiazole (NBD-Cl), 4-fluoro-7-nitro-2,1,3-benzooxadiazole (NBD-F) [4-15] etc. have been addressed as fluorogenic reagents and widely used in High-performance liquid chromatography (HPLC) coupled with various detection systems.

[a] *Department of Chemistry, College of Science, Sultan Qaboos University, Box 36, Al-khod 123, Sultanate of Oman. Email: fsuliman@squ.edu.om, alkindy@squ.edu.om; Phone: +968-24141480, fax: +968-24141469.*
[b] *Address here.*
[c] *Address here.*
† Footnotes relating to the title and/or authors should appear here.
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However, the question as to why compounds bearing pyrazolines are not much evaluated as potential candidates for pre - or post-column derivatization of biomolecules and its further application in different separation techniques. Even though, among the five membered heterocycles stand out due to their simple structure, blue light emitting efficiency, large extinction coefficient, high quantum yields and excellent medium sensitivity. Earlier works from our group reported the synthesis, spectroscopic characterization and photophysical response of novel pyrazoline-based fluorophores like 3-naphthyl-1-phenyl-5-(4-carboxyphenyl)-2-pyrazoline (NPCP) [16] and 3-naphthyl-1-phenyl-5-(5-fluoro-2-nitrophenyl)-2-pyrazoline (NPFP) [17] in different media and all the results provided a fruitful insight about the importance of pyrazoline based dyes in fluorophore family.

Following our interest to extend our preliminary results, in this work our early reported dye NPCP is evaluated as fluorescent labelling agent because both the amino and hydroxyl group of bio molecules offer possibilities for labelling and it has been demonstrated that NPCP can be easily converted to its corresponding derivatives by simple straightforward synthetic procedure. In this compound we are considering carboxylic acid moiety as a trigger, which has the potentiality to be tagged with biomolecules through functional group conversion. Even more it can be easily attacked by target groups on its conversion into acid chloride or by using activation reagents. To develop simple sensitive liquid chromatographic method with fluorimetric detection our attention was directed to some selected amino acids as model biomolecules to examine the feasibility of the new reagent. The study also included LC-ESI-MS/MS analysis that provides structural characterization of NPCP-Amino acids (NPCP-AAs) derivatives.

Experimental

Reagents and chemicals

All the chemicals and solvents used in this study were of analytical grade and purchased from Sigma-Aldrich Chemical Company (Sigma-Aldrich, USA). Ultrapure water was obtained in the laboratory using a Milli-Q water purification system (Millipore, Billerica, MA, USA). Synthesis of NPCP was reported in [16].

A stock solution of NPCP was prepared at a concentration of 0.5 mmol L^{-1} using acetonitrile as solvent and filtered through a 0.45 μm nylon membrane filter. The solution was stable at room temperature. 1 mmol L^{-1} stock standard solutions of AAs were prepared in phosphate buffer of pH 8.5 and stored at 4 ^{0} C. The working solutions of AAs were prepared by sequential dilutions with phosphate buffer. The activation agents 2, 2' – dipyridyl disulphide (DPDS) (50 mM) and triphenyl phosphine (TPP) (50 mM) were prepared with acetonitrile just prior to use.

Apparatus

A high-performance liquid chromatography system (Waters, Milford, MA, USA), which consists of Waters 1525 Binary HPLC Pump , Waters 2489 UV/Visible detector, Waters 2475 Multi λ fluorescence detector and Breeze 2 software was used for analysis and chromatographic data processing. Fluorescence was monitored at excitation and emission wavelengths of 370 and 480 nm respectively. Peak area was used as a measure of the detector's response. A Shimadzu (model multispec-1501) UV-Vis spectrophotometer (Shimadzu, Japan) and a Perkin Elmer (model LS 55) Luminescence spectrometer (Perkin Elmer, USA) were used to collect absorption and fluorescence spectra, respectively. A reversed phase Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 μm) purchased from Sigma Aldrich, USA was used for separation. The mass spectrometer Triple Quad LC/MS from Agilent Technologies, USA was equipped with an electrospray ionization (ESI) source (model 6460). The mass spectrometer system was controlled by Mass Hunter software.

Derivatization procedure

To a vial (2 mL) were added 1 mL of NPCP solution (0.25 mmol L^{-1}), 50 μL of DPDS and 50 μL of TPP solution (1.25 mM). While vortex mixing, 50 μL of amino acid mixture was added to this solution and made up to the mark with acetonitrile. The mixture was heated at 60 ^{0} C for 15 minutes and then left to cool at room temperature. A 10 μL volume of the crude solution was injected directly onto the HPLC.

Sample Preparation and recovery of amino acids from spiked juice samples

Fresh oranges were purchased from the local market and the orange juice were hand-squeezed in the laboratory just before the analysis. Then the extracts were centrifuged for 20 min at 10,000 rpm in a Fisher Scientific centrifuge (CLINISPIN 642 E, horizon, USA), and the supernatant was filtered through a 0.45-µm filter membrane, diluted 10-fold with phosphate buffer of pH 8.5 and stored at 4 ^{0} C.

Identification of amino acids was based on the comparison between the retention time of the standards of the amino acids and those in orange juice and was confirmed by spiking each individual standard amino acids of increasing concentrations. The recovery study was carried out by adding each amino acid at 3 concentration levels to an orange juice that was previously analysed (Table 4). The resulting spiked samples were analysed in duplicate.

HPLC and LC-MS/MS conditions

The separation of AAs after labelling with NPCP were carried out by reversed-phase chromatography using an isocratic elution of a 6:4 acetonitrile-water mixture containing 0.1% acetic acid at a flow rate of 0.5 mL min^{-1}. Chromatographic peaks were identified by injecting individually derivatized amino acid solution and confirmed by LC-MS/MS. The MS conditions were as follows: gas temperature 300 ^{0} C, Gas flow 5 L min^{-1}, Nebuliser gas 50 psi, Sheath gas temperature 375 ^{0} C, Sheath gas flow 12 L min^{-1}, Capillary voltage 4000 V (positive), Nozzle voltage 500 V, Chamber current 0.27 μA. The elution profile was isocratic using 6:4 acetonitrile – water containing 0.01% formic acid and the flow rate was 0.3 mL min^{-1}. The type of column, solvent system and MS parameters were optimized for better analysis of the chemical profiling of derivatives. Agilent EC18 column (2.1 cm × 100 mm × 1.8 μm) was selected for providing fast and efficient separations at lower column pressures. The fragmentor voltage and the cell accelerator voltage was kept at 5 V and 3 V respectively.

Method validation

The analytical method was validated to demonstrate the stability, response linearity, recovery, limit of detection (LOD), limit of quantitation (LOQ), precision (repeatability) and accuracy (recovery) of samples. Linearity was investigated at six levels in the range from 0.1-1 mg L^{-1} of amino acid concentration. Three replicates were performed at each level and the calibration curves were obtained with the average of peak area of three replicates. Standard curves were fitted by linear regression equation A = ac+ b, where c is the concentration and A is the peak area. The precisions were assessed by determining the relative standard deviations (RSD) of standard solutions. The accuracy of the method was evaluated considering two different parameters: the absolute percent error and % recovery. The absolute percent error was calculated for each well-recovered amino acid of unknown concentration. The LOD and LOQ were calculated on the basis of standard deviation of the response and the slope obtained from the linearity plot of calibration graph. For the proposed HPLC method, LOD and LOQ have been established by the determination of the signal/noise ratios of 3:1 (LOD) and 10:1 (LOQ).

Results and Discussion

Optimization of derivatization and separation conditions

Based on consideration of structural analogy and reactivity of NPCP-OCl with chloroformates [18], initially we were focussing on developing derivatization conditions involving the carbonyl chloride which readily undergoes nucleophilic substitution reaction with amino group to yield the amide derivative. Unfortunately it was found that the very broad by-product peak of NPCP-OH masks the adjacent peaks and also the chromatogram was disturbed by a lot of noisy signals with higher fluorescence intensity. To obtain the best potential of NPCP in HPLC, the derivatization of NPCP with seven amino acids namely, arginine, lysine, proline, serine, glycine, threonine and tyrosine were studied in detail.

Among the different catalysts tested in preliminary experiments, the combination of DPDS and TPP [19 - 20] was found to be suitable for using as an activation agents to achieve a rapid quantitative derivatization without interfering peaks. Both reagents are freely soluble in acetonitrile and was found that concentration in excess of 50 mmol L^{-1} did not offer any advantage to the reaction yield and time. Based on our previous experiences from synthesis, acetonitrile was selected as a reaction solvent for performing the derivatization reaction.

At first the effect of different concentration of the reagent (0.025-0.375 mmol L^{-1}) on the derivative peak area was investigated, as shown in Fig. 1. For the complete derivatization excess reagent was used, when the amount of reagent (0.25 mmol L^{-1}) was three times more than that of amino acid concentration (0.08 mmol L^{-1}), the derivatization peak area was found to be stable. The derivatization reaction appeared to be highly dependent on pH of reaction medium and several kinds of basic media were tested including borate buffer, phosphate buffer and carbonate buffer. Both, borate and phosphate were found to be suitable and the comparison of derivatization yield indicates that phosphate buffer was the best one (data not shown).

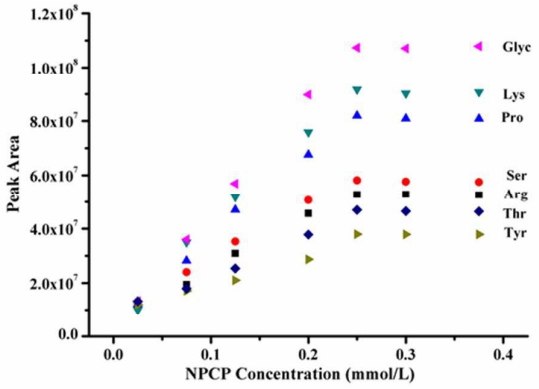

Fig. 1. Effect of NPCP concentration on the labelling reaction of NPCP with amino acids. Chromatographic conditions: Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 μm); column temperature 30 ^{0} C; flow rate 0.5 mL min^{-1}; λex/λem= 370/480 nm; [AAs] = 10 mg L^{-1}. Reaction conditions: DPDS/TPP, Phosphate buffer (pH 8.5), Acetonitrile, 60 ^{0} C, 15 min.

The influence of diverse pH values on the peak areas was also investigated using phosphate buffer and the effect of pH of

phosphate buffer (0.01 mol L^{-1}) on the labelling reaction of NPCP with the amino acid proline was shown in Fig. 2. The results indicated that the responses of NPCP-AAs derivatives were very low when the buffer pH<7 and all the examined amino acids achieved the maximum derivatization yield at pH 8.5. This was probably due to deprotonation of nitrogen in amino acids (pKa value of amino acids for ammonium ion = 8.95 – 10.60) at the basic condition and act as a strong nucleophile which readily undergoes nucleophilic acyl substitution reaction [21]. In order to manage both, the amount of buffer species in the reaction mixture and the solubility of analytes, the amino acids are dissolved in phosphate buffer (pH 8.5) and used in subsequent derivatization procedure.

The effect of reaction time on labelling reaction was examined at various temperatures to achieve the best derivative yield. The time course of derivatization reaction with the optimal conditions were measured at room temperature and found that a clean reaction occurred at room temperature, but times in excess of 45 minutes were needed for a maximum response. At first the effect of temperature on fluorescence intensity was investigated in the range of 20 ^{0} C to 80 ^{0} C. Results showed that the yields of derivatization increased quickly with the rising temperature and reached a plateau at 60 ^{0} C (Fig. 3).The time effect studies were carried out to a maximum of 45 minutes and the optimum reaction time for obtaining maximum peak area was found to be 15 min at 60 ^{0} C and also unidentified peaks were minimal in this reaction conditions.

Based on these observations, the reaction time of 15 min at 60 ^{0} C in acetonitrile- buffer medium containing 0.25 mmol L^{-1} of NPCP together with 50 mmol L^{-1} of DPP/TPP was selected as an optimal reaction conditions for tagging procedure. Scheme for the derivatization reaction of NPCP with model analyte proline is shown in Fig. 4.

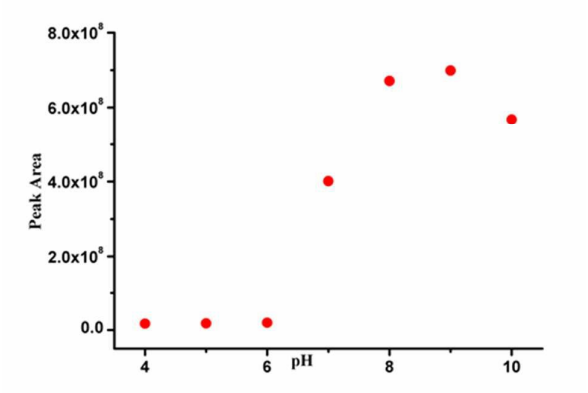

Fig. 2. Effect of pH of phosphate buffer (0.01 mol L^{-1}) on the labelling reaction of NPCP with amino acid Proline. Chromatographic conditions: Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 μm); column temperature 30 ^{0} C; flow rate 0.5 mL min^{-1}; λex/λem= 370/480 nm; [NPCP] = 0.25 mmol L^{-1}; [Proline] = 10 mg L^{-1}. Reaction conditions: DPDS/TPP, Acetonitrile, 60 ^{0} C, 15 min.

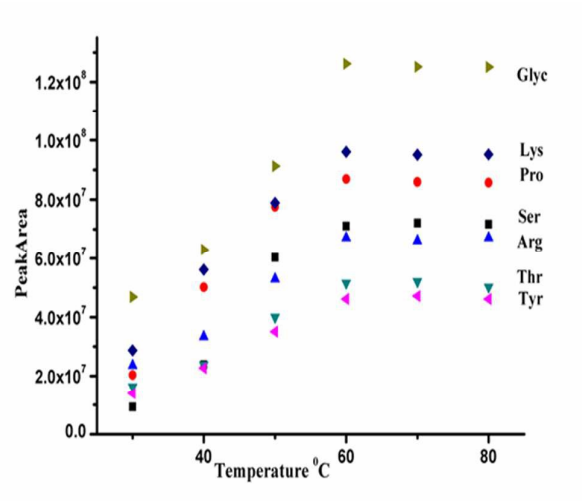

Fig. 3. Effect of temperature on the labelling reaction of NPCP with amino acids. Chromatographic conditions: Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 μm); column temperature 30 ^{0} C; flow rate 0.5 mL/min; λex/λem= 370/480 nm; [NPCP] = 0.25 mmol L^{-1}; [AAs] = 10 mg L^{-1}. Reaction conditions: DPDS/TPP, Acetonitrile, 15 min.

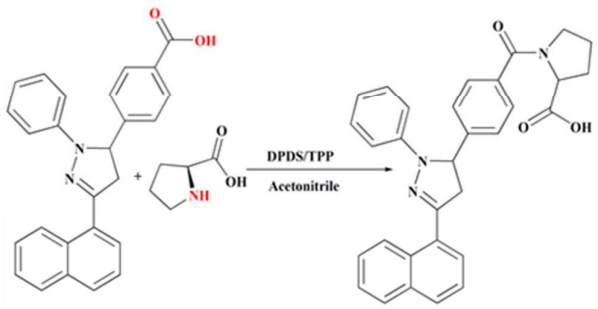

Fig. 4. Derivatization reaction of NPCP with proline

Reaction conditions: DPDS/TPP, Phosphate buffer (pH 8.5), Acetonitrile, 60 ^{0} C, 15 min.

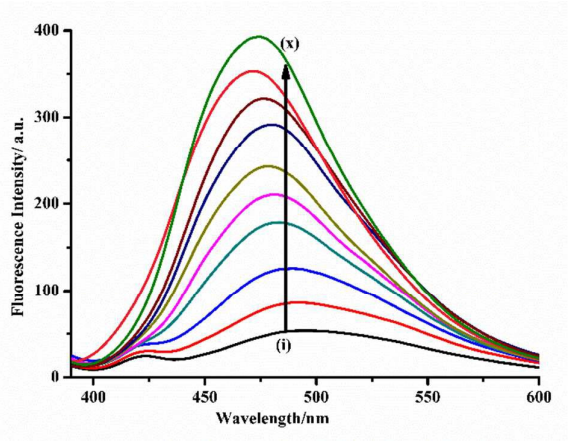

Fig. 5. Emission spectra of NPCP in water as a function of acetonitrile percentage composition. Curves (i) to (x) correspond to 100% , 90%, 80, 70% , 60%, 50%, 40%, 30%, 20%, 10% water (v/v), respectively.

The effect of organic solvent on the fluorescence emission of NPCP was further investigated by using water-acetonitrile mixture (Fig. 5). The emission maximum of NPCP in water (497 nm) is blue-shifted with a sharp increase in fluorescence intensity as the content of organic component (10 to 90%) increases showing a relationship between the emission maximum, fluorescence intensity and v/v % of solvent composition.

In order to establish the developed method in HPLC system, various parameters were optimized to achieve the maximum separation of analytes within short time interval. It was observed that the fluorescence of AAs derivatives was also strongest in acetonitrile and much weaker in water, regarding the fluorescence intensity and shift in emission maximum 6:4 acetonitrile-water composition was selected as a compromise. Also, it was found that the retention and resolution of these derivatives was significantly dependent on the pH of the mobile phase. The results showed that for most NPCP-AAs derivatives, the maximum excitation and emission wavelengths were at 370 and 480 nm respectively. Thus the fluorescence detection was carried out by setting the emission wavelength at 480 nm and the excitation wavelength at 370 nm. The separation of derivatized amino acids was studied by using a reversed-phase HPLC column and eluting with binary solvents.

The effect of buffers on the separation was also investigated by using phosphate and acetate buffer (pH 3-6) and it was found that the peaks of the derivatives were more intense on increasing the pH in line with our early observations demonstrating highly fluorescent NPCP derivatives in basic media. Unfortunately, under these mobile phase conditions poor separation and overlapping peaks were observed, particularly for early eluting AAs derivatives. To overcome this poor separation conditions, a number of acids (acetic, trifluoroacetic and phosphoric acid) were examined at various concentrations. While adding 0.1% acetic acid as acidic modifier, significant changes were observed on both separation and peak shape and an isocratic instead of gradient elution was found to be capable of furnishing a rapid separation at a flow rate of 0.5 ml min^{-1}. A typical chromatogram obtained under these conditions is shown in Fig. 6.

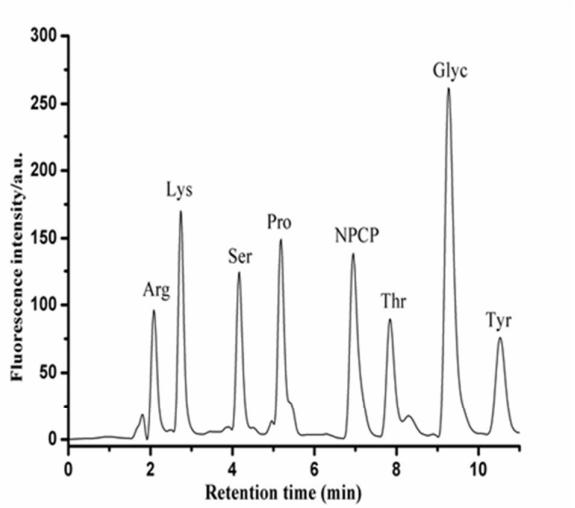

Fig. 6. Chromatograms for amino acid derivatives. Chromatographic conditions: Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 μm); column temperature 30 ^{0} C; flow rate 0.5 mL min^{-1}; λex/λem= 370/480 nm; [NPCP] = 0.25 mmol L^{-1}; [AAs] = 10 mg L^{-1}; Peaks are labelled with abbreviations: Arg (Arginine 2.0 min), Lys (Lysine 2.7 min), Ser (Serine 4.1 min), Pro (Proline 5.1 min), Thr (Threonine 7.8 min), Glyc (Glycine 9.2 min), Tyr (Tyrosine 10.5 min), NPCP (3-naphthyl-1-phenyl-5-(4-carboxyphenyl)-2-pyrazoline).

Mass spectral analysis of the derivatives

NPCP reacts with the -amino group of amino acids in basic medium by eliminating water molecule to give the corresponding fluorescent amide derivatives and these peaks were considered to be the products of the reaction of amino acids with NPCP. The mass spectrometric features of these amino acid derivatives were investigated using a tandem TQ-MS. The representative MS/MS profile of glycine derivative is shown in Fig. 7 and for other amino acids in Fig.S1.

NPCP derivatized amino acids, followed by LC-ESI-MS/MS enables the detection of each derivative by both SIM mode (selected ion monitoring) and scan mode. The protonated molecule $[M+H]^{+}$ of the arginine derivative was observed as the base peak (m/z 549), while the sodium adduct was represented by the peak at m/z 571. The proline derivative was identified as a highly intense peak at m/z 489. The loss of a phenyl group from the derivative resulted in the fragment with m/z 411. The spectrum of serine derivative showed the parent ion peak at m/z 481. The spectrum also included the

product ion at m/z 420 because of further loss of carboxylic group along with water molecule.

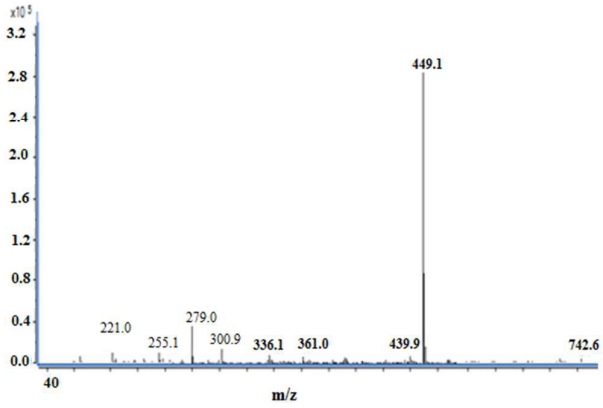

Fig. 7. The representative MS/MS profile for NPCP-glycine derivative.

The protonated molecule $[M+H]^+$ of the threonine derivative was observed at m/z 494. Cleavage of the derivative between amide HN-C produced fragment ion at m/z 391. Lysine possesses two potentially reactive amine groups that can be derivatized by NPCP. Lysine derivatized at one amino-group the parent ion peak is expected to be at m/z 521. Inspection of the spectrum of lysine-NPCP derivatives reveals the presence of a very small peak at m/z 521. However, the peak at m/z 893, which corresponds to double tagged lysine (NPCP-Lysine-NPCP), represents the most intense peak in the spectrum indicating that this is predominant species. The glycine derivative showed a prominent peak at m/z 449 of the parent ion. The spectrum of tyrosine derivative is characterized by intense peaks at m/z 556 and 578 corresponding to the respective $[M+H]^+$ and $[M+Na]^+$ ions. The peaks at m/z 221, 279 and 300 are common to all spectra. The peak at m/z 221 is attributed to a fragment containing pyrazoline ring attached to two phenyl groups. The unidentified peaks at m/z 279 and 301 found in all spectra may be due to the presence of the impurities like dibutylphthalate (plasticiser).

Stability of NPCP and its derivatives

Anhydrous acetonitrile solution of NPCP could be stored at 4 ^{0} C for at least two weeks, while comparing the derivatization yields for amino acids obtained with newly prepared NPCP solution showed no obvious difference. The stability of the NPCP-AAs in acetonitrile-buffer medium (each containing 5 mg L^{-1} of AA in phosphate buffer of pH 8.5) at room temperature after derivatization was studied over a period of two weeks, during which time they were analysed four times. The relative standard deviations for normalized peak areas varying by 1%-2% indicating no significant changes for the peak-area of derivatives over the time studied.

Quantitative analytical characteristics and comparison with other common used labelling reagents for amino acids in HPLC

As shown in Table 1, the linear regression coefficients (r^2) in the range of 0.9885-0.9963 for all the analytes showing a linear affiliation between the peak area and the amounts of the individual AAs (n=3) over the concentration ranges from 100-1000 µg L^{-1}. Detection limits is an important factor when the components in biological matrices were analyzed, particularly when they are present at low or trace concentrations.

The LOD and LOQ were established for individual amino acids and the minimum detectable amount for the proposed method is 12 - 34 µg L^{-1}, depending on the amino acid under consideration. The LOQ is estimated to be in the range of 39 - 116 µg L^{-1} for all the amino acids measured in this study. The reagent blank exhibited no potential interferences to the accurate quantitation of amino acids in derivatized solution. The accuracy of the method was evaluated at three unknown concentration levels (n=6) of AAs and quantify average peak area in calibration graph (Table 2). A good degree of accuracy was achieved for all analytes with recovery percentages ranging from 93 - 98%. The precision of the method was expressed as repeatability and % RSD in precision studies was found to be less than 3% indicating that the developed method is precise and accurate.

A wide variety of fluorescent labelling reagents have been proposed for the pre- and post-column derivatization of amino acids following by UV or fluorescence detection, certainly each with advantages and drawbacks. For example the o-phthaldialdehyde (OPA) method offers greater sensitivity but is only limited to primary amino acids and also OPA derivatives are often unstable. In case of reagents like phenyl isothiocyanate (PITC) and 9-fluorenylmethyl chloroformate (FMOC-Cl), the selectivity is relatively low and the procedure is lengthy, above all the excess reagent should be removed before the analysis and sometimes resulted in loss of their hydrophobic derivatives. 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) is highly sensitive to amino acids and alcohols but it undergoes rapid decomposition in aqueous media.

Table 1. Linearity, detection limits and precision data of NPCP-AA derivatives.

Amino acids	Linearity (r^2)	LOD (µg L^{-1})	LOQ (µg L^{-1})	Precision (%)
Arg	0.9987	15.1	50.3	2.6
Lys	0.9823	18.3	61.8	2.1
Ser	0.9893	11.9	39.7	1.6
Pro	0.9963	26.0	86.8	2.6
Thr	0.9895	14.8	49.4	2.0
Glyc	0.9885	34.7	115.8	2.2
Tyr	0.9912	16.7	55.8	2.1

Table 2. Recovery data for NPCP-AAs derivatives.

AA	Found			% Recovery		
Conc. (mg L^{-1})	0.3	0.7	0.9	0.3	0.7	0.9
Arg	0.28	0.67	0.87	95.5	96.4	97.5
Lys	0.27	0.67	0.83	91.4	95.8	92.9
Ser	0.28	0.64	0.83	96.5	92.08	93.1
Pro	0.27	0.63	0.82	92.2	95.8	91.8
Thr	0.28	0.63	0.82	94.2	91.2	91.8
Glyc	0.27	0.66	0.83	92.9	95.0	93.0
Tyr	0.27	0.68	0.87	91.6	97.8	96.7

7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) and 7-fluoro-4-nitrobenzooxadiazole (NBD-F) have also been developed for the determination of primary and secondary amino acids. It is reported that both had poor stability and are very expensive. While using 5-dimethylamino-1-naphthalenesulphonyl (dansyl) chloride (Dns-Cl), it amino acid derivatives lack stability with respect to time, solvents or exposure to light. 2, 4-Dinitrofluorobenzene derivatives (DNFB) have been found to be very stable under common laboratory conditions but the drawback of this reagent is its toxicity [22-29] and the overall comparison was given in Table 3.

While comparing the overall performance of the reported dye, it is clear that the proposed method shows a great improvement against previous methods published, with superior properties to currently employed reagents, including rapid, convenient derivatization, excellent sensitivity, stability and derivatization yields. One of the most attractive features of this method exhibits its simpleness for the preparation of amino acid derivatives and also simple preparation of reagent. NPCP can be prepared from available starting material via two synthetic steps, easily converted to the corresponding amide derivative by in situ nucleophilic acyl substitution of the corresponding acid chloride, and its purification by simple chromatographic techniques. The chromatographic conditions are simple, do not necessitate the use of complex buffered solutions, and ion-pairing reagents and yet facilitate the separation and complete resolution of model analytes in isocratic elution mode within a relatively short time. The LC separation for the derivatized amino acids showed good repeatability and the detection limits obtained are comparable to those obtained for the commonly used labelling reagents for amino acids. In fact, preliminary results indicate that the method may be suitable for the trace analysis of different amine containing compounds by HPLC with fluorimetric detection.

A very successful application of NPCP as a label for tagging amino group using HPLC, clearly indicates its usability a promising label for several group of compounds including proteins, peptides and alcohols. Moreover, further research may open up new avenues for the extensive application of this compound as a fluorescent brightening agent, fluorescent chemo sensor, organic light emitting diode and even as organic photonic materials.

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

Derivative reagent	Derivatization temperature (^{0}C)	Derivatization Time (min)	Detection Limit (µmol L^{-1})
OPA	RT	3	5.38-56.60
PITC	RT	20	3.00-50.00
FMOC-Cl	40	10	0.03-0.35
AQC	55	10	0.02-0.37
NBD-F	65	20	0.65-0.96
Dns-Cl	RT	60	0.75-0.77
DNFB	60	10	20.00
NPCP	60	15	0.02-0.07

Application to aromatic amino acids and orange juice sample

To further evaluate the comprehensive profile of NPCP as a labelling agent for amine group; neurotransmitters ephedrine and epinephrine were derivatized under the optimum derivatization conditions and the chromatogram were shown in Fig. 8. This indicates that NPCP is also suitable for analysis of other amine bearing biologically relevant compounds. For expanding the application of NPCP labelling for real samples, fresh orange juice were spiked with the early detected seven amino acids. All the seven amino acids were identified and the recoveries were from 98 % to 100.9 % as shown in Table 4. This substantiates the potentiality of NPCP as an efficient label for the quantification of AAs in real samples.

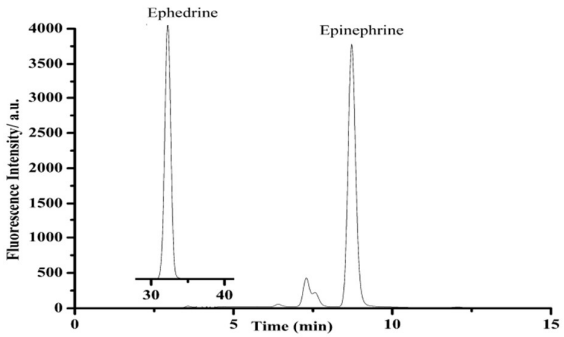

Fig. 8. Chromatograms for Epinephrine (8.7 min) & Ephedrine (32.2 min) derivatives. Chromatographic conditions: Ascentis Express C18 column (10 cm × 4.6 mm × 2.7 μm); column temperature 30 ^{0} C; flow rate 0.5 mL min^{-1}; λex/λem= 370/480 nm; [NPCP] = 0.25 mmol L^{-1}; [EPs] = 10 mg L^{-1}.

Table 4. Amino acids recovery from orange juice.

Amino acids	Added (μmol L^{-1})	Found (μmol L^{-1})	Recovery (%)
Arg	11.48	11.56 ± 0.62	100.6
	22.96	22.98 ± 1.35	100.0
	45.92	45.61 ± 2.10	99.3
Lys	13.68	13.60 ± 0.65	99.4
	27.36	27.40 ± 1.41	100.1
	54.72	54.73 ± 2.49	100.0
Ser	19.03	19.11 ± 0.71	100.4
	38.06	37.95 ± 1.43	99.7
	76.12	76.40 ± 2.81	100.3
Pro	17.37	17.38 ± 0.68	100.0
	34.74	34.68 ± 1.36	99.8
	69.48	69.51 ± 2.70	100.0
Thr	16.79	16.61 ± 0.69	98.9
	33.58	33.60 ± 1.34	100.0
	67.16	67.20 ± 3.06	100.0
Glyc	26.64	26.58 ± 0.85	99.7
	53.28	53.79 ± 1.76	100.9
	106.57	106.12 ± 3.4	99.5
Tyr	11.03	10.89 ± 0.42	98.7
	22.07	21.65 ± 0.89	98.0
	44.15	43.99 ± 1.81	99.6

Conclusions

NPCP has been found to be an attractive fluorigenic label suitable for the pre-chromatographic derivatization of biomolecules with amine functional group. It shows high selectivity and sensitivity towards amino group under milder reaction conditions such as moderate derivatization temperature, less derivatization time and no multiple derivatives or by-products. The limits of detection for the derivatized amino acids down to 35 μg L^{-1} proves that NPCP has as much as potential in relative to the main stream fluorescent labelling agents for tagging amino group. We believe that this pilot study provides a platform for the possible consumption of NPCP for the quantitative profiling of target analytes in complex biological and bio-medical as well as food samples and provides a good design idea for the development of new reagents from pyrazoline family.

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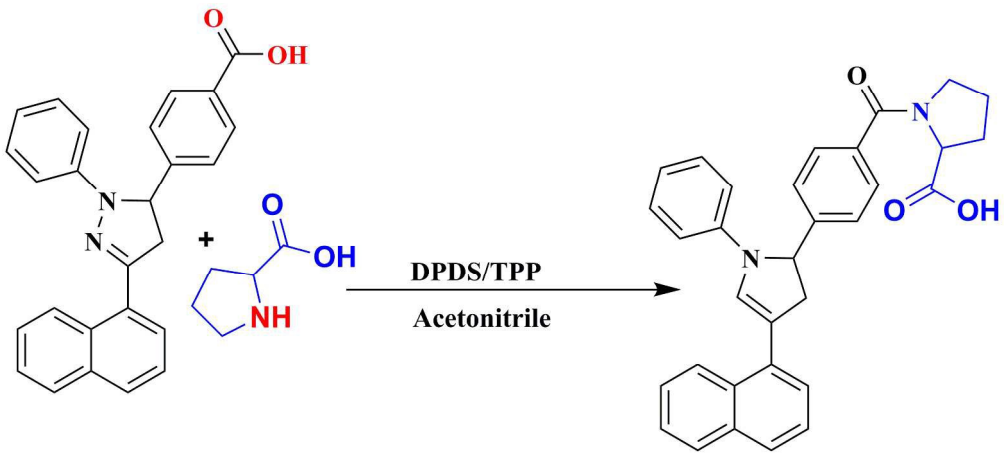

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