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Fluorescence properties of carbazole-*N*-(2-methyl)acetyl chloride and determination of amino compounds via high-performance liquid chromatography with pre-column fluorescence derivatization

Jinmao You[a], Wenjian Lao[a], Qingyu Ou[a,*], Xuejun Sun[b]

[a]*Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China*
[b]*Department of Chemistry, Qufu Normal University, Qufu, Shandong 273165, China*

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Abstract

A sensitive LC method for the determination of amino compounds with fluorescence detection has been developed. The reaction of fluorescent tagging reagent, namely carbazole-*N*-(2-methyl)acetyl chloride (CMA-Cl), with amino acids is reported. Emission maximum for the CMA derivatives of amino acids is 360 nm (λ_{ex}=335 nm). In all cases, the derivatives exhibit strong fluorescence, whereas the reagent itself also exhibits fluorescence. It is found that the labelled derivatives using CMA-Cl are very stable; <4% decomposition occurs after heating at 40°C for 24 h in neutral solution. Fluorescence intensity of amino acid derivatives is higher in neutral and alkaline than in acidic solutions. This method, in conjunction with a gradient elution, offers baseline resolution of 18 amino acids including Orn from a linear acetonitrile gradient (here, Asn, Gln and Trp are not tested, the principal reasons: Gln and Asn, in a real sample hydrolysed, have been changed into Glu and Asp; Trp gives as much as 60% loss of its monosubstituted derivative under proposed derivatization conditions.). Separation of derivatives is carried out on a reversed-phase C_{18} column with good reproducibility. Derivatization and chromatographic conditions are optimized. The relative standard deviations (n=6) for 50 pmol of each amino acid derivative are <4.5%. The detection limits, calculated by the corresponding peak heights (in cm) by injecting successively lower concentrations until a signal-to-noise of 3:1, are 10–65 fmol for the labelled amino acids.

Keywords: Derivatization, LC; Carbazolemethylacetyl chloride; Amino acids; Biogenic amines

1. Introduction

Fluorescence probes are extensively used in physical, chemical and biological sciences for investigating the structure and dynamics of living system. It is known that amino acids are important constituents of most living organisms. At present separations and quantitative determinations of amino acids by means of automatic analyzers and new fluorescent reagents, used for pre-column or post-column derivatization, are being intensively developed and summarized by King [1] and by Knapp [2]. Most amino acids show neither natural UV absorption nor fluorescence. Therefore, chemical derivatization is necessary to increase detection sensitivity and improve selectivity by means of pre-column or post-column HPLC separation.

Generally, the selectivity and sensitivity for common spectrophotometric detection for amino com-

*Corresponding author. Fax: +86-931-871-7088.
E-mail address: yjao@qfnu.edu.cn (Q. Ou)

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pounds are low [3–6]. Although a number of different types of fluorescent tagging reagents [7–11] have also been developed, there have also been many reports describing various shortcomings in application. For example, the *o*-phthaldialdehyde (OPA) method offers greater sensitivity widely and more selectivity [12–14], but is only limited to primary amino acids. The instability of the OPA derivatives makes manual derivatization difficult to reproduce, but satisfactory automated procedures have also been developed [15]. The 4-(2-phthalimidyl)benzoyl chloride (PIB-Cl) method offers also greater sensitivity, but is not appropriate to aromatic amine. At the same time, more interfering peaks were observed with standing of derivatized solution. 9-fluorenylmethylchloroformate (FMOC) [16–18], 1-(9-fluorenyl)ethyl chloroformate (FLEC) [19] and 2-(9-anthryl)ethyl chloroformate (AEOC) [20,21] reagents have also been developed for the derivatization of amino acids and peptides for chiral or non-chiral separation in LC or CE. These reagents result in good UV absorption and very high sensitivity with laser induced fluorescence detection, but the derivatized solution must be extracted with pentane to remove excess of reagent because it interferes with the separation of the amino acid derivatives and is detrimental to column performance [22–24]. Recently K. Ou et al. developed an improved LC method for the separation of amino acids derivatized with FMOC [25,26]. This problem has been overcome by adding a quenching reagent. This method yielded monosubstituted amino acid derivatives for the common protein amino acids, including histidine and tyrosine.

Newly, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [27] along with NBD-F [28,29] have also been developed for the determination of primary and secondary amino compounds. NBD-F is more reactive than NBD-Cl [30]. Data reported previously indicated that two regents themselves have about 50% decomposition in methanol–water solution exposed to daylight within 25 min [27,31] and a large number of fluorescent components have found which eluted later than the derivative itself.

With the developing of current techniques for separation of amino acids by LC or CE, an ideal reagent should thus fulfill several requirements [32]. First, it should be stable and give rapid reaction in high yields at low temperatures and the reaction products should be sufficiently stable. Excess reagent or by-products from the reaction should not disturb the separation. Further, the reagent should be selective for the target analysis and contain or produce a strong UV absorption or high fluorescent intensity.

On the basis of preliminary studies, carbazole-*N*-(2-methyl)acetyl chloride (CMA-Cl) was synthesized by microwave irradiation. In contrast to the acridine-*N*-acetyl chloride (ARC-Cl), carbazole-9-*N*-acetyl chloride (CRA-Cl) and carbazole-9-*N*-propionyl chloride (CRP-Cl) previously synthesized in our laboratory [33], the CMA itself is also fluorescent. However, this property is not a limiting factor. Provided the conditions of elution and composition of mobile phase, the disturbance of CMA itself on separation of other amino acid derivatives can be eliminated. By comparison the molecular structure with CRA-Cl and CRP-Cl, the fluorescence intensity of CMA-Cl tagging reagent is higher than that of CRA-Cl and CRP-Cl, but is lower than that of ARC-Cl. An interesting result is that CMA-Cl tagging reagent is also chiral derivatization reagent. Details with the regard to chiral separation will be reported later. Principal purpose in this study is to investigate the spectroscopic characteristics, stability and chromatographic behavior of the labelled compounds. The pre-column derivatization process developed in experiment does not require solvent extraction steps to remove excess derivatization agent by means of the method of K. Ou prior to chromatography. The synthesis and derivatization reaction process is shown in Fig. 1.

2. Experimental

2.1. Instrumentation

A model 655 liquid chromatograph equipped with an 650-10 S fluorescence spectrophotometer (Hitachi), a 7125 injection valve (USA), a 655 proportioning valve and a 644-61 integrator were used in experiments. Fluorescence excitation and emission spectra were also obtained on a 650-10 S fluorescence spectrophotometer. Excitation and emission bandpass are both at 15 nm. Amino acid derivatives were separated on a 200 mm×4.6 mm, 5

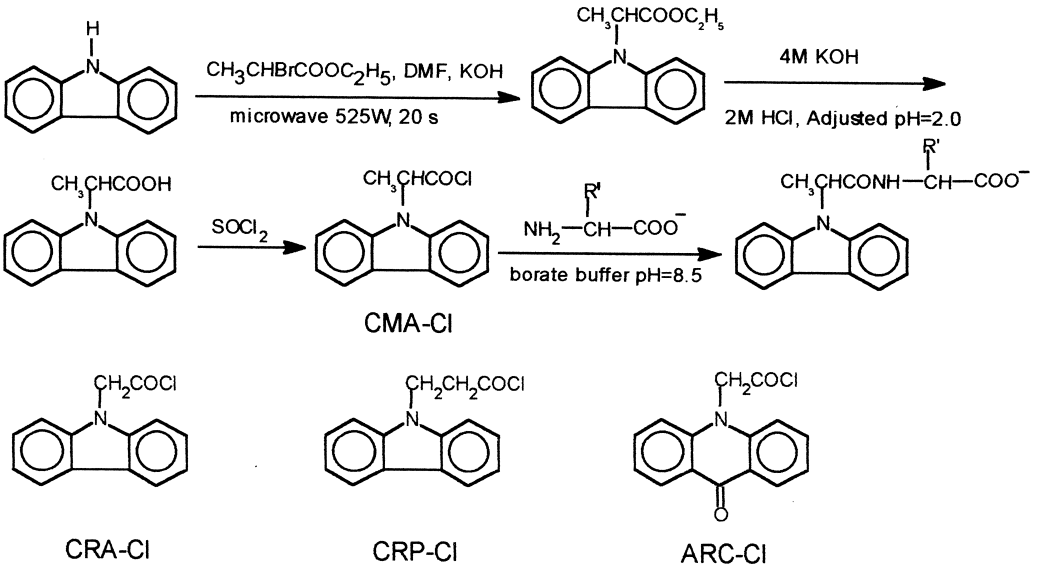

Fig. 1. The synthesis and derivatization processes of carbazole-*N*-(2-methyl)acetyl chloride with amino acids and the structures of CRA-Cl, CRP-Cl and ARC-Cl.

μm Spherisorb column (Dalian Institute of Chemical Physics, Chinese Academy of Sciences). A Paratherm U_2 electronic water-bath (Germany) was used to control column temperature. Galanz WP750B microwave, 2450 MH_2 (China) was used to synthesize carbazole-*N*-(2-methyl)acetyl acid. All mobile phase were treated ultrasonically for 15 min in order to remove gas bubbles prior to use.

2.2. Chemicals

Methanol, triethylamine, boric acid and ammonium dihydrogenorthophosphate were analytical grade from Jining Chemical Reagent Co. Doubly distilled water was used throughout. Amino acids were purchased from Sigma Co. Putrescine, cadaverine, spermidine and spermine were purchased from Chemical reagent Co. (Shanghai, China). Proteins were supplied by Department of Biology, Qufu Normal University (Qufu, Shandong). Ammonium dihydrogenorthophosphate stock solution (2.67 *M*), used for preparation of LC eluents, was adjusted to pH 5.5 with ammonia solution. Borate buffer was prepared from 200 m*M* boric acid solution adjusted to pH 8.8 with 4 *M* sodium hydroxide solution prepared from sodium hydroxide pellets. The alkaline cleavage reagent was prepared daily in 25 ml volumetric flask by mixing 17 ml of 0.850 *M* sodium hydroxide solution with 7.5 ml of 0.5 *M* hydroxylamine hydrochloride solution and 0.5 ml of 2-(methylthio)ethanol. The quenching reagent was acetonitrile–water–acetic acid (20:3:2). Triethylamine (0.36 *M*) stock solution used for preparation of LC eluent was adjusted to pH 5.5 with 4 *M* hydrochloric acid.

2.3. Synthesis of carbazole-n-(2-methyl)acetic acid (cma)

Carbazole 4.00 g was dissolved in DMF (10 ml), then (2-methyl)ethyl bromoacetate (5 ml) and potassium hydroxide (4.0 g) were added, the mixture reacts under microwave irradiation for 25 s at 525 W. The mixture was extracted by 20 ml of 4 *M* potassium hydroxide solution. The pH of extracted solution was adjusted to 2.0 with 2 *M* hydrochloric acid, the precipitation was removed and washed with 10% methanol (20 ml×2). The rough product was recrystallized from toluene to afford a white solid. yield 3.1 g (54%), m.p. 162–164. Found, C 75.31, H 5.44, N5.86; calculated, C 75.30, H 5.45, N 5.85; IR (KBr), 30002100 (COOH), 1715 (C=O), 1530 (ph),

1233, 741; *m/z*: 239(M^+), 240(M+1), 214(M–COOH); ^{1}H NMR, 1.66–1.73(–CH_3), 5.31–5.52 (–CH–CO), 7.08–8.08 (ph), 12.3 (–OH).

2.4. Preparation of CMA-Cl acetonitrile solution

24 mg carbazole-*N*-(2-methyl)acetic acid was added into a 10 ml of volumetric flask, then 4.0 ml of chloroform and 3.0ml of thionyl chloride redistilled were added. The content was heated at refluxing for 30 min to ensure a good conversion yield (in fact, the refluxing time>30 min, the derivatization yields of CMA-Cl with amino acids showed no remarkable increase). The solvent was evaporated by blowing N_2 to dryness, the dry sample was redissolved by addition of acetonitrile to 10 ml in a 10-ml volumetric flask as a $1.0 \cdot 10^{-3}$ *M* of CMA-Cl stock solutions for preparation of amino acid derivatives.

2.5. Chromatographic method

HPLC separation of CMA amino acid derivatives was carried out by a binary gradient. Eluent A was 4.5 m*M* ammonium dihydrogenorthophosphate+4.5 m*M* triethylamine (pH 5.5) and B was acetonitrile–water (90:10). The flow-rate was constant at 1.0 ml/min and the column temperature was kept at 35°C. The fluorescence emission wavelength at 360 nm (excitation at 335 nm). Unless stated otherwise, the gradient condition used for separation of amino acid and biogenic amine derivatives was shown in Table 1 (gradient 1 and gradient 2). Gradient 2 was used for rapid separation of polyamines in plant tissue.

2.6. Protein hydrolysis

A 3.0 μg of protein sample dissolved in water or dilute HCl (0.5 *M*) were pipetted into 50·6 mm test tube and dried under vacuum for 1.5 h. A 500 μl volume of 6 *M* hydrochloric acid was placed in the bottom of the vessel, and then the tube was inserted into another vial. The vial was sealed under vacuum after three alternate vacuum–nitrogen-flushing steps. Hydrolysis was then carried out at 115°C for 24 h [34–37]. Samples were then dried under vacuum and redissolved in the derivatization buffer.

2.7. Sample preparation of plant materials

Barley seedling and apple tissue: Extraction of barley seedling or apple tissue was carried out according to the previous method [38]. Fresh mass sample of plant tissue 0.5–1.0 g (barley seedling or apple tissue) was homogenized with 3 ml of 5% $HClO_4$. After extraction for 60 min in an ice-bath, the extracts were centrifuged at 0–4°C for 20 min at 25 000 *g*. The supernatant solution was flitered. The residue was extracted again twice with 1.5 ml of 5% $HClO_4$ solution. The resulting combined extracts were made up to 8 ml with 5% $HClO_4$ solution and stored at −30°C until HPLC analysis.

Table 1
Chromatographic gradient conditions

Gradient 1 for amino acids			Gradient 2 for rapid ployamines analysis		
Time (min)	A%	B%	Time (min)	A%	B%
0	90	10	0	95	5
6	90	10	5	90	10
16	85	15	10	80	20
25	75	25	20	70	30
30	60	40	25	60	40
35	40	60	30	50	50
40	30	70	35	1	99
45	20	80			
50	10	90			
55	1	99			
65	1	99			

2.8. Derivatization procedure

Standard solutions of amino acids were made from dilutions of commercial standard mixtures. A 10 μl of aliquot of amino acids was added in a centrifuge tube and adding 140 μl of 0.2 *M* borate buffer (pH 8.8), then adding 20 μl of CMA-Cl acetonitrile solution. The centrifuge tube agitated and the derivatization allowed for a reaction time of 90–120 s. A 20 μl volume of cleavage reagent was then added, and the mixed solution allowed to stand for a further 3.0 min. Finally, the reaction was stopped by adding 30 μl of quenching reagent. This mixture was directly used for HPLC analysis.

2.9. Preparation of representative cma-glycine derivative and its fluorescence quantum efficiencies

A 0.5 g amount of carbazole-*N*-(2-methyl)acetic acid was placed in a 10-ml round-bottomed flask and was dissolved in 5 ml of dichloromethane. To this flask, 1.5 ml of $SOCl_2$ was added and the mixture was allowed to reflux for 30 min. After the reaction was finished, the solvent and excess of $SOCl_2$ were removed by a rotary evaporator. The residue was redissolved in 2 ml of HPLC grade acetonitrile, and added a solution containing 0.30 g of glycine in 2 ml of acetonitrile, followed by the addition of 0.5 ml of 0.2 *M* borate buffer (pH 8.8). The mixture was well mixed and the reaction allowed to stand for 10 min, then extracted using 3·2 ml of ethyl acetate. Ethyl acetate was washed successively with 2 ml each of 0.1 *M* hydrochloric acid and water. The organic phase was dried with anhydrous sodium sulphate and was evaporated under a stream of nitrogen. The residue was redissolved in 2.0 ml of dichloromethane. A 10-ml volume of hexane was then added to precipitate CMA-glycine. The precipitate CMA-glycine was recrystallized by dichloromethane–hexane (1:1, v/v) until HPLC analysis showing no carbazole-*N*-(2-methyl)acetic acid remaining (HPLC conditions: mobile phase was 4.5 m*M* triethylamine (pH 5.5)–acetonitrile (70:30, v/v), the flow-rate was constant at 1.0 ml/min and the column temperature was kept at 30°C). Product: m.p. 138.6–140°C. Found, C 68.89, H 5.41, N 9.45; calculated, C 68.91, H 5.405, N 9.46; IR (KBr), 3000~2100 (COOH), 1692 (–CO–NH), 1530 (ph), 1346, 832; *m*/*z*: 296(M^+), 297(M+1); $\varepsilon = 2.24 \cdot 10^{-4}$.

A appropriate amount of CMA-glycine derivative was dissolved in various solvent system to yield a final concentration of 3 μ*M*. Corresponding fluorescence emission spectra were recorded with an excitation wavelength λ_{ex}335 nm in a 1 cm quartz cell on an 650-10S spectrofluorimeter. Detection of fluorescence efficiencies were accomplished by comparison with carbazole-*N*-(2-methyl)acetic acid as standard ($\phi_{f1'} = 1.0$). The symbol ϕ_{f1} value for a given derivative was calculated according to the equation:

$$\phi_{f1}/\phi_{f1'} = I_f(1 - 10^{-A'})/I_{f'}(1 - 10^{-A})$$

where ϕ_{f1} and $\phi_{f1'}$ are the fluorescence quantum efficiencies for the given derivatives and the standard. *A* and *A′* are the absorbency of the derivatives and the standard solutions (determined by UV). I_f and $I_{f'}$ are the areas under the emission curves of the derivative and the standard, respectively. Quantum efficiencies were measured in 100%, 50% acetonitrile and 50% methanol solutions, respectively.

3. Results and discussion

3.1. Ultraviolet absorption of the carbazole-N-(2-methyl)acetic acid

The ultraviolet absorption of CMA was investigated in acetonitrile–water (1:1) solution. The absorption maximum for CMA was 255 nm ($\varepsilon = 2.14 \cdot 10^4$). Molar absorption coefficients of CRA and CRP in the same conditions were $2.06 \cdot 10^4$ and $2.01 \cdot 10^4$, respectively. It was found that CMA exhibited a large molar absorption coefficient and made it more sensitive relative to that of CRA and CRP. This was probably due to the fact that the CMA molecule containing a α-methyl group led to a large conjugated system.

3.2. Stabilities of CMA-Cl and corresponding derivatives

CMA-Cl acetonitrile solution was stored at 0°C in darkness within 1 week, the derivatization yields of amino acids with CMA-Cl showed no obvious

difference. When CMA-Cl was stored in acetonitrile beyond 1 week, the corresponding derivatization yields of amino acids with CMA-Cl decrease 10% in comparison of that as obtained above. Generally, the stored CMA-Cl acetonitrile solution enough to allow further analysis of amine samples for at least 1 week. The stabilities of the corresponding CMA derivatives were also investigated by analyzing eighteen amino acid standards containing 50 pmol of each amino acid. The derivatives were stored at 10°C in darkness as well as in daylight for a period of 2 weeks, during which time they were analyzed four times. As expected, daylight had essentially no effect on stability. All the amino acid derivatives were stable during this time. The relative standard deviations for normalized peak areas varying between 1.0–4.5%.

Several vials of derivatives were heated in neutral solution at a constant temperature in a water-bath at 40°C for 3 days, during which time they were analyzed three times. The extent of decomposition was determined from the ratio of the peak height. The derivatives exhibited $<4\%$ decomposition. These results show that the stabilities of CMA derivatives are equal to that of CRA, CRP and ARC derivatives and enough to allow further analysis of derivatized samples at least 24 h at room temperature.

3.3. Fluorescence properties of derivatives

Fluorescence excitation and emission spectra of CMA are investigated. It is found that the excitation and emission of CMA in pure acetonitrile are 335 and 365 nm, respectively. The excitation and emission of representative CMA-glycine derivative shows no remarkable difference relative to that of CMA under the same operating conditions. It was found that the fluorescence intensities in acetonitrile (100%) was 18% stronger than in methanol (100%). The emission wavelength in acetonitrile–water was 2 nm shorter than that in methanol–water. There is a 18.2% difference for CMA derivatives in the fluorescence intensity between 50% of acetonitrile solution and 50% of methanol solution. Derivatives show a little change in fluorescence quantum efficiencies with increasing solvent polarity. The symbol ϕ_{f1} value for CMA-glycine derivative changes from 0.521 to 0.495 in going from 100% to 50% acetonitrile and from 0.462 to 0.434 in going from 100% to 50% methanol.

3.4. Optimization of reaction conditions for derivatization

In developing the CMA-Cl for separation of amino acids, it was important to assess the effect of CMA-Cl concentration and reaction time. The effect of CMA concentrations on the fluorescence intensity was investigated for representative aspartic acid derivative. For this experiment, a solution of 5 μM aspartic acid was prepared, which was mixed with corresponding derivatization agent at a concentration range from 0 to 100 μM, and dissolved in borate buffer (pH 8.8). A 90 s reaction time was achieved. Unless stated otherwise, the other conditions as described in the experimental section. It was found that the fluorescence intensity of aspartic acid derivative increased with the increasing derivatization agent concentrations from 0 to 80 μM. The fluorescence intensity was constant when the concentration of derivatization agent was over 80 μM. The effect of reaction time on fluorescence intensity for aspartic acid was also investigated. For this experiment, the concentration of derivatization agent was kept at 100 μM in order to react completely, the other conditions were similar to those as described above. The reaction time varied from 0.5 to 3 min, the fluorescent intensity steadily increased from 0.5 to 2 min and was constant after 2 min. The derivatization of CMA-Cl with amino acids was essentially completed within 90–120 s. The results indicated that the derivatization speed of CMA-Cl with amino acids was the same as CRA and CRP, but was slight slower relative to that of ARC.

In addition, It was found that if derivatization reaction time was over 2.5 min, amino acids with a hydroxyl group (–OH) or thiol group (–SH), such as Thr, Ser, Tyr or Cys, completely form disubstituted derivatives. The separation of disubstituted derivatives of Thr, Ser, Tyr and Cys was shown in Fig. 2B. As could be seen, the sensitivity for the elution of these disubstituted derivatives are low 62–74% relative to that of their mono-substituted derivatives. In addition, it was also found that if the derivatized solution was over 60 min, disubstituted Thr, Ser, Cys and Tyr derivatives produced 16–22% decomposi-

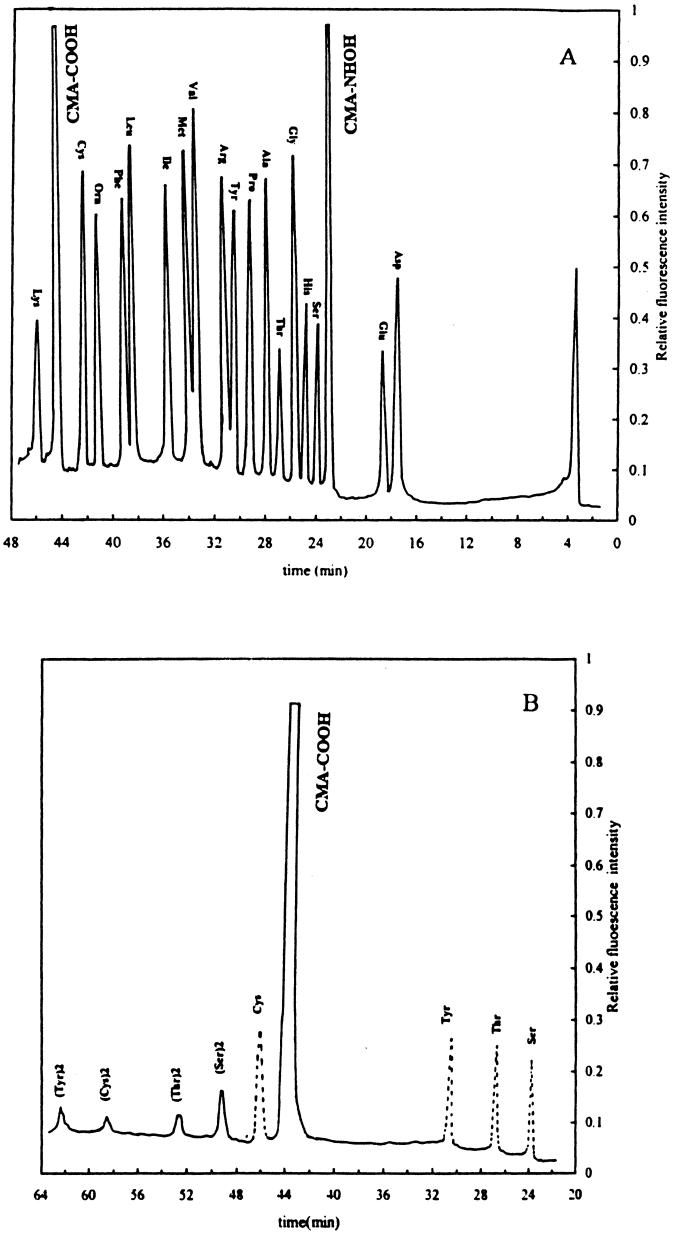

Fig. 2. (A, B) HPLC chromatograms of amino acid derivatives excitation wavelength 335 nm, emission 360 nm. Peaks are labelled with three-letter abbreviations for amino acids. Chromatographic conditions: Column, 200×4.6 mm I.D. Spherisorb 5 μm; Eluent A=4.5 m*M* ammonium dihydrogenorthophosphate (pH 5.5)+4.5 m*M* triethylamine; B=acetonitrile–water (90:10); flow-rate=1.0 ml/min; column temperature, 35°C; gradient 1 from Table 1. (**A**) 100 pmol of each amino acid standard derivatized with CMA-Cl, (**B**) 150 pmol of amino acid disubstituted derivative (peaks depicted with dotted lines stand for corresponding mono-substituted derivative).

tion in alkaline derivatizing solution (mainly ester saponification decomposition) and led to the difficult separation. This problem can be solved by two methods: (1) adjusting the pH of derivatized solution to neutral in order to avoid alkaline decomposition; (2) changing the disubstituted Thr, Ser, Cys and Tyr

derivatives into corresponding mono-substituted derivatives by means of alkaline cleavage reagent. For rapid separation, generally, disubstituted amino acid derivatives (containing a hydroxyl function group mentioned above) were changed into mono-substituted derivatives. In our experiment, it was found that only alkaline treatment of these disubstituted derivatives could produce the monosubstituted derivatives, but the conversion would cause as much as 15–20% loss of other amino acid derivatives. A cleavage reagent consisting of hydroxylamine and sodium hydroxide proved effective in converting these disubstituted derivatives to the mono-substituted derivatives. The effect of added cleavage reagent amounts on the conversion of representative disubstituted Tyr derivative was examined, The result was shown in Fig. 3. As could be seen from Fig. 3, the disubstituted Tyr derivative was essentially conversed when added cleavage reagent volume was >20 μl. It was also found that the peak height of corresponding mono-substituted derivative increased with progressively increasing cleavage reagent amount. Noted that a further increase cleavage reagent volume also resulted in a decrease in mono-substituted derivative, this was probably due to the fact that further increase cleavage reagent made the alkalinity in derivatizing solution dramatically enhanced led to hydrolysis of mono-substituted Tyr derivative. The cleavage reagent adopted proved optimal, as it had little effect on normal R-NH-CMA groups by appropriate controlling amounts of cleavage reagent. At the same time, hydroxylamine could rapidly react with excess CMA-Cl and changed into CMA-NHOH unaffected separation. In addition, if cleavage reaction time was >4.0 min, the derivatized amino acids, such as Try and Pro (simultaneously containing primary and secondary amino groups) would cause as much as 5% loss for their mono-substituted derivatives. However, these phenomena for other amino acid derivatives were not observed. therefore, a cleavage reaction time of 3.0 min was chosen.

The effect of pH on derivatization yields was also investigated for representative amino acids Asp, Ser, Glu, His and Arg. Reagent concentration was 100 μ*M*, reaction time was kept for 2 min. Individual amino acid concentration was 5 μ*M*, giving a total amine concentration of 25 μM. It was found that derivatization afforded maximum yields in the pH range from 8.5 to 9.5. When buffer solution pH was 7.5–8.5, derivatization yields for representative amino acids were only 95%. Generally, most sub-

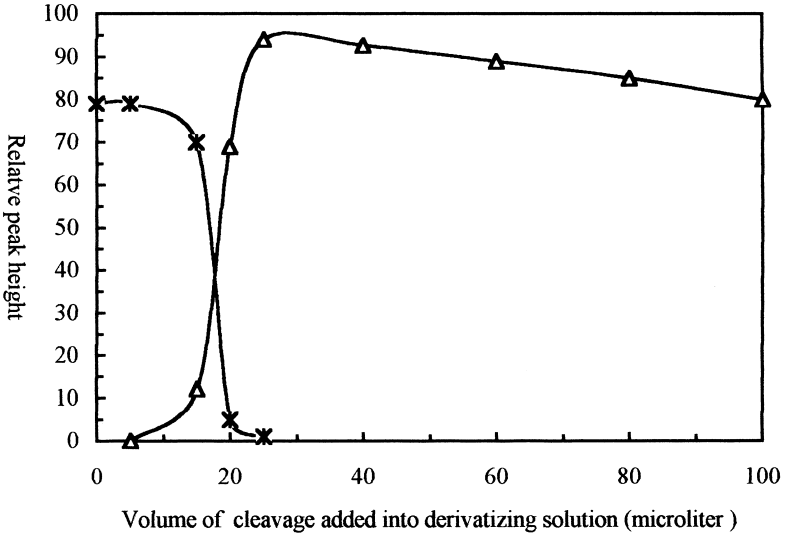

Fig. 3. Effect of added hydroxylamine cleavage reagent volume (μl) on representative Tyr derivative conversion; Condition: cleavage reaction time kept for 4 min. *, Conversion of disubstituted Tyr derivative with increasing cleavage reagent. Δ, After degradation, producing corresponding mono-substituted Tyr derivative.

sequent derivatization was carried out using 0.2 *M* borate buffer at pH 8.8.

Acetonitrile was used as the reaction cosolvent in preference to acetone as it avoided the problem of precipitation of the derivatives of hydrophobic amino acids and hydrophobic biogenic amines. But acetonitrile was used as the solvent for CMA-Cl, a separation of phases occurred at high buffer concentrations. This could be avoided by two methods: (1) Buffer concentration was controlled ≤ 0.2 *M*, or acetonitrile concentration in derivatized solution was kept at $<50\%$; (2) Use acetone instead of acetonitrile. We prefer the first possibility controlling the concentrations of buffer and acetonitrile, respectively.

In preliminary studies, borate, phosphate and bicarbonate buffers were investigated for the derivatization. Both borate and bicarbonate were satisfactory, but phosphate proved unacceptable as it produced a large interfering peaks. Generally, derivatization of amino compounds was carried out in borate buffer at pH 8.8 over the time from 90–120 s. Additionally, the derivatives of aspartic and glutamic acids were formed slowly than those of other amino acids, and the reaction was incomplete in acetone after 90 s. The use of acetonitrile as cosolvent evidently causes derivatization of them to proceed more slowly than that in acetone. However, it was found that adjustment of the pH of the borate buffer >8.5 gave complete derivatization over this time from 90–120 s.

In this study, it was also found that tryptophan (Trp) would be simultaneously given monosubstituted and disubstituted derivatives in the pH range of 8.5–10.0. It would be as much as 60% loss of its monosubstituted derivative with alkaline treatment by the cleavage reagent according to the experimental conditions mentioned above. Shortening the time of cleavaging treatment, the other disubstituted derivatives would not completely convert into monosubstituted. So Trp was not discussed in this experiment. Details on the conversion and separation of Trp would be reported later.

3.5. Linearity of derivatization

The linearities of the amino acid derivatives were established by the analysis of each amino acid standards containing 0.08, 0.1, 5.0, 50 and 100 pmol, respectively. The representative derivatives of Met, Gly, Ser, Phe, and Glu were investigated. It was found that they give linear derivatization at least three orders of magnitude range, and with the correlation coefficients >0.997.

3.6. Sensitivity and detection limits

The sensitivity are depend upon the size of column. Narrow or microbore columns give a further increase in sensitivity especially when baseline subtraction is used. The representative derivatives of 5 pmol of Glu, Gly, Thr, Phe and Met were investigated. Peak heights corresponding CMA derivatives are 1.3 and 1.2 times as high as CRA and CRP derivatives, respectively. this is probably due to the fact that CMA molecule contains a α-position methyl, which makes charge density of nitrogen atom enhanced relative to that of CRA and CRP. However, the peak heights for CMA derivatives are only 0.2 times as high as ARC derivatives possibly due to difference in molecular structure. Additionally, by comparison with FMOC, the peak heights for CMA derivatives mentioned as above are 1.7 times as high as FMOC derivatives for the same derivatized sample.

Detection limit is depend upon different detector and different elution conditions. Detection limits, calculated by the corresponding peak heights (in cm) by injecting successively lower concentrations until a signal-to-noise of 3:1, are 10–65 fmol. The low detection limit is directly attributable to the removal of impurities present in the reagents and blank water. These impurities can be eliminated by purifying the reagent and will give a further increase in sensitivity.

3.7. LC separation of derivatives

The LC separation of a standard containing eighteen amino acid derivatives was carried out using a reversed-phase Spherisorb-C_{18} column. It was found that eluent A was only 4.5 m*M* ammonium dihydrogenorthophosphate + 4.5 m*M* triethylamine (pH5.5); B was 90% aqueous acetonitrile, CMA-NHOH did not disturb the separation. If the concentration of derivatization agents was over 100 μM, CMA-OH would disturb the separation (no shown). The elution order of corresponding CMA derivatives at pH 5.5 is shown in Fig. 2A.

4. Applications

4.1. Compositional analysis of collagen

The analysis of the amino acids from a collagen hydrolysate was carried out and shown in Fig. 4. As could be seen, the established method was suitable for the determination of amino acids from collagen-type samples. The compositional analysis of the corresponding collagen hydrolysate is shown in Table 2.

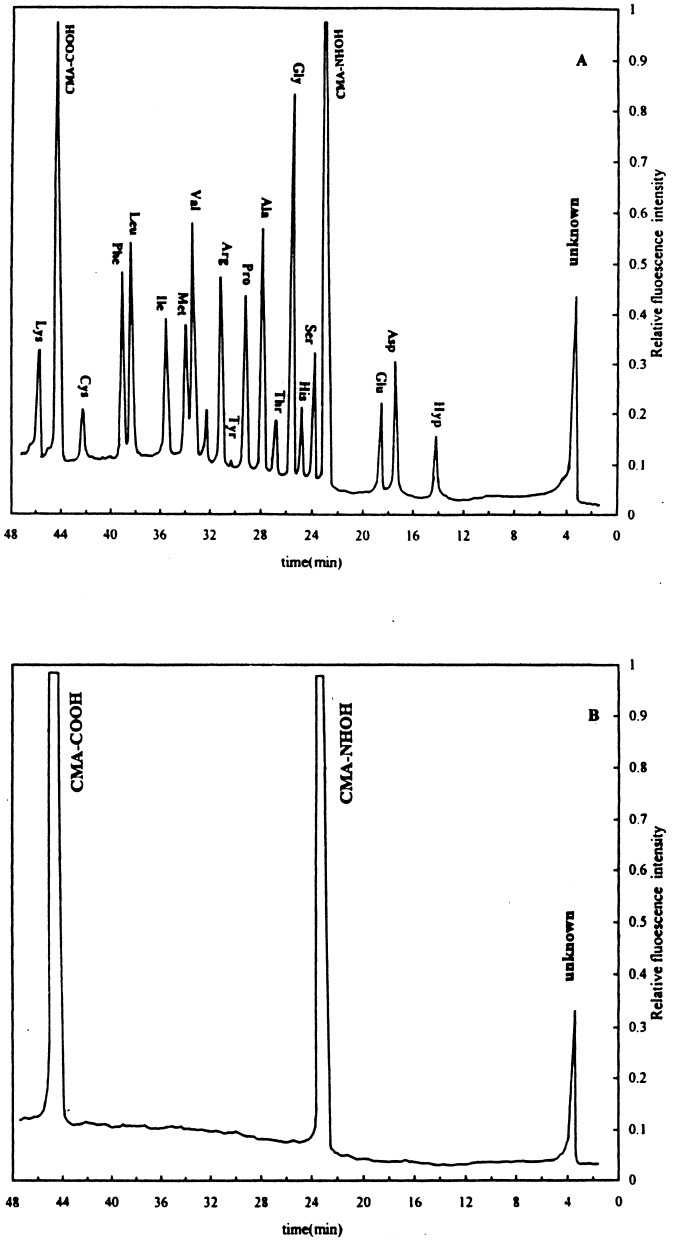

Fig. 4. Chromatograms of amino acid derivatives (**A**) Collagen hydrolysate, (**B**) hydrolysis blank. Chromatographic conditions and peaks as in Fig. 2.

Table 2
The composition analysis of hydrolyzed sample (3.0 μg, 10% was analyzed by HPLC)

Amino acid	Actual[a]	Sample[b]	Error	Retention time RSD (%) ($n=10$)
Asp	49	52.34	3.34	1.14
Glu	41	39.67	−1.33	1.32
Ser	51	56.78	5.78	1.10
His	28	31.67	3.67	1.31
Gly	61	58.33	−2.67	1.72
Thr	16	16.59	0.59	1.71
Ala	42	43.78	1.78	1.40
Pro	32	31.08	−0.92	1.18
Tyr	8	7.91	−0.09	1.19
Arg	51	52.34	1.37	0.86
Val	67	66.04	−0.96	0.87
Met	32	32.06	0.06	0.78
Ile	23	23.55	0.55	1.12
Leu	26	26.00	0	0.41
Phe	23	22.17	−0.83	0.38
Cys	19	20.41	−1.41	0.30
Lys	23	23.68	0.68	0.43
Hyp[c]				

[a] Actual sample was suppled by Department of biology, Qufu Normal University, Qufu Shandong, China.
[b] All data were normalized to Leu without correction for losses during hydrolysis.
[c] ND (Not determined).

4.2. Chromatographic separation of polyamines in plant tissue

Rapid separation of biogenic amines in plant tissue was also investigated using a multi-gradient elution. The mobile phase composition was: Eluent A, 10 m*M* ammonium dihydrogen-orthophosphate + 10 m*M* triethylamine pH 5.5–acetonitrile (52:48, v/v); B was acetonitrile–water (90:10, v/v). For determination of calibration curves, a standard solution containing 1.0 mg of each polyamine (of the corresponding hydrochloride) was dissolved in 100 ml of water. The resulting concentration of each amine was 10 μg/ml in the standard solution. The standard solution was derivatized as described in experimental section. Corresponding low concentration derivatized solutions were obtained by appropriate dilution with acetonitrile. Standard derivatized solutions containing 5–250 pmol/10 μl were injected five times. The optimized separation for a standard polyamines was obtained (Fig. 5A) with gradient 2 from Table 1. The regression analysis of calibration graphs and other quantitative data for polyamine derivatives were established and shown in Table 3.

A 20 μl volume of extracted polyamines solution was removed and derivatized as described in experimental section. The representative chromatogram for separation of biogenic amines in apple tissue was shown in Fig. 5B. Table 4 shows the results of biogenic amines contents in barely seedling and apple tissue.

5. Conclusions

In contrast to derivatizing reagents our previously developed such as CRA-Cl, CRP-Cl and ARC-Cl, the CMA derivatives also exhibited a high solubilities in derivatized solution. At the same time, it exhibited a large molar absorptivity and make it more sensitive relative to that of CRA and CRP derivatives. However, the sensitivity of CMA derivatives was lower than that of ARC derivatives, this was attributed to the difference in molecular structure. The present work has shown that CMA-Cl was effective derivatization reagent. Complete derivatization took less than 2 min at room temperature, and derivatives were stable for at least 24 h in neutral solution. Corresponding hydrolysates of reagent did not interfere with the separation by adjusting the

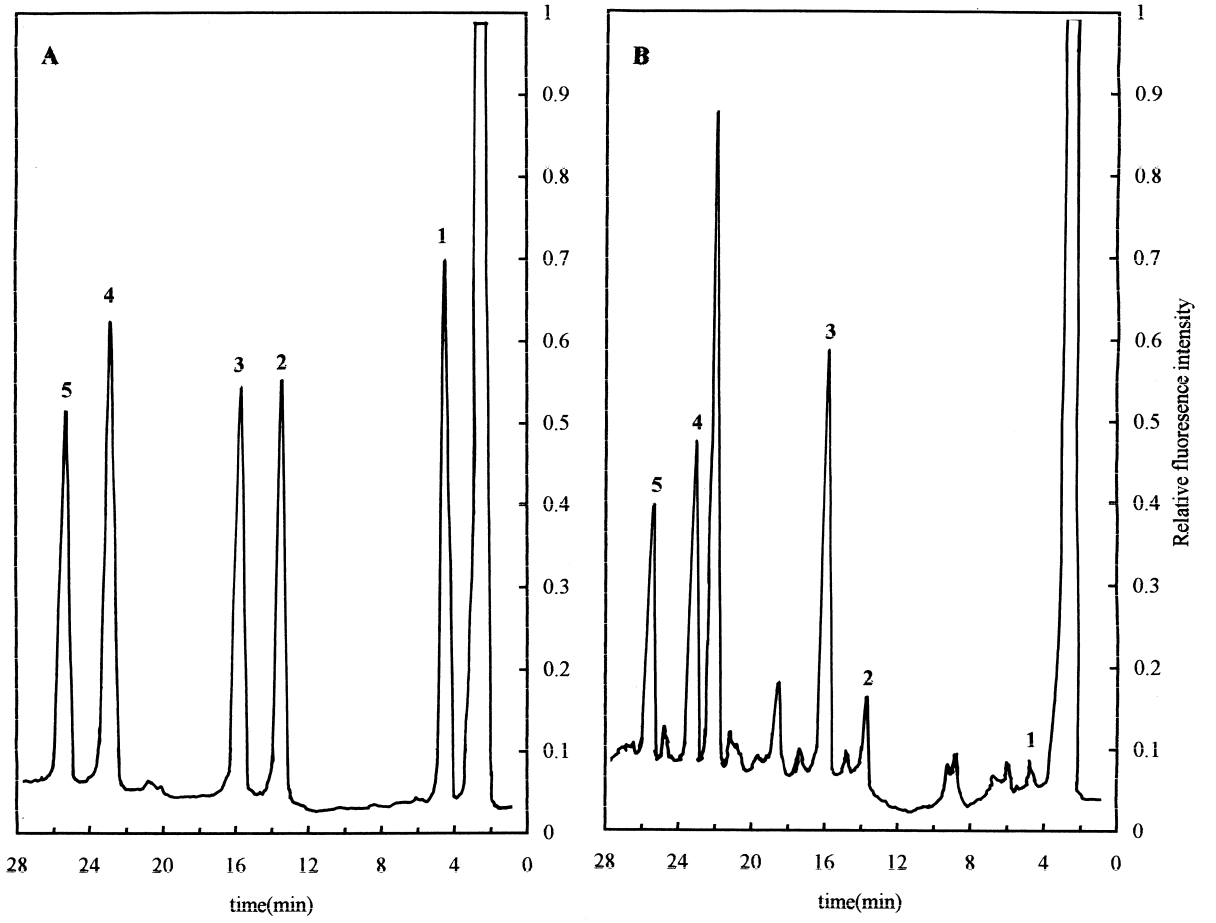

Fig. 5. (**A,B**) HPLC chromatograms of biogenic amines derivatized with CMA-Cl Column: Spherisorb-C_{18} (5 μm, 200×4.6 mm I.D.); column temperature 35°C. Eluent A=10 m*M* ammonium dihydrogenorthophosphate (pH5.5)+10 m*M* triethylamine (pH5.5)–acetonitrile (52:48, v/v) and B=acetonitrile–water (90:10); flow-rate=1.0 ml/min; excitation 335 nm; emission 360 nm; gradient condition as Table 1 (gradient 2). (**A**) Standard chromatogram 1=trimethylene diamine (80 pmol); 2=putrescine (100 pmol); 3=1,6-diamino hexane (100 pmol); 4=spermidine (120 pmol); 5=spermine (120 pmol). (**B**) Separation chromatogram of biogenic amines in apple flowers peaks as in **A**; 1,6-diamino hexane used as internal standard sample.

Table 3
Regression analysis of calibration graphs and other quantitative data for biogenic amines

Compound	Linear range injected amount pmol	Calibration graph[a]	Correlation coefficient	RSD (%)[b] ($n=6$)	Detection limit[c] (pmol)
Putrescine	10–400	$y=-0.43+0.0586x$	0.9996	3.2	0.23
Spermidine	10–400	$y=-0.68+0.0427x$	0.9998	4.0	0.25
Spermine	20–450	$y=-0.61+0.0375x$	0.9998	4.5	0.34

[a] y in cm, x in pmol injected amount.
[b] Relative standard deviation for 50 pmol of each CMA derivatives.
[c] Detection limits were obtained by the corresponding peak heights (in cm) by injecting successively lower concentrations until a signal-to-noise of 2:1.

Table 4
The analysis of biogenic amines in barley seedling and apple tissue

Compound		Amounts of biogenic amines in barley seedling (μg/g) ($n=6$)				
		Putrescine	Spermidine	Spermine	Total amount	Percentage of putrescine
Barley seedling	Upper	89.7±3.0	41.2±3.0	11.3±2.5	142.2	63.1
	Middle	45.2±3.5	23.8±2.0	7.8±1.5	76.8	58.8
	Lower	33.6±2.0	27.5±2.0	5.3±1.5	66.4	50.6
	Root	42.7±2.0	15.2±1.5	1.7±1.0	59.6	71.6
		Found (μg/g)	Added (μg/g)	Recovered (μg/g)	Recovery (%)	
Apple young fruits (Fuji)	Putrescine	65.8±3.0	20.0	86.8±3.0	105	
	Spermidine	74.6±3.0	35.0	107.4±3.0	93.7	
	Spermine	28.2±2.5	25.0	55.2±2.5	108	
Apple flowers (Fuji)	Putrescine	47.3±3.0	20.0	68.1±3.0	104	
	Spermidine	12.8±3.0	35.0	146.2±3.0	95.4	
	Spermine	18.7±2.5	25.0	44.6±2.5	104	

composition of mobile phase. Derivatization yields of close to 100% were achieved for derivatized amino acids. Separation on LC afforded the detection limits <100 fmol for amino acids and <0.35 pmol for polyamines. These characteristics made it an attractive derivatization agent for amino compounds.

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