Fluorescence properties of carbazole-9-ylpropionic acid and its application to the determination of amines *via* HPLC with fluorescence detection

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Fluorescence spectra of carbazole-9-ylpropionic acid (CRP), in the presence of different halide salts, organic solvents and surfactants and at various temperatures were investigated. The quenching constants of NaF, NaCl, NaBr, NaI and CH_3I to CRP are 9.4, 12.6, 104, 456 and 560, respectively. Studies on the fluorescence spectra of CRP in 25 solvents indicated that the CRP fluorescence intensity increases with increasing polarizability of solvents and decreases on elevating the temperature. The influence of surfactants on the fluorescence spectra of CRP can be classified as two types: monomer quenching (below the CMC) and the micelle effect (above the CMC). The monomer quenching constant K for cetyltrimethylammonium bromide (CTMAB) to CRP is 1.8×10^3 l mol^{-1}. The binding constants K of micelles for CTMAB and TX-100 to CRP are 2.6×10^3 and 1.2×10^3 l mol^{-1}, respectively. The fluorescence intensity and emission wavelength of CRP in the presence of the anionic surfactant SDS show no difference. As an application study the use of carbazole-9-ylpropionyl chloride (CRP-Cl) as derivatization reagent for the simultaneous separation of polyamines and amino acids with pre-column fluorescence derivatization *via* LC is described. The derivatization and chromatographic conditions were optimized on a reversed-phase C_{18} column using a binary gradient. Studies on derivatization conditions indicate that primary and secondary amines react very fast with CRP-Cl in alkaline solution to give the corresponding fluorescent derivatives, which exhibit excellent sensitivity and stability. This method, in conjunction with a multi-step gradient, offers a complete resolution of amino acid and polyamine derivatives. The separation of polyamines extracted from plant tissue takes less than 25 min. Excellent response linearity is demonstrated for amounts of polyamines injected in the range 50–250 pmol. The relative standard deviations ($n = 6$) at the picomole level are $< 5\%$ and detection limits (signal-to-noise ratio = 3) are at the femto mole level.

Fluorescence probes are extensively used in physical, chemical and biological sciences for investigating the structure and dynamics of living systems.[1,2] Biogenic amines and amino acids are natural compounds in different food products. Biogenic amines are also indicators of good food quality. In addition, some biogenic amines, including polyamines, such as putrescine, cadaverine, spermidine and spermine, play an important role in cell fission and nucleotide and protein biosynthesis in both animals and plants. Therefore, it is important to determine certain biogenic amines including polyamines and amino acids in different food matrices. Most amino acids and polyamines show no fluorescence. Therefore, chemical derivatization is necessary to increase the detection sensitivity and improve the selectivity by pre- or post-column liquid chromatographic (LC) separation. The selectivity and sensitivity for common spectrophotometric detection[3–6] are low. Other techniques used to improve detection limits are micro-column LC[7] and capillary electrophoresis[8] with laser induced fluorescence detection, which allows the detection of attomole amounts of material. Popular methods for the determination of amino acids are still pre-column and post-column derivatization with fluorescence detection. Several common fluorescent derivatization reagents[9–20] have also been developed for the determination of amino compounds. Despite the popularity of these pre-column methods, there have also been many reports describing various shortcomings in applications, and to date no one method has been reported that overcomes all problems. For example, *o*-phthalaldehyde (OPA) offers high sensitivity, but is limited to primary amino acids. The 4-(2-phthalimidyl)benzoyl chloride (PIB-Cl) method also offers high sensitivity, but is not appropriate for aromatic amines. Further, on standing the derivatized solution, more interfering peaks are observed, possibly due to decomposition during analysis.[21] Although 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) is more reactive than NBD-Cl for derivatizing amino compounds, data previously reported indicate that the two reagents themselves undergo about 30–50% decomposition in methanol–water solution within 25 min when exposed to daylight.[22] 9-Fluorenylmethyl chloroformate (FMOC),[23] 1-(9-fluorenyl)ethyl chloroformate (FLEC)[24] and 2-(9-anthryl)ethyl chloroformate (AEOC)[25] have also been developed as reagents for the derivatization of amino acids and peptides for chiral or non-chiral separations in LC or capillary electrophoresis (CE). These reagents result in good UV absorbance and very high sensitivity with laser induced fluorescence detection. Current procedures using derivatization with these reagents are also troublesome. The derivatized solution must be extracted with pentane to remove excess of reagent[26,27] because it interferes with the separation of amino acid derivatives and is detrimental to column performance.

In a previous paper,[28] we described the synthesis of several reagents and their application in common amino acids analysis. The principal goal of this work was to investigate systematically the effects of halide salts, pH, organic solvents, surfactants and temperature on the fluorescence properties of carbazole-9-ylpropionic acid (CRP). Further, application to the simultane-

ous separation of polyamines and amino acids was also investigated. Complete and reproducible separation of a multi-component mixture consisting of polyamines and amino acids, in conjunction with a multi-gradient program, was obtained with satisfactory results. To our knowledge, this is the first time that fluorescence characteristics of CRP and the application of CRP for the simultaneous determination of amino compounds have been reported.

Experimental

Instrumentation

A Model 655 liquid chromatograph equipped with Model 650-10S fluorescence spectrophotometer (Hitachi, Seisakusho, Tokyo, Japan), a Rheodyne (Cotati, CA, USA) Model 7125 injection valve, a Model 655 proportioning valve and a Model 644-61 integrator (Hitachi) were used. Fluorescence excitation and emission spectra were also obtained on a Model 650-10S fluorescence spectrophotometer. Amino acid and polyamine derivatives were separated on a 200 × 4.6 mm id 5 μm Spherisorb C_{18} column (Dalian Institute of Chemical Physics, Chinese Academy of Sciences). A Paratherm U_2 electronic water-bath (Hitachi) was used to control the column temperature.

Reagents

Methanol and triethylamine were of analytical reagent grade from Jining Chemical Reagents (Jining, Shandong, China), and redistilled prior to use. Doubly distilled water was used throughout. All solvents used for reversed phase LC were filtered through 0.45 μm filtration disks. Amino acids were obtained from Sigma (St. Louis, MO, USA). Putrescine hydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride were supplied by the Biology Department, Qufu Normal University (Qufu, Shandong, China). Acetonitrile was of chromatographic grade. All other reagents for the determination of CRP fluorescence spectra were of analytical-reagent grade from Jining Chemical Reagents. Borate buffer was prepared from 0.2 mol l^{-1} boric acid solution adjusted to pH 8.8 with 4 mol l^{-1} sodium hydroxide solution prepared from sodium hydroxide pellets. Triethylamine stock standard solution (0.36 mol l^{-1}) used for the preparation of the LC eluent was adjusted to pH 6.5 with 2.0 mol l^{-1} hydrochloric acid. Ammonium dihydrogenorthophosphate stock standard solution (2.67 mol l^{-1}), used for the preparation of the LC eluent, was adjusted to pH 6.5 with ammonia solution. All mobile phases were treated ultrasonically for 15 min to remove gas bubbles prior to use.

The alkaline cleavage reagent was prepared daily in a 25 ml calibrated flask by mixing 17 ml of 0.850 mol l^{-1} sodium hydroxide solution with 7.5 ml of 0.5 mol l^{-1} hydroxylamine hydrochloride solution and 0.5 ml of 2-(methylthio)ethanol. The quenching reagent was acetonitrile–water–acetic acid (20:3:2, v/v/v).

Standard CRP-Cl derivatization solution was prepared according to the method described previously.[28]

Stock standard solutions of 0.5 mol l^{-1} NaF, 0.5 mol l^{-1} NaCl, 0.5 mol l^{-1} NaBr, 0.5 mol l^{-1} NaI, 0.2 mol l^{-1} CTMAB, 0.2 mol l^{-1} TX-100 and 0.2 mol l^{-1} SDS were prepared in doubly distilled water. Working standard solutions were obtained by appropriate dilution with water. A 0.5 mol l^{-1} CH_3I solution was freshly prepared in acetonitrile and lower concentrations were obtained by appropriate dilution with acetonitrile.

Chromatographic method

The HPLC separation of derivatives was performed on a Spherisorb C_{18} column with a binary gradient. Eluent A was 0.02 mol l^{-1} ammonium dihydrogenorthophosphate + 0.009 mol l^{-1} triethylamine (pH 6.5)–methanol (88 + 12 v/v) and B was acetonitrile–water (95 + 5 v/v). The flow rate was constant at 1.0 ml min^{-1} and the column temperature was kept at 35 °C. The fluorescence emission wavelength was 370 nm (excitation at 340 nm).

Separation of polyamines extracted from plant tissue was also carried out with a multi-gradient program. Eluent A was 0.015 mol l^{-1} ammonium dihydrogenorthophosphate + 0.005 mol l^{-1} triethylamine (pH 6.5)–acetonitrile (53 + 47 v/v) and B was acetonitrile–water (85 + 15 v/v).

Fluorescence spectra in the presence of halide salts, organic solvents and surfactants

The effects of the addition of various concentrations of halide salts and surfactants on the fluorescence spectra of CRP were studied in the following manner. Progressively increasing amounts of different additives were added separately in small increments to solutions containing probe CRP. After each addition, the solution was mixed carefully, followed by recording the corresponding fluorescence emission spectrum.

Sample preparation

Food products. Cheese sample was treated according to a previously described method.[29] A 30 g amount of the paste (equivalent to 20.0 g of cheese) was suspended in 40 ml of 0.1 mol l^{-1} HCl. The mixture was vigorously stirred at 0–4 °C for 30 min. After centrifugation, the supernatant solution was filtered. The residue was extracted twice with 20 ml of 0.1 mol l^{-1} HCl. Filtrates of the extracts were made up to 100 ml with 0.1 mol l^{-1} HCl and stored at −30 °C until HPLC analysis.

Plant materials. Extraction of barley seedling was carried out according to the method described previously.[30] A sample of plant tissue was chopped and 0.1–0.5 g fresh mass of tissue was homogenized with 5 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 filtered and the residue was extracted with two 2.0 ml volumes of 5% $HClO_4$ solution. The resulting combined extracts were diluted to 10 ml with 5% $HClO_4$ solution and stored at −30 °C until HPLC analysis.

Derivatization procedure

An appropriate volume of amino acid in polyamine stock standard solution was transferred into a 5 ml centrifuge tube, pH 8.8 buffer was added to bring the volume to 40–400 μl, then 50 μl of CRP-Cl solution in acetonitrile were added. The concentration of borate buffer was kept at 0.1–0.2 mol l^{-1} in the derivatized solution. The centrifuge tube was agitated and derivatization allowed to proceed for 2 min. A 30 μl volume of cleavage reagent was then added and reaction was allowed to proceed for a further 3.0–3.5 min, then finally the reaction was stopped by the addition of 50 μl of quenching reagent and the mixture was used directly for analysis.

Results and discussion

Excitation and emission spectra of CRP

CRP is a fluorescent compound which is easily dissolved in organic solvents. The uncorrected excitation and emission spectra in ethanol and tetrahydrofuran are shown in Fig. 1. The excitation maxima are 295 and 340 nm, respectively, and the emission maxima are 355 and 370 nm, respectively. Generally, λ_{ex} 340 nm λ_{em} 370 nm are chosen for the determination of amino acid and polyamine derivatives in order to eliminate baseline drift.

Effect of different solvents on the fluorescence intensity and emission wavelength

Solvents are classified in five groups: group 1 = methanol, ethanol, propan-1-ol, butan-1-ol, pentan-1-ol, hexan-1-ol, heptan-1-ol and octan-1-ol; group 2 = methylamine, ethylamine, propylamine and butylamine; group 3 = formic acid, glacial acetic acid, propionic acid, butyric acid and pentanoic acid; group 4 = dichloromethane, chloroform and tetrachloromethane; and group 5 = dimethyl sulfoxide, tetrahydrofuran, *N*,*N*-dimethylformamide, acetonitrile and acetone (see Table 1). The effects of various solvents on the fluorescence spectra of CRP were investigated with an excitation wavelength λ_{ex} 340 nm. As can be seen from group 1, the fluorescence intensities of CRP in alcohols increase with increase in carbon number. This observation is new, as far as we know. This is probably due to the fact that the hydrogen bond forces between CRP and various alcoholic molecules decrease with increasing solvent viscosity. An interesting result is that the maximum fluorescence emission remains unchanged.

In group 2, it was found that the fluorescence intensities of CRP in methylamine, ethylamine, propylamine and butylamine decrease with increase in carbon number, probably because the solubilities of CRP in these solvents decrease significantly with increase in carbon number.

With the solvents in group 3, it is also found that for carbon numbers more than five, the fluorescence intensity of CRP decreases rapidly with increase in carbon number, which may be attributed to the progressively decreasing solubility of CRP in these solvents. A surprising result is that the fluorescence intensity of CRP in formic acid is nearly three times lower than that in glacial acetic acid, propionic acid and butyric acid, probably because the CRP molecule is partially protonated in the relatively strong formic acid, resulting in corresponding weak fluorescence emission.

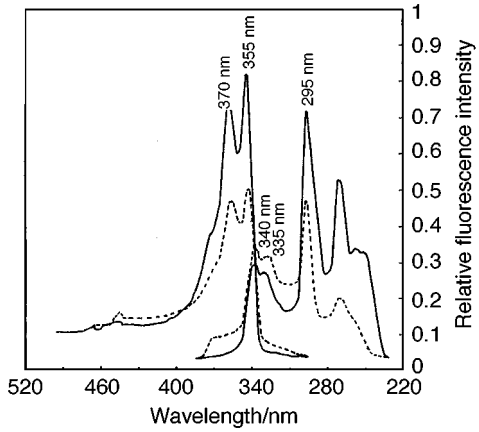

Fig. 1 Excitation and emission spectra of CRP. Solid lines represent excitation and emission spectra in ethanol solution and dotted lines those in tetrahydrofuran solution.

Another result is that solvents in group 4, in which the fluorescence intensities of CRP exhibit substantial differences, the fluorescence intensity is dramatically quenched with an increase in number of chlorine atoms. No emission intensity of CRP in tetrachloromethane is observed. The fluorescence emission intensity in chloroform is nearly five times less than that in dichloromethane. In group 5, it was found that the emission spectra of CRP show slight differences in emission maxima. As can be seen from Table 1, CRP exhibits a large fluorescence intensity in dimethylsulfoxide with a 4–10 nm red shift in emission wavelength. However, no emission intensity of CRP in acetone is observed. The fluorescence intensities of CRP in group 5 solvents decrease in the order dimethyl sulfoxide > tetrahydrofuran > *N*,*N*-dimethylformamide > acetonitrile >> acetone.

Effect of temperature on fluorescence intensity in non-aqueous solvents

Fluorescence-based temperature sensing is attracting increasing interest as it finds unique applications in monitoring the temperature within micro-sized domains (*e.g.*, a biological cell) or hostile environment (*e.g.*, a microwave-irradiated system).[31,32] The fluorescence intensity of fluorophores in non-aqueous solvents was investigated at a variety of temperatures with λ_{ex} 340 nm and λ_{em} 370 nm. It is suitable for thermometry at temperatures in the range 15–60 °C. Here, the excitation wavelength (λ_{ex} 340 nm) was chosen for recording fluorescence spectra. The temperature was controlled by a connected circulator. It is known that there is a direct correlation between the fluorescence measurements and the bulk viscosity of the medium surrounding the fluorescence probe. This type of

Table 1 Effect of the nature of the solvent on CRP spectral properties (the concentration of CRP in each solvent was kept at 1.0×10^{-6} mol l^{-1})

Solvent	Excitation/nm		Emission/nm		Relative intensity[a]
Group 1—					
Methanol	295	340	355	370	106.2
Ethanol	295	340	355	370	134.8
Propan-1-ol	295	340	355	370	135.6
Butan-1-ol	295	340	355	370	141.8
Pentan-1-ol	295	340	355	370	150.8
Hexan-1-ol	295	340	355	370	152.7
Heptan-1-ol	295	340	355	370	165.1
Octan-1-ol	295	340	355	370	167.4
Group 2—					
Methylamine	295	340	355	375	69.7
Ethylamine	295	340	355	375	63.2
Propylamine	295	340	355	375	31.8
Butylamine	295	340	—	—	—[b]
Group 3—					
Formic acid	295	340	354	370	20.9
Glacial acetic acid	295	340	350	367	132.1
Propionic acid	295	340	350	370	133.2
Butyric acid	295	340	350	370	134.3
Pentanoic acid	295	340	350	370	11.6
Group 4—					
Dichloromethane	295	340	352	367	96.9
Chloroform	295	340	355	370	23.2
Tetrachloromethane	295	340	—	—	—[b]
Group 5—					
Dimethyl sulfoxide	295	340	360	376	100*
Tetrahydrofuran	295	340	351	366	68.2
N,*N*-Dimethylformamide	295	340	357	371	45.7
Acetonitrile	295	340	355	371	39.5
Acetone	295	340	—	—	—[b]

[a] Fluorescence intensities of CRP in various solvents were calculated according to that of dimethylsulfoxide as 100% using λ_{em} = 370 nm. [b] Emission intensity not observed.

correlation was used to estimate the microviscosity afforded in different organic solvents at different temperatures. In this study, it was found that CRP is thermally stable and exhibits no significant decomposition over the temperature ranges investigated. A kinetic analysis of thefluorescence intensity of CRP at different temperatures in various solvents led to a linear correlation between ln I_{em} and 1/T. The slope of the Arrhenius plot yields the emission stabilization energies,[33] as shown in Table 2.

Additionally, the steady state fluorescence intensity of CRP in aqueous solution was also investigated at various temperatures from 15 to 60 °C. It was found that the emission intensity of CRP increases with increase in temperature (not shown), possibly owing to the electroionization of CRP molecules.

The effect of temperature on the fluorescence spectra of CRP in solvents in groups 1–5 were investigated over the range 15–60 °C in 15 increments. It was found that an increase in temperature does not cause any visible shift of the fluorescence spectra. Moreover, recording spectra from 15 to 60 °C allowed the five groups of solvents to be distinguished in terms of changes in emission intensity (Table 2). With solvents from group 1, the CRP fluorescence intensity (I = 100 at 15 °C) decreased considerably with increase in temperature up to 60 °C (I = 67–81); this decrease is almost completely reversed when the temperature is returned from 60 to 15 °C. With solvents from group 2, the fluorescence intensity of CRP decreased considerably (I = 68–76 at 60 °C) but was still reversible. With solvents from group 3, a substantial decrease in emission intensity was also observed (I = 67–69 at 60 °C), but this decrease was irreversible with formic acid. With solvents from group 4 (aprotic solvents), the decrease was slight (I = 87–90 at 45 °C); the solvents in group 4 are easily volatilized, so the investigated temperature range was 15–45 °C), and irreversible for dichloromethane. In addition, it was also found that the emission intensity of CRP in tetrachloromethane was completely quenched; however, an increase in temperature did not cause any visible shift of the fluorescence spectra. With solvents from group 5, the fluorescence intensities of CRP showed large differences, but an interesting result is that the decrease in emission intensity at 60 °C in dimethylsulfoxide, tetrahydrofuran and *N*,*N*-dimethylformamide showed no difference (I = 75 at 60 °C).

Table 2 Effect of temperature on CRP fluorescence intensity in various solvents

Solvent	Intensity 15 °C	30 °C	45 °C	60 °C	Stability energy/ kJ mol^{-1}	Regression coefficient
Group 1—						
Methanol	100	91	83	75	5.08	0.9978
Ethanol	100	91	82	73	5.57	0.9956
Propan-1-ol	100	92	84	77	4.71	0.9983
Butan-1-ol	100	91	82	73	5.57	0.9956
Pentan-1-ol	100	90	81	72	5.81	0.9975
Hexan-1-ol	100	92	83	74	5.34	0.9937
Heptan-1-ol	100	93	87	81	3.72	0.9989
Octan-1-ol	100	89	77	67	7.14	0.9963
Group 2—						
Methylamine	100	88	78	68	6.79	0.9979
Ethylamine	100	89	79	71	6.09	0.9996
Propylamine	100	90	83	76	4.82	0.9994
Butylamine	—	—	—	—[a]		
Group 3—						
Formic acid	100	87	78	69	6.52	0.9994
Glacial acetic acid	100	87	79	69	6.44	0.9972
Propionic acid	100	86	78	68	6.69	0.9972
Butyric acid	100	85	76	67	6.99	0.9986
Pentanoic acid	100	84	75	67	7.04	0.9973
Group 4—						
Dichloromethane	100	95	90	—[b]	2.69	0.9991
Chloroform	100	94	87	—[b]	3.55	0.9954
Tetrachloro-methane	—	—	—	—[a]		
Group 5—						
Dimethyl sulfoxide	100	95	97	75	5.02	0.9991
Tetrahydrofuran	100	94	87	75	4.97	0.9954
N,*N*-Dimethylfor-mamide	100	90	81	75	5.17	0.9993
Acetonitrile	100	91	82		5.03	0.9985
Acetone	—	—	—	—[a]		
Pure water	100	104	109	114		

[a] Emission intensity not oberserved. [b] No determination.

Effect of pH on fluorescence spectra of CRP

The CRP molecule contains a carboxylic group, hence the fluorescence emission spectrum may change with change in pH. The emission intensity is constant at pH < 3 because CRP is not dissociated in this range. In addition, the emission intensity is also constant at pH > 8.0 because it is completely dissociated in this range. It can be seen that the fluorescence emission intensity of CRP increases gradually with increasing pH in the range 3.0–8.0 (see Fig. 2). The fluorescence emission intensity is higher in basic solution than in acidic solution, probably because the nitrogen atom in the CRP molecule may also share its free electron pair with hydrogen ion to form a weak covalent bond, resulting in a decrease in fluorescence intensity. This statement disagrees with the data shown in Table 1, in which the fluorescence intensity of CRP in solvents from group 2 (amines) is lower than that in solvents from group 3 (acids). This is probably due to the fact that all these solvents are pure reagents in which the fluorescence intensity of CRP is primarily ruled by hydrogen bonding forces. On the other hand, CRP reacts partially with organic base (amines) to form the corresponding ammonium salt, resulting in low fluorescence. In addition, CRP is also a weak organic acid;[28] the dissociation constant K_{am} can be obtained *via* RP-HPLC from the capacity factor values k at different pH as $K_{am} = 4.67 \times 10^{-7}$.

Effect of halide salts and organic halides on CRP fluorescence spectra

In order to elucidate the influences of halide salts and organic halides on the fluorescence emission spectra, we chose five additives, NaCl, NaF, NaBr, NaI and CH_3I, to examine the effects of various amounts of additives on the fluorescence emission spectra of CRP. It was found that the addition of progressively increasing amounts of NaF and NaCl into CRP solution results in a slight quenching of the emission intensity. This is possibly due to the fact that there is only a weak intermolecular interaction between CRP molecules and the electronegative elements (F and Cl). However, the addition of

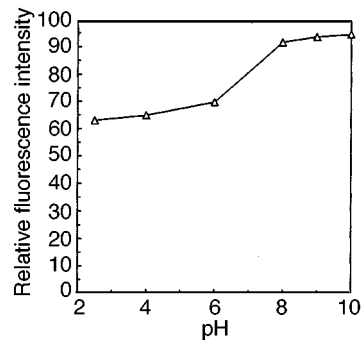

Fig. 2 Effects of pH on fluorescence spectra of CRP.

progressively increasing amounts of NaBr and NaI to CRP solution leads to a large decrease in emission intensity. It is believed that there is a strong intermolecular interaction between CRP molecules and the electronegative elements (Br and I); this interaction decreases the electron density of CRP molecules and leads to a corresponding extinction of the fluorescence intensity, but this interaction does not cause any visible shift in emission wavelength. In order to achieve the same extent of quenching as seen with NaI, nearly twice the concentration of NaBr with respect to NaI is required. In addition, note that the addition of progressively increasing amounts of CH_3I causes a large decrease in emission intensity. The same extent of quenching relative to that of NaI can be achieved even with a two orders of magnitude lower concentration of CH_3I. Furthermore, complete quenching was observed with increasing concentration of CH_3I beyond 4.0×10^{-2} mol l^{-1}. This may be due to cumulative effects of electrostatic binding and the formation of a quaternary ammonium salt between CRP and CH_3I. Although the fluorescence quenching phenomena are observed in both instances involving the addition of halide salts and CH_3I to CRP solution, the added amounts of halide salts or CH_3I to achieve an equal extent of fluorescence quenching show large differences. The effects of the addition of halide salts and organic halides on the CRP fluorescence intensity are shown in Table 3.

In order to estimate further the effects of salts and iodomethane on CRP fluorescence quenching, the fluorescence quenching data were plotted according to the Stern–Volmer equation:[34]

$$(F_0 - F)/F = K[D_0] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of salts, respectively, K is the quenching constant and $[D_0]$ is the concentration of the quencher. As can be seen from eqn. (1), the quenching constant K can be obtained by dividing $(F_0 - F)/F$ by $[D_0]$. The quenching constants for NaF, NaCl, NaBr, NaI, and CH_3I are 9.4, 12.6, 1.04, 456 and 560, respectively, *i.e.* they decrease in the order CH_3I > NaI > NaBr > NaF ≈ NaCl.

Effect of surfactants on CRP fluorescence spectra

The fluorescence emission intensity of CRP upon addition of progressively increasing amounts of surfactants to CRP solution was examined. We chose three additives, cetyl trimethylammonium bromide (CTMAB), TX-100 and SDS, for fluorescence studies.

The results indicate that the influence of the surfactant CTMAB on the fluorescence spectra of CRP can be classified as two types: fluorescence quenching [below the critical micelle concentration (CMC), 9.2×10^{-4} mol l^{-1}] and the micelle effect (above the CMC). They obey the Stern–Volmer relationship for static quenching and a solute–micelle interaction) linear relationship, respectively. The addition of progressively increasing amounts of CTMAB (the CTMAB concentration below the CMC) to CRP solution led to gradual fluorescent quenching. When the CTMAB concentration is near CMC, the emission intensity turns gradually from quenching to enhancement with further addition of increasing amounts of CTMAB, finally reaching saturation. The effects of progressively increasing amounts of the surfactant CTMAB on the CRP fluorescence spectra are shown in Fig. 3. Note that the addition of progressively increasing amounts of CTMAB to the CRP solution does not cause any visible shift in the emission spectra.

We also examined the effects of the addition of progressively increasing amounts of TX-100 solution to CRP solution on the fluorescence spectra. A similar kind of enhancement (above the CMC, 2.5×10^{-4} mol l^{-1}) in the fluorescence spectra was observed as for CTMAB. However, substantial quenching (below the CMC) with gradually increasing amounts of TX-100 was not observed. The maximum emission wavelength also remained unchanged (not shown).

The influence on the fluorescence emission spectra upon addition of progressively increasing amounts of the anionic surfactant SDS to the CRP solution on fluorescence spectra was also investigated. The results indicate that the emission spectra are not significantly altered with increasing amount of SDS. This is in marked contrast to what is observed in the experiments including the addition of either CTMAB or TX-100. This could be due to a lack of interaction between anionic surfactant aggregates and the CRP.

A theoretical study of CRP fluorescence quenching or enhancement on addition of increasing amounts of surfactants was also performed as follows.

It is well known that the fluorescence lifetime is of the order of nanoseconds and the micelle average lifetime is at about the

Table 3 Effects of the addition of progressive amounts of halide salts and organic halides on CRP fluorescence intensity (determined at 30 °C, pH 6.5)

	Salt concentration/10^{-3} mol l^{-1}					
Salt	0	0.625	1.25	1.875	2.5	3.125
NaF	100	99.4	98.8	98.3	97.7	97.0
NaCl	100	99.1	98.5	97.7	97.0	96.2
NaBr	100	93.9	88.5	83.7	79.8	75.5
NaI	100	77.8	63.7	54.1	46.9	40.3
CH_3I	100	74.1	58.8	48.8	41.6	36.6

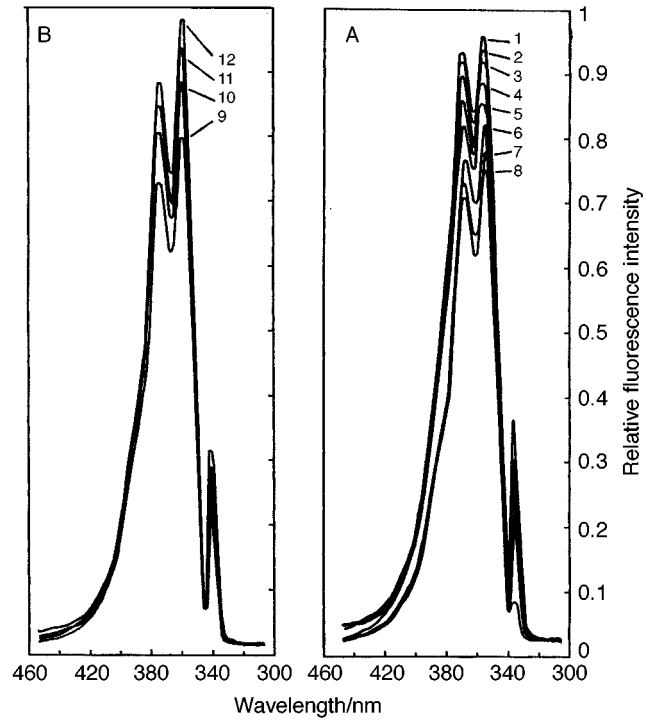

Fig. 3 Effect of addition of progressively increasing amounts of surfactant CTMAB on CRP fluorescence spectra. This experiment was carried out by adding increasing amounts of CTMAB to CRP solution and the fluorescence spectra were recorded after each addition. (A) Fluorescence quenching addition of progressively increasing amounts of CTMAB (concentration 0, 1.0×10^{-5}, 2.0×10^{-5}, 5.0×10^{-5}, 1.0×10^{-4}, 3.0×10^{-4}, 6.0×10^{-4} and 8.0×10^{-4} mol l^{-1}); (B) fluorescence enhancement with the addition of progressively increasing amounts of CTMAB (concentration 1.0×10^{-3}, 1.4×10^{-3}, 1.6×10^{-3} and 1.8×10^{-3} mol l^{-1}). The maximum excitation wavelength λ_{ex} 340 nm was used for the recording fluorescence spectra. The CRP concentration, in all cases, was maintained at 1.0×10^{-6} mol l^{-1}.

millisecond level.[35] Hence it can be assumed that micelles are relatively independent species existing in solution. According to the mass action law, a useful expression for solute distribution in micelle solution can be obtained:

$$S_w + M_n \leftrightharpoons S_m \quad (2)$$

The equilibrium constant (binding constant), K, is given by

$$K = \frac{[S_m]}{[S_w][M_n]} \quad (3)$$

where $[S_m]$and $[S_w]$ are the corresponding equilibrium concentrations in the micelle phase and the water phase, respectively and $[M_n]$ is the micelle concentration in which the aggregate number is N (N = 61 for CTMAB and 140 for TX-100).

$$[M_n] = \frac{c_{sf} - \mathrm{CMC}}{N} \quad (4)$$

where, c_{sf} is the concentration of surfactant. Generally, F_m and F_w are used for the fluorescence intensity in the micelle phase and the water phase, respectively. Hence the total fluorescence intensity (F) in the presence of micelles can be expressed quantitatively as

$$F = aF_m + (1 - a)F_w \quad (5)$$

where $a = [S]_m/[S]_T$ is the distribution coefficient of the solute in two phases and $[S]_T$ is the total concentration of solute in the two phases.

By combination of eqns. (3) and (5) we obtain

$$\frac{1}{F - F_w} = \frac{1}{F_m - F_w}\left(1 + \frac{1}{K[M_n]}\right) \quad (6)$$

As can be seen, the binding constant (K) for fluorescence enhancement can be obtained by dividing the intercept by the slope. By calculation, the monomer quenching constant K for CTMAB is 1.8×10^3 l mol^{-1}. The binding constants of micelles for CTMAB and TX-100 are 2.6×10^3 and 1.2×10^3 l mol^{-1}, respectively.

Optimization for derivatization

In developing CRP-Cl for the simultaneous separation of amino acid and polyamine derivatives, it is important to assess the effects of CRP-Cl concentrations and reaction times. The effect of CRP-Cl concentration on the derivatization yields of corresponding amino acid derivatives have been reported.[28] In this study, the effects of CRP-Cl concentration on the derivatization yields of representative polyamines, *viz.*, putrescine, spermidine and spermine, were investigated. A mixture consisting of putrescine (100 pmol), spermidine (100 pmol) and spermine (100 pmol) was prepared and treated with CRP-Cl in the concentration range 5.0–100 μmol in 0.2 mol l^{-1} borate buffer at pH 8.8. The other conditions were the same as described in the Experimental section. It was found that the fluorescent signal for each derivative steadily increases with increasing amount of CRP-Cl from 5.0 to 80 μmol, above which the intensity remained constant (not shown). It was also found that if the amount of CRP-Cl in the derivatized solution is lower than 40 μmol, there is not enough CRP-Cl for reaction, especially in samples in which many amino acids and polyamines are present.

The effect of the reaction time on the fluorescence intensity for the above polyamines was also investigated. The amount of CRP-Cl was kept at 100 μmol in order to react completely; all other conditions were the same as described above. The dependence of the peak heights of the amine derivatives on the reaction time indicates that the optimum derivatization time for achieving a high yield is about 2.0–2.5 min at 30 °C (pH 8.8). Under these reaction conditions, the derivatization of other amino compounds is also adequate. Complete derivatization for most amino acids is obtained after only 1.5 min. Therefore, the derivatization time adopted for the subsequent simultaneous derivatization of amino acids and polyamines was 1.5–2.0 min. If the derivatization time was > 2.5 min, amino acids with a hydroxyl functional group, such as Thr, Ser, Cys and Tyr, completely formed disubstituted derivatives, which was detrimental for their rapid and sensitive detection. Moreover, disubstituted Thr, Ser, Cys and Tyr derivatives were partially decomposed in alkaline buffer (mainly ester saponification decomposition), leading to a complex separation. Generally, disubstituted amino acid derivatives (amino acids containing a hydroxyl functional group mentioned above) are converted into monosubstituted derivatives in order to obtain a fast separation. In this study, it was found that alkaline treatment of these disubstituted derivatives with sodium hydroxide or potassium hydroxide solution can produce the monosubstituted derivatives, but the conversion will cause as much as a 25% loss of other amino acid derivatives. A cleavage reagent consisting of hydroxylamine and sodium hydroxide proved effective in converting these disubstituted derivatives into the monosubstituted derivatives. It was also proved optimal as it had little effect on normal RNH-CRP groups. At the same time, this cleavage reagent has the advantage of easily converting the unreacted CRP-Cl into CRP-hydroxylamine. However, it was also found that if the cleavage reaction time was > 4.0 min, the disubstituted amino acids, such as Tyr and Pro (simultaneously containing primary and secondary amino groups) will cause as much as a 5% loss of its derivatives (mainly secondary amino group decomposition), but these phenomena are not observed for other amino acid derivatives. Therefore, the cleavage reaction time adopted for converting the disubstituted derivatives (Thr, Ser, Cys and Tyr) into the corresponding monosubstituted derivatives was 3.0–3.5 min.

Acetonitrile is used as the reaction co-solvent in preference to acetone as it avoids the problem of precipitation of hydrophobic derivatives. At the same time, a separation of phases at high buffer concentrations is also observed. This can be avoided in two ways: (1) the buffer concentration is maintained at ≤0.2 mol l^{-1}, or the acetonitrile concentration in the derivatized solution is < 50% v/v; (2) the use of acetone instead of acetonitrile. Here, method (1) was chosen.

In preliminary studies, borate, phosphate and hydrogencarbonate buffers were investigated for derivatizing the amino acids. Both borate and hydrogencarbonate were satisfactory, but phosphate proved unacceptable as it decreased the speed of derivatization and led to partial precipitation and incomplete reaction. In this study, a similar phenomenon to that described above was observed. It was also found that there is little effect of buffer pH on derivatization yields in the pH range 8.5-9.5. Generally, most subsequent derivatizations were carried out using 0.2 mol l^{-1} borate buffer at pH 8.8.

Stability of CRP derivatives

The stability of CRP derivatives at room temperature was investigated previously by analyzing corresponding amino acid derivatives.[28] As expected, daylight had no effect on stability. In addition, derivatives from derivatized solution were diluted twice with mobile phase containing 0.02 mol l^{-1} ammonium dihydrogenorthophosphate + 0.009 mol l^{-1} triethylamine (pH 6.5) and kept at 40 °C in a water-bath for 2 h without substantial decomposition. These results showed that the stabilities of CRP derivatives were equal or superior to those of FMOC derivatives and that they were sufficiently stable to allow further analysis of derivatized samples after at least 24 h at room temperature.

Chromatographic separation of amino acids and polyamines

The LC separation of a mixture containing amino acid and polyamine derivatives was carried out on a reversed-phase

Spherisorb C_{18} column. The linearities and repeatabilities were as good as those as described previously for the separation of amino acids.[28] Different samples were successfully analyzed for both polyamines and amino acids. When the ammonium dihydrogenorthophosphate and triethylamine concentrations in mobile phase were >0.025 and 0.015 mol l^{-1}, respectively (with the methanol concentration maintained at 15% v/v in eluent A), it was found that Arg and Thr or Cys and Tyr were unresolved (not shown). In addition, Val and Met were also unresolved. At the same time, CRP-OH interfered with the separation of Lys when the concentration of methanol in eluent A was >15% v/v. However, this problem was easily resolved by varying the composition of the mobile phase. When the ammonium dihydrogenorthophosphate and triethylamine concentrations in mobile phase were <0.02 and 0.009 mol l^{-1}, respectively (with the methanol concentration maintained at 12% v/v in eluent A), the aforementioned derivatives were found to achieve complete resolution. Chromatograms of a standard mixture and an extract from cheese are shown in Fig. 4.

Chromatographic separation of polyamines in plant tissue

The separation of polyamines in plant tissue was investigated using multi-gradient elution. With a mobile phase composition of eluent A 0.015 mol l^{-1} ammonium dihydrogenorthophosphate + 0.005 mol l^{-1} triethylamine (pH 6.5)–acetonitrile (53 + 47, v/v) and B acetonitrile–water (85 + 15 v/v), the optimum separation of a standard polyamine mixture was obtained [Fig. 5(A)]. The by-products CRP-OH and CRP-NHOH from the derivatizing agent were consistently found not to affect the separation of polyamines extracted from barley seedings as they were eluted first due to the high acetonitrile concentration in eluent A. For the determination of calibration curves, standard solutions containing 5, 10, 15, 20 and 25 pmol μl^{-1} polyamines were derivatized and injected five times. The corresponding amounts injected were 50–250 pmol. The regression analysis of calibration graphs and other quantitative data for polyamine derivatives were established and given in Table 4. The separation of polyamines extracted from barley seedling is shown in Fig. 5(B). It was also found that an unknown component from the barley seedling extract seriously interfered with the separation of 1,6-diaminohexane (first used internal standard) in real samples and led to unsatisfactory results, so we chose cadaverine as the internal standard. The contents of polyamines extracted from barley seedlings are given in Table 5.

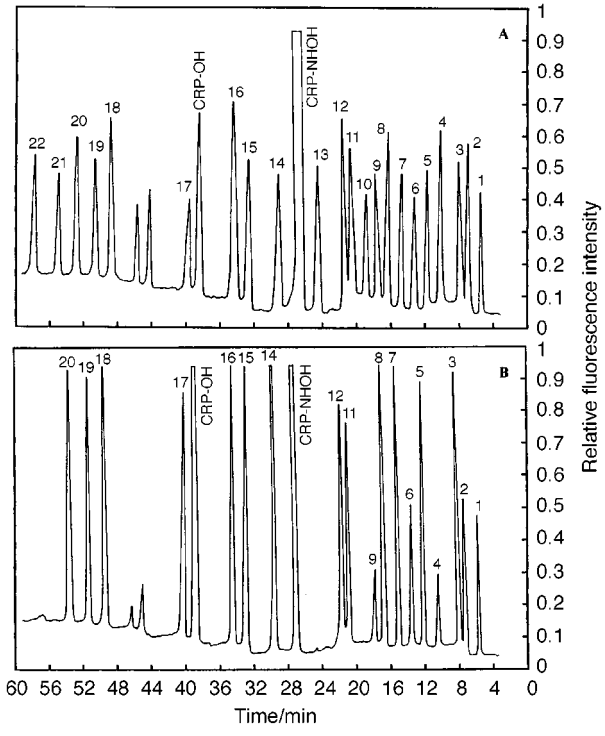

Fig. 4 Chromatograms of (A) 50 pmol of amino acid and polyamine standard mixtures and (B) cheese extract. Column temperature, 35 °C; excitation wavelength, λ_{ex}340 nm; emission wavelength, λ_{em} 370 nm; column, 200 × 4.6 mm id Spherisorb (5 μm); flow rate, 1.0 ml min^{-1}. Eluent A = 0.02 mol l^{-1} ammonium dihydrogenorthophosphate + 0.009 mol l^{-1} triethylamine–methanol (88 + 12 v/v); B = acetonitrile–water (95 + 5 v/v). Gradient conditions: 0–6 min = 95% A; 16 min = 85% A; 25 min = 75% A; 30 min = 70% A; 35 min = 60% A; 40 min = 50% A; 45 min = 40% A; 50 min = 10 % A; 60 −70 min = 1% A. Peaks: 1 = Asp; 2 = Glu; 3 = Ser; 4 = His; 5 = Gly; 6 = Thr; 7 = Ala; 8 = Pro; 9 = Tyr; 10 = Arg; 11 = Val; 12 = Met; 13 = Ile; 14 = Leu; 15 = Phe; 16 = Cys; 17 = Lys; 18 = putrescine; 19 = cadaverine; 20 = heptylamine; 21 = spermidine; 22 = spermine.

Conclusion

The derivatization reagent CRP-Cl for the quantitative analysis of amines and amino acids has been reported. This work has shown that CRP-Cl is an effective derivatization agent.

CRP-Cl reacts very fast with primary and secondary amines in alkaline solution to give the corresponding derivatives, which exhibit excellent sensitivity, stability and high derivative yield. Complete derivatization takes less than 2 min at room temperature, and the derivatives are stable for at least 2 d in neutral solution. By-products from the derivatizing agent do not interfere with the LC analysis when the mobile phase composition is suitably adjusted. In contrast to the molecular structure of FMOC, the CRP molecule contains a nitrogen atom, and its n–π conjugation (nitrogen atom is an electron pair donor) in molecule is dramatically augmented, which makes it more sensitive than FMOC with respect to fluorescence. Excess of derivatization reagent can be overcome by adding a quenching reagent. In fact, with FMOC, excess of reagent must be extracted prior to chromatographic analysis. Additionally, compared with AQC and CRP-Cl, AQC reacts with both primary and secondary amino acids only to form stable

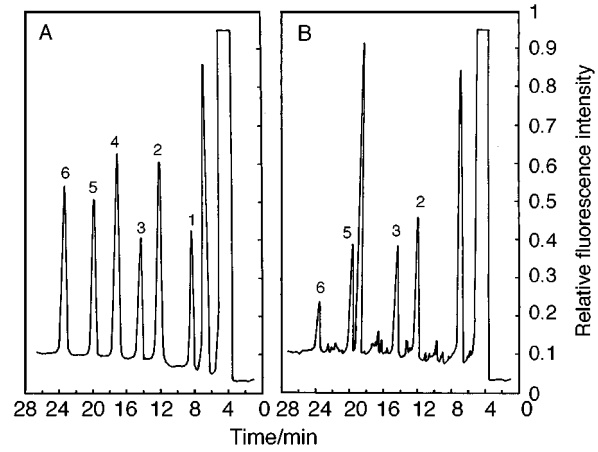

Fig. 5 HPLC of barley seedling extract derivatized with CRP-Cl. Eluent A = 0.015 mol l^{-1} ammonium dihydrogenorthophosphate + 0.005 mol l^{-1} triethylamine (pH 6.5)–acetonitrile (53 + 47 v/v); B = acetonitrile–water (85 + 15 v/v); flow rate, 1.0 ml min^{-1}; column temperature, 35 °C. Gradient conditions: initial = 95% A; 5 min = 90% A; 10 min = 80% A; 20 min = 70% A; 25 min = 60% A; 30 min = 50% A; 35 min = 1% A. (A) Standard chromatogram: 1 = trimethylene polyamine; 2 = putrescine; 3 = cadaverine; 4 = 1,6-diaminohexane; 5 = spermidine; 6 = spermine. (B) Sample analysis chromatogram: peaks as in A; cadaverine used as internal standard.

Table 4 Regression analysis of calibration graphs and other quantitative data for polyamines in plant tissue

Compound	Linear range amount injected/pmol	Calibration graph[a]	Correlation coefficient	RSD(%)[b] (n = 6)	Detection limit[c]/fmol
Putrescine	10–350	$y = -0.41 + 0.0576x$	0.999	3.2	23
Spermidine	10–400	$y = -0.66 + 0.0417x$	0.999	4.0	22
Spermine	20–450	$y = -0.58 + 0.0365x$	0.999	4.5	34

[a] y in cm, x in pmol amount injected. [b] relative standard deviation for 50 pmol of each CRP derivative. [c] Signal-to-noise ratio = 3.

Table 5 Determination of polyamines in barley seedlings

Barley seedling	Amounts of polyamine/μg g^{-1} (n = 6)				
	Putrescine	Spermidine	Spermine	Total amount	Putrescine (%)
Upper	87.2 ± 3.0	37.8 ± 3.0	9.2 ± 2.5	134.2	64.9
Middle	44.7 ± 3.5	20.6 ± 2.0	8.8 ± 1.5	74.1	60.3
Lower	31.6 ± 2.0	26.4 ± 2.0	4.3 ± 1.5	62.3	50.7
Root	42.7 ± 2.0	15.2 ± 1.5	1.7 ± 1.0	59.6	71.6

unsymmetric urea derivatives at room temperature, but the derivatization of Tyr is difficult. *o*-Phthalaldehyde offers greater sensitivity, but is limited to primary amino acids. PIB-Cl also offers high sensitivity, but is not appropriate for aromatic amines. At the same time, more interfering peaks are observed on standing the derivatization solution. Although NBD-F is more reactive than NBD-Cl for derivatizing amino compounds, data reported previously indicate that the two reagents themselves undergo about 30–50% decomposition in methanol–water solution within 25 min when exposed to daylight. In this study, CRP-Cl was developed as a fluorescence labeling reagent for the indirect determination of amino acids and polyamines, it is highly reproducible, and applicable to the determination of polyamines extracted from barley seedlings with satisfactory results. These characteristics make it an attractive derivatization agent for amino compounds. Note that organic halides, such as CH_3I, will cause a dramatic quenching on the fluorescence intensity of CRP.

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