Determination of Polyamines in Foods by Liquid Chromatography with On-Column Fluorescence Derivatization

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An on-column fluorometric derivatization method has been developed for the determination of polyamines using high-performance liquid chromatography. The system for the derivatization consisted of a single-plunger pump and a reversed-phase C₁₈ polymer column with a mobile phase of acetonitrile and alkaline borate buffer solution containing *o*-phthalaldehyde and *N*-acetyl-L-cysteine as derivatizing reagents. The polyamines injected onto the liquid chromatograph are derivatized to fluorophores at the inlet of the column, followed by chromatography on the same column. Optimization of the liquid chromatography conditions led to a simple analytical method for the simultaneous determination of polyamines with high reproducibility and linearity. Application of this method to the determination of polyamines in food samples resulted in overall mean recoveries greater than 85% at fortification values of 20–200 $\mu\text{g g}^{-1}$ of each polyamine.

Keywords On-column fluorescence derivatization, polyamines, *o*-phthalaldehyde, *N*-acetyl-L-cysteine, putrefaction, high-performance liquid chromatography

Polyamines exist widely in biological materials and also in many food materials. In foods most of them are produced by enzymatic degradation associated with putrefaction or fermentation and show physiological activity in humans.¹ Amine levels were shown to be a good index of food decomposition.² The polyamines usually do not cause any hazards to individuals unless large amounts are ingested. However, some of the polyamines may be nitrosated in the presence of nitrite or act as precursors for other compounds capable of forming carcinogenic nitrosamines.^{3–5} On the other hand, some of the polyamines are also known to potentiate histamine-toxicity^{6–8} and to act synergistically⁹ in allergy-like food poisoning caused by the intake of histamine-contaminated food. Therefore, the determination of polyamines in foods is important. There has also been a need to develop a simple analysis method to determine polyamines in order to control the quality and evaluate the safety of various foods.

A number of analytical methods, such as gas liquid chromatography (GC)^{10–12}, enzymatic oxygen electrode method¹³ and high-performance liquid chromatography (HPLC)^{14–19} have been used so far for the determination of polyamines in foods as well as biological materials. However, the GC methods require a tedious derivatization procedure, while the enzymatic oxygen electrode method cannot distinguish one polyamine from another. Most reports on the HPLC method have employed the

precolumn^{14–16} or postcolumn^{17–19} derivatization technique. However the precolumn methods suffer from some disadvantages¹⁶ such as requirements for carefully controlled reaction conditions and inferiority in reproducibility because of very limited stability. On the other hand, a postcolumn method is claimed to be less troublesome than a precolumn procedure and can eliminate its drawbacks. However, it requires expensive additional equipment for the chromatographic system, and the addition of a postcolumn reagent brings about peak broadening and dilution of samples. Furthermore, a gradient elution system has been required for the simultaneous analysis of various kinds of polyamines.

Recently, we have developed an on-column derivatization technique²⁰ for the determination of histamine which is derivatized at the inlet of the HPLC column with a mobile phase containing *o*-phthalaldehyde (OPA) reagents. This technique was designed to overcome some of the drawbacks of the current derivatization method. During the study of the on-column derivatization, we found that most of the primary amino compounds could also be derivatized to fluorophores by the on-column method. We thus attempted to apply this method further to the simultaneous determination of histamine and major putrefactive polyamines under isocratic HPLC conditions.

Experimental

Reagents and materials

Standard amines of histamine dihydrochloride (Him), tyramine hydrochloride (Tym), 1,3-diaminopropane dihydrochloride (Dap), putrescine dihydrochloride (Put), spermidine triphosphate (Spd), spermine diphosphate (Spm), cadaverine dihydrochloride (Cad), and diethylenetriamine (Det) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Though Him and Tym are not polyamines, these amines have been used as the major chemical indexes² for putrefaction. To prepare standard solutions, these polyamines were dissolved in 0.05 M hydrochloric acid (HCl). OPA was purchased from Nakalai Tesque (Kyoto, Japan), *N*-acetyl-L-cysteine (NAC) of biochemical grade from Merck (Darmstadt, Germany), and acetonitrile of HPLC grade from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

Amberlite CG-50 (type II., 200–400 mesh; Rohm and Haas, Philadelphia, PA, USA) for the clean-up column was conditioned with 0.1 M sodium acetate buffer (pH 5.6) and was then packed in a glass column (135×9 mm i.d.) at a height of 1 cm.

Apparatus and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-6A pump (Shimadzu, Kyoto, Japan), a Model 7125 injector (Rheodyne, Berkeley, CA, USA) with a 20 μ l volume loop, and a Model RF-535 fluorescence detector (Shimadzu) set at excitation and emission wavelengths of 330 nm and 430 nm, respectively. The analytical column for HPLC was an Asahipak ODP-50 (150×4.6 mm i.d.; Asahi Chemical Industry, Kanagawa, Japan), and was thermostated at 40°C throughout the experiment.

The HPLC mobile phase was a mixture of 50 mM sodium borate buffer (pH 9.9)–acetonitrile (77:23, v/v) containing 2 mM OPA and 2 mM NAC. Being less soluble in aqueous solvent, OPA was first dissolved in acetonitrile and then NAC and 50 mM borate buffer were added. The prepared mobile phase was transferred to an amber glass bottle, shielded from exposure to sunlight, and delivered isocratically at a flow rate of 0.5 ml min⁻¹.

Food sample preparation

A sample of 1 g was homogenized in a 10 ml test tube with 4 ml of 5% (w/v) trichloroacetic acid for 1 min, and then the homogenate was made up to 10 ml with water. After centrifugation at 1800g for 5 min, the supernatant was filtered with Toyo Roshi No. 5B filter paper. To 1 ml aliquot of the filtrate, 0.5 ml of 20 μ g ml⁻¹ Det as the internal standard was added, and the entire solution was neutralized to pH 6–7 with 1 M sodium hydroxide, followed by addition of 0.1 M sodium acetate buffer (pH 5.6) to a total volume of 5 ml. The solution was applied to an Amberlite CG-50 column, which was then

washed with 10 ml of 0.1 M acetate buffer (pH 5.6) and with 5 ml of water. Subsequently, polyamines were eluted with 5 ml of 0.1 M HCl. The flow rate of each elution was kept at less than 1 ml min⁻¹. The eluate was evaporated to dryness *in vacuo*, and the residue which formed was dissolved in 5 ml of 0.05 M HCl. A portion of the 10 μ l volume of this final solution was submitted to HPLC.

Results and Discussion

HPLC separation

The process of derivatization of each polyamine was presumed to be the same as in our previous work²⁰, *i.e.*, each of the polyamines introduced from an injector was derivatized spontaneously to its fluorophore at the inlet of the HPLC column in the presence of the OPA reagents in the mobile phase. Therefore, the chromatographic conditions were examined by modifying the method established previously.²⁰ That is, the column selected was an Asahipak ODP-50, which is superior in alkaline tolerance; the mobile phase was a mixture of borate buffer and acetonitrile containing OPA-NAC. The effects of the concentrations of acetonitrile and OPA-NAC, and of the pH of the mobile phase on the capacity factor (k') were examined in order to establish the optimum conditions for simultaneous determination of eight kinds of polyamines under isocratic elution.

Figure 1(A) shows that the acetonitrile concentration in the mobile phase influenced the k' values, *i.e.*, the k' values decreased with increasing acetonitrile concentration. When the acetonitrile concentration was *ca.* 23%, the k' values of the polyamines were in the range of 0.8–7, and the peaks on the chromatogram showed sufficient base line separation.

As for OPA-NAC, the molar ratio of OPA and NAC concentration was always equivalent. As Fig. 1(B) shows, the OPA-NAC concentration hardly affected the k' values of polyamines except for Spd and Spm, the values of which showed a slight tendency to increase with increasing concentration (0.5–3 mM). When the OPA-NAC concentration was 2 mM, all polyamines could be separated with base line separation.

The pH dependence of the separation was also investigated, since it might restrict the pH range of the eluent used for derivatization. Figure 1(C) shows that the increasing pH range of 9.1–11.6 tended to decrease the k' values for Tym significantly and to increase those for Spd and Spm. When the pH of the eluent was 10.6, all polyamines were well separated with base line separation. In this case, the pH of the mixed solution of 50 mM borate buffer (pH 9.9)–acetonitrile (77:23, v/v) containing 2 mM OPA-NAC was *ca.* 10.6.

As for the preparation of the mobile phase, it should be pointed out here that the background fluorescence intensity of the mobile phase was not settled immediately after preparation. This phenomenon was found to be more remarkable with increasing pH of the mobile phase.

Fig. 1 Effects of (A) acetonitrile concentration, (B) OPA-NAC concentration and (C) pH of the mobile phase on capacity factor of polyamines.

However, the background fluorescence intensity was found to become settled after overnight storage. Then we prepared the mobile phase on the previous day and stored it at *ca.* 10°C until to use.

Figure 2 shows a chromatogram of a mixture of polyamine standards under the conditions described above.

Linearity and reproducibility

In order to determine the applicability of this approach to routine quantification of polyamines, the precision and linearity of the on-column derivatization method were examined by using a standard solution of each polyamine (Table 1). The data were determined directly without compensation using an internal standard method. The repetitive analyses ($n=5$) of the polyamine standard solution gave corresponding relative standard deviations (RSD); all were below 2% for the peak area and below 0.2% for the retention times. These results indicate that the precision of this method is satisfactory.

The calibration graphs of the peak area *versus* polyamine concentration showed good linearity over the range of 0.4–8 ng for Him, 1–20 ng for Tym, 2–40 ng for Put, Spd and Cad, and 4–80 ng for Dap and Spm.

Extraction and cleanup

Sample extraction methods currently used for polyamine detection in various foods require a cleanup step before quantitative analysis. Our preliminary experiment revealed that the cleanup procedure for Him analysis described previously²⁰ should be modified in order to apply it to polyamine analysis, because the recovery of Tym from the Amberlite CG-50 (Type I, 100–200 mesh) column used for cleanup was inferior to

Fig. 2 Chromatogram of mixture of polyamine standards: Him, 4 ng; Det, 20 ng; Tym, 10 ng; Dap, 40 ng; Put, 20 ng; Spd, 20 ng; Spm, 40 ng and Cad, 20 ng.

those of other polyamines. This low recovery of Tym was attributable to the leakage of a part of Tym from the cleanup column during the washing operation. We then examined the elution behavior using an Amberlite CG-50 (Type II) with sodium acetate buffers of various pH values and concentrations. Consequently, the polyamines adsorbed on the Amberlite CG-50 (Type II) column were all recovered in 5 ml of 0.1 M HCl eluate after washing with 10 ml of 0.1 M acetate buffer (pH 5.6) and with 5 ml of water. Most of the impurities, such as

Table 1 Reproducibility of peak area (PA) and retention time (RT) of each polyamine

Amine/ $\mu\text{g ml}^{-1}$		RSD, % ($n=5$)	
		PA	RT
Histamine	0.04	1.0	0.1
	0.4	0.4	0.1
	4	0.7	0.1
Tyramine	0.1	0.6	0.2
	1	0.7	0.1
	10	1.7	0.1
1,3-Diaminopropane	0.4	2.3	0.2
	4	2.0	0.1
	40	1.1	0.1
Putrescine	0.2	1.7	0.1
	2	1.4	0.1
	20	2.0	0.2
Spermidine	0.2	1.5	0.2
	2	1.4	0.2
	20	2.0	0.2
Spermine	0.4	2.4	0.2
	4	2.2	0.2
	40	1.3	0.2
Cadaverine	0.2	1.2	0.2
	2	0.7	0.1
	20	1.8	0.2
Diethylenetriamine	0.2	1.8	0.1
	2	1.9	0.1
	20	0.4	0.1

amino acids present in foods, were eliminated by this procedure. Typical chromatograms are presented in Fig. 3 for some kinds of fermented samples (soy sauce, soybean paste and fermented soybeans) after impurity removal.

Recovery study and analysis of commercial samples

In order to evaluate the efficiency of the extraction and purification procedure described above, known amounts

Fig. 3 Typical chromatograms of polyamines from (A) soy sauce, (B) bean paste and (C) fermented soybeans.

of the polyamines were added to various Japanese fermented foods. Table 2 summarizes the recoveries of the polyamines from commercial samples of soy sauce, soybean paste, fermented soybeans, salted squid and spoiled milk fortified at 20–200 $\mu\text{g g}^{-1}$ of each polyamine. Overall mean recoveries greater than 85% and standard deviations less than 5% were obtained with every sample. These results ensure the usefulness of the proposed method in application to various foods.

The polyamine contents in various commercial foods and spoiled foods determined by the present method are summarized in Table 3. The amines present are the end products or intermediary metabolites of bacterial metabolism, and may reflect the history of the storage. It can readily be seen from the data that samples of soy sauce and bean paste which had a high Him content also had a high concentration of Tym, but no clear relationship has been observed for other polyamines in soy sauce and bean paste. On the other hand, fermented

Table 2 Recoveries^a (%) of polyamines added to various foods

Sample	Him (20)	Tym (50)	Dap (200)	Put (100)	Spd (100)	Spm (200)	Cad (100)
Soy sauce (Shoyu)	97.3 $\pm$ 1.6	95.6 $\pm$ 2.2	85.4 $\pm$ 3.1	96.3 $\pm$ 1.9	96.7 $\pm$ 2.7	94.6 $\pm$ 4.4	99.3 $\pm$ 1.8
Bean paste (Miso)	89.6 $\pm$ 2.6	92.8 $\pm$ 4.2	86.0 $\pm$ 1.9	93.3 $\pm$ 1.7	96.7 $\pm$ 1.7	96.8 $\pm$ 2.1	100.2 $\pm$ 0.9
Fermented soybeans (Natto)	95.1 $\pm$ 1.7	94.1 $\pm$ 2.2	85.7 $\pm$ 3.0	90.4 $\pm$ 1.6	93.9 $\pm$ 3.7	90.6 $\pm$ 1.9	96.2 $\pm$ 2.2
Salted squid (Ikano-shiokara)	100.0 $\pm$ 1.8	88.5 $\pm$ 4.5	90.6 $\pm$ 3.6	99.1 $\pm$ 1.9	100.0 $\pm$ 2.7	98.9 $\pm$ 1.2	99.5 $\pm$ 0.8
Spoiled milk	100.5 $\pm$ 1.3	94.3 $\pm$ 4.7	93.5 $\pm$ 2.1	98.8 $\pm$ 1.7	98.5 $\pm$ 2.2	97.2 $\pm$ 1.9	99.9 $\pm$ 1.8

a. Mean $\pm$ S.D. ($n=5$). Him, histamine; Tym, tyramine; Dap, 1,3-diaminopropane; Put, putrescine; Spd, spermidine; Spm, spermine; Cad, cadaverine. Each number designated in parentheses was an added polyamine concentration ($\mu\text{g g}^{-1}$).

Table 3 Polyamine content ($\mu\text{g g}^{-1}$) in various foods

Sample		Him	Tym	Dap	Put	Spd	Spm	Cad
Commercial foods								
Soy sauce	(A)	197	312	ND	44.8	11.8	ND	Tr
Soy sauce	(B)	159	288	ND	52.1	14.9	ND	ND
Soy sauce	(C)	144	310	ND	42.2	16.5	ND	2.6
Soy sauce	(D)	77.8	173	ND	18.2	14.9	ND	ND
Soy sauce	(E)	2.2	9.2	ND	5.2	Tr	ND	ND
Soy sauce	(F)	ND	5.2	ND	5.6	3.2	ND	ND
Bean paste	(G)	68.9	121	ND	21.5	19.3	ND	ND
Bean paste	(H)	3.1	10.7	ND	37.3	ND	ND	5.6
Bean paste	(I)	ND	96.3	ND	14.3	11.0	ND	ND
Bean paste	(J)	ND	4.4	ND	16.8	ND	ND	ND
Bean paste	(K)	ND	4.5	ND	14.8	ND	ND	ND
Fermented soybeans	(L)	ND	ND	ND	22.2	75.0	ND	59.7
Fermented soybeans	(L')	ND	ND	ND	19.0	75.5	ND	274
Fermented soybeans	(M)	ND	ND	ND	Tr	88.9	ND	3.9
Salted squid		ND	ND	ND	Tr	ND	ND	ND
Flaked salmon		ND	ND	ND	7.0	Tr	ND	ND
Dried bonito		1.8	Tr	ND	Tr	Tr	Tr	ND
Spiled foods								
Minched pork		5.4	72.3	ND	189	Tr	ND	272
Sea urchin eggs		ND	1440	ND	1730	ND	ND	2950
Spoiled milk		ND	ND	ND	36.3	ND	ND	24.4
Spoiled egg		ND	ND	ND	15.2	ND	ND	55.2

ND: not detected (Him, $<1 \mu\text{g g}^{-1}$; Tym, $<2.5 \mu\text{g g}^{-1}$; Dap, $<20 \mu\text{g g}^{-1}$; Put, $<5 \mu\text{g g}^{-1}$; Spd, $<10 \mu\text{g g}^{-1}$; Spm, $<20 \mu\text{g g}^{-1}$; Cad, $<5 \mu\text{g g}^{-1}$). Tr: trace.

soybean (Natto; as Japanese name), fermented by *Bacillus Natto*, had a high Spd content without Him and Tym. Yamamoto *et al.*¹¹ reported that a larger amount of Spd was present in fresh soy bean as compared with the amounts of other polyamines. It was unclear whether or not the Spd detected in the fermented soybeans was formed by bacterial enzymatic degradation. The sample (L') of fermented soybean listed in Table 3 was the same sample of fermented soybean (L) stored at 4°C for one month. The data of sample (L) and (L') showed that the amounts of Put and Spd hardly varied whereas the Cad content increased to about 4.6 times of its initial concentration. It is well known that Put and Cad can arise from enzymatic decarboxylation of the corresponding amino acids, ornithine and lysine, and that Spd is formed from Put. Therefore, the results suggested that the lysine decarboxylase of the bacteria in the fermented soybean (L) was active even at 4°C, while the ornithine decarboxylase was not active at this temperature.

Finally, we should like to make an additional remark about the durability of the HPLC column for the proposed method. So far we have analyzed more than 700 samples by injection for more than 400 h as the total running time without any trouble. The on-column derivatization method described here is simple, highly sensitive, specific and routinely useful for the quantitative analysis of polyamines in various foods with a simple cleanup procedure. The use of the on-column

derivatization method resolved the problem associated with the stability of the fluorophore produced in the reaction of OPA with a primary amino group. Moreover, this method is convenient and can be readily incorporated into existing laboratory HPLC systems as a new derivatization method.

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