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High-performance liquid chromatographic determination of polyamines in selected vegetables with postcolumn fluorimetric derivatization

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ABSTRACT

A rapid high-performance liquid chromatographic analysis for the simultaneous separation of five naturally occuring polyamines (agmatine, putrescine, cadaverine, spermidine and spermine) is described. Postcolumn derivatization with *o*-phthalaldehyde is used. The separation systems consisted of a strong cation-exchange column, an elution buffer consisting of 1 *M* sodium citrate (pH 5.4), a mixing coil for the chemical reaction and a spectrofluorimetric detector. The derivatized fluorescent compounds were detected at 340 nm (excitation) and 455 nm (emission). The recoveries of the polyamines were 94.5–107.0% with a standard deviation of 0.83–7.65%.

INTRODUCTION

Polyamines, such as agmatine, putrescine, cadaverine, spermidine and spermine, play an important role in cell fission, nucleotide biosynthesis and protein biosynthesis in both animals and plants. In fruits, many researchers have reported that free polyamine levels declined during fruit development and increased during fruit maturation and ripening [1–5]. These findings implied that free polyamine serves as an endogenous antisenescence agent in a number of plants [6]. On the other hand, ethylene is a senescence-promoting hormone and accelerates fruit and vegetable ripening. In contrast, free polyamines inhibited ethylene production in some tissues. It is suggested that polyamines and ethylene have opposite effects in relation to plant ripening and senescence [7–9].

Methods for the determination of polyamines in plants using thin-layer and high-performance liquid chromatography (HPLC) have been reported [10–14]. In these HPLC methods the polyamines were derivatized to either dansylates or benzoylates before the separation. Recently, *o*-phthalaldehyde reagent (OPA) has been successfully applied to polyamine assays as derivatization reagent in the precolumn [15] and postcolumn modes [16].

We have developed a rapid and sensitive HPLC method for the determination of the five natural polyamines agmatine (**I**), putrescine (**II**), cadaverine (**III**), spermidine (**IV**) and spermine (**V**) (Fig. 1) in vegetables with postcolumn fluorimetric derivatization. The objective was to establish the separation procedure and to determine polyamines of interest in vegetables in a single chromatographic run.

EXPERIMENTAL

Apparatus

The system consisted of two Model 880-51 degassers, two Model 880 PU LC pumps for the liquid chromatograph, a Model 850 AS autosampler, a

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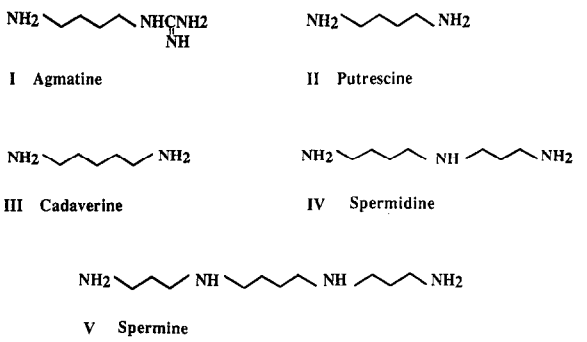

Fig. 1. Structures of polyamines I–V.

Polyaminepak (strong cation-exchange resin, 5-μm particle size) column (3.5 cm × 6 mm I.D.) (Japan Analytical Spectro, Tokyo, Japan), a Model 860 column over (70°C), a stainless-steel mixing coil (30 cm × 0.8 mm I.D.), an FP 210 spectrofluorimetric detector (excitation at 340 nm, emission at 455 nm) and a SIC Chromatorecordor 12 recorder. The system was controlled by a Model 801SC system controller. All instruments were purchased from Japan Analytical Spectro.

Reagents

Agmatine dihydrochloride and putrescine dihydrochloride were obtained from Wako (Osaka, Japan) and cadaverine dihydrochloride, spermidine trihydrochloride and spermine from Aldrich (Milwaukee, WI, USA). Other chemicals were of analytical-reagent grade (Wako).

Materials

Six vegetables, komatu-na (*Brassica rapa* L.), kaiware-daikon (Japanese radish; *Raphanus acanthiformis* Morel), broccoli (*Brassica oleracea* var. *italica, cv.* Charade), senpou-sai (*Brassica napus*), benri-na (*Brassica campestris* ssp.) and spinach (*Spinacia oleracea*), were grown and harvested at Chugoku National Agricultural Experiment Station, Ministry of Agriculture, Forestry and Fisheries of Japan.

Sample preparation

Leaves of komatu-na, benri-na and spinach, edible cotyledon of kaiware-daikon and buds of broccoli were taken by cutting with a knife and collected at random, then used as samples. A 1.0-g amount of sample was placed in a 30-ml glass vial containing 7 ml of 5% perchloric acid ($HClO_4$), then homogenized for 1 min with a Polytron (Nichion Rika Equipment, Tokyo, Japan). The homogenate was placed for 1 h in an ice-cold water-bath (0°C) to accelerate protein precipitation [17], followed by centrifugation at 35 000 *g* for 20 min (2°C). The supernatant was stored at −20°C until analysis. A portion of the defrosted supernatant was filtered with a 0.45-μm filter (Millipore Japan, Tokyo, Japan). A 10-μl volume of filtrate was applied to the HPLC column. All treatments were performed in triplicate and average values are given.

HPLC conditions

Fig. 2 presents a schematic diagram of the HPLC system. A mixture of 100 ml of acetonitrile and 900 ml of 1.0 *M* sodium citrate (adjusted to pH 5.4 with 60% $HClO_4$) was pumped at a flow-rate of 0.65 ml

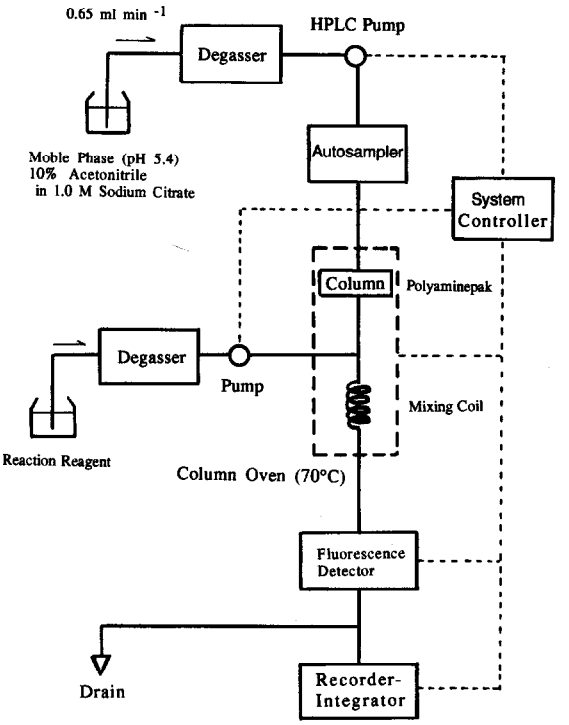

Fig. 2. Schematic diagram of the HPLC system. A postcolumn reaction solution was prepared by adding 2.0 ml of 2-mercaptoethanol, 2 ml of 30% Briji 35 and 0.8 g of OPA dissolved in 20 ml of ethanol to 980 ml of 0.4 *M* boric acid containing 0.4 *M* potassium hydroxide, and protected from light.

min^{-1} as the mobile phase. Duplicate 10-μl volumes of the sample filtrate described above were injected into the HPLC column. A postcolumn reaction solution was prepared by adding 2.0 ml of 2-mercaptoethanol, 2 ml of 30% Briji 35 and 0.8 g of OPA dissolved in 20 ml of ethanol to 980 ml of 0.4 *M* boric acid containing 0.4 *M* potassium hydroxide. The reaction solution was protected from light, pumped at a flow-rate of 0.7 ml min^{-1} and mixed with the column eluate to convert the polyamine into the fluorescent derivative. This compound was measured with a spectrofluorimeter. The column oven and the reaction coil were maintained at 70°C. The values of two injections were averaged.

Recovery studies

Recovery was estimated only using cotyledons of kaiware-daikon. Polyamines **I-IV** were dissolved in 5% $HClO_4$ to a final concentration of 0.2 m*M*. The samples were treated as described above, then 10 μl of the filtrate were injected into the HPLC column. The recoveries were determined by subtracting the values obtained for control vegetable tissue homogenization. The recovery experiment was performed with five replicates and mean values with the standard deviations are reported.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

Mobile phase. In order to separate five typical polyamines, we tried several elution systems, *i.e.*, acetonitrile–sodium dihyrogenphosphate and acetonitrile–sodium citrate, using a cation-exchange resin (PolyaminePak) and an isocratic elution system. The pH of the mobile phase was adjusted to 5.4 with 60% $HClO_4$. Both acetonitrile–1.0 *M* sodium dihyrogenphosphate (10:90, v/v) and acetonitrile–1.0 *M* sodium citrate (10:90, v/v) gave the best separation and resolution for all five compounds in 45 min. In this study, we selected 10% acetonitrile–1.0 *M* sodium citrate (pH 5.4) as the mobile phase. A typical chromatogram is shown in Fig. 3, the retention times for putrescine (**II**), cadaverine (**III**), spermidine (**IV**), agmatine (**I**) and spermine (**V**) being 4.8, 12.0, 16.5, 26.5 and 40.0 min, respectively.

Optimum concentration of reagent. We used OPA reagent as the reaction solution in order to convert polyamine into the fluorescent compounds. Fig. 4 shows the relationship between OPA concentration in the reaction solution and the peak area of four polyamines (**I–IV**) detected by spectrofluorimetry. In this study, 10 μl each of 125 m*M* polyamine stan-

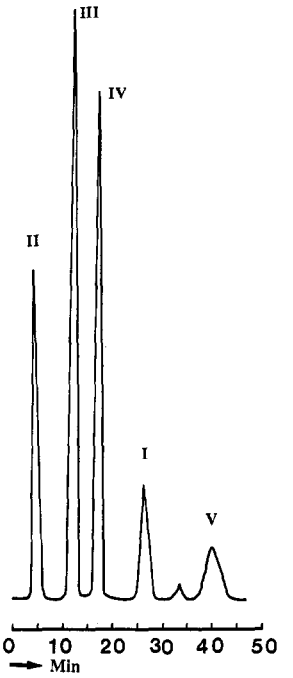

Fig. 3. Chromatogram of polyamines **I–V**. For operating conditions, see text.

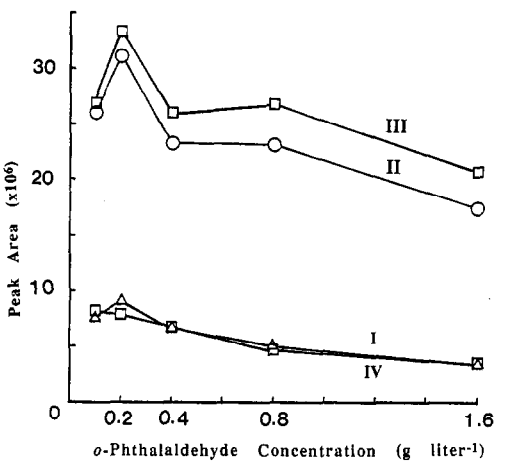

Fig. 4. Relationship between the peak area detected and the OPA reagent concentration. For operating conditions, see text.

dards were injected into the HPLC column. For the OPA concentration range studied (0.2–1.6 g l^{-1}), a 0.2 g l^{-1} concentration gave the maximum peak area except for **IV** in the concentration. However, the polyamine concentration studied was 1.25 nmol, and it would be necessary to increase the OPA reagent concentration in the presence of the higher concentrations of polyamines.

Linearity. In order to check the linearity of the relationship between the polyamine levels and the peak area using the above serparation system, 1 m*M* stock standard solutions of the five polyamines were prepared and suitably diluted with 5% $HClO_4$. Various amounts of the standard solution were injected into the HPLC column described above. Fig. 5 presents the calibration graphs for the five polyamines. All the graphs exhibited good linearity and obeyed Beer's law in the investigated concentration range of 0.06–5.00 nmol. The regression equation $y = ax + b$, where x is the concentration of polyamine (nmol) and y is the peak area, and the correlation coefficients (r) of the polyamines were as follows: for agmatine (**I**), $y = 4.573x + 0.0545$ ($r = 0.991$); for putrescine (**II**), $y = 10.882x + 2.2657$ ($r = 0.991$); for cadaverine (**III**), $y = 13.543x + 1.4795$ ($r = 0.996$); for spermidine (**IV**), $y = 4.838x + 0.5630$ ($r = 0.991$); and for spermine (**V**), $y = 0.481x + 0.0202$ ($r = 0.999$). These results suggest that the proposed HPLC method is sufficiently sensitive to detect the five polyamines. Spermine (**V**) had the lowest sensitivity.

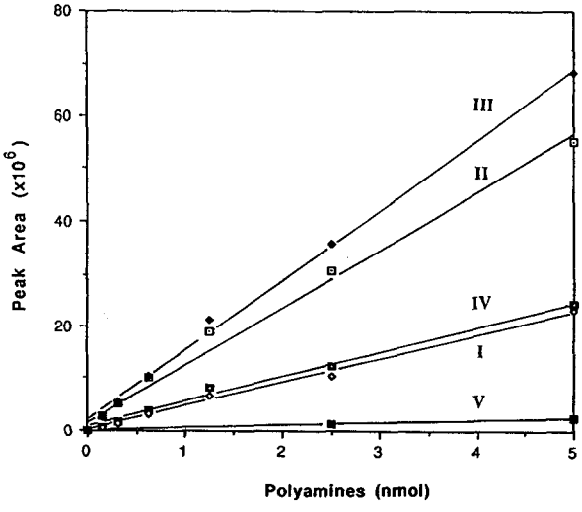

Fig. 5. Calibration graphs for polyamines **I–V** (peak area detected *versus* nanomoles of polyamines injected). **I** = Agmatine; **II** = putrescine; **III** = cadaverine; **IV** = spermidine; **V** = spermine. For conditions and regression equation, see text.

Recovery of polyamines from vegetable homogenates

Measured amounts of four polyamines (**I-IV**) were added to a sample homogenate of Japanese radish cotyledons (kaiware-diakon) with known levels of the compounds and were then determined by the proposed procedure. The recoveries of the polyamines were 94.5–107.0% with a standard deviation of 0.83–7.65% (Table I). Of the polyamines studied, it was appeared that the agmatine content varied more than the others.

Application

The contents of polyamines in the six selected vegetables were determined to demonstrate the validity of the method. The peaks were identified by adding a mixture of reference compounds to the sample solution before injection. Fig. 6 shows typical chromatograms of polyamines in the three vegetables. Table II gives the results expressed as nmol g^{-1} (fresh mass). Spermine (**V**) was not detected in any of the vegetables investigated. It has been reported that the contents of polyamines, particularly putrescine, spermidine and spermine, are abundant during seed germination of *Phaseolus mungo* [18] and that putrescine, cadaverine and agmatine are also abundant in bean seedlings [19]. In this study, the radish cotyledon (kaiware-daikon) contained more agmatine and putrescine, which agreed with the results reported by others [18,19]. Of the polyamines detected in the vegetables other than kaiware-daikon, spermidine was predominant, followed by cadaverine and/or putrescine in order of decreasing concentration.

CONCLUSIONS

A rapid useful HPLC analysis for five polyamines, agmatine, putrescine, cadaverine, spermidine and spermine, in vegetables was established. Polyamines were extracted from the vegetables by homogenization in 5% $HClO_4$, followed by centrifugation. The supernatant was filtered and injected into the HPLC column. The separated polyamines were converted into fluorescent derivatives reaction

TABLE I

RECOVERIES OF POLYAMINES I–IV FROM COTYLEDON OF KAIWARE-DAIKON (*RAPHANUS ACANTIFORMIS MOREL*)

For operating conditions, see text.

Polyamine	Added (nmol)	Recovered (nmol)	Recovery (%)	Mean recovery ± S.D. (%)
I	0.50	0.545	109.0	107.0 ± 7.65
	1.00	1.029	102.9	
	2.00	1.920	96.0	
	3.00	3.465	115.5	
	4.00	4.460	111.5	
II	0.50	0.480	96.0	96.2 ± 0.83
	1.00	0.975	97.5	
	2.00	1.922	96.1	
	3.00	2.856	95.2	
	4.00	3.852	96.3	
III	0.50	0.473	94.6	94.5 ± 0.93
	1.00	0.953	95.3	
	2.00	1.878	93.9	
	3.00	2.859	95.3	
	4.00	3.736	93.2	
IV	0.50	0.476	95.2	105.2 ± 6.41
	1.00	1.059	105.9	
	2.00	2.076	103.8	
	3.00	3.357	111.9	
	4.00	4.372	109.3	

TABLE II

CONTENTS OF POLYAMINES I-IV IN SIX VEGETABLES

For operating conditions, see text.

Vegetable	Polyamine[a] (nmol/g^{-1} fresh mass)				
	I	II	III	IV	V
Cotyledon of kaiware-daikon	4180	3887	–[b]	319.0	–
Broccoli	1.39	358.0	–	751.0	–
Komatsu-na	–	87.4	112.3	369.5	–
Senpou-sai	–	94.1	–	301.0	–
Benri-na	–	45.5	64.9	307.9	–
Spinach	–	27.5	–	98.4	–

[a] Average values of triplicate measurements.
[b] –, Not detected.

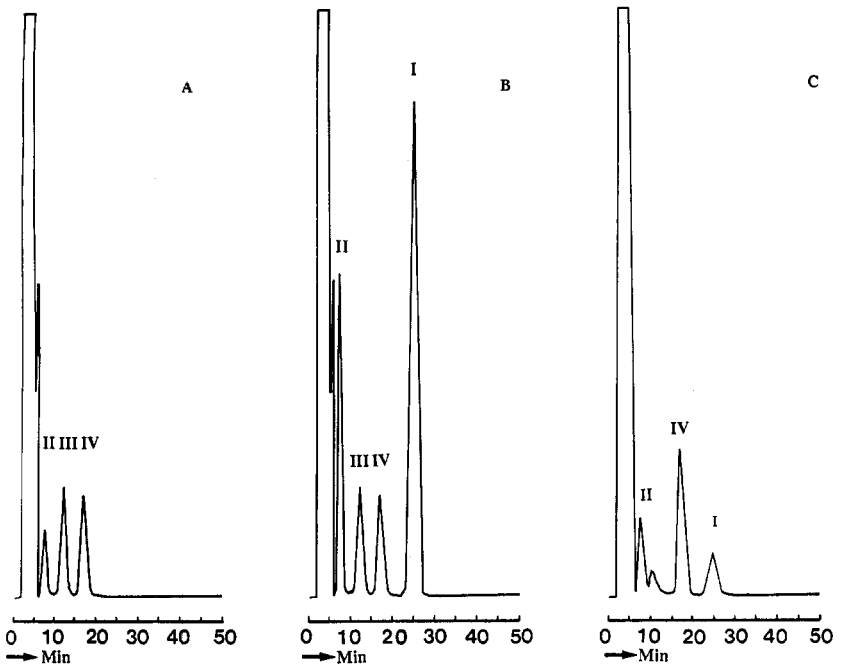

Fig. 6. Chromatograms of polyamines **I–IV** in (A) komatu-na, (B) kaiware-daikon and (C) broccoli. For the HPLC conditions, see text.

with OPA reagent, then detected by spectrofluorimetry.

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