Determination of polyamines in *Arabidopsis thaliana* by capillary electrophoresis using salicylaldehyde-5-sulfonate as a derivatizing reagent

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Herein, we report a novel method for the determination of polyamines in a sample extracted from *Arabidopsis thaliana* by capillary electrophoresis (CE) using salicylaldehyde-5-sulfonate (SAS) as a derivatizing reagent. An aldehyde group of SAS forms a Schiff base with amino groups of aliphatic polyamines, resulting in an anionic species with an absorption band in the ultraviolet region. The derivatization method was straightforward since the derivatives were formed by mixing a sample with the derivatizing reagent under a neutral pH. In addition, the negative charges induced by SAS led to a high resolution with a short analysis time. This method permitted the separation of five polyamines, which play important roles in plants. However, further improvement in sensitivity was needed for the determination of the polyamines in plant samples. Therefore, the CE method was coupled with solid-phase extraction (SPE) using an ion-pairing formation with sodium dodecyl benzene sulfonate. The SPE method improved the concentration limits of detection to sub-μM levels, which corresponded with a 10-fold enhancement. The calibration curves for cadaverine, putrescine, and spermidine were linear with concentrations that ranged from 1 to 20 μM and correlation coefficients (R^2) were greater than 0.998. The proposed method was applied to the determination of spermidine in a plant sample, *Arabidopsis thaliana*.

Introduction

Polyamines, which are found in eukaryotic cells, play important roles in biological systems. For example, polyamine levels correlate with tumor cell proliferation rates, and putrescine (PUT) is synthesized from arginine in microorganisms and plants, and is converted to spermidine (SPD) and spermine (SPM) in metabolism.[1,2] Thermospermine (TSPM) is considered to be a substance that is related to the growth factors of cells.[3]

A simple method for the determination of polyamines is needed not only for clinical testing and food analysis but also for clarification of the biological roles of polyamines, since the concentrations of polyamines vary in the process of cell division and suffer from environmental stress.[3-5] High performance liquid chromatography (HPLC) is conventionally used for the separation and determination of polyamines using derivatizing reagents such as dansyl chloride and *o*-phthalaldehyde (OPA).[6,7] The HPLC methods permitted the determination of polyamines with the limits of detection (LOD) ranging from 50 to 150 μg L^{-1}.[6] Mass spectrometry (MS) is also a useful detection technique in HPLC as demonstrated in the determination of polyamines with LOD of 22 μg L^{-1} for PUT, 15 μg L^{-1} for cadaverine (CAD), and 33 μg L^{-1} for SPD,[8] although the instrument is relatively expensive. Conversely, capillary electrophoresis (CE) is preferred to HPLC for the determination of biomolecules since it requires only a small amount of the sample for separation and determination. In addition, CE has an advantage that no or less organic solvent is needed for the preparation of a background electrolyte (BGE) and samples, i. e. CE is more green and economic than HPLC. Thus, CE has also been applied to the determination of polyamines using a labeling reagent of fluorescein isothiocyanate,[9] *p*-toluenesulphonyl chloride,[10] 1-pyrenebutanoic acid

succinimidyl ester,[11] 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate,[12] 4-fluor-7-nitro-2,1,3-benzoxadiazole,[13] and 1-(ε-succinimidyl-hexanoate)-1'-methyl-3,3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonate.[14]

Salicylaldehyde-5-sulfonate (SAS) is also a useful labeling reagent for primary amines in CE analysis, as we demonstrated in a previous study.[15,16] Since SAS reacts with aliphatic primary amines to form a stable Schiff base, the derivatives have negative charges that result in a selective manipulation of the separation. In the previous papers, a standard mixture of monoamines with different alkyl chains were separated after precolumn derivatization.[15] In addition, SAS permits the complete separation of six aliphatic diamines including putrescine and cadaverine within 17 min,[16] although the other polyamines like spermine, spermidine, and thermospermine, were not employed and no practical sample was measured. Therefore, SAS is a promising labeling reagent for the CE analysis of polyamines in practical samples.

In the present study, we demonstrated the separation of five biogenic polyamines that were known to exist in the plant samples. The polyamines were derivatized with SAS by mixing their aqueous solutions in the presence of ethanol. The derivatives were water-soluble so that it was possible to separate them in an aqueous phosphate buffer solution. To apply this method to the determination of polyamines in a plant sample, solid-phase extraction (SPE) was employed for preconcentration. The method showed good linearity and a low limit of detection when applied to the determination of polyamines in practical samples.

Experimental

a. Chemicals

All chemicals used in this study were of analytical grade. Deionized water was prepared by means of an Elix water purification system (Millipore Co. Ltd., Molsheim, France). Putrescine (PUT) dihydrochloric acid, spermine (SPM) tetrahydrochloric acid, spermidine (SPD), and sodium dodecylbenzene sulfonate (DBS) were purchased from Nacalai (Kyoto, Japan). Cadaverine (CAD) dihydrochloric acid was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 4-(2-Hydroxyethyl)piperazine-1-(ethanesulfonic acid) (HEPES) was purchased from Dojindo (Kumamoto, Japan). C18 cartridge columns (HyperSep C18 Columns, 100 mg mL^{-1}) were obtained from Thermo Fisher Scientific (MA, USA). Thermospermine (TSPM) was synthesized and graciously gifted by Prof. Niitsu's group at Joysai University.[17] Salicylaldehyde-5-sulfonate (SAS) was synthesized according to our previous paper.[15,16] Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Standard solutions of PUT, CAD, SPM, and TSPM were prepared by dissolving appropriate amounts of the polyamines in water. A standard solution of SPD was prepared by dissolving in ethanol. The concentrations of the standard solutions were adjusted to 10 mM. The standard solutions of PUT and CAD were kept in a refrigerator (4 ˚C) while the standard solutions of SPM, SPD, and TSPM were stored in a freezer (-20 ˚C). A phosphate buffer solution (pH 7.8) was prepared by mixing 0.1 M sodium hydrogenphosphate and potassium dihydrogenphosphate. To prepare 0.1 M HEPES buffer of pH 7.8, appropriate amounts of HEPES was dissolved in water and the pH was adjusted with sodium hydroxide.

b. Apparatus

A capillary electrophoresis system, model ^{3D}CE (Agilent Technologies, CA, USA) was used throughout the study. Fused-silica capillaries with an i.d. of 50 μm and an o.d. of 375 μm (total length, 64.5 cm; effective length, 56 cm) were obtained from Agilent Technologies. Samples were injected for 5 s at 5 kPa. Electropherograms were recorded via Hewlett-Packard ChemStation software, which permitted the automatic measurement of peak height, peak area, and migration time.

c. Derivatization of standard solutions with SAS

The derivatization procedure reported by our previous papers[15,16] was slightly modified for reacting polyamines with SAS. To optimize the derivatization conditions, 350 μL of a solution containing 0.1 μmol each of polyamines and 10 μmol phosphate (pH 7.8) was mixed with 50 μL of 50 mM SAS and 600 μL of ethanol, followed by incubation at 20 ˚C for 40 min. Finally, 1 mL of the sample solution contained 10 mM of phosphate buffer, 60% of ethanol, and 0.1 mM of each polyamine. In the determination of polyamines in a practical sample, HEPES buffer (pH 7.8) was used instead of phosphate buffer to prevent precipitation of buffer components in the reaction mixture containing more than 60% ethanol.

d. Determination of SPD in a plant sample

Polyamines in *Arabidopsis thaliana* (whole parts) were extracted according to the procedure reported by the references.[18,19] Plant samples frozen in liquid nitrogen were ground with a mortar, resulting in a powder sample. Cooled perchloric acid (5% aqueous solution) was added to the powder sample at a ratio of 2.5 mL of perchloric

acid to 0.5 g of the powder. After standing for 1 h on ice, the mixture was centrifuged at 15,000 *g* for 30 min under cooling conditions (4 ˚C). The supernatant was filtered using a 0.2 μm syringe filter, and the filtrate was used as a plant sample that contained 5% (v/v) perchloric acid.

To determine polyamines in a plant sample solution, the polyamines had to be concentrated due to the insufficient sensitivity of UV detection. Ion-pairing solid-phase extraction (SPE) was employed to concentrate the analytes. A C18 cartridge column was washed sequentially with 1 mL of acetone, methanol, water, and 10 mM acetate buffer (pH 5). To remove perchloric acid and adjust the pH to 5, potassium hydrogencarbonate powder was added to 3 mL of the sample, and the mixture was cooled to 0 ˚C, resulting in a solution with a precipitation of potassium perchlorate. After removing the precipitation by filtration, 600 μL of 0.1 M acetate buffer and 60 μL of 0.1 M DBS were added to the sample and then the mixture was passed through the C18 cartridge column to retain ion-pairs of polyamines and DBS. The C18 cartridge column was washed with 1 mL of water, and the ion-pairs were eluted by 270 μL of ethanol. The effluent was reacted with SAS by mixing it with 30 μL of 0.1 M HEPES buffer containing 50 mM SAS and maintaining the mixture at 20 ˚C for 40 min. The resultant solution was filtered with a 0.45 μm membrane filter and used for electrophoretic analysis.

Results and discussion

a. Separation conditions

In general, the pH of the BGE is an important factor for the separation conditions. The

electropherograms obtained by BGEs with different pH values are shown in Fig. 2. As noted in Fig. 2, unreacted SAS was detected after the migration times of any polyamine derivatives. This is a good characteristic of SAS as a labeling reagent for amines since the unreacted SAS showed no disturbance to the analytes. The electrophoretic mobilities of the polyamine derivatives with SAS were less sensitive to the pH of the BGE when the pH ranged from 6.0 to 9.0. This was attributed to the fact that the sulfonate group of SAS is completely anionic when pH ranges from 6.0 to 9.0. Conversely, peak areas or heights are influenced by the pH of the BGE. The effects of pH on the peak area of the four SAS derivatives (CAD, PUT, SPD, and SPM) are shown in Fig. 3. When the pH of the BGE was 6.0, only the peak of SPD was observed. Peak areas of CAD, PUT, and SPD were maxima at pH 7.0, while SPM showed the largest value at a pH of 7.8. The decrease in the peak area at higher pH was attributed to hydrolysis of the Schiff base in the BGE. Differences in the peak areas between pH 7.0 and 7.8 were small for CAD, PUT, and SPD, so pH 7.8 was selected for the separation of the polyamine derivatives.

Under the optimum conditions, TSPM was also separated from SPM, although they are structural isomers with two secondary amines at different positions. The separation of five polyamine derivatives is shown in Fig. 4. As reported in the previous paper, diamines were labeled with two SAS molecules, resulting in anionic species.[16] As seen in Fig. 2 and Fig. 4, the polyamine derivatives used in this study are also anionic species because they are detected after the negative peak corresponding to the electroosmotic flow. The migration order of SPM < SPD < CAD < PUT is similar to the order of decreasing in their molecular weight. As the solvated radius of these polyamines decrease in this order, the effective electrophoretic mobility increases.

Since polyamines migrate against the electroosmotic flow, the apparent mobility decreases for the smaller polyamines. Among these analytes, SPM and TSPM are structural isomers having two secondary amines. The different mobilities of these derivatives would be attributed to the difference in the degree of protonation in the secondary amines of these molecules. Although CAD and PUT were slightly overlapped due to the high concentrations of the analytes (100 μM each) in Fig. 2, they were also completely separated at lower concentrations. Thus, pH 7.8 was employed for the determination of polyamines in the practical samples.

b. Derivatization conditions

To optimize the derivatization conditions of polyamines with SAS, several factors including reaction time, pH of a reaction buffer, ethanol concentration, and temperature were examined using a standard mixture of CAD, PUT, SPD, and SPM. The derivatization reaction was completed after 40 min for all polyamines. Values for pH that ranged from 7.0 to 9.0 were attempted in 0.2 pH intervals. The optimal pH for the reaction was 7.8, which was similar to the pH of the BGE. It is preferable that the pH of the reaction buffer be the same as the pH of the BGE, since the sample has no influence on the buffering capacity of the BGE. The ethanol concentration in the reaction mixture showed a significant effect on the peak area of the polyamine derivatives. The peak areas increased as the ethanol concentration was increased by as much as 60%. However, further increases in the ethanol concentration led to a precipitation of the buffer components, which was sodium hydrogenphosphate. When HEPES buffer was employed instead of the phosphate buffer, the ethanol concentration could be increased as high as possible without precipitation. Thus, to increase the

concentration of ethanol, HEPES buffer was more suitable than phosphate buffer. So, we varied the concentration of ethanol at the range from 60 to 90% using HEPES buffer, and found that the optimized ethanol concentration was 90%. Consequently, HEPES buffer was used in the determination of a plant sample to increase the ethanol concentration to 90%. The reaction temperature was less effective at 18 to 24 ˚C. When the temperature was 20 ˚C, the peak areas of CAD and PUT were maxima. Conversely, the peak areas of SPD and SPM showed no significant change at 18 to 24 ˚C. Therefore, 20 ˚C was selected for the temperature of the derivatization reaction. The optimal conditions of the derivatization can be summarized as follows: the reaction time was 40 min, the buffer was 10 mM HEPES with pH 7.8, the ethanol concentration was 90%, and the reaction temperature was 20 ˚C.

c. Analytical performance

Several analytical parameters such as linearity and limits of detection (LOD) were evaluated under the optimum conditions. Calibration curves were prepared at a concentration range of 20 to 200 μM for CAD, PUT, SPD, and SPM, and 2 to 20 μM for TSPM. All the calibration curves were straight lines passing through the origin and showed good linearity with correlation coefficients, R^2, of 0.999 for CAD, 0.996 for PUT, and 0.999 for SPD. The calibration curves of SPM and TSPM showed slightly lower sensitivity and worse correlation coefficients of 0.952 and 0.959 than the others, respectively. The lower sensitivity may be due to their low labeling efficiency. The LODs, which were estimated by calculating the concentration showing a value of 3 for the signal-to-noise ratio (S/N), were 1.8 μM for CAD, 2.3 μM for PUT, 2.6 μM for SPD, 7.4 μM for SPM, and 6.2 μM for TSPM.

It should be noted that LODs were given by the concentration of the injected sample. So, the LODs for the sample used for the derivatization were estimated to be 5.1 μM for CAD, 6.6 μM for PUT, 7.4 μM for SPD, 21 μM for SPM, and 18 μM for TSPM. When the limits of quantification (LOQ) were defined as S/N=10, the analyte concentrations of the original sample were 17 to 70 μM. These concentrations were too high for the determination of polyamines in plant samples. For example, Naka and coworkers reported that plants contain several to several tens nmol/g fresh weight (FW) of polyamines depending on the parts such as seeds, leaves, stems, and flowers.[19] Thus, the concentrations of polyamines in our sample solution, which were extracted from 0.5 g of plant samples by 2.5 mL of perchloric acid, were estimated to be at sub-μM to μM levels. Therefore, further improvements in LODs must be achieved in the proposed method in order to apply it to the determination of polyamines in plant samples.

d. SPE

In the analyses of polyamines, SPE is frequently employed for pretreatment.[20] In the preliminary study, we tried to use a cation exchange resin column for preconcentration of the polyamines, however, the polyamines were not recovered by the column. On the other hand, a C18 cartridge was employed to remove the excess labeling reagent from a sample containing dansylated polyamines.[21] A C18 cartridge was also used for the extraction of ion-pairs between polyamines and ion-pairing reagents such as octanesulfonate, decanesulfonate, and dodecanesulfonate.[22] Conversely, Rodríguez and coworkers showed that higher recoveries were obtained using DBS than when using either octanesulfonate or decanesulfonate.[23] Therefore, to improve the sensitivity, ion-pairing SPE of polyamines with DBS was attempted using a C18 cartridge column.

Standard samples contained 5% (v/v) perchloric acid to equal that of the matrix of plant samples. The preparation method of the samples is described in section d of the experimental section, i. e., the addition of potassium hydrogencarbonate and the filtration of the sample are followed by ion-pairing SPE. In the SPE experiments, 3 mL of the samples were retained on the C18 cartridge column and eluted by 270 μL of ethanol, and then 30 μL of a derivatization solution was added to form SAS derivatives, that is, the sample was concentrated 10-fold by the SPE. Four polyamines, CAD, PUT, SPD and SPM, were measured at a concentration range of 1 to 20 μM in the sample solution before SPE. The results are summarized in Table 1. Unfortunately, no peak of SPM was found in the electropherogram. It was speculated that the interaction of SPM with the C18 solid phase might be too strong to elute with ethanol because of the hydrocarbon chain longer than CAD, PUT, and SPD. For CAD, PUT, and SPD, the calibration curves showed good linearity, and the LODs were improved more than 20-fold. Therefore, the method would be applicable to the determination of CAD, PUT, and SPD in plant samples.

e. The determination of polyamines in a plant sample

A plant sample, *Arabidopsis thaliana*, was determined according to the proposed method. The electropherograms of a standard mixture and the plant sample are shown in Fig. 3. A broad peak between SPD and CAD was confirmed to be the peak of DBS used as the ion-pairing reagent. As seen in Fig. 3, SPD was found in the plant sample with a concentration of 11.1±0.03 μM (n=3), which corresponded to 55.5±0.17 nmol/g FW. This value would be acceptable according to the results reported by Naka et al.,[19] who found a concentration of 20 to 70 nmol/g FW in *Arabidopsis thaliana*.

The concentrations of PUT and SPM were found to be 5 to 20 nmol/g FW in the literature[19] although they were not detected in the present study. The results in Fig. 3 indicate that the proposed method is applicable to the determination of biological polyamines.

Conclusions

Capillary electrophoresis was applied to the determination of biogenic polyamines in a plant sample. The labeling reagent, SAS, permitted UV absorbance detection of the polyamines and manipulated the separation selectivity by converting polyamines to anionic aromatic compounds. The method is considered to be a green methodology since no toxic organic solvent is required, and the consumption of solvents and reagents is much less than the other methods using HPLC.

To apply the method to a practical plant sample, preconcentration by ion-paring SPE was useful in improving the sensitivity. After ion-paring SPE, the calibration curves of polyamines showed good linearity with a LOD of less than 0.5 μM. The CE method coupled with ion-pair SPE was successfully applicable to the determination of polyamines in *Arabidopsis thaliana*. As a result, SPD was found in the plant sample at a concentration of 11.1 μM, which was consistent with the values reported previously. Therefore, the proposed method would be useful for the determination of polyamines in practical samples.

Acknowledgements

We thank Professor Masaru Niitsu of Joysai University for his generous sample of synthesized thermospermine. T.K. acknowledges support by Grants-in-Aids for

Scientific Research, Scientific Research (B).

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Table 1. Analytical parameters for CE coupled with SPE

Analyte	Regression curve	Correlation coefficient	LOD/ μM (S/N=3)	LOQ/ μM (S/N=10)	Recovery/ % (n=3)
CAD	y = 1.0048x + 0.2003	0.999	0.25	0.83	37±1.5
PUT	y = 0.7242x - 0.3388	0.998	0.37	1.2	31±1.5
SPD	y = 0.5092x - 0.2332	0.998	0.44	1.5	49±1.9

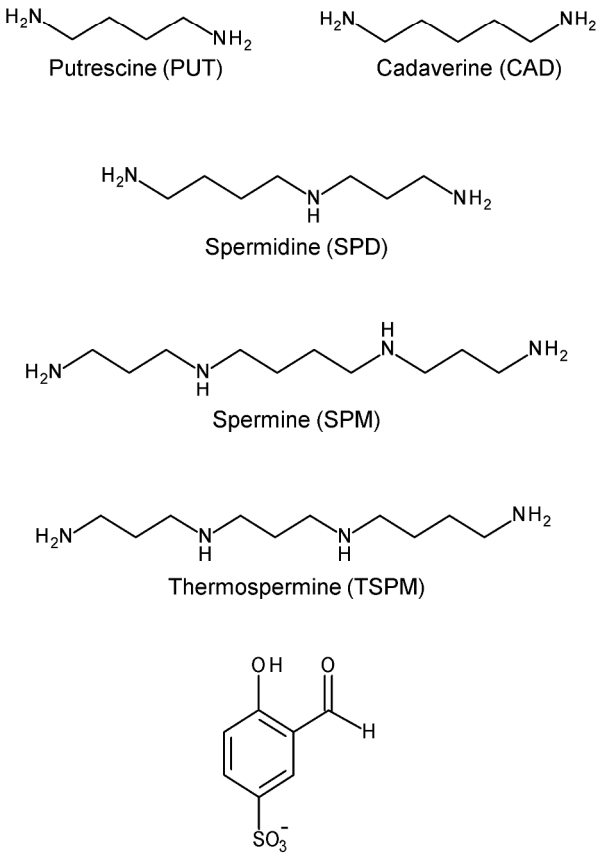

Figure 1. Structures of polyamines and derivatizing reagent.
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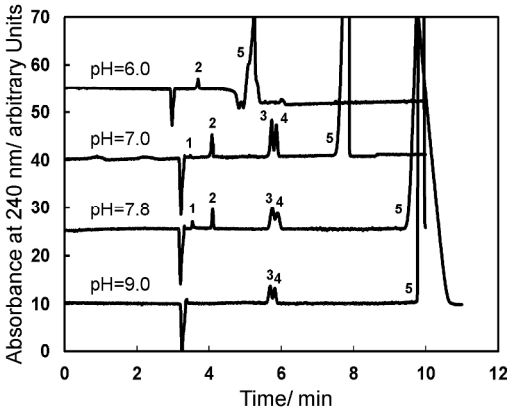

Figure 2. Electropherograms of polyamine labeled with SAS at different pH values. 1=SPM, 2=SPD, 3=CAD, 4=PUT, 5=SAS. Migration buffer, 10 mM acetate buffer (pH 6.0), 10 mM phosphate buffer (pH 7.0, 7.8) or 10 mM borate buffer (pH 9.0); sample solution; 500 μM CAD, PUT, SPD, SPM, and 5 mM SAS in 10 mM phosphate buffer (pH 7.8) containing 60% ethanol; capillary, total length = 64.5 cm, effective length = 56 cm, i.d. = 50 μm; injection, 5 kPa for 5 s; applied voltage, 30 kV; detection wavelength, 240 nm; and, capillary temperature, 20 °C. The unit of mAU is the milliabsorbance unit. The baselines are shifted upward to show four electropherograms.
209x297mm (300 x 300 DPI)

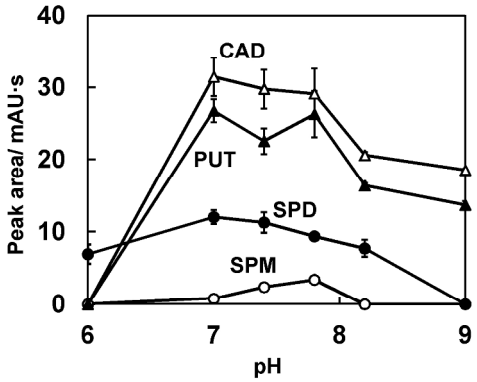

Figure 3. pH of the migration buffer and its effect on the peak areas of polyamines labeled with SAS. Conditions were the same as in Fig. 2.
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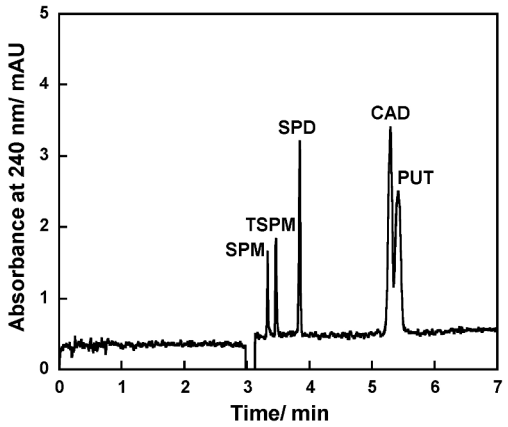

Figure 4. Electropherogram of standard polyamines labeled with SAS.
Sample solution; 50 μM CAD, PUT, SPD, SPM, TSPM, and 5 mM SAS in 10 mM phosphate buffer (pH 7.8) containing 60% ethanol; migration buffer, 10 mM phosphate (pH 7.8). Other conditions for electrophoresis were the same as in Fig. 2.
209x297mm (300 x 300 DPI)

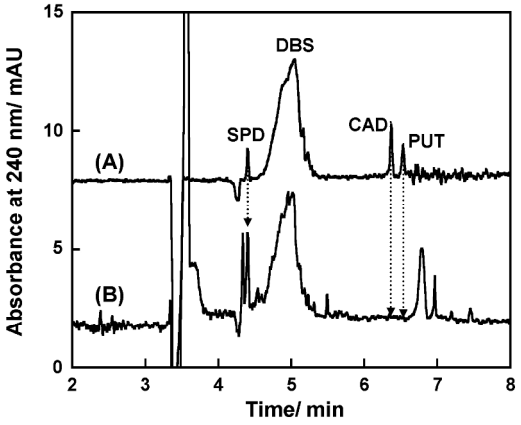

Figure 5. Electropherograms of polyamines preconcentrated by SPE.
A 3 mL aliquot of a sample solution was concentrated to 270 μL by ion-pairing SPE, and 30 μL of the derivatizing solution was added to the concentrated sample; (A) the standard solution containing 5 μM of CAD, PUT, and SPD, (B) a plant sample. Sample solutions contain 5 mM SAS, 10 mM HEPES (pH 7.8), and 90% ethanol. The baseline of (A) is shifted upward to show two electropherograms. Migration buffer, 10 mM phosphate (pH 7.8). Other conditions for electrophoresis were the same as in Fig. 2.
209x297mm (300 x 300 DPI)

Supplementary material

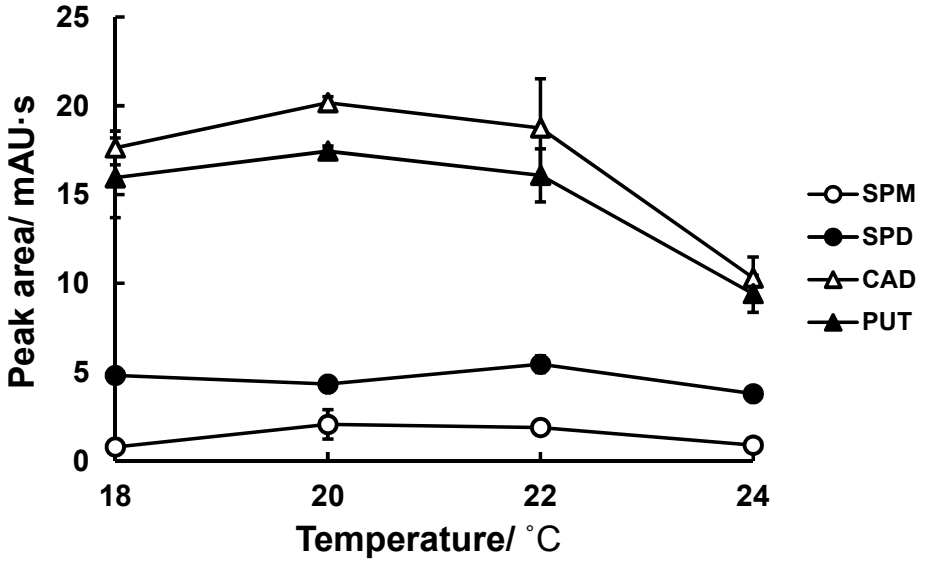

Fig. S1 Effect of temperature on the derivatization reaction.

Migration buffer, 10 mM phosphate buffer (pH 7.8); sample solution; 500 μM CAD, PUT, SPD, SPM, and 5 mM SAS in 10 mM phosphate buffer (pH 7.8) containing 60% ethanol; capillary, total length = 64.5 cm, effective length = 56 cm, i.d. = 50 μm; injection, 50 mbar for 5 s; applied voltage, 30 kV; detection wavelength, 240 nm; and, capillary temperature, 20 ˚C. The unit of mAU is the milliabsorbance unit.

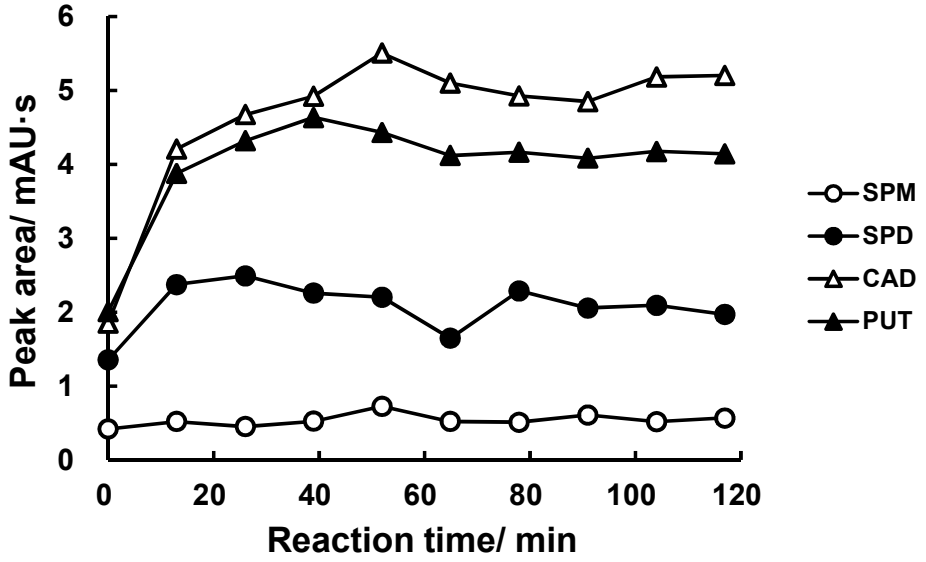

Fig. S2 Effect of reaction time on the derivatization reaction.

Sample solution; 500 μM CAD, PUT, SPD, SPM, and 5 mM SAS in 10 mM phosphate buffer (pH 7.8) containing 60% ethanol. Other conditions are similar to Fig. S1.

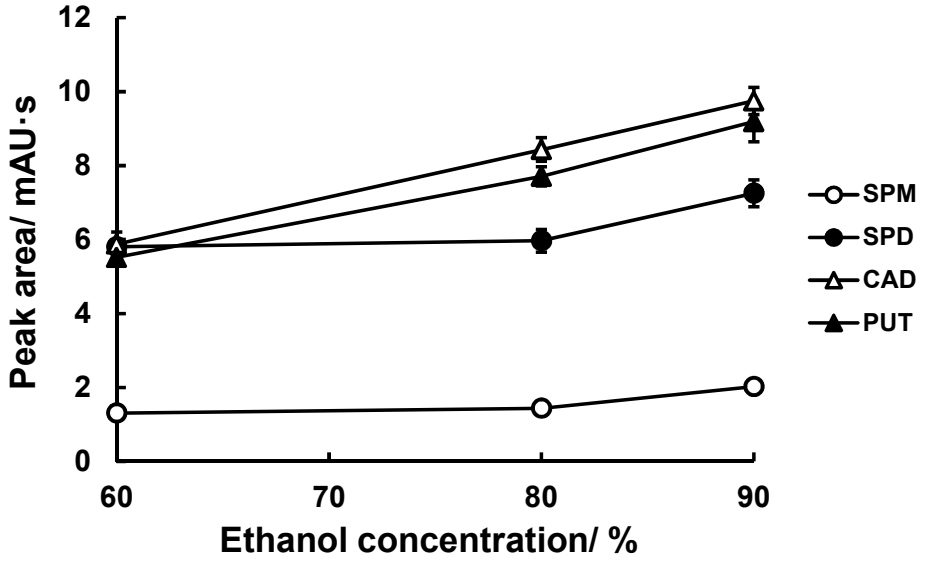

Fig. S3 Effect of ethanol concentration on the derivatization reaction.

Sample solution; 50 μM CAD, PUT, SPD, SPM, and 5 mM SAS in 10 mM HEPES buffer (pH 7.8) containing ethanol. Other conditions are similar to Fig. S1.