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AMINO ACIDS PROTOCOLS

Analysis of polyamines in biological samples by HPLC involving pre-column derivatization with *o*-phthalaldehyde and *N*-acetyl-L-cysteine

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Abstract Polyamines (putrescine, spermine and spermidine) play a crucial role in the regulation of cell growth, differentiation, death and function. Accurate measurement of these substances is essential for studying their metabolism in cells. This protocol describes detailed procedures for sample preparation and HPLC analysis of polyamines and related molecules (e.g., agmatine and cadaverine) in biological samples. The method is optimized for the deproteinization of samples, including biological fluids (e.g., 10 μl), plant and animal tissues (e.g., 50 mg), and isolated/cultured cells (e.g., 1×10^6 cells). The in-line reaction of polyamines with *o*-phthalaldehyde and *N*-acetyl-L-cysteine yields fluorescent derivatives which are separated on a reversed-phase C_{18} column and detected by a fluorometer at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The total running time for each sample (including column regeneration on the automated system) is 30 min. The detection limit is 0.5 nmol/ml or 0.1 nmol/mg tissue in biological samples. The assays are linear between 1 and 50 μM for each of the polyamines. The accuracy (the nearness of an experimental value to the true value) and precision (agreement between replicate measurement) of the HPLC method are 2.5–4.2 % and 0.5–1.4 %, respectively, for biological samples, depending on polyamine concentrations and sample type. Our HPLC method is highly sensitive, specific, accurate, easily automated, and capable for the analysis of samples with different characteristics and small volume/amount, and provides a useful research tool for studying the biochemistry, physiology, and pharmacology of polyamines and related substances.

Keywords Polyamines · Derivatization · *o*-Phthalaldehyde · *N*-Acetyl-L-cysteine · HPLC

Abbreviations

HPLC	High-performance liquid chromatography
NAC	*N*-Acetyl-L-cysteine
OPA	*o*-Phthaldialdehyde
PBS	Phosphate-buffered saline
SDS	Sodium dodecyl sulphate

Introduction

Major polyamines in cells are putrescine, spermidine, and spermine which are found in almost all living organisms (Hamana and Matsuzaki 1992; Ignarro et al. 2001; Wallace et al. 2003). Polyamines contain polycations that can interact with polyanionic macromolecules such as DNA, RNA, ion channels, and protein kinases, thus regulating gene expression, post-transcriptional modifications, cell cycle, membrane structure, and function (Pegg and Casero 2011; Koponen et al. 2012; Wallace et al. 2003; Wu 2009). As a result, polyamines play a crucial role in the regulation of growth, development, and function of animals, plants, and microorganisms (Agostinelli 2012; Wu et al. 2009).

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The composition and concentrations of polyamines in biological samples vary greatly with cell type and physiological conditions of the organism (Seiler and Raul 2005; Wei et al. 2001; Wu and Morris 1998). Therefore, there is growing interest in the biology and pathobiology of polyamines (Correa-Fiz et al. 2012; Do et al. 2013; Levillain et al. 2012; Tavladoraki et al. 2012), including amino acid biochemistry and nutrition (Wu 2013). Sensitive and accurate determination of polyamines is crucial for conducting biological science research.

The analysis of polyamines has been a challenge for scientists over the past decades because these molecules are not chromophores or fluorophores themselves and cannot be readily analyzed by spectrophotometric or fluorescent detection methods (Liu et al. 2012; Seiler 1971). Many techniques have been developed to determine polyamines, such as thin-layer chromatography, enzymatic assay, high-performance liquid chromatography (HPLC) coupled with pre-column or post-column derivatization methods [forming fluorophores with dansyl chloride, fluorescamine, and *o*-phthaldialdehyde (OPA); forming chromophores with benzoyl chloride, dabsyl chloride, and tosyl chloride] and liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) or high-performance liquid chromatography with quadrupole time-of-flight mass spectrometry (HPLC/Q-TOF MS) methods (Acheampong et al. 2011; Cerrada-Gimenez et al. 2012; Isobe et al. 1987; Jung et al. 2012; Liu et al. 2012; Marton and Lee 1975; Sánchez-López et al. 2009; Schenkel et al. 1995; Seiler 1971; Wu et al. 2000a, b). The HPLC methods have been used widely due to their high sensitivity and reproducibility, as well as ease of automation. However, the setbacks are also obvious, such as long derivatization times and instability of derivatives (Acheampong et al. 2011). The addition of sodium dodecyl sulfate (SDS) or phosphate buffer (pH 4) to the HPLC solvents or extraction procedures to stabilize the *o*-phthalaldehyde-derivatives has been reported to be ineffective (Campíns-Falcó et al. 2001). Also, the inclusion of SDS in the HPLC solvents is corrosive to the plunger of the HPLC pump.

Polyamines can react with OPA and *N*-acetyl-L-cysteine (NAC) to form relatively stable derivative products, namely OPA–NAC-polyamines (Campíns-Falcó et al. 2001). This reaction is very rapid to allow in-line pre-column derivatization which eliminates errors in manual operations and shortens the time interval between the formation of the reaction products and detection of fluorescence. Based on our experience, the amount of OPA (the final concentration of 1.95 mg/ml or 14.5 mM) used in the reaction with polyamines has been found to be optimal for rapid derivatization with substances containing amino or amine groups (e.g., Choi et al. 2014; Dai et al. 2013; Lei et al. 2013; Rezaei et al. 2013; Satterfield et al. 2013; Wu and Knabe 1994; Wu and Thompson 1987, 1988). Thus, the combined use of OPA and NAC offers advantages for rapid, sensitive, and reproducible analysis of polyamines in biological samples.

Sample-processing methods may also affect the accuracy of polyamine analysis. Relevant factors are (1) chemical composition and temperatures of buffers used to dilute the samples and dilution factor, (2) temperatures and containers (e.g. glass and plastic) used for grounding samples, (3) chemical characteristics and concentrations of acids used to deproteinize samples, (4) temperatures and speed of centrifugation of the acidified samples, (5) chemical characteristics and concentrations of alkalines used to adjust the pH of the acid extracts for derivatization, and (6) derivatization methods used (manually or automated) for HPLC analysis. It was reported that polyamines could bind to glass and, therefore, use of plastic tubes and vials for treating and storage of polyamine-containing samples is critically important for their analysis (Flores and Galston 1982). Also, deproteinization method, such as trichloroacetic acid–ether extraction, has been shown to affect the accuracy of polyamine analysis (Marton et al. 1974). Of note, perchloric acid is widely used for the deproteinization of various biological samples (including fluids, plant and animal tissues, isolated/cultured cells, as well as bacteria and solid food/feed stuff), and is known to stabilize polyamines for at lest 6 months (Acheampong et al. 2011; Codoñer-Franch et al. 2011; Flores and Galston 1982). Moreover, potassium carbonate (K_2CO_3) has been used with perchloric acid to remove extra amounts of acid and Cl^- in acidified samples in our and other laboratories (Chen et al. 1987; Masukawa et al. 2006; Sase et al. 2013; Wu and Knabe 1995). The combined use of liquid nitrogen, cold perchloric acid, and cold potassium carbonate to grind and deproteinize samples offers an advantage of preventing the degradation/transformation of polyamines in biological samples while removing proteins.

Therefore, this protocol optimizes and integrates sample processing methods that are suitable for the deproteinization and derivatization of a variety of biological samples with different characteristics and a small volume/amount, and also achieves simple, fast, sensitive, reproducible, and accurate analysis of polyamines in agricultural and biomedical research. The work flow diagram of the protocol is shown in Fig. 1.

Materials

Reagents

HPLC-grade water (Fisher Scientific; Cat. # W7-4).

HPLC-grade methanol (Fisher Scientific; Cat. # A452-4).

Tetrahydrofuran, CHROMASOLV® Plus, for HPLC, ≥99.9 % (Sigma-Aldrich; Cat. # 34865-1L.

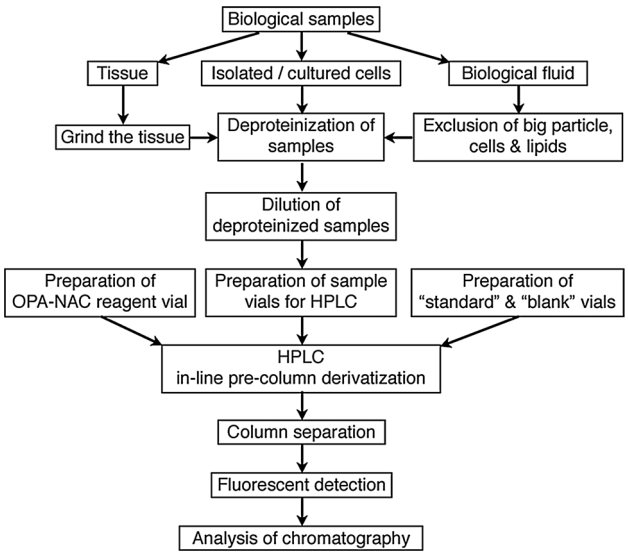

Fig. 1 Work flow diagram on the procedures described in this protocol for HPLC analysis of polyamines

Benzoic acid (Sigma-Aldrich; Cat. # 242381-500G).

Potassium tetraborate tetrahydrate ($K_2B_4O_7{\cdot}4H_2O$) (Sigma-Aldrich; Cat. # P5754-500G).

Sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$) (Sigma-Aldrich; Cat. # B9876-500G).

N-Acetyl-L-cysteine (Sigma-Aldrich; Cat. # A7250-5G).

o-Phthaldialdehyde (OPA) (Sigma-Aldrich; Cat. # P0657-5G). OPA should be stored in a brown bottle to protect it from light.

Brij® L23 solution (Brij® 35 solution) (Sigma-Aldrich; Cat. # B4184-100ML).

Sodium acetate trihydrate (Sigma-Aldrich; Cat. # S7670-1KG).

Perchloric acid ($HClO_4$; 70 %) (Sigma-Aldrich; Cat. # 244252-500ML; or Fisher Scientific, Cat # A229-8LB).

Potassium carbonate (K_2CO_3) (Sigma-Aldrich; Cat. # P5833-500G).

Hydrochloric acid (HCl) (Sinopharm Chemical Reagent; Cat. # 10011018).

Sodium chloride (NaCl) (Sigma-Aldrich; Cat. # S5886-1KG).

Potassium chloride (KCl) (Sigma-Aldrich; Cat. # P5405-500G).

Sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich; Cat. # S5136-500G).

Potassium phosphate monobasic (KH_2PO_4) (Sigma-Aldrich; Cat. # 60218-100G).

Putrescine dihydrochloride (Sigma-Aldrich; Cat. # P7505-25G).

Spermidine trihydrochloride (Sigma-Aldrich; Cat. # 85578-1G).

Spermine tetrahydrochloride (Sigma-Aldrich; Cat. # 85605-1G).

Agmatine sulfate salt (Sigma-Aldrich; Cat. # A7127-1G).

Cadaverine dihydrochloride (Sigma-Aldrich; Cat. # 33220-10G-F).

1,6-Hexanediamine (Sigma-Aldrich; Cat. # H11696-25G).

N-Acetylputrescine hydrochloride (Sigma-Aldrich; Cat. # A8784-25MG).

N^1-Acetylspermine trihydrochloride (Sigma-Aldrich; Cat. # 01467-100MG).

Equipment

Arium® pro VF Ultrapure Water System (Sartorius AG; http://www.sartorius.com).

Sorvall® Legend® Micro 21R Microcentrifuge (Thermo Scientific; http://www.thermoscientific.com).

Waters Alliance e2695 Separation Module (Waters; http://www.waters.com).

Waters 2475 Multi λ Fluorescence Detector (Waters; http://www.waters.com).

Empower 3™ software (Waters; http://www.waters.com).

Vortex-Genie 2 (Scientific Industries; http://www.scientificindustries.com).

Thermomixer® comfort (Eppendorf; http://www.eppendorf.com).

Analytical balance (METTLER TOLEDO; http://www.mt.com; Cat. # ML204).

FiveEasy™ pH meter (METTLER TOLEDO; http://www.mt.com; Cat. # FE20).

Preparation of reagents

HPLC-grade water is used to prepare the following solutions:

6 N HCl: add slowly 49.1 ml of concentrated HCl (37–38 %) to 50.9 ml H_2O.

1.5 M $HClO_4$: add 32.2 ml of 70 % $HClO_4$ to 150 ml H_2O. Make up to a final volume of 250 ml with H_2O.

2 M K_2CO_3 dissolve 69.11 g K_2CO_3 in 150 ml H_2O. Make up to a final volume of 250 ml with H_2O.

Phosphate-buffered saline (PBS): dissolve 8.01 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, and 0.27 g KH_2PO_4 in 800 ml H_2O. Adjust the pH of the solution to 7.4 using 6 N HCl. Make up the final volume of 1 l with H_2O. Autoclave the solution at 121 °C for 20 min and cool the solution to 25 °C before use.

1.2 % (w/v) benzoic acid: dissolve 8.4 g benzoic acid in 525 ml H_2O. Add 175 ml of saturated $K_2B_4O_7$ (saturated $K_2B_4O_7$ is prepared in H_2O).

40 mM sodium borate buffer (pH 9.5): dissolve 30.51 g $Na_2B_4O_7{\cdot}10H_2O$ in 2 l H_2O.

o-Phthalaldehyde–*N*-acetyl cysteine (OPA–NAC) reagents: dissolve 50 mg OPA and 50 mg NAC in 1.25 ml methanol. Add 11.2 ml of 40 mM sodium borate buffer

(pH 9.5) and 0.4 ml of Brij®-35 to the solution. Mix the solution gently. (Use a brown bottle to prepare and store the OPA–NAC solution. Prepare the solution on the day of HPLC analysis and store it at 4 °C for use within 24 h).

Solvent A (0.1 M sodium acetate; pH 7.2): add 27.3 g sodium acetate (trihydrate) and 96 µl of 6 N HCl to 1.6 l H_2O. Add 180 ml methanol and 10 ml tetrahydrofuran. Make up the final volume of 2 l with H_2O. Mix the solution well.

Solvent B: 100 % HPLC-grade methanol.

Polyamine standard solutions

HPLC-grade water is used to prepare the following solutions.

Polyamine standards are dissolved in H_2O in autoclaved tubes. The polyamine standard solution can be stored at − 80 °C for 6 months.

1. 20 mM Putrescine: dissolve 16.12 mg putrescine-2HCl (MW = 161.1) in 5 ml H_2O.

   1 mM Putrescine: mix 50 µl of 20 mM putrescine with 950 µl H_2O.

2. 20 mM Spermidine: dissolve 25.5 mg spermidine-3HCl (MW = 254.6) in 5 ml H_2O.

   1 mM Spermidine: mix 50 µl of 20 mM spermidine with 950 µl H_2O.

3. 20 mM Spermine: dissolve 34.9 mg spermine-4HCl (MW = 348.2) in 5 ml H_2O.

   1 mM Spermine: mix 50 µl of 20 mM spermine with 950 µl H_2O.

4. 20 mM Agmatine: dissolve 22.9 mg agmatine (sulfate salt; MW = 228.3) in 5 ml H_2O.

   1 mM Agmatine: mix 50 µl of 20 mM agmatine with 950 µl H_2O.

5. 20 mM N^1-Acetylputrescine: dissolve 6.7 mg *N*-acetylputrescine-HCl in 2 ml H_2O.

   1 mM *N*-Acetylputrescine: mix 50 µl of 20 mM *N*-acetylputrescine with 950 µl H_2O.

6. 20 mM N^1-Acetylspermine: dissolve 7.1 mg N^1-acetylspermine-3HCl in 1 ml H_2O.

   1 mM N^1-Acetylspermine: mix 50 µl of 20 mM N^1-acetylspermine with 950 µl H_2O.

7. 20 mM Cadaverine: dissolve 17.5 mg cadaverine-2HCl in 5 ml H_2O. 1 mM Cadaverine: mix 50 µl of 20 mM cadaverine with 950 µl H_2O.
8. 20 mM Hexanediamine: dissolve 11.6 mg 1,6-hexanediamine in 5 ml H_2O.

Table 1 HPLC gradients for the analysis of polyamines

Solvent (%)	Time (min)						
	0	12	16	18	23	25	30
A	70	35	30	0	0	70	70
B	30	65	70	100	100	30	30

Flow rate: 1.0 ml/min (or 0.8 ml/min under high pressure)

1 mM Hexanediamine: mix 50 µl of 20 mM hexanediamine with 950 µl H_2O.

Mixed polyamine standards (100 nmol/ml for each polyamine).

Add the following to a microcentrifuge tube:

100 µl of 1 mM Putrescine.
100 µl of 1 mM Spermidine.
100 µl of 1 mM Spermine.
100 µl of 1 mM Agmatine.
100 µl of 1 mM N^1-Acetylputrescine.
100 µl of 1 mM N^1-Acetylspermine.
100 µl of 1 mM Cadaverine.
100 µl of 1 mM Hexanediamine.
200 µl H_2O.

Mixed polyamine standards (10 nmol/ml for each polyamine).

Add 100 µl of the above mixed polyamine standards (100 nmol/ml) with 900 µl H_2O.

Chromatographic equipment

The HPLC system is a Waters Alliance e2695 HPLC system equipped with a guard column (5 cm × 4.6 mm ID) filled with Supelco Pellicular packing (40 µm, Cat. # 58232) and an analytical column (Supelco SUPELCOSIL™ LC-18 HPLC column; 15 cm × 4.6 mm ID, 3 µm, Supelco Cat. # 58985). The HPLC is run at a gradient mode (Table 1). The total running time for each sample (including column regeneration on the automated system) is 30 min. A Waters 2475 multi λ fluorescence detector is used to detect the fluorescence of each polyamine derivative, with excitation λ being set at 340 nm and emission λ at 450 nm. The Empower 3™ software is used to record and analyze the HPLC chromatography data.

Procedures

Sample preparations

Methods for preparation of samples depend on their characteristics (Li et al. 2001; Wu et al. 2000a, b). Here, effective procedures are given for biological fluids, tissues, and isolated/cultured cells. All the procedures described below are carried out on ice.

Preparation of biological fluids

(a) Transfer 200 µl of a biological fluid to a 1.5-ml Eppendorf tube. Centrifuge the tubes at 15,000×*g* at 4 °C for 10 min, and transfer 100 µl of the supernatant fluid to a new 1.5-ml eppendorf tube.
(b) Add 100 µl of ice-cold 1.5 M $HClO_4$ to a tube containing 100 µl of the supernatant fluid; vortex the tube for 1 min at 25 °C.
(c) Add 50 µl of ice-cold 2 M K_2CO_3 to the mixture and leave the cap open for 1 min to allow the evaporation of CO_2.
(d) Seal the cap of the tube and vortex the tube for 1 min at 25 °C. Open the cap of the tube to release extra gas and then re-seal the cap.
(e) Centrifuge the tube at 15,000×*g* at 4 °C for 10 min. Transfer 100 µl of the supernatant fluid to a new 1.5-ml Eppendorf tube. The dilution factor for the sample is 2.5.
(f) Add adequate amounts of H_2O to the resulting supernatant fluid from step (e) above to make the appropriate dilution for HPLC analysis of polyamines.

Preparation of tissues

(a) Snap-freeze small pieces of fresh tissue samples in liquid nitrogen and store the frozen tissues at −80 °C.
(b) Grind a small piece of the frozen sample (~200 mg) in liquid-nitrogen cooled mortar.
(c) Weight ~50 mg of the grounded sample powder in a 1.5-ml Eppendorf tube cooled by liquid nitrogen.
(d) Add 200 µl of ice-cold 1.5 M $HClO_4$ to a tube containing 50 mg sample and then seal the cap of the tube. Vortex the tube for 1 min at 25 °C.
(e) Add 100 µl of ice-cold 2 M K_2CO_3 to the mixture. Leave the cap open for 1 min to allow the evaporation of CO_2.
(f) Proceed with steps d–f described for the preparation for biological fluids.

Preparation of isolated/cultured cells

(a) Transfer medium containing ~5×10^6 cells to a new 1.5-ml Eppendorf tube, centrifuge the tube at 10,000×*g* at 4 °C for 1 min and discard the supernatant fluid. Wash the cell pellet twice with 0.5 ml of ice-cold PBS (pH 7.4) by centrifugation at 10,000×*g* at 4 °C for 1 min after each wash.
(b) Vortex the tube containing cells for 30 s at 25 °C, then add 200 µl of ice-cold 1.5 M $HClO_4$ to the tube, and seal the cap of the tube. Vortex the tube for 1 min at 25 °C.
(c) Add 100 µl of ice-cold 2 M K_2CO_3 to the mixture. Leave the cap open for 1 min to allow the evaporation of CO_2.
(d) Proceed with steps d–f described for the preparation for biological fluids.

Preparation of HPLC vials for auto-sampling and analysis

(a) To a 2-ml vial, add the following:

50 µl of a sample, polyamine standard solution or water (blank).
50 µl of 1.2 % (w/v) benzoic acid.
700 µl of H_2O.

(b) Vortex each vial for 10 s at 25 °C.
(c) Place the vials onto the autosampler: the OPA–NAC reagent vial at position #1, the blank vial at position #2, the polyamine standard vial at position #3, sample vials at positions #4 to #14, and the polyamine standard vial at position #15. The ratio of 1:1 (g/g) for OPA:NAC is optimal for the response factors of polyamines in terms of their peak areas.
(d) The in-line pre-column derivatization of polyamines is accomplished by setting the "auto addition" option in the sample set method of the software Empower 3, an injection volume of 10 µl was set for both the derivatization reagent (vial #1) and standard/sample (e.g., vial #2 to #15). After mixing in the derivatization loop, an injection (a total volume of 20 µl) was automatically performed with no delay time.
(e) After each sample set is completed, use the Empower 3 software to quantify polyamines in the samples.

HPLC chromatograms

All the polyamines in the assay mixture are well separated, as indicated in the HPLC chromatography of polyamine standards and related substances, including agmatine and cadaverine (Fig. 2). This method is specific for polyamines and is free of interference by amino acids. The detection limit for this method is 0.5 nmol/ml or 0.1 nmol/mg tissue in biological samples. OPA used in the derivatization solution has no adverse effect on the HPLC system, and one HPLC column can be used to analyze at least 500 samples. The procedures described in this protocol have been used successfully in our laboratory for the determination of polyamines in sow's milk, as well as tissue samples from the liver and small intestine of growing pigs (Table 2).

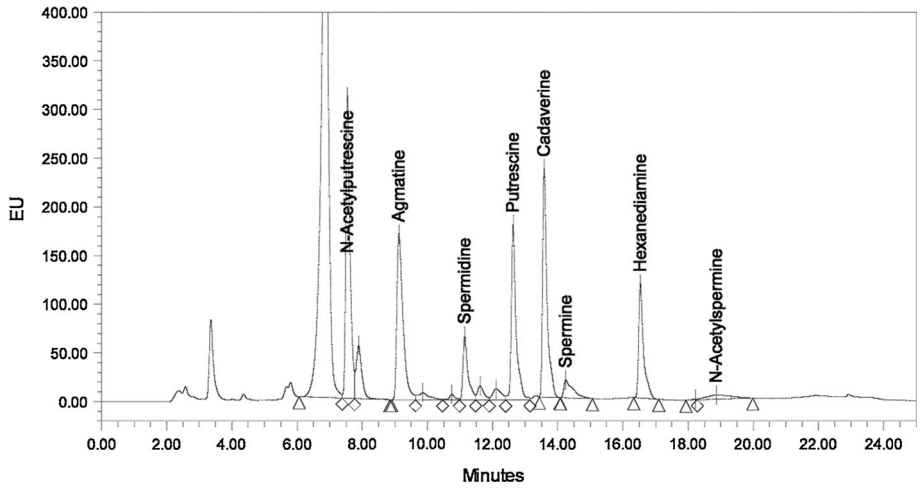

Fig. 2 HPLC chromatography for analysis of polyamine standards (10 nmol/ml)

Table 2 Concentrations of polyamines in various biological samples

	Sow's milk[a] (nmol/ml)	Piglet liver[b] (nmol/g tissue)	Piglet jejunum[b] (nmol/g tissue)	Piglet ileum[b] (nmol/g tissue)
N^1-Acetylputrescine	ND	ND	ND	ND
N^1-Acetylspermine	ND	ND	ND	ND
Agmatine	6.30 ± 1.11	ND	ND	ND
Cadaverine	0.26 ± 0.07	ND	ND	ND
Hexanediamine	ND	ND	ND	ND
Putrescine	2.22 ± 0.41	164 ± 10	171 ± 15	211 ± 12
Spermidine	28.4 ± 4.9	1244 ± 133	966 ± 72	1177 ± 93
Spermine	11.8 ± 1.4	6125 ± 355	2565 ± 106	2672 ± 137

Values are as mean ± SD, $n = 6$

ND not detectable

[a] Obtained from sows on Day 14 of lactation

[b] Obtained from 28-day-old piglets

The deproteinization procedures described in this protocol have been optimized for the processing and analysis of small volume samples. Examples for the volume or amount of a biological sample required for the analysis of polyamines are 10 μl of a physiological fluid (e.g., plasma and fetal fluids), 50 mg of a plant or animal tissue, and 1×10^6 cells. An insert within an HPLC vial may be used for a small volume or amount of a sample. Additionally, the volumes of $HClO_4$ and K_2CO_3 used for sample preparation can be reduced to minimize the dilution of a sample. For example, the sample to 1.5 M $HClO_4$ ratio can be 1:1 (v/v) for physiological fluids and 1:4 (w/v) for a tissue. The 1.5 M $HClO_4$ to 2 M K_2CO_3 ratio is 2:1 (v/v) for all types of samples. The OPA–NAC reagent to sample ratio is 1:1 (v/v) for the in-line pre-column derivatization. The rapid and sensitive analysis of polyamines is satisfactorily achieved through the automated in-line pre-column derivatization of polyamines using the OPA–NAC reagents. The assays are linear between 1 and 50 μM for each polyamine.

Accuracy and precision of the HPLC method

The accuracy (the nearness of an experimental value to the true value) of the HPLC method, as determined with known amounts of polyamine standards and expressed as the relative errors [(measurement value − true value)/true value × 100 %)], is 2.5–4.2 % for the biological samples, depending on polyamine concentrations and sample type (Supplemental Table 1). The precision (agreement between replicate measurement) of the analysis, as evaluated by the relative deviation (mean of absolute deviation/mean of replicate measurements × 100 %), is 0.5–1.4 %, depending on polyamine concentrations and sample type (Supplemental Table 2). Our HPLC method is highly sensitive, specific, accurate, easily automated, and provides a useful research tool for studying the biochemistry, physiology, and pharmacology of polyamines and related substances.

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Conflict of interest The authors declare that they have no conflict of interests.

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