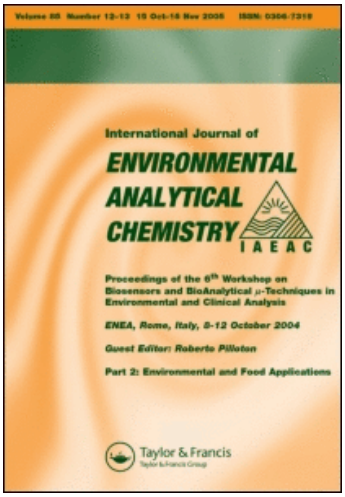

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A New High Performance Chromatographic Method for Polyamine Analysis in *Picea* Needles, Without Previous Extract Purification

V. R. Villanueva[a]; M. Mardon[a]; M. Th. Le Goff[a]
[a] Institut de Chimie des Substances Naturelles, C.N.R.S., Gif-sur-Yvette, France

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A New High Performance Chromatographic Method for Polyamine Analysis in *Picea* Needles, Without Previous Extract Purification†

V. R. VILLANUEVA, M. MARDON and M. Th. LE GOFF

Institut de Chimie des Substances Naturelles, C.N.R.S., 91190 Gif-sur-Yvette, France

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A fully automated, fast and sensitive method for the separation of 19 interrelated amino compounds is presented.

With the help of a high performance amino-acid analyser, ion-exchange chromatography was used to separate ornithine, lysine and arginine from its decarboxylated products: putrescine, cadaverine and agmatine. Also included are ammonia, ethanolamine, histamine, aminopropane, diaminopropane, acetyl and carbamyl putrescine as well as most of the known polyamines, i.e.: nor-spermidine, spermidine, homospermidine, thermine, spermine and 1,7-diaminoheptane which serves as internal standard for the quantification. The method uses 2 buffers, 1 single temperature and fluorimetric detection. It takes 72 minutes and is of picomole sensitive level. This method is well suited for the analysis of crude samples without preliminary purification, thus saving time and reducing preparative losses.

The reproducibility of the method and its application for the analyses of samples from different biological sources, such as microorganisms, plant and animal material has been tested in our laboratory for more than one year with excellent results. This method could serve as a powerful tool for the analysis of these amino compounds in which there is currently considerable interest.

Preliminary results concerning compared studies of healthy and injured needles of air polluted *Picea* are discussed.

KEY WORDS: Polyamines analysis, arginine, *Picea*, pollution.

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INTRODUCTION

Polyamines are natural substances ubiquitous in biological systems. They are ornithine- or arginine-derived compounds, whose cationic charge is responsible for their interaction with the polyanionic cell components (i.e. DNA, RNA, phospholipids, etc.). This property accounts for the multiplicity of biological roles ascribed to these substances in cell division, cell differentiation and cell growth.[1-4]

Although polyamines have been known since 1678[5] the first structure was only established 250 years later.[6]

Spermine and spermidine, the two polyamines known till the seventies received relatively poor attention, mainly owing to the difficulty of their analysis. However, considerable interest on these compounds arose after the finding in 1970[7] that urine of cancer patients contained higher quantities of polyamines compared to that of healthy organisms. Since then a great number of research groups all over the world have centred their interest in the identification and determination of the biological roles of polyamines in cells. Therefore sophisticated, rapid and very sensitive analytical methods were developed.[8] Their extensive utilization resulted in raising the number of known polyamines (Table I) and establishing the importance of their involvement in many biochemical and physiological processes, as reflected by the enormous amount of literature published in the recent years.[9]

Since 1976, work in this laboratory contributed to the development of analytical methods, the study of polyamine biosynthesis pathways and the physiological and biochemical approaches of their possible roles in microorganisms, animal and plant cells.[8,10-28]

This communication presents a new automatic analytical method for the separation and quantification in the same sample of the main polyamines and their precursors. This method has been utilized for more than one year in this laboratory and is currently employed in the study of pollution effects on *Picea* needles. Preliminary results of this work are discussed.

MATERIALS AND METHODS

Chemicals

Amino acids, mono-, di- and polyamines were obtained from Sigma (St. Louis, Mo., U.S.A.) and Merck (Darmstadt, F.R.G.). Mono-

TABLE I

Amine	Structure
1,3-Diaminopropane	$NH_2(CH_2)_3NH_2$
Putrescine	$NH_2(CH_2)_4NH_2$
Cadaverine	$NH_2(CH_2)_5NH_2$
Agmatine	$NH_2(CH_2)_4NH-\underset{\underset{NH}{\|}}{C}-NH_2$
3,3'-Diaminodipropylamine (sym-nor-spermidine or caldine)	$NH_2(CH_2)_3NH(CH_2)_3NH_2$
Spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH_2$
Homospermidine	$NH_2(CH_2)_4NH(CH_2)_4NH_2$
Aminopropyl cadaverine	$NH_2(CH_2)_3NH(CH_2)_5NH_2$
N,*N'*-bis-aminopropyl-1,3-propanediamine (sym-nor-spermine or thermine)	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$
Thermospermine	$NH_2(CH_2)_3NH(CH_2)_3NH(CH_2)_4NH_2$
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

acetylputrescine was synthetized according to reference 29. *O*-Phthalaldehyde was obtained from Fluka (Buchs, Switzerland). All other chemicals used for the preparation of buffers and reagents were obtained as the highest purity grade from Merck (Darmstadt, F.R.G.).

Sample preparation

Needles of seemingly healthy and damaged *Picea* trees were collected from an air polluted area in the Vosges region, France. They were weighed (Fr. wt.) and extracted twice with a pestle and mortar with 5% TCA in 0.05 N HCl (10 needles/1 ml) containing 5 nanomoles of 1,7-diaminoheptane per ml as internal standard according to reference 10. Proteins were determined by the Bradford method.[30]

Instrumentation and chromatographic conditions

An amino acid analyser (Liquimat-Labotron) equipped with a fluorimeter using a 50 μl flow cell was employed. An integrator

(ICAP-10, LTT, France) was coupled to the fluorimeter for amine quantification by the internal standard method. Separation of amines was carried out on a 9×0.45 cm I.D. column of BC X-12 cation exchange resin (Benson Co. Reno, Nev., U.S.A.). The composition of the eluting buffers and the elution time were as indicated in Table II. Buffers were prepared from freshly double-distilled deionized water, brought to the required pH and filtered through a millipore filter (0.22 μm pore size). After the filtration the alcohols were added. Flow rates were 33 ml/h for the buffers and 15 ml/h for the fluorogenic reagent. Column back pressure generated during analysis were 7 kg/cm^2. The column was thermostatized at 74°C. Regeneration of the column was achieved in 8 min with a solution of LiOH (0.3 N) containing 250 mg/L of EDTA and re-equilibration obtained in 24 min with the first buffer.

TABLE II

Composition of conditions of eluting buffers

	Buffers	
	First	Second
Lithium citrate	0.40 N (37.6 g/L)	0.60 N (56.4 g/L)
Potassium chloride	0.12 N (9 g/L)	2.0 N (150 g/L)
Boric acid	0.30 N (18.6 g/L)	—
pH (before addition of ethanol)	8.45	5.10
Ethanol added	2.50%	5.50%
Final pH	8.50	5.20
Time (min)	28	38

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram obtained with a standard mixture of 500 picomoles of amine compounds using our cationic exchange high performance column chromatographic technique. The components of the mixture, the retention time, the relative fluorescence peaks area and the constant response values are shown in Table III. The method using two buffers, one single temperature and fluorimetric detection with the help of *o*-phthalaldehyde achieves separation in a little more than one hour. The first buffer elutes together anionic and neutral compounds. Thereafter, as shown in the

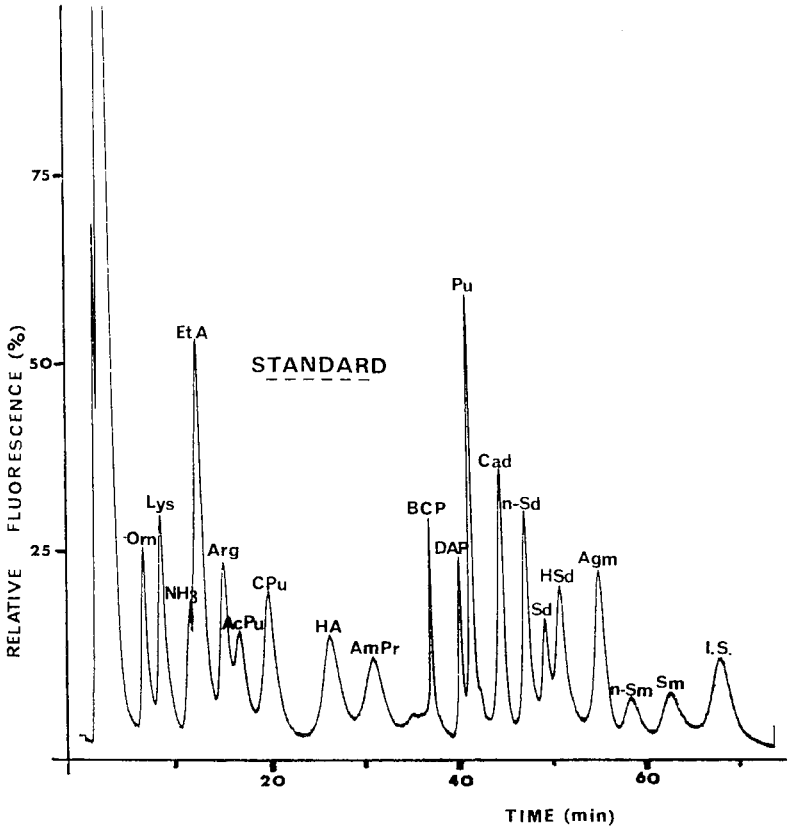

FIGURE 1 Chromatogram of a standard mixture of aminoacids, mono-, di- and polyamines, all at 500 pmoles (EtAM 250). See Table III for the abbreviations and their chromatographic behaviour and Material and Methods for the chromatographic conditions.

chromatogram, ornithine, lysine, ammonia, ethanolamine, arginine, acetyl- and carbamyl-putrescine, histamine and aminopropane are eluted in that order. The second buffer elutes diaminopropane, putrescine, cadaverine, nor-spermidine, spermidine, homospermidine, agmatine, nor-spermine, spermine and 1,7-diaminoheptane (internal standard).

The presence of ethanol in the buffers helps to centre and to improve the separation of arginine and histamine from their respec-

TABLE III

Precision and accuracy of the method[a]

Compound	Abbreviation	Retention time (min)	Surfce area of peaks			
			Mean	S.D.	RSD%	Constant
Ornithine	Orn	7.3±0.0	99,821	698	0.70	1,188
Lysine	Lys	9.3±0.1	154,553	1,379	0.89	806
Ammonia	NH_3	12.3±0.1	—	—	—	—
Ethanolamine	EtAm	13.4±0.1	325,487	669	0.21	339
Arginine	Arg	16.0±0.0	139,578	236	0.17	890
Acetylputrescine	AcPu	17.7±0.1	103,822	722	0.70	1,224
Carbamyl putrescine	CPu	20.8±0.1	159,063	677	0.43	765
Histamine	HA	27.3±0.3	115,258	236	0.20	946
Aminopropane	AmPr	32.1±0.2	72,627	638	0.88	1,253
1,3-Diaminopropane	DAP	40.5±0.0	49,057	733	1.49	2,190
Putrescine	Pu	41.8±0.1	157,703	641	0.41	706
Cadaverine	Cad	44.7±0.2	115,937	772	0.67	935
Nor-spermidine	n-Sd	47.2±0.1	133,111	1,093	0.82	919
Spermidine	Sd	49.2±0.1	65,339	979	1.50	1,518
Homospermidine	HSd	50.8±0.2	134,850	366	0.27	870
Agmatine	Agm	55.2±0.2	166,552	331	0.20	779
Nor-spermine	n-Sm	58.6±0.2	35,629	265	0.75	3,210
Spermine	Sm	62.8±0.3	42,360	295	0.70	2,471
Internal-standard	I.S.	68.5±0.2	116,793	941	0.81	1,000

[a]Average of ten determinations on a mixture containing 500 pmole of each of the products (EtAm 250 pmole). Quantification was effected with an ICAP-10 Integrator coupled to the fluorimeter. 1,7-Diaminoheptane was used as internal-standard (I.S.).

tive neighbour compounds. Ethanol accelerates also the elution of the internal standard and contributes to the sharpness of the peaks. The method is sensitive at picomole level and its reproductibility is comparable to that of the best methodologies published as yet for aminoacid analysis (as shown in Table III). An important improvement of the method is that samples from different biological sources can be analysed without previous purification, thus saving time and avoiding loss of amino compounds, particularly those present in trace amounts.

This method has been used in our laboratory for more than one year in different polyamine-concerned problems. It is now serving more particularly to compare amine contents between needles of healthy and damaged *Picea* subjected to air pollution. Table IV

TABLE IV

Amine content[c] in samples of healthy and polluted *Picea*

Samples				Orn	Lys	Arg	CPu	Pu	Sd	Sm	Total protein (μg)	Fresh weight (mg)
Healthy		1	1985[a]	11	18	101	0.5	1.5	12	13	744	26.2
		2	1984[b]	3	10	85	0.5	3	15	2.6	1,232	53.6
Polluted	(1)	3	1985[a]	trace	16	40	0.6	11	37	5	696	26.5
		4	1984[b]	traces	8	8	0.5	3	25	traces	756	52.6
	(2)	5	1985[a]	traces	5	8	0.5	1	4	5.6	552	13.6
		6	1984[b]	traces	1	1	0.2	1	3	traces	1,448	48.2
	(2)	7	1985[a]	traces	7	7	traces	5	14	4	628	18
		8	1984[b]	traces	5	5	0.4	8	22	traces	1,208	55
	(3)	9	1985[a]	traces	24	10	1.5	2	54	15	484	23
		10	1984[b]	traces	3	1	0.3	1	40	traces	564	28

(1) to (3): Increasing degree of injury (slight, medium and heavy damage).

[a,b]1985 and 1984 shoots collected on the same tree at the same time.

[c]Picomole/μg of protein.

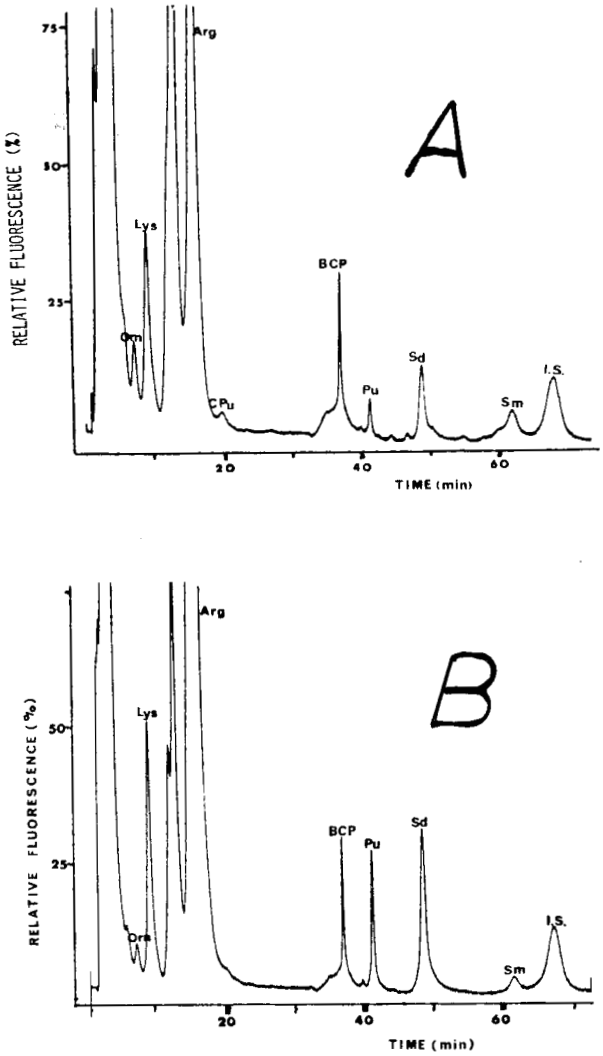

FIGURE 2 Chromatogram of a 50 μl TCA extract of healthy needles of *Picea*. *A*: needles from 1985 shoots; *B*: needles from 1984 shoots. Both collected from the same tree at the same time (see also Table IV).

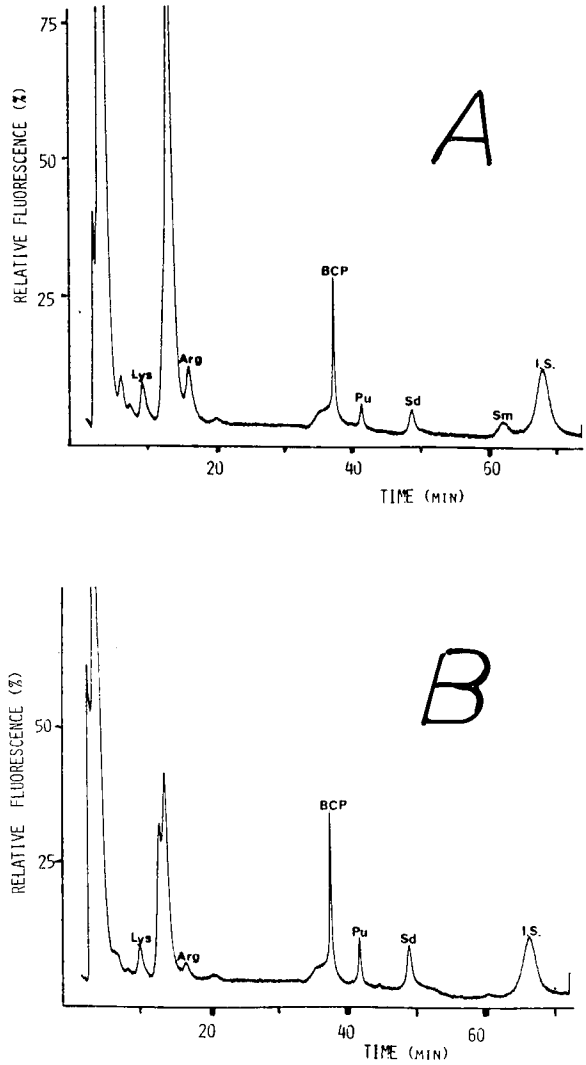

FIGURE 3 Chromatogram of a 50 μl TCA extract of damaged needles of *Picea*. *A*: needles from 1985 shoots; *B*: needles from 1984 shoots. Both collected from the same tree at the same time (see also Table IV).

summarizes preliminary results obtained with samples collected in the Vosges region of France. Considerable differences appear to exist in the content of the polyamine precursor arginine in needles from the young 1985 formed shoots. This difference is even more clear-cut when comparing needles from the 1984 shoots. (See also Figures 2 and 3.)

Also spermine could indicate some differences. These first results need to be verified. Further work in this area is currently in progress.

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