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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF METHYLATED AMINO ACIDS AND RELATED COMPOUNDS AND ITS APPLICATION TO THE ANALYSIS OF SAMPLES OF POLLUTED HEALTHY AND DAMAGED *PICEA* NEEDLES WITHOUT EXTRACT PURIFICATION

V. R. VILLANUEVA*, M. MARDON, M. Th. LE GOFF and F. MONCELON

Institut de Chimie des Substances Naturelles, CNRS, 91190 Gif sur Yvette (France)

SUMMARY

A fully automated, rapid and sensitive method for the separation of 24 inter-related amino compounds is presented. It uses a high-performance laboratory-made analyser, ion-exchange chromatography and fluorimetric with *o*-phthalaldehyde detection. All methylated known natural derivatives of histidine, lysine and arginine are well separated. The method uses three buffers and a single column temperature. An analysis requires 118 min and the method is sensitive to picomole levels of the compounds concerned. Ion-exchange chromatography and the composition of the buffers allow the application of the method to samples from different biological sources without previous purification, thus saving time and avoiding losses of amino compounds, particularly those present in trace amounts. It has been used for the analysis of crude polluted *Picea* needle samples.

INTRODUCTION

Because of their involvement in both the structure and function of various proteins, methylated amino acids (histidine, lysine and arginine) have attracted considerable interest particularly in connection with fast-growing tissue processes. Further, the protein methylation reaction is ubiquitous in nature, as evidenced by the fact that various well characterized and specialized proteins such as histones, myosin, actin, opsin, flagellin, cytochrome and ribosomal proteins of eukaryotic and prokaryotic organisms are found methylated in nature[1]. Methylation of proteins is one of the several post-translational modification reactions of polypeptides. Side-chains of amino-acids such as histidine, lysine and arginine and carboxyl groups are methylated with S-adenosylmethionine as the methyl donor by various specific methyl transferases to the amino acid residue involved[2,3].

Methylated amino acids are currently considered to be derived from the *in vivo* hydrolysis of methylated proteins. Nevertheless, evidence has been presented that in some instances methylation could also take place directly on the free amino acid or on a non-peptide derivative[4].

The biological significance of protein methylation has not been fully elucidated. However, as methylated amino acids are found in highly specialized proteins[1], the determination of these compounds may be important for the study of changes in cell metabolism caused by pathological and physiological conditions. However, detailed investigations in this field have been hampered by the lack of a routinely manageable method capable of the rapid and specific determination of these modified amino acids. Some methods have been published, but most of them concern only the determination of some of these compounds[5-10] or are too time consuming[11]. Previous work in our laboratory led to the development of a method for the automatic determination of all methylated histidine, lysine and arginine compounds in just over 3 h[12]. We have now improved this method in order to apply it to the determination of all basic methylated amino acids and some biosynthetically related compounds. Twenty-four compounds can be separated in less than 2 h at the picomole level using lithium citrate buffers and an inexpensive laboratory-made automated analytical system. This method has been utilized for more than 1 year in this laboratory for the determination of modified amino acids in crude biological extracts and is currently being employed in the study of physiological effects of pollution in *Picea* needles.

EXPERIMENTAL

Chemicals

Most amino acids and their methylated derivatives were obtained from Sigma (St. Louis, MO, U.S.A.), methylated arginines and methylated lysines from Calbiochem (San Diego, CA, U.S.A.) and *o*-phthalaldehyde (OPA) from Fluka (Buchs, Switzerland). All other chemicals used for the preparation of buffers and reagents were obtained in the highest grade available 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 (Massif du Donon, France). They were cooled by using dry-ice for transport and conserved in a deep-freeze at −30°C. They were weighed, reduced to a fine powder using liquid nitrogen with a pestle and mortar and extracted twice with a solution of 5% trichloroacetic acid in 0.05 M hydrochloric acid (10 needles per ml) containing 10 nmol/ml of ε-aminocaproic acid, which serves as an internal standard for quantification purposes.

Instrumentation and chromatographic conditions

The automated analytical system constructed previously for the analysis of sugar[10] was used with minor modifications (removal of one pump and the reaction coils), as shown in Fig. 1. A fluorimeter (Spectra-Glo; Gilson-France, Villiers le Bel, France) using a 45 μl flow cell was coupled to an integrator (SP 4200; Spectra-Physics, Orsay les Ulis, France) for quantification by the internal standard method. A 33 × 0.4 cm I.D. column of BC-X7 cation-exchange resin (7–10 μm diameter and 7% cross-linked) was used (Benson, Reno, NV, U.S.A.).

The composition of the eluting buffers and the elution time were as indicated in Table I. The buffers were prepared from freshly doubly distilled, deionized water, adjusted to the required pH and filtered through a Millipore filter (Type HA, 0.45
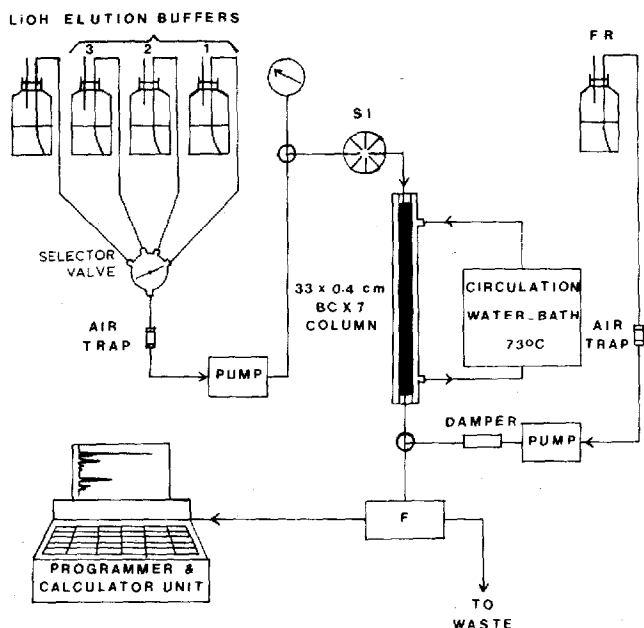
Fig. 1. Diagram of the automatic chromatographic system. Milton Roy precision piston pumps were employed. SI = sample injector; FR = fluorescence reagent; F = fluorimeter.

μm pore size; Millipore Waters, Saint Quentin and Yvelines, France). The flow-rates were 33 ml/h for the buffers and 20 ml/h for the OPA fluorogenic reagent, which was prepared as described previously[13]. One column temperature, 73°C, was used during the analysis. Regeneration of the column was achieved in 4 min with a solution of 0.6 M lithium hydroxide containing 250 mg/l of EDTA and re-equilibration was completed in 24 min with the first buffer. The column back-pressure generated during analyses was 40 kg/cm^2.

RESULTS AND DISCUSSION

Fig. 2 shows the chromatographic resolution of 24 compounds (500 pmol of most of them) from a standard mixture consisting of common amino acids present in proteins, ε-aminocaproic acid (internal standard), methylated derivatives of his-

TABLE I

COMPOSITION AND CONDITIONS OF ELUTING BUFFERS

Buffer	Lithium citrate (N)	pH	Elution time (min)
1	0.1	6.06	8
2	0.6	6.85	23
3	1.2	6.95	80

TABLE II

PRECISION AND ACCURACY OF THE METHOD

Average of ten determinations on a mixture containing 500 pmol of each of the products (CAR, ANS and CPU, 300 pmol; IS and TML, 1000 pmol). Quantification was effected by the internal standard method with an SP 4200 integrator coupled to the fluorimeter.

| Compound	Abbreviation	Retention time (min)	Peak area			Response factor (RF)
			Mean	S.D.	R.S.D. (%)	
Tyrosine	Tyr	11.9 ± 0.1	124 450	1660	1.33	0.741
Phenylalanine	Phe	14.5 ± 0.2	180 166	3284	1.82	0.512
Internal standard	IS	18.3 ± 0.2	179 850	1784	0.99	1.000
Hydroxytryptophane	OHTrp	20.7 ± 0.2	219 934	2228	1.01	0.405
Histidine	His	24.9 ± 0.3	234 079	1545	0.66	0.387
3-Methylhistidine	3Me-His	26.7 ± 0.2	190 465	1202	0.63	0.465
Tryptophan	Trp	28.4 ± 0.1	469 301	2118	0.45	0.187
1-Methylhistidine	1Me-His	33.3 ± 0.3	120 580	816	0.68	0.446
Carnosine	CAR	36.0 ± 0.3	179 067	160	0.10	0.486
Anserine	ANS	41.0 ± 0.2	60 837	569	0.93	0.865
Hydroxylysine	Hyl	47.5 ± 0.2	145 345	865	0.60	0.603
Diaminobutyric acid	DAB	50.2 ± 0.2	90 056	786	0.87	0.990
Ornithine	Orn	53.1 ± 0.3	114 185	1760	1.54	0.688
Lysine	Lys	55.6 ± 0.2	196 609	1597	0.81	0.453
Ammonia	NH$_3$	58.4 ± 0.1				
Ethanolamine	EtAm	60.6 ± 0.2	342 029	1055	0.31	0.260
Monomethyllysine	MML	64.4 ± 0.3	117 369	1357	1.15	0.732
Dimethyllysine	DML	74.1 ± 0.2	118 401	180	0.15	0.745
Trimethyllysine	TML	86.5 ± 0.3	421 823	2676	0.63	0.430
Arginine	Arg	93.6 ± 0.3	197 750	1622	0.82	0.440
Carbamoylputrescine	CPU	97.7 ± 0.3	70 208	708	1.00	0.756
μ-Dimethylarginine	μ-DMA	104.5 ± 0.3	115 955	557	0.48	0.776
Monomethylarginine	MMA	107.9 ± 0.3	95 820	686	0.71	0.800
s-Dimethylarginine	s-DMA	115.1 ± 0.3	83 308	916	1.09	1.103
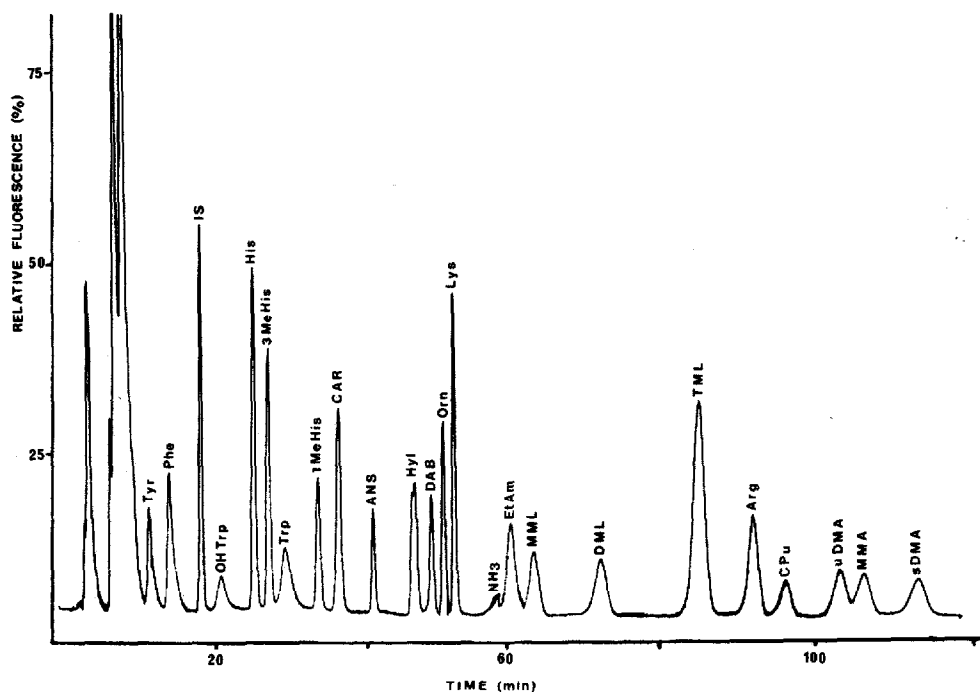
Fig. 2. Chromatographic analysis of a standard mixture of methylated derivatives of histidine, lysine and arginine and related amino compounds. For abbrevations, see Table II.

tidine, lysine and arginine and related compounds. The components of the mixture, the amount of each applied to the column, the retention time, the relative fluorescence peak areas and the response factors (*RF*) with respect to ε-aminocaproic as internal standard are shown in Table II.

The column and the elution programme employed gave a good overall resolution. The first buffer elutes in a group the acidic and neutral amino acids (aspartic acid to leucine) and then separates sharply tyrosine, phenylalanine and the internal standard. The second buffer separates hydroxytryptophan, tryptophan, histidine and its methylated derivatives, carnosine and anserine. The third buffer resolves hydroxylysine, diaminobutyric acid, ornithine, lysine, ammonia, ethanolamine, methylated derivatives of lysine, arginine and carbamoylputrescine and methylated arginines. Complete analysis is achieved in less than 2 h using a single column temperature, which represents a considerable improvement over previously published methods[12]. The method is sensitive at the picomole level and its reproducibility is comparable to that of the best methods published for amino acid analyses (as shown in Table II).

The system described here was developed in order to obtain a method for the direct analysis of crude deproteinized biological extracts. This was achieved by the use of ion-exchange chromatography and appropriate ionic strength and pH in each of the three eluting buffers. The first buffer elutes anionic and neutral compounds and initiates a sharp resolution from tyrosine and the other two buffers complete the elution of the remaining amino compounds. Prior purification of the samples, which

is often time consuming, is not necessary and thereby the risk of losing compounds that could be present in trace amounts was avoided. The retention times of different amine compounds were not altered. This was verified by co-chromatographing the crude samples with standard mixtures.

Compared with previously described methods, the proposed method has the advantage of separating a larger number of related amino compounds in a single chromatographic run in a relatively short time with high sensitivity. The use of an integrator and of an internal standard in the sample contributes to easier and reproducible quantification.

The method has been applied in our laboratory to study different problems concerning modified amino acids. It is now serving particularly for a comparative analysis between needles of healthy and damaged *Picea* subjected to air pollution. The chromatograms in Figs. 3 and 4 obtained from crude extracts of *Picea* needles show that small amounts of methylated derivatives can be detected. Also interesting is the fact that significant differences exist between the contents of histidine and tryptophan. The high content of tryptophan in damaged trees could indicate abnormal kinetics of the utilization of this compound, which is known to be the precursor of indoleacetic acid, the growth hormone of plants[14]. Further work on this aspect is in progress.
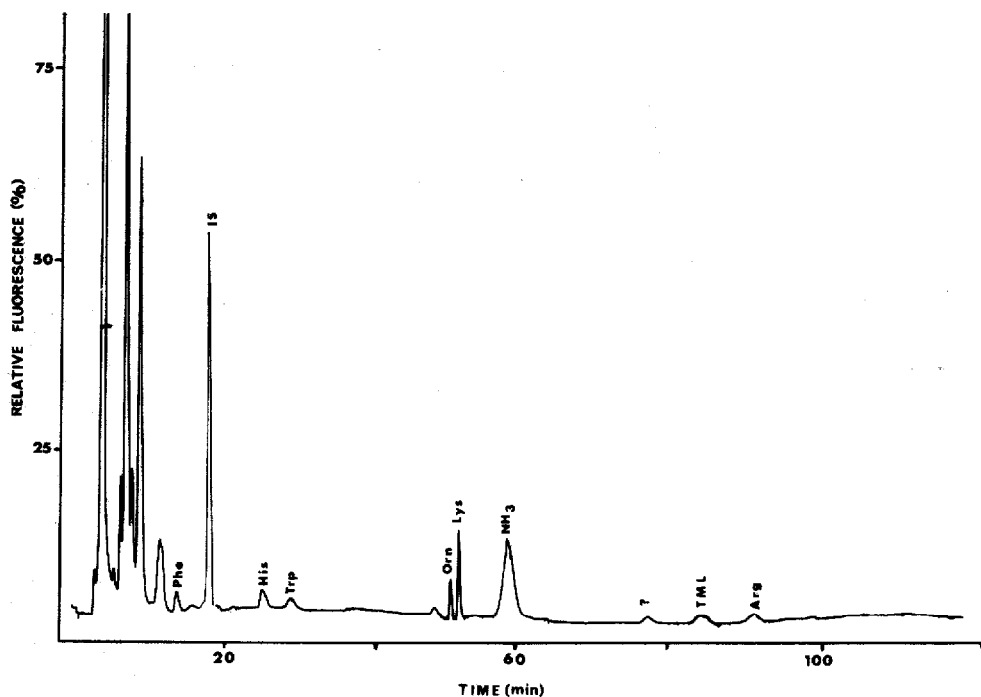

Fig. 3. Chromatographic analysis of crude extracts of healthy *Picea* needles. For abbreviations, see Table II.
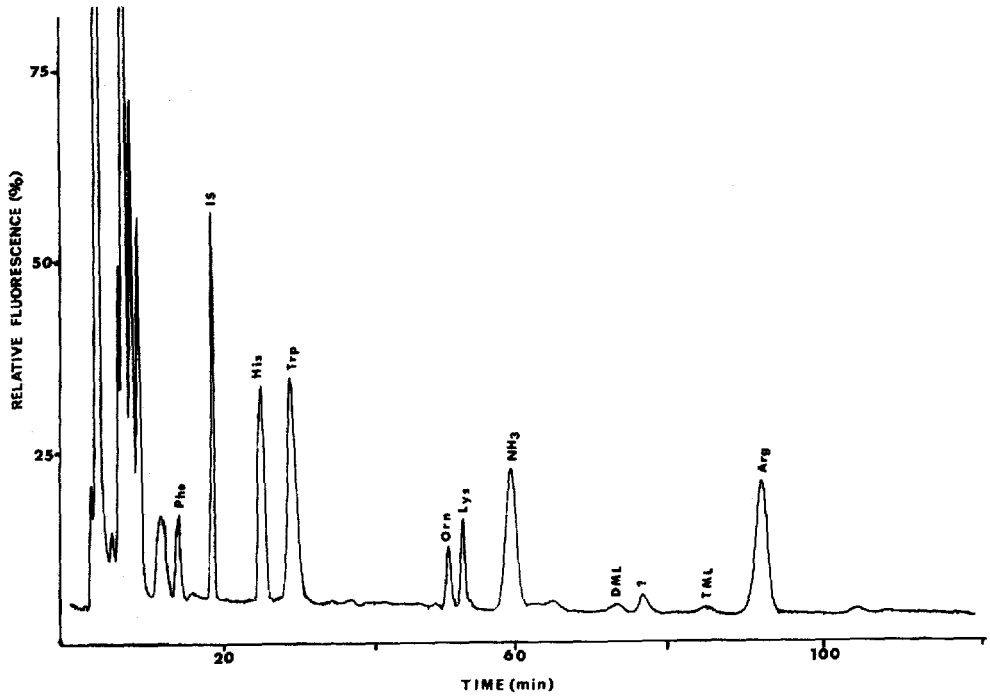
Fig. 4. Chromatographic analysis of crude extracts of damaged *Picea* needles. Noteworthy are the high levels of histidine and tryptophan compared with those in healthy trees (Fig. 3). For abbreviations, see Table II.

ACKNOWLEDGEMENTS

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