Journal of Chromatography, 286 (1984) 253–260
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 264

DETERMINATION OF VERATRIC ACID AND ITS METABOLITES IN BIOLOGICAL MATERIAL BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

ZDZISŁAW SUPRYNOWICZ* and BOGUSŁAW BUSZEWSKI
Department of Chemical Physics, M. Curie-Skłodowska University, 20-031 Lublin (Poland)
and
JERZY ROGALSKI and ELŻBIETA MALARCZYK
Department of Biochemistry, M. Curie-Skłodowska University, 20-031 Lublin (Poland)

SUMMARY

The optimization of the separation of veratric acid and its demethylation products, isovanillic and vanillic acids, by ion-suppression and ion-pair high-performance liquid chromatography is described. The influence of the eluent pH, composition of the mobile phase and concentration of complexing ion-pairing agent was investigated. Examples are given of the determination of methoxy acids in a test biological material from *Nocardia* culture and the detection limit of the separated compounds was determined.

INTRODUCTION

About $15 \cdot 10^{10}$ tonnes of lignocellulose waste are accumulated each year, and this is the biggest reserve of organic materials that can be utilized as a possible source of food or fuel[1]. The most frequent polysaccharide component is cellulose, which can be hydrolysed chemically or enzymatically. However, decomposition of this component in native substrates, such as wood or straw, is difficult owing to the protective layer of lignin, which prevents the decomposition of cellulose. This layer is chiefly destroyed by fungi such as *Basidiomycetes*[2], *Ascomycetes* and *Fungi imperfecti*[3] and by bacterial cultures[4,5], whose effect was tested in model cultures. Among these, *Nocardia* from the order Actinomycetales play an important role, as they can decompose not only lignin but also phenyl monomers[6,7] formed during the decomposition. Compounds such as veratric acid and other aromatic acids, produced naturally in the processes of microbiological decomposition of lignin, are frequently investigated in model experiments on biodegradation[3] and repolymerization of lignin into soil humus[8,9]. As a result of biotransformation, veratric acid may be demethylized, creating either vanillic or isovanillic acid, which undergo further degradation processes including cleavage of the aromatic ring and decomposition into carbon dioxide[6,7].

The method that has been most frequently used so far for investigating the decomposition of particular phenol monomers was the release of $^{14}CO_2$ from specifically labelled functional groups[6,7,10]. This method facilitated the determination of the loss of a given substrate and, to a small extent, observation of the products

0021-9673/84/$03.00

formed. Thin-layer chromatography is commonly used for the qualitative determination of the products formed after the decomposition of, among others, veratric acid[8,11]. This method is time consuming and does not yield quantitative results, so it is not very useful in routine investigations.

For the identification of the polymers (dimers to tetramers) of vanillic acid, created during the action of lacase on vanillic acid, Bollag *et al.*[8] applied reversed-phase high-performance liquid chromatography (HPLC) using water–propanol–acetic acid (72:24:4) as the eluent. The time of the analysis was 10–25 min. It was therefore desirable to develop a rapid and simple method for the qualitative and quantitative analysis of methoxyphenol decomposition products in biological material, using as an example veratric acid and the basic products of its decomposition.

In this investigation, the conditions for the separation of methoxy acids forming in the processes of lignin biotransformation were examined and optimized. Tetrabutylammonium chloride was used as the complexing agent (counter ion). The influence of the eluent pH, counter-ion concentration and the amount of methanol and acetonitrile in the mobile phase was examined[12].

EXPERIMENTAL

Apparatus

A Pye Unicam Model LC-20 liquid chromatograph with a UV detector (254 nm) of 8-μl cell capacity and a Model 7120 sampling valve (Rheodyne, Berkeley, CA, U.S.A.), with a 10-μl loop, were used. A stainless-steel column (10 cm × 4 mm I.D.) was used, protected with *ca.* 3-μm porous steel sinters. The sampler was connected with the column and the detector by stainless-steel capillaries of 0.2 mm I.D.

Column and reagents

A sorbent with a chemically bonded C_{18} stationary phase prepared on the basis of LiChrosorb Si 100 of particle size 10 μm (Merck, Darmstadt, F.R.G.) was used[13].

The column was packed according by the upward slurry-packing technique, as described by Suprynowicz *et al.*[14]. After packing, the column was washed with 100 ml of methanol at a flow-rate of 2–3 ml/min, and then tested according to Bristow and Knox[15]. The mobile phase was vacuum degassed for 15 min in an ultrasonic bath.

The following reagents, all of analytical-reagent grade, were used: methanol and acetonitrile (Merck) veratric acid and tetrabutylammonium chloride (Fluka, Buchs, Switzerland), vanillic and isovanillic acids (Sigma, St. Louis, MO, U.S.A.), benzyl alcohol (Carlo Erba, Milan, Italy), sodium acid phosphate, phosphoric acid, tetrachloromethane and acetone (POCH, Gliwice, Poland). Doubly distilled water was used.

Preparation of standard mixtures and biological samples

Standards of each of the three methoxy acids were dissolved in methanol or acetonitrile to obtain definite concentrations mixed in the ratio of 1:1:1 and used as model test mixture.

The biological material came from a *Nocardia* culture, cultivated on a mineral

medium with the addition of 0.1% of succinic acid as the only source of carbon[5]. The cultivation was interrupted in the logarithmic growth phase and 0.02% of veratric acid was added for the induction of demethylases. Subsequently 1-ml samples were taken at certain intervals, centrifuged, and the methoxy acids in the supernatant were determined by ion-pair HPLC. Before the chromatographic measurements, the 1-ml samples were acidified with 0.1 ml of 5 *N* sulphuric acid and extracted from the acid medium with two 2-ml volumes of diethyl ether. The ether layers were combined and dehydrated by the addition of anhydrous magnesium sulphate evaporated under vacuum and then dissolved in pure methanol.

Quantitative determination

Peak-height measurements were used for quantitative determination with benzyl alcohol as an internal standard. Linearity of the detection response was established in the investigated range of concentrations. The concentration of the solute was calculated according to the equation

$$c = h \cdot \frac{c_s}{h_s} \cdot \frac{h_y}{c_x}$$

where c = the concentration of the solute in a biological sample, h = the peak height of the solute in a biological sample, c_s = the concentration of the standards (benzyl alcohol) in a biological sample, h_s = the peak height of the standard in a biological sample, h_y = ratio of the peak heights of the standard and solute in a test mixture and c_x = ratio of the concentrations of the standard and solute in a test mixture.

RESULTS AND DISCUSSION

Optimization of separation conditions of the test mixture of methoxyacids was carried out in three stages: (a) evaluation of the effect of pH; (b) determination of the optimal composition of the mixed mobile phase (organic component–water); and (c) establishment of the concentration of the complexing agent.

pH showed the greatest influence on k' values. A pH of *ca.* 5 seems to be optimal for an adequate resolution (R_s) and a short analysis time (t_A) (Fig. 1).

Replacement of methanol with acetonitrile led to lower k' values and even to shorter analysis times.

About the same values of t_A and R_s and the highest selectivity of the isovanillic and vanillic acid peaks can be obtained with the use of *ca.* 10% methanol or *ca.* 5% acetonitrile in water (Fig. 2 and Table I). A change in the concentration of the complexing agent, tetrabutylammonium chloride, has a stronger effect on the k' values of the investigated substances when methanol–water than when acetonitrile–water is used as the mobile phase (Fig. 3).

To avoid side reactions, it seems advisable to use acetonitrile or *ca.* 0.015 *M* or slightly lower concentrations of tetrabutylammonium chloride. Mobile phases with acetonitrile seem preferable to phases with methanol because the reproducibility of k' and α values is better. For instance, the mean standard deviations of k' and α for the mobile phase containing 5% of acetonitrile are $\sigma_{k'} = 0.095$ and $\sigma_\alpha = 8 \cdot 10^{-4}$, respectively, whereas the respective values for the mobile phases containing

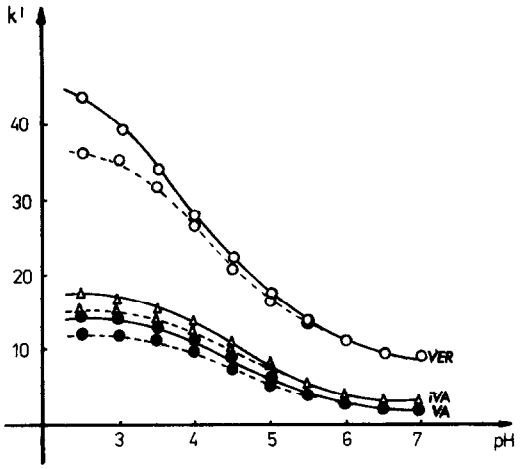


Fig. 1. Influence of pH on k' of methoxy acids. Mobile phase: methanol–water (——); acetonitrile–water (– – – –), containing 0.015 *M* tetrabutylammonium chloride. VA, Vanillic acid; iVA, isovanillic acid; VER, veratric acid.

10% of methanol are: $\sigma_{k'} = 0.1118$ and $\sigma_{\alpha} = 0.2801$.

Peak asymmetry factors (A_s) give an indication of separation systems. The numerical values of A_s do not exceed the acceptable value of *ca.* 1.2 much, and the results in Table II confirm the optimized conditions chosen.

The detection limits of the methoxy acids obtained under different conditions are compared in Table III. The use of acetonitrile instead of methanol provides a lower detection limit, of the order 10^{-7} g/ml.

TABLE I

SELECTIVITY (α) AND RESOLUTION (R_s) USING DIFFERENT MOBILE PHASE COMPOSITION AND A CONSTANT CONCENTRATION OF MODIFYING AGENT

Modifying agent: phosphate buffer (pH 5.5) and 0.015 *M* tetrabutylammonium chloride. α and R_s determined for the pair of peaks isovanillic acid and vanillic acid.

Organic component of mobile phase	*Concentration (%, v/v)*	*k'*		α	R_s
		Vanillic acid	*Isovanillic acid*		
(pure water)		11.20	12.69	1.13	2.59
Methanol	5	7.72	9.5	1.23	2.00
	10	5.06	6.26	1.24	1.95
	15	2.85	3.26	1.15	1.14
	20	1.76	2.00	1.13	0.53
	25	1.13	1.13	1.00	–
Acetonitrile	5	5.06	6.30	1.25	1.76
	10	2.29	2.70	1.16	1.02
	15	1.07	1.18	1.10	0.43
	20	0.85	0.85	1.00	–

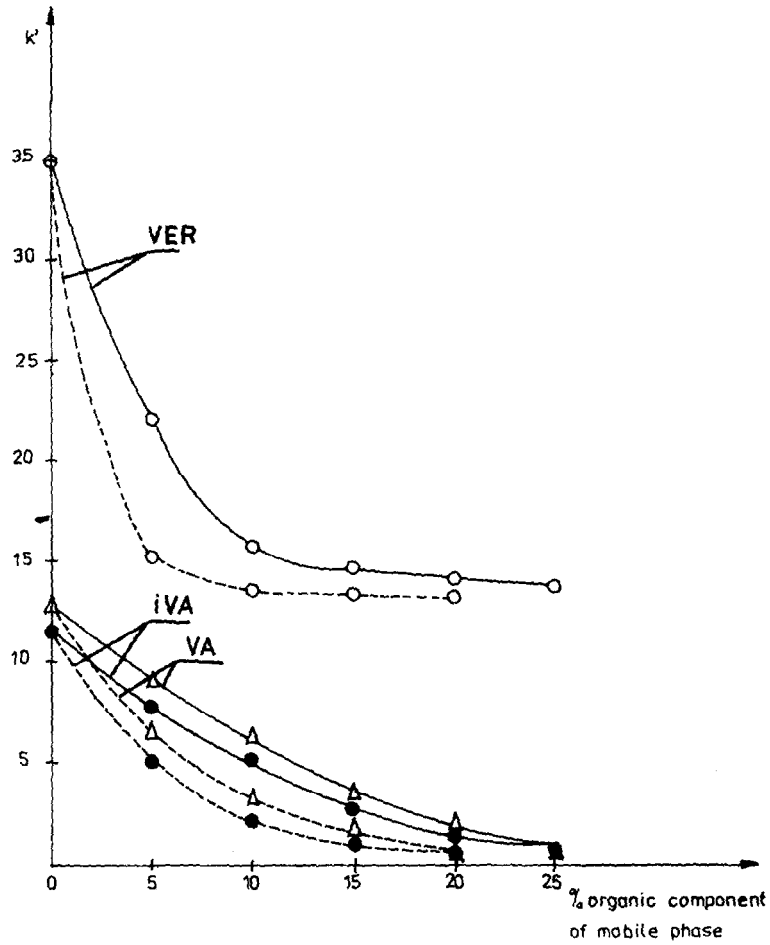


Fig. 2. Influence of mobile phase composition on k' of methoxy acids. Compounds: methanol–water (——), acetonitrile–water (- - - - -), pH 5.5, containing 0.015 *M* tetrabutylammonium chloride. Abbreviations as in Fig. 1.

TABLE II

COMPARISON OF PEAK ASYMMETRY FACTORS OF METHOXY ACIDS

Peak asymmetry factors (A_s) measured at 10% of the maximum peak height. Complexing agent: 0.015 *M* tetrabutylammonium chloride.

Organic component of mobile phase	*Concentration (%, v/v)*	*pH*	A_s		
			Vanillic acid	*Isovanillic acid*	*Veratric acid*
Methanol	10	3.5	1.20	1.03	1.25
	10	5.5	1.20	1.25	1.28
Acetonitrile	5	3.5	1.15	1.18	1.10
	5	5.5	1.10	1.05	1.25

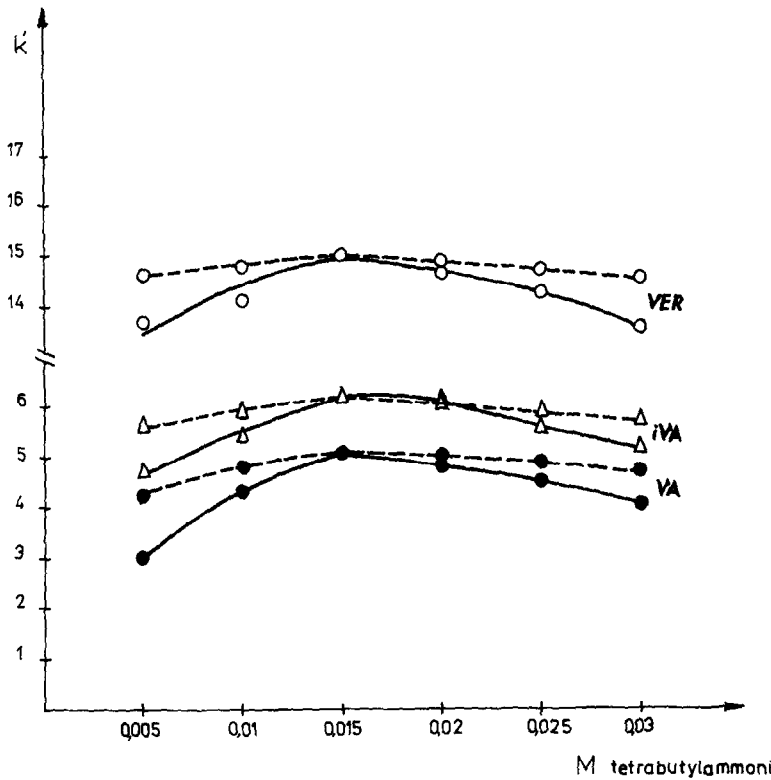
TABLE II

LIMITS OF DETECTION OF METHOXY ACIDS

Complexing agent: 0.015 *M* tetrabutylammonium chloride.

Organic component of mobile phase	*Concentration (%, v/v)*	*pH*	*Detection limit* (g/ml)*					
			Vanillic acid		*Isovanillic acid*		*Veratric acid*	
			Value	*S.D.**	*Value*	*S.D.**	*Value*	*S.D.**
Methanol	10	3.5	$1 \cdot 10^{-6}$	$4.300 \cdot 10^{-8}$	$2 \cdot 10^{-6}$	$7.483 \cdot 10^{-8}$	$1 \cdot 10^{-6}$	$3.240 \cdot 10^{-8}$
	10	5.5	$5 \cdot 10^{-7}$	$1.489 \cdot 10^{-8}$	$1 \cdot 10^{-6}$	$9.117 \cdot 10^{-8}$	$5 \cdot 10^{-7}$	$1.351 \cdot 10^{-8}$
Acetonitrile	5	3.5	$4 \cdot 10^{-7}$	$1.630 \cdot 10^{-8}$	$1.5 \cdot 10^{-6}$	$4.637 \cdot 10^{-8}$	$4 \cdot 10^{-7}$	$1.068 \cdot 10^{-8}$
	5	5.5	$2 \cdot 10^{-7}$	$7.910 \cdot 10^{-9}$	$7.5 \cdot 10^{-7}$	$1.352 \cdot 10^{-8}$	$2 \cdot 10^{-7}$	$1.823 \cdot 10^{-8}$

* Standard deviation, in all instances calculated from at least eight measurements.



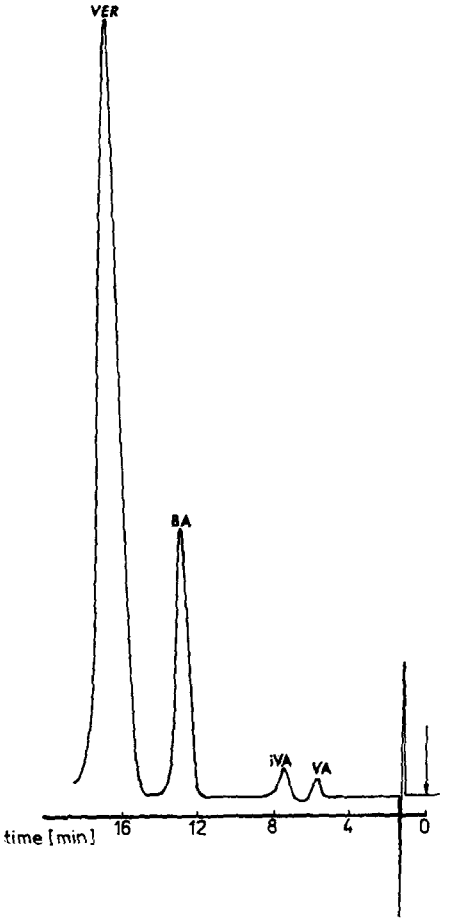


Fig. 3. Influence of concentration of complexing agent on k' of methoxy acids. Mobile phase: methanol–water (——); acetonitrile–water (- - - -), pH 5.5. Abbreviations as in Fig. 1.

Fig. 4. Chromatogram of the methoxy acids present in the supernatant after centrifugating *Nocardia* cells induced with 0.02% veratric acid. Internal standard: benzyl alcohol (BA). Mobile phase: methanol–water (10:40), pH 5.5, containing 0.015 *M* tetrabutylammonium chloride. Abbreviations as in Fig. 1.

The chromatogram in Fig. 4 shows an example of the separation of methoxy acids in a biological sample.

CONCLUSIONS

Veratric acid and its metabolites in biological samples can be separated and determined by ion-pair HPLC. The limit of detection with a UV detector (254 nm) is of the order of 10^{-6}–10^{-7} g/ml of a single methoxy acid. The time of analysis does not exceed 20 min. The developed method may be used for the investigation of lignin biotransformation products.

REFERENCES

1 J. M. Nyston and R. K. Andren, *Process Biochem.*, 11 (1976) 26.
2 J. Trojanowski, *Int. Biodet. Bull.*, 5 (1969) 119.
3 K. Haider and J. Trojanowski, *Arch. Microbiol.*, 105 (1975) 33.
4 N. T. Cartwright and K. S. Holdom, *Microbios*, 8 (1973) 7.
5 K. Haider, J. Trojanowski and V. Sundman, *Arch. Microbiol.*, 119 (1978) 103.
6 K. Gradziel, K. Haider, J. Kochmańska, E. Malarczyk and J. Trojanowski, *Acta Microbiol. Polon.*, 27 (1978) 103.
7 J. Trojanowski, K. Haider and V. Sundman, *Arch. Microbiol.*, 114 (1977) 149.
8 J. M. Bollag, S.-Y. Liu and R. D. Monard, *Soil Biol. Biochem.*, 14 (1982) 157.
9 S.-Y. Lieu, E. D. Monard and J. M. Bollag, *Soil Biol. Biochem.*, 45 (1981) 1100.
10 J. Rogalski, J. Trojanowski, M. Deryło. M. Głowacka and Z. Lorkiewicz, *FEMS Microbiol. Lett.*, 13 (1982) 423.
11 F. H. Bernhardt, H. Staudinger and V. Ullrich, *Hoppe-Seyler's Z. Physiol. Chem.*, 351 (1970) 467.
12 B. A. Bidlingmeyer, *J. Chromatogr. Sci.*, 18 (1980) 525.
13 Z. Suprynowicz and B. Buszewski, in preparation.
14 Z. Suprynowicz, B. Buszewski, R. Lodkowski and J. Trocewicz, *Chem. Anal. (Warsaw)*, 25 (1980) 749.
15 P. A. Bristow and J. H. Knox, *Chromatographia*, 10 (1977) 279.