Journal of Chromatography, 362 (1986) 47–53
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 642

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR DETERMINING ORGANIC ACIDS IN WINES

FEDERICA CACCAMO, GUIA CARFAGNINI, ANTONIO DI CORCIA* and ROBERTO SAMPERI

Dipartimento di Chimica, Universita' "La Sapienza" di Roma, Piazza Aldo Moro, 00185 Rome (Italy)

(Received March 4th, 1986)

SUMMARY

Lactic, acetic, tartaric, malic, succinic and citric acids in wine samples were determined as phenacyl esters. The sample was buffered at pH 6.8, mixed with a solution of phenacyl bromide and a crown ether in acetone, and heated at 100°C for 40 min. An aliquot of the reaction mixture was subjected to reversed-phase high-performance liquid chromatography. Recoveries of the six acids were higher than 95%. The specificity of this assay was good as assessed by quantifying wine acids with other chromatographic techniques. The within-run coefficient of variation ranged from 1.7% for acetic acid to 3.1% for citric acid. When compared with a similar, recently reported assay, our assay gave more reliable results, especially for citric acid.

INTRODUCTION

Wines contain a complex mixture of organic acids. Among these, tartaric, malic, succinic, citric, lactic and acetic acids are the most important as their balance has major implications both in sensory terms and for the stability of wine.

Various attempts have been made to determine simultaneously these acids by means of chromatographic techniques. Paper and thin-layer chromatography provide only a qualitative assessment of the balance of the individual acids. Several derivatization procedures have been proposed for the analysis of polycarboxylic acids by gas chromatographic methods[1–4]. In our experience, however, none of these methods meets the requirements of simplicity, rapidity and, mostly, reliability.

A recent trend is the analysis of intact wine organic acids by high-performance liquid chromatography (HPLC) by exploiting different retention mechanisms, such as ion exchange[5], ion exclusion[6] and ion suppression[7]. Rapidity and simplicity are claimed for these procedures. To date, however, all the chromatographic systems employed for the direct determination of organic acids in wine samples have revealed the problem of coelution of neutral compounds with the analytes of interest. Various, alternative clean-up procedures have been developed to improve the accuracy but at the expense of rapidity and simplicity.

0021-9673/86/$03.50

Crown ether-assisted derivatization of carboxylic acids to phenacyl esters is simple to perform and the resulting derivatives show excellent chromatographic properties[8]. Moreover, the presence of water does not seriously affect the derivatization yield, and non-exctractive preparative procedures for organic acids in aqueous solution have been proposed[9,10].

Very recently, Mentasti *et al.*[11] described an HPLC method for the determination of organic acids in wine samples, which couples their conversion into phenacyl esters without recourse to problematic solvent extraction with the ease of application of reversed-phase liquid chromatography.

This report describes an improved HPLC procedure for the assay of organic acids in wine, which involves substantial modifications of the method mentioned above. The main modification is that of buffering the reaction mixture which results in a better yield from the derivatization reaction, thus eliminating a source of error in the determination of polycarboxylic acids, especially citric acid.

EXPERIMENTAL

Reagents

Acid standards were obtained from various commercial sources. A 0.08 *M* phosphate buffer, pH 6.8, was prepared by dissolving 5.44 g KH_2PO_4 and 14.3 g $Na_2HPO_4 \cdot 12H_2O$ in 1 l of distilled water. To this solution, methylmalonic acid, used as internal standard, was added to give a concentration of 1 g/l. The esterification agent was prepared by dissolving in acetone both phenacyl bromide (Fluka, Buchs, Switzerland) and dicyclohexane-18-crown-6 (Fluka) to give concentrations of 30 and 1.5 g/l, respectively. The working standard was prepared by dissolving the six acids of interest in water to give individual concentrations of 0.5 g/l, except for tartaric acid the concentration of which was 2 g/l. The pH of this solution was adjusted to 7 by adding potassium hydroxide. The same was done for wine samples.

Procedure

In a PTFE-lined screw-capped glass tube, 0.2 ml of a neutralized wine sample or of the working standard were mixed with 0.8 ml of the phosphate buffer solution and 3 ml of the esterifiction agent. The mixture was heated at 100°C for 40 min, and on cooling a 6-μl aliquot was used for chromatography.

Instrumentation

A Varian Model 5000 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with a Rheodyne Model 7125 injector and with a Model 2050 UV detector (Varian) was used. A 25 cm × 4.6 mm column filled with 5-μm C_{18} reversed-phase packing and a guard column containing Pelliguard, both from Supelco (Bellefonte, PA, U.S.A.), were used. The chromatographic elution of derivatized acids was performed in a way similar to that reported elsewhere[11]. Briefly, solvent A was water, solvent B methanol. The flow-rate was 1.5 ml/min. Linear gradient elution was carried out by increasing the methanol percentage from 30 to 90% in 17 min. Phenacyl esters were detected at 254 nm.

RESULTS AND DISCUSSION

Optimization studies of the esterification of the acids considered were performed. In Table I the derivatization yield for selected acids dissolved in the acetone–water mixture, both unbuffered and buffered with two different phosphate concentrations, is reported as a function of the reaction time. The conversion efficiency was assessed according to the criterion adopted by Mentasti *et al.*[11]. Initially, following a previously reported analytical procedure[11], the reaction was carried out in an unbuffered acetone–water mixture. As is seen, under these conditions, the extent of conversion of the acids to phenacyl esters was not satisfactory. Surprisingly, we observed that, after heating at 100°C for 40 min, the final apparent pH of the reaction mixture was even lower than 1.5. Such a low value is probably due to decomposition of phenacyl bromide by water resulting in the formation of hydrobromic acid, which blocks the esterification reaction by converting carboxylate anions into their corresponding molecular forms. Performing the esterification reaction at temperatures lower than 100°C avoided to a large extent decomposition of the esterification agent but for polycarboxylic acids the yield was poor.

Buffering the reaction mixture significantly improved the conversion efficiency for polycarboxylic acids. However, the concentration of the phosphate buffer in the reaction mixture is a critical parameter. Maximum esterification occurs when the phosphate buffer solution is 0.08 *M*. Under this condition, the percentage conversion of the other acids not reported in Table I ranged between 98 and 100%. By decreasing the buffer concentration, a slight, constant decrease in the yield of derivatization was observed. On the other hand, at phosphate concentrations higher than 0.08 *M*, there is a drastic loss of efficiency. This seems to be due to an excessive inorganic salt concentration in the reaction mixture rather than to the specific nature of the phosphate ions. Under the most favourable experimental conditions, a low degree of conversion of carboxylic acids into their phenacyl esters was obtained by adding 0.15 *M* sodium chloride to the reaction mixture.

TABLE I

PERCENTAGE YIELD OF SOME ACID DERIVATIVES FROM THE ESTERIFICATION REACTION AT VARIOUS REACTION TIMES AND BUFFER CONCENTRATIONS

Compound	*Buffer concn. (M)*	*Yield (%)*		
		20 min	*40 min*	*60 min*
Acetate	0	84	87	79
	0.08	100	100	97
	0.20	100	80	79
Tartrate	0	50	47	46
	0.08	36	98	95
	0.20	55	66	72
Citrate	0	29	34	36
	0.08	37	96	91
	0.20	39	50	56

TABLE II

MEAN RECOVERY OF SIX ACIDS FROM WINE

Analyte	*Concentration (g/l)*		
	Added	*Found ± S.D. (n = 10)*	*Recovery (%) and range*
Lactate	1.00	0.981 ± 0.027	98.1 (95.1–100.7)
Acetate	1.00	0.973 ± 0.020	97.3 (94.6–99.9)
Tartrate	2.00	1.90 ± 0.075	94.9 (91.2–98.9)
Malate	1.00	0.954 ± 0.028	95.4 (91.8–97.9)
Succinate	1.00	0.970 ± 0.022	97.0 (95.2–100)
Citrate	1.00	0.958 ± 0.042	95.8 (90.3–101)

The analytical recovery of the acids of interest was evaluated by adding known amounts of them to ten different wine samples and reassaying (Table II).

To evaluate the precision of the method, a wine specimen was analyzed eight times. The results are reported in Table III. A typical chromatogram obtained by this procedure is shown in Fig. 1.

The extent of background interference from impurities in the reagents and by-products of the reaction was evaluated by heating the reaction mixture in the absence of acids. The corresponding chromatographic profile showed a series of small peaks appearing in the vicinity of those for malic, succinic and methylmalonic acids. However, none of these interfered with the analysis. Moreover, one peak was eluted just after that for citric acid, which disappeared when the reaction was carried out in an unbuffered solvent. Probably, this peak is due to partial esterification of the phosphate ion, as purification by recrystallization of the salts used to prepare the buffer solution failed to eliminate the artifact. Under the chromatographic conditions selected, this artifact did not interfere with the analysis of citric acid. By decreasing the steepness of the elution gradient, a tendency of the artifact peak to overlap that for citric acid was observed.

To assess whether unknown endogenous compounds in wine might affect the measurement of the carboxylic acids of interest, we compared the results obtained by chromatographing the reaction mixture on both reversed and normal phases. The

TABLE III

WITHIN-RUN PRECISION FOR SIX ACIDS IN A WINE SAMPLE

Analyte	*Mean ± S.D. (n=8) (g/l)*	*C.V. (%)*
Lactate	2.60 ± 0.065	2.5
Acetate	0.46 ± 0.008	1.7
Tartrate	1.22 ± 0.023	1.9
Malate	0.22 ± 0.440	2.0
Succinate	0.44 ± 0.008	1.8
Citrate	0.58 ± 0.018	3.1

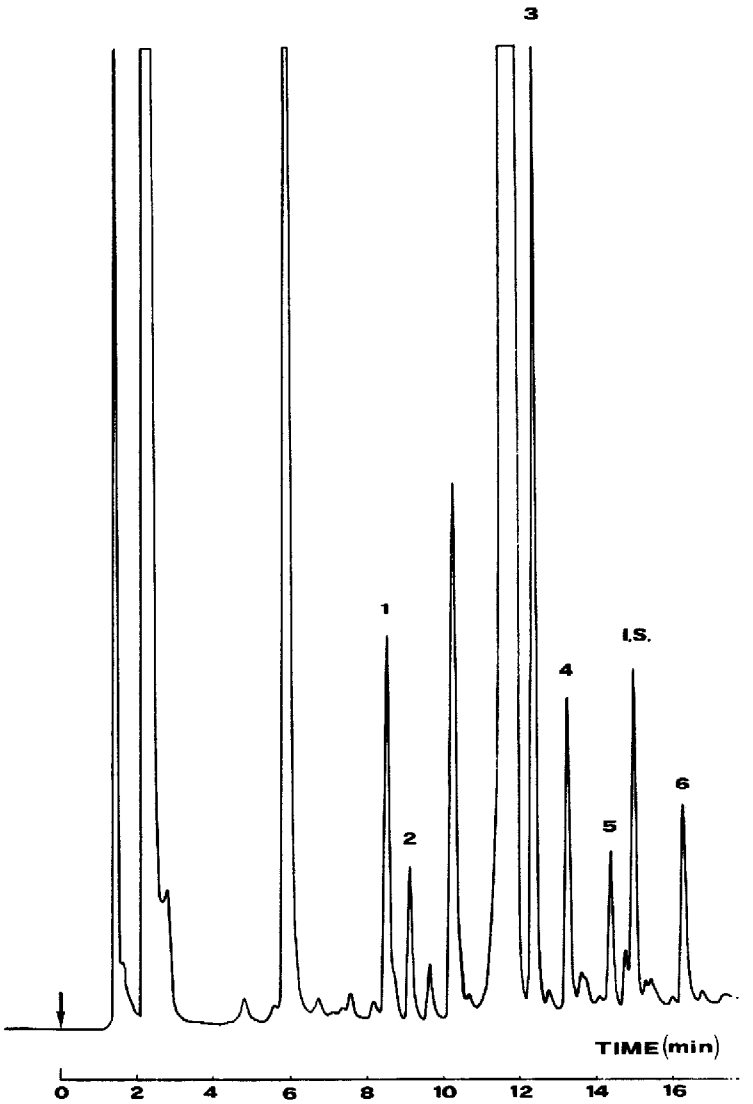

Fig. 1. Chromatogram of a commercial sample of Italian wine (Asti Spumante). Peaks: 1 = lactate (1.54 g/l); 2 = acetate (0.4 g/l); 3 = tartrate (2.83 g/l); 4 = malate (0.74 g/l); 5 = succinate (0.30 g/l); 6 = citrate (0.50 g/l). I.S. = Internal standard.

latter chromatographic mode was performed by using a silica column with hexane–isopropanol as mobile phase. This chromatographic system was unable to separate the acetic acid derivative from the solvent front. Therefore, data obtained for acetic acid by the reversed-phase technique were compared with those obtained by a gas chromatographic technique which determines acetic acid by direct injection of an aqueous sample[12]. The results are shown in Table IV. Linear regression analysis shows that the values obtained by the three methods are in good agreement, and all correlation coefficients were approximately unity.

TABLE IV

RESULTS OBTAINED FOR WINE SAMPLES ($n = 13$) BY NORMAL (x) AND REVERSED-PHASE (y) CHROMATOGRAPHY

Analyte	*Concentration (g/l)*			*Slope*	*r*
	$\bar{y}$	$\bar{x}$	*Intercept*		
Lactate	1.96	2.10	0.106	0.806	0.989
Acetate	0.320	0.338*	−0.022	1.012	0.978
Tartrate	1.87	1.91	0.250	0.851	0.970
Malate	0.429	0.452	0.013	0.920	0.989
Succinate	0.456	0.481	0.021	0.904	0.965
Citrate	0.747	0.810	−0.011	0.909	0.983

* Obtained by gas chromatography.

TABLE V

COMPARISON OF RESULTS OBTAINED BY OUR METHOD (y) AND BY ANOTHER HPLC ASSAY[11] (x)

Analyte	*Concentration (g/l)*			*Slope*	*r*
	$\bar{y}$	$\bar{x}$	*Intercept*		
Lactate	1.92	1.91	−0.092	1.05	0.980
Acetate	0.340	0.310	0.038	1.03	0.910
Tartrate	1.89	1.50	−0.068	1.30	0.863
Malate	0.551	0.492	0.005	1.22	0.979
Succinate	0.469	0.410	−0.018	1.22	0.813
Citrate	0.750	0.331	0.280	1.41	0.600

Results obtained by our method were compared with those obtained by the previously reported method[11] which involves esterification of carboxylic acids in an unbuffered solvent. Linear regression analysis of our data, y, and those obtained by the previous method, x, for 20 wine specimens gave the results shown in Table V. For lactate, acetate and malate, the two methods were well correlated and the relative mean values obtained were in fair agreement. For the other acids, the two methods did not compare well, especially for citrate, for which the mean values were very different. This discrepancy is probably due to the fact that, as shown above, the derivatization of citrate to its phenacyl ester is particularly affected by the reaction conditions. It has to be stressed that the determination of citrate in wines is of importance because, in some cases, it is added intentionally and is therefore subject to regulation in many countries.

REFERENCES

1 M. Gee, *Anal. Chem.*, 37 (1965) 926.
2 H. H. Luke, T. E. Freeman and L. B. Kier, *Anal. Chem.*, 35 (1963) 1916.
3 N. W. Alcock, *Anal. Biochem.*, 11 (1965) 335.
4 G. G. McKeown and S. I. Read, *Anal. Chem.*, 37 (1965) 1780.

5 U. J. Kaiser, *Chromatographia,* 6 (1973) 387.
6 K. Tanaka, T. Ishizuka and H. Sunahara, *J. Chromatogr.*, 174 (1979) 153.
7 Cs. Horvath, W. Melander and I. Molnar, *Anal. Chem.*, 49 (1977) 142.
8 H. D. Durst, *Tetrahedron Lett.*, (1974) 2421.
9 S. C. Chan, *Clin. Chem.*, 26 (1980) 1528.
10 J. P. Moody and S. M. Allan, *Clin. Chim. Acta*, 127 (1983) 263.
11 E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Baiocchi and M. Savigliano, *J. Chromatogr.*, 323 (1985) 177.
12 A. Di Corcia, R. Samperi and C. Severini, *J. Chromatogr.*, 198 (1980) 347.