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DERIVATIZATION, IDENTIFICATION AND SEPARATION OF CARBOXYLIC ACIDS IN WINES AND BEVERAGES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the derivatization, identification and separation of carboxylic acids in beverages such as wines and other commercial drinks or natural fruit juices has been developed. The accuracy and precision of the method are discussed with reference to specific methods for the determination of single acids. Applications to the analysis of different wines and beverages are demonstrated.

INTRODUCTION

Several papers have been published on the separation procedures applicable to high-performance liquid chromatography (HPLC) for the analysis of carboxylic acids[1]. Four main methods have commonly been used, namely ion-exchange and ion-exclusion separation, solvophobic chromatography, ion-pair chromatography and reversed-phase chromatography of derivatized products.

Ion-exchange and ion-exclusion separations are generally performed on silica-based ion exchangers and on exchangers with a styrene–divinylbenzene copolymer structure[2,3]. In solvophobic chromatography, the addition of acids or acidic buffers to the mobile phase lowers the pH and suppresses the dissociation of the carboxylic groups of the solutes. Under these conditions, hydrophobic interaction of the organic structure of the solutes with reversed-phase stationary phases is induced and in this instance a solvophobic chromatographic separation may be obtained[4,5].

Ion-pair chromatography has also been applied to the separation of acids, using silica gel and cellulose coated with a reagent capable of forming ion pairs and a non-polar mobile phase[6]. Non-polar counter ions have also been used in reversed-phase ion-pair chromatography[7].

Derivatizing agents have been used in liquid chromatography for the reversed-phase separation of organic acids. The products mainly investigated are differently substituted phenacyl[8], naphthacyl[9], *p*-nitrophenyl[10] and *p*-nitrobenzyl esters[11].

The nature and concentration of carboxylic acids in wines are of interest in several aspects of wine chemistry[12,13]. These compounds have an important influence

on the organoleptic properties and they also act as substrates or products in various enzymatic transformations.

Tartaric, malic and citric acids are present as major acid components in wines, musts and grapes. Tartaric acid (TA) is the major acid (1–4 g/l) which regulates wine acidity, and is the only acidic compound that is allowed as an additive for acidity adjustment by the EEC regulations, the maximum level being 1.5 g/l[14]. TA undergoes degradation by lactic bacteria (such as *Lactobacillus brevis*) to lactic acid and acetic acid, with a concomitant increase in volatile acidity.

Malic acid (MA) also undergoes a series of degradations, the main one being the malolactic fermentation[12,13]. This takes place in the first few months after wine making, with a concomitant reduction in MA concentration and an increase in lactic acid (LA).

Citric acid (CA) is present in wines within the limits 0.1 g/l for red wines to 0.6 g/l for white wines. EEC legislation[14] allows the addition of CA to wines, in order to avoid iron salt precipitation, up to a total limit of 1.0 g/l. Hence the evaluation of CA content in wines is also of great interest as regards biological stability and the observation of regulatory limits.

Acetic acid (AA) is formed during alcoholic fermentation by disproportionation of acetaldehyde, which is derived from carbohydrate fermentation. Volatile acids, mainly AA, are also formed during MA and CA degradation by bacteria. The AA level does not increase beyond 0.5–0.7 g/l. Above this limit, the action of pathogenic bacteria on glycerine carbohydrates or the action of acetic bacteria able to oxidize ethyl alcohol have to be considered.

Lactic acid (LA) is the final product of malolactic fermentation, which takes place during the first few months after wine making. Its formation may be repressed by treatment with metabisulphite or sulphur dioxide. Its determination may be of interest in the evaluation of wine origins.

Several other acids are minor components present in the range 0–100 mg/l, such as dicarboxylic, hydroxy, keto and phenolic acids[12,13].

Some acids are also added to wines as preservatives or in order to control acidity and stability. Ascorbic acid (limit allowed by Italian regulations, 150 mg/l)[12,13] is an antioxidant and sorbic acid (limit 200 mg/l)[12–14] has antiseptic action. Other compounds have been found in wine but are not allowed by EEC regulations; *e.g.*, benzoic and salicylic acids have been used as anti-fermentation additives.

We present here an HPLC procedure for the identification, separation and determination of acids in wines and beverages. Derivatization with phenacyl bromide[15–17] has been optimized for application to acids in wines and separation has been accomplished on standard octadecylsilica columns. The method is compared with other HPLC methods for the determination of carboxylic acids in wines.

EXPERIMENTAL

Chemicals

Standard solutions of the investigated acids were prepared from analytical-reagent grade chemicals (Fluka, Merck). Phenacyl bromide and 18-crown-6 for the derivatization reactions were supplied by Fluka. Phenacyl bromide was recrystallized from *n*-heptane (5 g dissolved in 150 ml at 50°C and allowed to cool to 0°C).

Apparatus

Analyses were carried out using a Varian LC 5060 chromatograph equipped with a UV-100 spectrophotometric detector and a Vista 401 Data System. Chromatographic-grade methanol (Merck) and doubly distilled and filtered (0.45 μm) water were used as the mobile phase.

The chromatograms were performed on different commercial HPLC columns, namely Merck Hibar RT 250-4 RP-18 (7 μm), Hibar RT 250-4 RP-8 (7 μm) and Waters Assoc. μBondapak RP-18 (10 μm) (300 × 3.9 mm I.D.). A Waters Assoc. guard column was used throughout, with Waters Assoc. Bondapak-C_{18}/Corasil (37–50 μm) as packing material.

Derivatization

The derivatization of acids by phenacyl bromide:

$$RCOOH + BrCH_2COC_6H_5 \rightarrow RCOOCH_2COC_6H_5 + HBr$$
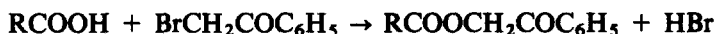
takes place with satisfactory yields only in the presence of a catalyst. Among those previously suggested, we have tested triethylamine[16], fluoride ions[17] and 18-crown-6[15]. Both triethylamine and fluoride allow activation of the RCOO$^-$ anion by hydrogen bonding, but preliminary measurements showed that triethylamine gives very low yields with acids such as CA, TA and MA. In contrast, fluoride is an effective catalyst for the derivatization but its strong interaction with dissociable protons makes it poorly selective; in fact, phenolic compounds, present in wines, undergo derivatization.

18-Crown-6 proved to be very effective on promoting the derivatization of carboxylic acids previously converted into potassium salts. Complexation of K$^+$ by the ether allows dissolution of the carboxylate salts in a suitable solvent. Preliminary neutralization to pH 7–8 with KOH or KHCO$_3$ ensures the absence of interference from phenols and esters (under these conditions neither neutralization nor hydrolysis occurs). The solvent that gave the best derivatization yields was water–acetone (1:3). At higher water contents the derivatization was less effective, and at higher acetone contents the reaction became very slow. A heating period of 75 min at 100°C was satisfactory for all the acids investigated.

The recovery for the esterification process was checked by comparing the absorption of the derivatizing agent and of the single derivatized acids at 254 nm (at this wavelength the equivalent molar absorptivities of phenacyl bromide and the esters are identical). For AA and LA the ester formation yield was ≥97% and for dicarboxylic (MA and TA) and tricarboxylic acids (CA) it was *ca.* 85% and *ca.* 76%, respectively.

After these preliminary studies, the following derivatization procedure was adopted throughout.

Solution A: prepare a 0.170 *M* solution of recrystallized phenacyl bromide in acetone (equivalent to 10 g/l of AA).

Solution B: prepare a 0.0170 *M* solution of 18-crown-6 in acetone.

To a 10-ml Pyrex test-tube with a screw-cap add 1.00 ml of wine previously adjusted to pH 7–8 with KHCO$_3$, 1.00 ml of solution A, 1.00 ml of solution B and 1.00 ml of acetone. Place the test tube in a boiling water-bath for 75 min, then cool.

The cooled solution is ready for the chromatographic analysis.

No other pre-treatment or clean-up was necessary. The solutions, after derivatization, were stable for several weeks.

Chromatographic conditions

The chromatographic conditions generally adopted were as follows: mobile phase, solvent A water, solvent B methanol; flow-rate, 2.00 ml/min; detection, UV absorption, 254 nm; volume injected, 10 μl; elution programme, starting composition 65% A, 35% B, linear gradient increase of B concentration at 2.5%/min; and column, Merck Hibar RT 250-4 RP-18 (7 μm).

RESULTS AND DISCUSSION

Chromatographic behaviour

A mixture of the six major acids present in wines served as a standard solution for optimizing the chromatographic conditions. Using water–methanol as the eluent, the single acids showed a noticeable increase in retention times with increasing number of carboxylic groups. Hence a gradient elution was found to be convenient (see Fig. 1). Each acid was identified by comparison with standard solutions of single pure compounds. Retention times are reported in Table I.

Fig. 2 shows the chromatogram of a sample of Dolcetto di Diano d'Alba, D.O.C. wine, 1983 (D.O.C. = controlled origin denomination), derivatized according to the above procedure.

A Van Deemter plot obtained for the elution of acetic acid (one of the more easily eluted acids) showed that the highest theoretical plate number was achieved at
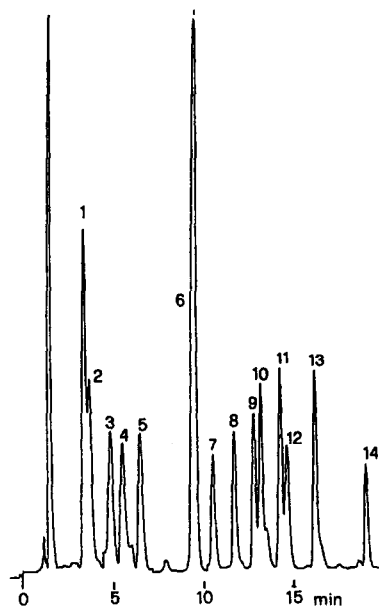

Fig. 1. HPLC separation of standard carboxylic acids (0.500 g/l) according to the described procedure. For identification of acids, see Table I.

TABLE I

RETENTION TIMES OF THE INVESTIGATED ACIDS DERIVATIZED ACCORDING TO THE DESCRIBED PROCEDURE

Dead time (t_0) = 1.10 min.

| No.	Acid	t_R (min)	No.	Acid	t_R (min)
	Galacturonic acid	2.5	9	Citramalic	13.6
1	Acetone	3.7	10	Succinic	13.7
2	Glycolic	3.9		Phenylacetic	14.2
3	Glyoxylic	5.2		Cinnamic	14.6
	Pyruvic	5.9		Benzoic	14.7
4	Lactic	6.0	11	Glutaric	14.8
5	Acetic	6.9	12	Valeric	15.0
	Propanoic	9.6		Sorbic	15.2
6	Phenacyl bromide	10.0		Fumaric	15.4
	Mandelic	10.5		Anisic	15.6
7	Tartaric	10.9		Gallic	16.3
	Ascorbic	11.1		Isocitric	16.7
	Salicylic	11.7	13	Citric	16.9
	p-Hydroxybenzoic	11.7		Benzilic	17.1
	Vanillic	11.9		Protocatechuic	17.9
	Butyric	12.2	14	Enanthic	19.7
8	Malic	12.4		Caprylic	21.1
	α-Chetoglutaric	13.1			
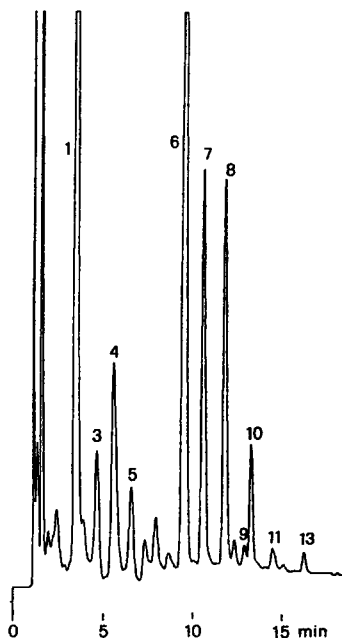
Fig. 2. HPLC separation of carboxylic acids in Dolcetto di Diano d'Alba wine (1983) according to the described procedure. For identification of acids, see Table I. The main acid composition is reported in Table VI.
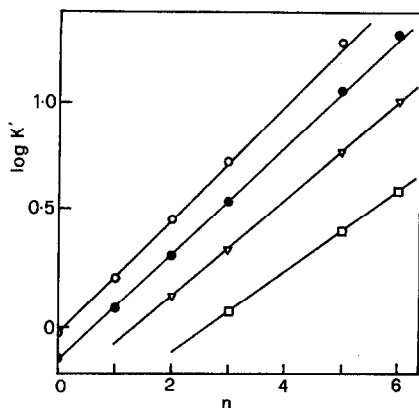
Fig. 3. Variation of log k' with n for the homologous series $CH_3(CH_2)_nCOOH$ eluted under different isocratic conditions. Methanol–water (%, v/v): O, 60:40; ●, 65:35; ▽, 70:30; □, 75:25. Merck Hibar RT 250-4 RP-18 (7 μm) column.

a flow-rate of 0.7 ml/min. The very small increase in theoretical plate height with increasing flow-rate (small value of the C term in the Van Deemter equation) and the need to reduce elution times for acids such as CA and SA suggested the higher flow-rate chosen. Under such conditions all 30 acids investigated showed retention times between 2.5 and 22 min.

In order to characterize the chromatographic behaviour of the investigated solutes, the variation of capacity factors with mobile phase composition and with chemical structure within homologous series was investigated. As can be seen from Fig. 3, plots of log k' against molecular weight were linear within the series of car-

TABLE II

VARIATIONS IN CAPACITY FACTORS IN THE INVESTIGATED HOMOLOGOUS SERIES UNDER DIFFERENT ISOCRATIC CONDITIONS FOR ELUTION ON THE MERCK HIBAR 250-4 RP-18 (7 μm) COLUMN

Series	Structural unit	$CH_3OH:H_2O$ (%, v/v)	Δ (log k')
$CH_3(CH_2)_nCOOH$ (n = 0–6)	CH_2	60:40	0.25
		65:35	0.23
		70:30	0.21
		75:25	0.19
$HOOC(CH_2)_nCOOH$ (n = 2–4)	CH_2	50:50	0.18
		55:45	0.15
		60:40	0.13
		65:35	0.11
HOOCCHRCHR'COOH	OH	45:55	−0.21
		50:50	−0.19
		55:45	−0.18
		60:40	−0.16
		65:35	−0.15

TABLE III

ANALYTICAL RESULTS FOR THE DETERMINATION OF ACETIC, TARTARIC AND SUC-
CINIC ACIDS IN A SAMPLE OF DOLCETTO WINE BEFORE AND AFTER THE ADDITION OF
"SPIKES"

Each value represents the mean of seven independent determinations.

Acid	Amount originally present (g/l)	Amount added (g/l)	Total content in "spiked" wine (g/l)	"Spike" found (g/l)	Error (%)
Acetic	0.557	1.000	1.517	0.960	−4.0
Tartaric	2.114	2.000	4.246	2.132	+6.6
Succinic	0.483	1.000	1.538	1.055	+5.5

boxylic acids $CH_3(CH_2)_nCOOH$ for n ranging between 0 and 6 at each mobile phase composition. The same linear behaviour was found for the series $HOOC(CH_2)_nCOOH$ with $n = 2$–4 and the series $HOOCCHRCHR'COOH$ with $R = R' = OH$, $R = OH$ and $R' = H$ and $R = R' = H$.

For each series, the slope of the above plots represents $\Delta\log k'$ for a unit increase in the basic structural moiety for each component in the homologous series. Table II reports the values of such slopes for different isocratic conditions and for each of the three homologous series. The CH_2 unit has a strong effect within the aliphatic monocarboxylic acid series, whereas in the dicarboxylic acid series it has a much smaller effect [compare, for example, $\Delta\log k' = 0.25$ with 0.13 using water–methanol (40:60)]. Hence the effect of the basic moiety also depends on the type of overall structure of the homologous series concerned.

The presence of two carboxylic groups in the second latter instance strongly reduces interactions of the CH_2 moiety with the column. In contrast, the effect of an OH group leads to a decrease in retention (negative slopes in Table II). It is worth mentioning that the slope of such plots represents the logarithm of the separation efficiency for an adjacent pair in a homologous series, so the reported slopes values

TABLE IV

DETERMINATION OF MAJOR CARBOXYLIC ACIDS IN DOLCETTO DI DIANO D'ALBA
WINES BY THE CALIBRATION GRAPH METHOD (CGM) AND THE STANDARD ADDITIONS
METHOD (SAM)

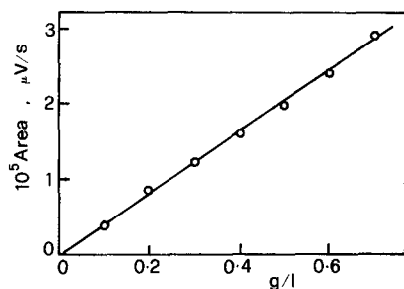| Acid	Dolcetto wine (1982)		Dolcetto wine (1983)	
	CGM (g/l)	SAM (g/l)	CGM (g/l)	SAM (g/l)
Lactic	1.00	1.00	0.60	0.55
Acetic	0.56	0.56	0.29	0.28
Tartaric	2.11	1.95	1.53	1.50
Malic	0.50	0.60	1.55	1.40
Succinic	0.45	0.48	0.42	0.35
Citric	0.24	0.21	0.16	0.17

Fig. 4. Calibration graph for the determination of acetic acid derivative eluted according to the procedure described.

can be considered as an index of separation efficiency for a combination of solutes, mobile phase and column.

The separation efficiency for the same series of carboxylic acid derivatives was also investigated with two other columns, *viz.*, a Merck Hibar RT 250-4 RP-8 (7 μm) column (the only difference from the standard column is the reversed-phase chain length) and a Waters Assoc. μBondapak RP-18 (10 μm) column. A small decrease in separation efficiency, as tested from the slope for the corresponding log k' *vs.* ΔMW plots, was found for each of the two columns. Also, the theoretical plate height was least for the Hibar RT 250-4 RP-18 (7 μm) column (6400 TP/m).

Quantitative determination

In order to determine each acid component, preliminary measurements were made by adding known amounts of AA, TA and SA to a sample of Dolcetto wine. The good agreement between the results for the amounts added and the values found (see Table III) indicates a very low "matrix effect" of wine on the measurements performed.

Another interesting comparison was made between, on the one hand, the analytical results obtained from direct peak-area evaluation and a standard calibration

TABLE V

COMPARISON OF RESULTS FOR THE DETERMINATION OF ACETIC AND TARTARIC ACIDS IN DIFFERENT WINES OBTAINED BY THE PRESENT CHROMATOGRAPHIC METHOD WITH THOSE OBTAINED BY VOLATILE ACIDITY AND THE BLOUIN-REBELEIN METHODS

Wine	Acetic acid (g/l)		Tartaric acid (g/l)	
	Chromatographic method	Volatile acidity method	Chromatographic method	Blouin–Rebelein method
Dolcetto 1982	0.56	0.54	1.90	2.10
Dolcetto 1983	0.29	0.32	1.53	1.73
Nebbiolo 1982	0.47	0.59	1.40	2.20
Nebbiolo 1983	0.28	0.34	1.18	1.40
Barbera 1982	0.51	0.56	2.16	2.16
Barbera 1983	0.26	0.35	1.50	1.42

graph and, on the other, the quantitative results obtained with the internal standard additions method. Also in this instance (see Table IV), the good agreement between the two series of measurements indicates the absence of noticeable matrix effects. Hence the simpler method based on the use of standard calibration lines (see Fig. 4 as an example) was adopted. Under these conditions a relative mean accuracy of ±8% for each of the six major acids was found. Typical values (± S.D.; seven independent derivatizations) were as follows: TA 2.12 ± 0.11 g/l, AA 0.557 ± 0.025 g/l and SA 0.483 ± 0.021 g/l, with a relative reproducibility of *ca.* 5%.

Interferences from esters

Some acids, in particular acetic and succinic acids, are present in wines as monoethyl esters. In order to check the absence of interferences from such components, synthetic standard solutions containing 100 mg/l of AA and 50 mg/l of ethyl acetate were derivatized according to the present procedure. The evaluation of AA
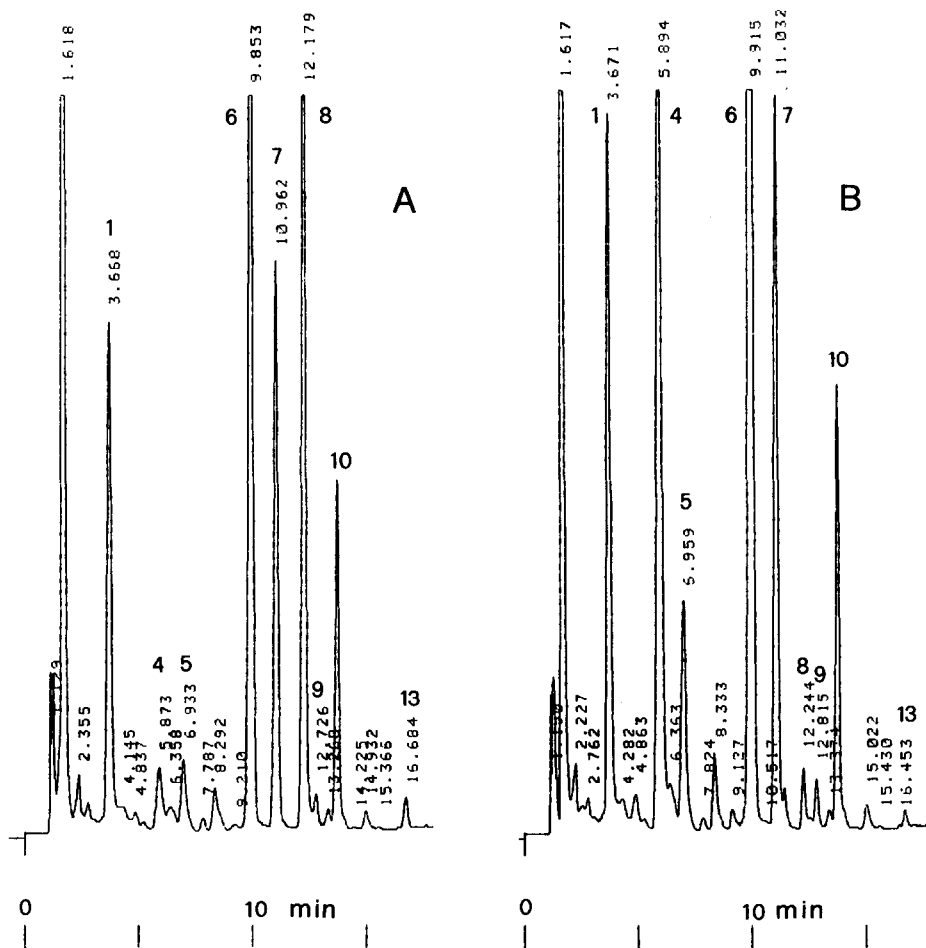

Fig. 5. Chromatogram of a sample of Grignolino del Monferrato wine (1983). The determination of the single acids is reported in Table VI. For identification of acids, see Table I.

did not show any significant difference from the results obtained with a corresponding solution of AA that did not contain the ester.

Comparison with other official determination methods

Some single acids present in wines can be analysed by standard methods[13,14,18], such as an enzymatic method (enzymatic assay coupled to NAD/NADH[+] indicator reaction) for MA; acetic acid is often evaluated by acid–base titration of the acid fraction obtained by steam distillation of wine; tartaric acid can be determined by the Blouin–Rebelein method based on the reaction of TA with ammonium metavanadate[18].

Table V compares the results obtained for AA. Slightly higher values obtained by the volatile acidity evaluation seem to derive from the presence of volatile acidic components such as formic, propionic and butyric acids, which, being steam distilled,
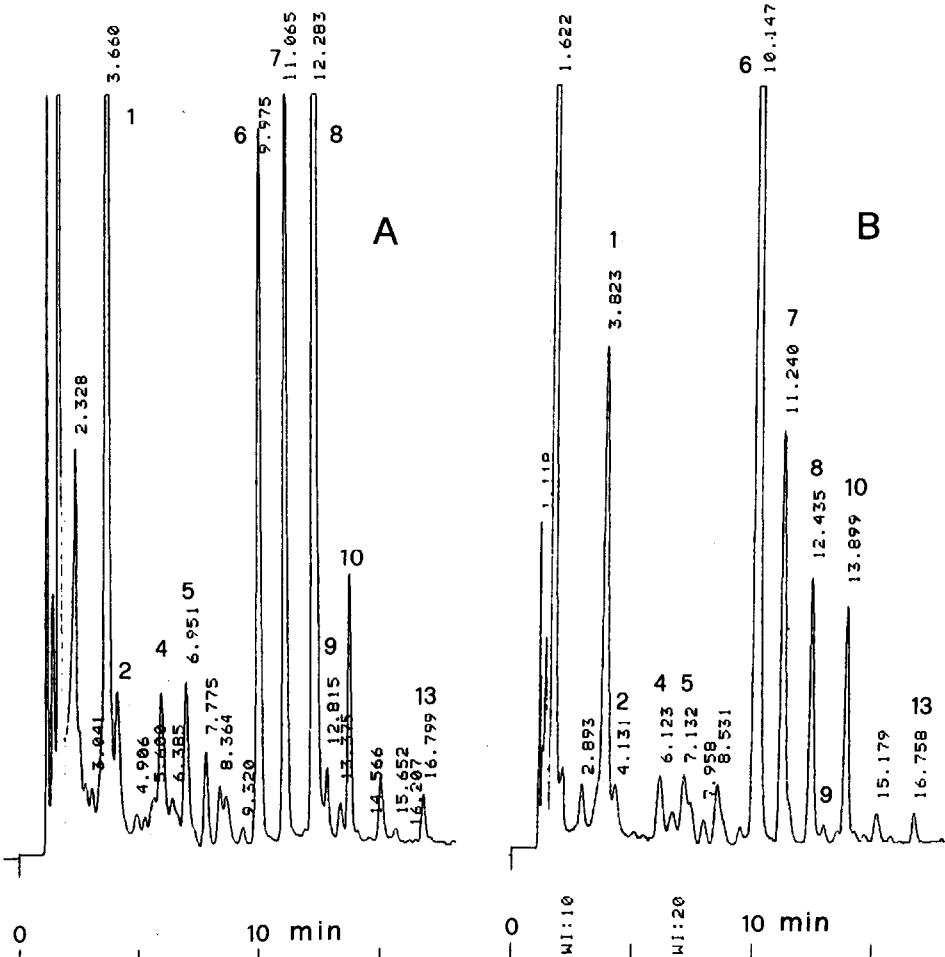

Fig. 6. Chromatograms of (A) Barbera d'Alba wine (1983) and (B) Alicia di Salemi wine (1983). The determination of the single acids is reported in Table VI. For identification of acids, see Table I.

are titrated and evaluated as AA. The chromatographic and enzymatic methods also gave similar results (within 2%). Only for the Blouin–Rebelein method were systematically higher values obtained by the chromatographic method. This discrepancy may be clearly assigned to a specific redox interaction of vanadate ion[19] with the vicinal diol moiety present not only in TA but also in several other wine components such as, MA, glycerine and carbohydrates.

A series of chromatograms of wines may now be considered in order to illustrate the possibilities of the proposed method. Fig. 5A and B show the chromatogram of samples of Grignolino del Monferrato wine (1983). Sample A was kept from the alcoholic fermentation up to the spring of 1984 at 5°C, whereas sample B was kept at a constant temperature of 24°C (optimum conditions for the enzymatic reactions of the malolactic transformation)[13]; the evaluation of the relative amounts of MA and LA allows the effect of conservation conditions to be evaluated.

Fig. 6A shows a chromatogram of Barbera wine (1983); the high concentration of malic acid is characteristic of this quality of wine in its first year. The high concentration of MA is responsible for the sour taste of this wine.

The quantitation of acids in wines can also provide information on the characteristics of typical wines. The lower acid content of wines obtained from grapes grown in very hot regions (e.g., Southern Italy) may be observed from the chromatogram in Fig. 6B where the results for an Alicia di Salemi wine are shown.

The effect of ageing of wine may also be monitored by the present method. An example is given in Table VI where Nebbiolo wines, obtained from the grapes of the same crù in different years from 1978 to 1983, were derivatized and chromatographed in April 1984. The different rate of acid decrease clearly shows the effect of wine ageing (note the sudden decrease in MA, with a concomitant increase in LA, the slower decrease of TA, etc.).

TABLE VI

DETERMINATION OF MAJOR CARBOXYLIC ACIDS IN WINES AND BEVERAGES BY THE PROPOSED CHROMATOGRAPHIC METHOD

| Beverage	Acid (g/l)					
	Lactic	Acetic	Tartaric	Malic	Succinic	Citric
Dolcetto di Diano d'Alba wine (1983)	0.60	0.29	1.53	1.55	0.42	0.16
Grignolino del Monferrato wine (1983):						
(A) kept at 5°C	0.25	0.23	2.20	2.73	0.75	0.18
(B) kept at 24°C	1.62	0.51	1.87	0.38	0.65	0.11
Barbera d'Alba wine (1983)	0.39	0.26	1.50	3.73	0.40	0.24
Alicia di Salemi wine (1983)	0.24	0.20	1.82	1.10	0.76	0.24
Nebbiolo d'Alba wine:						
1983	0.36	0.28	1.18	2.40	0.38	0.14
1982	1.10	0.47	1.40	0.67	0.46	0.17
1981	1.83	0.62	1.04	0.20	0.44	Traces
1979	1.72	0.54	1.04	—	0.41	—
1978	1.72	0.44	1.02	—	0.41	—
Natural orange juice				1.10		3.80
San Pellegrino orange juice				0.20		2.30
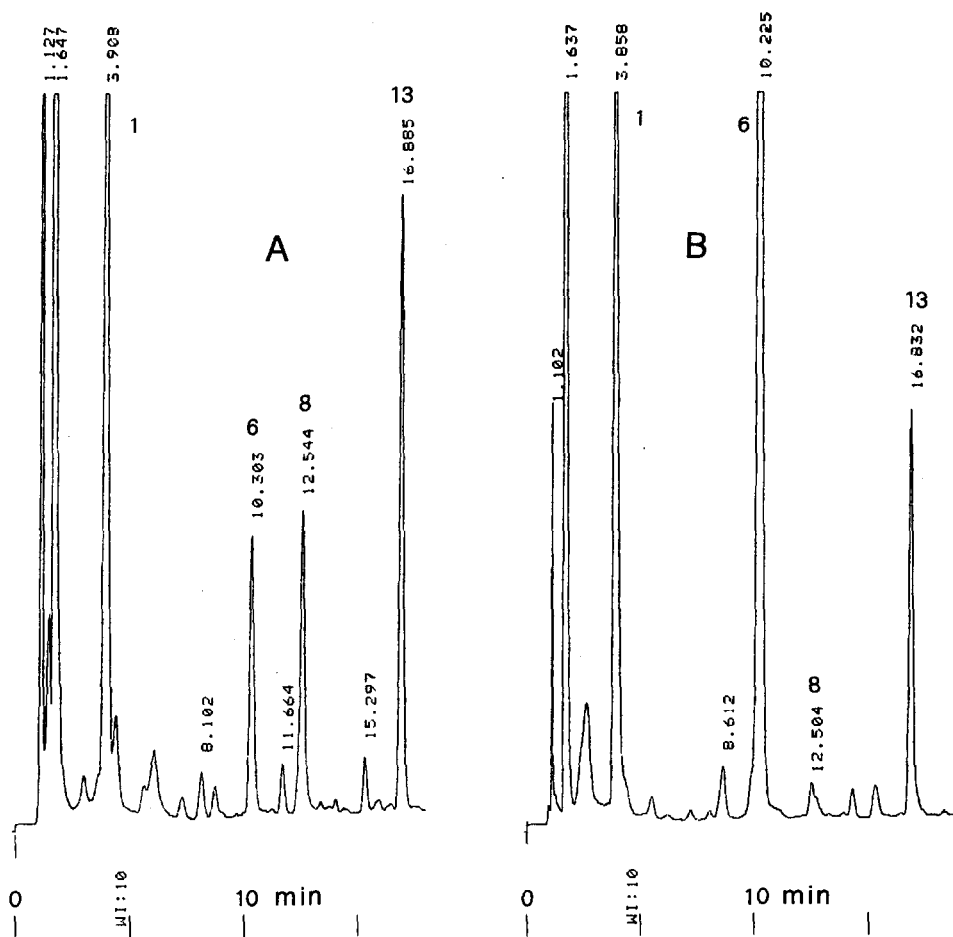
Fig. 7. Chromatograms of (A) natural orange juice from pressed fruits and (B) commercial Aranciata San Pellegrino orange juice, derivatized according to the described procedure. For identification of acids and quantitation, see Tables I and VI.

The proposed method was also tested for the identification and determination of acidic compounds in beverages such as beer, soft drinks and fruit juices. As examples, Fig. 7A and B show the chromatograms for the analysis of a commercial orange juice and of a juice from freshly pressed oranges.

In conclusion, the proposed method allows the identification, separation and determination of carboxylic acids as constituents of various beverages and natural products. The analysis, which is simple and rapid, is performed at 254 nm, avoiding interferences that occur at lower wavelengths[20]; the eluent is a simple water–methanol mixture without buffers, salts or acids, as in other cases[21], which make the column life very short. Also, the sensitivity, accuracy and precision may be comparable to those in other suggested methods[22–25].

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