Journal of Chromatography, 395 (1987) 323–333
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1097

ROUTINE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CARBOXYLIC ACIDS IN WINES AND CHAMPAGNE

D. TUSSEAU* and C. BENOIT
Comité Interprofessionnel du Vin de Champagne, 5 Rue Henri-Martin, 51204 Epernay Cédex (France)

SUMMARY

Carboxylic acids have an important influence on the biological stability and the organoleptic properties of wines. A simple, rapid and reproducible high-performance liquid chromatographic method for the determination of the main carboxylic acids (tartaric, malic, shikimic, lactic, acetic, citric, succinic, fumaric and propionic acids) in wines is described. There is no interference from either sugars or amino acids. The method does not need any preparation or extraction of the sample. The linearity, the level of detection, the repeatability and the reproducibility were studied for each acid. The results are compared with those obtained by chemical and enzymatic assays.

INTRODUCTION

Physical and chemical analyses of grape juices and wines have become one of the most important aspects of modern quality control. The composition and quality of the finished wine depend largely on the composition of the must (freshly crushed grapes), so it is important to have a thorough knowledge of its analytical parameters.

Wine is a very complex matrix of substances[1,2], and it is very important for the winemaker to know both the carboxylic acid and sugar contents of the grape, as they provide data for deciding on the best time to harvest the grapes and for controlling the fermentation and also provide for the overall quality of the winer's production[1,2].

The nature and concentration of organic acids in musts and in wines are of interest because they have an important influence on the organoleptic properties[1,2]. Tartaric acid (TA) is the specific acid of the grape and consequently of the wine. Its quantity depends on the grape variety, the region and the season. The must may contain 2–15 g/l of TA. Its concentration decreases both during the alcoholic fermentation and during cold stabilization, so the wine may contain 1–5 g/l of TA. TA may undergo degradation by lactic bacteria to lactic and acetic acids. This unwanted spoilage is called "tourne".

Malic acid (MA), the major acid of fruit, is a ubiquitous compound in the vegetable kingdom. From the grape to the aged wine, the amount of MA decreases to negligible levels because of a series of biological degradations, the main one being

0021-9673/87/$03.50

malolactic fermentation (MLF), which takes place after the alcoholic fermentation and results in an increase in lactic acid. Musts may contain 5–20 g/l and wines 0–10 g/l of MA.

Citric acid (CA) is present in musts (0.2–0.5 g/l) and in wines (0–0.5 g/l). In order to avoid precipitation of the iron(III) salt, the EEC allows the addition of CA up to a maximum concentration of 1 g/l. Hence the evaluation of CA in wines is also of great interest in maintaining biological stability and in remaining with legal limits.

Lactic acid (LA) occurs in fermented beverages, but generally not in musts. The two enantiomers may be found in wine. Alcoholic fermentation produces a small amount of D(−)-LA (180–400 mg/l). The major isomer is L(+)-LA (0–5 g/l). It is the final product of MLF (which is caused by a bacterium). MLF is often wanted by the winemaker, because it decreases the acidity of the wine while increasing its stability, but if it takes place after bottling the wine may become cloudy.

Acetic acid (AA) is another important acid that must be monitored. AA is formed in small amounts during the alcoholic fermentation, but if it is present in large amounts it may indicate that something is wrong. EEC rules define a maximum amount of AA in wines.

Alcoholic fermentation of carbohydrates always produces small amounts of succinic acid (SA). The amount produced depends on the conditions of fermentation and may vary from 0 to 1.5 g/l.

Shikimic acid (ShA) comes from the skin of the grape and is always present in musts and in wines.

Many other acids (such as fumaric, formic, pyruvic and uronic acids) are minor components present in musts and wines in the range 0–100 mg/l.

The main acids are usually determined by complex chemical analyses[1–3]. A few of them [MA, LA, D(−)- and L(+)-LA, AA and CA] can be individually quantified by enzymatic methods[1–3].

Several papers have been published on separation procedures applicable to high-performance liquid chromatography (HPLC) for the determination of carboxylic acids[4–6]. Ion exchange has been used for a long time[7], but has now been replaced by ion-exchange and ion-exclusion separation[8–19]. The latter gives good results, but neutral compounds (such as polyols) may co-elute with the acids. Sweet wines and musts contain appreciable amounts of sugar. As fructose co-elutes with malic acid, the acids must be separated before being analysed, thus increasing the analysis time and decreasing the reproducibility[15]. Another disadvantage of this technique is that the calibration graphs plotted against concentration are not always linear.

Reversed-phase chromatography is a simple and therefore very attractive process, and many papers on this technique have been published[20–26]. Some of the techniques described require derivatization of the acids. Phenacyl[27], naphthacyl[28], *p*-nitrophenyl[29] and *p*-nitrobenzyl ester[30–32] derivatives have been described. They have very high UV absorbances but they give excessive peaks caused by the formation of secondary products or the presence of impurities in the reagents[26].

Ion-pair chromatography[33–35], neutral polymeric columns[36], two-phase (ion-exchange, reversed-phase) columns[37] and temperature programming with cation-exchange columns[38] have also been investigated, but with little success.

The choice must be made on the basis of the aim of the analysis. In our work we wanted to obtain the best possible separation of the main carboxylic acids (mean

TA, MA, LA, Sha, AA, CA and SA) in musts and wines. Moreover, we wanted a method that is rapid and easy to use, as short as possible, involves a minimum of preparation and of course is usable for routine analysis.

The most convenient method of separation seemed to be reversed-phase chromatography. Although several methods for the determination of organic acid in wines have already been proposed, they generally do not produce good separations for all wines. For instance, chromatograms of red wines always show many unresolved peaks. An insufficient resolution always leads to imprecise quantification even when integration is used. We describe here an HPLC procedure for the quantification of carboxylic acids in musts, wines and champagne. It does not need any derivatization and is accurate enough to be used in routine analyses. We have calculated the repeatability and the reproducibility of the analyses. The repeatability and reproducibility are statistical analyses defined by the ISO 5725 standard. Roughly, repeatability (r) represents the intrinsic errors of the apparatus (valves, column, detector and integrator) and reproducibility (R) additionally takes the errors in manipulation into consideration.

The method has been compared with chemical and enzymatic methods of analysis.

The eluent was adjusted to pH 2.1 in order to have maximal protonation of the acids. This seems very low for bonded silica columns, but they remain in good condition for at least 4 weeks with full utilization, and several hundred analyses can be run before renewal is necessary.

EXPERIMENTAL

Apparatus

Analyses were carried out with a Spectra-Physics SP 8100 chromatograph equipped with a Spectra-Physics SP 8110 autosampler. The injector was a Valco valve with a 10-μl sampling loop. The detector was a Pye Unicam PU 4020 UV detector connected with a Spectra-Physics SP 4270 computing integrator, which in turn was connected to an IBM PC AT via a Labnet network.

The chromatographic separations were performed on two Merck LiChrospher 100 CH 8/II 5 μm (250 $\times$ 4 mm I.D. RP-8 spherical phase columns.

Chemicals

Water was obtained from a Millipore Milli-Q water purification system. Analytical-reagent grade chemicals from Merck were used exept where indicated otherwise.

Chromatographic conditions

The mobile phase was composed of 70 g/l (0.52 M) potassium dihydrogenphosphate and 14 g/l (0.10 M) ammonium sulphate ajusted to pH 2.1 with phosphoric acid. The flow-rate was 0.8 ml/min at room temperature. Detection was effected by measurement of UV absorption at 210 nm.

Sample preparation

The samples were filtered through a 0.45-μm Millipore filter and purified

through Sep-Pak C_{18} cartridges (Millipore/Waters). This did not alter the carboxylic acid composition of the samples.

RESULTS AND DISCUSSION

Chromatographic behaviour

Fig. 1 shows the separation of the nine major acids of interest. Each acid was identified by its retention time in comparison with standard solutions of pure compounds. Retention times of other authentic known acids are reported in Table I.

Plots of the integrated peak area against concentration of the acids were all linear (Fig. 2).

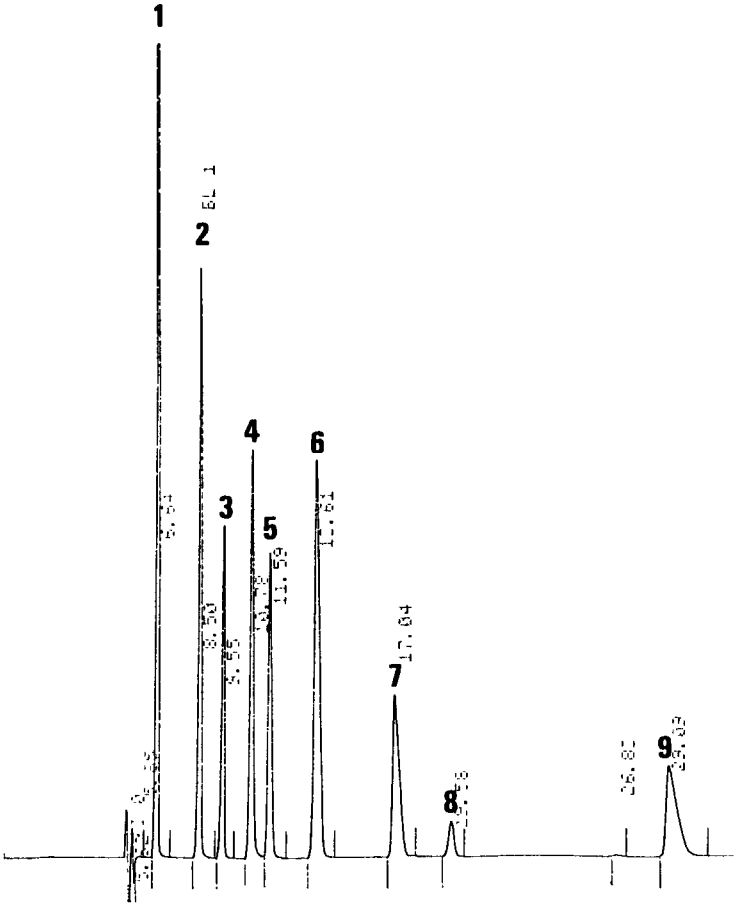

Fig. 1. Chromatogram of a standard mixture. Ten microlitres of 5 μg/μl of each acid [except shikimic acid (3), 0.05 μg/μl, and fumaric acid (8), which is an impurity of malic acid] were injected through two LiChrospher 100 CH 8/II 5 μm (250 × 4 mm I.D.) columns. Eluent, 70 g/l KH_2PO_4 and $(NH_4)SO_4$ adjusted to pH 2.1 with H_3PO_4. Flow-rate, 0.8 ml/min at room temperature. Detection, UV absorption at 210 nm. Numbers correspond to the compounds listed in Table I.

TABLE I

RETENTION TIMES OF DIFFERENT ACIDS

No.	*Acid*	t_R *(min)*	*No.*	*Acid*	t_R *(min)*
	Mucic	5.9		α-Ketoglutaric	9.7
	Glucuronic	5.9	4	Lactic	10.8
	Galacturonic	5.9		Sodium azide	11.2
	Saccharic	5.9	5	Acetic	11.6
	Glyceric	5.9	6	Citric	13.6
	Arginine and other amino acids	5.9		Gentisic	14.6
	α-Ketogluconic	6.05	7	Succinic	17.0
	Gluconic lactone, gluconic	6.05		Sorbic	17.4
	Glyoxylic–fructose	6.05		Citramalic	15
1	Tartaric	6.6	8	Fumaric	19.6
	Glycolic	6.85		*trans*-Aconitic	25.5
	Quinic	7.1	9	Propionic	29.0
2	Malic	8.5		Tannic	29.0
	Oxaloacetic	8.6		Gallic	34.3
	Pyruvic	8.6		Mesoxalic	38.33
	Malonic	8.6		Phenylalanine	41.15
	Ascorbic	8.6		Protocatechic	54.6
3	Shikimic	9.5		Mandelic	55.2

Quantitative determination

We used an external standard prepared using tartaric acid, 5.0 g/l (Sigma), malic acid, 5.0 g/l (Sigma), shikimic acid, 0.050 g/l (Sigma), lactic acid, 5.0 g/l (Fluka) (as sodium lactate), acetic acid, 5.0 g/l (Fluka, as sodium acetate), citric acid, 5.0 g/l (Merck), succinic acid, 5.0 g/l (Fluka) and propionic acid, 5.0 g/l (Prolabo) (as sodium propionate. All these chemicals were the highest quality available from the producers (malic acid always contains trace amounts of fumaric acid).

Figs. 3–6 show chromatograms of a must (Fig. 3), a wine after alcoholic fermentation (Fig. 4), a wine after malolactic fermentation (champagne) (Fig. 5) and a red wine (Fig. 6). The calibrations and the quantitative analyses are means of nine replicates. Grubb's test did not indicate any aberrant individual difference (5% level). Bartlett's test show that the variances were homogeneous (5% level), so we could calculate the repeatability (r) and the reproducibility (R) (Table II).

In none of the wines were we able to quantify the citric acid, nor was it possible to measure acetic acid in the white wine after alcoholic fermentation.

In contrast to methods using reversed-phase chromatography[20–25], our method allows a separation of amino acids, fructose and uronic acids from tartaric acid. It is faster than that using three C_{18} packed columns[26].

The repeatability for tartaric acid varies from 24 to 66 mg/l (for concentrations in the range 1.3–6.9 g/l) when the reproducibility varies from 40 to 283 mg/l. These results are better than those of the collaborative analysis of the Office International de la Vigne et du Vin[39]. Collaborative analyses are inter- and intra-laboratory studies of value of methods of wine analysis[40–45].

For malic acid, r varies from 32 to 48 mg/l (for concentrations in the range 0.2–6.4 g/l) when R varies from 58 to 95 mg/l. Hence r and R are lower than those of the collaborative analysis for the higher concentrations (must and white wine after

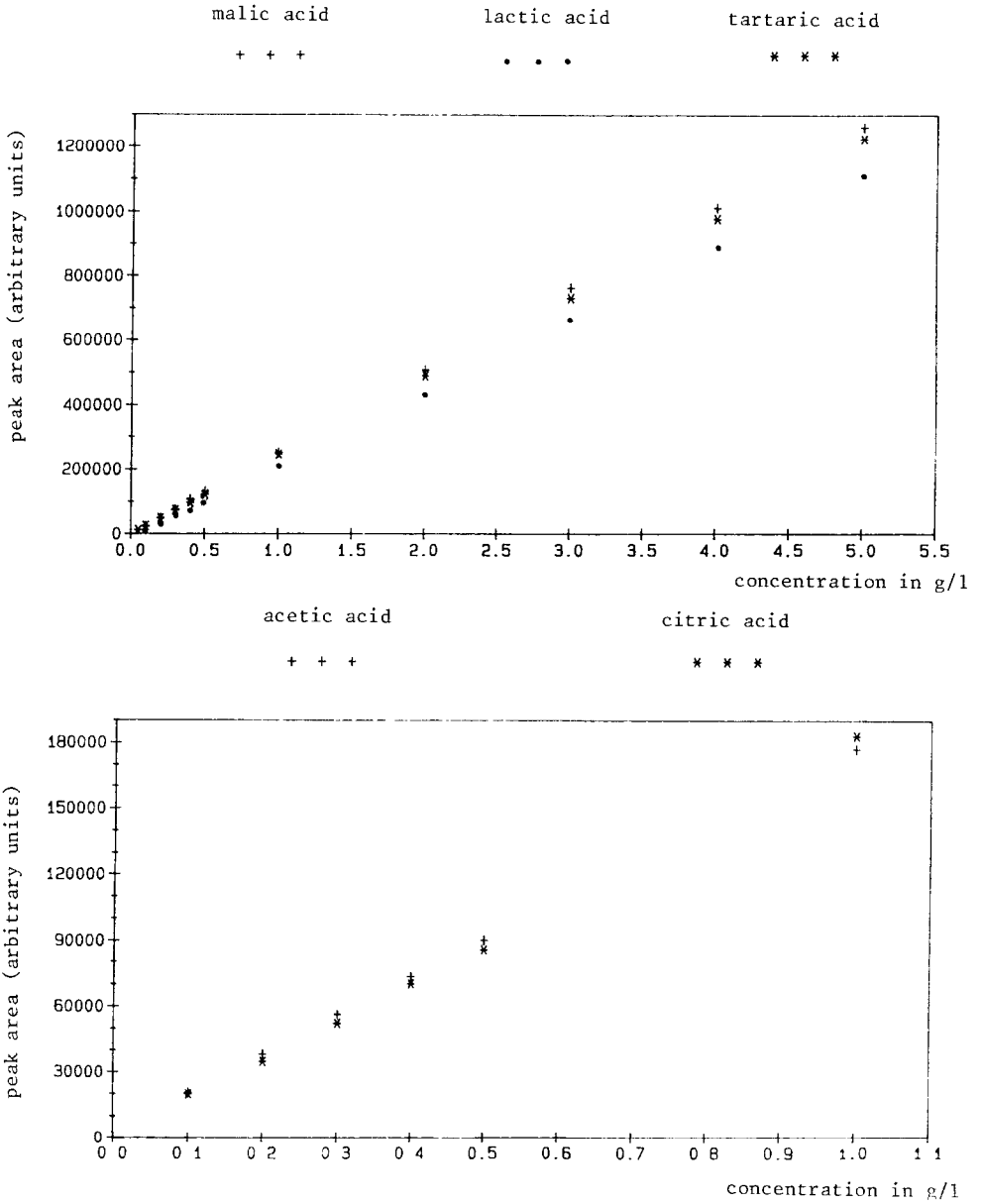

Fig. 2. Calibration graphs for the determination of the acids eluted according to the described procedure.

alcoholic fermentation), but are larger for low concentrations (below 200 mg/l).

However, we should point out that r and R are constant with our method, whereas they vary considerably with concentration for the collaborative analysis[39,46–48], so the values below 200 mg/l cannot be well quantified with an HPLC procedure.

TABLE II

REPEATABILITY (r) AND REPRODUCIBILITY (R) OF ANALYSES OF DIFFERENT WINES USING THE PROPOSED HPLC METHOD

$\bar{m}$ = average concentration of nine replicates.

Sample	*Acid*	$\bar{m}$ (mg/l)	r (mg/l)	r (%)	R (mg/l)	R (%)
Must	Tartaric acid	6983	65	1.0	134	2.0
	Malic acid	6402	32	0.5	95	1.5
Wine after alcoholic fermentation	Tartaric acid	5666	53	0.9	283	5.0
	Malic acid	5712	44	0.8	90	1.5
	Lactic acid	135	33	24.6	59	44
	Acetic	345	58	17	74	21
	Citric acid	NQ*	–	–	–	–
Wine after malolactic fermentation (champagne)	Tartaric acid	2943	66	2.2	105	3.6
	Malic acid	197	34	20	82	48
	Lactic acid	2655	4	0.2	8	0.3
	Acetic acid	NQ	–	–	–	–
	Citric acid	NQ	–	–	–	–
Red wine	Tartaric acid	1346	24	1.8	40	3.0
	Malic acid	688	48	7.0	58	8.5
	Lactic acid	2282	80	3.5	201	8.8
	Acetic acid	657	51	7.8	129	19.7
	Citric acid	NQ	–	–	–	–

* NQ: Not quantified.

For lactic acid, r varies from 4 to 80 mg/l (for concentrations in the range 0.13–2.7 g/l) and R varies from 8 to 20 mg/l. For the higher concentrations [wine after malolactic fermentation (champagne) and red wine], r and R are lower than the collaborative analysis[39,46–48], but are greater for low concentrations (wine after alcoholic fermentation). This is the same as for the results obtained with malic acid.

Several unknown compounds have their retention times close to those of AA and CA, which decreases the quality of the analysis. Unfortunately, as they are present only in trace amounts we are not able to quantify them.

Comparison with other methods

Some acids present in wines can be determined by standard techniques such as enzymatic methods (enzymatic assay coupled with an NAD–$NADH^+$ indicator reaction) for MA, D(−)- and L(+)-LA, AA and CA[46–51]. TA can be determined by the Blouin–Rebelein method based on its reaction with ammonium metavanadate[1,3,39].

Table III compares the results obtained with our HPLC method with those using standard methods. The results are similar, except for TA, where the Blouin–Rebelein method gives systematically higher values (from 0.2 to 1.0 g/l). This has been found by other workers, who explained this discrepancy by the interactions of the vanadate with other wine components such as MA, glycerol and carbohydrates[27].

The quantification of MA by HPLC gives the same results as those obtained

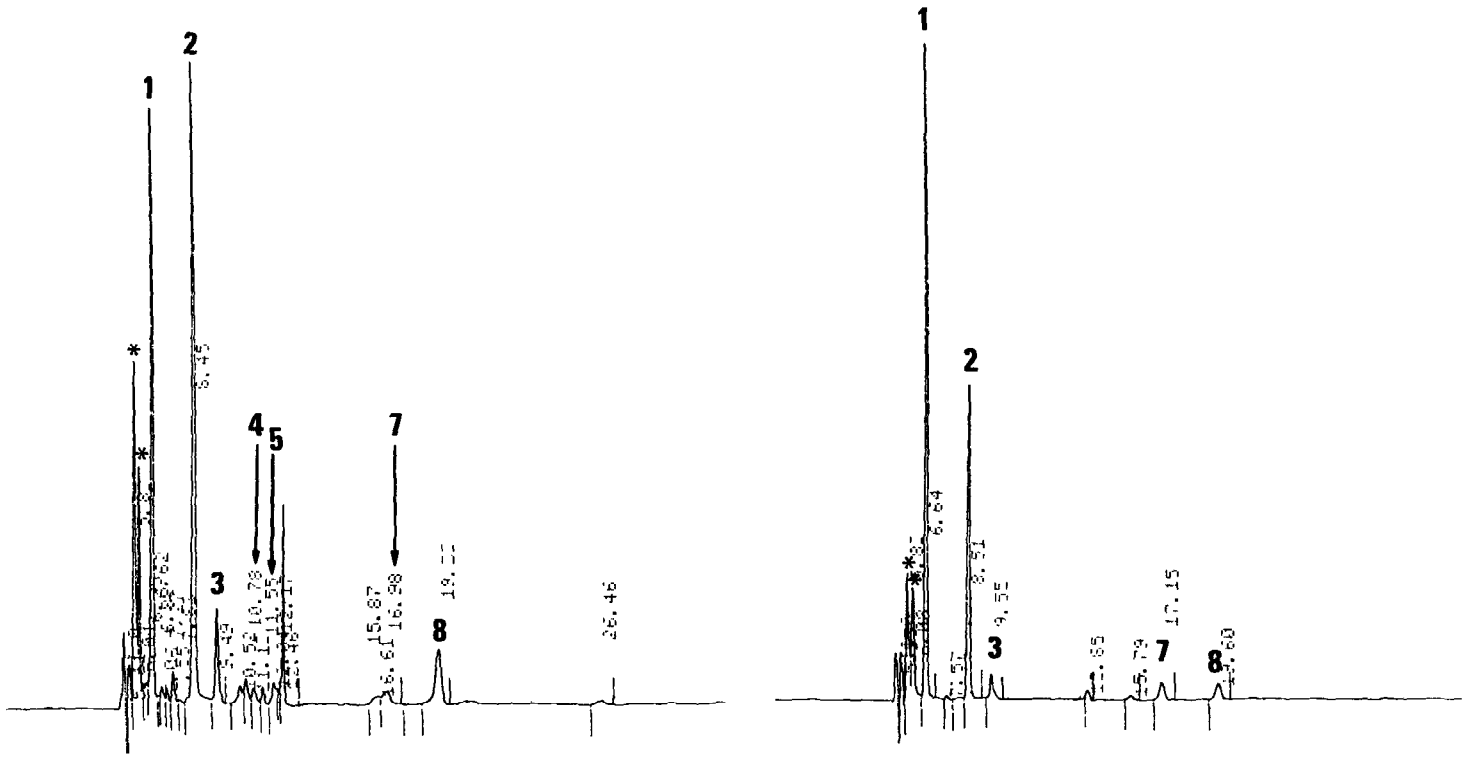

Fig. 3. Chromatogram of a must. For identification, see Table I. Asterisks indicate either amino acids or fructose. Quantification of the acids is reported in Table III. Analytical conditions in Fig. 1.

Fig. 4. Chromatogram of a wine after alcoholic fermentation. For identification, see Table I. Asterisks indicate either amino acids or fructose. Quantification of the acids is reported in Table III. Analytical conditions as in Fig. 1.

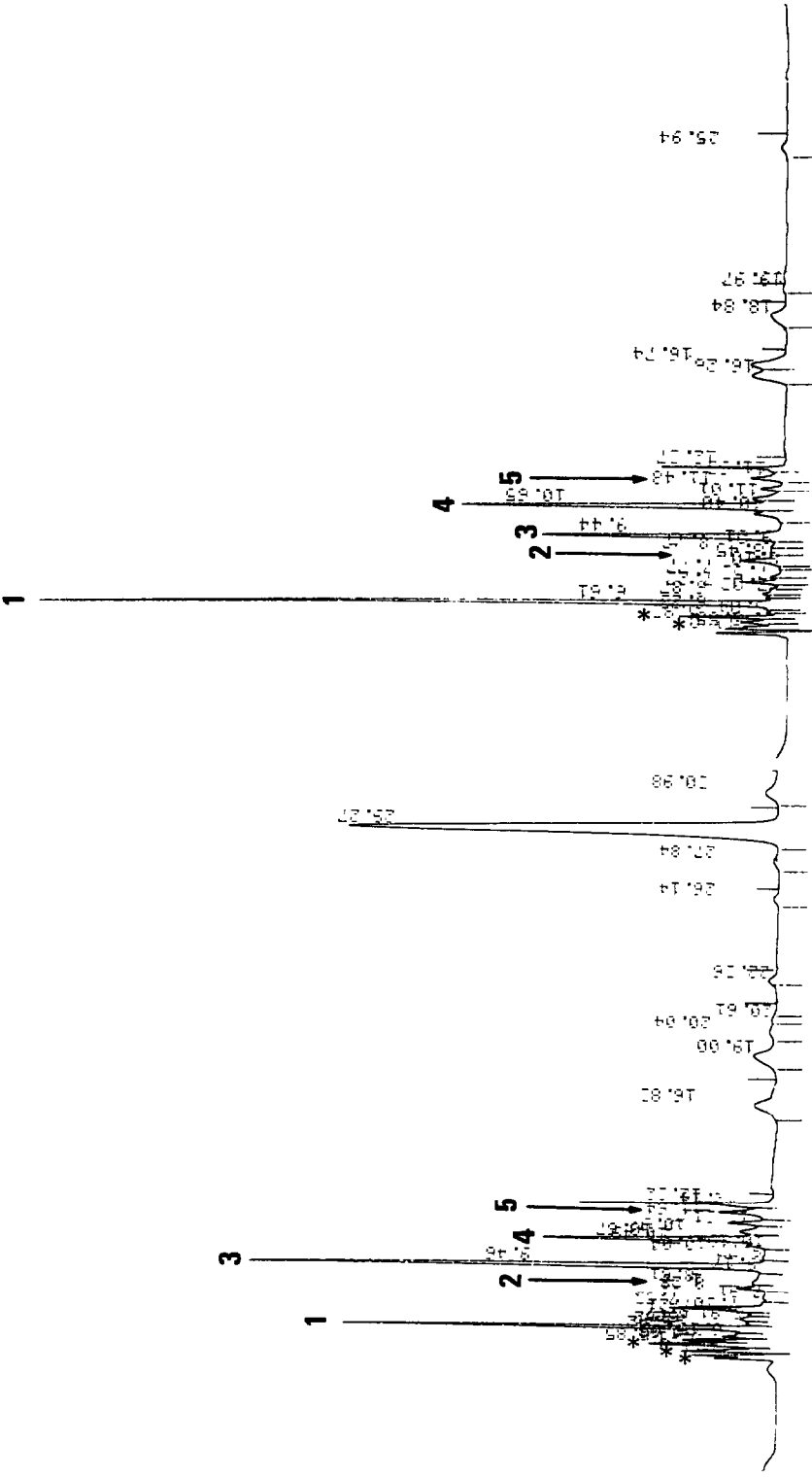

Fig. 5. Chromatogram of a wine after malolactic fermentation (champagne). For identification, see Table I. Asterisks indicate either amino acids or fructose. Quantification of the acids is reported in Table III. Analytical conditions as in Fig. 1.

Fig. 6. Chromatogram of a red wine. For identification, see Table I. Asterisks indicate either amino acids or fructose. Quantification of the acids is reported in Table III. Analytical conditions as in Fig. 1.

TABLE III

COMPARISON OF RESULTS OF ANALYSES OF DIFFERENT WINES OBTAINED WITH STANDARD METHODS (BLOUIN–REBELEIN FOR TARTARIC ACID AND ENZYMATIC ASSAYS FOR THE OTHERS) AND WITH PROPOSED HPLC METHOD

$\bar{m}$ = average concentration of nine replicates; σ^2 = variance.

Sample	*Acid*	*Standard method*		*HPLC method*	
		$\bar{m}$ *(mg/l)*	σ^2	$\bar{m}$ *(mg/l)*	σ^2
Must	Tartaric acid	7400	4634	6402	8620
	Malic acid	6793	1497	6983	1076
Wine after alcoholic fermentation	Tartaric acid	6160	3000	5666	9261
	Malic acid	5709	3259	5712	841
	D-Lactic acid	120	12	135	369
	L-Lactic acid	17	39	135	369
	Acetic acid	260	283	NQ*	–
	Citric acid	192	26	NQ	–
Wine after malolactic fermentation (champagne)	Tartaric acid	3220	4502	2943	1089
	Malic acid	146	56	170	778
	D-Lactic acid	139	12	2655	256
	L-Lactic acid	2461	2670	2655	256
	Acetic acid	239	410	NQ	–
	Citric acid	68	3	NQ	–
Red wine	Tartaric acid	1570	12927	1346	185
	Malic acid	NQ	–	688	576
	D-Lactic acid	276	2680	2282	4692
	L-Lactic acid	2177	4732	2282	4692
	Acetic acid	604	532	657	1927
	Citric acid	226	8	NQ	–

* NQ: Not quantified.

with the enzymatic method. For LA, the sum of the amounts of D(−)- and L(+)-LA is equal to the amount of LA quantified by HPLC.

CONCLUSION

The method reported here is a good alternative to other HPLC methods based either on ion-exchange or reversed-phase systems (with or without derivatization). It allows the identification, separation and quantification of carboxylic acids in musts and wines. The analysis, which is simple and rapid, does not require any complicated preparation of the sample, so that the method may be used routinely.

The precision of the quantification is comparable to the traditional methods except for the determination of trace amounts of acetic and citric acid.

REFERENCES

1 J. Ribereau-Gayon, E. Peynaud, P. Sudraud and P. Ribereau-Gayon, *Science et Technique du Vin*, Tome 1, Dunod, Paris, 1976, p. 671.

2 M. A. Amerine and C. S. Ough, *Methods for Analysis of Musts and Wines*, Wiley, New York, 1980, p. 341.
3 *Recueil des Méthodes Internationales d'Analyse des Vins*, Office International de la Vigne et du Vin, Paris, 348 pp.
4 R. Macrae, *J. Food Technol.*, 16 (1981) 1.
5 M. E. Evans, *J. Liq. Chromatogr.*, 6 (1983) 153.
6 R. Schwarzenbach, *J. Chromatogr.*, 251 (1982) 339.
7 P. Symonds, *Ann. Nutr. Aliment.*, 32 (1978) 957.
8 R. T. Marsili, H. Ostapenko, R. E. Sommons and E. D. Green, *J. Food Sci.*, 46 (1981) 52.
9 E. Rajakylä, *J. Chromatogr.*, 218 (1981) 695.
10 P. Vrátný, O. Mikeš, P. Štrop, J. Čoupek, L. Rexová-Benková and D. Chadimová, *J. Chromatogr.*, 257 (1983) 23.
11 S. H. Ashoor and J. Welty, *J. Chromatogr.*, 287 (1984) 452.
12 S. H. Ashoor and M. J. Knox, *J. Chromatogr.*, 299 (1984) 288.
13 J. R. Benson and D. J. Woo, *J. Chromatogr. Sci.*, 22 (1984) 386.
14 R. J. Bushway, J. L. Bureau and D. F. McGann, *J. Food Sci.*, 49 (1984) 75.
15 A. C. de la Harpe, personal communication, 1984.
16 S. A Kupina, *Am. J. Enol. Vitic.*, 35 (1984) 59.
17 J. D. McCord, E. Trousdale and D. D. Y. Ryu, *Am. J. Enol. Vitic.*, 35 (1984) 28.
18 R. Schwarzenbach, *Mitt. Geb. Lebensmittelunters. Hygg.*, 75 (1984) 51.
19 C. Gancedo and B. S. Luh, *J. Food Sci.*, 51 (1986) 571.
20 S. N. Deming and M. L. H. Turoff, *Anal. Chem.*, 50 (1978) 546.
21 C. Gonnet, M. Marichy and H. Philippe, *Analusis*, 7 (1979) 370.
22 J. Schneyder and W. Flak, *Mitt. Klosterneuburg*, 312 (1981) 57.
23 B. S. Buslig, C. W. Wilson and P. E. Schaw, *J. Agric. Food Chem.*, 30 (1982) 34.
24 W. Flak and G. Pluhar, *Mitt. Klosterneuburg*, 33 (1983) 60.
25 A. Houllemare, personal communication, 1983.
26 J. P. Goiffon, A. Blachere and C. Reminiac, *Analusis*, 13 (1985) 218.
27 E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Baiocchi and M. Savigliano, *J. Chromatogr.*, 322 (1985) 177.
28 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1974) 1849.
29 E. Grushka, H. D. Durst and E. J. Kikta, Jr., *J. Chromatogr.*, 112 (1975) 673.
30 I. Roorda, C. Gonnet and J. L. Rocca, *Analusis*, 10 (1982) 409.
31 W. Steiner, D. Fröblich and R. Battaglia, *Mitt. Geb. Lebensmittelunters. Hyg.*, 75 (1984) 37.
32 R. Badoud and G. Pratz, *J. Chromatogr.*, 360 (1986) 119.
33 E. Tomlinson, C. M. Riley and T. M. Jefferies, *J. Chromatogr.*, 173 (1979) 89.
34 P. Jandera and H. Engelhardt, *Chromatographia*, 13 (1980) 18.
35 C. Droz and H. Tanner, *Schweiz. Z. Obst. Weinbau*, 118 (1982) 434.
36 P. E. Shaw and C. Q. Wilson, III, *J. Sci. Food Agric.*, 34 (1983) 1285.
37 A. Clement and B. Loubinoux, *J. Liq. Chromatogr.*, (1983) 1705.
38 G. Chiu, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9 (1986) 410.
39 R. Garcia Faure, *Feuillet vert No. 754*, Office International de la Vigne et du Vin, Paris, 1982.
40 F. J. Goëtsch, W. Krönert, D. Olschimke, U. Otto and S. Vierkötter, *Feuillet vert No. 667*, Office International de la Vigne et du Vin, Paris, 1978.
41 R. Garcia Faure, *Feuillet vert No. 622*, Office International de la Vigne et du Vin, Paris, 1977.
42 D. Olschimke, *Feuillet vert No. 682*, Office International de la Vigne et du Vin, Paris, 1979.
43 C. Junge, *Feuillet vert No. 711*, Office International de la Vigne et du Vin, Paris, 1980.
44 G. Sarwar, R. Blair, M. Friedman, M. R. Gumbmann, L. R. Hackler, P. L. Pellett and T. K. Smith, *J. Assoc. Off. Anal. Chem.*, 1 (1985) 52.
45 C. Junge, *J. Assoc. Off. Anal. Chem.*, 1 (1985) 141.
46 C. Junge, *Feuillet vert No. 783*, Office Interntional de la Vigne et du Vin, Paris, 1984.
47 C. Junge, *Feuillet vert No. 500/600*, Office International de la Vigne et du Vin, Paris, 1976.
48 S. Van den Driessche, *Feuillet vert No. 755*, Office International de la Vigne et du Vin, Paris, 1982.
49 C. Junge, *Feuillet vert No. 753*, Office International de la Vigne et du Vin, Paris, 1983.
50 G. Henninger, L. Mascaro, *J. Assoc. Off. Anal. Chem.*, 5 (1985) 1024.
51 A. Herranz, I. Mareca, *Anal. Bromatol.*, 34 (1982) 219.