Journal of Chromatography, 542 (1991) 277–293
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 024

Optimized isocratic separation of major carboxylic acids in wine

R. M. MARCÉ*, M. CALULL, J. C. OLUCHA, F. BORRULL and F. X. RIUS
Department of Chemistry, University of Barcelona, Pça. Imperial Tarraco 1, 43005 Tarragona (Spain)
(First received July 18th, 1990; revised manuscript received October 23rd, 1990)

ABSTRACT

The reversed-phase high-performance liquid chromatographic separation of major carboxylic acids in wine was optimized. The separation was carried out by isocratic elution, and the optimization of the mobile phase composition with a constant solvent strength was studied. The optimization was carried out by using the overlapping resolution mapping approach employing previously developed software. The best mobile phase consisted of tetrahydrofuran, methanol and acetonitrile as organic modifiers and water as a carrier solvent to maintain a constant solvent strength.

INTRODUCTION

The determination of carboxylic acids in wine has considerable importance in enology as these compounds are used to control the vinification process. Carboxylic acids also have a great influence on the biological stability and organoleptic properties of wines, and are therefore determined routinely in many enological laboratories.

Various chromatographic methods offer alternatives to the time-consuming traditional methods of analysis, especially high-performance liquid chromatography (HPLC) employing either reversed-phase partition equilibria or ion-exchange equilibria [1–3]. Of the methods based on reversed-phase chromatography, the direct method, without derivatization and detection by UV spectrophotometry at about 210 nm, and the derivatization method, using different reagents such as phenacyl [4,5], naphthacyl [6], *p*-nitrophenyl [7] or *p*-nitrobenzyl [8] bromides and detection at 254 nm, are commonly used. The method using derivatized compounds is preferred to the direct method owing to the higher sensitivity achieved.

Good resolution between the different peaks is usually obtained when using a gradient of solvent strength, but isocratic elution has two basic advantages in routine analysis, *viz.*, the equipment is simpler and the overall time of analysis for a series of samples is shorter. In this paper, an optimized isocratic method for determining carboxylic acids in wine with precolumn derivatization with phenacyl bromide is reported.

Two factors that depend on the mobile phase composition, the capacity factor k' and the separation factor α, influence the resolution in isocratic systems. The

0021-9673/91/$03.50

separation factor is influenced by solvent composition and, for a constant solvent strength, different resolutions are obtained when different solvents are used. Three solvents (methanol, acetonitrile and tetrahydrofuran) as organic modifiers and water as the carrier to maintain a constant solvent strength have been used to find the optimum solvent composition by using the overlapping resolution mapping (ORM) [9,10] technique, which gives the best overall separation for a chosen resolution level.

Although some software implementing overlapping resolution mapping has been reported [11], in this work we used our own program that includes high-quality graphics for this application. The program runs on an IBM PC or compatible computer and is available from the authors.

EXPERIMENTAL

Equipment

A Hewlett-Packard Model 1050 modular chromatograph with a Hewlett-Packard Series 1050 variable-wavelength detector and a workstation with a Model 35900 interface was used. The chromatographic separation was carried out with a Spherisorb ODS-2 column (250 × 4.6 mm I.D.) of 5-μm particle size and a precolumn (30 × 3.9 mm I.D.) filled with Bondapak C_{18}/Corasil (37–50-μm particle size).

Reagents and standards

Phenacyl bromide (Fluka), 18-crown-6 (Fluka) and phosphate buffer solution (pH 6.8) were used in the derivatization reaction. All solvents in the derivatization process (acetone) and in the chromatographic separation (methanol, acetonitrile and tetrahydrofuran) were of HPLC quality from Merck and water was purified in a Milli-Q apparatus (Millipore).

The study was carried out with the most frequent carboxylic acids found in wine, *i.e.* tartaric, malic, acetic, lactic, succinic and citric acid (Aldrich). Methylmalonic acid was used as an internal standard.

Chromatographic conditions

The derivatization procedure has been optimized elsewhere [12] and the chromatographic conditions (flow-rate, 1 ml/min; detection, UV absorption at 254 nm; volume injected, 5 μl; and temperature, 30°C) were chosen on the basis of previous work [12].

RESULTS AND DISCUSSION

The first step in the optimization of the solvent composition for isocratic elution consists in determining a suitable solvent strength that gives acceptable values of k' for all the carboxylic acids considered. Among different approaches developed for determining an adequate solvent strength [13–15], one based on an initial gradient separation was chosen [13]. From the chromatogram obtained by using a binary water–methanol linear gradient, sufficient information can be extracted to calculate the percentage of methanol in the mobile phase that gives acceptable values of k' and, from this value, three different mixtures of acetonitrile, methanol and tetrahydrofuran with water were derived that define the three vertices of an optimization triangle [16].

Taking into account the experience from previous work in our laboratory on the optimization of a linear gradient separation of carboxylic acids present in wine [12], a linear gradient from 30 to 90% methanol in 20 min allowed good resolution to be obtained for all the peaks studied (Fig. 1). The sample injected consisted of a white wine to which pure carboxylic acids had been added because some of them were not present naturally in the actual wine sample analysed. The injected sample was obtained by mixing 1 ml of the white wine sample with 1 ml of a 0.5 g/l standard solution of carboxylic acids.

In the optimization process, peaks 1 (system peak), 2 (lactic acid), 3 (acetic acid), 4 (reagent peak, phenacyl bromide), 5 (tartaric acid), 6 (malic acid), 7 (succinic acid), 8 (internal standard, metylmalonic acid) and 9 (citric acid) were considered. The first peak appearing in Fig. 1 corresponds to acetone, the solvent used in the derivatization solution, and as its retention time is shorter than those of the system peaks, it was not considered.

From the chromatographic data, the program calculates the percentage of the first isocratic solution derived from the gradient elution which determines the solvent strength from the expression [13]

$$c_o = (c_1 c_2 ... c_n)^{1/n}$$

where c_i is the solvent composition at which each solute i leaves the column, calculated from the following equation [13]

c_i = initial concentration of selectivity-adjusting solvent + (time of elution for each peak − distance between gradient generator and column inlet in time units) × (gradient rate in % of selectivity-adjusting solvent per minute)

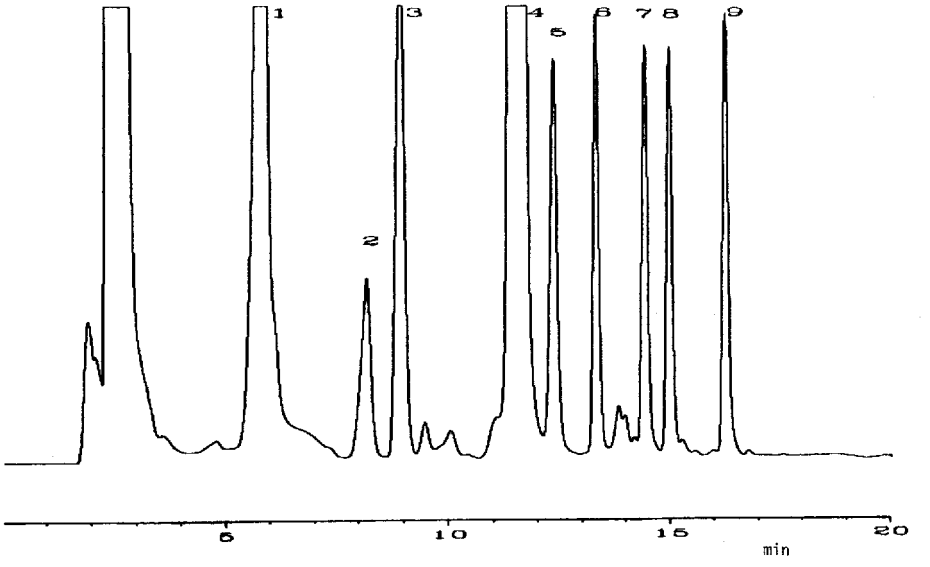


Fig. 1. Chromatogram of the carboxylic acids obtained with linear gradient elution from 30 to 90% of methanol in 20 min.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE DIFFERENT EXPERIMENTS TO BE CARRIED OUT TO DEFINE THE RESPONSE SURFACE

Experiment	Methanol (%)	Acetonitrile (%)	Tetrahydrofuran (%)	Water (%)
1	52.75	0.00	0.00	47.25
2	0.00	42.86	0.00	57.14
3	0.00	0.00	30.48	69.52
4	26.38	21.43	0.00	52.19
5	0.00	21.43	15.24	63.33
6	26.38	0.00	15.24	58.38
7	17.58	14.29	10.16	57.97
8	35.17	7.14	5.08	52.61 Validation[a]
9	8.79	28.57	5.08	57.55 Validation[a]
10	8.79	7.14	20.32	63.74 Validation[a]

[a] These experimental conditions were used on the validation of the mathematical expression found.

However, as poor retention of solutes is sometimes found with this value, it is multiplied by a correction factor [13]. The c_o value found using the software developed was 63.3% of methanol, which was multiplied by a factor of 5/6 [13], resulting a mobile phase constituted by 52.8% methanol and 47.2% water.

The compositions of all other experimental points were computed by the program (Table I) by using the expression

$$S = \sum s_i \varphi_i$$

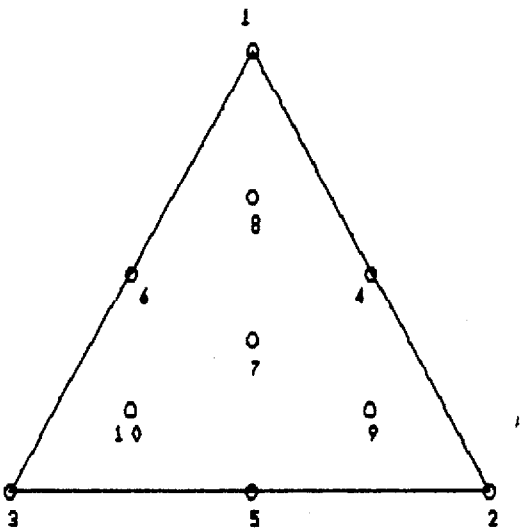


Fig. 2. Simplex lattice experimental design used. Point 1 (52.8% methanol, 47.2% water), point 2 (42.9% acetonitrile, 57.1% water) and point 3 (30.5% tetrahydrofuran, 69.5% water).

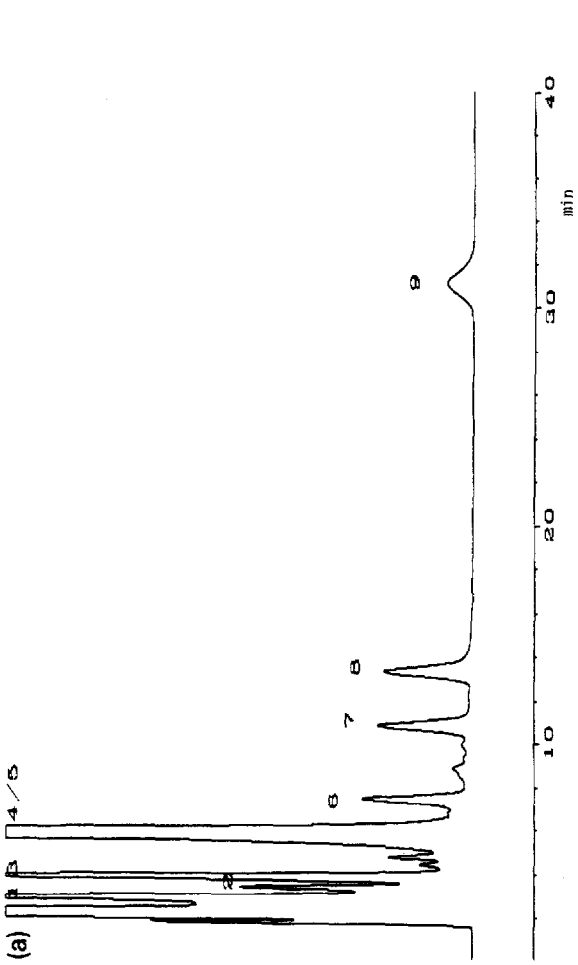
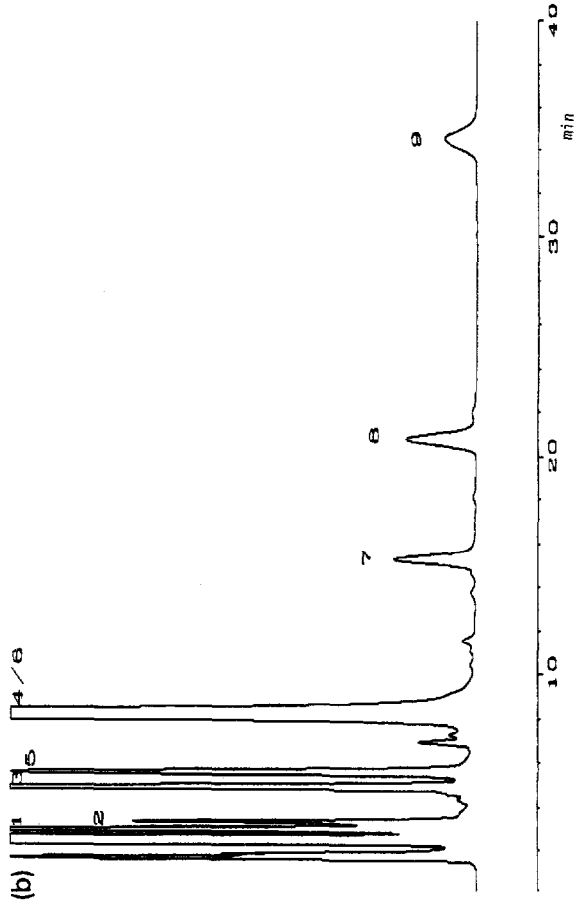


Fig. 3.

(*Continued on p. 282*)


(b)
1
2
3
5
4 / 6
7
8
9
10
20
30
40
min

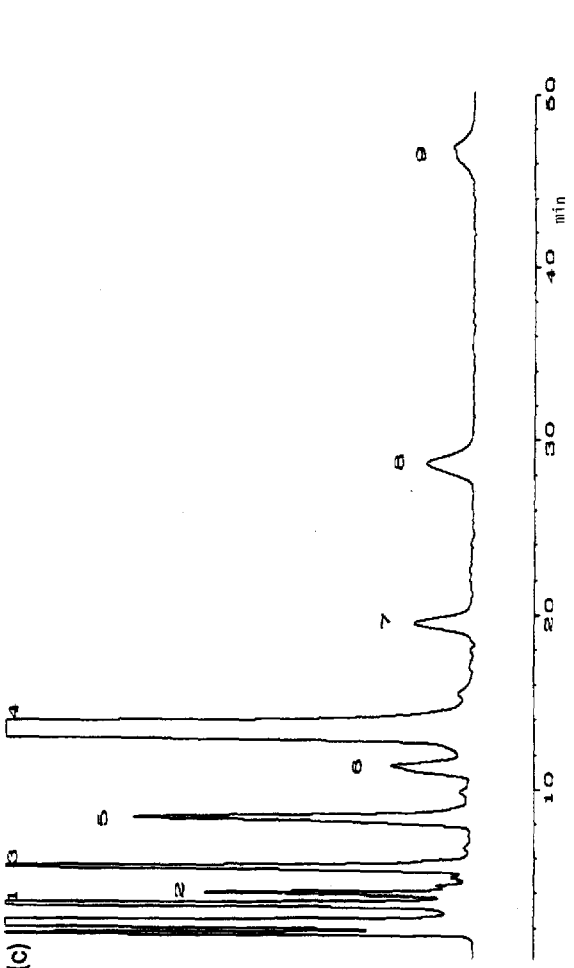
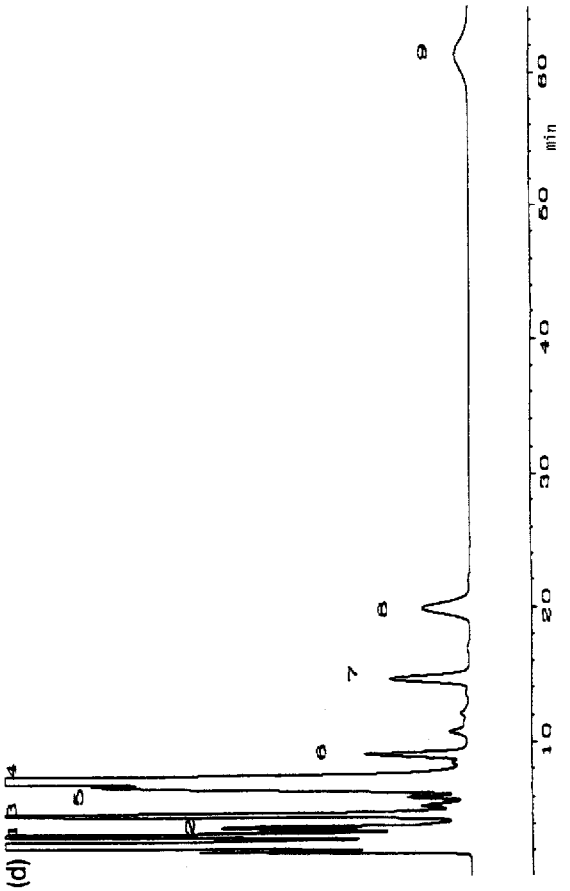


Fig. 3.

(*Continued on p. 284*)


(d)
1
2
3
4
5
6
7
8
9
10
20
30
40
50
60
min

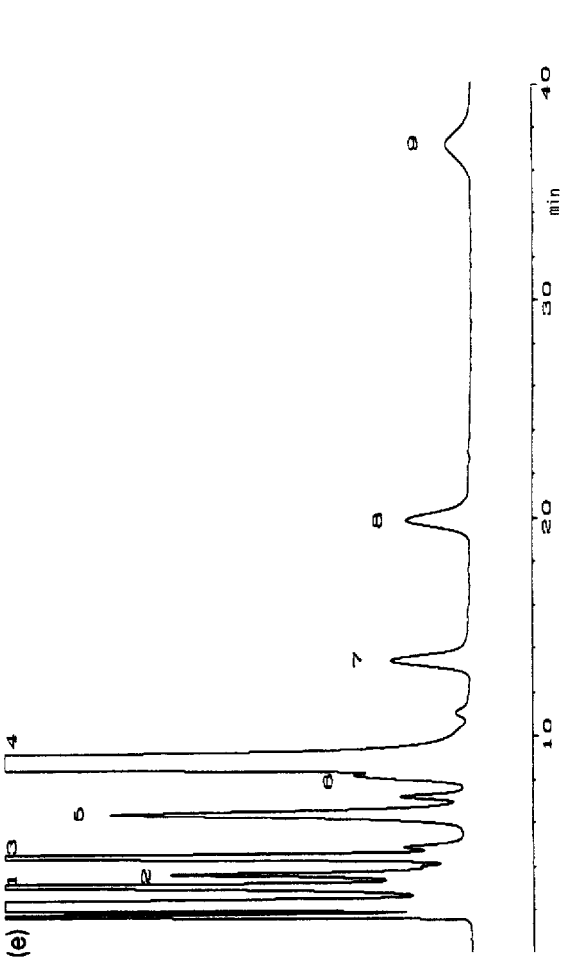
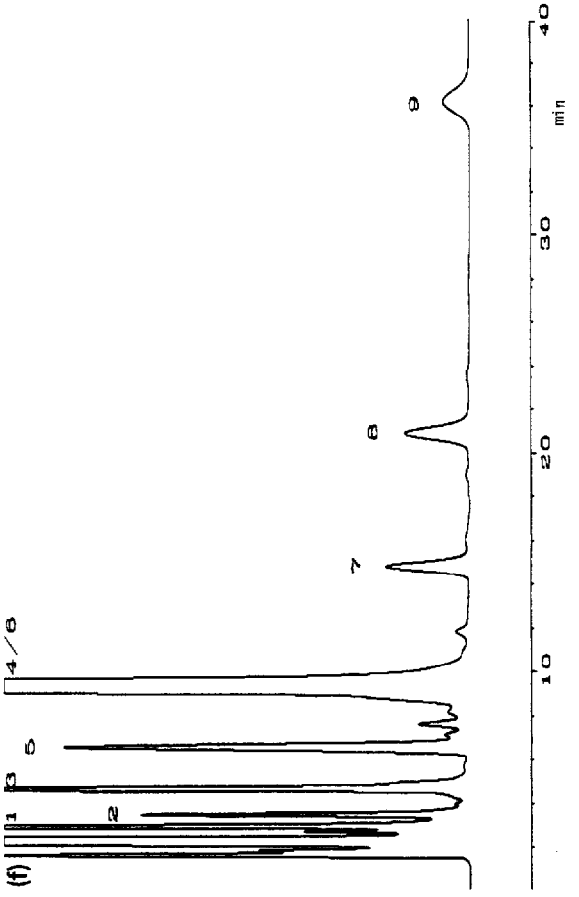


Fig. 3.

(Continued on p. 286)


(f)
1
2
3
5
4/6
7
8
9
10
20
30
40
min

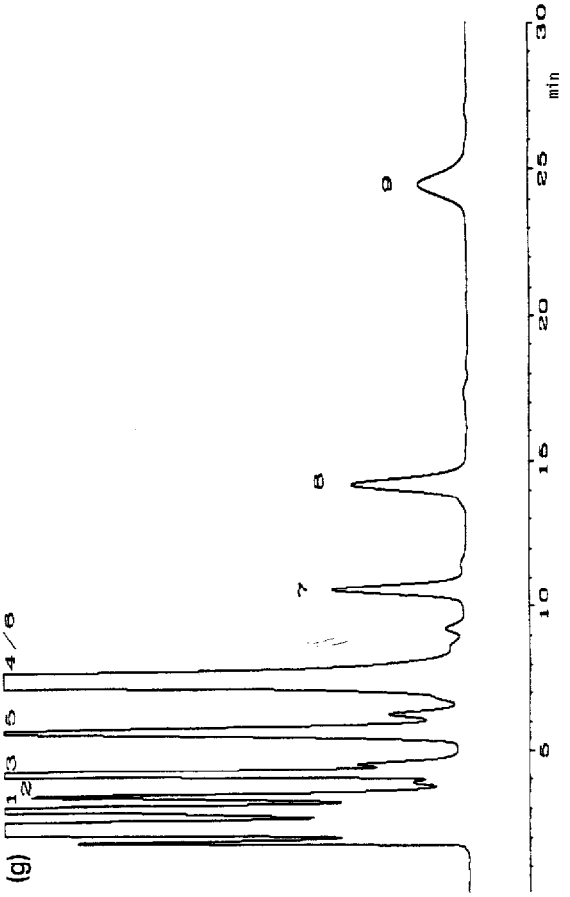


Fig. 3. Chromatograms obtained for the different isocratic mobile phase compositions.

where S is the solvent strength of the mobile phase, s_i is the solvent strength weighting factor of each solvent i (2.6, 3.2 and 4.5 for methanol, acetonitrile and tetrahydrofuran, respectively) and φ_i is the solvent fraction.

Fig. 2 shows the parameter space defined in which the optimization will take place. The three binary mobile phases derived are the vertices of a simplex lattice experimental design and points 4, 5 and 6 define the compositions of the ternary mobile phase and point 7 defines the quaternary mobile phase. Points 1–7 were used to calculate the response surface and points 8–10 to check the lack of fit between the model and the experimental results. All mobile phase compositions involved will give rise to approximately the same solvent strength, although specific retention times and therefore orders of elution and selectivities will change.

The different chromatograms obtained for each experiment are shown in Fig. 3. Peaks 1, 2, 3, 7, 8 and 9 do not change their elution orders in any of the experiments carried out, whereas peaks 4, 5 and 6 change their position when the composition of the mobile phase varies.

The seven chromatograms were evaluated by using the overlapping resolution mapping approach by using the SMR[a] program. The program models the resolution between each pair of peaks as a function of the percentages of methanol, acetonitrile and tetrahydrofuran along the parameter space defined by the experimental design used. The following equation has been considered:

$$R_{m,n} = a + bx + cy + dz + ex^2 + fy^2 + gz^2 + hxy + ixz + jyz$$

where x, y and z are the percentages of methanol, acetonitrile and tetrahydrofuran, respectively, m and n refer to the pairs of peaks 1–2, 2–3, 3–4, 4–5, 5–6, 6–7, 7–8, 8–9, 3–5, 4–6 and 4–7 and a, b, c,...,j are the coefficients of the model.

First, the different peaks of each pair are tracked by the software by comparing the different retention times. Prior to the introduction of the retention time and width, each peak of the chromatogram is assigned to a compound. The order with the first composition is considered to be the initial order and the elution order obtained for the other compositions are compared with this one. When the elution order obtained changes in a composition, a crossover is considered.

Taking into account the seven experiments carried out, the computer program calculates the coefficients of the model by a non-linear multivariate regression algorithm.

Once a minimum threshold value for the resolution between all peaks has been defined, the SMR program allows the visualization of those areas (unshaded zones) in which the mobile phase compositions give rise to chromatograms in which the resolution is higher than the fixed resolution value. In Fig. 4 the different response surfaces obtained for a resolution higher than 1.5 for each pair of peaks can be observed. It can be seen that the pairs of peaks 3–4, 5–6, 6–7, 7–8, 8–9, 3–5 and 4–7 have a resolution higher than the minimum established value of 1.5 for all possible compositions at the solvent strength considered. At this resolution value, the pairs of peaks 1–2, 2–3, 4–5 and 4–6 have a resolution lower than 1.5 for some mobile phase compo-

[a]SMR is the name of the program used. They are the initials of the Catalan translation of ORM (overlapping resolution map).

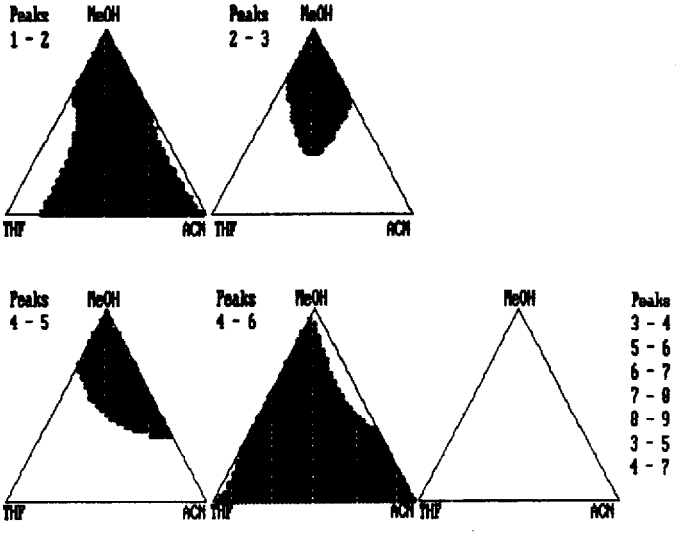


Fig. 4. Peak-pair resolution map for a resolution higher than 1.5. Here and in subsequent figures, MeOH = methanol, ACN = acetonitrile and THF = tetrahydrofuran.

sitions. The pair of peaks 2–3 and 4–5 have a resolution lower than 1.5 when the percentage of methanol in the mobile phase is higher and the pairs of peaks 1–2 and 4–6 have only a small zone where the resolution is higher than 1.5. These two zones are localized when a high percentage of tetrahydrofuran is present in the mobile phase and when the mobile phase is composed of a mixture of methanol and acetonitrile.

By superposing all the computed response surfaces, a unique overlapping resolution map is obtained in which the unshaded zones indicate the mobile phase compo-

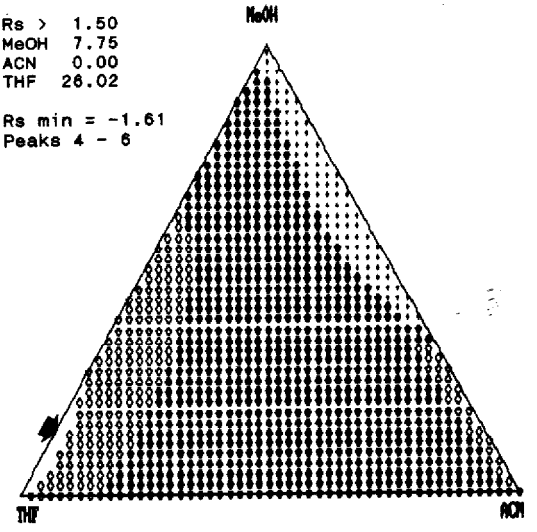


Fig. 5. Overlapping resolution map for a resolution of 1.5.

TABLE II

THEORETICAL RESOLUTION VALUES OBTAINED UNDER THE OPTIMUM CONDITIONS

Mobile phase: methanol–tetrahydrofuran (7.75:26.02).

Resolution, R_s	Peaks	Resolution, R_s	Peaks
2.0661	1–2	5.1407	7–8
2.8516	2–3	7.4442	8–9
6.7576	3–4	4.0742	3–5
−3.8675	4–5	1.6138	4–6
2.7942	5–6	3.2587	4–7
6.0116	6–7		

sitions which give rise to resolutions higher than 1.5 for all pairs of peaks considered (Fig. 5). This zone is located in the present instance at a high concentration of tetrahydrofuran and low concentrations of both acetonitrile and methanol. The optimum conditions for the isocratic analysis can be deduced from the graph obtained. The program allows the identification by means of an arrow of the optimum zone and it calculates the concentrations of each solvent in the mobile phase at each considered point, and the pair of peaks with the lowest resolution which is indicated at the top left of the figure. The optimum conditions considered for isocratic elution are 26% of tetrahydrofuran, 7.8% of methanol and 66.2% of water. Under these conditions the lowest resolution of 1.6 is obtained for peaks 4–6. The program allows one to see all other resolution values obtained under these conditions for the remainder of the peaks. The values obtained can be seen in Table II. Under these conditions all other peaks show a resolution higher than 2.7. The chromatogram obtained under these conditions is shown in Fig. 6.

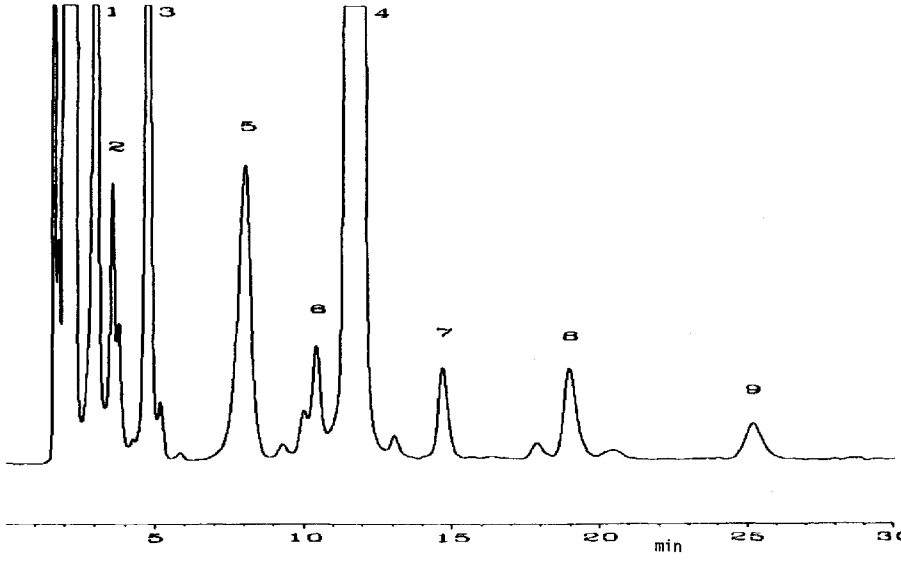


Fig. 6. Chromatogram obtained under the optimum conditions.

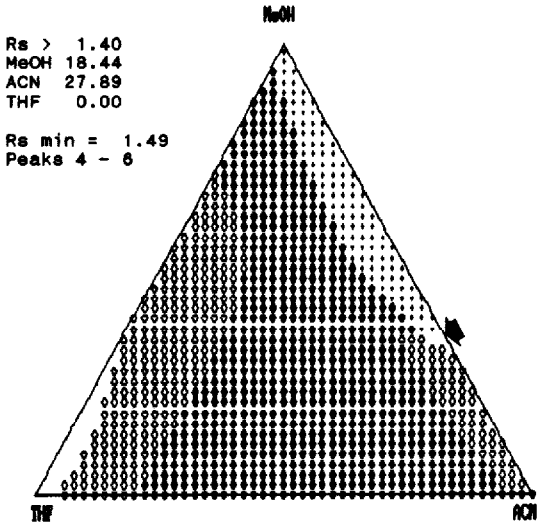


Fig. 7. Overlapping resolution map for a resolution of 1.4 without tetrahydrofuran in the mobile phase.

An alternative mobile phase composition can be found by slightly reducing the minimum value of the resolution between peaks. If the threshold value is set to 1.4, the response surface depicted in Fig. 7 is obtained. As indicated by the arrow, a solvent composition which does not include tetrahydrofuran can be used in an isocratic elution (18.4% methanol, 27.9% acetonitrile and 53.7% water), giving rise to the chromatogram shown in Fig. 8. Clearly, peaks 1 and 2 and 5 and 4 are coeluted to

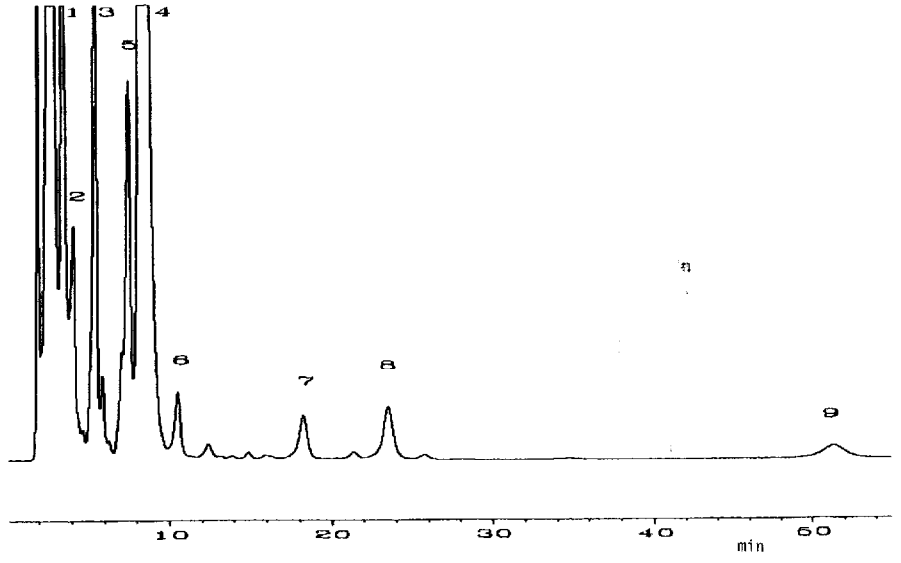


Fig. 8. Chromatogram obtained with the conditions in Fig. 7.

TABLE III

EXPERIMENTAL AND THEORETICAL RESOLUTION VALUES AND RESIDUAL VALUES FOR THE PAIR OF PEAKS 1–2 FOUND WITH THE FIRST SEVEN EXPERIMENTS

Experiment	Exptl. response, $R_{exp.}$	Calc. response, $R_{calc.}$	$R_{calc.} - R_{exp.}$
1	0.966	0.963	0.0030
2	1.560	1.557	0.0030
3	2.188	2.184	0.0030
4	1.683	1.695	0.0120
5	1.514	1.526	0.0120
6	0.800	0.812	0.0120
7	1.297	1.270	0.0271
			$\sum(R_{calc.} - R_{exp.})^2 = 0.0012$

a greater extent than in the previous zone and the retention time of citric acid (peak 9) has greatly increased.

When the first seven experimental points are used to check the lack of fit, the values of the residuals obtained for each pair of peaks by subtracting the experimental from the theoretical values are very similar and the error calculated, the sum of squared residuals, is acceptable for the method used. An example of the residuals obtained for the pair of peaks 1–2 can be seen in Table III. When replications of the three points corresponding to experiments 8, 9 and 10 are considered in the analysis of lack of fit of the computed model, the sum of squared residuals obtained increases but its value is acceptable for the method used. Different values obtained in the statistical analysis are given in Table IV.

CONCLUSIONS

The optimum mobile phase composition for determining carboxylic acids in wine by reversed-phase HPLC using precolumn derivatization with phenacyl bromide and isocratic elution has been found. A resolution greater than 1.5 between all

TABLE IV

VALIDATION OF THE RESPONSE SURFACE FOR THE PAIR OF PEAKS 1–2 WITH EXPERIMENTS 8, 9 and 10.

Each experimental value is the mean of three determinations.

Experiment	Exptl. response, $R_{exp.}$	Calc. response, $R_{calc.}$	$R_{calc.} - R_{exp.}$
8	1.306	1.320	0.014
9	1.392	1.224	0.168
10	1.531	1.489	0.042
			$\sum(R_{calc.} - R_{exp.})^2 = 0.0302$

pairs of peaks was considered as the optimization criterion. The mobile phase found consists of tetrahydrofuran (26%), methanol (7.8%) and water (66.2%) and the retention time for the last peak is 26 min.

An alternative mobile phase which avoids the use of tetrahydrofuran is composed of acetonitrile (27.9%), methanol (18.4%) and water (53.7%) and the resolution for all the peaks is greater than 1.4, but the retention time of the last peak increases to 52 min.

When the developed method is compared with that employing gradient elution, a shorter time for elution of all compounds is observed for the latter, but it must be taken into account that the overall analysis time can increase considerably because of the time to adapt the column between to determinations.

The SMR program has been used to determine the optimum mobile phase composition which gives a minimum resolution for all the peaks of interest and allows one to visualize the overlapping resolution maps with satisfactory results.

ACKNOWLEDGEMENTS

The authors thank CICYT (project 86-0029) for financial support given and R. M. M. gratefully acknowledges a research grant from CIRIT (project AR89) of Catalonia.

REFERENCES

1 R. F. Frayne, *Am. J. Enol. Vitic.*, 37 (1986) 4.
2 A. Schneider, V. Gerbi and M. Redoglia, *Am. J. Enol. Vitic.*, 38 (1987) 2.
3 J. P. Goiffon, A. Blachere and C. Reminiac, *Analusis*, 13 (1985) 218.
4 E. Mentasti, M. C. Gennaro, C. Sarzanini, C. Baiocchi and M. Savigliano, *J. Chromatogr.*, 322 (1985) 177.
5 F. Caccamo, G. Carfagnini, A. Di Corcia and R. Samperi, *J. Chromatogr.*, 362 (1986) 47.
6 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1974) 1849.
7 E. Grushka, H. D. Durst and E. J. Kikta, *J. Chromatogr.*, 112 (1975) 673.
8 R. Badoud and G. Pratz, *J. Chromatogr.*, 360 (1986) 119.
9 J. L. Glajch, J. J. Kirkland and K. M. Square, *J. Chromatogr.*, 199 (1980) 57.
10 P. J. Schoenmakers, *Optimization of Chromatographic Selectivity*, Elsevier, Amsterdam, 1986.
11 J. L. Glajch, J. L. Kirkland and J. M. Minor, *J. Liq. Chromatogr.*, 10 (1987) 1727.
12 R. M. Marcé, M. Calull, F. Borrull and F. X. Rius, *Am. J. Enol. Vitic.*, 41 (1990) 289.
13 M. D. Smet, G. Hoogewijs, M. Putemans and D. L. Massart, *Anal. Chem.*, 56 (1984) 2662.
14 J. C. Berridge, *Techniques for the Automated Optimization of HPLC Separations*, Wiley, Chichester, 1985.
15 L. R. Snyder, J. L. Glajch and J. J. Kirkland, *Practical HPLC Method Development*, Wiley-Interscience, New York, 1988.
16 R. Leher, *Int. Lab.*, Nov.–Dec. (1981) 76.