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Determination of additives in wine by high-performance liquid chromatography

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ABSTRACT

Two methods for determining additives in wine samples by reversed-phase high-performance liquid chromatography using UV–visible detection were studied. One method used gradient elution for the separation of the different additives in a short time (less than 12 min). Before the injection of the sample, a solid-phase extraction was applied to obtain better results when a red wine was analysed. The other method effected the separation of these compounds by isocratic elution using cetyltrimethylammonium bromide (CTAB) as an ion-pair reagent, without sample pretreatment.

INTRODUCTION

Different methods have been developed for the determination of food additives, including UV spectrophotometry [1,2], thin-layer chromatography [3,4], gas chromatography [5–7] and high-performance liquid chromatography (HPLC) [8–16]. The HPLC technique is the most commonly used method for the determination of possible additives in different foods, *e.g.*, cheese [13], mayonnaise [10], yoghurt [14,16], cosmetic products [11], liquid foods [15], powdered milk [15], sauces, mustard and coconut cream [15].

The control of different additives is important owing to the strict control of wine quality. Among different additives, antiseptics and antioxidants are commonly used in wine treatment, and their addition is regulated. Only the addition of sulphur dioxide, sorbic acid and ascorbic acid is permitted, and only up to a certain concentration. The Office International de la Vigne et du Vin (OIV) established two HPLC methods to determine different possible compounds by isocratic elution (one allowed the separation of the most polar and the other the least polar compounds) [17].

In this work, two methods to determine a group of antiseptics (sorbic acid, benzoic acid, *p*-chlorobenzoic acid, salicylic acid, *p*-hydroxybenzoic acid, ethyl *p*-hydroxybenzoate), an antioxidant (ascorbic acid) and a sweetener (saccharin) by reversed-phase HPLC using UV–VIS detection were developed, one involving gradient elution and the other isocratic elution. Simultaneous separation of these additives by isocratic elution is difficult because of their different polarities. For this reason ion-pair chromatography was used.

In the gradient elution method, the determination of saccharin and ascorbic acid was not possible because of their high polarity and their co-elution with other polar compounds in the wine sample at the beginning of the chromatogram.

When the gradient method was applied to determine these compounds in red wine samples, pretreatment of the sample was needed to decrease the interference of the matrix. In this work, a solid-phase extraction with strong anion exchange (LC-SAX) cartridges was applied, and the different conditions of treatment were optimized.

Although good resolution can be obtained in a short time when a gradient of solvent strength is used, isocratic elution has some advantages in routine analysis. In this work, an isocratic method to determine all compounds, including ascorbic acid and saccharin, was also optimized using ion-pair

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chromatography. Using this method, salicylic acid could not be determined because a peak distortion appeared.

When the ion-pair method was used, different variables influencing the separation were taken into account and their influence was studied to obtain the optimum conditions.

EXPERIMENTAL

Equipment

A Hewlett-Packard (Avondale, PA, USA) liquid chromatograph with an HP 1040M diode-array detector was used. Separation was carried out using a 5-μm Spherisorb ODS-2 column (250 × 4.6 μm I.D.) with a precolumn (30 × 3.9 mm I.D.) packed with μBondapack C_{18}/Corasil (particle size 37–50 μm) (Teknokroma, Barcelona, Spain). Chromatographic data were collected and recorded using an HP 7999A workstation.

Reagents and standards

In both methods studied, acetic acid, phosphoric acid, sulphuric acid and 0.05 *M* acetate–0.05 *M* phosphate buffer solution (Merck, Darmstadt, Germany) were used as modifiers of the pH, acetonitrile (HPLC quality from Merck) as organic modifier and cetyltrimethylammonium bromide (CTAB) (Sigma, St.Louis, MO, USA) and tetrabutylammonium bromide (Fluka, Buchs, Switzerland) as ion-pair reagents. Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

The additives studied were ascorbic acid, benzoic acid, salicylic acid, sorbic acid, *p*-chlorobenzoic acid, *p*-hydroxybenzoic acid, ethyl *p*-hydroxybenzoate and saccharin (Sigma). The study was carried out with a standard solution of additives at a concentration of 10 ppm in water–acetonitrile (50:50).

Chromatographic conditions

Gradient elution. The chromatographic conditions adopted were as follows: flow-rate, 1 ml/min; detection, UV absorption at 240 nm; volume injected, 5 μl; temperature, constant at 60°C; and mobile phase, acetic acid at pH 3 as solvent A and acetonitrile as solvent B with a gradient programme (Table I).

Isocratic elution. The chromatographic conditions were as follows: flow-rate, 1 ml/min; detection, UV absorption at 235 nm; volume injected, 5 μl; and temperature, constant at 40°C. The mobile phase composition was optimized and the best conditions obtained were 2 m*M* CTAB, 35% of acetonitrile and 10% of buffer solution (0.05 *M* H_3PO_4– and 0.05 *M* acetic acid adjusted with 2 *M* NaOH at pH 5.5).

TABLE I

GRADIENT ELUTION PROGRAMME

Time (min)	Solvent A (%)	Solvent B (%)
0	85	15
2	85	15
8	60	40
15	0	100
20	85	15
30	Next injection	

Sample preparation

All wine samples were filtered with a 0.45-μm nylon membrane. When the gradient elution method was applied to a red wine, a solid-phase extraction to remove the coloured compounds and decrease the interference of the matrix with the anion exchange LC-SAX cartridges (quaternary amine-bonded silica, strong anion exchanger) (Supelco, Bellefonte, PA, USA) was necessary. The cartridge was conditioned with 5 ml of Milli-Q purified water and then 1 ml of sample diluted 1:2 was slowly passed through the extraction tube. In order to obtain a good recovery of additives, it was necessary to pass 1.5 ml of 0.5 *M* sulphuric acid through the cartridge. After homogenization of the two fractions the sample can be injected.

RESULTS AND DISCUSSION

Gradient elution

Simultaneous separation of some of these compounds using gradient elution have been reported [10,11,14–16]. In this work, a preliminary study was carried out to determine sorbic acid, salicylic acid, benzoic acid, *p*-hydroxybenzoic acid, *p*-chlorobenzoic acid and ethyl *p*-hydroxybenzoate with gra-

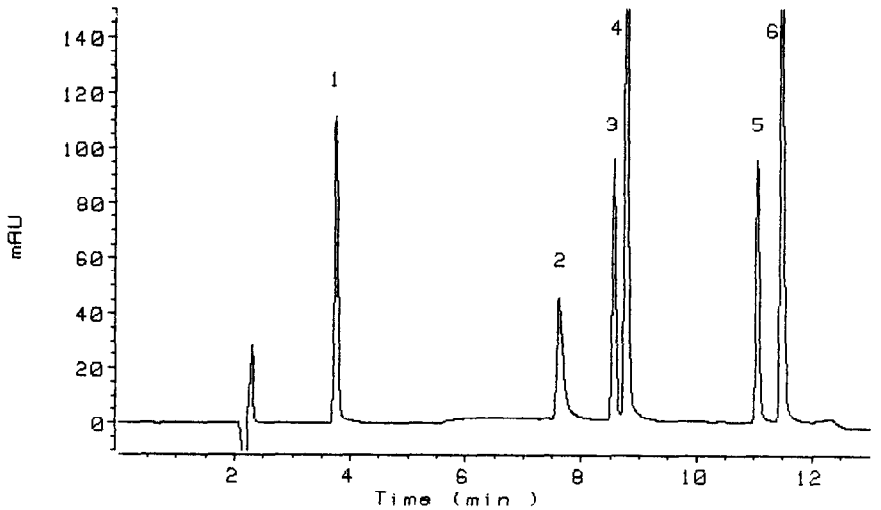

Fig. 1. Chromatogram of standards obtained by gradient elution. Peaks: 1 = *p*-hydroxybenzoic acid; 2 = salicylic acid; 3 = benzoic acid; 4 = sorbic acid; 5 = ethyl *p*-hydroxybenzoate acid; 6 = *p*-chlorobenzoic acid.

dient elution. The mobile phase was acetic acid (pH 3) with acetonitrile as organic modifier.

Different acids were used to adjust the pH of solvent A to 3. When sulphuric or phosphoric acid was used, distortion of the peaks occurred. A good baseline in wine analysis was obtained when acetic acid was used. The pH was established at 3 because higher values increased peak distortion and shoulders on the peaks appeared; on the other hand, low values increased absorption of the mobile phase.

Fig. 1 shows a chromatogram of a standard mixture of compounds obtained with the above experimental conditions. Under these conditions a good separation of the different compounds was obtained within 12 min.

In all the experiments carried out to obtain the optimum conditions in the gradient elution, ascorbic acid and saccharin eluted with short retention times, owing to the high polarity of these compounds. When a wine sample spiked with a standard solution was analysed, these compounds co-eluted with other compounds present and for this reason the determination of these two additives was not possible by the gradient elution method. Under the experimental conditions chosen, only six of the eight additives studied appeared in the chromatogram.

Fig. 2 shows the chromatogram obtained when a red wine sample spiked with a standard solution was analysed. A baseline distortion can be observed caused by the large number of compounds present in wine and which absorb at this wavelength when the gradient elution method is used. This distortion of the baseline can cause difficulties with determinations, mainly for salicylic acid.

Solid-phase extraction can be used to clean up the sample before injection. The great difference in the polarities of the compounds studied made it difficult to carry out this extraction with a Sep-Pak C_{18} cartridge. Only Terada and Sakabe [12] used this method after the formation of an ion pair with CTAB. In this study, the clean-up of the sample was carried out with LC-SAX tubes. The chromatogram of the same red wine spiked with the standard solution after solid-phase treatment is shown in Fig. 3.

In order to optimize the extraction conditions with the SAX cartridge, after 1 ml of the sample spiked with the standard solution had passed through the cartridge, different volumes of sulphuric acid were tested to elute the compounds; 1, 1.5 and 2 ml of 0.5 *M* sulphuric acid were compared (Table II), and good results were obtained with 1.5 ml. Higher elution volumes involved greater dilu-

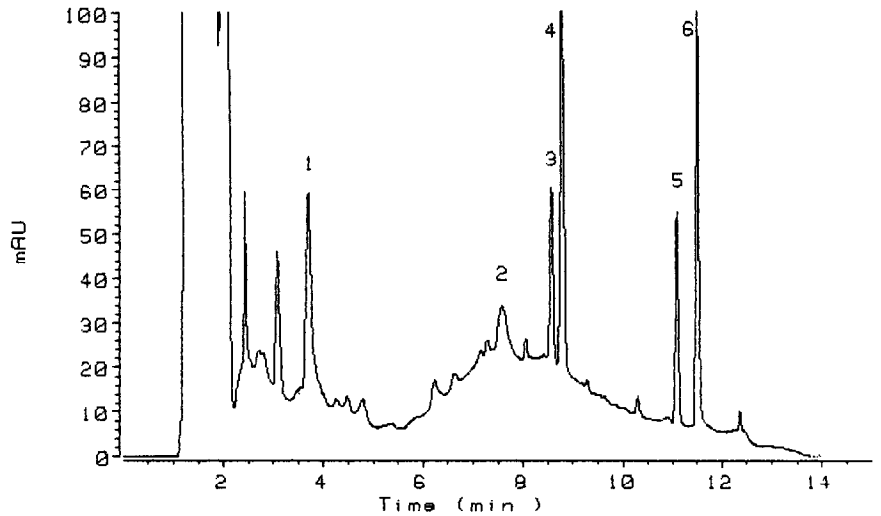

Fig. 2. Chromatogram of red wine spiked with standards obtained by gradient elution without SAX extraction. Peaks as in Fig. 1.

tion of the sample, and the results were not improved. A concentration of sulphuric acid lower than 0.5 *M* did not elute compounds with good recoveries.

The purity of the peaks was checked with the HP 1040 diode-array detector. The peaks for a wine sample were compared with those for the standard and the match factor was determined. In all instances the match factors were higher than 990, indicating that the peaks were pure.

Before this method can be applied to real samples it is necessary to validate the method and determine the recovery, repeatability, reproducibility, linearity and detection limit. To determine the recovery of the method, including SAX treatment, a red wine sample was spiked with different concentrations of

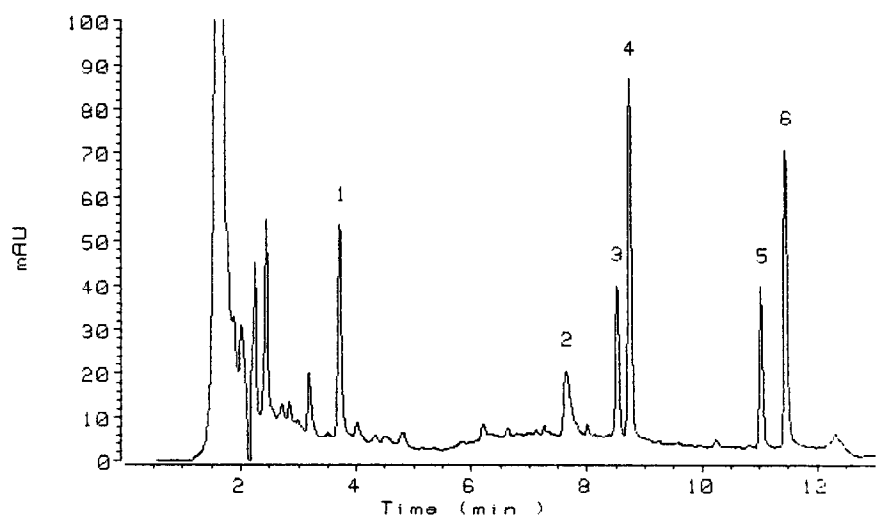

Fig. 3. Chromatogram of the same red wine as in Fig. 2 spiked with a solution of standards obtained by gradient elution after SAX extraction. Peaks as in Fig. 1.

TABLE II

EFFECT OF THE VOLUME OF 0.5 *M* SULPHURIC ACID IN THE SAX TREATMENT

Compound	Volume of H_2SO_4 (ml)	Amount added (ppm)	Found (ppm)	Recovery (%)
p-Hydroxybenzoic acid	1	10.41	9.50	91.3
	1.5		9.30	89.3
	2		9.23	88.7
Salicylic acid	1	10.07	7.75	77.2
	1.5		9.38	93.2
	2		9.24	91.8
Benzoic acid	1	10.51	9.76	92.9
	1.5		10.76	102.4
	2		11.24	107.0
Sorbic acid	1	10.80	8.89	83.1
	1.5		9.13	84.5
	2		9.44	87.4
Ethyl *p*-hydroxybenzoate	1	9.90	8.74	88.3
	1.5		8.94	90.3
	2		8.97	90.6
p-Chlorobenzoic acid	1	10.33	7.01	67.9
	1.5		8.96	86.7
	2		9.35	90.5

the standard solution and the results are given in Table III.

A study of the repeatability of the method and its reproducibility between days was performed. The results for repeatability showed a relative standard deviation ($n = 10$) ranging from 1.8 to 4% and those for reproducibility between days from 2.5 to 5%.

Good linearity of response was obtained for all the compounds studied between 5 and 50 ppm. For sorbic acid, whose addition is allowed up to 200 ppm, linearity was studied from 5 to 300 ppm.

The detection limit of the method was 0.5 ppm. However, this could be enhanced for a particular analysis by using the wavelengths of maximum absorption (benzoic acid, 225 nm; sorbic acid, *p*-hydroxybenzoic acid and its ethyl ester, 260 nm; and salicylic acid and *p*-chlorobenzoic acid , 235 nm). In this work a wavelength of 240 nm chosen for the simultaneous detection of all these compounds.

Isocratic elution

The different polarities among the additives requires ion-pair formation in order to determine them by isocratic elution. As a preliminary step, it was necessary to select the ion-pair reagent. Two ion-pair reagents, CTAB and tetrabutylammonium bromide, were studied. After preliminary experiments, CTAB was chosen because it was not possible to determine sorbic acid with tetrabutylammonium bromide under the conditions chosen for this study.

Different variables influencing the separation were studied. In the first step, pH was optimized because it had the greater influence on the resolution, especially in the separation of sorbic and benzoic acids.

The influence of pH on k' is shown in Fig. 4. The influence of pH was similar for all the acids but different from that observed for saccharin and ethyl *p*-hydroxybenzoic acid. Fig. 4 shows the different k' values of *p*-chlorobenzoic acid at different pH values and its high value with respect to the others. After different experiments a pH of 5.5 was chosen.

The concentration of the buffer solution was studied. Fig. 5 shows a decrease in k' at higher percentages of buffer for all the compounds except of ethyl *p*-hydroxybenzoate, which showed a different

TABLE III

STUDY OF THE RECOVERY WITH SAX TREATMENT

Compound	Amount added (ppm)	Found (ppm)	Recovery (%)
p-Hydroxybenzoic acid	5.20	5.18	99.7
	10.41	9.30	89.3
	31.23	27.67	88.6
	52.05	46.06	88.5
Salicylic acid	5.04	3.97	78.7
	10.07	9.38	93.2
	30.21	27.58	91.3
	50.35	46.22	91.8
Benzoic acid	5.26	6.00	114.1
	10.51	10.76	102.4
	31.53	31.40	99.6
	52.55	52.34	99.6
Sorbic acid	5.40	5.02	92.9
	10.80	9.13	84.5
	32.40	27.67	85.4
	54.00	45.74	84.7
Ethyl *p*-hydroxybenzoate	4.95	5.00	100.9
	9.90	8.94	90.3
	29.70	26.05	87.7
	49.50	43.31	87.5
p-Chlorobenzoic acid	5.16	4.99	96.7
	10.33	8.96	86.7
	30.99	26.90	86.8
	51.65	45.19	87.5

behaviour. The best separation was obtained at 10%, as the separation between ascorbic, *p*-hydroxybenzoic acid and ethyl *p*-hydroxybenzoate was better than that obtained at 5%, and *p*-hydroxybenzoic acid and its ester overlapped at 15%.

Fig. 6 shows the effect of CTAB concentration on the capacity factors. An increase in k' is observed with increase in CTAB concentration, except for ethyl *p*-hydroxybenzoate, which showed only a slight increase. Overlapping of different peaks appeared at CTAB concentrations less than 2 m*M* but concentrations higher than 2 m*M* resulted in higher k' values. Therefore, a concentration of 2 m*M* was chosen as the optimum.

The effect of the percentage of acetonitrile was studied and the results are shown in Fig. 7. A value

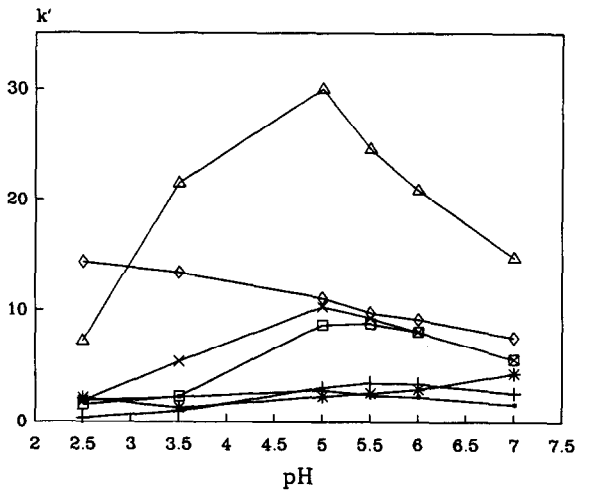

Fig. 4. Effect of the pH of the buffer solution added to the mobile phase containing 2 m*M* CTAB as an ion-pair reagent, 5% of buffer solution and 35% of acetonitrile on the k' of the additives. ● = Ascorbic acid; + = *p*-hydroxybenzoic acid; ∗ = ethyl *p*-hydroxybenzoate; □ = sorbic acid; × = benzoic acid; ◇ = saccharin; △ = *p*-chlorobenzoic acid.

of 35% was chosen because this is the maximum concentration that involved no co-elution of peaks and gave an acceptable analysis time.

From the different experiments carried out, the optimum conditions chosen were 35% of acetoni-

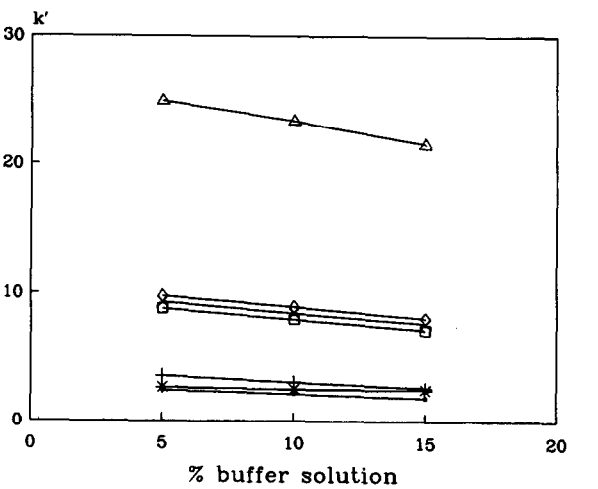

Fig. 5. Effect of the concentration of buffer solution (pH 5.5) on the k' of the additives. Mobile phase containing 2 m*M* of CTAB and 35% of acetonitrile. Symbols as in Fig. 4.

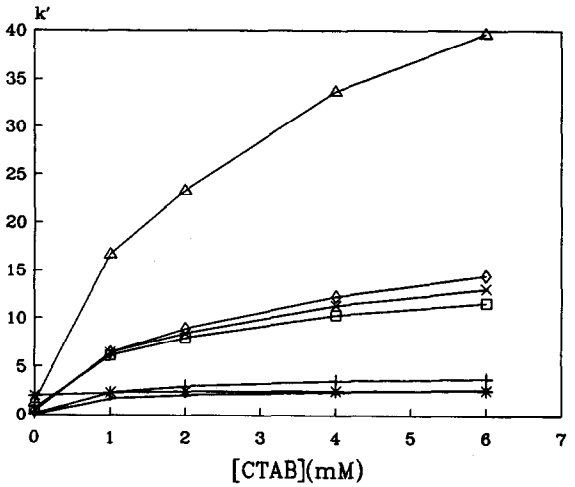

Fig. 6. Effect of the concentration of CTAB on k' of the additives. Mobile phase, acetonitrile–water–buffer (pH 5.5) (35:55:10). Symbols as in Fig. 4.

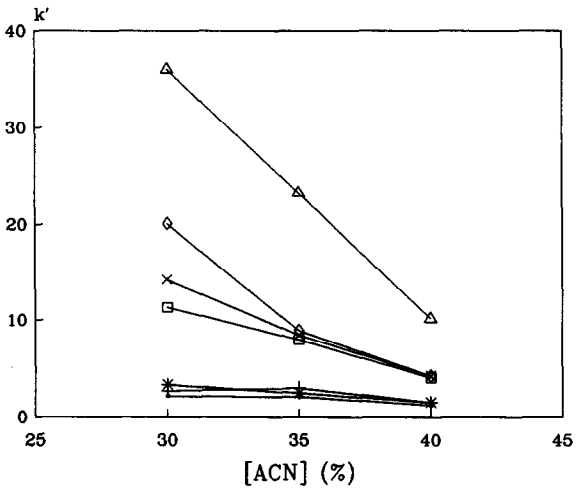

Fig. 7. Effect of the concentration of acetonitrile on the k' of the additives. Mobile phase containing 2 mM CTAB and 10% of buffer solution at pH 5.5. Symbols as in Fig. 4.

trile, 10% of buffer solution at pH 5.5 and an ion-pair concentration of 2 mM. The analysis time under these conditions was 40 min and good resolution between the different peaks was obtained. A chromatogram of a standard solution is shown in Fig. 8. As can be seen, the long analysis time is due to the determination of *p*-chlorobenzoic acid. This is not, however, the most important additive, and when its determination is not required, the analysis time decreases to 18 min.

Salicylic acid could not be determined by this method because of the distortion of the peak and its low sensitivity under the conditions adopted.

The optimization of the mobile phase was carried

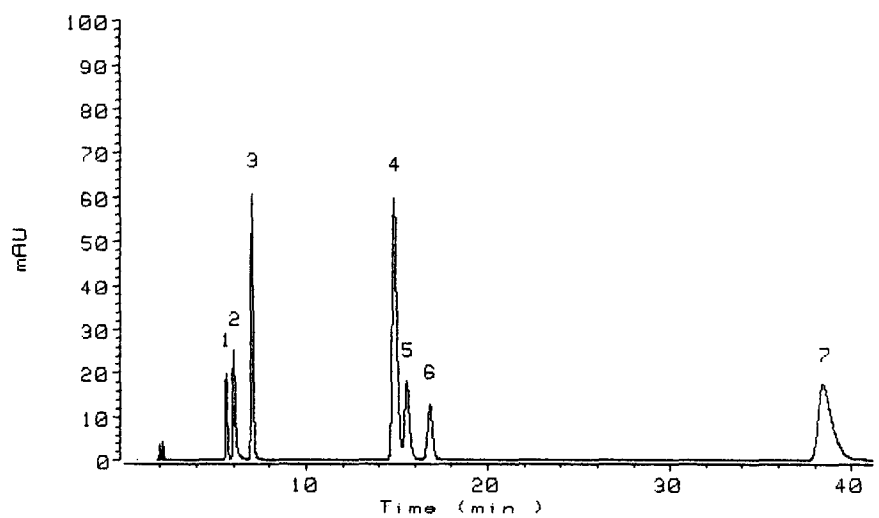

Fig. 8. Chromatogram of standards obtained under the optimum experimental conditions. Peaks: 1 = ascorbic acid; 2 = ethyl *p*-hydroxybenzoate; 3 = *p*-hydroxybenzoic acid; 4 = sorbic acid; 5 = benzoic acid; 6 = saccharin; 7 = *p*-chlorobenzoic acid.

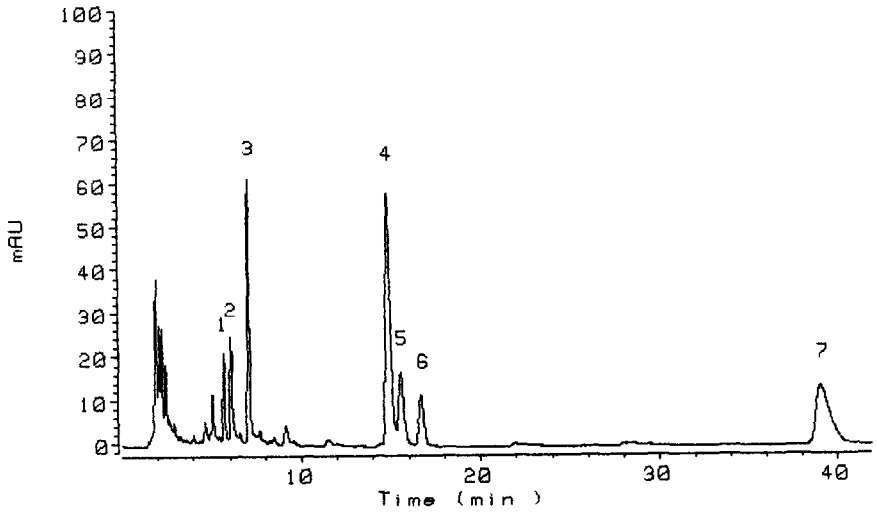

Fig. 9. Chromatogram of a red wine spiked with standards. Peaks as in Fig. 8.

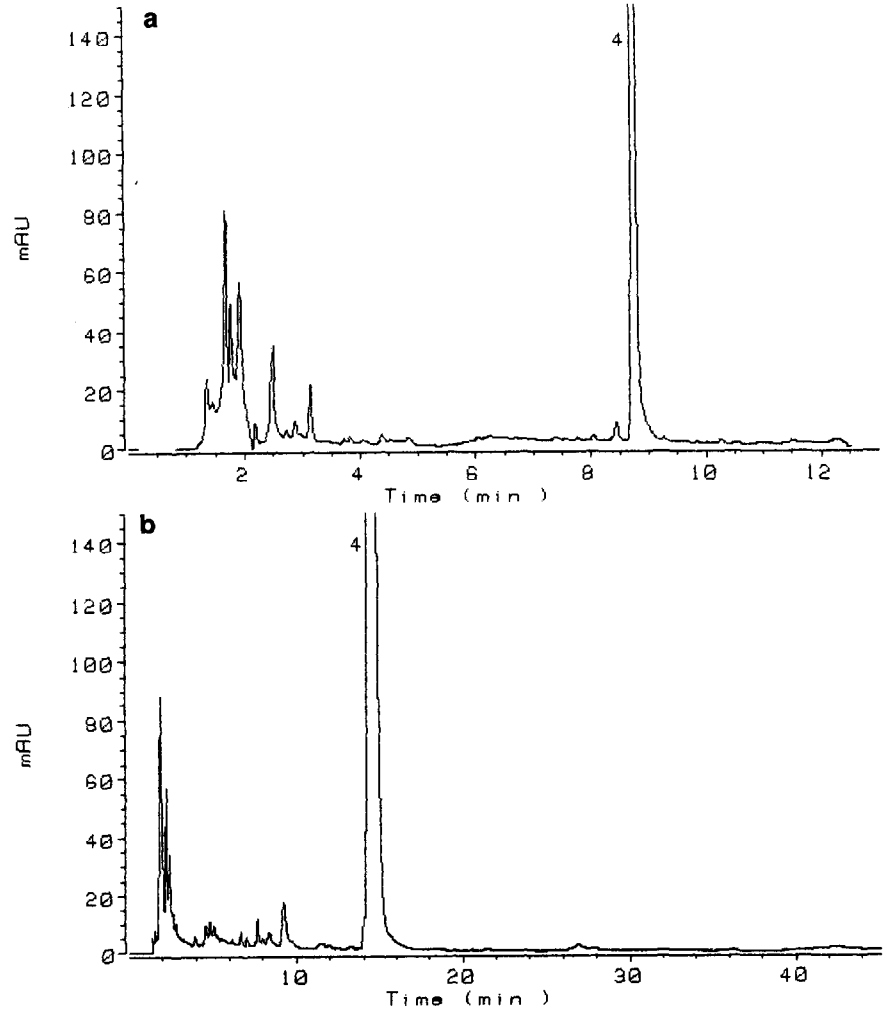

Fig. 10. Chromatogram of a white wine in which sorbic acid was detected (peak 4). (a) Gradient elution method; (b) isocratic method.

out taking into account the possible co-elution of other substances when a wine is analysed. The elution of a red wine sample spiked with a standard solution is shown in Fig. 9.

Similar studies carried out with the gradient method were applied to validate this isocratic method. Matrix interference was studied and no interference was obtained with either by red wine or white wine, so no sample pretreatment is necessary. The repeatability of the method showed a relative standard deviation (n = 10) between 2.0% and 4.2% and the reproducibility between days was between 2.7% and 6.1%.

The detection limit of the method was established as 0.5 ppm for all the substances except ascorbic acid (3 ppm). Good linearity of response was obtained between the same range of concentrations. Linearity for ascorbic acid was obtained from 5 to 300 ppm and for saccharin from 5 to 50 ppm.

After both methods had been validated, several wine samples were analysed to determine these compounds. Only in some of them was sorbic acid detected, at a very low concentration. The chromatograms obtained by the two methods for one of the white wines analysed, in which sorbic acid was detected, are shown in Fig. 10. The result obtained by the gradient method was 170 ppm and by the isocratic method 162 ppm.

The purity of the sorbic acid peak was checked and factors of 995 and 994 were obtained for the peak obtained by the gradient method and the isocratic method, respectively. To confirm the identification, each spectrum was compared with one recorded in a UV–VIS spectral library and factors of 992 and 994 were obtained.

CONCLUSIONS

Two methods for determining additives in wine samples were developed. The gradient method showed good results and a short analysis time, but no determination of the most polar compounds was possible under the conditions studied. In this event, if a red wine is analysed, pretreatment of the sample is required to obtain good results. On the other hand, ion-pair chromatography allows the separation of the additives, including saccharin and ascorbic acid, by isocratic elution with no pretreatment of the sample, but the analysis time is longer if *p*-chlorobenzoic acid is to be determined.

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