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Optimization of the separation of phenolic compounds by micellar electrokinetic capillary chromatography

M.A. Rodríguez-Delgado*, M.L. Pérez, R. Corbella, G. González, F.J. García Montelongo

Department of Analytical Chemistry, Nutrition and Food Science, University of La Laguna, 38071 Tenerife, Spain

Abstract

A group of phenolic compounds including phenolic aldehydes, acids and flavonoids are separated by micellar electrokinetic chromatography (MECC). The influence of buffer (concentration and pH), concentration of sodium dodecylsulphate (SDS) and applied voltage were studied. To increase the selectivity of the separation and the resolution of the solutes organic solvents are added to the separation buffer, the best results were obtained when methanol was used at lower percentages. An optimized buffer (150 m*M* boric acid (pH 8.5)–50 m*M* SDS–5% methanol) provides the optimum separation with regard to resolution and migration time. This method was applied to the determination of these compounds in wine samples with good results.

Keywords: Wine; Optimization; Phenolic compounds

1. Introduction

Phenolic compounds are a wide group of substances that have particular importance in enology. These substances contribute to several sensorial characteristics, such as colour, flavour, astringency and hardness of wine, directly or by the formation of complexes with proteins, the formation of insoluble polymers and the interaction with other phenolic compounds. Furthermore, these compounds are important in food hygiene due to their bactericidal effects and consequently they are essential in the quality of a wine [1–4]. The types and concentrations of the phenolic compounds depend on the grape variety and ripening, atmospheric conditions and the techniques employed in producing the must. It is well-known that the contact of the must and wine with a wood barrel during the aging process accounts for the presence of some phenols in wine.

The similar chemical characteristics of these compounds and the complexity of wine samples make the use of chromatographic methods with high resolution, such as thin layer chromatographic (TLC) and high-performance liquid chromatographic (HPLC), necessary for their determination [5–10]. However, these methods are very expensive, use toxic solvents, have high separation times and sometimes need the development of extremely complex gradients for the separation. Capillary electrophoresis (CE) has been shown to be a fast, powerful, clean and efficient separation technique for a wide variety of compounds [11–15]. These characteristics are related to the use of high separation voltages and efficient dissipation of Joule heat in a narrow fused-silica capillary (typically 25–100 μm I.D.). Micellar electrokinetic capillary chromatography (MECC) continues to gain popularity as a useful technique for separating analytes difficult to separate by capillary zone electrophoresis (CZE). MECC, which is a modification of CZE, has extended the utility of CE

*Corresponding author.

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to the separation of neutral analytes under the influence of an electric field [16]. In this migration mode a surfactant is added to the buffer at a concentration above its critical micellar concentration. The separation principle is based on the differential partition of analytes between the micelle, which is a pseudostationary phase and the surrounding aqueous phase. Compounds such as flavonoids strongly interact with micelles and consequently selectivity may be varied by modifying micelle concentration. Micellar liquid chromatography (MLC) [17] shows that small amounts of organic solvents may improve separation efficiency and modify the selectivity, thus the use of an organic modifier is commonly accepted in CE. Therefore, the type and concentration of the surfactants and the type and concentration of the organic modifier of the aqueous phase is important for the optimization of the efficiency and separation selectivity [18–22].

The development of photodiode-array detectors has increased the advantages of CE in the study of phenolic compounds [23,24], as UV–visible spectra provided by these detectors can be used for identification of peaks by comparison with standards, and for checking peak purity in complex samples such as wines samples. Modifications in selectivity can be detected with minimal experiences, which is very useful to develop the optimization procedure quickly.

The aim of this investigation was to develop a method for the rapid analysis of a group of phenolic compounds by MECC using sodium dodecylsulfate as the surfactant. The influence of buffer pH, concentration of the electrolyte, concentration of the surfactant, type and concentration of the organic modifier (e.g. methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, acetone and acetonitrile) and voltage on the migration behaviour was studied. Finally, using the optimised method examples of analyses of phenolic compounds in Spanish wine samples are presented.

2. Experimental

2.1. Apparatus

The analysis were carried out in a P/ACE 5510 HPCE (Beckman Instruments, Fullerton, CA, USA) electrophoresis apparatus equipped with a diode array detector. The fused-silica capillary used was 57 cm (50 cm effective length)×75 μm I.D. (Beckman Instruments, Fullerton, CA, USA). Data were collected and analysed using the System Gold software from Beckman running on a 486DX2-66 MHz computer.

2.2. Reagents

All chemicals were of analytical grade. All phenol (Sigma Chemical Co., St. Louis, MO, USA), standard solutions were prepared in HPLC grade methanol (Montplet and Esteban, Barcelona, Spain). Stock 1000 mg/l standard solutions of each compound was prepared in methanol and stored at −4°C. Working standard solutions were prepared by diluting with methanol. Sodium dodecylsulphate (SDS), 2-(*N*-cyclohexylamino)-ethanesulfonic acid (CHES) and *N*-(2-hydroxyethyl)piperazine-*N′*-(2-ethanesulfonic acid) (HEPES) were obtained from Aldrich (Milwaukee, WI, USA). Acetonitrile, ethanol, 1-propanol, 2-propanol, 1-butanol and acetone, used as solvent modifiers, were obtained from Merck (Darmstadt, Germany). Buffer solutions were prepared from boric acid (Merck, Darmstadt, Germany) (concentration range: 10–150 m*M* and SDS (10–100 m*M*) by dissolving them in ultrapure water from Milli-Q system (Millipore, Bedford, USA) with a conductivity of 18 MΩ. The final pH values were adjusted with 1.0 *M* sodium hydroxide. The final volume was adjusted by the addition of water and the organic modifier (3–20%). All buffer solutions were filtered through a 0.22 μm poliamide filter (Sartolon, Sartorius) and sonicated prior to usage.

2.3. Electrophoretic procedure

The capillary was conditioned prior to use by flushing with 1.0 *M* NaOH for 10 min, with water for 2 min and finally with the running buffer for 15 min. Injections were made using N_2, pressure 0.5 p.s.i. for 2 s (1 p.s.i.=6894.76 Pa). The external temperature of the capillary was 25°C. Detection took place at 280 nm. In order to increase the migration time reproducibility, the capillary was rinsed between injections with 0.1 *M* NaOH for 2 min, ultrapure water for 1 min and fresh buffer for 3

min. If drastic drifts in electrophoretic current and/or migration times were observed, the capillary was rinsed with 1.0 *M* NaOH solution for 15 min followed by 10-min rinses with deionized water, methanol, deionized water again and the running buffer.

Duplicate injections of the solutions were performed and average peak areas corrected (area/migration time) were used for the quantitation.

2.4. Mobility calculations

The electrophoretic mobility of each analyte was calculated from the experimental migration time by the equation:

$$\mu_{ep} = \mu - \mu_{eo} = \frac{L_d L_t}{V}\left(\frac{1}{t_m} - \frac{1}{t_{eo}}\right)$$

where μ_{ep} is the electrophoretic mobility of the analyte, μ is the apparent mobility, μ_{eo} is the electroosmotic mobility, t_m is the migration time measured from the electropherogram, t_{eo} is the migration time for an uncharged solute (i.e. methanol), L_t is the total length of capillary, L_d is the length of capillary between injection and detection points and V is the applied voltage.

2.5. Sample preparation

Samples of comercially available wines from different Spanish regions were analysed with the proposed method. Wine (5 ml) was extracted with diethyl ether (5 ml) for 15 min using a Selecta Rotabit (Selecta, Barcelona, Spain) at 120 u min^{-1}. The ether layer was separated and evaporated to dryness using a nitrogen stream. The dry residue was dissolved in 0.5 ml of methanol and aliquots injected into the CE system.

3. Results and discussion

3.1. Effect of buffer pH

In CE separation the buffer pH is one of the most important parameters since its control determines the extent of ionisation and mobility of each solute. The inclusion of flavonoids, phenolic acids and aldehydes in the mixture tested makes pH optimization necessary, which depends on the different structures of these compounds. The influence of the pH was studied using three buffer systems in the neutral and basic region: 10 m*M* boric–borate at pH 8.0, 8.5 and 9.0; 10 m*M* HEPES at pH 7.0 and 10 m*M* CHES at pH 10.0. Every buffer was 50 m*M* SDS. Fig. 1 shows the influence of pH in the separation of this group of compounds. It is evident that at lower pH values (<8.5) the peaks are broader and with low resolution. The same effect is observed at pH 10.0 when CHES is used as buffer electrolyte. However, pH 9.0 leads to sharp peaks but with limited resolution. Although, the analysis time is higher when pH 8.5 is used, the resolution for the first and the last peaks is improved. So, the resolution between the pairs caffeic acid–kaempferol, vanillic acid–myricetin, (+)-catechin–(−)-epicatechin and quercetin–rutin is better than obtained at pH 9.0. When pH increases the migration time becomes shorter, but the resolution diminishes drastically at higher pHs. Thus, a pH of 8.5 was selected as optimum in order to minimise analysis times and good resolution between peaks.

3.2. Effect of micelle concentration

Fig. 2 shows the electrophoretic mobility of each phenolic compound at different concentrations of SDS. Different behaviour is observed when the concentration of surfactant is varied in the range of 10–100 m*M* at pH 8.5. The electrophoretic mobility of caffeic acid increases with increasing surfactant concentration, with a maximum at 50 m*M* SDS. Kaempferol and (−)-epicatechin present the same behaviour, while myrecitin and vanillic acid mobility diminish slightly as surfactant concentration increases. The electrophoretic mobility of the rest of the solutes diminishes markedly with increasing surfactant concentration. This diminution can be justified by the formation of borate complexes with the hydroxyl groups on the phenol ring resulting in one negative charge in the complex. Although, the increased size of the complex should facilitate its partitioning into the micellar phase, the strong electrostatic repulsion between the complex and the micelles of SDS results in longer migration time [24]

Although, the migration order of phenolic com-

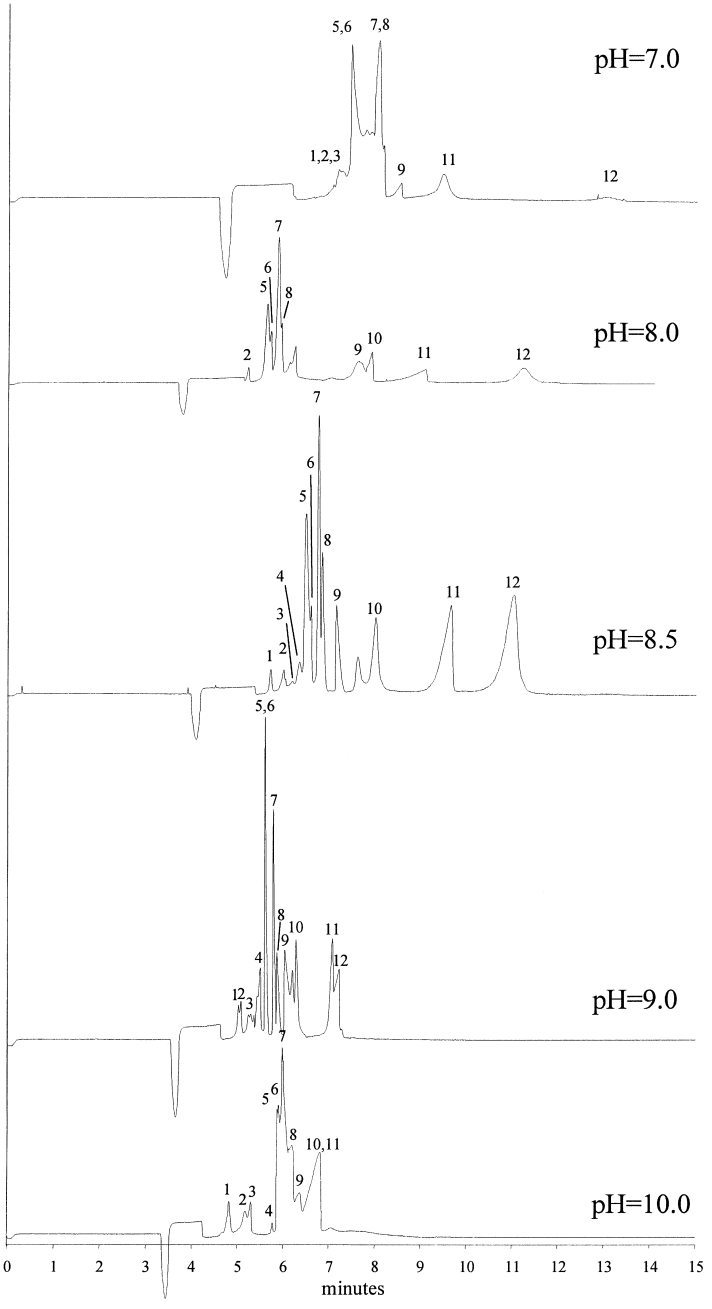

Fig. 1. Capillary electropherograms of a set of 12 phenolic compounds with 50 m*M* SDS as EOF modifier at: pH 7.0 (10 m*M* HEPES); pH 8.0, 8.5 and 9.0 (10 m*M* boric acid); pH 10.0 (10 m*M* CHES). Operating conditions: fused-silica capillary (57 cm×50 cm, 75 μm I.D.), temperature=25°C, voltage=20 kV, detection=280 nm, hydrodinamic injection=2 s. Peak identification: (1) (+)-catechin, (2) (−)-epicatechin, (3) quercetin, (4) rutin, (5) protocatechuic-aldehyde, (6) syringealdehyde, (7) ferulic acid, (8) *p*-coumaric acid, (9) vanillic acid, (10) myricetin, (11) kaempferol, (12) caffeic acid.

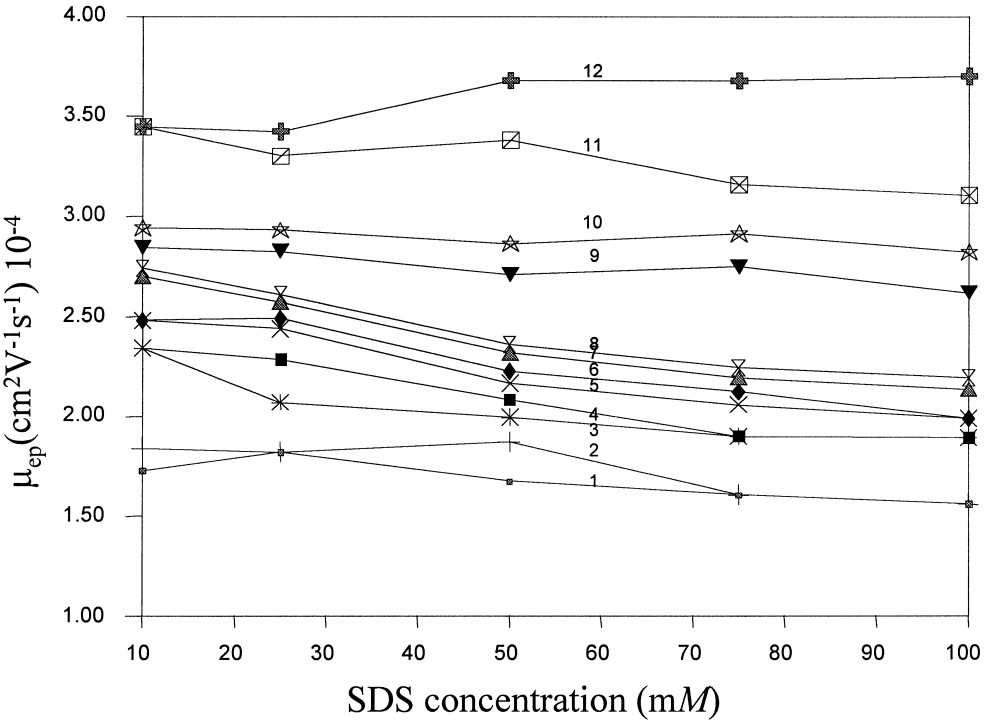

Fig. 2. Effect of SDS concentration on the electrophoretic mobility of phenolic compounds with a fixed concentration of 10 m*M* boric acid at pH 8.5. Analyte identification and other operating conditions are the same as for Fig. 1.

pounds with surfactant concentration remains the same, the resolution varies considerably as SDS concentration increases from 10 to 50 m*M* (Fig. 2). It can be observed that at low SDS concentration the couples of quercetin–rutin, protocatechuic-aldehyde–syringealdehyde and caffeic acid–kaempherol comigrate. However, the pairs can be resolved when SDS concentration increases until 50 m*M*. At higher SDS concentrations the couples quercetin–rutin and protocatechuic-aldehyde–syringealdehyde comigrate again. Therefore, the best separation was obtained with an intermediate SDS concentration (50 m*M*) in the buffer.

3.3. Effect of the electrolyte concentration

The influence of the concentration of boric acid in the range 10–150 m*M* on the separation of 12 phenolic compounds was examined with 50 m*M* SDS at pH 8.5. Fig. 3 shows the electrophoretic mobility of these compounds at different concentrations of boric acid. The mobility diminishes slightly with increasing boric acid concentration, but better selectivity for the separation is attained when boric acid concentration is increased. So a reversal migration order for the couples protocatechuic-aldehyde–rutin and caffeic acid–kaempherol at higher boric acid concentrations can be observed. Although, the separation between the couple protocatechuic-aldehyde–syringealdehyde becomes worse with higher boric acid concentrations a baseline separation for quercetin–rutin and ferulic acid–*p*-coumaric acid couples was obtained. Hence, 150 m*M* was chosen as the optimum concentration of boric acid.

3.4. Effect of applied voltage

The effect of applied voltage on the resolution was examined in the range 10–30 kV at pH 8.5. Although, the increase of the EOF results in shorter analysis time and an improvement of the efficiency, the resolution decreases when the applied field strength increases. Furthermore, the intensity increases drastically to more than 100 μA at higher

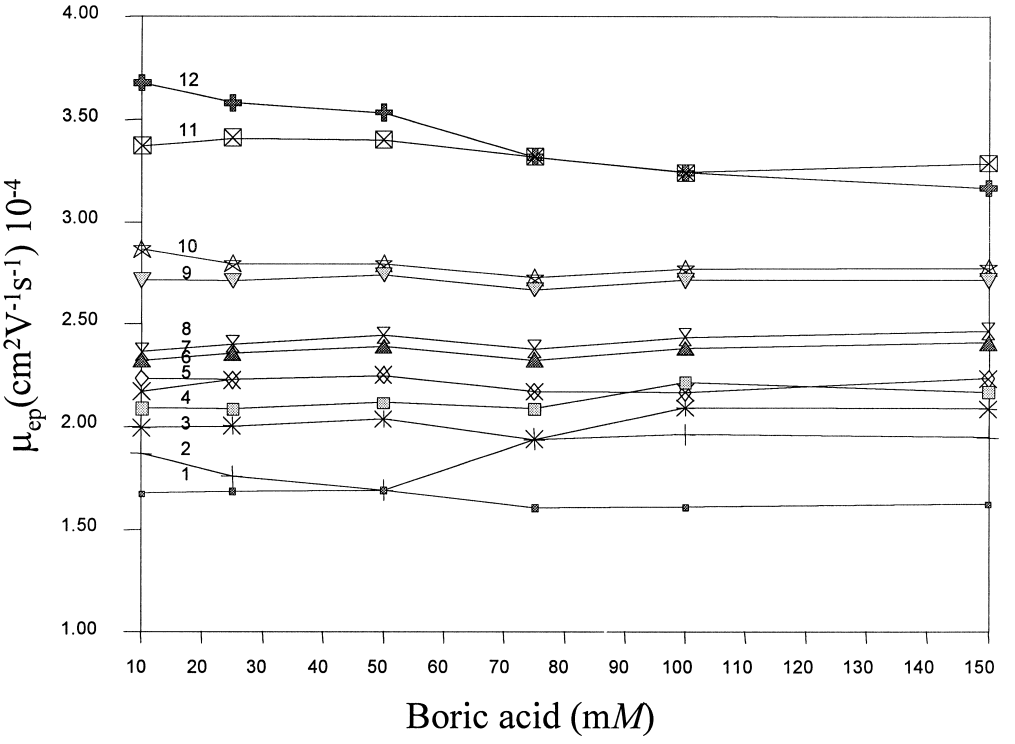

Fig. 3. Effect of the concentration of boric acid on the electrophoretic mobility of phenolic compounds with a fixed concentration of 50 m*M* SDS at pH 8.5. Analyte identification and other operating conditions are the same as for Fig. 1.

applied voltages, which suggests that Joule heating cannot be ignored at voltages higher than 20 kV. So, the separation has been achieved at 20 kV with an analysis time lower than 18 min.

3.5. Effect of organic modifier

Organic solvents can be added to the buffer solution in order to improve separation, resolution and to increase the elution window by reducing the EOF [25–28]. The influence of organic modifiers was examined using 150 m*M* boric acid–50 m*M* SDS buffer of pH 8.5. Fig. 4 shows the separation of this group of compounds in the presence of methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, acetone and acetonitrile. Although, the addition of alcohols resulted in a decreasing of the EOF and, consequently, an increasing in time of analysis, an improvement in the separation efficiency was obtained. An exception was caffeic acid, which highly increased its electrophoretic mobility when organic modifiers were added to the buffer. Furthermore, the resolution diminished when the chain length of the alcohol increased (Fig. 4). So, with 1-butanol the efficiency for the last peak (kaempferol) was very bad as an overlapped peak was obtained and the resolution for the couple (+)-catechin–(−)-epichatechin was lost. Although, 2-propanol reduced the analysis time, the resolution for the last peaks diminished and so, vanillic acid, myricetin and caffeic acid were overlapped. The addition of acetonitrile reduced the efficiency of the last peak and the resolution for the couple vanillic acid–myricetin was null.

The best results were obtained when methanol was added to the buffer. So, the efficiency and resolution between the couples rutin–syringealdehyde, ferulic acid–*p*-coumaric acid and kaempferol–caffeic acid were improved drastically. The elution order between myrecitin and caffeic acid was altered, which can be justified in terms of the partition equilibrium of solute and alcohol with SDS micelles.

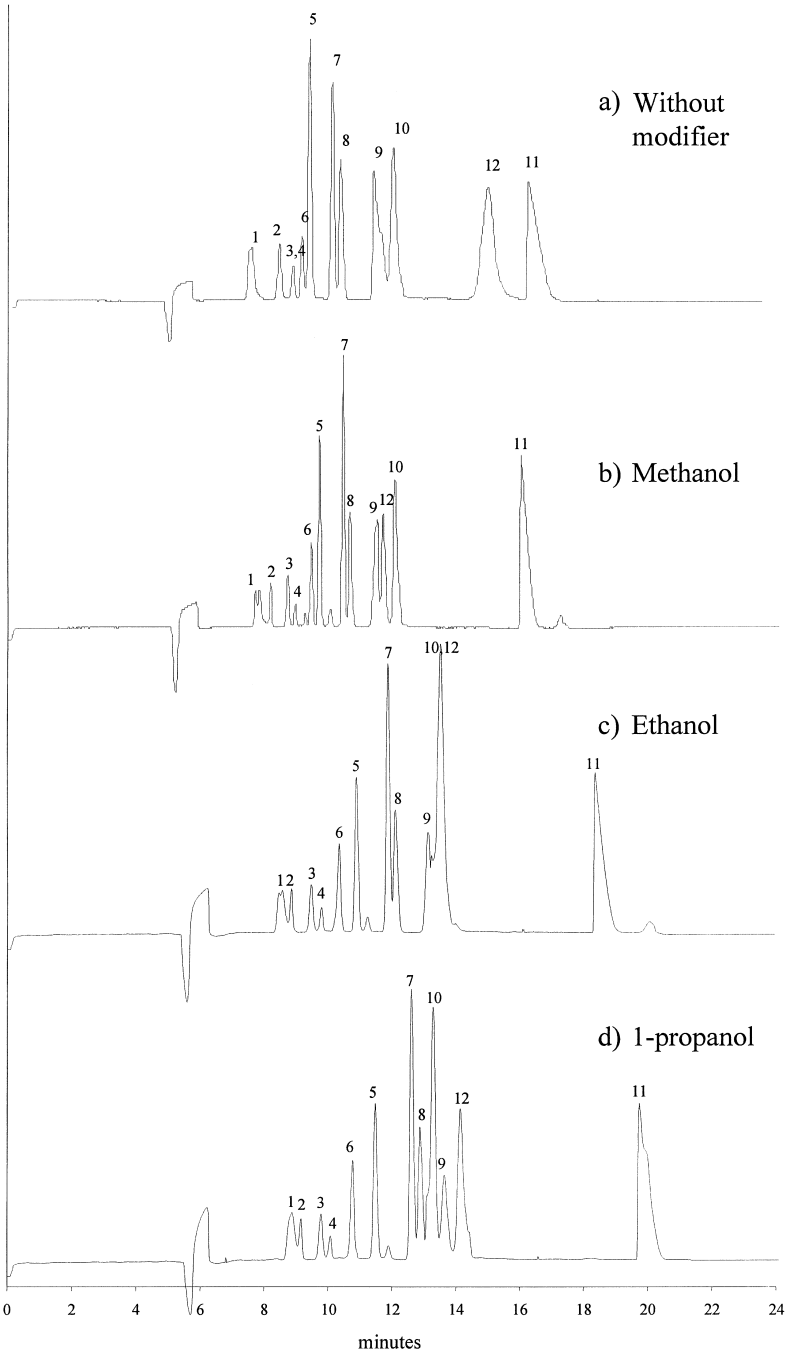

Fig. 4. Influence of various organic solvents at 5% (v/v) on the separation of phenolic compounds: (a) without modifier, (b) methanol, (c) ethanol, (d) 1-propanol, (e) 2-propanol, (f) 1-butanol, (g) acetone, (h) acetonitrile. Buffer solution: 150 m*M* boric acid–50 m*M* SDS at pH 8.5. Peak identification and other operating conditions are the same as for Fig. 1.

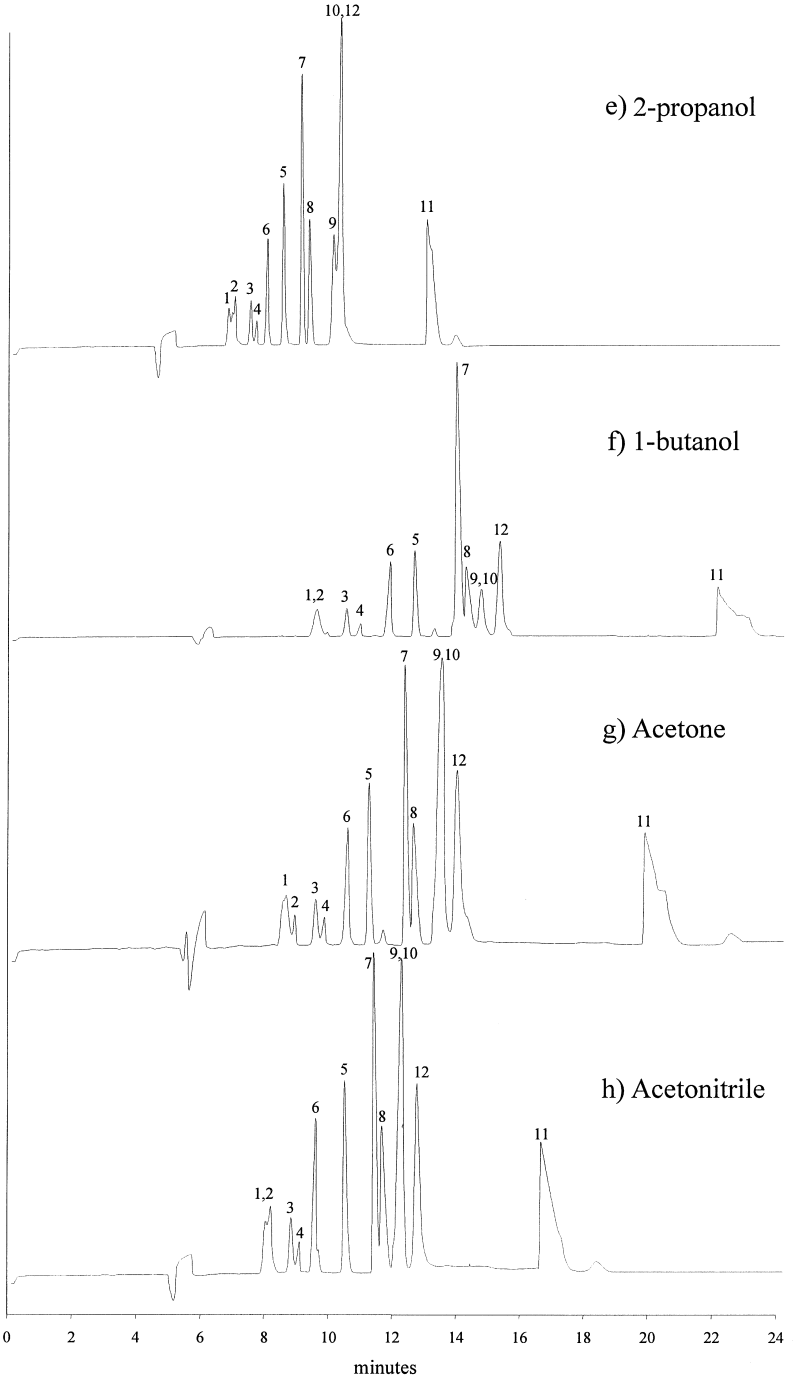

Fig. 4 (*continued*).

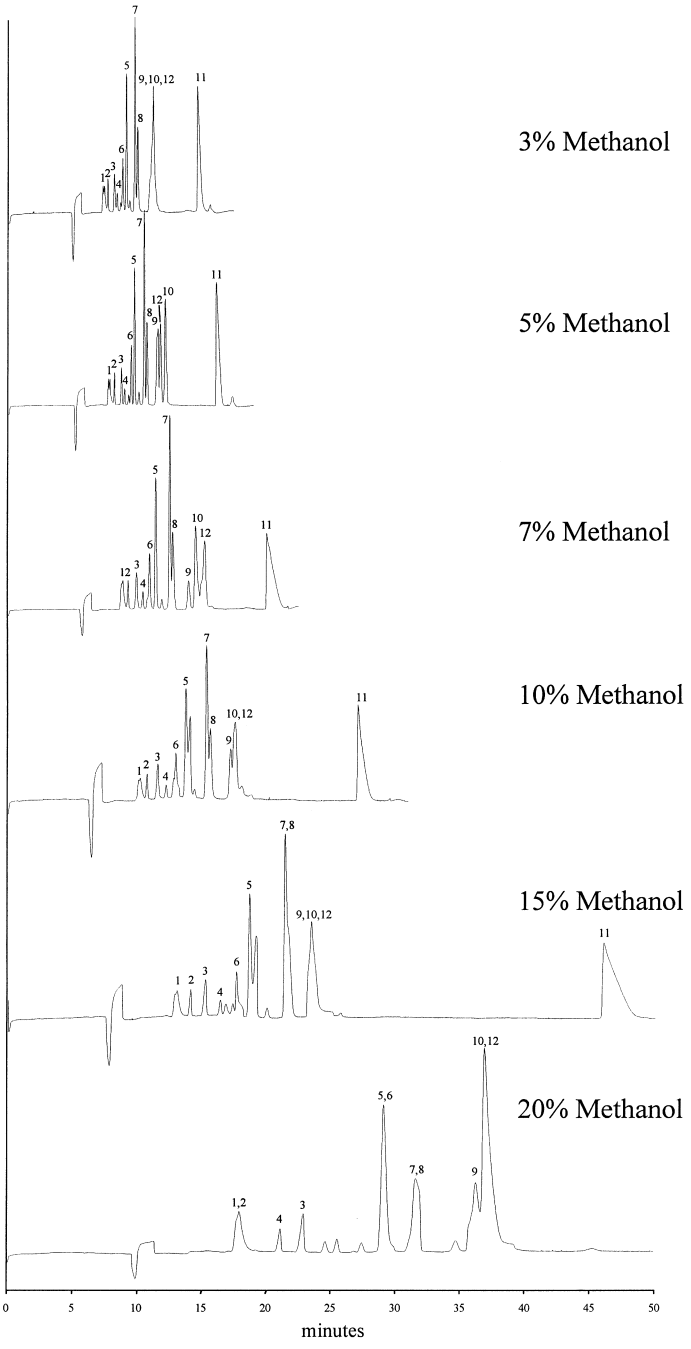

Fig. 5. Influence of methanol percentage on the separation. Buffer solution: 150 m*M* boric acid–50 m*M* SDS at pH 8.5. Peak identification and other operating conditions are the same as for Fig. 1.

In order to find the best compromise between resolution and analysis speed, the effect of the concentration of methanol as organic modifier was investigated, and the results are shown in Fig. 5. It is observed that high methanol contents drives unsatisfactory separations, which is agreement with the statement of Janini and Isaaq [29] that the addition of organic solvent above 15% drops the column ef-

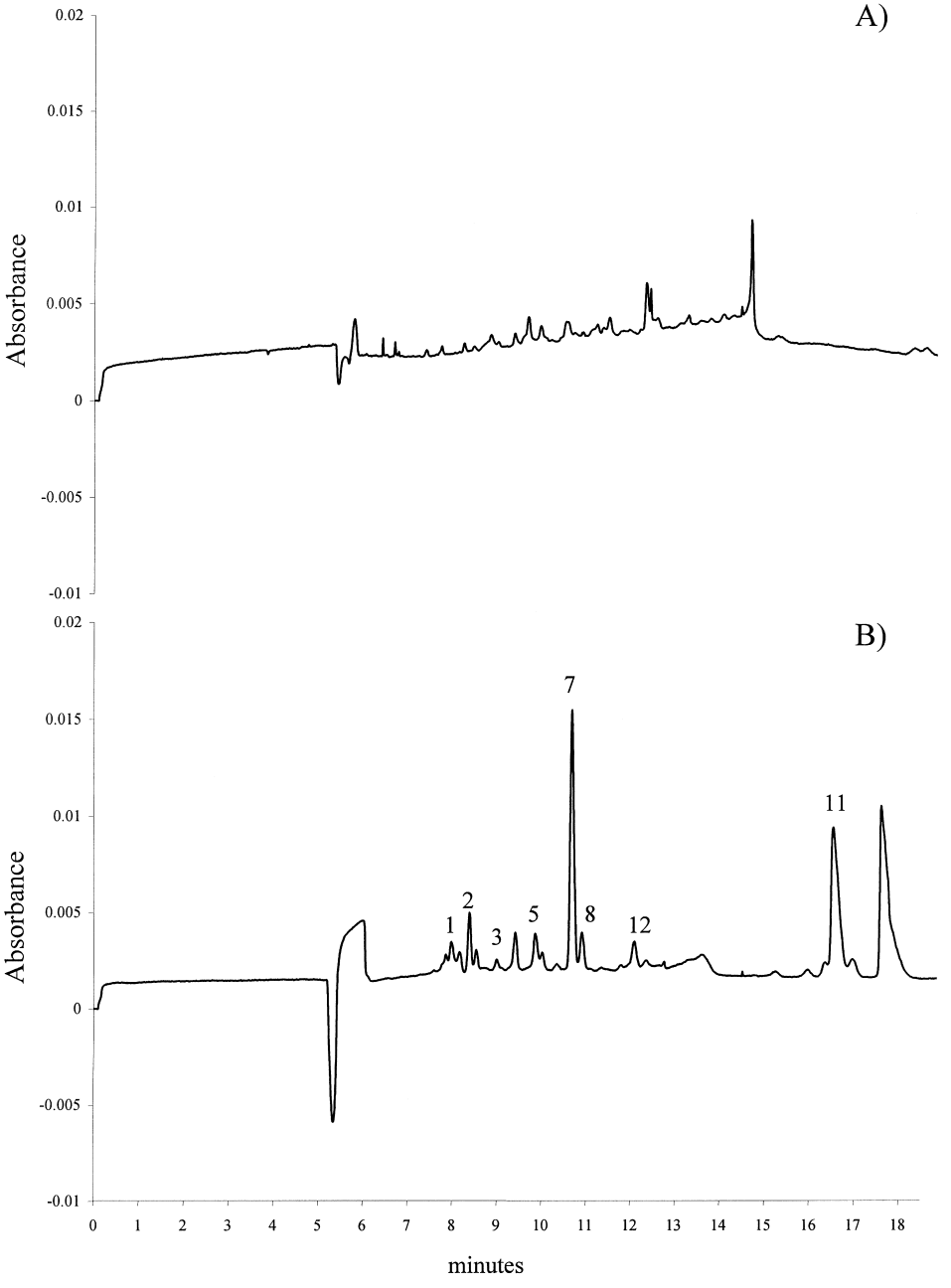

Fig. 6. Electropherograms of a wine sample injected: (A) directly, (B) after an extraction/preconcentration procedure. Buffer solution: 150 m*M* boric acid–50 m*M* SDS–5% methanol at pH 8.5. Peak identification and other operating conditions are the same as for Fig. 1.

ficiency, and that migration times become impractical. So, the analysis time is higher than 50 min for 20% methanol and about 50 min for 15% methanol. The best resolution is obtained using a 5% methanol in the buffer. The efficiency of the last peak (kaempferol) increases markedly and peaks 9, 10 and 12 which comigrate at 3% methanol can be resolved at 5% of this modifier. Higher percentages of methanol, i.e. 7% diminishes the resolution and efficiency of peaks 7, 8, 9, 10, 12 and 11 and led to long analysis times.

3.6. Determination of phenolic compounds in wine samples

The developed method has been applied for the determination of phenolic compounds in different wine samples. Calibration curves for the quantitative analysis were obtained at 50 m*M* SDS–150 m*M* boric acid–5% of methanol at pH 8.5. Calibration graphs show excellent correlations (linear correlation coefficients ≥ 0.995) for all analytes in a wide concentration range of 0.1–50 mg/l. Repeatability was examined by ten replicate injections of a mixture of the 12 phenolic compounds at concentrations of 1.0 mg/l. The relative standard deviation (RSD) fell below 6.8%. Fig. 6 shows the electropherogram of a sample of wine directly injected and injected after extraction with diethyl ether. When the sample is injected directly it is impossible to quantify the phenolic compounds and peak overlapping is observed, but the use of an stage of extraction/preconcentration allows quantifying the analytes. This extraction stage is also necessary for other chromatographic methods such as HPLC or TLC in order to extract this group of compounds. Table 1 shows the concentration of some of the phenolic compounds found in the wine samples, where the content of these phenols varies depending on the sample. UV–visible spectra for the different phenolic compounds in the wine samples were always compared with the spectra of standard solutions with the same solvent composition.

4. Conclusions

The influence of surfactant and electrolyte concentration and the type and concentration of the organic modifier on the migration behaviour was studied. It was demonstrated that using intermediate concentrations of SDS (50 m*M*), high concentrations of electrolyte (150 m*M*) and low concentrations of methanol (5%) as the organic modifier at pH 8.5 allows the separation of this group of compounds with good resolution, high efficiency and low analysis time. The optimised method was successfully applied to wine samples.

Acknowledgements

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Table 1
Results obtained in the determination of phenolic compounds in wine samples

No.	Compound	S1 (mg/l)	S2 (mg/l)	S3 (mg/l)	S4 (mg/l)	S5 (mg/l)
1	(+)-Catechin	23.64	22.30	16.54	–	–
2	(−)-Epicatechin	32.18	26.43	33.90	33.60	22.25
3	Quercetin	8.92	9.75	7.42	7.55	–
4	Rutin	–	–	–	–	–
5	Protocatechuic-aldehyde	2.59	7.01	4.65	9.79	2.68
6	Syringealdehyde	–	–	–	–	–
7	Ferulic acid	41.22	40.65	5.01	42.75	46.92
8	*p*-Coumaric acid	11.67	12.11	–	14.19	12.90
9	Vanillic acid	–	6.50	7.20	–	5.10
10	Myricetin	–	4.34	5.43	–	–
11	Kaempferol	1.33	2.77	2.59	3.33	3.60
12	Caffeic acid	6.60	18.16	18.45	7.65	21.26

– = Not detected.

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