Eur Food Res Technol (2002) 215:255–259
DOI 10.1007/s00217-002-0528-8

ORIGINAL PAPER

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Analysis of sugar acids by capillary electrophoresis with indirect UV detection. Application to samples of must and wine

Received: 18 December 2001 / Revised: 1 March 2002 / Published online: 26 April 2002

Abstract A study has been conducted of the separation of sugar acids (gluconic, galacturonic and glucuronic acids) by capillary electrophoresis using indirect UV detection. We have tested various background electrolytes: *p*-hydroxibenzoic acid, sorbic acid, potassium hydrogen phthalate, protocatechuic acid, α-resorcylic acid and β-resorcylic acid. The choice between these electrolytes was made on the basis of studies of electrophoretic mobility and absorbance values at the wavelengths of our CE system. Of all the electrolytes tested, it was found that β-resorcylic acid best met the required characteristics. The best separation of the three acid was achieved with a time of 8 min, for an electrolyte consisting of 5 mM β-resorcylic acid, 1 mM TTAOH (pH 3.0), indirect detection at 214 nm, -20 kV of run voltage and capillary of 60 cm length and 75 µm i.d. It was found that the proposed method is applicable to samples of both must and wine, and that the samples do not require any prior treatment apart from centrifugation (only in the case of must), filtration and dilution in suitable proportion.

Keywords Sugar acid · Indirect UV detection · Capillary electrophoresis · Must · Wine

Abbreviations BGE background electrolyte · CE Capillary electrophoresis · DMSO dimetilsulfoxide · TTAOH tetradecyltrimethilammonium hydroxide

Introduction

The sugar acids (gluconic, galacturonic and glucuronic acids), are non-volatile acids formed by the microbial oxidation of sugars. These acids have particular importance in enology, since they constitute part of the acidity of musts and wines. Their presence is often associated with processes of infection by bacteria and fungi; specifically they are associated with the vine disease caused by the fungus *Botrytis cinerea* [1].

Gluconate and the cetogluconates may be produced by the bacterial oxidation of glucose. Gluconic acid is not metabolized by yeasts or bacteria, and can be used as an indicator of the deterioration of the fruit. Apart from this function, gluconic acid does not present any other enological interest. Galacturonic acid, in turn, has been found to be associated with the phenomenon of browning in white wines. This acid is the main constituent of the pectins that exist as a polymer of galacturonic acid joined via α-1,4 glycoside by methanol molecules. The formation of these polymers is induced by the presence of metallic ions, such as iron and copper, in the must or the wine [2].

In summary, it can be stated that the content of these three acids in vitivinicultural samples is related to processes of deterioration that lead to financial losses and damage to product reputation in the market.

Traditionally, gluconic acid is determined either enzymatically or by liquid chromatography [3]. We also find in the bibliography methods that determine gluconic acid by CE. In most of the cases they are methods of analysis of low molecular weight organic acids where the gluconic acid presents a high time of migration

Glucuronic and galacturonic acids are not very common substrates, and their determination is most frequently performed by high performance liquid chromatography (HPLC) [4, 5].

There are several studies describing the determination of these three acids by capillary electrophoresis [6, 7, 8, 9, 10, 11]. However, due to the similarity of their chemical structure to that of sugars, and to their low absorbances in the UV-vis zone (principally glucuronic and galacturonic acids), their analysis requires methods more closely related to those of glucides than of acids [8, 9]. The determination of carbohydrates has been overcome by derivatization [12, 13], by electrochemical detection [8, 9], or by indirect detection [14, 15].

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In the present study, we have devised a method using capillary electrophoresis, for the determination of these acids of enologic importance. We have selected the best electrolyte among a series of possible substances, based on their nature, concentration and pH.

Materials and methods

Apparatus

The separation was performed using a Waters Capillary Ion Analyser (Milford, MA, USA) with UV-Vis detection at 185, 214 and 254 nm. The injection was by means of a hydrodynamic system at 10 cm height and 30 s injection time. The capillary utilized had an effective length of 57 cm and a total length of 60 cm, and with 75 µm of i.d. The separation voltage was –20 kV.

The UV-Vis spectros of possible BGEs were performed using a Helios Gamma UV-Vis spectrometer (Unicam, Cambridge, UK).

Standards and reagents

All reagents were of analytical grade. The glucuronic and galacturonic acids were obtained from Fluka (Buchs, Switzerland), and the gluconic acid (sodium gluconate) from Sigma (Darmstadt, Germany). Several compounds were tested as possible BGEs: the α-resorcylic, β-resorcylic, *p*-hydroxibenzoic and protocatechuic acids were from Fluka, the potassium hydrogen phtalate and sorbic acids were obtained from Panreac (Barcelona, Spain). The sodium hydroxide used to adjust the pH, and DMSO were obtained from Panreac and the methanol from Scharlau (Barcelona, Spain).

The TTAOH flow modifier was obtained from Waters (Milford, MA, USA)

Buffers, standard solutions and samples

The final conditions of the electrolyte were 5 mM of β-resorcylic (dissolved in 0.5% of methanol by volume), pH 3 (with NaOH) and 1 mM of TTAOH.

The standard solutions of acids were prepared immediately before each analysis was performed, from concentrated solutions (2500 ppm) of the acids separately. Before introducing them into the CE system, they were filtered using a Nylon filter of 0.45 µm.

The real samples used were musts and wines from the Jerez region. They had not been submitted to any prior treatment apart from being centrifuged (in the case of the musts, to eliminate coarse material), diluted by 1/10 with Milli-Q water, and filtered through Nylon filters of 0.45 µm.

In the CE technique, the peaks do not all pass across the detector at the same speed and, therefore, the more slowly moving peaks spend more time in the detector, giving rise to larger peak areas. To obtain peak areas independent of time, it is usual to divide these areas by the time of migration; the areas thus calculated are designated normalized areas [16]. These normalized areas have been used in the present study.

Results and discussion

The most important aspects to take into account in the separation with indirect detection of different analytes are, first, the mobility of the electrolyte (probe); second, the mobility range of the analytes to be separated; and third, the sensitivity of the detection [17, 18].

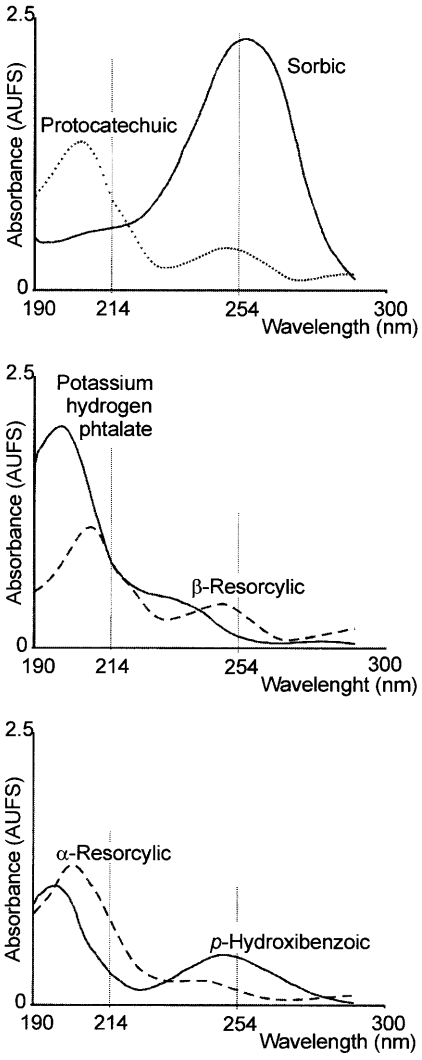

Fig. 1 UV-Vis spectra of possible BGEs (0.05 mM aqueous solutions)

Selection of the background electrolyte

We have selected a series of substances that, either from the bibliography [7, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26] or from their spectral conditions, (Fig. 1) could be considered good BGEs. These substances are *p*-hydroxibenzoic acid, sorbic acid, potassium hydrogen phthalate, protocatechuic acid, α-resorcylic acid and β-resorcylic acid.

Determination of the electrophoretic mobility

The effective mobility of the various BGEs was determined by injecting 0.1 mM solutions of these substances and using DMSO 0.05% (v/v) as a neutral marker [7]. The electrolyte used was formed by a phosphate buffer solution of 10 mM at pH 8. The other electrophoretic conditions were: wavelength of 214 nm, capillary length of 60 cm and internal diameter of 75 µm; the samples

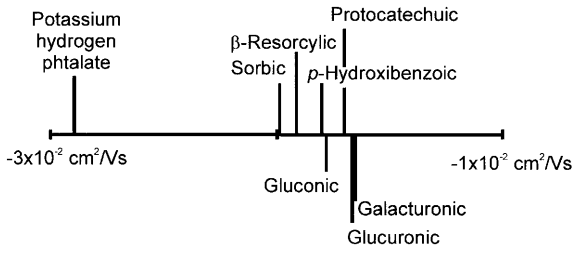

Fig. 2 Effective mobilities of several BGEs and analytes at pH 8

were injected hydrostatically for 30 s, and the voltage applied was +20 kV.

The analytes of interest were also submitted to the same mobility calculations; the electrolyte used was also formed by a phosphate buffer solution of 10 mM at pH 8, wavelength of 185 nm, capillary length of 60 cm and internal diameter of 75 µm; the samples were injected hydrostatically for 30 s, and the voltage applied was +20 kV. In this case, it was necessary to use fairly concentrated solutions of these due to the low absorbance of the sugar acids at this wavelength; specifically the solutions of sugar acids used were 100 ppm. Figure 2 gives the results of sampling the mobility of the substances considered as BGEs, together with the mobility of the species to be analyzed.

Bearing in mind that the CE system used is a unit of fixed wavelength, equipped with lamps of 214 and 254 nm., it was logical to study those substances that present absorbance at these wavelengths, specifically protocatechuic and β-resorcylic acids for a wavelength of analysis of 214 nm and sorbic acid for that of 254 nm. Because the sugar acids present absorbance at 185 nm, this value of wavelength was not considered in the study.

Choice of the optimum concentration of electrolyte

A study has been conducted of the variation of the absorbance with the concentration, for each of the probes selected. Absorbance measurements were performed by filling the capillary with the desired probe solution, then stopping the flow and measuring the absorbance under static conditions. Measures were performed at 214 nm for protocatechuic and β -resorcylic acids, and 254 nm for sorbic acid.

Sensitivity data (response/concentration) were calculated from the measured absorbances [27, 28]. A plot of sensitivity versus concentration was constructed to show when the detector linearity limit is reached (Fig. 3). The concentration at which sensitivity declined by more than 15% was used to define the upper limit of detector linearity. These concentrations are 5 mM for protocatechuic and β -resorcylic acids, and 3 mM for sorbic acid.

In this figure it can also be observed that β -resorcylic acid presents higher sensitivity values than protocatechuic acids, which indicates a higher molar absorbance capability of this BGE at 214 nm. This characteristic

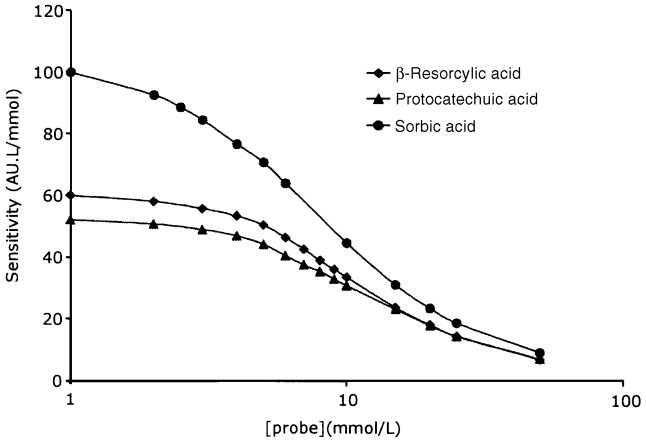

Fig. 3 Sensitivity detector vs concentration at 254 nm for sorbic acid, and 214 nm for protocatechuic and β -resorcilic acids

leads to a greater response of the analytes being determined.

Study of the pH

Since the three substances selected as background electrolytes are weak organic acids, this means that their concentration in the electrophoretic medium will tend to be determined by the pH of the medium. In addition, the acids we wish to determine, the sugar acids, are also weak organic acids, therefore their concentration will depend on the pH of the medium we use. For these reasons, several experiments were conducted aimed at obtaining the optimum pH values for each of the three probes, using the selected concentrations in the previous section.

To achieve a better analysis of negative species by capillary electrophoresis, it is necessary to apply a negative voltage, so that the ions migrate together with the electro osmotic flow towards the detector, situated on the anodic extreme. To do this, species that invert the direction of electro osmotic flow (i.e. modifiers of electro osmotic flow) must be used [29]. Since the three analytes, the sugar acids, are weak organic acids, all the experiences were made using an organic flow modifier, more concretely TTAOH 1 mM.

Three different tests were performed, each with a different probe, keeping constant all the conditions of the medium apart from the pH.

When protocatechuic acid was used as probe, it can be observed that at high values of pH (10–12), the protocatechuic acid precipitates out. This makes it impossible to carry out the experiment at these values of pH. At lower pH values, a clear separation of the analytes is not observed, and only one single peak is obtained for the three species.

When sorbic acid is used as probe, it is not observed an optimum resolution between obtained peaks. But if the test is performed using β -resorcylic acid as the probe, it is observed that there exists an acceptable reso-

Table 1 Accuracy and linearity for the analytes. Sample 20 ppm of each acid. Electrophoretic conditions same as Fig 4

Acid	RSD (%) n=4			Linearity		
	Migration time	Peak area	Normalized area	Linear range (ppm)	Ecuation of regression line	Correlation Coefficient (r)
Glucuronic	1.21	0.89	0.70	5–50	y=74.03x+114.95	0.9996
	1.38	0.43	2.05	5–50	y=58.95x+43.08	0.9997
Galacturonic	1.37	0.96	1.07	5–50	y=80.76x–43.13	0.9991
	1.58	1.64	1.99	5–50	y=50.43x–23.12	0.9989
Gluconic	1.44	2.12	0.59	5–50	y=110.90x+119.36	0.9995

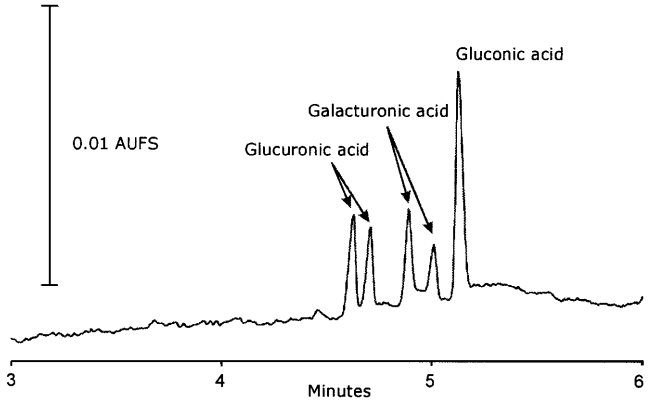

Fig. 4 Separation of sugar acids. Electrophoretic conditions: electrolyte 5 mM β-resorcylic acid, 1 mM TTAOH, pH 3.0; detection wavelength 214 nm (indirect detection); separation voltage: –20 kV, hydrostatic injection: 10 cm height and 30 s injection time; temperature 200 °C; capillary 60 cm length × 75 µm i.d. Sample 20 ppm of each acid

lution between peaks, which diminishes when increase pH; it was found that a pH of 3.0 was best for carrying out the experiment.

Under these conditions, five peaks are obtained for the three acids analyzed (Fig. 4). The glucuronic and galacturonic acids, present two peaks each, while gluconic acid presents a single peak. The three acids studied have six carbon atoms in their molecule and their chemical structure is that of hydroxylate acid; in all cases the formation of lactone is possible, but the glucuronic and galacturonic acids (uronic acids) can form hemiacetals (intramolecular esters between an aldehyde or ketone group and an alcohol group). In an acid medium, such as the musts and wines of the samples being analyzed, or the electrolyte used in the analysis (pH 3.0), the ring opens, with equilibrium existing between the hemiacetal and lactone forms.

Study of the injection time

Once the optimum conditions of the electrolyte have been established, the next step is to select the optimum injection time. A sample of internal standards of 20 ppm was injected over four different times (10, 15, 20 and 30 s) with four repetitions. Although good values of RSD were obtained over 20 s (RSD values lower than 2.5, except for the peak 2 of the glalacturonic acid that it presents a RSD of 9.7), a period of 30 s was selected for the injection time, owing to the nature of the samples to be injected and the small quantities of analytes present in the samples.

The quantities of these acids present in musts and wines are relatively small, except in the rare cases of diseased grape or browned wines; and the normalized area of the peak is directly proportional to the injection time. Both of these reasons led us to select 30 s as the optimum injection time.

Validation of the method: accuracy and linearity

The accuracy (Relative Standard Deviation, RSD) of the present method for the various analytes are summarized in Table 1. The RSDs obtained from four repetitions with a solution of 20 ppm show values of less than 2.2% with respect to the peak area, of less than 2.0% in normalized area and less of 1.6% in migration times. To study the linearity of the method, five standard samples containing mixtures of the acids studied with concentrations ranging from 5 to 50 ppm were analyzed. The results of the regression lines, obtained with six points and three repetitions per point, are given in Table 1. According to these results, the method is linear in the range of concentrations studied for the three acids (and five peaks), with values of r>0.999 being obtained.

Application to real samples

The method has been applied to real samples of the year's musts and wines of the Jerez region. Figure 5 shows examples of two typical electropherograms, one for a must, the other for a wine. From a comparison between these chromatograms and those obtained for standards, it can be observed that, in the case of both must and wine, the glucuronic acid is only quantifiable in the first of the two peaks presented, since the second peak co migrates with another compound or compounds, thus

Table 2 Concentrations of the sugar acids found in must and wine samples

Acid	Must (1/10)			Wine (1/10)		
	Quantities		Recovery (%)	Quantities		Recovery (%)
	Initial (ppm)	Added (ppm)		Initial (ppm)	Added (ppm)	
Glucuronic	6.0	30.0	107	–	30.0	114
Galacturonic	6.0	30.0	87	17.9	30.0	110
Gluconic	10.9	30.0	104	52.0	30.0	105

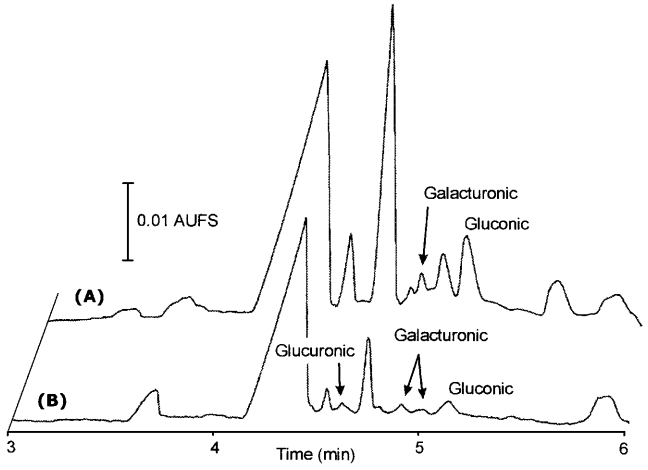

Fig. 5 Electrophoregrams in vinic beverage samples: wine (*A*) and must (*B*). Electrophoretic conditions same as Fig 4

making it impossible to quantify the glucuronic acid. Galacturonic acid can be quantified by either of the two peaks in the case of the must, but only by the second of the two peaks in the case of the wine; this is a similar situation to that encountered with glucuronic acid. Gluconic acid, however, does not migrate at the same time as any other compound, therefore it is possible to identify it directly in the two types of sample.

Together with the quantification of the acids in the real samples, a study of recoveries was also conducted. Table 2 presents the data of the quantities found, those added, and the percentage of recovery, for all the samples studied. It can be appreciated that almost all the values are somewhat higher than 100%.

Conclusions

A rapid and easy methods has been devised for determining gluconic, glucuronic and galacturonic acids in samples of grape must and wine.

The three acids to be analyzed emerge in 6 min, in both the standard and the problem samples, giving five peaks: the uronic acids present two peaks each, and the gluconic acid one peak. The linearity and repeatability of the method are found to be good, the first presenting values of correlation coefficient better than 0.999 for the five peaks obtained, and the second presenting values of RSD lower than 2%.

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