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ORIGINAL PAPER

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Method devised for determining low molecular weight organic acids in vinic samples by capillary electrophoresis: validation of the method with real samples

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Abstract The validation has been carried out of a method devised for determining by capillary electrophoresis the low molecular weight organic acids present in various types of vinic sample. The organic acids specifically studied are those most commonly found in samples of vinic origin: formic, fumaric, succinic, oxalic, malic, tartaric, acetic, lactic and citric acids. The electropherograms were obtained using an electrolyte consisting of tetraborate buffer (10 mmol/l) at pH 9.3, an electroosmotic flow moderator (TTAOH), and Ca^{2+} and Mg^{2+} as complexing agents, for better resolution of some of the acids studied. Other conditions of the method are hydrostatic injection (10 cm height for 30 s), UV detection at a wavelength of 185 nm and temperature of 200 °C.

The method used for the validation was comparison of the slopes of the curves obtained with standards and the regression curves obtained by standard addition. For this comparison, t and F statistics were used.

Keywords Organic acids · Must · Wine · Brandy · Capillary electrophoresis · Validation

Introduction

The organic acids of low molecular weight are an important group of compound in these types of samples of grape-derived products; these acids are involved in such important aspects as the organoleptic characteristics (flavour, colour, aroma,...), the stability and the microbiological control of the products [1, 2].

These organic acids have diverse origins: they may derive from the raw material itself (the grape) or else from the various processes to which it is subjected, principally fermentation. They are intermediate metabolites or ends of metabolic routes involved in the fermentation of sugars, malolactic fermentation, ethanol oxidation, etc.

The predominant acids in the grape are usually tartaric and malic acids, which are synthesised by the grape itself during its process of maturation. Other acids such as citric and succinic are also present but in smaller quantities [3]. The evolution of these acids in the grape and their content in the must are useful data for monitoring the process of maturation of the grape and the organoleptic properties of the must. Other acids present in the wine and in other grape-derived products include lactic acid, which is the final product of the malolactic fermentation, acetic acid, which is formed by the oxidation of ethanol during vinification, among other metabolic routes, and formic and fumaric acids, which are formed from pyruvic acid.

All the acids cited influence the organoleptic characteristics of the final product, together with other phenomena such as the precipitations of tartaric salts (tartrates and bitartrates), or acetic sharpness (high contents of acetic acid due to acetic bacteria). In respect of their presence in brandy, organic acids are particularly important for Brandy de Jerez, due to its specific method of production: this Brandy is aged in oak wood barrels that have previously contained wine, and presents traces of organic acids in its composition. During the ageing period in which the barrels hold the typical wine of the Jerez region, the internal walls of the barrels absorb components of the wine, including several of these organic acids. Subsequently the acids are passed to the Brandy, forming part of its composition, by solubilisation of the deposits on the walls of the oak barrels, during the ageing of the Brandy [4].

Each acid can be determined either enzymatically or spectroscopically, after it has been separated from the other components [5], but for some of them, such as fumaric acid, there is no official analytical method [1].

Considering the analysis of enological drinks, references are found to applications of the analysis by capillary electrophoresis for the determination of diverse types of organic acid. There are studies of the analysis of

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the organic acid content of samples of red wine by capillary isotachophoresis [6], of 4-methyl imidazol in colouring caramel by capillary isotachophoresis [7], of organic acids in wine by capillary electrophoresis of zone with inverse detection, and utilising a flow modifier and potassium phthalate acid as the base for the buffer solution [8].

It is notable that, except for the study by Guillén et al. [9] on the analysis of organic acids in samples of Brandy de Jerez by ion exchange chromatography, we have not found any study that makes mention of the analysis of these compounds, by either EC or by any other analytical technique, in brandies or similar products such as cognac.

Materials and methods

Standards and reagents. All the reagents used in this study were of reagents quality or superior. The citric, lactic, formic and oxalic acids were supplied by Fluka (Buchs, Switzerland); the fumaric, malic and succinic acids by Sigma (St. Louis, MO, USA); the acetic and tartaric acids, and the calcium chloride and magnesium chloride ($MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$) by Merck (Darmstadt, Germany); the ethanol and sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) by Panreac (Barcelona, Spain) and the electro-osmotic flow modifier TTAOH (tetradecyltrimethylammonium hydroxide) by Waters (Milford, MA, USA). All the samples were prepared using Milli-Q quality water (Millipore, Bedford, MA, USA).

Conditions of electrophoresis. The experiments carried out were conducted using a Waters Quanta 4000 capillary electrophoresis system equipped with a UV-Vis detector of wavelength set at 185 nm, together with Millennium 2100 software for control and data acquisition.

The fused silica capillary tube was of 75 µm internal diameter and 60 cm in total length (53 cm of effective length). Before each injection of sample the capillary was washed with first a solution of sodium hydroxide 0.01 mol/l (1 min), then de-ionised water (1 min), and finally the electrolyte (3 min), immediately before proceeding to the injection of the samples.

The samples were hydrostatically introduced into the capillary (height 10 cm). The voltage applied was 7 kV using a negative feed source, injection time 30 s, UV detection at 185 nm and temperature of 200 °C. The parameter used to carry out the quantification is the normalised area of the peak (quotient between the area of the peak and the time of retention).

The electrolyte used consisted of tetraborate buffer (10 mmol/l), at a pH of 9.3, electro-osmotic flow modifier (TTAOH) 0.5 mmol/l, 10 ppm of Ca^{2+} and 10 ppm of Mg^{2+} as complexing agents (added in the form of chlorides). Having prepared the electrolyte, and before its utilisation, it was filtered.

Preparation of the standard solutions. Individual standard solutions of each acid were prepared, at a concentration of 1000 ppm. Immediately before performing the various analyses, working solutions of a mixture of acids were prepared, to be employed as stock solutions. By diluting these stock solutions the rest of the solutions of standards were prepared. The samples were filtered through Millipore filters of 0.45 µm before being injected.

Preparation of the real samples. All the real samples analysed in the present study are of organic origin; specifically, all are derived from the grape. The effect of this is that the samples present a high degree of salinity, this making difficult their direct determination by means of the technique utilised. Of the various methods available for the desalinisation of the samples, we have selected dilution with water. This offers several advantages: it reduces the saline content of the samples, it is easy to perform, it does not require excessive manipulation of the samples and it does not saturate the detector with the high concentrations of some of the target organic acids that some of the samples studied present.

Therefore several different dilutions were tested for each of the types of sample analysed (Fig. 1).

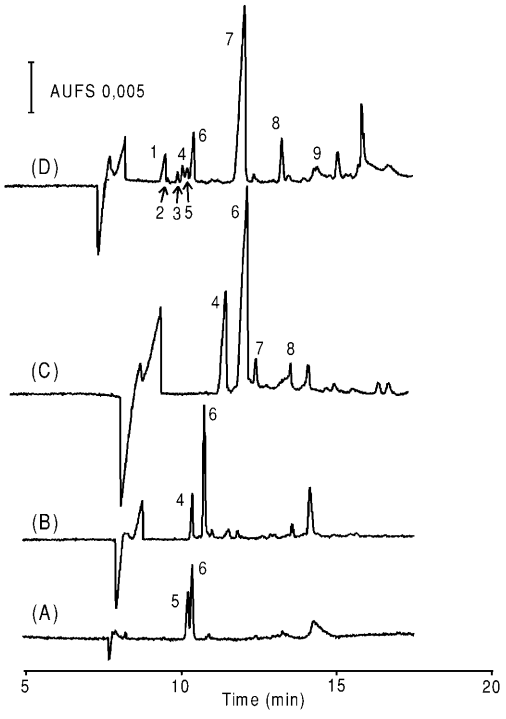

Fig. 1A–D Electropherograms of real samples analysed: **A** must dilution 1/100; **B** wine dilution 1/10; **C** wine dilution 1/100; **D** brandy dilution 2/5. Organic acids: 1 formic, 2 fumaric, 3 succinic, 4 oxalic, 5 malic, 6 tartaric, 7 acetic, 8 lactic, 9 citric. For electrophoretic conditions see materials and methods

Statistical techniques used. It must be borne in mind that the samples used in this study are of great chemical complexity and are difficult to reproduce accurately; thus they are very susceptible to presenting the matrix effect: the decrease or increase of the absorbance signal due to the presence of other components in the sample. Consequently we used the method of standard additions to the real samples in this validation of the method. The slope values of the regression curves obtained with standard solutions were then compared with the slope values obtained by the method of standard additions, by means of the F and t statistics [10]. The least squares method was used to calculate the regression curves between the concentration of the various acids in the samples studied, and the normalised area of the peak.

Results and discussion

Validation of the method: accuracy and linearity

The linearity of the present method was confirmed by the analysis of standard solutions of samples containing a mixture of the acids studied in concentrations similar to those we expected to find in vinic samples. The data shown in Table 1 correspond to the curves obtained for each acid with the concentrations at which the measure-

Table 1 Summary of calibration data and precision of the optimised method of CE

Acid	Concentration range (ppm)	Equation of regression lines	R	Detection limit (ppm)	Linearity
Formic	5–40	y=166.1x+64.0	0.9998	0.65	99.69
Fumaric	0.5–4	y=239.1x+88.4	0.9982	0.08	98.84
Succinic	1–8	y=240.1x+88.3	0.9977	0.44	98.80
Oxalic	1–8	y=239.7x+121.5	0.9910	1.06	97.44
Malic	4–32	y=200.7x+32.6	0.9990	1.55	99.11
Tartaric	5–40	y=142.0x+202.9	0.9993	1.39	99.29
Acetic	3–24	y=148.1x+228.1	0.9992	0.91	99.19
Lactic	5–40	y=120.3x+237.9	0.9890	4.75	97.17
Citric	4–32	y=124.5x+115.7	0.9990	1.42	99.14

Table 2 Data of addition standards for must. Lines with five point and four repetitions

Acid	Concentration ad. (ppm)	Equation of regression lines	R	Recovery
Malic	0–32	y=200.3x+3781.7	0.9984	98.82
Tartaric	0–10	y=144.1x+1476.3	0.9972	98.40

Table 3 Data of addition standards for wine. Lines with five point and four repetitions

Acid	Concentration ad. (ppm)	Equation of regression lines	r	Recovery
Succinic	0–5	y=243.7x+2108.2	0.9966	98.28
Tartaric	0–50	y=143.9x+6092.9	0.9951	97.92
Acetic	0–6	y=296.4x+1317.1	0.9964	98.22
Lactic	0–7	y=117.2x+647.4	0.9964	98.24

ments were taken. Each curve has been obtained with five points and four repetitions per point.

Determination of organic acids in real samples

Having selected all the conditions of the method of analysis, it was applied to real samples of grape-derived products of the Jerez-Xérèz-Sherry Denomination of Origin region.

Method of adding the standards

Given the great complexity of the real samples to be analysed, it was decided to validate the method by standards, comparing the slopes obtained using standards, with the slopes obtained by means of the method of standard additions. For this, different studies of standard additions were conducted, one for each type of sample studied.

Must. The two acids studied in this case are the most predominant acids usually found in this type of sample: tartaric and malic acids. The samples of must studied were diluted to a concentration of 1/100: 1 ml of must in 100 ml of total solution. Table 2 shows the values of the regression curve obtained for the acids studied in a sample of must. The two acids present standard deviations of less than 0.5%, and recoveries close to 100%, as well as very similar values of correlation coefficient.

Wine. In the case of the study of samples of wine, two dilutions were performed, one to determine acids found in relatively large concentrations in the sample, the predominant acids such as tartaric and succinic, the other dilution for acids found in lower concentrations, such as acetic and lactic. Table 3 gives the values of the regression curve obtained for the four acids studied in the wine.

In this study it is observed that in general the correlation values are somewhat lower than those previously obtained for must, with tartaric acid being the one presenting the lowest value (0.9951), although in general the four acids present similar r values. In respect of the recovery values, the finding is similar to that for the study of must: values close to 100% are obtained for the four acids and all the concentrations added. The repeatability values for wine are less than 3% and the recovery values range around 100%, except for lactic acid which presents very high values (124%) at the lowest additions.

Brandy. The organic acids present in the samples of brandy are all found to be in similar ranges of concentration. This means that all the acids can be quantified in one single injection, without having to carry out several different dilutions.

These are the samples presenting the worst repeatability and recovery values. The mean repeatability values are around 2%; in this case it is lactic acid that presents the lowest values (<0.1%) and succinic acid that presents the highest value (5%).

Recovery values are again close to 100%, with oxalic acid presenting the highest and lowest values (around

Table 4 Data of addition standards for brandy. Lines with five point and four repetitions

Acid	Concentration ad. (ppm)	Equation of regression lines	r	Recovery
Formic	0–18	y=169.5x+3241.9	0.9941	97.72
Fumaric	0–1.6	y=235.8x+294.2	0.9970	98.37
Succinic	0–2.4	y=244.3x+650.1	0.9901	97.05
Oxalic	0–4.8	y=260.1x+1137.5	0.9755	95.30
Malic	0–8	y=203.5x+1293.2	0.9873	96.64
Tartaric	0–24	y=140.0x+3981.1	0.9973	98.46
Acetic	0–6	y=506.8x+20804.6	0.9887	96.84
Lactic	0–4	y=123.0x+2390.6	0.9758	95.33
Citric	0–16	y=127.9x+973.2	0.9900	96.32

Table 5 Data of organic acid concentration (ppm) from Brandy of Jerez samples (n=6). Commercial categories of *Brandy de Jerez*: S=*Solera*, SR=*Solera Reserva* and SGR=*Solera Gran Reserva*

	Formic	Fumaric	Succinic	Oxalic	Malic	Tartaric	Lactic	Citric
S	3.8	0.3	0.3	–	2.6	7.1	8.1	–
SR	13.7	0.2	1.3	1.5	2.2	16.9	10.4	3.4
SGR	18.1	0.3	4.2	5.6	3.9	89.8	53.8	6.7

109% and 95% recovery). Considering again the values of the coefficient of correlation (Table 4), it can be observed that the situation found in the case of must again occurs with brandy: all are lower than those obtained for standards, with some falling below 0.98.

Comparison of the slopes

On comparing the slopes of the curves obtained with standards with those obtained by standard additions, for each acid and sample studied, it is observed that there are no significant differences between them, with the exception of those obtained for acetic acid, where the differences are significant; it is found that the value of the slope increases in proportion to the increase in the alcohol content of the sample studied. In other words, wine presents a slope value intermediate between standards and brandy. It is supposed therefore that a matrix effect exists in the determination of acetic acid, according to the type of electrolyte used, and that this could be associated with the alcohol content of the sample injected.

Applications to enological products

Having established the analytical method, it was then applied to real samples, specifically to samples of Brandy de Jerez of different qualities, samples of must and wine taken during the course of the fermentation process, and to commercial samples of tartaric acid used in wine-making.

Applications to samples of Brandy de Jerez

One objective of this study was to check the increase of organic acids during the brandy production process, due to the use of oak barrels previously wined for the ageing of the brandy. This involved the study of the presence of organic acids from both the qualitative and quantitative point of view.

The various organic acids present in samples of the three commercial categories of Brandy de Jerez (*Solera*, *Solera Reserva* and *Solera Gran Reserva*, in ascending order of quality and price) were quantified by this method. Samples were provided by the Regulatory Council of Brandy de Jerez.

Table 5 presents the data of mean concentration found for the various different samples and acids analysed. As expected, an increase is observed in the concentrations of the organic acids, moving from the *Solera* category through *Solera Reserva* to *Solera Gran Reserva*. This tendency is due to the increasing length of required minimum time spent ageing in the oak barrels.

Applications to samples of must and wine

This method was also applied to the determination of organic acids in samples of must of the Sauvignon Blanc grape variety. In particular, it was used to monitor the evolution of malic and tartaric acids from the maturation of the grape through to the production of the wine, passing through all the stages of the vinification process, including maturation, pressing, oxidation, racking, etc. (Fig. 2).

Applications to tartaric acid used in wine-making

Lastly, the method was used to determine the presence of citric acid in commercial samples of tartaric acid. Tartaric acid is the only acid allowed under Spanish legislation that may be added to must and wine to correct its acidity. The only source of origin of this acid is the grape

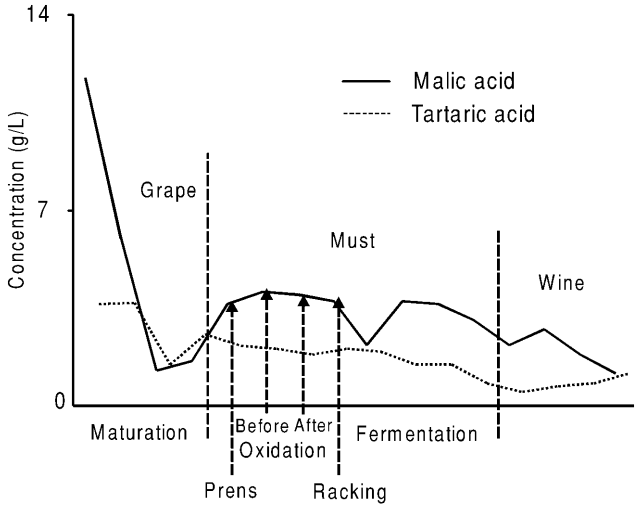

Fig. 2 Evolution of malic and tartaric acids concentration from the maturation of the grape through to the production of the wine

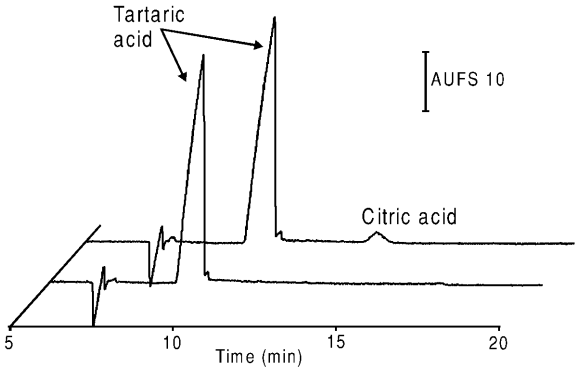

Fig. 3 Electrophoretograms obtained for a solution of 1000 ppm prepared from the commercial tartaric acid (A) without citric acid added and (B) with citric acid added (30 ppm). For electrophoretic conditions see materials and methods

itself: it is obtained from the lees and the residue generated in the winery, which makes it very costly to produce, and hence expensive to use. A customary and fraudulent technique consists of adulterating this acid with some 3–5% of citric acid, which is much cheaper; such an addition at this level is very difficult to detect by most HPLC methods. In these methods both acids present very close retention times, and so the enzymatic test is the only way of checking this possible contamination of tartaric acid. The method proposed in this paper has been used to determine possible contamination by citric acid in commercial samples of tartaric acid.

In Fig. 3 two electrophoretograms are shown, one obtained for a solution of 1000 ppm prepared from commercial tartaric acid, and the other for a similar solution to which had been added 30 ppm of citric acid. It can be observed that a peak appears in the corresponding zone. This result demonstrates the usefulness of the proposed method for resolving accurately the two acids at such different degrees of concentration, enabling this type of fraud to be checked rapidly due to the difference in migration time between one acid and the other.

Conclusions

In the light of the data obtained in the study of the validation of the method, by comparison of the slopes obtained with standards and those obtained by the method of standard additions, we can state that the method is valid for all but one of the acids studied, and presents in all cases satisfactory regression values for the technique proposed and the nature of the samples studied. The one exception is acetic acid, which must be quantified by the method of standard additions.

This is a rapid and versatile method that allows the analysis of samples of diverse composition, with the minimum prior treatment of dilution and filtration. Furthermore, the method involves a low consumption of low cost reagents.

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