Ion-exclusion chromatographic determination of organic acids in vinegars

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Abstract

A new ion-exclusion HPLC procedure for the determination of organic acids in vinegars under isocratic elution was developed. Optimum experimental conditions for pH of the eluent, column temperature and mobile phase flow-rate were estimated from a factorial design. The method was successfully validated and enables the reliable separation of major organic acids in wine vinegars (i.e. tartaric, citric, malic, lactic and acetic acid) in about 25 min. It was applied to the analysis of different vinegar samples. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Organic acids are interesting compounds in wine-derived products. Some of them are originally present in the grape but others may appear during alcoholic, acetic or another kind of fermentation. The level and nature of the organic acids present in vinegars may provide information concerning the origin of the raw material, microbiological growth and even processing techniques. The total acidity of vinegars is expressed as acetic acid, the major organic acid in vinegars. Tartaric acid is the major acid in wine, originally occurring in the grape and subsequently in wine and wine vinegars. It represents the fixed portion of acidity and its presence in vinegars (up to 4 g/l) is an indication of the origin of the wine. Malic acid appears in wine vinegars in low amounts and is very variable depending on the wine

origin and enological treatments; for cider vinegars, malic acid is the most representative. It is therefore an important compound to be monitored during the maturation of the grape and wine production. Malic acid is converted into lactic acid during malolactic fermentation, usually produced after alcoholic fermentation. The final content of this acid in wine vinegars depends on the substrate wine, ranging up to 1 g/l [1]. Lactic acid may be oxidised by some species of Acetobacter and Gluconobacter; therefore its level may decrease during acetic fermentation [2]. Citric acid is formed during alcoholic fermentation and may occasionally be used as a substrate by some microorganisms, producing acetic acid [3].

An attempt to determine organic acids in wine vinegars has been carried out by reversed-phase high-performance liquid chromatography (RP-HPLC), using two C_{18} columns (arranged in tandem), UV detection at 210 nm and sample filtration through Sep-Pack C_{18} [1]. However, the complete

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separation of the organic acids was not achieved with this procedure.

Ion-exclusion chromatography has also been used in order to determine organic acids. It is a valuable technique for separating neutral and weakly acidic or basic substances from ionic compounds based on an ion-exclusion mechanism rather than ion-exchange. The stationary phase is an ion-exchange resin that acts as a semipermeable membrane, enabling the non-ionic species to penetrate the resin and excluding, almost completely, the charged species. The hydrophobic interactions between the hydrocarbon matrix of the resin and neutral organics (nonexcluded) and the degree of access of the sample to the pore volume of the packing material play an important role [4]. Ion-exclusion chromatography is particularly useful for the separation of organic acids (according to the differences in their pK_a) by using a strong acid ion-exchange resin in its acidic form as stationary phase. Weak acids behave as nonelectrolytes at low pH, and consequently they are strongly retained by the resin and more strong acids are eluted first. Thus, the pH of the mobile phase is the most relevant factor for the separation. Thus, ion-exclusion chromatography has been used to determine organic acids in vinegars. Ashoor et al. [5] used an Aminex HPX87 column with a diluted sulphuric aqueous solution ($4.5 \cdot 10^{-3}$ M) as eluent and UV detection at 210 nm in order to perform a rapid monitoring of acetic acid in various foods, among them vinegars. Non-volatile organic acids in various types of vinegars have been determined using a Dionex (HPICE/AS 1) column, volatile acids being previously separated and determined in the distillate [6]. Bertolini et al. [7] used an Aminex column, sulphuric acid solution (pH 1.88) as mobile phase and UV detection at 210 nm could separate the main organic acids in vinegars: citric, tartaric, malic, succinic, lactic and acetic. The sample is subjected to a pretreatment consisting in a decoloration with active carbon and subsequently filtering.

In this paper an ion-exclusion HPLC method which separates in a single run the main organic acids in vinegars is reported. This method does not require any sample preparation and any interferences due to other components present in vinegars are detected.

2. Experimental

2.1. Chemicals

All the chemicals used were of analytical-reagent grade. Citric acid, tartaric acid, malic acid, lactic acid and acetic acid were supplied by Merck (Darmstadt, Germany), acetic acid by Carlo Erba (Barcelona, Spain). Sulphuric acid for the preparation of the mobile phase was from Merck. Other chemicals used to prepare synthetic wine vinegar were: glycerol, diethyl acetal, methyl acetate, acetaldehyde, acetoin, proline, ethyl acetate, 3-methyl-1-butanol, ethanol (Merck), gallic acid (Fluka, Buchs, Switzerland), and methanol (Tecnokroma, Barcelona, Spain). Must caramel was kindly provided by a wine vinegar winery.

Double distilled water purified with a Milli-Q (Millipore, Bedford, MA, USA) system was used throughout.

A synthetic vinegar matrix (for validation purposes) was prepared by dissolving in water the major characteristic compounds usually present in wine vinegars as indicated in Table 1.

A test solution containing 0.40 g/l citric, 0.50 g/l tartaric, 0.15 g/l malic, 0.30 g/l lactic and 10.75 g/l acetic in the synthetic vinegar matrix was prepared.

2.2. Equipment

Two HPLC systems were used a HP-1050 liquid

Table 1
Chemical composition of the synthetic vinegar matrix

Compound	Spiked amount[a]
Ethyl acetate	24.3
Glycerol	3.74
Methanol	1.58
3-Methyl-1-butanol	134
Methyl acetate	0.923
Acetaldehyde diethyl acetal	0.891
Acetaldehyde	0.624
Acetoin	0.392
Proline	0.5
Gallic acid	25.2
Ethanol	2
Must caramel	Just until suitable colour

[a] In g/l except for gallic acid (mg/l) and ethanol (% v/v).

chromatograph (Hewlett-Packard, Palo Alto, CA, USA) consisting in a Model 7125 manual injector (Rheodyne, Cotati, CA, USA), equipped with a 20-µl loop, a HP1050 variable-wavelength UV–Vis detector and an HP CHEMSTATION data processing system (Hewlett-Packard). For the study of the peak purity, a Waters 600E system controller (Milford, MA, USA) with a Model 7125 manual injector (Rheodyne) connected to a Waters 996 photodiode array detector and a MILLENIUM 2.0 data station. A 420A Orion pH meter (Boston, MA, USA) was also utilized.

2.3. Column and mobile phase

The column used was Aminex HPX87-H, 300× 7.8 mm I.D. (Bio-Rad, Richmond, CA, USA) packed with styrene copolymerized with divinylbenzene (9-µm particle diameter) operating at a temperature of 60°C.

The mobile phase was an aqueous sulphuric acid solution adjusted to pH 1. The flow-rate was 0.5 ml/min. Detection was performed by UV absorption measurement at 214 nm.

2.4. Vinegar samples and sample preparation

Ten vinegar samples were used in this study: three wine vinegars obtained by quick acidification methods (submerged culture), three sherry wine vinegars made by traditional slow acidification methods (surface culture) in wooden barrels, one aceto balsamico di Modena and three cider vinegars.

The vinegars were injected onto the column without any handling except for dilution and microfiltration through Millex-GV$_{13}$ 0.22 µm filters. After the method development, some experiments were performed for setting the suitable sample dilution. Direct injection of vinegars led to excessively broad peaks (specially for acetic acid) accompanied by shifts to shorter retention times, which indicates sample overload [8]. Thus, vinegars were diluted four times before injection. In some cases, for sherry vinegars with a higher concentration of acetic acid, ten-fold dilution was needed exclusively for acetic acid quantitation.

3. Results and discussion

3.1. Method development

3.1.1. Choice of chromatographic operational conditions

Considering the chromatographic conditions proposed by Falqué and Fernández [9] and Bertolini et al. [7] for the determination of organic acids, a two-level full factorial design was applied in order to reach a proper separation of citric and tartaric acids. The factors considered at this stage were the pH and the flow-rate of the mobile phase and the column temperature. A two-level full factorial design was performed. The chromatographic runs were obtained using a randomized arrangement in order to avoid systematic errors produced by uncontrolled factors [10]. A standard solution containing the organic acids studied at the concentrations typically found in wine vinegars was injected in triplicate for each run. Resolution was calculated according to Schoenmakers [11], the citric–tartaric peak resolution was taken as the analytical response since the remaining peak pairs are well resolved.

The factorial design was performed by applying multiple linear regression models [12] and the coefficients obtained indicate that the main effects are due to pH and flow-rate, and the unique significant interaction relates these two factors. The resolution is not affected by temperature in the assayed range (60–70°C). Since the maximum advisable temperature for this column is 65–70°C, 60°C was chosen to avoid damage to the packing material. The best resolution values appeared at pH 1 and a flow-rate of 0.5 ml/min. Thus, a mobile phase consisting of an aqueous sulphuric solution of pH 1 at a flow-rate of 0.5 ml/min and a column temperature of 60°C were the selected chromatographic conditions to develop the ion exclusion HPLC method. The output corresponding to these conditions is presented in Fig. 1a.

3.1.2. Peak identification

Organic acids were identified by retention times and by matching their spectra with those of standard solutions. To test peak purity the analytical column was placed in a liquid chromatograph equipped with a photodiode array detection (PDA) system; spectral
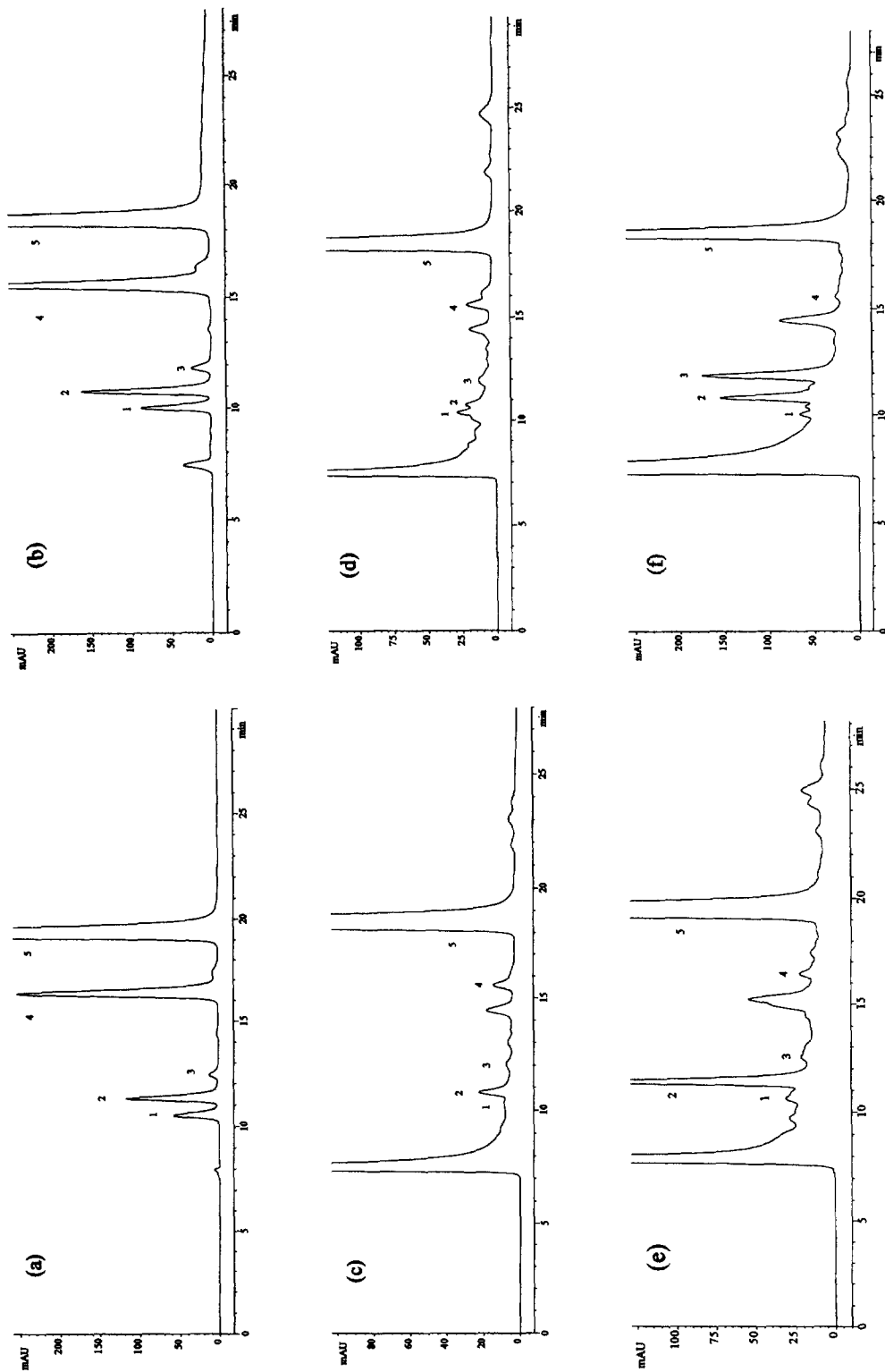
Fig. 1. (a) Chromatogram of a standard solution of organic acids. Isocratic elution using Aminex HPX- 87H column (300×7.8 mm I.D.). Mobile phase: aqueous sulphuric acid solution adjusted to pH 1; flow-rate, 0.5 ml/min; temperature, 60°C; detection: UV absorption at 214 nm. Peaks: 1=citric; 2=tartaric; 3=malic; 4=lactic; 5=acetic. (b) Chromatogram of a vinegar matrix spiked with the organic acid studies. (c) Chromatogram of a wine vinegar (sample code QV3) diluted 1:4 (v/v). (d) Chromatogram of a Chromatogram of a sherry wine vinegar (SV1) diluted 1:4 (v/v). (f) Chromatogram of a Aceto Balsamico vinegar diluted 1:4 (v/v). cider vinegar (CV1) diluted 1:4 (v/v). (e) Chromatogram of a sherry wine vinegar (SV1) diluted 1:4 (v/v). (f) Chromatogram of a Aceto Balsamico vinegar diluted 1:4 (v/v).

data were taken each 2.4 nm in a wavelength range set between 200 and 400 nm. The identification of citric, tartaric, malic and acetic acid was verified by both methods; lactic acid seems to coelute with another compound but it was demonstrated that at 214 nm the absorbance was maximum for lactic acid and negligible for the coeluting compound. At the selected operational conditions, the retention times (in min) rounded up to the last significant figure for the studied organic acids are indicated in the following: 10.4 (citric acid), 11.3 (tartaric acid), 12.2 (malic acid), 16.2 (lactic acid) and 19.7 (acetic acid). Some discussion of the nonidentified peaks is given in the following. In Fig. 1a two unidentified peaks appear: peak at 8 min that corresponds to injection and peak at 17.5 min that may correspond to one impurity of the commercial lactic acid which also contains 2-hydroxipropionic acid, lactoil lactic acid and polyacetic acid [13].

3.2. Method validation

Method validation entails evaluation of various parameters of the method such as selectivity, accuracy, precision, linearity (concentration–detector response relationship), sensitivity, detection and quantitation limit and recovery from the matrix. For the mentioned parameters, the works of Metha [14], Green [15] and Rodríguez et al. [16] were followed.

3.2.1. Specificity

The chromatogram of a test mixture (the studied organic acids spiked into the synthetic vinegar matrix) is depicted in Fig. 1b. The specificity criterion for an assay method is that analyte peaks will have baseline chromatographic resolution of at least 1.5 from all the other sample components. In our case, all the peak pairs are well resolved with resolutions greater than 1.7.

3.2.2. Linearity

The peak area using absorbance detection at 214 nm, was studied for each organic acid. Five levels of concentrations were tested in triplicate; these concentrations correspond to the expected values in vinegars once they have been suitably diluted (see Section 2.4). Using a zero intercept, excellent linearity was obtained in all cases with correlation co-

efficients higher than 0.99990. Rough linear ranges are as follows (in g/l): 0.006– 0.825 (citric), 0.025–1.25 (tartaric), 0.012–0.5 (malic), 0.005–0.625 (lactic) and 0.72–14.5 (acetic).

3.2.3. Accuracy and recovery studies

Based on our experience in vinegar analysis and literature data [1,7,17], a test solution was prepared containing 0.40 g/l of citric acid, 0.50 g/l of tartaric acid, 0.15 g/l of malic acid and 0.30 g/l of lactic acid and 10.75 g/l of acetic acid. The average recovery was calculated as the mean value obtained by three different additions of the organic acids studied at three levels: level 0 (the concentrations in the test solution), level −1 (75% the concentrations in the test solution) and level +1 (125% the concentrations in the test solution). Each solution was injected in triplicate. Average recoveries ranged within 97±3 and 103±6%. By applying the student t-test to the average recoveries [16,18], the null hypothesis (the recovery is close to unity and the method is accurate) was accepted at a significance level of 5%.

3.2.4. Limits of detection and determination

Both detection (LOD) and quantitation (LOQ) limits need to be determined only for impurity methods [15]. In our case, the proposed method is for assaying of organic acids in vinegars, and consequently the detection and quantitation limits are not required. However, the LOD and LOQ values obtained from the calibration curve [10] were (in g/l): citric (0.02, 0.05), tartaric (0.02, 0.05), malic (0.002, 0.007), lactic (0.004, 0.014) and acetic (0.25, 0.82).

3.2.5. Uncertainty studies

To test the precision of the method, the test solution was used for testing the instrument precision (injection repeatability) and the intra-assay reproducibility precision. The instrument precision (repeatability) expressed as coefficient of variation or relative standard deviation (R.S.D.) was evaluated from ten successive injections and yielded values ranging from 3.8% for acetic acid to 5.7% for lactic acid. The intra-assay precision (reproducibility) was tested over 10 working days by injecting the test

solution five times per day; values ranged from 4.3% for tartaric acid to 8.6% for acetic acid.

3.2.6. Robustness

The robustness of the proposed method was partially determined. Sample, elevated pH along with a reduced temperature were overcome. In our case the robustness criterion selected is that if the following changes in the chromatographic conditions: pH of eluent 1 ± 0.2 units; flow-rate 0.5 ± 0.1 ml/min; column temperature $60\pm3°C$, produce unaffected results in the peak quantitation, then the method is considered robust. Column lifetime should also be taken into account for robustness but with care, because column lifetimes are provided as averaged values and not for particular use.

Robustness was tested by injecting the test solution in triplicate at the operational conditions I (pH 1.2, flow-rate 0.6 ml/min, column temperature 63°C) and II (pH 0.8, flow-rate 0.4 ml/min, column temperature 57°C). The concentration of organic acids found in each case were statistically equivalent and, consequently, the method may be considered robust.

3.3. Determination of organic acids in vinegar samples

The method was applied to different vinegar samples as mentioned in Section 2.4. The organic acids were quantified using external calibrations based on peak area. Samples were diluted four times prior microfiltration and injection; for the determination of acetic acid in sherry wine vinegars a tenfold dilution was performed. In exceptional cases whenever the concentration of a single acid was lower than the LOQ, the vinegar was directly injected in order to quantify this acid, for example tartaric acid in cider vinegars. Each sample was injected in triplicate. The results obtained are presented in Table 2. Chromatograms for wine vinegar, sherry wine vinegar, cider vinegar and Aceto Balsamico are shown in Fig. 1c–f, respectively.

Citric acid was the least abundant, specially in cider vinegars. Tartaric acid is well represented in grapes derived products; it is present in considerable amounts in all wine vinegar samples, including Aceto balsamico and in low concentrations in cider vinegars. In cider vinegars as well as in all categories of nonwine vinegar, the low level or absence of tartaric acid suggests its possible use as an origin indicator for vinegars [19,20]. Malic acid is present in all the samples analysed. Cider vinegars and sherry vinegars contain this acid in appreciable amounts; the highest concentration is found for Balsamic vinegar as has been pointed out by Plessi et al. [21]; in this case to quantify malic acid a dilution higher than tenfold was needed. Lactic acid is produced by bacteria from malic acid during malolactic fermentation; the high malic acid content

Table 2
Determination of organic acids in different vinegar samples

Vinegar samples	Organic acids (g/l)				
	Citric	Tartaric	Malic	Lactic	Acetic
QV 1	0.288±0.004	0.34±0.01	0.079±0.006	0.181±0.007	59.05±0.01
QV 2	0.035±0.001	0.363±0.001	0.059±0.000	0.120±0.001	58.6±0.2
QV 3	nq	0.271±0.007	0.109±0.001	0.42±0.02	57.1±0.6
CV 1	nq	0.071±0.001	0.292±0.005	0.572±0.009	46.2±0.2
CV 2	nq	0.020±0.001	0.39±0.01	0.648±0.004	46.9±0.5
CV 3	nq	0.059±0.001	0.30±0.02	0.58±0.01	46.7±0.6
SV 1	0.073±0.005	2.9±0.2	0.23±0.01	0.32±0.03	76.8±0.5
SV 2	nq	1.65±0.02	0.248±0.005	0.30±0.02	69.4±0.1
SV 3	0.062±0.004	2.57±0.01	0.24±0.01	0.21±0.01	86.3±0.8
ABM	0.300±0.001	2.25±0.01	6.74±0.06	0.263±0.003	48.2±0.3

Results expressed as means±S.D. from triplicate measurements.
Abbreviations: QV, wine vinegar produced by submerged fermentation; CV, cider vinegar; SV, sherry vinegar and ABM, Aceto Balsamico Modena; nq=not quantitated, values under the LOQ.

of apples is commonly reduced in ciders by this malolactic fermentation but the high amount of lactic acid formed is partially oxidised by acetic bacteria, thus, it is present at levels ranging 0.572–0.648 in cider vinegars. As expected, levels of acetic acid are very high in sherry vinegars.

A peak at 8 min is observed in all samples and it corresponds to caramel, a normal component of vinegars. This peak is large in the balsamic vinegar of which it is an important component.

4. Conclusions

A new ion-exclusion HPLC method for determination of organic acids in vinegars was developed; its advantages are good linearity, high precision and accuracy, ease and speed operation. This method does not require any complicated sample preparation, so may be used routinely.

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