Organic acid profiling by liquid chromatography for the characterization of base vines and sparkling wines

Anais Izquierdo-Llopart, Aida Carretero and Javier Saurina✉

Department of Chemical Engineering and Analytical Chemistry, University of Barcelona. Martí i Franquès, 1-11, E-08028 Barcelona, Spain.

✉ Corresponding author: Javier Saurina

e-mail: xavi.saurina@ub.edu

Abstract

A rapid and reliable method based on liquid chromatography with UV detection has been developed here to determine the main organic acids in base and sparkling wines of the protected designation of origin Cava. Compounds have been separated by reversed-phase mode with a water/acetonitrile solution (95:5 v/v adjusted to pH 2). Figures of merit established at 210 nm are fully compatible with the wine analysis, with correlation coefficients better than 0.996, repeatabilities around 2% and detection limits generally below 1 g L^{-1}. A total of 53 base wine and 140 cava samples from different *coupages* have been analyzed. Compositional profiles of organic acids have been used as the source of analytical information for characterization and classification purposes. Results have shown that varietal and blending issues, malolactic fermentation and tartaric acid stabilization affect the composition of organic acids.

Keywords: Organic acids; Liquid chromatography; Base wine; Sparkling (cava) wine; Characterization; Principal component analysis.

Introduction

Low molecular weight organic acids are important natural constituents of wines. Some of them are originally present in the grape while others appear during subsequent fermentation processes as a consequence of (bio)chemical reactions. For instance, tartaric, gluconic, malic and citric acids, come directly from the grape while succinic, fumaric, lactic and acetic acids are mainly produced during the winemaking processes (Chidi et al. 2018). Tartaric acid is the main acid of wine, accounting for ca. 30% of the total acids (Sweetman et al. 2009). Tartaric acid is resistant to decomposition by bacteria, so its transformation into lactic and acetic acid is quite residual. Malic acid is microbiologically labile, thus resulting in lactic acid in the course of malolactic fermentation (Maicas 2001; Versari et al. 1999). Citric acid is another subtract of lactic bacteria so its concentration typically decays in the course of winemaking processes. Succinic and acetic acids are other secondary fermentative products, the latter being related to unwanted vinegary spoilage (Chidi et al. 2018). Gluconic acid is a minor component typically associated to an excessive fruit ripening so its occurrence at high concentration is often a sign of poor grape quality .

Organic acids strongly influence on some organoleptic features such as taste and equilibrium. In this way, acids give to wines a slightly tart flavor, but this can be modulated by alcohol, sugars, minerals and other components. Organic acids are also relevant chemical descriptors of interest for quality control purposes (Ragone et al. 2015; Saurina 2010), providing information on origin, grape variety, microbiological growth and oenological practices. Levels of acids may affect the color, taste and aroma of the wine. Also, they influence on the stability and microbiological quality of the wine, stopping or, at least, delaying the growth of harmful microorganisms that could cause wine spoilage. The evolution of the acidity during the several stages of wine and

cava production is used by the winemakers to know about the quality of the final product.

Traditionally, the wineries used potentiometric and volumetric methods to assess the total and volatile acidity of wines. The quantification of individual compounds such as tartaric, malic, lactic, acetic and gluconic acids has been carried out by enzymatic, spectroscopic and chromatographic methods (Mato et al. 2005; Sochorova et al. 2018). Enzymatic approaches are highly selective but may result in time-consuming and expensive analyses due to the need of specific reagents for each species (Sochorova et al. 2018; Zeravik et al. 2016; Mazzei et al. 2007). New devices based on gold and nanocomposite technologies have contributed to improve the detection (Monosik et al. 2012). Flow-injection analysis has been used to facilitate the automation of the enzymatic processes combined with spectroscopic detection (Mataix et al. 2001). Spectroscopic methods for multianalyte determination rely on Fourier Transform Infrared (FTIR) with further chemometric analysis by Partial Least Square (PLS) regression (Regmi et al. 2012; Pizarro et al. 2011).

Separation techniques result in one of the most convenient approaches for the simultaneous determination of a wide range of the organic acids in wine samples (Mato et al. 2005; Sochorova et al. 2018). Among them, HPLC is the most common technique since the pioneering studies by Palmer et al. (Palmer et al. 1973). Regarding the separation in HPLC, reversed-phase (RP) and ion exchange modes have been extensively used, combined with UV spectrophotometric, refractive index and electrochemical detection (Li et al. 2018; Coelho et al. 2018; Zheng et al. 2009; Kerem et al. 2004). Recently, new RP stationary phases with alkyl (e.g., C18) groups have been especially designed to retain a wide range of hydrophilic species using eluents with high percentages of water (up to 100%). These RP alkyl columns rely on silanol endcapping

with trimethylsilyl groups to provide good stability and full compatibility with polar solvents like water. As a result, the use of this type of columns in current analytical laboratories has been consolidated and numerous studies have been published in this regard (Long et al. 2009; Dos Santos Lima et al. 2019). Anyway, despite the excellent performance of these columns, the separation of food components is difficult and various analytical issues remain still unresolved, such as the complex retention behavior of analytes as a function of the pH of the mobile phase, and the diversity of interfering species occurring in the sample. In this regard, sample pretreatments such as dialysis and electrodialysis coupled to HPLC can provide better results (Kritsunankul et al. 2009; Ohira et al. 2014). Apart from HPLC, gas chromatography (GC) and capillary electrophoresis (CE) have also been used for the determination of organic acids in wines. In the case of GC, analytes must be derivatized to decrease their polarity and increase volatility using, for instance, silanization reactions (Zhang et al. 2018). CE, in contrast, is envisaged as a natural separation mode for charged molecules such as organic acids so that several papers have been published on this topic (Rovio et al. 2011; Peres et al. 2009; Mato et al. 2007).

Cava is a type of sparkling wine of high quality with Protected Designation of Origin (PDO) produced by the *Champenoise* method. Cava is gaining popularity in our society because of its excellent organoleptic features thus, currently, resulting in the most exported Spanish wine (Buxaderas et al. 2012; http://www.institutdelcava.com/en/). Cava starts from base wines conveniently blended which are subjected to a second fermentation taking place in the bottle, followed by an aging period for a minimum of 9 months in the cellar before commercialization. Although the classical *coupage* is composed of Macabeu (Ma), Xarel.lo (Xa) and Parellada (Pa) varieties, in the last years new varieties of white and red grapes have

been introduced, such as Chardonnay (Cha), Pinot Noir (PN), Trepat (Tr) and Garnacha (Ga), the latter producing rosé products (Izquierdo-Llopart et al. 2019).

In this paper, a new HPLC method with UV detection has been developed to determine organic acids of low molecular weight in base wine and cava samples. The analytical method has been optimized carefully to improve detection and separation features. Analytical parameters such as linearity, detection limits and repeatability have been established under optimal working conditions. Here, fingerprints from by HPLC-UV and compositional profiles related to organic acids have been exploited as the source of information for characterization purposes. The corresponding data sets have been analyzed using radial diagrams and principal component analysis (PCA). Patters among chemical composition and oenological features have encountered, thus demonstrating the applicability of the method to the characterization and quality control of these products.

Materials and methods

Chemicals and solutions

Phosphoric acid (85% w/w, Merck), acetonitrile (UHPLC PAI-ACS SuperGradient, Panreac, Castellar de Valles, Barcelona, Spain) and Milli-Q water (Millipore Bedford) were the components of the mobile phase. Reagents for the preparation of organic acids standards were tartaric, malic, citric, succinic, fumaric, gluconic, acetic and lactic acids (analytical reagent grade, Merck, Darmstadt, Germany). Stock solutions at a concentration of 10 g L^{-1} were prepared in Milli-Q water (from Milli-Q system, Millipore Bedford, USA). Standard working solutions were prepared in the range 1 to

8000 mg L^{-1}. The highest one was prepared with Milli-Q water and the others by the appropriate dilution with the mobile phase.

Samples

53 base wines and 140 cava samples of different blends (*coupages*) were kindly provided by the winery Raventós Codorníu (Sant Sadurni d'Anoia, Barcelona, Spain). Base wines resulting from a first alcoholic fermentation in tanks were made with 10 different blends (see Table 1) of the following grape varieties: Macabeu (Ma), Xarel·lo (Xa), Parellada (Pa), Chardonnay (Cha), Monastrell (Mo), Pinot Noir (PN), Garnatxa negra (Ga) and Trepat (Tr). All blends were subjected to malolactic fermentation (MLF), with the exception of *coupage* I. Cava samples resulting from a second fermentation of base wines consisted of 11 *coupages* as indicated in Table 2. They were the same as those previously defined in Table 1 except for the additional *coupage* K, analogous to *coupage* A but with 15-30 months of aging period. A quality control (QC) for the set of base wines was prepared by mixing 100 µL of each wine sample. In the same way, another QC for the series of cava samples was prepared. QCs were analyzed repeatedly every 10 sample injections to detect and minimize possible chromatographic variations and evaluate the soundness of PCA models. All the samples were degasified and filtered through 0.45 µm nylon filters (Whatman, Clifton, NJ, USA) prior to the analysis.

Chromatographic method

An Agilent HPLC 1100 LC system (Agilent Technologies, Santa Clara, CA, USA) equipped with quaternary pump (G1311A model), vacuum degasser (G1379A model), autosampler (G1392A model) and diode array detector (DAD, G1315B model) was

used. Data was processed with an Agilent ChemStation for LC 3D (Rev. A. 10.02) offline software.

Analytes were separated in a C_{18} polar analytical column Zorbax SB-Aq (4.6 mm ID × 150 mm, 5 µm particle size, Agilent Technologies) under isocratic elution with acidified water/acetonitrile solution (95/5, *v/v*) adjusted to pH 2 with phosphoric acid. The column was set at room temperature, the injection volume was 10 µL, the flow rate 1 mL min^{-1} and the run time 5 min. The UV detection was performed at 210 nm. Apart from the selected column, the performance of the following columns was investigated during the optimization process: Kinetex C_{18} polar (Phenomenex, Torrance, CA, 100 mm x 4.6 mm I.D. with 2.6 µm particle size), Spherisorb S10 NH2 (Waters Corporation, Milford, MA, 250 mm x 4.6 mm I.D. with 5 µm particle size), XTerra® C_{18} (Waters, 150 mm x 4.6 mm I.D. with 3.5 µm particle size), Rezex Roa (Phenomenex, 150 mm x 7.8 mm I.D. with 8 µm particle size), and Syncronis TM HILIC (Thermo Fisher Scientific Inc., 100 mm x 4.6 mm I.D. with 5 µm particle size).

Data analysis

Base wines and cavas were characterized according to their levels of organic acids as the source of analytical information. Samples were preliminary evaluated by radial plots obtained with Excel (Microsoft, Redmon WA, USA). Principal component analysis (PCA) using the PLS-Toolbox (working under MATLAB, Applied Chemometrics, Inc, PO Box 100 Sharon, USA) was further applied to relate the organic acid contents with the wine classes.

Two different types of data matrices were analyzed by PCA under profiling and fingerprinting approaches, which consisted of organic acid concentrations and chromatograms at 210 nm, respectively. As the pretreatment, concentrations were

autoscaled to equalize the descriptive ability of each variable; in fingerprinting, data was smoothed with a Savitzky–Golay filter (second degree fitting, 11-point window) and normalized (vector normalization of each chromatogram within the working time window). In any case, the plot of scores showed the distribution of the samples on the principal components (PCs), thus revealing trends on the varieties and blends of base wines and cavas. The variability of the experimental data was assessed from the dispersion of the QCs which should appear in a compact group in the middle of the scores plot. The plot of loadings showed the distribution of variables and their impact on the sample features.

Results and discussion

Optimization of the chromatographic conditions

First studies were focused on the optimization of the detection and separation conditions of the HPLC-UV method. The detection of organic acids by UV spectroscopy is, in general, difficult because of the quite poor absorption features of these analytes in UV range. Apart from fumaric and lactic acids which displayed a reasonable absorptivity above 240 nm, the other analytes were detected at 210 nm.

The separation of organic acids by HPLC was envisaged as a complex issue owing to the high polarity of analytes. Here, separation conditions were first optimized using pure analyte standards, including, acetic, lactic, fumaric, tartaric, malic, succinic, gluconic and citric acids. Several analytical columns were investigated, covering a wide range of interaction mechanisms such as hydrophobicity, hydrophilicity and anion exchange. A preliminary study was carried out to select the most promising columns and disregard the less satisfactory ones.

In the first screening, RP columns (e.g., Kinetex C_{18}, Kinetex C_{18} polar and XTerra C_{18}) were tested using hydro-organic solutions (acetonitrile percentage from 0 to 1% *v/v*) acidified with phosphoric and sulfuric acids in the pH range from 1.0 to 7.0 (pH adjusted with a sodium hydroxide solution). Some of these columns were successfully proposed for the study of polar compounds so they were considered here for a preliminary evaluation (Snow et al 2015). In our study, the elution mode was isocratic, the flow rate was 0.5 mL min^{-1} and the column temperature ranged from 23 to 60ºC. Results obtained indicated that the separation was not entirely satisfactory and several compounds co-eluted.

The weak anion exchange column assayed (Spherisorb S10 NH2) consisted of aminopropyl groups chemically linked on silica particles. The separation was investigated at different pH values in the range from 1.4 to 8.0 using a mobile phase of 0.5 mmol L^{-1} phosphoric acid (pH adjusted with sodium hydroxide solution). The elution mode was isocratic and the flow rate was 0.5 mL min^{-1}. The retention behavior depended on both the protonation of the exchanger occurring below pH 8 and deprotonation of analytes. As a result, it was found that analytes co-eluted at pH 1.5 because of their poor interaction with the exchanger; the interaction increased with pH up to 6.5 due to the formation of carboxylate anions of analytes, and finally decayed at pH 8.0 due to the loss of exchange ability of the column. The retention behavior was complex, especially for polyprotic compounds as multiple charged species were involved. Besides, proper separation conditions without peak overlapping could not be found. Other conditions such as column temperature (in the range 20 to 80ºC) and addition of acetonitrile as an organic modifier (from 0 to 10% *v/v*) were also investigated. Anyway, although the retention varied with these factors, the selectivity was seldom modified so that the separation of co-eluting compounds was not improved.

The possibilities of size exclusion as the separation mechanism were investigated using a sulfonated polymeric Rezex ROA column. The mobile phase consisted of 2.5 mmol L^{-1} sulfuric acid solutions (pH was adjusted to 1.4, 2.5 and 6.0 using sodium hydroxide solution). The column temperature was set to 20 and 80ºC and the flow rate was 1.5 mL min^{-1}. The separation of most organic acids, evaluated from pure standards, was successful, with chromatograms displaying good resolutions and peak symmetries. The best separation was obtained at pH 2.5, although double peaks were obtained for various compounds. Anyway, chromatographic results were not fully satisfactory when dealing with wine samples. This finding was attributed to the higher complexity of the wine matrix and the occurrence of interferences, possibly from phenolic acids. This column was finally discarded because of the coelution of the analytes with other matrix components.

The separation performance of a polar alkyl-based column (Zorbax SB-Aq) was also investigated in detail as follows. In recent years, the use of this type of columns has become more popular and numerous studies have been published in this regard. In particular, this RP stationary phase was conceived to retain a wide range of compounds, especially hydrophilic species, using high percentages of water in the mobile phase (Long et al. 2009). In this case, analytes should be neutral to enhance their interactions with the stationary phase. The mobile phase consisted of 20 mmol L^{-1} phosphoric acid solution and pH was varied from 2.0 to 3.0 to protonate the carboxylic groups. The flow rate was 1 mL min^{-1}. It was found that at pH 2.0 all the components were reasonably separated while at pH 3.0 the retention decreased and some overlapping peaks occurred. The effect of the addition of acetonitrile to the organic phase was studied in the range 0 to 10% *v/v*. Results shown in Fig.1 indicate that retention decreased with increasing the organic solvent content. An optimal compromise among separation and analysis time

was obtained with 5% acetonitrile, so this composition was chosen for further experiments. As an example, chromatograms of standard solutions of the organic acids, and representative white and rosé cavas are depicted in Fig. 2. It can be seen that compounds were successfully resolved and compositional profiles of samples showed differences that could be exploited for descriptive purposes.

Method validation

Quality parameters of the proposed HPLC-UV method were established with pure organic acid standards and selected wine and cava samples. Results have been summarized in Table 3. The linear range of the calibration was established from the injection of 10 standard solutions with different analyte concentrations, namely: 20 to 2000 mg L^{-1} for acetic, succinic and gluconic acids; 10 to 2000 mg L^{-1} for citric acid; 10 to 5000 mg L^{-1} for lactic acid; 5 to 5000 mg L^{-1} for malic acid; 10 to 8000 mg L^{-1} for tartaric acid; 1 to 500 mg L^{-1} for fumaric acid. Calibration models obtained by least square regression displayed excellent linearity, with determination coefficients better than 0.993. The repeatability of the method was evaluated from 10 replicated injections of a standard mixture of 200 mg L^{-1} for tartaric and succinic acids, 50 mg L^{-1} for acetic acid, 25 mg L^{-1} for citric, lactic, gluconic and malic acids and 2.5 mg L^{-1} for fumaric acid. The relative standard deviation (RSD%) in terms of retention time was below 0.3% and around 6% in terms of peak area. Limits of detection (LODs) and quantification (LOQs) were estimated from 10 replicated injections of a standard solution of the different analytes at 50 mg L^{-1} each, except for fumaric acid which was assayed at 5 mg L^{-1}. LODs and LOQs were calculated at signal-to-noise ratios of 3 and 10, respectively. It should be pointed out that these values were in the order of magnitude of mg L^{-1}, fully compatible with the typical levels of organic acids in the wine and cava samples. The accuracy of the method was studied from a

spiking/recovery procedure in which a representative sample was spiked with the organic acids at the levels specified for the study of the method repeatability. The mean recovered concentration from a series of 6 independent replicates calculated as a percentage ($c_{recovered}/c_{spiked} \times 100$) was used to express the accuracy values, $c_{recovered}$ and c_{spiked} being the calculated and added concentrations, respectively. Results were in the range 89 to 111%, thus indicating that the method proposed was suitable for the analysis of wine and cava samples.

The performance of the proposed method was compared with other recent publications dealing with the determination of organic acids in wines by HPLC and related techniques (see Table 4). As can be seen, tartaric, malic, lactic, acetic, citric and succinic acids were commonly quantified as they were the most relevant compounds. Anyway, in some cases, other specific acids were investigated such as shikimic, glucuronic, glucaric, etc. In genral, RP mode with stationary especially adapted for polar species was the choice of several authors. Alternatively, ion exchange mode was another explored possibility. Regarding detection, UV at 210 nm was widely used, providing LODs in the range 0.1 to 10 mg L^{-1}; in general these values were higher than those reported here. Electrochemical and refractive index detectors improved LOD values in one order of magnitude, approx. LC-MS platforms provided additional advantages such as improved sensitivity and selectivity, and allowed new compounds to be identified, of course, at the expense of more complex and expensive assays. Other analytical parameters of our method, such as linearity, repeatability and accuracy were similar values previously published (see Table 4). Regarding to runtime, our proposed method allowed quite fast analyses thus being especially suitable for dealing with the study of large series of samples for quality control and authentication issues. In summary, our proposal seems to be a good option for the determination of organic acids

in wine matrices given its great analytical performance, standing out for its simplicity, low cost, robustness and speed.

Characterization of base wine and cava samples from the protected designation of origin Cava

Compositional profiles of organic acids

Average concentrations of organic acids in each *coupage* of the base wine and cava samples are given in Tables 5 and 6, respectively. This data has been used to plot several radial diagrams of organic acids concentrations depending on the classes (see Fig. 3). By far, tartaric acid is the most abundant acid in base wines and cavas due to the high levels occurring in the grapes. Tartaric acid concentrations in base wines were quite disperse, ranging from 6.48 to 10.18 mg L^{-1}, with highest concentrations for PN and BN (without MLF). More homogeneous values were found in cavas (from 4.6 to 5.7 mg L^{-1}), thus indicating that the descriptive ability of this variable for cava discrimination was poor. Besides, a noticeably decrease in tartaric acid concentration was found (ca. 35% lower) when comparing cavas and base wines. This decay was attributed to the tartaric stabilization process to which the base wine was subjected before performing the second fermentation. This oenological process was focused on limiting the quantity of potassium bi-tartrate and neutral calcium tartrate in the final products to avoid further precipitation.

From the quantitative point of view, malic acid was the second most important acid in these samples. Before MLF, this compound occurred at concentrations from 2 to 4 g L^{-1}, approximately, base wines and below 1 g L^{-1} in cavas. Exceptionally, *coupage* I, which was not subjected to MLF, displayed similar concentrations in both base wine and in cava samples. Regarding the influence of grape varieties on the malic acid

content, it was evidenced that base wines from Cha variety were especially rich while the classical blend (Ma, Xa and Pa) and the rosé combination (Mo, Ga and Tr) showed the lowest levels. Inversely correlated with the evolution of malic acid, lactic acid was mainly generated by the action of lactic bacteria during the MLF process. Thus, the amount of lactic acid in c*oupage* I was lower than in other blends because of the absence of MLF.

Citric acid was present in base wines at concentrations ranging from 0.5 to 1.7 g L^{-1} depending on the blend. This acid decays significantly when the wine is fermented by lactic bacteria because it is a sensitive substrate to this type of microorganisms. Levels from 0.10 to 0.32 g L^{-1} were found in cava samples. For varietal comparison, base wines of c*oupages* elaborated with high percentages of the Cha variety (e.g., G, S and A) displayed high values of citric acid since the corresponding grapes are richer in this component.

Succinic acid is very stable in front of microbiological processes, so its evolution throughout vinification and aging is quite irrelevant. As it can be seen, concentrations in base wines and cavas are similar, in the range from 0.5 to 0.7 g L^{-1}, approximately. In cavas, for instance, the comparison of 9-months and 18-months aged samples with the same varietal composition (e.g., *coupages* A and K) revealed almost identical concentrations.

Fumaric and acetic acids were below the detection limits so they were irrelevant for descriptive purposes. In the acetic acid case, this finding indicates that MLF was done under optimal conditions. Gluconic acid appears in ripe fruits as an indicator of grape putrefaction. Values higher than 0.6 g L^{-1} have been associated to spoiled grape which is not recommendable for wine production. In our sets of samples, gluconic acid

concentrations were lower than 0.5 g L^{-1}. For descriptive purposes, for instance, combinations with red grapes and Cha varieties presented higher values than those from classical blends.

Principal component analysis

In order to carry out a more comprehensive characterization of wines as a function of the contents of organic acids, PCA was applied using both chromatographic fingerprints and concentration profiles as the source of information.

First models were established from chromatograms recorded at 210 nm. Data was preprocessed by smoothing and normalization to minimize the influence of the overall intensity on the description. In the case of base wines, the distribution of samples as a function of blends was better visualized from the scatter plot of PC1 versus PC4 (see Fig. 4a). Results showed that QC samples appeared in a compact group in the middle of the graph, thus indicating the excellent reproducibility of chromatographic data as well as the descriptive ability of the model. A good separation between rosé and white wines was observed across PC1, with wines rich in red grape varieties predominating on the left part and those from white varieties (blends of Ma, Xa, Pa and Cha) located on the right side. PC4 mainly discriminated among white *coupages*, with the three classical varieties to the bottom and Cha to the top part.

Regarding cava wines, Figure 4b shows the corresponding plot of scores. In contrast to the base wine description, here the discrimination of samples as a function of grape varieties and blends was not so well defined. This finding was attributed to the correction in the organic acids contents before the second fermentation of the traditional *Champenoise* method from which levels of acids tended to be more similar. As a result, although samples belonging to the same *coupage* were clustered, some overlapping

among classes was found so that the relevance of organic acids as the descriptors of cava classes was limited.

Profiling data consisting of concentrations of organic acids in base wine and cava samples was also evaluated by PCA. For base wines, the data matrix consisted of the contents of the 8 acids in the 53 samples plus the QCs regularly injected (every 10 samples). Data was autoscaled before PCA to equalize the influence of major and minor components on the description. 3 PCs were able to explain ca. 90% of the experimental variance (with PC1 retaining a 36.98%, PC2 a 25.76% and PC3 a 19.71%). The plot of scores (Fig. 5a) suggested that PC1 mainly separated wines according to the content of Cha in the blends. Hence, the monovarietal Cha was on the right while the Ma, Xa and Pa mixing appeared in the opposite site. Correspondingly, *coupages* with a low percentage of Cha appeared close to the classical varieties while those richer in Cha tended to the right. The application of MLF was clearly distinguished form PC3, with base wines, with BN without MLF found in a compact group to the top and the treated ones to the bottom part. Information gained for the loadings plot indicated that malic acid was abundant in BN without MLF, citric acid in classes with the Cha and succinic acid in classical *coupages* (Ma, Xa and Pa). Finally, wines from red grapes presented increased levels of gluconic acid. These results were in agreement with preliminary conclusions extracted from radial diagrams (see in section 3.3.1).

In the case of cavas, the corresponding matrix was composed of organic acid concentrations of 140 samples belonging to 11 classes of different blends and the QCs. Results by PCA (not given here) showed that PC1 and PC2 explained 38.80% and 17.94% of the experimental variance, respectively. Regarding the sample distribution, QCs were clustered in the center of the model and cavas from the same *coupage* were grouped together. Unfortunately, although some general patterns could be deduced no

clear separation among blends was encountered with the exception of non MLF (right side) versus MLF (left side) classes. As above indicated, this finding was attributed to the fact that organic acid concentrations in the cava samples were quite similar regardless of blends since they were oenological corrected to obtain more homogeneous lots from an organoleptic point of view.

Conclusions

The HPLC-UV method developed here was applied successfully to the determination of organics acids in base wine and cava samples. Among the diverse separation possibilities that could be suitable to address the separation, including reversed-phase, HILIC and anion exchange, the reversed-phase mode especially adapted to polar compounds provided the best results. Hence, analytes were chromatographically resolved without interferences from other endogenous wine species. The exploratory study of compositional profiles of organic acids revealed important differences among base wines and cavas. In particular, analyte amounts in base wines depended on the blends and grape varieties while their composition in the set of cavas was quite homogeneous (and lower). This behavior was attributed to the corrective actions applied to the cava production (including malolactic fermentation, tartaric stabilization, second fermentation, etc.) which equalized the acidity features.

Data from both chromatographic fingerprints and compositional profiles were treated by PCA to gain overall information on organic acid descriptors. Results from the two approaches were similar, in agreement with previous conclusions extracted from radial diagrams. Hence, we believe that organic acids may result in useful descriptors of varieties and blends at the stage of base wine but they offer limited possibilities to

discriminate among cava classes because of the corrective oenological processes applied to the winemaking procedure.

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Compliance with Ethical Standards

Conflict of Interest: The first author Anaïs Izquierdo Llopart, the second author Aida Carretero and the corresponding author Javier Saurina declare that they have no conflict of interest.

Ethical Approval: This article does not contain any studies with humans and animals performed by any of the authors.

Informed Consent: Not applicable.

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Table 1.Characteristics of base wine samples studied.

Class	Blends	Number of samples	Malolactic fermentation	Aging process	pH	Ethanol (% v/v)
C	Classical (Ma / Xa / Pa)	6	YES	NO	3.1 ± 0.1	10.6 ± 0.2
G	Cha	6	YES	NO	3.0 ± 0.1	10.7 ± 0.2
I	BN	4	NO	NO	3.0 ± 0.1	10.7 ± 0.2
P	PN	4	YES	NO	3.0 ± 0.1	11.0 ± 0.2
W	BN	6	YES	NO	3.2 ± 0.1	10.9 ± 0.2
A	Classical (30%) / Cha (70%)	6	YES	NO	3.1 ± 0.1	10.9 ± 0.2
E	Classical (85%) / Cha (15%)	6	YES	NO	3.1 ± 0.1	10.7 ± 0.2
S	Ma (25%) / Xa (25%) / Cha (50%)	6	YES	NO	3.2 ± 0.1	10.8 ± 0.2
T	PN (70%) / Cha (30%)	6	YES	NO	3.1 ± 0.1	10.9 ± 0.2
V	Mo / Ga / Tr	3	YES	NO	3.0 ± 0.1	10.9 ± 0.2

Blend reference: Ma, Macabeu; Xa, Xarel·lo, Pa, Parellada; BN, Blanc de Noirs (made from Pinot Noir grapes); PN, Pinot Noir; Tr, Trepat; Ga, Garnatxa; Mo, Monastrell; Cha, Chardonnay.

Table 2. Characteristics of cava samples studied. See Table S1 for blend identification.

Class	**Blends**	**Number of samples**	**Malolactic fermentation**	**Aging process** (months)	**pH**	**Ethanol** (% v/v)
C	Classical (Ma / Xa / Pa)	10	YES	9	2.9 ± 0.1	11.7 ± 0.2
G	Cha	10	YES	9	2.8 ± 0.1	12.0 ± 0.2
I	BN	10	NO	9	2.9 ± 0.1	11.8 ± 0.2
P	PN	10	YES	9	2.9 ± 0.1	12.2 ± 0.2
W	BN	10	YES	9	3.0 ± 0.1	11.6 ± 0.2
A	Classical (30%) / Cha (70%)	15	YES	9	2.9 ± 0.1	12.1 ± 0.2
E	Classical (85%) / Cha (15%)	15	YES	9	2.9 ± 0.1	11.9 ± 0.2
S	Ma (25%) / Xa (25%) / Cha (50%)	15	YES	9	2.8 ± 0.1	12.0 ± 0.2
T	PN (70%) / Cha (30%)	15	YES	9	2.9 ± 0.1	12.2 ± 0.2
V	Mo / Ga / Tr	15	YES	9	2.9 ± 0.1	12.1 ± 0.2
K	Classical (30%) / Cha (70%)	15	YES	>15	3.0 ± 0.1	11.5 ± 0.2

Table 3. Validation parameters of the proposed HPLC-UV method established at 210 nm under optimal experimental conditions.

Compound	**RT** (min)	**Linear range*** ($g\ L^{-1}$)	**Sensitivity** ($mAU\ min\ L\ g^{-1}$)	**R^2**	**Repeatability** **peak area** (*sd*)	**LOD** ($mg\ L^{-1}$)	**LOQ** ($mg\ L^{-1}$)
Gluconic acid	1.61±0.005	0.02-1.0	0.0072	0.9948	0.021	0.22	0.75
Tartaric acid	1.80±0.004	0.005-8.0	0.0164	0.9974	0.267	0.81	2.7
Malic acid	2.04±0.004	0.01-3.5	0.0129	1.0000	0.018	0.16	0.55
Lactic acid	2.16±0.005	0.01-3.5	0.0276	0.9999	0.023	1.4	4.8
Acetic acid	2.34±0.005	0.005-1.0	0.0089	0.9930	0.018	0.10	0.33
Citric acid	2.57±0.000	0.01-2.0	0.0139	0.9999	0.012	0.26	0.87
Succinic acid	2.88±0.005	0.02-0.8	0.0077	0.9982	0.023	0.13	0.43
Fumaric acid	3.80±0.004	0.001-0.1	0.8403	0.9973	0.023	0.023	0.07

(*) High limit of each compound corresponds to the maximum tested concentration. RT, retention time; ±, standard deviation of retention time; R^2, determination coefficient, *sd*, standard deviation (n=10) from repeated injections of a standard solution (200 $mg\ L^{-1}$ tartaric and succinic acids, 50 $mg\ L^{-1}$ acetic acid, 25 $mg\ L^{-1}$ citric, lactic, gluconic and malic acids and 2.5 $mg\ L^{-1}$ fumaric acid); LOD, limit of detection; LOD, limit of quantification.

Table 4. Summary of recent publications dealing with the determination of organic acids in wines by HPLC and related techniques.

Reference	Analytes	Samples	Analytical Technique	Separation conditions	Figures of merit	Comments
Ivanova-Petropulos, 2020	Tartaric, malic, shikimic, lactic, citric and succinic acids	Chardonnay and Merlot wines	RP-HPLC-DAD; detection 210 nm	Column: Shimadzu Shim-pack GIST C18 (250 mm × 4 mm ID, 5 μm). Isocratic mode: 0.005 M H_3PO_4 (pH 2.1). Flow rate: 1 mL min^{-1}	r > 0.998; LODs 0.02 – 2,5 mg L^{-1}; LOQs 0.06 – 8,5 mg L^{-1}; repeatability RSD < 10%; reproducibility RSD < 15%; recovery 94.8 – 108%	PCA provided discrimination according to the wine variety
Ricciutelli, 2019	3-IPMA and 2-IPMA	Red and white Italian wines	LC-MS systems, LC-IT and LC-Q-Orbitrap	Column: Grace RP (150 × 2.1 mm ID, 3 μm). Gradient mode: 1% aqueous formic acid and ACN. Flow rate: 0.2 mL min^{-1}	Linear range 5-320 mg L^{-1}; r > 0.991; repeatability RSD < 15.1%; recovery > 86.7%	Identification of new isomers
de Souza, 2019	Gluconic, glucuronic and glucaric	Commercial wines	HPAEC-PAD at gold electrode; detection potential 0.26 V vs Pd/PdO	Column: DIONEX CarboPac PA1 anion-exchange (4 × 250 mm ID, 10.0 μm). Gradient mode: 0.28 mol L^{-1} acetate + 0.10 mol L^{-1} NaOH and ultrapure water. Flow rate: 1 mL min^{-1}	For gluconic acid: Linear range 5×10^{-6} – 2.0×10^{-4} mol L^{-1}; r = 0.9996; LOD 7.0×10^{-8} mol L^{-1}; sensitivity 3.7×10^{6} (nA mol L^{-1}); repeatability RSD 2.3%	Method for the simultaneous detection of sugars (glucose, fructose and arabinose), organic acids and arabitol
Li, 2018	Lactic, tartaric, succinic, citric, maleic, fumaric, and isocitric acids	Red and white wines	HPAEC with PAD and conductivity detection	Column: IonPac AS11-HC Analytical column (4 × 250 mm ID, 10.0 μm). Isocratic mode: 500 mmol L^{-1} NaOH. Flow rate: 0.4 mL min^{-1}	r > 0.99; repeatability RSD 0.62 – 6.18%; reproducibility RSD 0.34 – 3.48%; LODs < 0.03 mg L^{-1}; LOQs < 0.10 mg L^{-1}; recovery 83 – 113%	Simultaneous Determination of Organic Acids and Alditols by a Valve-Switching approach
Coelho, 2018	Tartaric, malic, lactic, citric and acetic acids, and sugars	Commercial wines and juices from Northeast Brazil	HPAEC-RID-DAD; detection 210 nm	Column: Agilent Hi-Plex H ion exchange (300 × 7.7 mm ID, 8.0 μm). Isocratic mode: 4.0 mmol L^{-1} H_2SO_4. Column temperature: 70°C. Flow rate: 0.5 mL min^{-1}.	r > 0.9982; precision RSD < 1.4%; LODs 0.003 – 0.044 g L^{-1}; LOQs 0.008 – 0.199 g L^{-1}; recovery 76 – 106%; run time 20 min	PCA for quality control of the products
Tasev, 2016	Tartaric, malic, shikimic, lactic,	Macedonian red and	RP-HPLC-UV; detection 210nm	Column: LiChrosorb RP-18 column (250 x 4.6 mm ID, 5 μm).	r > 0.99; LODs 0.0007 – 0.0136 g L^{-1}; LOQs 0.0026 – 0.0448 g L^{-1};	Other RP columns were assayed and

	citric and succinic acids	white wines		Isocratic mode: 5 mM H_3PO_4 (pH 2.1). Flow rate: 1 mL min^{-1}.	recovery 94.5 – 105 %; RSD < 3.5%	compared
Ohira, 2014	Acetic, propionic, tartaric, malic, lactic, citric, pyroglutamic, succinic, butyric, valeric, caproic, levulinic, isobutyric and isovaleric acids	Red and white wines	On-line electrodialytic IEC-HPLC-UV; detection 210 nm	Column: ion exclusion RSpak KC-811 (300 × 8.0 mm ID, 5 μm). Isocratic mode: 3 mM $HClO_4$. Flow rate 0.7 mL min^{-1}	Linear range 2 – 50 mmol L^{-1}; recovery 80 – 109%	On-line electrodialytic matrix isolation of organic acids by means of ion transfer device. Removal of multiple matrix components
Pereira, 2010	Tartaric, malic, succinic, lactic, acetic, citric and oxalic acids, polyphenols and furanic compounds	Red, white and rosé wines	RP-HPLC-DAD; detection 210 nm (organic acids)	Column: Atlantis dC18 (250 x 4.6 mm ID, 5 μm) difunctionally bonded C18. Gradient mode: 10 mM KH_2PO_4 (pH 2.70) and ACN. Column temperature: 30°C. Flow rate: 1 mL min^{-1}.	Linear range 0.060 – 1.512 g L^{-1}; r > 0.9997; LODs 0.001 – 0.046 g L^{-1}; recovery 97 – 105%; RSD < 9.0%; run time 12 min	Sequential determination of organic acids, furans and phenolic compounds in different wine matrices
Kritsunankul, 2009	Tartaric, malic, lactic, acetic, citric and succinic acids	Thai wines	Flow injection on-line dialysis sample pretreatment and RP-HPLC-UV; detection 210 nm	Column: Aquasil C18 column (100 x 3.0mm ID, 5 μm). Isocratic mode: 0.05 mol L^{-} KH_2PO_4 (pH 2.5) and ACN (99:1 v/v). Flow rate: 0.8 mL min^{-1}	For tartaric acid: linear range 0.25 - 7.5 g L^{-1}; r 0.9997; RSD < 5.4%; LOD 135 mg L^{-1}; run time 8 min	Flow injection on-line dialysis sample pretreatment improved the method performance
Zheng, 2009	Oxalic, tartaric, pyruvic, malic, ascorbic, lactic acetic, citric and succinic acids	Cabernet Sauvignon and Beichun red wines	RP-HPLC-UV; detection 210 nm except for ascorbic acid (243 nm)	Column: Atlantis dC 18 (4.6 x 150 mm ID, 5 μm). Isocratic mode: 0.01 mol L^{-1} KH_2PO_4 (pH 2.7) / ACN 95:5 (v/v). Flow rate: 0.8 mL min^{-1}	For tartaric acid: linear range 0.002 – 2.3 g L^{-1}; r = 0.9994; LODs 0.02 - 3.9 mg L^{-1}; RSD < 0.15% for retention time and <4% for peak area; recovery 85 - 109%; run time 15 min.	Comparison with some already existing methods indicated that the developed method is suitable for determination of most organic acids in wine.
Kerem, 2004	Citric, tartaric,	Cabernet	RP-HPLC-DAD;	Column: Synergi™ Polar-RP™	Linear range 0.5 - 8 g L^{-1};	polar reversed-phase

	malic, lactic and acetic acids, and some polyphenols	Sauvignon red wines	detection 210 and 280 nm (polyphenols)	column (250 mm × 4.6 mm ID 5 μm). Gradient mode: 0.2% aqueous TFA (pH 1.9) and ACN. Flow rate: 1.5 mL min^{-1}	repeatability RSD 1.0%; between-day precision RSD 5.0%	phenyl end-Encapped column adapted to aqueous low-pH solvent, high flow and rapid analyses
Zotou, 2004	Galacturonic, tartaric, malic, lactic, acetic, citric and succinic acids	White and red Greek wines	RP-HPLC-DAD; detection 230 nm	Column: RP C18 ODS-2 (250 x 4 mm ID, 5 μm). Isocratic mode: 0.02 M KH_2PO_4 (pH 2.9) / methanol (98:2 v:v). Flow rate: 1.5 mL min^{-1}	Linear range 0.003 - 2.0 g L^{-1}; LODs 0.001 - 0.05 mg L^{-1}; recovery78.0 - 106.8%	Clean-up with polyvinylpyrrolidone, followed by SAX

ACN acetonitrile; DAD diode array detector; HPAEC High performance anion-exchange chromatography; ID internal diameter; IEC ion exclusion chromatography; IPMA isopropylmalic acid; IT ion trap; LOD Limit of detection; LOQ limit of quantification; ODS octadecylsilica; PCA principal component analysis; RP reversed-phase; RSD relative standard deviation; RID refractive index detection; SAX strong ion exchange; TFA trifluoroacetic acid.

Table 5. Average concentrations of organic acids in each base wine class.

Concentrations expressed in g L^{-1}. ± indicates the standard deviation of concentrations among the wines belonging to the same class.

Class	Gluconic acid	Tartaric acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid	Fumaric acid
C	0.25 ± 0.03	6.5 ± 0.5	0.7 ± 0.1	2.1 ± 0.4	ND	0.6 ± 0.1	0.77 ± 0.01	ND
W	0.40 ± 0.08	7.1 ± 0.3	0.35 ± 0.08	3.2 ± 0.2	ND	0.9 ± 0.4	0.58 ± 0.04	ND
P	0.46 ± 0.02	10.2 ± 0.1	0.5 ± 0.1	1.6 ± 0.7	ND	0.56 ± 0.08	0.52 ± 0.04	ND
I	0.28 ± 0.04	9.2 ± 0.7	1.6 ± 0.1	0.50 ± 0.09	ND	0.7 ± 0.1	0.6 ± 0.1	ND
G	0.43 ± 0.04	7.9 ± 0.3	1.5 ± 0.1	3.1 ± 0.3	ND	1.7 ± 0.1	0.48 ± 0.08	ND
A	0.36 ± 0.03	6.5 ± 0.6	0.92 ± 0.08	3.1 ± 0.3	ND	1.2 ± 0.0	0.6 ± 0.2	ND
E	0.34 ± 0.09	7.5 ± 0.6	0.9 ± 0.1	1.7 ± 0.5	ND	0.5 ± 0.2	0.8 ± 0.2	ND
S	0.36 ± 0.07	6.9 ± 0.9	0.65 ± 0.05	2.8 ± 0.3	ND	1.06 ± 0.06	0.54 ± 0.07	ND
T	0.46 ± 0.08	7.3 ± 0.4	0.67 ± 0.1	2.2 ± 0.3	ND	0.6 ± 0.2	0.55 ± 0.04	ND
V	0.4 ± 0.1	7.7 ± 0.1	0.9 ± 0.2	2.2 ± 0.5	ND	0.7 ± 0.2	0.6 ± 0.1	ND

Table 6. Average concentrations of organic acids in each cava class. Concentrations expressed in g L^{-1}. ± indicates the standard deviation of concentrations among the wines belonging to the same class.

Class	Gluconic acid	Tartaric acid	Malic acid	Lactic acid	Acetic acid	Citric acid	Succinic acid	Fumaric acid
C	0.35 ± 0.02	5.1 ± 0.2	0.60 ± 0.05	1.9 ± 0.1	ND	0.10 ± 0.03	0.56 ± 0.04	ND
W	0.42 ± 0.03	4.8 ± 0.3	0.42 ± 0.07	2.2 ± 0.3	ND	0.20 ± 0.05	0.58 ± 0.06	ND
P	0.40 ± 0.02	5.7 ± 0.4	0.42 ± 0.04	2.6 ± 0.1	ND	0.14 ± 0.01	0.66 ± 0.03	ND
I	0.37 ± 0.02	5.6 ± 0.3	1.55 ± 0.07	0.62 ± 0.06	ND	0.22 ± 0.06	0.82 ± 0.06	ND
G	0.35 ± 0.06	5.3 ± 0.3	0.96 ± 0.08	2.1 ± 0.1	ND	0.16 ± 0.03	0.72 ± 0.06	ND
A	0.40 ± 0.03	4.8 ± 0.3	0.6 ± 0.1	3.05 ± 0.23	ND	0.12 ± 0.03	0.45 ± 0.06	ND
K	0.37 ± 0.03	4.7 ± 0.3	0.51 ± 0.04	2.7 ± 0.3	ND	0.14 ± 0.05	0.49 ± 0.09	ND
E	0.36 ± 0.02	5.3 ± 0.4	0.51 ± 0.06	1.7 ± 0.1	ND	0.3 ± 0.3	0.74 ± 0.06	ND
S	0.32 ± 0.05	4.8 ± 0.2	0.5 ± 0.1	2.3 ± 0.3	ND	0.15 ± 0.04	0.71 ± 0.09	ND
T	0.41 ± 0.04	5.0 ± 0.1	0.63 ± 0.09	3.4 ± 0.1	ND	0.18 ± 0.06	0.67 ± 0.07	ND

Figure captions

Figure 1. Chromatograms of a standard solution of 200 mg L^{-1} each organic acid as a function of the acetonitrile percentage in the mobile phase. Conditions: Agilent Zorbax SB-Aq column; 20 mmol L^{-1} phosphoric acid at pH 2; injection volume 10 µL; flow rate 1 mL min^{-1}; run time 5 min; detection at 210 nm. Peak assignation: (1) Gluconic acid, (2) Tartaric acid, (3) Malic acid, (4) Lactic acid, (5) Acetic acid, (6) Citric acid, (7) Succinic acid.

Chromatograms of a standard solution (a) and a white base wine (b) and a rosé base wine (c). Conditions: Agilent Zorbax SB-Aq column; 20 mmol L^{-1} aqueous phosphoric acid (pH 2)/acetonitrile 95:5 *v:v*; injection volume 10 µL; flow rate 1 mL min^{-1}; run time 5 min; detection at 210 nm. Standard composition: 200 mg L^{-1} tartaric and succinic acids, 50 mg L^{-1} acetic acid, 25 mg L^{-1} citric, lactic, gluconic and malic acids and 2.5 mg L^{-1} fumaric acid. Peak assignation: (8) Fumaric acid, see Fig. 1 for the others.

Figure 3. Radial diagrams of organic acid concentrations in the different *coupages*. *Coupage*: Ma/Xa/Pa, Macabeu, Xarel·lo and Parellada; Cha, Chardonnay; PN, Pinot Noir; Mo/Ga/Tr, Monastrell, Garnatxa and Trepat; BN, Blanc de Noirs; ML, Malolactic fermentation. Compound assignation: (a) tartaric acid, (b) malic acid, (c) lactic acid, (d) citric acid, (e) succinic acid, (f) gluconic acid. Black color corresponds to base wines and orange to cava samples.

Figure 4. Plots of scores from principal component analysis from the study of chromatograms of base wines (a) and cava classes (b). Wine assignation: Ma, Macabeu; Xa, Xarel·lo, Pa, Parellada; BN, Blanc de Noirs; PN, Pinot Noir; Tr, Trepat; Ga, Garnatxa; Mo, Monastrell; Cha, Chardonnay.

Figure 5. Results of principal component analysis from the study of organic acid of base wines. Plot of scores (a) and plot of loading (b). Wine assignation: see Fig. 4.