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Determination of Carboxylic Acids in Vinegars and in Aceto Balsamico Tradizionale di Modena by HPLC and GC Methods

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The presence of carboxylic acids in grape products has been investigated for a long time by researchers, from both the qualitative and quantitative points of view. Evaluation of carboxylic acids requires the study and optimization of some operative variables which are strictly related to the matrix. In particular, the determination of organic acids in real matrixes such as Aceto Balsamico Tradizionale of Modena (ABTM; a traditional balsamic vinegar made from cooked grape must) is often difficult because of the presence of numerous interferences that need to be removed by separation techniques. To this aim, in the present work a solid-phase extraction (SPE) method with C18 and NH_2 exchangers was used to clean the ABTM samples prior to analysis or further treatments. Both HPLC and GC techniques were used to determine organic acids. The efficiency of these two different analytical techniques in the study of ABTM acidic composition has been evaluated. Both methods separately were not able to supply all the data related to carboxylic acids. In particular, HPLC allows acetic and lactic acids quantification, but gluconic and succinic acids are better determined by GC. As far as tartaric, citric, and malic acids are concerned, both HPLC and GC methods give statistically equivalent results. The variation of the single acidic species composition along a series of casks furnished interesting information regarding the chemical transformations taking place during the aging process of this product.

KEYWORDS: ABTM; vinegar; carboxylic acids; SPE; HPLC; GC

INTRODUCTION

Aceto Balsamico Tradizionale of Modena (ABTM) is one of the most well-known and appreciated food products of the district of Modena. It is an ancient sauce, whose production still continues by traditional processes, handed down from generation to generation. ABTM is a traditional balsamic vinegar obtained through alcoholic and acetic fermentation of cooked musts of selected grapes, after a long aging period (at least 12 years), in sets of wooden casks. In this way a valuable product of unique taste is obtained. ABTM received PDO certification from the European Union (EU) in 2000 because of its typical production procedures and the well-defined geographical areas of its production.

The PDO and PGI regulations have increased consumer interest in regional foods, such as ABTM, and consequently research attention has also been growing. However, among all the published literature about ABTM, detailed studies on carboxylic acids determination have not yet been performed.

Organic acids represent an important fraction of this product: besides acetic acid, which is the principal product of acetic fermentation, ABTM contains many other carboxylic acids which are either produced by microbial fermentation or originated directly from grapes. Qualitative and quantitative characterization of ABTM organic acids could be of particular interest regarding study of both the product evolution during aging and its typicalness.

Furthermore, the total acidity of ABTM (expressed as tartaric acid equivalent) represents one of the most important chemical parameters of the product for both marketing and the biological safety of the product. However, even if the quantification of total acidity by titration is rather easy, analysis of the single acids in this matrix is difficult because of the presence of abundant sugars and phenolic compounds that interfere during the separation and quantification steps (*1*). For these reasons, it is necessary to pretreat the ABTM samples in order to eliminate interferences and to enrich the solution in acids.

Among the various techniques proposed in the literature, solid-phase extraction (SPE) has emerged as a powerful tool

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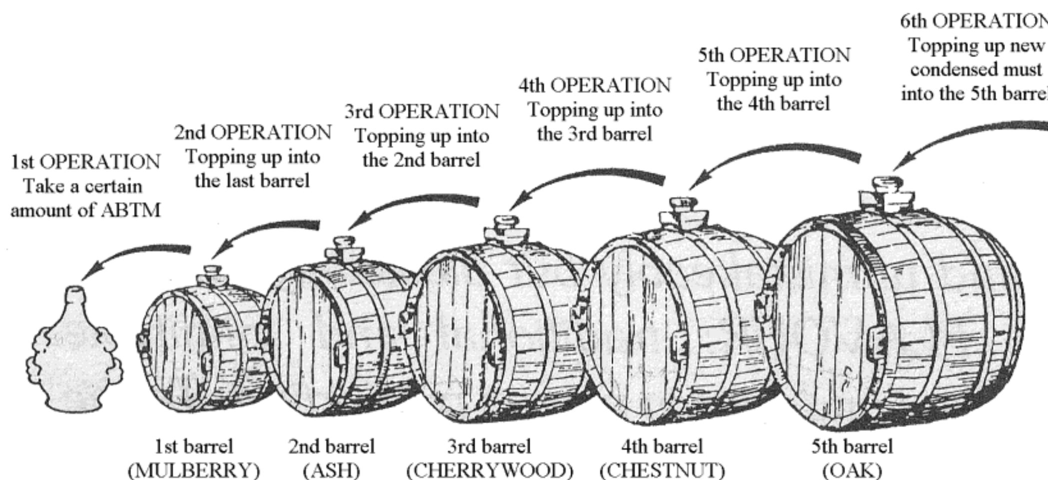

Figure 1. Example of an ABTM production set of barrels made of different sizes and woods with the topping-up procedure shown schematically.

for chemical isolation and purification. The SPE technique allows the elimination of interferences, thus obtaining a solution which is ready for HPLC analysis with UV detection. Gas chromatographic analyses were carried out on the same solutions after silylation. The objective of this work is to identify and determine the amount of carboxylic acids by using HPLC, GC, and GC/MS techniques, both in ABTM samples (aged at least 12 years) and in samples taken during different stages of the aging process, aiming at following their evolution during time.

For the sake of clarity it is necessary to give a few details on the nomenclature utilized throughout the paper. In particular, we use the term "wine vinegar" to indicate the product coming from the acetic fermentation of wine; the term "ABTM" to indicate the marketable traditional balsamic vinegar made from cooked must and aged at least 12 years; and the term "vinegar" to indicate any traditional ABTM products aged less than 12 years and therefore not marketable. In this context the term sample is used in its broadest meaning indicating the vinegar matrix, the wine vinegar, or the ABTM.

MATERIALS AND METHODS

Materials. Water for samples and standards preparation was purified by a Millipore Milli Q185 Plus system (Millipore, Bedford, MA).

Total acidity was evaluated by titration with standard 0.1 M sodium hydroxide, NaOH, supplied from Carlo Erba. Titer was determined by a Normex solution of 0.05 M sulfuric acid, H_2SO_4, also supplied from Carlo Erba.

Citric, tartaric, and succinic acids were supplied by Fluka with purity greater than 99.5%. Glutaric, formic, and malonic acids were also supplied from Fluka and had purity greater than 98%. Malic, fumaric, and maleic acids were furnished from Aldrich-Chemie with purity greater than 99%. Lactic acid was a Fluka reagent with purity greater than 99%. Gluconic and para-hydroxybenzoic acids (the latter used as internal standard in GC) were supplied by Sigma with purity greater than 99.8%. Acetic acid was a Carlo Erba product with purity greater than 99.8%. Pyridine was provided by Pierce, and silylation reactive (BSTFA plus 1% TMCS) was from Supelco. Suprapur H_2SO_4 96% by Merck, was used to prepare eluent solution for HPLC analysis with a 0.005 M final concentration. Eluent solution for SPE amino columns was produced by dilution of 30% RPE ammonium hydroxide, NH_4OH, by Carlo Erba. The final concentration was 1.5%.

Alltech Corp. supplied C18 and NH_2 SPE columns with 500 mg of active phase. Methanol used for conditioning of the SPE columns was supplied by Carlo Erba as a HPLC grade solvent.

Water content of all samples was determined by Karl Fischer (KF) titration. The KF reagent was supplied by Fluka (1 mL sol. KF $\cong$ 5−6 mg water, H_2O). The reagent was standardized with a primary standard of sodium tartrate bihydrate provided by the same firm with a purity of 99.5%.

ABTM Production. To better understand the nature of the samples that are the object of this study, the procedures for making ABTM are briefly described in this section.

Cooked must is the raw starting material for making ABTM. It is obtained from a must of selected grapes that is condensed by simmering gently over an open fire in uncovered pans. The aging process is carried out in a set of barrels composed by a variable number (ranging from 5 to 10) of wooden casks of different volumes. During the aging process the liquid in each cask is kept constant by transferring a certain amount of vinegar from one cask to another in a decreasing progression. This procedure is called "topping up" and it is illustrated in **Figure 1**.

The first operation consists of taking from the oldest cask an aliquot of aged ABTM (more than 12 years old), which is devolved to the market, then from the next elder cask, vinegar is topped up to the oldest one in order to replace the volume that is lost. This procedure goes on by topping up from one cask to the neighboring one until the youngest cask is reached. This one is then fed with the new cooked must (*2*).

Sampling. In this study we have analyzed two ABTM samples, eight vinegar samples coming from a series of casks, a sample of cooked must, and a sample of wine vinegar used in the vinegar factory during the start-up procedure of the set of barrels. The analyzed ABTM (A1 and B2) and vinegar samples (A3, B4, A5, B6, A7, B8, A9, and B10, in decreasing order) came from a set of 10 barrels obtained by crossing 2 independent series of 5 casks each, named A and B, to form a new longer set of barrels. This operation introduced a variation in the chemical and physical properties of the vinegars contained in each single series of casks, which probably would disappear over time. An aliquot of 100 mL each of wine vinegar, cooked must, ABTM samples A1 and B2, and vinegar samples A3, A5, A7, A9, B4, B6, B8, and B10 was taken from the barrels and stored in sterile Pyrex bottles. All the samples were stored at 4 °C, while cooked must was conserved at −20 °C. The samples are stable for a long time in these conditions.

Sample Preparation. An aliquot of 6 g of sample was diluted to 20 g with water. To eliminate phenolic compounds 5 mL of this solution was passed through a C18 SPE cartridge, which was previously activated with 2 mL of a 9:1 water/methanol (H_2O/CH_3OH) mixture. The eluate was brought to 25 mL final volume. To separate organic acids, 10 mL of this solution was passed through an amino (NH_2) SPE column which was previously activated with 5 mL of CH_3OH. The column was then washed with deionized water to remove interfering substances, and the isolated organic acids were eluted with 2 mL of 1.5% NH_4OH. The eluate was brought to 10 mL final volume and this solution was used for HPLC assay.

For gas chromatographic analysis 400 μL of the same solution was evaporated in N_2 current, added with 20 μL of a 5000 mg L^{-1} solution of *p*-hydroxybenzoic acid (IS) in pyridine and derivatized by adding 250 μL of silylation reactive and 250 μL of pyridine. The final solution was heated at 70 °C for 20 min before injection. The entire analytical procedure was always applied at least on two replicated samples. All the experimental data were obtained from twice-replicated inde-

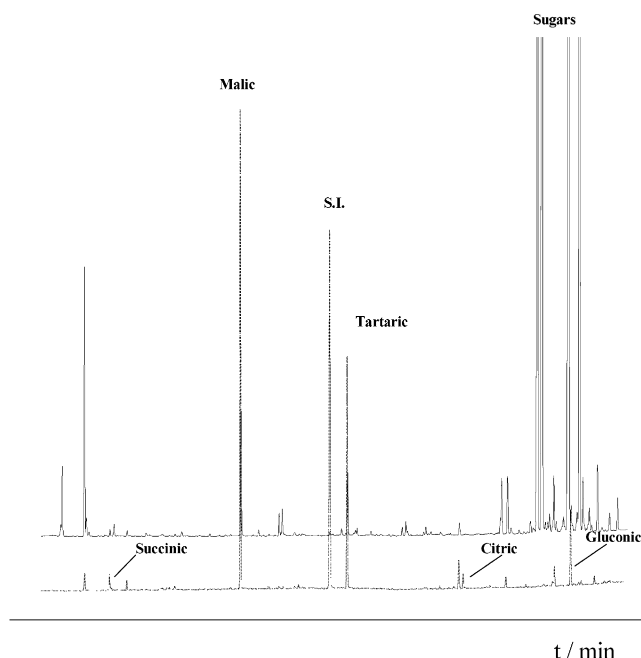

Figure 2. GC chromatogram obtained from an ABTM sample after SPE (C18 and NH_2) treatment (dotted lower line) and by direct silylation of an ABTM sample (solid upper line).

pendent samples. Each sample was always analyzed at least twice by replicated injections both in HPLC and GC technique.

The effect of the solid-phase extraction procedure is particularly evident by comparing the GC profile measured on the silylated vinegar sample with the profile corresponding to the silylated SPE-treated sample. **Figure 2** shows the two chromatograms. In particular, in the first chromatogram organic acids represent the minor constituents (ranging from 5 to 9%) of the product, and shows sugars present as the major species (ranging from 65 to 80%). For this reason SPE treatment was necessary to isolate organic acids and to achieve a better identification and quantification.

Equipment. Samples and standards were weighed using a Sartorius Research R160P analytical balance with a sensitivity of ±0.01 mg. Standards and sample dilutions were prepared by weight, using a Mettler PM 4800 Δ Range balance with a sensitivity of ±0.01 g.

Samples were dissolved with a trans-sonic thermostatable ultrasound bath supplied by Elma. Use of the trans-sonic bath helped dissolution of the densest vinegars and gave homogeneous solutions.

Total acidity of the samples was determined by volumetric titration with an EA940 digital pH-meter from Orion, which was fitted with combined glass electrodes (Ross type, supplied by Orion).

High-Performance Liquid Chromatography. A Beckman System Gold HPLC was used. The system was formed by a model 116 isocratic single pump, a 210A injection valve, equipped with a 20-μL injection loop, provided with a magnetic sensor to automatic start, a model 166 UV-Vis detector, a model 406 analogical to digital interface, and an Eppendorf TC-50 thermostat for HPLC column. The system was managed by System Gold chromatographic software, version 3.0, also supplied by Beckman.

A Bio-Rad HPX-87H Aminex column (length 30 cm, diameter 7.8 mm), thermostated at 55 °C, was used for all the work. Mobile phase was a 0.005 M H_2SO_4 solution, and the separation was conducted by isocratic technique with a flux of 0.6 mL min^{-1}. Organic acids were detected by the UV equipment at 210 nm.

Gas Chromatography. Derivatized carboxylic acids were analyzed in a Varian 3400 gas chromatograph provided with flame ionization detection. A nonpolar capillary column of fused silica (Heliflex AT-1; length 30 cm; internal diameter 0.25 mm; film thickness 0.1 μm) was used.

The column head pressure of the carrier gas (He) was 14 PSI, and the linear velocity of the gas was 25 cm/s. Split ratio in the injector was 90:1. The injector temperature was 265 °C. The oven starting temperature was 60 °C and it was increased at a rate of 4 °C/min until 250 °C, and then held at constant temperature for 5 min. The FID temperature was 300 °C. FID air flow was 300 mL/min, while the H_2 flow was 30 mL/min.

Table 1. Response Factor $\bar{K}_i$, Standard Deviation s_{Ki}, and Relative Error er % Values for the Investigated Acids Relative to Internal Standard (IS) *p*-Hydroxybenzoic Acid

parameter[a]	succinic	malic	tartaric	citric	gluconic
$\bar{K}_i$	1.2830	1.103	0.9859	1.3377	0.8848
s_{Ki}	0.0160	0.006	0.0200	0.0700	0.0050
er %	1.27	0.55	2.47	9.54	0.61

[a] The response factor $\bar{K}_i$ is reported as mean value of 7 replicates.

Acids were quantified in GC by means of the internal standard method. The response factors relative to the internal standard method were calculated using the relation

$$K_i = \frac{gr_i}{gr_{IS}} \times \frac{area_{IS}}{area_i} \tag{1}$$

where K_i is the response factor for the *i*-th species, gr_i is the weight of the *i*-th species, gr_{IS} is the weight of the internal standard, $area_i$ is the peak area of the *i*-th species, and $area_{IS}$ is the peak area of the internal standard. The standard solution used for the calculation of K_i was a mixture of acids solved in pyridine, and the calculated response factors are listed in **Table 1**.

The same standard solution utilized for the response factors evaluation was also used for periodic monitoring of the instrumental efficiency, i.e., of the reproducibility and the repeatability of the experimental and instrumental conditions over time.

The identity of the carboxylic acids peaks was confirmed through the measurement of the fragmentation spectra, using a GC coupled to a mass spectrometer (MS). The GC−MS system was formed by a 3400 Varian GC connected to a Finningan SSQ710A MS, capable of electron impact and chemical ionization.

A nonpolar capillary column (J&W DB-5ms, length 30 cm, internal diameter 0.25 mm, film thickness 0.25 μm) was used.

The column head pressure of the carrier gas (He) was 14 PSI. Split ratio in the injector was 100:1. Injector temperature was 250 °C. The oven starting temperature was 80 °C. Initial temperature was kept for 1 min, then it was increased at a rate of 8 °C/min until 280 °C. The temperature of the transfer line was kept at 285 °C.

RESULTS AND DISCUSSION

Sample Treatment. Carbohydrates, organic acids, and phenolic compounds represent the main organic matrix of ABTM. Because our main interest focused on organic acids determination, we first optimized the analytical method for isolation of the acidic species. In fact, to eliminate interfering substances and to preserve the efficiency of the analytical column, the samples were treated before chromatographic analysis by the SPE technique, using C18 (*3*−*5*) and NH_2 SPE columns in sequence.

Carbohydrates are very polar compounds soluble only in aqueous solvent. Organic acids, which are present in vinegars, are small, very polar compounds that are ionized at neutral pH. Phenolic compounds are moderately polar species that will be retained on nonpolar sorbents. Because of the differences in molecular structure, however, it is not possible to extract all three classes with a single, simple procedure using traditional techniques. Sorbent extraction addresses the problem effectively through the use of multiple extraction mechanisms applied simultaneously, and in this case the selected analytical methodology required the use of C18 and NH_2 SPE columns. The first column retains polyphenols through a solvophobic mechanism, because the retention is regulated by the repulsion

Table 2. Peak Area and Standard Deviation Values of Aqueous Standard Acids Mixture before and after Treatment with SPE C18 and NH_2 Columns

acid	peak area[a]	
	$\bar{X}^b \pm s_X$	$\bar{Y}^c \pm s_Y$
citric acid	4.9 ± 0.5	5.0 ± 0.5
tartaric acid	6.9 ± 0.5	7.0 ± 0.5
malic acid	0.7 ± 0.1	0.8 ± 0.1
succinic acid	4.2 ± 0.5	4.3 ± 0.5
lactic acid	4.2 ± 0.5	4.3 ± 0.5
fumaric acid	48 ± 1	48 ± 1
acetic acid	1.8 ± 0.5	1.8 ± 0.5

[a] Mean values of 5 replicates. [b] Peak area measured before the SPE treatments. [c] Peak area measured after the SPE treatments with C_{18} and NH_2.

between polar mobile phase and solute. This phenomenon can be forced by the incomplete derivatization of fixed phase with C18 groups and by the consequent presence of hydroxyl groups, which increase the entity of polar type interactions. As a consequence, polyphenols are retained on the C18 column, while sugars and acids are eluted in the liquid phase.

The NH_2 SPE retention mechanism is based on ionic interactions. Moreover, this fixed phase has basic properties that permit retention of the most acidic species. This allows the retention of carboxylic acids and their consequent separation from sugars, which are present in large amount in the vinegar matrix. However, this retention mechanism is strongly correlated to the pK_A values of the acidic species: acids with high pK_A, in fact, are not quantitatively retained. The initial pH values of vinegar samples have fundamental importance. In fact, the pH affects the dissociation mechanism of acids; in this way, the strongest acids, being the most dissociated species, interact immediately with the amino groups of the stationary phase and are therefore retained in the upper side of the SPE column, while the weakest species interact mainly through hydrogen bonding and dipolar forces and are more poorly retained.

Aiming at validating this experimental approach in sample treatment, two different types of recovery tests were made. The first was carried out using an aqueous mixture of eight standard carboxylic acids, which was analyzed by HPLC before and after SPE treatment. The peak area values of the carboxylic acids are shown in **Table 2**. The good agreement between the values determined before and after the separative treatment evidences a good retention and elution in the SPE step even for those species with higher pK_A, as confirmed by the Student's *t* test.

The initial pH of the aqueous solutions does not affect the retention mechanism, and, if pH is lower than two units with respect to pK_A of amino stationary phase ($pK_A = 9.8$), it is possible to retain also the weakest acids.

To verify matrix effects on the SPE procedure, a further recovery test was executed by means of the standard addition method using vinegar samples (*6*). For each acidic species, five aliquots of a middle-age vinegar sample were taken, and four of these were added to an increasing quantity of the corresponding standard acid solution, with final concentrations ranging from 50 to 600 ppm, depending on the acidic species. Then the five aliquots were treated as previously described and analyzed.

The calibration curve relative to the aqueous standard solutions of each acidic species allows us to calculate the concentrations ($C_{calc.}$) of the investigated species. The linear regression of these values as a function of the concentrations of added standards in the vinegar matrix ($C_{add.}$), give the recovery function, whose slope represents the recovery value

Table 3. Recovery Values (rec) and Standard Deviations (s_{rec}) for the Investigated Organic Acids

acid	rec ± s_{rec}
tartaric acid	0.95 ± 0.04
citric acid	1.01 ± 0.01
malic acid	1.01 ± 0.04
succinic acid	0.81 ± 0.03
lactic acid	0.91 ± 0.02
acetic acid	1.07 ± 0.01

Table 4. Statistical Comparison between Slope Values b_1 and b_2 Relative to the Calibration Curves with Aqueous Standard and Standard Addition Methods, Respectively

acid	std b_1	std addition b_2
tartaric acid	2.66×10^{-4}	2.55×10^{-4}
citric acid	3.46×10^{-2}	3.49×10^{-2}
malic acid	1.32×10^{-4}	1.33×10^{-4}
succinic acid[a]	2.36×10^{-2}	1.57×10^{-2}
lactic acid[a]	6.65×10^{-5}	6.12×10^{-5}
acetic acid[a]	1.80×10^{-2}	1.92×10^{-2}

[a] Significantly different values by two-tailed *t* test ($p \leq 0.05$).

of the analytical method for a given acid (*7*). The calculated recovery values are shown in **Table 3**. As previously mentioned, the recovery values were established by HPLC and then applied also to the acids concentrations determined by GC.

In fact, the analytical scheme utilized for the recovery evaluation represents, as a first approach, a measure of the efficiency of the SPE treatment.

However, further considerations about these data will be made later in the paper. From the standard addition curve, it is also possible to infer the relative error of each measure, this value giving an evaluation of the precision of the HPLC measure (*8*−*10*).

The calibration curve relative to aqueous standards and the one obtained with the standard addition method on the vinegar matrix were compared through a Student's *t* test in order to establish which of them it is possible to use for the evaluation of the concentration of acids in the samples *(9, 10)* (**Table 4**). The Student's *t* test permits evaluation of the eventual presence of systematic errors of proportional type; in this case, the determination of the acid concentration cannot be carried out with the aqueous standard curve and the addition technique would be more appropriate.

Sample preparation for GC analysis implies evaporation of the sample solutions under N_2 current. To evaluate possible losses of sample during this phase, as reference solution, an aqueous mixture of standard carboxylic acids was prepared by analytical weighing and an aliquot of this was silylated as described in the sample preparation section and then injected in GC. Comparison between the acidic species content of the reference solution before and after the drying procedure showed a perfect coincidence of the data, showing that no loss of analytes occurred. Furthermore, evaluation of possible inconsistencies due to matrix effects was tested by adding known quantities of the standard mixture to a fixed aliquot of sample after the SPE treatment. The solution was treated with the complete analytical method and after silylation was injected in GC. In this case also, the experimental data showed that during the drying procedure no remarkable errors occurred.

Determination of the Carboxylic Acids. *High-Performance Liquid Chromatography*. The carboxylic acids that have been

Table 5. Organic Acid Concentration, Expressed as g kg^{-1} with Associated Absolute Errors, Measured by HPLC Technique, and Water Content, Expressed as % with the Relative Error, Determined by Karl Fisher Titration

sample[a]	tartaric	citric	malic	succinic	lactic	acetic	water
B10	7.3 ± 0.3	0.99 ± 0.02	16.4 ± 0.7	6.2 ± 0.7	0.5 ± 0.2	19.1 ± 0.3	42.65 ± 0.4
A9	7.2 ± 0.3	0.67 ± 0.02	13.0 ± 0.5	7.0 ± 0.8	0.7 ± 0.2	29.1 ± 0.4	46.95 ± 0.4
B8	9.4 ± 0.4	0.54 ± 0.02	13.0 ± 0.5	7.6 ± 0.8	0.8 ± 0.2	27.7 ± 0.4	39.38 ± 0.4
A7	8.3 ± 0.3	0.74 ± 0.02	14.8 ± 0.6	8.1 ± 0.8	0.5 ± 0.2	27.5 ± 0.4	35.01 ± 0.4
B6	7.6 ± 0.3	0.92 ± 0.02	14.8 ± 0.6	9.6 ± 0.9	0.5 ± 0.2	40.1 ± 0.5	37.17 ± 0.4
A5	6.9 ± 0.3	0.87 ± 0.02	16.5 ± 0.7	9.6 ± 0.9	0.4 ± 0.2	30.6 ± 0.4	29.79 ± 0.3
B4	7.5 ± 0.3	0.89 ± 0.02	17.4 ± 0.7	10.0 ± 0.8	0.5 ± 0.2	41.3 ± 0.6	31.66 ± 0.3
A3	6.8 ± 0.3	0.84 ± 0.02	17.4 ± 0.7	11.2 ± 1.0	0.3 ± 0.2	29.9 ± 0.4	29.35 ± 0.3
B2[b]	7.4 ± 0.3	0.63 ± 0.02	16.4 ± 0.7	12.8 ± 0.9	0.8 ± 0.2	22.9 ± 0.3	13.43 ± 0.2
A1[b]	6.9 ± 0.3	1.01 ± 0.02	17.6 ± 0.8	11.7 ± 0.7	1.1 ± 0.2	16.3 ± 0.2	11.22 ± 0.2
cooked must	8.5 ± 0.3	0.56 ± 0.02	7.8 ± 0.3	5.1 ± 0.7	0.0 ± 0.2	5.4 ± 0.1	56.16 ± 0.5
wine vinegar	3.4 ± 0.1	0.56 ± 0.02	1.5 ± 0.1	6.5 ± 0.7	0.4 ± 0.2	106 ± 1	78.98 ± 0.5

[a] Samples designated A and B are listed in order of increasing age. [b] ABTM marketable products.

Table 6. Organic Acids Concentration, Expressed as g kg^{-1} with Associated Absolute Errors, Measured by GC Technique

sample[a]	tartaric	citric	malic	succinic	gluconic
B10	7.3 ± 0.7	1.0 ± 0.1	15 ± 1	0.27 ± 0.02	1.9 ± 0.1
A9	6.6 ± 0.6	0.8 ± 0.1	13 ± 1	0.41 ± 0.04	2.2 ± 0.1
B8	9.7 ± 0.9	0.8 ± 0.1	15 ± 1	0.46 ± 0.04	2.8 ± 0.1
A7	8.9 ± 0.9	0.9 ± 0.1	15 ± 1	0.52 ± 0.05	2.3 ± 0.1
B6	8.1 ± 0.8	0.9 ± 0.1	15 ± 1	0.74 ± 0.07	2.9 ± 0.1
A5	6.6 ± 0.6	0.9 ± 0.1	16 ± 1	0.79 ± 0.07	2.8 ± 0.1
B4	6.9 ± 0.7	0.9 ± 0.1	15 ± 1	0.86 ± 0.08	2.9 ± 0.1
A3	7.3 ± 0.7	0.9 ± 0.1	18 ± 1	0.85 ± 0.08	2.4 ± 0.1
B2[b]	7.2 ± 0.7	0.9 ± 0.1	16 ± 1	0.91 ± 0.08	3.4 ± 0.1
A1[b]	5.5 ± 0.5	0.6 ± 0.1	12 ± 1	0.56 ± 0.05	1.5 ± 0.1
cooked must	8.7 ± 0.8	0.6 ± 0.1	8 ± 1	0.05 ± 0.01	0.3 ± 0.1
wine vinegar	2.7 ± 0.3	0.2 ± 0.1	3 ±1	0.54 ± 0.05	5.3 ± 0.2

[a] Samples designated A and B are listed in order of increasing age. [b] ABTM marketable products.

determined and quantified in the experimental samples by HPLC are tartaric, citric, malic, succinic, lactic, and acetic acids, whose concentrations are reported in **Table 5** with their estimated standard errors. Acetic acid is characterized by a retention time higher than those of any other acid or sugar present in the vinegars and a low K_A value, and, as a consequence, it has been possible to determine it in the sample solution treated only with SPE C18.

Liquid chromatography has given very good results in the quantification of tartaric, citric, and malic acids, as confirmed by the high analytical recovery values and by the coherence between the values calculated on the aqueous standard solutions and on vinegars sample with the standard addition method (**Table 4**). As regards acetic, succinic, and lactic acids, although the recovery test on the aqueous solutions gave satisfactory results, the information obtained by the t test suggests an influence of the matrix.

In particular, succinic acid is characterized by a low recovery value relative to the standard addition method in vinegar solutions. This is the direct consequence of the nonparallelism condition stressed by the slopes data reported in **Table 4**. It is thus evident that the vinegar matrix strongly interferes with the retention mechanism of the amino SPE producing a loss of analyte on succinic acid determination, with the recovery for this species in the aqueous solvent being almost quantitative (**Table 2**).

Gas Chromatography. The carboxylic acids that have been determined and quantified in vinegars by GC are tartaric, citric, malic, succinic, and gluconic acid, whose concentrations are reported in **Table 6**.

Acetic acid and lactic acid have not been determined by GC because of their volatility, which causes losses during preparation steps. Moreover, the retention times of their silyl derivatives are too short to ensure a good quantification at the adopted chromatographic conditions.

Because the GC analyses have been conducted on the same solutions used for HPLC determinations, the previously calculated recovery factors have been applied to calculate the final concentrations of the acids.

It is now possible to make some considerations on the analytical data from the different analytical methods. As far as malic and tartaric acids are concerned, the concentrations quantified by GC analysis are very close to those obtained by the HPLC technique and can be considered equal from a statistical point of view by applying a Student's t test at a confidence level of $p \leq 0.05$.

Different considerations can be drawn about citric acid quantification, because of its own chemical characteristic of hydroxylated polyprotic acid. During the silylation procedure both the ter- and tetra- derivatives are present in the sample solution. This fact can be monitored in the chromatographic trace, where it is possible to observe both the peaks corresponding to the ter- (minor species) and tetra- (major species) silyl derivatives. The contemporary presence of the two species, probably related to the kinetics of the derivatization process, may result in an increasing error associated with the analytical determination. However, the GC data calculated for citric acid are statistically equivalent to the values obtained with HPLC (Student's t test at a confidence level of $p \leq 0.05$).

GC chromatography permits, moreover, determination of succinic acid, whose quantification in HPLC is troublesome, probably owing to the SPE procedure and matrix effect due to coelution of an interfering species. The concentrations measured by GC are lower than the ones obtained by HPLC , but these

Table 7. Comparison between Measured and Calculated Total Acidity

sample[a]	total acidity	
	titrated acidity (gr‰ tartaric acid)	calculated acidity (gr‰ tartaric acid)
B10	51.06	52.15
A9	60.30	60.87
B8	62.20	61.53
A7	61.96	61.99
B6	79.80	77.81
A5	69.25	67.03
B4	80.08	79.99
A3	67.10	66.86
B2[b]	60.04	58.20
A1[ab]	51.99	50.42
cooked must	22.93	23.62
wine vinegar	123.25	117.87

[a] Samples designated A and B are listed in order of increasing age. [b] ABTM marketable products.

data are in good agreement with the values reported from other authors for similar matrixes (*5*, *11−14*).

Gluconic acid was determined only by GC because of its closeness with tartaric acid in the HPLC elution.

To confirm the determination of the examined compounds, some injections in GC/MS have been executed, which have permitted establishment of the correctness of the peaks attribution.

The different analytical methods that we adopted in this work are comparable only for the determination of tartaric, citric, and malic acids. In fact, for these three species, taking also into account the experimental errors, the results obtained by both GC and HPLC are in excellent agreement, as shown in **Tables 5** and **6** and previously stated.

Although all the steps of the analytical method, have been validated with the standard addition method, it is well-known that the presence of systematic errors cannot be verified. For this reason, aiming at checking the accuracy of the analytical data, for all the samples the instrumental values have been compared with the ones from the volumetric titration, which is the official UE method for determination of total acidity in wines, and cooked and rectified musts and vinegars. **Table 7** reports the total acidity values, as weight (g) of tartaric acid for 1000 g of vinegar, determined by titration and by instrumental methods. The instrumental total acidity was calculated considering either the GC or the HPLC data when acids were analyzed by both methods; only the GC values for gluconic and succinic acids, and the HPLC data for acetic and lactic acids.

Generally, instrumental and volumetric data are in good agreement and this confirms the goodness of experimental determinations and the correctness of the executed treatments. The good agreement between the two data series has been confirmed by the linear regression model of the calculated total acidity on the titrated total acidity, which gave a correlation coefficient equal to 0.9935. A t test has been performed on the intercept and on the slope to check if they were equal to 0 and to 1, respectively: in both the cases the null hypothesis at the 0.05 probability level has been retained, confirming that there is no evidence for systematic differences between the two sets of results.

Further considerations can be drawn from the data relative to the different acidic species by taking into account the water content of the investigated samples, which is reported in the last column of **Table 5**. Just as expected, water content in vinegars decreases during aging because of natural evaporation passing from cooked must to ABTM. Now, if we consider the acidic content expressed on dry samples it is possible to supervise the dynamics of variation over time. In this way, samples are not affected by the dehydration phenomena that occur during aging of the product relative to each species.

In particular, tartaric acid is present in high percentage in vinegars and ABTM, and considering the water content of samples it is evident that its concentration decreases sensibly, passing from young to old vinegars, probably because of its precipitation as a potassic or a calcic salt.

This behavior is not observable if the concentration data of vinegar samples are not corrected for the water content, consistent with the fact that, even though there is a decrease of the absolute content of acid in time, water evaporation causes an increase in concentration.

Citric and malic acids concentrations, on the contrary, do not undergo high variations during the aging phase, remaining almost constant in the different samples.

Succinic acid increases in the young samples and decreases in the old ones, probably because of reactions that form other products such as esters. This trend is well detectable both in the whole and in the dried samples.

The content of gluconic acid evaluated in the vinegar samples is in agreement with other bibliographic data (*11*). However, as a matter of fact, there is in the literature a remarkable variability of measured concentrations of this acid. This is probably due to the different analytical approaches used for its determination, and to strong matrix interferences that are likely to occur. The observed trend in the gluconic acid for the analyzed vinegar samples is rather oscillating, probably because of oxidative phenomena occurring to different extents in the two series of casks A and B. In fact, if vinegar samples coming from the two different series of casks, sample B10 to B2 and A9 to A1, are considered separately, the two deriving trends are much more uniform. This fact underlines that the set of casks obtained by crossing series' A and B has not yet achieved uniformity of chemical and physical properties.

Similar conclusions may be drawn for acetic acid. The particular trend of the concentration values is probably due to a change of the bio-oxidation phenomena in the first stage of the product aging and to a slow evaporation of the acid during the maturation phase. The trend of the data could be easily explained if the samples coming from the two series of casks are considered separately as previously stated for gluconic acid.

CONCLUSIONS

The two analytical methods chosen to investigate ABTM, i.e., HPLC and GC, were separately not able to supply all the data related to carboxylic acids.

In fact, GC did not allow determination of acetic and lactic acids because of the losses incurred during sample preparation. On the other hand, liquid chromatography did not permit quantification of gluconic and succinic acids correctly because of the numerous interferences that are present in both the SPE treatment and the sample matrix.

As regards the method validation, because of the absence of an appropriate standard sample, it was necessary to use the standard addition approach to determine the analytical recovery. This technique permits an indirect validation of the methodology, but not the individuation of systematic errors and additive interferences during quantification. For this reason a comparison between the titrated total acidity and the instrumental data has been performed in order to evaluate the accuracy of the procedure. Although these considerations allow recognition of

the presence of some problems for the carboxylic acids quantification, instrumental information allowed a higher and much more detailed control of the product in the casks with respect to titration data only. On the other hand, knowledge of the total acidity is of fundamental importance because it constitutes an essential reference value for a crossed control of the instrumental results.

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LITERATURE CITED

(1) Tusseau, D.; Benoit, C. Analyse Collaborative des Acides Organiques des Hoût et des Vins par HPLC. *O. I. V. F. V. 800,* **1986**.
(2) Gazzetta Ufficiale della Repubblica Italiana no 44 del 23/02/1987 D. M. 9/02/1987.
(3) Garcia Romero, E.; Sanchez Muñoz, G. Determination of organic acids, in grape musts, wines and vinegars by high performance liquid chromatography. *J. Chromatogr. A* **1993**, *655*, 111−117.
(4) Zerbinati, O.; Aigotti, R.; Daniele, P. G. Analysis of chiral carboxylic acids in wine by high performance liquid chromatography with coupled UV and circular dichroism detection. *J. Chromatogr. A* **1994**, *671*, 281−285.
(5) Calull, M.; Marcè, R. M.; Borrull, F. Determination of carboxylic acids, sugars, glycerol and ethanol in wine and grape must by ion exchange high performance liquid chromatography with refractive index detection. *J. Chromatogr.* **1992**, *590*, 215−222.
(6) Community Bureau of Reference. *BCR Reference Materials*; Commission of the European Communities: Brussells, Belgium.
(7) Funk, W., Dammann, V., Donnevert, G., Eds. Establishing a new analytical procedure. In *Quality Assurance in Analytical Chemistry*; VCH: Weinheim, Germany, 1995; pp 15−57.
(8) Miller, J. C., Miller, J. N., Eds. Calibration methods in instrumental analysis: regression and correlation. In *Statistics and Chemometrics for Analytical Chemistry*, 4th ed.; Pearson Education Limited (Prentice Hall): Dorchester, Dorset, Great Britain, 2000; pp 107−150.
(9) Armitage, P., Berry, G., Eds. Regression and correlation; Further analysis of straight-line data. In *Statistical Methods in Medical Research*, 2nd ed.; Blackwell Scientific Publications: Oxford, Great Britain, 1987; pp 141−159 and pp 264−295.
(10) Goldsmith, P. L., Davies, O. L., Eds. Linear relationships between two variables. In *Statistical Methods in Research and Production*, 4th ed.; Oliver and Boyd Tweeddale Court: Edinburgh, Great Britain, 1972; pp 178−236.
(11) Pallotta, U.; Amati, A.; Minguzzi, A. Composizione del vino. In *Lezioni di Enologia*; Clueb: Bologna, Italy, 1977; pp VII/1−32.
(12) Mentasti, E.; Gennaro, M. C.; Sarzanini, C.; Baiocchi, C.; Savigliano, M. Derivatisation, identification and separation of carboxylic acids in wines and beverages by high performance liquid chromatography. *J. Chromatogr.* **1985**, *322*, 177−189.
(13) Caccamo, F.; Carfagnini, G.; Di Corcia, A.; Samperi, R. Improved high performance liquid chromatography assay for determining organic acids in wines. *J. Chromatogr.* **1986**, *362*, 47−53.
(14) Vonach, R.; Lendl, B.; Kellner, R. High performance liquid chromatography with real time Fourier transform infrared detection for determination of carbohydrates, alcohols and organic acids in wines. *J. Chromatogr. A* **1998**, *824*, 159−167.

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