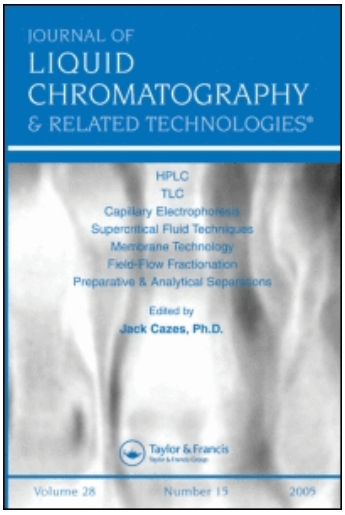

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HPLC Determination of Organic Acids in Traditional Balsamic Vinegar of Reggio Emilia

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ABSTRACT

The major constituents of Traditional Balsamic Vinegar (TBV) of Reggio Emilia (including citric, malic, tartaric, lactic, acetic, gluconic, and succinic acids, fructose, and glucose) were quantified in a single HPLC run. A cation exchange column was used, and the analytes were quantified by the standard addition method. These conditions provided a reliable method, which was applied to twenty-one samples. Glucose and fructose were the main constituents. Acid concentration showed a great variability, and it was characterized by the presence of gluconic acid. Except in one sample, acetic acid was the main constituent of this class of compounds.

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Key Words: TBV; Vinegar; Acids; Aceto balsamico tradizionale; Ion exchange HPLC.

INTRODUCTION

Traditional Balsamic Vinegar (TBV) of Reggio Emilia is an ancient product traditionally used as a food condiment, recognized through Protected Denomination of Origin (PDO) certification. It is obtained from "must cooked over a direct flame (i.e., concentrated), coming from the pressing of grapes traditionally grown in the zone of Reggio Emilia." The ageing period (at least 12 years) is carried out in five or more casks of decreasing capacity. During this period aliquots of the product are periodically transferred from one cask to the following one. Alcoholic fermentation and acetic oxidation are the main biological transformation, but they occur in the first two casks only. In the others, because of the high solute concentration, no biological activity occurs, and the product increases its concentration owing to the evaporation of water. The result is a dark, sweet acid syrup, greatly appreciated by a large number of domestic consumers, as well as all over the world.

Up to 50–60% w/w of reducing sugars (i.e., glucose and fructose) and acidity often higher than 60-g L^{-1} of acetic acid equivalents, are some of the characteristics of this product. These substances are extremely important because they affect stability, color, and flavor of the final product.

Total vinegar acidity is expressed as acetic acid, which is usually the main compound of this group. However, other acids are also present. They have different origins; some come directly from the biosynthesis of the vine (tartaric, malic, and citric acid), others from metabolic pathways related to sugar metabolism (succinic, lactic, gluconic acid), while acetic acid is mainly due to ethanol oxidation.

Among the others, gluconic acid is particularly important. In fact, this acid is a genuine factor for TBV.[1] It comes from glucose and fructose oxidation by *Acetobacter* spp. and *Gluconobacter* spp., which are some bacteria responsible for TBV production.

Separation of sugars and acids in a single HPLC run has been used in many beverages. In particular, hydrogen sulfonated polystyrene–divinylbenzene columns have been applied to must and wines.[2–4] Also, vinegar acids have been studied with comparable methods.[5–7] However, the particular composition of TBV is claimed for optimization of previous reported techniques. In particular, the presence of high sugar concentration and a large number of by-products of unknown nature coming from long aging, make such techniques unsuitable. In fact, the determination of these substances was recently achieved by using both a GC and HPLC method. In

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particular, gluconic and succinic acids were better determined as trimethyl silyl derivatives by capillary GC, while acetic and lactic acids were better quantifies by ion-exclusion HPLC. The two techniques were equivalent for the quantification of the other acids.[8]

The aim of this work was to study and optimize the determination of the main organic acids along with reducing sugars in TBV with a single run, in order to elaborate on a reliable and repeatable method.

EXPERIMENTAL

Vinegar Samples

Twenty one TBVs provided by the Consorzio tra i Produttori di Aceto Balsamico Tradizionale di Reggio Emilia were analyzed for their content in acids and sugars. Before analysis, each sample was diluted 50 times and filtered through a 0.22-μm membrane filter.

Standard Solutions

Citric, malic, tartaric, lactic, acetic, calcium gluconate, succinic acids, fructose, and glucose were supplied by Aldrich Chemical Co. (Milwaukee, USA).

Standard stock solutions were prepared in bidistilled water and five different mixtures, with all acids at increasing solutions prepared in order to spike samples for standard addition method.

Chromatographic Conditions

A Perkin Elmer HPLC Series 200 apparatus was equipped with an isocratic pump (Perkin Elmer series 200 LCP), a UV/Vis detector set at 210 nm for acid determinations (Perkin Elmer UV/Vis detector LC295), a refractive index detector for sugar quantification (Perkin Elmer Series 200 refractive index), and an injection valve (Rheodyne Inc., Cotati, CA) fitted with a 20-μL loop.

The samples were separated isocratically using a Bio-Rad Aminex HPX 87H Hydrogen form cation exchange resin-based column (300 × 7.8 mm i.d.). The column temperature was set at 42°C with a Series 200 oven (Perkin Elmer). The mobile phase was 0.004 N sulfuric acid at 0.6 mL min^{-1}.

Chromatograms were acquired and processed with Total Chrom Workstation software, through a PE Nelson 900 Series interface; identification was carried out by a spiking technique and by comparing retention times; the method of standard addition for quantification was carried out.

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RESULTS AND DISCUSSION

General Considerations

Sugar quantification was carried out without difficulty. In fact, the refraction index trace was characterized by two large early-eluting peaks, which corresponded to glucose and fructose, respectively (Fig. 1). Ethanol and glycerol were also detectable. Moreover, ethanol was present only in trace amounts and glycerol determination was not for our purposes.

Acids eluted within 15 min and, in another 20 min, the column was ready for following injections. The first 10 min of the HPLC trace was particularly crowded. In fact, the wavelength of 210 nm was not selective enough and during this time a large peak of unresolved substances overlapped, citric acid, tartaric acid, gluconic acid, and malic acid (Fig. 2). Moreover, a poor resolution of this last acid and fructose was verified. This fact was already reported in literature,[9] and other authors[4] proposed the use of THF as mobile phase modifier in order to improve this separation. The use of a standard addition method minimizes the problem of interfering substances as well as malic quantification. The remaining acids eluted without any interference.

The identity of each organic acid was carried out by comparison with the retention time of pure standards. Spiking a real sample with pure diluted standards was also used for uncertain identifications.

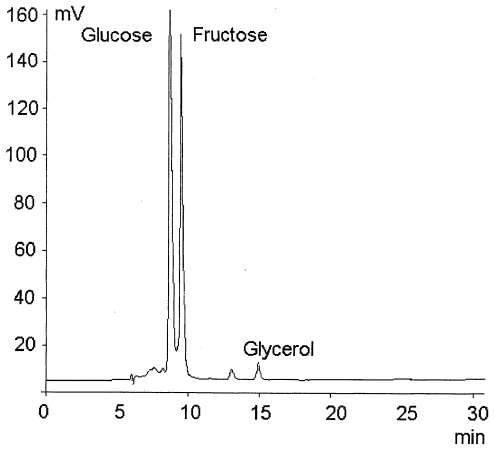

Figure 1. HPLC trace of reducing sugars of a real sample.

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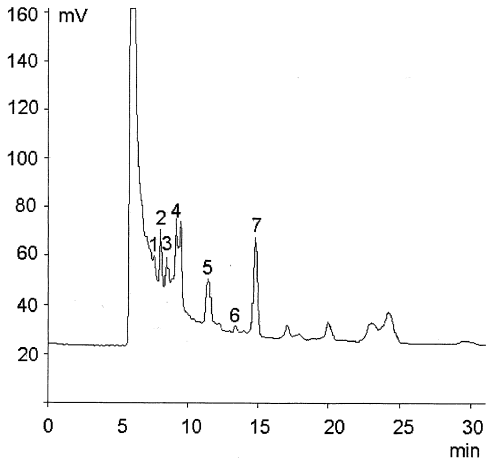

Figure 2. HPLC trace of acids of a real sample. 1, citric acid; 2, tartaric acid; 3, gluconic acid; 4, malic acid; 5, succinic acid; 6, lactic acid; 7, acetic acid.

Sample Pretreatments

In order to overcome the problem of matrix interference, some pretreatment were tried.

At first, decoloration with active charcoal was carried out. A sample aliquot (10 mL) of diluted TBV (1 : 50) was added with different charcoal amounts. After 10 min, the sample was filtered through a membrane filter (0.22 μm) and it was directly injected into the HPLC system without further treatments. Unfortunately, charcoal was too active. In fact, even if a quantity of charcoal as low as $30\,mg\,100\,mL^{-1}$ was not able to eliminate the interference completely, acid areas were already strongly affected. As a consequence, higher charcoal amounts caused a severe decrease of all peaks. The gluconic acid area was the most penalized (−72%), while other acid areas were halved. Moreover, the content of the interferences vary from one sample to one another. For this reason, a standardization of the method was not possible.

SPE acid preseparation was also tried. A 1g-SAX cartridge was conditioned with 10 mL of MeOH followed by 5 mL of H_2O. Then, 10 mL of 1 : 50-diluted sample was applied and the column was rinsed with 3 mL of water. Acids were collected with 2 mL of 0.5 M H_2SO_4. Probably, the high sugar content and the contemporary presence of the same substances that interfered with HPLC analyses, make this technique ineffective. In fact, the

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recovery of the acids was not satisfactory. Gluconic acid was particularly penalized, but also weaker acids were not completely retained by the SPE cartridge.

Sample dilution was also studied. Traditional Balsamic Vinegar was diluted 1 : 50, 1 : 75, and 1 : 100 in order to eliminate the interference. The most diluted samples did not show HPLC traces particularly clearly. On the contrary, many acids were below the detection limit. For this reason 1 : 50 dilution was chosen. As a consequence, a standard addiction method was used, as described in the next section.

Method Evaluation

Five levels of concentrations, and a blank, were tested in triplicate (Table 1). These concentrations ranged within the expected quantities in vinegars of each acid after proper dilutions.

Linear ranges, regression equations, R^2 values, and detection limits for the analytes are reported in Table 2.

As expected, relative standard deviation of each standard addition (Table 3) showed decreasing values for higher concentration. The means were lower than 2%, with the only exception of lactic acid, which showed a very high variability also in the blank.

Method Validation

In order to validate the proposed method, total acidity, was carried out with the official titrimetric method,[10] and it was compared with the sum of the acids

Table 1. Concentrations ($g\,100\,g^{-1}$) of each analyte for each standard addition.

	Blank	1st	2nd	3rd	4th	5th
Citric acid	0	0.0020	0.0040	0.0060	0.0080	0.0100
Tartaric acid	0	0.0110	0.0140	0.0170	0.0200	0.0230
Gluconic acid	0	0.0200	0.0280	0.0360	0.0440	0.0520
Malic acid	0	0.0240	0.0350	0.0460	0.0570	0.0680
Succinic acid	0	0.0100	0.0160	0.0220	0.0280	0.0340
Lactic acid	0	0.0010	0.0015	0.0020	0.0025	0.0030
Acetic acid	0	0.0210	0.0290	0.0370	0.0450	0.0530
Glucose	0	0.3800	0.4300	0.4800	0.5300	0.5800
Fructose	0	0.3800	0.4300	0.4800	0.5300	0.5800

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Table 2. Regression equations, R^2 values, linear ranges, and percent mean esteemed error for each analyte.

	Equation	R^2	Linear range (g 100 g^{-1})	Mean esteemed error (%)
Citric acid	$y = 1.36 \times 10^7 x + 23201$	0.9997	0.02–0.71	4
Tartaric acid	$y = 2.41 \times 10^7 x + 190205$	0.9995	0.38–1.92	3
Gluconic acid	$y = 5.33 \times 10^6 x + 78230$	0.9994	0.02–9.28	4
Malic acid	$y = 1.06 \times 10^7 x + 92815$	0.9959	0.22–4.67	19
Succinic acid	$y = 9.60 \times 10^6 x + 197053$	0.9994	0.67–3.48	2
Lactic acid	$y = 1.15 \times 10^7 x + 9920$	0.9916	0.01–1.27	13
Acetic acid	$y = 9.97 \times 10^6 x + 567342$	0.9994	0.50–6.44	2
Glucose	$y = 4.56 \times 10^6 x + 2126367$	1	9.5–58.5	2
Fructose	$y = 4.63 \times 10^6 x + 2087527$	1	7.0–53.2	4

for each sample recalculated as acetic acid-equivalent. Data were in good agreement ($y = 1.01x - 0.02$; $R^2 = 0.7$). Student tests were carried out on a and b parameters of the straight line equation. The H_0 hypothesis was accepted at a significance level of 5%, confirming the comparability of the two sets of data.

Determination of Sugar and Acids in the Samples

Reducing sugars (43–63 g 100 g^{-1}) were the main TBV constituents (Table 4). A prevalence of glucose vs. fructose was verified in almost all samples (Fig. 3). In grapes and musts, these two sugars are equimolar.

Table 3. Relative standard deviation for blank and for each standard addition.

	Blank	1st	2nd	3rd	4th	5th	Mean
Citric acid	2.86	1.67	0.76	0.83	0.69	0.27	1.18
Tartaric acid	0.68	1.10	0.21	0.60	0.88	0.87	0.72
Gluconic acid	3.28	2.48	1.12	1.57	1.32	0.72	1.75
Malic acid	2.42	1.25	0.13	0.52	0.85	1.25	1.07
Succinic acid	4.43	0.63	1.54	0.29	0.98	0.12	1.33
Lactic acid	20.33	5.02	4.64	5.32	3.97	6.12	7.57
Acetic acid	0.69	0.60	0.85	0.49	0.42	0.18	0.54
Glucose	0.63	0.91	0.48	0.04	0.52	0.04	0.44
Fructose	0.57	0.87	0.52	0.10	0.40	0.05	0.42

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Table 4. Concentrations ($g\,100\,g^{-1}$) of organic acids and reducing sugar in TBV.

	Citric acid	Tartaric acid	Gluconic acid	Malic acid	Succinic acid	Lactic acid	Acetic acid	Glucose	Fructose
1	0.07	0.46	0.11	0.79	0.89	0.06	2.05	30.9	31.6
2	ND	0.75	0.94	0.97	1.09	0.04	1.64	27.1	24.8
3	ND	0.44	0.55	0.69	1.04	0.06	1.93	27.2	25.6
4	0.09	0.70	6.68	0.54	1.29	0.06	1.21	27.0	25.5
5	0.21	0.72	0.20	0.59	1.42	0.05	2.51	30.7	28.8
6	ND	0.57	0.97	0.69	0.96	0.10	2.26	26.5	23.9
7	0.06	0.38	1.17	0.65	1.48	0.08	2.35	24.1	25.1
8	ND	0.77	0.27	0.98	0.93	1.17	1.88	25.4	22.8
9	0.04	0.50	0.57	0.59	1.02	0.06	1.94	27.7	24.9
10	0.05	0.49	0.74	0.62	1.02	0.07	1.85	26.6	23.6
11	ND	0.68	0.89	0.89	0.92	0.06	2.13	26.1	22.2
12	0.08	0.47	0.35	0.75	0.88	0.06	2.32	24.6	21.1
13	0.09	0.48	0.51	0.57	0.68	0.06	2.13	22.7	21.9
14	0.07	0.47	0.34	0.63	0.95	0.07	2.94	22.9	20.0
15	0.14	0.59	0.92	0.54	0.81	0.04	2.93	25.5	21.7
16	0.05	0.56	0.09	1.20	1.04	0.07	3.08	24.3	21.7
17	0.07	0.66	0.15	1.10	0.77	0.05	2.31	22.9	22.8
18	0.08	0.39	0.73	0.44	1.03	0.04	2.84	23.3	22.5
19	0.07	0.41	0.71	1.47	1.77	0.05	2.59	24.1	19.2
20	0.08	0.63	0.02	0.59	0.67	0.04	2.69	21.0	19.4
21	0.11	0.38	0.47	0.51	0.87	0.05	2.40	22.1	19.4

Note: ND, not detectable.

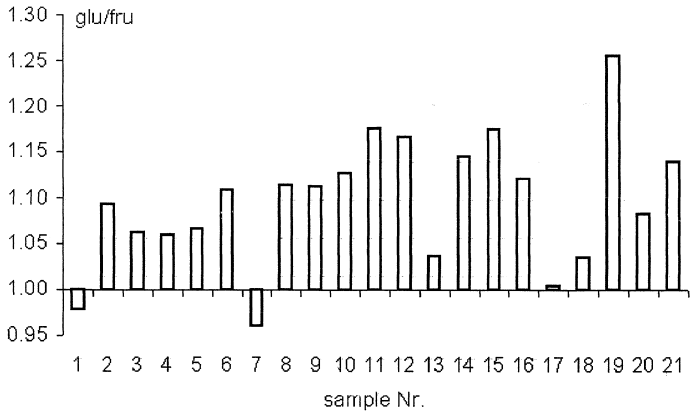

Figure 3. Glucose–fructose ratio in TBV samples.

The partially selective oxidation of fructose by TBV microorganism was responsible for these differences.

The amount of each acid (Table 4) was in good agreement with those of other authors,[8] with exception of succinic acid and gluconic acid. Some samples showed concentrations one order of magnitude higher. Furthermore, in general, our samples presented a great variability as already reported,[8,11] The low manipulation of the sample and the accuracy of a standard addiction technique could explain these discrepancies. Moreover, TBV is characterized by a great variability, which is quite common in such products. A great number of samples should be necessary to reach a more definitive answer.

In many cases, the sum of sugars and acids was over the 50% of the whole composition (Fig. 4), but no correlation was verified between the two classes of substances.

Citric acid was the least abundant, and in two samples was not detectable because of the interference of the first part of the chromatogram. In any case, it never exceeded 0.21 g $100\,g^{-1}$.

Tartaric acid (0.38–0.77 g $100\,g^{-1}$) is the main grape acid. However, in TBV this preponderance was not always respected. Probably, as a consequence of the concentration process occurring during the long ageing and because of the poor water solubility of potassium bitartrate (0.6% w/v), a significant amount of tartaric acid has been precipitated.

Malic acid (0.43–1.47 g $100\,g^{-1}$) is lower compared to other data reported in literature.[11] However, the high acidity of the media and its concentration do not allow malolactic fermentation. As a consequence, lactic acid (0.03–1.17 g $100\,g^{-1}$) came from sugar metabolism. Data from literature are comparable.[8]

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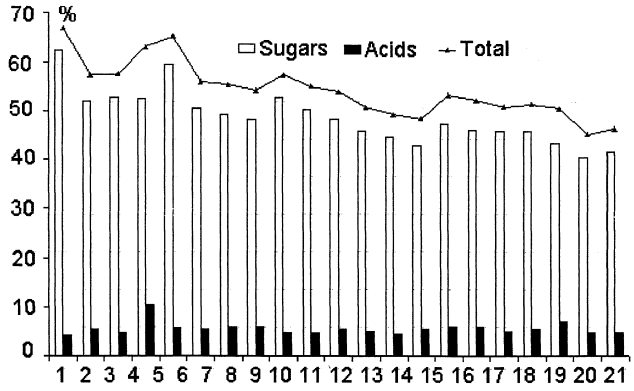

Figure 4. Percent composition of TVB main classes of compounds.

Acetic acid (1.20–3.08 g $100\,g^{-1}$) was the main acid with the only exception of sample 4, which showed an anomalous high quantity of gluconic acid (6.68 g $100\,g^{-1}$). The balance of these two acids is particularly important. In fact, both contribute to the sourness sensation, but acetic acid is volatile and is characterized by a strong pungent note. However, other authors[11] reported acetic acid concentration up to 13 g $100\,g^{-1}$, which was typical in products of some decades ago, while today consumers prefer less aggressive TBV.

CONCLUSIONS

The proposed method is fast and it requires scarce sample preparation, allowing the contemporary determination of sugars and acid. For these reasons, it can be used for routine analyses, which need robust methods and have a large number of samples.

In some cases, sugars are the main compounds followed by water and acids. For this reason and because of their sensory properties, their monitoring is particularly important. Moreover, gluconic acid is particularly relevant in assessing quality, and authenticity of TBV.

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