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Simultaneous determination of sugars and organic acids in aged vinegars and chemometric data analysis

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Abstract

Aceto Balsamico Tradizionale of Modena (ABTM) is a typical product (PDO denomination) of the province of Modena produced by cooked grape must which undergoes a long ageing period (at least 12 years) in series of wooden casks (batterie). The study of the transformations of this product during ageing is extremely relevant in order to control the authenticity of ABTM towards succedaneous products and mislabelling of age.

This paper presents the results of the investigation of sugars and fixed organic acids in ABTM samples of different ages, coming from different *batterie*. The analytes were simultaneously determined by a gas chromatographic method optimised for this peculiar matrix.

The method shows good separation and resolution of the investigated chemical species and allows their determination in the concentration ranges reported in brackets: malic (7.6–15.5 g kg^{-1}), tartaric (4.0–9.7 g kg^{-1}), citric (0.6–1.5 g kg^{-1}) and succinic (0.36–0.62 g kg^{-1}) acid and glucose (153–294 g kg^{-1}), fructose (131–279 g kg^{-1}), xylose (011–0.39 g kg^{-1}), ribose (0.078–0.429 g kg^{-1}), rhamnose (0.061–0.195 g kg^{-1}), galactose (0.136–0.388 g kg^{-1}), mannose (0.41–1.46 g kg^{-1}), arabinose (0.33–1.00 g kg^{-1}) and sucrose (0.46–6.84 g kg^{-1}), with mean associated errors ranging from 5 to 19% depending on the analytes.

Moreover, the recovery values are always satisfactory, being close to one for most of the analytes.

Furthermore, in order to assess the degree of variability of the different analytes content with vinegar ageing and the similarity/dissimilarity among series of casks a three-way data analysis method (Tucker3) is proposed. The chemometric technique applied on the data set shows differences between the samples on the bases of their different ageing period, and between the *batterie,* which traditionally have an own peculiar production procedure.

Keywords: Organic acids; Sugars; ABTM; Chemometrics; Tucker3

1. Introduction

In the last few years, the scientific interest towards the issue of authentication of regional products, characterised by a small production area and by peculiar production procedures, has been continuously growing [1,2]. This interest has been mainly determined by the need of supporting authenticity and traceability by more objective analytical methodologies, with respect to paper certifications.

Aceto Balsamico Tradizionale of Modena received the Protected Designation of Origin (PDO) certification in 2000 [3,4]. The PDO implies that once the name is registered, it is protected against the sale of any other competing imitation product seeking to use the reputation of the name of origin. ABTM is a traditional balsamic vinegar obtained through alcoholic fermentation and acetic bio-oxidation of cooked musts of selected grapes, after a long ageing period (varying from 12 to 25 years) in sets of wooden casks, called "*batterie*" [3,5]. According to its making technique this condiment is also characterised by the topping up procedure, with freshly prepared cooked must, which is performed every year [3,5]. These characteristics definitely make ABTM unique among any other aged foodstuff.

The interest towards this product is also testified by several publications [5–15]. In particular, the attention of researchers has been pointed on the investigation of authenticity [7,11] and ageing markers [8,10], in order to characterize ABTM and to differentiate it from similar products or to discriminate vinegar samples of different ages or production techniques. Neverthe-

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less, it should be pointed out that ABTM is a complex matrix and therefore, it is plausible that its authenticity could not be ascertained through the individuation of a restricted group of markers.

In particular, remarkable variations take place during the ageing period of ABTM. Moreover, also considering the traditional making procedure, the *batterie* coming from different producers might have their own characteristics and this gives reason of the importance of analysing products of different ages coming from the barrels of various *batterie*, in order to evaluate several compositional parameters and their variability ranges [5,13–15].

Carbohydrates have been poorly investigated in ABTM, although they are one of its major constituents. As a matter of fact only the major sugars, i.e. glucose and fructose, were determined in aged products [12] by liquid chromatography technique, while no data are available on the minor carbohydrates concentration evaluated neither in aged ABTM nor along the *batterie*. Moreover, it is worthwhile noticing the lacking of official analytical methods, in the European Community, for the determination and speciation of sugars in vinegars, cooked musts or wine-based matrices. On the contrary, some volumetric methods are available for the quantification of the total amount of sugars [16], while the speciation of glucose and fructose can be performed by liquid chromatography technique [16].

The aim of this work is two-fold: (i) to optimise a gas chromatographic (GC) method for the simultaneous identification and the analytical quantification of non-volatile organic acids and carbohydrates in ABTM and (ii) to investigate the evolution of the compositional profile of these species in vinegar samples of different ages coming from different producers.

As regards the first item, even if many techniques [17–27] have been described in the literature for the analysis of sugars and acids in different matrices, capillary GC with flame detection seems to be one of the most interesting and widely applied [23–27]. GC technique shows excellent detection limits, separation capacity, simplicity and low cost. The required sample preparation is relatively short time consuming and relatively inexpensive and it has been used for the analysis of the compounds of interest in different products [28,29].

The data obtained for the vinegar samples coming from the *batterie* of different producers were processed by three-mode principal component analysis [29–32], in particular Tucker3 method [21–33] was applied. In fact, the three-way chemometric approach well represents the structure of the experimental variables being characterised by three distinct sources of systematic variability, i.e. samples age, compositional profile and the different *batterie*.

2. Experimental

2.1. Chemicals

Water for samples and standards preparation was purified by a Millipore Milli Q185 Plus system (Millipore, Bedford, MA). Organic acids such as citric, tartaric, succinic and malic, and sugars such as glucose, sucrose, fructose, arabinose, xylose, ribose, rhamnose, mannose and galactose, were supplied by Fluka (Milan, Italy) with a purity greater than 99.5%. Phenyl-β-D-glucopyranoside (internal standard), *p*-hydroxybenzoic acids (internal standard) and gluconic acid (sodium salt), with purity greater than 99%, were purchased from Sigma–Aldrich (Milan, Italy). The oximant reagent, hydroxylamine hydrochloride, was supplied by Carlo Erba (Milan, Italy), with purity greater than 99%. Pyridine Ultrapur® reagent, used to prepare oximant and standard solutions, was obtained from Fluka. Silylation reagent, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) was provided by Supelco (Milan, Italy). Solid phase extraction (SPE) cartridges C18 500 mg, were supplied by Supelco. Methanol (HPLC grade) and Karl Fischer (KF) titration reagent (1 mL solution KF ≅ 5–6 mg H_2O) were provided by Fluka.

2.2. Equipment

Samples were homogenised by using an Elma (Milan, Italy) trans-sonic thermostatable bath.

Samples and standards were weighted using a Mettler AE 200 balance with a sensitivity of ±0.1 mg.

The amount of water in the samples was determined on a Crison 431 model KF Titrator.

Gas chromatographic determinations were performed on a Varian 3400 gas chromatograph provided with a flame ionisation detector (FID).

A non polar capillary column (Alltech AT-5 length 60 m, i.d. 0.25 mm, film thickness 0.20 μm) was used. Helium was used as carrier gas (linear velocity 30 cm s^{-1} evaluated at 150 °C). The split injection mode was used with a split rate 1:30. Flow rates of H_2 and air to the FID were 30 and 300 mL min^{-1}, respectively. The FID and injector temperature were setted at 310 and 280 °C, respectively.

The column starting temperature was 80 °C and it was increased at 4 °C min^{-1} to 210 °C and then programmed to 250 °C at 2 °C min^{-1}. The temperature was finally increased to 280 °C at 4 °C min^{-1} and held for 10 min.

The identity of all the chemical species was also confirmed through the measurement of their mass-fragmentation spectra. The capillary column and the experimental conditions were the same as for the GC-FID instrument; the temperature of the transfer line was kept at 290 °C.

The GC–MS system was formed by a 3400 Varian GC connected to a Finningan single quadrupole mass spectrometer SSQ710A model. Electron beam impact spectra were recorded in the 40–1400 amu range in full scan mode with a sampling frequency of 1 scan s^{-1} and at 70 eV ionisation energy.

The temperature of the electron ionization source and the mass quadrupole analyzer are 230 and 150 °C, respectively.

The identification of unknown compounds was based on matches to the NIST and GP libraries, also supplied by Finningan.

2.3. Sampling

Vinegars were sampled from each barrel of 3 *batterie*, namely *batterie* C, M and S, coming from three different producers. All the *batterie* are composed by six casks, cask 1 contains the

Table 1
Volume (L) and approximate age (years) of the wooden casks relatively to each *batteria*

Batteria	Cask volume (L)						Age of *batteria* (years)
	6	5	4	3	2	1	
M	60	50	40	30	20	18	25
C	50	35	35	30	25	20	20
S	40	32	25	22	20	14	27

oldest vinegar and cask 6 contains the youngest one. The characteristics of the 3 *batterie* are reported in Table 1. *Batteria* S was started in 1976 and the first topping up procedure [5] was carried out the following year. In 1988, the producer started to collect the aged ABTM from the oldest barrel, to be approved for the commercialisation [34]. *Batteria* M was started in 1979 and after 12 years the producer started the commercialisation of the product. On the contrary, *batteria* C was started in 1984, but the aged ABTM has not been yet collected for marketing and the producer has only carried out the topping up procedure to compensate the natural evaporation of the liquid.

With the aim of obtaining a representative sample of the bulk for each barrel, five aliquots of 100 mL of product, collected at different depth of the cask, were homogenised; then a sample of 100 mL was taken, while the remaining volume was poured back in its original cask. Every sample was stored in sterile polystyrene bottles and kept at 4 °C.

2.4. Sample preparation

Before analysis the sample was warmed up at room temperature and then an aliquot of 6 g was diluted to 20 g with water. To eliminate phenolic compounds, 5 mL of this solution were passed through a C18 SPE cartridge, previously activated with 2 mL of a 9:1 (v:v) H_2O:CH_3OH mixture.

The cartridge was then washed with 2 mL of water and the collected eluates were diluted to a 25 mL final volume. An aliquot of 100 μL of this solution was evaporated under N_2 flow and the sugars converted to their oxime derivatives by adding 500 μL of oximant reagent (hydroxylamine hydrochloride 30 mg mL^{-1} in pyridine) containing phenyl β-D-glucopyranoside (2 mg mL^{-1} in pyridine) and *p*-hydroxybenzoic acid (1 mg mL^{-1} in pyridine), as internal standards, and heated at 70 °C for 30 min. The oximes and the acids were then silylated by addition of 500 μL of silylation reagent and re-heated at 70 °C for 30 min. An aliquot of 1 μL of this final solution was injected for the chromatographic analysis. Each sample was analysed three times by three repeated injections.

2.5. Quantification

Individual analytes in the samples were identified by comparing their retention times and fragmentation mass spectra with those of the corresponding standard compounds. In particular, standards were injected both singly and in multiple solution. The investigated species were quantified by means of the internal standard method using *p*-hydroxybenzoic acid and phenyl β-D-glucopyranoside as internal standards for acids and sugars [5], respectively.

Table 2
Concentration of the standard mixture solutions, used for the evaluation of the mean response factors, $\bar{K}_i$ values, and recovery data (expressed as %) relatively to each investigated analyte determined by GC method, with the associated standard deviations, *s*

Compound	Concentration solution A (g kg^{-1})	Concentration solution B (g kg^{-1})	Mean $\bar{K}_i \pm s$	Recovery ± s (%)
Xylose	0.02	0.04	0.40 ± 0.02	108 ± 2
Arabinose	0.02	0.04	2.9 ± 0.1	115 ± 8
Ribose	0.02	0.04	0.69 ± 0.03	99 ± 2
Rhamnose	0.02	0.04	0.91 ± 0.03	97 ± 3
Fructose	1.00	2.00	1.20 ± 0.02	100 ± 3
Galactose	0.02	0.04	0.99 ± 0.03	97 ± 2
Mannose	0.02	0.04	1.12 ± 0.04	124 ± 2
Glucose	1.00	2.00	0.90 ± 0.03	100 ± 2
Sucrose	0.02	0.04	1.1 ± 0.1	64 ± 4
Succinic acid	0.10	0.20	1.16 ± 0.04	95 ± 1
Malic acid	0.30	0.60	1.11 ± 0.04	100 ± 2
Tartaric acid	0.15	0.30	1.06 ± 0.08	101 ± 4
Citric acid	0.10	0.20	1.7 ± 0.2	96 ± 6

The response factors K_i, were calculated using two different concentration mixtures of standard analytes dissolved in pyridine (Table 2). The K_i values, obtained for the two different concentrations, are statistically equivalent, according to Student's *t*-tests and then the average $\bar{K}_i$ values (Table 2) were used to quantify the analytes in the samples.

3. Three-way principal component analysis

In the present case a standard two-way table (samples versus variables) was not the best way to describe the experimental data. Though the number of samples, i.e. 54, equals to 18 (6 casks × 3 repetitions) × 3 (*batterie*), is not prohibitive, the peculiarities of each *batteria* lead to confound the variation along the casks with the inter-*batterie* variation and, as a result, the ageing trend is overwhelmed and could not emerge.

Fig. 1 shows a schematic representation of how the data were arranged in a 3-MODE array: the casks, i.e. samples 1–6, are on the MODE 1 (*I*), the variables, i.e. sugars and acids concentrations on MODE 2 (*J*), and the *batterie* i.e. C, M and S on MODE 3 (*K*).

3.1. Data pretreatment

It has been recently outlined [35] that, when centering and scaling are performed on *N*-way arrays, care must be paid to perform centering across a specific MODE and to apply scaling by a transformation within a specific MODE.

There are several possibilities to perform centering and scaling when dealing with *N*-way analysis. In the present work, the data were centered across the first MODE, i.e. casks, by rearranging the three-way array to a bi-dimensional $I \times JK$ matrix and then removing the column averages. In this way, for each producer, any constant difference among the samples has been

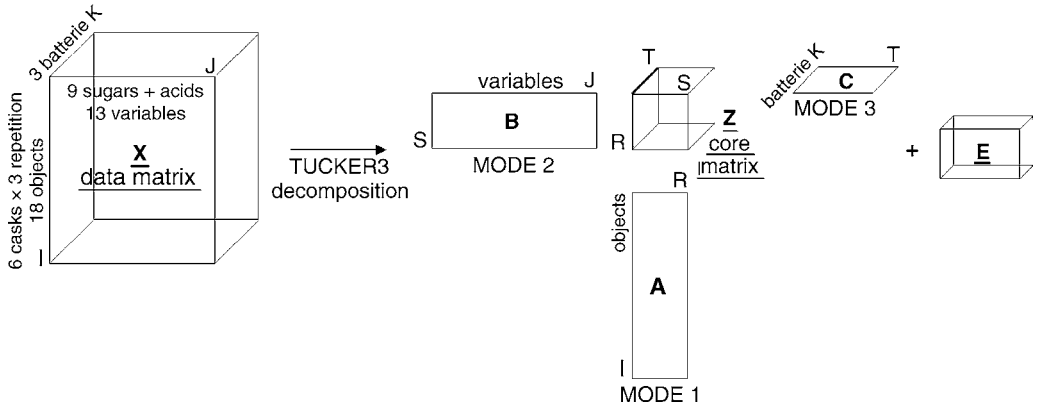

Fig. 1. Schematic representation of how the three-way data matrix is arranged and decomposed by the application of the Tucker3 analysis.

removed, while the variation among the different producers is preserved. In fact, each sample is anchored to its *batteria* average (one producer average).

As far as scaling is concerned a blockscaling procedure defined as "block-adjusted non scaled data" [36], within the second MODE, i.e. variables, was applied.

In Appendix A, this blockscaling procedure is discussed in detail.

This scaling allows minor components to contribute to the model without altering the relative scales of variables belonging to the same block.

3.2. Tucker3 decomposition

Tucker3 analysis was carried out by using the *N*-way Toolbox for MATLAB©, freely accessible via Internet [37].

The Tucker3 method may be considered as an extension of principal component analysis to three-way data arrays. For a detailed description of the method the reader is referred to the relevant literature [29–33]. Briefly the Tucker3 decomposition of a three-way array $\underline{\mathbf{X}}$, of dimension $I \times J \times K$, furnishes three sets of loadings (the MODE 1 loadings, in analogy with bi-dimensional PCA are also called scores), one for each MODE (matrices **A**, **B** and **C**), together with a core matrix ($\underline{\mathbf{Z}}$) describing the relationships among them, see Fig. 1.

Mathematically the Tucker3 decomposition can be expressed by the following equation:

$$x_{ijk} = \sum_{r=1}^{R}\sum_{s=1}^{S}\sum_{t=1}^{T}\sum_{r=1}^{R} a_{ir} b_{js} c_{kt} z_{rst} + e_{ijk}$$

where a_{ir}, b_{js} and c_{kt} are elements of the loading matrices **A** ($I \times R$), **B** ($J \times S$) and **C** ($K \times T$); z_{rst} is the element of the core matrix $\underline{\mathbf{Z}}$ of order $R \times S \times T$; e_{ijk} denotes the error term for the element x_{ijk}. Here R, S, T are the number of the latent factors retained for each of the three MODEs, respectively.

In order to choose the dimensionality of the Tucker3 model, different combinations of R, S, T were evaluated by using the *tucktest* routine implemented in the *N*-way Toolbox and the best compromise between good fit and small number of factors has been selected.

The elements of the core matrix $\underline{\mathbf{Z}}$ represent the entity of the interaction among the factors of the different MODEs. The squared elements of the core matrix are proportional to the variation explained by the combination of the factors corresponding to their indices, i.e. if z_{112} is the largest core element, special attention in interpreting the model has to be given to the interaction between factor 1 of MODE 1, factor 1 of MODE 2 and factor 2 of MODE 3 [33].

4. Results and discussion

For the sake of clarity, few details on the terminology utilised throughout the paper are necessary. In particular, the term "ABTM" is used to indicate the marketable traditional balsamic vinegar aged at least 12 years. The term "vinegar" is used 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 as well as the ABTM.

4.1. Analytical method optimization

The simultaneous determination of carbohydrates and organic acids by GC methods requires the conversion of these compounds to volatile molecules such as methyl-, acetyl-, silyl-, oxime- or oxime-silyl-derivatives [25,28,29]. The oxime-silyl-derivatisation is commonly used and implies a two steps process: (1) conversion of the sugars to their oximes by reaction with hydroxylamine in pyridine solution and (2) conversion of oximes and carboxylic acids to their silyl derivatives by reaction with silylation reagents. The first step (oxime formation) is recommended to avoid the formation of isomeric forms of the sugars, which can interfere during the quantification step of the analytes, because of the presence of a high number of overlapped peaks.

To optimise the procedure for the determination of sugars and acids in ABTM samples, i.e. to achieve the best conditions for sample preparation and chromatographic separation, different experiments were carried out on a representative test vinegar sample, obtained by mixing equal aliquots of product coming from each barrel of a battery. ABTM matrix consists mainly of a complex mixture of carbohydrates, organic acids, polyphenols

and other minor species. Since polyphenol compounds strongly interfere with sugars and acids determination, purification of the vinegar sample by using C18 SPE was always performed to avoid interferences during the quantification steps [14]. This procedure does not involve carbohydrates and organic acids, which are not retained on the columns, since C18 working mechanism is based on hydrophobic interactions. Carbohydrates, in fact, are neither anionic nor non polar and they pass through the column unretained; in the same way organic acids are also polar and do not interact with the sorbent.

As regard the silylation reaction, different derivatisation temperatures (50, 70 and 90 °C) and reaction times (15, 30 and 60 min at 70 °C) were tested. It was observed that the peak areas of the investigated species slightly increased from 50 to 70 °C, but no significant differences were observed between 70 and 90 °C and, as a consequence, 70 °C was selected as derivatisation temperature. Moreover, the yields of derivatised compounds increase from 15 to 30 min and do not significantly change when time is further increased; therefore 30 min was selected as reaction time.

When direct silylation is performed on the vinegars samples, sucrose and all the acids are resolved as single peaks, with the exception, eventually, of gluconic and citric acids, while every reducing sugar provides multiple peaks corresponding to trimethylsilyl (TMS) derivatives. These peaks are due to anomers formation and pyranose ↔ furanose interconversion, that lead to a low resolution and a rather difficult quantification of the analytes. On the contrary, a two steps oximation-silylation reaction allowed a remarkable simplification of the chromatogram and an higher peak's resolution, as each reducing sugar gives rise to only two peaks corresponding to the *syn* and *anti* oximes, in agreement with data obtained by other authors [26].

The two steps derivatisation reaction allows obtaining a minor number of peaks, but some overlaps are still observed in the chromatograms (Fig. 2). This behaviour is further confirmed by the GC–MS spectra obtained in the same experimental conditions. In fact, the isomeric forms of oxime-TMS derivatives of different sugars have almost similar mass spectra fragmentation patterns. Table 3 reports the main ions fragments obtained by electron impact ionization for the investigated analytical species. The data show the similarities between the isomeric sugars: xylose, arabinose, ribose and glucose, galactose, mannose as reported from other authors [38].

Table 3
Main ion fragments, *m/z*, obtained by electron impact ionization of sugars and organic acids derivatives as oxime-TMS (O-TMS) or TMS compounds

Compound	Ions fragmentation (*m/z*)
Xylose O-TMS	307, 217, 205, 147, 103, 73
Arabinose O-TMS	307, 217, 205, 147, 103, 73
Ribose O-TMS	307, 217, 205, 147, 103, 73
Rhamnose O-TMS	524, 321, 231, 219, 147, 117, 73
Fructose O-TMS	612, 422, 307, 217, 147, 103, 73
Galactose O-TMS	612, 524, 422, 319, 205, 147, 103, 73
Mannose O-TMS	612, 524, 422, 319, 205, 147, 103, 73
Glucose O-TMS	612, 524, 422, 319, 205, 147, 103, 73
Sucrose-TMS	378, 318, 258, 216, 174, 156, 147, 73
Succinic acid-TMS	262, 247, 147, 73
Malic acid-TMS	335, 233, 147, 73
Tartaric acid-TMS	438, 423, 292, 219, 189, 147, 73
Citric acid-TMS	465, 363, 273, 245, 204, 147, 73

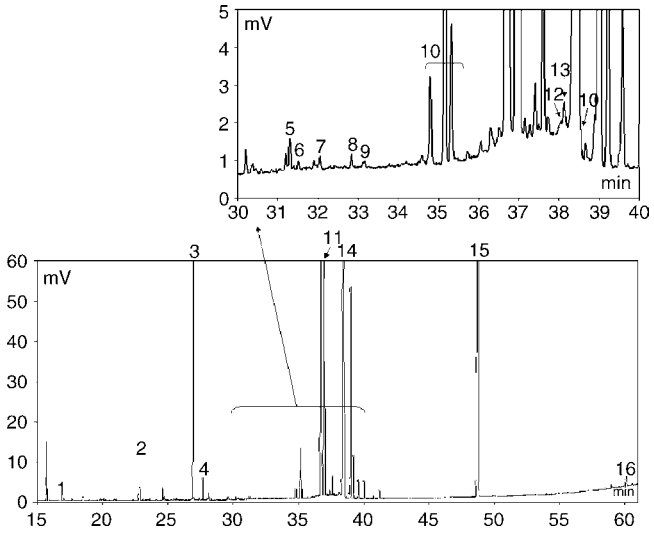

Fig. 2. Chromatogram of a representative ABTM sample. Listed peaks correspond to the oxime-TMS derivatives of sugar and TMS derivatives of acids used for the quantitation as follow: 1, succinic acid; 2, malic acid; 3, *p*-hydroxybenzoic acid (*I.S.*); 4, tartaric acid; 5, xylose; 6, arabinose; 7, ribose; 8, citric acid; 9, rhamnose; 10, gluconic acid; 11, fructose; 12, galactose; 13, mannose; 14, glucose; 15, phenyl-β-D-glucopyranoside (*I.S.*); 16, sucrose.

Therefore, the possibility to discriminate between different analytical species and their *syn* and *anti* forms is mainly due to the differences in the retention times rather than their mass spectra.

Moreover, the ratio between the two isomers, *syn* and *anti*, of each sugar is always constant, as confirmed by the injection of mixtures of standards at a different concentration, see Table 2. Generally, the *syn* form is more stable and produces a more intense peak with respect to the *anti* form, therefore, the *syn* form was used to calculate the sugars concentration; for example, glucose, xylose, mannose and galactose showed the two isomeric forms in an approximately 4:1 (*syn*:*anti*) ratio, as observed by other authors [27]. Furthermore, whenever an overlap between an *anti* and a *syn* peak of sugars with similar concentration occurred, such as the case of the *anti* peak of arabinose and the *syn* one of xylose, the constancy of the isomeric ratio was used to determine the sugars concentration of both species. On the contrary, when the concentrations of the sugars with overlapped peaks are rather different, the evaluation of the concentration was not possible with this approach; such as the case of sorbose with fructose (the *syn* peak of sorbose is co-eluted with the *anti* peak of fructose). Further, in the case of sorbose, although the *syn–anti* ratio is well established from standards injection, the impossibility to detect the *anti* peak of the analyte in the samples leads to the conclusion that this sugar is eventually present at an extremely low concentration in the vinegar matrix.

The quantification of gluconic acid with this analytical method it is not possible. In fact, gluconic acid gives four deriva-

tives after the oximation step and GC–MS analysis identified these molecules as lactonic forms of differently silylated gluconic acid molecules, peaks 10 of Fig. 2. Moreover, the ratio between the peaks is not constant with gluconic acid concentration as observed from the analysis of the standards solutions (data not reported). However, the amount of gluconic acid in ABTM samples of different age was determined in a previous work [5] and was find to range between 1 and 3 g kg^{-1}. Similarly to gluconic acid, with the present method it is not possible to quantify acetic acid and other low boiling species such as lactic acid. In fact, owing to their high volatility, these analytes are completely loss during the sample dryness step. For this reason, to date, the HPLC technique remains the only instrumental approach for acetic acid and low boiling species determination in vinegars and other related matrices.

Because of the matrix complexity and the absence of an appropriate certificate reference material, the recovery values of the investigated analytes were evaluated by the standard additions method and the recovery function [5,14,39]. In the standard additions method, for each analytical species, four aliquots of the representative test vinegar sample were taken, and three of these were spiked with an increasing amount of the corresponding standard solution, with final concentrations ranging from 5 to 25 mg g^{-1}, for the acidic species, and ranging from 10 to 400 mg g^{-1}, for the sugar ones. Recovery values are obtained from the slope of the recovery function [39] and are listed in Table 2.

For almost all the investigated species the recovery values are satisfactory, being close to 100%, and no significant matrix effects were observed [39,40].

The recovery values of sucrose and mannose significantly differ from 100%. In particular, the low recovery value observed for sucrose is probably due to a partial hydrolysis of the added sucrose induced by the low pH of ABTM. On the contrary, the high recovery value of mannose is probably due to an overestimation of its peak area during the integration process, due to a peak overlapping with galactose.

4.2. Carboxylic acids and sugars content

The mean concentration, corresponding to three injections of the measured acids and sugars are reported in Tables 4 and 5, respectively. Table 4 also reports the water content measured by Karl Fischer titration.

In particular, as regards the organic acids content, succinic and citric acids are present at low concentration and show a similar trend in all the *batterie* passing from cask 6 to 1. These data are also in agreement with our previous studies [5]. On the other side, the malic and tartaric acids concentration trends show some differences when considering the three *batterie* C, M and S. In this context, series M shows an increasing concentration trend with the product age for both the organic species higher than expected on the basis of the water content decrease (as estimated by the last column of Table 4). The concentration of tartaric acid in series C and S is almost constant along the *batteria* and it is lower than in series M. In fact, as the water content decrease (Table 4) with the product ageing, tartaric acid should remain almost constant due to the precipitation of potassium as tartaric salt.

Table 4
Mean organic acids concentration (three repeated injections, g kg^{-1}) and water content, expressed as %, with associated absolute errors measured in the C, M and S *batterie*

Cask	Succinic acid	Malic acid	Tartaric acid	Citric acid	Water (%)
C					
1	0.66 ± 0.04	13.1 ± 0.8	5.3 ± 0.8	1.5 ± 0.3	36.6 ± 0.3
2	0.64 ± 0.03	13.7 ± 0.8	4.7 ± 0.5	1.5 ± 0.3	38.8 ± 0.4
3	0.40 ± 0.01	12.4 ± 0.7	4.6 ± 0.7	1.5 ± 0.3	42.5 ± 0.3
4	0.64 ± 0.04	14.3 ± 1.0	5.0 ± 0.6	1.5 ± 0.2	39.8 ± 0.5
5	0.51 ± 0.04	12.7 ± 0.9	5.3 ± 0.6	1.2 ± 0.2	50.7 ± 0.2
6	0.38 ± 0.02	7.6 ± 0.4	4.0 ± 0.9	0.7 ± 0.1	60.6 ± 0.4
M					
1	0.57 ± 0.03	15.2 ± 1.3	9.6 ± 1.2	1.1 ± 0.5	23.1 ± 0.5
2	0.58 ± 0.08	14.6 ± 1.3	9.6 ± 1.1	1.5 ± 0.3	28.7 ± 0.5
3	0.53 ± 0.03	15.4 ± 0.9	9.3 ± 0.8	1.4 ± 0.3	32.3 ± 0.3
4	0.43 ± 0.03	15.5 ± 0.9	8.2 ± 0.9	1.2 ± 0.4	38.2 ± 0.4
5	0.45 ± 0.02	14.0 ± 1.2	7.7 ± 1.0	1.1 ± 0.4	42.7 ± 0.2
6	0.46 ± 0.03	10.7 ± 1.1	5.2 ± 0.9	0.7 ± 0.4	51.0 ± 0.4
S					
1	0.45 ± 0.04	9.9 ± 0.8	5.4 ± 0.6	0.8 ± 0.2	27.7 ± 0.4
2	0.47 ± 0.04	10.2 ± 0.8	6.4 ± 0.9	1.4 ± 0.2	26.8 ± 0.2
3	0.50 ± 0.03	10.4 ± 0.7	6.0 ± 0.7	1.2 ± 0.2	28.4 ± 0.4
4	0.49 ± 0.05	10.5 ± 0.7	6.1 ± 0.8	1.0 ± 0.1	31.9 ± 0.2
5	0.48 ± 0.05	10.8 ± 1.3	6.0 ± 1.2	1.0 ± 0.3	39.0 ± 0.3
6	0.65 ± 0.04	8.9 ± 0.6	5.2 ± 0.6	0.6 ± 0.1	46.3 ± 0.5

These differences may be mainly due to the type of grape used to obtain the cooked must, i.e. white or red grape, and may vary yearly as consequence of the topping up procedure as well. However, as any information about grape type is lacking, any other consideration about the trend of organic acids concentration is only speculative.

With reference to the glucose and fructose quantity in the analysed samples, in addition to the increasing concentration effect observed along the *batterie*, passing from cask 6 to 1, mainly due to the natural evaporation of water, one more consideration can be stressed out. While grape juice contains a comparable amount of glucose and fructose (glucose/fructose $\cong$ 1), the analysed *batterie* showed a glucose/fructose ratio greater than 1 for all the series C, M and S. In particular, the ratio decreases with different extent, during ageing, in *batterie* C and M, while *batteria* S shows an almost constant glucose/fructose ratio. Several reasons might be responsible for these trends. In particular, heat treatment of foods containing reducing sugars in alkali or acid condition, triggers a sequence of non-enzymatic reactions that lead to the formation of different compounds such as furan derivatives. Moreover, as the sugars concentration increases due to the loss of water by the heating process, brown coloured products are obtained through caramelisation reaction.

In both cases the major intermediate product, obtained by the same chemical reactions pathways of the caramelisation and Maillard reactions, that occur during the must cooking step, is in agreement with the hypothesis of a minor stability of fructose towards hydroxymethylfurfural (HMF) and furfural formation

Table 5
Mean sugars concentration (three repeated injections, g kg^{-1}) with associated absolute errors measured in the C, M and S *batterie*

Cask	Xylose	Arabinose	Ribose	Rhamnose	Fructose	Galactose	Mannose	Glucose	Sucrose
C									
1	0.37 ± 0.03	0.85 ± 0.06	0.24 ± 0.01	0.15 ± 0.01	220 ± 11	0.27 ± 0.02	1.02 ± 0.06	248 ± 14	2.6 ± 0.5
2	0.35 ± 0.03	1.00 ± 0.06	0.12 ± 0.02	0.12 ± 0.01	200 ± 10	0.24 ± 0.01	1.01 ± 0.07	233 ± 14	2.5 ± 0.4
3	0.32 ± 0.03	0.79 ± 0.05	0.12 ± 0.01	0.11 ± 0.02	187 ± 9	0.26 ± 0.02	0.98 ± 0.06	232 ± 15	1.8 ± 0.4
4	0.29 ± 0.02	0.89 ± 0.06	0.10 ± 0.02	0.08 ± 0.01	170 ± 8	0.27 ± 0.02	0.91 ± 0.05	234 ± 14	1.8 ± 0.3
5	0.25 ± 0.02	0.66 ± 0.06	0.12 ± 0.01	0.075 ± 0.004	149 ± 8	0.22 ± 0.01	0.61 ± 0.04	201 ± 11	1.1 ± 0.2
6	0.15 ± 0.01	0.37 ± 0.02	0.08 ± 0.01	0.061 ± 0.005	131 ± 7	0.14 ± 0.02	0.41 ± 0.03	153 ± 10	0.5 ± 0.1
M									
1	0.39 ± 0.03	0.93 ± 0.05	0.31 ± 0.03	0.19 ± 0.02	244 ± 13	0.32 ± 0.03	1.46 ± 0.09	293 ± 16	6.8 ± 1.2
2	0.33 ± 0.03	0.89 ± 0.06	0.25 ± 0.05	0.13 ± 0.01	236 ± 13	0.33 ± 0.02	1.20 ± 0.08	285 ± 20	5.5 ± 1.0
3	0.27 ± 0.02	0.73 ± 0.05	0.31 ± 0.02	0.13 ± 0.01	215 ± 11	0.31 ± 0.02	1.06 ± 0.08	277 ± 16	4.3 ± 0.7
4	0.23 ± 0.03	0.55 ± 0.04	0.43 ± 0.04	0.11 ± 0.01	204 ± 12	0.28 ± 0.02	1.02 ± 0.07	282 ± 17	3.0 ± 0.5
5	0.19 ± 0.01	0.48 ± 0.03	0.27 ± 0.03	0.10 ± 0.01	173 ± 9	0.26 ± 0.02	0.85 ± 0.05	257 ± 15	2.0 ± 0.4
6	0.11 ± 0.01	0.33 ± 0.02	0.21 ± 0.02	0.09 ± 0.01	131 ± 7	0.20 ± 0.01	0.57 ± 0.05	224 ± 13	0.7 ± 0.1
S									
1	0.28 ± 0.02	0.70 ± 0.05	0.27 ± 0.03	0.12 ± 0.02	280 ± 17	0.33 ± 0.02	1.02 ± 0.07	301 ± 19	3.9 ± 0.8
2	0.26 ± 0.03	0.71 ± 0.05	0.20 ± 0.02	0.18 ± 0.01	275 ± 14	0.39 ± 0.03	0.97 ± 0.06	294 ± 17	4.5 ± 0.8
3	0.26 ± 0.02	0.79 ± 0.05	0.14 ± 0.02	0.15 ± 0.01	271 ± 14	0.28 ± 0.02	0.97 ± 0.06	287 ± 17	4.9 ± 0.8
4	0.27 ± 0.03	0.69 ± 0.05	0.26 ± 0.02	0.13 ± 0.01	264 ± 15	0.34 ± 0.02	0.97 ± 0.07	290 ± 16	3.5 ± 0.5
5	0.22 ± 0.02	0.87 ± 0.05	0.13 ± 0.01	0.10 ± 0.02	231 ± 12	0.26 ± 0.02	0.75 ± 0.04	253 ± 15	2.3 ± 0.4
6	0.21 ± 0.02	0.47 ± 0.03	0.20 ± 0.02	0.09 ± 0.01	194 ± 10	0.27 ± 0.02	0.64 ± 0.04	219 ± 12	1.7 ± 0.3

with respect to glucose [41,42]. A further possibility could be due to the fermentation phenomena that utilise glucose and fructose following diverse pathways during the first ageing years, or the precipitation of glucose caused by its minor solubility with respect to fructose [43].

4.3. Data analysis

In the previous section, the compositional data of the minor and major constituents relatively to organic acids and sugars have been discussed on the basis of the variability of each single chemical specie, and relevant differences in the concentration trend of each analyte, among the different *batterie,* were highlighted. In order to simultaneously analyse all the collected data and to gain an overall overview of the existing relationships among compositional profile, product ageing and *batterie* similarity/dissimilarity, the data reported in Tables 4 and 5 have been elaborated with three-mode principal component analysis, i.e. Tucker3 analysis. This is a powerful explorative data analysis tool suitable when, as in this case, the structure of the experimental data is characterised by three distinct sources of systematic variability, i.e. the different ageing of product belonging to different casks, the different *batterie* (series owned by a given producer), and the different compositional profiles. In fact, interpretation and visualization of the data are appreciably improved by extracting the information content relevant to the different variability sources, in distinct subspaces, i.e. by using three-mode PCA distinct sets of loadings are obtained for each variability source.

In this case, the more classical approach of using principal component analysis after unfolding is not recommended since the variability among the different *batterie*, due to the traditional making procedure, such as varying extent of "topping up", use of different musts and casks capacities, will be overlaid to the intra casks variability, mainly due to product ageing (data not reported).

The values reported in Tables 4 and 5 were organised in a three-way array with: 18 rows (I) representing the vinegar samples (including replicates) on the first mode, MODE 1, 13 columns (J) corresponding to the concentrations, expressed as g kg^{-1} referred to the dry matter for each sample, of the investigated chemical species, (four organic acids and nine sugars) on the second mode, MODE 2, and 3 thickness terms (K) corresponding to the different *batterie* on the third mode, MODE 3, obtaining an $\underline{\mathbf{X}}$ array of dimension $18 \times 13 \times 3$ (Fig. 1). The $\underline{\mathbf{X}}$ array has been pre-treated as explained in the Section 4.3 and then Tucker3 analysis was performed.

Different Tucker3 models were calculated and core consistency analysis and percentage of explained data variance were considered to identify the right number of factors in each mode. A model with dimensionality (3 3 2), i.e. three factors on MODE 1 (samples), three factors on MODE 2 (compositional variables) and two factors on MODE 3 (*batterie*), was selected. This model explains 68% of the total data variance. The core matrix, obtained by the Tucker3 analysis, is characterised by the highest core elements: z_{111} (with negative sign) which explains 48% of the core matrix variance; followed by core elements z_{232} and z_{221} (both with negative sign) each one explaining about 7% of the core matrix variance. In the following discussion the loadings plots relative to each MODE will be first discussed separately then, taking into account the most relevant interaction terms ($z_{i,j,\underline{k}}$), factor 1 of each MODE will be discussed comparatively, followed by joint analysis of factor 2 of MODE 1 and MODE 2 and factor 1 of MODE 3.

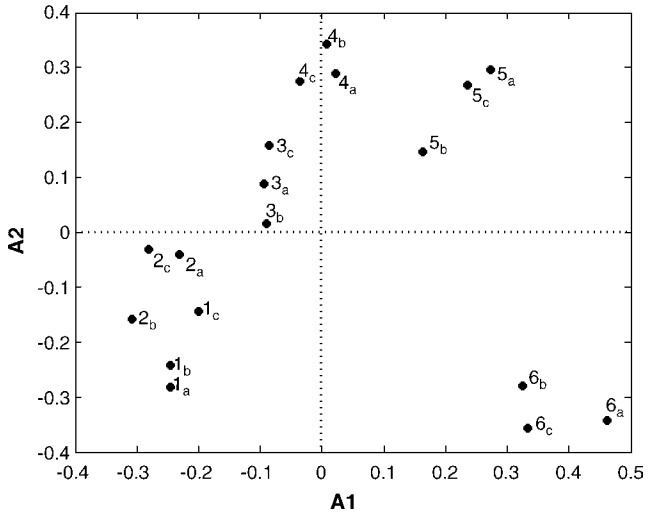

Fig. 3. Scores plot A1 vs. A2 for MODE 1 (samples). Numbers indicate the cask while the letters a–c indicate repeated sample.

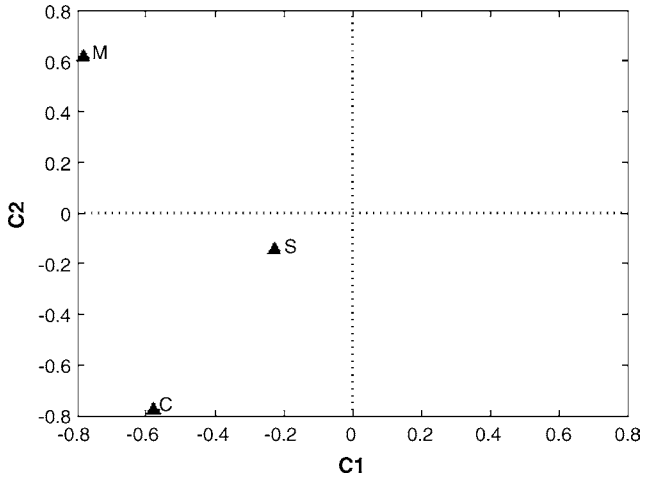

Fig. 5. Loadings plot C1 vs. C2 for MODE 3 (*batterie*).

Figs. 3–5 show the factor 1 versus factor 2 scores plot for MODE 1 (samples with repetitions), MODE 2 (compositional variables) and MODE 3 (*batterie* C, M and S) loadings plot, respectively.

The score plot of the first two factors (A1 versus A2) for MODE 1 (Fig. 3), i.e. samples mode, shows the presence of three main groups: the first one includes the youngest samples (belonging to the fifth, $5_{a,b,c}$, and the sixth, $6_{a,b,c}$, casks); the second group includes samples from casks $3_{a,b,c}$, $4_{a,b,c}$ and $5_{a,b,c}$, and the third one the oldest samples (belonging to the first, $1_{a,b,c}$, and the second, $2_{a,b,c}$, casks). The first factor clearly distinguishes samples from the first and third group, being located on the opposite side of the plot, while the middle aged samples, $3_{a,b,c}$ and $4_{a,b,c}$, are close to each other and near to the origin. Factor A1 thus depicts the ageing trend along the series of casks.

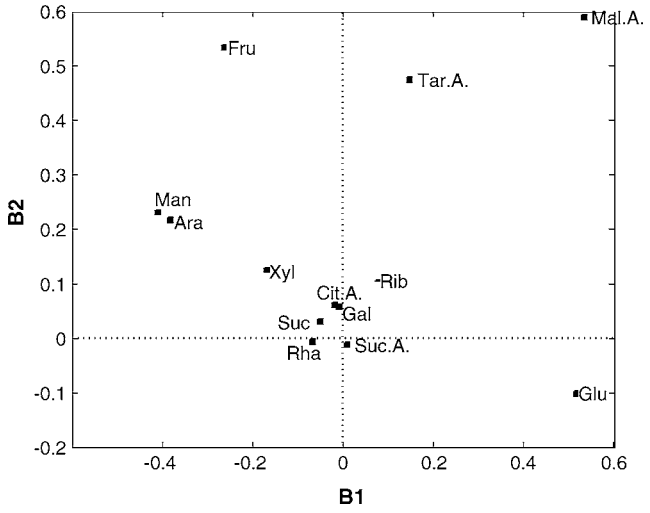

Fig. 4. Loadings plot B1 vs. B2 for MODE 2 (variables). Variables are as follow: Suc.A., succinic acid; Mal.A., malic acid; Tar.A., tartaric acid; Cit.A., citric acid; Xyl, xylose; Ara, arabinose; Rib, ribose; Rha, rhamnose; Fru, fructose; Gal, galactose; Man, mannose; Glu, glucose; Suc, sucrose.

The MODE 2 loadings plot, B1 versus B2 (Fig. 4) shows the distribution of the investigated variables, i.e. sugars and organic acids. Most of the minor components (with the exception of mannose and arabinose) are close to the origin and therefore do not vary significantly along the casks series. Glucose and fructose lie almost in opposite position with respect to the axis origin, highlighting a different chemical behaviour of these species in the ABTM matrix along the cask series. As it has been previously pointed out, several mechanisms, such as different degradation and fermentation rates and different solubility, may be responsible of the observed dissimilarity.

Malic and tartaric acids have similar loadings values on the second factor (B2) while on the first factor (B1) tartaric acid is close to the origin indicating a modest variability of this species along the casks series.

The MODE 3 loadings plot C1 versus C2 (Fig. 5) shows the distribution of the *batterie*; these share negative values on factor C1 indicating a common trend with ageing.

The first important core element z_{111} indicates a dominant interaction among the factors A1, B1 and C1 of the three MODEs. The axis A1 distinguishes the vinegar samples on the basis of their ageing and, taking into account the position of the *batterie* on C1 axis, it is possible to appreciate the different degree of variability of the samples within each series; which is higher for *batteria* M, intermediate for C and negligible for S (*batteria* S is close to the plot origin) and it is somehow consistent with the peculiar features of each *batteria*. In fact, S is the oldest *batteria* and from C it has not spilled ABTM from the last cask for marketing purposes yet.

Besides, taking into account the negative sign of the z_{111} element of the core matrix, the following conclusions can be drawn: (i) along factor 1, B1, in the chemical variables mode, glucose and malic acid contents (tartaric acid content to a less extent), relative to dry matter, get the highest loading values, highlighting their probably major content in the younger samples with respect to the older ones, above all for *batteria* M; (ii) on the other side, the opposite holds for fructose, mannose and arabinose.

The different "quality and/or variety" of the cooked must poured yearly during the topping up procedure may be invoked as a possible explanation for these observations. Besides glucose and fructose are subjected to different chemical transformation during ageing (see previous discussion).

As far as interaction among A2, B2 and C1 is concerned (z_{221} also with negative sign), it takes into account the replicates variability, and mostly the lower contents in cask 6, mainly for *batteria* M, of fructose, malic and tartaric acids, that make cask 6 not following the ageing order on factor A2. This peculiar behavior may be interpreted on the basis of cask 6 being the one where each year newly prepared cooked must is added and therefore is subjected to the most relevant chemical and physical transformations.

Thus three-mode PCA gives an effective data display, which allows depicting the relevant features of the investigated system. In particular, on one hand the peculiarities of each investigated *batteria*/producer emerge but on the other hand, a common ageing trend is observed, i.e. it is possible on the basis of the compositional profile of the bulk, to differentiate the starting phase (casks 6 and 5), the maturation period (casks 4–2) and the final ageing step (cask 1).

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Appendix A

When, as in the present case, there is the risk of dramatically upweighting components present in very small amount and showing little variation, unscaled *X*-data may be preferable. However, in the present case, given the presence of the two major sugars (glucose and fructose) at concentration at least of one order of magnitude higher with respect to every others components, we want still to make the different categories of components comparable in influence on the Tucker3 model. This was accomplished as follow: three blocks of variables are defined: (block 1) major sugar components: glucose, fructose and sucrose (three variables); (block 2) minor sugar components: arabinose, xylose, ribose, rhamnose, mannose and galactose (six variables); and organic acids as (block 3) (four variables).

Blockscaling has been accomplished by rearranging the three-way array to a bi-dimensional $IK \times J$ matrix and then weighting each variable belonging to the same block by:

$$w_{j_B} = \sqrt{\frac{\mathrm{SS_{TOT}}}{\mathrm{SS_{BLOCK}} \cdot n_{\mathrm{BLOCK}}}}$$

where SS_{TOT} is the total sum of squares over all J's variables, SS_{BLOCK} is the sum of squares over the J's variables belonging to the given block and n_{BLOCK} is the number of blocks, i.e. three in our case.

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