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^{1}H NMR Studies on Italian Balsamic and Traditional Balsamic Vinegars

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In the present work Principal Component Analysis applied to ^{1}H NMR spectra of balsamic and traditional balsamic vinegars is used to establish a simple and rapid aging determination protocol. Chemical composition of vinegar is dominated by carbohydrates even though several small components can be clearly observed in the proton NMR spectrum. Quantitative determination of some selected metabolites such as ethanol, acetic acid, malic acid, glucose, and HMF, considered as potential aging indicators, has been performed. ^{1}H NMR spectroscopy provides noninvasive characterization of such compounds, and our data demonstrate the validity of this approach, giving very promising results for assessing the quality of the final product.

KEYWORDS: Vinegars; NMR; principal component analysis

Balsamic and traditional balsamic vinegars (BV and TBV, respectively) from both Modena and Reggio Emilia are the most appreciated Italian vinegars at the international level and have been recently acknowledged as "origin denomination protected" products (D. O. P.) by the European Union (CE n. 813/2000 April 17, 2000). The qualitative characteristics and the peculiarity of these products are essentially dependent from the geographical environment of production, which is affected either by natural (climatic factors) or by human factors (handed down production techniques) giving rise to a unique food product.

The differences between balsamic and traditional balsamic vinegars are mainly due to the aging period and to the production procedures, which are regulated by set rules (G. U. 124 of May 3, 2000). TBV is produced from cooked must of selected local grapes, such as Trebbiano, Lambrusco, Ancellotta, Sauvignon, Berzemino, Occhio di Gatta and Sgavetta; the aim of must cooking is to interrupt any fermentation in progress. The use of any extra additive is forbidden, only yeast addition (*Zygosaccaromices*) can be done providing alcoholic fermentation of sugars, which will be transformed again by further addition of acetic bacteria, known with the name of "MADRE". This latter process is performed into barrels arranged in set of 5/7 casks each of varying capacity and made of different woods: durmast, chestnut, ash tree, cherry tree, and mulberry tree. After few years of aging, typically during the cold season, the operation called "RINCALZO" (ridging) takes place. The largest barrel receives the year's simmered must, while from the smallest, the TBV ready for use is spilt; the unfinished product is added in the intermediate barrels. As the contents of the smallest barrel diminishes because of spilling, it is replaced for the correct quantity by taking from the barrel immediately preceding, and this one in turn will have to be fed from the next preceding one, until the first and largest is reached. This, as said above, is replenished with the must obtained from the year's harvest. For TBV, this procedure is usually repeated at least 13 years up to 25−30 years. BV, on the contrary, can be produced from cooked must blended with wine, acetic acid, and flavors and can experience different aging process into barrels, which can last from two months up to 3 years, giving rise to the so-called "red stamp", or over 3 years up to 20 years, the "white stamp", for best quality products. Low commercial quality vinegars can be produced industrially by blending different vinegars, flavors and dyes.

BV and TBV contain many different chemical compounds and several analytical methods have been employed to attempt the vinegar characterization and age determination. Some of these compounds, like carboxylic acids, have been determined by HPLC and GC methods (*1*), while the phenolic compounds content has been analyzed by HPLC methods in aged sherry vinegars, and it was shown to increase during aging (*2*). NMR and MS isotopic ratio (D/H) methods for acetic acid determination were also used for natural vinegars authentication (*3*, *4*). Among all the possible components, sugars (glucose and fructose) are the most abundant, and their composition affects both the final product quality and the alcohol concentration because of the biochemical activities of yeast.

Sugars can quite easily experience chemical modifications as a consequence of must heating and aging processes, employing different mechanisms that involve both glucose and fructose. Water elimination gives rise to both aliphatic and aromatic rings containing oxygen. Among these products, the most abundant and important is 5-hydroxymethyl-2-furfurale (HMF), which has

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been quantified in different food products as thermal damage indicator (*5*). Other compounds, whose presence is due to thermal degradation of HMF (*6*), can be present in much smaller quantities (up to 200 mg/L); among them, 5-acetoxymethyl-2-furfurale (5AMFA) have been proposed as aging marker (*7*). Different analytical methods have been developed so far for HMF determination in different food products, like fruit juice (*8*), wine (*9*−*13*), honey (*14*, *15*), milk (*16*), etc. HMF concentration control in commercial food products is related to its possible role as initiator and promoter of colon cancer (*17*, *18*). Food prepared in households under individual conditions makes the estimation of HMF intake impossible. Fortunately, the daily use of vinegar contributes a negligible amount of HMF intake.

Determination of HMF by HPLC in different vinegars has been recently published (*19*) and showed that HMF can reach high quantity in balsamic vinegars (up to 5500 mg/L in TBV) with respect to commercial vinegars, due to the high initial content of sugars, while other aromatic derivatives of HMF are present in one or two concentration order lower. These results suggest that HMF is a good marker for must concentration procedure and age estimation of TBV and BV vinegars. During the aging process, pH decrease and spontaneous evaporation can further increase the sugar degradation with consequent HMF accumulation. Other age indicators, and in particular the racemization values of free amino acids and acetoine, have been suggested for discrimination of aged vinegars (*20*).

The aim of this work is to investigate the presence of some specific metabolites in balsamic and traditional balsamic Italian vinegars by ^{1}H NMR, as to provide a protocol that can quickly check the declared aging. The advantages of the NMR technique with respect to other analytical methods are the non invasive approach, the high specificity and selectivity reachable, and the possibility to provide information on a wide range of metabolites in a single experiment; finally, the sample preparation is almost negligible. The metabolites we have monitored by ^{1}H NMR were ethanol, acetic acid, malic acid, glucose, and HMF in several TBV, BV vinegar samples and some commercial vinegar samples. There should be even more metabolites that can be monitored, but actually these were readily detectable, and moreover, they seemed to be sufficient for a rapid aging determination. Combination of NMR data and statistical analysis can also give interesting results for authentication purposes, as already demonstrated in other similar works (*21*−*25*). Authentication tests are usually done by a judges panel via sensorial analysis, giving specific points to the samples tested. A spectroscopical NMR determination of metabolites can corroborate the sensorial analysis and furthermore overcome the low specificity of the other analytical methods.

MATERIALS AND METHODS

Samples. A set of 17 samples, either obtained commercially or by gift, have been used for quantitative metabolite determination. Among them, samples 1, 2, 11, 14, 15, 16, and 17 have been obtained directly from the producers, with certified origin and aging process. Sample 13, marked with RM (rich must) consist of balsamic vinegar obtained with addition of larger amount of concentrated must. All other samples have been purchased in commercial stores.

NMR Analysis. NMR experiments have been recorded on a Bruker DMX 500 spectrometer operating at 11.7 T and equipped with a 5-mm reverse probe with z-gradient. Samples were prepared by dissolving 50 μL of vinegar in 450 μL of DMSO-$^2H_6$. An external calibrated TSP reference (trimethylsylyl [2,23,3-$^2H_4$] propionate) has been used to obtain quantitative measurements. All spectra have been phase and baseline corrected before FT transformation and standard integration routine from XWINNMR program (version 2.6 Bruker) have been used for signal quantification. Mono dimensional ^{1}H spectra have been acquired with and without low power water signal irradiation. Selective 1D TOCSY experiments have been obtained with Gaussian shaped pulse of 80 ms length and 120 ms mixing times. The aging of commercial samples has been declared by the producer on the bottle label. We have tested different aged balsamic vinegars and five samples of TBV (samples 1, 2, 15, 16, and 17), all samples from different producers.

Statistical Method. ^{1}H NMR data have been evaluated with Statgraphics Statistical Computer Package "Statgraphics Plus 7.11" software and the multivariate statistical analysis performed was in terms of principal component analysis (PCA). (*26*)

RESULTS AND DISCUSSION

NMR spectra of different samples have been analyzed, and **Figure 1A** represents a typical ^{1}H NMR spectrum of balsamic vinegar sample while Figure 1B shows assignment of some selected metabolites indicated by arrows.

The ^{1}H resonances of ethanol, acetic acid, malic acid, α- and β-glucose anomeric protons, and HMF have been readily identified on the basis of their typical spectral pattern and chemical shift values, while for glucose side chain assignment, a selective 1D TOCSY has been employed (**Figure 2**).

The aim of these "selective" NMR techniques is to solve ambiguity in resonance assignment and to give useful information in the metabolite recognition. An example of this technique is given in **Figure 2**, traces B and C, where the hindered sugar region (between 4.3 and 3.3 ppm) is "cleaned" by selective excitation of α- and β-glucose anomeric protons respectively, thus giving rise to the complete glucose side chain resonances. Unambiguous confirmation of proton assignment for other selected metabolite resonances have been obtained by addition of pure single compound to the vinegar sample. The selected peaks (ethanol, acetic acid, malic acid, glucose, and HMF) have been quantified against the calibrated TSP peak, in terms of signal integration, and the results (expressed in gr/L) are summarized in **Table 1**. In particular, we have detected different concentration for α- and β-glucose in vinegar samples, but a constant ratio of them seem to be present in all samples analyzed. We decided then to report only α-glucose content in vinegar samples, as indicated in **Table 1**. The highest values of HMF, glucose, and malic acid content, were found for the older BV and TBV, while much lower values were found for the younger balsamic vinegars, in agreement with previous statements (*19*), most likely due to the particular production procedure, like the cellulose degradation of the wood barrels. Furthermore, it was supposed that heat treatment of the must during vinegar production mostly influence the HMF formation and even evaporation time, which occurs during vinegar aging. Thus, high HMF concentration in older samples is expected. Contrarily, the ethanol concentration was found to decrease with an increase in the vinegar age in TBV samples is at minimum values. Balsamic vinegar samples obtained with rich must ripening present completely altered metabolite contents with respect to balsamic vinegar samples of the same age. This clearly suggests that the quantification of a single metabolite is not sufficient for age determination; a direct correlation between single metabolite concentrations and aging process seems not

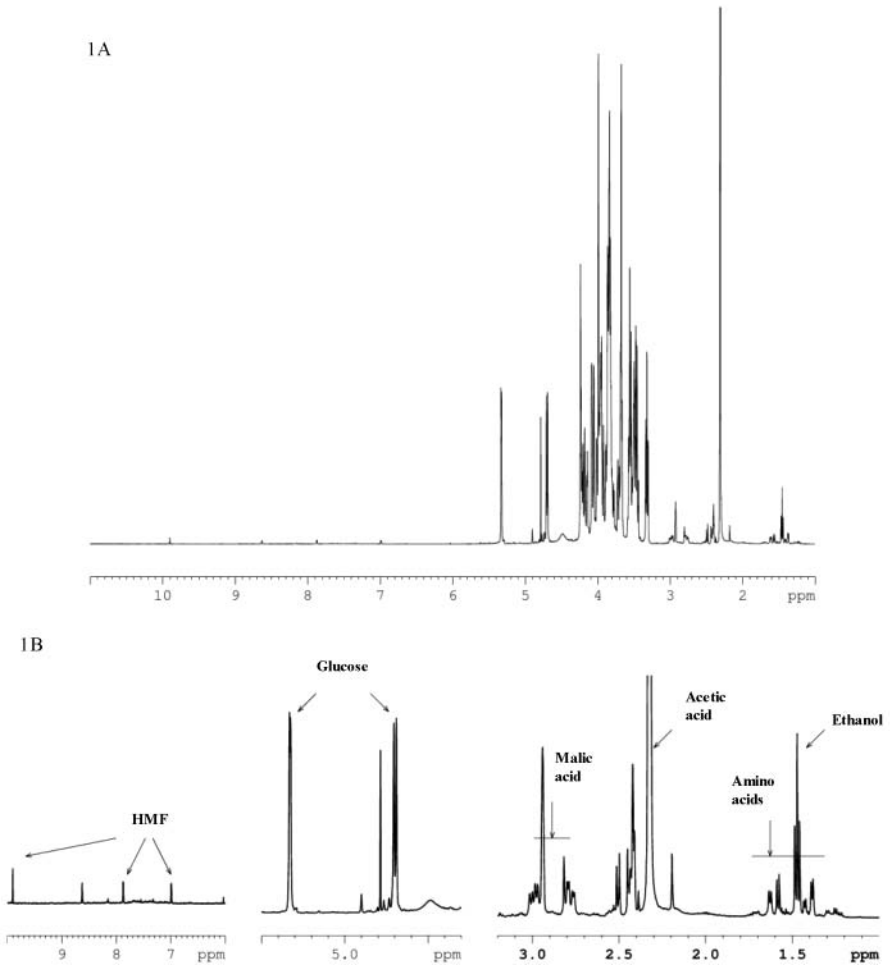

Figure 1. ^{1}H NMR spectra recorder with water suppression pulse program. (**A**) Typical spectrum of balsamic vinegar sample. (**B**) Expanded regions (aromatic, anomeric, and aliphatic regions, from left to right) with metabolite assignments.

to be present. The richness of information resulted in this complex metabolite concentration variation correlated with aging process appears to be a typical problem that PCA can rationalize.

The integrated values of selected resonances have been submitted to PCA analysis, and the result is depicted in **Figure 3**.

The graphical representation of the scores scatter plot obtained by selecting two PCs as axes shows the similarities/differences among the samples. The first component (PC1) describes 64% of the variance, while the second component (PC2) describes only 18%; the two PCs together express 82% of the total variability, constituting an accurate representation of the products disposition and are related to the length of the aging period. It is important to underline that two or more products must be considered more similar to each other the closer they are (Euclidean distances). The left end of the graphic clusters vinegar samples with less aging period (less than 3 years) together with "commercial" balsamic vinegar samples (vinegars declared under 1 year age). These samples are characterized by high acetate and low HMF, malic acid, and glucose concentrations, with respect to all other balsamic vinegar samples. The age discrimination among these "young" samples is not feasible actually, most likely due to lack of characteristic metabolites. Moving along the principal component on the right side, increasing aged vinegar samples are clustered up to the oldest one (30 years). In the middle position, medium aged (12−15 years old) balsamic vinegar samples can be found, and finally the oldest on the extreme right side of the score plot. The typical elements of these vinegars are low acetate quantity and high HMF, malic acid, and glucose concentrations. Ethanol was found not particularly discriminating for vinegar aging: In fact, this variable is explained only by the second principal component, which does not give any additional information with respect to the samples differentiation. As can be observed, the sample 13 in **Table 1** (denoted ">8 RM") is positioned in a anomalous region of the score plot with respect to the other samples. This sample has been obtained by addition of large amount of concentrated must, thus altering some metabolite concentration, especially ethanol and HMF. Its position with respect to the PC2 axis is mainly due to the highest ethanol content, while its projection on the PC1 axis respects the correct age value. Furthermore, a position discrepancy for samples labeled 15 and 16 in **Table 1** is observed in the score plot of **Figure 3**. This misplacement suggest the requirement of more variables for a completely determined PCA analysis, and not a wrong approach, because all other samples are significantly discriminated on the basis of their aging process. We can define the principal component as the "quality component", along which the vinegar samples reported are distributed with respect to their aging process. In particular, we can notice that the similarity among younger samples are more evident (much closer in space) for less than 3 years and "commercial" vinegar samples. On the contrary, increasing sample aging, separation among samples increases as well: one for all, the oldest TVB sample is completely isolated.

High field NMR was already recognized as an important tool in validation procedures of food, like oils (*21*), wines and fruit juice (*27*). Furthermore, authentication studies with the use of isotope ratios determined by NMR have been appeared in the

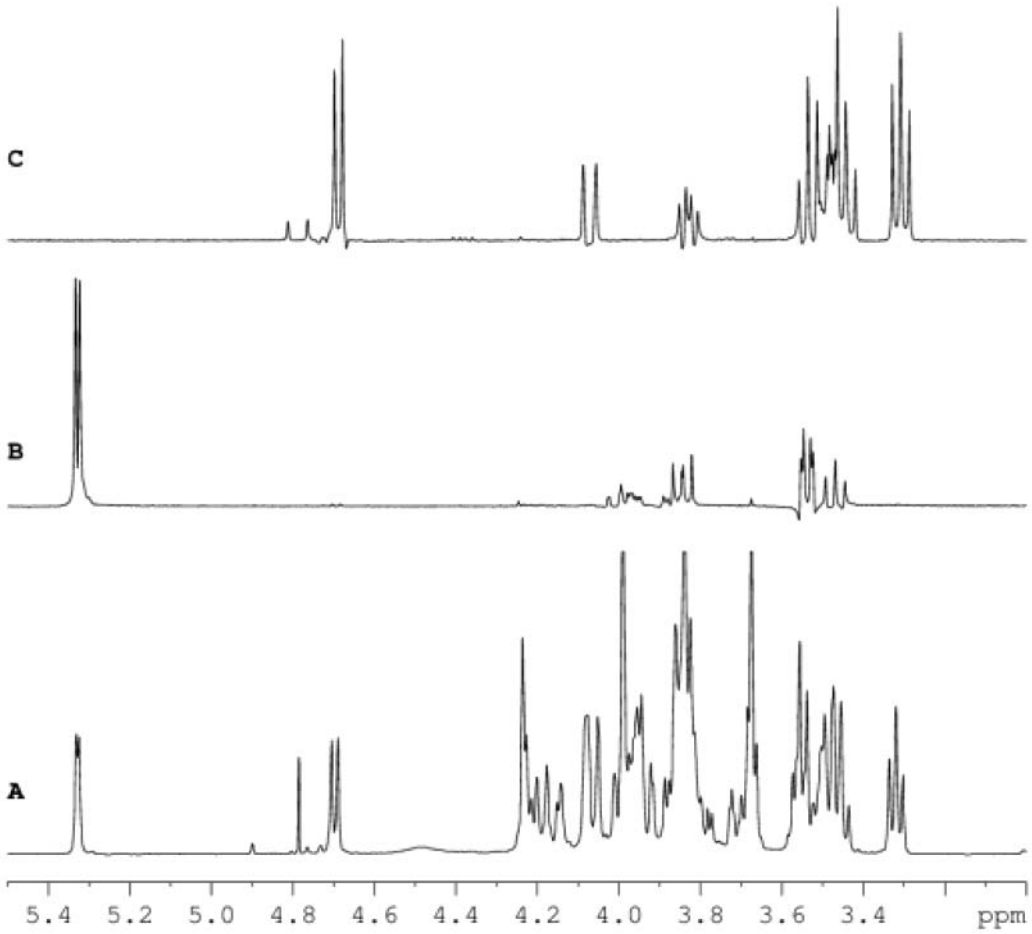

Figure 2. [1]H NMR spectra of vinegar sample (trace A). Expanded region of **1D** TOCSY experiments obtained with selective excitation of α-glucose anomeric proton at 5.3 ppm (trace B) and β-glucose anomeric proton at 4.69 ppm (trace C).

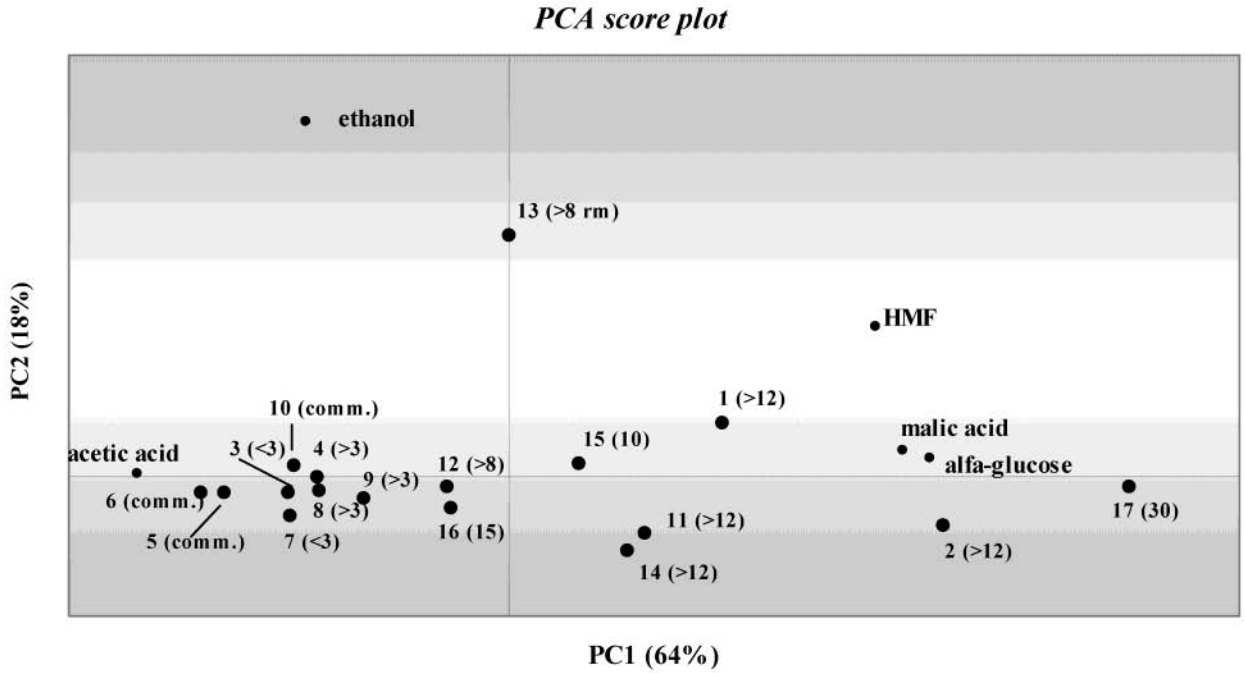

Figure 3. PCA score plot of the studied samples; label numbers refer to declared sample aging (see text).

literature (*4*, *28*−*29*). We have found that following by [1]H NMR, the concentration of specific metabolites, and the use of these data into statistical multivariate methods, it is possible discriminate the vinegar aging among different samples of BV, TBV, and commercial vinegars. In the present work, the components analysis by [1]H NMR in combination with statistical methods seems to be a useful and very promising tool for the investigations of adulteration and over processing processes. To address completely the complex aging process of BV and TBV, and furthermore to rationalize the authentication concern, more

Table 1. Concentration (gr/L) of Selected Metabolites[a]

sample	age (years)	EtOH	AcOH	malico	glucose	HMF
1	>12	4.55	105.38	60.22	535.39	13.55
2	>12	0.00	60.41	77.24	441.63	18.95
3	<3	3.72	148.87	16.39	216.67	2.62
4	>3	4.27	161.19	23.65	294.18	2.74
5	comm	2.50	178.24	8.44	234.30	1.20
6	comm	3.23	179.21	17.57	278.14	3.05
7	<3	3.66	151.25	15.05	215.16	2.77
8	>3	3.94	148.78	21.26	278.42	1.92
9	>3	3.37	158.62	23.33	355.68	2.21
10	comm	5.21	166.64	18.81	365.32	0.58
11	>12	0.00	107.92	22.73	516.55	18.73
12	>8	3.09	163.65	32.72	565.69	7.76
13	8 RM	11.81	130.58	32.22	439.20	25.81
14	>12	0.00	85.37	19.81	463.48	13.35
15	10	4.03	135.87	55.74	512.66	7.44
16	15	2.88	143.98	34.11	417.48	2.78
17	30	0.00	82.31	84.29	858.64	28.27

[a] comm stands for less than one year age and RM stands for rich must vinegar. Glucose concentration has been calculated considering the α-glucose anomeric proton integral value.

metabolites have be to be found and processed with PCA, to be able to discriminate among vinegars differing for closer (single year) aging process. We are actually developing methods in this direction.

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