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Abstract	The aim of this work is to study the chemical and functional characterization of balsamic vinegar of Modena (BVM) and traditional balsamic vinegars from Modena (TBVM), using different methods to assay the phenolic content and the antioxidant activity. Besides, NMR analysis was used to obtain information about the principal substances in the samples. One hundred and nine samples of both TBVM and BVM were analyzed in all. Despite the observed high intragroup variability, the statistical analysis showed statistically significant differences between TBVM and BVM. The TBVMs are richer in phenolics, flavonoids, and tannins and show higher antioxidant capacity than BVMs. The general discriminant analysis (GDA) model including all the compositional and NMR data was able to group the samples according to the type of vinegar. The first canonical discriminant function explains 92.2 % of the total variance, and the leave-one out cross-validation show a predictive capacity of 89.6 %.

Keywords separated by ' - '	Traditional balsamic vinegar of Modena - Balsamic vinegar of Modena - NMR - Antioxidant activity
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Antioxidant Activity, Phenolic Compounds, and NMR Characterization of Balsamic and Traditional Balsamic Vinegar of Modena

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Abstract The aim of this work is to study the chemical and functional characterization of balsamic vinegar of Modena (BVM) and traditional balsamic vinegars from Modena (TBVM), using different methods to assay the phenolic content and the antioxidant activity. Besides, NMR analysis was used to obtain information about the principal substances in the samples. One hundred and nine samples of both TBVM and BVM were analyzed in all. Despite the observed high intragroup variability, the statistical analysis showed statistically significant differences between TBVM and BVM. The TBVMs are richer in phenolics, flavonoids, and tannins and show higher antioxidant capacity than BVMs. The general discriminant analysis (GDA) model including all the compositional and NMR data was able to group the samples according to the type of vinegar. The first canonical discriminant function explains 92.2 % of the total variance, and the leave-one out cross-validation show a predictive capacity of 89.6 %.

Keywords Traditional balsamic vinegar of Modena · Balsamic vinegar of Modena · NMR · Antioxidant activity

Introduction

Balsamic and traditional balsamic vinegars from Modena (BVM and TBVM, respectively) are unique products, produced from the alcoholic fermentation and acetic bioxidation of cooked and concentrated locally grown grape must. The differences between balsamic and traditional balsamic vinegars are mainly due to the aging period and production procedures.

TBVM is a natural product, prepared with cooked and concentrated must, natural yeasts, which provide alcoholic fermentation of sugars, and acetobacters which transform alcohol in acetic acid. The latter process is performed in a series of barrels of varying capacity and made of different woods for at least 12 years. For TBVM, the use of any extra additives is forbidden.

BVM, on the contrary, is prepared with cooked and concentrated must, wine vinegar, and eventually caramel as color stabilizer, no other flavoring or food colors are admitted. Its aging period ranges usually from 2 months to more than 3 years; only in this case the appellation "old" can be used.

To obtain the traditional balsamic appellation, vinegar producers must follow a rigid protocol stated by an Italian Act (Gazzetta Ufficiale della Repubblica Italiana 2000) and approved by the European Council Regulation (EC 813/2000). TBVM obtained the Protected Designation of Origin (PDO) certification from European Union in 2000. Even if the BVM are less fine, they also are quality products obtaining the Protected Geographical Indication (PGI) certification from EU (Reg. CE no. 583/2009 July 3, 2009), so BVMs from Modena have a large national and international consumption.

BVM and TBVM are rich in phenolics such as catechins, phenolic acids, flavonols, tannins (Verzelloni et al. 2007), and compounds synthesized during must cooking, as a result of caramelization or of Maillard reaction such as melanoidins (Falcone and Giudici 2008) that give to them a strong antioxidant activity (Verzelloni et al. 2010).

For the different production procedures, aging period and phenolic composition, several works explored the possibility to correlate some antioxidant parameter, easily measured with

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conventional techniques, with composition (Verzelloni et al. 2007), appellation (Greco et al. 2013), and aging as declared by producers (Greco et al. 2013; Verzelloni et al. 2010). Total phenolic and flavonoid contents are classically and generally determined with Folin–Ciocalteu reagent and colorimetric assay, respectively, while antioxidant capacity with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, ferric reducing ability of plasma (FRAP) assay, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

The obtained results not always are positively correlated, due to different reaction mechanisms with different groups of antioxidant compounds and to restricted sample's number. Verzelloni et al. (2007, 2010) demonstrated that the FRAP assay underestimates the antioxidant capacity of TBVM and BVM with respect to the ABTS assay. For the authors, this difference could be due to the different reaction conditions: ABTS assay is carried out at neutral pH, and the FRAP assay is conducted at acidic pH 3.6 to maintain iron solubility. Another possible explanation of the underestimation of the FRAP respective to the ABTS assay could be that FRAP assay detects compounds that act by the single electron transfer mechanism, while ABTS assay detects compounds that act either by direct reduction via the electron transfers or by radical quenching via the hydrogen atom transfer mechanism (Prior et al. 2005).

In the ABTS assay as well as in the DPPH assay, the steric accessibility is a major determinant of the radical reaction. Thus, small molecules that have better access to the radical site have a higher apparent antioxidant activity.

In this work, conventional techniques for antioxidant capacity determination are compared to photochemiluminescence (PCL) assay. The assay involves the photochemical generation of superoxide $O_2^{\bullet -}$ free radicals combined with chemiluminescence detection. The PCL method is based on a photo-induced chemiluminescence accompanied and antioxidant inhibitable autoxidation of luminol. The luminol is a photosensitizer, generating superoxide radicals, and also a chemiluminogenic probe for free radicals (Popov and Lewin 1996). In contrast to other commonly used AOC assays, the PCL method is not restricted to a specific pH value or temperature range.

The aim of this work is to study the chemical and functional characterization of three different vinegars: extra old and old TBVM, and BVM, using different methods to assay the phenolic content and the antioxidant activity. Besides, NMR analysis was used to obtain information about the principal substances in the samples. At our best knowledge among many analytical studies focused on the TVBM and BVM characterization, none of them used compositional and functional data coupled with ^{1}H NMR spectroscopy and multivariate data analysis in the attempt to obtain models able to classify the samples. In this way, this work aims to give a further contribute to the quality evaluation and valorization of these typical and unique products.

Materials and Methods

Chemicals and Apparatus

All reagents were purchased from Sigma-Aldrich (Milan, Italy). The absorbances were measured with a Beckman DU730 UV-Vis spectrophotometer (Palo Alto, CA). The luminol PCL assay was carried out using the Photochem® instrument with the ACL kit (Analytikjena, Jena, Germany). Wilmad NMR tube, 5 mm, Ultra-Imperial Grade, 7 in.L, 526-PP was purchased from Sigma-Aldrich (Milan, Italy). One-dimensional and bidimensional NMR spectra were acquired with a Bruker FT-NMR Avance 400 spectrometer (Ettlingen, Germany).

Samples

One hundred and nine samples of both TBVM and BVM were supplied in all. Among them, 28 were extra old (>25 years of aging) TBVM, 40 were old (>12 and <25 years of aging) TBVM and 41 were BVM of unknown aging. All the TBVMs and several BVMs were provided by local vinegar houses, while the other BVMs were purchased on the market in 2012. All TBVMs and BVMs are labelled as PDO and PGI products respectively.

Phenolic Compounds

Total Phenolics Determination

Total phenolic content was determined using the Folin–Ciocalteu method described by Singleton and Rossi (1965), partially modified. First, 1 mL of deionized water and 500 μL of Folin-Ciocalteu reagent were added to 50 μL of 1:10 diluted vinegar. The mixture was allowed to stand for 5 min, and then, 2 mL of a 10 % aqueous Na_2CO_3 solution was added. The final volume was adjusted to 10 mL. Samples were allowed to stand for 90 min at room temperature before measurement at 700 nm versus the blank. The amount of total phenolics is expressed as micrograms (+)-catechin equivalents (CE)/milliliters of vinegar, through the calibration curve of (+)-catechin in the range of 0.5–7.5 ppm.

Total Flavonoids Determination

Total flavonoid content was determined using the method described by Dewanto et al. (2002) modified in our laboratory. To 100 μL of 1:10 diluted vinegar, 2 mL of deionized water, 150 μL of 5 % $NaNO_2$ solution, 300 μL of 10 % $AlCl_3$ solution, and 1 mL of NaOH 1M were added. The volume was adjusted to 5 mL, and the absorption was measured at 510 nm versus the blank. Total flavonoids are expressed as micrograms (+)-catechin equivalents (CE)/milliliters of

vinegar, through the calibration curve of (+)-catechin. The calibration curve linearity range was 0.5–10 ppm.

Total Condensed Tannins Determination

The determination of total condensed tannins was obtained using the method described by Broadhurst and Jones (1978), partially modified. Three milliliters of vanillin (4 % in MeOH, *w*/*v*) and 1.50 mL of HCl were added to 100 μL of 1:10 diluted vinegar. The final volume was adjusted to 5 mL with methanol, and the absorption was measured at 500 nm versus the blank. The amount of total condensed tannins was expressed as micrograms (+)-catechin equivalents (CE)/milliliter of vinegar, through the calibration curve of (+)-catechin comprised between 0.5–10 ppm.

Antioxidant Activity

Photochemiluminescence Assay

The luminol PCL assay was carried out with the procedure described by Popov and Lewin (1999). 2–3 mL of reagent 1 (solvent and dilution reagent), 200 μL of reagent 2 (buffer solution), 25 μL of reagent 3 (photosensitizer), and 10 μL of standard or sample (diluted vinegar) solution were mixed and measured. A light emission curve was stopped at 180 s using inhibition as the parameter to evaluate antioxidant effect. The antioxidant capacity was then determined by use of the integral under the curve and was expressed as micrometers of Trolox equivalents (TEs)/milliliters of vinegar. Trolox was used as standard to obtain a calibration curve (0.5–2 nM).

ABTS Assay

The ABTS (7 mM) was dissolved in distilled water. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting to the ratio of 1:1 ABTS stock solution with 4.9 mM potassium persulfate solution and leaving the mixture to stand in the dark at room temperature for 24 h before use (Re et al. 1999). The concentration of the resulting blue–green ABTS radical solution was adjusted to an absorbance of 0.700±0.020 at 734 nm. Fifty microliters of appropriately diluted vinegar was added to 1,450 μL of the resulting blue–green $ABTS^{\bullet+}$. The mixture, protected from the light, was measured at 734 nm after 30 min of incubation. The percent inhibition of $ABTS^{\bullet+}$ radical was calculated according to the equation: percent inhibition of $ABTS^{\bullet+}$ radical=[1−(Asample/Acontrol)]×100, where Asample was the sample absorbance after 30 min of incubation and Acontrol was the absorbance of $ABTS^{\bullet+}$ solution. The antioxidant activity was expressed as micrometers of Trolox equivalents (TEs)/milliliters of vinegar. The calibration curve considered was comprised between 10–400 μM.

DPPH Assay

The DPPH assay was evaluated according to Aadil et al. (2013). Fifty microliters of appropriately diluted vinegar was added to 1,450 μL of 0.06 mM of methanolic DPPH radical solution. The reaction mixture was left to stand at room temperature in the dark for 15 min. The absorbance for the sample (Asample) was measured at 515 nm against methanol blank. Acontrol was the absorbance of DPPH solution. The percent inhibition of DPPH radical was calculated according to the equation: percent inhibition of DPPH radical=[1−(Asample/Acontrol)]×100. Methanolic solutions with different Trolox concentrations (0.05−1 mM/L) were analyzed. The free radical scavenging capacity was expressed as micrometers of Trolox equivalents (TEs)/milliliters of vinegar using calibration curve.

NMR Spectroscopy

Sample Preparation

To prepare NMR samples, 0.1 g of each vinegar was exactly weighed directly into the NMR tube and dissolved with 500 μL of dimethyl sulphoxide-d_6 (DMSO-d_6). A volume of 20 μL of tetramethylsilane (TMS) was added as reference compound. Also standard samples, prepared by dissolving 15 mg of each considered compounds in 0.5 mL of DMSO-d_6 and by adding 20 μL of TMS as reference compound, were analyzed.

NMR Experiments

To obtain comparable spectra, the balsamic vinegar and standard samples were analyzed using the same acquisition parameters. ^{1}H NMR spectra were acquired with a Bruker FT-NMR Avance 400 spectrometer operating at 400.13 MHz for ^{1}H. All of the experiments were performed at 300 K and nonspinning. ^{1}H NMR data were acquired using the Bruker spin-echo sequence "cpmg1d" (Carr−Purcell−Meiboom−Gill, Bruker Library), this sequence allows to suppress all broad signals, including the water signal, which may be removed without direct suppression and to enhance narrow resonances. The following acquisition parameters were applied: tdn (number of data points), 32 K; dummy scans, 4; acquisition time, 3.4210 s; delay time, 3.0 s; number of scans, 64; spectral width, 4,789.27 Hz; fidres, 0.1461. Total acquisition time was 7 min and 46 s. The assignments of the metabolites have been confirmed on the basis of the ^{13}C NMR, ^{1}H−^{13}C heteronuclear multiple-bond correlation (HMBC), and ^{1}H−^{13}C heteronuclear single-quantum coherence (HSQC) analyses. The acquisition parameters of the ^{13}C NMR experiments were as follows: number of scans, 8 K; dummy scans, 4; tdn (number of data points), 32 K; spectral

width, 22,075.055 Hz; acquisition time, 0.7422 s; delay time, 1.5 s; fidres, 0.6737 Hz. Total acquisition time was 5 h, 14 min, and 59 s. The acquisition parameters of the HMBC experiments were as follows: number of scans, 32; dummy scans, 16; td, 3 K in the acquisition or direct HMBC dimension F2 (^{1}H) and 100 in indirect HMBC dimension F1 (^{13}C); spectral width, 5592.841 Hz in F2 (^{1}H) and 20,124.465 Hz in F1 (^{13}C); digital resolution, 1.8206 Hz in F2 (^{1}H) and 201.245 Hz in F1 (^{13}C); acquisition time, 0.2747 s; delay time, 0.5 s; HMBC delay time, 62.5 min. The acquisition parameters of the HSQC experiments were as follows: number of scans, 4; dummy scans, 12; tdn, 1 K in the acquisition or direct HSQC dimension F2 (^{1}H) and 256 in indirect HSQC dimension F1 (^{13}C); spectral width, 5,995.204 in F2 (^{1}H) and 19,118.721 F1 (^{13}C); digital resolution, 5.855 Hz in F2 (^{1}H) and 74.682 Hz in F1 (^{13}C); acquisition time, 0.0854 s; delay time, 1.5 s. Total acquisition time was 27 min and 50 s. The chemical shifts were reported as δ_H (ppm) relative to TMS.

Spectral Calculation

The application of the ^{1}H NMR technique to balsamic vinegar samples generates very complicated spectra that need to be previously processed and subsequently analyzed by chemometric methods. All ^{1}H NMR spectra were phased and calibrated using the TMS signal by the XWinNMR software package (Bruker BioSpin GmbH Rheinstetten). To reduce the inhomogeneous proton NMR chemical shift, primarily concerning pH-dependent signals, all spectra were aligned using the toolbox Icoshift 1.0 for MATLAB (Mathworks Inc., Natick, MA, USA) (Savorani et al. 2010). Finally, the spectra were baseline corrected by PLS_Toolbox version 5.2.2 for use with MATLAB (eigenvector Research Inc., Wenatchee, WA, USA). The ^{1}H NMR spectra were integrated using the software Amix 3.7.10 (Bruker BioSpin GMBH, Rheinstetten, Germany). The integration was performed by considering the principal metabolites present in the spectra obtained from all the samples analyzed. Nineteen signals were integrated in all and were normalized respect to the integral of the TMS, used for the spectral calibration and as reference compound.

Statistical Analysis

All the results were collected in three data sets and normalized before the analyses. The first one refers to the composition and functional activities and consists of six variables and 109 samples. The second one contains the principal integrated NMR peaks and consists of 19 variables and 109 samples. The last one, derived from the previous two data sets, includes the data of the chemical characterization, contains the compositional data (total phenolics, flavonoids, and tannins) and the integrated NMR peaks and consists of 22 variables and 109 samples. These data sets were statistical analyzed by analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) to assess the statistical significance of measured differences. To evaluate the most important variables which discriminate between the vinegar samples, post hoc test was performed using Tukey 'Honest Significant Difference Test' (HSD). For all these monovariate tests, the *P* level was set at 0.05.

To achieve a reliable classification of the vinegar samples, unsupervised and supervised pattern recognition procedures were applied. Principal component analysis (PCA) was performed to verify the intrinsic variation in the data sets and identify possible outliers. General discriminant analysis (GDA) (McLachlan 1992) was also used in this work to determine whether a given classification of cases into a number of groups is an appropriate one. After the construction of the models, to evaluate the classification performance, the leave-one out method was used as a validation procedure (Henrion and Henrion 1994). All statistical calculations were performed using the PLS_Toolbox version 5.2.2 for MATLAB, Statistica 6.1 (StatSoft® Italia, Vigonza, Italy) and SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Phenol Content and Antioxidant Activity

Table 1 shows the phenolic contents and the antioxidant activities of the vinegar samples. From these results, the high variability between the observed measurements is evident and demonstrated by the SD values. The CV% indeed, in some cases, is well above the 50 % for both TBVM and BVM samples, but these are not surprising result. TBVMs, in fact, are unique and unmistakable typical Italian vinegars produced by local vinegar houses, often founded by small family-run business, according to the product specification, but not produced according to standardized industrial processes. The observed variability confirms this peculiarity and explains the difficulty of classifying these products. As regards BVMs, it is important to clarify that the samples analyzed come from different suppliers. Some of these were purchased on the market; therefore, they must be considered large-scale products, other ones were provided by local vinegar houses. Besides, the age of these samples is unknown, and the set rules state that the maturation process is performed for at least 60 days; however, it is allowed to extend the aging period, thus reaching higher quality parameters. Unlike TBVMs, for which there is a clear distinction and classification of the vinegar depending on the aging period defined by the set rules, for BVMs it is allowed only the word "aged," if aging has been extended for at least 3 years in wooden barrels.

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Q5 t1.1 **Table 1** Phenol content and antioxidant activity of the vinegar samples[a]

t1.2 t1.3 Sample	Total phenolics (μg CE/mL)	Total flavonoids (μg CE/mL)	Total tannins (μg CE/mL)	PCL (μM TEs/mL)	ABTS (μM TEs/mL)	DPPH (μM TEs/mL)
t1.4 TBVM extra old	11,468±2,542	2,609±951	1,989±768	41.26±9.1	33.04±21.8	19.61±14.0
t1.5 TBVM old	7,646±2,554	1,435±734	1,205±534	27.12±11.1	33.52±19.3	13.59±6.6
t1.6 BVM	4,688±2910	1,528±838	899±481	17.16±11.9	16.88±13.6	12.83±9.9
t1.7 Overall mean	7,515±3,768	1,771±963	1,291±724	27.01±14.4	27.14±19.6	14.85±10.4
t1.8 CV%						
t1.9 ABTM extra old (%)	22.2	38.6	36.5	22.0	66.0	71.5
t1.10 ABTM old (%)	33.4	44.3	51.2	40.8	57.5	48.4
t1.11 ABM (%)	62.1	53.5	54.8	69.4	80.4	77.2
t1.12 *P* (ANOVA)[b]	*<0.05*	*<0.05*	*<0.05*	*<0.05*	*<0.05*	*<0.05*
t1.13 *P* (MANOVA)[b]	*<0.05*					
t1.14 HSD test[c]						
t1.15 ABTM extra old	A	A	A	A	A	A
t1.16 ABTM old	B	B	B	B	A	AB
t1.17 ABM	C	B	B	C	B	B

[a] Results are reported as mean ± SD

[b] Statistically significant values are reported in italic

[c] Same letter in the same column indicates no significant differences ($P<0.05$)

However, BVMs differently refined can be found on the market, and only in few cases the aging process is directly indicated on the bottle labels. The high phenolic content of TBVM probably is due to the extraction of some phenolics

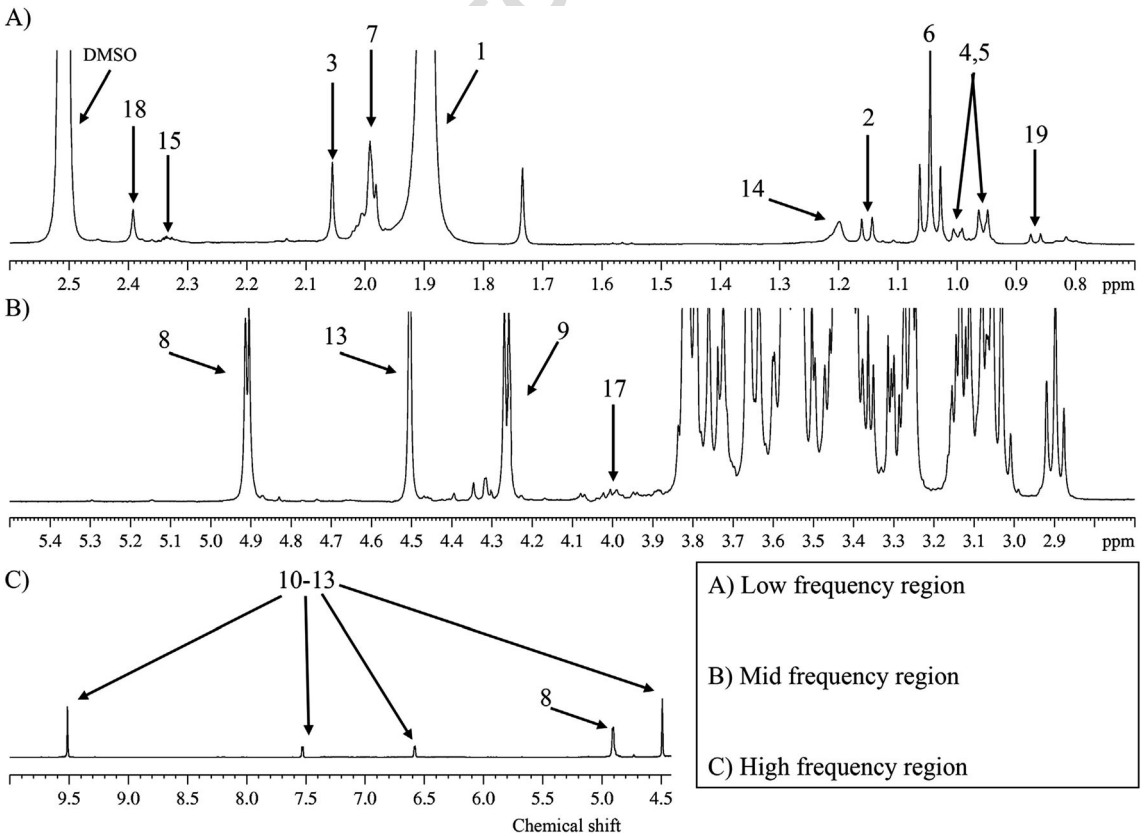

Fig. 1 Typical expanded ^{1}H NMR spectra recorded at 400 MHz of a TBVM sample with highlighted integrated signals (see Table 2 for chemical shift references)

from the wood during aging and to the evaporation that concentrates the product. BVM is a mixture of cooked and concentrated grape must and wine vinegar, and this may be the main reason of the lower phenolic content.

Despite the observed high intragroup variability, the ANOVA and the post hoc tests (Table 1) show statistically significant differences between TBVM and BVM for all the analyses. The TBVMs are richer in phenolics, flavonoids, and tannins and show higher antioxidant capacity than BVMs. Considering these results, the regression and correlation analyses of the antioxidant activities against total phenolics, total flavonoids, and total tannins were performed. As regards the antioxidant capacity determined by the PCL assay, the correlation coefficients, r^2, were 0.668 ($P<0.05$) for total phenolics, 0.416 ($P<0.05$) for total flavonoids, and 0.412 ($P<0.05$) for total tannins. For the $ABTS^{\bullet+}$ radical scavenging activity, r^2 were 0.239 ($P<0.05$) for total phenolics, 0.124 ($P<0.05$) for total flavonoids, and 0.010 ($P=0.287$) for total tannins. For the $DPPH^{\bullet}$ radical scavenging activity, r^2 were 0.378 ($P<0.05$) for total phenolics, 0.524 ($P<0.05$) for total flavonoids, and 0.017 ($P=0.174$) for total tannins. These results demonstrated that very poor correlations were observed between the phenolic compound contents and the antioxidant activities; nevertheless, several of them are statistical significant ($P<0.05$). The antioxidant activity is often attributed to phenolic compounds; however, the absence of strong correlation has been reported also by other authors (Velioglu et al. 1998; Kahkonen et al. 1999; Maisuthisakul et al. 2007). The interpretation of the results obtained by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activity assays is often complicated. As reported in the literature, these two radicals may be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (Jimenez et al. 2004). Reactivity patterns and mechanisms are difficult to interpret without detailed information about the composition and structures of antioxidants being tested. Steric accessibility is another factor that may interfere with the interpretation of the results. Small molecules that have better access to the radical site have higher apparent antioxidant activity. Many antioxidants that react quickly with peroxyl radicals, and therefore have a high antioxidant capacity, may react slowly or may even be inert to $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals due to steric inaccessibility (Prior et al. 2005). Probably, in our case, the major determinant of the poor correlations between antioxidant activity and phenolics is the high molecular weight melanoidin content. These colored and uncolored polymeric molecules are the final products of the Maillard reactions (Martins et al. 2001). The presence of these compounds in TBVMs is reported by several authors (Falcone and Giudici 2008), and it is also reported that they may contribute until to 40–50 % of the total antioxidant activity of aged TBVM (Tagliazucchi et al. 2010). The concentration, the chemical characteristics, and therefore the activity of this kind of compounds are strictly related with the production and aging processes. This probably also explains the high variability of antioxidant activity results obtained from similar but not identical samples, which may contribute to the poor correlation data. Nevertheless, the quantification of these compounds was not the purpose of this work.

NMR Spectroscopy

A representative ^1H NMR spectrum recorded at 400 MHz of a TBVM sample, with expansions of aliphatic/alcoholic, and sugar regions, is shown in Fig. 1. The NMR assignments of the principal metabolites are reported in Table 2. The metabolites were assigned on the basis of additional NMR experiments, by recording NMR spectra of pure compounds, and confirmed by comparing our results with literature data (Cirlini et al. 2009; Consonni et al. 2008a, b; Consonni and Cagliani 2007). Table 3 shows the signal integration results for some selected and well-resolved peaks of the principal metabolites identified. As reported for the phenolic compound contents and the antioxidant activities, also from these results, the high variability between the integrated values is evident and demonstrated by the SD values. In general, the same considerations above reported about the characteristics and peculiarities of TBVMs and BVMs can be made. As evident,

Table 2 NMR integrated signals[a]

	Compound	Group	δ (ppm)
1	Acetic acid	$C2H_3$	1.90
2	Acetoin	$C4H_3$	1.13
3		$C1H_3$	2.08
4	2,3-Butanediol	$C1H_3$	0.93
5		$C4H_3$	0.98
6	Ethanol	$C2H_3$	1.04
7	6-Acetyl glucose signals[b]	CH_3CO	1.93–2.01
8	α−Glucopyranose	αC1H	4.89
9	β-Glucopyranose	βC1H	4.26
10	5-HMF	C1H	9.56
11		C3H	7.52
12		C4H	6.63
13		$C6H_2$	4.47
14	Lactic acid	$C3H_3$	1.19
15	Malic acid	C2H	2.38
16		C2′H	2.59
17		C3H	4.07
18	Succinic acid	$C2H_2$ $C3H_2$	2.40
19	Valine	CγH3	0.87

[a] Assignments were from HSQC and HMBC experiments. The chemical shifts were expressed as relative values to those of TMS at 0 ppm

[b] Esters of glucose (6-acetylglucose) in the two anomeric forms. See Cirlini et al. for more details

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t3.1 **Table 3** Signal integration results for the principal metabolites identified[a]

t3.2		TBVM extra old[b]	TBVM old[c]	BVM	Overall mean	*P* (ANOVA)[b]	HSD test[c]			t3.3
							TBVM extra old	TBVM old	BVM	
t3.4	1: Valine	0.248±0.11	0.213±0.11	0.186±0.07	0.212±0.10	*<0.05*	A	AB	B	
t3.5	2: 2,3-Butanediol ($C1H_3$)	7.080±4.06	5.224±5.02	1.452±0.64	4.282±4.33	*<0.05*	A	A	B	
t3.6	3: 2,3-Butanediol ($C4H_3$)	5.212±3.62	3.947±4.55	0.811±0.47	3.092±3.77	*<0.05*	A	A	B	
t3.7	4: EtOH	2.348±1.58	4.111±4.64	7.278±8.28	4.849±6.15	*<0.05*	A	A	B	
t3.8	5: Acetoin ($C4H_3$)	1.061±0.59	1.327±0.76	1.299±0.65	1.248±0.68	0.237	A	A	A	
t3.9	6: Lactic acid	2.921±1.79	2.147±1.22	0.926±0.41	1.887±1.43	*<0.05*	A	B	C	
t3.10	7: Acetic acid	187.3±27.9	191.2±17.7	195.4±9.71	191.8±18.8	0.209	A	A	A	
t3.11	8: 6-Acetyl glucose	48.12±9.97	30.98±13.2	10.95±7.92	27.85±18.2	*<0.05*	A	B	C	
t3.12	9: Acetoin ($C1H_3$)	1.304±0.35	1.376±0.48	1.204±0.61	1.293±0.51	0.316	A	A	A	
t3.13	10: Malic acid (C2H)	0.491±0.20	0.422±0.17	0.288±0.08	0.389±0.17	*<0.05*	A	A	B	
t3.14	11: Succinic acid	1.874±0.72	1.760±1.02	0.746±0.18	1.408±0.89	*<0.05*	A	A	B	
t3.15	12: Malic acid (C2′H)	0.357±0.26	0.335±0.18	0.238±0.06	0.304±0.18	*<0.05*	A	A	B	
t3.16	13: β-Glucopyranose (βC1H)	38.90±15.9	32.50±17.8	12.61±5.71	26.66±17.8	*<0.05*	A	A	B	
t3.17	14: Malic acid (C3H)	11.72±4.62	7.831±5.89	2.114±1.08	6.680±5.77	*<0.05*	A	B	C	
t3.18	15: 5-HMF (C6H2)	14.64±6.45	7.443±5.07	2.701±4.25	7.508±6.95	*<0.05*	A	B	C	
t3.19	16: α-Glucopyranose (αC1H)	27.49±12.3	23.35±12.5	9.859±4.39	19.34±12.6	*<0.05*	A	A	B	
t3.20	17: 5-HMF (C4H)	4.859±2.19	2.481±1.73	0.898±1.51	2.496±2.35	*<0.05*	A	B	C	
t3.21	18: 5-HMF (C3H)	4.771±2.33	2.467±1.77	1.003±1.64	2.508±2.38	*<0.05*	A	B	C	
t3.22	19: 5-HMF (C1H)	9.305±4.25	4.600±3.25	1.492±2.68	4.640±4.52	*<0.05*	A	B	C	

[a] For each sample results are reported as mean ± SD

[b] Statistically significant values are reported in italic

[c] Same letter in the same row indicates no significant differences ($P<0.05$)

the TBVM samples show higher integration results than BVMs for all the signals, except for ethanol, which is more concentrated in BVMs, and acetoin, for which the differences are not statistically significant. These compounds are described in the literature as the most significant ones for discriminating the balsamic vinegars and for monitoring the aging process. (Caligiani et al. 2007; Masino et al. 2005; Theobald et al. 1998; Cirlini et al. 2009; Consonni et al. 2008).

The PCA was performed to verify the intrinsic variation in the datasets and to identify possible outliers. As regards the composition and functional activities (original dataset consists of six variables and 109 samples), the PCA model resulted in four PCs explaining 97.82 % of the total variance, and it was not able to discriminate the samples. Two TBVMs extra old and one BVM samples were omitted from the further analyses because resulting as strong outliers. The resulting dataset therefore consists of six variables and 106 samples. As regards the integrated NMR peaks (original dataset consists of 19 variables and 109 samples), the resulted five PCs model explained 90.65 % of the total variance, and also in this case, it was not able to discriminate the samples. One TBVM extra old, one TBVM old and one BVM sample, were omitted from the further analyses because resulting as strong outliers. The resulting dataset therefore consists of 19 variables and 106 samples. PCA was performed also on the third data set which includes all the compositional data (original dataset consists of 22 variables and 109 samples). The resulting six PCs model explained 91.27 % of the total variance, and also in this case, it was not able to discriminate the samples. Two TBVMs extra old and one TBVM old samples were omitted from the further analyses because resulting as strong outliers. The resulting dataset therefore consists of 22 variables and 106 samples.

These results demonstrated the considerable complexity of the system. In order to give a clearer interpretation and to verify whether the application of a supervised multivariate statistical analysis was able to classify the samples, GDA was applied on each dataset. The GDA model obtained from the composition and functional activities data set was able to partially group the samples. The first canonical discriminant function (DF) explains 86.5 % of the total variance, and the results of the leave-one out cross-validation show a predictive capacity of 81.1 %. The first two DFs are particularly correlated with phenolics and flavonoids. By analyzing the integrated NMR peak dataset, a GDA model able to group the samples according to the type of vinegar was obtained. The first DF explains 93.6 % of the total variance, and the results of the leave-one out cross-validation show a predictive

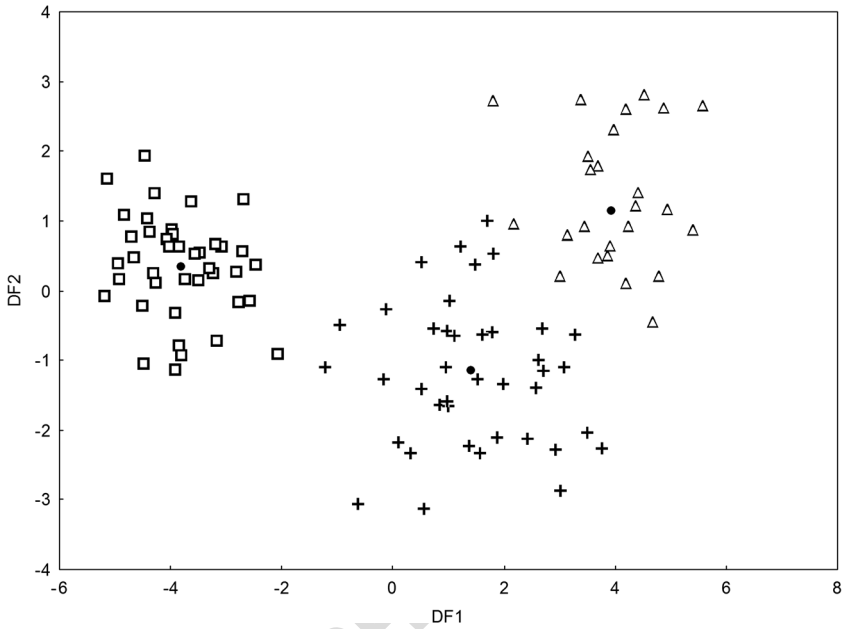

Fig. 2 Vinegar classification using GDA on the third dataset, which includes the compositional data and the integrated NMR peaks; the separation of samples in three clusters is shown: TBVM extra old (*white triangle*), TBVM old (*plus symbol*), BVM (*white square*), and group centroids (*black-filled circle*).

capacity of 87.7 %. The first two DFs are particularly correlated with the integrated signals of 6-acetyl glucose, malic acid (C2H and C2′H), succinic acid, α-glucopyranose (αC1H), β-glucopyranose (βC1H), and 5-HMF (all the signals). The GDA was performed also on the third data set, which includes all the compositional and NMR data. This model was able to group the samples according to the type of vinegar (Fig. 2). The first canonical discriminant function (DF) explains 92.2 % of the total variance, and the results of the leave-one out cross-validation show a predictive capacity of 89.6 %. The first two DFs are particularly correlated with total phenolics, total flavonoids, and the integrated signals of malic acid (C2H and C2′H), succinic acid, α-glucopyranose (αC1H), β-glucopyranose (βC1H), and 5-HMF (all the signals). Although the clusters are partially overlapped, the score plot (Fig. 2) showed that the information contained in the dataset was suitable to classify and distinguish the BVMs from the TBVMs. The model is able to differentiate the extra old and the old samples among the TBVMs, except for few samples. In this regard, it is important to remark that the TBVMs are not produced according to standardized industrial processes. As already mentioned, the observed variability confirms this peculiarity and explains the difficulty of classifying these products.

The proposed methods to assay the phenolic content and the antioxidant activity and NMR analysis, used to obtain information about the principal substances in the samples, represented here a powerful combination in chemical and functional characterization of TBVMs (extra old and old) and BVMs. The wide variability observed in the data is a direct consequence of the different making procedures employed to obtain BVMs and TBVMs and represents the peculiarity that make these typical Italian vinegars unique and unmistakable. Although the difficulty of classifying these products, here a good GDA model is reported, able to discriminate the BVMs from the TBVMs and to differentiate the extra old and the old samples among the TBVMs. The most statistically significant variables were found to be total phenolics, total flavonoids and the integrated signals of malic acid (C2H and C2′H), succinic acid, α-glucopyranose (αC1H), β-glucopyranose (βC1H), and 5-HMF (all the signals). On the basis of these findings and the additional knowledge on typical Italian vinegars reported in this work, new information usable to reinforce and safeguard the wealth of Modenese traditions, represented by these unique products, was here provided. Furthermore, this work also highlights the high content and good antioxidant capacity of these products. These characteristics together with their pleasant aroma and intense taste make TBVM and BVM excellent taste enhancers in low-sodium salt diets very attractive for the health protection.

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Conflict of Interest Davide Bertelli declares that he has no conflict of interest. Annalisa Maietti declares that she has no conflict of interest. Giulia Papotti declares that she has no conflict of interest. Paola Tedeschi declares that she has no conflict of interest. Gianpiero Bonetti declares that he has no conflict of interest. Riccardo Graziosi declares that he has no conflict of interest. Vincenzo Brandolini declares that he has no conflict of interest. Maria Plessi declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

References

Aadil RM, Zeng XA, Han Z, Sun DW (2013) Effects of ultrasound treatments on quality of grapefruit juice. Food Chem 141:3201–3206

Broadhurst WT, Jones RB (1978) Analysis of condensed tannins using acidified vanillin. J Sci Food Agric 29:788–794

Cirlini M, Caligiani A, Palla G (2009) Formation of glucose and fructose acetates during maturation and ageing of balsamic vinegars. Food Chem 112:51–56

Consonni R, Cagliani LR (2007) NMR relaxation data for quality characterization of Balsamic vinegar of Modena. Talanta 73:332–339

Consonni R, Cagliani LR, Benevelli F, Spraul M, Humpfer E, Stocchero M (2008a) NMR and Chemometric methods: a powerful combination for characterization of Balsamic and Traditional Balsamic Vinegar of Modena. Anal Chim Acta 611:31–40

Consonni R, Cagliani LR, Rinaldini S, Incerti A (2008b) Analytical method for authentication of Traditional Balsamic Vinegar of Modena. Talanta 75:765–769

Dewanto V, Wu X, Adom KK (2002) Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 50:3010–3014

Falcone PM, Giudici P (2008) Molecular size and molecular size distribution affecting traditional balsamic vinegar aging. J Agric Food Chem 56:7057–7066

Greco E, Cervellati R, Litterio ML (2013) Antioxidant capacity and total reducing power of balsamic and traditional balsamic vinegar from Modena and Reggio Emilia by conventional chemical assays. Int J Food Sci Technol 48:114–120

Henrion R, Henrion G (1994) Uberwachte klassifikation. In: MultiVariate Datenanaly. Springer-Verlag, Berlin (Germany), pp 71–73

Jimenez A, Selga A, Torres JL, Julia L (2004) Reducing activity of polyphenols with stable radicals of the TTM series. Electron transfer versus H-abstraction reactions in flavan-3-ols. Org Lett 6:4583–4586

Kahkonen MP, Hoppia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, Heinonen M (1999) Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 47:3954–3962

Maisuthisakul P, Suttajit M, Pongsawatmanit R (2007) Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. Food Chem 100:1409–1418

Martins SIFS, Jongen WMF, Van Boekel MAJS (2001) A review of Maillard reaction in food and implications to kinetic modeling. Trends Food Sci Technol 11:364–373

McLachlan GJ (1992) Discriminant analysis and statistical pattern recognition. Wiley, New York

Popov I, Lewin G (1996) Photochemiluminescent detection of antiradical activity; IV: testing of lipid-soluble antioxidants. J Biochem Biophys Methods 31:1–8

Popov I, Lewin G (1999) Antioxidative homeostasis: characterization by means of chemiluminescent technique. Methods Enzymol 300:96–100

Prior RL, Wu X, Schaich K (2005) Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290–4302

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans CA (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237

Savorani F, Tomasi G, Engelsen SB (2010) Icoshift: a versatile tool for the rapid alignment of 1D NMR spectra. J Magn Reson 202:190–202

Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16:144–158

Tagliazucchi D, Verzelloni E, Conte A (2010) Contribution of melanoidins to the antioxidant activity of traditional balsamic vinegar during aging. J Food Biochem 34:1061–1078

Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 46:4113–4117

Verzelloni E, Tagliazucchi D, Conte A (2007) Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar. Food Chem 105:564–571

Verzelloni E, Tagliazucchi D, Conte A (2010) Changes in major antioxidant compounds during aging of traditional balsamic vinegar. J Food Biochem 34:152–171

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