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Molecular Size and Molecular Size Distribution Affecting Traditional Balsamic Vinegar Aging

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A first attempt at a semiquantitative study of molecular weight (MW) and molecular weight distribution (MWD) in cooked grape must and traditional balsamic vinegar (TBV) with increasing well-defined age was performed by high-performance liquid size exclusion chromatography (SEC) using dual detection, that is, differential refractive index (DRI) and absorbance (UV−vis) based detectors. With this aim, MW and MWD, including number- and weight-average MW and polydispersity, were determined with respect to a secondary standard and then analyzed. All investigated vinegar samples were recognized as compositionally and structurally heterogeneous blends of copolymers (melanoidins) spreading over a wide range of molecular sizes: the relative MW ranged from 2 to >2000 kDa. The extent of the polymerization reactions was in agreement with the TBV browning kinetics. MWD parameters varied asymptotically toward either upper or lower limits during aging, reflecting a nonequilibrium status of the balance between polymerization and depolymerization reactions in TBV. MWD parameters were proposed as potential aging markers of TBV.

KEYWORDS: Traditional balsamic vinegar; melanoidins; size exclusion chromatography; molecular weight distribution; browning; aging marker

INTRODUCTION

Traditional balsamic vinegar (TBV) from both Modena and Reggio Emilia was historically prepared starting from a cooked grape must and following a quasi-dynamic technology (*1*), which consisted of transferring vinegar from aone barrel to another in a set of five to seven barrels of decreasing capacity and different woods for at least 12 years to produce the precious product deserving of the "Protected Denomination of Origin" (PDO) label by the European Union (*2*). To date, however, scientific knowledge on how the composition and process may affect the age-related properties of TBV is lacking or of poor quality, but, on the other hand, the Disciplinary of Production has established neither standard procedures nor standard samples for evaluating objectively TBV quality and age, although the commercial value increases greatly with the claimed age and special prices are requested for very old vinegars (25 years old or older). Some authors have proved that the rheological properties of a TBV, and therefore any end properties related to the vinegar viscosity, cannot be explained on the basis of only the composition of the main constituents such as sugars and organic acids (*20*): a deviation from the Newtonian behavior was proved to be determined in a synergic way by the presence of minor constituents affecting viscosity (*21*). Numerous attempts were made to individuate chemical markers of the vinegar age, but all of these studies failed due to the lack of guarantee about the age of the investigated vinegar samples (*3*). On the other hand, it is very difficult both to define and to assess the age of a TBV, because each barrel contains a mix of products of different ages. Recently, Giudici and Rinaldi (*3*), on the basis of a theoretical study of the quasi-dynamic process followed to produce TBV, proposed a mathematical model to calculate the age of TBV only accounting for the volume of vinegar in the set of barrels and for the volume of the vinegar withdrawn and/or transferred from barrel to barrel. The proposed approach is gaining interest in vinegar science due to the fact that it may offer the possibility to individuate in a reliable way an aging marker for TBV. Any chemical and/or physical property varying continuously throughout the entire process of vinegar production, from cooked must to end product, could be proved to be a reliable aging marker and, therefore, it might be able to reflect objectively the time evolution of TBV peculiarities.

From a biotechnological perspective, the TBV production involves a complex combination of microbial and chemical−physical reactions able to transform a cooked grape must to a dark, thick, and tasty sauce. The cooking of grape must was proved to be responsible for the nonenzymatic browning reactions giving to the musts the typical brown color and caramel-like flavor (*17–19*). The quasi-dynamic process followed in the TBV production imposes a nonequilibrium status for any chemical and physical properties that may extend over the entire period of aging; consequently, a great extent of nonenzymatic browning reactions is expected during long-term storage (aging). It is well accepted that sugar-containing foods under thermal treatments undergo a complex series of chemical reactions, that is, caramellization, sugar degradation, and Maillard reactions as a

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Table 1

PEG/PEO standard mixture	MWD parameters[a] M_p (Da)	M_w (Da)	M_n (Da)	PDI	mass (mg)	concentration (g/L)
m1	1010000	932000	843000	1.1	1.125	7.5−1.5−3
	118999	118000	98200	1.2	2.25	1.5−3−6
	6690	6550	6170	1.06	2.25	1.5−3−6
	628	636	547	1.16	2.25	1.5−3−6
m2	478000	496000	339000	1.46	2.25	1.5−3−6
	44700	42700	34000	1.25	2.25	1.5−3−6
	1960	2010	1840	1.09	2.25	1.5−3−6
m3	263000	271000	208000	1.3	2.25	1.5−3−6
	18600	17900	14900	1.2	2.25	1.5−3−6
	232	232	232	1	2.25	1.5−3−6

[a] M_p indicates the molecular weight at the maximum absorption of a peak; M_p values were used for the calibration procedure; PDI was calculated as the ratio between M_w and M_n, indicating the average polydispersity of a population.

Table 2

MWD parameter	formula[a]
number-average MW	$M_n = \Sigma(N_iM_i)/\Sigma(N_i)$
1st-order weighted-average MW	$M_w = \Sigma(W_iM_i)/\Sigma(W_i)$
2nd-order weighted-average MW	$M_z = \Sigma(W_iM_i^2)/\Sigma(W_iM_i)$
3rd-order weighted-average MW	$M_{z+1} = \Sigma(W_iM_i^3)/\Sigma(W_iM_i^2)$
viscosity-average MW	$M_v = [\Sigma(W_iM_i^a)/\Sigma(H_i)]^{1/a}$
polydispersity index	PDI $= M_w/M_n$

[a] N_i is the number of molecules of molecular weight M_i; M_n is the average molecular weight per molecule; W_i is the weight (or concentration) of molecules of molecular weight M_i; a is the tabulated exponent value appearing in the Mark−Houwink equation fitted to PEG/PEO size exclusion data obtained in the same operative conditions used for TBV samples. In the Mark−Houwink equation, i.e., intrinsic viscosity $= KM^a$, the exponent a is related to the polymer chain conformation.

function of their composition, temperature, pH, water activity, and other factors. Due to the high reactivity of the intermediates, complex dehydration, condensation, and radical reactions can take place, resulting in a wide variety of polymeric products, so-called "melanoidins", whose functionalities strictly depend on the structure and size of macromolecules that, in turn, depend on both the source of reactants and reaction conditions (*4*). Generally, melanoidins are expected to have significant effects on the end quality and in consumer acceptance of widely consumed dietary goods (e.g., coffee, cocoa, bread, malt, and honey) thanks to their antioxidant properties (*4–8, 26*), antimicrobial activity (*9*), antihypertensive properties (*10, 11*), prebiotic activities (*11*), browning properties (*12, 13*), and foam stability (*14*). Sometimes, melanoidins are considered to be potentially undesirable compounds playing a strong role in the binding of nutritionally important metals (*15*) and flavor compounds (*16*). All of these functionalities are presumably derived from the fact that the melanoidin structures are sufficiently diverse to have complex functional behavior.

Decoupling the TBV melanoidins into discrete polymeric distributions should aid our understanding of how and what fraction of macromolecules affects a targeted end properties. Really, the nonequilibrium status of TBV, mainly due to the quasi-dynamic technology used to produce it (*1*), imposes fractioning polymeric compounds, and macromolecule features must be analyzed upon long-term storage. Unfortunately, the fractioning of naturally occurring melanoidins from food matrices is very difficult and, indeed, most of the chemical and biological studies about melanoidin functionalities have been done on synthetic melanoidins. Generally, melanoidins are grouped rather arbitrarily as high molecular weight (HMW) or low molecular weight (LMW) with the latter by a lower limit of 3500 Da, which is the nominal cutoff value in the dialysis systems used to obtain them. Unfortunately, to date, no scientific works have been focused on the development of melanoidins during an extended period of storage/aging and on their potential use as reliable aging markers for TBV.

The present work supports the idea that there is a need of new knowledge and achievements with a clear exploitation potential to improve vinegar quality accounting for consumer protection in terms of guarantees on the claimed TBV age. With this perspective, molecular size distribution analysis was performed as the first attempt to gain knowledge on the polymeric nature of TBV and the mechanism of polymer formation as well as their relationships with the age-related properties of vinegar. Many naturally occurring polymers, such as polysaccharides, cellulosics, natural rubber, and some proteins, have defined molecular size distributions, that is, macromolecules of differing molecular weights, each with a finite concentration. Generally, these macromolecules are considered to be polydisperse because they possess distributions in a variety of physical properties simultaneously such as the molar mass, branching, density, chemical composition, melt properties, specific heat capacity, and viscosity. Each of these distributions is governed by the polymerization mechanism, monomer reactivity ratio, monomer availability, processing conditions, and they may, in turn, affect a wide number of end-quality properties. Various distributions may also combine synergistically to affect product properties in a fashion that the individual distributions would not.

The goal of this research was double. First was to determine the molecular weight (MW) and molecular weight distribution (MWD) from cooked grape must to final product upon TBV aging by size exclusion high-performance liquid chromatography (SEC) with dual detection, that is, using simultaneously differential refractive index (DRI) and ultraviolet−visible (UV−vis) based detectors. Second was to perform the statistical MWD analysis with the understanding of the reliability of using the distribution parameters as new potential aging markers and as quality descriptors in a design perspective of the end properties of TBV. The proposed work is expected to have a definable impact on the advancement of vinegar science and technology and innovative vinegar chemistry.

MATERIALS AND METHODS

Sample Retrieval. Samples of cooked grape must and vinegar samples were withdrawn from a dedicated set of wood barrels consisting of seven wood casks of descending order of capacity. For each barrel two samples were withdrawn and analyzed: all data were the mean of these two replicates. A direct-flame heating stainless steel was used to concentrate to 50 °Brix a volume of grape must from "Trebbiano" grape cultivars harvested with glucose to fructose ratios approaching 1.10. The set of barrels was managed over a period of about 14 years following the accurate and systematic refilling procedure proposed by Giudici and Rinaldi (*3*) to reach a steady-state volumetric flow of product into the set of barrels.

Size Exclusion Chromatography. SEC separation was carried out using a series of two polymer-based columns, that is, TSK-gel GMPW$_{XL}$ (30 cm length, 7.8 mm i.d., 13 μm particle size, <100−1000 Å pore size) and TSK-gel G3000PW$_{XL}$ (30 cm length, 7.8 mm i.d., 6 μm particle size, 200 Å pore size) connected to a TSK-gel PWH guard column. An Intelligent UV detector (model UV-2070 Plus, Jasco Corp., Tokyo, Japan) and a refractive detector (model RI-2031Plus, Jasco Corp.) were used simultaneously in series as mass concentration sensitive devices by fixing two wavelengths to detect the light absorptions, that is, 280 and 420 nm. HPLC-grade water was used as eluent after it was filtered with 0.22 μm Millipore cellulose nitrate filters

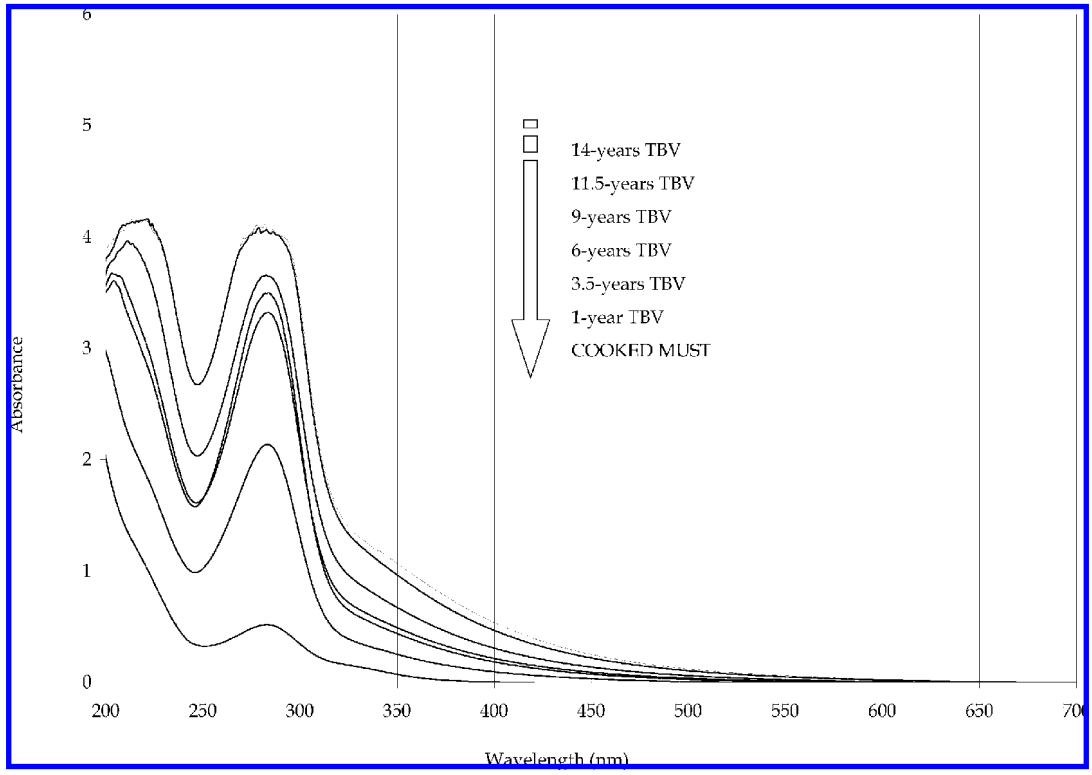

Figure 1. UV−vis spectra covering the ultraviolet and visible wavelengths. The shift factor was the TBV age. The color band indicates the relationship between the visible color band and absorption wavelength (nm).

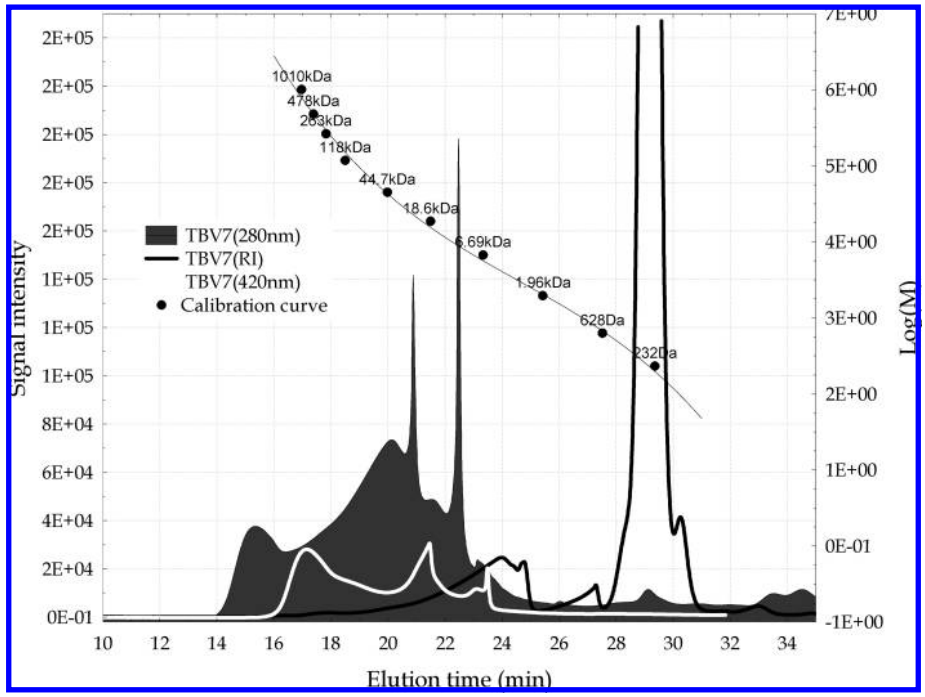

Figure 2. SEC elution pattern showing the molecular weight distribution of the 14-year TBV as detected by RI and UV−vis detectors (280 and 420 nm). The cubic curve indicates the theoretical relationship between the elution time and PEG/PEO standard molecular weights (solid circles) used for secondary calibration (the right axis can be used to examine the relative molecular weights of macromolecules).

and degassed before. The system of columns was placed in the column oven, and the optimum size exclusion conditions were experienced with an oven temperature, injection volume, column pressure, and isocratic flow rate of 30 °C, 20 μL, 40 kg/cm^2, and 0.7 mL/min, respectively. A Rheodyne volumetric injector was used to start the SEC separation. To avoid effects of polymer viscosity, the lowest detectable sample concentration was preferred. Consequently, the sample preparation for injection was obtained as follows: a weighted amount of TBV of about 1 g was diluted in 10 mL of HPLC-grade water, and then it was filtered with 0.22 μm Millipore cellulose nitrate filters and degassed before use.

The pH of TBV samples was determined before and after their aqueous dilution using a digital pH-meter (model 8417, Hanna Instrument, Padova, Italy; with probe for thick fluids). The observation of the fact that the pH did not change significantly (pH 2.75, likely due to the high content in organic acids having buffer capacity), allowed

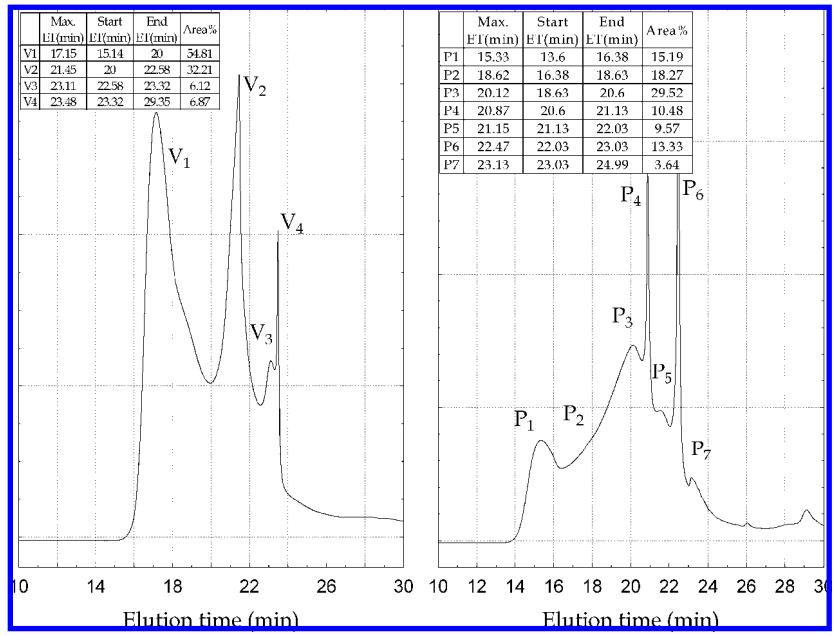

Figure 3. SEC elution patterns of the molecular weight distribution for the 14-year TBV as detected at both 420 nm (**a**) and 280 nm (**b**). Max ET indicates the elution times at the maximum absorption; Start RT and End RT indicate the peak integration limits. The relative molecular weight of brown melanoidins ranged on average between 5 and 1010 kDa, whereas that of the uncolored ones ranged from 2 to >5 kDa. Oligomeric melanoidins were detected by DRI, and their molecular weights ranged between 2 and 5 kDa.

us to believe that all ionic links into the polyanionic polymers, which melanoidins are, were not modified with respect to those occurring in the original vinegar samples.

Calibration Procedure. Because of the different concepts of the structure of melanoidins, consistent standards simulating melanoidins were not available for calibration stage. Consequently, a secondary calibration-based approach was carried out preferring standard mixtures of polyethyleneglycol/polyethyleneoxide (PEG/PEO) polymers (Sigma Aldrich Production GmbH) due to their wide range of molecular weights and narrow molecular weight distributions. In our work, size exclusion chromatography was used as a relative, not absolute, technique to calibrate the SEC system and determine MWD parameters. In this way, it was possible to examine only the trend of MW upon aging and compare any relative difference among TBV samples. The analytical characteristics of standard polymers are reported in **Table 1**.

Data Collection and Processing. SEC data were acquired and processed with an advanced chromatographic software (version 2.5.6.99; DataApex Ltd. 2007, Prague, Czech Republic). A value equal to twice the baseline noise was chosen as starting point for peak identification; a baseline-separated method was followed for peak integration. A polynomial cubic function ($R^2 = 0.999$) was fitted to the integrated data (area of peaks). Then, a calibration curve was constructed by plotting log(MW) versus elution volumes (V_i) and then used to convert the detector output (response versus elution time) into a differential MWD as

$$\frac{d(W_i)}{d(\log M_i)} = \frac{dV_{R,i}}{d(\log M_i)} \cdot \frac{H_i}{\sum H_i} \cdot \frac{1}{d} \qquad (1)$$

where $dV_R/d(\log M)$ is a reverse slope of the calibration curve used for the SEC system and the term $H_i/[(\Sigma H_i) \times d]$ equals $dW_i/dV_{R,i}$. The division interval (d) indicates the space between data points on SEC chromatograms in which MW is practically constant; H_i is the signal intensity at each division interval (d). To determine the weight fraction of a sample at a given molecular weight, a cumulative distribution was obtained from the integrated form of the differential MWD.

Molecular Weight Averages and Distribution. The shape, width, and magnitude of the relative MWD were characterized by estimating the classical statistical parameters used in material science as reported in **Table 2**. As far as the physical meanings of MWD parameters are concerned, notoriously the M_n parameter is known to be sensitive to the presence of LMW species and, therefore, to influence the properties that are affected by polymer chain ends such as colligative properties, refractive index, density, and specific heat capacity of a sample. The M_w parameter is sensitive to the presence of the HMW components and, therefore, influences properties that are affected by large chains such as melt and solution viscosity. M_z and M_{z+1} parameters are sensitive to the presence of very HMW species (at the peak tail) and, therefore, influence viscoelastic properties (time-dependent mechanical properties) of the sample. The M_v parameter is sensitive to the presence of MW with high intrinsic viscosity affecting the average viscosity of the sample. Finally, PDI is known to be sensitive to the broadness of size distribution in the sample and to the mechanism of polymer formation (generally, for single MW polymers, PDI = 1; for condensation polymers, PDI = 2; and for polymers from free radical polymerization, PDI > 2).

5-Hydroxymethyl-2-furfural (HMF), glucose, and fructose were purchased from Sigma Aldrich Co. (St. Louis, MO) and used as received to detect the elution time in the same working conditions applied for TBV samples.

To eliminate effects by initial differences due to the refilling procedure by cooked must, all MWD parameters were normalized on the basis of their corresponding value in the cooked must.

Sampling and SEC analysis were performed in triplicate. Significant differences between samples were analyzed with Statistica for Windows (StatSoft, Tulsa, OK). In this paper, all observed differences between samples represent significant differences with at least $p < 0.05$.

Browing Development. To account for differences in cooked grape must, a normalized browning index (BI) was defined as the ratio between the 420 nm absorptions of the TBV samples and those of cooked must.

RESULTS AND DISCUSSION

Due to the lack of knowledge about the naturally occurring molecular size distribution in TBV, SEC analysis was first carried out for a preliminary investigation (data not shown) of the overall range of molecular sizes using alone TSK-gel $GMPW_{XL}$, a mixed-bed polymer-based column able to separate soluble polymers over a wide range of molecular sizes spreading from 50 to 8000 kDa. Successively, TSK-gel $G3000PW_{XL}$ was used simultaneously in series with the TSK-gel $GMPW_{XL}$ column to improve the resolution. Before performing TBV

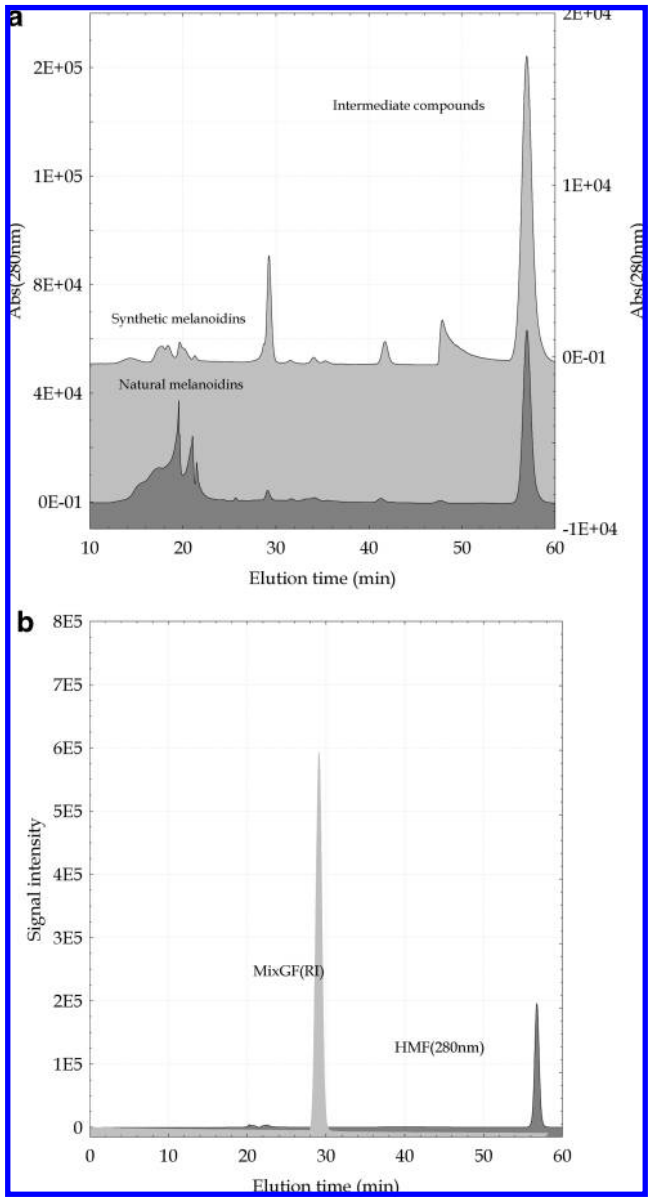

Figure 4. SEC elution profile of cooked must and synthetic melanoidins as detected at 280 nm (**a**) and of standard solution of 5-hydroxymethyl-2-furfural (HMF) and glucose/fructose mixture (**b**).

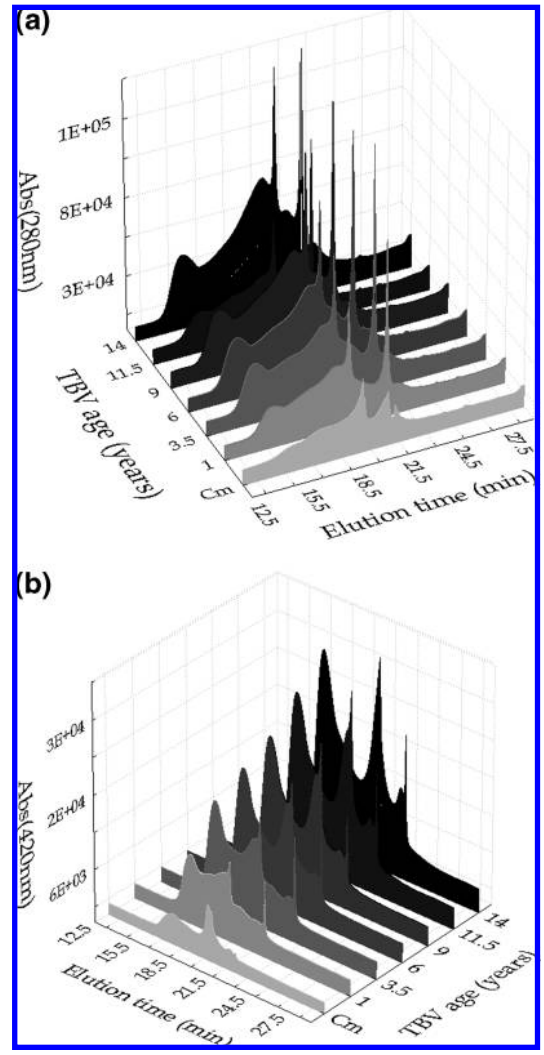

Figure 5. SEC elution profiles of TBV samples upon aging (**a**) as detected at 280 nm (peaks represent all melanoidins containing uncolored chromophores) and (**b**) as detected at 420 nm (peaks represent all brown melanoidins naturally occurring in cooked must and newly formed upon TBV aging).

Table 3. Time Course of Relative Amount of the Relative Concentration for Brown Melanoidins (420 nm) during TBV Aging

	relative percentage			
	V1	V2	V3	V4
cooked must	42.29	44.46	6.22	7.03
1-year TBV	30.54	55.56	6.26	7.64
3.5-year TBV	45.67	42.81	4.64	6.88
6-year TBV	51.49	37.39	5.02	6.10
9-year TBV	52.06	35.39	6.31	6.24
11.5-year TBV	52.52	33.85	6.95	6.69
14-year TBV	54.81	32.21	6.12	6.87

analysis, the performance of the TSK-gel SEC column system was judged by evaluating both the theoretical plates and asymmetry factor so that high-performance and reproducible results were guaranteed. Consequently, chromatograms were analyzed according to the principle of SEC theory for which the greater molecular hydrodynamic volume (effective size), the smaller the elution time (or volume) as well as compounds of the same size elute together.

UV−Vis Spectrum Properties of TBV. Figure 1 shows the whole UV−vis spectrum, from 200 to 700 nm, for each investigated sample. Spectra exhibited a well-defined absorption peak at 280 nm and featureless end absorptions as the wavelength decreases, according to what was found for other naturally occurring melanoidins (*13, 27*). Again, there was evidence for another absorption peak around the 218 nm wavelength at the 10th year of aging, whereas a bathochromic effect took place for this peak over the remaining aging period. This wavelength shift was supposedly due to a possible increase of the extent of chromophore conjugation in melanoidins resulting in a copigmentation effect. A similar dependence of the spectrum on the aging time was observed in ref *26* for synthetic melanoidins from glycine and glucose.

Molecular and Structural Heterogeneity of TBV. In this work, we arbitrarily refer to as melanoidins all copolymers with a molecular weight above 2 kDa showing colored and/or uncolored chromophores. In particular, the brown melanoidins of TBV spread from 5.2 to >1000 kDa as they were compared with PEG/PEO standard mixtures. **Figure 2** shows the SEC elution patterns for the 14-year TBV samples. The calibration

Table 4. TimeCourse of Relative Amount of the Uncolored Melanoidins (280 nm) during TBV Aging

	relative percentage						
	P1	P2	P3	P4	P5	P6	P7
cooked must	10.84	20.68	33.61	6.36	13.28	6.71	8.52
1-year TBV	12.8	19.79	22.48	15.86	7.92	17.29	3.86
3.5-year TBV	17.15	19.54	22.2	11.03	12.08	14.18	3.82
6-year TBV	15.88	22.2	22.11	11.61	11.38	13.49	3.32
9-year TBV	15.25	18.43	27.38	12.98	8.61	13.9	3.44
11.5-year TBV	15.19	18.27	29.52	10.48	9.57	13.33	3.64
14-year TBV	15.19	18.27	29.52	10.48	9.57	13.33	3.64

curve for PEG/PEO standard mixtures is also reported (cubic curve). Notoriously, SEC separates soluble compounds on the basis of their hydrodynamic volume so that the elution order of macromolecules in TBV was expected to be not proportional to their compositional difference, and different macromolecules eluting at the same retention volume have the same molecular size but different chemical structure. Consequently, only an average composition can be inferred at each elution time by SEC analysis of TBV. The fact that the signal intensity from dual detector systems produced not superimposable elution profiles reflects the different chemical natures and distributions of chromophores on the macromolecules. As can be see from **Figure 2**, at the higher molecular sizes there is a comparatively large vis (420 nm) and much higher UV (280 nm) response and a low or nonexistent DRI response, with the first eluted polymer population absorbing only UV radiations. At high elution time the situation is reversed: RI detection was much more sensitive to trace the presence of low molecular sizes and oligomers. On the basis of the absorbing properties, the investigated TBV sample was recognized as a compositionally heterogeneous blend of copolymers. As detected at 420 nm, four discrete polymeric populations containing brown chromophores were recognized; in **Figure 3a** they are referred to as V1, V2, V3, and V4 peaks. As detected at 280 nm, seven discrete populations containing uncolored chromophores were recognized; in **Figure 3b** they are referred to as P1, P2, P3, P4, P5, and P6 peaks. Copolymers containing brown chromophores were eluted with a decreasing broadness of molecular size distributions and a decreasing relative concentration, that is, 54.8, 32.2, 6.1, and 6.8%, respectively. The relative MW of brown melanoidins ranged on average from 2 to >1000 kDa, whereas the MW of the uncolored ones ranged from 2 to >2000 kDa. Oligomeric melanoidins were detected with DRI, and their molecular weights ranged mainly between 2 and 5 kDa (data not shown).

We attempted to compare the average composition of chromophores naturally occurring in cooked must with those from a synthetic solution of melanoidins produced by Maillard reaction. With this aim, a dual detection profile was acquired for a synthetic solution of melanoidins after it was prepared following a modified version of method by Shore et al. (*22*). The synthesis procedure was adapted to reproduce reaction conditions more closely resembling those existing in the grape must cooking as follows: glucose (72 g), fructose (72 g), and lysine (30 g) were dissolved in 100 mL of HPLC-grade water and incubated at 50 °C for 72 h; the mixture was maintained at pH 3 by the addition of a mixture of acetic and tartaric acids. It is interesting to note that in **Figure 4a**, the interaction between a simple amino acid and two simple monosaccharides, that is, glucose and fructose, leads to chromophore and molecular size distributions very closely resembling that naturally occurring in the cooked must, especially for molecular weights under 100 Da, including more likely all Maillard intermediates with low molecular weights. In **Figure 4b** the SEC elution profile of standard solution for HMF and G/F mixture (1:1 in weight) is represented. On the basis of the UV−vis and DRI detector responses, the standard peaks were comparatively associated with the peaks eluted at the same retention time in the TBV and synthetic melanoidin chromatograms (**Figure 4a**). Both sugar and HMF peaks were used as "flow markers" in a flow correction procedure eliminating the shift in the elution time due to the interdetector volume.

SEC Elution Pattern as "Structure Fingerprinting" of TBV. Panels **a** and **b** of **Figure 5** show, respectively, the UV and vis elution profiles for the investigated TBV samples throughout the aging. As can be seen in these profiles, the elution profile changed quantitatively from one vinegar to another. In particular, the total area under the chromatograms (from 12 to 30 min) increased with aging over 4 and 7 times as detected at 280 and 420 nm, respectively. It should be noted that the ratio between peak areas changed significantly during aging. There is strong evidence of the fact that the heterogeneity degree affecting both the molecular sizes and chromophore distribution as detected in the later stages of TBV aging is very close to the heterogeneity occurring in the early stage vinegars. On the other hand, there is evidence that the shape of melanoidin peaks was a time-independent property: this fact suggests that the SEC elution patterns can be considered a reliable "structure fingerprinting" of the TBV age.

An attempt to establish a possible mechanism for melanoidin development in TBV during aging was made by HPSEC analysis. With this aim, areas under chromatogram peaks detected at 420 nm due to LMW melanoidins (calculated as the sum of the V3 and V4 populations) were plotted against areas under chromatogram peaks detected at the same wavelength and due to the HMW melanoidins (calculated as the sum of the V1 and V2 populations) for each TBV age. Linear correlation was found with $R^2 > 0.989$: the absorption ratio between HMW and LMW melanoidins was constant upon aging. On the basis of this evidence, it was reasonably hypothesized that melanoidins develop during TBV aging as a result of a time-dependent combination of isolated, low molecular weight polymers containing colored chromophores only or together with low molecular weight polymers containing uncolored chromophores. This hypothesis is in agreement with results obtained by Wedzicha and Kaputo (*23*), who suggested that the melanoidin formation in a model solution was a result of a built-up mechanism of brown LMW into preformed HMW structures in such a way that did not affect the chromophore distribution.

Development of TBV Browning. Tables 3 and **4**, respectively, show the relative concentration for brown and uncolored melanoidins at each TBV age. Surprisingly, a complex trend for melanoidin development was observed. The percent value of V1 and V2 followed an opposite trend, the former increasing and the latter decreasing with aging time, whereas the percent values of V3 and V4 remained practically unchanged. On the other hand, among the uncolored melanoidins only the relative concentration of P5 decreased, whereas P7 remained unchanged. These findings are in line with the hypothesis on the possible mechanism proposed for melanoidin formation during TBV aging for which all degradation products from LMW molecules are expected to become available to act as monomers in the formation of the HMW melanoidins.

Figure 6 shows the time course of the browning index (absorption at 420 nm) during vinegar aging. In the same figure

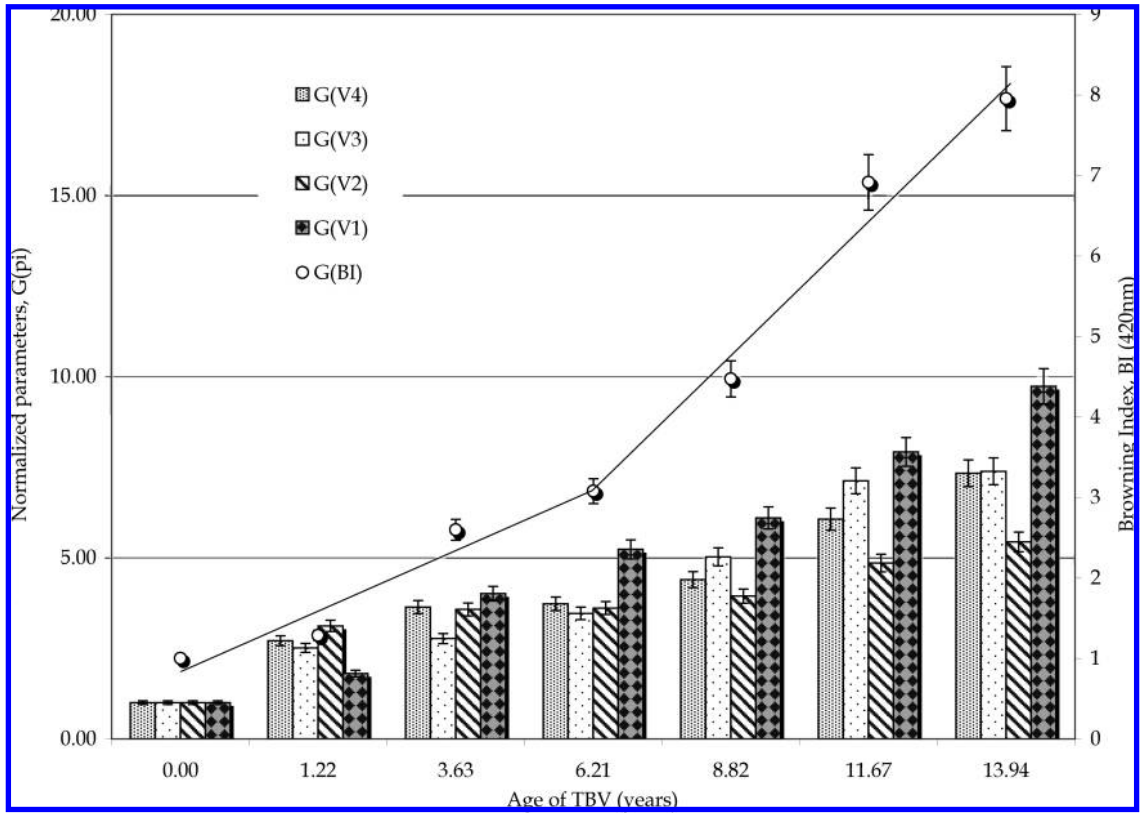

Figure 6. Time course of the browning index (absorbance at 420 nm) in the TBV samples. Bars represent the relative concentration of the brown melanoidin population. All data were normalized with respect to their corresponding values in cooked must and referred as to G(V1), G(V2), G(V3), G(V4), and G(BI).

the relative fraction of colored melanoidins was reported also. The overall yield of melanoidins was proportional to the total color formation. Also, the visual observation of the vinegar color was consistent with the trend observed for the browning index. The development of browning in TBV samples followed a two-rate determining kinetics with the second stage starting at about the sixth year of aging. The average accumulation rate of melanoidins followed the order V3 ≈ V4 > V1 ≈ V2, which is in line with the observed browning kinetics. The fact that LMW melanoidins contributed to TBV browning much more than the HMW ones was in agreement with what was found by other authors in sugar−amino acid solutions (*24*) and in vinegars (*25*).

MWD Parameters As Aging Markers of TBV. In **Figure 7** the relative fraction of each melanoidin population is plotted against the density levels of TBV; a theoretical concentration line representing water loss during TBV production is reported also. The rate of formation of melanoidins in TBV was markedly greater than that of the concentration process of TBV due to the water loss from the set of barrels. As can be inferred from this figure, the increasing melanoidin amount cannot be explained by the simple concentration process of the TBV: it grows exponentially upon aging. This means that sugar degradation reactions started during cooking of the grape must and strongly progressed during the aging of TBV, being favored by water evaporation. The fact that, in the range of the calibrated MW, a continuous profile was observed for melanoidin peaks (**Figure 1**) indicates the presence of macromolecules differing from one each other by a few units (they cannot be separated into distinguishable peaks) and, therefore, the presence of very broad polydisperse compounds in TBV.

All MWD parameters and relative concentrations of melanoidin populations in the 14-year TBV samples are listed in **Tables 5** and **6** for uncolored and brown melanoidins, respectively. As can be inferred from these data, P1, P2, and P3 as well as V1 and V4 can be recognized as highly polydisperse polymers.

The time course of the MWD parameters for V1 and V2 as well as for P1 and P7 melanoidin populations is represented in **Figure 8**: all distribution parameters decreased with TBV age, reaching asymptotically lower limits. This finding indicates the coexistence of polymerization and depolymerization mechanisms running with a decreasing rate during TBV aging: they allowed the chemical heterogeneity of melanoidins to increase and, concomitantly, the molecular sizes to decrease over the entire aging period. Similar behavior was observed for the other colored and uncolored melanoidin populations detected in TBV (data not shown). These results were corroborated by the polydispersity index (PDI) of some melanoidin populations (see **Figure 9**). However, the PDI data calculated for the V1 and V2 melanoidin populations followed an opposite trend with TBV age, going asymptotically toward upper and lower limits, respectively. The fact that MWD varied over the entire aging period under study, reflecting a nonequilibrium status of the balance between polymerization and depolymerization reactions, makes it possible to propose the MWD parameters as potential descriptors of the time-dependent reactions affecting TBV quality and, therefore, as potential aging markers of the TBV age.

All findings fully agree with the theoretical model for the TBV aging proposed by Giudici and Rinaldi (*3*). These authors proved that the age of TBV is strictly increasing in any single barrel and reaching a finite upper limit as the number of years of the barrels set goes theoretically to infinity. Authors calculated these limits for TBV age starting only from the volume of

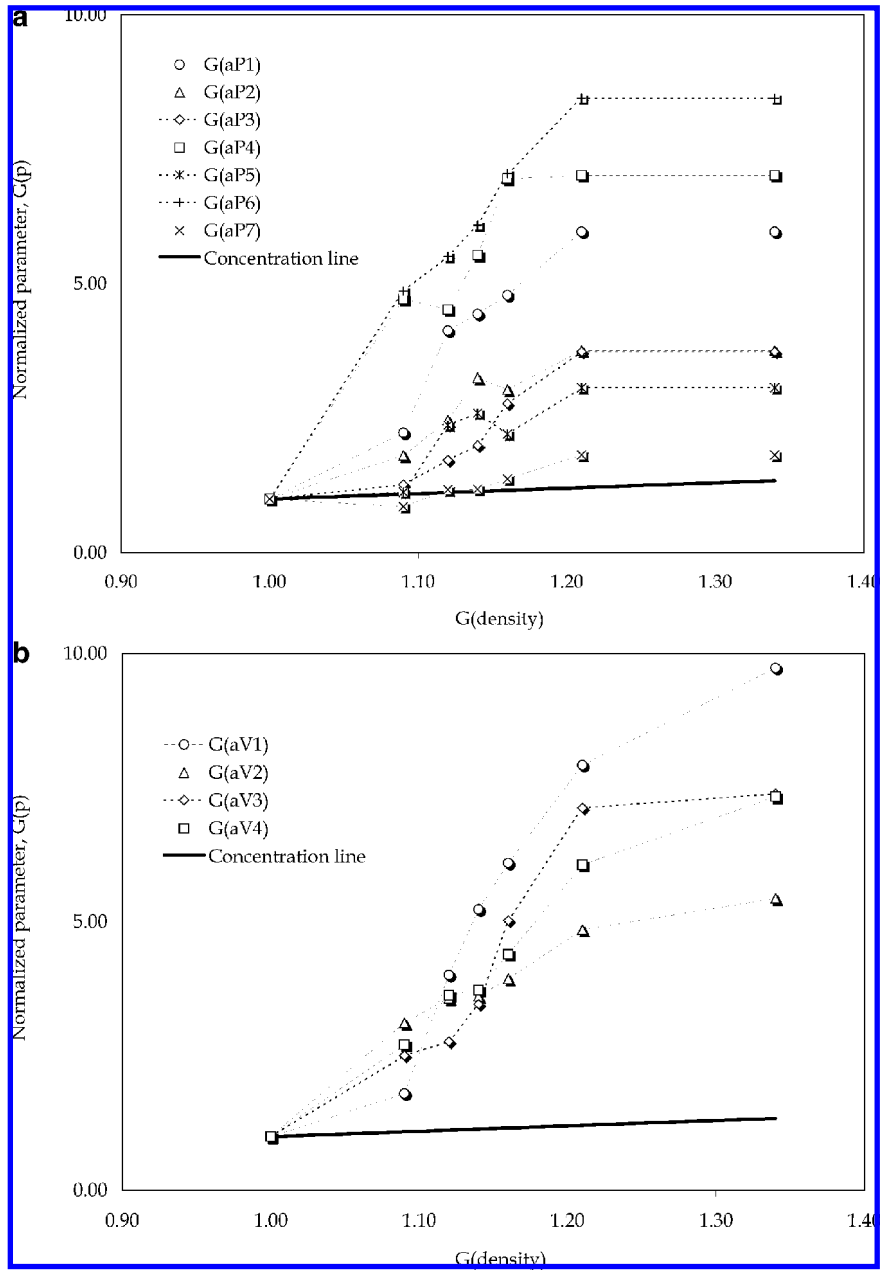

Figure 7. Time-dependent accumulation of the melanoidins of TBV samples, detected at 280 nm (**a**) and 420 nm (**b**). The dotted line indicates the theoretical concentration line: any value below or above this line indicates the consumption of the new formation of the compound, respectively. The relative concentration of melanoidins was expressed as a normalized index (calculated as the ratio of the area under each peak in the SEC chromatogram and that of the corresponding value in cooked must samples).

Table 5

	M_p	M_n	M_w	M_z	M_{z1}	M_v	PD	area %
P1	6.5E+06	4.2E+06	8.1E+06	1.6E+07	2.9E+07	7.3E+06	1.91	15.19
P2	1.3E+05	2.8E+05	4.4E+05	7.0E+05	9.4E+05	4.1E+05	1.58	18.27
P3	3.9E+04	4.8E+04	5.8E+04	7.0E+04	8.2E+04	5.6E+04	1.20	29.52
P4	2.3E+04	2.3E+04	2.3E+04	2.3E+04	2.4E+04	2.3E+04	1.01	10.48
P5	1.9E+04	1.5E+04	1.5E+04	1.5E+04	1.6E+04	1.5E+04	1.02	9.57
P6	8.9E+03	8.9E+03	9.0E+03	9.1E+03	9.2E+03	9.0E+03	1.01	13.33
P7	6.2E+03	4.9E+03	5.1E+03	5.3E+03	5.4E+03	5.1E+03	1.04	3.64

Table 6

	M_p	M_n	M_w	M_z	M_{z1}	M_v	PD	area %
V1	3.5E+05	1.9E+05	3.5E+05	6.4E+05	1.1E+06	3.1E+05	1.86	54.81
V2	1.6E+04	1.6E+04	1.9E+04	2.2E+04	2.6E+04	1.9E+04	1.16	32.21
V3	6.3E+03	6.8E+03	6.8E+03	6.9E+03	7.0E+03	6.8E+03	1.01	6.12
V4	5.2E+03	2.7E+03	3.9E+03	4.5E+03	4.8E+03	3.8E+03	1.47	6.87

vinegar in the barrel set as well as from the volume of vinegar withdrawn and/or transferred one from barrel to barrel.

The present study proved that TBV undergoes profound chemical transformation during the long-term aging, resulting in the formation of new heterogeneous macromolecules (melanoidins), colored and uncolored, spreading from high polymeric to oligomeric and monomeric sizes. The size distribution of these macromolecules was recognized as a "structure fingerprinting" of the TBV age: it was expected to affect structure-related properties of TBV including viscosity, colligative properties, refractive index, density, specific heat, glass transition temperature, and others. From a theoretical standpoint, the investigated TBV samples behaved as kinetically unstable liquids, because polymerization and depolymerization reactions occurred continuously from cooking grape must to final product during the

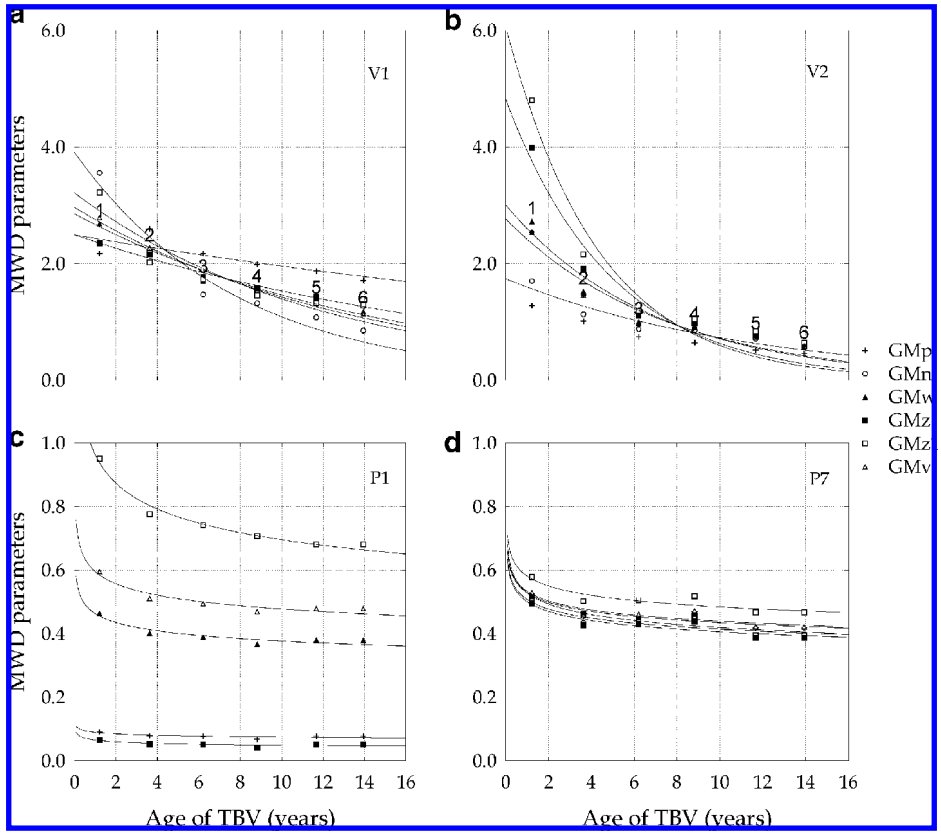

Figure 8. Time course of the MWD parameters for brown melanoidin populations V1 (**a**) and V2 (**b**) and for uncolored melanoidin populations P1 (**c**) and P7 (**d**). The exponential trend was proved by a nonlinear regression procedure of a logarithmic function of the experimental data. All data were normalized with respect to their corresponding value in cooked must.

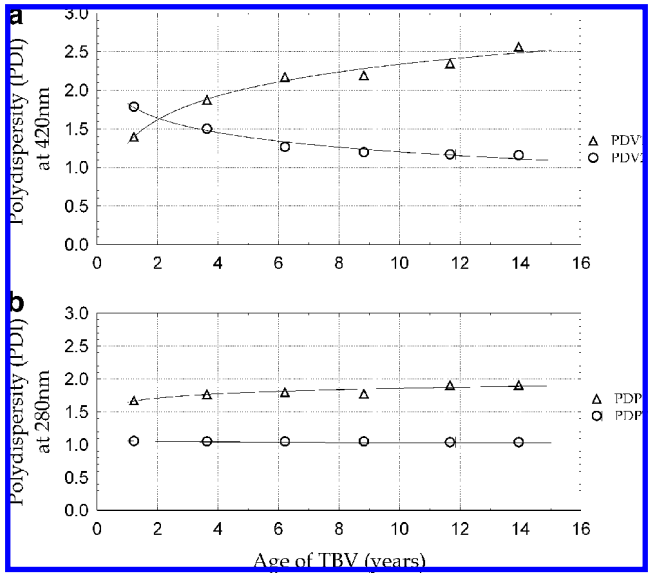

Figure 9. Time course of polydispersity index (PDI) for the melanoidins detected at 280 nm (**a**) and 420 nm (**b**) during TBV aging.

entire aging period, affecting continuously the structure, composition, and distribution of molecular weight constituents as well as the chemical and physical properties of TBV. If the features of the set of wood barrels used for vinegar production as well as the working conditions including refilling procedure should be changed with respect to those used in our work, then both the extent and kinetics of age-related reactions will be expected to vary strongly.

The proposed approach to study TBV opens up the possibility of understanding how the polymerization reaction extent and/or the polydispersity of the newly formed compound may affect any age-related properties of TBV. We believe that the future availability of standardized procedures aimed at controlling the polymerization reactions in TBV will allow new ground to be broken in both vinegar science and trade: the use of the proposed aging markers will open possibilities for authorities to an objective validation of the TBV age, whereas for vinegar producers, possibilities will open in terms of design perspective of the end-product quality, taking any structure-related properties into account as key factors for agreement to the disciplinary requirements and in consumer acceptance

ABBREVIATIONS USED

MW, molecular weight; MWD, molecular weight distribution; SEC, high-performance liquid size exclusion chromatography; DRI, differential refractive index; UV−vis, ultraviolet−visible; TBV, traditional balsamic vinegar; NEB, nonenzymatic browning reactions; HMW, high molecular weights; LMW, low molecular weights; PEG/PEO, polyethyleneglycol/polyethyleneoxide (PEG/PEO); HMF, 5-hydroxymethyl-2-furfural; G, glucose; F, fructose; BI, brown index.

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