

Relaxometric Studies for Food Characterization: The Case of Balsamic and Traditional Balsamic Vinegars

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NMR spectroscopy is a powerful technique for investigating the structure and composition, as well as the physicochemical properties, of foodstuff. NMR-field cycling modality reports about the relaxation times of solvent molecules as a function of the applied magnetic field strength. In the case of aqueous solutions, this methodology is particularly valuable in assessing the interactions of water molecules with paramagnetic and large-size macromolecular systems. ¹H NMR field cycling relaxometry has been used to characterize traditional balsamic vinegars and balsamic vinegars of Modena. It has been found that the longitudinal relaxation time (T_1) of the water proton resonance is mainly determined by the water molar fraction and the occurrence of dissolved macromolecules and paramagnetic metal ions. Actually, the observed ¹H nuclear magnetic resonance dispersion (NMRD) profiles appear markedly affected by the formation of paramagnetic macromolecular adducts. It has been shown that counterfeit specimens can be identified on the basis of the comparison of their T_1 and T_2 (transverse relaxation time) values with respect to the corresponding values of genuine samples. For the latter ones, a relationship has been found that relates the observed T_1 to the age of the vinegar.

KEYWORDS: NMR; fast-field cycling relaxometry; nuclear magnetic resonance dispersion profile; balsamic vinegar

INTRODUCTION

The last decades have witnessed a growing interest in the search for new tools for food characterization. Any progress in analytical instrumentation is, early or later, exploited to obtain a more advanced description of foodstuff. Often the diffusion of a new methodology is hampered only by the complex sample preparation and the associated costs. Therefore, methods that do not require any (or minimal) sample treatment are highly desired.

Over the years, high-resolution NMR spectroscopy has gained an important position among the spectroscopic methods for food characterization thanks to its unique ability to identify (and quantify) all the major low- to medium-molecular weight components by a single spectrum acquisition without the need for any separative procedure.

Besides the high-resolution approach, NMR has provided important contributions to the field by the use of its low-

resolution (or wide line) modality. Low-resolution methods lack chemical shift information, and the acquired ¹H signal consists of a single absorption containing the information arising from all protons present in the specimen. This approach has been shown to be particularly useful in investigating systems consisting of different phases (solid–liquid, water–fat, etc.) (*1*). However, also in the case of aqueous solutions, the relaxation characteristics of the single-water absorption bring about a wealth of information about the solute molecules that is highly relevant in the characterization of a given specimen. Moreover, the possibility of recording the proton relaxation rate over an extended range of magnetic fields further improves the potential of this approach as it allows us to identify the occurrence of different contributions that can be assigned to various components of the foodstuff (*2, 3*).

The results presented here aim to show the potential of the field cycling relaxation approach in food analysis. The methodology has been applied to the characterization of balsamic vinegars of different aging process, but it can be easily extended to any other foodstuff containing dissolved paramagnetic metal complexes.

Over the past few years, traditional balsamic vinegar (TBVM) and balsamic vinegar of Modena (BVM) have received a great deal of attention from different research groups (*4–10*), mainly aimed at focusing through NMR studies, the characterization

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Table 1. List of the BVM, TBVM, and Suspicious Counterfeit TBVM (italic style) Samples Analyzed^a

	type	age (years)	longitudinal relaxation rate, ^b $R_{1,oss}$ (s ⁻¹)		transverse relaxation rate, ^b $R_{2,oss}$ (s ⁻¹)	
			20 MHz	70 MHz	20 MHz	70 MHz
1	BVM	<3	1.14	1.31	1.95	3.00
2	BVM	<3	1.80	1.97	2.70	3.95
3	BVM	<3	1.28	1.41	2.09	3.18
4	BVM	>3	2.78	2.93	3.83	5.41
5	BVM	>3	4.30	4.83	7.25	9.43
6	BVM	>3	4.30	4.86	5.88	8.70
7	BVM	>3	2.89	2.98	4.55	6.90
8	BVM	>8	6.34	7.12	8.33	11.63
9	TBVM	>12	44.26	39.19	55.87	68.03
10	TBVM	>12	41.93	43.29	51.28	67.11
11	TBVM	20	69.88	67.36	90.09	115.34
12	TBVM	20	38.98	39.79	49.50	66.67
13	TBVM	>25	86.3	59.80	132.45	148.37
14	TBVM	34	75.00	53.97	113.25	128.87
15	TBVM	>50	116.28	62.09	232.56	263.16
16	<i>TBVM?</i>	12–25	47.18	38.44	62.50	72.99
17	<i>TBVM?</i>	>12	48.19	27.51	71.43	74.63
18	<i>TBVM?</i>	12–25	47.97	27.08	73.53	76.34
19	<i>TBVM?</i>	12–25	70.05	37.37	135.69	146.20

^a The longitudinal and transverse relaxation rates, measured at 20 and 70 MHz (25 °C), are reported. ^b The maximum absolute uncertainty in the measurement is $\pm 1\%$.

of aging process, and fraud detection (11). TBVM and BVM are much appreciated Italian products all over the world. Balsamic vinegar is essentially made of wine vinegar supplemented with caramel with possible aging in casks for fewer or more than three years (on this basis, they are labeled with a red or white stamp, respectively). TBVM is a completely different product, made of cooked must and aged in series of decreasing volume casks made of different woods for at least 12 years and up to 25 years or longer. TBVM is a high-quality product, the production of which is regulated by law (Ministerial Decree, February 9, 1987, Italy), and its cost on the market is rather high depending on its aging process. In this respect, fraud may occur by altering young products that may be sold as old or extra old upon modification of their physicochemical properties, like density, color, etc.

The aim of this work is to demonstrate the ability of field cycling relaxometry to provide an in-depth characterization of both TBVM and BVM.

EXPERIMENTAL PROCEDURES

The interaction of water molecules with solutes' components in foods can be investigated by the measurement of NMR relaxation times, the spin–lattice (T_1) and spin–spin (T_2) relaxation times of water protons.

The relaxation times report about the modulation of magnetic interactions of water protons with the surrounding environment and are therefore dependent on the molecular reorientational time of water molecules. Moreover, the effective reorientational time is related to the extent of association the water molecules have with immobilized or slowly moving macromolecules. In general, the larger the macromolecule content, the larger the reorientational time felt by the water protons. The relationship between the relaxation time and the dynamics of the magnetic interactions is dependent on the frequency of observation (i.e., on the applied magnetic field as $\omega = \gamma B_0$). The field dependence of T_1 is represented in the nuclear magnetic resonance dispersion (NMRD) profiles that report about the changes in $1/T_1$ in the function of the applied magnetic field strength. This technique provides information about structural and dynamic features of molecules and on electron relaxation (12).

A dedicated NMR spectrometer allows us to acquire relaxation data over an extended range of Larmor frequencies (from 0.01 to 20 MHz). Additional relaxation data can be obtained on commercially available instruments operating at higher field strengths.

In the presence of a paramagnetic solute, the observed water proton relaxation rates, $(1/T_i)_{obs}$, are the sum of two contributions (13):

$$(1/T_i)_{obs} = (1/T_i)_d + (1/T_i)_p \quad i = 1 \text{ or } 2 \quad (1)$$

where $(1/T_i)_d$ is the diamagnetic water relaxation rate in the absence of a paramagnetic species and $(1/T_i)_p$ represents the additional paramagnetic contribution. In the absence of solute–solute interactions, the water relaxation rates are linearly dependent on the concentration of paramagnetic species, [M]. The relaxivity, r_{ip} , is defined as the slope of this dependence in units of $\text{mM}^{-1} \text{s}^{-1}$:

$$(1/T_i)_{obs} = (1/T_i)_d + r_{ip}[M] \quad i = 1 \text{ or } 2 \quad (2)$$

For a given paramagnetic species, the value of r_{ip} is a function of temperature and magnetic field strength.

The large and fluctuating local magnetic field in the proximity of a paramagnetic center provides an additional relaxation pathway for solvent nuclei. Since these fields fall off rapidly with distance, random translational diffusion of water molecules and the paramagnetic species as well as specific chemical interactions that bring the solvent molecules near the metal ion (e.g., within 5 Å) is important in transmitting the paramagnetic effect. Each type of chemical interaction can yield different relaxation efficiencies as governed by the distance and time scale of the interaction; the sum of these contributions and that due to translational diffusion gives the total relaxivity of the paramagnetic species. In general, fluctuations of the magnetic field can arise by (i) conformational changes within the molecule or detachment of a ligand bearing the nucleus of interest, e.g., a coordinated water molecule, (ii) reorientation of the molecules with respect to the external magnetic field, and (iii) longitudinal and transverse electron spin relaxation.

Materials. Eight BVM and seven TBVM samples were analyzed; four suspected counterfeit TBVM samples were also investigated (Table 1). BVM samples were obtained directly from the manufacturer or bought at a local supermarket; TBVM samples were obtained from Consorzeria di Spilamberto di Modena, and the suspected counterfeit TBVM samples (labeled as old) were obtained from a governmental control agency. They have undergone an in-depth characterization by NMR spectroscopy (9, 11, 14). TBVM samples were selected to cover a large range of aging processes, as well both red and white stamps (fewer and more than 3 years, respectively) for BVM samples.

Instrumentation. Water proton T_1 and T_2 measurements at a fixed frequency were carried out on a Stellar SpinMaster spectrometer [Stellar Snc, Mede (PV), Italy] operating in the range from 20 to 80 MHz, by means of the inversion–recovery method and the Carr–Purcell–Meiboom–Gill (CPMG) sequence, respectively.

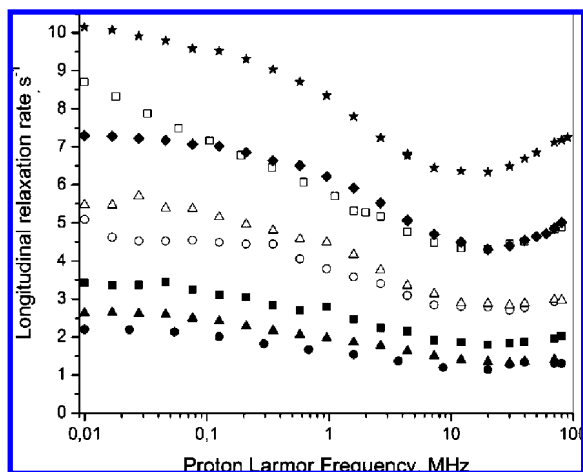


Figure 1. NMRD profile of BVM samples: samples 1 (●), 2 (▲), and 3 (■) with an aging process of fewer than 3 years; samples 4 (○), 5 (□), 6 (◆), and 7 (△) with an aging process of more than 3 years; and sample 8 (★) with an aging process of more than 8 years.

^1H NMRD profiles were recorded at 25 °C on a Stellar Spinmaster-FFC field cycling relaxometer (Stellar Snc) by measuring water proton longitudinal relaxation rates at magnetic field strengths in the range from 2.4×10^{-4} to 0.47 T (corresponding to 0.01–20 MHz proton Larmor frequencies). The relaxometer was able to switch the magnetic field strength in the millisecond time scale by working under complete computer control with an absolute uncertainty in $1/T_1$ of $\pm 1\%$.

The temperature was controlled by a Stellar VTC-91 airflow heater (Stellar Snc), equipped with a copper-constantan thermocouple; the actual temperature in the probe head was measured with a Fluke 52 kJ digital thermometer (Fluke AG, Zurich, Switzerland), with an uncertainty of ± 0.3 °C.

RESULTS AND DISCUSSION

Figure 1 shows the NMRD profiles of the BVM samples that have experienced an aging process ranging between fewer than 3 and 8 years. The observed NMRD profiles resemble those obtained from aqueous solutions of paramagnetic metal complexes that show dispersion in the 1–10 MHz range (15, 16).

Generally, the dissolved paramagnetic metal ions are represented mainly by Fe(III), Mn(II), and Cu(II) (17). BVM is rich in species able to coordinate paramagnetic metal ions, in particular organic acids and sugars, thus yielding an improvement in their ability to relax water protons.

With an increase in the age of the BVM specimen, there is a progressive increase in the observed relaxation rates, and in the high-field region (>20 MHz), the relaxation hump becomes more pronounced because of the elongation of the molecular reorientational time of water protons upon interacting with a paramagnetic metal ions. Such elongation may result from either the interaction with macromolecular substrates or the overall increase in the viscosity of the solution. The association between the high-frequency hump and the presence of paramagnetic ions has been checked by adding aliquots of a Mn(II) concentrated solution to a BVM specimen (data not shown). Invariably, the addition of the paramagnetic ions caused a more pronounced hump at the high-field side of the NMRD profile.

As BVM consists of caramel-supplemented vinegars, it seems likely that the observed behavior can be ascribed to the interaction of Mn(II) ions with caramel. Moreover, when an aliquot [50% (w/w) final solution] of a concentrated solution of caramel is added to a commercial BVM (<3 years old), the characteristic relaxation hump (at ca. 30 MHz) is detected.

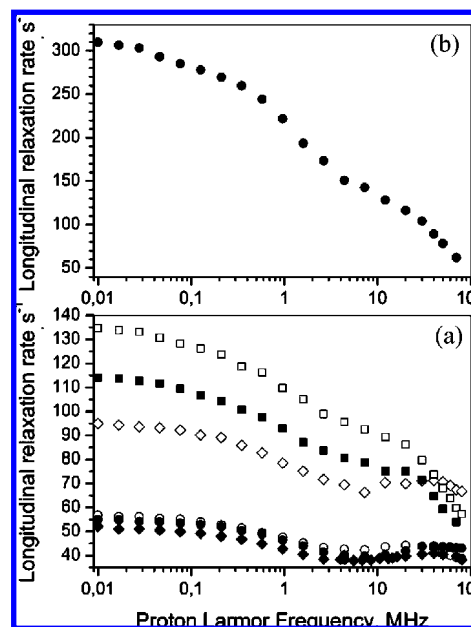


Figure 2. NMRD profile of TBVM samples. (a) Samples 9 (○) and 10 (●) with an aging process of more than 12 years, samples 11 (◇) and 12 (◆) with an aging process of 20 years, sample 13 (□) with an aging process of more than 25 years, and sample 14 (■) with an aging process of 34 years. (b) Sample 15 (●) with an aging process of more than 50 years.

The NMRD profiles for TBVM samples, characterized by an aging process of 12 to >50 years, are reported in **Figure 2**. In general, these samples exhibited much higher longitudinal relaxation rates with respect to the BVM samples over the whole proton Larmor frequency range explored. Furthermore, with respect to BVM samples, there is a shift to a lower frequency of the high-field relaxation hump. The higher longitudinal relaxation rates reflect the differences in the production process of TBVM with respect to BVM. In fact, must cooking gives rise to a concentrated solution of many constituents (sugars, organic acids, nitrogen compounds, metal ions, and polyphenols), together with the formation of neo-compounds, such as melanoidines, heterogeneous polymers formed from sugars and amino acids also through the Maillard reaction. When heated, polyphenol-containing systems form condensed tannins (18). Then, during the aging process, besides the slow degradation processes, TBVM undergoes a further slow concentration process by water evaporation through the staves that contributes to the matrix modification.

As for BVM discussed above, a characteristic feature of the TBVM sample is represented by the high-frequency relaxation hump whose position is mainly determined by the relative values of the electronic relaxation time of the paramagnetic ion and the reorientational correlation time of the complex (τ_R). The elongation of τ_R may be so dramatic that it causes a larger low-frequency shift of the hump that now appears as a shoulder of the main dispersion (samples 13 and 15 in **Figure 2**). Sample 12 (claimed to be 20 years old) shows an anomalous behavior, being more similar to younger samples than to the other 20-year-old specimen (sample 11). This finding parallels an analogous observation made in high-resolution NMR experiments (9) and leads us to suggest that the reported age represents an overestimation of the aging process undergone by this sample. Finally, in the NMRD profile of the >50 -year-old TBVM sample, the observed relaxation rates are very high, typical of an extremely viscous solution with a marked decrease in the

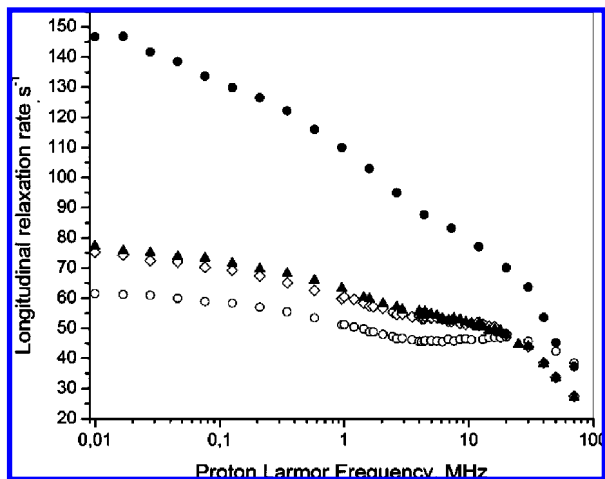


Figure 3. NMRD profile of suspected counterfeit TBVM samples: samples 16 (○), 17 (◇), 18 (▲), and 19 (●).

water molar fraction with respect to the previous ones. The dispersions at ca. 1 and 20 MHz are still well detected.

In conclusion, our data indicate that the shift to lower frequencies of the high-field hump associated with the occurrence of a slowly reorienting paramagnetic adduct is a good marker for the aging process.

In agreement with one of the aims of this work, suspicious counterfeit samples have been analyzed. **Figure 3** reports the NMRD profiles for four such samples. In particular, the counterfeit TBVM sample profiles showed the same order of relaxation rates (ca. 25–80 s⁻¹) as the TBVM (aged for 12–20 years) samples. Their behavior is characterized by a steep decrease at high magnetic field strengths. Sample 19 presents a type of behavior that is more similar to that of the oldest TBVM (>50 years old) sample than the youngest ones (12–20 years old). However, sample 19 does not present the same high

relaxation rate values across the whole field range and the dispersion at ~1 MHz. Tentatively, one may associate the observed behavior with a higher molar fraction of the counterfeit specimen with respect to the real TBVMs.

To gain more insight into the relationship between aging and paramagnetic relaxation, we acquired the transverse relaxation rates of both BVM and TBVM samples. For slowly moving paramagnetic systems, it is expected that R_2 reaches a “plateau” value that is determined by the reorientational time, τ_R . In **Table 1**, the transverse relaxation rates measured at 20 and 70 MHz are reported. The longitudinal relaxation rates at the same magnetic field strengths are included, for the sake of comparison. In general, the increase in both the relaxation rates with aging is observed. The comparison between longitudinal and transverse relaxation times measured at the same magnetic field (70 MHz in this case, selected for the best discrimination between samples) revealed a correlation with aging process. By plotting the longitudinal against the transverse relaxation time values (**Figure 4**), we can observe a good linear correlation for BVM samples. Moreover, three (samples 17–19) of the four counterfeit samples are well separated from the main group of the TBVM samples.

By using T_1 values measured at 70 MHz (**Table 1**), we can derive the following equation:

$$T_2(\text{ms}) = (0.46246 \pm 0.01086)T_1(\text{ms}) \quad (R^2 = 0.98535)$$

Thus, for genuine TBVM samples, it is possible to gain insight into the age of a given specimen by measuring the T_1 of its water proton resonance.

Although information can be gained by measurement at a single frequency, it is evident that the acquisition of the full $1/T_1$ NMRD profile is more informative as the high-field relaxivity hump and the major dispersion change their frequency from sample to sample.

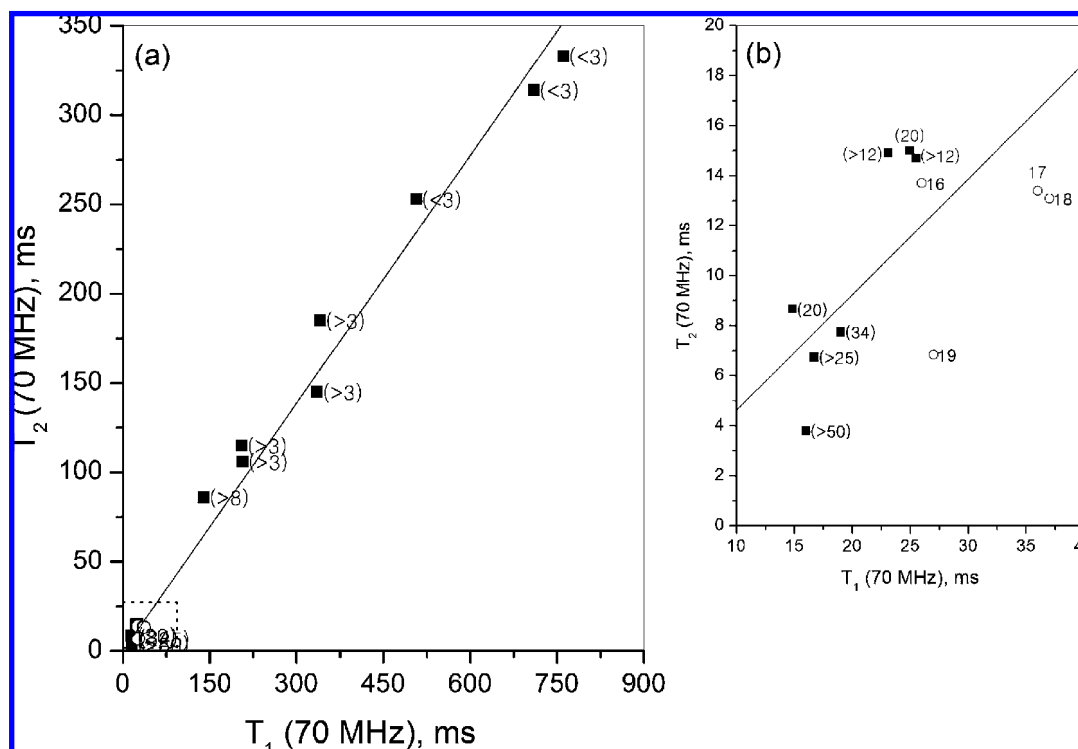


Figure 4. (a) Longitudinal relaxation rate values vs transverse relaxation rate values of BVM and TBVM (■) and suspected counterfeit TBVM (○). (b) Zoomed region of panel a (here indicated by the square inset region). Numbers in parentheses indicate the age of the samples; numbers without parentheses indicate the suspected counterfeit samples, as reported in **Table 1**.

In conclusion, these studies clearly show that the use of field cycling NMR relaxometry may be a powerful tool for characterizing BVM and TBVM samples. The aging process experienced by the samples, in particular by TBVM samples, is mostly characterized by water loss and progressively concentration that affected the longitudinal and transverse proton relaxation times of this product. A relationship may be extracted that relates the age of the sample and the observed T_1 and T_2 values. Further improvement in the assessment of the lack of doping in the investigated samples arises from the comparison between T_1 and T_2 (Figure 4). Three of the four counterfeit samples are clearly out of the region which characterizes genuine vinegars. However, we have no suggestion to explain why counterfeit specimen 16 lies on the line of genuine vinegars. In general, very useful insights can be gained from the frequency-dependent hump that occurs in the high-field region of the NMRD profile. We assign this hump to the occurrence of slowly moving paramagnetic adducts because of both the higher viscosity with aging and the interaction with macromolecular substrates. Clearly, this hump may also be generated by adding macromolecular species, most likely sugar syrup, Arabic gum, or caramel, to young vinegar, but the overall shape of the resulting profile does not fit with those of the genuine samples.

In summary, we think that the NMRD profile acquisition can be of high utility in gaining a better characterization of genuine vinegars, and it may provide useful insights for recognizing fraudulent products.

ACKNOWLEDGMENT

Prof. Amorotti and Consorteria di Spilamberto are acknowledged for providing TBVM samples.

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Received for review December 1, 2008. Revised manuscript received February 18, 2009. Accepted February 19, 2009.

JF803727D