



Formation of glucose and fructose acetates during maturation and ageing of balsamic vinegars

Martina Cirilini *, Augusta Caligiani, Gerardo Palla

Dipartimento di Chimica Organica e Industriale, Università di Parma, Via Usberti 17A, 43100-Parma, Italy

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ABSTRACT

We report for the first time the detection of glucose acetates in balsamic vinegars. It is well known that during traditional balsamic vinegar (TBV) ageing several modifications occur. In this paper it was experimentally determined that some modifications take also place during the short maturation time of balsamic vinegar of Modena (BVM), with an increase of the esters content due to the reaction between acetic acid and sugars, in particular glucose. The formation of esters was observed both on reference solutions containing glucose and acetic acid, subjected to an accelerated ageing by heating at 50 °C, and on real balsamic vinegar samples. Glucose esters were mainly detected as α - and β -glucopyranose acetates. The formation of fructose acetates in reference solutions, was also studied, recording a more complex pattern of products. The structures of the esters were determined by GC/MS analyses and NMR spectroscopy.

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1. Introduction

Balsamic vinegars of Modena (BVM) and traditional balsamic vinegar of Modena (TBV) are considered typical Italian products (European Council Regulation, 2000, G.U. Repubblica Italiana, 2000, G.U. Unione Europea, 2007). BVM is a vinegar, while TBV is a condiment. They are either obtained from the alcoholic and acetic fermentation of cooked and concentrated grape musts, but in BVM it is allowed to add wine vinegar (10% v/v minimum) and caramel color (2% v/v maximum), while for TBV it is allowed to use only cooked grape must and in case inoculation of colonies of acetic bacteria. Another very important difference between BVM and TBV regards the maturation and ageing process, that for the balsamic vinegar of Modena is performed in wooden barrels for at least 60 days, while for the traditional balsamic vinegar of Modena it takes place in characteristic wooden barrels of different size for at least 12 years.

The modifications occurring in TBV during ageing were extensively studied (Antonelli, Chinnici, & Masino, 2004; Caligiani, Silva, & Palla, 2007; Chiavaro, Caligiani, & Palla, 1998; Plessi, Monzani, & Coppini, 1989; Sanarico, Motta, Bertolini, & Antonelli, 2003), while the modifications that take place during the short maturation of the BVM have not been studied at all.

Sugars, in particular glucose and fructose, are the main components of both balsamic and traditional balsamic vinegars (Cocchi,

Ferrari, Manzini, Marchetti, & Sighinolfi, 2007; Plessi, Monzani, & Coppini, 1988). The initial sugar concentration depends on the quantity of cooked grape must used, based on the experience choice of the vinegar makers. Actually, the Production Disciplinary only fixes the minimum concentration value but not the maximum one. For TBV of Reggio Emilia, the minimum is referred to the final sugar concentration (30° Bx); for TBV of Modena only the minimum cooking time and temperature are fixed (30 min at 80 °C), without any reference to the final sugar concentration (European Council Regulation, 2000; Gazzetta Ufficiale della Repubblica Italiana No. 124, 2000). This fact could explain the great composition variability found in TBV from different producers (Plessi, Bertelli, & Miglietta, 2005).

Regarding BVM, the Production Disciplinary fixes the minimum density of the cooked must to be used for the production ($d = 1.240$ at 20 °C) (Gazzetta Ufficiale dell'Unione Europea No. 152 & 06 July, 2007).

Moreover, the sugar concentration increases during ageing of TBV, as a consequence of the progressive water evaporation in the set of the wooden barrels. Organic acids are the other important components of balsamic vinegar: acetic acid is the main product of acetic fermentation by *Acetobacter* species, while other carboxylic acids are either produced by microbial fermentation (lactic acid, succinic acid) or originally present in grapes (malic acid, tartaric acid, and citric acid). The qualitative and quantitative characterization of the organic acids in balsamic vinegar is of particular interest for the study of the product evolution during ageing and for its typicalness and many studies were dedicated to the

* Corresponding author. Tel.: +39 0521 905407; fax: +39 0521 905472.

E-mail address: martina.cirilini@nemo.unipr.it (M. Cirilini).

characterization of these compounds (Cocchi et al, 2006; Cocchi, Lambertini, Manzini, Marchetti, & Ulrici, 2002; Giudici, 1993; Giudici, Barbagallo, Altieri, & Masini, 1994; Masino, Chinnici, Franchini, Ulrici, & Antonelli, 2005).

From a chemical point of view, compounds carrying hydroxylic groups can react with organic acids to give esters with a kinetic that mainly depends on pH, concentration and temperature. In TBV the slow formation of acetoxymethylfurfural from hydroxymethylfurfural and acetic acid was demonstrated during ageing (Giaccio and Del Signore, 1997), but the formation of sugar esters has never been reported.

For this reason, the aim of this work was to study the formation of glucose and fructose acetates in balsamic vinegar samples, in order to better understand the modifications of the product occurring during maturation and ageing and to improve the knowledge of chemical composition, that is important to determine quality, authenticity, and safety of vinegar.

2. Materials and methods

2.1. Materials

Standard of glucose, fructose, phenyl- β -D-glucopyranoside, and 3-(trimethylsilyl)-propionate-d4 (TSP, internal standard for NMR analysis) were purchased from Sigma–Aldrich (Milan, Italy), acetic acid from Carlo Erba (Milan, Italy), silica gel from Merck (Darmstadt, Germany).

2.2. Sampling

Determination of glucose and fructose acetates was carried out on experimental balsamic vinegars with known different sugar content (120, 150, 200 and 350 g L⁻¹) and on traditional balsamic vinegars, supplied by a Modena region producer and declared to be aged in a range of 7–30 years.

2.3. Simulations of maturation/ageing

Reference solutions containing glucose/acetic acid and fructose/acetic acid in distilled water were subjected to accelerated ageing

by heating in a laboratory oven. The simulation conditions (solute concentration, heating temperature, and time) were chosen in order to simulate the range of real composition of balsamic vinegars, and are summarized in Table 1.

2.4. Isolation of glucose and fructose acetates

The samples obtained from heating of reference solutions of glucose/acetic acid and fructose/acetic acid were fractionated on silica (43–60 μ m) column utilizing methylene chloride and methanol 3:1 (v/v) as eluents. The fractions containing glucose acetates and those containing fructose acetates were dried under vacuum for the subsequent characterization by high resolution NMR spectroscopy and GC–MS analysis.

2.5. NMR conditions

NMR spectra were recorded on a VARIAN INOVA-600 MHz spectrometer. The dried samples of isolated glucose acetates and isolated fructose acetates were dissolved in 0.8 ml of deuterated water containing 0.1% standard solution of sodium 3-(trimethylsilyl)-propionate-d4 (TSP). The ¹H NMR spectra were registered utilizing a triple resonance inverse probe, while ¹³C NMR spectra were recorded with proton decoupling (WALTZ 16), utilizing a Nalorac probe.

2.6. GC–MS analysis

The dried samples of isolated glucose acetates, isolated fructose acetates or those of balsamic vinegar samples (10–100 mg, depending on the vinegar concentration) were added to 0.5 mL of internal standard solution (phenyl- β -D-glucopyranoside, 500 ppm), evaporated to dryness under vacuum and dissolved in 0.9 mL of dimethylformamide. The samples were then silylated adding 0.3 mL of trimethylchlorosilane and 0.6 mL of hexamethyldisilazane and heating at about 60 °C for a few minutes. Samples were extracted with 1 ml hexane and injected (split mode) into GC–MS (Agilent Technologies, Santa Clara, CA, USA) on a DB5 capillary column (30 m, 0.25 mm i.d., 0.25 μ m film thickness; J&W Scientific, Folsom, CA) in the following chromatographic conditions:

Table 1
Concentration (g L⁻¹) of glucose, fructose and esters in synthetic solutions

Sample	Glucose (g L ⁻¹)	Fructose (g L ⁻¹)	Real time (days)	Corresponding age (months)	Glucose acetates (g L ⁻¹)	Fructose acetates (g L ⁻¹)
G1a	100	0	7	2	1.16 ± 0.01	0
G1b	100	0	14	4	1.31 ± 0.02	0
G1c	100	0	21	6	1.72 ± 0.05	0
G1d	100	0	42	12	1.73 ± 0.05	0
G2a	200	0	7	2	3.3 ± 0.1	0
G2b	200	0	14	4	4.2 ± 0.1	0
G2c	200	0	21	6	5.2 ± 0.2	0
G2d	200	0	42	12	5.2 ± 0.2	0
G3a	300	0	7	2	6.2 ± 0.2	0
G3b	300	0	14	4	7.3 ± 0.2	0
G3c	300	0	21	6	8.4 ± 0.3	0
G3d	300	0	42	12	8.4 ± 0.3	0
F1a	0	100	7	2	0	0.36 ± 0.03
F1b	0	100	14	4	0	0.71 ± 0.05
F1c	0	100	21	6	0	1.11 ± 0.08
F1d	0	100	42	12	0	1.10 ± 0.08
F2a	0	200	7	2	0	1.24 ± 0.09
F2b	0	200	14	4	0	1.34 ± 0.09
F2c	0	200	21	6	0	1.5 ± 0.1
F2d	0	200	42	12	0	1.5 ± 0.1
F3a	0	300	7	2	0	1.7 ± 0.1
F3b	0	300	14	4	0	2.2 ± 0.2
F3c	0	300	21	6	0	2.5 ± 0.2
F3d	0	300	42	12	0	2.6 ± 0.2

Note: Each solution contains 60 g L⁻¹ of acetic acid and was heated at the constant temperature of 50 °C.

oven temperature programmed from 60 °C for 3 min, then increased to 270 °C at 20 °C min⁻¹; head pressure 6.93 psi; injector temperature 250 °C; detector temperature 280 °C; carrier gas: helium. MS conditions: ion source temperature: 230 °C; electron impact: 70 eV; acquisition mode: scan ($m/z = 40\text{--}550$).

To determine the Response Factor for the quantitative analyses of sugar acetates the isolated fraction of glucose acetates was used as reference, assuming that for fructose acetates the response was the same. The Response Factor value referred to phenyl- β -D-glucopyranoside resulted 0.9.

3. Results and discussion

3.1. Synthesis and characterization of sugar esters

It was made the hypothesis that a Fisher esterification reaction occurred between acetic acid and sugar of balsamic vinegar during ageing. Firstly, it was investigated the reaction between glucose and acetic acid and the reaction between fructose and acetic acid in reference solutions, preparing six solution of different concentration of glucose or fructose (Table 1), respectively 100, 200, and 300 g L⁻¹, and acetic acid, 60 g L⁻¹, in distilled water, simulating the concentration normally found in different balsamic vinegars. Each solution was heated (50 °C) in a laboratory oven for 7, 14, 21, and 42 days. It is well known that, according to Arrhenius equation, the rate of a reaction doubles if the temperature increases of about 10 °C. So it was calculated that heating reference solutions at 50 °C (in respect of room temperature, 20 °C) increases the reaction

rate of a factor of 8. For this reason, one week of heating at 50 °C correspond to two months of maturation at 20 °C.

All reference solutions, inclusive “zero time”, were analysed by GC/MS. The qualitative results obtained from GC/MS analyses of solutions containing glucose and acetic acid show that, at zero time only the glucose signals were present, but after 7, 14, 21, and 42 days of heating treatment the presence of two signals (Fig. 1a), that are compatible with glucose acetic esters, were observed. The two compounds can be recognized by mass spectra (Fig. 1b), in which the characteristic fragments of glucose ($m/z = 217, 204, 191, 147$) and the fragment of acetyl group ($m/z = 43$) are present. The results obtained from GC/MS analyses of solutions containing fructose and acetic acid show that, after the heating treatment, two signals appears, which corresponds to acetic esters of fructose, as shown in Fig. 2. The quantitative results reported in Table 1 show that the formation of acetic esters in the synthetic solutions analysed is directly proportional to the initial quantity of sugar and to the heating time. After 21 days of heating, the concentration of the esters does not increase, that means that the equilibrium was reached.

In order to verify and confirm the structures of these compounds, the reference solutions were fractionated on silica column to obtain the separation between the fraction containing glucose (or fructose) and the fraction containing acetates. The isolated fractions were analysed by ¹H NMR and ¹³C NMR. The ¹H NMR spectrum of the fraction containing glucose acetates shows the presence of two partially overlapped singlet at 2.135 and 2.145 ppm, shifted to low field respect to acetic acid (2.045 ppm), and a shift of all glucose signals to low fields respect to the

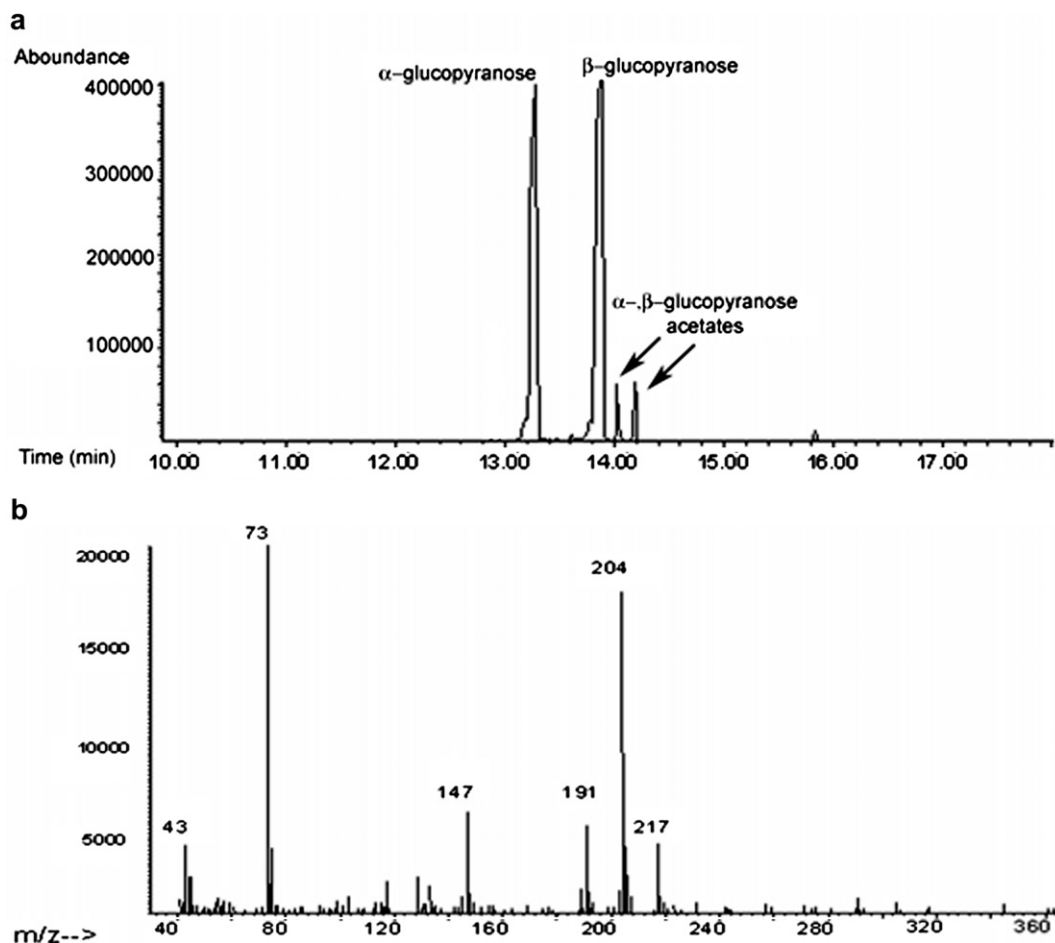


Fig. 1. (a) Gas chromatographic profile of the synthetic solution containing 300 g L⁻¹ of glucose and 60 g L⁻¹ of acetic acid after seven days heating and (b) mass spectra of α -glucose acetate.

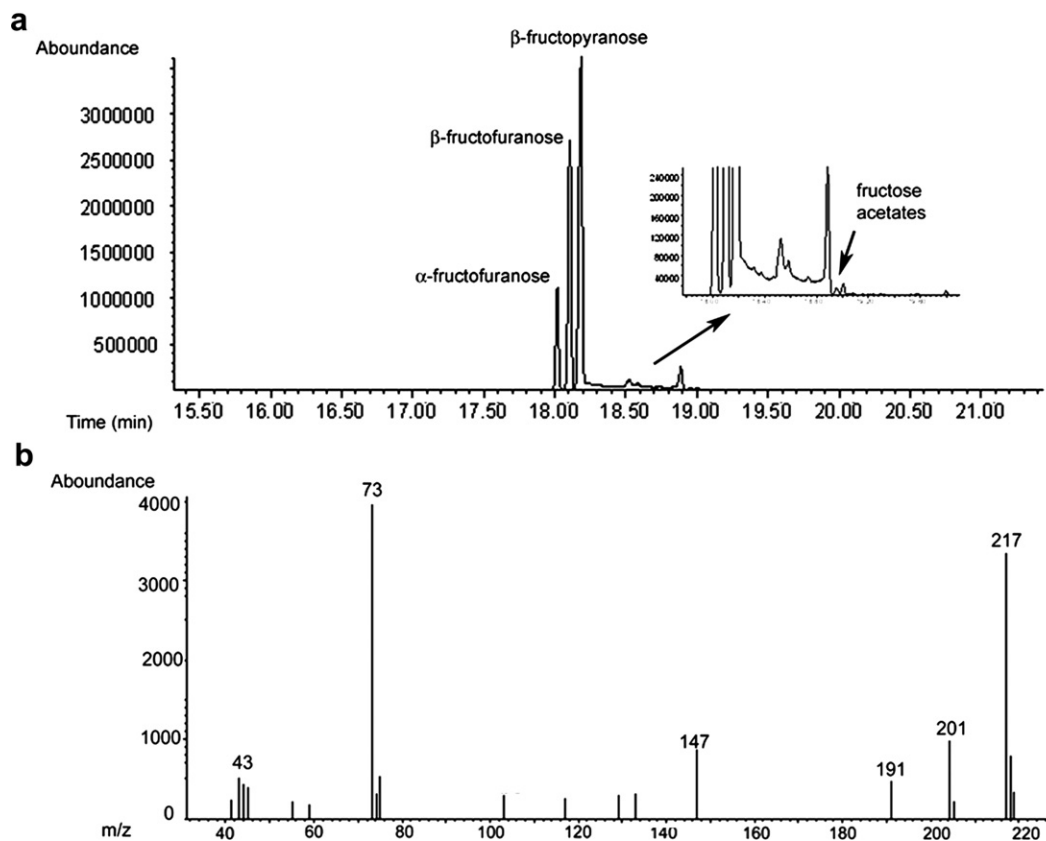


Fig. 2. (a) Gas chromatographic profile of the synthetic solution containing 300 g L^{-1} of fructose and 60 g L^{-1} of acetic acid after seven days heating and (b) mass spectra of fructose acetate.

corresponding signals of α and β -glucose, as shown in Fig. 3. The shifts were attributed to the presence of ester linkage in the compounds and because they were particularly marked for the signals around 3.8 ppm, which correspond to protons of C6 of glucose, it was possible to conclude that the esterified group was the $-\text{OH}$

in position 6. The presence of two anomeric signals in ^{13}C NMR (Fig. 4a) confirm the formation of only one ester of glucose (6-acetylglucose) in the two anomeric forms.

The NMR spectra of fructose acetates fraction were more complicated: seven signals related to acetic groups were detected in

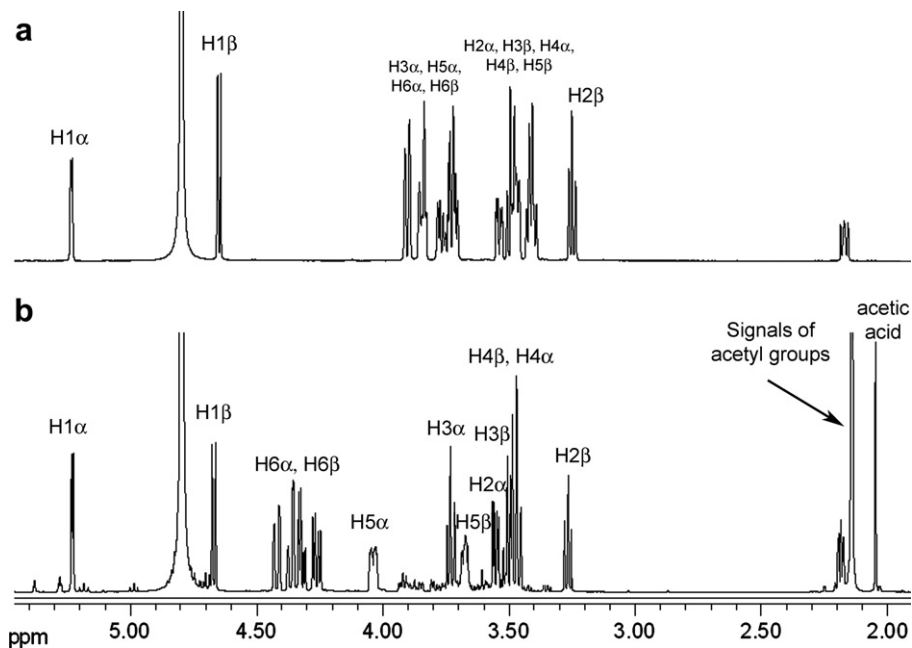


Fig. 3. Comparison between ^1H NMR spectra (600 MHz) of (a) glucose and (b) glucose acetate.

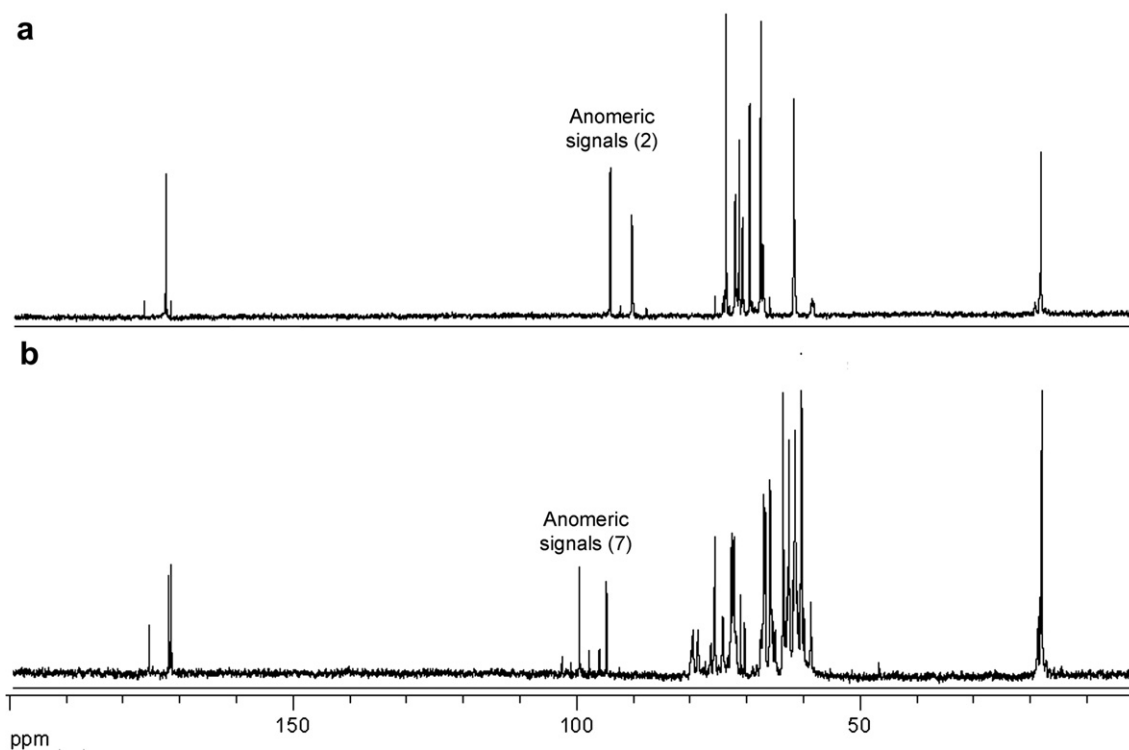


Fig. 4. ^{13}C NMR spectra (600 MHz) of (a) glucose acetate and (b) fructose acetate.

Table 2

Glucose acetates amounts (reported as g L^{-1}) in BVM samples at different time of maturation

Sample	Zero time	2 months	4 months	6 months	8 months	10 months
BVM (120 g L^{-1} of sugar)	0	0.75 ± 0.02	0.61 ± 0.02	1.01 ± 0.03	0.92 ± 0.03	1.19 ± 0.04
BVM (150 g L^{-1} of sugar)	0	0.83 ± 0.03	0.73 ± 0.02	0.87 ± 0.03	1.00 ± 0.03	1.47 ± 0.05
BVM (200 g L^{-1} of sugar)	0	0.99 ± 0.03	1.17 ± 0.04	1.40 ± 0.04	1.45 ± 0.05	2.02 ± 0.06
BVM (350 g L^{-1} of sugar)	0.030 ± 0.001	1.40 ± 0.04	1.88 ± 0.06	2.90 ± 0.09	3.2 ± 0.1	5.4 ± 0.2

the ^1H NMR spectrum (not shown) and nine signals of anomeric carbon in the ^{13}C NMR spectrum (Fig. 4b), showing that in the case of fructose the acetylation positions were more than one. No assignment was possible in the sugar zone of ^1H NMR spectrum because of the strong overlaps. The formation of many fructose esters can explain the difficult to detect all of them both in reference solutions and in real balsamic vinegar samples.

3.2. Determination of acetic esters in real samples of balsamic vinegar

The presence of glucose acetates in samples of balsamic vinegar of Modena of different sugar content and also in samples of traditional balsamic vinegar of different ages was investigated utilizing GC/MS technique. The results (Tables 2 and 3) show that acetates were present in all the samples analysed.

Table 3

Glucose acetates amounts (reported as g L^{-1}) in TBV samples of different age

Sample	Glucose (g L^{-1})	Glucose acetates (g L^{-1})
TBV 7 years	130 ± 4	3.1 ± 0.1
TBV 9 years	144 ± 4	3.4 ± 0.1
TBV 11 years	174 ± 5	4.6 ± 0.1
TBV 13 years	199 ± 6	4.9 ± 0.1
TBV 14 years	233 ± 7	7.5 ± 0.2
TBV 16 years	290 ± 15	7.4 ± 0.2
TBV 17 years	288 ± 9	5.3 ± 0.2
TBV 31 years	273 ± 9	4.2 ± 0.1
TBV 36 years	247 ± 8	4.1 ± 0.1

The balsamic vinegar of Modena was analysed at different time of maturation in order to verify if the formation of esters was dependent by time. The results, reported in Table 2, show an increase of glucose acetate during ageing of BVM. Moreover, the glucose acetate formation is strictly related to the initial glucose amount in the samples, as observed for the reference solutions.

From the traditional balsamic vinegar analysis, higher amounts of glucose acetate were observed, but their increase during ageing is not regular, as reported in Table 3: the amount of glucose acetate initially correlates with the age and the parallel glucose increase, but in the most aged sample it tends to decrease. The presence of fructose acetates in balsamic vinegar samples was also investigated, but the detection of these products resulted very difficult because of coelution with other compounds present in real matrix.

To confirm the results, commercial vinegar samples of other origin, such as wine vinegar, apple vinegar, tomato vinegar, rice vinegar were also analysed (data not shown): the glucose acetate was present only in small amount in rice vinegar, (which contains significative amount of glucose), confirming that the presence of this compound is strictly related to the presence of sugar.

4. Conclusions

The hypothesis of formation of glucose and fructose acetates during maturation and ageing of balsamic vinegar was experimentally verified by spectroscopic analyses (NMR) and gas-chromatographic analyses (GC–MS) of the reaction products obtained by

forced ageing of reference solutions containing glucose/acetic acid and fructose/acetic acid. The analyses confirmed the effective formation of glucose and fructose acetates. These products were checked by GC/MS analyses also in samples of balsamic vinegar of Modena of different sugar content, verifying that the formation of glucose acetates is strictly related to the initial glucose amount. In the samples of traditional balsamic vinegar, the increase of esters during ageing is not regular. However, a greater amount of glucose acetate in TBV respect to BMV was observed. So, the application of the analysis to a more representative number of samples could give better information about the possibility of discrimination of the two kinds of vinegars on the basis of ester content.

This study represents a further contribute to the understanding of the modification reactions that occur in a complex matrix such as balsamic vinegars during maturation and ageing.

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