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XXI CICLO

Development of New Analytical Methods for the Characterization, Authentication and Quality Evaluation of Balsamic Vinegar of Modena

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1. INTRODUCTION

Chapter 1: Introduction

1.1 Vinegars

Vinegar is defined as ‘‘a liquid fit for human consumption, produced from a suitable raw material of agricultural origin, containing starch, sugars, or starch and sugars, by the process of double fermentation, alcoholic and acetous, and contains a specified amount of acetic acid’’ (Joint FAO/WHO Food Standards Programme, 1987).

The English term “vinegar” derived from the French word “*vin aigre*”, that means “sour, acid wine”.

Currently, the international definition of vinegar corresponds to the product obtained by the biological oxidation of fermented agricultural products (apple, rice, honey, malt etc.), not only that obtained from wine. In Italy, on the contrary, almost all the commercial vinegar is obtained from wine.

Vinegar was known by most ancient civilizations, and its use as a seasoning or preserving agent is as ancient as the use of wine. Although it is a spontaneous process which takes place in wines and musts in contact with air, vinegar is far from being the simple spoilage of wine. Vinegar, as a food side-product from wine, has lately acquired an important role as salad dressing, ketchup and other sauces.

Vinegar processing is an ancient technology (Egypt, 8000 B.C.) that follows wine production. Vinegar has been used by Greeks as a medicine, while by Romans it was commonly utilised as a beverage (called *oxicrat*, composed by vinegar, water and eggs), as a flavouring (the famous *Apicio* recipes were based on vinegar), and as a medicine and cosmetic.

Several vinegar therapeutically applications are mentioned on historical documents; during cholera diffusion, it was recommended for disinfection. Even for epidemic plague and leprosy, subjects soaked with vinegar apparently did not show any contagiousness.

Today, vinegar is used exclusively for gastronomy and food technology, for dressing and pickled food, and as sauces ingredient (mayonnaise).

While the quality attributes of wine and olive oil are traditionally accepted and diffused, for vinegar more promotion and valorisation are needed. It is easy and quite normal to find a wine and olive oil list at the restaurant, while a “vinegar list” is just an exception.

This is because vinegar was in the past considered a side-product of the wine industry and often was produced using raw materials of poor quality, such as not marketable wines. This

was a big mistake, because vinegar is not only a food with nutritional properties, but it is principally used to confer particular sensorial properties to food products. For this reason it should be of excellent quality and taste.

Fortunately, in the last years something is changing, and many vinegar producers are starting to understand the concept of vinegar quality, and then, beside the particular case of balsamic vinegars, some other vinegars of excellent quality and selected raw materials can be found on the market (for examples Barolo and Moscato vinegars, Vinsanto vinegar, aromatized vinegars etc.).

1.1.1 Production and consumption

From data of Permanent Committee for International Vinegar (C.P.I.V.), during 2000, 6.8 millions hectolitres of vinegar have been produced by the EU (the 26% from wine, the 61% from alcohol and the remaining from other products).

In the EU, Italy is the first producer of wine vinegar (38% of the whole amount, produced by 30 factories) while in Belgium there is the highest consumption (2.7 litres pro capite per year).

1.1.2 Biochemical aspects of acetification

Vinegar is produced by two steps of fermentation; the first is the conversion of fermentable sugars to ethanol by yeasts, usually *Saccharomyces* species, and the second is the oxidation of ethanol by bacteria, usually *Acetobacter* species.

Until the beginning of XIX century it was thought that vinegar was derived from the spontaneous acidification of wine. In 1864 Pasteur discovers that vinegar was produced by the action of some bacteria, called *"Mycoderma aceti"* for their capacity to create biological film on the surface, and produce vinegar on the liquid where they growth. Later, several new strains with these characteristics were isolated; in 1898 Beijerinck proposed the introduction of the new *Acetobacter* genus. Following the new taxonomic analytical techniques, in 1994 Sievers classified *Acetobacter* as reported in Table 1.1.

Table 1.1: Sievers classification's of *Acetobacter.*

Acetobacter	*aceti*
Acetobacter	*liquefaciens*
Acetobacter	*pasteurianus*
Acetobacter	*hansenii*
Acetobacter	*diazotrophicus*
Acetobacter	*xylinum*
Acetobacter	*methanolicus*
Acetobacter	*europaeus*
Acetobacter	*oxydans*

Acetic acid bacteria are strictly aerobic microorganisms with a strong ability to oxidize sugars and alcohols to the corresponding organic acids. Also, they can grow in media containing high concentrations of acetic acid. This properties are industrially employed to obtain wine vinegar, especially by using bacteria of the genera *Acetobacter* and *Gluconobacter* (Maestre et al., 2008).

Acetobacter are Gram negative, aerobic and acidophylis microrganisms. Their metabolic characteristic is the ability for ethanol oxidation. When we speak of acetic fermentation, it is not exact because actually this process is an incomplete ethanol oxidation. This bioprocess can be summarized as following reported:

$$CH_3 - CH_2OH + O_2 \rightarrow CH_3 - COOH + H_2O$$

The acetic acid obtained can react with ethanol with the consequent formation of ethyl acetate, contributing to the typical vinegar flavour.

The intermediate compound of this reaction is acetaldehyde. Ethanol is dehydrogenated stepwise to acetic acid; the resulting reduced form of the co-substrate methoxatin ($PQQH_2$) is oxidised during the respiratory chain of *Acetobacter,* that uses the energy produced from this reaction for growing (Fig. 1.1).

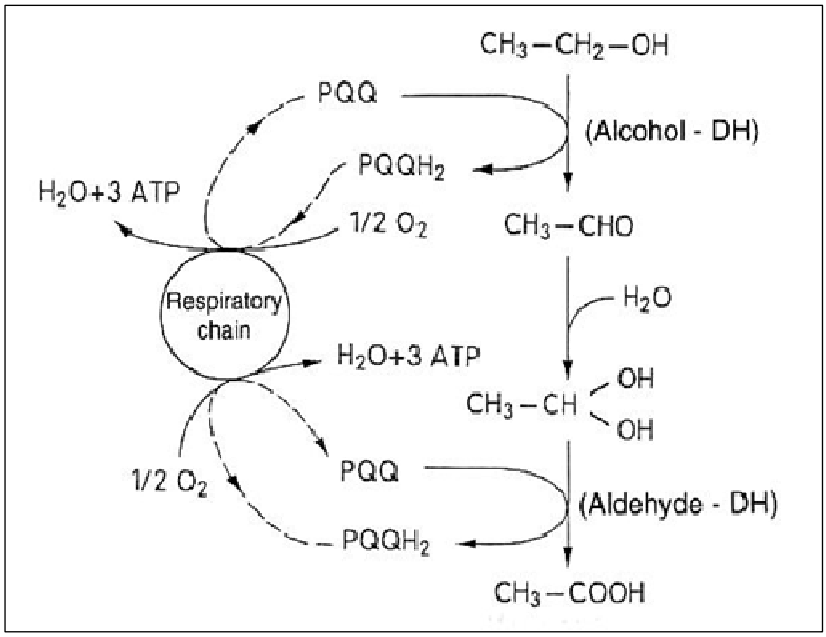


Fig. 1.1: Oxidation of ethanol to acetic acid by *Acetobacter* species (from Belitz, 2004).

Acetobacter bacteria need the presence of oxygen for their grown and reproduction. For this reason, the reaction can stop at the first oxidation step if there is not a sufficient quantity of oxygen in the reaction ambient, obtaining only acetaldehyde. In presence of a surplus of oxygen, acetic acid could be oxidated to give water and carbon dioxide, with a consequent reduction of process yield.

The optimal temperature for *Acetobacter* growth is about 30° C. Dupuy (1957) found that the rate of acetic acid production during acetification increases of two times, from 23 to 28° C. The optimal pH range for *Acetobacter* species growth is 5,4-6,3, but they grow without any problem also in wine, at lower pH (2,2-3,0). They are particularly sensible to SO_2, even at the concentration used for wine-making, but not to potassium sorbate and other anti-mycotic substances and they have not complex nutritional needs.

1.1.3 Processing technology

From a technological point of view, there are two well defined methods of vinegar production: the traditional process (slow acetification) and the submerged method (quick acetification) (Tesfaje et al., 2002).

The first one is the so called “surface culture fermentation”, where the acetic acid bacteria are placed on the air-liquid interface in a direct contact with atmospheric air (oxygen). The presence of the bacteria is limited only to the surface of the liquid, this is also considered a static method. Nowadays this particular method is employed for the production of traditional and selected vinegars and a very long period of time is required to obtain an high acetic degree. This method allows a simultaneous acetification and ageing.
This method employs for the acetification a barrel composed of a wooden container (50000-70000 l) inside of which there is a porous membrane. The wood shavings are posed on the internal membrane to 50 cm from the upper side of the container. The wine is drip through the wood shavings. The liquid in exit from the container is sent to the upper side and passed through the membrane until it reaches the acetic value fixed. The entire cycle of acetification takes 7-9 days.
This particular technology is used for the production of high quality vinegars, obtaining a product more in rich volatile compounds.

The quick method is employed for the production of most commercial vinegars of major consumption, the so called “submerged culture system”. This method implies that acetic bacteria are in direct contact with the fermenting liquid in which a strong aeration is applied to assure the oxygen demand. This method was introduced for the production of vinegar at the beginning of XX century.
This method is called “quick acetification” because it takes only one day. The process takes place in a refrigerated stainless steel tube (10000-30000 l); in one day of production 8000-9000 l of vinegar are obtained.
The vinegars produced with this technology have to by clarified, filtered and pasteurized before bottling.

In Fig. 1.2, a scheme of the acetification by surface culture and an image of an industrial submerged culture system are reported.

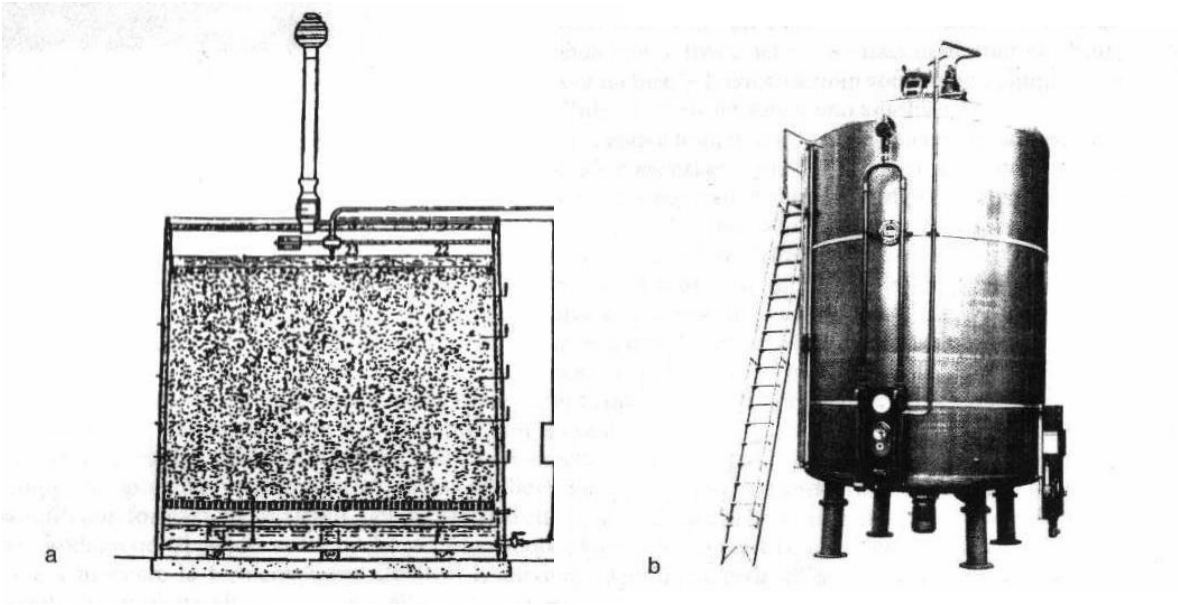


Fig. 1.2: Acetification by a) surface culture (wood shaving) and b) submerged culture (from Cabras, 2004).

1.1.4 Different typologies of vinegar

Wine vinegar

Wine vinegar is mostly produced in countries with enological traditions.

The wine utilized for vinegar production is normal wine, wine with high volatile acidity and wine with alcoholic degree less than 8%. Altered wines can not be utilized.

For common vinegar production, the alcohol content of wine has to be between 7 and 10%, for quality vinegar it should be higher. To reach the correct alcoholic degree, dilutions of wine with water or with less alcoholic wines are performed. The acetification process is stopped when the alcoholic degree is about 0.2-0.3% for common vinegars, and 0.6-1.5% for quality vinegars.

Aromatized wine vinegar

For the production of aromatized wine vinegar only high quality vinegar can be used. The aromatization can be obtained through infusion of spices (cinnamon, nutmeg, black pepper, paprika, cumin, mustard, ginger) and aromatic herbs (garlic, laurel, dill, basil, onion, tarragon, sweet marjoram, mint, rosemary, rue, sage, shallot) for a period of 40-60 days.

This products can be obtained also by adding of infusions (5% maximum).

An example of aromatized vinegar is the raspberry red wine vinegar, in which natural raspberry flavour is added to red wine vinegar. This product can be used for fruit salads, as a marinade or sauces for meats.

Special wine vinegars

Special wine vinegars are the aged and filtered products obtained through the acetous fermentation of a selected blend of wines or of single wine typology. The taste of this vinegars is reminiscent of the wine from which they come.

Examples are Barolo wine vinegar, but also champagne vinegar; Cabernet Sauvignon wine vinegar of high quality and rich in colour; Chardonnay wine vinegar with distinctive flavours and aroma, light to medium gold in colour; Merlot wine vinegar, of unique flavour and aroma, high quality, dark red in colour.

Sherry wine vinegar

Sherry wine vinegar is a vinegar obtained from red wine, a special product from Jerez (Spain). It is produced by peculiar traditional methods: the "solera" system and the static method. A solera system consist on a series of buts arranged in step whose number may vary from three to eight. The substrate arrives at the step on the top of the system and the final product is withdraw from the step at the bottom which is the most aged, but the volume transferred will never exceed one-third of the total volume. Barrels in stage 1 are filled with vinegar from stage 2, which are filled with vinegar of barrel in step 3, and so on. This is the dynamic method of production, in contrast with the static one, in which vinegar is produced in a single but (Garcia-Parilla et al., 1999).

A minimum of 6 months of ageing is required for a vinegar to be considered a Sherry vinegar. Those vinegars aged 2 years are called "Reserva" and those aged for more than 2 years are called "Gran Reserva".

Fruit vinegar

The fruit for vinegar production has to be fresh and fully ripe. The juice obtained after pressing is fermented by selected starters. The product obtained has the flavours of the fruit used for the production. The technology used for these particular kinds of vinegar is the slow fermentation through wood shavings.

An example of fruit vinegar is apple cider vinegar, made from cider or apple must, and is often sold unfiltered, with a brownish-yellow colour. It is currently very popular, partly due to

its alleged beneficial properties. In terms of cooking, cider vinegar is not good for delicate sauces or vinaigrettes, but it is excellent for use in chutneys and marinades.

Cereal vinegar

The two more important examples of cereal vinegars are malt vinegar and rice vinegar.

Malt vinegar is an aged and filtered product obtained from the acetous fermentation of distilled infusion of malt. Malt has a distinctive flavour that contributes to the flavour of the deriving vinegar and brewed beverages such as beer. Malt vinegar is popular for pickling, especially walnut pickles. It is most famous as condiment for fish and chips.

Rice or rice wine vinegar is the aged and filtered product obtained from the acetous fermentation of sugars derived from rice. Rice vinegar is excellent for flavouring with herbs, spices and fruits due to its mild flavour. It is light in colour and has a clean, delicate flavour. Widely used in Asian dishes, it is popular because it does not significantly alter the appearance of the food.

Balsamic vinegar

The balsamic vinegar is very different from wine vinegar. The starting materials for balsamic vinegar are not wine but cooked grape must.

Even this product has a very old tradition (XI-XII century), but the term "balsamic" was firstly reported in 1747 in a letter written by Antonio Boccolari from Modena in order to describe the therapeutic properties of this vinegar produced in Modena and Reggio Emilia provinces. Nowadays this very valuable product is used for high quality gastronomy. There are two different kinds of balsamic vinegar: the traditional balsamic vinegar of Modena and Reggio Emilia (Aceto balsamico tradizionale di Modena e Reggio Emilia, TBV) and the balsamic vinegar of Modena (Aceto balsamico di Modena, BVM). A specific section will be dedicated to this product.

1.1.5 Vinegar composition

The data on chemical composition of vinegars, of Italian vinegars in particular, are very poor. Few are, in particular, the studies about the determination of those parameters that can define the origin and the quality of vinegar.

In Table 1.2, data from chemical and physical analyses of wine vinegar, apple vinegar and traditional balsamic vinegar of Modena and Reggio Emilia are reported.

Table 1.2: Chemical composition of Italian vinegars (Average ± SD) (from Cabras, 2004) (n.d. = not detectable; *detected on 16 samples; **detected on 5 samples).

		Wine vinegar	Apple vinegar	TBV of Modena	TBV of Reggio Emilia
N° of samples		61	1	66	61
Density	g ml^{-1}	1.013	1.015	1.275 ± 0.070	1.206 ± 0.083
Alcohol	% (v/v)	0.30 ± 0.260	1.54	0.77 ± 0.71*	0.50 ± 0.39
Total acidity	% (w/v)	6.70 ± 0.33	5.46	7.22 ± 1.43	5.54 ± 2.26
Volatile acidity	% (w/v)	6.48 ± 0.49	4.80	3.77 ± 0.98	2.77 ± 1.73
pH		2.75 ± 0.15	2.95	2.79	2.64 ± 0.18
Tartaric ac.	g L^{-1}	1.52 ± 0.43	n.d.	4.78 ± 0.25**	-
Malic ac.	g L^{-1}	0.29 ± 0.17	0.76	11.34 ± 1.57**	-
Lactic. ac.	g L^{-1}	0.52 ± 0.39	0.92	44.58 ± 2.85**	-
Gluconic ac.	g L^{-1}	0.28 ± 0.32	n.d.	9.00 ± 1.75**	-
Sugars	g L^{-1}	-	-	552 ± 141	219 ± 118
Dry matter	g L^{-1}	13.68 ± 2.64	17.69	-	-
Ashes	g L^{-1}	2.02 ± 0.41	2.73	8.57 ± 2.41	5.12 ± 2.32
Glycerol	g L^{-1}	3.46 ± 1.06	3.71	13.2 ± 2.9**	6.6 ± 3.1

Vinegar composition is strictly related with its raw materials from which vinegar is obtained. In the case of a wine vinegar, the product derives from a dilution of wine; for the balsamic vinegar, the raw material is cooked must that is fermented and naturally concentrated during maturation and ageing of the product. It is important to remember that the wood is a kind of molecular sieve, allowing the selective permeation of small molecules (ethanol, H_2O), with concentration of the bigger ones.

In wine vinegar and in apple vinegar, the reducing sugars amount is generally negligible, while in TBV their amount is very high because they derive from the cooked must that is concentrated during ageing.

Glycerol presents a major content in TBV than in wine and apple vinegar and the glycerol increases during TBV ageing.

High concentration of gluconic acid can be present in TBV because of chemical and microbial oxidation of glucose during the cooking and the seasoning processes, while it remains at low levels in wine vinegar. Malic acid level is quite low in wine vinegars as it is reduced from

malolactic fermentation, while in TBV its levels are higher as a consequence of the concentrated must utilization and ageing.
Lactic acid, D- and L- forms, in TBV, shows very high concentrations, while in wine D-lactic acid presents very low values. For this reason, it is possible to affirm that the D-lactic acid in TBV derives from glucose fermentation.

1.1.6 Italian legislation about vinegar

The denomination "vinegar" or "wine vinegar" is reserved to the product obtained by acetic fermentation of wines.
Total acidity of vinegar has to be not less than 6% (g 100 ml^{-1}) and alcohol content has to be less than 1.5% (Law n° 991 of 09.10.1964). Vinegar can be added with aromatizing substances (max 5%) and marketed as aromatized wine vinegar. Vitamin B1 (thiamine) has to be added as denaturant (5g 100 l^{-1}) to wine for acetification. The addition of colouring substances to vinegar is forbidden. In the Italian Official Journal of the 27 March 1986 (G. U. n°76, 27 March 1986) the limits for some parameters of vinegar are reported: total acidity, alcohol quantity, dry matter, ashes, metals (Zn, Cu, Pb, Br), boric acid and sorbitol.
Vinegar can also be produced from other alcoholic liquids of agricultural origin; in this case the denomination has to be "vinegar of…" indicating the raw material utilized.

Balsamic vinegars of Modena (BVM) and traditional balsamic vinegar of Modena and Reggio Emilia (TBV) are considered special Italian vinegars.
Balsamic vinegar of Modena is more diffused in respect to traditional balsamic vinegar; it was recognized as "special vinegar" already in 1933. The denomination "Aceto Balsamico di Modena" was fixed in the DL n°162 of 3 December 1965. For traditional balsamic vinegars, the denomination "Aceto balsamico tradizionale di Modena e Reggio Emilia" was recognized in the Law n°93 of 03.04.1986, and classified as "aged dressing"
TBV received the PDO (Protected Denomination of Origin) certification from the European Union (EU) in 2000 (European Council Regulation (EC) 813/2000) because of its typical production procedure and the well-defined geographical areas of production.
The most recent document about balsamic vinegar of Modena dates 2007. This disciplinary fixed all parameters for the production of BVM (Gazzetta Ufficiale dell'Unione Europea, 2007).

1.1.7 Authentication and quality evaluation of vinegar

The characterization of vinegar encompasses different purposes, including food authentication and classification of the products on the basis of quality criteria. To protect the consumer from being sold an inferior product with a false description, and in addition, to defend honest traders from unfair competition, are crucial issues in food quality control. In this way, vinegar, as the rest of the food, is verified as complying with its label description.

The final quality of vinegar is determined by raw material used as substrate, the acetification system used and eventually wood ageing (Morales et al., 2001). Chemical composition and physicochemical parameters are influenced by these factors and one of the main problem in the authentication of vinegar is the wide range of values obtained for the main physicochemical and sensorial parameters. Moreover, some researches in the field have focused on setting up validated methods which can ensure the authenticity of food and differentiate defective or adulterated vinegars from the authentic ones.

For example, it was possible to use the polyalcohol content in order to ascertain the vinegar origin, in case of a suspicion of an adulteration of wine vinegar with less expensive alcohol vinegar (Antonelli et al., 1994).

There is a remarkable interest in differentiating among wine vinegars made by quick acetification or by traditional methods in which surface culture is involved, since the price of the latest is much higher. Good results have been achieved using different analytical parameters such as: acidity, total extract, glycerol, alcohol and sulfates as well as mineral elements.

Volatile components of vinegar, such as ethyl propionate, acetoin, as well as other parameters, have been used to distinguish between quality and defective or adulterated samples of wine vinegar (Nieto et al., 1993). Volatile profile is clearly influenced by the acetification process employed.

D- and L- amino acids as R- and S- acetoin levels can be used to characterize some particular kind of vinegar, such as balsamic vinegar of Modena and traditional balsamic vinegar of Modena and Reggio Emilia. D/L ratio of proline, for example, was useful for evaluating the age, while the R/S ratio of acetoin allowed traditional balsamic vinegars (TBV) to be discriminated from balsamic vinegar (BVM) (Chiavaro et al., 1998).

Phenols are present in wine vinegars due to their natural content in grapes or as a result of contact with wood during the ageing process, and seem to be an important group of substances to accomplish the differentiation by origin and technology involved. Multivariate analysis of data revealed that phenolic compounds of wine vinegars are a good tool to classify

and predict the membership of samples according to the elaboration method applied or the geographical origin of the substrate wine (Garcıa-Parrilla et al., 1996).

Regarding the quality evaluation of a given food, many parameters can be measured by taking into account nutritional value, food safety and sensory properties. In the case of vinegar the quality is strongly determined by sensory properties as it may modify the overall appreciation of a given food or meal. Sensory analysis is a valuable tool by which organoleptic properties of foods are analysed by our senses.

However, one of the difficulties of tasting this product is the strong contribution of acetic acid to the overall sensation. Literature concerning wine vinegar sensory studies is poor. There are two models for vinegar sensory analysis (Tesfaye et al., 2002). The first one consists in preparing vinegar in most approximate way as it is normally consumed. The second model encompasses testing vinegar as it is, using wine glasses. This model is the usual procedure in vinegar cellars to perform sensory analysis.

1.2 Balsamic Vinegar of Modena

1.2.1 History and Italian legislation

Balsamic vinegar of Modena (BVM) and traditional balsamic vinegar of Modena and Reggio Emilia (TBV) are considered typical Italian products. BVM is a vinegar, while TBV is considered a condiment.

Balsamic vinegar is a symbol of the culture and history of Modena. Its existence is due to the particular climatic characteristics of the territory and to the knowledge and competence of the human factor, that create an exclusive product, distinctive of the countries of Modena and Reggio Emilia, the ancient Estense Duchy.

The origin of this product comes from the ancient Romans.

The term "balsamic" was used for the first time in ancient registers of Duchy of Modena and Reggio Emilia in 1747 and, probably, derives from a therapeutic use of the product.

At the end of 1800, balsamic vinegar of Modena appears in the most important manifestations, becoming of international interest. The most important producer in that time was Giuseppe Giusti, whose productions are present in history since 1605.

From the legal point of view, the first ministerial authorization to produce balsamic vinegar of Modena goes back up at 1933, in order to regulate and safeguard the production and the producers of this particular product . Then, in 1965, a set of rules, the first Disciplinar of production (D.M., December 3, 1965) described the preparation procedure for BVM, which consists in a mixture of wine vinegar, caramel and eventually aged wine vinegar.

A recently list of rules has been made, in which are mentioned all the parameters for production of balsamic vinegar of Modena (Gazzetta Ufficiale dell'Unione Europea, 6 Luglio 2007).

These rules regard:

- Name: "Aceto Balsamico di Modena" (Balsamic Vinegar of Modena);
- Some analytic parameters such as: density (1.06 g ml^{-1} minimum), ethanol amount (1.5% v/v maximum), total acidity (6% w/v minimum), sulphur dioxide (100 mg L^{-1} maximum), reducing sugar concentration not less than 110 g L^{-1}, dried matter minimum 30 g L^{-1} and ashes 0,25% minimum;

- Some organoleptic parameters such as colour (intense brown), clearness (clear and brilliant), odour (persistent, soft, acetic, with oak smell), taste (bitter-sweet);
- Geographical origin: the production of BVM can be carried out only in the districts of Modena and Reggio Emilia;
- Production method: BVM is obtained from partially fermented and/or cooked and/or concentrated grape musts, derived from Lambrusco, Sangiovese, Trebbiano, Albana, Ancellotta, Fortana and Montuni tendrils (Emilia Romagna), by addition of 10 years aged wine vinegar and wine vinegar (10% v/v minimum). The concentration of must has to be not less than 20% of total mass with a minimum density of 1.24 g ml^{-1}. Addition of caramel is permitted to a maximum of 2% v/v. Addition of any other substance is forbidden;
- Fermentation process must be conducted with the slow acetification method or by addition of selected starters;
- Labelling: on the bottles, the name "Aceto Balsamico di Modena" has to be coupled with the diction PGI (Protected Geographical Indication) written in abbreviated form or extensively.

There are also two certified production Consortia (CABM: "Consorzio per l'Aceto Balsamico di Modena" and CPCABM: "Consorzio per la Produzione Certificata dell'Aceto Balsamico di Modena"), but the producers are not obliged to ask for the consortium certification.

1.2.2 Commercialization of balsamic vinegar of Modena

Since 1994, producers activated themselves to safeguard and protect the balsamic vinegar of Modena production. For this reason the first certified production Consortium was formed: CABM.

The producers of BVM, by means of the CABM, applied for the Protected Geographical Indication (P.G.I.) status to the UE Commission. In the meantime, a D.M. of August 3, 2006, temporally permits the use of P.G.I. for this product (Consonni et al., 2007). Nowadays, the P.G.I. denomination has been approved and used for balsamic vinegar of Modena (Gazzetta Ufficiale dell'Unione Europea, 6 Luglio 2007).

The Consortium, as nowadays described in the 2007 Disciplinar, has also drafted a set of important rules to guarantee production standards (Voluntary Product Certification, DT 003.1, 2001): the musts used for production can come only from grapes grown in Emilia Romagna;

the product has to be matured for a period of at least 60 days in wooden barrels (bordeaux red stamp); the AGED product has to be seasoned for at least 3 years in wooden barrels (white stamp); Modena Balsamic Vinegar bearing the seal is produced and bottled in the zone of origin; before bottling, the vinegar is analysed by a laboratory approved by the CABM.

The official bottle of CABM is of 250 ml volume and reports the Consortium seal but each associated producer can utilize different bottles (normally of 250 or 500 ml).

Fig. 1.3: Official bottle of CABM for balsamic vinegar of Modena.

Fig. 1.4: Bordeaux red stamp of CABM: BVM 60 days aged.

Fig. 1.5: White stamp of CABM: BVM 3 years aged.

Nowadays, there are other two associations of BVM producers that work in parallel to CABM.

Recently, another consortium was formed: the CPCABM (Consorzio per la Produzione Certificata dell'Aceto Balsamico di Modena).

Other seals, supplied from CPCABM, can be found in commerce, such as:

- Brown stamp: BVM matured for at least 60 days in oak barrels;
- Green stamp: BVM produced from grape must deriving from biological agriculture;
- White and gold stamp: BVM aged for at least 3 years in oak barrels.

Fig. 1.6: Brown, green and white/gold stamps of CPCABM.

The CABM and CPCABM are the most important association of producers of balsamic vinegar of Modena, but exists, also, a committee of producers of less importance named: Committee of Independent Producers of balsamic vinegar of Modena. The symbol of this association is reported in the following figure.

Fig. 1.7: Stamp of Committee of Independent Producers of balsamic vinegar of Modena.

It is important to highlight that producers are not obliged to ask for the consortium certification. Italian market admits also the commercialisation of BVM without any stamp;

this does not indicate necessarily a lower quality of the products. In fact, good quality and ageing could characterize both BVM sample with white stamp or without stamp. Furthermore, within BVM with bordeaux red stamp, lower quality samples (called "first price") can be found and they are sold at lower price because essentially obtained by mixing wine vinegar, cooked grape must and caramel.

1.2.3 Production and biological aspects

Balsamic vinegar of Modena is an original and typical product whose consumption undergoes to a large increase of production; in the years between 1995 and 2003 a 300% of increase has been observed (Consonni et al., 2007). Almost 21 millions of litres of concentrated must were produced in 2005 with a corresponding amount of 60 millions of litres of vinegar. BVM is exported in more than 60 nations, and in 2003 the invoice was more than € 200 millions.
Balsamic vinegar of Modena is produced in a large scale and is obtained by adding wine vinegar to concentrated must and caramel (maximum 2% v/v) for the colour correction. In Fig. 1.8 a scheme of production of BVM is reported.

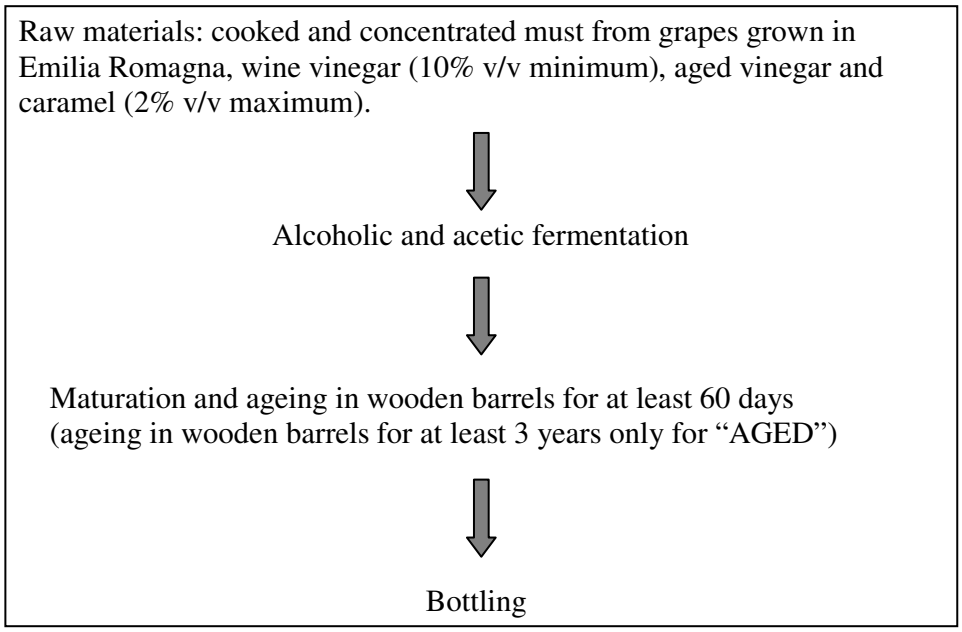


Fig. 1.8: Scheme of production of BVM.

For the production of balsamic vinegar of Modena two acetification process can be used. The first method requires a must concentration until a reduction of 1/3 of its volume, as well as for the production of traditional balsamic vinegar, and acetification by adding wine vinegar. The

second method regards the slow fermentation through wood shavings, inoculated with *Acetobacter* species with a continuous addition of concentrated must.

The microbiological aspects of the alcoholic and acetic fermentations that occur in balsamic vinegars have been recently reviewed by Turtura (2003).
In general, the microorganisms involved in the fermentation process of balsamic vinegar, both balsamic vinegar of Modena and traditional balsamic vinegar, have been isolated and characterized. The yeasts isolated were osmophylic strains with fermentative capacity, such as *Zygosaccharomyces, Schizosaccharomyces, Saccharomyces* and *Hanseniaspora*, and also with oxidative capacity, such as *Candida*.
Among the ten genera of acid acetic bacteria now recognized, vinegar oxidation as well as spoilage of wine and beer is due mainly to strains belonging to the *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* species. Recently, strains of acid acetic bacteria have been isolated from must for TBV production and identified by physiological and molecular methods. In particular strains belonging to the following species were detected: *Gluconacetobacter europaeus* (25 strains), *Gluconacetobacter hansenii* (1 strain), *Gluconacetobacter xylinus* (1 strain), *Acetobacter pasteurianus* (2 strains), *Acetobacter aceti* (1 strain) and *Acetobacter malorum* (7 strains) (Gullo et al., 2008).
Yeasts and *Acetobacter* are able to live together in the ambient of cooked and concentrated grape must, utilizing the nutritive substances in mutual symbiosis without interfere each other. Probably the alcoholic and acetic fermentations occur contemporary in balsamic vinegars. In particular, Giudici (1990) demonstrated that growth of osmophilic yeast isolated (predominantly *Zygosaccharomyces rouxii*) from balsamic vinegar was completely inhibited by 1% of acetic acid.
In musts of high concentration or in must added with wine vinegar, yeasts and *Acetobacter* are present in low quantity and in some case can be totally absent, hindering the correct development of the fermentative step in the balsamic vinegar production. The greatest obstacle to acetic acid bacteria growth was the high sugar concentration, since the majority of the isolated strains were inhibited by 25% of glucose. On the contrary, ethanol concentration of the cooked and fermented must was less significant for acetic acid bacteria growth.

Balsamic vinegar of Modena is completely different from the traditional one (TBV) both in terms of raw material and processing method, while some organoleptic characteristics could be similar, such as colour, density, taste etc. Giving an idea of this difference, a bottle of 500

ml of balsamic vinegar of Modena costs € 3, while a bottle of 100 ml of traditional balsamic vinegar costs € 60-75.

1.2.4 Chemical aspects of balsamic vinegars: principal components and state of art

It is important to consider that the legislation about BVM is very recent, and, for this reason, data found in literature about this product are very poor. So, the data about BVM are reported by comparing with those of traditional balsamic vinegar (TBV).

Sugars and furanic compounds

Sugars, in particular fructose and glucose, are the main components of balsamic vinegar of Modena, because they derive from the grape must used for the production of vinegar. In fact, grape juice contain two main sugars, fructose and glucose (Cocchi et al., 2007). The initial sugars concentration in a vinegar depends on the quantity of grape must used that is a based-experience choice of the single producer. The Production Disciplinar only fixes the minimum density of the must used for the production of BVM (d=1,24 g ml^{-1} at 20°C) and the minimum amount of cooked or concentrated grape must that can be used (20% in respect of the total mass used for production) (Gazzetta Ufficiale dell'Unione Europea, July 6, 2007). The final product must present a concentration of reducing sugars of at least 110 g L^{-1}, but a maximum is not fixed.

The sugar concentration increases during ageing of traditional balsamic vinegar, as a consequence of the progressive water evaporation in the set of barrels. For this reason, the literature data on sugar concentration and/or dry matter shows a great variability, depending on the provenience of the samples analysed. In fact, Masino (2005) found, analysing sugar amount, in a set of barrels of TBV, that sugar concentration increased from 21.3° Brix of the first cask to 72.5° Brix of the last cask.

It is important to remember that glucose and fructose are the sugars more abundant in fruit, being present in grapes practically in equal amounts (Plessi et al, 1988). Sanarico (2003) found that reducing sugars are the main components of TBV, from 43 g $100g^{-1}$ to 63 g $100g^{-1}$, with a prevalence of glucose versus fructose in almost all samples, while in the corresponding grapes and musts the two sugars are equimolar. The partially selective oxidation of fructose by TBV microorganism is responsible for these differences. Cocchi (2006) found also the

presence of other sugars in TBV samples, such as xylose, arabinose, ribose, mannose and sucrose.

It is very difficult to find in literature studies about sugars of BVM, for this reason the composition of BVM is studied by a comparison with data about sugars of TBV.

Heat treatment of food containing reducing sugars, in alkali or acid condition, triggers a sequence of non-enzymatic reactions that lead to the formation of different compounds; in particular in acid media, furan derivatives are produced through several reaction steps. Moreover, as the sugars concentration increases due to the loss of water by heating process, brown coloured products are obtained through caramelisation reaction. In both cases, the major intermediate product is 5-hydroxymethylfurfural (HMF) which may lead to the formation of furfural (Belitz et al., 2004). Among furan compounds, HMF is the most abundant in cooked must and, in general, it is found at significant levels in processed food. Besides HMF, must cooking yields some other furanic congeners: furoic acid (FA), furaldehyde (Fal) and acetoxymethylfurfural (AMFA) (Antonelli et al., 2004). HMF is often used as an index of heat treatment and of deteriorative changes in food such as tomato paste, honey and fruit juices. In addition, HMF is an indicator of adulteration of food products with acid-converted invert sugar (Theobald et al., 1998). Another possible source of HMF is the addition of caramel.

As said before, the heating of monosaccharides under acidic conditions gives rise to a large number of furan and pyran compounds (Belitz et al., 2004). The formation of these compounds can be explained by enolizations and dehydrating reactions of carbohydrates. The reaction pathway in acid starts slowly with enolization to important intermediates called enediols. Glucose gives rise to 1,2-enediol and fructose to 2,3-enediol (Fig. 1.9). The steps of the formation of HMF from 1,2 enediol are shown in Fig. 1.10. 2-hydroxyacetylfuran, which is preferentially formed from fructose, can be obtained starting from the corresponding 2,3-enediol by water eliminations (Fig. 1.11).

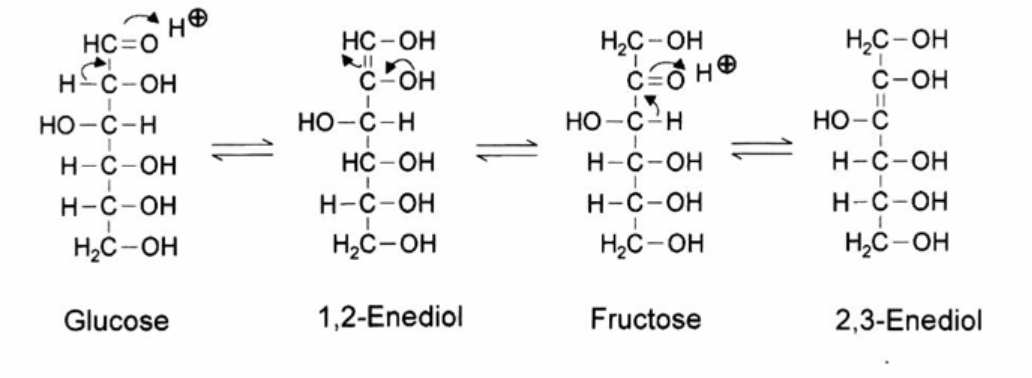


Fig. 1.9: Formation of enediols from glucose and fructose.

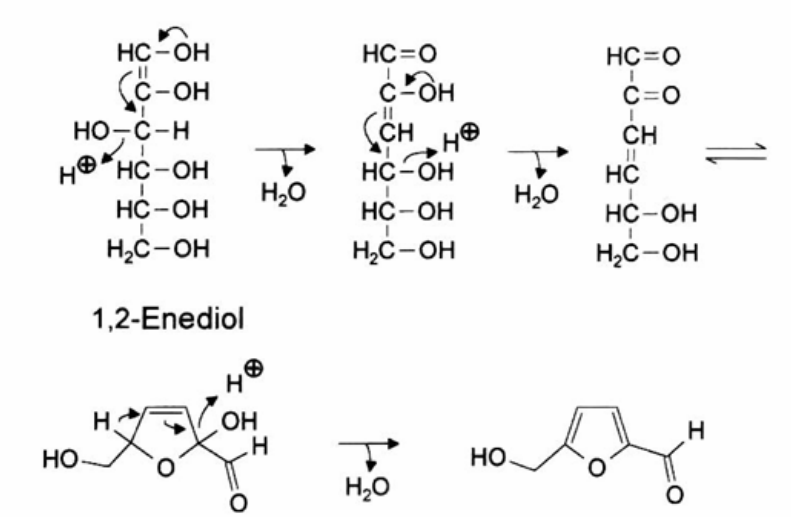


Fig. 1.10: Formation of HMF.

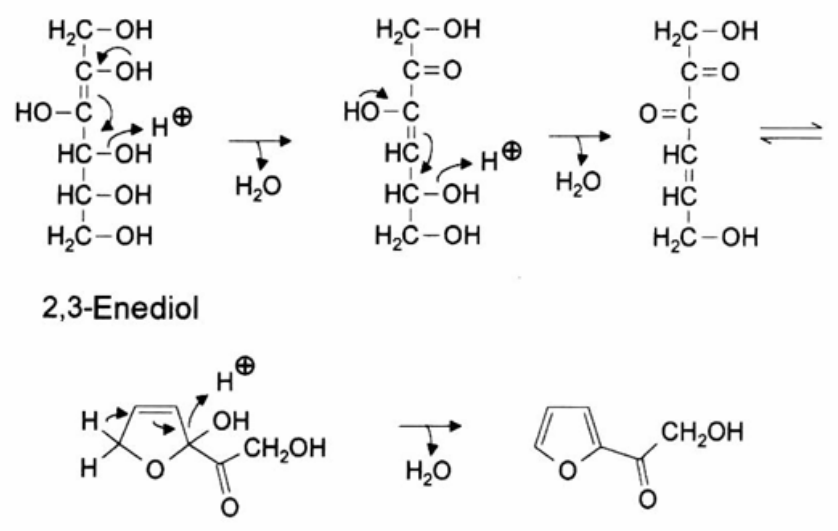


Fig. 1.11: Formation of 2-hydroxyacetylfuran.

The first investigation about the presence of furanic compounds in balsamic vinegar goes back up to 1996. Giacco (1996) found by GC-MS a non well characterized furanic compound in traditional balsamic vinegar but not in balsamic vinegar. The concentration of this compound showed also an increasing trend related to ageing. The identification of this substance was carried out later by the same authors as 5-acetoxymethylfurfural (Giacco and Del Signore, 1997). Later, in 1998, Theobald et al. analysed several samples of vinegar by HPLC, founding that in balsamic vinegar of Modena samples, HMF reached a concentration included in a range from 300 mg L^{-1} to 3.3 g L^{-1}, while in traditional balsamic vinegars samples the concentration of HMF depended on their age and reached values up to 5.5 g Kg^{-1} after a maturation time of 25 years. These high values could be due to several reasons: a high starting concentration of HMF in concentrated must, the long fermentation process and the storage in wooden barrels. In addition, the concentration due to natural evaporation with time also contributes to high values.

Del Signore (2001) obtained a discrimination of balsamic vinegars and traditional balsamic vinegars, showing that one of the most discriminating compound corresponds to 5-acetoxymethylfuraldehyde (AMF).

A significant correlation was also found between HMF and AMF, as an obvious consequence of their biochemical relation (Masino et al., 2005). AMF concentration relies not only on HMF amount, but also on bacterial activity. To better understand the trend of furanic compounds during ageing of traditional balsamic vinegar of Reggio Emilia, a multivariate statistical approach has been adopted. The PCA performed utilizing as variables hydroxymethylfurfural, furoic acid, furfural, 5-acetoxymethylfurfural, pH, total acidity and soluble solids content allowed to discriminate the different ageing time of TBV.

Acidity and organic acids

For balsamic vinegar of Modena, total acidity has to be not less than 6% (w/v) (G. U. 2007, July 6). The total acidity represents one of the most important chemical parameters of the product for both marketing and biological safety.

Total acidity is due to the contemporary presence of acetic acid and other carboxylic acids. Acetic acid is the main product of acetic fermentation, but BVM contains many other carboxylic acids which are either produced by microbial fermentation or originated directly from grapes. Qualitative and quantitative characterization of BVM and TBV organic acids could be of particular interest for the study of these products evolution during maturation and ageing and of its typicalness.

The presence of carboxylic acids in grape products has been investigated for a long time by researches, from both qualitative and quantitative point of view. The first studies on acidic fraction of balsamic vinegars were limited to the determination of the total acidity, as a sum of fixed and volatile acidity (Coppini et al., 1973, Turtura and Benfenati, 1988; Stacchini et al., 1990). The first determination of composition of organic acid in balsamic vinegar and other vinegars was made by Plessi et al. (1989) by enzimyc techniques.

Giudici (1993) found that the quantity of gluconic acid, product of the catabolism of glucose by the acetic acid bacteria, was higher for traditional balsamic vinegars than in balsamic and wine vinegars. For this reason, gluconic acid was proposed as genuineness criterion for traditional balsamic vinegars. This research was then extended to differently aged samples of traditional balsamic vinegar and to other organic acids (Giudici et al, 1994), determined by enzymic methods. Experimental results confirmed the higher value of gluconic acid in TBV (0.68-1.14g $100g^{-1}$) compared to BVM (0.20g $100g^{-1}$) and wine vinegars (0.02g $100g^{-1}$), showing also that its quantity increased during ageing. Tartaric acid was present in amounts between 0.24 and 0.86 g $100g^{-1}$ and in every set of TBV remains constant, probably in consequence of precipitation phenomena. Malic acid showed higher values for TBV (0.89-1.32 g $100g^{-1}$) compared with BVM (0.27g $100g^{-1}$) and wine vinegar (0.08g $100g^{-1}$). Lactic acid, both D- and L- forms, was detected for TBV at very high concentrations (about 2g $100g^{-1}$ of each form) remaining quite constant during ageing.

Cocchi (2002) determined the amounts of different organic acids in several TBV samples of different ages both by HPLC and GC techniques, founding that tartaric acid was present in high concentration showing a decreasing passing from young to old samples; citric and malic acid concentrations, on contrary, did not undergo high variations during the ageing time, remaining almost constant in the different samples; succinic acid increased in young samples and decreased in the old one, because of the reaction that forms other products such as esters.

Sanarico (2003) analysed, at the same time, sugars and organic acids in TBV of Reggio Emilia samples by HPLC, founding no correlations between the two classes of substances.

Some organic acids allowed the differentiation of vinegars produced from materials of different origin and different acetification methods. In fact, Natera te al. (2003) analysed different vinegar samples, such as wine vinegar (red and white), balsamic vinegar, apple vinegar and malt vinegar, founding that in apple vinegar, citric and malic acid were the organic acids present in largest amount. For malt vinegar, lactic acid was the only non-volatile organic acid found, while wine and balsamic vinegars were characterized by their content of tartaric acid and their relative low amount of malic acid.

More recently, Masino et al. (2005) analysing different TBV samples of different ages, found a significant correlation between total acidity and furoic acid. Acidity was also correlated with the concentration of the product (°Brix).

Alcohols and volatile substances: the flavour

When a balsamic vinegar is fully matured, it possesses numerous volatile and non-volatile organic and inorganic substances. Among the numerous alcohols found in vinegar, glycerol is present in large quantity as a by-product of the alcoholic fermentation of monosaccharides, particularly it is generated by certain varieties of osmophilic yeasts.

Glycerol helps to impart a soft, velvety flavour to the vinegar and is considered an indicator of quality for balsamic vinegar, as it is for wine vinegar.

Xylitol, which is always present in fruit, may be formed in the fermentation metabolism of aerobic yeasts from pentose such as xylose.

Plessi et al. (1988) showed that glycerol and xylitol were present in several vinegar and balsamic vinegar samples analysed, in particular they found that xylitol was present in very small amounts. They found also that ethanol was present in all samples, in accordance with its particular formation, but the quantities were always modest.

It is important to remember that in balsamic vinegar of Modena, the presence of ethanol is permitted below 1,5% (v/v) (G.U. 2007, July 6).

Volatile compounds and alcohols are generally very important because they play a primary role in the aromatic fraction of wine and vinegar.

To study the volatile fraction, samples must be free from the matrix, free of interfering substances and must be concentrated to a suitable degree for analytical detection. In fact, Gerbi et al. in 1992 found a very good results by extracting volatile compounds from vinegar by an Extrelut resin and identifying several substances by GC analyses.

The most abundant volatile compound in traditional balsamic vinegar is 2,3 butanediol (45.06-431.4 mg 100 ml^{-1}), followed by acetoin (19.18-133.79 mg 100 ml^{-1}), discovered since 1973 by Coppini et al. The very volatile compounds diacetyl and acetaldehyde were found in lower quantity (1.96-6.06 and 7.43-28.16 mg 100ml^{-1} respectively). These researches were then extended (Coppini et al., 1978) to balsamic vinegar and wine vinegar and results showed that TBV contained the highest values of all the compounds previously mentioned, followed by BVM and wine vinegar, demonstrating the prevalent effect of vinegar concentration.

In 1998, Chiavaro et al. found that the R/S acetoin ratio allowed TBV to be distinguished from BVM, in particular TBV showed very high racemisation for acetoin, not found in commercial balsamic vinegar such as BVM.

Balsamic vinegar flavour is very complex and still largely unknown. In 2002, Zeppa et al., analysed the volatile fraction of TBV of Reggio Emilia. They wanted to evaluate the wood acetification battery effect on the volatile components of the final product. They subdivided the volatile compounds in 15 groups: ketones, ethyl esters, acetates, aldehydes, alcohols, furan derivates, enolic derivates, lactones, nitrogen compounds, hydrocarbons, fatty acids, sulphur compounds, phenols, miscellaneous and also unidentified compounds. They found that in each battery, the compound concentrations produced during alcoholic fermentation decreased from the first barrel to the last one, while on the contrary, the concentration of acetic acid, oxidative ageing and Maillard reactions products increased from the first barrel to the last barrel. Moreover the wood used for casks manufacturing and their age seemed to have a significant effect on the aroma components of the final products.

More recently, Morales et al. (2004) showed that in vinegars aged in wooden barrels, the volatile compounds were enriched, as a result of two important processes: the concentration due to water lost through the wood, and the formation of new compounds, such as esters. Moreover, volatile compounds from wood can enrich the aromatic fraction of vinegar. In fact, in several aged vinegars they found the presence of vanillin, that seems to be the main marker for oak-chip aging.

Cocchi et al. (2004) found that the volatile fraction of vinegar, analysed by HS-SPME/GC technique, can be useful to characterize BVM and TBV and to show the differences between the two products. More recently, Pizarro et al. (2008) showed that it is possible to discriminate between wine vinegar, balsamic vinegar, sherry vinegar and cider vinegar by analysing the volatile fraction with the same technique.

Monitoring the evolution of the volatile organic compounds of TBV during ageing, Cocchi et al. (2008) found that compounds which concur to the volatile fraction of the product are extremely transformed from young to aged samples: the discrimination among vinegars of different age is due mainly to the different amount of acetic acid, ethyl acetate, ethanol, furfurals and other minor compounds.

Amino acids

The information about amino acidic composition of balsamic vinegars are not very abundant.

It is well known that acetic bacteria in wine use ethanol, free amino acids and ammonium as a source of carbon and energy. Some amino acids are the intermediates of some volatile compounds that can influence vinegar quality (Maestre et al., 2008). L-proline is present at high concentration in grape must and cannot be degraded in the absence of molecular oxygen; as a result, this in the most abundant amino acid in wine, wine vinegar and balsamic vinegar of Modena.

Firstly, Coppini et al. (1973) determined the hystidine content of samples aged between 15 and 100 years, showing that its quantity was higher in traditional balsamic vinegar than in must and wine (13.74-22.19 mg 100 ml^{-1}). Later, they extended this investigation evaluating the proline content of amino acid fraction (Coppini et al., 1978). The results showed that proline was more abundant in BVM (51.51 mg $100ml^{-1}$) than in TBV (27.34 mg $100ml^{-1}$). The first complete investigation on amino acids of balsamic vinegars and other vinegars was made by Erbe and Brueckner (1998) showing that the more abundant amino acids were proline and alanine. Chiavaro et al. (1998) found a correlation between D/L proline ratio and TBV age, in fact the D/L proline ratio decrease during ageing.

Free L-proline is the major amino acid in wine vinegars and high amounts of this amino acid indicate the use of grape must for vinegar production (Tesfaye et al., 2002). It is assumed that D-proline might be used as an indicator of ageing and consequently for a quality and authenticity controls. The relative high amounts of D-proline in balsamic vinegars was explained by the Maillard reaction and it is not attributable to acid-catalysed optical isomerisation of L-proline. The characterization of different types of balsamic vinegars, traditional (TBV) and industrial (BVM), has been possible by the determination of 23 amino acids by an automatic analyser and subsequent a multivariate statistical approach (Del Signore et al., 2000).

Minerals

Balsamic vinegar contains Mg, Ca, Fe, Mn, Co, Zn, Cu, and Pb (Del Signore et al., 1998).

From the multivariate statistical elaboration of Cr, Mn, Co, Ni, Cu, Zn, Cd and Pb results, the discrimination between traditional balsamic vinegar, balsamic vinegar and wine vinegar was possible.

Generally, wine and balsamic vinegar can contain relatively high levels of leads. Conversely, the lead, as other elements, may come from contamination during the vinegar production process (Ndung'u et al., 2004).

Trace elements was determined by Del Signore et al. (1998). Lead is known for its toxicity effects in living organisms; the concentration of this metal was somewhat higher if compared to the reported law limit. Manganese concentration was in the trace range. The Fe concentration was significantly high, probably because of the formation of stable complexes in solution. Cobalt was present in vinegar and TBV samples at ultra trace levels. The concentration of Cu was almost constant, passing from the youngest vinegar to the oldest one. Zinc was present at high concentration in old TBV. Cadmium is a toxic metal for human health and it was detected in the ultra trace range.

1.2.5 Authentication and quality evaluation of balsamic vinegar of Modena

As well known, the quality of food is the result of a harmonious equilibrium among parameters having a different origin: chemical, physical, biological, organoleptic (in addition to those cultural and economical). The organoleptic characteristics, and in particular flavour, are the properties which make balsamic vinegar of Modena and traditional balsamic vinegar of Modena and Reggio Emilia typical products. Italian regulations give importance to this aspect, even if the flavour of the various kind of vinegar is defined using adjectives that presuppose an exclusively subjective judgement. For example: the flavour of BVM in defined as "aromatic, pleasant and typical", while that of TBV "characteristic fragrant bouquet, complex but at the same time well blended, penetrating and persistent".

The study regarding the flavour of food is restricted because of some difficulties: to define the contribution that each component gives to the flavour and the current analytical techniques that have a lower sensibility than human olfactory organs.

There are some studies that show some possible ways for the discrimination between the balsamic vinegar of Modena, industrial product, and the traditional one. These studies were based on determination, for example, of amino acids (Del Signore et al., 2000), of D/L proline ratio or R/S acetoin ratio (Chiavaro et al., 1998), or of volatile compounds (Del Signore, 2001).

As already described, recently, headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC) and multivariate data analysis were applied to classify different vinegar types (white and red, balsamic, sherry and cider vinegar) on the basis of their

volatile profiles (Pizarro et al., 2008) or to evaluate the evolution of volatile organic compounds of different samples of traditional balsamic vinegar during the ageing (Cocchi et al., 2008).

Nowadays, unfortunately, there are not sufficient data that allow to have a satisfying determination of quality and authenticity of BVM, probably because of its recent regulation, its large production and low cost respect to TBV. For this reason, more investigations are required in order to reach this objective.

2. AIM OF THE WORK

Chapter 2: Aim of the work

Balsamic vinegar of Modena (BVM) is produced and consumed in large scale. The regulation for its production is very recent (2007) as the acquisition of P.G.I. denomination. For this reason, it is necessary to discriminate between authentic products, produced under the specific parameters fixed by laws, and the vinegars produced without rules.
The traditional balsamic vinegar (TBV) is a very interesting product, studied under several points of view, while the chemical and physical modifications that take place during the short maturation and ageing period of balsamic vinegar of Modena are not completely known. Moreover, for BVM, there are not molecular markers of origin, quality and authenticity reported in literature.

So, the aim of this work is the characterization of the chemical and physical properties of balsamic vinegar of Modena, by chromatographic analyses (GC/MS, TLC, HPLC/UV/MS) and by high resolution nuclear magnetic resonance spectroscopy (HR-NMR), in order to better understand the modifications that take place in this product during maturation and ageing.
In particular, the attention was focused to the research of some analytical parameters, according to those fixed by laws, which can allow the authentication and quality evaluation of this particular vinegar.

The work is divided in three parts. Each step of the work has been developed in order to find out some analytical parameters or methods that can allow to understand if a balsamic vinegar of Modena was really produced by observing the parameters fixed by law.

In particular, the work was divided in three principal parts:

1) Study of modifications that take place during the short maturation time of BVM: determination of total acidity, determination of fixed acidity and study of the formation of sugar acetates with consequent chemical characterization and determination by GC/MS and NMR techniques. In this way, it is possible to improve chemical information about BVM, useful to the authentication and quality evaluation of the product.

2) Research of an analytical method for qualitative and quantitative determination of caramel content by TLC/UV-Visible spectroscopy, HPLC/UV/MS and ^{1}H-NMR techniques. A method for the determination of caramel content is particularly important in order to evaluate the quality and the authenticity of a balsamic vinegar of Modena, because of the addition of caramel is allowed to a maximum of 2% in volume.

3) Characterization of the natural aromatic profile and determination of possible added flavours by HS-SPME/GC/MS technique. The addition of flavours to BVM is forbidden by law, for this reason this kind of analysis is very important in order to control and safeguard the authentic balsamic vinegars.

3. CHEMICAL MODIFICATIONS OF BALSAMIC VINEGAR OF MODENA: FORMATION OF SUGAR ESTERS

Chapter 3: Study of chemical modifications during maturation and ageing of Balsamic Vinegar of Modena: the formation of sugar esters

3.1 State of the art

Balsamic vinegar of Modena (BVM), as already described (Paragraph 1.2), is a product obtained by adding wine vinegar and caramel to concentrated or cooked grape must. BVM is a very complex product that contains several compounds such as sugars, amino acids, furanic compounds, organic acids, volatile substances and alcohols.

The several chemical and physical modifications that occur during the long ageing time of the traditional balsamic vinegar (TBV) were studied by a large number of researchers (Antonelli et al., 2004, Caligiani et al., 2007, Chiavaro et al., 1998, Cocchi et al., 2007, Plessi et al., 1989, Sanarico et al., 2003). Many physical and chemical reactions can occur also during the short maturation time of BVM, but, nowadays, these modifications are not completely studied. For this reason we are so far from a complete characterization of balsamic vinegar of Modena.

Sugars, mainly glucose and fructose, are the main components of balsamic vinegars, either of the traditional and of the industrial one. In traditional balsamic vinegars, a concentration of sugars during ageing occurs (Masino et al., 2005). A sugars concentration during ageing and maturation time is not reported for BVM, for which only the minimum sugars concentration is fixed by law.

Organic acids, as some researchers showed (Cocchi et al., 2002, Masino et al., 2005, Sanarico et al., 2003), represent an important fraction of balsamic vinegars. The most abundant organic acid is acetic acid; the other acids are present in smaller quantities: citric acid, malic acid, succinic acid, tartaric acid and lactic acid. Total acidity represents one of the more important parameters both for marketing and biological safety of the balsamic vinegars. Total acidity of BVM is fixed by law as 6g 100 ml^{-1} minimum and an eventual decrease of this parameter is not admitted.

As well known, in balsamic vinegars several esters are present. During the primary alcohol fermentation of grape juice, a number of odorous esters are formed; in fact the spontaneous fermentation of grape must involves various microbial species, that produce higher alcohols

and esters (Plata et al., 2003). These esters are essentially ethyl esters of organic acids, alcohol acetates and ethyl esters of fatty acids (Diaz-Maroto et al., 2005).
The contemporary presence of sugars and organic acids, in particular acetic acid, in balsamic vinegars could give rise to an esterification reactions that brings to the formation of non-volatile acetate esters. For this reason, it was supposed that glucose and fructose can react with acetic acid during the maturation time of BVM and the ageing of TBV.

The aim of this first part of the work is to characterize balsamic vinegar of Modena samples through the determination of total acidity, fixed acidity and to correlate the acidity variations to the formation of sugar esters, in order to improve the chemical information about this product, useful for its authentication and quality evaluation.

3.2 Materials and methods

3.2.1 Preparation of standard solutions

The esterification study and the determination and characterization of sugar acetates were carried out on a set of six reference solution prepared in laboratory. These solutions contained fructose (100, 200, 300 g L^{-1}) and acetic acid (6%, w/v) or glucose (100, 200, 300 g L^{-1}) and acetic acid (6%, w/v) in distilled water. All the solutions were subjected to an accelerated maturation by heating at 50°C in a laboratory oven, for a time ranging from 7 to 42 days. According to the Ahrrenius equation, the rate of a reaction doubles when temperature increases of 10°C: so, for example, a reference solution heated for 7 days at 50°C is comparable to a vinegar maturated two months at 20°C. This experimental plan is summarised in Table 3.1.

Table 3.1: Summary of experimental conditions.

Solution name	**Fructose ($g\ L^{-1}$)**	**Glucose ($g\ L^{-1}$)**	**Heating time (days)**	**Corresponding maturation time (months)**
Fructose 1a	100	0	7	2
Fructose 1b	100	0	14	4
Fructose 1c	100	0	21	6
Fructose 1d	100	0	42	12
Fructose 2a	200	0	7	2
Fructose 2b	200	0	14	4
Fructose 2c	200	0	21	6
Fructose 2d	200	0	42	12
Fructose 3a	300	0	7	2
Fructose 3b	300	0	14	4
Fructose 3c	300	0	21	6
Fructose 3d	300	0	42	12
Glucose 1a	0	100	7	2
Glucose 1b	0	100	14	4
Glucose 1c	0	100	21	6
Glucose 1d	0	100	42	12
Glucose 2a	0	200	7	2
Glucose 2b	0	200	14	4
Glucose 2c	0	200	21	6
Glucose 2d	0	200	42	12
Glucose 3a	0	300	7	2
Glucose 3b	0	300	14	4
Glucose 3c	0	300	21	6
Glucose 3d	0	300	42	12

3.2.2 Vinegar samples

The analyses (determination of total acidity, fixed acidity and sugar acetates amount) were carried out initially on a set of experimental balsamic vinegar of Modena "home made" having known different sugar content (4 samples) (Table 3.2); these samples were analysed at different maturation time at room temperature, 25° C (2, 4, 6, 8, 10 months).

Table 3.2: Summary of experimental BVM samples.

Sample name	Sugar content (g L^{-1})	Density (g ml^{-1})
BVM 1	120	1.06
BVM 2	150	1.07
BVM 3	200	1.10
BVM 4	350	1.17

The sugar acetates determination was also carried out on a set of commercial balsamic vinegars of Modena (9 samples) (Table 3.3), with different consortium stamps (Figure 3.1), on two sets of traditional balsamic vinegars of different ages (Table 3.4 and Table 3.5) and on a set of vinegars derived from raw materials different from grape must, such as white wine vinegar, red wine vinegar, apple vinegar, malt vinegar, rise vinegar and tomato vinegar.

Table 3.3: Summary of commercial BVM samples.

Sample name	Stamp colour	°Bx	Density (g ml^{-1})
BVM AG	/	31.7	1.12
BVM FE	/	41.0	1.17
BVM FI	Bordeaux red	23.2	1.08
BVM CO	Brown	40.0	1.16
BVM OR 1	Bordeaux red	33.2	1.13
BVM OR 2	Green	28.0	1.11
BVM MF 1	Brown	26.2	1.10
BVM MF 2	White	38.5	1.15
BVM MF 3	White/gold	51.7	1.23

Figure 3.1: Examples of commercial balsamic vinegars of Modena with different Consortium stamps.

Table 3.4: Summary of TBV samples from "acetaia Galletti".

Sample name	Age (years)	°Bx	Density (g ml^{-1})
TBV G7	7	43.5	1.10
TBV G9	9	44.5	1.08
TBV G11	11	50.5	1.14
TBV G13	13	51.5	1.15
TBV G14	14	73.75	1.28
TBV G16	16	73.25	1.29
TBV G17	17	71	1.23

Table 3.5: Summary of TBV samples from Consortium of traditional balsamic vinegar of Modena and Reggio Emilia.

Sample name	Age (years)	°Bx	Density (g ml^{-1})
TBV C16	16	53.5	1.17
TBV C17	17	48.2	1.17
TBV C18	18	51.5	1.19
TBV C19	19	55.6	1.19
TBV C20	20	65.3	1.21
TBV C21	21	63.8	1.28
TBV C22	22	62.6	1.25
TBV C23	23	65.1	1.26
TBV C31	31	67.25	1.27
TBV C36	36	65.5	1.22

3.2.3 Materials

For the organic acids analysis, standards of lactic acid, succinic acid, malic acid, tartaric acid, citric acid and glutaric acid (used as internal standard) were purchased from Sigma-Aldrich (Milan, Italy), acetic acid from Carlo Erba (Milan, Italy), Amberlite IRA-958 resin from Fluka (Milan, Italy).

For NMR analyses, standard of TSP (3-(trimethylsilyl)-propionate-d4) was purchased from Sigma-Aldrich (Milan, Italy).

For sugar acetates analyses, standard of β-D-phenylglucopyranoside, fructose and glucose were purchased from Sigma-Aldrich (Milan, Italy).

3.2.4 Determination of total acidity

The total acidity was determined by titration of 10 g of BVM with NaOH 1 M to a pH value of 8.2 and expressed as % of acetic acid (g 100 ml^{-1}).

3.2.5 Determination of fixed acidity: the organic acids

Sample preparation

For each vinegar sample, dry matter was determined by refractometry at 25° C, thus samples were diluted to 25 °brix. Then 0.2 ml of the diluted vinegar was added with the internal standard (1 ml of aqueous glutaric acid 1000 ppm). The solution was poured into a column filled with 1 ml anion exchange resin (Amberlite IRA-958) previously regenerated with 8 ml of NaOH 1,5 N and washed with distilled water to reach neutral pH. Resin was then washed with 25 ml of distilled water to clean the resin from sugars and then with 25 ml of methanol. Organic acids were recovered from the resin with 4 ml of 4 N HCl in methanol; the solution obtained was heated at 50° C for 45 minutes in order to obtain the complete esterification of the organic acids. The solution was neutralized with solid $NaHCO_3$, filtered and directly injected in GC/MS (1 µl) for the analysis on a Chirdex capillary column.

For the quantitative analyses of organic acids, Response Factors (RF) were calculated referred to glutaric acid:

- RF of lactic acid = 0.93
- RF of succinic acid = 0.71
- RF of malic acid = 0.97
- RF of tartaric acid = 0.32
- RF of citric acid = 1.55

GC/MS conditions

GC/MS was performed on an Agilent Technologies 6890N gas-chromatograph coupled to an Agilent Technologies 5973 mass spectrometer.

The analysis conditions are summarized in the following table (Table 3.6).

Table 3.6: Instrumental conditions for the determination of organic acids.

Instrumental parameter	Characteristic/value
GC conditions	
Capillary Column	CHIRASIL-DEX (J & W Scientific, 25 m x 0.25 mm, f.t. 0.25 μm)
Oven temperatures	Oven temperature increased from 50°C to 160°C, at 10°C/min after an initial hold at 50°C for 3 minutes. Final temperature is maintained for 5 minutes.
Injector mode	Split 20:1
Injector temperature	230°C
Carrier	He
Carrier flow	10 ml min^{-1}
MS conditions	
Ion source temperature	230°C
Detector temperature	230°C
Electron impact	70 eV
Acquisition mode	SIM. Selected ions: 45, 71, 90, 100, 101, 103, 115, 119, 129, 143.

3.2.6 Determination of glucose, fructose and relative acetates by GC/MS

Sample preparation

For the reference solutions 10 mg of each samples was used, while for vinegars sample variable amounts ranging from 10 mg to 100 mg were used, depending on the vinegar concentration. The samples were added with 1 ml of a solution of β-D-phenylglucopyranoside (500 ppm in distilled water) as internal standard and evaporated to dryness under vacuum. Then, the samples were dissolved in 1 ml of dimethylformamide, added with 0.6 ml of hexamethyldisilazane and 0.3 ml of trimethylchlorosilane and maintained at room temperature (25° C) for 30 minutes. In this way, the derivatisation of hydroxylic groups was obtained. The sugars and sugar acetates were analysed as trimethylsilyleters. The reaction is shown in Figure 3.2.

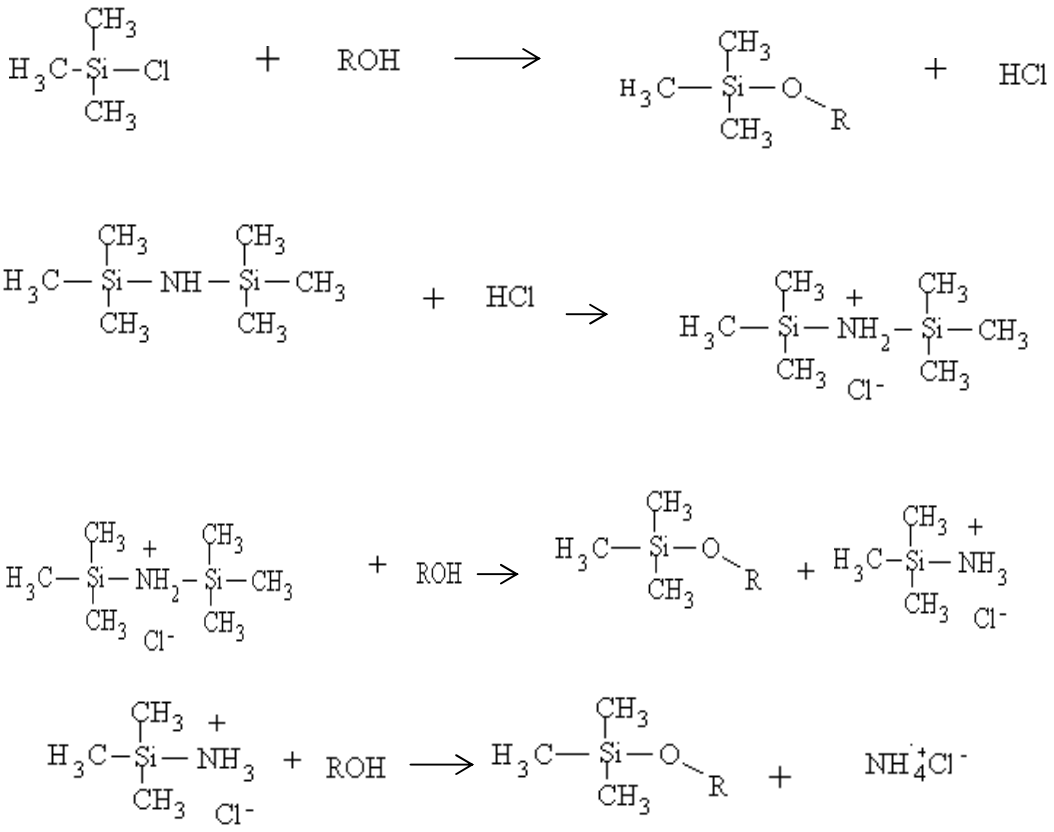


Figure 3.2: Reaction of derivatisation of hydroxylic groups.

The samples were, then, extracted with 2 ml of hexane and analysed by GC/MS, by injection of 1 μl on a SLB5 capillary column.

For the quantitative analyses of sugar acetates, the isolated fractions of fructose acetate and glucose acetate (Paragraph 3.2.7) were used in order to determine the response factor (RF) referred to β-D-phenylglucopyranoside. The RF value for fructose acetate was 0.8 and the RF value for glucose acetate was 0.9. The RF value for fructose was 1.18 and RF value for glucose was 1.43.

GC/MS conditions

GC/MS was performed on an Agilent Technologies 6890N gas-chromatograph coupled to an Agilent Technologies 5973 mass spectrometer.

The analysis conditions are summarized in the following table (Table 3.7).

Table 3.7: Instrumental conditions for the determination of sugar acetates.

Instrumental parameter	Characteristic/value
GC conditions	
Capillary Column	SLB5 (SUPELCO, 30 m x 0.25 mm, f.t. 0.25 µm)
Oven temperatures	Oven temperature increased from 60°C to 260°C, at 10°C/min after an initial hold at 60°C for 3 minutes. Final temperature is maintained for 12 minutes.
Injector mode	Split 20:1
Injector temperature	280°C
Carrier	He
Carrier flow	20 ml min^{-1}
MS conditions	
Ion source temperature	230°C
Detector temperature	280°C
Electron impact	70 eV
Acquisition mode	Full scan (m/z = 40-500)

3.2.7 Characterization of glucose and fructose acetates by NMR

Sample preparation: isolation of acetates

The reference solutions summarized in Table 3.1 were subjected to an accelerated maturation treatment by heating at 50° C in a laboratory oven for different times. The samples obtained were fractionated on a silica gel column (43-60 µm). The eluents utilized were methanol and methylene chloride (1:3). The different fractions containing fructose acetates and glucose acetates were evaporated to dryness under vacuum, dissolved in 0.8 ml of deuterated water containing, as internal standard, 0.1% solution of TSP (3-(trimethylsilyl)-propionate-d4) and analysed by NMR spectroscopy.

NMR conditions

NMR spectra were registered on a VARIAN INOVA-600 MHz spectrometer.

Instrumental parameters for registration of ^{1}H NMR and ^{13}C NMR spectra are summarised in the two following tables (Table 3.8 and 3.9)

Table 3.8: Instrumental parameters for the registration of ^{1}H NMR spectra.

Parameter	Value
Probe	Triple resonance inverse probe (^{1}H, ^{13}C, ^{15}N)
90° pulse	5.8 μs
Observed pulse (pw)	2.9 μs (45°)
Spectral width (sw)	9611.9 Hz
Spectrum data point (np)	77984
Acquisition time (at)	4.06 s
Recycle delay (d1)	1 s
Transients (nt)	32
Transmitter Power (tpwr)	63
Temperature	25°C
Sample spin	20 Hz
Transmitter Offset (tof)	576 Hz
FT size	128K

Table 3.9: Instrumental parameters for the registration of ^{13}C NMR spectra.

Parameter	Value
Probe	Nalorac
90° pulse	13 μs
Observed pulse (pw)	6.5 μs (45°)
Spectral width (sw)	37735.8 Hz
Spectrum data point (np)	128000
Acquisition time (at)	1.696 s
Recycle delay (d1)	1 s
Transients (nt)	256
Transmitter Power (tpwr)	61
Temperature	25°C
Sample spin	On
Transmitter Offset (tof)	3123.82 Hz
Decoupler Offset (dof)	-2100 Hz
Decoupler power	43
Line broadening	1 Hz
FT size	128K

3.3 Results and discussion

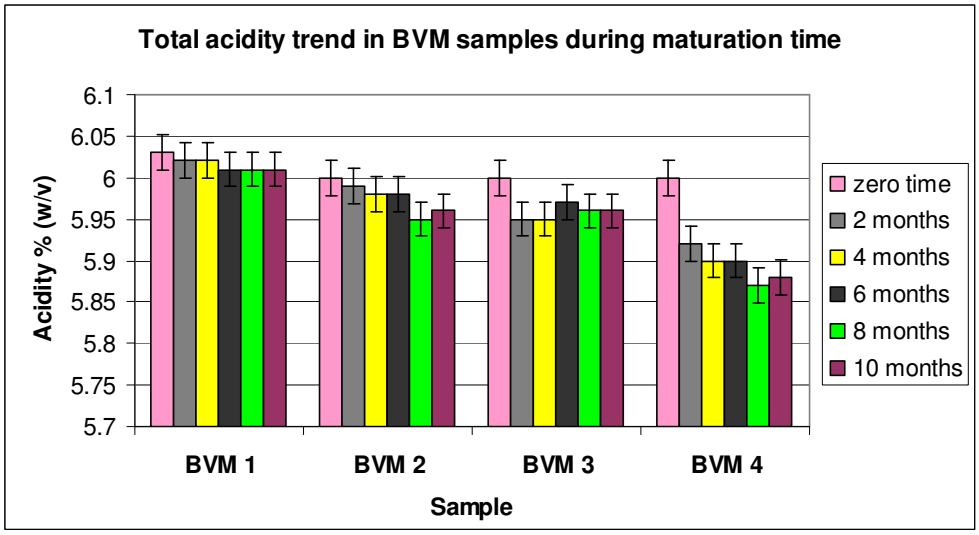
3.3.1 Determination of total acidity

The determination of total acidity was carried out on experimental balsamic vinegar samples with different sugar content (Table 3.2), every 2 months starting from their production (zero time), for a quality control of the products. Data obtained are shown in Table 3.10. The % standard deviation, as instrumental error, was also estimated, and resulted of 0.35%.

Table 3.10: Total acidity of experimental balsamic vinegar samples reported as % of acetic acid (g 100 ml^{-1}).

Sample	Zero time	2 months	4 months	6 months	8 months	10 months
BVM 1	6.03±0.02	6.02±0.02	6.02±0.02	6.01±0.02	6.01±0.02	6.01±0.02
BVM 2	6.00±0.02	5.99±0.02	5.98±0.02	5.98±0.02	5.95±0.02	5.96±0.02
BVM 3	6.00±0.02	5.95±0.02	5.95±0.02	5.97±0.02	5.96±0.02	5.96±0.02
BVM 4	6.00±0.02	5.92±0.02	5.90±0.02	5.90±0.02	5.87±0.02	5.88±0.02

The results showed that for the experimental BVM samples with different sugar content, in particular those with an high concentration of sugars, there is a reduction of the total acidity during maturation, as shown in the following graphic (Graphic 3.1).



Graphic 3.1: Total acidity trend in experimental BVM samples during maturation.

The total acidity is a very important parameter for the quality evaluation of the product. In fact, the total acidity is fixed by law (6 g 100 ml^{-1} minimum) and a decrease of this parameters is not acceptable for the commercialisation of a vinegar.

Total acidity is the result of the sum of fixed acidity and volatile acidity. In order to better understand if the observed trend was due to fixed or volatile acidity, the attention was focused on the determination of the organic acids content.

3.3.2 Determination of organic acids

The major organic acids in balsamic vinegar are malic, tartaric, citric, succinic and lactic acid (Fig. 3.3).

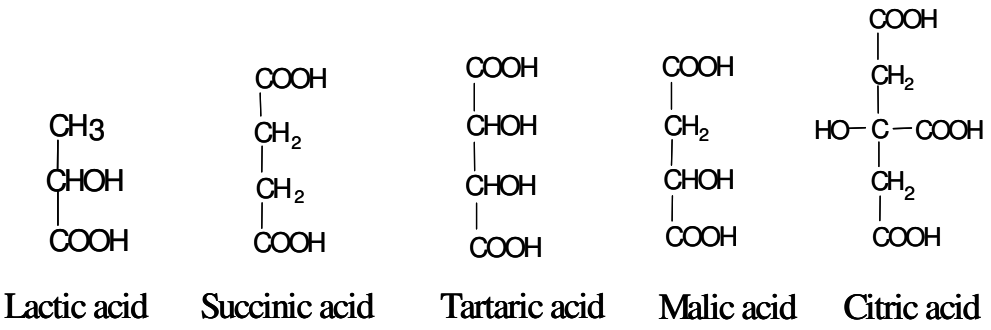


Fig. 3.3: Structures of the main organic acids in vinegar.

Evaluation of carboxylic acids by GC in BVM is often difficult because of the presence of numerous interferences that need to be removed by separation techniques. Analysis of the single acids in this matrix is difficult because of the presence of abundant sugars and phenolic compounds that interfere during the separation and quantification steps. For these reasons, it is necessary a pre-treatment of the samples in order to eliminate interferences and to enrich the solution in acids. The purification was achieved by anionic exchange column, organic acids were recovered from the column by CH_3OH/HCl, that was also the derivatization reagent. Methyl esters of organic acids were then analysed by GC/MS. Quantification was made by comparison with an internal standard (glutaric acid). Analyses were carried out, every 2 months starting from the production time, on the experimental balsamic vinegar samples with different sugar content (Table 3.2).

In Figure 3.4 a GC/MS chromatogram of the organic acids is shown.

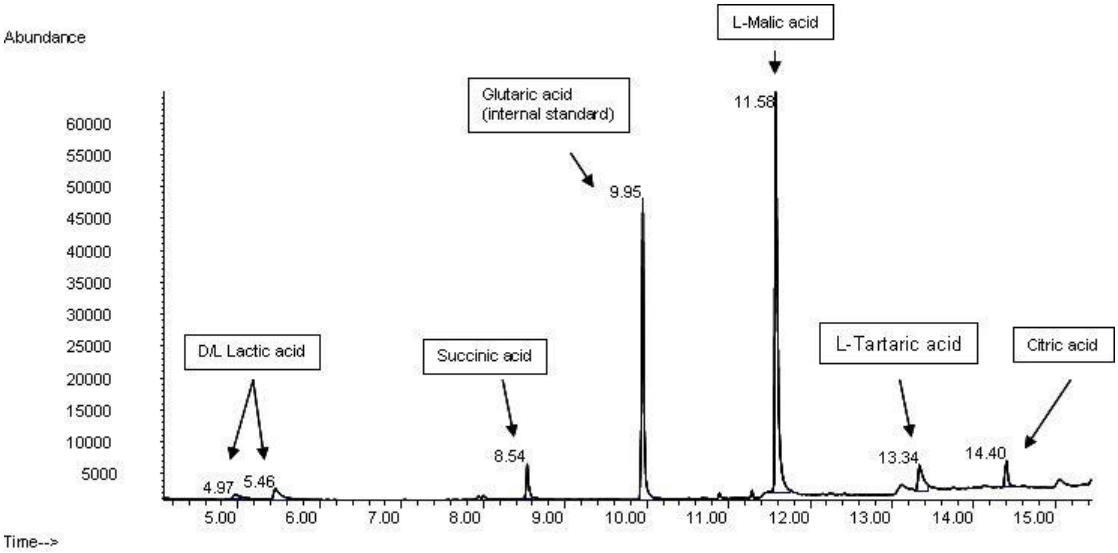


Figure 3.4: GC/MS chromatogram of the organic acids obtained from the analysis of a BVM sample.

The GC/MS chromatogram obtained from the analysis of a BVM sample, shows that the most abundant organic acid in balsamic vinegar of Modena is malic acid.

The results, reported as total sum of all organic acids present in the samples, show that there is not a significant difference between the samples at production time (zero time) and the samples at 10 months of maturation. Data are shown in Table 3.11.

Table 3.11: Total organic acids content expressed as g L^{-1} in experimental BVM samples during maturation time.

Sample	Zero time	2 months	4 months	6 months	8 months	10 months
BVM 1	3.4±02	3.0±0.1	3.4±0.2	3.6±0.2	3.2±0.2	3.1±0.2
BVM 2	3.9±0.2	3.8±0.2	4.9±0.3	5.6±0.3	4.9±0.2	4.5±0.2
BVM 3	4.2±0.2	4.6±0.2	4.5±0.2	4.9±0.3	4.3±0.2	4.4±0.2
BVM 4	10.2±0.5	10.4±0.5	9.4±0.5	10.7±0.5	10.1±0.5	10.2±0.5

In conclusion, it is possible to affirm that the total acidity reduction that occurred in these samples during maturation, was not due to a decrease of the fixed acidity but it was probably due to a decrease of the volatile acidity. The samples, during maturation time, were kept perfectly closed, then there was no possibility of loss of volatile compounds by evaporation.

For this reason, it is possible to conclude that the total acidity decrease was caused by some reactions between acetic acid and other compounds occurring during maturation of balsamic vinegar.

The next step of the work was to investigate the reactions responsible of the acidity reduction.

3.3.3 Formation of fructose and glucose acetates: characterization by GC/MS and NMR techniques

In the previous paragraph, a reduction of the total acidity in experimental balsamic vinegars during maturation was experimentally demonstrated and attributed to a decrease of the volatile acidity. Because acidity is mainly due to acetic acid, it was supposed that a decrease of acetic acid content occurred, and that this reduction was due to the reaction of acetic acid with other compounds naturally present in the matrix.

From a chemical point of view, acetic acid and alcohols can react to give esters. Because the most abundant substances containing hydroxylic groups in BVM are sugars, it was supposed that a Fisher esterification reaction occurred between sugars (fructose and glucose) and acetic acid.

In order to confirm this hypothesis, the reaction between fructose and acetic acid and glucose and acetic acid was investigated in different solutions prepared in laboratory (Table 3.1), three of which contained fructose and three contained glucose at increasing concentrations. The six solutions were analysed by GC/MS at the same day of the preparation (zero time) and then were placed in a laboratory oven at 50° C. As expected, at zero time, the GC/MS chromatograms related to sugar analysis showed, for the solutions containing fructose and acetic acid, only the characteristic signals of fructose, and for the solutions containing glucose and acetic acid only the signals characteristic of glucose.

The same analyses were repeated after seven days of heating treatment, recording the presence of others signals in the GC/MS chromatograms. These signals reported the same characteristic mass fragments of fructose or glucose (217, 204, 191, 147 m/z) and other fragments corresponding to acetyl group (43 m/z) as shown in Figure 3.5 and 3.6.

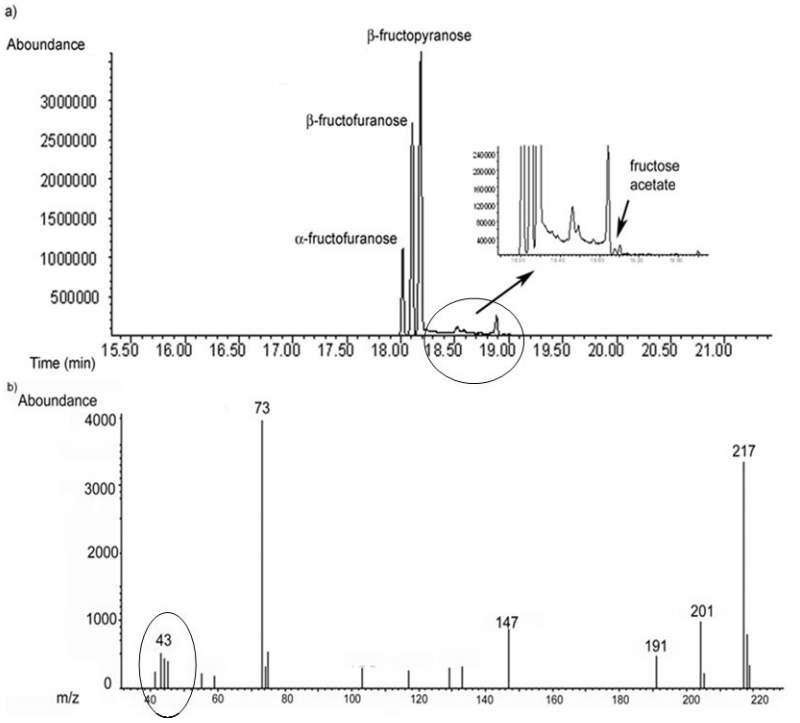


Figure 3.5: GC/MS chromatogram of a reference solutions containing fructose and acetic acid after one week heating (a) and mass spectra of fructose acetate signal (b).

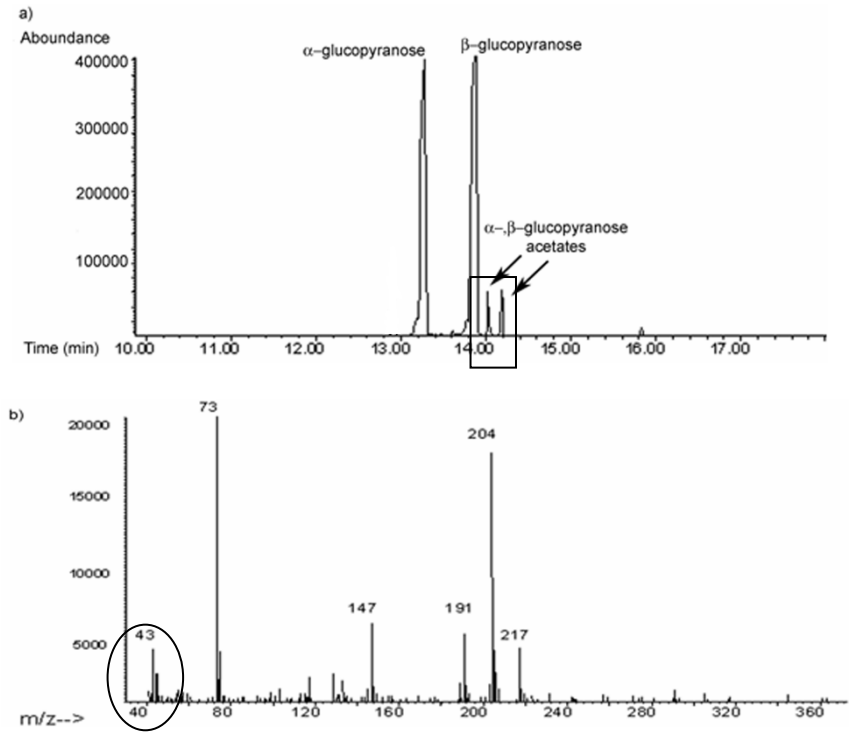


Figure 3.6: GC/MS chromatogram of a reference solutions containing glucose and acetic acid after one week heating (a) and mass spectra of glucose acetates signals (b).

Observing these GC/MS chromatograms, we can conclude that we have the presence of a signal corresponding to fructose acetate (Figure 3.5a) and two signals corresponding to glucose acetates (Figure 3.6a). For this reason, it is possible to confirm the formations of a fructose acetate and of a glucose acetate, this one in the two anomeric forms (α and β).

Quantitative analyses were carried out on every solutions after 7, 14, 21 and 42 days of heating. The results, reported in the Table 3.12, show that the concentrations of fructose and glucose acetates increase during heating treatment, but after 21 days the equilibrium was reached because the quantities of sugar acetates do not increase. Moreover, the data show that the formation of fructose or glucose acetates is strictly related to the initial amount of fructose or glucose, respectively, and to the heating time.

Table 3.12: Concentrations (g L^{-1}) of fructose and glucose acetates in reference solutions after 7, 14, 21 and 42 days of heating at 50° C.

Solution name	**Fructose (g L^{-1})**	**Glucose (g L^{-1})**	**Heating time (days)**	**Fructose acetates (g L^{-1})**	**Glucose acetates (g L^{-1})**
Fructose 1a	100	0	7	0.36±0.03	
Fructose 1b	100	0	14	0.71±0.05	
Fructose 1c	100	0	21	1.11±0.08	
Fructose 1d	100	0	42	1.10±0.08	
Fructose 2a	200	0	7	1.24±0.09	
Fructose 2b	200	0	14	1.34±0.09	
Fructose 2c	200	0	21	1.47±0.11	
Fructose 2d	200	0	42	1.48±0.11	
Fructose 3a	300	0	7	1.72±0.13	
Fructose 3b	300	0	14	2.18±0.15	
Fructose 3c	300	0	21	2.51±0.19	
Fructose 3d	300	0	42	2.58±0.18	
Glucose 1a	0	100	7		1.16±0.01
Glucose 1b	0	100	14		1.31±0.02
Glucose 1c	0	100	21		1.72±0.05
Glucose 1d	0	100	42		1.73±0.05
Glucose 2a	0	200	7		3.35±0.10
Glucose 2b	0	200	14		4.19±0.12
Glucose 2c	0	200	21		5.16±0.16
Glucose 2d	0	200	42		5.21±0.16
Glucose 3a	0	300	7		6.21±0.19
Glucose 3b	0	300	14		7.31±0.22
Glucose 3c	0	300	21		8.42±0.26
Glucose 3d	0	300	42		8.45±0.26

Starting from equivalent conditions (same heating temperature and sugar concentration), glucose acetate amounts were higher than those of fructose acetate.

In order to study the chemical structures of fructose and glucose acetates, NMR experiments were performed. The reference solutions containing fructose or glucose acetates were fractionated on a silica gel column, as explained in Paragraph 3.2.7. In this way, the separation of sugar acetates from sugars was obtained. The samples obtained were characterized by NMR spectroscopy recording ^{1}H-NMR and ^{13}C-NMR spectra.

The ^{1}H-NMR spectrum of the isolated fraction of glucose acetates, compared with ^{1}H-NMR spectrum of glucose, showed the presence of two overlapped singlets at 2.135 and 2.145 ppm, shifted to low fields in respect to acetic acid signal (2.045 ppm). In the glucose acetates spectrum, there is also a shift to low fields of all glucose signals in respect to the corresponding signals of α and β-glucose, because of the presence of the acetyl group in the compounds. These shifts were particularly marked for signals of protons of C 6 at 3.8 ppm. For this reason, it was possible to conclude that the –OH group of glucose that preferentially reacts with acetic acid to give esters is that on C 6, so the glucose acetate formed in the reaction was really one (6-acetylglucose) in the two anomeric forms, α and β, as shown in Figure 3.8.

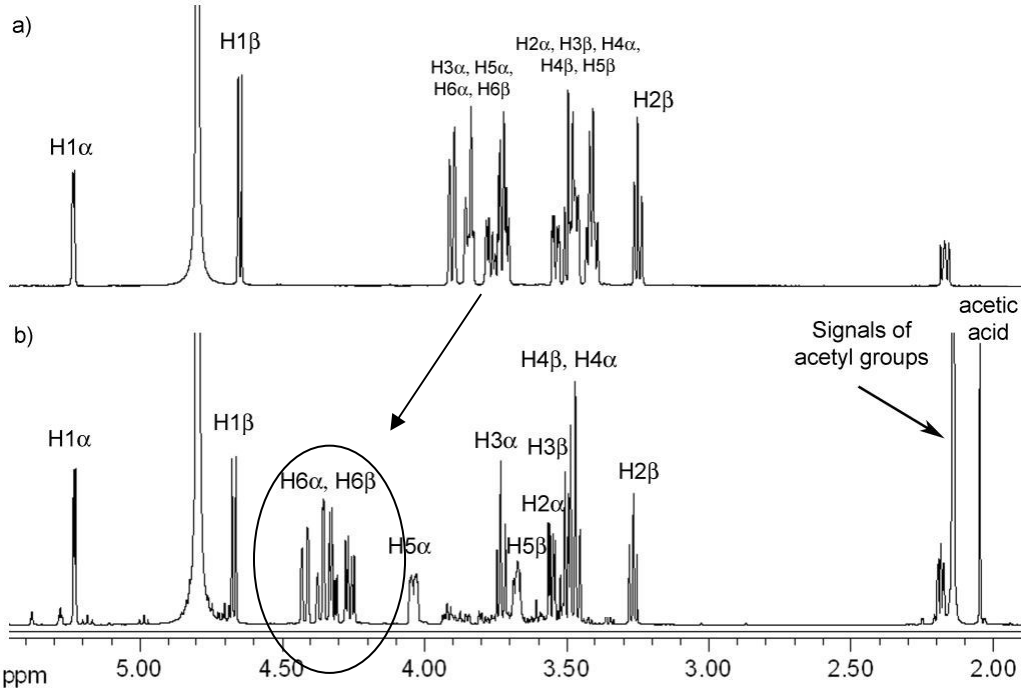


Figure 3.7: ^{1}H-NMR (600 MHz) spectrum of glucose (a) in comparison with glucose acetates spectrum (b).

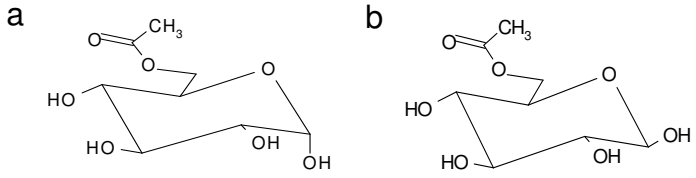


Figure 3.8: α glucose acetate (a) and β glucose acetate (b).

^{1}H-NMR spectrum of the isolated fraction of fructose acetate was also recorded, but the spectrum resulted very complicated because of the presence of many overlapped signals corresponding to different acetylation positions on fructose. For this reason, it was possible to conclude that fructose acetate compounds formed by reaction between fructose and acetic acid were more than one, as found by GC/MS technique.

In order to better understand how many compounds were formed by esterification reaction, ^{13}C-NMR spectra were registered on isolated sugar acetates fractions.

For the fraction containing glucose acetates, ^{13}C-NMR spectrum (Figure 3.9a) showed the presence of two anomeric signals, confirming the hypothesis of formation of one acetic ester of glucose in two anomeric forms. In the case of fructose acetates, the spectrum (Figure 3.9b) showed the presence of nine signals due to anomeric carbons, proving that the esterification positions of fructose were more than one.

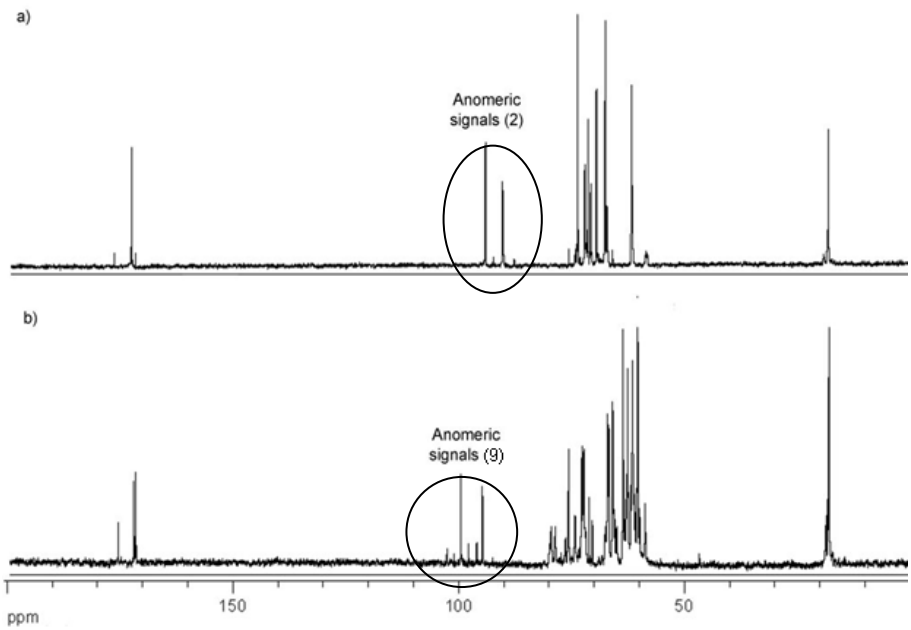


Figure 3.9: ^{13}C-NMR (600 MHz) spectrum of glucose acetate (a) and fructose acetates (b).

It is possible to conclude that there was the formation of several fructose acetates, that were difficult to be detected by GC/MS technique.

Starting from the affirmation that the formation of esters by the reaction between sugar (fructose and glucose) and acetic acid, effectively, occurred in BVM, it was investigated the possibility of the formation of other esters by the reaction between fructose or glucose with other organic acids, such as malic and tartaric acid.
The study was developed in the same way used for sugar acetates. Four solutions were prepared (Table 3.13):

Table 3.13: Concentrations (g L^{-1}) of fructose, glucose, malic acid and tartaric acid in reference solutions.

Solution name	**Fructose (g L^{-1})**	**Glucose (g L^{-1})**	**Malic acid (g L^{-1})**	**Tarataric acid (g L^{-1})**
Fructose M	200	0	40	0
Fructose T	200	0	0	40
Glucose M	0	200	40	0
Glucose T	0	200	0	40

The concentration of sugar used for the reference solutions was chosen in order to simulate a real sugar concentration of a BVM, while the concentration of malic and tartaric acid was chosen in order to have an excess of organic acids in the reference solutions.
These solutions were placed in a laboratory oven at 50° C for one month. The samples were analysed by GC/MS at time zero and after the heating treatment.
In this case, after one month of heating treatment, it was not possible to detect any reaction product, in fact, the chromatograms of the starting solutions were perfectly overlapped with those of treated solutions (Figures 3.10, 3.11, 3.12 and 3.13). For this reason, it was possible to conclude that there was no evidence of the formation of esters between fructose and malic or tartaric acid and between glucose and malic or tartaric acid, probably because these reactions were more difficult than those with acetic acid.
The GC/MS chromatograms of the reference solutions containing fructose and malic acid or glucose and malic acid overlapped with the GC/MS chromatogram of the same solutions heated at 50° C for one month are reported in the following figures, showing that GC/MS

chromatogram of the reference solutions analysed at time zero overlapped perfectly with GC/MS chromatogram of the same solutions analysed after heating treatment.

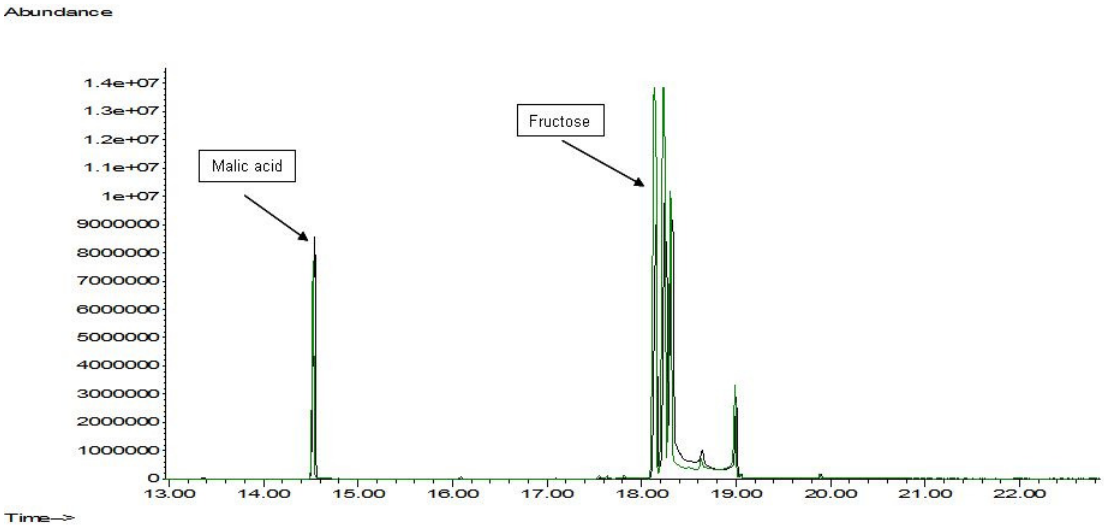


Figure 3.10: GC/MS chromatogram of a reference solutions containing fructose and malic acid (black) overlapped with the GC/MS chromatogram of the same solution heated at 50° C for one month (green).

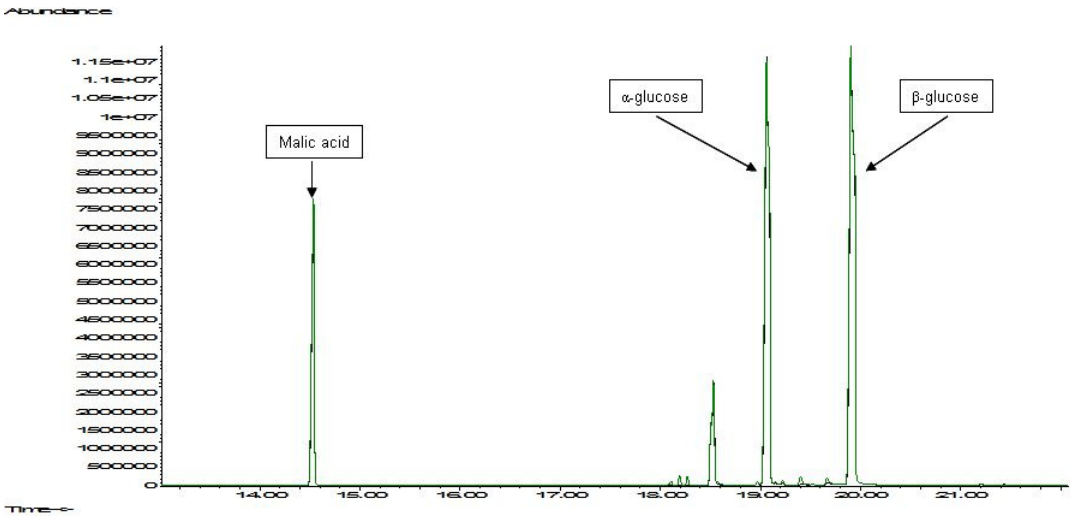


Figure 3.11: GC/MS chromatogram of a reference solutions containing glucose and malic acid (black) overlapped with the GC/MS chromatogram of the same solution heated at 50° C for one month (green).

The GC/MS chromatograms of the reference solutions containing fructose and tartaric acid or glucose and tartaric acid overlapped with the GC/MS chromatogram of the same solutions heated at 50° C for one month are reported in the following figures, showing that GC/MS chromatogram of the reference solutions analysed at time zero overlapped perfectly with GC/MS chromatogram of the same solutions analysed after heating treatment.

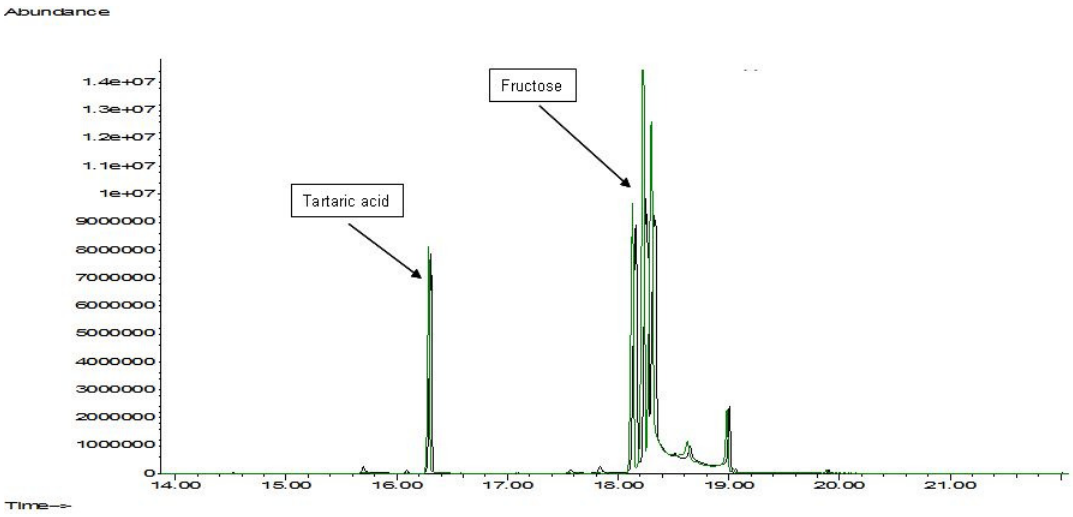


Figure 3.12: GC/MS chromatogram of a reference solutions containing fructose and tartaric acid (black) overlapped with the GC/MS chromatogram of the same solution heated at 50° C for one month (green).

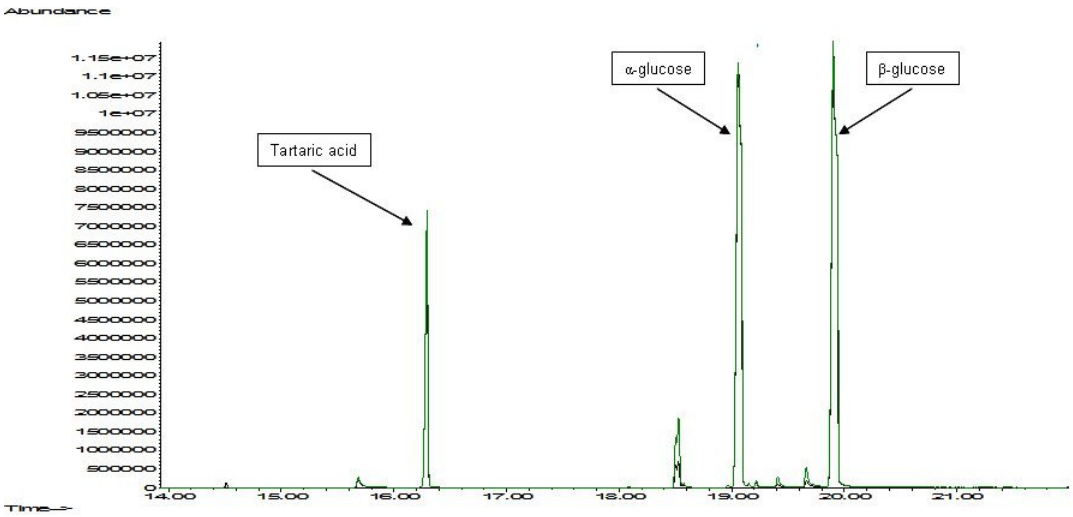


Figure 3.13: GC/MS chromatogram of a reference solutions containing glucose and tartaric acid (black) overlapped with the GC/MS chromatogram of the same solution heated at 50° C for one month (green).

3.3.4 Determination of sugar acetates in balsamic vinegar samples by GC/MS analysis

The determination of fructose and glucose acetates was carried out on balsamic vinegar samples and on vinegars produced using raw materials different from grape must. The technique used for these analyses was the GC/MS, as described in paragraph 3.2.6.

First, the presence of sugar acetates was investigated on experimental balsamic vinegar samples. The samples were analysed during maturation in order to confirm that the esters formation was dependent by time.

The results (Table 3.14) showed that in these particular samples only glucose acetate was present and the concentration of this ester increased during maturation time, while the total acidity decreased. Moreover, the concentration of glucose acetate was related to the initial amount of sugar present in BVM samples, as found for the reference solutions.

Table 3.14: Concentrations (g L^{-1}) of glucose acetate in experimental BVM samples during their maturation.

Sample	Initial sugar content	Zero time	2 months	4 months	6 months	8 months	10 months
BVM 1	120	/	0.75±0.02	0.61±0.02	1.01±0.03	0.92±0.03	1.19±0.04
BVM 2	150	/	0.83±0.03	0.73±0.02	0.87±0.03	1.00±0.03	1.47±0.05
BVM 3	200	/	0.99±0.03	1.17±0.04	1.40±0.04	1.45±0.05	2.02±0.06
BVM 4	350	/	1.40±0.04	1.88±0.06	2.90±0.09	3.21±0.10	5.36±0.17

The study was then extended to some commercial balsamic vinegar of Modena with different colour stamps and to two samples without any stamp.

The results, reported in Table 3.15, showed that in all samples there was the presence of glucose acetates, while fructose acetates were not detectable in all vinegars. The amounts of sugar acetates was related to the sugar concentration of the samples. The concentration of glucose acetates, in particular, was higher in the two samples marked with white stamp and white/gold stamp (BVM MF 2 and BVM MF 3) that were the more aged products. It is important to remember that the white or white/gold stamp is the mark of a vinegar aged for at least three years, while the bordeaux red, the brown and the green stamps are marks of vinegar matured for 60 days. The glucose acetate amounts were less abundant in bordeaux red, green and brown BVM samples than in white and white/gold BVM samples, also,

because in the less aged samples the reaction of esterification did not reach the equilibrium, as demonstrated for the synthetic solutions, in which the equilibrium was reached after 21 days of heating treatment at 50°C, corresponding to 6 months of ageing.

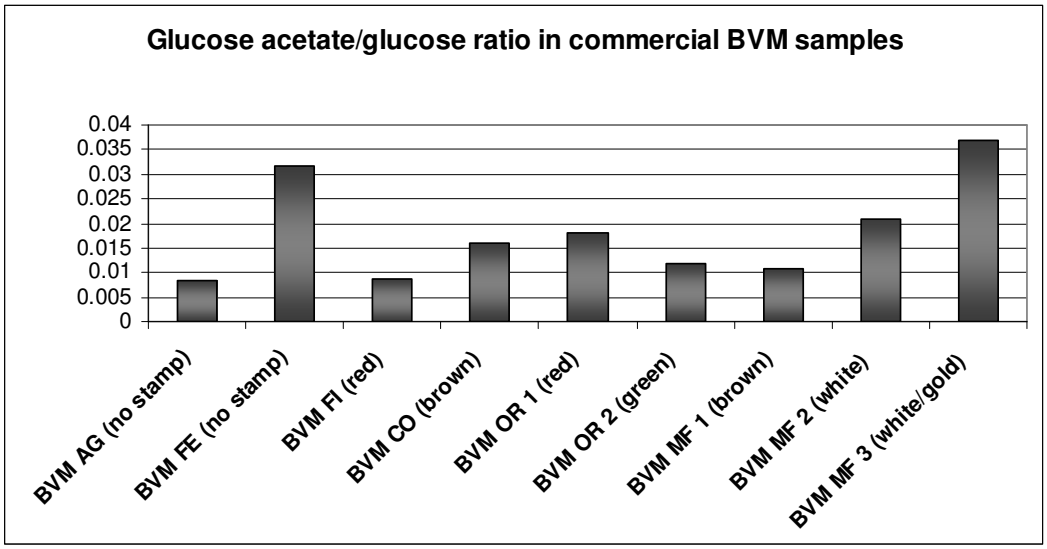
It is possible to use the glucose acetate/glucose ratio to distinguish an aged product (3 years) from a less matured product (60 days), as shown in Graphic 3.2.

For samples without any stamp, it is possible to conclude that BVM AG sample is comparable with the samples matured for 60 days, while the vinegar named BVM FE is comparable with a more aged vinegar.

Vinegars that have the same seal can present different amounts of fructose and glucose, depending on the raw materials, that are cooked and concentrated grape must, and, consequently, also different amounts of sugar acetates.

Table 3.15 Concentrations (g L-1) of fructose, fructose acetates, glucose and glucose acetate in commercial BVM samples.

Sample name	**Stamp colour**	**Fructose (g L^{-1})**	**Fructose acetates (g L^{-1})**	**Glucose (g L^{-1})**	**Glucose acetates (g L^{-1})**
BVM AG	/	109.84±7.44	/	140.78±4.45	1.19±0.04
BVM FE	/	175.00±11.85	/	165.52±5.23	5.26±0.16
BVM FI	Bordeaux red	108.14±7.32	/	111.93±3.54	0.97±0.03
BVM CO	Brown	195.08±13.21	/	176.76±5.59	2.81±0.09
BVM OR 1	Bordeaux red	153.23±10.37	0.10±0.01	135.31±4.28	2.43±0.08
BVM OR 2	Green	154.93±10.49	0.05±0.00	152.61±4.82	1.83±0.06
BVM MF 1	Brown	119.23±8.07	0.08±0.01	123.59±3.91	1.35±0.04
BVM MF 2	White	173.41±11.74	0.14±0.01	156.56±4.95	3.25±0.10
BVM MF 3	White/gold	234.00±15.84	0.18±0.01	210.86±6.66	7.77±0.24



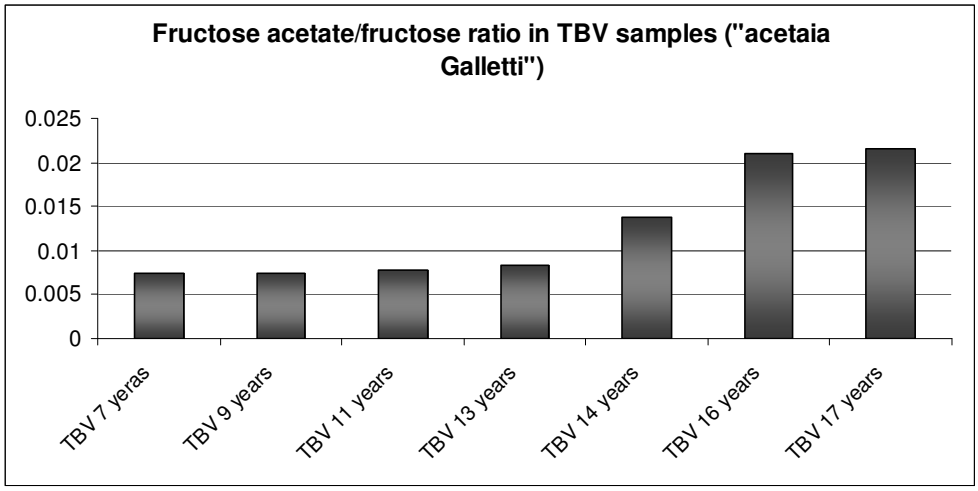
Graphic 3.2: Glucose acetate/glucose ratio in commercial BVM samples.

The determination of sugar esters was then extended to several traditional balsamic vinegars (TBV) of different ages, in order to verify the increasing of sugar acetates during a very long ageing period. In these samples an higher concentrations of glucose acetate was found in respect of BVM samples. In all samples fructose acetates were also detected. The increase of sugar acetates during ageing was not always regular. In fact for "Galletti" TBV, the fructose acetate/fructose ratio increased during ageing (Graphic 3.3), while glucose acetate/glucose ratio presented an irregular trend (Graphic 3.4), as well as for the TBV samples from Consortium in which the trends of fructose acetate/fructose and glucose acetate/glucose ratios resulted irregular (Graphic 3.5 and 3.6).

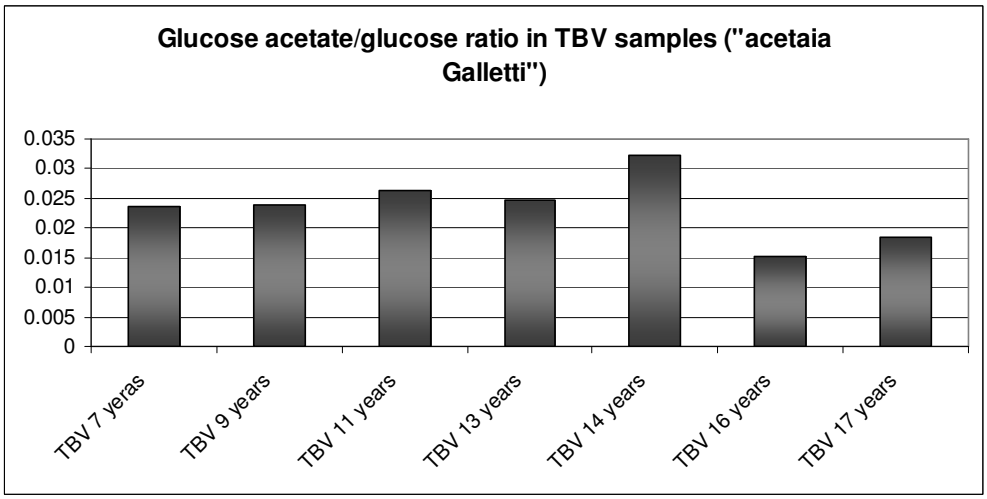
The sugar acetates concentration was related to the sugar amount of the samples. Moreover, the amount of fructose and glucose acetates seems, initially, related to sample age, but in the most aged TBV their amount seems to decrease. The higher amounts of sugar esters in TBV samples can be related to the higher concentration of sugars that the vinegar achieves during its maturation and ageing. The results are shown in Table 3.16 and 3.17.

Table 3.16: Concentrations (g L^{-1}) of fructose, fructose acetates, glucose and glucose acetates in TBV samples of different ages from "acetaia Galletti".

Sample name	Age (years)	Fructose (g L^{-1})	Fructose acetates (g L^{-1})	Glucose (g L^{-1})	Glucose acetates (g L^{-1})
TBV G7	7	112.66±7.63	0.84±0.06	130.18±4.11	3.07±0.10
TBV G9	9	129.26±8.75	0.95±0.07	143.71±4.54	3.44±0.11
TBV G11	11	146.94±9.95	1.15±0.08	174.13±5.50	4.58±0.14
TBV G13	13	164.85±11.16	1.36±0.10	199.08±6.29	4.91±0.15
TBV G14	14	208.78±14.13	2.87±0.21	232.91±7.36	7.49±0.23
TBV G16	16	208.45±14.11	4.39±0.32	489.90±15.48	7.43±0.23
TBV G17	17	187.82±12.72	4.05±0.30	288.48±9.12	5.35±0.17



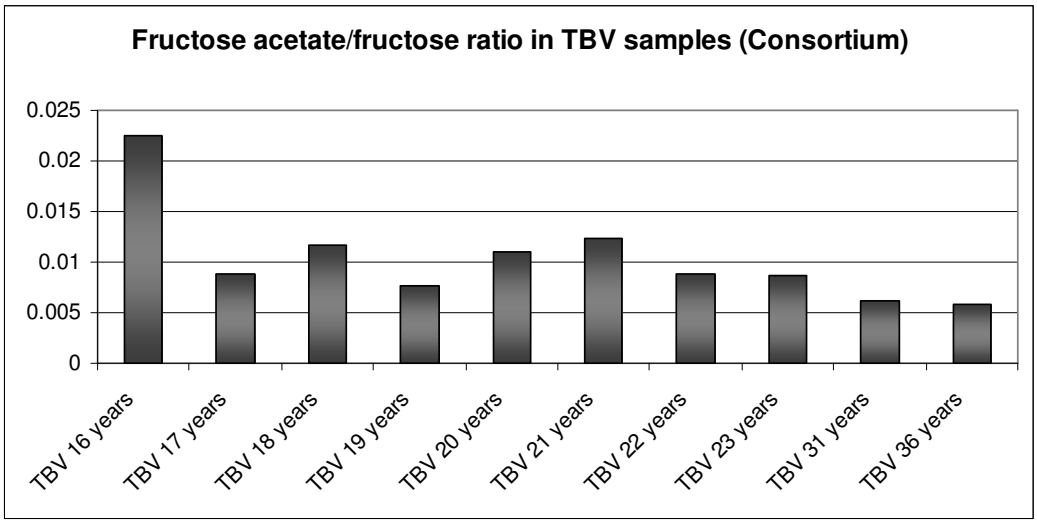
Graphic 3.3: Fructose acetate/fructose ratio in "Galletti" TBV samples of different ages.



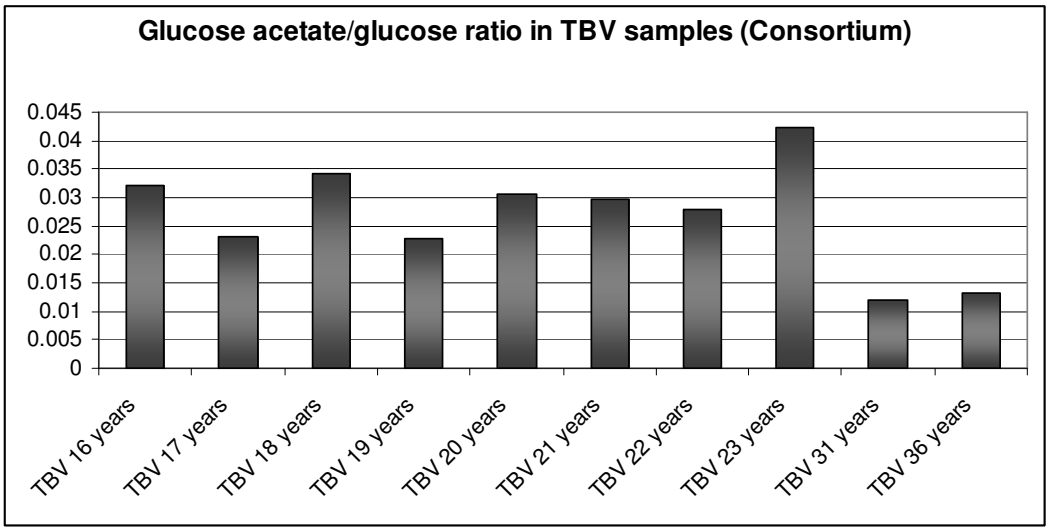
Graphic 3.4: Glucose acetate/glucose ratio in "Galletti" TBV samples of different ages.

Table 3.17: Concentrations (g L^{-1}) of fructose, fructose acetates, glucose and glucose acetates in TBV samples of different ages from Consortium of traditional balsamic vinegar of Modena and Reggio Emilia.

Sample name	Age (years)	Fructose (g L^{-1})	Fructose acetates (g L^{-1})	Glucose (g L^{-1})	Glucose acetates (g L^{-1})
TBV C16	16	197.97±13.40	4.47±0.33	244.48±7.73	7.86±0.25
TBV C17	17	181.15±12.26	1.61±0.12	222.84±7.04	5.17±0.16
TBV C18	18	116.63±7.90	1.36±0.10	139.30±4.40	4.78±0.15
TBV C19	19	177.36±12.01	1.35±0.10	212.77±6.72	4.83±0.15
TBV C20	20	140.37±9.50	1.54±0.11	172.66±5.46	5.29±0.16
TBV C21	21	138.35±9.37	1.70±0.13	171.60±5.42	5.09±0.16
TBV C22	22	147.84±10.01	1.31±0.10	186.19±5.88	5.21±0.16
TBV C23	23	143.98±9.75	1.24±0.09	151.07±4.77	6.37±0.20
TBV C31	31	205.00±13.88	1.28±0.09	272.76±8.62	3.29±0.10
TBV C36	36	166.80±11.29	0.98±0.07	247.12±7.81	3.26±0.10



Graphic 3.5: Fructose acetate/fructose ratio in "Consortium" TBV samples of different ages.



Graphic 3.6: Glucose acetate/glucose ratio in "Consortium" TBV samples of different ages

The amounts of fructose acetates were probably under estimated in all samples analysed because of the difficulty to detect all these compounds by GC/MS technique.

In order to extend these data, other vinegar samples were analysed. These vinegar samples derived from starting materials different from grape must, such as wine vinegar, apple vinegar, malt vinegar, rice vinegar and tomato vinegar.

The results showed that fructose acetates were not present in these samples, while glucose acetate was present only in the samples with an higher amount of glucose, such as malt vinegar and rice vinegar. For this reason, it is possible to conclude that the presence of glucose acetate in a vinegar is strictly related to the natural amount of glucose present in the product.

Table 3.18: Concentrations (g L^{-1}) of fructose, fructose acetates, glucose and glucose acetates in vinegar different from BVM.

Vinegar kind	**Fructose (g L^{-1})**	**Fructose acetates (g L^{-1})**	**Glucose (g L^{-1})**	**Glucose acetates (g L^{-1})**
White wine	0.89±0.06	/	0.46±0.01	/
Red wine	1.89±0.13	/	0.74±0.02	/
Apple	5.45±0.37	/	0.76±0.02	/
Malt	2.03±0.14	/	16.47±0.52	0.29±0.01
Rice	1.00±0.07	/	9.22±0.29	0.16±0.01
Tomato	6.28±0.42	/	0.86±0.03	/

3.5 Conclusions

The study confirm the formation of fructose and glucose acetates during maturation and ageing of balsamic vinegar.

The reaction investigated on reference solutions showed that the formation of acetic esters was strictly related to the initial sugar amounts and to maturation time of the solutions. The presence of fructose and glucose acetates was confirmed both by GC/MS analysis and NMR spectroscopy.

These acetic esters were determined by GC/MS on balsamic vinegar of Modena samples (experimental and commercial samples). The results showed that, also in this case, the amount of sugar esters is related to the initial amount of sugar present in the vinegar and to the maturation time of the vinegar. Moreover, a correlation between the total acidity decrease and the formation of glucose acetates in experimental BVM was found and the glucose acetate/glucose ratio could be used for the determination of the age of a not marked vinegar.

The products of the reaction between fructose and acetic acid and between glucose and acetic acid were also determined by GC/MS on two sets of traditional balsamic vinegar. The obtained data showed that also in this case the sugar acetate amounts were related to the sugar concentration of the samples, but their increase during ageing was not regular. However, higher amounts of both fructose acetates and glucose acetates were found in TBV samples in respect to BVM samples.

The presence of glucose acetate was also observed in malt vinegar and rice vinegar, confirming that the formation of this ester is strictly related to the natural amount of glucose present in the product.

Fructose acetates remained difficult to be checked by GC/MS technique.

This study is useful to understand the possible chemical modifications that occur during the maturation and ageing of balsamic vinegar of Modena.

4. DETERMINATION OF CARAMEL IN BALSAMIC VINEGAR OF MODENA

Chapter 4: Research of an analytical method for the determination of caramel in Balsamic Vinegar of Modena

4.1 State of the art

Caramel is a colouring food additive. It is the-dark brown liquid or solid material resulting from carefully controlled heat treatment of the following food-grade carbohydrates: dextrose, invert sugar, lactose, malt syrup, molasses, starch hydrolysates and fractions thereof, or sucrose.

Several kinds of caramel exist, but four classes, classified by the guideline of U. E. (Direttiva dell'Unione Europea 94/36/CE), are the principal ones. Each caramel typology is characterized by the use. The four classes of caramel are differentiated by the different food to which the caramel is added and for the production process.

Caramel of class I or E150A:

It is called "simple caramel", because it is the most natural. It is produced by cooking at 180°C sugars, such as glucose, fructose, sucrose, invert sugar, glucose syrup or dextrose. Acids, alkali or salts can be added, while the addition of ammonia, ammonia salts and sulphites is not permitted. It is used mainly for the production of liquors and cakes.

Caramel of class II or E150B:

It is called "caustic sulphite caramel". It is produced by the same method of caramel E150A, with or without addition of acids, alkali or salts, by adding sulphites such as sodium sulphite, potassium sulphite etc. The addition of ammonia or ammonia compounds is not permitted. It is used mainly for the production of liquors and ice creams.

Caramel of class III or E150C:

It is called "ammonia caramel". It is produced by the same method of caramel E150A, with or without addition of acids, alkali or salts, by adding ammonia derivates but the addition of sulphite compounds is not permitted. It is used mainly for the production of dark beers, cooked products (cakes, breads, etc.) and sauces for meat.

Caramel of class IV or E150D:

It is called "ammonia-sulphite caramel". It is produced by the same method of caramel E150A, with or without addition of acids, alkali or salts, adding ammonia salts and sulphite compounds. It is used mainly for the production of non-alcoholic drinks.

Caramel is used to colour and flavour a different range of food. The reaction conditions and the chemical reagents used for caramel production are selected in order to give it the desired characteristics. Because of the many variables in ingredients and process conditions involved in the manufacture, nowadays the exact chemical composition of caramel is not completely known.

Various low molecular weight compounds have been detected in the four kinds of caramel and some of them are considered markers of caramel colour. For example, the compound 5-(hydroxymethyl)-2-furaldehyde has been detected in all four classes of caramel (Litch et al, 1992a,b), while 4-methylimidazole has been detected in class III and in class IV (Litch et al., 1992c).

In 1997, Coffey et al., presented an HPLC/UV method for the estimation of class III caramel added to food. The analyses were carried out on biscuits and beer. They found that the food samples added with this particular kind of caramel presented a characteristic marked peak with a corresponding adsorbance at 275 nm, but no molecular information was given.

Caramel can be added to balsamic vinegar for colour correction and it can belong to all classes (D. M. 209/96, Articolo 6, Comma 2, Allegato V) even if it is more preferable an addition of caramel III or IV, that are the most dark in trade.

It is important to remember that for the balsamic vinegar of Modena (BVM) the maximum amount of caramel that can be added is fixed by law at 2% in volume (Gazzetta Ufficiale dell'Unione Europea, 2007). For this reason, it is important to find an analytical method for the quantitative determination of the caramel content in BVM, in order to control and find out the vinegars not produced with the characteristics reported by laws.

Nowadays, there is no specific method for the estimation of caramel in balsamic vinegar reported in literature. So, the aim of this work is to research an analytical method for the qualitative and quantitative determination of caramel of all the classes in balsamic vinegar of Modena, in order to evaluate the authenticity of this product and to prevent food frauds.

4.2 Materials and methods

4.2.1 Caramel and vinegar samples

Characterization of caramel was carried out on 4 caramel samples of different classes: one sample of caramel E150A (class I), one sample of caramel E150B (class II), one sample of caramel E150C (class III) and one sample of caramel E150D (class IV). In order to better understand the behaviour of the caramel colour in real samples of BVM, the analyses were carried out on 4 solutions prepared starting from a caramel free balsamic vinegar, adding the colorant of different class to this vinegar at different concentrations (from 0.2% to 3% v/v) chosen in order to simulate the real concentrations of colorant of a BVM. These solutions were used to study and perform an analytical method for the determination of caramel in real samples of BVM.

The analyses were then extended to 30 BVM samples of different producers recovered from several markets of Parma and Reggio Emilia. The samples were named "BVM" followed by a progressive number.

4.2.2 Characterization and determination of caramel by TLC and UV-Visible spectrometry

Sample preparation

The reference solutions of caramel in balsamic vinegar, the sample of caramel free balsamic vinegar and the samples of commercial BVM, were diluted in distilled water in order to obtain a final solution with 5% of dry matter. Then, 10 µl of the diluted solution were pointed up a RP-TLC plate and developed with an eluent composed of methanol and water (9/1).

For samples containing caramel, a coloured spot remains at the base of the deposition on TLC plate. The silica gel corresponding to this coloured spot was recovered and extracted with a solution of aqueous ethanol (20% v/v). The resulting solutions were filtered and analysed by UV-Visible spectrometry.

UV-Visible analyses

UV-Visible spectra were performed with a Perkin Elmer UV/Vis spectrometer Lambda Bio 20.

The samples were analysed in the range of 800-220 nm, registering the adsorbance at 275 nm (point of maximum). The temperature of the measurement cells was maintained at 25° C during the analyses.

4.2.3 Characterization of caramel by HPLC/UV/ESI/MS analysis

Sample preparation

Three samples were prepared:

- Solution 1: caramel free balsamic vinegar;
- Solution 2: caramel free balsamic vinegar added with 2% (v/v) of caramel E150A;
- Solution 3: caramel free balsamic vinegar added with 2% (v/v) of caramel E150C;

The samples were diluted with distilled water (1/10) and analysed by HPLC/UV/ESI/MS.

HPLC/UV/ESI/MS conditions

HPLC/UV/ESI/MS was performed on a Waters 2695 separation module coupled with a Waters 996 photodiode array detector, and a Waters SQ detector with ESI interface.

The analysis conditions are summarized in Table 4.1.

Table 4.1: Instrumental condition for detection of caramel.

Instrumental parameter	Characteristic/value
HPLC conditions	
Column	C18: Waters SPHERISORB 5 µm ODS2, 4.6x250 mm
Oven temperature	40° C
Mobile phase	The starting eluent was composed of methanol 90% and distilled water 10%. Initial conditions were maintained for 10 minutes. Eluent composition increased to 100% of distilled water in 30 minutes, then returned to initial condition in 10 minutes.
Injection volume	10 µl
Flow	1.0 ml min^{-1}
UV conditions	
Acquisition mode	Scan. Range: 600-220 nm
MS conditions	
ESI ion mode	Positive
Gas	Nitrogen
Capillary voltage	2.5 kV
Cone voltage	28 V
Cone pressure	80 l h^{-1}
Source temperature	150°C
Desolvation temperature	180°C
Desolvation pressure	600 l h^{-1}
Acquisition mode	Scan. Range: 100-1000 m/z

4.2.4 Characterization of caramel by ^{1}H-NMR spectroscopy

Sample preparation

Two series of three samples were prepared:

- Solution 1: caramel free balsamic vinegar;
- Solution 2: caramel free balsamic vinegar added with 2% (v/v) of caramel E150A;
- Solution 3: caramel free balsamic vinegar added with 2% (v/v) of caramel E150C or E150D;

100 µl of solution were put into a 5 mm NMR tube and added with 100 µl of a standard solution of TSP, 3-(trimethylsilyl)-propionate-d4 (1% in D_2O) purchased from Sigma-Aldrich (Milan, Italy) and 800 µl of deuterated water. The samples were analysed by ^{1}H-NMR.

NMR conditions

NMR spectra were recorded on a VARIAN INOVA-600 MHz spectrometer.

Instrumental parameters used for the registration of ^{1}H NMR spectra are summarised in Table 4.2.

Table 4.2: Instrumental parameters for registration of ^{1}H NMR spectra.

Parameter	**Value**
Probe	Triple resonance inverse probe (^{1}H, ^{13}C, ^{15}N)
90° pulse	5.8 µs
Observed pulse (pw)	2.9 µs (45°)
Spectral width (sw)	9611.9 Hz
Spectrum data point (np)	77984
Acquisition time (at)	4.06 s
Recycle delay (d1)	1 s
Transients (nt)	32
Transmitter Power (tpwr)	63
Temperature	25°C
Sample spin	20 Hz
Transmitter Offset (tof)	576 Hz
FT size	128K

4.3 Results and discussion

4.3.1 Characterization and determination of caramel by TLC and UV-Visible spectrometry

Several studies were carried out in order to determine the caramel in food, using the evaluation of compound naturally present in the colorant such as HMF, 4-methylimidazole or other compounds (Litch et al., 1992a, b, c). In the case of balsamic vinegar of Modena, it is not possible to use some molecular markers as, for example, HMF, because it is naturally present in the matrix. In fact, it is important to remember that BVM is produced starting from cooked and concentrated grape must. During cooking, the must becomes dark and dense and many chemical changes occur, due to the prolonged thermal treatment (80-95° C). Furans such as HMF can be formed (Cocchi et al, 2007). Moreover, alpha-amino acids, present in must, can undergo thermal condensation with sugars and form Maillard reaction products such as melanoidins (Piva et al., 2008). For these reasons, it is necessary to research a method for the determination of caramel in balsamic vinegar of Modena not based on these molecular markers.

In order to study the behaviour of the caramel in a complex matrix such as balsamic vinegar, four solutions containing the four different kind of caramel of each class were prepared using as starting material a caramel free balsamic vinegar. The caramel concentration used for the experiments was 2% (v/v), the maximum permitted by law in balsamic vinegar of Modena.

The first step of the experiment was to understand how to obtain a separation between the caramel colour and the coloured components of balsamic vinegar, in order to recover and determine the caramel fraction added to vinegar. The test were carried out by TLC (Reversed Phase) chromatography using several eluent compositions, such as:

- Methanol/Ethyl acetate (1/1);
- Methanol;
- Methanol/water (9/1).

The best performance was obtained using the mixture methanol/water (9/1) as eluent.

By RP-TLC chromatography, it was observed that the solutions of balsamic vinegar containing class III and class IV caramel, E150C and E150D respectively, left a brown coloured spot at the base of the deposition on silica (TLC) after the elution with aqueous methanol. Then, it is possible to conclude that caramel E150C and E150D have the same

behaviour. In Figure 4.1. the TLC plate of the solution of a balsamic vinegar added with 2% (v/v) of caramel E150C in which the brown spot is clearly visible is reported as example.

Figure 4.1: TLC of the solution of balsamic vinegar containing class III caramel at 2% (v/v) after elution with aqueous methanol.

For the caramel free balsamic vinegar sample, no coloured spot remained after elution, proving that the brown coloured spot observed for samples containing caramel E150C and E150D was effectively due to the presence of caramel. The samples of balsamic vinegar containing class I and class II caramel did not leave any coloured spot on silica after elution, showing the same result of the caramel free balsamic vinegar.

These results were probably due to the different characteristics of the four kinds of caramel, in particular the different reagents used for their production. It is important to remember that there is a significant difference between the manufacturing process used for production of class I and class II caramel and the process used for class III and IV caramel, that is the absence or the presence of ammonia. In fact, class III and class IV of caramel are produced by adding ammonia or ammonia salts, while for the production of class I and class II the addition of ammonia or ammonia compounds is forbidden. For this reason, it is possible to conclude that the brown coloured spot on silica gel left by caramel E150C and E150D is related to the use of ammonia or ammonia derivates in their production. It is probably that these compounds are the products of the reactions occuring between sugars and ammonia or ammonia compounds to form Maillard products. In caramels of class I and II it is not possible to detect Maillard products, because the addition of ammonia or ammonia derivates during the manufacturing process is forbidden.

However, by RP-TLC chromatography, it is possible to detect the presence of caramel of class III and IV in balsamic vinegar samples. This is an easy procedure to have a qualitative analysis of balsamic vinegar of Modena and it is a useful method of screening.

A quantitative determination of class III and IV caramel amount is also possible. Considering that caramel E150C and E150D have the same behaviour if analysed by RP-TLC and that they are produced in a similar way, it was decided to focus the attention on the caramel E150C.

In order to determine the E150C or E150D caramel content in real balsamic vinegar of Modena samples, standard solutions with different caramel concentration were prepared starting from the balsamic vinegar without caramel. The concentrations used are summarise in the Table 4.3.

Table 4.3: Reference solution concentrations of caramel E150C.

Solution	**% of E150C caramel (v/v)**
S. 1	0
S. 2	0.26
S. 3	0.53
S. 4	1.06
S. 5	1.51
S. 6	2.12
S. 7	2.51
S. 8	3.02

These solutions were analysed on RP-TLC. The silica gel at the base of depositions in correspondence of the brown coloured spot, was recovered and extracted with aqueous ethanol, as describe in paragraph 4.2.2.

The eight solutions obtained were analysed by UV-Visible spectrometry observing a point of maximum absorption at the wavelength of 275 nm, as shown in Figure 4.2.

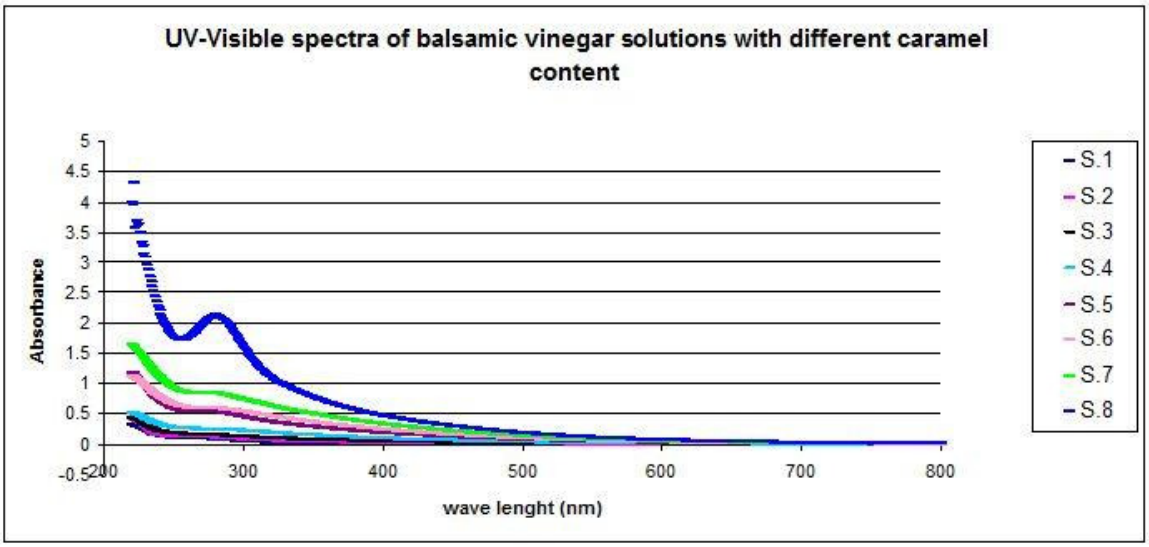


Figure 4.2: UV-Visible spectra of solutions of balsamic vinegar containing different E150C caramel concentrations.

Recording the absorbance measured at the wavelength of 275 nm, it was possible to built a calibration curve for E150C caramel.

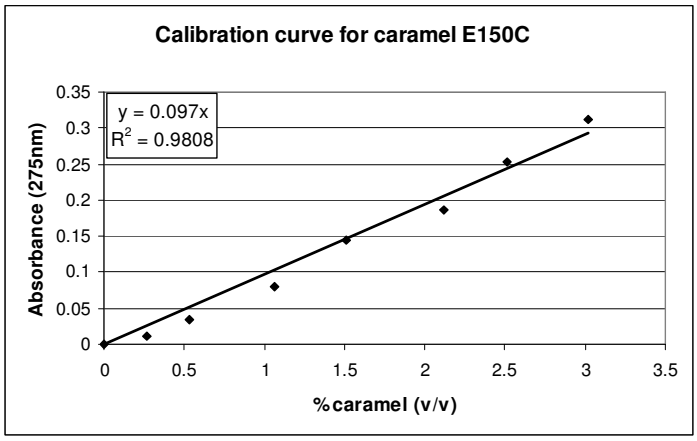


Figure 4.3: Calibration curve obtained for class III caramel (E150C).

The % standard deviation, as instrumental error, was also estimated, and resulted 0.5%.

The calibration curve obtained for class III caramel allowed to determine the caramel content in real BVM samples. Then, this particular method was applied to several commercial BVM in order to evaluate the possible presence of caramel and to evaluate the colorant content, if present.

The results, summarised in Table 4.4, showed that only three commercial BVM samples were not added with class III or IV caramel. The other samples present a caramel concentration

under the maximum permitted by low (2% v/v), except for two samples (BVM 14 and BVM 27).

Table 4.4: Caramel of class III or IV content in real BVM samples.

BVM sample	Qualitative evaluation	% caramel (v/v)
BVM 1	Negative	N.Q.*
BVM 2	Positive	1.131±0.006
BVM 3	Positive	1.025±0.005
BVM 3	Positive	0.660±0.003
BVM 4	Positive	0.525±0.003
BVM 5	Positive	0.596±0.003
BVM 6	Positive	1.597±0.008
BVM 7	Positive	0.366±0.002
BVM 8	Positive	1.446±0.007
BVM 9	Positive	1.532±0.008
BVM 10	Positive	0.997±0.005
BVM 11	Negative	N.Q.*
BVM 12	Positive	1.636±0.008
BVM 13	Positive	1.242±0.006
BVM 14	Positive	2.531±0.013
BVM 15	Negative	N.Q.*
BVM 16	Positive	1.452±0.007
BVM 17	Positive	0.873±0.004
BVM 18	Positive	1.709±0.008
BVM 19	Positive	0.967±0.005
BVM 20	Positive	1.050±0.005
BVM 21	Positive	1.621±0.008
BVM 22	Positive	0.848±0.004
BVM 23	Positive	1.682±0.008
BVM 24	Positive	1.837±0.009
BVM 25	Positive	0.451±0.002
BVM 26	Positive	0.699±0.003
BVM 27	Positive	3.312±0.016
BVM 28	Positive	1.709±0.008
BVM 29	Positive	1.442±0.007
BVM 30	Positive	1.490±0.007

* N.Q.: not quantifiable.

It is important to remember that this method can not be used for the estimation of caramel of class I and II (E150A and E150B) that do not leave any coloured spot on silica gel of TLC plate, because of their chemical composition different from that of caramels III and IV, produced by adding ammonia. For this reason, other analytical methods were investigated.

4.3.2 Characterization and determination of caramel by HPLC/UV/ESI/MS analysis

A method that could allow the characterization and the determination of all classes of caramel was investigated: HPLC/UV/ESI/MS technique.

Three solutions were prepared: one of caramel free balsamic vinegar, one by adding 2% of caramel E150A to caramel free balsamic vinegar and one by adding 2% of caramel E150C to caramel free balsamic vinegar. The solutions were diluted in distilled water (1/10 in volume) and analysed by HPLC/UV/ESI/MS.

The results showed that the sample of balsamic vinegar without caramel do not present any signal in the UV chromatogram (figure 4.4 a), while in the chromatogram of BVM added with caramel E150A a peak appeared (figure 4.4 b). In the chromatogram of BVM added with 2% of E150C caramel, there were two signals (figure 4.4 c): one of these signals corresponded to that present in the UV chromatogram of sample added with class I caramel, while the signal of lower intensity was characteristic of this kind of caramel, even if its integration and consequent estimation was difficult because of its low abundance.

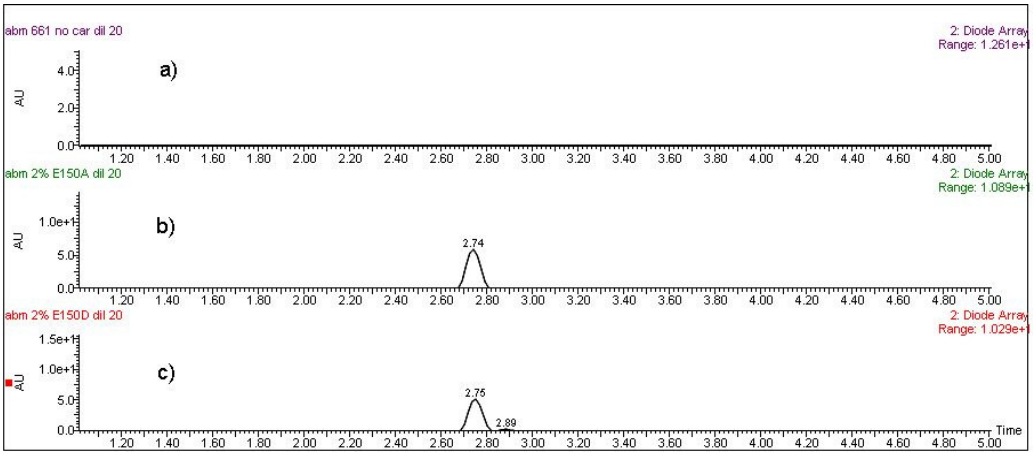


Figure 4.4: HPLC/UV chromatograms of BVM caramel free (a), of BVM added with caramel E150A (b) and of BVM added with caramel E150C (c).

The HPLC/UV technique presents an advantage in respect to the RP-TLC method coupled with UV-Visible spectroscopy, because it allows also the qualitative detection of class I caramel.

Using only HPLC coupled with UV detector, it is not possible to have any information about the compounds that caused the presence of the observed signals. The differentiation of caramels can be difficult because the observed characteristic signal of caramel E150C is few abundant and, probably, at low concentrations is not detectable.

For this reason, a MS detector coupled with HPLC/UV system with ESI interface was tested. The MS chromatograms obtained from the analyses of the BVM added with caramel E150A caramel and of the BVM added with 2% of E150C caramel were very complicated and presented a low signal at the same retention time of the corresponding signals in the UV chromatogram, as shown in Figure 4.5.

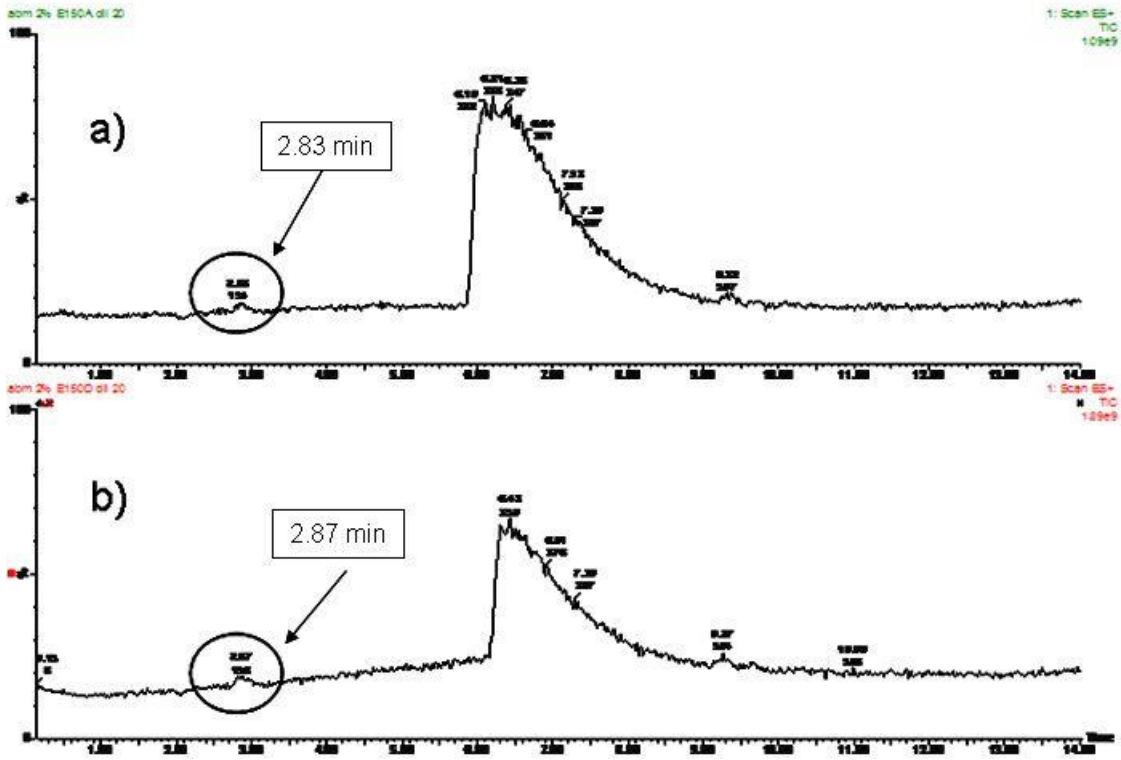


Figure 4.5: MS chromatograms of BVM added with caramel E150A (a) and of BVM added with caramel E150C (b).

The mass spectra of the signals present in the MS chromatograms, corresponding to that of UV chromatograms, are shown in Figure 4.6 and 4.7.

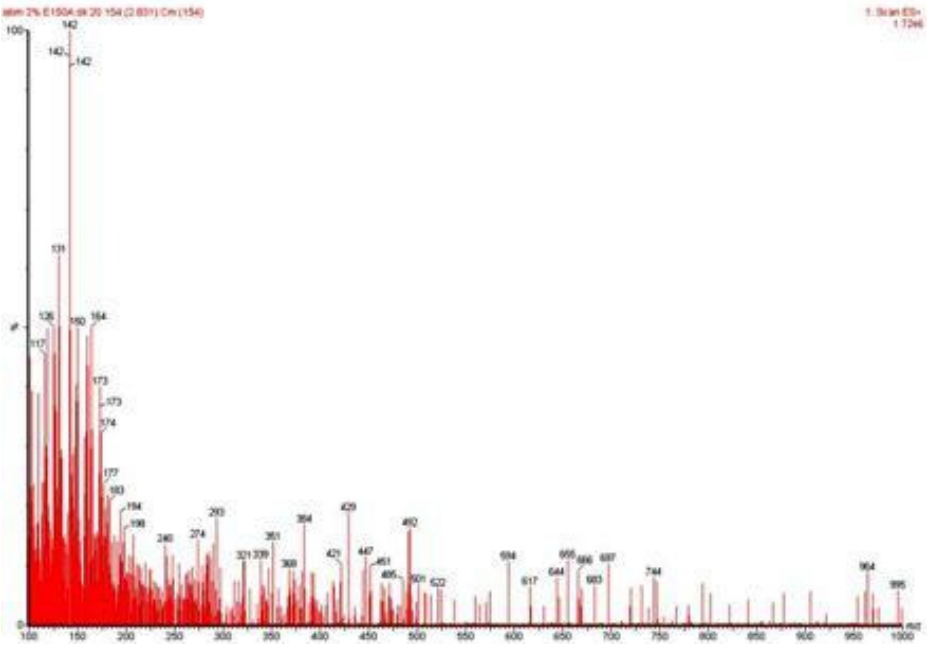


Figure 4.6: MS spectrum of the signal at retention time of 2.83 min in the MS chromatogram of BVM added with caramel E150A.

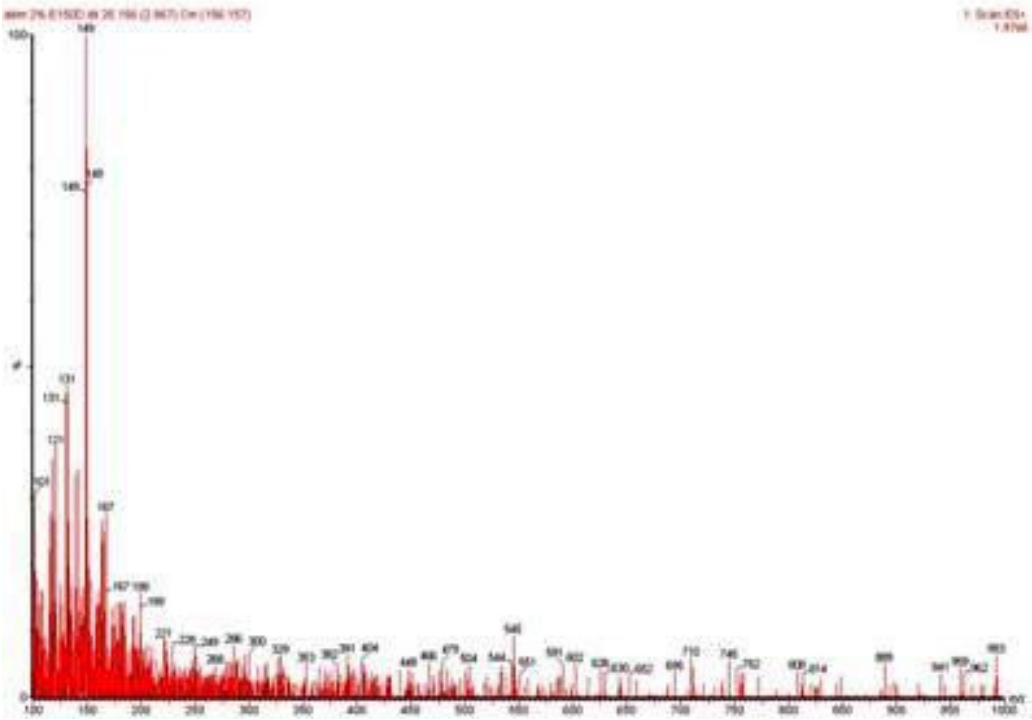


Figure 4.7: MS spectrum of the signal at retention time of 2.87 min in the MS chromatogram of BVM added with caramel E150C.

The interpretation of these spectra resulted very difficult. For this reason, it was decided to adopt another method of analysis in order to understand the chemical composition of the two kinds of caramel.

4.3.3 Characterization and determination of caramel by ^{1}H-NMR spectroscopy

It was decided to try another analytical method: ^{1}H-NMR spectroscopy.

Also in this case, it was analysed the caramel free balsamic vinegar, the caramel free balsamic vinegar added with 2% of caramel E150A and the caramel free balsamic vinegar added with 2% of caramel E150C. For each NMR analyses, 100 µl of vinegar solution were used, adding 100 µl of a standard solution of TSP (1% in D_2O) and 800 µl of deuterated water for sample preparation.

The registered spectra showed that with this method it was possible to detect caramel and, also, to have a clear differentiation between the kinds of caramel added to vinegar. In fact, by comparing the spectrum of the caramel free balsamic vinegar (figure 4.8 a) with that of the balsamic vinegar added with caramel E150A (figure 4.8 b), it is possible to observe the presence of two signals in the spectrum of the sample added with class I caramel, not presents in the spectrum of the sample caramel free. Besides, observing the spectrum of balsamic vinegar added with caramel E150C (figure 4.8 c), it is possible to find several signals not present in the spectrum of the vinegar caramel free. These signals were different from that present in spectrum of the vinegar added with class I caramel.

For this reason it is possible to conclude that this particular method allows to detect any kind of caramel and to differentiate clearly the caramels from each others. It is also possible to have some information about the chemical structure of the compounds responsible of these signals and then of the colour of the caramel. The signals appeared in the zone of the spectrum characteristic of furan compounds.

In order to confirm these results, another set of samples was prepared and analysed, starting from another sample of caramel free balsamic vinegar. In this way, it was possible to control that the signals found with the first experiments were really due to the presence of caramel in the sample and not to a matrix effect. The results showed the presence of the same signals found in the first experiment, that were two signals for the sample of caramel free balsamic vinegar added with 2% of caramel E150A (signals at 9.48 and 6.78 ppm) (Figure 4.8b) and two groups of signals for the sample of caramel free balsamic vinegar added with 2% of

caramel E150C or E150D (signals at about 8.57 ppm and signals between 7.31 and 7.21 ppm) (Figure 4.8c).

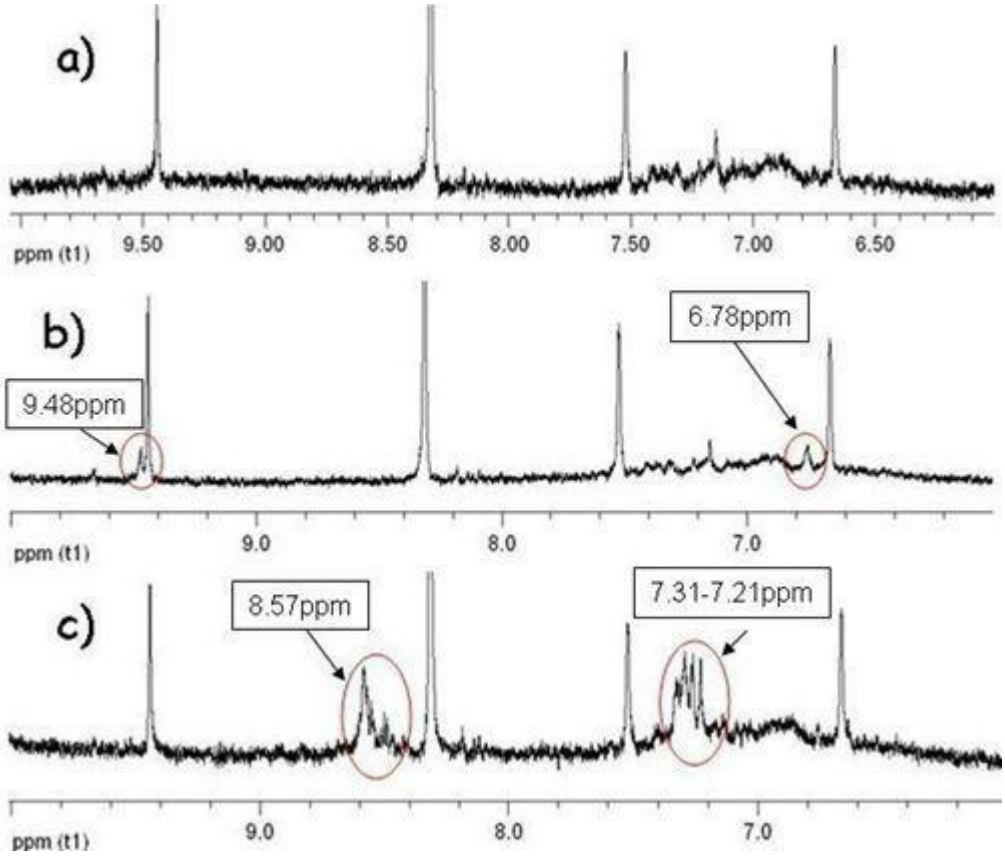


Figure 4.8: ^{1}H-NMR spectra (600 MHz) of a BVM sample caramel free (a), of a BVM added with E150A caramel (b) and of a BVM added with E150C caramel (c).

This method of analysis seems to be the more performing for caramel determination, because the sample preparation is very short and easy, the analysis time takes only few minutes and permits to have information about molecular structure of compounds that give the characteristic colour to the caramel.

4.4 Conclusions

The method of RP-TLC showed that a qualitative determination of caramels of class III and IV in balsamic vinegar of Modena was possible. This technique is very easy and can be used as a screening method. With this method a quantitative determination of caramel is also possible, after elution, recovery and extraction of silica gel at the base of deposition on TLC plate, using UV-Visible spectrometry technique. In this way only caramel E150C and E150D amount can be evaluated. Besides, this analysis technique requires several steps and for this reason is too subjected to errors.

The HPLC/UV method was more convenient, because it was possible to detect also class I caramel, but it did not allow to have some information about molecular components of each kind of caramel. Coupling HPLC/UV with an MS detector, it was possible to have chemical information about the characteristic compounds of the caramels, but the interpretation of the obtained spectra resulted very difficult.

NMR spectroscopy presented more advantages, because the sample preparation was very fast, all kinds of caramel were detected and clearly differentiated from each others and, also, this particular technique allows to have some molecular information about compounds responsible of caramel colour of different class.

In conclusion, the NMR technique seems to be the best method in order to determine qualitatively, and eventually quantitatively, caramel of every class in balsamic vinegar of Modena.

5. STUDY OF THE AROMATIC PROFILE OF BALSAMIC VINEGAR OF MODENA

Chapter 5: Characterization of the aromatic profile of Balsamic Vinegar of Modena

5.1 State of the art

Vinegar is used for flavouring and preserving a wide range of food. The chemical and organoleptic properties of a vinegar are determined by the acetification system used, the raw materials employed for the production and, in some cases, by the length of maturation and ageing time in wood. For this reasons, the flavour of balsamic vinegar depends on the raw materials, on the composition of the grape must, on the temperature of must cooking and on the compounds formed during the maturation and ageing; so it is logical to suppose that the balsamic vinegars may be characterized and differentiated by the qualitative and quantitative analysis of their volatile components.

The characterization and the determination of volatile compounds of balsamic vinegar is an important parameter for the evaluation of quality and authenticity of the product. As reported by law (Gazzetta Ufficiale dell'Unione Europea, 2007) the addition of any kind of flavours to balsamic vinegar of Modena is forbidden. Nowadays, it is possible to find in commerce sauces produced starting from balsamic vinegar. These sauces, used as meat or cheese dressing, are concentrated products, and can be added of some fruit flavours such as strawberry, raspberry, wild berries, lemon etc. For this reason, in order to safeguard the authenticity of balsamic vinegar of Modena, it is important to control, in the product, the absence of added flavours. The aromatic profile of BVM can give this kind of information.

In order to analyse the aromatic fraction of a balsamic vinegar, the most critical phase is the extraction of volatile substances. Volatile compounds are normally analysed by head space or by purge and trap methods. In 1990s, the solid-phase microextraction (SPME) has been developed. It is a fast and less expensive sample preparation method, used routinely in combination with GC or GC/MS, and successfully applied to a wide variety of compounds, specially for the extraction of volatile organic compounds from environmental, biological and food samples, including water, wine, vinegar, coffee and different fruits. Two types of SPME techniques can be used to extract analytes: head-space technique (HS-SPME), in which a special fibre is exposed to the vapour above the gaseous, liquid or solid sample, or direct immersion technique (DI-SPME), in which the fibre is directly immersed in the liquid sample.

The most important parameters affecting the SPME method are: kind of fibre employed, extraction temperature and sample volume.

As regards balsamic vinegars, several studies were carried out in order to characterize the volatile fraction. In 2001, Del Signore applied the dynamic head-space gas chromatography technique in order to characterize balsamic vinegars from Modena, dosing several volatile compounds. The data collected were elaborated with multivariate statistical techniques, allowing a good discrimination among the different kinds of vinegar, in particular traditional and industrial vinegars, because of the different concentration of the volatile substances. One year later, in 2002, Natera et all., published a validation study for the analysis of aroma compounds of vinegar with head-space solid-phase microextraction. They found that the HS-SPME technique, using a Carboxen/polydimethylsiloxane fibre, is an appropriate method for the analysis of the aroma fraction of vinegar.

Moreover, Zeppa et al., 2002, studied the effect of acetification battery in a set of wooden cask of traditional balsamic vinegar (TBV), analysing the volatile components of the products obtained with the same acetification method. They detected several compounds, extracted by C_{18} cartridge and analysed by GC/MS, such as ketones, aldehydes, ethyl esters, acetates and furan derivates from Maillard reaction, such as furfural, 5-methyl-2-furaldehyde, 5-hydroxymethylfuraldehyde or HMF, 5-acetoxymethylfurfural and 2-furoic acid.

In 2004, Cocchi et al, showed that the discrimination between traditional balsamic vinegar (TBV) and balsamic vinegar of Modena (BVM), was possible using HS-SPME technique coupled with GC/MS analysis. More recently (Cocchi et al., 2008), it was monitored, by the same technique, the evolution of the volatile organic compounds of different samples of TBV during ageing, finding a separation of vinegar samples of different age, due to the different amount of acetic acid, ethyl acetate, ethanol and furfurals. The HS-SPME extraction coupled with GC/MS technique, followed by multivariate data analysis, can also be used as a method for the classification of different vinegar types, such as wine vinegar (red and white), balsamic vinegar (traditional or industrial), sherry vinegar and cider vinegar (Pizarro et al., 2008).

Nowadays, no studies about the determination of flavour added to balsamic vinegar of Modena are reported in literature. So, the aim of this work is to characterize the aromatic profile of balsamic vinegar of Modena samples by HS-SPME extraction technique, coupled with GC/MS analysis, in order to evaluate the possible addition of flavour. The data obtained have been analysed by principal components analysis (PCA), in order to classify the BVM samples, and to obtain a quality evaluation of the product.

5.2 Materials and methods

5.2.1 Flavour and vinegar samples

In order to evaluate the possible addition of flavours to balsamic vinegar of Modena, several flavour samples were analysed by HS-SPME coupled with GC/MS technique. Previously, flavour samples were analysed in aqueous solution prepared in laboratory by adding 100 µl of flavour to 10 ml of distilled water, obtaining a flavour solution of 1% concentration. In this way, it was possible to detect the characteristic components of each flavour.

The flavour samples are summarized in the following list:

- Lemon;
- Raspberry;
- Blueberry;
- Strawberry;
- Wild berries;
- Mandarin;
- Cinnamon;
- Oak.

These flavours were recovered from a chemical industry dedicated to flavours production.

A genuine balsamic vinegar of Modena was analysed in order to detect the characteristic flavour compounds of the vinegar.

The analyses were extended to 40 balsamic vinegar of Modena samples of different origin and from different producers recovered from several markets of Parma and Reggio Emilia. These vinegars did not report any stamp or mark on the bottle. The samples were named "BVM" followed by a progressive number.

The analyses were also extended to some commercial balsamic vinegar of Modena samples with a different consortium stamp (Table 5.1).

Table 5.1: Summary of commercial BVM samples.

Sample name	Stamp colour	Vinegar kind
RED 1	Bordeaux red	BVM Fini
BROWN 1	Brown	BVM Conad
RED 2	Bordeaux red	BVM Ortalli
GREEN	Green	BVM Ortalli
BROWN 2	Brown	BVM Monari-Federzoni
WHITE	White	BVM Monari-Federzoni
W-GOLD	White/gold	BVM Monari-Federzoni

These vinegars were analysed in order to find a correlation between the aromatic profile and the time of maturation of the products and to evaluate the quality of the vinegars.

5.2.2 Determination of aromatic profile of BVM by HS-SPME and GC/MS analysis

Sample preparation

In order to characterize the flavours composition, 1% (v/v) aqueous solutions of each flavour were prepared.

For the detection of the characteristic flavour compounds in a complex matrix such as the balsamic vinegar of Modena, solutions at different concentration of each flavour were prepared, by adding the flavours to a genuine BVM "flavours free". The flavour concentrations of vinegar solutions are summarised in the following table (Table 5.2).

Table 5.2: Summary of the solution concentrations of flavours added to BVM.

Flavour	**Concentrations (v/v)**
Lemon	1%, 0.5%, 0.1%, 0.05%, 0.01%
Raspberry	1%, 0.5%, 0.1%, 0.05%, 0.01%
Blueberry	1%, 0.5%, 0.1%
Strawberry	1%, 0.5%, 0.1%, 0.05%
Wild berries	1%, 0.5%, 0.1%, 0.05%, 0.01%
Mandarin	1%, 0.5%, 0.1%, 0.05%
Cinnamon	1%, 0.5%, 0.1%, 0.05%
Oak	1%, 0.5%, 0.1%

These solutions were also analysed in order to find the lowest concentration of each flavour detectable in BVM solutions.

For each SPME analysis, 10 ml of sample (aqueous solution of flavour and natural or synthetic vinegars) were placed in a 30 ml glass vial, adding Na_2CO_3 (purchased from Fluka, Milan, Italy) necessary to reach pH 7. A small magnetic stirring bar was also added. The vial was capped with a seal with Black Viton septa and placed in a thermostatated block on a stirrer. The sample was maintained at 40° C for 30 minutes, inserting the fibre in the sample head space. During the sampling time, the sample was stirred at constant speed. The fibre was then removed from the vial and inserted into the GC/MS injector for the desorption of compounds. The fibre was kept into the GC/MS injector for 2 minutes at 220° C.

SPME fibre

The silica fibre used for this study was purchased from Supelco (Bellefonte, PA, USA). The fibre was coated with 50/30 µm of Divinylbenzene-Carboxen-Polymethylsiloxane (DVB/Carboxen/PDMS). The fibres used for the analyses were previously conditioned by inserting them into the GC/MS injector at 220° C for 2 minutes.

GC/MS conditions

GC/MS was performed on an Agilent Technologies 6890N gas-chromatograph coupled to an Agilent Technologies 5973 mass spectrometer.

The analysis conditions are summarized in the following table (Table 5.3).

Table 5.3: Instrumental conditions for the determination of aromatic profile of BVM.

Instrumental parameter	**Characteristic/value**
GC conditions	
Capillary Column	SUPELCOWAX 10 (Supelco, 30 m x 0.25 mm, f.t. 0.25 µm)
Oven temperatures	Oven temperature increased from 50°C to 200°C, at 5°C/min after an initial hold at 50°C for 3 minutes. Final temperature is maintained for 18 minutes.
Injector mode	Splitless (2 minutes)
Injector temperature	220°C
Carrier	He
Total flow	18 ml min^{-1}
MS conditions	
Ion source temperature	230°C
Detector temperature	220°C
Electron impact	70 eV
Acquisition mode	Full scan (m/z = 41-500)

Data elaboration

All data obtained were statistically analysed by the principal components analysis method (PCA), in order to check some characteristic compounds related to the maturation and ageing of vinegars.

5.3 Results and discussion

5.3.1 Determination of the characteristic compounds in flavour samples by HS-SPME and GC/MS analysis

The first step of the study was the investigation of the characteristic composition of the flavours described in Paragraph 5.2.1, such as lemon, mandarin, raspberry, blueberry, strawberry, wild berries, cinnamon and oak.

The choice of these particular flavours was based upon the flavours usually employed to produce some particular sauces containing balsamic vinegar found in several local markets.

For the characterization of each flavour, the aqueous solutions were analysed by HS-SPME and GC/MS techniques. In this way, it was possible to detect the characteristic components of the flavours.

The choice of the fibre for flavours analysis was based upon several experiments made using three kinds of fibre: the PDMS fibre (coated with Polydimethylsiloxane, non-polar material), the DVB/Carboxen/PDMS fibre (coated with Divinylbenzene-Carboxen-Polymethylsiloxane, medium polar material, used for an expanded range of analytes) and CW-PEG fibre (coated with Carbowax-Polyethylene Glycol, polar material). The best results were obtained using the DVB/Carboxen/PDMS fibre, that was able to extract both polar and non-polar compounds.

Flavour of Lemon

The flavour of lemon presented five characteristic signals corresponding to Linalool (3,7-dimethyl-1,6-octadien-3-ol), 4-terpineol (1-p-menthen-4-ol), Neral (cis-citral or (Z)-3,7-dimethyl-2,6-octadienal), α-terpineol (1-p-menthen-8-ol) and α-citral (3,7-dimethyl-2,6-octadienal). These signals were recognized by comparison of their mass spectra with library mass spectra (WILEY275).

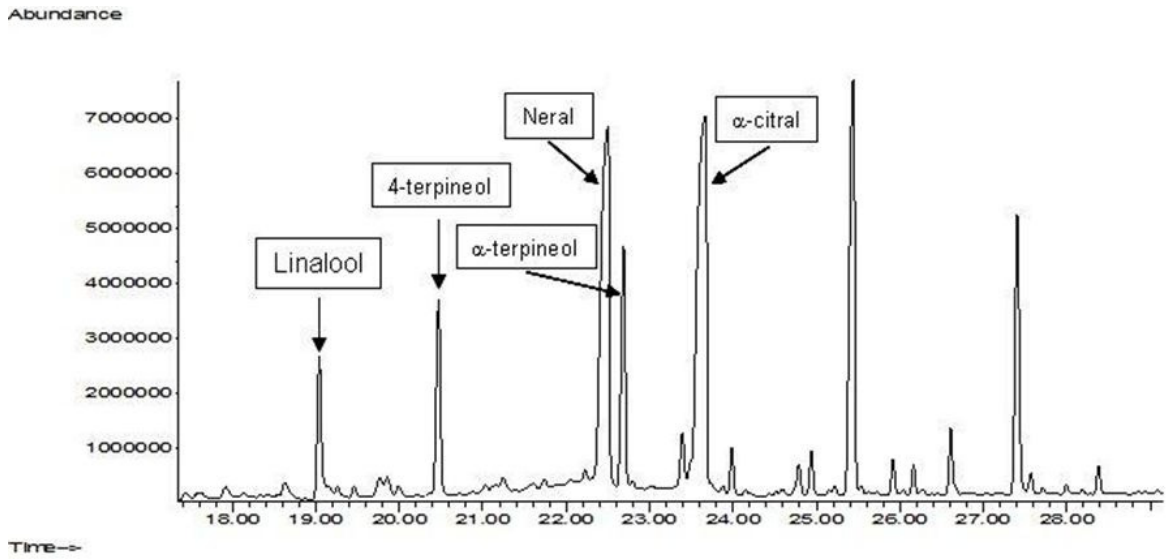


Figure 5.1: GC/MS chromatogram of head space of lemon flavour.

In the following figures the mass spectra with the relative molecular structure of the characteristic compounds of lemon flavour are reported.

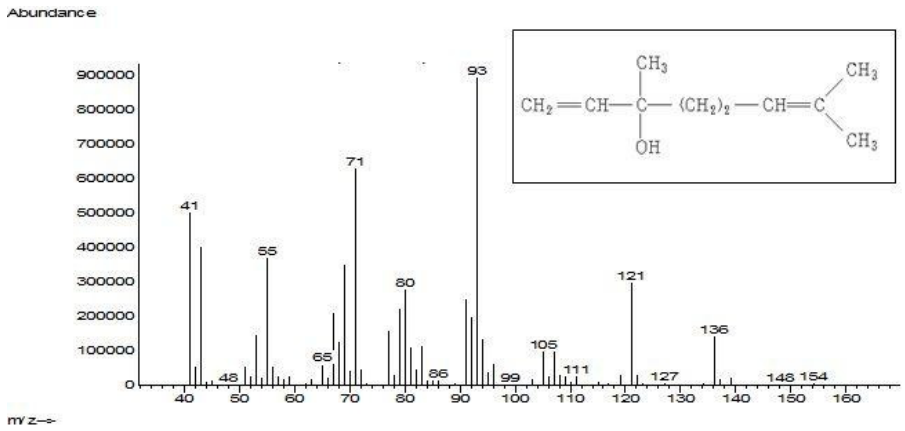


Figure 5.2: Mass spectrum of Linalool with relative molecular structure.

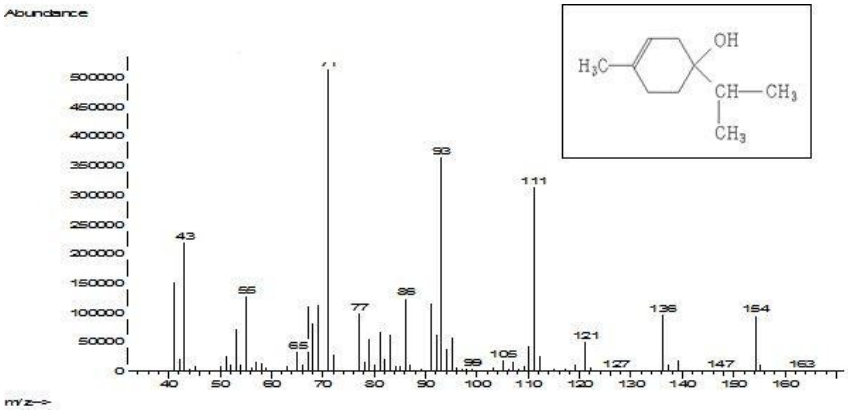


Figure 5.3: Mass spectrum of 4-terpineol with relative molecular structure.

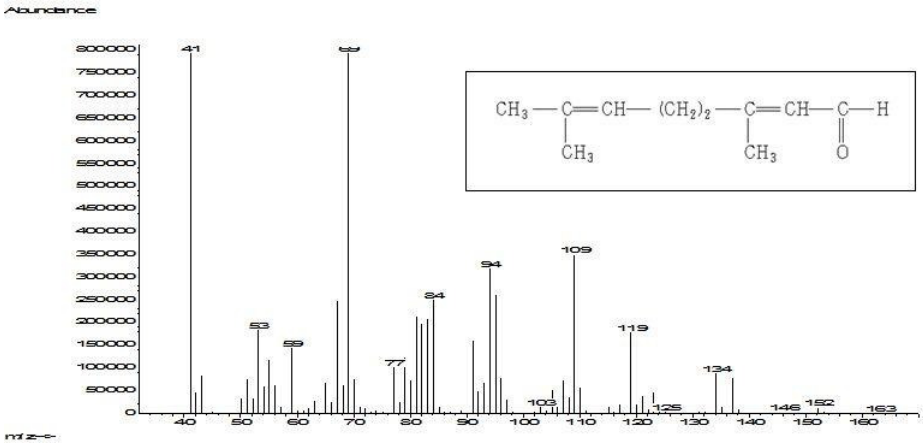


Figure 5.4: Mass spectrum of Neral with relative molecular structure.

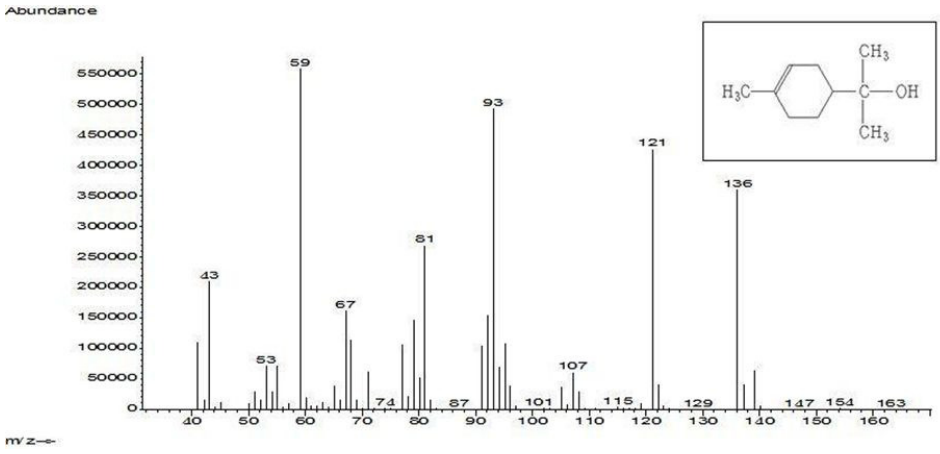


Figure 5.5: Mass spectrum of α-terpineol with relative molecular structure.

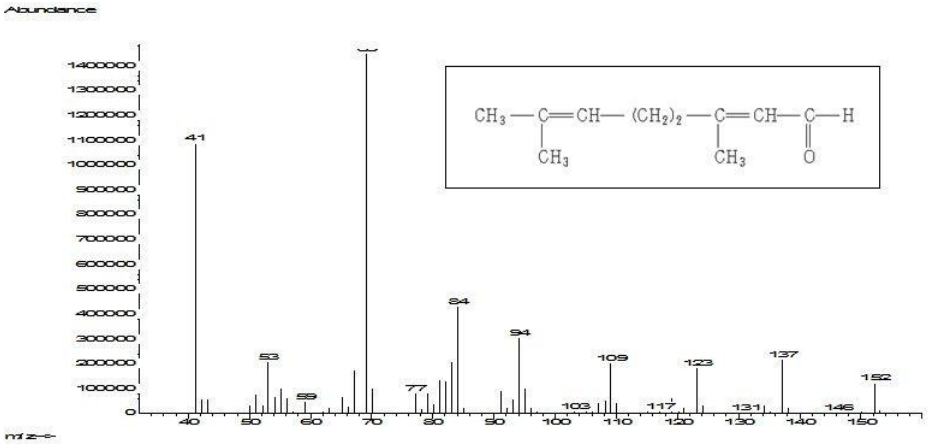


Figure 5.6: Mass spectrum of α-citral with relative molecular structure.

These identified signals, corresponding to the characteristic lemon flavour compounds, have been manually integrated, in order to find the relative percentages of each compound in the flavour. The results are summarized in the following list:

- Linalool 7%;
- 4-terpineol 10%;
- Neral 33%;
- α-terpineol 11%;
- α-citral 39%.

In particular, the Linalool/4-terpineol ratio was 2/3 and the Neral/ α-terpineol ratio was 7.5/2.5.

Flavour of Mandarin

The flavour of mandarin presented six characteristic signals corresponding to Linalool, 4-terpineol, Neral, α-terpineol, α-citral and citronellol (3,7-dimethyl-6-octen-1-ol) (Figure 5.7). These signals were recognized by comparison of their mass spectra with library mass spectra (WILEY275).

As expected, the characteristic compounds of mandarin were very similar to those of lemon, but in mandarin also citronellol was found. The mass spectrum of citronellol is shown in Figure 5.8.

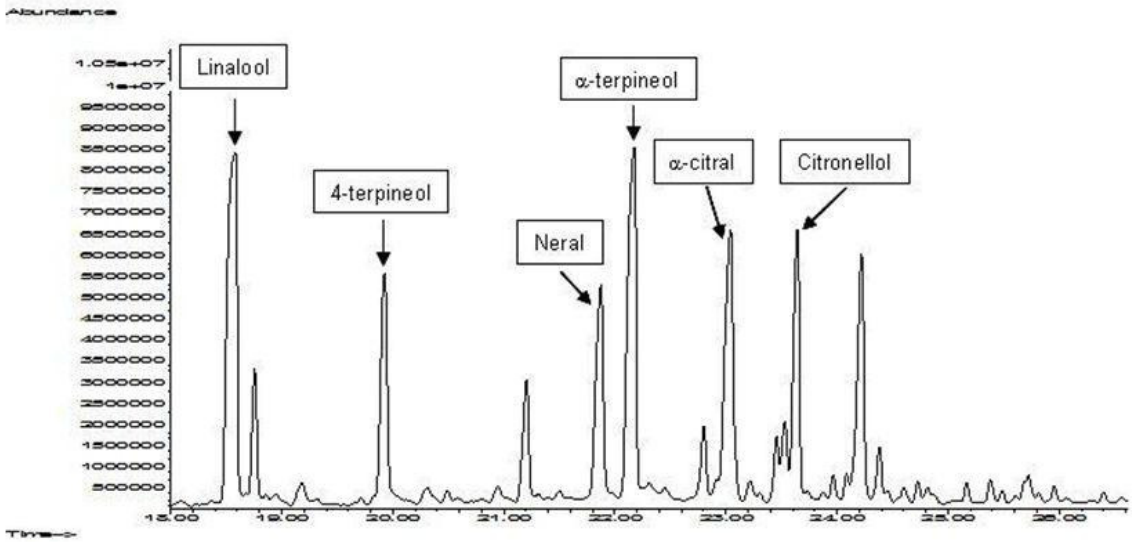


Figure 5.7: GC/MS chromatogram of head space of mandarin flavour.

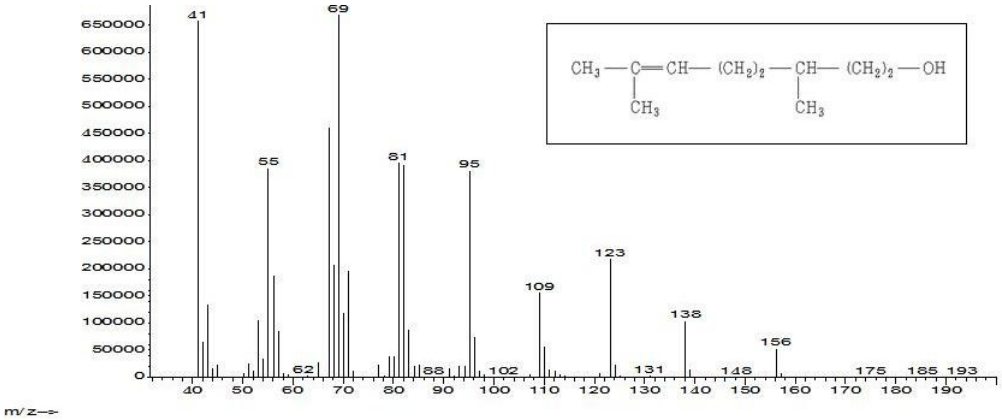


Figure 5.8: Mass spectrum of Citronellol with relative molecular structure.

The identified signals, corresponding to the characteristic mandarin flavour compounds, already found in lemon flavour, have been integrated in order to find the relative percentages of each compound. The results are summarized in the following list:

- Linalool 28%;
- 4-terpineol 18%;
- Neral 10%;
- α-terpineol 30%;
- α-citral 14%.

In particular, the Linalool/4-terpineol ratio was 3/2 and the Neral/α-terpineol ratio was 2.5/7.5, that is the contrary in respect to the ratios found in lemon flavour.

These ratios could be used to distinguish the flavour of mandarin from that of lemon in not clear cases.

Flavour of Raspberry

The flavour of raspberry presented three characteristic signals corresponding to α-ionone (4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one), β-ionone (4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one) and α-irone ((E)-4-(2,5,6,6-tetramethyl-1-cyclohex-2-enyl)but-3-en-2-one), as shown in Figure 5.9. These signals were recognized by comparison of their mass spectra with library mass spectra (WILEY275).

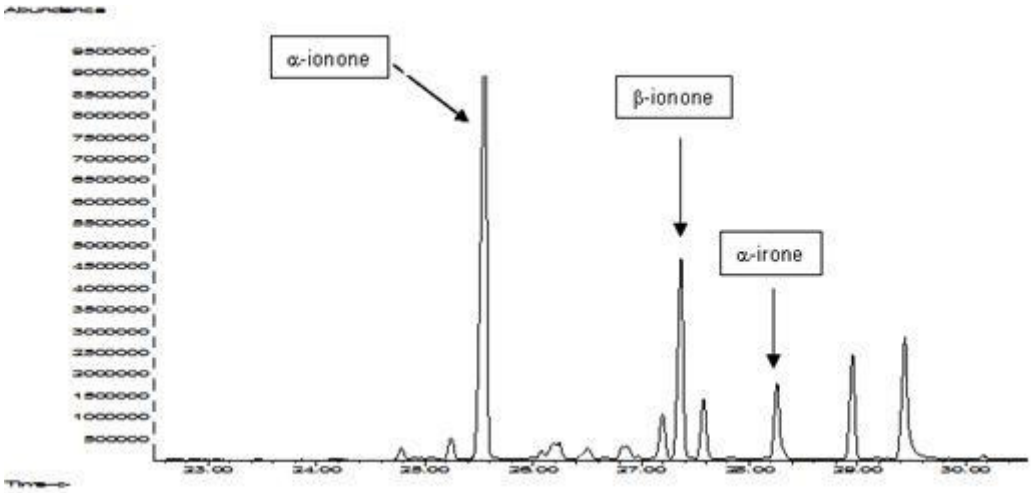


Figure 5.9: GC/MS chromatogram of head space of raspberry flavour.

In the following figures the mass spectra with relative molecular structure of the characteristic compounds of raspberry flavour are reported.

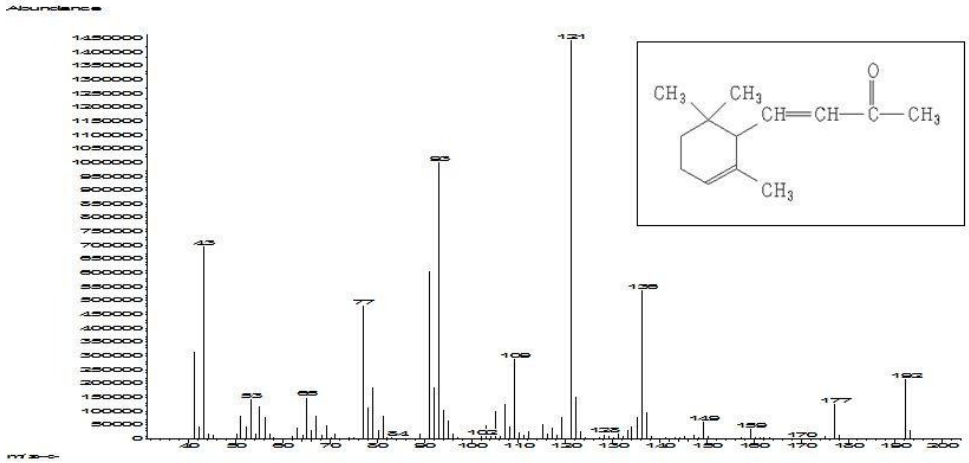


Figure 5.10: Mass spectrum of α-ionone with relative molecular structure.

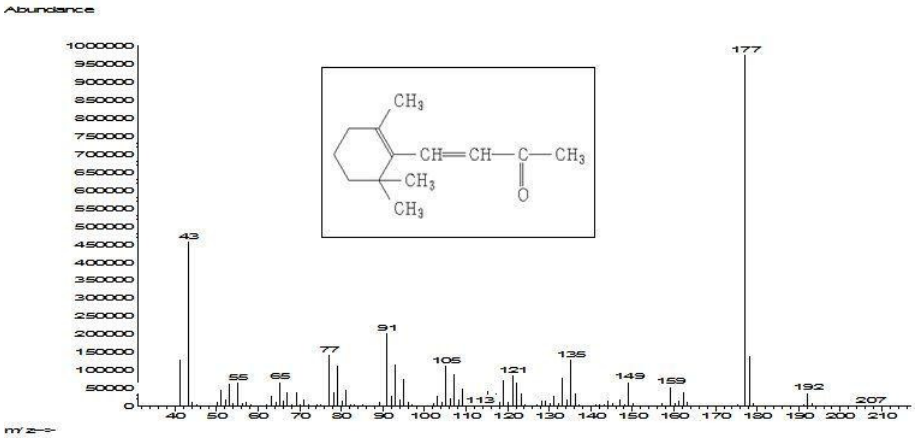


Figure 5.11: Mass spectrum of β-ionone with relative molecular structure.

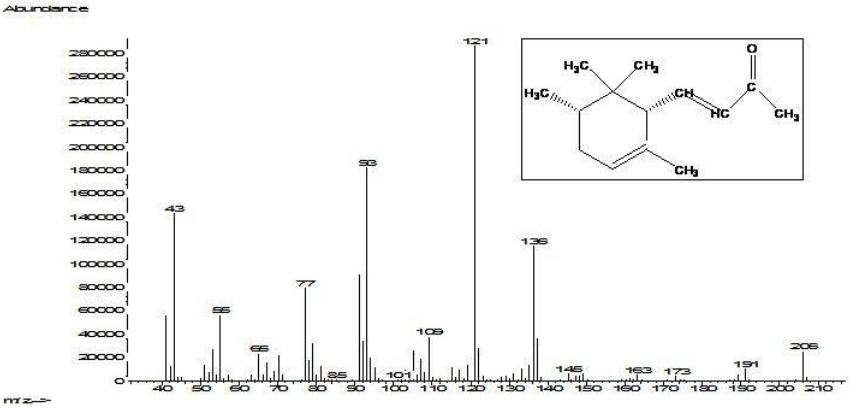


Figure 5.12: Mass spectrum of α-irone with relative molecular structure.

In particular, integrating the two signals corresponding to α-ionone and β-ionone, the α-ionone/β-ionone ratio resulted 8/2.

Flavour of Blueberry

The flavour of blueberry presented five characteristic signals corresponding to Menthone (2*S*,5*R*)-2-Isopropyl-5-methylcyclohexanone), Isomenthone (5-methyl-2-propan-2-ylcyclohexan-1-one), Linalool, Menthol (2-isopropyl-5-methylcyclohexanol) and β-fenchylalcohol (1,5,5-trimethylbicyclo[2.2.1]heptan-6-ol). These signals were recognized by comparison of their mass spectra with library mass spectra (WILEY275).

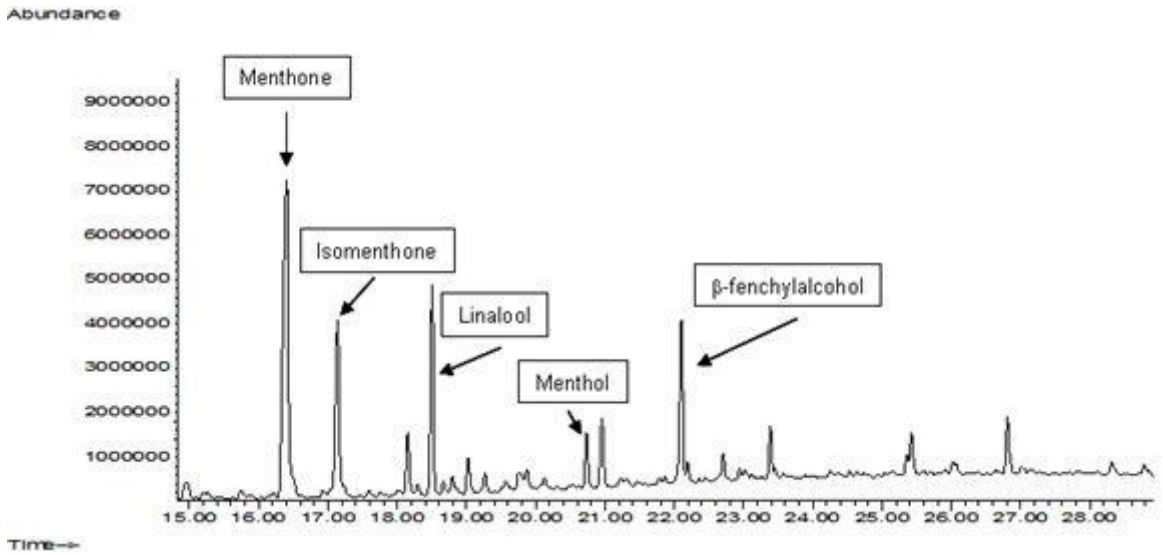


Figure 5.13: GC/MS chromatogram of head space of blueberry flavour.

In the following figures the mass spectra of Menthone, Isomenthone, Menthol and β-fenchylalcohol are reported.

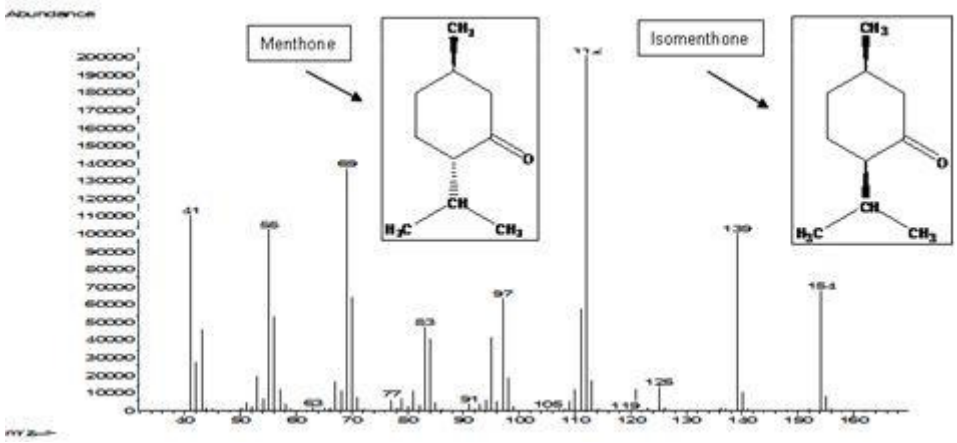


Figure 5.14: Mass spectrum of Menthone and Isomenthone with relative molecular structure.

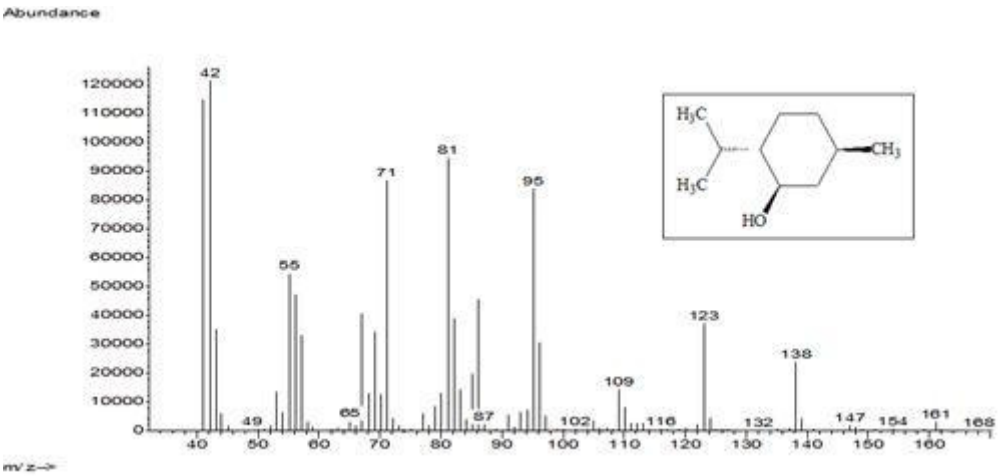


Figure 5.15: Mass spectrum of Menthol with relative molecular structure.

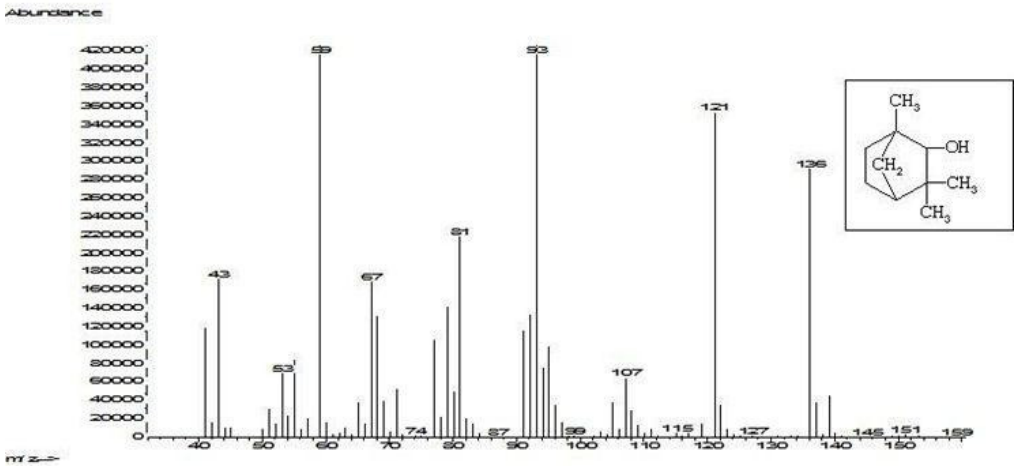


Figure 5.16: Mass spectrum of β-fenchylalcohol with relative molecular structure.

The two GC/MS signals corresponding to Menthone and Linalool has been manually integrated. In this way it was possible to obtain the Menthone/Linalool ratio, that resulted 7/3.

Flavour of Wild berries

The flavour of wild berries presented five characteristic signals corresponding to Menthone, Linalool, Pulegone ((R)-2-isopropylidene-5-methylcyclohexanone), α-ionone and β-ionone. These signals were recognized by comparison of their mass spectra with library mass spectra (WILEY275). As expected, the flavour of wild berries contained some compounds characteristic of raspberry (α-ionone and β-ionone) and some others characteristic of

blueberry (Menthone and Linalool). The mass spectrum of Pulegone is reported in Figure 5.18.

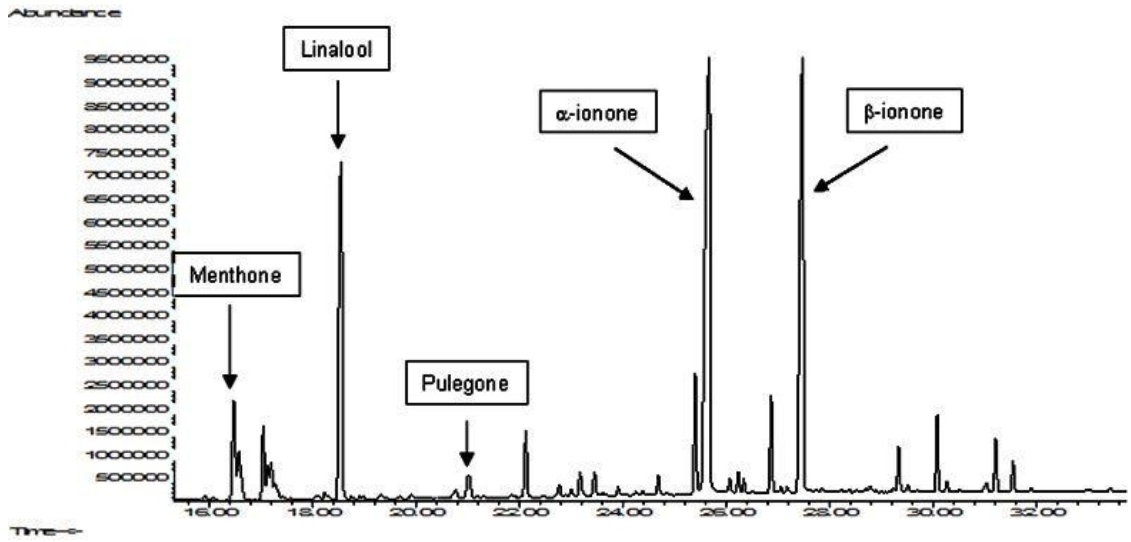


Figure 5.17: GC/MS chromatogram of head space of wild berries flavour.

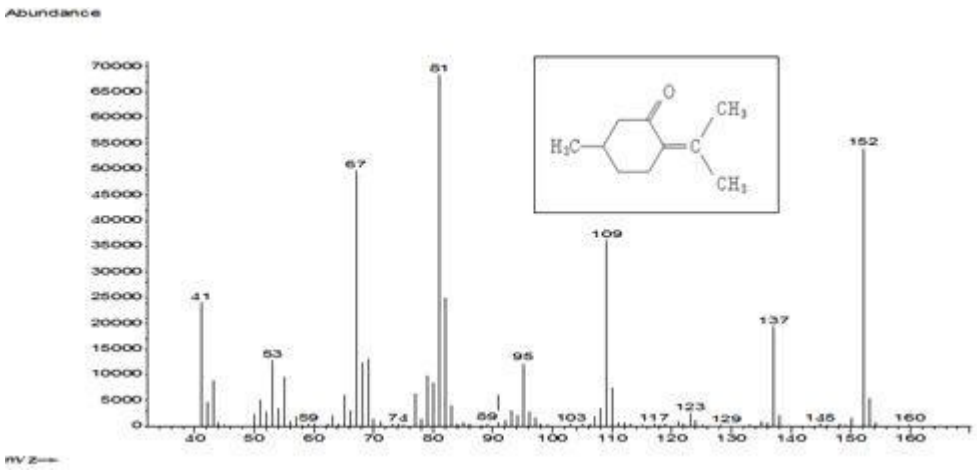


Figure 5.18: Mass spectrum of Pulegone with relative molecular structure.

Considering that wild barriers flavour contained some characteristic compounds already found in raspberry and in blueberry, it became necessary to focus the attention on the ratios of the compounds contained in these flavours. In particular, in wild berries the α-ionone/β-ionone ratio was 1/1, while in raspberry was 8/2, and, in wild berries, the Menthone/Linalool ratio was 1/9, while in blueberry was 7/3. Using these ratios, it is possible to better discriminate between wild berries, raspberry and blueberry flavour.

Flavour of Cinnamon

The flavour of cinnamon presented five characteristic signals corresponding to Furfural, Linalool, Cinnamic aldehyde ((E)-3-phenylprop-2-enal), Cinnamyl acetate (3-phenylprop-2-enyl acetate) and Eugenol (4-Allyl-2-methoxyphenol). These signals were recognized by comparison of their mass spectra with library mass spectra (WILEY275).

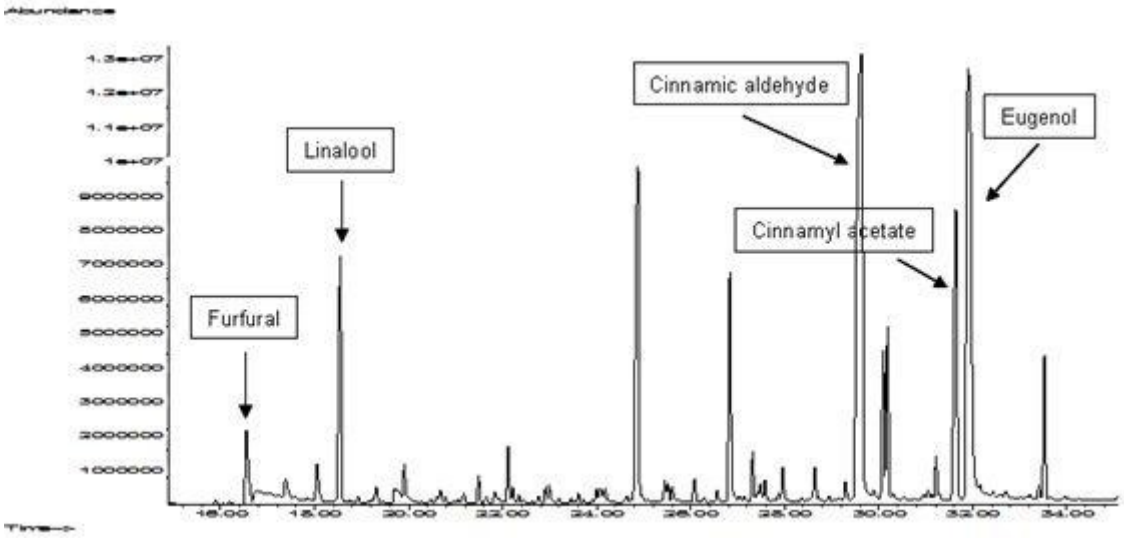


Figure 5.19: GC/MS chromatogram of head space of cinnamon flavour.

In the following figures the mass spectra of Furfural, Cinnamic aldehyde, Cinnamyl acetate and Eugenol are reported. Furfural can not be used as molecular marker of the presence of cinnamon in BVM, because it is naturally present in the balsamic vinegar.

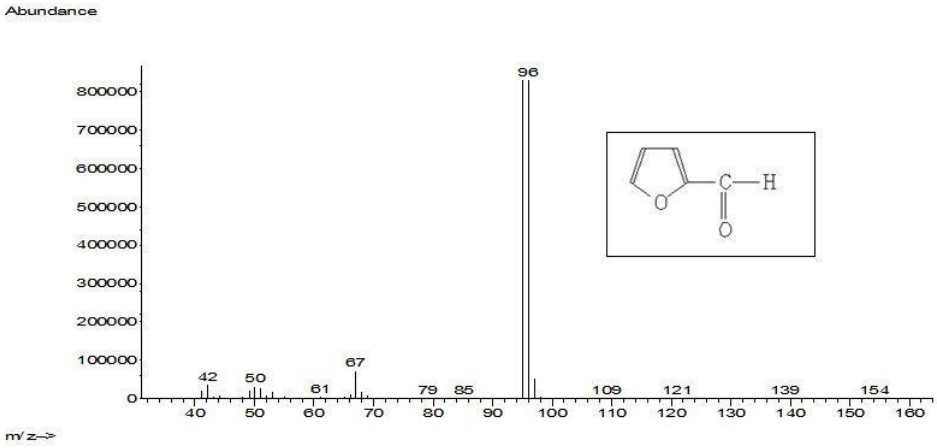


Figure 5.20: Mass spectrum of Furfural with relative molecular structure.

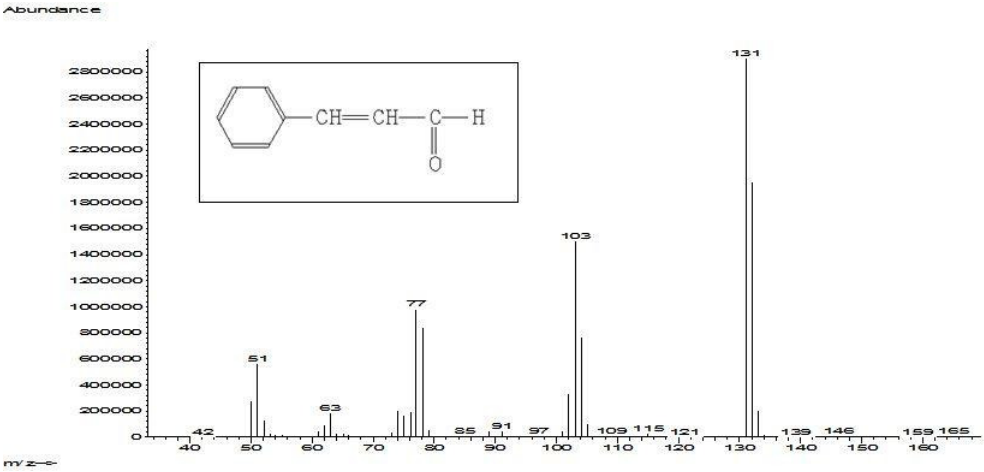


Figure 5.21: Mass spectrum of Cinnamic aldehyde with relative molecular structure.

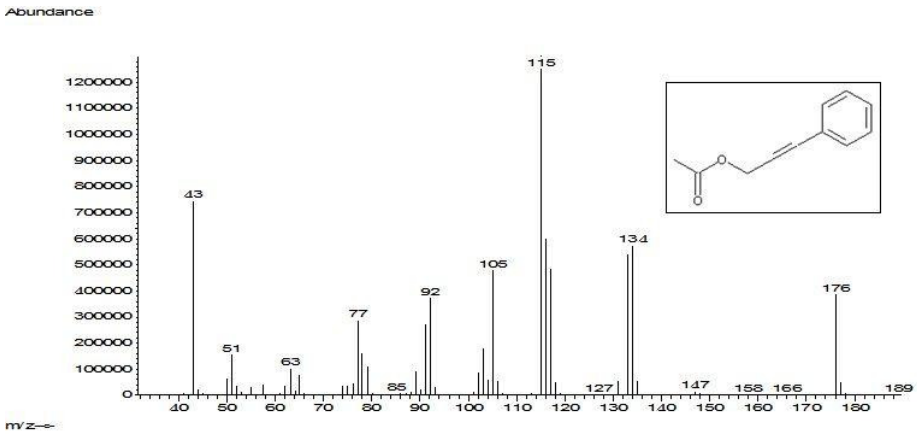


Figure 5.22: Mass spectrum of Cinnamyl acetate with relative molecular structure.

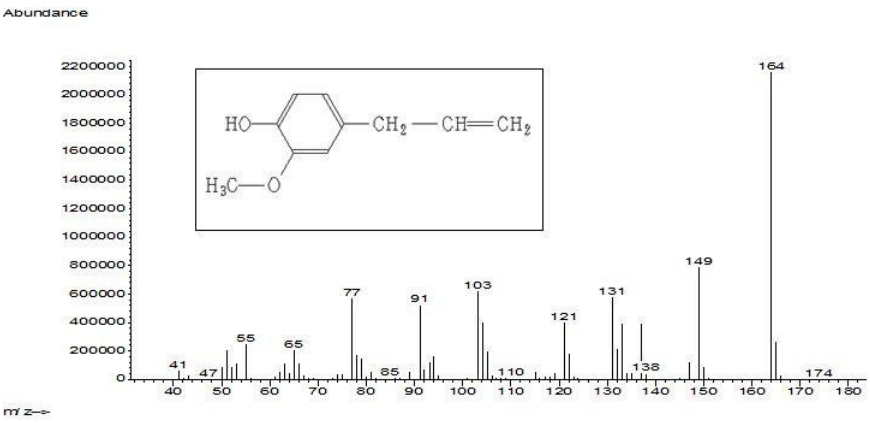


Figure 5.23: Mass spectrum of Eugenol with relative molecular structure.

The ratio between Cinnamic aldehyde, Cinnamyl acetate and Eugenol, obtained from the integration of the respectively GC/MS signals, resulted 4.5/1.5/4.

Flavour of Oak

The flavour of oak presented four characteristic signals corresponding to Geranial ((E)-3,7-dimethyl-2,6-octadienal), Cinnamic aldehyde, Cinnamyl acetate and Eugenol. These signals were recognized by comparison of their mass spectra with the library mass spectra (WILEY275). This flavour can be differentiated from that of cinnamon by the presence of Geranial. The mass spectrum of Geranial is reported in Figure 5.25 and it is identical to that of Citral, in fact, Geranial is named also trans-citral.

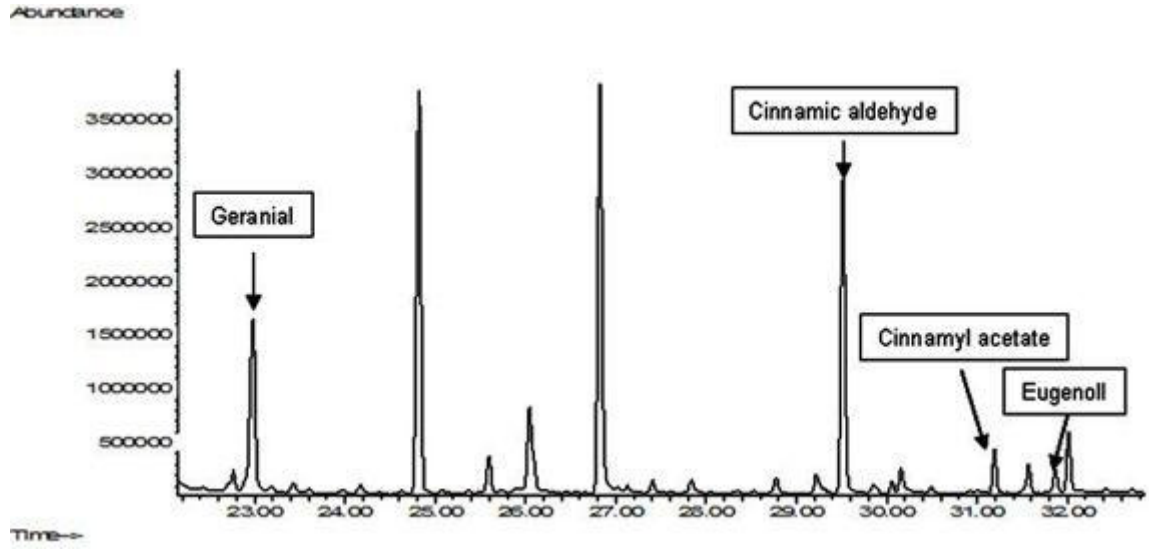


Figure 5.24: GC/MS chromatogram of head space of oak flavour.

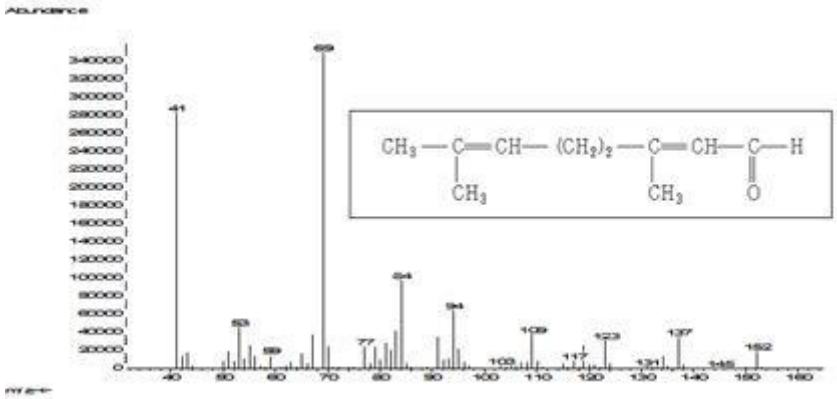


Figure 5.25: Mass spectrum of Geranial with relative molecular structure.

In this case, from the integration of the GC/MS signal it is possible to note that, Cinnamic aldehyde, Cinnamyl acetate and Eugenol ratio resulted 8/1/1, very different from that of cinnamon flavour.

Flavour of Strawberry

The flavour of strawberry presented four characteristic signals corresponding to 1-acetyloxy-2-propanone (Acetol acetate), Linalool, 1,3,8-p-menthatriene (1-Isopropenyl-4-methyl-1,3-cyclohexadiene) and Cis-3-hexenyl cis-3-hexenoate (3-Hexenoic acid, (3Z)-3-hexenyl ester). These signals were recognized by comparison of their mass spectra with the library mass spectra (WILEY275).

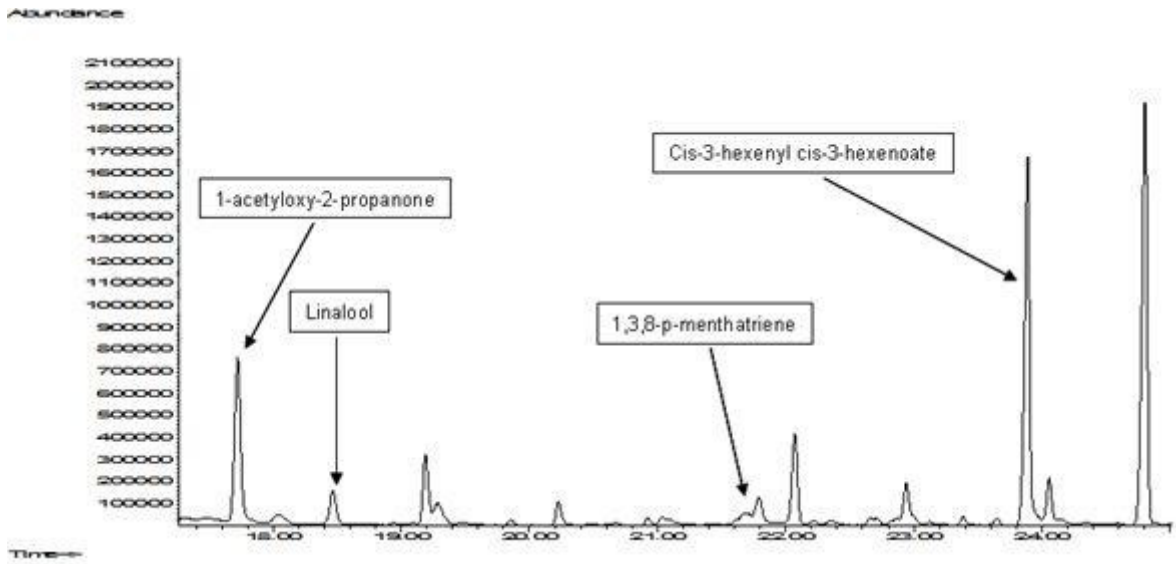


Figure 5.26: GC/MS chromatogram of head space of strawberry flavour.

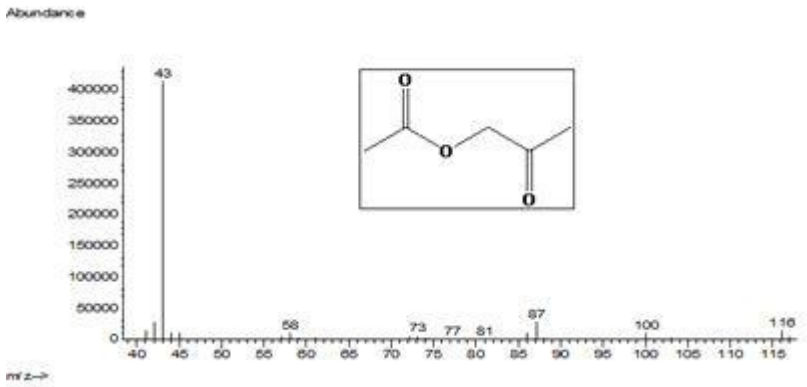


Figure 5.27: Mass spectrum of 1-acetyloxy-2-propanone with relative molecular structure.

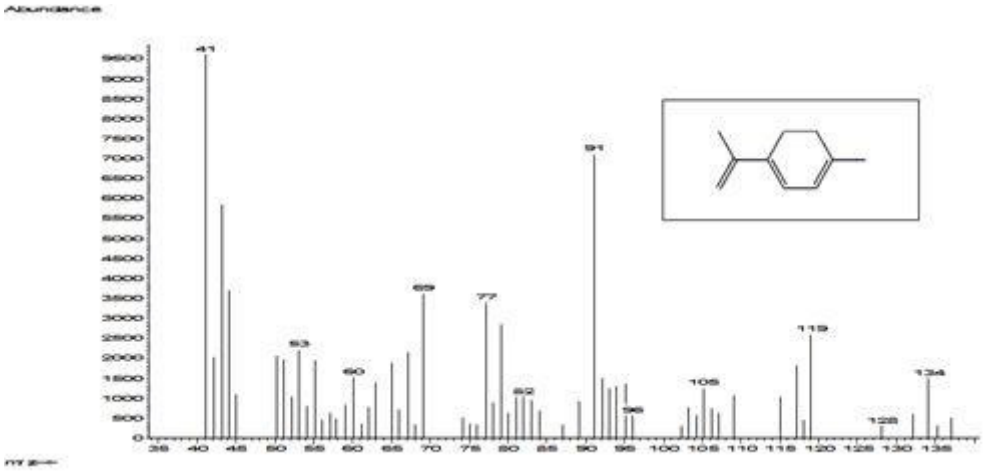


Figure 5.28: Mass spectrum of 1,3,8-p-menthatriene with relative molecular structure.

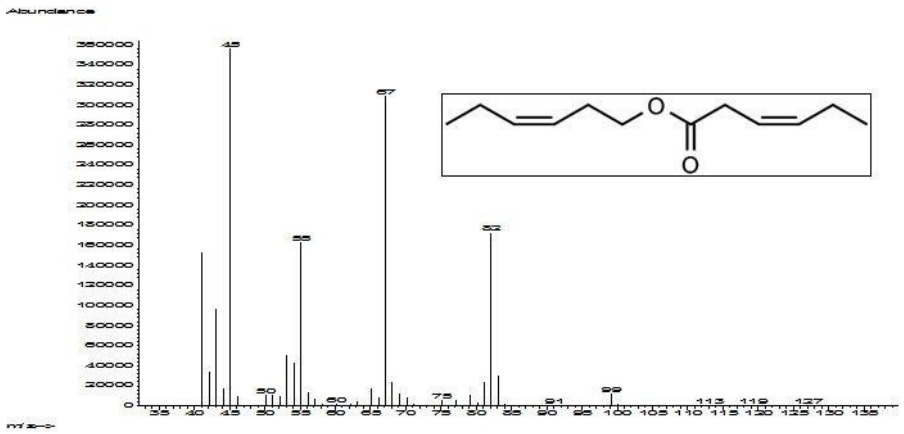


Figure 5.29: Mass spectrum of Cis-3-hexenyl cis-3-hexenoate with relative molecular structure.

It is possible to conclude that each flavour can be differentiated from the others, by means of one or more characteristic compounds and their characteristic ratios.

The Linalool is common to several flavour samples, as well as in several fruits such as raspberry, strawberry and also in grape, for this reason, it is not possible to use it as a marker of some particular flavour or as a marker of a flavour added to vinegar.

It is interesting to note that the flavours analysed do not contain the characteristic compounds of the relative fruits, for example, limonene was not detected in lemon, etc. This particular composition is due to the production process of the flavours, that were not obtained from an extraction of fruits but, probably, by chemical synthesis.

5.3.2 Determination of flavours in BVM samples by HS-SPME and GC/MS analysis

The composition of balsamic vinegar of Modena flavour is, nowadays, still largely unknown. The aroma profile of a BVM is dependent on the raw materials employed in the production, on the cooking time of the must, on the kind of wood of the barrels used for the maturation and ageing and on the acetification process.

The second step of this work was the determination of the characteristic aroma profile of the balsamic vinegar of Modena and the investigation of the presence of added flavours to BVM. This kind of analysis is very important because the addition of flavours to BVM is forbidden by law (Gazzetta Ufficiale dell'Unione Europea, 2007).

In order to determine the possible addition of flavours to BVM, the first part of the work was focused on the characterization of the aromatic profile of a genuine balsamic vinegar of Modena sample, in order to detect the compounds responsible of the characteristic flavour of the product.

The aromatic profile of a genuine BVM sample was obtained by analysing the head space of the sample by GC/MS, extracting the volatile components of the BVM by SPME technique in the same conditions used for flavour solutions.

The aromatic profile, shown in Figure 5.30a and 5.30b, showed the presence of several characteristic compounds of BVM flavour. These compounds, listed in Table 5.4, were recognized by comparing their mass spectra with library mass spectra (WILEY275).

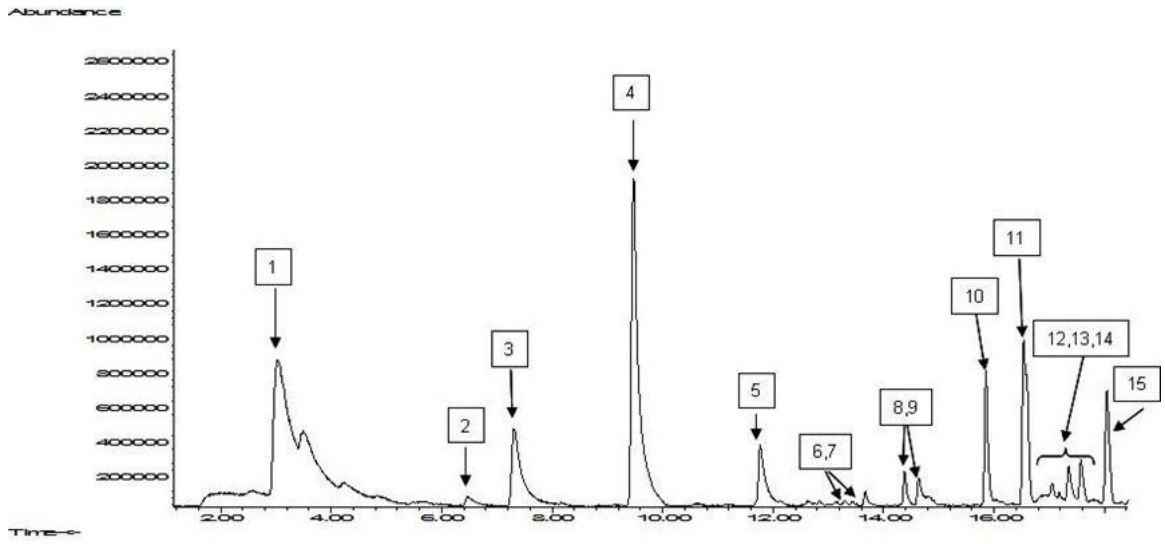


Figure 5.30a: GC/MS chromatogram of head space of a genuine BVM sample (first part).

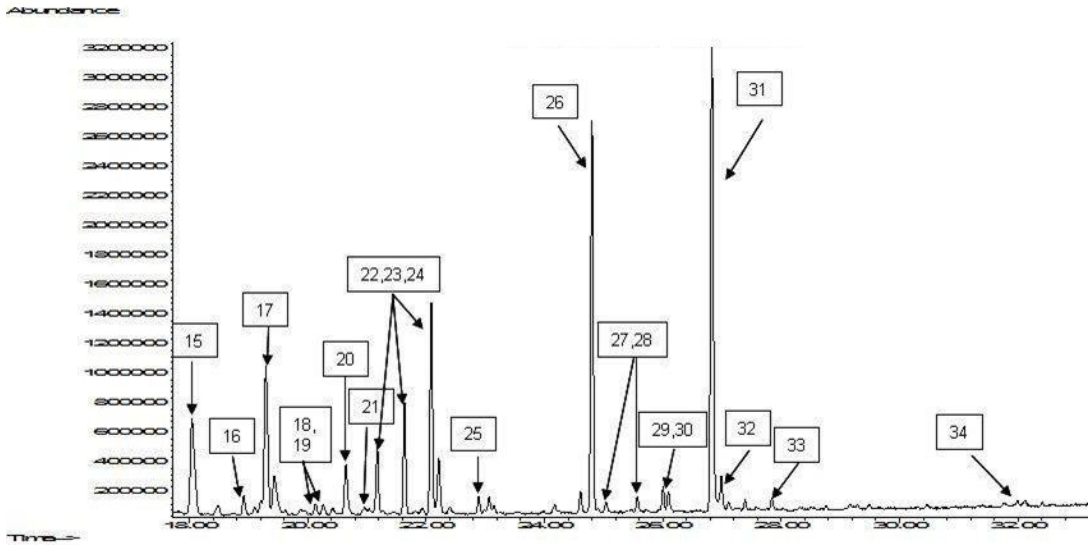


Figure 5.30b: GC/MS chromatogram of head space of a genuine BVM sample (second part).

Table 5.4: Retention time for each identified compound in the GC/MS chromatogram of the genuine BVM sample.

Signal number	Retention time (min.)	Relative percentage (%)	Compound
1	3.04	9.8	Ethyl acetate
2	6.70	0.0	Isobutanol
3	7.15	0.6	3-methyl-1-butanol acetate
4	9.56	12.8	3-methyl-1-butanol
5	11.83	6.8	3-hydroxy-2-butanone (Acetoin)
6	13.13	0.1	6-methyl-5-hepten-2-one
7	13.67	0.3	Unidentified
8	14.39	1.4	Ethyl acetoacetate
9	14.62	0.9	Nonanal
10	15.86	2.0	Unidentified
11	16.59	11.4	Furfural
12	17.06	0.3	2,3-butanediol diacetate
13	17.34	1.8	Decanal
14	17.57	1.7	2-acetylfuran
15	18.05	3.1	Benzaldehyde
16	18.92	1.0	2,3-butanediol
17	19.30	6.0	5-methylfurfural
18	20.13	0.2	Unidentified
19	20.27	0.2	2-acetyl-5-methylfuran
20	20.64	0.4	Phenol derivate
21	20.97	0.0	Phenylacetaldehyde
22	21.18	0.9	1,3-propanediol diacetate
23	21.64	0.7	Butanedioic acid, diethyl ester
24	22.08	0.1	Camphene
25	22.89	0.2	Benzyl acetate
26	24.80	12.1	Acetic acid, 2-phenyl ethyl ester
27	25.03	0.1	Unidentified
28	25.57	0.6	Neryl acetone
29	26.00	0.1	Unidentified
30	26.09	0.6	Benzyl alcohol
31	26.88	23.3	Phenylethyl alcohol
32	26.98	0.4	Unidentified
33	27.38	0.1	Unidentified
34	31.99	0.1	4-ethyl phenol

The compounds detected derive in part from the raw materials used for the BVM production, in fact some alcohols such as 3-methyl-1-butanol and phenylethyl alcohol have already been identified in wine. Other compounds can be originated from alcoholic and acetic fermentation (for example: ketones, aldehydes, ethyl esters and ethyl acetates), from the Maillard or pyrolysis reactions that occur during the cooking of the must (furan derivates), from oxidative

processes, or eventually extracted from the wooden barrels, and finally obtained from enzymatic reactions occurring during the balsamic vinegar of Modena production.

In order to evaluate the possible addition of flavours to BVM, each flavour was added to genuine BVM at different concentration, starting from 1% in volume (Table 5.2). In this way, it was possible to obtained the detection of the characteristic components of each flavour, previously detected in aqueous solutions, in BVM.

The GC/MS chromatograms of the samples composed of genuine BVM added with flavours, were compared with those of the single flavours diluted in water and with those of the genuine BVM, in order to check the presence of the characteristic signals of each flavour. In this way, it was possible to define the behaviour of each flavour in a complex matrix such as balsamic vinegar.

The results showed that, it was possible to distinguish the genuine BVM from those added with flavours and to identify each single flavour in BVM, because all the characteristic components of each flavour were detected in BVM samples, as summarized in Table 5.5.

Table 5.5: Characteristic compounds of flavours detected in BVM solutions.

Flavour	**Compounds detected in BVM solutions**
Lemon	Linalool, 4-terpineol, Neral, α-terpineol, α-citral
Mandarin	Linalool, 4-terpineol, Neral, α-terpineol, α-citral, citronellol
Raspberry	A-ionone, β-ionone, α-irone
Blueberry	Menthone, Isomenthone, Linalool, Menthol, β-fenchylalcohol
Wild berries	Menthone, Linalool, Pulegone, α-ionone, β-ionone
Cinnamon	Linalool, Cinnamic aldehyde, Cinnamyl acetate, Eugenol
Oak	Geranial, Cinnamic aldehyde, Cinnamyl acetate, Eugenol
Strawberry	1-acetyloxy-2-propanone, Linalool, 1,3,8-p-menthatriene, Cis-3-hexenyl cis-3-hexenoate

It is important to remember that Linalool can not be used as molecular marker of one particular flavour, because it is present in several samples. All flavours can be distinguished from each others for at least one compound.

In order to evaluate the lowest flavour addition detectable for each flavour, several BVM solutions at different flavours concentrations were analysed (Table 5.2).

In order to find a clear contamination and to determine the lowest flavour addition detectable, it was necessary to verify, in the GC/MS chromatogram, the contemporary presence of all compounds of the tested flavour in BVM.

Good results were obtained for lemon, wild berries and raspberry flavours, which the lowest detectable addition was 0.01% (v/v), while for oak and blueberry flavour the lowest detectable addition was 0.1% (v/v). For the other flavours the lowest detectable addition was 0.05% (v/v), that was an acceptable result.

Different flavours had, then, different responses, probably, because of their different characteristics, such as the analytes concentrations inside the product, that was undeclared, and the different response of each compounds in the GC/MS analysis.

The results are shown in Table 5.6.

Table 5.6: Lowest flavour concentrations detectable in BVM solutions; the concentrations are expressed as % in volume.

Flavour	**Lowest concentration detectable (% in volume)**
Lemon	0.01%
Mandarin	0.05%
Raspberry	0.01%
Blueberry	0.1%
Wild berries	0.01%
Cinnamon	0.05%
Oak	0.1%
Strawberry	0.05%

This study was, then, extended to 40 samples of balsamic vinegar of Modena without any stamp or mark, of different origin and different producers, recovered from several markets of Parma and Reggio Emilia. For each sample, the aromatic fraction was extracted by SPME technique and, then, analysed by GC/MS.

In order to evaluate a possible addition of flavours, the gas-chromatograms obtained by the analyses of BVM samples were compared with that of the genuine BVM, in order to check some unmatched signals. Moreover, for each gas-chromatogram obtained from BVM

analyses, the presence of the characteristic compounds of flavours was checked, by extracting the characteristic mass fragments. In the case of the presence of one or more compounds corresponding to one or more flavours in the BVM analysed, their retention times were compared with those obtained in the gas-chromatograms of genuine BVM added with flavours. This control was made by overlaying each gas-chromatogram of real BVM samples with those obtained for BVM added with flavours.

The results (Table 5.7) showed that, among the samples analysed, six samples presented the addition of flavours. It is important to highlight that these six samples were not suitable with the characteristics fixed by law, because the addition of flavours to balsamic vinegar of Modena is forbidden.

It is important to remember that to be sure of a real contamination, it is necessary that the tested BVM presents all the components of one or more flavours. In this case, only BVM 2 present a clear contamination due to the presence of oak flavour, in fact in this sample all the characteristic components of oak flavour are present.

Table 5.7: Results of the determination of possible added flavour to BVM sample.

BVM sample	Qualitative result	Notes
1	Negative	
2	**Positive**	The sample contained Geranial, Cinnamic aldehyde, Cinnamyl acetate, Eugenol. Presence of oak flavour.
3	Negative	
4	Negative	
5	**Positive**	The sample contained Menthol. Probable presence of blueberry flavour.
6	**Positive**	The sample contained Menthol. Probable presence of blueberry flavour.
7	Negative	
8	Negative	
9	Negative	
10	Negative	
11	Negative	
12	Negative	
13	Negative	
14	Negative	
15	Negative	
16	Negative	
17	Negative	
18	Negative	
19	Negative	
20	Negative	
21	**Positive**	The sample contained L-carvone and Methyl-eugenol. Probable presence of unidentified flavour.
22	Negative	
23	Negative	
24	Negative	
25	Negative	
26	**Negative/Positive**	The sample contained Linalool. The contamination is not sure.
27	Negative	
28	Negative	
29	Negative	
30	Negative	
31	Negative	
32	Negative	
33	Negative	
34	Negative	
35	Negative	
36	**Positive**	The sample contained Linalool and 4-terpineol. Probable presence of lemon or mandarin flavour.
37	Negative	
38	Negative	
39	Negative	
40	Negative	

The sample 36 contained Linalool and 4.terpineol, already found in lemon and mandarin flavours. In order to understand which flavour was added to this BVM, it was necessary to integrate the two GC/MS signals corresponding to Linalool and 4.terpineol in the

chromatogram of the sample. The Linalool/4-terpineol ratio resulted nearly 3/2. Comparing this result with those obtained by analysing the single flavours, the sample 36 resulted probably contaminated by mandarin flavour, in which Linalool/4-terpineol ratio was 3/2.

The analyses were then extended to a set of balsamic vinegar of Modena samples marked with different Consortium stamps, as reported in Table 5.1, in order to verify the real authenticity of these vinegars.
The results (Table 5.8) showed that, effectively, none of the BVM samples presented the addition of flavours.

Table 5.8: Results of the determination of possible added flavour in BVM sample with different stamps.

BVM sample	Stamp colour	Qualitative result
RED 1	Bordeaux red	Negative
BROWN 1	Brown	Negative
RED 2	Bordeaux red	Negative
GREEN	Green	Negative
BROWN 2	Brown	Negative
WHITE	White	Negative
W-GOLD	White/gold	Negative

It is possible to conclude that, this particular kind of analysis can be used for the authentication and quality evaluation of balsamic vinegar of Modena and, in particular, to control and safeguard the balsamic vinegars of Modena certified by the Consortiums, in order to determine eventual possible frauds.

5.3.3 Multivariate statistical elaboration of SPME-GC/MS signals of balsamic vinegars of Modena

In this part of the work a statistical elaboration of the GC/MS signals of the flavour profile of balsamic vinegars was performed, in order to discriminate between products of different maturation and ageing.

The signals in the GC/MS chromatograms were identified, as shown in Table 5.4, manually integrated and the results were elaborated with a multivariate statistical technique: the principal component analysis (PCA).

First, the GC/MS signals obtained from analyses of the samples of balsamic vinegar of Modena with different coloured stamps (Table 5.1) were statistically elaborated, in order to have a separation between the more aged samples (white and white/gold stamps) and the other samples reporting bordeaux red, brown and green seals. For each vinegar, two samplings were made and analysed. The samples analysed are summarized in the following table, in which the samples matured for at least 60 days were identified with number “1”, while the samples aged for at least 3 years were identified with number “2”.

Table 5.9: BVM sample with different stamps analysed.

BVM sample	Stamp colour	Sampling	Identification number
RED 1 a	Bordeaux red	1°	1
RED 1 b	Bordeaux red	2°	1
BROWN 1 a	Brown	1°	1
BROWN 1 b	Brown	2°	1
RED 2 a	Bordeaux red	1°	1
RED 2 b	Bordeaux red	2°	1
GREEN a	Green	1°	1
GREEN b	Green	2°	1
BROWN 2 a	Brown	1°	1
BROWN 2 b	Brown	2°	1
WHITE a	White	1°	2
WHITE b	White	2°	2
W-GOLD a	White/gold	1°	2
W-GOLD b	White/gold	2°	2

PCA was then performed on a matrix of 34 GC/MS signals, corresponding to the signals identified (Table 5.4), and three components were extracted with covariance method, describing 93.83 % of variance. In Figure 5.31 the scatter plot of the scores of PC1 versus PC2, explaining 89.75% of variance is reported.

The score plot shows that the BVM samples seem to cluster into two principal groups, that correspond to the group of BVM aged for at least 3 years (group 2) and to the group of BVM matured for at least 60 days (group 1).

The separation was based principally on the component 2 (PC 2).

The variables with high positive values on component 2 are characteristic of group 2. These variables correspond to Furfural, 5-methylfurfural and 2,3-butanediol. The values of these variables are listed in the following table (Table 5.10). The group 2 is characterized also by the variables with an high negative values on component 1; the variable with the higher negative value on PC1 is Ethyl acetate (Table 5.10).

The variables with high positive values on component 1 (PC 1) resulted characteristics for the group 1. These variables correspond to Benzyl alcohol, Phenylethyl alcohol and Acetoin. The values of these variables are listed in Table 5.10.

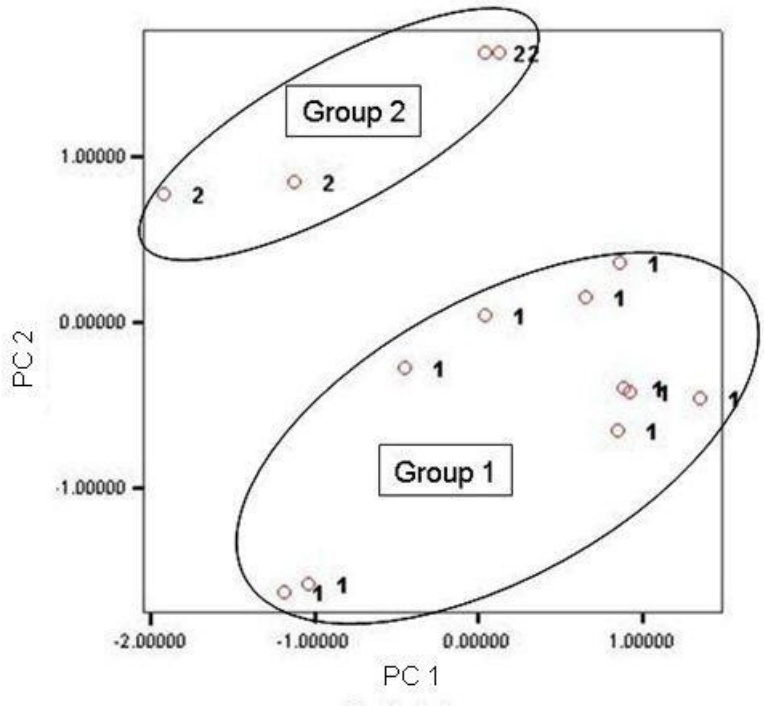


Figure 5.31: Scatter plot of the scores of the first two principal components obtained using GC/MS data of BVM samples, identified by number of the group.

Table 5.10: Coefficients of the two principal components for grouping of the vinegar based on the different maturation and ageing.

Variable name	PC1 value	PC2 value
Furfural	-0.332	+0.927
5-methylfurfural	-0.091	+0.838
2,3-butanediol	-0.210	+0.787
Ethyl acetate	-0.968	-0.246
Benzyl alcohol	+0.906	-0.005
Phenylethyl alcohol	+0.829	-0.360
Acetoin	+0.865	-0.233

Moreover, it is possible to note a separation based on component 1 in the group of the less aged BVM (group 1). In particular, there is a separation between the brown BVM, matured for at least 60 days in oak barrels, and the bordeaux red and green BVM, matured for at least 60 days in not specified wooden barrels (Figure 5.32).

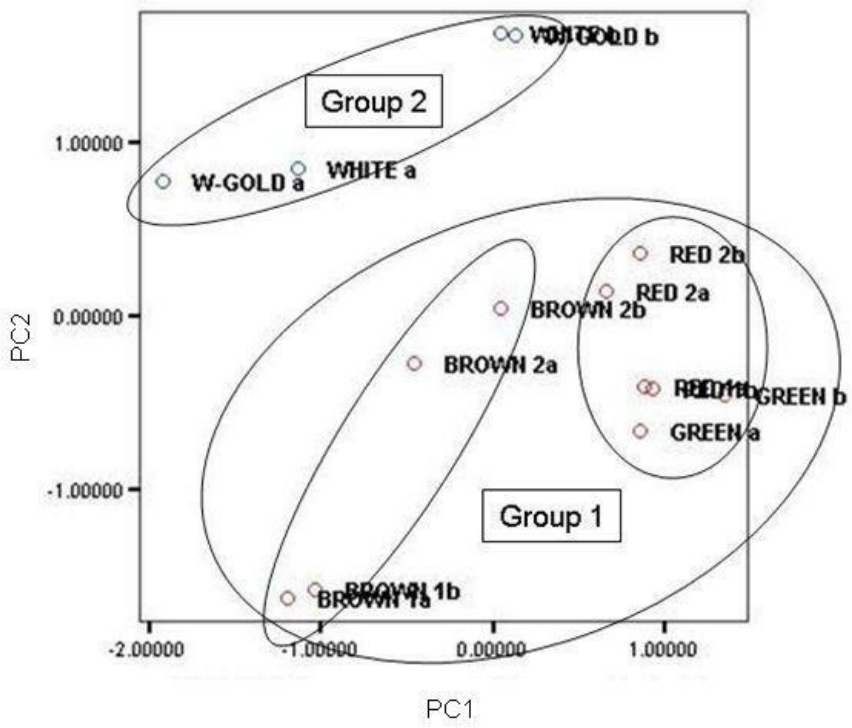


Figure 5.32: Scatter plot of the scores of the first two principal components obtained using GC/MS data of BVM samples, identified by colour of the stamps.

It is possible to conclude that multivariate statistical elaboration of the GC/MS signals obtained from the analysis of BVM samples head-space, permits to have a separation between BVM aged for at least 3 years and BVM matured for at least 60 days.
In order to confirm these results, it should be necessary to extend this particular analyses to a larger number of BVM samples marked with different coloured stamps.

The principal components analysis was, then, extended to others 40 BVM samples that did not reported any kind of stamp or mark on the bottle, in order to evaluate the maturation or ageing time of these samples. Also in this case, PCA was performed on a matrix of 34 GC/MS signals (Table 5.4), three components were extracted with covariance method, describing 79.26 % of variance. In Figure 5.33 the scatter plot of the scores of PC1 versus PC2, explaining 67.19% of variance is reported. In the matrix, the BVM samples of different coloured stamps were also considered, in order to have a reference.
The score plot shows that, also in this case, there is the separation of samples in 2 principal groups. Group 2 corresponds to that of more aged products, because of the presence in this

group of the white and the white/gold samples. Group 1 is formed by the BVM samples with brown, green and red seals and by others BVM, corresponding to a balsamic vinegar of Modena matured for at least 60 days. There are also three samples clearly not pertaining to a group: BVM 31, BVM 28 and BVM 24, probably because of a maturation or ageing treatment different from that of all other samples.

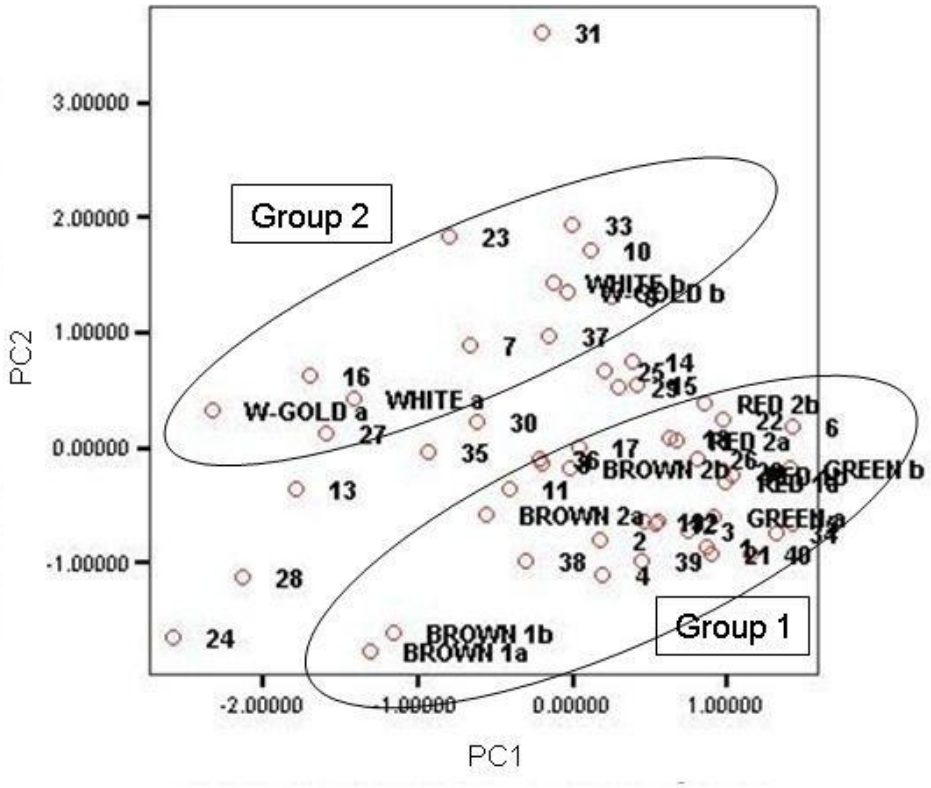


Figure 5.33: Scatter plot of the scores of the first two principal components obtained using GC/MS data of BVM samples, identified by colour of the stamps and by sample numbers.

It is possible to conclude that, the principal component analysis applied to SPME-GC/MS signals obtained from the analysis of head space of balsamic vinegar is useful to evaluate the maturation degree of the products with undeclared age.

5.4 Conclusions

The SPME technique coupled with the GC/MS analysis represented a very useful analytical method for the characterization of the aromatic profile of balsamic vinegar of Modena.
Moreover, with this particular technique it was possible to characterize several kind of flavour used for food aromatization. The characteristic compounds of each flavour analysed have been detected and identified. It was possible, then, to determine the addition of these flavours to balsamic vinegar of Modena, checking the presence of the characteristic flavour compounds in the GC/MS chromatograms of the BVM samples. In this way, it is possible to obtain an evaluation of the quality and the authenticity of the balsamic vinegars.

SPME technique coupled with GC/MS analysis allowed also to evaluate the differences between 3 years aged BVM (white or white/gold stamps) and 60 days matured BVM (Bordeaux red, green and brown stamps). The GC/MS signals were elaborated by the method of principal components analysis (PCA). In this way, a good separation between the two group of balsamic vinegars with different age of maturation was obtained, founding that the Furfural, 5-methylfurfural, 2,3-butanediol and Ethyl acetate amounts were discriminant for aged products, while Benzyl alcohol, Phenylethyl alcohol and Acetoin were discriminant for matured balsamic vinegars.
This method can be also used in order to evaluate the maturation and ageing degree of balsamic vinegars of undeclared age.

6. FINAL CONCLUSIONS

Chapter 6: Final conclusions and future perspectives

Results obtained allow to conclude that a characterization of balsamic vinegar of Modena (BVM) through its chemical and physical properties, by chromatographic analyses (GC/MS, TLC, HPLC/UV/MS) and by high resolution nuclear magnetic resonance spectroscopy (HR-NMR) is successful. This work allowed to find several analytical methods able to detect some molecular markers useful to verify the accordance with the legal parameters fixed by law (Gazzetta Ufficiale dell'Unione Europea, 2007) for this special vinegar.

The determination of sugar acetates allowed to better understand the chemical modifications that occur during maturation and ageing of BVM and to explain an eventual acidity decrease, that can cause a lacking of accordance with legal parameters for total acidity.
The formation of fructose and glucose acetates during maturation and ageing of balsamic vinegar of Modena was demonstrated both by ^{1}H-NMR, ^{13}C-NMR spectroscopy and GC/MS technique. The acetates were found and determined by GC/MS on balsamic vinegar of Modena, on traditional balsamic vinegar and on other vinegars rich of sugars. The results showed that the amount of sugar acetates is related to the initial amount of sugar present in the vinegar and to the maturation time of the vinegar. So, the glucose acetate/glucose ratio can be used for the determination of the maturation or ageing time of a balsamic vinegar.
Studies about the formation of other sugar esters, such as malates and tartrates, are in progress.

Another useful aspect for the authentication and quality evaluation of balsamic vinegar of Modena is the determination of the caramel content, that is fixed by law (2% v/v maximum).
The method of RP-TLC coupled with UV-Visible spectroscopy allowed a qualitative and quantitative determination of caramels of class III and IV in balsamic vinegar of Modena. The HPLC/UV method allowed to detect also class I caramel. Coupling HPLC/UV with an MS detector, it was possible to have chemical information about the characteristic compounds of the caramels, but the interpretation of the mass spectra resulted very difficult. ^{1}H-NMR spectroscopy presented more advantages, because the sample preparation was very easy and fast, all classes of caramel were detected and clearly differentiated from each others. For this

reason, the NMR technique seems to be the more promising method for the qualitative and quantitative determination of caramel of every class in balsamic vinegar of Modena.
A future perspective is the complete interpretation of NMR and MS spectra, in order to identify the characteristic caramel compounds, already detected by these techniques.

Another analytical method that improves the authentication and the quality evaluation of balsamic vinegar of Modena is the determination of the characteristic aromatic profile and the eventual addition of flavours extraneous to the product.
The SPME technique coupled with the GC/MS analysis represented a very useful analytical method for the characterization of the aromatic profile of balsamic vinegar of Modena. Moreover, with this particular technique it has been possible to characterize several kinds of flavour used for food aromatization and, also, to determine the addition of these flavours to balsamic vinegar of Modena, checking, by GC/MS analysis, the presence of the characteristic flavour compounds in the BVM samples.
The elaboration of GC/MS signals by the method of principal components analysis (PCA) allowed to obtain a good separation between 3 years aged BVM and 60 days matured BVM. So, this method is promising also to evaluate the maturation and ageing degree of balsamic vinegars of undeclared age. Analyses on more vinegar samples with different coloured stamps are in progress, in order to built a predictive model.

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Curriculum Vitae

Martina Cirlini was born in 1979 in Parma.

She went to school in Reggio Emilia where she received her high school degree from the *Liceo Scientifico Aldo Moro* in 1998.

She graduated in Parma in November 2004 with a major in Analytical and Food Chemistry in the laboratory of Prof. Palla and Dr. Caligiani, studying a method for the research of some analytical molecular markers for the characterization of different varieties of cocoa beans.

In 2005 she worked at *Parmalat* as laboratory analyst.

In 2006 she became a Ph. D. student in the group of Prof. Palla and Dr. Caligiani, focusing her scientific interest to balsamic vinegar of Modena, researching some analytical method for the characterization, the authentication and the quality evaluation of this product.

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