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METABOLOMIC APPROACH FOR CHARACTERIZATION AND DISCRIMINATION OF RED, WHITE AND ROSÈ ITALIAN WINES BY NUCLEAR MAGNETIC RESONANCE (NMR) AND HIGH RESOLUTION MASS SPECTROMETRY (HRMS)

Relatore
Prof. P. MASTRORILLI
Dipartimento di Ingegneria Civile,
Ambientale, del Territorio, Edile e di Chimica
Politecnico di Bari

Laureando
Javier Najarro Fernández

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1. Introduction

1.1 Wine and table grapes

The grape is a fruit that grows in tight clusters. Its skin is white or purple color and has a sweet taste. The grapes are consumed as fresh fruit or fruit juice, although it's main use is the production of wines. The grape has various vitamins and minerals, and it is believed to have antioxidant and anticancer powers.

1.1.1 Grape morphology

As depicted in Figure 1 each berry is made up of exocarp (the skin), mesocarp (pulp), and endocarp (which cannot be distinguished, however, from the rest of the pulp); this set of these three tissues is globally said pericarp, and surrounds the seeds. The fruit is fed by the ramifications of the vascular bundles of grape bunches entering through the pedicel.

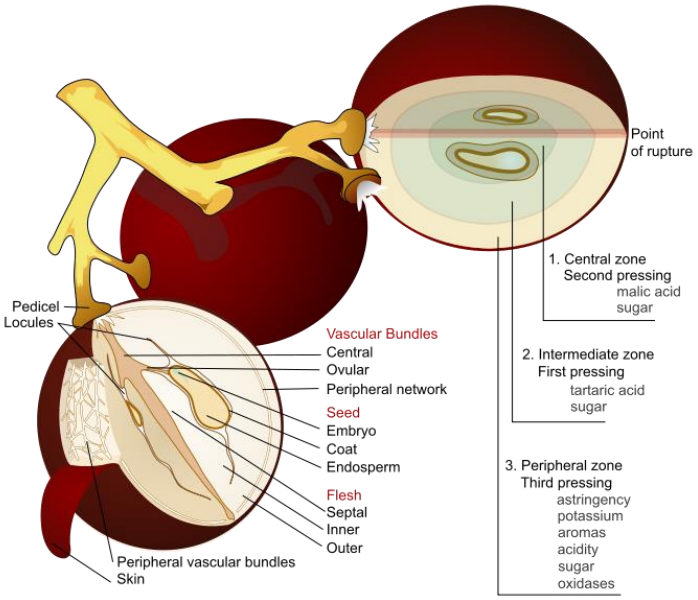


Figure 1. Grape morphology.

The skin is in turn made up of cuticle, epidermis and hypodermis. The cuticle is the outermost layer which is continuous and of variable thickness depending on the variety. It is generally covered by a waxy layer called the bloom. The epidermis is under the cuticle and it is consisting of one or two layers of cells elongated in the tangential direction; also for this layer thickness tends to vary depending on the variety. In the epidermis and in the outer layers of the hypodermis are concentrated tannins needed to

defend the berry from pests; they are linked to polysaccharides of the cell wall, or free in the vacuolar juice.

The pulp is made up of polygonal cells of large size, with thin and tense walls; the layers of cells ranging from 25 to 30 μm de longitud are organized into three distinct regions.

The grape seed, finally, is formed by a bulge and a thinnest part called spout. The main physiological function of the seeds is the production of hormones which are the promoters of the transformation and development of the ovary in berry.

The grape is the raw material for the production of wine, which comes from a natural process in which sugar for the fermentation of the most turns into alcohol. We have identified three zones when the grape development occurs during the wine formation.

First zone: the internal part of the seeds where the seeds are surrounded by a very high concentration of sugars and malic acid (in fact, sometimes this acid is converted to a sugar by gluconeogenesis). This area usually has a light green hues.

Second zone: the area concentric to the above one where the sugar concentration progressively decreases and increases the presence of tartaric acid which is the second chemical component in grapes after sugars. Malic acid and tartaric acid play an important role in winemaking and winemakers pay attention to the control of the presence of any of them in the final product.

Third zone: this area is the peripheral zone of the berry. It contains minerals, especially potassium, polyphenols such as tannins (located mainly in the outer of the skin), anthocyanins (responsible for the colors in wines), flavorings, etc. The characteristic flavors of the grapes are stored in this third area within the skin.

The way in which the grapes are crushed may affect the organoleptic properties of wine, for example, if you press the center of the grape berries some sugars are extracted, yielding few polyphenols, but if you continue to press the grape berries tannins begin to be extracted.

1.2 Wine

Wine is the product obtained by a total or partial alcoholic fermentation of fresh grapes or grape must reaching an alcohol content of not less than three-fifths of the total alcohol content.

Fermentation occurs by the metabolic action of yeasts which transform sugars contained in the grape fruit in ethyl alcohol and carbon dioxide. Sugars are the source for the development of fermentation. However, the wine represents the sum of a set of environmental factors: climate, latitude, altitude, hours of light, or temperature.

1.2.1 Historical development of wine

The ancient Greeks called Italy Enotria, "land of wine". Italy is the first wine producer in the world offering the most different kinds and brands (see Figure 2).

Each Italian regions are faithful to its viticulture traditions and tends to promote their own denominations. As a result there are more than two hundred officers' viticulture areas and about two million producers.

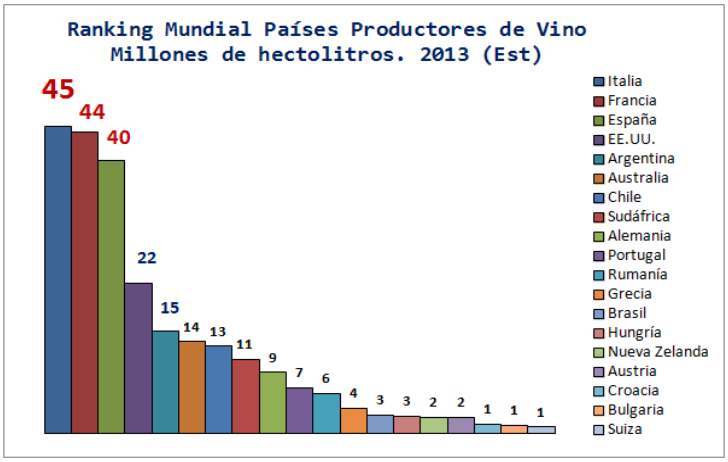


Figure 2. Countries with a high production of wine.

Depending on the time, modern Italy is the largest or second largest producer of wine in the world. In 2005, production was around 20% of the world total, second only to France, which produces 26%. In the same year, Italy's participation in the dollar value of imports of table wine in the US the market share in Italy has increased rapidly in recent years (see Figure 3).

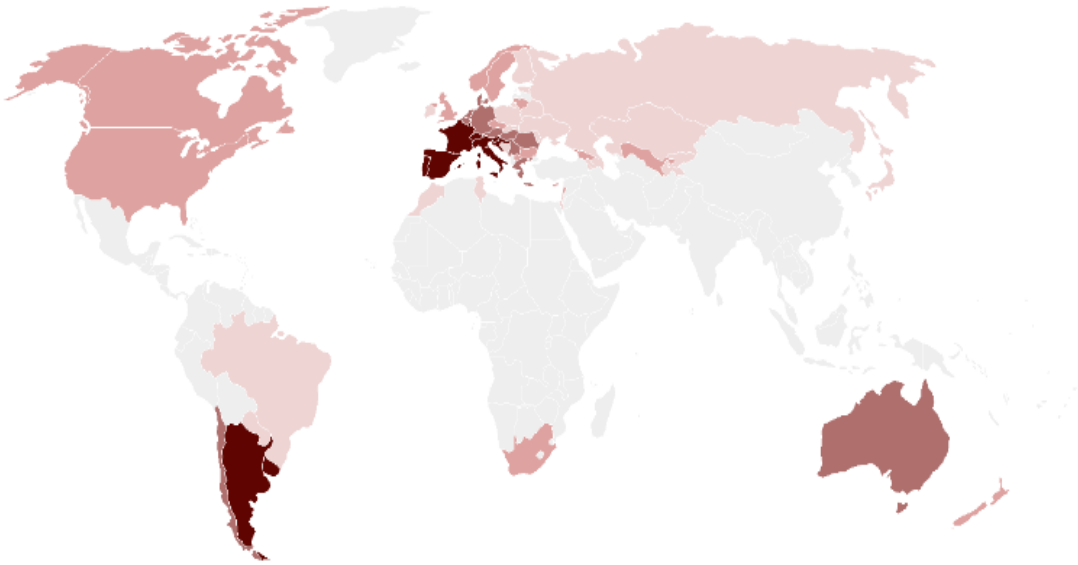


Figure 3. Wine *per capita* consumption world map per year, (- less than 1 liter; - from 1 to 7 liters; - from 7 to 15 liters; - from 15 to 30 liters; - more than 30 liters).

1.2.2 Vinification

- **Vinification of red, white and rosé wines**

The vinification is the process by which the must of grapes become wine and the process has these stages:

Harvest is the period when the ripe fruit are picked up (when the sugar content is between 200-250 g/liter). In the northern hemisphere harvest is done between September and October while in the southern hemisphere is performed between February and March.

Crushing and destemming consist of gently squeezing of the beans in order to extract the juice, being careful to not break the seeds. The rest of the grain are scraped.

Fermentation is the transformation of the sugar into alcohol. The fermentation lasts from 8 to 15 days, and during this period the yeasts are capable of transforming 17 grams of sugar in 1 degree or 1% of alcohol.

Separation of the marc from the wine.

Malolactic fermentation is a biochemical change of malic acid from wine.

Racking involves to pass the wine in different tanks in order to separate the wine from the sediment part, clarifying the wine.

Clarification and filtration consist to add beaten egg white to clean quickly and remove sediment.

Wine analysis and tasting panel are important steps in which wine is tasted and analyzed. According to these results wines take two paths:

Young wines: those which have completed light fruity and with moderate structure, bottled and rest a few weeks before going to market.
Wines of aging: those which are with great body and great structure and go through a process called aging that can last years.
Finally the bottling which is the final process before the placing in the market.

Scheme reported in Figure 4 show the different vinification in order to obtain red, white and rosé wines. Unlike red wines, which are obtained by alcoholic fermentation in the presence of solid parts (skins, seeds and stalks eventually), white wines come from the fermentation of grape juice only. For this reason, in the elaboration of white wines, the extraction of must and their clarification always come before the alcoholic fermentation. It is not the grape color, but the absence of maceration during the alcoholic phase that distinguishes the white vinification from the red ones. You can also obtain white wines from red grapes with white skins, if the grapes are pressed in appropriate conditions.

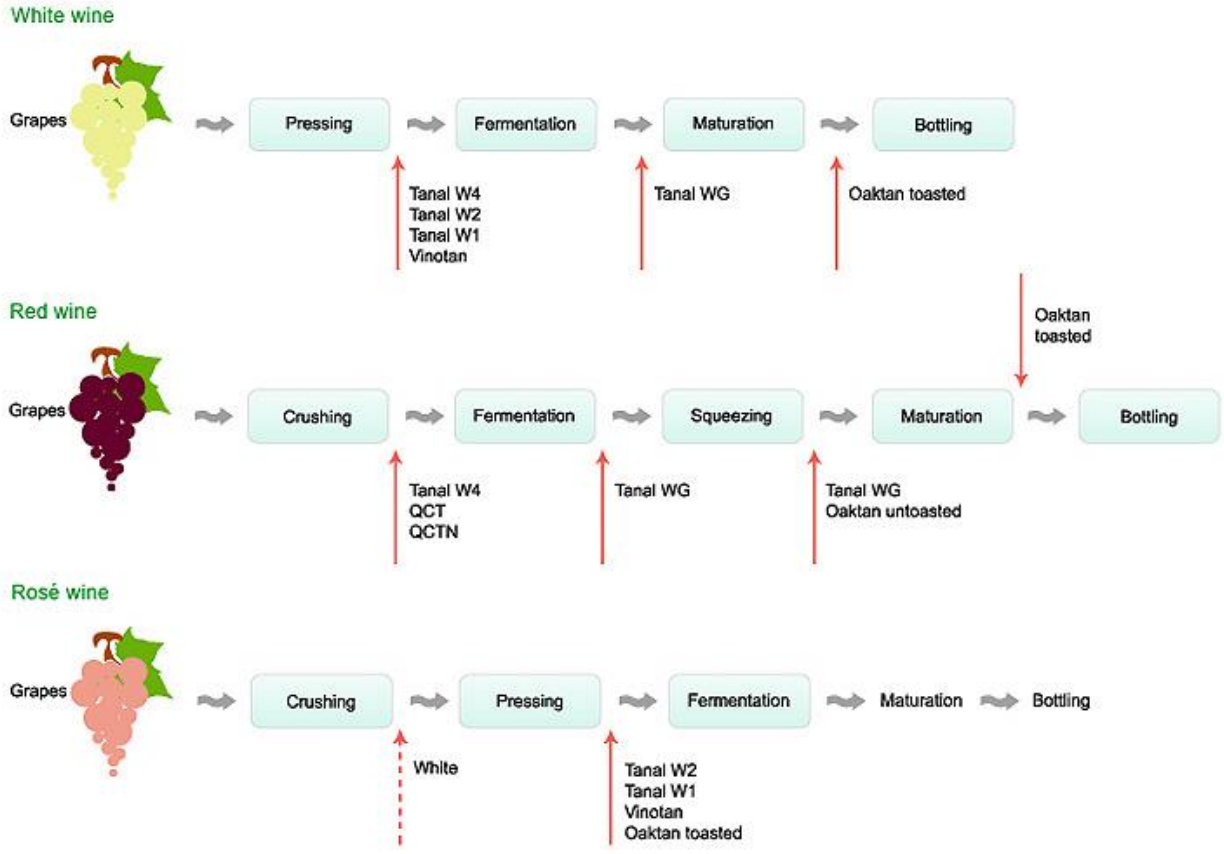


Figure 4. Three types of winemaking.

A limited maceration of the solid parts of the bunch is permitted, albeit with appropriate caution. The maceration to extract better constituents of grape skin participating aroma, structure and attitude to

aging of dry white wines. This type of maceration consists in the realization of a conscious and controlled phase of contact between the juice and the skins. Fill a tub of grapes were crushed and moderately; bear few hours collects the free run juice and drained the marc is pressed.

White wines are obtained away more or less complete from oxygen, limiting of all or part of oxidative phenomena, especially in the load of the phenols, during vinification and aging, in order to protect the fruity aromas of young wines and to avoid browning of color.

Just extract, grape must is more or less turbid. There are several particles in suspension: earth, fragments of husk and stalk, cellular debris resulting from the pulp and macromolecules in solution or colloidal ongoing precipitation. The racking is to separate, for example by decanting, the clear juice from their lees, before fermentation. Immediately after the necessary corrections are made of sugar content and acidity of the musts.

The fermentation tanks are filled with clarified must; aeration must be made during the replacement of the tank. The fermentation of a dry white wine, if well conducted, should not exceed ten days, unless an exceptionally high sugar content.

Unlike red wines, white wines malolactic fermentation is less practiced: its realization depends on the grape and wine-growing regions. Sometimes, to be protected from accidental malolactic fermentation in the bottle, you prefer to get it after the alcoholic fermentation, in perfectly controlled conditions.

The vinification of rosé is completed with different techniques. The first consists of a white vinification of black grapes, through direct pressing of fresh marc. To obtain the color, it is necessary a short period of maceration, which is performed directly in the cage of the press, during the draining.

The conduct of pressing has an impact on the essential quality of the wine. With the increase of pressure carried out, increases the extraction of total phenolic compounds. Later, must contains sulphite to be protected from oxidation. The practice of racking is less important than the white winemaking, but leads to a refinement of the aroma and the decrease of iron content; not necessary clarification of the must push as for white wines.

Alcoholic fermentation is made to occur especially with selected yeasts, to improve both its capacity to ferment that the organoleptic characteristics.

For this type of wines, for which the freshness and fruitiness are indispensable, the malolactic fermentation was not customary in the past. Today, however, despite the implementation is far from simple, the search for a certain softness leads to practice this secondary fermentation.
For most colorful and more structured rosé wines you can make another type of winemaking, which provides a maceration of the skins and seeds of the must, in order to increase the extraction of anthocyanins and tannins. Excessive maceration, however, can lead to a high colored wine with sharp perceptions of astringency and bitterness. This can be conducted either directly in the cage of the press for a period between 2 and 20 hours, one speaks of maceration, both in the tank for a period between 10 and 36 hours, is the case of vinification for bloodletting. The first is exclusively used for making rosé wines; the second has the aim to enrich and improve the red wines residues that remain in the tub after subtraction of wine designed for rosé wine production. The vinification for maceration brings softness to the wine and fruit character, with decrease in acidity.
The grapes are crushed, de-stemmed and the sulphite formation initiate in the tank. Then the juice is separated by pressing or bloodletting whole or in part and vinified according to the principles of winemaking for direct pressing. In some cases bloodletting regards rate of 10- 20% of the total, then the tank is filled with the must: on one hand it makes wine rosé wine and on the other hand a red wine enriched in phenolic compounds and color, for the effect of the increase of the proportion of marc. The contact time, temperature and sulfitation are the factors that influence the dissolution of the phenolic compounds and the final color.
The success of rosé wine is based on the use of good, fine and perfectly ripe grapes. [1]

1.2.3 Metabolic profile of wine

Organic acids

The acids in wine are an important component in both winemaking and the finished product of wine. They are present in both grapes and wine, having direct influences on the color, balance and taste of the wine as well as the growth and vitality of yeast during fermentation and protecting the wine from bacteria.

The measure of the amount of acidity in wine is known as the “titratable acidity” vs “total acidity”, which refers to the test that yields the total of all present acids, while strength of acidity is measured according to pH, with most wines having a pH between 2.9 and 3.9. Generally, the lower the pH, the higher the acidity in the wine. However, there is no direct connection between total acidity and pH (it is possible to find wines with a high pH for wine and high acidity).

In wine tasting, the term “acidity” refers to the fresh, tart and sour attributes of the wine which are evaluated in relation to how well the acidity balances out the sweetness and bitter components of the wine such as tannins. Three primary acids are found in wine grapes: tartaric, malic and citric acids. During the course of winemaking and in the finished wines, acetic, butyric, lactic and succinic acids can play significant roles.

Most of the acids involved with wine are fixed acids with the notable exception of acetic acid, mostly found in vinegar, which is volatile and can contribute to the wine fault known as volatile acidity. Sometimes, additional acids, such as ascorbic, sorbic and sulfurous acids, are used in winemaking.

Tartaric acid is the most important metabolite in wine due to the prominent role it plays in maintaining the chemical stability of the wine and its color and finally in influencing the taste of the finished wine. In most plants, this organic acid is rare, but it is found in significant concentrations in grape vines. Along with malic acid, and to a lesser extent citric acid, tartaric is one of the fixed acids found in wine grapes. The concentration varies depending on grape variety and the soil content of the vineyard.

The vine progresses through ripening, tartaric does not get metabolized through respiration like malic acid, so the levels of tartaric acid in the grape vines remain relatively consistent throughout the ripening process. [2]

Malic acid along with tartaric acid, is one of the principal organic acids found in wine grapes. It is found in nearly every fruit and berry plant, but is most often associated with green apples, the flavor it most readily projects in wine. In the grape vine, malic acid is involved in several processes which are essential for the health and sustainability of the vine. Its chemical structure allows it to participate in enzymatic reactions that transport energy throughout the vine. [3]

Lactic acid is often associated with “milky” flavors in wine. It is produced during winemaking by lactic acid bacteria. These bacteria convert both sugar and malic acid into lactic acid, the latter through malolactic fermentation. This process can be beneficial for some wines, adding complexity and softening the harshness of malic acidity, but it can generate off flavors and turbidity in others. [4]

Citric acid common in citrus fruits, such as limes, citric acid is found only in very minute quantities in wine grapes. It often has a concentration about 1/20 that of tartaric acid.

It is used less frequently than tartaric and malic due to the aggressive citric flavors it can add to the wine. In the European Union, use of citric acid for acidification is prohibited, but limited use of citric acid is permitted for removing excess iron. [5]

Acetic acid is a two-carbon organic acid produced in wine during or after the fermentation period. It is the most volatile of the primary acids associated with wine and is responsible for the sour taste of vinegar. During fermentation, activity by yeast cells naturally produces a small amount of acetic acid. If the wine is exposed to oxygen, Acetobacter bacteria will convert the ethanol into acetic acid. This process is known as the “acetification” of wine and is the primary process behind wine degradation into vinegar. An excessive amount of acetic acid is also considered a wine fault. A taster's sensitivity to acetic acid will vary, but most people can detect excessive amounts at around 600 mg/l.

Ascorbic acid also known as vitamin C, is found in young wine grapes prior to veraison, but is rapidly lost throughout the ripening process. In winemaking, it is used with sulfur dioxide as an antioxidant, often added during the bottling process for white wines. In the European Union, use of ascorbic acid as an additive is limited to 150 mg/l. [6]

Sorbic acid is a winemaking additive used often in sweet wines as a preservative against fungi, bacteria and yeast growth. Unlike sulfur dioxide, it does not hinder the growth of the lactic acid bacteria. In the European Union, the amount of sorbic acid that can be added is limited — no more than 200 mg/l. Most humans have a detection threshold of 135 mg/l, with some having a sensitivity to detect its presence at 50 mg/l. Sorbic acid can produce off flavors and aromas which can be described as “rancid”.

Succinic acid is most commonly found in wine, but can also be present in trace amounts in ripened grapes. While concentration varies among grape varieties, it is usually found in higher levels with red wine grapes. The acid is created as a byproduct of the metabolization of nitrogen by yeast cells during fermentation. The combination of succinic acid with one molecule of ethanol will create the ester mono-ethyl succinate responsible for a mild, fruit aroma in wines. [1]

Alcohols

Along with ethanol wines possess small quantities of other alcohols such as methyl alcohol (CH_3OH) which are not the direct result of the fermentation, but of enzymatic pectin (existing in the skin of grapes) hydrolysis. Because pectin is found more in the skin white wines contain less methyl alcohol than red wines. Sometimes it pre-heats the worth to delete this methyl content and stay in concentrations below 30 ppm. Reports of methanol content in wines from around the world indicate concentrations of 60 mg / liter (in a range from 40-120 mg / liter) for white wines and 150 mg / liter (range of 120-250 mg / liter) for red wines. Despite being the toxic methanol, quantities possessing wine are not quite as malignant lethal dose of 340 ml / kg, causes an average 70 kg person having to take approximately two hundred liters.

There are other alcohols in very small concentrations, such as polyols, one of the most important tri-alcohols is glycerol (glycerin) and their concentration is directly related to the fermentation temperature, with the total content of alcohols (greater Alcohol greater amount of glycerol) and the color of wine (higher in red wine than in white wine). The concentration of this alcohol is higher in table wines. The average glycerin content in wine is usually between 15 to 25 g / liter. Glycerin is synthesized in large part due to the fungus *Botrytis cinerea*, although there is some presence in healthy grapes. There is usually a higher content of glycerol fermentation at high temperature (this is the reason why red wines tend to have a higher content of glycerol). Glycerol is a dense, with a sweet taste (approx. 70% glucose) liquid and its presence adds sweetness and a feeling of fullness in the mouth.

Another poly-alcohol in wine is erythritol and its concentration depends on the strain of yeast that ferments the wine. Arabitol, mannitol, sorbitol (hexa mannitol alcohol isomer), inositol (hexa frequent alcohol in fruits). Almost all of these polyols provide sweetness to wine and have the

characteristic of being highlighted their concentrations when the noble grape rot is present. [7]

Esters

Alcohols play an important role in the maturation operation, after fermentation, and which react with natural grape acids to form esters (esterification). Of all those in the wine functional groups, esters are the most abundant: about 160 different identified. The esters are often categorized in enology into two categories: those from enzymatic reactions (butanoate, exanoato) and those chemically formed by esterification. Esters are the main components responsible for providing the wine a bouquet.

Many esters have a characteristic fruity aroma, making fruit fragrances during the tasting. However there are other classifications of esters oriented wine tasting, and are divided into volatile and non-volatile esters. One of the most important volatile esters and that is present in wine is ethyl acetate. Generally young wines usually have a higher concentration of volatile esters. Each ester has a threshold below which it is not perceptible by most humans.

Sugars

The major carbohydrates present in the juice are glucose and fructose, other carbohydrates are found in grapes but negligible proportions. The concentration of sugars is critical to the growth of yeasts during fermentation, the main wine yeast (*Saccharomyces cerevisiae*) feeds mainly glucose and fructose. Sugars unconsumed after fermentation are often called residual sugars (usually pentoses, such as arabinose, rhamnose and xylose). The concentration of these residual sugars during ripening can increase wood.

The residual sugar is important in the sweet tone of a wine, while the presence of residual sugars not only affects the fermentation. The presence of residual sugar in the wine results in a classification between dry and sweet wines. Generally the presence of a concentration of sugars of less than 1.5 g / liter makes the palate not detect the sweet taste, above 0.2% volume begin to sense detect the sweet taste of wine. Most people detect sweetness if it reaches a concentration of 1%.

Nitrogen containing compounds

Nitrogenous compounds are essential in the must to make it possible to correct fermentation. Among the predominant amino acids in grapes is proline and arginine. The reason for proline / arginine varies significantly in different varieties of *Vitis Vinifera*. Proline is an important part of nitrogen metabolism in yeast. As a second group of amino acids having dominant glutamine and alanine. As presumably the amino acid content is less after fermentation: partly because most of them somehow enter the yeast metabolism.

Among the nitrogenous compounds possessing wine proteins are found in concentrations of must ranging from 100 mg / L at 840 mg / l. During fermentation, the protein content may fall nearly 40%. Proteins act as zwitterions, can under certain circumstances instability resulting coagulate in the wine. Remove these unstable wine proteins is one of the objectives of clarification, one of the most used agents is bentonite and the other is silica gel.

Inorganic constituents

The mineral substances are represented by the ashes, which correspond to the set of products of incineration of the evaporation residue of the wine, conducted so as to obtain the totality of the cations in the form of carbonates and other mineral salts anhydrous. The minerals are located mostly in the solid parts of the grape.

The main mineral anions of musts and wines correspond to more or less soluble salts. The nitrates are present state of tracks, and are very soluble, as well as chlorides and anions phosphoric and sulfuric. Other anions minerals that exist in wine in the state of traces are bromide, iodide, fluoride, and the derivatives of silicic acid and boric acid.

The cations must be kept under control since they are involved in the formation of precipitates: the case of potassium (present in a dominant manner), calcium, iron (III) and copper (I), which may precipitate as bitartrates, phosphates, tartrates and chlorides.

Phenolic compounds

In Figure 5 the classification of the phenolic compounds are reported.

Polyphenols play a primary role in winemaking; are mainly responsible for the differences between white and red wines, in particular color and taste. During vinification are extracted from different parts of the grape and

undergo considerable changes in the structure during aging and the aging of the wine.

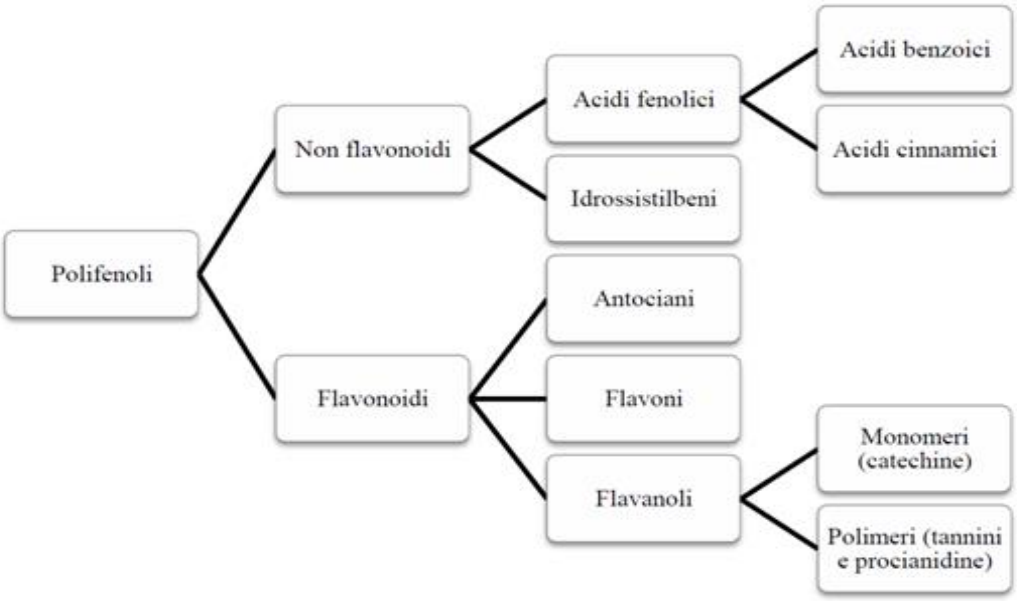
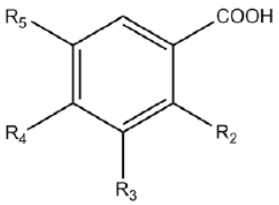
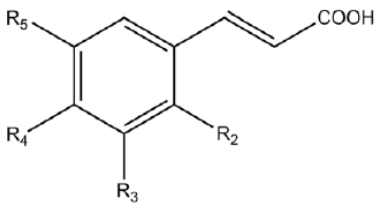


Figure 5. Phenolics classification.

Phenolic compounds play an essential role especially in the formation of the flavor of red wines. They are responsible for both characters gustatory positive, both negative characteristics; the feeling global sensorial results from a balance, directly related to the nature and concentrations of the different compounds present in wine.

Phenolic acids: they are mainly constituted by benzoic and cinnamic acids derivatives. As shown in Figure 6 there are seven known benzoic- type acids, such as salicylic acid and gentisic acid. They differ by the degree and nature of the substituents of the benzene ring and they can be found in grapes in the form of glycosides and esters. Free forms are more present in red wines. The cinnamic acids are essentially in the esterified form with tartaric acid or as glycosides of glucose.





Acidi benzoici	R_2	R_3	R_4	R_5	Acidi cinnamici
acido p-idrossibenzoico	H	H	OH	H	acido p-cumarico
acido protocatechico	H	OH	OH	H	acido caffeico
acido vanillico	H	OCH_3	OH	H	acido ferulico
acido gallico	H	OH	OH	OH	acido sinapico
acido siringico	H	OCH_3	OH	OCH_3	
acido salicilico	OH	H	H	H	
acido gentisico	OH	H	H	OH	

Figure 6. Phenolic acids.

Stilbenes: a family of compounds which is present in the grapes and represent more complex polyphenols, is stilbenes which are characterized by two benzene rings joined by an ethylenic chain. Among them, the *trans* isomer of resveratrol (3, 5, 4'-trihydroxystilbene) would be produced from the vine in response to attack by fungal parasites. Positive influences on human health and different pharmacological properties have been recently attributed to resveratrol. This compound is located in the skins, and extracted especially during the vinification of red wines. [7b]

Anthocyanins: pigments of red grapes, derived from anthocyanidins (see Figure 7) for glycosylation at position 3; are basically in the peel, rarely in the pulp, but they are also present in the leaves. The molecule of anthocyanins is constituted by benzene rings joined by an oxygenated and unsaturated heterocycle. In grapes and wines five basic molecules forms are distinguished (anthocyanins).

The color of these pigments is a function of the composition of the medium and depends not only on the substituents on the rings, but also by glycosylation.

The acidic solutions containing these substances are red and will discolor with pH increasing.

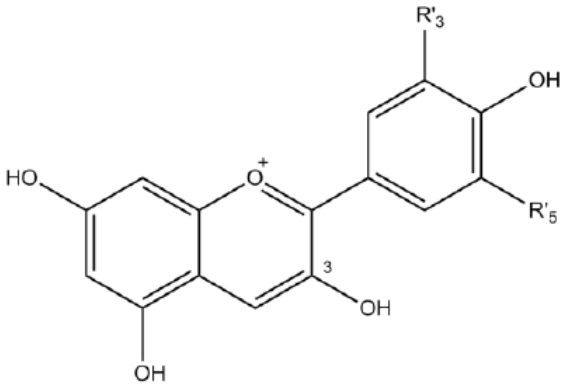
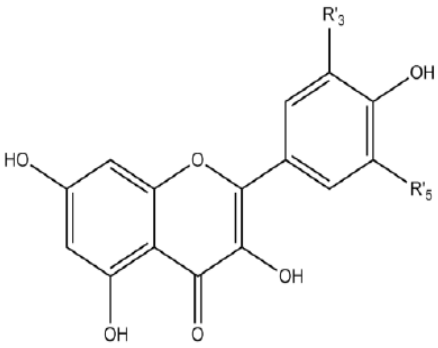


Figure 7. Anthocyanidin.

Flavonols: they are pigments of more or less deep yellow-quercetin and have a structure characterized by two benzene rings joined by an oxygenated heterocycle. The most common compounds are flavonols, yellow pigments of the skins of red and white grapes and flavanonols. In grapes these molecules are found in different forms (see Figure 8)**.**



R'3	R'5	
H	H	campferolo
OH	H	quercetina
OH	OH	miricetina

Figure 8. Flavonols.

Tannins: they are defined as substances able to originate combinations with proteins and other natural polymers, such as polysaccharides, via hydrophobic interactions and hydrogen bonds. When these compounds react with the glycoproteins rich in proline of the saliva, determine the sensory sensation of astringency. The tannins are derived from the polymerization of monomers containing phenolic functions; so that they can produce stable combinations with proteins which are sufficiently large.

Depending on their nature, they are divided into hydrolyzable tannins, condensed tannins and complex tannins.

The condensation products of the tannins are called procyanidins, and they can be dimers, trimers or oligomers. The condensed tannins, particularly procyanidins, are present in all the solid parts of the grape,

and pass into the wine during the maceration step. Their concentration depends on the variety, but especially from the winemaking conditions.

Terpenes (grape aroma and flavor)

The odorous compounds of the grapes belong to the category of terpenes. They are present in grapes as glycosylated form and also as compounds with thiol functions (responsible for olfactory defects).

1.3 European and Italian legislation wine

The enhancement of the quality of a wine is also based on legislative frameworks to ensure the identity of the products, through systems of national recognition. The discipline of the appellations of origin of wines ranking designations of origin and geographical indications typical.

For designation of origin of wines means the geographical name of a wine-growing area particularly suited used to describe a renowned quality product, whose characteristics are due to the natural environment and the human factor.

For IGT wine is the name of a geographic area used to describe the product that results.

The designations of origin and geographical indications typical are classified as:

- Appellations of Origin Controlled and Guaranteed (DOCG)
- Appellations of Origin (DOC)
- Typical Geographic Indications (I.G.T.)

For each of the above mentioned three categories and, in particular for each type of wine, there are the production regulations, which consist of a set of articles, which contain indications as to which the manufacturer must make reference to get a wine with specific characteristics.

The production rules are composed by an average of 8 items, which specify, in order, the type of wine, the geographical area of origin of the grapes, the rules for viticulture and wine-making, the characteristics of wine consumption , labeling and packaging. The characteristics of the wine consumption include the following parameters: color, smell, taste, alcoholic strength, total acidity and extracted minimum non-reducing. The

specifications for wines D.O.C.G. have characteristics more stringent than those for DOC, in turn more specific than those for IGT.

In order to use a specific appellation, the wines produced in accordance with the rules laid down for the description and presentation of DOCG and D.O.C. and the specific production rules, at the production stage, according to the rules of the EU, must be subjected to a preliminary chemical-physical analysis and an organoleptic test. The DOCG wines, in addition, the organoleptic examination must be repeated, batch by batch, during bottling.

The chemical analyzes performed on the must are of two types:

- Determination of acidity
- Determination of sugar content
- The main chemical analyzes on wine are:

  a) Determining the alcoholic strength: The alcoholic strength by volume (% vol) expresses the liters of ethanol per 100 L of wine, at 20 ° C. The measurement is made by distilling wine and subsequent determination of the relative density, with a hydrostatic balance, or specific gravity, with a pycnometer. In both cases, by means of the density tables goes back to alcohol.

  Another type of measurement can be completed by ebulliometer of Malligand. This method is based on the different boiling water (100 ° C) and ethanol (78.3 ° C), which together give a mixture with a boiling point intermediate between the two substances.

La formula sería:

$$\text{gramos alcohol} = \frac{\text{volumen (expresado en c.c.)} \times \text{graduación} \times 0{,}8}{100}$$

Figure 9. Calculation of alcohol consumed

  b) Determination of total acidity and volatile.
  c) Determination of sulfur dioxide and free combined.
  d) Determination of the dry.
  e) Determination of reducing sugars.

The EEC regulation. 2676/90 determines the methods of analysis to be used in the wine industry, listing the types of analyses to be performed, with related procedures.

To make the determinations listed above are required numerous analyses, each of which requires a specific pre-treatment of the test sample. These analyses provide information on a number of properties of a general nature, but are not sufficient to ascertain characteristics of the product more sophisticated as, for example, the origin or geographical varietal. In fact, a study conducted at SAMER, the chemical laboratory of the Chamber of Commerce of Bari, has shown that the traditional analysis conducted on wine and extra virgin olive oils according to production specifications (DOC, DOCG, PDO, etc.). Although used for classification of the same products, in reality do not allow the distinction varietal origin, geographic origin and vintage production. It is desirable, therefore, the development of analytical methods which are able to describe the sample in more detail. Metabolomic approach supported by means of nuclear magnetic resonance and mass spectrometry analytical techniques is well suited for this purpose, being able to provide information about a large number of substances contained in the sample, with a simple analysis. [8b]

1.4 Metabolomics

Metabolomics is the scientific study of chemical processes involving metabolites. Specifically, metabolomics is the systematic study of the unique chemical fingerprints that specific metabolic processes leave behind, obtaining their small-molecule metabolite profiles. The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes. This approach can be applied to the study of wine samples to investigate the metabolic profiles at the end of the vinification process.
Among the spectroscopic techniques NMR and Mass spectrometry are the most used in the metabolomic analysis: NMR, with the main use of the NMR spectroscopy of proton ^{1}H, and spectrometric mass (MS), in particular coupled to high performance liquid chromatography.

1.4.1 High Performance Liquid Chromatography (HPLC) coupled to Mass Spectrometry

The high performance liquid chromatography (HPLC) is the most used of all the techniques of analytical separations, due to the high sensitivity versatility and applicability.

The instrumental method HPLC is the result of the technological evolution of the techniques of column chromatography. The principles are the adsorption and distribution of the analytes in stationary phases which are packed in closed columns with very fine materials. The columns are controlled in a such way that as the contact area between the mobile phase and the stationary phase increases and the packing becomes more homogeneous. For these types of columns the mobile phase needs to flow at high pressure because, through columns with packing size particle so fine, the eluent flow becomes very slow. With the use of special pumps pressures of 50-150 atm are applied obtaining flow from 0.2 to 1.0 mL/min, sufficient to reach the elution in a reasonably short time.

The HPLC can be classified in several ways:

- Direct chromatography is performed with polar stationary phase and non-polar mobile phase.
- Reverse phase chromatography where stationary phase is a non-polar compound (often a hydrocarbon) and the mobile phase is relatively polar (such as water, methanol or acetonitrile).

On one hand in direct phase chromatography first the less polar component is eluted, because they are more soluble in the mobile phase; increasing the polarity of the mobile phase a decreasing the elution time of the polar compounds occurs. On the other hand in reverse phase, the component more polar appears first. Then increasing the polarity of the mobile phase the elution time of the polar compound decreases.

The mobile phase used in a HPLC separation have some general requirements: low viscosity, immiscibility with the stationary phase, low cost and easy availability, low volatility, compatibility with the detector, the minimum possible toxicity, low corrosivity, and especially high purity. The containers of the mobile phases are often equipped with a system for the elimination of dissolved gases, particularly oxygen and nitrogen, forming bubbles that interfere in the detector. Often, in addition to, the systems of degassing there is a dust filter for the particulate suspended in the solvent.

When using an eluent for separation at constant composition, it is called isocratic elution. It is possible to improve the efficiency of a separation using a gradient elution. In this case there are two or more solvents, which differ significantly in their polarity. When starts the elution we can vary the ratio of the solvents, in a continuous form or with a step change. The solvents are transported into a mixing chamber, with variable speed, in continuous and the ratio between solvents volumes can be changed as a linear function or exponential with respect to time.
Most of the analytical columns varies in length from 10 to 30 cm. Normally are straight, with the possibility of increasing the length, when is necessary by coupling two or more columns.

The HPLC separation apparatus can be coupled to a mass spectrometer. The mass spectrum of a sample is obtained by converting the components in the gaseous ions, and then separate them according to their mass to charge ratio. Mass spectrometry is very applicable, because it can provide a series of information about the qualitative and quantitative composition of organic and inorganic analytes in complex mixtures or the structure of a big range of molecular species or the isotopic ratios of the atoms in the samples.
The main components of a mass spectrometer are: the introduction system, the ion source (converts components of the sample into ions), the mass analyzer and the detector.

There are different types of sources, depending on the mode of ionization, which can be electronic (EI), chemical (CI), field (FI) for desorption of field (D), for fast atoms bombardment (FAB), for desorption using a laser and assisted by the matrix (MALDI) and electrospray (ESI).

The most source used for coupling the HPLC with the mass spectrometer is the one that allows the electrospray ionization of the analyte. The mobile phase comes from the HPLC column and is introduced into a capillary or metallic needle with a high potential difference (a few kV) with respect to a screen provided with a central hole, which is the entrance to the mass spectrometer. In ESI the analyte is usually present as an ion (positive or negative) in the HPLC mobile phase, thanks to the action of acids and/or bases. In case it is positively charged, his counter ion (negative) is discharged on the walls of the capillary spray, while the accumulation of positive charges do that the solution take at the output a shape of a cone (Taylor cone), due to the mutual repulsion electrostatic. When from the vertex of the Taylor cone the electrostatic repulsion

overcomes the surface tension of the mobile phase, the liquid filaments are separated and then, appear drops relatively with large size. The process leading to the formation of individual ions of the analyte, optionally solvated, this starting from the drops and can happen in two mechanisms:

- Coulomb Fission: the larger droplets are reduced by evaporation of the solvent; as, the concentration of positive charges in a smaller volume increases until the repulsion to overcome the surface tension: the droplet explodes into smaller droplets. The process continues in the chain up to the single ion, which can be equipped with more charges.
- Evaporation ion: an ion can be expelled from the surface of a small drop, before it is reduced further, due to the electrostatic repulsion of the adjacent ions

The ESI is a technique of "soft" ionization: in ESI-MS spectra the main ion is in almost all cases the molecular ion.

In most of the interfaces ESI a main inert gas flow (nebulizing), usually nitrogen, and optionally one auxiliary (drying) are used to facilitate evaporation of the solvent from the droplets, as well as to direct the aerosol towards the entrance hole of the mass spectrometer.

The analyzer is the key component of the instrument. Several methods for separating ions with different mass to charge ratios. In theory, a mass analyzer should be able to distinguish even very small differences in mass and allow the passage to a number of ions sufficient to provide the ionic streams easily measurable. The ability of a mass spectrometer to differentiate the masses in terms of resolving power, defined as: $R = m/\Delta m$ where Δm is the mass difference between two adjacent peaks resolved, is the nominal mass of the first peak. Two peaks are considered separate if the height of the vale between them is below a certain percentage of their height (usually 10%).

Among the various types of analyzers on the market, you can make a distinction based on the form in which they operate:

- Continuous mode: the analyzer is crossed by a continuous flow of ions. The magnetic field and the quadrupole operate in this mode.
- Pulsed mode: the ions are transferred to the analyzer periodically, for short times. The most common example is the time of flight (TOF).

- Trapping mode: the ions are trapped, through electromagnetic fields, in a spatial region defined. There are different types of traps, including the three-dimensional ion trap, linear, Fourier transform in the cyclotron and the Orbitrap.

The ions that reach the analyzer in TOF pass through a drift tube, about a meter long, in which there is absence of electric fields. After all ions that cross the tube have formally the same kinetic energy, their speeds are inversely proportional to the masses: the lighter ions reach the detector earlier than the heavier ions. The temporal separation occurs due to the differences in the speed of the ions.

In spectrometers time of flight detector is generally an electron multiplier; the digital data acquisition electronics requires a very fast, since the times of the flights of a few microseconds. [1]

1.4.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

The Nuclear Magnetic Resonance (NMR) is based on the measurement of absorption of electromagnetic radiation in the radio frequency region, including approximately between 400 and 600 MHz, by the nuclei of atoms of a sample immersed in a strong magnetic field.

Many atomic nuclei possess a property called spin that causes them to rotate about themselves. This rotation creates an electric field which in turn creates a magnetic moment, and makes them susceptible to resonance Magnetic.

Creating the magnetic moments with spin nuclei may be as small dipoles that under normal conditions (without applying any field), have random directions.

If a static magnetic field applied externally is enough powerful, the dipoles are aligned in the field direction, resulting in two possible different configurations:

High energy state (α-spin) where the vector field and dipole are antiparallel.

Low power state (β-spin) in which the vectors of field and dipole parallel.

Under these conditions there is greater number of spins in low power state, compared to spins at high energy state. The ratio between them follows a Boltzmann statistics.

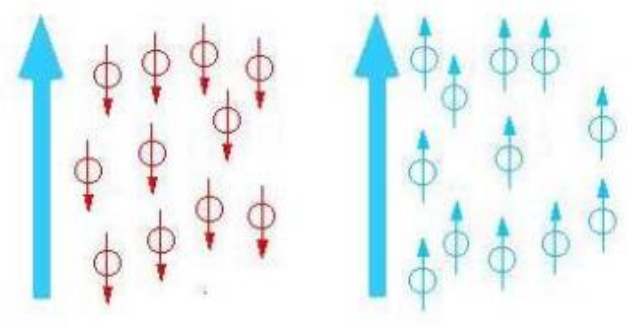


Figure 10. High energy state (red) and low energy state (blue).

By stopping the magnetic field, the nuclei that had changed status, return to their original state issuing the difference in photon energy as well as the Larmor frequency.

The resonance frequency of each nucleus is largely influenced by the chemical neighborhood in which nucleus is located: Each core is then screened by an electron cloud whose density depends from the neighborhood in which it is inserted. The position of the nucleus in the NRM spectrum is defined by σ, the chemical shift (expressed in ppm) which is a parameter that measures the effect of the displacement and is given by the shift of the resonance frequency of each core with respect to a frequency used as a reference. [11]

The NMR spectrometer is constituted by a magnet, a radio frequency oscillator and a receiver. All modern tools are based on superconducting magnets cooled in liquid helium. The cryomagnetism today represent the most effective method to produce magnetic fields: the field is produced by a coil (solenoid) consists of a thin of an Nb-Ti alloy. The sample is introduced into the probe (probe) that contains the windings of the transmitter, the receiver, and a spinner which rotates the tube near its vertical axis, so as to mediate the inhomogeneity of the field.

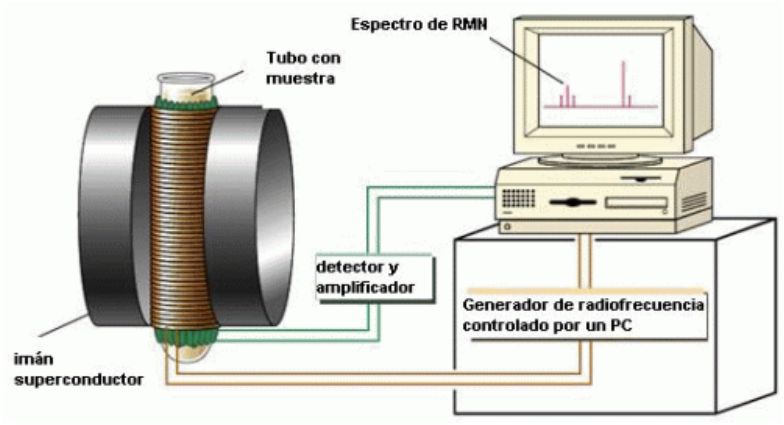


Figure 11. Schema of NMR instrument.

NMR is a technique that provides greater structural information. In the presence of a magnetic field, the nuclei of atoms possessing a magnetic moment spin which have two or more directions under the field. The behavior of the nuclei in the magnetic field may be influenced by multiple ways to give various kinds of information, but the basic information you get is:

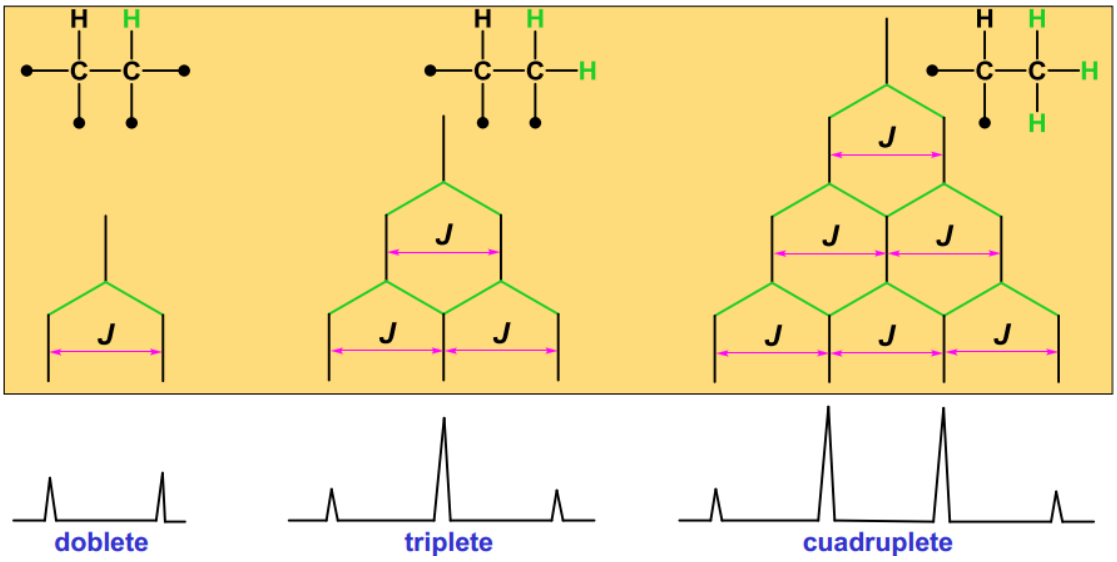
a) Frequency at which every nucleus is positioned: **displacement**;

b) Number of cores of the same nuclei type: **integral**;

c) Chemical neighborhood of the nuclei: **multiplicity**.

The signal of a nucleus type can be split into different signal groups due to coupling with other not identical nuclei.

If the coupling, for example, is with one, two or three identical protons the signal is split in a duplet, triplet and quartet respectively (see Figure 12).



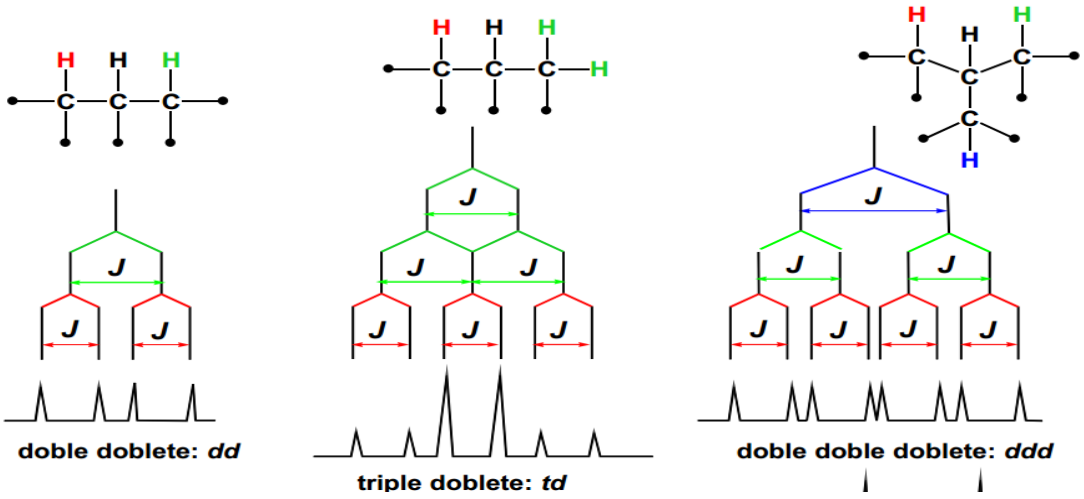


Figure 12. Way to find the structure that exists in our spectrum.

The magnitude of the coupling expressed in Hz is variable, depending on the relative disposition of the coupled protons giving important structural compound informations (chemical bonds, functional groups, spatial distribution, etc.).

For example, the coupling between neighboring aliphatic protons (in contiguous carbons), when there is free rotation has a value of 6 Hz as shown in Figure 13. [12]

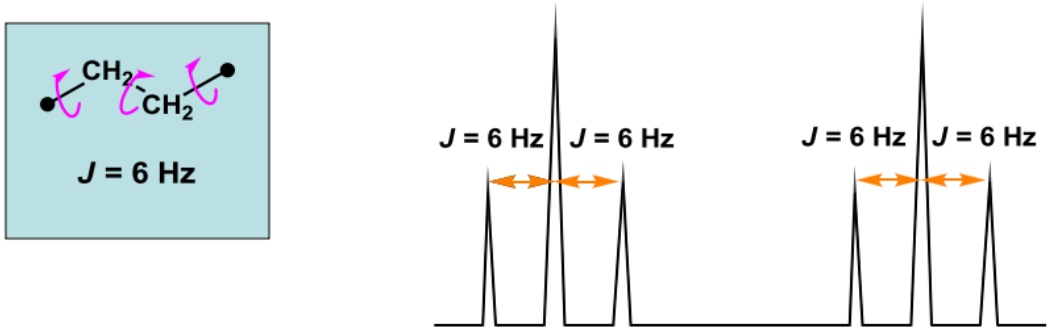


Figure 13. Definition illustrated the coupling constant J.

1.5 Principal Component Analysis (PCA)

The principal component analysis Principal Component Analysis (PCA) is a technique used in the context of multivariate statistics for the simplification of the original data. The primary purpose of this technique is the reduction of a number higher or lower than physically measurable variables (representing as many characteristics of the analyzed

phenomenon or properties of the samples analyzed) in new latent variables (called principal components, PC), among them uncorrelated (orthogonal), easily interpretable and able to highlight and summarize the information inherent in the initial matrix. Using this technique it is possible to assess the correlations between the variables and their relevance, view objects (identify any classes), summarize the description of the data, reducing the dimensionality of the data, querying the main and define a model of data representation in a space orthogonal.

The PCA consists of a rotation process of the original data defined by a matrix X of dimension n x p carried out so that the first new axis is oriented in the direction of maximum data variance, the second is perpendicular to the first and is in the direction of the subsequent maximum variance of the data, and so on for all p new axes.

The mathematical procedure for the determination of the main components consists in the calculation of the eigenvalues and eigenvectors of the covariance matrix (or correlation) of the data X, that is, in the diagonalization of the covariance matrix S of X.

The diagonalization of the covariance matrix involves the determination of a diagonal matrix said matrix of the eigenvalues, whose diagonal elements are the eigenvalues in descending order, and a matrix of the loadings L, whose columns are the eigenvectors of the covariance matrix. The eigenvectors are the unit vectors of the new space; the axes of the new space (major components) are the axes relative to the directions of maximum variance, in descending order. The covariance matrix S can be decomposed into two matrices by the technique of Single Value Decomposition (SVD). It is therefore possible to represent the initial data matrix X in a new orthogonal space, according to the following relationship:

$$T_{(n \times m)} = X_{(n \times p)} L_{(p \times m)},$$

Where L has the function of a rotation matrix, and T is called the matrix of scores.

The matrix L of the *loadings* is the matrix whose columns are the eigenvectors of the covariance matrix (or correlation) and where the rows represent the original variables. The *loadings* are standardized linear coefficients, the sum of squares of the *loadings* of an eigenvector is equal to 1, and the eigenvectors have unit variance.

The value of the scores is the result of a linear combination, in which the variables are the original variables (scaled or unscaled) and whose multiplication coefficients are the loadings.
The PCA provides the possibility of a graphical view of the results: it is possible to obtain both a representation of the objects (using the scores plot) that the variables (loadings plot). The *loadings plot* allows to analyze the role of each variable in the different components, their direct and inverse correlations and their importance. Groups of variables that appear in the neighboring *plot loadings* indicate that, limited to information carried by these components, they bring information common or similar (are related): if this happens for all the components considered as a model, you can represent the contents of information carried by this group of variables with one of them. This also applies to variables that appear in a position opposite to each other relative to the origin: also in this case, these variables are correlated or more correctly, inversely related. The scores plot allows to analyze the behavior of objects in the various components and their similarities. The *scores plot* allows to analyze the behavior of objects seen in the light of the components considered, that is, in the light of their meaning and values of the variables that best characterize them. In this way it is possible to see groupings of similar objects (*clusters*), the presence of particular objects (*outliers*), the occurrence of any regularity and distributions.
The correlation matrix R explicitly contains all the information regarding the correlations between the variables considered. The main diagonal of the correlation matrix is composed of values all equal to one (each variable is perfectly correlated with itself). The non-diagonal inform us about the correlation between all pairs of variables: these values are between -1 and +1. In the first case there is a perfect inverse correlation (when a variable increases, the other decreases); in the second case it has a perfect direct correlation (when a variable increases, the other also grows).

1.5.1 AMIX and MZMine

AMIX and MZMine are the softwares used to conduct our multivariate analysis of the mass data, in order to obtain the PCA for the discrimination of different agronomical product samples.

The first software used is AMIX. AMIX is designed to be machine independent, where the mass data are represented netCDF files which do not give information on alignments, artifact removal, etc, as the standard input. The key idea in AMIX is to do the bucketing along m/z close to the expected (machine dependent) variation of peak positions (within spectrum and within ensemble of spectra, the larger one counts) but due to the bucketing technique still get a table of acceptable size. As usual, the validation consists in finding relevant loadings from the loadings plot, turn the corresponding m/z values into sum formula and validate by looking into biochemistry.
The second software used is MZmine which is an open-source project delivering for mass-spectrometry data processing, with the main focus on LC-MS data. It is based on the original MZmine toolbox described in 2006 Bioinformatics publication, but it has been completely redesigned and rewritten.
MZmine maximum detection is performed in a three-step manner: first, the total values are detected within each spectrum (several methods are available, depending on the nature of the data). In the second step, a chromatogram is constructed for each of the total values that cross over a certain range of time. Finally, deconvolution algorithms are applied to each chromatogram to recognize the actual chromatographic peaks. The complete procedure which includes an alignment process (not performed by the AMIX processing) will be explained in the multivariate analysis sections.

1.6 Objectives

In this thesis the research was focused on the application of a metabolomic analysis of agronomical products such as Italian red, white and rosè wines. On the basis of this metabolomic approach, this work was aimed to the processing of the NMR and Mass data previously acquired by the chemical researchers of the laboratories where this study was carried out. The NMR and Mass data instrumental acquiring conditions were reported in the experimental sections. The investigation permitted the definition of the metabolic profiles of the typical Italian wine samples by the identification of the metabolites on the HPLC-Mass chromatograms and NMR spectra. Multivariate statistical analysis was used for the discrimination of the wines grouped by colors: red, white and rosè.

Moreover for the LC-Mass data two different softwares were applied to 42 wine samples divided in three groups of 14 samples for each color, in order to demonstrate that the "standard" peak-picking alignment routine followed by MZMine software leads to the same results obtained by AMIX software.

2. Development and experimental part

2.1 Materials and methods

The wine samples were stored at -20 ° C, respectively, at 4 ° C until analysis. The lyophilization was carried out with a lyophilizer Christ GmbH, Model 87 Alpha 1-4 LSC; the centrifugations were carried out with a centrifuge ALC PK 110; mechanical agitation were performed with a Pulsing Vortex Mixer VWR.
The D_2O (99.9%$_D$) and acetonitrile (HPLC purity) were bought from Sigma Aldrich, TSP (99%$_D$) was bought from Armar Chemicals, formic acid (HPLC purity) was bought from Fluka. The water was deionized with a system Millipore MILLI-DI to reach a resistivity of 18.2 MΩ cm at 25 ° C.
The NMR spectra were recorded using a spectrometer Bruker Avance I 400 MHz, equipped with a 5 mm inverse probe and an autosampler. Each spectrum was obtained and processed using software TopSpin 3.0 (Bruker BioSpin GmbH, Rheinstetten, Germany).
The HPLC-HRMS were performed with an Agilent 1200 series coupled to a spectrometer micrOTOF-Q II (Bruker Daltonik GmbH). Each spectrum was obtained using the software HyStar 3.2 and processed using the software Data Analysis 4.0 (Bruker Daltonik GmbH).

2.2 Supply of samples

In this thesis were analyzed by NMR and HRMS 340 commercially wines. The wines were ordered according to color: red, white and rosé wines. Other informations of the wines are the denomination of origin, the variety and the year of production.

In the collection there are: 201 red wines, 81 white wines and 58 rosé wines from different regions.

In red wines predominate Puglia wine because have 179, among which Castel del Monte, Gioia del Colle, Puglia rosso and Salento rosso and date from 2002 to 2011. Three of them (Aglianico del Vulture), from the region of Basilicata dating in 2005. Two of them (Lambrusco) from the region of Emilia Romagna where the year is unknown. One (Capriano del Colle) from

the region of Lombardy dating in 2011. Five of them (Montepulciano d'Abruzzo), from the Abruzzo region dating from 2005 to 2010.
Two of them (Solopaca rosso), from the Campania region dating in 2007. One of Trentino (Trentino Rotaliano), from the region of Trentino dating in 2007. One of Tuscany, two of Veneto, and five of Sicily complete the list, varying between 2007 and 2010.

In white wines Puglia wines dominate again again with some like these (Locorotondo, Castel del Monte Bianco, Gioia del Colle, Puglia bianco, Salento bianco, or Tarantino bianco) dating from 2007 to 2011. There are also wines from regions such as Lazio (Montefiascone), Campania (Fiano di Avellino) and Sicily (Inzolia di Sicilia).

Finally, in rosé wines all are from Puglia (Salento rosato, Puglia rosato, Daunia rosato, San Severo rosato and Murgia rosato) and dating from the years 2007 to 2011. It also appears one of the Abruzzo region (Cerasuolo d'Abruzzo - 2010).

2.3 Nuclear Magnetic Resonance Spectroscopy

2.3.1 Wine NMR analysis and preparation of the sample

800 μL of wine were added to 100 μL of a solution at pH = 2.0 (the pH value was reached adding about 55 μL of HCl (37 %) to 100 mL of a solution which contained 0.01 g of NaN_3 in H_2O) and to 100 μL of TSP in D_2O (0.15%$_p$). The mix obtained was shaken (with Vortex for 1.5 minutes at 1500 rpm) and afterwards the sample was transferred in a NMR analysis tube.

2.3.2 Parameters used (NMR instrumentation and experiments)

The NMR experiment was performed on wines was the one-dimensional ^1H NOESY with presaturation multiple (8 frequency) signals of water and ethanol, at a temperature of 298 K. The pulse sequence, using a pulse shape presaturation, rather than rectangular. This allows you to get one proton spectrum with an effective reduction of water and ethanol signals. The spectrum was acquired with 64 scans of 64 K points, with spectral width of 6793 Hz, a pulse angle of 90 °, an acquisition time of 4.82 s, a mixing time of 10 ms and a recycling delay of 2.0 s.

All spectra were recorded with an automatic procedure (the duration of which is about 8 minutes), comprising: loading the sample, stabilization of the temperature for 5 minutes, tuning, matching, shimming and calibration of the pulse at 90°. The phase of the spectra was corrected manually and it was made of the alignment of signals compared to the singlet of TSP at 0 ppm.

2.4 High Resolution Mass Spectrometry

2.4.1 Selection protocols for wine analysis

500 µL wine were added to 1000 µL of deionized H_2O. The sample was centrifuged at 4000 rpm for 30 minutes and the supernatant was transferred to a vial for HPLC-MS analysis.
In in this case the chromatogram peaks appears more intense, with a better resolution and a consequent improvement of the signal to noise ratio.

2.4.2 Instrumentation used

The chromatographic separation and analysis were performed using an Agilent 1200 series HPLC with column Phenomenex Synergy Fusion RP80A (100mm × 3mm, 4µm), equipped with a guard column and coupled to a spectrometer micrOTOF-Q II (Bruker Daltonik GmbH) equipped with ESI interface, which operates in negative modes.

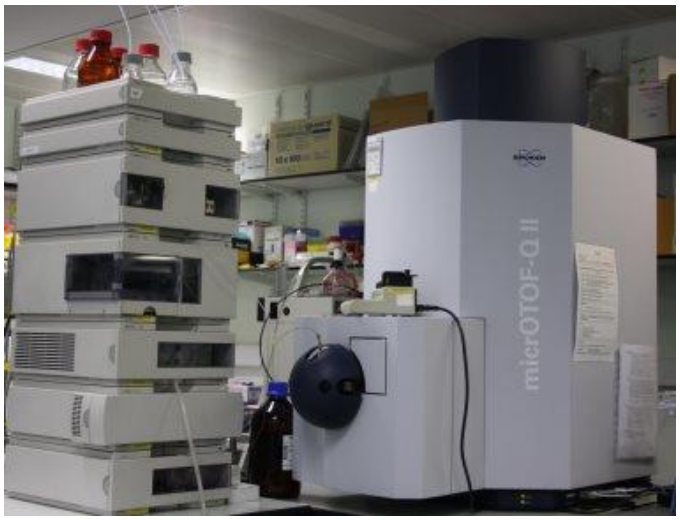


Figure 14. MicrOTOF-Q II.

2.4.3 HPLC-MS experiments for wine and parameters

The gradient conditions consisted of two solutions: A (H_2O) and B (acetonitrile), both with 0.1%v of formic acid. The gradient conditions are summarized in table 1:

tempo (min)	%A	%B
0 - 1	99	1
1 - 16	0	100
16 - 19	0	100
19 - 20	99	1
20 - 27	99	1

Table 1: Elution gradient used for wines.

The ESI parameters were optimized keeping the temperature of the drying gas (N_2) at 200 ° C, the flow of drying gas to 8.5 L / min and the gas pressure nebulizer (N_2) at 3 bar. In the positive mode, the capillary voltage is 3200 V and the voltage of the endplate is -500 V, while in the negative mode the two parameters have been optimized, respectively, at 4500 V and -500 V.

The calibration was carried out with clusters of sodium formate (1 mM NaOH in water: isopropanol $1:1_{(v/v)}$ with 0.1% formic acid), and in enhanced quadratic mode; the calibrant solution was injected at the beginning of the race through the use of a divert valve.

2.5 PCA parameters and data processing

Statistical analysis of data was performed using the software Amix-Viewer 3.9.4 (Bruker BioSpin GmbH, Rheinstetten, Germany). Mass spectra were divided into small regions (buckets) amplitude constant or variable, depending on the spectral regions examined.

The buckets were generated in two different modes: scaled to total intensity (TSI), scaling the intensity of each signal with respect to the total sum of the intensities, and no scaling (NS), that is, without making any scaling of the bucket. Since the NMR signals are proportional to the concentration of protons in solution, and the sample volume was kept

constant for all the measurements, the bucket generated in mode NS are related to the absolute amount of the protons, and that of the corresponding metabolites. For this reason, the intensity of the signals can be related to the concentration of metabolites, understood as moles per unit mass of the grapes. On the other hand, the bucket generated in STI mode may be related to the molar fraction of the metabolites. The set of buckets obtained for each sample are to be to define a row vector, while the set of row vectors go to define the bucket table, a matrix containing a number of rows equal to the number of samples analyzed and a number of columns equal to number of buckets generated. Being processed data were generated bucket removing regions of signal suppression water or ethanol.

Once generated the data matrix, the PCA was performed in two modes: either scaling the individual variables with respect to unit variance, is not scaling the variables.

When all the 340 wines are analyzed can introduced a number of parameters such as: the start time and end time 1.19 min to 12 min. This is so because there are sampled signals between these minutes. There is also the delta time whose value is 20. This can be seen in properties of the bucket.

Then, the same will be done but with 14 red wines, 14 white and 14 rosè in order to reach a new PCA with the 42 samples.

3. Results and Discussion

3.1 NMR analysis

3.1.1. Characterization of the metabolites

The ^{1}H NMR spectra can be ideally divided into three regions, each of which contains the typical signals of different classes of compounds. The region between 0 and 3 ppm contains most of the signals generated from amino acids and organic acids (or their salts); the region between 3 and 5.5 ppm contains predominantly the signals generated by the sugars and finally, the region between 5.5 and 9 ppm essentially contains the signals of the phenolic compounds.
Then, the list of metabolites detected is reported in table 2.

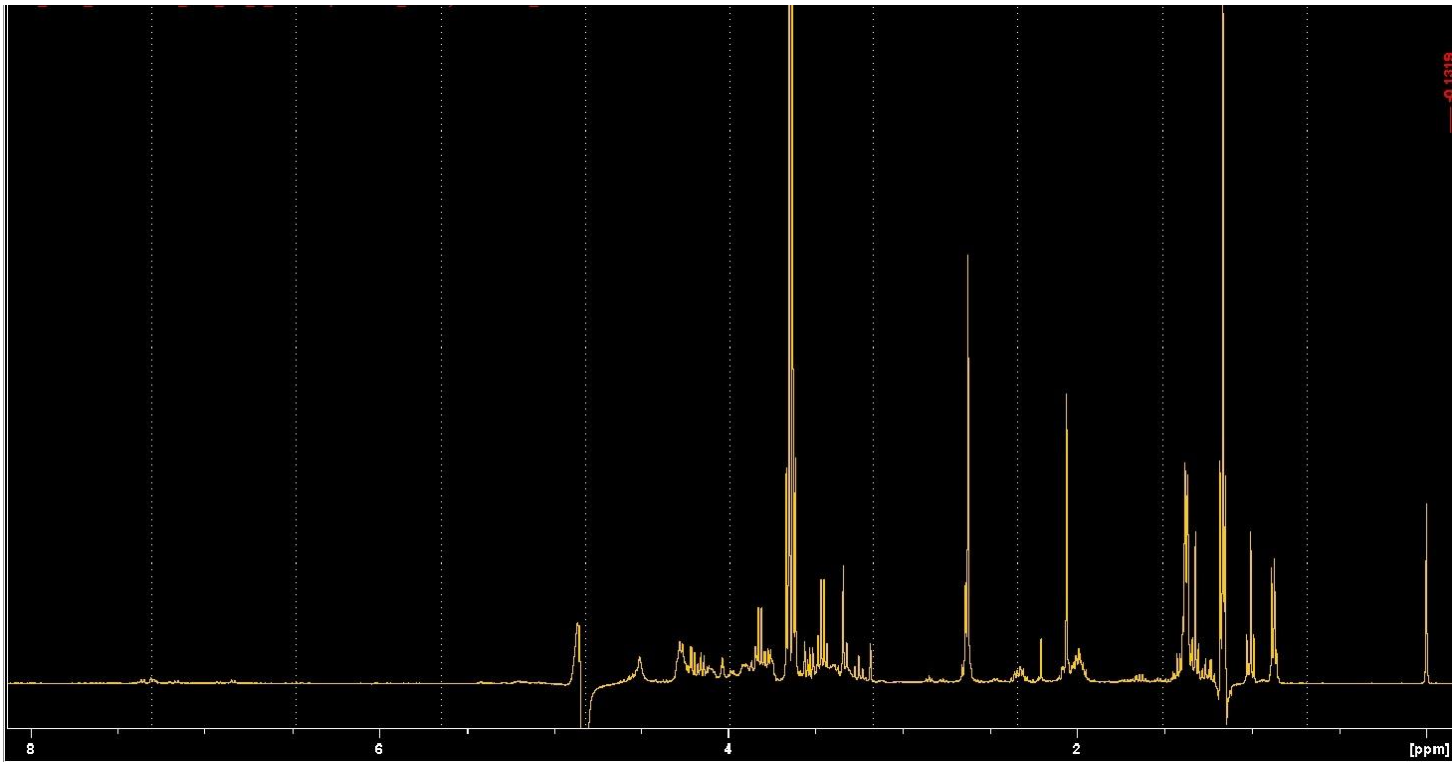


Figure 15. ^{1}H NMR red wine spectrum.

Figure 15 shows the ^{1}H NMR spectra of a typical wine sample. In all visible spectra signal suppression H_2O to ca. 4.7 ppm; spectra in wines is also the signal suppression ethanol: a quartet at ca. 3.6 ppm and a triplet at ca. 1.1 ppm triplet.

Table 2: signals present in the ^{1}H NMR spectra and attributions.

Metabolite	*Multiplicity (J,Hz)*	*$\delta^{1}H$ (ppm)*
Lactic acid	d (6.83) q (7.21)	1.39 4.21
Alanine	d (7.3)	1.48
Leucine	d (6.9) d (6.9) m	1.01 1.03 1.65
Proline	m m	2.00 2.34
Acetic acid	s	2.07
Succinic acid	s	2.64
Kaempferol	d (8.6) d (8.6)	7.17 6.82
Tartaric acid	s	4.52
Threonine	d (6.76)	1.34
α-Glucose	d (3.70)	5.20
β-Glucose	d (7.96) dd (9.1, 9.1)	4.61 3.23

Ethanol	t (7.09) q (6.92)	1.17 3.65
Malic acid	dd (10.63) dd (16.30) dd (16.30)	4.28 2.77 2.88
Arginine	m t (4.62)	1.68 3.76
Fructose	m m d (4.87)	3.97 3.88 4.12

3.2 HPLC-MS analysis

HPLC-MS analyses of the wine samples were performed in the negative ionization mode.

3.2.1 Characterization of the metabolites

HRMS analysis was performed in negative detection mode which permitted the annotation of 40 compounds belonging to several metabolite classes, including phenolic glycosides, sugars, alcohols, purine base, phenolic acids (hydroxybenzoic and hydroxycinnamic acids), aminoacids, organic acids, flavonoids (flavanols and flavonols), terpenes and derivatives. Figure 16 shows the LC-Mass chromatogram of a typical wine sample.The list of detected metabolites are reported in table 3. Mass spectra were processed using Data Analysis 4.0. The SmartFormula tool within DataAnalysis^{TM} (Bruker Daltonik GmbH, Bremen, Germany) was

used to obtain the elemental composition, errors and sigma values for each detected compound.

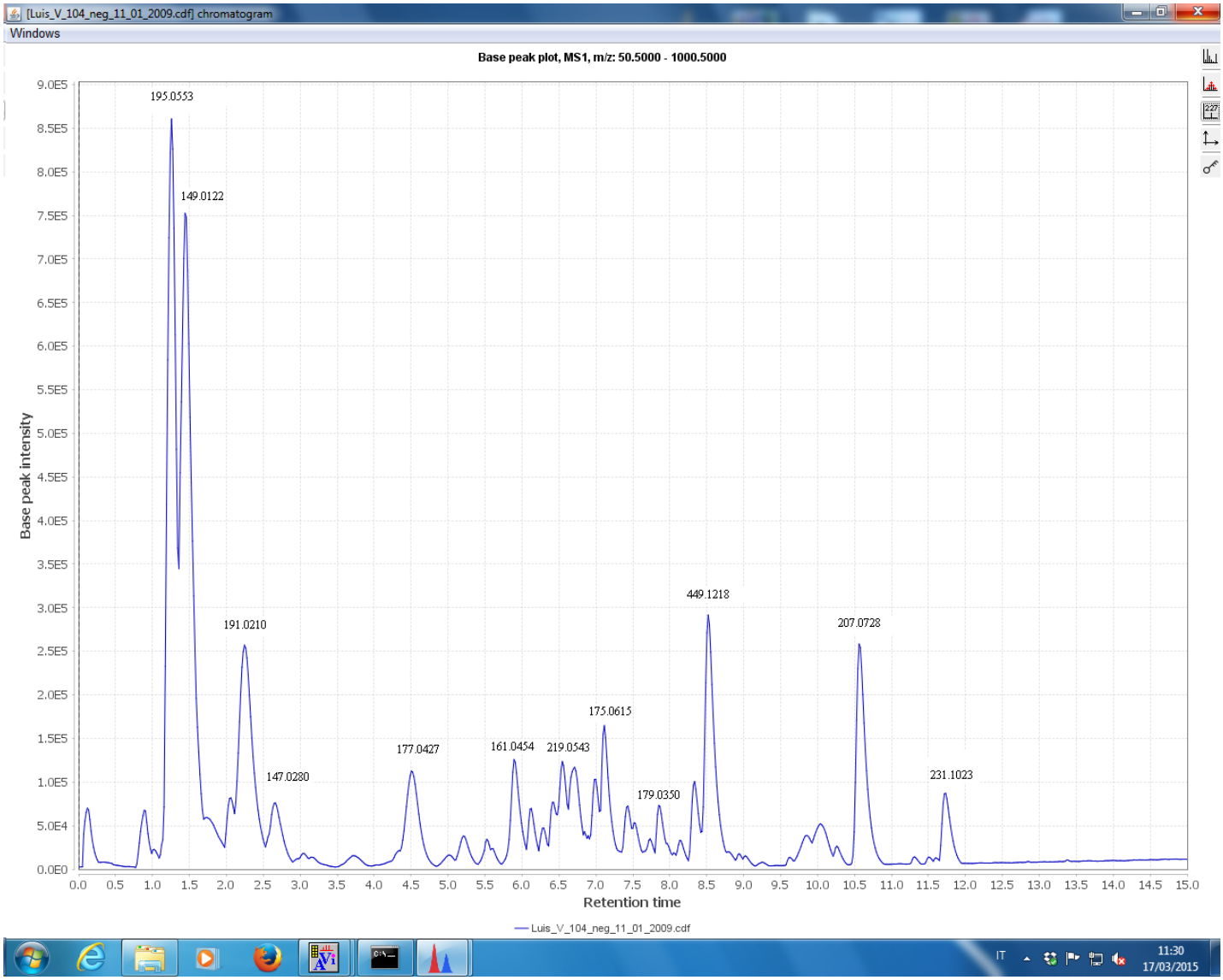


Figure 16. Red wine HRMS spectrum.

MS data were assigned to metabolites on the basis of accurate mass, isotopic distribution and fragmentation pattern in negative ion mode. Assignments were confirmed after comparison with literature data [15, 16, 17] and with on-line public metabolite databases [18, 19, 20] using Compound Crawler 1.0 (Bruker Daltonik GmbH, Bremen, Germany).

Table 3: Metabolites found in HRMS analysis.

*Peak label**	*Retention Time [min]*	*[M-H]⁻ m/z*	*Fragments or adducts formed in the MS source*	*MS/MS ions*	*Error (mDa)*	*mSigma Value*	*Formula [M-H]⁻*	*Compound*
Aminoacids								
10	3.35	513.1553 (100)			6.14	17.9	$C_{19}H_{25}O_{11}N_6$	Aminoacid derivative
13	3.82	485.1501 (100)			7.38	7.7	$C_{18}H_{25}O_{10}N_6$	Aminoacid derivative
19	5.54	499.1752 (100)			2.00	19.7	$C_{18}H_{27}O_7N_8S$	Aminoacid derivative
Hydroxybenzoic acids and derivatives								
16	5.21	169.0196 (100)		124.0494 (26)	-1.19	6.6	$C_7H_5O_5$	Gallic acid
Organic acids and derivatives								
1	1.22	195.0553 (100)			-2.90	1.8	$C_6H_{11}O_7$	Gluconic galactonic acid
2	1.44	149.0122 (100)			-3.02	5.1	$C_4H_5O_6$	Tartaric acid
3	1.59	133.0178 (100)			-2.32	2.7	$C_4H_5O_5$	Malic acid
4	2.03	147.0280 (93)			-1.80	6.0	$C_5H_7O_5$	Hydroxyglutaric acid/ citramalic acid
5	2.26	191.0210 (100)	111.0087(62)	111.0087 (11)	-3.49	2.4	$C_6H_7O_7$	Citric acid
6	2.60	117.0201 (100)			-1.01	4.7	$C_4H_5O_4$	Succinic acid
7	2.66	147.0280 (93)			-1.69	4.6	$C_5H_7O_5$	Hydroxyglutaric acid/ citramalic acid
9	3.18	161.0454 (100)			-1.58	6.7	$C_6H_9O_5$	2 hydroxy adipic acid
11	3.65	191.0561 (100)	145.0494 (31)	178.0895 (12) 145.0494 (18)	-1.12	7.5	$C_7H_{11}O_6$	Quinic acid
12	3.75	147.0666 (76)			-0.33	5.7	$C_6H_{11}O_4$	Mevalonic acid
14	4.37	147.0666 (35)			-0.49	5.0	$C_6H_{11}O_4$	Mevalonic acid

20	5.91	161.0454 (80)			-1.57	1.7	$C_6H_9O_5$	2 hydroxy adipic acid
22	6.43	219.0543 (100)	111.0087 (85)	111.0020 (55)	-2.97	2.6	$C_8H_{11}O_7$	R-(homo)$_2$ citrate
24	6.55	219.0543 (100)	111.0087 (62)	111.0087 (98)	-3.14	2.7	$C_8H_{11}O_7$	R-(homo)$_2$ citrate
26	7.00	175.0615 (100)			-2.24	2.3	$C_7H_{11}O_5$	2-isopropyl-malic acid
32	7.99	366.1280 (97)			-6.10	14.1	$C_{17}H_{20}O_{18}N$	Indole 3-lactic acid hexose
33	8.14	189.0765 (100)	129.0549 (25)	129.0621 (7)	-2.53	13.0	$C_8H_{13}O_5$	Diethyl L-malate
Hydroxycinnamic acids								
23	6.53	616.1229 (27)		211.0039 (24) 272.0909 (21) 149.0020 (96) 167.0309 (41) 143.0437 (22) 128.0319 (12)	-8.82	2.7	$C_{23}H_{26}N_3O_{15}S$	Grape Reaction Product
27	7.12	311.0476 (100)	179.0321 (56) 149.0097 (37) 135.0421 (12)	149.0175 (8) 179.0321 (24) 135.0421 (100)	-5.41	1.0	$C_{13}H_{11}O_9$	Caftaric acid
29	7.72	295.0547 (78)	163.0554 (48) 149.0097 (77) 119.0494 (9)	163.0392 (22) 149.0097 (11) 119.0494 (100)	-5.50	2.6	$C_{13}H_{11}O_8$	Cis-Coutaric acid
30	7.85	179.0350 (83)	135.0421 (65)	135.0495 (34)	-3.18	7.4	$C_9H_7O_4$	Caffeic acid
31	7.93	325.0642 (45)	193.0520 (63)	-	-7.7	8.3	$C_{14}H_{13}O_9$	Fertaric acid
38	10.56	207.0728 (100)	133.0484 (22)	133.0337 (100)	-3.19	3.3	$C_{11}H_{11}O_4$	Dimethyl caffeic acid
39	11.83	231.1023 (100)	169.1233 (52)	169.0903 (5)	-3.14	7.8	$C_{14}H_{15}O_3$	Tetrahydrofurfuryl cinnamate
Monosaccharides								
15	4.55	177.0427 (100)			-1.56	2.3	$C_6H_9O_6$	Gluconolactone
Purine base								

8	3.03	151.0303 (100)			-1.60	3.8	$C_5H_3N_4O_2$	Xanthine
Flavonoids and derivatives								
21	6.28	315.0836 (100)		123.0413 (24)	-1.35	21.4	$C_{17}H_{15}O_6$	Dihydroxydimethoxyflavanone
35	8.69	449.1218 (19)		285.0500 (49) 178.9811 (33) 151.0177 (80) 125.0228 (21)	-4.37	31.3	$C_{21}H_{21}O_{11}$	Astilbin
Mixed Pentose/Hexose Disaccharides								
18	5.51	383.1602 (27)			-3.04	5.5	$C_{15}H_{27}O_{11}$	2,3 butanediol apiosylglucoside
Alcohols and derivatives								
28	7.30	401.1521 (95)			-5.23	15.2	$C_{18}H_{25}O_{10}$	Benzyl alcohol hexose-pentose
Terpenes and derivatives								
25	6.83	443.2032 (100)			-6.48	3.9	$C_{21}H_{31}O_{10}$	Dihydrophaseic acid 4-O-β-D-glucoside
40	12.02	347.1824 (100)			4.52	15.5	$C_{20}H_{27}O_5$	Gibberellic acid derivative
Other metabolites								
17	5.39	188.0905 (100)			-1.35	8.0	$C_8H_{14}NO_4$	Unknown
34	8.49	247.0850 (100)			-4.57	4.9		Unknown
36	9.02	339.0732 (10)	177.0396 (85) 161.0171 (20)	177.0480 (42) 161.0171 (69) 133.0484 (18)				Unknown
37	9.81	268.0670 (100)						Unknown

*Peak label assigned according to overall temporal elution order.

[14]

3.3 PCA applied to HRMS data for all wines

PCA analysis applied to LC-mass data of all wine samples was performed using AMIX software. Figure 17 (a) shows the resulting PC1/PC2 score plot. The wine samples are differentiated into three groups with PC1 explaining 38.9 % and PC2 15.8% of the total variance. The largest group are the red wines that are shown in black circles, forming a large and distinguished group on the side of the positive values of PC1. The white wines, described as blue circles, are forming a significant group on the side of negative values of PC1. Finally the rosé wines, which are shown in green circles, are clearly grouped in an intermediated position between the previous two groups.

As ascertained by PC1/PC2 loadings plot depicted in Figure 17 (b), the discriminating metabolites along PC1 were tartaric acid (m/z 149.0081), gluconic or galactonic acid (m/z 195.0490), fertaric acid (m/z 325.0642), gallic acid (m/z 169.0124), grape reaction product (m/z 143.0346), R-$(homo)_2$ citrate (m/z 219.0565), 2 hydroxy adipic acid (m/z 161.0786), quinic acid (m/z 191.0543), dimethyl caffeic acid (m/z 207.0686).

It is noted therefore that the tartaric acid is rather more present in white wines, the dimethyl caffeic acid is predominant in rosé wines and fertaric acid is more present in red wines.

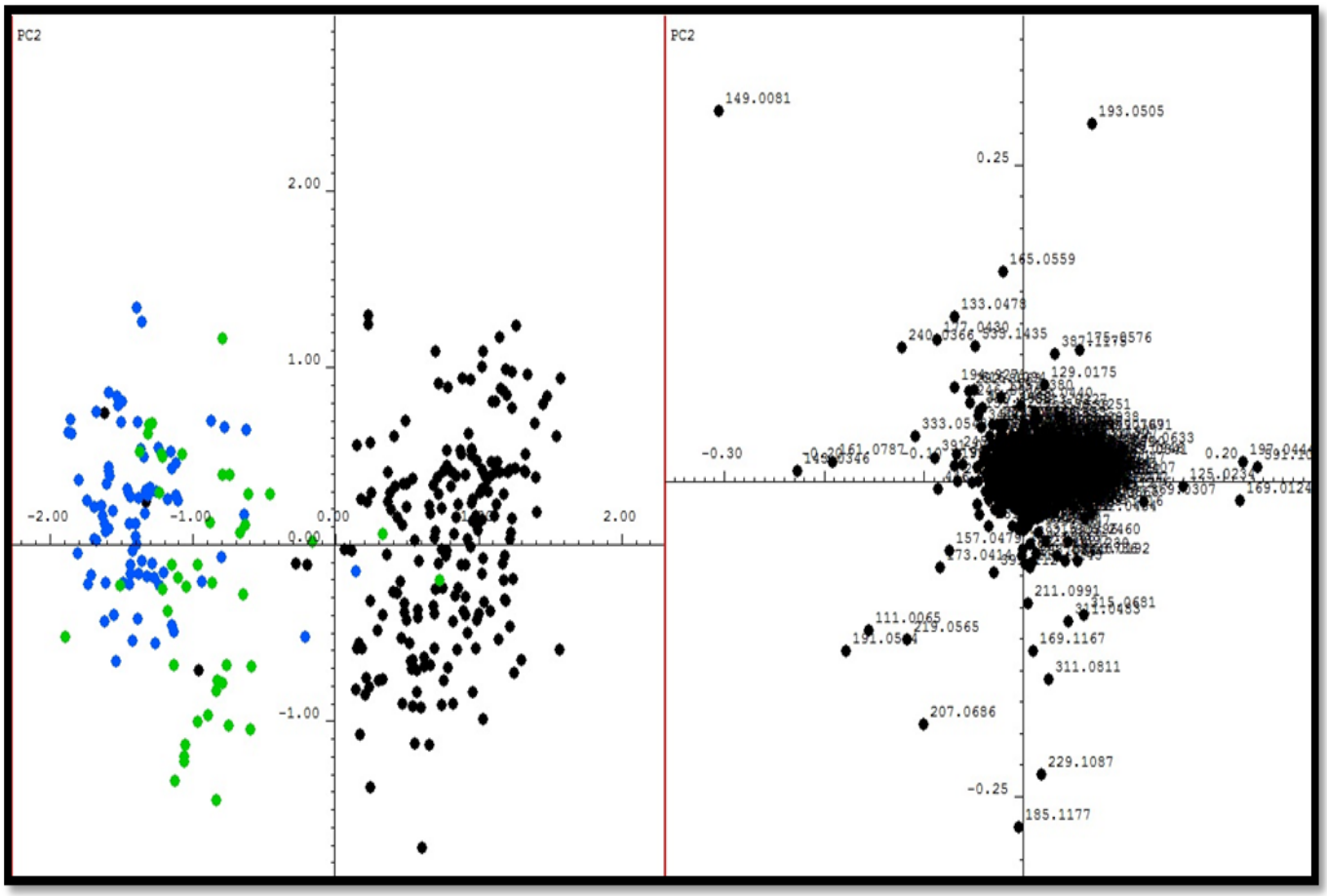


Figure 17 (a) PC1/PC2 score plot **Figure 17 (b) PC1/PC2 loading plot obtained applying PCA to all the wine samples**

This statistical analysis based on a metabolomic approach applied to LC-MS data can be considered a successful method for the assessment of the real color of wine samples.

3.4 Amix and MZMine: do they give the same result?

MZMine and AMIX are independent of each other and the user should select one to perform a PCA on LC-Mass data. AMIX do not follow the "standard" peak-picking alignment routine on which is based MZMine.
The advanced bucketing in AMIX does an internal peak picking and it allows peaks movements in the order of the chosen bucket with along the m/z axis. AMIX does not allow bucketing along the time axis, takes the whole retention time as bucket height. Alignment of peaks along the retention time axis is not needed.

The key idea in AMIX is to do the bucketing along m/z close to the expected variation of peak positions (within spectrum and within ensemble of spectra, the larger one counts) but due to the bucketing technique still get a table of acceptable size. As usual the validation is to find relevant loadings from the loadings plot, turn the corresponding m/z values into sum formula and validate by looking into biochemistry.
In short, Amix imports the HRMS row and directly processes in order to prepare the bucket based on only two variables m/z and intensity. On the contrary MZmine needs to go step by step with precise alignment of wine samples HRMS data variables (retention time and m/z). A comparison of a PCA applied to the same set of LC-Mass data and obtained using AMIX and MZMine could be newsworthy in order to control the authenticity of the results obtained with the two procedures. In fact, 42 wines are chosen from the total collection of the analyzed wines. They are 14 samples of red wines, 14 white wines and 14 rosés. Then the differentiation by multivariate statistical analysis applied to these data is performed using the Amix and MZmine softwares.

3.4.1 PCA applied to selected wine samples LC-Mass data performed with MZMine

For the MZMine procedure the 42 LC-Mass chromatograms in .cdf format were loaded the retention time range was set from 1.17 to 12.40 minutes. A Mass detection process was applied (Figure 18).

The Mass detection generates a list of points to be scanned per sample. Within this option, an algorithm for mass detention called Centroid is used, fixing the noise level which indicate the lower intensities at a given value as noise at 1500.

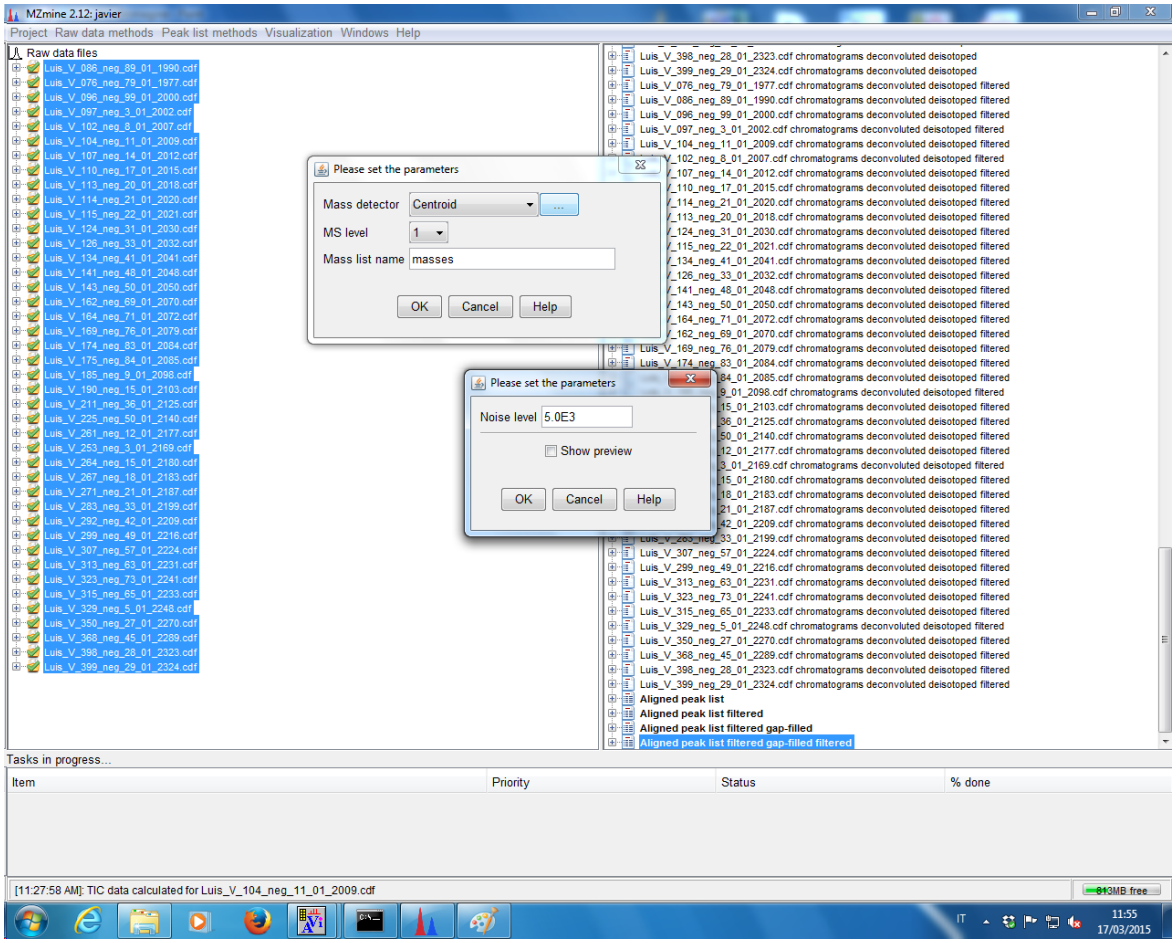


Figure 18. Mass detection.

Subsequently a Chromatogram Builder process was performed (Figure 19). This application takes the mass lists generated by the Mass detection process and builds a chromatogram for each mass that can be detected continuously over the scans.

In this step some parameters can be adjust. “Min time span” (minimum time span over which the same ion must be observed in order to be recognized as a chromatogram) which is in our case set at 0.3; “min height”, (minimum intensity of the highest data point in the chromatogram) which is in our case set at 8000; “m/z tolerance”, (maximum allowed difference between two m/z values to be considered same) ranging in our case from 0.005 or 5 ppm.

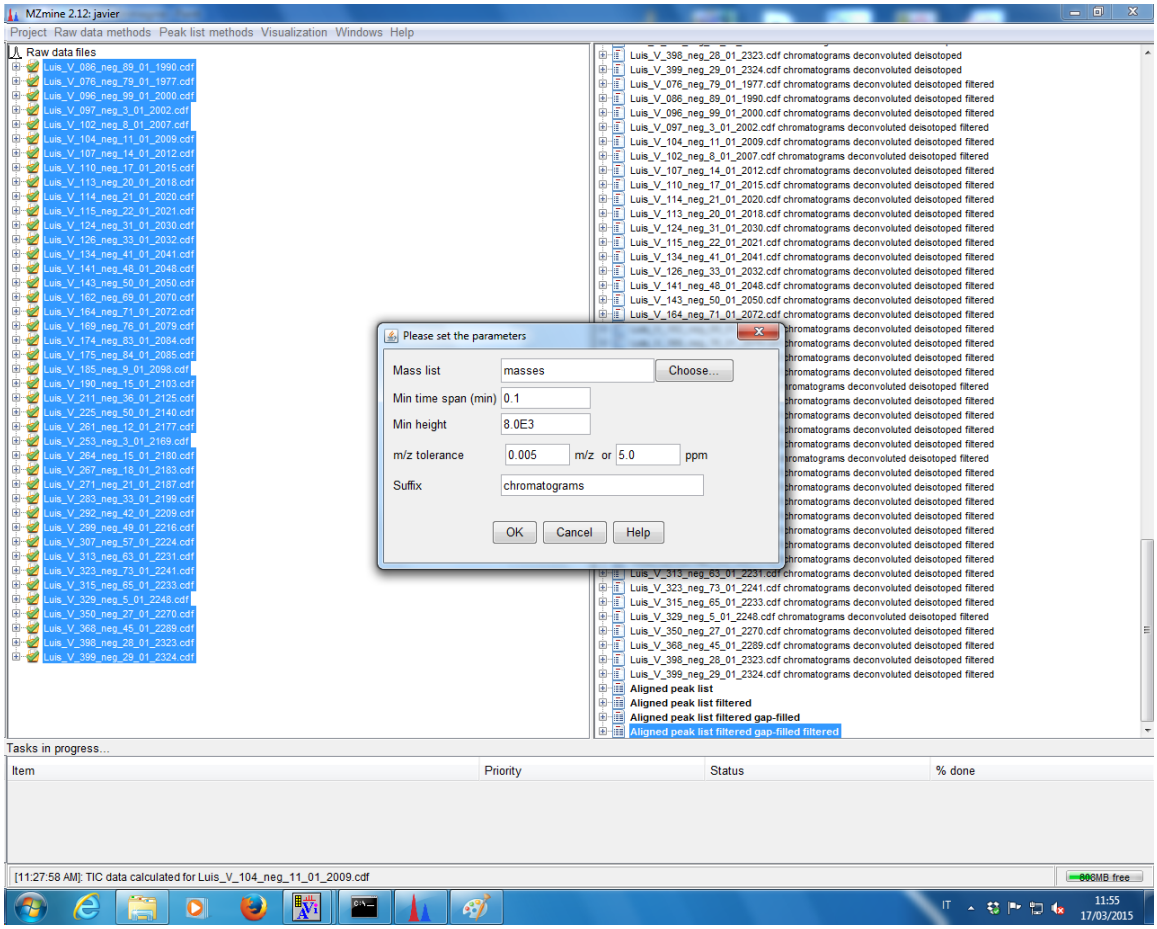


Figure 19. Chromatogram builder.

The third step was the Peak deconvolution (Figure 20). This module separates each detected chromatogram into individual peaks. This part used the algorithm Local Minimum Search and the following parameters were set: chromatographic threshold which is the threshold for removing noises and it was set at 65%; search minimum in RT range was set to 0.1; minimum relative height which is the minimum height of a peak relative to the chromatogram top data point and it was set to 5%; min absolute height was set at 50.000 and min ratio of peak top to edge was set to 2.

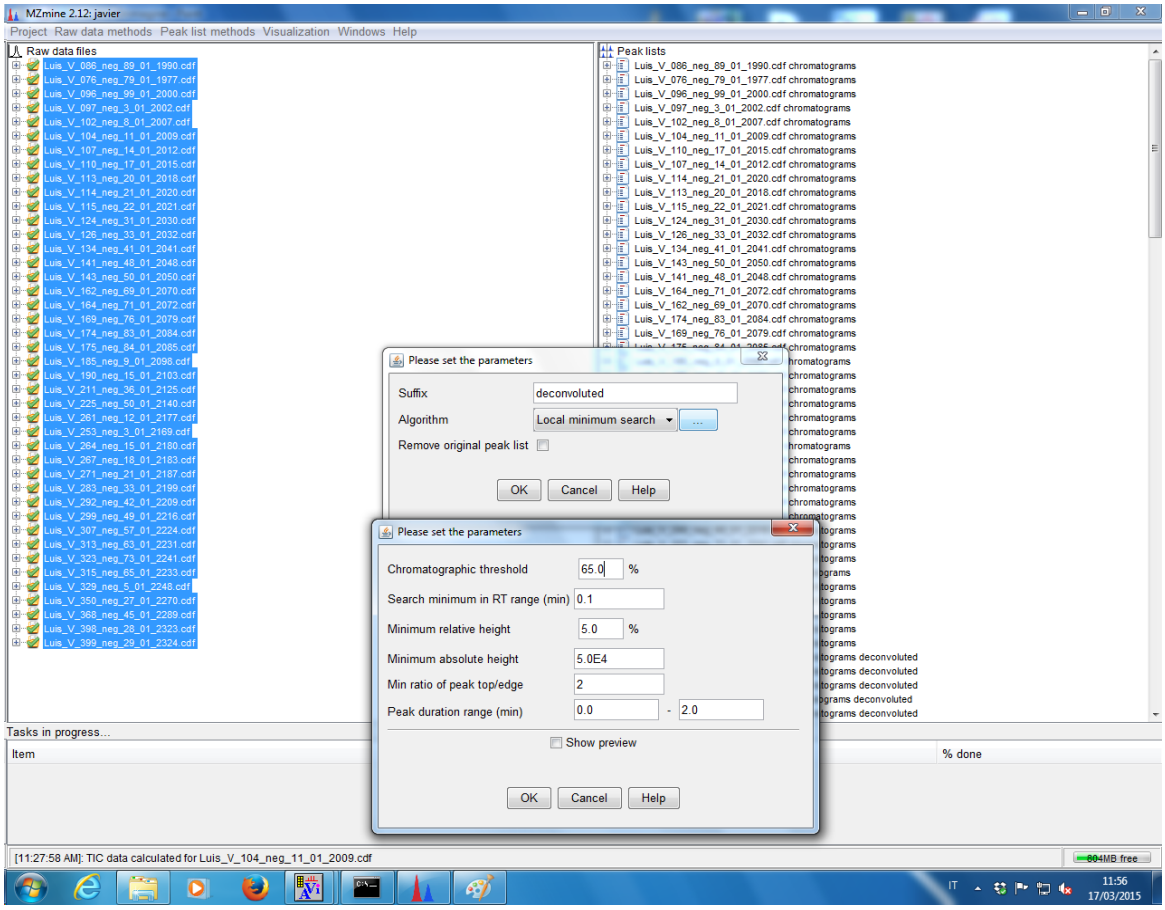


Figure 20. Peak deconvolution.

The fourth step is Isotopic peaks grouping (Figure 21). This step detects isotopic peaks and groups them together into isotopes patterns. Within this step a m/z tolerance from 0.005 to 5 ppm was insert, a retention time tolerance of 0.1, a maximum charge of 2 and representative isotope of lowest m/z were set.

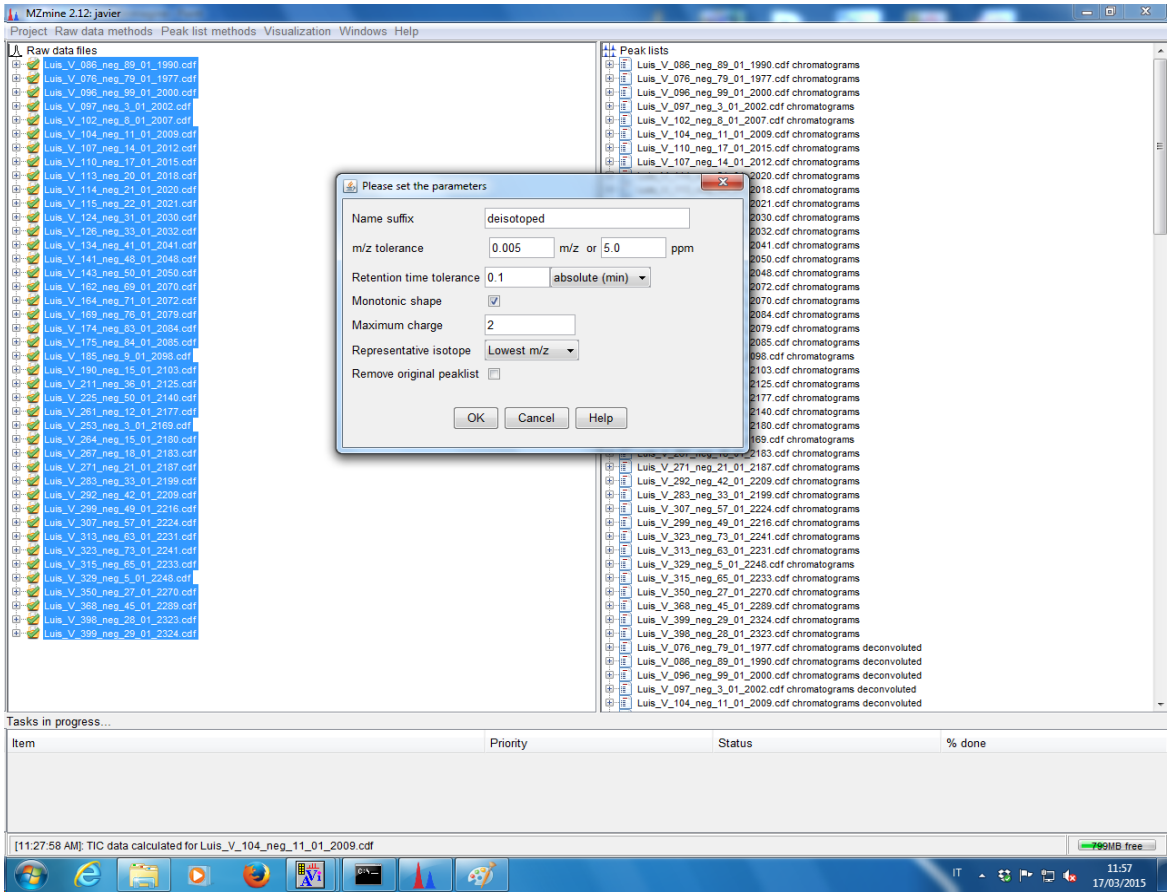


Figure 21. Isotopic peaks grouping.

Then the Peak list rows filtering was performed (Figure 22). This method removes certain entries for a peak list based on given restrictions. These restrictions were: the retention time range selected was from 1.17 to 12.40 minutes and the peak duration range was between 0 and 2.

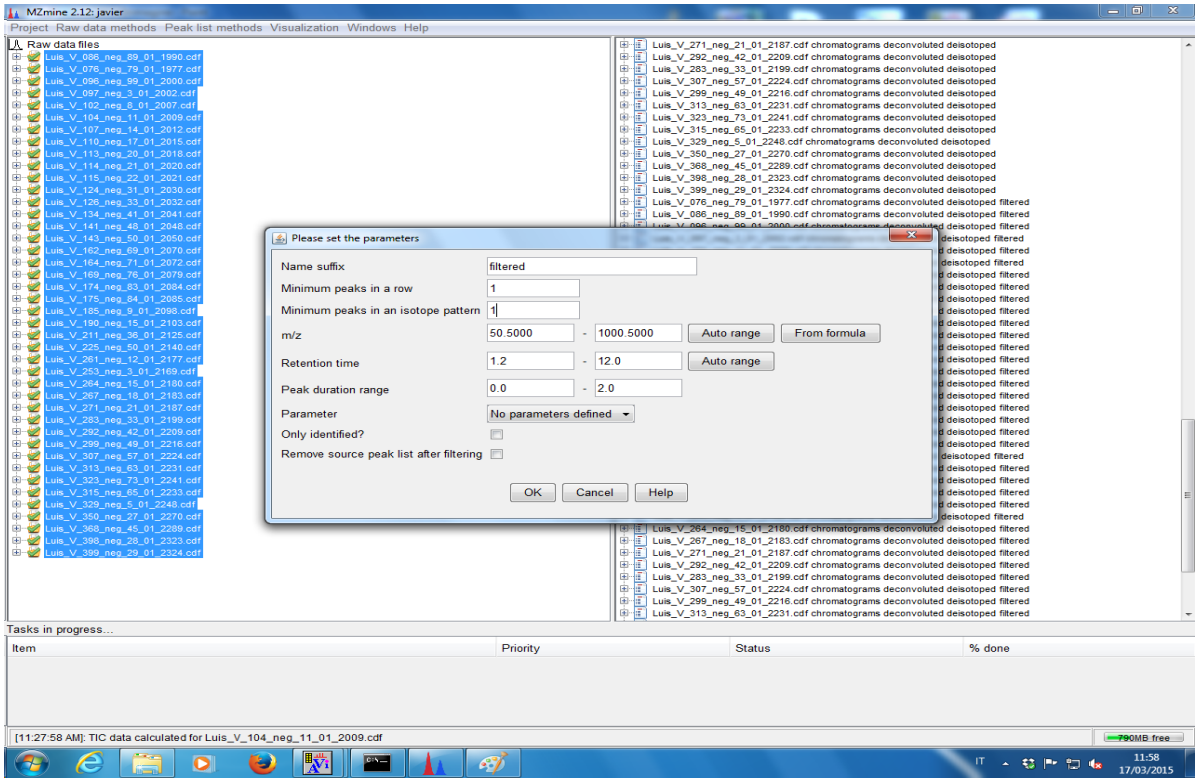


Figure 22. Peak list rows filtering.

A this stage the above mentioned Alignment procedure (Figure 23) was performed in order to obtain a peak list aligned in terms of m/z and time retention based on the RANSAC algorithm.

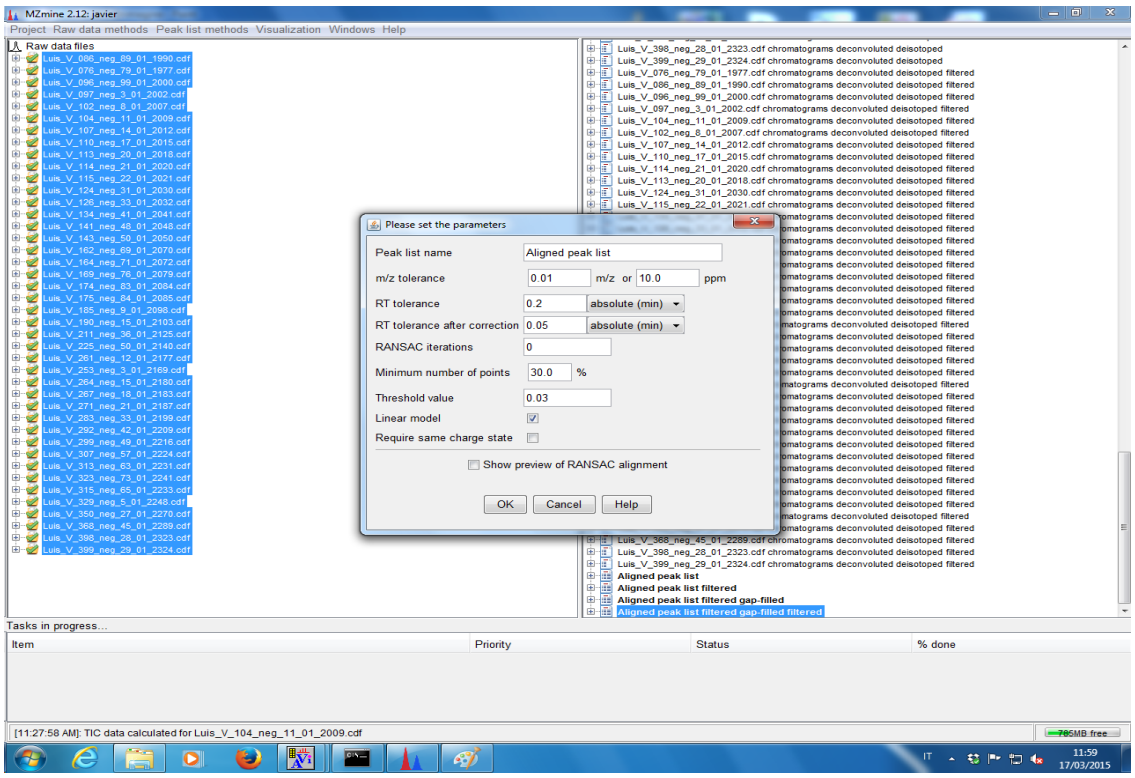


Figure 23. Alignment procedure.

A Peak list rows filtering (Figure 24) was performed for a second time after the Alignment procedure.

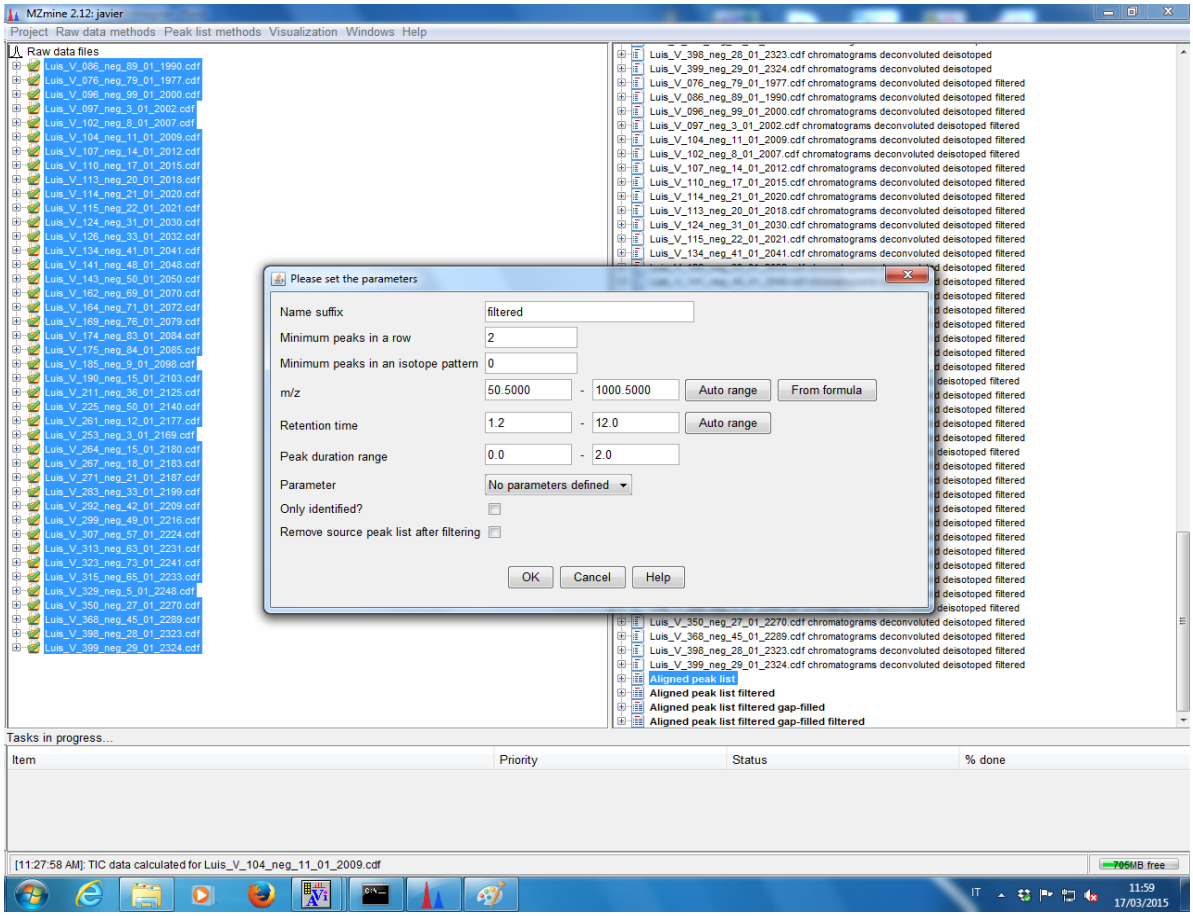


Figure 24. Peak list rows filtering (second time).

Then a Gap filling process was applied. This method filled the missing peaks (gaps) in the peak list by looking at the whole m/z and retention time range of the peak list row and adding all raw data points in the same range as shown in Figure 25.

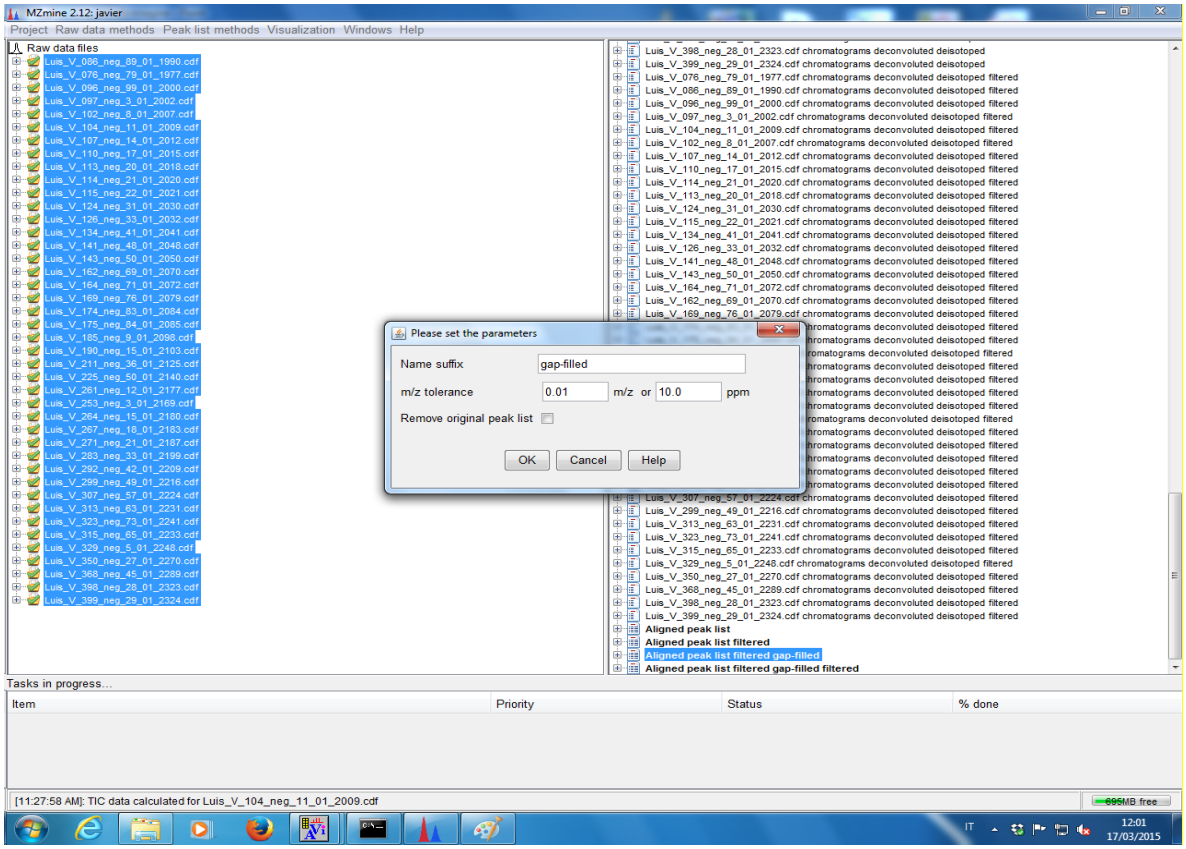


Figure 25. Gap filling.

Finally, the Duplicate peak filtering (Figure 26) removed duplicate peaks, in particular peaks with same retention times and m/z values from the peak list.

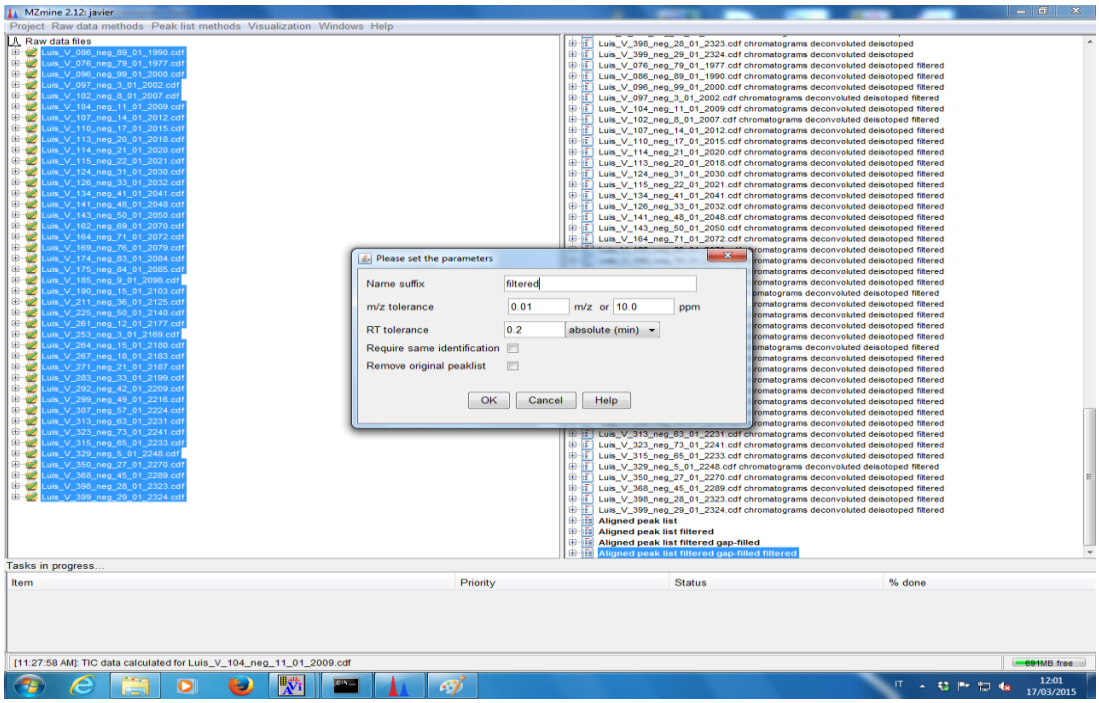
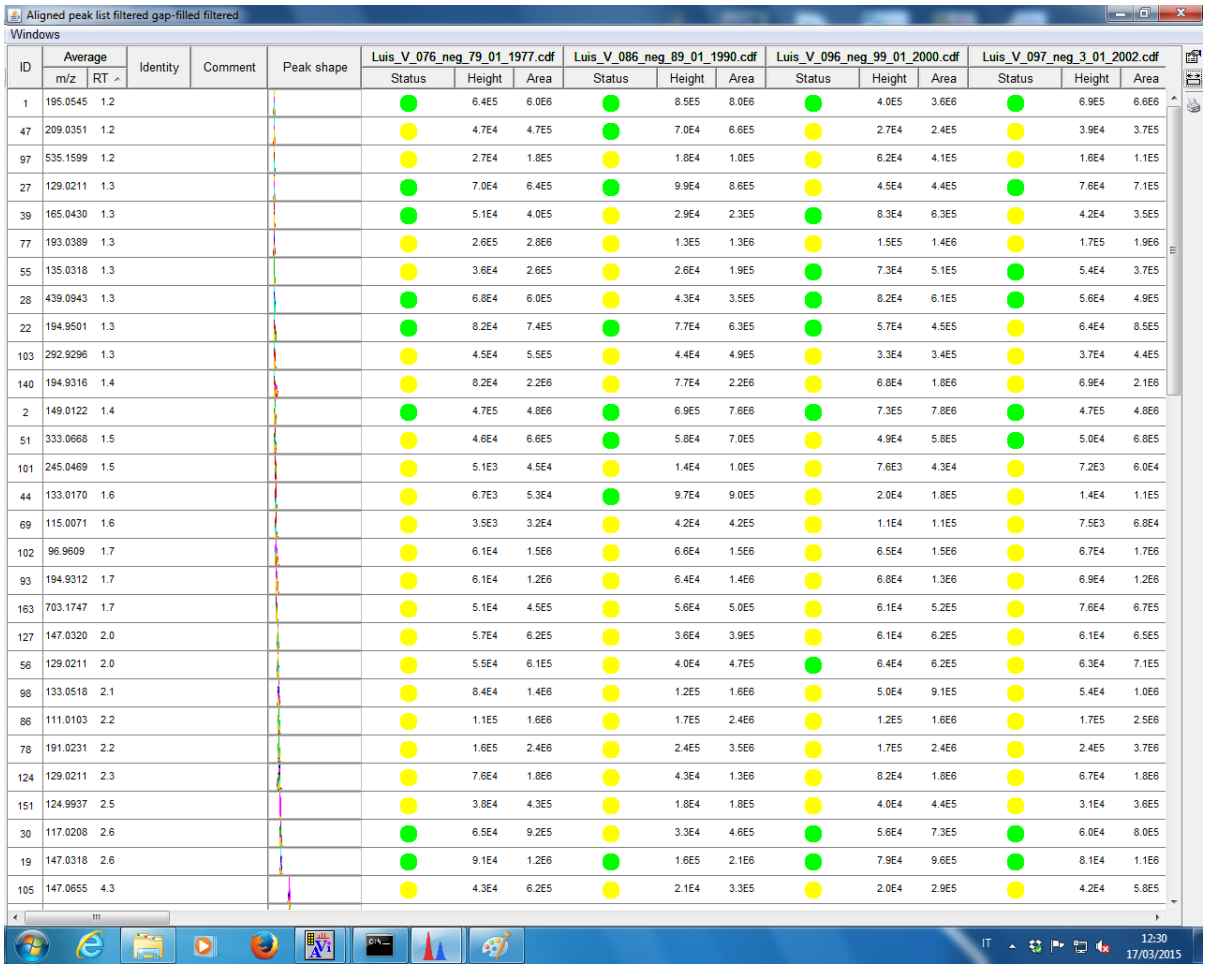


Figure 26. Duplicate peak filtering.

As shown in Figure 27, at the end of the whole procedure a list representing the retention time and the corresponding m/z of the metabolites annotated for the 42 wine samples was created. The green symbol indicates the metabolite detected in the analyzed samples while the yellow symbol indicates the estimation of the metabolite presence in the analyzed samples.



ID	Average		Identity	Comment	Peak shape	Luis_V_076_neg_79_01_1977.cdf			Luis_V_086_neg_89_01_1990.cdf			Luis_V_096_neg_99_01_2000.cdf			Luis_V_097_neg_3_01_2002.cdf		
	m/z	RT				Status	Height	Area	Status	Height	Area	Status	Height	Area	Status	Height	Area
1	195.0545	1.2					6.4E5	6.0E6		8.5E5	8.0E6		4.0E5	3.6E6		6.9E5	6.6E6
47	209.0351	1.2					4.7E4	4.7E5		7.0E4	6.6E5		2.7E4	2.4E5		3.9E4	3.7E5
97	535.1599	1.2					2.7E4	1.8E5		1.8E4	1.0E5		6.2E4	4.1E5		1.6E4	1.1E5
27	129.0211	1.3					7.0E4	6.4E5		9.9E4	8.6E5		4.5E4	4.4E5		7.6E4	7.1E5
39	165.0430	1.3					5.1E4	4.0E5		2.9E4	2.3E5		8.3E4	6.3E5		4.2E4	3.5E5
77	193.0389	1.3					2.6E5	2.8E6		1.3E5	1.3E6		1.5E5	1.4E6		1.7E5	1.9E6
55	135.0318	1.3					3.6E4	2.6E5		2.6E4	1.9E5		7.3E4	5.1E5		5.4E4	3.7E5
28	439.0943	1.3					6.8E4	6.0E5		4.3E4	3.5E5		8.2E4	6.1E5		5.6E4	4.9E5
22	194.9501	1.3					8.2E4	7.4E5		7.7E4	6.3E5		5.7E4	4.5E5		6.4E4	8.5E5
103	292.9296	1.3					4.5E4	5.5E5		4.4E4	4.9E5		3.3E4	3.4E5		3.7E4	4.4E5
140	194.9316	1.4					8.2E4	2.2E6		7.7E4	2.2E6		6.8E4	1.8E6		6.9E4	2.1E6
2	149.0122	1.4					4.7E5	4.8E6		6.9E5	7.6E6		7.3E5	7.8E6		4.7E5	4.8E6
51	333.0668	1.5					4.6E4	6.6E5		5.8E4	7.0E5		4.9E4	5.8E5		5.0E4	6.8E5
101	245.0469	1.5					5.1E3	4.5E4		1.4E4	1.0E5		7.6E3	4.3E4		7.2E3	6.0E4
44	133.0170	1.6					6.7E3	5.3E4		9.7E4	9.0E5		2.0E4	1.8E5		1.4E4	1.1E5
69	115.0071	1.6					3.5E3	3.2E4		4.2E4	4.2E5		1.1E4	1.1E5		7.5E3	6.8E4
102	96.9609	1.7					6.1E4	1.5E6		6.6E4	1.5E6		6.5E4	1.5E6		6.7E4	1.7E6
93	194.9312	1.7					6.1E4	1.2E6		6.4E4	1.4E6		6.8E4	1.3E6		6.9E4	1.2E6
163	703.1747	1.7					5.1E4	4.5E5		5.6E4	5.0E5		6.1E4	5.2E5		7.6E4	6.7E5
127	147.0320	2.0					5.7E4	6.2E5		3.6E4	3.9E5		6.1E4	6.2E5		6.1E4	6.5E5
56	129.0211	2.0					5.5E4	6.1E5		4.0E4	4.7E5		6.4E4	6.2E5		6.3E4	7.1E5
98	133.0518	2.1					8.4E4	1.4E6		1.2E5	1.6E6		5.0E4	9.1E5		5.4E4	1.0E6
86	111.0103	2.2					1.1E5	1.6E6		1.7E5	2.4E6		1.2E5	1.6E6		1.7E5	2.5E6
78	191.0231	2.2					1.6E5	2.4E6		2.4E5	3.5E6		1.7E5	2.4E6		2.4E5	3.7E6
124	129.0211	2.3					7.6E4	1.8E6		4.3E4	1.3E6		8.2E4	1.8E6		6.7E4	1.8E6
151	124.9937	2.5					3.8E4	4.3E5		1.8E4	1.8E5		4.0E4	4.4E5		3.1E4	3.6E5
30	117.0208	2.6					6.5E4	9.2E5		3.3E4	4.6E5		5.6E4	7.3E5		6.0E4	8.0E5
19	147.0318	2.6					9.1E4	1.2E6		1.6E5	2.1E6		7.9E4	9.6E5		8.1E4	1.1E6
105	147.0655	4.3					4.3E4	6.2E5		2.1E4	3.3E5		2.0E4	2.9E5		4.2E4	5.8E5

Figure 27. List of the annotated metabolites resulting from the MZMine procedure applied to the wine samples.

This list was the bucket table used for the PCA generation.

3.4.2 PCA applied to selected wine samples LC-Mass data performed with AMIX

PCA applied to the 42 wine samples LC-Mass data using AMIX was performed with the same parameters used for the all wine samples and already described in the experimental section.

In Figure 28 the PC1/PC2 score plots resulting from the statistical analysis of the 42 wine samples performed by MZMine (left side) and AMIX (right side) are reported. The PC1/PC2 score plots are practically comparable. The three distinct groups representing the red, white and rosé wines were established and discriminated. Therefore, the results obtained using two different procedures lead to the same classification.

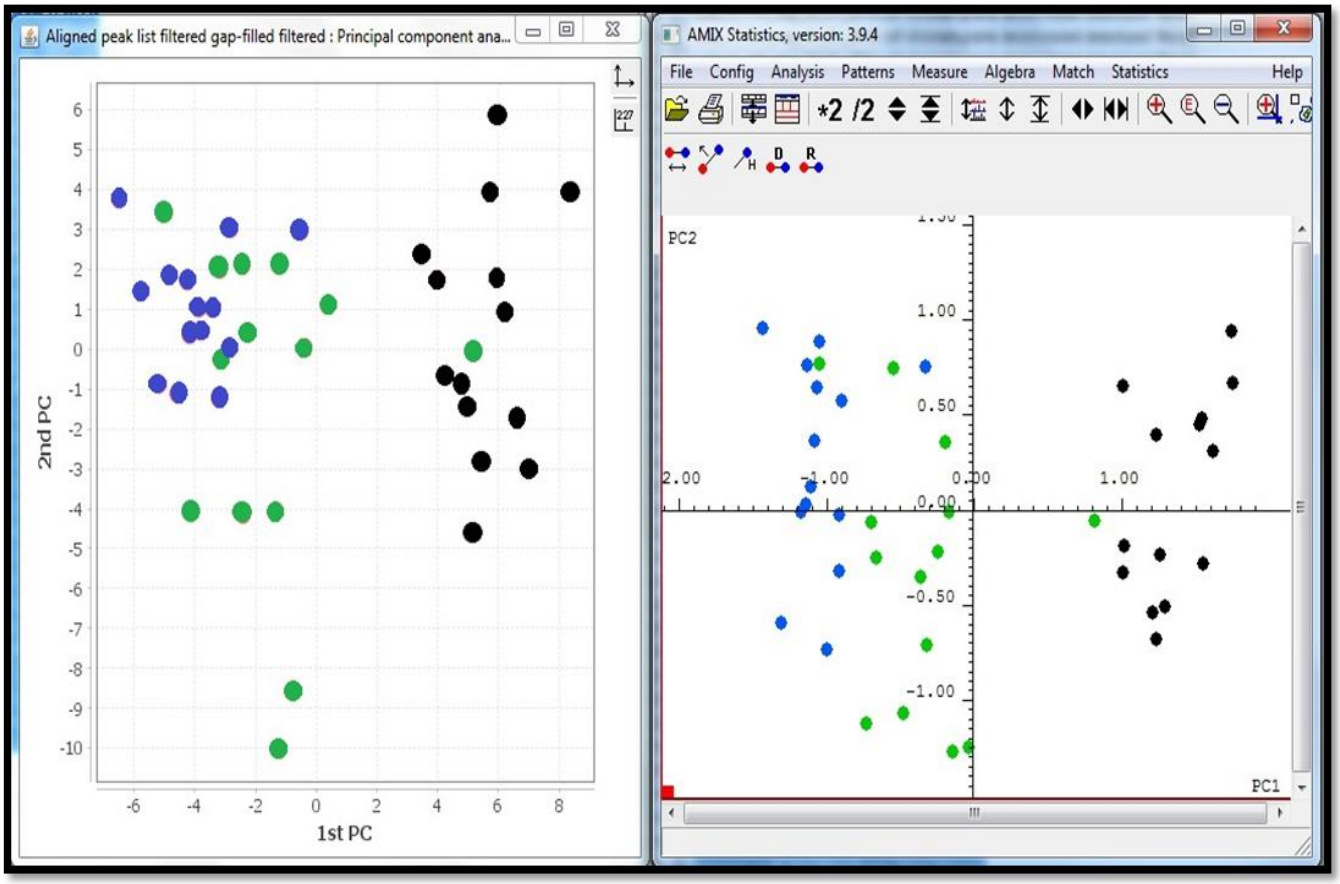


Figure 28. Comparison of PCA result through MZMine and AMIX.

4. Conclusions

In this thesis, samples of wines were analyzed by NMR and HR-MS.

- More metabolites have been found by HRMS than the NMR technique. The metabolites found are mainly belong to organic acids and sugars. A typical case is represented by sugars such as glucose and fructose, characterized exhaustively with the NMR, but that generate a unique signal in the mass spectra chromatograms. As evident from the results it is important to be able to clearly distinguish the signals of alpha-glucose, beta-glucose and fructose, because the ratio of these sugars in the pulp of the grape represents a parameter for a satisfactory varietal.
- PCA allowed a good distinction along the color in the total samples of wine. PCA can perfectly distinguishes 3 groups: red, white and rosé. This discrimination of color of wine samples allowed good quality control in the winemaking process, especially for the most particular rosé wines .
- The loading plot of the PCA of all wine samples provides the information that fertaric acid is characteristic in red wines, tartaric acid is characteristic in white wines and dimethyl caffeic acid is predominant in rosé wines.
- AMIX and MZMine lead to the same results. Amix imports the HRMS row and directly processes in order to prepare the bucket based on only two variables m/z and intensity. On the contrary MZmine needs to go step by step with precise alignment of wine samples HRMS data variables (retention time and m/z).

The thesis work shows that the NMR and HRMS, applied to the analysis of wines, are two complementary techniques and allow to evaluate aspects not appreciable with the techniques traditionally used for the analysis of these products.
Finally, the results obtained in this thesis consolidate the foundation for the use of NMR and HRMS for the varietal certification of origin of wine products.

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