FOODINTEGRITY HANDBOOK

A GUIDE TO FOOD AUTHENTICITY ISSUES AND ANALYTICAL SOLUTIONS

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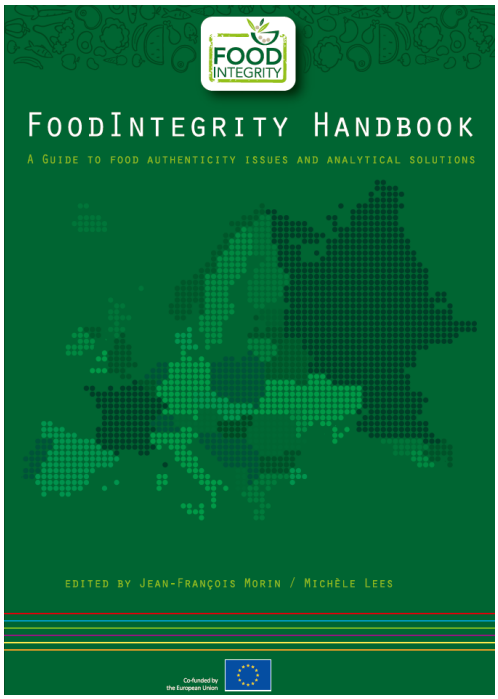

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Wine and must

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General overview of the product

Approximately 8 000 years ago and at the same time, in an area situated between the Black and Caspian seas (corresponding to modern Georgia and Armenia) and in Mesopotamia (modern Iraq and Iran) were once domesticated, or, at least grown as part of an ancestral cultivation, wild European grape vines (*Vitis vinifera*) [1]. Evidences of probably the earliest known winery, dating back 6000 years, hosting relicts of a press and several fermentation and storage vessels, were found in a cave in Areni (Armenia) [2].

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For its intoxicating and exciting properties, rapidly wine became far more than an ordinary beverage, and was often used as a ritual libation for priests and royalty in religious ceremonies, or as a votive offering to gods. Later, the expansion of the Greek civilization, and also that of the Roman Empire, led to the diffusion of the cult of Dionysus (or Bacchus for the Romans), the god of wine, and the vine growing culture, in all the coastal regions around the Mediterranean Sea. Under Celtic and Roman influence, viticulture was then introduced to the continental European temperate regions, notably to France and Germany. After the fall of the Roman civilization, when Europe was afflicted by mass migration and invasions, inside scattered monasteries was seeded and nursed the first embryo of modern winemaking knowledge.

Nowadays, the European Union is the world's largest wine producer and consumer, with roughly 70 % of global production and 60 % of global consumption. All 27 EU member states produce wine to some extent, and each has its own language, traditions and wine classifications. World wine production was around 246.7 mhl in 2017 (OIV report), with Italy, France and Spain as the leading world producers.

According to the European Commission's Directorate General for Agriculture and Rural Development (DG AGRI), European wine production can vary a lot from year to year (with yields ranging from +20 % to -20 %), highly influenced by weather conditions and/or the sanitary conditions of the vines. This has an important impact on price levels and hence on the number and types of adulteration. The price of wine also depends on its production area and label.

Wine exports are increasing year by year and accounted in 2017 for over 25 % of the volumes produced, whereas imports remain constant. Five main destinations (USA, Switzerland, Japan, Canada, China-Hong Kong) account for up 70 % in value of all wines exported outside the EU.

Outside Europe, the main wine producer is the USA, followed by Australia and China. Wine production in China is increasing year by year, from being absent in 2005 and taking its place as the world's 6th largest wine producer in 2016.

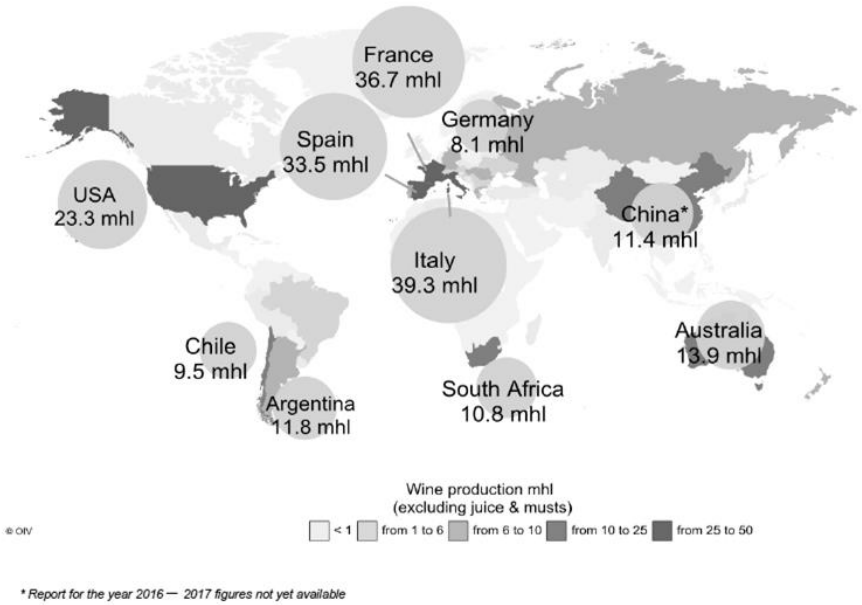

Figure 1: 2017 wine production, OIV Report

1. Product Identity

1.1. Definition of the product and manufacturing process

The most relevant constituents of must and wine are water, carbohydrates, acids, alcohols, phenolics, nitrogenous compounds (proteins, amino acids and ammonium salts), inorganic substances (metals and anions) and flavours. The chemical composition of grapes is affected by many factors, particularly grape variety or cultivar, environmental factors such as climate and soil (the concept of 'terroir'), viticultural management and seasonal variations (the concept of 'vintage'), and also on the variability of winemaking practices.

1.1.1. The winemaking process

Harvesting

Grapes are naturally rich in fermentable sugars, organic acidity, aroma precursors, protective tannins and coloured anthocyanins and flavonoids, making possible an easy transformation to a naturally stable beverage, wine. Moreover, grapes are rich in minerals and nitrogen compounds that are essential to promote the biochemical fermentation to wine. The choice of when to pick the grapes will determine acidity, sugar content and the potential richness in flavour of the musts. This decision was traditionally performed on the basis of a tasting directly in the vineyard, although today it is usually the result of a more conscious chemical evaluation of fruit composition.

Crushing and Pressing

After the grapes are sorted, if they are to be used in the production of white wines, they are generally destemmed and crushed, whereas for red wines, the stems are often not removed. Must is the freshly pressed grape juice that contains also the skins and seeds. For white wines, the juice is quickly separated from the skins and seeds, unless a greater extraction of aroma precursors from the skins is sought using a cold maceration technique. Red wines, on the other hand, are left in contact with their skins to extract as much as possible colour, tannins and aroma compounds. Nowadays, all these processes are automatically performed using mechanical equipment.

Fermentation

After crushing and pressing, the must can start to ferment at room temperature either within 8-12 hours when indigenous or wild yeasts are naturally present, or in a shorter time when selected yeast strains or a traditional 'pied de cuve' are added as inoculum. The latter practice provides an effective control of fermentation and prediction of the organoleptic features of wines, also reducing the risk of blocking and off-flavour deviations. Fermentation generally ends within 10-20 days, when fermentative sugars are totally converted into alcohol and a dry wine is obtained, whereas, for sweet wines, they are cooled to stop fermentation and filtered through a textile filter to remove yeasts. To create a sweet wine, wine makers will sometimes stop the process before all of the sugar is converted. Fermentation can take from 10 days to one month or more. Often, also a secondary bacterial fermentation of malic acid to lactic acid is promoted, especially for red wines or some specific white wines.

Clarification

This corresponds to the physical practices which are necessary at the end of fermentation to remove the solid fraction from the wine, such as dead yeast cells, precipitates of insoluble salts, and organic aggregates of polyphenols and proteins. After a period of static sedimentation, the

wine is periodically transferred into new containers, such as stainless-steel tanks or oak barrels. Wine can be also clarified using fining agents and filtration equipment.

Aging and Bottling

The ageing of wine, using variable periods of maturation in oak barrels or of aging in glass bottles, represents a crucial winemaking step, potentially able to improve the fineness of wine, making its aroma and taste more complex and pleasing to consumers. A shorter aging in steel tanks before bottling is instead commonly used for fresh white wines.

1.1.2. Legal definition

The legal definition of must and wine is provided by the OIV (The International Organisation of Vine and Wine), which is the body of reference in the area of vine and wine.

'**Grape must** is the liquid obtained from fresh grapes, whether spontaneously or by physical processes such as: crushing, removing stems from grape berries or crushed grapes, draining, pressing.' When alcoholic fermentation has been prevented by sulphiting or addition of carbon dioxide or by sorbic acid, the must is defined as **preserved grape must** and can contain up to 1 % vol ethanol. **Concentrated grape must** is obtained by its partial dehydration and has a density higher than 1.24 g/mL, whereas **caramelized grape must** is obtained by its partial dehydration on direct heat and has a density higher than 1.3 g/mL.

'**Wine** is the beverage resulting from the complete or partial alcoholic fermentation of fresh grapes, whether crushed or not, and from the grape must. Its acquired alcoholic strength should not be less than 8.5 p. 100 vol. Nevertheless, considering climatic conditions, soil or grape variety, special qualitative factors or individual traditions specific to certain vineyards, the total minimum alcoholic strength can be reduced to 7 p. 100 vol. by special legislation of the region in question'.
Wine is then defined as dry, demi-sec, semi-sweet and sweet, depending on the content of sugar, and still and semi-sparkling, depending on the carbon dioxide concentration.

As regards **wine labelling**, the EU classified wine quality into two categories: 'QWPSR' (Quality Wine Produced in a Specific Region) and 'Table Wine'. These were replaced in 2011 with PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication), as explained below.

PDO (Protected Designation of Origin) wine are "produced, processed and prepared in a given geographical area, using recognised know-how". Their quality and properties are significantly or exclusively determined by their environment, in both natural and human factors. Each EU country has its own quality categories which correspond to PDO. The most significant are: France: AOC (Appellation d'Origine Contrôlée); Italy: DOC (Denominazione di Origine Controllata) and DOCG (Denominazione di Origine Controllata e Garantita); Spain: DO (Denominación de Origen) and DOCa (Denominación de Origen Calificada).

PGI (Protected Geographical Indication) wine is linked to the geographical area in which it is produced, processed or prepared, and has specific qualities attributable to that geographical area. The category is named VDP (Vin de Pays) in France, IGT (Indicazione Geografica Tipica) in Italy and VT (Vino de la Tierra) in Spain.

Table Wine and Table Wine with a Geographical Indication were collectively replaced by PGI in 2011. The aim of this was to remove the word 'Table', along with its connotations of low quality, from the EU wine nomenclature. Thus the phrases Vin de Table (France), Vino da Tavola (Italy), Vino de Mesa (Spain), Vinho de Mesa (Portugal) and Tafelwein (Germany and Austria) are now legally obsolete.

1.2. Current standards of identity or related legislation

The International Organisation of Vine and Wine, originally named 'International Wine Office' was created in 1924 as an agreement among eight nations, but today accounts for 46 international member states.

OIV activity is focused on publishing methods of analysis and quality assurance in oenological laboratories, for the determination of the analytical composition of wines, musts and spirit beverages of vitivinicultural origin and wine vinegars. The first collection of analytical methods, the Compendium of International Methods of Wine Analysis, was published in 1962, while the present Compendium of International Methods of Wine and Must Analysis is annually revised and amended since 2000.

Many member countries, in order to facilitate international trade, have adopted the Compendium introducing its definitions and methods into their own regulations. In this way, the European Union (Regulation No 479/2008) recognised all the methods of the Compendium making them binding in all Member States for establishing the composition of the products covered by that Regulation. Regulation (EC) No 606/2009, laid down that the list and description of these analysis methods must be published also at Community level (C Series of the Official Journal of the European Union).

2. Authenticity issues

2.1. Identification of current authenticity issues

Food and beverage authenticity issues fall into one of the following categories:

i. Non-compliance with the established legislative standards,

ii. Adulteration of high value products, through substitution by cheaper but similar ingredients or extension adulterant

iii. Misdescription and/or mislabelling of geographical, botanical or species origin.

In the case of wine/must, category (i) corresponds to the non-compliance with the legislative reference standards and limits of European regulations and OIV, Codex and specification rules of each PDO or IGP in terms of the chemical-physical composition of the product. Some examples are given in Table 1. The authenticity of the samples is determined by using quantitative analyses which quantify the amount of the compounds present: if the actual values are outside the limits quoted in the table, the samples are non-authentic.

The category (ii) relates to the unpermitted addition of exogenous sugars and water in order to increase the alcoholic degree and the yield of the product, and the unpermitted addition of exogenous compounds, such as flavours, glycerol, dyes, tartaric acid and CO_2 in order to improve the poor quality of the product.

In these cases, the authenticity of the product is evaluated using analytical approaches able to trace the source of the compound (from grape, from exogenous products or synthetic). Maximum acceptable limits do not exist, but a reference database on the basis of the analysis of authentic samples has to be built.

Table 1: Maximum acceptable limits of various substances contained in wine (mainly from Compendium of International Methods of Analysis-OIV, 2015/1 Issue)

Substance	Maximum acceptable limits	Notes
Citric acid	1 g/L	
Volatile acidity	20 milliequivalents/L	The volatile acidity of various specially fortified old wines (wines subject to special legislation and controlled by the government) may exceed this limit.
Arsenic	0.2 mg/L	
Borom	80 mg/L (expressed as boric acid)	
Bromine	1 mg/L	Limit exceeded by way of exception in wines from certain vineyards with a brackish subsoil.
Cadmium	0.01 mg/L	
Copper	1 mg/L 2 mg/L	For liqueur wines produced from unfermented or slightly fermented grape must (Oeno 434-2011)
Diethylene glycol	≤ 10 mg/L, to the Quant. Limit	
Malvidol diglucoside	15 mg/L	
Silver	< 0.1 mg/L	
Total sulphur dioxide (at the time of sale to the consumer)	150 mg/L	For red wines containing a maximum of 4 g/L of reducing substances.
	200 mg/L	For white and rosé wines containing a maximum of 4 g/L of reducing substances.
	300 mg/L	For red, rosé and white wines containing more than 4 g/L of reducing substances.
	400 mg/L	In exceptional cases some sweet white wines.(Oeno 9/98)
Ethanediol/Ethylene glycol	≤ 10 mg/L	
Fluoride	1 mg/L	Except for wines coming from vineyards treated in conformity with national law, with cryolite in which case, the level of fluoride must not exceed 3 mg/L (Oeno 8/91)
Methanol	400 mg/L	For red wines
	250 mg/L	For white and rosé wines(Oeno 19/2004)
Ochratoxin A	2 µg/L	For wines obtained as from the 2005 harvest (CST 1/2002).
Lead	0.15 mg/L	For wine made, starting from the 2007 harvest year (Oeno 13/06).
Propan-1,2-diol Propylene glycol	150 mg/L	Still wines
	300 mg/L	Sparkling wines (Oeno 20/2003)
Excess sodium	80 mg/L	(Oeno 12/2007)
Sulfates	1 g/L (expressed as potassium sulfate)	

Misdescription and mislabelling (iii) concern false declaration of origin and grape variety, harvest year and wine category. The aim of this adulteration is to give premium price and value to products with low quality.

In addition, for these types of adulteration, reference databases have to be built on the basis of the analysis of authentic samples in order to define the ranges of values that are characteristic of a particular production area, vintage or variety.

2.2. Potential threat to public health

In the very long history of wine fraud several adulterations have posed severe health risks and harm to consumers. One of the oldest examples is the addition of lead acetate (sugar of lead) as a sweetener, which was already reported in Ancient Rome and again in the 17th century. This practice particularly occurred when "good" wine was rare and led to severe health damage by lead intoxication. The determination of lead acetate addition by the precipitation of black lead sulphide was one of the early official test methods established in Germany (1788: "Württembergische Weinprobe") in the fight against food fraud.

More recent examples of health risks related to wine fraud were the addition of diethylene glycol and methanol in the mid-1980s. In 1985 it was uncovered that diethylene glycol (an anti-freeze agent) was added to Austrian wine in a large scale in order to imitate a better wine quality by its sweet taste and increasing the extract. Acute diethylene glycol intoxications lead to nephrotoxic effects. In 1986, several cases of death and severe intoxications were reported after the consumption of Italian wine which contained high concentrations of methanol. Methanol, cheaper and free of tax compared to ethanol, was added intentionally in order to reach the former required minimum alcoholic degree for table wine with low-grade starting material.

As for allergens, according to the European Regulations, there are maximum limits for sulphur dioxide content depending on the type of wine, and wines containing sulphite must be labelled with "contains sulphites". Moreover, if egg or dairy products are used, these must be declared on the label.

For wine there is risk of contamination with Ochratoxin A and lead. Ochratoxin A is formed when grapes are contaminated by certain mould species and its maximum allowed level is 2.0 µg/kg. For lead there is a threshold limit of 0.20 mg/kg.

3. Analytical methods used to test for authenticity

3.1. Officially recognised methods

According to the Resolution OIV Oeno 9/2000, analytical methods are classified in 4 categories on the basis of criteria of robustness and metrological traceability (I, Criterion Benchmark Method; II, Benchmark Method; III, Approved Alternative Methods; IV, Auxiliary Method) and they should be recommended for different uses: from tests in cases of disputes or calibration purposes, to monitoring, inspection and regulatory purposes.

The OIV Compendium consists of 5 sections and 6 annexes, where physical and chemical analyses are grouped in the second and third sections, respectively.

Physical tests are used to define very different characteristics of wines and musts. Some of these methods are basic and principally devoted to checking the general compliance with legal or trade specifications: Density, Total Dry Matter, Ash and its alkalinity, Chromatic Characteristics, Folin-Ciocalteau Index, and Turbidity. Others are very specific, such as the determination of the $^{18}O/^{16}O$ isotope ratio of water from wine and must after equilibration with CO_2, using isotope ratio mass spectrometry (IRMS).

The chemical tests of section 3 are divided into 2 subsections: Organic compounds (Sugars, Alcohols, Acids, Gas, Other organic compounds) and Non-organic compounds (Anions, Cations, Other non-organic compounds).

3.1.1. Sugars

The determination of fermenting sugars in must and wine represents a fundamental issue for oenology. Different approaches are provided: the most practical for use in the winery, but not very accurate, is the determination of reducing sugars as an estimation of fermentable ones. It is indeed of the lowest category. The determination of glucose and fructose by an enzymatic method, and the determination of sugars, including glycerol and sucrose, by HPLC, are both regarded as being of superior accuracy and selectivity, and are considered as belonging to category II. Of a lower classification are the two approaches that use differential pH sensors for the joint determination of glucose and fructose or, separately, of glucose, fructose and sucrose.

Polyols derived from sugars and residual sugars in dry wines (fructose, glucose, mannitol, sorbitol, dulcitol, and mesoinositol) are determined using gas chromatography after formation of their trimethylsilylated derivatives.

The source of sugar (whether from grape or from cane or beet) is determined using Site Specific Nuclear Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR) which determines the deuterium distribution and the D/H ratios in the methylic and methylenic sites of ethanol derived from the fermentation of grape musts, concentrated grape musts, grape sugar (rectified concentrated grape musts) and wines. The $^{13}C/^{12}C$ isotope ratios of glucose, fructose, glycerol, ethanol in products of vitivinicultural origin (dry wine, sweet wine, grape juice, and rectified concentrated must) are determined by HPLC/IRMS. This method belongs to category II for glucose, fructose and glycerol, and III for ethanol.

3.1.2. Alcohols

Accurate measurement of alcoholic strength (by volume) was, for a long time, both a technical challenge and a practical need for establishing the commercial value of wine. Two methods (categories I and IV) are available. The first measures the alcoholic strength of wine determining the density of its distillate using, alternatively, a pycnometer, an electronic densimeter, or a hydrostatic balance. The second method, definitely less accurate, uses a hydrometer or refractometer to determine the alcoholic strength of the wine distillate.

Two possible methods for methanol quantitation are also considered. The first determines methanol in the wine distillate using GC/FID, while the second measures it on the base of the violet colour intensity at 575 nm after its oxidation to formaldehyde by potassium permanganate and reaction with chromotropic acid in a sulphuric medium.

In this section are also reported 2 isotopic methods. The first determines the $^{13}C/^{12}C$ isotope ratio of wine ethanol or that obtained through the fermentation of musts, concentrated musts or grape sugar by IRMS, enabling the detection and quantification of sugars of C_4 origin (sugar cane or corn isoglucose) which are added to products derived from grapes. The second method is for the determination of the $^{13}C/^{12}C$ isotope ratio of glycerol in wines by GC/C or HPLC coupled to IRMS, and which is used to detect the addition of glycerol from maize (C_4 plant) or from synthesis (fossil sources) to wines or to spirit drinks.

Moreover, the absolute content of glycerol in wine can be investigated using two different approaches: one method based on the colorimetric measure at 480 nm of the reaction products of formaldehyde, obtained by the oxidation of glycerol, with phloroglucinol; or using an enzymatic approach.

3.1.3. Acids

Total and volatile acidity (and their difference, fixed acidity) methods both belong to category I, and are based on titrimetric measurements, directly or after distillation of the wine.

For the determination of the single organic acids, several chromatographic approaches are proposed: by thin-layer chromatography (sorbic acid); by HPLC (tartaric, malic, shikimic, lactic, acetic, citric, succinic and fumaric acids; sorbic, benzoic and salicylic acids; shikimic acid; L-ascorbic acid and D-iso-ascorbic acid); by GC (sorbic acid); by Capillary Electrophoresis (sorbic acid; tartaric, malic and lactic acids) and by ionic chromatography (malic, citric and tartaric acids).

Enzymatic methods are also provided for the selective measuring of enantiomeric forms (D-lactic and L-lactic acids, D-malic and L-malic acids, L-ascorbic acid) and citric acid.

A method for the identification of L- tartaric acid origin (plant or fossil) using ^{14}C activity is also proposed.

3.1.4. Carbon dioxide

Carbon dioxide content in still, semi-sparking and sparkling wines can be measured by titration and is carried out using an acid solution in the presence of carbonic anhydrase, while a direct overpressure measurement in bottles of semi- sparkling and sparkling wines can be performed, after thermal stabilisation and agitation of the bottle, using a specific pressure gauge (aphrometer).

An IRMS method can also be used to discriminate the origin of CO_2 in the headspace of bottled sparkling wines on the basis of stable carbon isotope ratio ($^{13}C/^{12}C$).

3.1.5. Other organic compounds

The main compounds of this class are detected using chromatographic methods: by thin-layer chromatography (artificial sweeteners such as saccharine, dulcin, cyclamate, and P4000), by HPLC (hydroxymethylfurfural by reversed-phase chromatography at 280 nm; ochratoxin A using an immunoaffinity column and fluorescence detection; 9 anthocyanins on reverse phase column and VIS detection at 518 nm; lysozyme on reverse phase column with combined spectrophotometric and spectrofluorimetric detection; 17 biogenic amines on reverse phase column after ophthalaldehyde derivatisation and fluorimetric detection, or 8 of the most frequently present in wine, on reverse phase column after derivatisation with diethyl 2-(ethoxymethylene)malonate (DEEMM) and UV detection at 280 nm; α-dicarbonyl compounds, such as glyoxal, methylglyoxal, diacetyl and pentane-2,3-dione, on a reverse phase column after 1,2-diaminobenzene derivatisation and UV detection at 313 nm), by GC (ethyl acetate on wine distillate using flame ionisation detection; ethyl acetate after purification on a solid phase extraction column and mass analysis; 3-methoxypropane-1,2-diol and 6 cyclic diglycerols, as impurities of 'synthetic' glycerol (plant and animal triglycerides), after extraction and mass analysis; polychlorophenols and polychloroanisols after pre-concentration on head space/solid phase microextraction or solid/liquid extraction, and mass analysis or electron-capture detection; α-dicarbonyl compounds after 1,2-diaminobenzene derivatisation and mass analysis; 27 volatile compounds in wines using flame ionisation detection; 1,2-propanediol and 2,3-butanediol after 'salting out' extraction and mass analysis).

Capillary electrophoresis is proposed for: glutathione using fluorimetric detection; lysozyme using high performance capillary electrophoresis and UV detection at 214 nm). Immunoblotting test permits to check the presence of plant proteins in must and wine, while residues of allergenic

proteins from fining agent can be detected in wine applying the direct and indirect ELISA methods. Immunological methods of immunoprinting are also available for testing the presence of unstable proteins in white wines.

3.1.6. Non-organic compounds

Specific methods are indicated for single anions: by colorimetric test at 590 nm after ashing and treatment with chloramine T and phenolsulfonephthalein (total bromide); by titrimetry using Ag/AgCl electrode potentiometry (chlorides); by selective ion electrode (fluorides); by colorimetry measuring the yellow phospho-vanadomolybdate complex (total phosphorous); and by gravimetry (sulphates).

Different analytical methods are also proposed for cations: by atomic absorption spectrophotometry (AAS) or by flame photometry (FP) (potassium, sodium); by AAS (calcium, iron, cooper, magnesium, zinc, silver); by graphite furnace atomic absorption (GFAA) (cadmium); and by a method that fulfils required performance criteria (e.g. GFAA or ICP-MS; lead). A multi-element method using inductively coupled plasma / atomic emission spectrometry is also provided for potassium, calcium, magnesium, sodium, iron, copper, zinc, manganese, strontium, aluminium, and barium.

A final section is also provided for 'other non-organic compounds' analysis: arsenic can be analysed by atomic absorption spectrometry after ethyl alcohol evaporation, As (V) and As (III) reduction to hydride, or by flameless atomic absorption spectrophotometry after acid mineralization and reduction to arsenic hydride; total nitrogen by direct Dumas method or, after acid mineralization and basic distillation, by titration of ammonia; boron by spectroscopic analysis at 420 nm after alcohol evaporation distillation, decolouration on polyvinylpolypyrrolidone, and complexation with azomethine H; mercury by florescence after wine mineralisation and its reduction with stannous chloride; natamycin by HPLC in combination with DAD or MS detection; phthalates in wines after extraction by gas chromatography/mass spectrometry.

For sulphur dioxide in wine, 2 different approaches are proposed: by titration with sodium hydroxide, after 10 °C and roughly 100 °C distillation (free and total sulphur dioxide, respectively) and oxidation; by titration, direct and after alkaline hydrolysis, of wine with iodine (free and total sulphur dioxide, respectively).

Multielement quantitative determination of aluminium, boron, bromine, cadmium, cobalt, copper, strontium, iron, lithium, magnesium, manganese, nickel, lead, rubidium, sodium, vanadium, and zinc in wines (after mineralisation of the sweetest ones) using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is also defined.

Finally, for pesticide residues in wine, the OIV have adopted the extraction method QuEChERS (Quick Easy Cheap Effective Rugged and Safe) necessary to prepare the sample before GC/MS and/or LC/MS-MS analysis.

These methods allow in the majority of cases to detect adulteration linked to the (i) category: non-compliance with the established legislative standards, based on the comparison of data with reference limits (cf. Table 1). Some of these methods are also used commonly to verify other types of adulteration belonging to the other 2 categories (cf. section 3.2.1).

3.2. Application and interpretation of official methods

Some of the officially-recognised methods listed above have been the subject of studies investigating the factors involved in the variability of their data or concerning their application for detecting mislabelling of must and wine, in terms of declared grape variety or geographic origin or unpermitted addition of exogenous components.

3.2.1. Stable isotope ratio analysis

The stable isotope ratios of H, C and O have been analysed using IRMS and SNIF-NMR in wine and must since 1987, using official standards that are listed as OIV methods. They are expressed in D/H ppm when analysed using SNIF-NMR [$(D/H)_I$ for the methylic site and $(D/H)_{II}$ for the methylenic site] and in $\delta^{13}C$ ‰ and $\delta^{18}O$ ‰ when analysed using IRMS. This analysis enables the detection of sugar and water addition as well as mislabelling, on the basis of a comparison of data with an official reference databank, set up according to the current European Regulation 555/2008. According to this, every year a number of samples that are representative of the wine production of each Member State are officially collected by the relevant national competent authority. The sampling design has to take into account both the geographical distribution and the harvest period due to the geographical and climatic variability of the isotopic values. For each sample, about 10 kg of fresh grapes are harvested, vinified under controlled conditions and the resulting reference wines analysed in accredited laboratories. The data plus a number of metadata related both to the harvest and the vinification are registered in one official databank that is managed by the European Directorate General, Joint Research Centre (DG JRC). The isotope databank comprises reference data for each year. This allows definition of limits for authentic wines and musts in terms of isotopic data, for each country, each sub-area (e.g. region) and each protected designation of origin (PDO-IGP) as well as general limits [3].

Recently the effect of some oenological practices, such as dealcoholisation, grape withering and the stopping of fermentation on these isotopic ratios has been investigated. The variations in wine water $\delta^{18}O$ and $\delta^{13}C$ ethanol encountered have to be considered when interpreting the isotopic values of actual samples.

The reduction of ethanol levels in wine (= dealcoholisation) is today an important topic for many different reasons, including climate change, health and social matters. Dealcoholisation of up to 2 % vol. is allowed by the legislation (EC Reg. 606/2009). Of the available dealcoholisation techniques, the membrane contactor is one of the most efficient and commonly used. The physical phenomena occurring is called osmotic distillation as the compounds extracted are actually migrating through the membrane pores in a gas physical state. In recent works [4,5] variations of wine water $\delta^{18}O$ of up to -1 ‰ and of ethanol $\delta^{13}C$ of up to +1 ‰ have been encountered for 2 % v/v dealcoholisation. The drop in $\delta^{18}O$ water is mainly caused by isotopic diffusion, which involves $H_2{}^{18}O$ migration from the wine to the extracting solution. The increase of $\delta^{13}C$ is due to the fact that ^{13}C has vapour pressure lower than the ^{12}C, and this causes a prevalent transfer of ethanol with ^{12}C.

Withering involves postharvest drying of grapes and can be performed in a dedicated ventilated or unventilated fruit drying room, (called a “fruttaio”) during autumn-winter, or withering grapes on the plant (‘plein-air’). In both cases, during this period the grapes lose water, and this causes a variation of wine water $\delta^{18}O$ [6]. In ‘fruttaio’, $\delta^{18}O$ decreases significantly from fresh to dry grapes, with differences from -2 to -6 ‰. The decrease in $\delta^{18}O$ is coherent with the decrease in temperature and is due to a chemical exchange between grape water and atmospheric water vapour according to equilibrium isotope fractionation. For Passito produced ‘en plein air’, $\delta^{18}O$

increased with withering in southern Italy, since, due to the relatively higher temperature in these areas, kinetic evapo-transpiration takes place.

Stopping of alcoholic fermentation up to 4.5–10 % of ethanol, is used for the production of some traditional Italian sweet wines (such as Moscato d'Asti) in order to leave a pleasant amount of residual sugar in the wine. It was found [7] that the $\delta^{13}C$ and, in particular, the $(D/H)_{II}$ values of ethanol of wine were positively related to the stage of fermentation, while $(D/H)_{I}$ and $\delta^{18}O$ of ethanol were not. The partially fermented musts were characterized by lower isotopic values, which, in the case of $(D/H)_{II}$, are outside the range of variability of natural wines.

Moreover, more innovative isotopic methods, based on the analysis of the stable isotope ratio of other elements or of other components have been developed.

The $\delta^{18}O$ of wine ethanol was measured directly on dry-ethanol using TC/EA-IRMS in pyrolysis conditions, after having trapped residual water using a molecular sieve [8]. It was found to be significantly correlated with the $\delta^{18}O$ of wine water and can therefore be considered as an internal reference to improve the detection of wine watering, as is the case for fruit juice [9]. As the addition of water to wine changes only the $\delta^{18}O$ of water and not that of ethanol, the watering of wine changes this relationship, which can then fall outside the threshold value, even if the water $\delta^{18}O$ is not outside the limit defined by the wine databank. Thus, measuring the $\delta^{18}O$ of ethanol improves the detection of the watering of wine.

Internal reference was found also for $\delta^{13}C$ to potentially improve the detection of sugar or alcohol addition to wine [10]. The compound specific analysis of the main higher alcohols in wine showed indeed a strong relationship between their $\delta^{13}C$ and that of ethanol, that might help to identify exogenous ethanol sources. However, additional experiments verifying the possible refinement were not performed.

Recently also a method to measure $\delta^{15}N$ in must, wine and in the extracted proline was developed [11]. For proline, the most abundant amino acid in grape and wine and not used by yeast as nitrogen source, $\delta^{15}N$ was measured after N-acetylisopropyl derivatisation using gas chromatography − combustion − isotope ratio mass spectrometry (GC-C-IRMS). $\delta^{15}N$ values of leaves, grapes, wine and particularly must and wine proline were found to be related to those of $\delta^{15}N$ in the growing soil. The addition of inorganic or organic adjuvants was able to influence the $\delta^{15}N$ of bulk wine, but not the $\delta^{15}N$ of wine proline, which is therefore the best marker for tracing the geographical origin of wine.

A GC-c-IRMS method for analysing vanillin in distillates after dichloromethane extraction was developed [12]. Storage in oak barrels release different degradation products such as vanillin, which plays an important role in the flavour and aroma of the distillates. The addition of vanillin, as well as other aroma compounds, of different origins is prohibited by European law. $\delta^{13}C$ values are able to distinguish natural vanillin extracts (-21.0 ‰ to -19.3 ‰), vanillin from lignin and also from tannin (-29.5 ‰ to -26.7 ‰) and synthetic vanillin (-32.6 ‰ to -29.3 ‰).

3.2.2. Trace element profile

Several studies have shown that the trace element profile can be used to classify wines according to their geographic provenance [13,14]. Factors such as soil geochemistry influence the elemental composition of crops. On the other hand, anthropogenic factors such as viticultural practices and processing methods have a strong effect as well.

In 1994, Latorre et al. [15] differentiated the PDO Rias Baixas Spanish wine from Galicia from its imitations. Pattern recognition analysis, performed on ICP-MS data, revealed that Li and Rb were the most discriminating variables. Similar studies were carried out by Baxter et al. [16] on wines from different regions of Spain and England. Taylor et al. [17] studied soils and wines from the Canada's two major wine-producing regions. They found that, among trace elements, strontium was able to differentiate both soils and wines from the two regions. The fingerprint of REE was kept unaltered in the passage soil–grapes–must, while fractionation occurred in wine [18] after the clarification with bentonites. In addition, analysis of Moscato musts from 102 samples showed that is possible to classify their geographic origin, building a basis for identification of possible addition of foreign musts.

3.2.3. Shikimic acid content

Shikimic acid occurs naturally in wine in different concentrations. It is derived from caffeic acid and is a precursor of different amino acids in the biochemistry of the grape plant. Its concentration in wine has been linked to its grape variety.

The analytical method originally proposed by Holbach et al. [19] became an officially-recognised Category II method adopted by the OIV in 2004 (OIV-MA-AS1-02, Oeno 33/2004) [20], fully validated in a collaborative trial.

Based on the publication of Holbach 2001 [19], the official German wine control authorities published reference data for the so-called burgundy group of varieties which are characterized by a low content of shikimic acid (< 30 mg/l) in 2003. Since then, in 2018 the data collection consists of almost 14 000 data – including a broad range of varieties, entries which still the early findings. Wines from many growing regions over the world are implemented, although some sample collectives for certain more local varieties derive mainly from Germany.

For some questions the shikimic acid concentration gives interesting information on the authenticity of the wine variety. For example, Riesling wines are characterised by a high content (with an arithmetic mean of 58 mg/L, n=3346) in contrast to the burgundy wines which show a low concentration of shikimic acid. Therefore, shikimic acid is an indicator for certain varieties and can be indicative for some others. Further authors have confirmed the suitability of shikimic acid for the verification of certain wine varieties [21], reported for low shikimic acid concentrations for the variety Semillon, and Merlot showed a lower shikimic acid concentration than Cabernet sauvignon. Furthermore, the authors showed that the combination of shikimic acid with protein/anthocyanin profiles led to a successful verification of different varieties grown in France (Chardonnay, Chenin, Petit Manseng, Sauvignon Semillon and Ugni Blanc). The low content of shikimic acid for Burgundy varieties was confirmed also for Chilean wines [22].

3.2.4. Anthocyanin composition

The analysis of anthocyanins and particularly their ratios has been successfully used for verifying the identity of grape varieties. Although similar types of anthocyanins are found in different grape varieties, the relative amounts of the individual compounds and their ratios differ. The analytical method was adopted by the OIV as a Category II method (reference method) with Resolution 22/2003: "HPLC-Determination of nine major Anthocyanins in red and rosé wines" (MA-E-AS315-11-ANCYAN) [23].

Thus Individual grape varieties can be verified from one or more anthocyanin compounds in some cases. The acylated anthocyanins have proven to be particularly characteristic for certain grape varieties, with considerable practical significance being attached to the ratio of acetylated to p-

coumaroylated anthocyanins (Rac/cou) and the sum of acylated anthocyanins (Sac) in the assessment of the variety ([18]. For example, it has been noted that Pinot Noir grapes contain no acylated anthocyanins and this feature of burgundy wines is successfully applied for their variety control. For example, the German speciality “Weißherbst” which is a rosé wine produced from 100 % Pinot Noir grapes, should show no significant proportion of acetylated anthocyanins. It should be noted, however, that measurement uncertainty of the wine in question, the typical authorized blending (e.g. 15 % in the EU) and, in the case of sweetened wines, the addition of further products (such as must), should all be considered appropriately before drawing conclusions on the variety in question. In addition, the ageing of wine gives rise to the degradation and polymerisation of the anthocyanins which leads to the absence of the analytes.

Brunello di Montalcino, one of the flagship products of Italian oenology, must be produced from Sangiovese grapes grown in Montalcino, a specific area in Tuscany. Sangiovese grapes are poor in acetylated anthocyanins, one property that in principle promotes the makes it possible to authenticate these premium wines by analysis of the anthocyanins, but as these wines typically aged up to 10 years, sophisticated mass spectrometric approaches give more reliable results as shown by Arapitsas et al. 2012 [24].

One example related to grape variety fraud -the so-called “Pinotgate” incident- was uncovered 2010 in California where Pinot Noir wine imported from France sold in the United States was identified to contain large amounts of Merlot and Syrah [25]. According to information available, the wine was first suspected because of its sensory properties.

3.3. New prospective

There are moreover analytical methods in the literature that are showing promising applications for wine characterization mainly in terms of its geographical and varietal origin.

3.3.1. NMR profiling

^{1}H NMR spectroscopy in combination with multivariate data analysis can be successfully used also to achieve information on various aspects of wine quality such as the authenticity, grape variety, geographical origin, and the year of vintage [26].

This technology, called Wine-Profiling™, has been developed and validated in a joint effort by Bruker BioSpin GmbH and a consortium of analytical laboratories with expertise in wine analysis. The comparison of the spectroscopic fingerprint obtained for each individual sample with that of a large database of authentic wine samples provides answers to questions on the composition, geographical origin, grape variety and vintage. This procedure had been already developed with success for fruit juice analysis (SGF-Profiling™), and it was further optimized for wine and alcoholic beverages in general. In particular, to overcome the need to eliminate the major signals (water and ethanol), a methodology was developed which can suppress both signals from water and ethanol during the NMR experiment without losing signals outside those regions. Similarly to SGF-Profiling™, Wine-Profiling™ provides both targeted and untargeted analysis. The former is performed through the quantification of 56 parameters per sample and their comparison with official reference values, while the latter is carried out through verification models able to detect any deviation from authentic reference data. Models are still under construction to enlarge and maintain the database but at the date of this publication the methodology is well established to control the origin for the major producing countries (France, Italy, Spain, Germany, Chile, Austria), even at the regional level for some major regions (France: Bordeaux, Burgundy, Languedoc, Rhone

Valley, Loire Valley ; Italy : Piemonte, Toscana, Sicilia, Puglia ; Spain : La Rioja, Ribera des Duero) and also to control the major varieties (Red : Pinot Noir, Tempranillo, Garnacha Tinta, Syrah, Merlot Noir, Cabernet Sauvignon, Sangiovese, Nebbiolo, Montepulciano, Primitivo, Dornfelder, Portugieser Blau, Zweigeltrebe Blau ; White : Chardonnay Blanc, Sauvignon Blanc, Riesling, Pinot Blanc/Gris, Silvaner, Verdejo, Mueller Thurgau, Veltliner, Moscatel, Welschriesling. This analytical technique gives information on unforeseen deviations and is a multivariate untargeted analysis useful for a screening control of the market. Interlaboratory comparison is monitored with a dedicated Proficiency Testing Scheme, Pro-PTS, organised by Eurofins Analytics France which controls not only the quantitative parameters but also the interpretation of the sample.

The Wine screener provides additional answers in the control of the authenticity of wines, and in combination with other methods, such as stable isotope analysis as described above, it can offer a performant solution using data fusion. The benefit of fusing NMR data with alternative techniques has been provided by [27]. The authors evaluated the combination of discrete isotopic data with the untargeted NMR spectrum to have better control of wines. Both techniques are known to provide useful information to the characterization of wine: ^{1}H NMR spectroscopy can be used to build robust classification models for grape variety, year of vintage and geographical origin, while stable isotope ratio analysis is a good source of chemical information for the authenticity assessment of food products. By combining these two methodologies, an improvement of classification rates of wine was achieved: 100 % for the determination of geographical origin (60–70 % correct prediction was obtained with stable isotope data alone and 82–89 % with ^{1}H NMR spectroscopy) and 99 % for the vintage of wine (from 88 to 97 % with ^{1}H NMR).

3.3.2. MS metabolomics

Metabolomics represents one of the most recent analytical approaches used in wine authentication. Since wine is a very complex matrix and all its metabolites are physically and chemically diverse, it is not possible to identify all of them in a single platform measurement. Therefore, it is necessary to use different, complementary analytical techniques. Besides NMR mentioned above, mass spectrometry (MS) is frequently used, either ambient or coupled to separation techniques such as gas chromatography (GC) and liquid chromatography (LC) [28–30].

Among mass spectrometry techniques used in wine metabolomics, LC-MS is the most common. It is suitable for determination of non-volatile, thermolabile compounds (e.g. phenolic compounds). One of its main advantages is, that before analysis of the wine, no pre-treatment or extraction of the sample is necessary. However sometimes, simple steps like filtration, dilution or pre-concentration of the sample might be desirable [28].

LC provides metabolite separation based on the different distribution between the mobile (liquid) and stationary phases. For this purpose, the LC system can be equipped with different types of columns, although usually reverse phase columns are used. The ionisation sources frequently used in conjunction with LC-MS are electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), or atmospheric pressure photoionisation (APPI), however, ESI is the most common. Considering that most metabolites ionise in one ionisation mode (positive or negative), not in both, it is necessary to analyse the samples in both of them, in order to cover a wider metabolome. After ionisation, ions pass through the mass analyser. Since the metabolomics approach is focused on characterisation of the entire composition of small metabolites (metabolome), mass analysers capable of whole metabolome analysis within single analytical run (with good dynamic range, fast scan speed, sensitivity and high mass resolution and mass accuracy) such as Time of flight (TOF) and orbital ion traps are usually used. The resolution achieved is closely associated with the ability of the instrument to measure the accurate mass,

which is crucial for the identification of unknown compounds. The mass error is usually below 5 ppm in the case of TOFs and below 2 ppm in the case of orbital ion traps. Extensive technological improvements have been achieved with the new generation of hybrid instruments, Q-TOFs and Q-Orbitraps, allowing performance of the specific ion fragmentation, bringing an additional dimension by enabling the identification of unknown compounds (MS/MS spectra, i.e. spectra of fragment ions by HRMS) [31]. It should be mentioned that by metabolomics analysis large amount of data is obtained. In order to extract valuable information from the data, pre-treatment steps (data mining, retention time and m/z alignment etc.) and also effective statistical software tools are required for effective data handling (not only for LC-MS, but also for GC-MS and ambient MS) [28,30,32,33] .

LC-MS in combination with metabolomics could be used for different wine authentication purposes, such as classification/discrimination of wine samples according to their variety [34–36], origin/producer [34,37], vintage [34], and quality [34].

Since in most of the cases, this goal is achieved using statistical evaluation of data, marker identification does not represent a necessary step in non-targeted metabolomics studies aimed at sample classification/discrimination [36]. However sometimes, it might be useful to know the identity of a compound related to the sample differentiation. In the following paragraph, examples of several markers are listed.

In the study of Rubert et al. [35], astilbin was identified as a marker of Pinot Noir and different flavonol glucosides as markers of Merlot and Tempranillo (among varieties Pinot Noir, Tempranillo, Merlot, Shiraz, Riesling, Sauvignon Blanc, Silvaner and Chardonnay Blanc). In the study carried out by Roullier-Gall et al. [37], polyphenols, fatty acids, carbohydrates, and amino acids were identified as markers of different wine samples according to the geographical origin/producer (among Chablis, Meursault 1, Meursault 2, and Corton Charlemagne wines).

Another technique frequently used in metabolomics is GC-MS. Unlike LC-MS, this technique is limited to detection of thermostable, sufficiently volatile compounds. Therefore, the main drawback of GC-MS-based metabolomics is the need for sample handling prior to the analysis. The most important of these are procedures enhancing the volatility and the thermal stability of the metabolites (e.g. derivatisation) and procedures (extraction processes) isolating metabolites and enhancing their concentration (e.g. liquid-liquid extraction - LLE, solid-phase extraction - SPE, solid-phase microextraction - SPME) [28,38].

As with LC-MS, a chromatographic separation based on different distribution between two phases is used. However this time, the mobile phase is gaseous. The ionisation source dominantly used in conjunction with GC-MS is electron ionisation (EI) with an ionisation energy of 70 eV. In combination with standardised protocols of data acquisition, the use of EI results in reproducible, rich fragmentation mass spectra. These can then be recorded in large user libraries (e.g. NIST 14 Mass Spectral Library) or compared (matched) with mass spectra (and other additional information) already present in the libraries, in order to confirm the compound identification. This is undoubtedly one of the main advantages of the GC-EI-MS-based techniques [30,38].

Since the requirements for the mass analysers capabilities in GC-MS are similar to LC-MS, mass analysers such as TOF or hybrid Q-TOF are suitable for wine metabolomics. However, the most frequently used mass analyser (due to its relatively low price, high sensitivity and good dynamic range) in GC-MS-based metabolomics is the quadrupole [30,38].

GC-MS in combination with metabolomics is often used to authenticate wine variety [39,40], producer[39] and vintage [39]. In the following paragraph, examples of several markers are listed.

In the study of Kruzlicova et al. [39], various terpenes and alcohols (e.g. α-terpineol, linalool, 1-hexanol and (E)-3-hexen-1-ol) were identified to be the most important markers for wine classification according to the wine variety (among varieties Welsch Riesling, Gruener Veltliner and Mueller Thurgau) Most important markers for wine classification according to the producer/origin (producers located in West and South West Slovakia) were esters, alcohols and carboxylic acids (e.g. diethyl succinate, 2-ethylphenylacetate, (E)-3-hexen-1-ol, 1-hexanol and hexanoic acid) and according to the vintage (years 1996, 1997 and 1998) were alcohols, esters and carboxylic acids (e.g. (E)-3-hexen-1-ol, hexanoic acid, 1-hexanol and ethyl-3-hydroxy butanoate).

Ambient mass spectrometry represents a group of MS-based techniques, which are not coupled with chromatographic separation. In other words, analytes are directly injected / transferred into the mass spectrometer, without prior separation. In comparison with the GC- and LC-MS based approaches, ambient MS is far less informative. It is not capable of isomer separation, quantification of individual metabolites is less accurate and it does not provide additional data such as retention time/factor/index etc. Also, the absence of chromatographic separation prior to MS analyses may increase matrix effects and cause ionisation suppression. However, ambient MS is much faster than GC- and LC-MS-based approaches and for large sample sets analysis it is often the only possible/reasonable option [30].

The ionisation sources frequently used in ambient MS are desorption ESI (DESI), extractive ESI (EESI), direct analysis in real time (DART) or matrix-assisted laser desorption / ionization (MALDI). For the purposes of ambient MS analysis, advanced high-resolution tandem MS instruments such as Fourier-transform ion cyclotron resonance MS (FT-ICR-MS), TOF-MS or Orbitrap MS are usually used [30,41].

Ambient MS was used for example for the authentication of wine variety or to detect adulteration of wine by illegal wine mixing or by colouring [42]. In the following paragraph, examples of several markers are listed.

In the study of Hartmanova et al. [42], different anthocyanins (e.g. malvidin-3,5-diglucoside, malvidin-3-acetylglucoside and peonidin-3-acetylglucoside) were used for authentication of wine according to the variety.

3.3.3. DNA Molecular analysis

The metabolic composition of grapes and wines depends on external factors whereas each grapevine cultivar displays a unique genotype which is independent of growing conditions, such as soil composition, environmental conditions, vintage and cultural practices. For this reason, DNA is the ideal target molecule for efficient variety identification also of wines as an alternative to, or in combination with, chemical profiling.

Identification of grapevine varieties from direct plant material - leaves, fruits, canes and roots - through DNA-based markers is a well-established practice. Simple Sequence Repeats (SSRs or microsatellites) have proved to be the best genetic markers for grapevine DNA typing because of their high degree of polymorphism, species-specificity, co-dominant Mendelian inheritance, reproducibility and simple data. Due to the extensive use of this fingerprinting technology worldwide, large international *Vitis* databases of SSR profiles are now available as references for grapevine varietal identification (Vitis International Variety Catalogue, VIVC - www.vivc.de).

Wine is a complex matrix where the DNA found comes not only from the grapes used for its elaboration, but also from the spontaneous microbiota, or which have been inoculated for alcoholic and malolactic fermentations, as well as from the additives of biological origin and the

concentrated musts that may have been used. The potential of a genetic traceability approach in a such heterogeneous matrix, indeed, is almost unlimited, since the molecular analysis would enable the identification, not only of the grape varieties from which it has been produced, but also the yeasts and/or bacteria strains used for fermentation and to establish if genetically modified organisms (GMO) have been used [43–45]. Thus, the development of genetic analysis would make possible the traceability of a wine at all levels and in all stages of the winemaking process.

However, winemaking implies several processing steps which limit the quantity and quality of DNA available in wine. On one hand the DNase from the microorganisms degrade DNA during fermentation generating denatured and fragmented residues. On the other hand, decantation, clarification, filtration and other fining treatments may contribute to the decrease of the final DNA concentration available. In addition, the co-existence of polysaccharides and proteins interfere with DNA isolation, and other substances — such as polyphenols — act as inhibitors of the polymerase chain reaction (PCR) methodology used for genetic fingerprinting analysis.

Genotyping for grapevine varietal identification can be roughly described in four main steps: DNA isolation from plant material, DNA markers amplification by PCR, analysis of the PCR products by capillary gel electrophoresis and results interpretation [46]. This methodology was first applied for grape juice varietal identification by [47], and then for varietal wine authentication by Siret el al. [48,49], who analysed experimental wines from the start to the end of fermentation. These authors performed successful varietal identification by SSR genotyping in musts, but reported difficulties for the authentication of the cultivars in finished wines due to the scarce DNA isolated. Successive studies have been performed in order to improve DNA isolation from wine, but in all cases, although varietal identification of musts was possible, reproducibility problems for the systematic authentication of finished experimental and/or commercial wines were always reported again due to the extraction of low DNA quantity and quality from a wine matrix [50–60]. Analysis of other marker types, such as chloroplast SSR markers or Single Nucleotide Polymorphism (SNP) markers, has been proposed [51,59]. Multivarietal must mixtures and blended experimental wines have been analysed as well to detect the discriminatory power of the DNA marker technology for determining the varieties used in the mixtures [49–51,55,61]. Although it could be determined if more than one variety was used, the identification of unknown additional cultivars used became impossible, especially when the blends consisted of more than two varieties. Preliminary results obtained using a TaqMan SNP genotyping approach highlighted the potential of Real Time PCR for wine varietal authentication and quantification [59]. The TaqMan assay is much more sensitive than SSR genotyping, not only because it requires a smaller amount of DNA for the analysis, but also because it is based on the analysis of cultivar-specific SNPs. Moreover, this method is more sensitive and precise for relative quantification of each variety in a mixture because is based on specific allele probes.

Despite all the studies performed up to date, the main limiting factor for the development of a standard method for wine varietal authentication remains the quality of grape nucleic acids extracted from wine. The PCR amplification of shorter fragments of DNA that allows access to minute traces of nucleic material seems more promising at least for authentication purposes.

4. Overview of methods for authenticity testing

The following table provides a summary of the methods and the authenticity issues they address.

Analytical technique	Indicative data or analyte	Authenticity issue / information
Enzymatic, HPLC, differential pH sensors	Sugars (glucose, fructose, sucrose)	Non-compliance with the established legislative standards, based on the comparison of data with reference limits
Pycnometry, electronic densimeter, hydrostatic balance	Alcoholic strength	
GC-FID	Methanol	
Colorimetry, enzymatic	Glycerol	
Titrimetric measurements	Total and volatile acidity	
TLC, HPLC, GC, CE, IC	Organic acids	
Aphrometer	CO_2	
HPLC	Biogenic amines	
Colorimetry, titrimetry, gravimetry	Anions	
AAS, FP, GFAA, ICP-MS	Cations	
Direct & indirect ELISA	Proteins	Residues of allergenic proteins from fining agent
TLC	Saccharine, dulcin, cyclamate	Use of artificial sweeteners
Stable isotope analysis SNIF-NMR & IRMS	(D/H)I ppm, $\delta^{13}C$, $\delta^{18}O$	Detection of sugar and water addition; application to withering, dealcoholisation, stopping of alcoholic fermentation
IRMS	$\delta^{13}C$ of CO_2	Origin of CO_2 in sparkling wines
ICP-MS	Trace element profile	Geographical provenance
HPLC/UV	Shikimic acid	Grape varieties
HPLC	Anthocyanins	Grape varieties
^1H NMR screening	Overall profile of ^1H NMR spectrum + selection of compositional parameters	Grape variety, vintage, geographical origin
Metabolomics using LC or GC-MS with ESI, APCI, APPI ionisation and TOF, Q-TOF, Q-Orbitrap mass analysers	Various metabolites in wine	Classification of wine samples according to variety, origin/producer, vintage, quality
DNA-based techniques (SSR, microsatellites)	DNA extracted from wine	Grape varieties ; yeast/bacterial strains

5. Conclusion

The authenticity of oenological products appears to be well guaranteed by a complex and robust analytical control system. However, the high value of these commodities generates continuous attack to their genuineness. Botanical and geographical, as well as varietal origins, probably represent the main issues for the sector. Further innovative methods using isotope, mineral and metabolomic profiles integrated with DNA molecular analysis can represent the future of this challenge. Availability of specific and extensive compositional databases and of validated and recognised analytical protocols are required. However only a higher awareness of these new approaches from the competent governmental control bodies and courts will make it possible to reach a superior control of frauds and mislabelling.

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