Hyphenated Techniques in Grape and Wine Chemistry

Edited by

Riccardo Flamini
CRA, Viticulture Research Center, Conegliano Veneto, Italy

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To Emanuela and Vittorio, my family

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Preface

In the last twenty years, the increased production and consumption of table grapes and wines has partly been encouraged by the amply demonstrated beneficial effects of these foods on human health. The effects of moderate wine consumption on some categories of illness, such as cardiovascular diseases, degeneration of the brain due to ageing, and certain carcinogenic diseases, have been studied. The *Organisation Internationale De la Vigne et Du Vin* (O.I.V.) reports that raisins are one source of food with which to fight hunger in the world.

The efforts of the largest grape- and wine-producing countries are focused on improving product quality, rather than on increasing production, in order to remain competitive with emerging countries by better systematic positioning of 'premium' and 'super premium' wines in market niches. In this sphere, viticulture aims at improving grape quality by means of appropriate growing techniques, selection of the best clones and varieties, and study of environmental influences on vineyards. The main efforts of enology are devoted toward optimizing industrial processes: enhancement of grape compound extraction in wine-making, the best ways of achieving alcohol and malolactic fermentation, and barrel- and bottle-ageing all aim at obtaining products with particular characteristics and well-defined identities.

The legislation of the European Community and of single countries is devoted to protecting consumer health and internal markets from the sometimes harmful effects which may be caused by low-quality products. As a consequence, in order to export wines and wine-derived products, quality certificates are required (analysis of contents of pesticides, heavy metals, toxins, etc), for which legal limits are defined. The current activity of researchers and control organisms is also devoted to detecting adulterated products and illegal additives, and to verifying the proper match between the true characteristics of products and their producers' declarations (variety, geographic origin, quality, vintage, etc).

For all these aims, knowledge of the chemical composition of grapes and wines is essential, and analytical chemistry is the proper tool with which to achieve it.

In my opinion, research in Italy has played and still plays an important role in the field of grape and wine chemistry. So when John Wiley & Sons asked me to edit this volume, I promptly contacted some of my Italian research colleagues and asked each of them to contribute one or more chapters on the main topics of their research. Surprisingly, in view of their workloads, they all gave me an enthusiastic 'Yes!'.

The result is in your hands: a work on the most recent analytical approaches and techniques applied to grape and wine chemistry, perceived not only as support to Research and Quality Control Laboratories, but also as an up-to-date source for students beginning work in the field of oenological analytical chemistry.

This volume is divided into eight chapters, by subject. Due to its multidisciplinary nature, analytical techniques are not described, apart from brief introductions to Chapters 7 and 8, to which readers are referred for more specific analytical chemistry books. Again, due to the enormous number of subjects discussed, only a brief summary of methods, including materials and instruments used, is given, and readers are referred to single publications. The main parameters of wine fermentation are treated in the first chapter, but discussion of volatile esters and higher alcohols were deemed to be more suitably located in Chapter 5 on aromatic compounds.

In order to avoid any form of advertising, wherever possible the trade names of products and producers have been omitted, and only a general product description is given.

I hope that this work may at least partially fill a gap in the literature on the subject, which has been particularly enriched in recent years by the development of new analytical techniques and technologies.

I would like to thank all the authors and contributors whose work made this volume possible – in particular, Mirko De Rosso and Antonio Dalla Vedova, for their support in organizing the text, and Rocco Di Stefano, Paolo Cabras, Andrea Curioni, Roberto Larcher and Giorgio Nicolini for their faith in me, which they demonstrated by so willingly writing their contributions.

A separate acknowledgment goes to Giuseppe Versini who, with his contribution, spirit of enterprise, and belief in the project right from the beginning, enormously assisted me in completing this work.

Lastly, I would like to thank John Wiley & Sons Ltd for giving us this opportunity to present Italian research in the field of oenology.

Riccardo Flamini
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Conegliano Veneto, Italy, September 2007

1

Principal Parameters of Grape Ripening and Wine Fermentation

Rocco Di Stefano and Riccardo Flamini

1.1. Introduction

1.1.1. Main Parameters of Grape Ripening

Determination of ripening parameters is important to evaluate the quality and maturity state of the grape. The principal parameters usually considered for this aim are pH, total acidity, tartaric, malic, and citric acid, fructose and glucose in the grape must, anthocyanins, tannins and other compounds in the grape whose contents change during ripening. The date to start the harvest is decided mainly on the basis of pH, total acidity, sugar and organic acid content of the must. An index utilized to estimate the technological maturity is the sugars/total acidity ratio of must, which becomes constant close to the full maturation of the grape. Trends of the organic acids and sugars content in the grape during ripening are shown in Figure 1.1.

The principal organic acids in grape are L(+)-tartaric, L(−)-malic, shikimic and citric acid. Gluconic, 2-keto-D-gluconic and mucic acids are present in rot grapes as metabolites of *Botrytis Cinerea*. The structures of these compounds are reported in Figure 1.2.

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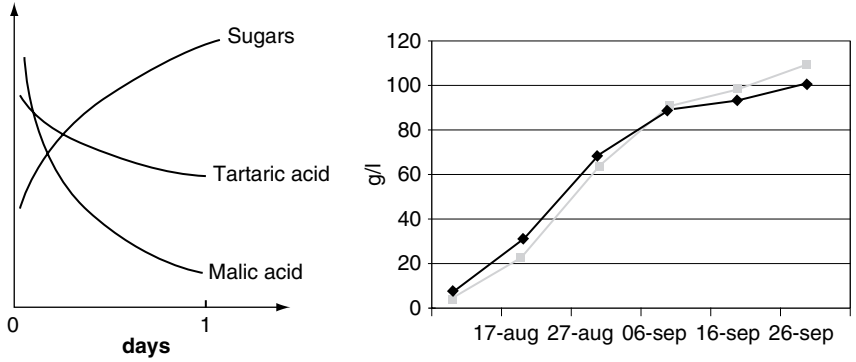

Figure 1.1 Trends of organic acids and sugars in grape during ripening (♦ glucose; ■ fructose)

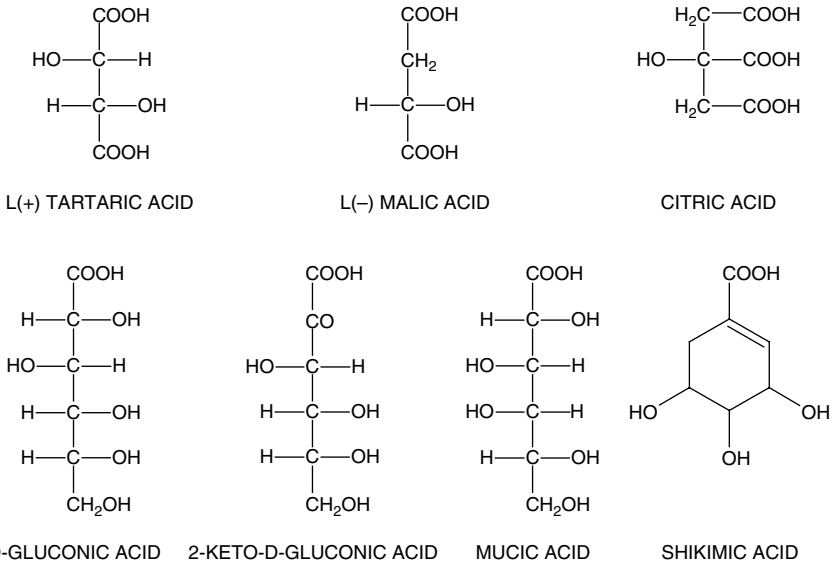

Figure 1.2 The main organic acids in grape

In the production of monovarietal wines, even if a single variety is used, grapes are usually collected from different vineyards which are often sited in zones characterized by different microclimates and are grown with different cultural practices. As a consequence, to correctly program the harvest it is necessary to know the ripening parameters of all the different grapes. Moreover, on the basis of the sugar levels of the must the alcoholic content of wine is predicted, while the anthocyanin

profile and content of grape skins suggest the more suitable practices to be used in winemaking. Knowledge of the pH value, total acidity and organic acids of must provides important information about the acidic and sensorial characteristics of the final wine and about the need to correct acidic parameters, and allows planning of the technological practices such as tartaric stabilization and correction of the wine acidity, and controlling the course of malolactic fermentation. In particular, intensity and duration of acidic sensation play an important role as quality parameters of white wines, as well as in astringency perception of red wines. In grapes affected by *Botritys cinerea* (grey rot) attack a significant level of gluconic acid is usually present. Affection of grape by others moulds (e.g. *Aspergillus niger*) may induce formation of some organic acids such as glucuronic and citric acid.

The singular organic acids and sugars in the grape juice can be determined quickly and with high precision and accuracy by high-performance-liquid-chromatography (HPLC) analysis. HPLC analysis of skin extracts also provides the anthocyanin profile of red grapes. By titration and spectrophotometry methods total acidity, pH, total polyphenols and extractable phenolics indexes of the must are determined.

In wine, total organic acids content, total acidity (free organic acids) and ashes alkalinity (organic acids in salt form) are interrelated as follows:

$$\text{total organic acids (meq/L)} = \text{total acidity (meq/L)} + \text{ashes alkalinity (meq/L)}$$

It means the sum of organic acids in wine determined by HPLC have to be lower than the sum total acidity and wine ashes alkalinity determined by titration.

The grape sampling

By using HPLC coupled with modern sample preparation methods determination of tartaric, malic, and citric acids, of fructose and glucose and of the anthocyanin profile, can be performed with high precision and accuracy. Nevertheless, grape sampling in the vineyard is often affected by a high variability, due to the fact that the grapes collected may be at different stages of ripening. To minimize this undetermined error, particular attention has to be devoted to the choice of the method used to obtain a representative sample for analysis. The sampling method needs to be rapid and reproducible; on the other hand the clusters collection requires high quantities of grape to ensure a representative sample.

Picking of a suitable number of berries from some clusters directly in the vineyard, rather than part of clusters that require large samples and selection of berries in the laboratory, can in part overcome these problems and provide a representative sample. In the case of berry sampling, it is necessary that the plants selected for the collection are representative of the vineyard, and the size of the cluster and plants has to be the same in all sampling. Consequently, it is very important that the person collecting the samples has experience and knowledge of the vineyard in question – its cultural practices, treatments and general condition. Following these rules, a few hundred berries can be sufficient to have a representative sample for determination of the mean ripening state of the grapes.

1.1.2. Fermentation Parameters of Wine

Alcoholic fermentation

The microorganisms responsible for alcoholic fermentation are mainly *Saccharomyces cerevisiae* yeasts. Nevertheless, in the initial phase of the process the non-*Saccharomyces* yeasts can contribute, positively or negatively, to the composition and quality of wines. The principal event occurring in alcoholic fermentation is transformation of sugars into ethanol. Many volatile and non-volatile secondary metabolites, such as ethanol homologous alcohols, fatty acids and esters, aldehydes, ketones, hydroxyacids, ketoacids and dicarboxylic acids, are produced by the yeasts. Alcohols, ketoacids and hydroxyacids are by-products of proteic metabolism of yeasts. Even if yeasts can directly incorporate the must aminoacids, most of these compounds are synthesized by transfer of α–NH_2 amino group of the must aminoacids to specific ketoacids produced in the glyceropyruvic fermentation (Figure 1.3). Amination of ketoacids occurs by a transamination reaction in which the couples glutamic acid/pyridoxal phosphate and 2-ketoglutaric acid/pyridoxamine phosphate are –NH_2 transporters. As a result, glutamic acid transfers the α–NH_2 group from a must aminoacid to a ketoacid from glycolysis and a new aminoacid, that can enter in proteic synthesis, is produced. The excess ketoacids undergo loss of carboxyl group and the aldehydes generated are then reduced to alcohols such as 2-methyl-propan-1-ol, 2-methyl-butan-1-ol, 3-methyl-butan-1-ol, 3-methylthio-propan-1-ol, 2-phenylethanol, tyrosol, tryptophol. It is hypothesized that even γ-butyrolactone is formed from ketoglutaric acid through this pathway.

Propan-1-ol is directly originated by the loss of the 2-ketobutyric acid carboxyl group and subsequent reduction of propionic aldehyde formed.

It is interesting to note that in fermented products the same alcohols always form, even if different sources of nitrogen, but not singular aminoacids, are available. Ketoacids can also be directly reduced to hydroxyacids such as lactic, 2-hydroxy-3-methyl butyric, 2-hydroxy-3-methyl pentanoic, 2-hydroxy-4-methyl pentanoic, 2-hydroxy-3-phenyl propionic and 2-hydroxy glutaric acids. The corresponding ethyl esters can dramatically influence the wine aroma.

It was hypothesized that succinic acid, the principal fermentative dicarboxyl acid in wine, is formed in the tricarboxyl acids cycle (Figure 1.4). Even if this metabolism is not used by microorganisms in fermentation, the enzymes involved are able to operate liberation of succinic acid.

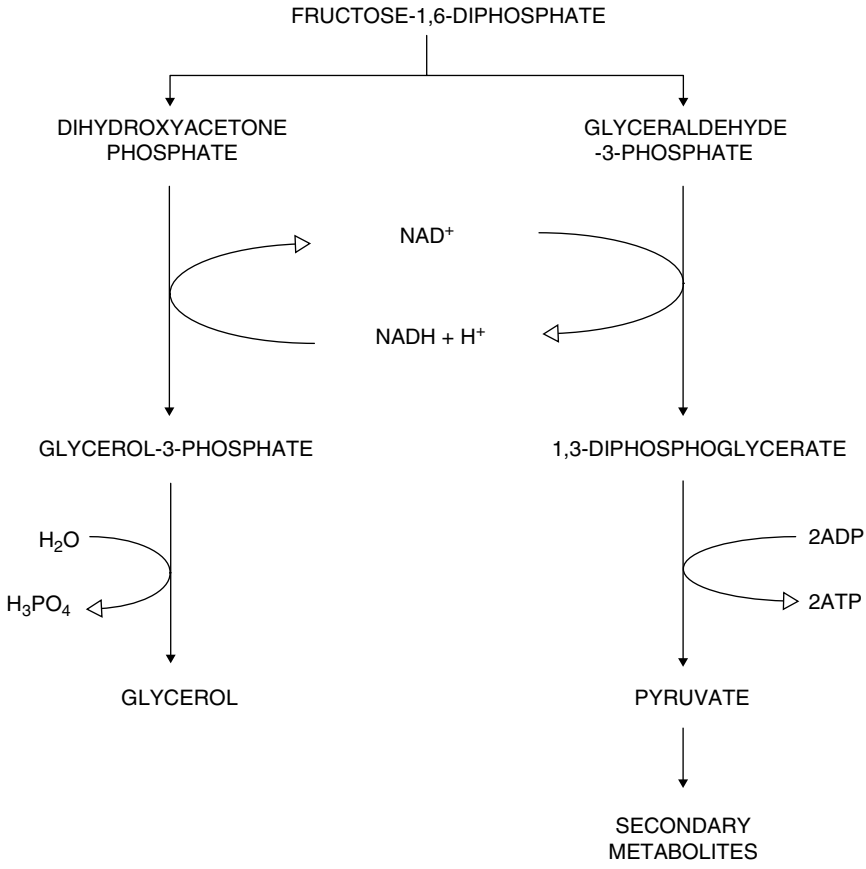

Figure 1.3 Glycero-pyruvic fermentation pathway

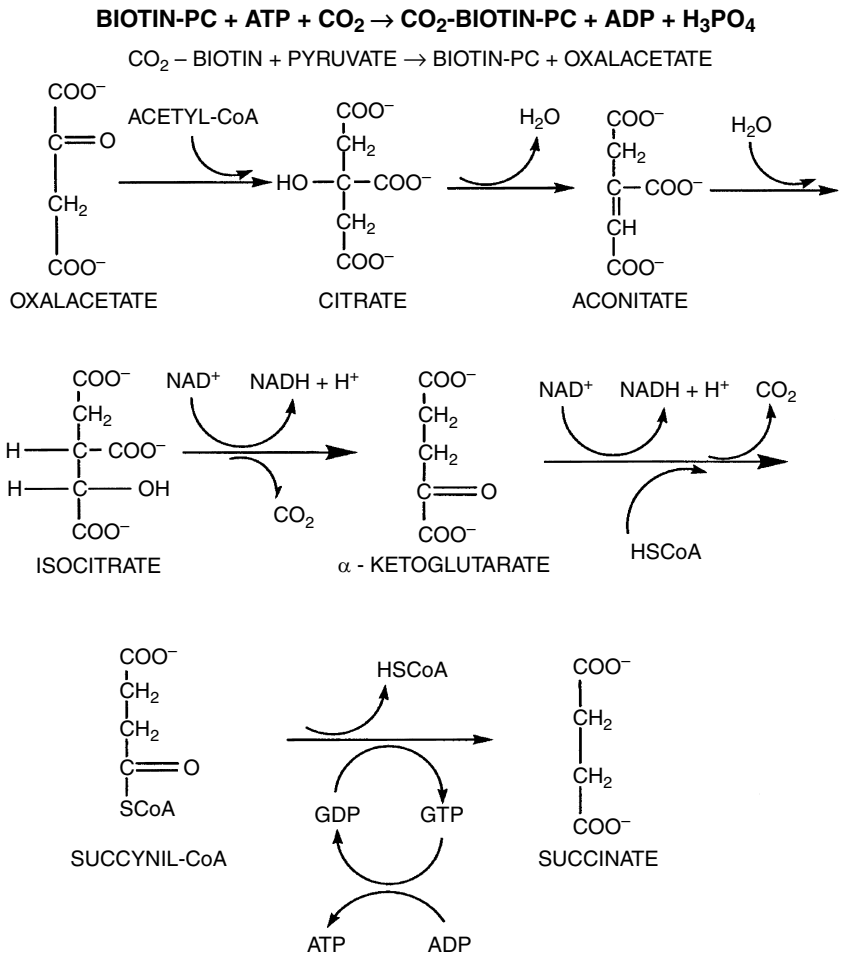

Figure 1.4 Synthesis of succinic acid. PC: pyruvate carboxylase

Production of oxalacetate by CO_2 addition to pyruvic acid, operated by pyruvate carboxylase (biotin coenzyme), allows the cycle to continue.

Short- and middle-chain fatty acids are formed in the lipids metabolism. Relevant levels of acetic acid are usually present in wine (several hundred mg/L) due to the fact that this compound can be formed by different metabolic pathways. On the contrary, the levels of short and middle-chain fatty acids are relatively low, with a maximum of a few mg/L in red wines and tens mg/L in white wines.

It was observed that yeast production of middle-chain fatty acid esters, and acetates of alcohols formed by reduction of the correspondent

aldehydes derived from decarboxylation of ketoacids, is promoted by conditions such as strong clarification of the must, lower fermentation temperature, higher α-NH_2 nitrogen content, lower oxygen and lipids availability. This phenomenon can be due to the arrest of lipid synthesis at the level of middle-chain fatty acids: if synthesis would continue, the low availability of oxygen in the fermenting must, necessary for oxidation of C-16 and C-18 fatty acids would promote accumulation of long chain fatty acids with problem for the yeast cell membrane. As a consequence, part of the middle chain fatty acids are converted into esters in order to avoid their accumulation in the cell and the consequent citoplasmatic pH alteration.

After ethanol, glycerol is the main product of alcoholic fermentation. This compound forms at the start of fermentation due to low availability of pyruvate decarboxylase and alcohol dehydrogenase enzymes. As a consequence, re-oxidation of $NADH^+$ to NAD^+, necessary to convert glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate to continue glycolysis, is too slow, and re-oxidation of NAD^+ occurs with the transformation of dihydroxyacetone phosphate into glycerol. Not all pyruvic acid molecules undergo decarboxylation into acetaldehyde, ketoacids, succinic acid and 2,3-butanediol form from these non-decarboxylated molecules. Pyruvic acid is also used in the production of acetyl-CoA, an important intermediate involved in the synthesis of most secondary metabolites of fermentation. The glycero-pyruvic fermentation pathway is reported in Figure 1.3. The scheme in Figure 1.4 shows synthesis of succinic acid.

Malolactic fermentation

Malolactic fermentation (MLF) is an important process, nowadays also conducted on an industrial scale, aimed at improving organoleptic characteristics and conferring microbiological stability to quality wines (Davis *et al.*, 1985). The main transformation of the wine occurring in this process operated by lactic bacteria, is decarboxylation of L(−)-malic acid with formation of L(+)-lactic acid (Figure 1.5).

Moreover, changes in the composition of carbonyl compounds in wine occur. Some of these compounds, such as diacetyl and acetoin, confer aromatic complexity to the wine; acetadehyde at high level confers a pungent note to the wine (Di Stefano and Ciolfi, 1982). Glyoxal, methylglyoxal and hydroxypropandial are produced by *Oenococcus oeni* (De Revel and Bertrand, 1993; Guillou *et al.*, 1997; Guillou, 1997),

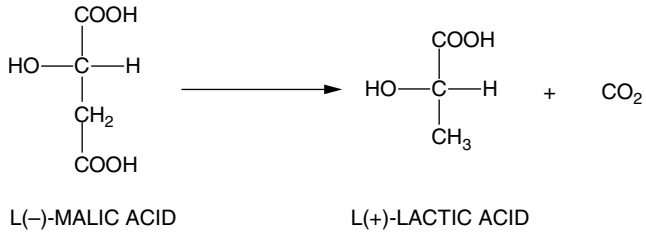

Figure 1.5 Transformation of L(−)-malic acid into L(+)-lactic acid occurring in malolactic fermentation (MLF)

the lactic bacterium responsible for MLF. Aliphatic aldehydes such as hexanal, (*E*)-2-hexenal, (*E*)-2-heptenal, octanal and (*E*)-2-octenal, produced by oxidation of unsaturated fatty acids by grape lipooxigenases, are responsible for herbaceous odours (De Revel and

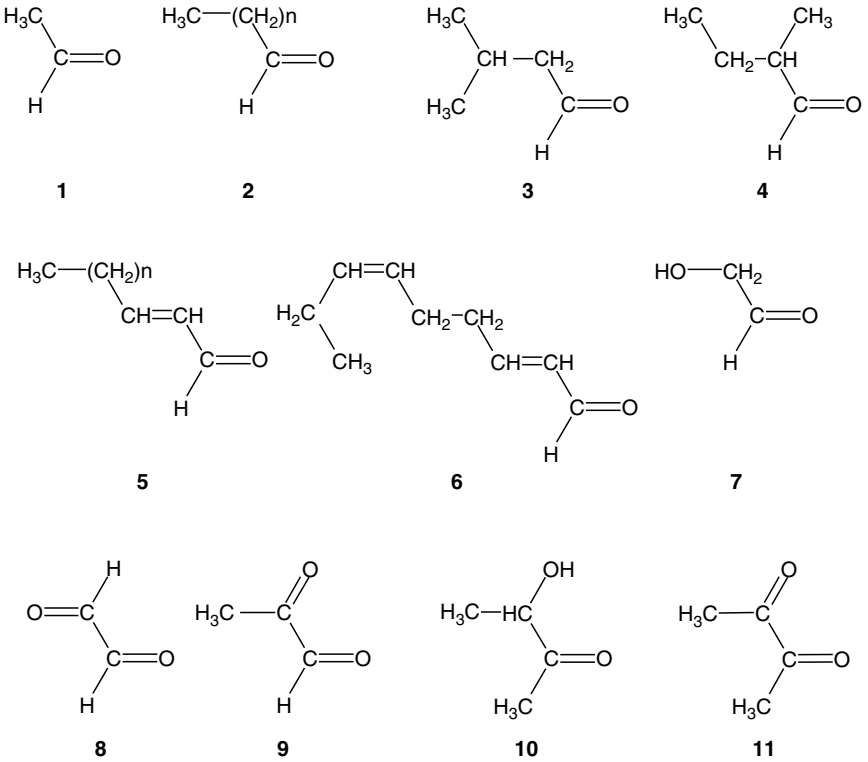

Figure 1.6 Principal carbonyl compounds involved in MLF. (1) acetaldehyde; (2) n = 2 butyraldehyde, n = 4 hexanal, n = 5 heptanal, n = 6 octanal, n = 7 nonanal, n = 8 decanal; (3) isovaleraldehyde; (4) 2-methylbutyraldehyde; (5) n = 2 (E)-2-hexanal, n = 5 (E)-2-nonenal; (6) (E,Z)-2,6-nonadienal; (7) glycolaldehyde; (8) glyoxal; (9) methylglyoxal; (10) acetoin; (11) diacetyl

Bertrand, 1994; Allen, 1995); decanal and (*E*)-2-nonenal, on the other hand, are associated with 'sawdust' or 'plank' odour (Chatonnet and Dubourdieu, 1996; 1998). The principal carbonyl compound formed in MLF is 2,3-butanedione (diacetyl), whose level can improve, or affect, the wine with its butter-like or 'fat' note (Davis *et al*., 1985). Diacetyl and 3-hydroxy-2-butanone (acetoin, the reduced form of diacetyl) are produced by pyruvate metabolism of yeasts and lactic bacteria, and their levels may increase two or three fold with MLF depending on the lactic bacteria strain involved (Davis *et al*., 1985; Martineau and Henick-Kling, 1995; Radler, 1962; Fornachon and Lloyd, 1965; Rankine *et al*., 1969; Mascarenhas, 1984). For diacetyl in wine sensory thresholds ranging from 0.2 mg/L (in *Chardonnay*) to 0.9 mg/L (*Pinot noir*), and 2.8 mg/L (*Cabernet Sauvignon* wine), are reported (Martineau *et al*., 1995).

Structures of the principal carbonyl compounds involved in MLF are reported in Figure 1.6 (Flamini *et al*., 2002a).

1.2. Determination of Grape Ripening Parameters

1.2.1. Sample Preparation

Determination of pH, total acidity and organic acids is usually performed on the must sample prepared by hand pressing of the grape. Due to the fact that in white grape winemaking no contact between must and berry skins occurs, this method of must preparation is more suitable for white rather than for red grapes. Contact with skins induces a partial extraction of cations with a consequent change of pH and total acidity of must with respect to the sample prepared by hand pressing. As a consequence, for preparation of the must of red grapes it is better to homogenize the grapes and recover the must by centrifugation. To clarify the must, a treatment with pectolitic enzyme can be performed.

Clarification of the must in winemaking is made by treatments with silica gel, filtration, centrifugation, or by the use of enzymes. During filtration and centrifugation, oxidation of polyphenols may occur; loss of protective colloids occurring with enzymes can promote precipitation of potassium bitartrate affecting the tartaric acid, pH and total acidity data. For determination of organic acids either in skins or in the

whole berry, the sample is prepared by performing extraction using a mineral acid solution.

For analysis of organic acids and sugars, about 400 grape berries are picked in vineyard as described in section 1.1.1 in order to ensure a well-representative sample. Two hundred berries are weighed and homogenized for a fixed time (1–2 min), the solution is centrifuged at 4000 *g* for 15 min and the must is used for LC analysis.

In general, to preserve musts from the sugars and organic acids degradation due to fermentation, addition of allyl isothiocyanate at a concentration of 50 mg/L in the sample is suitable to preserve the sample for three months, and allyl isothiocyanate or ethyl bromoacetate at 100 mg/L for six months' storage. The use of sodium azide does not prevent degradation of malic and tartaric acids, but at 75 mg/L inhibits fermentation for six months. Pasteurization or octanoic acid addition do not guarantee the preservation of a must sample (Flamini and Dalla Vedova, 2007).

1.2.2. Organic Acids Analysis

Sample preparation for analysis of organic acids in must is performed by solid-phase-extraction (SPE) in order to remove phenolic compounds. 1 mL of must is diluted 1:5 with a H_3PO_4 5×10^{-3} M solution and passed through a C_{18} 300-mg cartridge previously activated by passage of 2 mL methanol followed by 3 mL of H_3PO_4 5×10^{-3} M. The eluate is recovered in a 20-mL volumetric flask, the stationary phase is washed with 2 mL of H_3PO_4 5×10^{-3} M and eluate is collected in the same volumetric flask; finally, the volume is adjusted with the H_3PO_4 solution. HPLC is usually performed by a reverse-phase (RP) C_{18} column. An HPLC chromatogram from analysis of organic acids in a grape must sample is shown in Figure 1.7.

Analysis of organic acids can be also performed by using a polystyrene-divinylbenzene primary amine cation exchange resin (Aminex) column (Schneider *et al*., 1987). The grape must sample is diluted 1:2 with the LC elution solvent (H_2SO_4 0.013 N for musts, 0.026 N for wines), filtered on membrane 0.22 μm and directly injected into the column. Also in this case, to remove phenolic compounds in particular in red grape musts, SPE can be performed as described above by using a C_{18} cartridge previously activated with passage of methanol and H_2SO_4 0.013 N (0.026 N for wines).

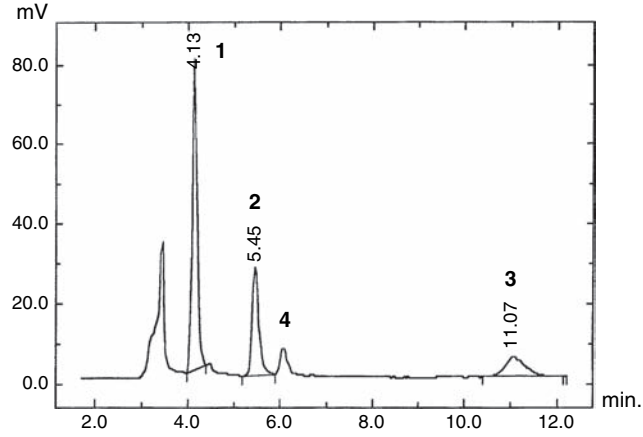

Figure 1.7 HPLC chromatogram of organic acids analysis of *Prosecco* grape must sample. 1. tartaric acid, 2. malic acid, 3. citric acid, 4. shikimic acid. Analytical conditions: Lichrospher 100 RP-18 (250×4 mm, 5 μm) column (Merck, Darmstadt, Germany) at room temperature, detection at wavelength 210 nm, sample volume injected 20 μL; solvent H_3PO_4 5×10^{-3} M with isocratic elution at flow rate 0.6 mL/min

1.2.3. Analysis of Sugars

Analysis of sugars in must is usually performed by using an Aminex column and refractometer as detector. The HPLC chromatogram from a grape must analysis performed in this way is shown in Figure 1.8.

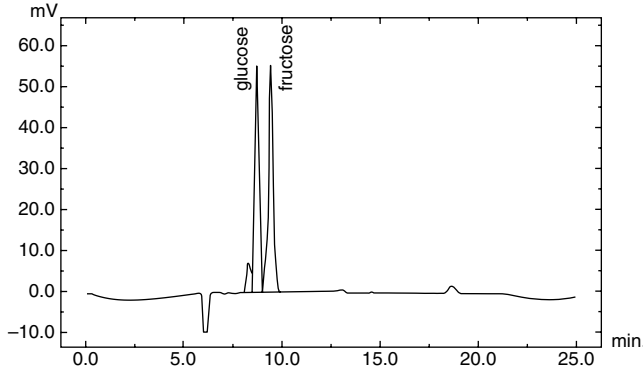

Figure 1.8 Chromatogram relative to HPLC analysis of sugars in a grape must sample. Analytical conditions: column Aminex HPX-87H (300×7.8 mm, 9 μm) (Bio-Rad Laboratories, Richmond, CA) at 60 °C; detector refractometer; sample volume injected 20 μL; solvent H_2SO_4 0.013 N with isocratic elution at flow rate 0.6 mL/min

The HPLC method for analysis of glucose, fructose, sucrose and glycerol in musts and wines proposed by the Organisation Internationale de la Vigne et du Vin (O.I.V.), uses an alkylamine (250 × 4 mm; 5 μm) column (Compendium of International Methods of Wine and Must Analysis OIV, 2006). A volume of 9 mL of must sample is diluted 1:5 with water and passed through a C_{18} cartridge previously activated. The first 3 mL of the eluate are lost, the last 6 mL are collected in a 10-mL volumetric flask. Calibration curves are calculated with a solution of glucose, fructose and glycerol at concentration 10 g/L. Table 1.1 summarizes the analytical conditions used. For glucose and fructose, limits of detection (LOD) of 0.18 and 0.12 g/L, respectively, and limits of quantification (LOQ) of 0.6 and 0.4 g/L respectively, are reported.

Table 1.1 Analytical conditions in the sugars and glycerol analysis by using alkylamine column (*Compendium of International Methods of Wine and Must Analysis OIV*, 2006).

column:	alkylamine (250 × 4 mm; 5 μm)
column temperature:	room
solvent:	acetonitrile/H_2O (80:20 v/v)
sample volume inj.:	20 μL
elution:	isocratic
flow:	1 mL min^{-1}
detector:	refractometer
column retention time (min)	
glycerol	5.0
fructose	8.5
glucose	9.5
sucrose	14.0

1.3. Analysis of Fermentation Parameters of Wine

1.3.1. Alcoholic Fermentation

Organic acids analysis

Preparation of the sample for analysis of organic acids in wine is analogous to the method reported for preparation of the must sample by using SPE C_{18} cartridge (paragraph 1.2.2) but, instead of collecting the eluate of a 1 mL diluted sample in a 20-mL volumetric flask, a 10-mL volumetric flask is used (the final sample is diluted 10-fold). To improve

separation between acetic acid and ethanol peaks in the HPLC chromatogram using a C_{18} column, a second SPE step can be performed in order to isolate organic acids from the matrix. The solution from the first C_{18} SPE cartridge is passed through a second cartridge containing an anionic exchange quaternary-amine resin (e.g. 500 mg). Prior to the passage through the second cartridge, the pH of the solution is adjusted to 7–8 by addition of some drops of a concentrated NaOH solution, and the cartridge is activated by the passage of 2 mL methanol followed by 20 mL of NaF 0.5 M solution and 5 mL water (Schneider *et al*., 1987; Hunter *et al*., 1991; Flamini and Dalla Vedova, 1999). A 2.5 mL volume of sample solution is passed through the anionic exchange resin, then the cartridge is washed with 2 mL of water to remove inorganic compounds and analytes are recovered with 4 mL of HCl 1% (v/v) aqueous solution collecting the eluate in a 5-mL volumetric flask. Finally, the volume is adjusted by H_3PO_4 5×10^{-3} M and the solution is filtered on membrane 0.22 μm. A scheme of sample preparation is shown in Figure 1.9.

Quantitative recoveries of tartaric, malic, citric, citramalic and succinic acids are achieved, and are higher than 90% for acetic and

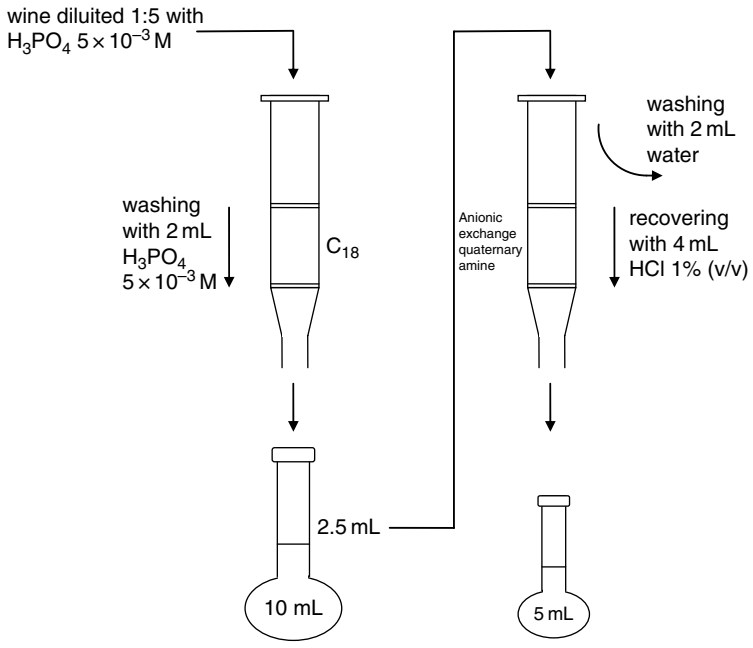

Figure 1.9 Isolation of organic acids from the wine by 2-step solid-phase-extraction

lactic acids; recoveries of pyruvic and shikimic acids are not quantitative. Figure 1.10 shows the HPLC chromatogram recorded in the analysis of a *Cabernet Sauvignon* wine after the 2-step SPE sample preparation.

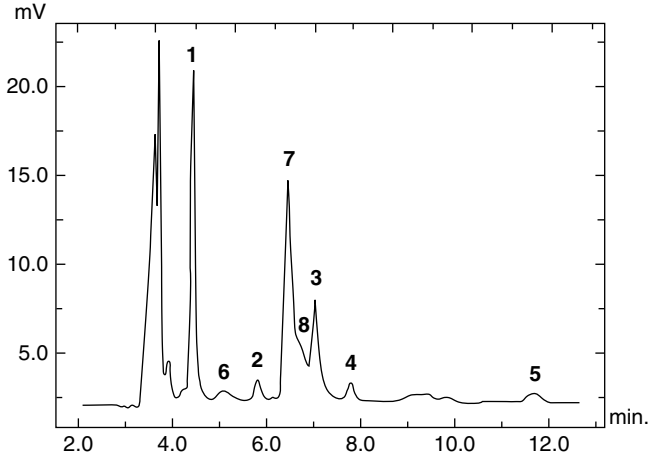

Figure 1.10 HPLC analysis of organic acids in *Cabernet Sauvignon* wine using after sample preparation by C_{18} SPE followed by isolation of organic acids a 500-mg amine-quaternary resin (Figure 1.9). 1. tartaric acid, 2. malic acid, 3. lactic acid, 4. acetic acid, 5. citric acid, 6. pyruvic acid, 7. shikimic acid. Analytical conditions: column C_{18} (250×4 mm, 5 μm) at room temperature, detection at wavelength 210 nm, sample volume injected 20 μL, solvent H_3PO_4 5×10^{-3} M with isocratic elution at flow rate 0.6 mL/min (Flamini and Dalla Vedova, 1999)

By performing HPLC analysis using the Aminex column, the chromatograms shown in Figure 1.11 (standard solution) and 1.12 (wine sample), are recorded.

By using both C_{18} and Aminex HPLC column, succinic, citramalic and fumaric acid can also be determined in the same run of the other organic acids. With C_{18} columns, these compounds exit in the chromatogram after citric acid in the sequence succinic-citramalic-fumaric; in the chromatogram using the Aminex column, succinic acid exits close to shikimic acid, the fumaric acid peak falls between those of lactic acid and acetic acid.

If a manual injection is performed, it may be useful to introduce an internal standard into the sample. For analysis using Aminex, formic acid can be used; this compound exits in the chromatogram between lactic acid and acetic acid. Using a C_{18} column, either maleic acid or acrylic acid

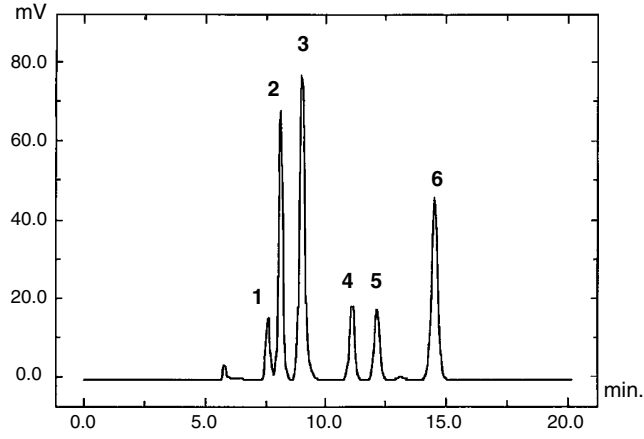

Figure 1.11 Analysis of an organic acids standard solution. 1. citric acid, 2. tartaric acid, 3. malic acid, 4. succinic acid, 5. lactic acid, 6. acetic acid. Analytical conditions: column Aminex HPX-87H (300 × 7.8 mm, 9 μm) at 65 °C (Bio-Rad Laboratories, Richmond, CA); detection at wavelength 210 nm; sample volume injected 10 μL; solvent H_2SO_4 0.026 N with isocratic elution at flow rate 0.8 mL/min

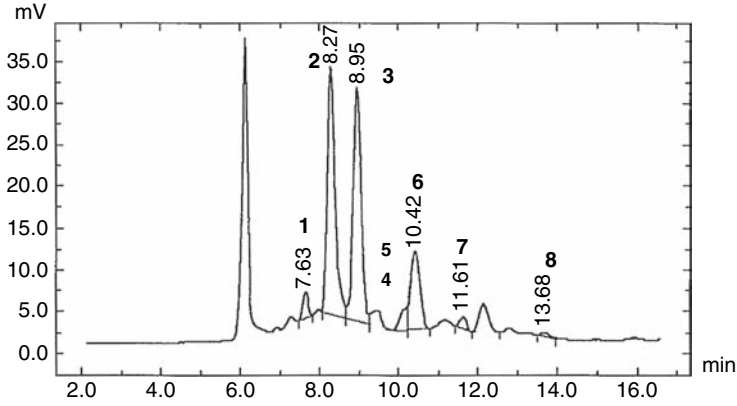

Figure 1.12 Chromatogram relative to analysis of organic acids in a *Pinot blanc* wine sample. 1. citric acid, 2. tartaric acid, 3. malic acid, 4. pyruvic acid, 5. succinic acid, 6. shikimic acid, 7. lactic acid, 8. acetic acid. Analytical conditions: column Aminex HPX-87H (300 × 7.8 mm, 9 μm) at 65 °C (Bio-Rad Laboratories, Richmond, CA); detection at wavelength 210 nm; sample volume injected 10 μL; solvent H_2SO_4 0.02 N/acetonitrile (91.5:8.5 v/v) with isocratic elution at flow rate 0.6 mL/min

can be used: the first elutes from the column after acetic acid (retention time ca. 7.5 min) and it is more nonsuitable for analysis of fermented musts and wines; acrylic acid exits from the column in about 16 min after succinic and fumaric acid and it is more suitable in wine analysis. At wavelength

210 nm maleic acid shows an absorbance 4-fold higher than acrylic acid (Flamini and Dalla Vedova, 1999).

Recently, an increased interest in shikimic acid (SHA) in grapes and wines as genotypic variety tracer, is observed (Fischleitner *et al.*, 2004; Symonds and Cantagrel, 1982; Holbach *et al.*, 2001). Methods for determination of SHA are to perform silylation and GC-FID analysis, or to perform direct injection in a reverse phase HPLC column and detection at 210 nm. The latter method was reported to be less time consuming and more robust, with a detection limit half of that obtained by GC, and a better repeatability of analyses at low concentrations (Versini *et al.*, 2003). Sample preparation for HPLC analysis is performed by diluting 5 mL of wine 1:4 (v/v) with H_3PO_4 0.07% solution and filtration on membrane 0.22 μm. Analysis is performed by two C_{18} (250 × 4 mm, 5 μm) columns in sequence operating at 40 °C and aqueous H_3PO_4 0.07% as eluent at flow rate 0.6 mL/min and detection at 210 nm. SHA exits from the columns after 10.8 min. By using a diode-array-detector (DAD), simultaneous detection at 223 and 243 nm is performed to verify the presence of malonic and ascorbic acid co-eluting with SHA. The latter compound can be eliminated from the sample prior to analysis by treating wine with H_2O_2. In these conditions, the detection limit in standard solution is 0.9 mg/L.

Gas chromatographic analysis of organic acid methyl esters

Another method for determination of the principal organic acids in musts and wines is by synthesis of their methyl (Di Stefano and Bruno, 1983) or silyl esters (De Smet *et al.*, 1981) and GC analysis of derivatives. For the sample preparation, a volume of 0.5 mL wine is transferred in a 50-mL distillation flask and some drops of ammonia 0.1 M solution are added. The solution is evaporated to dryness under vacuum, re-dissolved in 1 mL of methanol and 0.28 mL of HCl concentrate solution is added. One millilitre of a maleic acid 1 g/L methanolic solution is added as internal standard. The reaction is carried out at 55 °C for 4 h. After cooling at room temperature, 5 mL of $NaHCO_3$ 0.5 M aqueous solution is added, and transferred in a separatory funnel. Three consecutive liquid–liquid extractions using 5 mL of dichloromethane each, are performed. The organic phases are combined, the resulting solution is dried over Na_2SO_4 anhydrous and reduced to small volume prior to GC analysis. To improve the reaction yield, HCl can be substituted with a H_2SO_4 concentrate drop. Figure 1.13 shows the GC chromatograms

from analysis of a standard solution, a *Chardonnay* wine and an *Asti* sparkling wine.

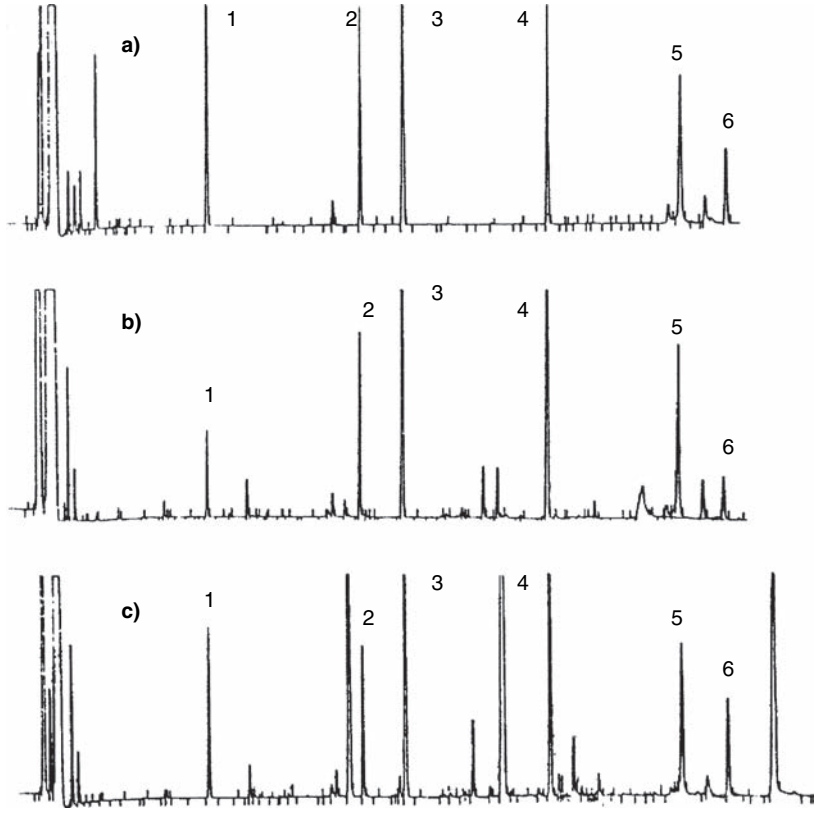

Figure 1.13 GC analysis of organic acid methyl esters in: a) standard solution, b) *Chardonnay* wine, c) *Asti* sparkling wine. 1. methyl lactate, 2. dimethyl succinate, 3. dimethyl maleate (i.s.), 4. dimethyl malate, 5. dimethyl tartrate, 6. trimethyl citrate. Chromatographic conditions: poly(ethylene) glycol fused silica capillary column (30 m × 0, 25 mm; 0.25 μm), injector and detector temperature 250 °C, flame ionization detector. Oven program: 2 min at 50 °C, from 50 to 200 °C at rate 4 °C/ min, 200 °C isotherm for 10 min (Di Stefano and Bruno, 1983)

HPLC analysis of sugars and alcohols

The method proposed by Shneider *et al.* (1987) for analysis of organic acids in wine can be applied to perform the simultaneous determination of organic acids, glucose and fructose, glycerol and ethanol by using the

instrumental configuration showed in figure 1.14 containing two detectors – a spectrophotometer (UV) and a refractometer (RI) – on-line.

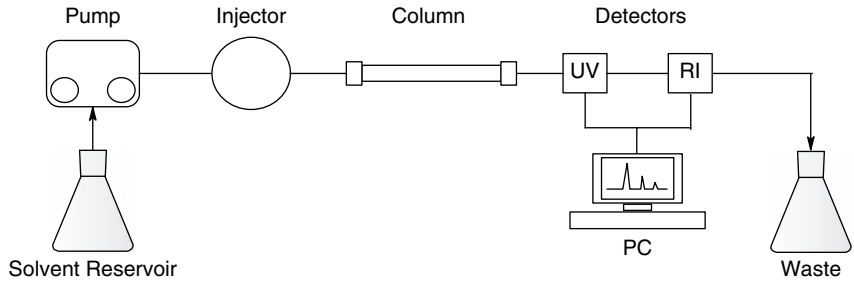

Figure 1.14 Scheme of HPLC system with detectors UV-Vis and RI on-line used for simultaneous determination of organic acids, sugars and alcohols in wine

Depending on the chromatographic conditions used, glucose or fructose co-elute from the column with malic acid and it is necessary to correct the malic acid contribution to the sugar peak area in the RI chromatogram. Due to the lower absorption at wavelength 210 nm of hexoses with respect to malic acid, the peak area measured in the UV chromatogram is, practically speaking, due to malic acid. To perform quantification of glucose, the area of the peak in RI chromatogram formed from overlapping of the two compounds is subtracted from the area value corresponding to half malic acid concentration calculated from the UV chromatogram (Testa, 1991). To avoid the sample dilution resulting from SPE sample preparation using a C_{18} cartridge, phenolic compounds can be removed by treating the wine with carbon 4–8 g/L. However as a consequence of this treatment, a significant loss only of succinic acid was reported (Shneider *et al.*, 1987).

An HPLC-RI chromatogram from analysis of wine sugars and glycerol by using an Aminex column is shown in Figure 1.15. In the same run, organic acids are determined by UV detection (chromatogram in Figure 1.12).

Traditional methods of analysis for determination of principal organic acids, glycerol and sugars in wine are based on enzymatic or colorimetric reactions; ethanol is determined by distillation of wine and density measurement of the distillate. In Table 1.2, data of organic acids, glycerol, glucose, fructose and ethanol determined by HPLC are reported as a percentage of results obtained by the traditional methods.

As may be seen from the data in Table 1.2, HPLC analysis well fit with those obtained by traditional methods in both musts and wines,