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Basic Protocols in Enology and Winemaking

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Basic Protocols in Enology and Winemaking

Edited by

Maurício Bonatto Machado de Castilhos

Department of Agricultural Sciences and Biology, Minas Gerais State University, Frutal, Minas Gerais, Brazil

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Editor
Maurício Bonatto Machado de Castilhos
Department of Agricultural Sciences and Biology
Minas Gerais State University
Frutal, Minas Gerais, Brazil

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Preface

Welcome to the "Basic Protocols in Enology and Winemaking." This book provides valuable information regarding technical and analytical methods applied in wine analysis worldwide. The book presents 14 chapters and each one is dedicated to a punctual wine chemical property. Each chapter provides insights into traditional and advanced methods used for major and minor component wine analysis, the latter quantitated at trace levels. This book aims to facilitate wine analysis through a set of pre-established analyses that promote safe, accurate, and precise results. All methods are based on established literature and can be relied upon for any wine type.

The principal subject of this book is centered on methods using classical apparatus and mechanisms such as titration, distillation, spectrophotometry, and advanced methods applying high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MSn), gas chromatography coupled with mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR). It is a set of easy-to-read and easy-to-understand analyses, making it an ideal tool for analysts who need to perform more accurate and precise analyses for their projects and experiments. The central subject of each chapter is described as follows:

Chapter 1, "Total and Volatile Acidity: Traditional and Advanced Methods," written by Brazilian researchers, begins with a presentation of wine acidity and all the acids responsible for total and volatile acidity. This chapter brings methods using potentiometric titration, distillation, chemical indicators, and simple reagents.

Chapter 2, "Alcohol Content: Traditional and Advanced Methods," written by researchers from Portugal begins with an introduction regarding the alcohols found in wine, highlighting ethanol, which is the most relevant alcohol in the wine matrix. The authors reported methods using ebulliometry, gas chromatography, liquid chromatography, enzymatic assays, and infrared spectroscopy, among others.

Chapter 3, "Total and Reducing Sugars: Traditional and Advanced Methods," written by Brazilian researchers, presents methodologies using refractometry and hydrometer, chemical and enzymatic methods, liquid chromatography (HPLC), and information regarding other methods concerning the identification and quantitation of sugars in wines.

Chapter 4, "Total Phenolic Content: Traditional Methods," written by Brazilian researchers, presents information about the importance of phenolic compounds identification and quantitation in wines since these chemical substances provide health benefits to consumers. The chapter shows the classical spectrophotometric methods and their variations for comprehensive analysis.

Chapter 5, "Color Indexes: Traditional and Advanced Methods," written by researchers from Italy and Chile, contains a brief description of the importance of wine color for quality and sensory appeal, showing the modus operandi to identify and quantitate the wine color indexes using spectrophotometry and the well-known CIELab space methodology.

Chapter 6, "Anthocyanin Identification and Quantitation by High-Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)," written in partnership between researchers from Brazil and Spain, reports the importance of anthocyanins for red wines since these compounds to respond for wine appearance and antioxidant activity. The

chapter brings methods using the spectrophotometry approach and HPLC-MSn methodology with high precision and accuracy for identifying and quantitating these minor compounds that have crucial importance for wine analysis.

Chapter 7, "Flavonol Identification and Quantitation by High-Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)," written by Spanish researchers, provides information regarding the flavonols' chemical structure and their importance for wine color due to their copigmentation effect, also related with sensory properties such as bitterness, astringency and color intensity of young red wines. The authors report methods based on the HPLC-MS^n approach, bringing detailed information concerning the application of this method.

Chapter 8, "Flavan-3-ol (Flavanol) Identification and Quantitation by High-Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)," written by Brazilian researchers, contains information regarding the chemical structure of flavan-3-ols and their contribution to wine antioxidant capacity and sensory properties such as bitterness and astringency. The authors reported detailed information regarding the use of HPLC coupled with mass spectrometry for accurate wine analysis.

Chapter 9, "Hydroxybenzoic and Hydroxycinnamic Acid Derivatives (HCAD) Identification and Quantitation by High-Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)," written by Spanish researchers, provides valuable information regarding the phenolic acids and their contribution to wine chemistry. The authors described, rich in detail, the protocol to perform an HPLC-MS^n analysis for phenolic acids identification and quantitation.

Chapter 10, "Stilbene Identification and Quantitation by High-Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS)," written by researchers from Uruguay, brings information regarding the stilbenes and their high antioxidant activity in wines. The authors informed details of HPLC-MS application for stilbenes identification and quantitation.

Chapter 11, "Analysis of the Free and Bound Fraction of Volatile Compounds in Musts and Wines by GC/MS: Results Interpretation from the Sensory Point of View by OAV Technique," written by Spanish researchers, reports the importance of volatile compounds for wine aroma. They explained that the concentration of volatile compounds needs to be compared with the odor activity values (OAV) to observe which volatile compounds are responsible for wine aroma quality. The method using gas chromatography coupled with mass spectrometry (GC-MS) is the most used for this analysis.

Chapter 12, "Identification of Wine Compounds by Nuclear Magnetic Resonance," written by Brazilian researchers, gives valuable information about the wine components that can be identified and quantitated using the advanced method of nuclear magnetic resonance (NMR). The use of NMR for wine components is recent, and this chapter is extremely useful for analysts who have an interest in using the NMR approach to identify and quantitate all the wine components even those with lower concentrations.

Chapter 13, "Ethanol Suppression on Wine Analysis Using Nuclear Magnetic Resonance (NMR)," written by Brazilian researchers, is a continuation of Chap. 12 since ethanol suppression is mandatory for wine analysis. The ethanol must be suppressed for NMR wine analysis due to its higher concentration which provides a huge peak in the NMR spectra, hindering the identification of the other wine chemical compounds.

Chapter 14, "Methods to Determine Biogenic Amines in Wine by RP-HPLC," written by researchers from Portugal, reports the importance of identifying and quantitating biogenic amines in wines since they can cause beverage safety problems due to their high toxicity for humans. The authors reported an advanced method using RP-HPLC for identifying and quantitating these compounds with precision and accuracy.

The book "Basic Protocols in Enology and Winemaking" is considered an analytical guide for wine researchers and analysts to facilitate the laboratory routine and deliver simple and advanced methods that can provide results with high precision, accuracy, and repeatability.

I hope you appreciate the content of this book. Enjoy the moment and read without moderation!

Cheers!

Frutal, Brazil *Maurício Bonatto Machado de Castilhos*

Contents

Contributors

Aline Alberti • *Department of Food Engineering, State University of Ponta Grossa (UEPG), Ponta Grossa, PR, Brazil*

Fabio C. L. Almeida • *Institute of Medical Biochemistry (IBqM) and National Center for Structural Biology and Bioimaging (CENABIO), Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

Danieli Cristina Alves • *Postgraduate Program in Food, Nutrition and Food Engineering, São Paulo State University, São José do Rio Preto, São Paulo, Brazil*

Eduardo Boido • *Enology and Fermentation Biotechnology, Faculty of Chemistry, University of the Republic, Montevideo, Uruguay*

Edmundo Bordeu • *Department of Fruit Production and Enology, School of Agricultural and Forest Sciences, Pontifical Catholic University of Chile, Santiago, Chile*

Werner F. Brandão • *Multidisciplinary Research Center UFRJ – Xerém in Biology (NUMPEX-Bio), Federal University of Rio de Janeiro, Duque de Caxias, RJ, Brazil*

Natalia Brossard • *Department of Fruit Production and Enology, School of Agricultural and Forest Sciences, Pontifical Catholic University of Chile, Santiago, Chile*

Icaro P. Caruso • *Multiuser Center for Biomolecular Innovation (CMIB) and Department of Physics, Institute of Biosciences, Letters and Exact Sciences (IBILCE), São Paulo State University (UNESP), São José do Rio Preto, SP, Brazil; Institute of Medical Biochemistry (IBqM) and National Center for Structural Biology and Bioimaging (CENABIO), Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

Fernanda Cosme • *CQ-VR, Chemistry Research Centre – Vila Real, Food and Wine Chemistry Lab, School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

Marcel M. L. da Cunha • *Multidisciplinary Research Center UFRJ – Xerém in Biology (NUMPEX-Bio), Federal University of Rio de Janeiro, Duque de Caxias, RJ, Brazil*

Romina Curbelo • *Organic Analytical Area, Faculty of Chemistry, University of the Republic, Montevideo, Uruguay*

Danilo Davyt • *Organic Analytical Area, Faculty of Chemistry, University of the Republic, Montevideo, Uruguay*

Gisele C. de Amorim • *Multidisciplinary Research Center UFRJ – Xerém in Biology (NUMPEX-Bio), Federal University of Rio de Janeiro, Duque de Caxias, RJ, Brazil; Institute of Medical Biochemistry (IBqM) and National Center for Structural Biology and Bioimaging (CENABIO), Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*

Maurício Bonatto Machado de Castilhos • *Department of Agricultural Sciences and Biology, Minas Gerais State University, Frutal, Minas Gerais, Brazil; Food Science and Technology Department, Institute of Biosciences, Humanities and Exact Sciences, São Paulo State University, São José do Rio Preto, São Paulo, Brazil*

Letícia Barbosa de Melo • *Postgraduate Program in Environmental Sciences, Minas Gerais State University, Frutal, Minas Gerais, Brazil*

Celso Guarani Ruiz de Oliveira • *Brazilian Agricultural Research Corporation, Embrapa Grape and Wine, Bento Gonçalves, RS, Brazil*

Juliane Barreto de Oliveira • *Minas Gerais Agricultural Research Corporation – EPAMIG, Belo Horizonte, Minas Gerais, Brazil*

Matheus Felipe de Oliveira Silva • *Postgraduate Program in Environmental Sciences, Minas Gerais State University, Frutal, Minas Gerais, Brazil*
Eduardo Dellacassa • *Flavor Biotechnology Laboratory, Faculty of Chemistry, University of the Republic, Montevideo, Uruguay*
Laura Fariña • *Enology and Fermentation Biotechnology, Faculty of Chemistry, University of the Republic, Montevideo, Uruguay*
Leonor C. Ferreira • *CQ-VR, Chemistry Research Centre – Vila Real, Food and Wine Chemistry Lab, School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*
Luís Filipe-Ribeiro • *CQ-VR, Chemistry Research Centre – Vila Real, Food and Wine Chemistry Lab, School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*
Esteban García-Romero • *Castilla-La Mancha Grapevine and Wine Institute, Regional Institute for Agri-Food and Forestry Research and Development (IRIAF), Tomelloso, Ciudad Real, Spain*
Sergio Gómez-Alonso • *Regional Institute for Applied Scientific Research (IRICA), University of Castilla-La Mancha, Ciudad Real, Spain; Faculty of Chemical Sciences & Technologies, University of Castilla-La Mancha, Ciudad Real, Spain*
Pedro Miguel Izquierdo-Cañas • *Castilla-La Mancha Grapevine and Wine Institute, Regional Institute for Agri-Food and Forestry Research and Development (IRIAF), Tomelloso, Ciudad Real, Spain; Science and Technology Park of Castilla-La Mancha, Albacete, Spain*
Isabella O. Lourenço • *Multiuser Center for Biomolecular Innovation (CMIB) and Department of Physics, Institute of Biosciences, Letters and Exact Sciences (IBILCE), São Paulo State University (UNESP), São José do Rio Preto, SP, Brazil*
Maria Eduarda da Mata Martins • *Postgraduate Program in Food, Nutrition and Food Engineering, São Paulo State University, São José do Rio Preto, São Paulo, Brazil*
Juliana Milheiro • *CQ-VR, Chemistry Research Centre – Vila Real, Food and Wine Chemistry Lab, School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*
Carolina Pavez Moreno • *Department of Agricultural and Food Sciences, University of Bologna, Cesena, Italy*
Alessandro Nogueira • *Department of Food Engineering, State University of Ponta Grossa (UEPG), Ponta Grossa, PR, Brazil*
Fernando M. Nunes • *CQ-VR, Chemistry Research Centre – Vila Real, Food and Wine Chemistry Lab, School of Life Sciences and Environment, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*
Tania Paniagua-Martínez • *Regional Institute for Applied Scientific Research (IRICA), University of Castilla-La Mancha, Ciudad Real, Spain; Faculty of Chemical Sciences & Technologies, University of Castilla-La Mancha, Ciudad Real, Spain*
Giuliano Elias Pereira • *Brazilian Agricultural Research Corporation, Embrapa Grape and Wine, Bento Gonçalves, RS, Brazil*
José Pérez-Navarro • *Regional Institute for Applied Scientific Research (IRICA), University of Castilla-La Mancha, Ciudad Real, Spain; Higher Technical School of Agronomic Engineering, University of Castilla-La Mancha, Ciudad Real, Spain*
Leda Atílio Pita • *Postgraduate Program in Food, Nutrition and Food Engineering, São Paulo State University, São José do Rio Preto, São Paulo, Brazil*

Luisina Rodríguez • *Organic Analytical Area, Faculty of Chemistry, University of the Republic, Montevideo, Uruguay*
Lia Lucia Sabino • *Postgraduate Program in Food, Nutrition and Food Engineering, São Paulo State University, São José do Rio Preto, São Paulo, Brazil*
Gilson C. Santos Júnior • *Department of Genetics, Rio de Janeiro State University, Rio de Janeiro, RJ, Brazil*
Acácio Antonio Ferreira Zielinski • *Department of Chemical Engineering and Food Engineering, Federal University of Santa Catarina (UFSC), Florianópolis, SC, Brazil*

Chapter 1

Total and Volatile Acidity: Traditional and Advanced Methods

Lia Lucia Sabino and Maurício Bonatto Machado de Castilhos

Abstract

One of the most relevant wine sensory attributes is acidity, which is also considered a parameter of the quality and microbiological stability of the beverage. The acidity is represented by different organic acids synthesized directly from the grape or resulted from the alcoholic fermentation process. The acidity assessment at various stages of winemaking is crucial to ensure a final product with high quality. Different procedures ranging from conventional titrations to alternative methods are applied for the acid profile determination. This chapter explains the principal methods to perform the total and volatile acidity in wines, describing the methodology and discussing their advantages.

Key words Total acidity, Volatile acidity, Organic acids, Titration, pH, HPLC, Microbial stability

1 Introduction

Considering the incessant search for obtaining the maximum quality results in wine production throughout the history of winemaking, innovative practices and technologies have been used [1], a premise used primarily regarding the determination of acidity since it is an attribute that plays a crucial role in the area of enology. Wine acidity is considered a parameter that affects the sensory properties and quality of wines, helping analyze the microbiological stability through the volatile acidity parameter [2].

Acidity is considered one of the most essential sensory attributes in wine, the sensation caused by this component derives primarily from the mixture of organic acids transferred from the grape pulp to the wine during the winemaking process [3], the acids formed during and after the alcoholic fermentation [4], and the acids resulting from the malolactic fermentation process that can occur spontaneously with the participation of lactic acid bacteria (LAB), naturally present in grapes, or be induced by commercial starter cultures [5]. The LAB degrades malic acid into lactic acid,

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metabolizing other substances, such as sugars, citric acid, and amino acids, into substances that may be undesirable, such as acetic acid [6].

All acids present in wines are classified into two categories: the fixed acidity, represented by tartaric, malic, lactic, succinic, and citric acids, with tartaric and malic acids representing about 90% of the wines' fixed acidity; and the volatile acidity represented by compounds such as acetic, formic, butyric, propionic, and fatty acids with chains longer than 12 carbons [7]. The total acidity is determined by the sum of the fixed and volatile acidity [8].

Tartaric acid, representative of fixed acidity, plays a crucial role in the acidity stability and the beverage's sensory quality, especially in the perception of astringency [3]. Acetic acid, the principal representative of the volatile acidity, works as an indicator of wine microbial stability and sanity, that is, high volatile acidity values are a wine microbial spoilage indicative, primarily from acetic acid bacteria, causing the off-flavor of acetic acid in wine. The production of acetic acid is also linked to the contamination of the fruit or must by acetic bacteria, resulting from the oxidation of the wine [8], and consequently its production is carefully monitored and controlled throughout the winemaking process [9].

The total and volatile acidity in wine can be determined using different procedures ranging from conventional titrations to alternative and advanced methods (Table 1).

Table 1
Conventional and alternative methods for the total and volatile acidity determination

	Conventional Methods	Alternative Methods
Total acidity	Titration using standard alkaline solution with bromothymol blue indicator (OIV-MA-AS313-01) [13]	FTIR spectroscopy—Interferometer WineScan FT 120 [10]
	Titration using standard alkaline solution and pH meter (IAL 235/IV) [14]	Sequential injection analysis (SIA) with spectrophotometric detection [4]
		High-performed liquid chromatography (HPLC) [11]
Volatile acidity	Titration of wine distillate obtained from distillation process with alkaline solution (OIV-MA-AS313-02) [13]	Sequential injection analysis (SIA) with spectrophotometric detection [4]
	Titration of wine distillate obtained from steam distillation process with alkaline solution (IAL 236/IV) [14]	FTIR spectroscopy—Interferometer WineScan FT 120 [12]

2 Methods

2.1 Total Acidity by International Organization of Vine and Wine (OIV-MA-AS313-01) [13]

The wine total acidity is the sum of its titratable acidities when it is titrated to pH 7.0 using a standard alkaline solution. Carbon dioxide is disregarded in the total acidity analysis. The principle of this method is based on a potentiometric titration or titration with bromothymol blue as chemical indicator and comparison with an end-point color standard.

2.1.1 Chemicals

1. Buffer solution pH 7,0 (*see* **Note 1**).
2. Sodium hydroxide solution, NaOH, 0.1 mol/L.
3. Bromothymol blue indicator solution, 4 g/L (*see* **Note 2**).

2.1.2 Apparatus

1. Water vacuum pump.
2. Vacuum flask 500 mL.
3. Potentiometer with scale graduated in pH values and electrodes. The glass electrode must be kept in distilled water. The calomel/saturated potassium chloride electrode must be kept in a saturated potassium chloride solution.
4. Beakers.

2.1.3 Procedure

Sample Preparation

1. Elimination of the carbon dioxide (if existent): Place approximately 50 mL of wine in a vacuum flask and apply vacuum to the flask using a water pump for 1–2 min, while shaking continuously. Other CO_2 elimination systems may be used if the CO_2 elimination is guaranteed.

Potentiometric Titration

1. Calibration of the pH meter: The pH meter is calibrated for use at 20 °C, according to the manufacturer's instructions, with the pH 7.0 buffer solution at 20 °C.

Measurement: Into a beaker, introduce a volume of the sample, prepared as described in Subheading 2.1.3.1, equal to 10 mL in the case of wine and 50 mL in the case of rectified concentrated grape must. Add about 10 mL of distilled water and then add sodium hydroxide solution, 0.1 mol/L, from a burette until the pH is equal to 7.0 at 20 °C. The sodium hydroxide must be added slowly and the solution stirred continuously. Let n mL be the volume of sodium hydroxide, 0.1 mol/L, added.

Titration with Indicator (Bromothymol Blue)

1. Preliminary test for end-point color determination: Into a beaker, place 25 mL of boiled distilled water, 1 mL of bromothymol blue solution, and a volume prepared as in Subheading 2.1.3.1 equal to 10 mL in the case of wine, and 50 mL in the case of rectified grape concentrated must. Add sodium hydroxide solution, 0.1 mol/L, until the color changes to blue-green. Then add 5 mL of the pH 7.0 buffer solution.

2. Measurement: Into a beaker place 30 mL of boiled distilled water, 1 mL of bromothymol blue solution, and a volume of the sample prepared as described in Subheading 2.1.3.1to 10 mL in the case of wine and 50 mL in the case of rectified grape concentrated must. Add sodium hydroxide solution, 0.1 mol/L, until the same color is obtained as in the preliminary test above. Let *n* mL be the volume of sodium hydroxide solution, 0.1 mol/L, added.

2.1.4 Calculation

The total acidity expressed in milliequivalents per liter is given by:

1. A = 10 **n**. Data expressed with one decimal place.
2. The total acidity expressed in grams of tartaric acid per liter is given by: A' = 0.075 × A. Data expressed with two decimal places.
3. The total acidity expressed in grams of sulfuric acid per liter is given by: A' = 0.049 × A. Data expressed with two decimal places.

2.2 Total Acidity by Adolfo Lutz Institute (IAL 235/IV) [14]

This method is based on the neutralization of acids using titration with standardized alkali solution, using a phenolphthalein indicator for white and rosé wines or with the pH meter for red wines.

2.2.1 Chemicals

1. Sodium hydroxide solution 0.1 N.
2. Phenolphthalein solution.

2.2.2 Apparatus

1. pH meter.
2. Magnetic shaker.
3. Magnetic stirring bar.
4. Volumetric pipette 10 mL.
5. Erlenmeyer flask 250 mL.
6. Beaker 250 mL.
7. Burette 25 mL.
8. Graduated pipette 1 mL.

2.2.3 Procedure

With the graduated pipette, take 10 mL of the decarbonated wine into a 250 mL Erlenmeyer flask containing 100 mL of water. Add 0.5 mL of phenolphthalein and titrate with standardized sodium hydroxide solution until persistent pink coloration (for white and rosé wines) or transfer the sample to a beaker and titrate it to the turning point (pH 8.2–8.4) using a pH meter (for red wines).

2.2.4 Calculation

The total acidity expressed in milliequivalents per liter is given by:

$$\text{Total acidity}\left(\frac{\text{mEq}}{\text{L}}\right) = \frac{n.f.N.1000}{V}$$

n: volume in mL of sodium hydroxide solution spent in the titration.

f: correction factor (standardization) of the sodium hydroxide solution.

N: concentration of the sodium hydroxide solution

V: sample volume

2.3 Volatile Acidity by International Organization of Vine and Wine (OIV-MA-AS313-02) [13]

The volatile acidity is determined by the volatile acids of the acetic series present in wine in the free state and combined as salts. Carbon dioxide is first removed from the wine. Volatile acids are separated from the wine by steam distillation and titrated using standard sodium hydroxide. The acidity of free and combined sulfur dioxide distilled under these conditions should be subtracted from the acidity of the distillate. The acidity of any sorbic acid, which may have been added to the wine, must also be subtracted (*see* **Note 3**).

2.3.1 Chemicals

1. Tartaric acid, crystalline.
2. Sodium hydroxide solution 0.1 M.
3. Phenolphthalein solution 1% in neutral alcohol 96% (m/v).
4. Hydrochloric acid (U20 = 1.18–1.19 g/mL) diluted 1/4 with distilled water.
5. Iodine solution 0.005 M.
6. Potassium iodide crystalline.
7. Starch solution 5 g/L (*see* **Note 4**).
8. Saturated solution of sodium tetraborate, Na2B4O7.10H2O about 55 g/L at 20 °C.
9. Acetic acid 0.1 M.
10. Lactic acid solution 0.1 M (*see* **Note 5**).

2.3.2 Apparatus

1. Steam distillation apparatus consisting of: a steam generator (the steam must be free of carbon dioxide), a flask with steam pipe, a distillation column, a condenser (*see* **Note 6**).
2. Water aspirator vacuum pump.
3. Vacuum flask.

2.3.3 Procedure

1. Eliminate the sample carbon dioxide placing about 50 mL of wine in a vacuum flask.
2. Apply vacuum to the flask with the water pump for 1–2 min while shaking continuously. Other CO_2 elimination systems may be used if the CO_2 elimination is guaranteed.

3. Place 20 mL of wine, free from carbon dioxide, into the flask. Add about 0.5 g of tartaric acid. Collect at least 250 mL of the distillate.
4. Titrate with the sodium hydroxide solution using two drops of phenolphthalein as indicator. Let n mL be the volume of sodium hydroxide used.
5. Add four drops of the dilute hydrochloric acid, 2 mL starch solution and a few crystals of potassium iodide.
6. Titrate the free sulfur dioxide with the iodine solution 0.005 M.
7. Let n^* mL be the volume used.
8. Add the saturated sodium tetraborate solution until the pink coloration reappears.
9. Titrate the combined sulfur dioxide with the iodine solution 0.005 M. Let n^{**} mL be the volume used.

2.3.4 Calculation

The volatile acidity, expressed in milliequivalents per liter, is given by (with one decimal place):

$$5\ (n - 0.1\ n^* - 0.05\ n^{**})$$

The volatile acidity, expressed in grams of sulfuric acid per liter, is given by (with two decimal places):

$$0.245\ (n - 0.1\ n^* - 0.05\ n^{**})$$

The volatile acidity, expressed in grams of acetic acid per liter, is given by (with two decimal places):

$$0.300\ (n - 0.1\ n^* - 0.05\ n^{**})$$

2.4 Volatile Acidity by Adolfo Lutz Institute (IAL 236/IV) [14]

This method determines the volatile titratable acidity of wines and other fermented beverages by volumetry after steam distillation.

2.4.1 Chemicals

The chemicals used in this method are the same as in Subheading 2.2.

2.4.2 Apparatus

1. Electric hotplate.
2. 10 mL volumetric pipette.
3. Cazenave-Ferré distillation apparatus or similar assembly.
4. Steam generator.
5. Erlenmeyer flasks 250 and 500 mL.
6. Liebig's or serpentine condenser.
7. Burette 10 mL.
8. Pipette 1 mL.

2.4.3 Procedure

1. Transfer, with a volumetric pipette, 10 mL of the sample into the bubbler and 250 mL of CO_2-free water into the Cazenave-Ferré steam generator apparatus or transfer the sample to a similar steam distillation set.
2. Connect the condenser.
3. Heat up the Cazenave-Ferré apparatus on an electric plate and bring to a boil with the steam tap open to eliminate air from the condenser and possibly carbon dioxide from the distilled water.
4. Then close the stopcock, so that the water vapor bubbles up into the sample dragging the volatile acids.
5. Collect at least 100 mL of the distillate in a 250 mL Erlenmeyer flask containing 20 mL of distilled water.
6. Add 1 mL of phenolphthalein indicator solution.
7. Quickly titrate with sodium hydroxide solution until the pink coloration persists for 30 s.

2.4.4 Calculation

The calculation of volatile acidity follows the same protocol as in Subheading 2.2.

2.5 Malic and Tartaric Acids Using High-Performance Liquid Chromatography (HPLC) [11]

Tartaric and malic acids are essential wine constituents. These two acids come from the grape, and their levels enhance during ripening, alcoholic fermentation, and malolactic fermentation, when it occurs.

2.5.1 Chemicals

1. Tartaric acid solution L(+) 1 g/L prepared with ultrapure water.
2. Malic acid solution L(−) at 1 g/L prepared with ultrapure water.
3. Phosphoric acid solution (pH 2.5) prepared by diluting 1.2 mL of phosphoric acid in a 1 L flask with ultrapure water.

2.5.2 Apparatus

1. Liquid chromatograph equipped with a Rheodyne 20 μL Rheodyne injector, operating under isocratic condition.
2. Diode-array detector at 212 nM wavelength.
3. Varian® MCH-NCAP-5 column, 15 cm long by 4.6 mM internal diameter.
4. Microsyringe 100 μL.
5. Volumetric flask 100 mL.
6. Volumetric pipettes 5 mL and 10 mL.
7. Membrane filtration equipment.
8. Cellulose filter membrane 25 μM.

2.5.3 Analytical Conditions

1. Mobile phase: solution of ultrapure water and phosphoric acid (pH 2.5) in the ratio 98.8:1.2 v/v at a flow rate of 0.9 mL min^{-1}.
2. Temperature: ambient.
3. Pressure: 100 atmospheres.
4. Injection volume: 20 μL.
5. UV detector: 212 nM.
6. Sensibility: 0.5 O.D.

2.5.4 Procedure

1. Dilute the wine sample to 10% with ultrapure water.
2. Homogenize and filter using a cellulose membrane with a pore size of 25 μM.
3. Inject the sample into the liquid chromatograph.
4. The same procedure is carried out with the standard solutions of tartaric and malic acids.
5. The separation of the tartaric and malic acid peaks is indicated in the chromatogram in Fig. 1.

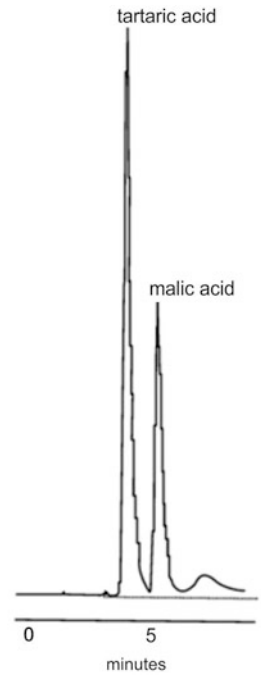

Fig. 1 Tartaric and malic acid peaks using liquid chromatography

3 Conclusion

The wine acid profile is determined by numerous factors depending on climatic conditions during vine management to the technologies applied during the production and maturation process. Due to the importance of total and volatile acidity on several wine features, they are directly related to wine microbiological and chemical stability with a consequent impact on the sensory attributes. Hence, acidity evaluation at various stages of the winemaking process, whether by conventional or alternative methods, becomes indispensable to ensure a high-quality final product.

4 Notes

1. Potassium di-hydrogen phosphate, KH_2PO_4 (107.3 g), sodium hydroxide solution, NaOH, 1 mol/L (500 mL), and water to 1000 mL. Alternatively, commercial buffer solutions are available.
2. Bromothymol blue (4 g), neutral ethanol 96% (v/v) (200 mL), dissolve and add water free of CO_2 (200 mL), sodium hydroxide solution (1 mol/L) enough to produce blue green color at pH 7.0 (7.5 mL) and water to 1000 mL.
3. Part of the salicylic acid used in some countries to stabilize the wines before analysis is present in the distillate. This must be determined and subtracted from the acidity.
4. Mix 5 g of starch with 500 mL of water, stirring continuously and boil for 10 min. Add 200 g sodium chloride and when cool, make up to one liter (1 L).
5. 100 mL of lactic acid is diluted in 400 mL of water. This solution is heated in an evaporating dish over a boiling water bath for 4 h, topping up the volume occasionally with distilled water. After cooling, make up to a liter (1 L). Titrate the lactic acid in 10 mL of this solution with 1 M sodium hydroxide solution. Adjust the solution to 1 M lactic acid (90 g/L).
6. This equipment must pass the following three tests:
   (a) Place 20 mL of boiled water in the flask. Collect 250 mL of the distillate and add to it 0.1 mL sodium hydroxide solution (0.1 M) and two drops of phenolphthalein solution. The pink coloration must be stable for at least 10 s (i.e., steam to be free of carbon dioxide).

(b) Place 20 mL acetic acid solution (0.1 M) in the flask. Collect 250 mL of the distillate. Titrate with the sodium hydroxide solution (0.1 M): the volume of the titer must be at least 19.9 mL (i.e., at least 99.5% of the acetic acid entrained with the steam).

(c) Place 20 mL lactic acid solution (1 M) in the flask. Collect 250 mL of the distillate and titrate the acid with the sodium hydroxide solution (0.1 M). The volume of sodium hydroxide solution added must be less than or equal to 1.0 mL (i.e., not more than 0.5% of lactic acid is distilled).

References

1. Pretorius IS (2020) Tasting the terroir of wine yeast innovation. FEMS Yeast Res 20(1):1–22
2. Vahj K, Kahlert H, Von Mühlen L, Albrecht A, Meyer G, Behnert J (2013) Determination of the titratable acidity and the pH of wine based on potentiometric flow injection analysis. Talanta 111:134–139
3. Picariello L, Rinaldi A, Martino F, Petracca F, Moio L, Gambuti A (2019) Modification of the organic acid profile of grapes due to climate changes alters the stability of red wine phenolics during controlled oxidation. Vitis 58(5): 127–133
4. Vidigal SSMP, Rangel AOSS (2017) A flow-based platform for measuring the acidity parameters in wine. Talanta 168:313–319
5. Jeromel A, Kovačević Ganić K, Mihaljević Žulj M, Maslek M, Puhelek I, Jagatić Korenika AM (2018) Biogenic amine production during spontaneous and inoculated MLF of Zweigelt Wines. Agric Cons Sci 83(3):239–242
6. Soufleros EH, Bouloumpasi E, Zotou A, Loukou Z (2007) Determination of biogenic amines in Greek wines by HPLC and ultraviolet detection after dansylation and examination of factors affecting their presence and concentration. Food Chem 101(2):704–716
7. Barnabé D, Venturini Filho WG, Bolini H (2007) Quantitative descriptive analysis of wines produced from Niágara Rosada and Bordô grapes. Braz J Food Technol 10(2): 122–129
8. Jackson RS (2020) Wine science: principles and applications. Academic Press, San Diego
9. Vilela-Moura A, Schuller D, Falco V, Mendes-Faia A, Côrte-Real M (2010) Effect of fermentation conditions and micro-oxygenation on the reduction of volatile acidity by commercial *S. cerevisiae* strains and their impact on the aromatic profile of wines. Int J Food Microbiol 141:165–172
10. Chen H, Liu Y, Chen J, Fu X, Suo R, Chitrakar B, Wang J (2022) Effects of spontaneous fermentation on microbial succession and its correlation with volatile compounds during fermentation of Petit Verdot wine. LWT 168:113890
11. Rizzon LA (2010) Methods for wine analysis. Embrapa Informação Tecnológica, Brasília
12. Ntuli RG, Saltman Y, Ponangi R, Jeffery DW, Bindon K, Wilkinson KL (2023) Impact of skin contact time, oak and tannin addition on the chemical composition, color stability and sensory profile of Merlot wines made from flash d'etente treatment. Food Chem 405:134849
13. International Organisation of Vine and Wine (OIV) (2022) Compendium of international methods of wine and must analysis. Paris, France
14. Adolfo Lutz Institute (IAL) (2008) Physicochemical methods for food analysis. Adolfo Lutz Institute, São Paulo

Chapter 2

Alcohol Content: Traditional and Advanced Methods

Fernanda Cosme, Luís Filipe-Ribeiro, and Fernando M. Nunes

Abstract

Ethanol is the major compound produced during wine alcoholic fermentation from grape juice sugars (glucose and fructose). Due to its toxicity, legal limits, and taxes applied in many countries, the alcohol content is one of the most relevant parameters, that is controlled in wine production all around the world. This chapter clearly explains the principal methods used to perform ethanol analysis in the enology field, describing the methodologies, protocols, and discussing their advantages and disadvantages.

Key words Ethyl alcohol, Ebulliometry, Distillation, Hydrometry, Enzymatic method, Chromatographic methods, FTIR

1 Introduction

Ethanol is the major alcohol in wine, being also present small quantities of other higher alcohols (fusel alcohols) such as propanol, butanol, and glycerol [1]. Ethanol (ethyl alcohol) is the major product in wine produced by the alcoholic fermentation of sugars by yeast, and it can be indicated in terms of percent by volume (% vol), percent by weight (% wt), grams per 100 mL or density [1]. In alcoholic beverages, such as wine, the formal expression of alcohol concentration is alcoholic strength by volume, defined as the number of liters of ethanol contained in 100 L of wine, % vol., being measured at a temperature of 20 °C [2]. In wine, the normal alcohol levels range from 7% to 24%, depending on its classification [1]. Usually, it is a parameter of mandatory labeling in the different types of wines produced all around the world.

Analytical methods to determine the alcohol content need to be precise, accurate, and not expensive since it is a routinely measured parameter. There are several methods to determine the alcoholic content in wines: by chemical oxidation (dichromate oxidation), measuring the wine boiling point (ebulliometry), and by distillation followed by the measurement of the alcohol content

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by densitometry, pycnometry, hydrometry, refractometry, by enzymatic methods, or by chromatographic methods such as high-performance liquid chromatography (HPLC) and gas chromatography (GC), and by spectroscopic methods such as near-infrared (NIR) or mid-infrared (MIR) spectroscopy, and nuclear magnetic resonance (NMR) [3]. Each of the methods has its own advantages and disadvantages. For example, using GC, ethanol can be measured without interference; however, when using ebulliometry, the ethanol content also influences the measured value. On the other hand, NIR determines the light absorbed by a wine sample in the near-infrared range [1]. The NIR method is also fast, accurate, and user-friendly for wine alcohol determination. The accuracy of NIR determinations is ±0.01% (v/v) [1]. Pycnometry is an Association of Official Analytical Chemists (AOAC)-approved method for analyzing the ethanol of wines by density [4], and the International Organization of Vine and Wine (OIV) official method for ethanol analysis consists of the distillation of the wine alkalinized by a suspension of calcium hydroxide, followed by determination of the alcoholic strength of the distillate by pycnometry, electronic densimetry, aerometry, or refractometry [2].

The ethanol analysis by ebulliometry is not an approved AOAC or OIV method, however, this method is still used frequently. According to Son et al. [5], in small winery laboratories, the most inexpensive and suitable methods are the boiling point method (ebulliometry) or the distillation method followed by density measurement. Nevertheless, the boiling point method often lacks accuracy, whereas the distillation method is problematic when multiple samples need to be analyzed. Therefore, as measurement technology develops, ebulliometry will most likely become outdated, substituted with faster and more accurate methods that will also be cost-effective for small wineries. So, as diverse methods are available to determine the wine alcohol content, it is essential to know how the methods differ for understanding the differences among the results obtained [1, 6]. In the wine industry, these aspects are relevant as the winemakers need, for example, to monitor ethanol during the alcoholic fermentation process and fortified wine production.

2 Wine Alcohol Determinations

2.1 Alcohol Determination by Ebulliometry

Ebulliometry is the most usual method for determining the alcohol content in wine due to its simplicity [1, 6]. The vapor pressure of ethanol in a solution differs according to its content. As the content of alcohol in the wine rises, the boiling point decreases and the boiling point is relative to the boiling point of pure water. Therefore, the water and wine boiling points are determined [6].

Fig. 1 Ebulliometer apparatus

Ebulliometers generally measure the boiling point of wine using the boiling point of water as a reference. As the boiling point changes with atmospheric pressure and the atmospheric pressure in the laboratory is not always at one-atmosphere pressure, regular calibrations are required (at least twice daily and more on days with unstable weather). As the ebulliometric method uses a sliding scale that lets one adjust for the real boiling point of pure water, the water boiling point sets the zero, and the wine boiling point determines the alcohol content [1, 6]. This determination is performed in an ebulliometer, an apparatus that heats the wine and measures the temperature at which the ethanol/water mixture begins to boil (a chilled condenser avoids the loss of the water/ethanol vapor) (Fig. 1).

Several interferences can occur in ebulliometry, essentially associated with non-volatile compounds that affect wine extract and its volatility, such as sugars. The presence of reducing sugars in the wine sample impacts the boiling point, and wine samples with >2% reducing sugars must be diluted to less than 2% before the analysis [7]. Higher alcohols concentrations decrease the boiling point of the mixture. The method is accurate until 16% (v/v) alcohol content. Therefore, also wine with alcohol content >16% (v/v) needs to be diluted [1].

It is a simple method regardless of its limited analytical quality, and it is considered the less accurate of all enumerated methods [1, 6]. The accuracy is generally ±0.5% (v/v), while some modern versions state a value of ±0.15% (v/v). Some modern versions use a digital or an electronic thermometer to enhance precision.

In the ebulliometry method, it is also relevant to regularly verify the sensitivity and stability of the thermometer reading during the wine analysis and to verify if the water refrigeration is open and working well. It is also important to regularly verify the boiling point, specifically if the atmospheric pressure is changing during the day, affecting the water boiling point and increasing the wine analysis errors. This method presents a usual deviation in dry wines of about ±0.2% (v/v) [6].

2.1.1 Materials

Ebulliometer.

Distilled water.

2.1.2 Method

Determination of the Water Boiling Point

1. Measure nearly 30 mL of distilled water into the boiling chamber.
2. Insert the thermometer into the boiling chamber.
3. Once the temperature in the thermometer reaches a stable point, take the water boiling point. The disk "Degré Alcoolique du Vin" establishes the 0.0% (v/v) alcohol on the outer scale (Fig. 2).
4. Cool and drain the ebulliometer.

Determination of the Wine Boiling Point

1. Wash the boiling chamber with a few milliliters of the wine to be analyzed, and drain it.
2. Measure nearly 50 mL of wine in the boiling chamber.
3. Insert the thermometer into the boiling chamber.

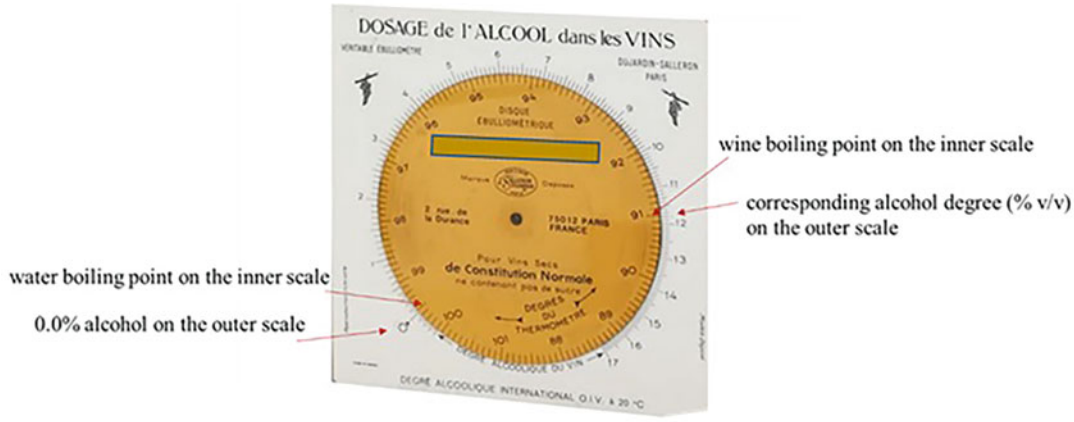

Fig. 2 "Degré Alcoolique du Vin" disk

4. Once the temperature in the thermometer reaches a stable point, take the wine boiling point. In the disk "Degré Alcoolique du Vin" put the boiling point of the wine on the inner scale and record the corresponding alcohol degree (% v/v) on the outer scale (Fig. 2).
5. Cool and rinse the ebulliometer.

2.2 Alcohol Content Determination by Distillation Followed by Density Determination by Hydrometry

Distillation involves the conversion of a liquid into the gaseous phase, condensing the vapor back to the liquid phase, and collecting the distillate in a receiving vessel. Once the wine is boiled, the ethanol starts evaporating before the water. If the wine is boiled for an optimal period, all the alcohol will be evaporated from the wine sample before the volatilization of other wine components. The condensed distillate collected at this point comprises practically all the ethanol from the wine sample but in a lower volume. So, the distillate is diluted with distillate water to the same volume as the wine sample used. This reconstituted distillate will have the same percentage of alcohol (by volume) as the initial wine sample. The alcohol percent in the reconstituted distillate can be accurately measured using an alcohol hydrometer [2].

Distillation is an accurate method that reduces the interference from reducing sugars as they are not distillable [1], but sulfur dioxide and acetic acid are usual interferences and may cause problems if wine is directly distilled. Hence, it is suggested to neutralize the wine sample before distillation to avoid these interferences. Young and sweet wines can produce foam during the distillation process; hence it is recommended to add small quantities of the antifoaming agent before distillation or put a small glass or metal balls or small ceramic pieces to minimize this effect. The temperature control of the wine sample at the beginning of the distillation process and the final distillate for an accurate measurement are critical points in this analysis.

In young and sparkling wines, the carbon dioxide should be removed by stirring 250–300 mL of the wine in a 1000 mL flask for a few minutes or removed by ultrasounds. The time spent involving the distillation of the wine sample and the analysis of the respective distillate are disadvantages of this method [1, 6]. However, fast distillation systems (Fig. 3) perform distillation between 5 and 15 min, presenting energy consumption as a disadvantage. The quality of thermometers and hydrometers are also critical points in this method. Some laboratories stabilize the wine sample temperature and wine-distilled solution using a water bath at 20 °C for temperature stabilization and control (using a digital and precise thermometer); on the other hand, others use the official tables to do the temperature correction for the ethanol parameter. It is recommended to proceed with the wine stabilization using a temperature-controlled water bath.

Fig. 3 Distillation system

2.2.1 *Materials*

Distillation equipment.
Graduated thermometer, 0–40 °C (±0.1 °C).
200 mL volumetric flask.
Measuring cylinder: 36 mm diameter and 320 mm height.
Alcohol hydrometer (0–10 and 10–20% (v/v) Alcohol).
Boiling stones (antifoaming agents).
Distilled water.
Suspension of calcium hydroxide at 2 M.

2.2.2 *Methods*

Wine Sample Distillation

1. Rinse the volumetric flask with the wine sample before use. Add, to a volumetric flask, a volume of 200 mL of wine at room temperature. Control the temperature at 20 °C.
2. Transfer the wine into the distillation flask containing about 25 mL of distilled water.

3. Rinse down the walls of the volumetric flask four times with approximately 5 mL of distilled water and add rinsing water to the distillation flask.
4. Neutralize the contents of the distillation flask with 10 mL of calcium hydroxide at 2 M (120 g of $Ca(OH)_2$/L).
5. Add a small number of boiling stones to the boiling flask, especially in the case of young or sweet wines to prevent excessive foam formation during the distillation.
6. Turn on the cold water to the condenser and adjust the heat input to yield moderate boiling.
7. Place the 200 mL volumetric flask with approximately 10 mL of distilled water under the condenser's output to collect a volume of distillate approximately equal to 75–80% of the initial volume.
8. Complete the distillation at about 35 min.
9. Remove the volumetric flask from the apparatus. Adjust the distillate temperature to 20 °C.
10. After cooling, carefully add distilled water to the distillate in the volumetric flask until the volume reaches 200 mL and mix thoroughly.

Note: The distillation equipment must ensure that the ethanol-water mixture with an alcoholic strength of 10% (v/v), distilled five times successively, does not present an alcoholic strength lower than 9.9% (v/v) after the fifth distillation; that is, the loss of alcohol during each distillation should not be more than 0.02% (v/v) [2]. In the case of wine samples with high alcohol content, it may be advisable to pre-dilute them before the distillation operation.

Determination of the Alcohol Content of the Distillate by Hydrometry

1. Rinse a measuring cylinder with a small quantity of distillate. Reject the rinse distillate.
2. Rinse the alcohol hydrometer with a small quantity of distillate.
3. Transfer the remaining distillate to a measuring cylinder.
4. Gradually immerse the hydrometer into the distillate while slightly spinning the hydrometer stem. Guarantee that the hydrometer is floating freely into the distillate. Insert the thermometer into the distillate.
5. Read the temperature of the distillate on the thermometer 1 min after stirring to equilibrate the temperature of the measuring cylinder, the thermometer, the alcoholmeter, and the distillate-preferably hydrometer readings should be taken at 20 °C; however, a temperature correction can be made if needed. At least three readings should be taken, if necessary,

with the help of a magnifying glass. Results are rounded to the nearest decimal place.

6. Remove the thermometer and read the apparent alcohol content after 1 min at the point corresponding to the base of the meniscus. Take at least three readings.

Note: The distillation method is an official method for the alcohol content determination in the wine of the OIV [2] and of the AOAC [4].

2.3 Gas Chromatography (GC) Analysis of Ethanol

Ethanol in a wine sample can be separated from other volatile compounds on a gas chromatograph column, such as a Carbowax type with a bonded polar phase. The ethanol content in wine is measured by mixing a known amount of internal standard (2-propanol) and injecting it into the gas chromatograph. Peak responses of ethanol and internal standard are compared and determined (AOAC 983.13-1988, [4]). Ethanol determination by gas chromatography has a precision of ±0.2% (v/v) for wines containing 20% (v/v) ethanol [7]. Stackler and Christensen [8] developed a method for ethanol determination in wine by gas chromatography. According to the authors, this method, which uses an internal standard (n-butanol) and flame ionization detector (FID), is more accurate and precise than methods commonly used for ethanol determination. The standard error estimate for the method is 0.07% (v/v) over the range of 7–24% (v/v). In this method, a gas chromatograph with a flame ionization detector equipped with a Carbowax column is used. The wine sample is injected into a heated chamber (120–125 °C). Carrier gas (helium) transports the compounds volatilized in the injector through a heated (80 °C) column, allowing the compounds present to separate according to their affinity for the stationary phase. The molecules will then pass through a heated (125 °C) detector, which will yield a signal directly proportional to the amount of compounds being burned [1, 7]. This method has several advantages over other methods since it can be automated, is fast, and the precision is excellent [7].

2.3.1 Materials

Gas chromatograph equipped with flame ionization detector (FlD), computer, heated injector.

Hypodermic syringe (10 μL).

Internal standard solution of 2-propanol (0.2% v/v) [4, 9] or n-butanol (0.1% v/v) in H_2O [10].

Distilled water.

Standard ethanol-water solution (in the concentration range of the wine samples being analyzed).

Table 1
Gas Chromatography parameters for wine alcohol determination

Conditions	Column—Carbowax
Carrier gas	N_2
Flow rate (mL/min)	15
Oven temperature (°C)	105
Injector temperature (°C)	175
Detector temperature (°C)	175

Adapted from AOAC 983.13-1988 [4]

2.3.2 Method

1. Gas chromatograph operating conditions for ethanol analysis (adapted from AOAC 983.13-1988, [4]), Table 1.
2. Regulate the air and H_2 gas flows to the FID detector to those specified in the equipment working instructions (nearly 300 and 30 mL/min, respectively).
3. Dilute the alcohol standard solution 1 + 99 with the internal standard solution. Inject at minimum three separate 1.0-μL aliquots into the equipment, and record the resulting chromatograms. Calculate the peak area for the alcohol peak comparing it with the internal standard peak (determine the average of the three response ratios—RR′).
4. Dilute the wine sample 1 + 99 with the internal standard solution. Inject a minimum of three 1.0-μL aliquots into the equipment, and record the resulting chromatograms. Calculate the suitable response ratios for the alcohol peak to the internal standard peak and determine the average of the several response ratios (RR).
5. Determine the % alcohol in the wine sample.

$$\%\text{Alcohol} = (\text{RR} \times \%\text{Alcohol in standard})/\text{RR}'$$

Notes

A calibration curve may be constructed by preparing five or more alcohol standard solutions covering the range of expected sample alcohol concentrations.

Gas chromatography is an AOAC [4, 10]-approved method for determining ethanol.

2.4 Analysis of Ethanol by Liquid Chromatography (LC)

The high-performance liquid chromatography (HPLC) method allows the analysis of ethanol simultaneously with acids, sugars, and glycerol using a cation-exchange column (packed with

hydrogen sulfonated divinyl benzene-styrene copolymer). Elution is performed with 0.045 N H_2SO_4 with 6% acetonitrile (v/v). Two procedures can be used for sample preparation. Sample clean-up with a SAX cartridge (strong anion exchange) and by direct injection of diluted wine (1:20) with the mobile phase, filtered through a 0.22 μm cellulose-acetate membrane before injection of the diluted wine. This latter technique gives the best results in terms of precision, accuracy, and time [11]. The substitution of the organic modifier in the mobile phase with 4% tetrahydrofuran (THF) and 5% n-propanol, and the application of 0.01 N NH_3PO_4 improve the separation of the organic acids [12]. This method shows good linearity and precision.

2.4.1 Materials (Adapted from [12])

HPLC system equipped with a pump, a UV detector, a refractive index detector, and a column oven.

Data acquisition and peak processing software.

Aminex HPX-87H column (300 × 7.8 mm) (or equivalent).

Commercial standards.

Stock solutions of organic acids, sugars, and alcohols.

0.22 μm membrane filter (cellulose-acetate).

SAX cartridge (strong anion exchange).

Milli-Q water.

50 μL syringe.

2.4.2 Method (Adapted from [12])

This HPLC method makes a simultaneous quantitation of the carboxylic acids, namely, citric, tartaric, malic, shikimic, succinic, lactic, fumaric, and acetic acids, sugars such as glucose and fructose, and alcohols, namely, glycerol and ethanol in grape must and in wine.

1. Prepare stock solutions of organic acids, sugars, and alcohols in redistilled water at concentrations commonly found in grape musts and wines.
2. Dilute wine samples (up to five times for white grape must and wine, 10 times for red wine) and filter (0.22 μm) before direct injection in the HPLC.
3. Use an HPLC system equipped with a pump, a variable wavelength UV-detector set at 215 nm, and connected with a refractive index detector.
4. Install an Aminex HPX 87H (300 × 7.8 mm) column or equivalent thermostated at 30 °C, and allow the system to stabilize.

5. Use the following elution conditions—0.01 NH_3PO_4, with 4% tetrahydrofuran (THF) and 5% n-propyl alcohol (n-PA), with a flow rate of 0.45 mL/min at 30 °C.
6. Identify the sample peaks based on the retention times and spiking technique.
7. Perform the quantitation through an external standard calibration curve with adequate range.

2.5 Enzymatic Analysis

The enzyme alcohol dehydrogenase catalyzes the oxidation of ethanol using nicotinamide-adenine dinucleotide (NAD^+) as a co-factor to produce reduced nicotinamide-adenine dinucleotide (NADH). It is a stoichiometric reaction, and the NADH formed can be quantified by spectrophotometry at 334 nm [9] or 340 nm [7]. The molar extinction coefficient at 340 nm for NADH (also NADPH) at 25–37 °C is 6.3×10^3 L/mol/cm [6]. The solution is buffered in the alkaline region (pH 8–9), and semicarbazide is added to remove the acetaldehyde formed, thus forcing the reaction to completion. The test is very sensitive. It is useful for solutions of less than 1% (v/v) of ethanol [7]. The more NADH formed, the higher the alcohol concentration. This method is more time-consuming and labor-intensive than some of the other methods mentioned previously [1].

2.5.1 Preparation of the Following Solutions (Adapted from Ough and Amerine [7])

1. Buffer solution (pH 9.0)—Dissolve 10 g of $Na_4P_2O_7.10H_2O$, 2.5 g of semicarbazide hydrochloride, and 0.5 g of glycine in 250 mL of distilled water in a 300 mL volumetric flask. Bring to volume with distilled water at 20 °C (stable during 3 weeks at 5 °C).
2. In 6 mL of distilled water, dissolve 72 mg of NAD^+ (stable during 4 weeks at 4 °C).
3. Alcohol dehydrogenase solution 30 mg/mL of distilled water (stable for 1 week at 4 °C).
4. Diluted ethanol standards should be prepared daily.
5. Samples should be diluted to be in the range of 0.01–0.15 g/L.
6. A blank spectrophotometric sample should be prepared by the addition of 2.5 mL of buffer, 0.1 mL of NAD^+ solution, and 0.5 mL of water to a test tube, mix, and after 2 min, read the optical density (E_1), then introduce 0.02 mL of NADH solution and mix again. Put the sealed tube in a water bath at 37 °C for 25 min. The same procedure is performed for the wine sample; however, add 0.10 mL of the wine sample and only 0.40 mL of water. Read the optical density of the wine sample (E_2) at 340 nm using a 1 mm cell. Both samples must be read with the same cell.
   The alcohol content from the samples is calculated as [7]:

$E = Es + Eb$.

Es = sample E_2 – sample E_1.

Eb = blank E_2 – blank E_1.

Ethanol (g/100 mL) = $[(V)\,(MW)\,(\Delta E)\,(F)]/[(\varepsilon)\,(d)\,(v)]$.

V = final volume (3.12 mL).

MW = molecular weight 46.07 (g/mol) for ethanol.

$e\varepsilon$ = molar extinction coefficient at 340 nm (6.3 L/mmol/cm),

d = light path (cm) (0.1),

v = sample volume (100 mL),

F = dilution factor (1000 for dry wine).

Notes

There are already available commercial enzymatic kits to perform ethanol analysis in wine samples from various companies.

Enzymatic methods are considered limited concerning their accuracy in sample dilution. Furthermore, other alcohols are oxidized to some extent, producing positive errors. According to McCloskey and Replogle [13], the enzyme assay does not present enough precision; however, it offers speed and low sample quantities or reagent preparation for estimating wine ethanol concentrations.

2.6 Infrared Spectroscopy Methods

Infrared spectroscopy on the near-infrared (NIR) or mid-infrared (MIR) range of the electromagnetic spectrum can be a simple, fast, environmentally friendly, and reliable technique for the determination of the alcohol content of wines, among other enological parameters, without the need for costly and time-consuming sample preparation [14–17]. The NIR spectrum is characterized by overtones and combinations of bands of N–H, O–H, C–H, and S–H bonds and molecular vibrations within the spectral range of 14,000–4000 cm^{-1} [18]. In the MIR region (4000–400 cm^{-1}), fundamental stretching and bending vibrations of C–H, C–O, O–H, and N–H bonds result in strong absorption bands [19].

The strong absorption in the MIR region is on one hand of analytical advantage, being observed various information-rich sharp peaks. Nevertheless, samples containing high amounts of water and organic compounds, such as wine or grape juice, are highly absorptive in the MIR region. Dedicated FT-MIR instruments are now available and are used extensively in the routine analysis of wine by the industry [20] (Fig. 4). The use of FT-MIR spectroscopy has been proposed and implemented by several research groups for routine analysis of a large number of wine parameters such as alcohol content, volatile acidity, pH, tartaric acid, lactic acid, malic acid, total acidity, glucose and fructose, acetic acid, and polyphenols and others. In contrast, sulfur dioxide analysis is not performed with accuracy in FTIR equipment [21].

Fig. 4 FTIR equipment

Most of the liquid sampling devices for infrared spectroscopic analysis are still based on measurements employing transmission cells using CaF_2 cuvettes. A potential alternative to transmission measurements is the application of attenuated total reflection (ATR) FTIR spectroscopy, which is usually applied for highly absorptive liquid samples or surface analysis [22]. In ATR spectroscopy, penetration depth into the medium is dependent on both the wavelength and the refractive sample index (up to 3 μm, dependent on the setup), which is lower in comparison with transmission-type spectrometers (10–50 μm). Therefore, even regions that show total absorption by water in transmission-type spectra are accessible for analysis in ATR sampling.

Extraction of relevant information from the spectra can be achieved by linear multivariate calibration techniques such as partial least squares regression (PLS), multiple linear regression (MLR), or nonlinear methods such as artificial neural networks (ANN) or support vector machines (SVM) [23]. PLS is the most common calibration tool in spectroscopy [24]. While in some cases, the whole MIR spectrum is used for calibration (FullPLS), a selection of relevant spectral areas (filters, features, variable subsets) is often performed before building a model. The variable selection is performed for the spectral regions' identification, showing which are relevant for the prediction of the analyte while excluding noisy variables or carrying little information about the analyte or interferences [25].

2.6.1 *Materials*

Each equipment and sampling device has its particularities; nevertheless, the OIV has established some guidelines (RESOLUTION OIV/OENO 390/2010, [2]) concerning the calibration and quality control of the obtained results. Nevertheless, some examples are

given below following the most used set-ups available on the market.

Near-Infrared Analyzers

The apparatuses work by reflection. The bottom of the flow cell containing the wine or the grape must that will be analyzed is equipped with a reflector that reflects the incident infrared ray, thus crossing the sample a second time before being analyzed by the detector.

The apparatus includes the following pieces:

1. Pumping system for the sample: a peristaltic pump is used to fill the measuring cell. The pumping system is generally complemented by a constant-temperature water bath, enabling adjustment of the sample temperature to the value required for the measurement.
2. Light source: a tungsten lamp producing polychromatic light with a spectrum ranging from 320 to 2500 nm. The power supply must be perfectly stabilized to ensure constant intensity.
3. Wavelength selector: the instrument used in enology uses interferential filters with known wavelengths or array monochromators to select wavelengths characterizing the target compounds.
4. Measuring cell: the part that the incident and reflected radiations pass is composed of quartz maintained at a constant temperature, commonly through a Peltier effect system. The bottom of the cell can be made of a reflectable gold-plated ceramic.
5. Detector: two lead-supplied photocells collect the reflected radiation.
6. Data acquisition: the computer carries out the mathematical and statistical processing, ensuring the comparisons with the instrument calibration and the required concentration.

Methods

1. Calibration.

The calibration is performed using the highest possible number (at least 50) of wines or grape musts of known analyte concentrations. The values of these concentrations must be distributed over the entire measuring range. The matrices must be as close as possible to those of the wines or grape musts that will be analyzed. For each calibration sample, a measurement is collected for a maximum number of wavelengths covering the near-infrared spectrum. A multilinear regression is carried out from the recorded results, making it possible to establish the following relation:

$$C = K_0 + K_1R_1 + K_2R_2 + K_3R_3 + \ldots + KiRi$$

Where: C is the target concentration value, K_0 is a typical constant of the instrument for a target compound, regardless of the wavelength, Ki is a constant for an instrument, a target compound, and a given wavelength, Ri is the expression of the spectral measure for Li wavelength.

For each target analyte, two to ten wavelengths are selected for measurement. The calibration quality is then tested by running a new series of samples of reference wine or grape musts of known concentrations.

2. Periodic calibrations are necessary when routine checks show a drift in the results, mainly attributable to the equipment's natural variation (aging of the electronic components, repair, and the substitution of parts, among others). This procedure does not consider the wavelength selection; however, it provides a new measurement of the K0 and Ki constants.
3. Routine bias corrections before each use of the equipment, one (or more) control sample(s) of known concentration in the analyte is/are analyzed. If there is a bias concerning the expected value, corrections can be made to fit the values as required.

Mid-Infrared Analyzers

The circuit begins with a sample needle, which can be operated manually or controlled by an auto-sampler. A peristaltic pump transfers the sample into a heating chamber reaching 40 °C. After filtration, it crosses through the measuring cup. The latter is an essential part of the instrument. The sample is then drained into the sink. The complete cycle for a sample lasts around 30 seconds. The automated version allows an effective rate of analysis of about 120 samples per hour.

Methods

1. Sample preparation.

The sample for analyses does not require any specific preparation. In the case of wine musts or wines with high sediments, centrifugation or filtration will be carried out to avoid clogging. The samples with higher carbon dioxide amounts, near 750 mg/L, demand a preliminary removal is necessary to avoid degasification problems in the analysis circuit.

2. Calibration.

Generally, the wine or grape must middle infrared spectrum contains information of analytical interest that is not extracted immediately. In most cases, it requires sophisticated mathematical/statistical processing methods. Numerous chemometric methods can be applied to obtain the results described above. They may be divided into two groups according to their adjustment of the linear or non-linear model. The methods can be characterized as

simple statistical tools such as principal component analysis (PCA) to highly sophisticated tools such as neural networks. While all manufacturers offer optical measuring instruments, which satisfactorily bring relevant results, the standardization *modus operandi* of these measurements, the tools used to process spectrum data, and the quality of chemometric tools available to users present crucial variations. The difficulties encountered by laboratories are often related to this point. The quality of the chemometric treatment used to read the spectral data of a wine or grape must is of central importance to reliable and accurate analytical results.

3 Notes

The MIR methodology used for determining the alcohol content works very well; however, it is relevant noticing that the initial calibration with the type of wines that will be analyzed is crucial for the success of the analysis due to wine matrix interferences. The presence of carbonic gas in the sample is also a critical point since it could provoke a high deviation in the result; for example, a wine with 14% (v/v) of ethanol, with the presence of carbonic gas in the sample, could present a result of 10% (v/v) of ethanol. Generally, this situation can decrease the results in almost all conventional enological parameters, such as alcohol, total and volatile acidity, and organic acids; on the contrary, the density remains stable. This situation is not easily detected, which is a central problem of this analysis, and the equipment does not present any control to detect those deviations. In this context, the analyst must have the experience to detect these types of situations; nevertheless, a result with a considerable deviation could be obtained in the analysis. It is imperative to validate the decarbonization procedures in the laboratory, especially for sparkling wines.

Acknowledgments

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References

1. Jacobson JL (2006) Introduction to wine laboratory practices and procedures. Springer, New York
2. OIV (2021) Compendium of international methods of wine and must analysis. In: International Organization of Vine and Wine (OIV), Paris
3. Margalit Y (2005) Winery technology and operations. Wine Appreciation Guild, South San Francisco, pp 165–169
4. AOAC (2012) Official Methods of Analysis of AOAC International, 19th edn, Gaithersburg

5. Son HS, Hong YS, Park WM, Yu MA, Le CH (2009) A novel approach for estimating sugar and alcohol concentrations in wines using refractometer and hydrometer. J Food Sci 74(2):C106–C111
6. Curvelo Garcia A (1988) Controlo de qualidade dos vinhos. Instituto da Vinha e do Vinho, Lisboa
7. Ough CS, Amerine MA (1988) Methods of analysis of musts and wines, 2nd edn. Wiley, New York
8. Stackler B, Christensen E (1974) Quantitative determination of ethanol in wine by gas chromatography. Am J Enol Vitic 25:202–207
9. Zoecklein BW, Fugelsang KC, Gump BH, Nury FS (1990) Production wine analysis. An AVI Book Published by Van Nostrand Reinhold, New York
10. Caputi A, Mooney DP (1983) Gas chromatographic determination of ethanol in wine: collaborative study. AOAC 66:1152–1157
11. Castellari M, Versari A, Spinabelli U, Galassi S, Amati A (2000) An improved HPLC method for the analysis of organic acids, carbohydrates, and alcohols in grape musts and wines. J Liq Chrom Rel Technol 23:2047–2056
12. Chinnici F, Spinabelli U, Amati A (2002) Simultaneous determination of organic acids, sugars, and alcohols in musts and wines by an improved ion-exclusion HPLC method. J Liq Chrom Rel Technol 25:2551–2560
13. McCloskey LP, Replogle LL (1974) Evaluation of an enzymatic method for estimating ethanol in wines using an "enzyme kit". Am J Enol Vitic 25:194–197
14. Patz C-D, David A, Thente K, Kürbel P, Dietrich H (1999) Wine analysis with FTIR spectrometry. Vitic Enol Sci 54:80–87
15. Dubernet M, Dubernet M (2000) Utilisation de l'analyse infrarouge à Transformée de Fourrier pour l'analyse œnologique de routine. Rev Fr OEnol 181:10–13
16. Gishen M, Holdstock M (2000) Preliminary evaluation of the performance of the Foss WineScan FT 120 instrument for the simultaneous determination of several wine analyses. Aust Grapegrow Winemaker Ann Technol:75–81
17. Kupina SA, Shrikhande AJ (2003) Evaluation of a Fourier transform infrared instrument for rapid quality-control wine analyses. Am J Enol Vitic 54:131–134
18. Reichenbächer M, Popp J (2012) Vibrational spectroscopy. In: Reichenbächer M, Popp J (eds) Challenges in molecular structure determination. Springer, Berlin
19. Bauer R, Nieuwoudt H, Bauer FF, Kossmann J, Koch KR, Esbensen KH (2008) FTIR spectroscopy for grape and wine analysis. Anal Chem 80(5):1371–1379
20. Patz D, Blieke A, Ristow R, Dietrich H (2004) Application of FT-MIR spectrometry in wine analysis. Anal Chim Acta 513(18):81–89
21. Moreira JL, Santos L (2003) Spectroscopic interferences in Fourier transform infrared wine analysis. Anal Chim Acta 513(1): 263–268
22. Ewing AV, Clarke G, Kazarian S (2016) Attenuated total reflection-Fourier transform infrared spectroscopic imaging of pharmaceuticals in microfluidic devices. Biomicrofluidics 10(2): 024125
23. Ami D, Mereghetti P, Doglia SM (2013) Multivariate analysis for Fourier transform infrared spectra of complex biological systems and processes. In: Freitas LV, Freitas APBR (eds) Multivariate analysis in management, engineering and the sciences. IntechOpen, London
24. Haaland DM, Thomas EV (1988) Partial least-squares methods for spectral analyses. 1. Relation to other quantitative calibration methods and the extraction of qualitative information national laboratory. Anal Chem 60:1193–1202
25. Mehmood T, Sæbø S, Liland KH (2020) Comparison of variable selection methods in partial least squares regression. J Chemom 34(6): e3226

Chapter 3

Total and Reducing Sugars: Traditional and Advanced Methods

Aline Alberti, Acácio Antonio Ferreira Zielinski, and Alessandro Nogueira

Abstract

In the wine industry, the analysis of sugars in grapes, grape must, alcoholic fermentation, and final product are relevant information in the manufacture of wines. This chapter consists of the compilation and discussion of classical methods (refractometry, chemical, enzymatic, and liquid chromatography) and those of high analytical complexity such as infrared spectroscopy (FTIR), gas chromatography, and nuclear magnetic resonance (NMR). In addition, the development of a methodology for glucose and fructose analysis by HPLC is described. This chapter contains information aimed at both the wine industry and alcohol beverage researchers.

Key words Grape must, Wine, Enzymatic method, HPLC, FTIR, RMN

1 Introduction

Wine has been consumed for several years. Its production precedes recorded history, and the earliest evidence of enology or winemaking from cultivated grapevines can be dated back to between 5400 and 5000 BCE in Iran [1]. In 2018, global wine production, which occurs on almost every continent, was 290 million hectoliters [2], an 8% increase compared with 2016.

Wine is a very complex alcoholic beverage containing a wide range of components capable of influencing wine's chemical properties and inducing sensory perceptions. These compounds are influenced by several oenological factors, including the grape type and grape structure, climatic factors, and fermentation techniques [3–5]. According to Cabanis et al. [6], wine is composed primarily of water (between 750 and 900 g/L), alcohols (69–121 g/L), polyols (5–20 g/L), organic acids (3–20 g/L), nitrogenous compounds (3–6 g/L), and polyphenolic compounds (2–6 g/L). Also,

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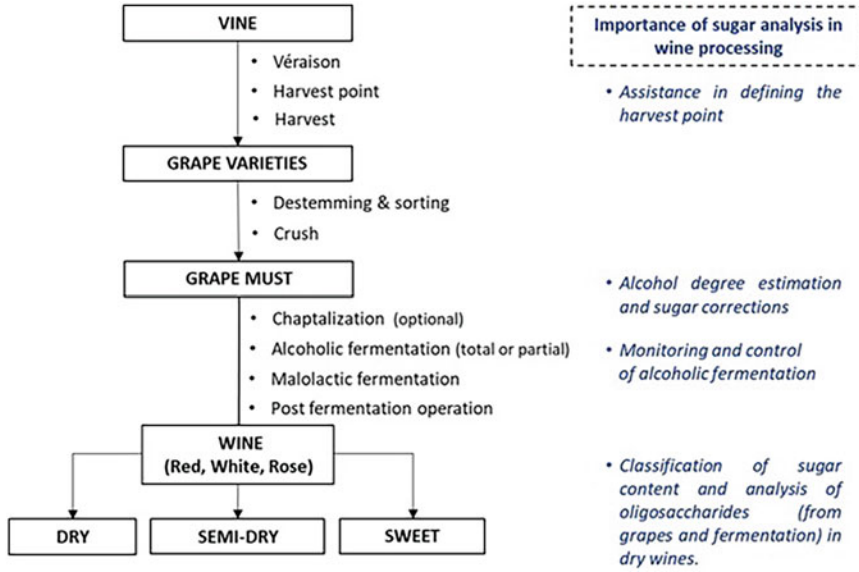

Fig. 1 Importance of sugar analysis in wine processing

there are complex carbohydrate molecules, including polysaccharides and oligosaccharides from grapes, yeasts, and bacteria during winemaking [7, 8].

The analysis of sugars in grapes, grape must, alcoholic fermentation, and final product are relevant information in the manufacture of wines (Fig. 1). This analysis is one of the most important for defining the ideal grape harvesting point during the Véraison period. A representative sampling in the vineyard is used to determine the soluble solids content, analyzed by refractometry. In grapes, the primary sugars are hexoses: fructose and glucose, typically occurring in concentrations of 0.2–4.0 and 0.5–1.0 g/L, respectively. Sucrose is hydrolyzed by enzymatic action resulting in fructose and glucose and it is typically found in concentrations of 0–0.2 g/L, making it a minor and rare wine constituent [9].

After destemming and crushing the grapes, the grape sugar content is determined to calculate the possible alcoholic degree of the wine (Fig. 1). If the alcohol content is below the minimum established by legislation, the chaptalization operation can be carried out. This operation consists of adding sucrose to the grape must during alcoholic fermentation to increase the final alcohol content by a maximum of 3 °GL (%v/v). This correction is made using the relation of 18 g of sucrose per liter of grape must is equivalent to 1.0 °GL after alcoholic fermentation. Correction of the ethanol content in the final product is not allowed [10]; however, chaptalization can be illegal, especially in Africa, where the warmer condition makes grapes develop adequate sugar content [11].

Sugars are the primary substrate of the production of ethanol in wine during alcoholic fermentation. Residual sugars from incomplete fermentation or non-fermentable sugars are responsible for adding sweetness to wines. Grape sugars provide metabolic energy to the primary wine yeast, *Saccharomyces cerevisiae*, which converts the primary grape sugars to ethanol and CO_2, in cases leaving some residual sugar content. Dry table wines have a residual sugar content of around 1–4 g/L, which is the threshold for classifying wines as "dry" [12]. Sugars can also be metabolized to higher alcohols, aldehydes, and fatty-acid esters, which add aromatic features to the wine.

Several analytical methods have been used in carbohydrate determination in foods, including spectroscopy [13], capillary electrophoresis [14], gas chromatography, and liquid chromatography [15–17]. Liquid chromatography (LC) stands out among the most used techniques in carbohydrate analysis, and due to its simplicity and precision, numerous methods have been developed to improve the sensitivity and resolution of the analysis. However, in some cases, depending on the matrix, the difficulty in the sugar chromatographic analysis lies in the sample preparation to eliminate possible interferences.

According to OIV (International Organisation of Vine and Wine) [18], reducing sugars can be determined by chemical, enzymatic, and high-performance liquid chromatography (HPLC) methods. Then, this chapter will focus on the HPLC method, providing information on the proper preparation of samples and mobile phase, the operation of a chromatograph, and the steps involved in validating a methodology for analyzing sugars during wine processing. Also, a selection of methods for analyzing sugars will be presented (Table 1).

2 Refractometer and Hydrometer in Grapes

The concentration of sugars, expressed as either Brix or %SS, increases during the grape maturation and it can be measured with a refractometer or a hydrometer. The Brix refractometer measures the degree to which sugar molecules bend light as it passes through a prism considering the degree of bending directly associated with the concentration of sugars. The hydrometer measures the specific gravity of the sugars (soluble solids) in the grape must. In both methods, the sample temperature must be at 20 °C to avoid errors. The refractometer is easy to use and can be taken into the field to analyze the sugar content in grape berries, while the hydrometer requires enough juice to be floated. Sugars are crucial for winemaking since 1% sugar converts to 0.55% alcohol (%SS × 0.55 = % alcohol). Sugars are easy to measure and they are a common measurement in the wine industry; however, it is not a feasible measure of grape maturity when used by themselves.

Table 1
Different methods used in sugar analyzes in winemaking

Fruit/ Beverage	Sugars	Method	Conditions	Referência
Grape and grape must	Total soluble solids (TSS)	TSS, °Brix	Keep the sample temperature at 20 °C.	Previtali et al. [19]
Grape must and wine	Glucose and fructose	Enzymatic method	Determined according to the organisation Internationale de la Vigne et du Vin (OIV).	García-Martín et al. [20]
Grape juice and wine	Glucose, fructose, maltose, and rhamnose	HPLC	RI detector, column: Agilent Hi-Plex H (300 × 7.7); Mobile phase: 4 mM H_2SO_4 (0.5 mL/min).	Coelho et al. [21]
Wine	Glucose and fructose	HPLC	ELSD detector (nebulizer gas flow 2.74 L/min); column: Spherisorb NH_2 (250 mM · 4.6 mM i.d., 5 μM); mobile phase: Acetonitrile-water (87/13, v/v) at 1.1 mL/min.	Villiers et al. [22]
Wine	Rhamnose, xylose, fructose, glucose, sacarose, lactose, maltose	HPLC	ELSD detector (40 °C, 250 kPa, 2.0 mL/min); column: Prevail carbohydrate ES (250 mM × 4.6 mM i.d., 5 μm); mobile phase: Acetonitrile-water (80/20, v/v) at 1.0 mL/min.	La Torre et al. [23]
Wine	Rhamnose, xylose, fructose, arabinose, glucose, mannose, and galactose	GC-MS	Previous solvolysis with anhydrous methanol containing 0.5 M HCl (80 °C, 16 h). 2 DB-1 capillary column (30 m × 0.32 mM i.d., 0.25 μM film), H_2 as the carrier gas.	Apolinar-Valiente et al. [24]
Wine	Total reducing sugars	FTIR spectroscopy	Winescan™ FT-120 instrument (FOSS).	Comuzzo et al. [25]
Wine	Glucose, rhamnose, arabinose, trehalose, galactose, xylose, and sucrose	Nuclear magnetic resonance (RMN)	Bruker DMX 500 spectrometer operating at 11.7 T with a 5 mM reverse probe with z-gradient.	Consonni et al. [26]

3 Chemical Method to Determine Reducing Substances

All sugars that exhibit ketone and aldehyde functions show reducing substances that are determined by their reducing action on an alkaline solution containing a copper salt. The official method proposed by OIV (method OIV-MA-AS311-01A) [18] is based on the reduction of Cu(II) in a boiling alkaline medium determining the remaining copper. The limitation of this chemical method (the ability of sugars to reduce copper (II) ions in solution) that compromises its use is related to the fact that other compounds can also react with copper and give an erroneous result.

4 Glucose and Fructose by the Enzymatic Method

A method approved to determine glucose and fructose in wine and grape must by OIV [18] is the enzymatic method (method OIV-MA-AS311-02).

The method consists of the phosphorylation of glucose and fructose by adenosine triphosphate (ATP) in a reaction catalyzed by hexokinase (HK), producing glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P). Then, G6P reacts with nicotinamide adenine dinucleotide phosphate (NADP) combined with glucose-6-phosphate dehydrogenase (G6PDH) to form the gluconate-6-phosphate and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The amount of NADPH produced corresponds to the amount of glucose-6-phosphate and, therefore, glucose. At the end of this reaction, F6P is transformed into G6P by the action of phosphoglucose isomerase (PGI), then the G6P cycle is restarted as described. The resulting NADPH is determined by a spectrophotometer at 340 nM.

5 High-Performance Liquid Chromatography (HPLC)

The chromatographic analysis consists of the differential migration of the components of a mixture between two immiscible phases: the mobile and the stationary. The functioning of a basic HPLC system is shown in Fig. 2. In this process, the mobile phase is pumped through the system and it is responsible for transporting the sample to the column [27]. The column consists of packed solid particles (stationary phase), which interact physically and chemically with the analyte. The difference in the magnitude of these forces determines the resolution and, therefore, the separation of the individual solutes. Usually, a guard column is placed before the analytical column to avoid damage and consequently increase the service life. The most used columns in chromatographic systems in sugar

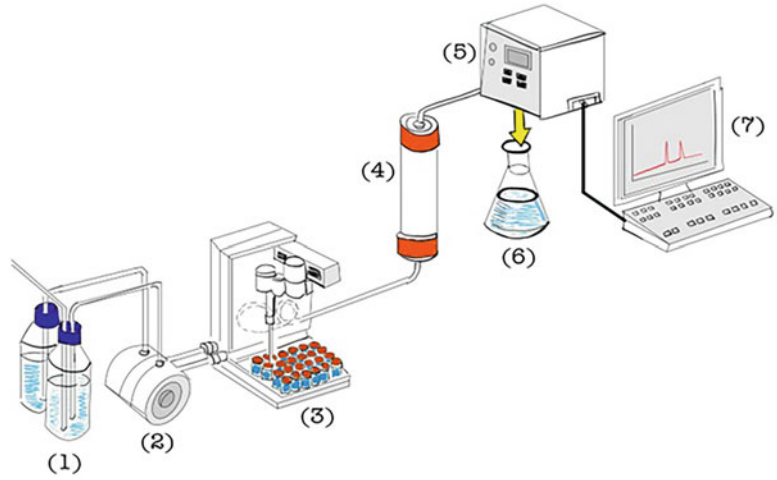

Fig. 2 Diagram of the operation of a high-performance liquid chromatography (HPLC) system: (1) mobile phase, (2) pump, (3) sample injection, (4) column, (5) detector, (6) disposal, and (7) data processor

analysis are based on ion exchange mechanisms and silica-gel columns with alkyl groups or amines. Cation exchange columns usually use ultrapure water as the mobile phase, while anion exchange columns use alkaline solutions or those containing salts. Silica-gel columns with amine groups are usual, and their eluent consists of water and acetonitrile.

After the separation step that takes place in the column, the analytes are directed to the detectors, which emit a signal that is registered when detecting its presence. The signal obtained by the detector is converted into a graph over time called a chromatogram, enabling the identification and quantitation of the different compounds present in the wine sample.

5.1 Detectors

The determination of individual carbohydrates is not possible without the use of appropriate detectors coupled to the chromatographic system. Among the most common are the refractive index (RI), evaporative light scattering (ELSD), and electrochemical (PAD). The choice is based on the desired selectivity and sensitivity.

5.1.1 Refractive Index Detector (RI)

The IR detector works as a differential refractometer that measures changes in the deflection of a light beam due to the difference in the refractive index of the eluent induced by the solute, considering that all solutes affect the eluent in the refractive index [28]. However, sensitivity varies, given the compounds lacking absorption or other properties that allow selective detection and thus may affect RI detection. The disadvantage of RI is that the signal is highly dependent on the wavelength and solute density. Furthermore, the RI signal is very sensitive to the composition of the eluent, and it opposes the elution gradient, causing a baseline shift when using gradients [29].

5.1.2 Evaporative Light Scattering Detector (ELSD)

The ELSD, compared to refractive index detectors, provides better sensitivity, and more significant baseline stability; also, it does not require the use of temperature for detection [30]. Its operation is based on the vaporization of the solvent producing an aerosol, the gas transports the particles until detection, and the amount of scattered light is measured [29]. Due to their size, therefore, carbohydrates (when in solution form) are good light scatterers. ELSD detector can be used to detect carbohydrates in grapes [32, 33] and wines [22, 23]; however, this detector has low selectivity [30].

5.1.3 Electrochemical Detector (PAD)

Electrochemical detection, also called pulsed amperometric detection (PAD), is superior to RI and ELSD detection regarding the selectivity, sensitivity, and the possibility of elution gradient [30]. In this type of detection, the electrochemical sensor is kept at an adequate constant potential, which undergoes electrochemical oxidation or reduction with the electroactive compounds of interest [33]. Cataldi and Nardiello [34] developed a method for monosaccharide determination and proline in wine by high-performance anion-exchange chromatography coupled with PAD.

5.2 Sample Preparation for Sugar Analysis in Wine

According to the method of analysis and the matrix type, it is necessary to prepare the sample, which may involve different physical, chemical, and enzymatic processes. It is an essential part of the analysis, and the choice of sample preparation method should be made to obtain the highest possible accuracy response. In some grape juice and wine, the sample preparation step is often restricted to sample dilution and filtration. Different filter types, including paper, fiberglass, and membranes, can be used [35].

Given the complexity of wine and grape must, in addition to the fact that several sugar isomers exist in the same sample, fractionation could be necessary. The use of membranes, ultra or nanofiltration, dialysis, and solid-phase extraction cartridges are examples of methods employed to purify the extracts. Castellari et al. [36] compared wine samples fractionated with a SAX cartridge and proceeded with the dilution (1:20) to separate neutral from the acidic compounds. The fractionation was satisfactory; however, only diluted wines provided better precision in the results. In the assessment carried out by Liu et al. [31] in grape juices of different varieties, the samples were pre-treated with a solid-phase extraction cartridge (SPE-C18) and then diluted at a ratio of 1:5. Solid phase extraction (SPE) cartridges are commonly used for sample clean-up before phenolic analysis since the hydrophobic substances are retained and the analytes are eluted with an aqueous solution. Therefore, this procedure can be feasible for sample preparation for the analysis of sugar, organic acids, and phenolic compounds [22].

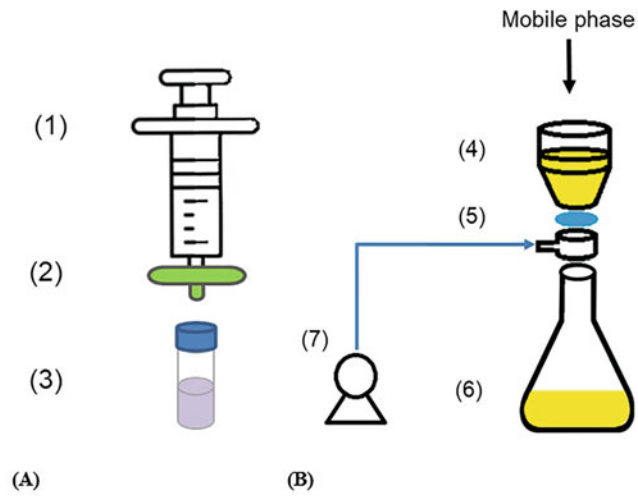

Fig. 3 Scheme of a sample (**a**) and mobile phase (**b**) filtration system: (1) syringe, (2) syringe filter, (3) vial, (4) mobile phase before filtration, (5) membrane, (6) filtered mobile phase, and (7) vacuum pump

5.3 Filtration: An Indispensable Unit Operation for Samples and Mobile Phases

Regardless of how the samples are prepared, it is necessary to filter them to prevent insoluble materials from blocking the passage through the column (clogging).

The most common way of filtering samples is the use of syringe filters, that is, the sample inside a syringe (1) passes through the filter (2) to the vial (3) (Fig. 3a). Different filter types with different membrane materials are commercially available (e.g. nylon, polyvinylidene difluoride—PVDF, polytetrafluorethylene polymer—PTFE), and your choice depends on the polarity of the solvent. As with samples, mobile phases must also be periodically filtered before analysis. Figure 3b shows the apparatus used for mobile phase filtration, in which the mobile phase (4) under suction (7) passes through the membrane (5) filtering it (6).

6 Case Study: HPLC Method to Determine Glucose and Fructose in Grape Must

The literature reported many HPLC analytical methods used to determine sugars in grape musts and wines. Once glucose and fructose are the principal sugars present in the grape must, in this section the liquid-chromatographic method for monosaccharides quantitation will be presented.

6.1 Method Validation

After choosing the quantitation method for sugar analysis in grape musts and wines, the analytical method must present reliable information on the sample. It must, therefore, undergo an assessment called validation. According to IUPAC [37], validation aims to demonstrate that the method is suitable for the intended purpose, that is, for qualitative, semi-quantitative, and/or quantitative determinations. Typical properties of the analytical methods are

specificity and selectivity, range, linearity (calibration curve), the limit of detection (LOD), the limit of quantification (LOQ), accuracy, precision, and robustness.

Selectivity The sugar analysis can be performed by comparing the retention time of compounds in the sample with and without standard addition. The peaks must be well-defined and separated from the other compounds.

Linearity Consists of an internal or external calibration curve with the compounds of interest (standards) to be quantitated in the sample within the target range (calibration curve intervals, minimum 7 points). In the case of glucose and fructose analysis, the application of solutions at different concentrations is analyzed in the method. In methods that the chromatograms are obtained, at least three points in each concentration are required. Thus, the area is determined by each peak integration, and the curve is plotted (area × standard concentration). Linear models are used for evaluating the sugar calibration curve. The regression coefficients (R^2 and R^2 adjusted) are evaluated, and analysis of variance (ANOVA) assess the model's lack of adjustment and significance.

Limit of Detection (LOD) and Limit of Quantification (LOQ) They can be obtained through the signal-to-noise ratio where a comparison is made between the signals of samples in low concentrations of the compounds of interest and a blank sample. Thus, the 3:1 (signal: noise) ratio is accepted for LOD and 10:1 for LOQ.

Accuracy After performing the linearity procedures, the accuracy of an analytical method is necessary, as it represents the proximity of the individual results found concerning a so-called true value. Several methodologies are available for accuracy testing; however, the one presented is recovery testing. For example, two different concentrations of glucose and fructose are added within the linear range of the sample. Results are calculated by dividing the glucose/fructose peak area in the standard-added sample by the sum of the peak area of the sample and standard from different runs, as the following equation:

$$\text{Recovery } (\%) = \frac{\text{Area (sample + standard)}}{\text{Area (sample)} + \text{Area (standard)}} \times 100$$

Precision Precision is obtained by analyzing the dispersion of results over a series of continuous measurements from the same sample.

Robustness The method ability to resist discrete variations in the analytical test. For example, in glucose and fructose analysis, robustness can be calculated with a discrete variation in column chromatography temperature (± 5 °C).

6.2 Analysis

1. Sample preparation.

   Before analysis, the grape juice sample is diluted (1:20, v:v) in ultrapure water.

   Then, the mixture is filtered in a nylon (0.22 μM) syringe filter (Fig. 3a) directly into the vial.

2. Mobile phase preparation.

   A solution containing 5 mM H_2SO_4 in ultrapure water is used as a mobile phase.

   Then the solution is filtered (**item 3**, Fig. 3b) on a nylon membrane (0.45 μM). The choice of the mobile phase will depend on the column chosen for analysis. It is relevant to emphasize that the analyst reads the instructions in the column manual for more information. After the filtration step, the mobile phase is degassed in a sonicated bath for 15 min.

3. Chromatographic system and analysis condition.

   A chromatographic system coupled with a refractive index detector is used. The separation took place in a cation exchange column [Aminex HPX-87H, Bio-Rad (300 × 7.8 mM i.d.)] under an isocratic flow of the mobile phase at 0.5 mL/min after injection of 10 μL of the sample.

   During the analysis, both the column and detector temperatures are maintained at 30 °C.

4. Data analysis.

After 25 min, the chromatogram shown in Fig. 4 is obtained. In the comparison of the sample retention time with the standard, it is assumed that peaks number (I) and (II) correspond to glucose and fructose, respectively. After the identification step, the peaks'

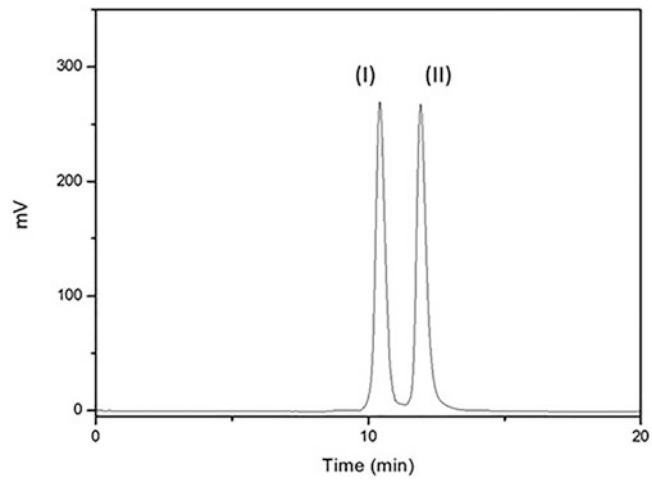

Fig. 4 Chromatogram of the grape must. Peak (I) corresponds to glucose and peak (II) to fructose. (AROUND HERE)

areas are obtained by integration. As the analysis is performed in triplicate, three areas of each peak are obtained. Considering an average area of glucose 4,141,497, and fructose 3,838,020, and replacing these values on the calibration curves:

Glucose: y (area) = 962,786 * (concentration, g/L) – 2742.3.

Fructose: y (area) = 925,797 * (concentration, g/L) – 66,006.

Multiplying by the dilution factor (twenty times in this case), the mean concentrations are 86.09 g/L and 84.34 g/L for glucose and fructose, respectively.

7 Other Methods

Besides the classical methods (chemical, enzymatic, and liquid chromatography) used to determine sugars in the wine, other methods have been used, such as infrared spectroscopy, gas chromatography, and nuclear magnetic resonance (NMR).

The Fourier transform infrared (FT-IR) and Fourier-transform near-infrared (FT-NIR) spectroscopy are instrumental methods based on the measurement of the vibration of a molecule excited by infrared radiation at a specific wavenumber range. FT-IR technology allows taking advantage of distinct infrared intervals, namely, NIR (near-infrared) and MIR (medium-infrared), coupled with different instrumentation [38]. The infrared spectroscopy associated with multivariate data analysis enables qualitative and quantitative analysis. A qualitative assessment of the spectra can be performed by principal components analysis (PCA), while partial least squares (PLS) regression allows the enhancement of calibration models based on spectral and analytical data [39]. However, to obtain reliable predictions, exhaustive calibrations should be performed in the same conditions for sample processing to remain consistent throughout an individual study. There is equipment based on this technique that performs reducing sugars, alcoholic degree, dry extract, total acidity and pH, volatile acidity, malic, lactic, and tartaric acid, glycerol, and sulfates at the same time.

Gas chromatography is also used for sugar analysis [24, 40, 41]. The sample preparation involves the derivatization of the sample as methanolysis and trimethylsilylation. Methanolysis may be preferable to classical acid hydrolysis due to its higher recoveries of both neutral sugars and uronic acids, whereas trimethylsilylation has been recognized as a quantitative, rapid derivatization method for a wide range of carbohydrates [42].

Several studies aim to identify malpractice and fraud, and various physical and chemical analytical techniques have been employed for this purpose [43–45]. The analytical methods used include chromatography, isotopic ratio mass spectrometry, optical spectroscopy, and NMR spectroscopy. NMR spectroscopy with

chemometric analysis has gathered more attention due to its simplicity and fastness. The detection of beet sugar illegally added before fermentation can be detected by analyzing the wine alcohol using a ^{2}H NMR [46].

8 Conclusion

Monitoring the sugar content in beverage manufacturing is essential to ensure the quality of the final beverage. Among the various techniques, high-performance liquid chromatography is the most used technique in the quantitation of carbohydrates in foods. However, there is no ideal method, but techniques with different characteristics with better adjustment according to the type of sample. The use of advanced technologies, such as those based on infrared and NMR spectroscopy, which can be a limitation for producers, can be achieved through partnerships with research centers, laboratories, and universities that have the infrastructure and knowledge of these techniques.

References

1. Johnson H (2005) The story of wine. Mitchell Beazley, London
2. FAOSTAT (2021) Food agriculture organization of the United Nations. Statistical database. http://faostat.fao.org/site/291/default.aspx. Accessed 01 Nov 2021
3. Margalit Y (2012) Concepts in wine chemistry. Board and Bench Publishing
4. Soleas GJ, Diamandis EP, Goldberg DM (1997) Wine as a biological fluid: history, production, and role in disease prevention. J Clin Lab Anal 11:287–313
5. McGovern PE (2003) Ancient wine: the search for the origins of viniculture. Princeton University Press
6. Cabanis JC, Cabanis MT, Cheynier V, Teissedre PL (1998) Tables de composition. In: Flanzy C (ed) OEnologie: fondements scientifiques et technologiques. Lavoisier Technique & Documentation
7. Dols-Lafargue M (2018) Polysaccharide production by wine lactic acid bacteria: negative trait or potential advantage? A review. Appl Microbiol 4:1–8
8. Apolinar-Valiente R, Williams P, Doco T (2021) Recent advances in the knowledge of wine oligosaccharides. Food Chem 342: 128330
9. Waterhouse AL, Sacks GL, Jeffery DW (2016) Understanding wine chemistry. Wiley, London
10. Guerra CC (2016) Vinho tinto. In: Venturini-Filho WG (ed) Bebidas Alcoólicas. Ciência e Tecnologia. Edgar Blücher, São Paulo
11. Villiers A, Alberts P, Tredoux AGJ, Nieuwoudt HH (2012) Analytical techniques for wine analysis: an African perspective; a review. Anal Chim Acta 730:2–23
12. Rupasinghe HPV, Joshi VK, Smith A, Parmar I (2017) Chemistry of fruit wines. Sci Technol Fruit Wine Prod:105–176
13. Hall MB (2003) Efficacy of reducing sugar and phenol–sulfuric acid assays for analysis of soluble carbohydrates in feedstuffs. Ani Feed Sci Technol 185:94–100
14. Cabálková J, Žídková J, Přibyla L, Chmelík J (2004) Determination of carbohydrates in juices by capillary electrophoresis, high-performance liquid chromatography, and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry. Electrophoresis 25:487–493
15. Belscak-Cvitanovic A, Komes D, Dujmovic M, Karlovic S, Biskic M, Brncic M, Jezek D (2015) Physical, bioactive and sensory quality parameters of reduced sugar chocolates formulated with natural sweeteners as sucrose alternatives. Food Chem 167:61–70
16. Shanmugavelan P, Kim SY, Kim JB, Kim HW, Cho SM, Kim SN, Kim SY, Cho YS, Kim HR (2013) Evaluation of sugar content and composition in commonly consumed Korean

vegetables, fruits, cereals, seed plants, and leaves by HPLC-ELSD. Carbohydr Res 380: 112–117

17. Ouchemoukh S, Schweitzer P, Bachir Bey M, Djoudad-Kadji H, Louaileche H (2010) HPLC sugar profiles of Algerian honeys. Food Chem 121:561–568
18. International Organization of Vine and Wine. (OIV) (2016) Compendium of international methods of wine and must analysis. 13th Assemblee Generale. Mainz (Germany)
19. Previtali P, Dokoozlian NK, Pan BS, Wilkinson KL, Ford CM (2022) The effect of ripening rates on the composition of Cabernet Sauvignon and Riesling wines: further insights into the sugar/flavor nexus. Food Chem 373: 131406
20. García-Martín N, Perez-Magariño S, Ortega-Heras M, González-Huerta C, Mihnea M, González-Sanjosé ML, Palacio L, Prádanos P, Hernández A (2010) Sugar reduction in musts with nanofiltration membranes to obtain low alcohol-content wines. Sep Purif Technol 76: 158–170
21. Coelho EM, Valéria C, Padilha S, Miskinis GA, Gomes Barroso De Sá A, Pereira GE, Cavalcanti De Azevêdo L, Dos M, Lima S (2018) Simultaneous analysis of sugars and organic acids in wine and grape juices by HPLC: method validation and characterization of products from Northeast Brazil. J Food Compos Anal 66:160–167
22. Villiers A, Lynen F, Crouch A, Sandra P (2004) Development of a solid-phase extraction procedure for the simultaneous determination of polyphenols, organic acids and sugars in wine. Chromatographia 59:403–409
23. La Torre GL, La Pera L, Rando R, Lo Turco V, Di Bella G, Saitta M, Dugo G (2008) Classification of Marsala wines according to their polyphenol, carbohydrate and heavy metal levels using canonical discriminant analysis. Food Chem 110:729–734
24. Apolinar-Valiente R, Williams P, Mazerolles G, Romero-Cascales I, Gómez-Plaza E, López-Roca JM, Ros-García JM, Doco T (2014) Effect of enzyme additions on the oligosaccharide composition of Monastrell red wines from four different wine-growing origins in Spain. Food Chem 156:151–159
25. Comuzzo P, Rauhut D, Werner M, Lagazio C, Zironi R (2013) A survey on wines from organic viticulture from different European countries. Food Control 34(2):274–282
26. Consonni R, Cagliani LR, Guantieri V, Simonato B (2011) Identification of metabolic content of selected Amarone wine. Food Chem 129:693–699
27. Nollet LML, Toldra F (2012) Food analysis by HPLC. Taylor & Francis
28. Magwaza LS, Opara UL (2015) Analytical methods for determination of sugars and sweetness of horticultural products: a review. Sci Hortic 184:179–192
29. Raessler M (2011) Sample preparation and current applications of liquid chromatography for the determination of non-structural carbohydrates in plants. Trend Anal Chem 30:1833–1843
30. Molnár-Perl I (2000) Role of chromatography in the analysis of sugars, carboxylic acids and amino acids in food. J Chromatogr A 891:1–32
31. Liu HF, Wu BH, Fan PG, Li SH, Li LS (2006) Sugar and acid concentrations in 98 grape cultivars analyzed by principal component analysis. J Sci Food Agr 86:1526–1536
32. Ma C, Sun Z, Chen C, Zhang L, Zhu S (2014) Simultaneous separation and determination of fructose, sorbitol, glucose and sucrose in fruits by HPLC-ELSD. Food Chem 145:784–788
33. Santos WTP, Gimenes DT, Richter EM, Angnes L (2011) Análise por injeção em fluxo com detecção amperométrica de múltiplos pulsos: potencialidades e aplicações. Quím Nova 34(10):1753–1761
34. Cataldi TRI, Nardiello D (2003) Determination of free Proline and monosaccharides in wine samples by high-performance anion-exchange chromatography with pulsed Amperometric detection (HPAEC-PAD). J Agric Food Chem 51:3737–3742
35. Sanz ML, Martínez-Castro I (2007) Recent developments in sample preparation for chromatographic analysis of carbohydrates. J Chromatogr A 1153:74–89
36. Castellari M, Versati A, Spinabelli U, Galassi S, Amati A (2000) An improved HPLC method for the analysis of organic acids, carbohydrates, and alcohols in grape musts and vines. J Liq Chromatogr RT 23:2047–2056
37. Thompson M, Ellison SLR, Wood R (2002) Resulting from the symposium on harmonization of quality Assurance Systems for Analytical Laboratories. Pure Appl Chem 74:835–855
38. Carbas B, Machado N, Oppolzer D, Queiroz M, Brites C, Rosa EAS, Barros AIRNA (2020) Prediction of phytochemical composition, in vitro antioxidant activity and individual phenolic compounds of common beans using MIR and NIR spectroscopy. Food Bioprocess Technol 13:962–977
39. Brás LP, Bernardino AS, Lopes JA, Menezes JC (2005) Multiblock PLS as an approach to compare and combine NIR and MIR spectra in calibrations of soybean flour. Chemometr Intell Lab 75:91–99
40. Guadalupe Z, Martínez-Pinilla O, Garrido Á, Carrillo JD, Ayestarán B (2012) Quantitative determination of wine polysaccharides by gas

chromatography–mass spectrometry (GC–MS) and size exclusion chromatography (SEC). Food Chem 131(1):367–374

41. Apolinar-Valiente R, Romero-Cascales I, Williams P, Gómez-Plaza E, López-Roca JM, Ros-García JM, Doco T (2015) Oligosaccharides of cabernet sauvignon, Syrah and Monastrell red wines. Food Chem 179:311–317
42. Mejanelle P, Bleton J, Tchapla A, Goursaud S (2002) Gas chromatography-mass spectrometric analysis of monosaccharides after Methanolysis and Trimethylsilylation. Potential for the characterization of substances of vegetal origin: application to the study of museum objects. J Chromatogr Libr 66:845–902
43. Amargianitaki M, Spyros A (2021) NMR-based metabolomics in wine quality control and authentication. Chem Biol Technol Agric 4(1):1–12
44. Ranaweera KR, Capone DL, Bastian SEP, Cozzolino D, Jeffery DW (2021) A review of wine authentication using spectroscopic approaches in combination with chemometrics. Molecules 26(14):1–15
45. Rubert J, Lacina O, Fauhl-Hassek C, Hajslova J (2021) Metabolic fingerprinting based on high-resolution tandem mass spectrometry: a reliable tool for wine authentication? Anal Bioanal Chem 406(27):6791–6803
46. Solovyev PA, Fauhl-Hassek C, Ried J, Esslinger S, Bontempo L, Camin F (2021) NMR spectroscopy in wine authentication: an official control perspective. Compr Rev Food Sci F 20(2):2040–2062

Chapter 4

Total Phenolic Content: Traditional Methods

Maurício Bonatto Machado de Castilhos, Lia Lucia Sabino, Matheus Felipe de Oliveira Silva, Danieli Cristina Alves, Maria Eduarda da Mata Martins, Letícia Barbosa de Melo, and Leda Atílio Pita

Abstract

Phenolic compounds are considered one of the most important chemical substances in wines due to their antioxidant activity and efficiency in minimizing cardiovascular and neurodegenerative diseases, inflammation, and cancer incidence. Due to this health benefits, consumers are seeking foods and beverages with a natural composition and low processing, rich in phenolic compounds to improve the quality of their diet. The phenolic compounds are divided into flavonoid (anthocyanins, flavonols, flavan-3-ols) and non-flavonoid (phenolic acids and stilbenes) substances, and the antioxidant capacity of a food matrix depends on the concentration and the diversity of these compounds since all of them promote antioxidant properties. Traditional methods are used to determine total phenolic compounds in grapes and wines, and the most known apply spectrophotometric assays. This chapter aims to provide relevant information regarding the principal spectrophotometric methods used to determine the total phenolic compounds in wines.

Key words Phenolic compounds, Anthocyanin, Flavonol, Flavan-3-ol, Phenolic acids, Stilbenes, Spectrophotometry, Wines, Grapes

1 Introduction

Consumers have become more exigent regarding food ingredients since they are concerned about natural products that bring health benefits. The food industry has developed new and alternative technologies to obtain products near their natural composition minimizing the macro and micronutrient loss due to food and beverage processing. In this context, the wine sector is growing its consumers worldwide since wine is a beverage that, in moderate intake, can provide health benefits due to its high phenolic composition [1, 2]. Phenolic compounds are extracted from grape skins and seeds and they present high antioxidant activity, a property that

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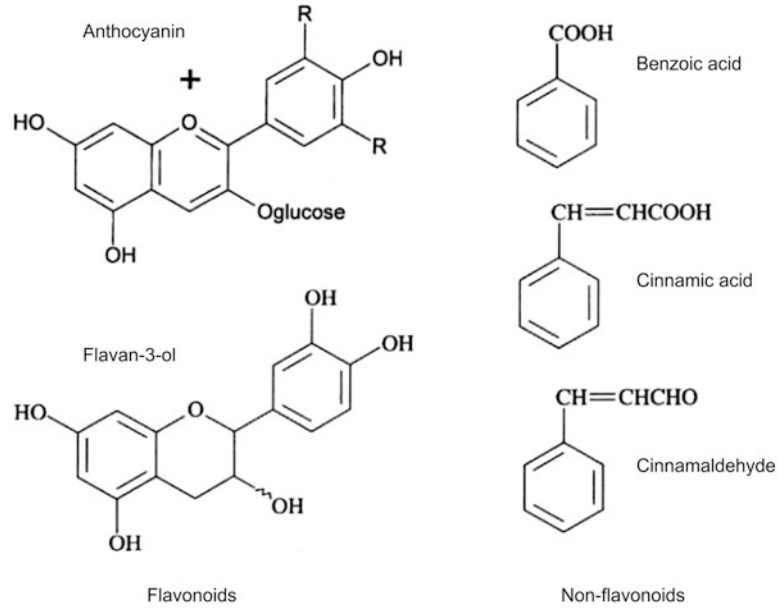

Fig. 1 Flavonoid and non-flavonoid chemical structure. Adapted from Jackson (2020) [6]

reduces oxidative cell damage, cardiovascular and neurodegenerative diseases, inflammation, and chronic diseases [3, 4]. These health benefits are closely linked to the increase of phenolic compound consumption in the diet, and wine is a great option [5].

According to their chemical structure, phenolic compounds are divided into two groups: flavonoids and non-flavonoids. Flavonoids are chemical compounds characterized by a C6-C3-C6 chemical structure since they present two phenolic rings and one heterocycle pirano ring at the center of the molecule, and non-flavonoids are composed of C6-C1 and C6-C2 chemical structures with one phenolic ring and carbon radicals linked to the molecule (Fig. 1). Flavonoids are composed of anthocyanins, flavonols, and flavan-3-ols, and non-flavonoids are composed of hydroxycinnamic and hydroxybenzoic acids and stilbenes [6, 7].

The anthocyanins are the red pigments in grapes and wines, located primarily in grape skins and less usually in pulp (except for "tenturier" grape varieties that present colored pulp) [8]. Anthocyanins are more chemically stable in glucosidic form instead of their aglycone form (anthocyanidin), and they can also be identified in wines as mono- or diglucosidic anthocyanins, depending on the grape variety. Normally, *Vitis vinifera* grapes produce monoglucosidic anthocyanins since the gene responsible for the synthesis of the diglucosidic form is defective; however, *Vitis labrusca*, *Vitis rotundifolia*, and grape hydrides produce mono- and diglucosidic anthocyanins promoting higher chemical stability for wine color [6, 9, 10].

Flavonols occur in the lowest concentration on grapes and wines, representing 1–10% of the total concentration of phenolic compounds, and this chemical class is represented by quercetin, myricetin, laricitrin, kaempferol, and isorhamnetin. The synthesis of flavonols in grapes is activated by UV exposure and blue radiation [6]. Flavonols are also responsible for the yellow color of red and white wines. In grapes, these molecules are present in glycoside form, and in wines, they can be identified as their aglycone form since the glycoside form was hydrolyzed during alcoholic fermentation. Their concentration is near 100 mg/L, depending on the grape variety, winemaking, vine management, and climatic conditions [8].

Flavan-3-ols are grape seed components and they are extracted with ethanol. Flavan-3-ols (or flavanols) with lower molecular weight can provide bitterness for wine, and the flavan-3-ols with high molecular weight are responsible for determining wine astringency. The flavan-3-ols can interact with salivary proteins promoting the astringent mouthfeel since the proteins' precipitation cause a decrease of the mouth lubrification, resulting in palate constriction [11, 12].

Phenolic acids are found in grape skin and seeds and gallic acid is one of the primary compounds of the hydroxybenzoic acids, and the hydroxycinnamic acids are composed of coumaric, caffeic, ferulic, and sinapic acids. They enhance and stabilize red wine color by inter- and intramolecular reactions, also contributing to wine flavor and present potential biological activities [13, 14]. Stilbenes are synthesized in the grape skin and seeds, and factors such as vine stress, microbial spoilage, mechanical damage, and UV radiation incidence are responsible for their concentration enhancement in grapes [11, 15]. Resveratrol is the principal stilbene followed by piceid and astringin. Some studies have revealed that resveratrol and other stilbenes are responsible for the higher antioxidant activity of wines and it is responsible for decreasing the incidence of degenerative diseases [16, 17]; however, it is well known that all the phenolic compounds present antioxidant activity and their concentration, as well as their diversity in the matrix, can influence the high or low antioxidant property in wine [18].

In this context, several methods have been applied to identify and quantitate total phenolic compounds in grape extracts and wines; however, the spectrophotometric methods are the most used by analysts. Therefore, this chapter brings valuable information about conventional methods to identify and quantitate the total phenolic compounds in wines using spectrophotometric procedures.

2 Total Phenolic Using Folin–Ciocalteu Reagent

This method is the most used in determining the total phenolic content in wines and it consists in determining the total phenolics using the Folin–Ciocalteu reagent at 765 nm wavelength. It can be used directly in cuvettes, using a few quantities of samples and reagents, or in test tubes of about 10 mL capacity. Good quality micropipettes should be used to obtain adequate reproducibility. The method described below is adapted to test tubes of 10 mL capacity, adapted from OIV-MA-AS2-10 [19].

2.1 Chemicals

1. Folin–Ciocalteu reagent (*see* **Note 1**).
2. Gallic acid solution (*see* **Note 2**).
3. Sodium carbonate solution (*see* **Note 3**).
4. Distilled water.

2.2 Apparatus

1. Semi-analytical balance.
2. UV-vis spectrophotometer.
3. 10 mM cuvettes.
4. Vortex for reagents and samples homogenization.
5. Beakers 50 mL.
6. Test tubes.
7. Automatic pipette in the range of 100–1000 μL.

2.3 Method

1. The calibration curve is prepared by adding the following volumes of the gallic acid solution: 0 mL (0 mg/L); 0.1 mL (50 mg/L); 0.2 mL (100 mg/L); 0.3 mL (150 mg/L); 0.5 mL (250 mg/L); 1.0 mL (500 mg/L); 2 mL (1000 mg/L); and 3 mL (1500 mg/L) into a 10 mL volumetric flask and complete with distilled water.
2. For each dissolution of the calibration curve, pipette 100 μL into separate tubes.
3. Add 7.9 mL of distilled water.
4. Add 500 μL of Folin–Ciocalteu reagent, stirring well.
5. Wait for about 4 min for the reaction.
6. Add 1500 μL of sodium carbonate solution, stirring to mix the reagents well.
7. Leave the dissolutions at 40 °C for about 30 min and determine the absorbance of each dissolution at 765 nm (*see* **Note 4**).
8. Plot the absorbance on a graph along with the concentrations and determine the concentration of total phenolics in mg/L using linear regression, using the straight-line equation from the calibration curve graph (Fig. 2).

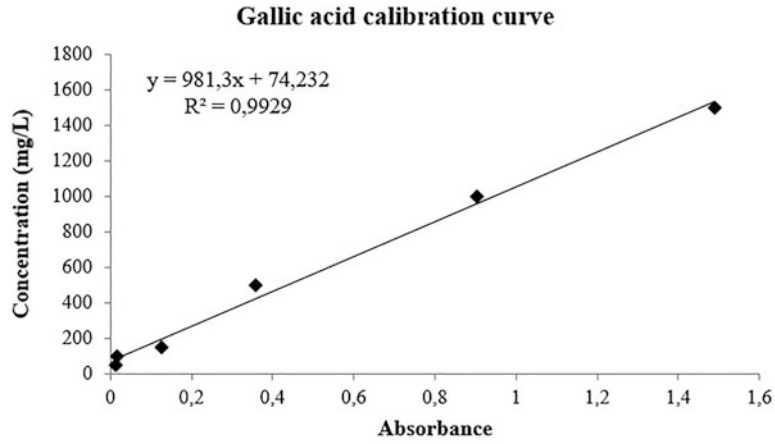

Fig. 2 Calibration curve of the gallic acid solution in different concentrations. (AROUND HERE)

9. Repeat the same protocol for each wine sample beginning from **Step 2**.
10. After 30 min at 40 °C, read the absorbance of each sample and each point of the gallic acid calibration curve in a spectrophotometer at 765 nm.

2.4 Calculation

The absorbance obtained for the sample (measured in triplicate) will be entered into the analytical expression obtained after building the standard curve (in mg equivalents of gallic acid per liter of the sample). Using the standard curve of gallic acid (Fig. 2), we have the following equations, where total phenolic content (*TPC*) is the total phenolic content for each replicate and *A* is the absorbance of each wine sample at 765 nM:

– Total phenolic content (expressed in mg/L of gallic acid equivalents):

$$TPC = (A \times 981.3) + 74.232$$

3 Total Phenolic Content by Optical Density at 280 nm (OD 280 Value)

This method presents advantages to the Folin–Ciocalteu method since it is rapid and has high reproducibility. This method is based on Ribéreau-Gayon et al. (2006) [8]. Specific molecules such as cinnamic acids and chalcones have no absorption maximum at this wavelength; however, they present small concentrations in wines, and any error in the value will be insignificant.

3.1 Chemicals

1. Wine samples.
2. Distilled water.

3.2 Apparatus

1. UV-vis spectrophotometer.
2. 10 mM cuvettes.
3. Vortex for reagents and samples homogenization.

3.3 Method

1. Dilute red wine and white wine samples 1/100 and 1/10 with distilled water, respectively.
2. Read the absorbance of the sample at 280 nM wavelength.
3. Multiply the absorbance with the dilution factor.
4. It is possible to define the relative contributions of phenolic acids and other non-phenolic substances in wine using this determination. It is considered constant at around 7 for both red and white wines using the Folin Ciocalteu reagent.
5. This is a crucial factor, especially for white and rosé wines, as it represents practically 50% of the value. In red wines, it may be considered that

$$\text{OD } 280 = 7 + \text{DA} + \text{DT}$$

Furthermore, the average anthocyanin and tannin coefficients in wine is determined as follows:

DA (anthocyanin absorption at 280 nM) = 20 × anthocyanin concentration expressed in g/L.

DT (tannin absorption at 280 nM) = 12 × tannin concentration expressed in g/L.

4 Tannin Determination by Spectrophotometry

This method involves the reduction of the Folin–Dennis reagent, in a basic medium, by the tannin present in the sample, producing an intense blue coloration that is measured in the visible region. The result is expressed as tannic acid [16].

4.1 Chemicals

1. Folin–Dennis reagent (*see* **Note 5**).
2. Sodium carbonate saturated solution (*see* **Note 6**).
3. Tannic acid standard solution (*see* **Note 7**).

4.2 Apparatus

1. UV/Vis spectrophotometer.
2. Cuvette 10 mM.
3. Analytical balance.
4. Glass wool.

5. Hot plate.
6. Volumetric flasks 100 mL, 500 mL, and 1000 mL.
7. Flask with ground-glass joint 1000 mL.
8. Reflux condenser.
9. Volumetric pipettes 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, and 10 mL.
10. Beaker 50 mL.

4.3 Standard Curve Preparation

Pipette aliquots of 1 to 10 mL of tannic acid standard solution into 100 mL volumetric flasks containing 75 mL of distilled water. Add 5 mL of the Folin–Dennis reagent and 10 mL of saturated sodium carbonate solution and complete with water. Shake well and read after 30 min at 760 nM against the blank. Plot the standard curve, relating the absorbance values with the concentration in mg/100 mL.

4.4 Procedure

Pipette 5 mL of the sample into a 100 mL volumetric flask containing 75 mL of distilled water. Add 5 mL Folin–Dennis reagent and 10 mL saturated sodium carbonate solution and complete with water. The solution should be filtered in case of turbidity. Stir well and read at 760 nM after 30 min, using a blank prepared in the same way with distilled water in place of the sample.

4.5 Calculation

After the determination of the tannic acid concentration using the standard curve, use the following equation for the correction of the tannin concentration in mg/100 mL:

$$\frac{C \times 100}{A}$$

C: tannic acid concentration in the sample corresponding to the standard curve.

A: sample volume in mL.

5 Notes

1. This reagent is bought ready-made from specialized chemical companies.
2. Dissolve 0.50 g of gallic acid in 10 mL of ethanol in a 100 mL volumetric flask and complete it with distilled water. This dissolution can be opened daily but should be kept under refrigeration for up to 2 weeks after preparation.
3. Dissolve 200 g of anhydrous sodium carbonate in 800 mL of distilled water and bring it to boiling point. After it has cooled down, insert some sodium carbonate crystals, and after 24 h filter and complete to 1 L with distilled water.

4. Use the blank as a sample to zero the spectrophotometer (the blank is the dissolution with 0 mL of the wine sample).
5. Add 100 g sodium tungstate hydrate, 20 g phosphomolybdic acid, and 50 mL phosphoric acid in 750 mL distilled water. Reflux for 2 h, cool, and dilute to 1000 mL in a volumetric flask.
6. Take 35 g of anhydrous sodium carbonate and dissolve in 100 mL of distilled water at 70–80 °C, cool overnight, and sieve the supersaturated solution with sodium carbonate decahydrate crystal ($Na_2CO_3.10H_2O$). After crystallization, filter through glass wool.
7. Dissolve 100 mg tannic acid in a 1000 mL volumetric flask with distilled water. This solution has a concentration of 0.1 mg of tannic acid per mL.

References

1. Gaona IJA, Fanzone ML, Galmarini MV, Chirife J, Ferreras-Charro R, García-Estévez I, Escribano-Bailón MT (2022) Encapsulation of phenolic compounds by spray drying of Ancellotta and Aspirant Bouchet wines to produce powders with potential use as natural food colorants. Food Biosci 50:102093
2. Di Giacomo G, Romano P (2021) Advanced fractionation process for wine-based products diversification. J Food Sci Technol 58(12): 4685–4692
3. Cianciosi D, Forbes-Hernández TY, Ansary J, Gil E, Amici A, Bompadre S et al (2020) Phenolic compounds from Mediterranean foods as nutraceutical tools for the prevention of cancer: the effect of honey polyphenols on colorectal cancer stem-like cells from spheroids. Food Chem 325:126881
4. Olas B (2018) Berry phenolic antioxidants-implications for human health? Front Pharmacol 9
5. Chen Y, Wen J, Deng Z, Pan X, Xie X, Peng C (2020) Effective utilization of food wastes: bioactivity of grape seed extraction and its application in food industry. J Funct Foods 73:104113
6. Jackson RS (2020) Wine science: principles and applications. Academic Press, San Diego
7. Fernandes I, Pérez-Gregorio R, Soares S, Mateus N, De Freitas V, Santos-Buelga C, Feliciano AS (2017) Wine flavonoids in health and disease prevention. Molecules 22(2):292
8. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D (2006) Handbook of enology: the chemistry of wine – stabilization and treatments. Wiley, New York
9. Acevedo A, Hilbert G, Rivière C, Mengin V, Ollat N, Bordenave L et al (2012) Anthocyanin identification and composition of wild *Vitis* spp. accessions by LC-MS and LC-NMR. Anal Chim Acta 732:145–152
10. Zhu L, Zhang Y, Deng J, Li H, Lu J (2012) Phenolic concentrations and antioxidant properties of wines made from North American grapes grown in China. Molecules 17:3304–3323
11. De Castilhos MBM, Del Bianchi VL (2021) Vinhos. In: Moraes IO (ed) Biotecnologia Industrial: Biotecnologia na Produção de Alimentos. Blucher, São Paulo
12. Gonzalo-Diago A, Dizy M, Fernández-Zurbano P (2014) Contribution of low molecular weight phenols to bitter taste and mouthfeel properties in red wines. Food Chem 154:187–198
13. Zhang B, Shi X, Zhang Y, Wang Q, Zhou P, Li Y, Tao Y (2022) The implication of phenolic acid matrix effect on the volatility of ethyl acetate in alcohol-free wine model: investigations with experimental and theoretical methods. Food Chem 378:132114
14. Zhang B, He F, Zhou P, Liu Y, Duan C (2015) Copigmentation between malvidin-3-O-glucoside and hydroxycinnamic acids in red wine model solutions: investigations with experimental and theoretical methods. Food Res Int 78:313–320
15. Bravo AE (1996) Resveratrol in wine: contribution to potential cardiovascular protective activity. Alimentaria 269:71–72
16. De La Lastra CA, Villegas I (2007) Resveratrol as an antioxidant and pro-oxidant agent:

mechanisms and clinical implications. Biochem Soc Trans 35:1156–1160

17. Nixdorf SL, Hermosín-Gutiérrez I (2010) Brazilian red wines made from the hybrid grape cultivar Isabel: phenolic composition and antioxidant capacity. Anal Chim Acta 659(1–2): 208–2015
18. De Castilhos MBM, Corrêa OLS, Zanus MC, Garcia Maia JD, Gómez-Alonso S, García-Romero E, Del Bianchi VL, Hermosín-Gutiérrez I (2015) Pre-drying and submerged cap winemaking: effects on polyphenolic compounds and sensory descriptors. Part II: BRS Carmem and Bordô (*Vitis labrusca L.*). Food Res Int 76:697–708
19. International Organisation of Vine and Wine (OIV) (2022) Compendium of international methods of wine and must analysis. Paris, France

Chapter 5

Color Indexes: Traditional and Advanced Methods

Carolina Pavez Moreno, Natalia Brossard, and Edmundo Bordeu

Abstract

Color is a relevant wine sensory feature easily perceived by wine consumers and directly associated with its quality. Both red and white wines present the polyphenols as the primary chemical compound responsible for wine color. In red wines, the principal component responsible for their color is the anthocyanin located in the vacuole of the cells in the grape berry skin. On the other hand, the characteristic pale-yellow color in white wines is due to enzymatic reactions affecting polyphenolic compounds such as hydroxycinnamic acids, flavanols, and flavonols, followed by non-enzymatic browning produced by oxidation reactions. Due to the importance of color for wine quality, its management and control are crucial for the wine industry. Usually, wine color is determined using two different spectrophotometric approaches, measurement of color density, and description of color in the CIELAB space, which is detailed in the present chapter. Color density measurement is the most widely used method and is recommended by the International Organization of Vine and Wine (OIV).

Key words Wine, Color, CIELAB, Spectrophotometry, Polyphenols

1 Introduction

Color is a relevant wine feature of red wines primarily associated with quality by wine consumers [1]. In red wines, the principal component responsible for their color is a class of polyphenols compounds known as anthocyanins [2–4]. Anthocyanins are secondary metabolites responsible for the color of several fruits, vegetables, and derived products such as wine [5]. Normally, these compounds are located in the vacuoles of the epidermal cells of the grape berry skin. However, "teinturier" grapes are an exception because in these grapes it is possible to find anthocyanins in both skin and pulp [2, 6].

In the same way as white wines, their characteristic pale-yellow color is due to the presence of polyphenolic compounds. The phenolic composition of white wines depends on several factors, such as the grape variety, growth, climatic conditions, and winemaking practices [7]. For instance, pre-fermentation techniques

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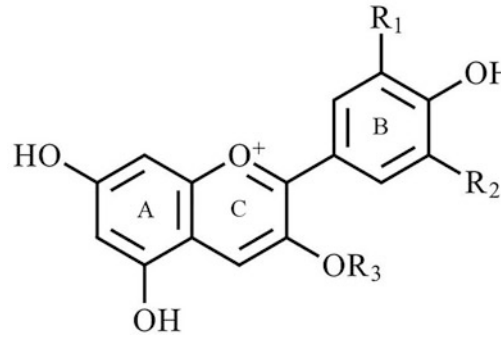

Fig. 1 Chemical structures of anthocyanidins (monomers) and anthocyanins (glycosylated form) in wines

such as skin maceration and pressing are among the most employed to enhance polyphenolic extraction [7, 8]. However, the polyphenolic content is lower compared with those reported in red wines and is composed primarily of hydroxycinnamic acids, flavanols, and flavonols [8]. The color of white wine begins to change in the early stages of winemaking via enzymatic reactions caused by the active polyphenol oxidase, in which hydroxycinnamic esters are crucial in these reactions.

After fermentation, non-enzymatic reactions occur that progressively change the initial pale-yellow color to brown, mediated by the oxidation reaction of hydroxycinnamates and flavanols (catechin and epicatechin). These originated compounds are yellow xanthylium salt pigments with maximum visible absorption at 440 and 460 nm [9]. However, the color of white wines is determined using 420 nm wavelength to avoid interference with the possible pinking of leucoanthocyanins [10].

Anthocyanins found in red wines produced by *Vitis vinifera* grapes are part of the family of flavonoid polyphenols. The flavonoids are divided into several classes according to the oxidation degree and substitution pattern of ring C [5] (Fig. 1). The principal monomeric anthocyanins present in wines are in their glucoside forms as 3-O-monoglucosides linked at the hydroxyl group of the C ring in the flavonoid chemical structure [1], and the most important are cyanidin, delphinidin, peonidin, petunidin, and malvidin [2, 11], and recently it was also found in *Vitis vinifera* a pelargonidin-3-O-glucoside in Cabernet Sauvignon and Pinot Noir skin berries (Table 1) [12].

These anthocyanins differ from each other in the substitution pattern of their chemical structure in the B ring of the flavonoid structure, which can directly affect the hue and color stability due to the effect on the delocalized electrons in the molecule [1]. Among the monomeric anthocyanins, malvidin-3-O-glucoside is the most abundant and the most responsible for the red color in young red wines, varying from more than 90% in Grenache and less

Table 1
Most important anthocyanidins and anthocyanins found in red wines

	Compound name	R_1	R_2	R_3
Anthocyanidins	Pelargonidin	H	H	H
	Cyanidin	OH	H	H
	Delphinidin	OH	OH	H
	Peonidin	OCH_3	OH	H
	Petunidin	OCH_3	OH	H
	Malvidin	OCH_3	OCH_3	H
Anthocyanins	Pelargonidin-3-*O*-glucoside	H	H	Glu
	Cyanidin-3-*O*-glucoside	OH	H	Glu
	Delphinidin-3-*O*-glucoside	OH	OH	Glu
	Peonidin-3-*O*-glucoside	OCH_3	OH	Glu
	Petunidin-3-*O*-glucoside	OCH_3	OH	Glu
	Malvidin-3-*O*-glucoside	OCH_3	OCH_3	Glu

Glu Glucose molecule

than 50% in Sangiovese wines. In this context, anthocyanin monomeric distribution is strongly dependent on the grape variety, growing conditions, and viticulture practices [13].

Anthocyanins exhibit a wide range of colors, that is, depending on their structure they can change with the pH from orange to blue. At very acidic conditions (pH ≈ 1), anthocyanins are present in their flavylium cation presenting a red color. When the pH of the solution rises between 3 and 4, the flavylium cation is involved in two parallel equilibrium reactions. One side is characterized by the deprotonation of the flavylium cation producing a violet quinoidal structure, and on the other side the hydration of the cation at the C-2 position occurs, yielding a non-colored hemiketal structure [1, 11, 14]. Furthermore, the addition of bisulfite can promote a reaction to form a colorless compound. This reaction of anthocyanins with bisulfite is known as bleaching [14] (Fig. 2).

According to the equilibrium reactions of anthocyanins, it would be expected that at wine pH these compounds were present mostly in their colorless form. However, the self-association of anthocyanins and other compounds present in wine can intensify and give stability to the color [15].

This association of anthocyanins with other compounds (intermolecular) and with themselves (intramolecular) is called copigmentation [16]. The copigmentation is a phenomenon that colored anthocyanins in their planar structures, such as the red flavylium or blue quinoidal base, interact with other planar species (i.e., B ring of the flavan-3-ol compound) through hydrophobic interactions [17, 18]. The color stability promoted by copigmentation is explained by the water and hydration protection of the flavylium cation, displacing the equilibrium at wine pH from the colorless hydrated form toward the red flavylium or blue quinoidal base [1, 16–19].

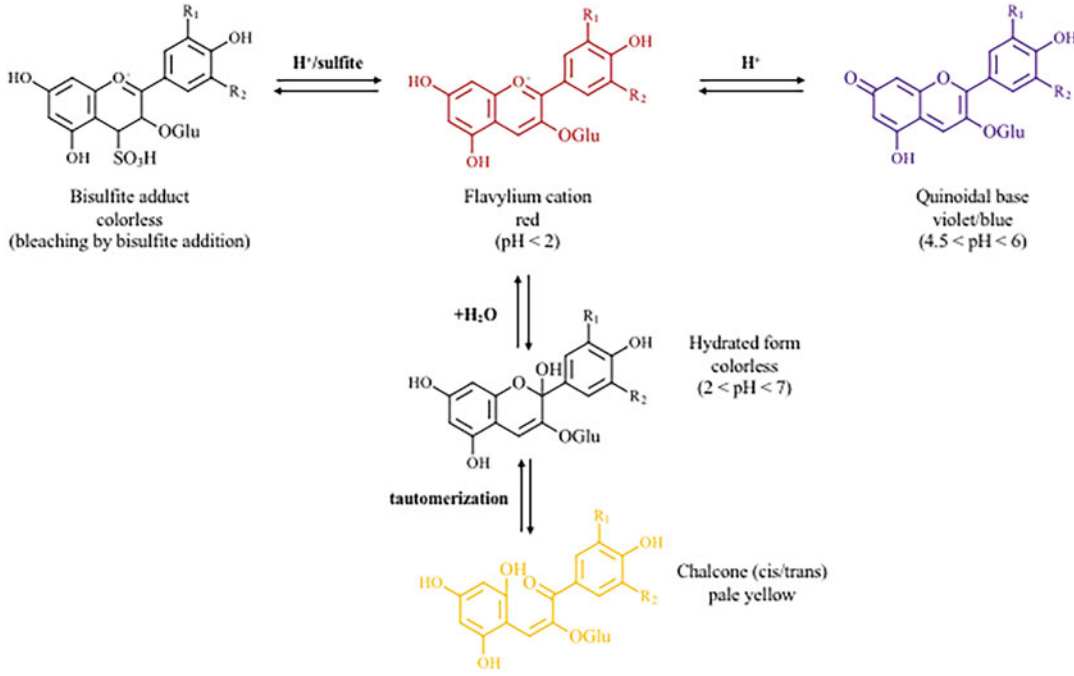

Fig. 2 Reaction of anthocyanins in wine with pH variations. Adapted from [1, 14, 17, 19] (AROUND HERE)

Among the reactions causing anthocyanin transformation, those that originate a new pyran ring in the resulting structure are very relevant for color stability, especially in aged red wines. These compounds known as pyranthocyanins are the resulting addition of acetaldehyde, pyruvic acid, vinyl phenol, vinyl catechol, vinyl guaiacol, acetone, and monomeric and dimeric procyanidins in the anthocyanin structure to the carbon in position 4 and the hydroxyl group in position 5, that cause a hypochromic shift in the visible absorption of anthocyanins, which produce a change in the wine color toward oranges hues [20]. The presence of a pyran ring confers high color stability since it prevents water and bisulfite addition at carbon C-4 that avoids the formation of the colorless structure of anthocyanins [21].

According to the International Organization of Vine and Wine (OIV), the chromatic features of wine are its luminosity and chromaticity. Luminosity depends on the transmittance and varies inversely with the intensity of the wine color. On the other hand, chromaticity depends on the dominant wavelength and purity that characterize the wine hue [22, 23]. Young red wines exhibit a maximum spectral absorption at a wavelength of 520 nm (red) and a minimum absorption at 420 nm (yellow). However, aged red wines exhibit the opposite behavior with maximums of absorption more shifted to 420 nm. For white wines the situation is

different, and they do not exhibit a defined spectral maximum of absorption. They just exhibit light absorption at 420 nm according to their color. In most wineries, the absorbance at different wavelengths, 420 nm (yellow), 520 nm (red), and 620 nm (blue), are used to characterize the wine color [23].

2 Materials

Spectrophotometer enables to perform measurements between 300 and 700 nm.

Glass cuvettes with an optical path adequate to give reasonable absorbances. For red wines, 0.1 cm is the most frequently used.

Polyvinylidene fluoride (PVDF) membranes of 0.45 μm.

Distilled water as the reference liquid.

3 Methods

3.1 Color Intensity

The color intensity or color density is a spectrophotometric method that describes the chromatic features conventionally expressed by the sum of absorbances of wavelengths at 420, 520, and 620 nm expressed at a 1 cm cuvette optical path. Additionally, the hue is expressed as the ratio of absorbance at 420 and 520 nm and represents the proportion of yellow and red in red wines [22, 23].

The method herein described is applicable to red and rosé wines, for white wines the color density is given by the absorbance determined at 420 nm, and the same is usually used to describe the oxidation degree in white wines [23].

3.1.1 Analytical Procedure

1. Filter all the samples using a cellulose acetate membrane of 0.45 μm to eliminate any particles in suspension.
2. Use distilled water as a reference liquid to set the blank on the absorbance scale of the spectrophotometer using a cuvette with the same optical path as the sample.
3. Read the absorbance of each sample at 420, 520, and 620 nm using an optical path adequate to give reasonable absorbance readings, ideally between 0.3 and 0.7 (1, 2, 5, 10, and 20 mm), and convert absorbance readings to what it would be if measured in a 10 mm cuvette.

The color intensity (I) is conventionally expressed by the sum of all absorbances:

$$I = A_{420} + A_{520} + A_{620}$$

On the other side, the hue N is given by:

$$N = \frac{A\ 420}{A\ 520}$$

According the OIV, both color intensity and hue results should be expressed using three decimal digits.

3.2 CIELAB

The Commission Internationale de L'Eclairage (CIE) proposed a method to determine the chromatic characteristics of wines and other food. The method that attempts to simulate the perception of color by real observers is based on the determination of tristimulus values, which established a nonlinear three-dimensional space with coordinates X, Y, and Z [24, 25]. The calculation of these coordinates is possible using the following expressions [26]:

$$X = k\sum_{\lambda} \tau\lambda\ S\lambda\ \overline{x}\lambda\Delta\lambda$$

$$Y = k\sum_{\lambda} \tau\lambda\ S\lambda\ \overline{y}\lambda\Delta\lambda$$

$$Z = k\sum_{\lambda} \tau\lambda\ S\lambda\ \overline{z}\lambda\Delta\lambda$$

where k is a normalization constant; τ_λ the spectral transmittance; S_λ the spectral emission of the illuminant chosen; $\overline{x}\lambda, \overline{y}\lambda, \overline{z}\lambda$ the color matching functions of the standard observed used; and $\Delta\lambda$ the measurement interval.

The colorimetric characteristics are defined by clarity (L) (L* = 0 black; L* = 100 colorless), the color component red/green (a^*) ($a^* > 0$ red; $a^* < 0$ green), and the yellow/blue color component (b^*) ($b^* > 0$ yellow; $b^* < 0$ blue). Additionally, there are the derived magnitudes chroma (C*) and hue (H*) [25, 27, 28]. The magnitude C* is the chromatics of a color object judged relative to the white, and on the other hand, H* is the attribute of appearance by which color is identified according to its similarities to red, yellow, green, blue, or a combination of two of these color parameters [29] (Fig. 3).

The official method proposed by the CIE [30] for determining the color of wine involves the measurement of the transmittance spectrum every 5 nm over the whole of the visible spectrum (380–780 nm) in specific conditions using a spectrophotometer [24, 26].

For the use of the CIELAB color space optimized in 1986, the CIE recommended performing the spectrophotometric measurements using a spectrophotometer with illuminant D65, instead of the original illuminant C. The reason for this optimization is that the illuminant D65 is a more appropriate representation of

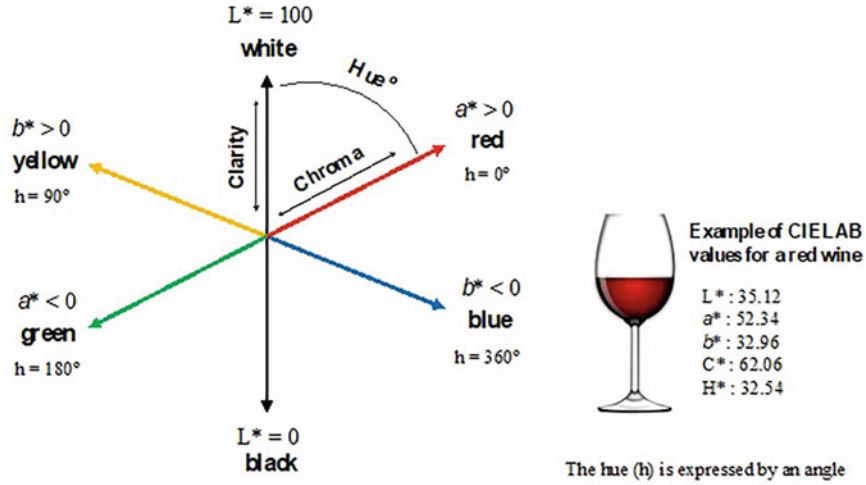

Fig. 3 CIELAB space. Adapted from [33, 34] (AROUND HERE)

daylight. Additionally, the visual field of the observer should be 10° instead of 2° of the original method [24, 31].

Further optimization of this model was proposed by Pérez-Caballero et al. (2003) [26] to reduce the data of the spectrum by the application of statistical characteristic vector analysis (CVA). This method allows the calculation of the CIELAB coordinates by the measurements of only four wavelengths ($A_{450\ nm}$, $A_{520\ nm}$, $A_{570\ nm}$, and $A_{630\ nm}$). The equation used to determine the X, Y, and Z coordinates are the following expressions using transmittance values (τ) at each wavelength [26]:

$$X = 19.717\tau_{450} + 1.884\tau_{520} + 42.539\tau_{570} + 32.474\tau_{630} - 1.841$$

$$Y = 7.950\tau_{450} + 34.764\tau_{520} + 42.736\tau_{570} + 15.759\tau_{630} - 1.180$$

$$Z = 103.518\tau_{450} + 4.190\tau_{520} + 0.251\tau_{570} + 1.831\tau_{630} - 0.818$$

To obtain the color coordinates L*, a^* and b^*, the tristimulus (X, Y, and Z) obtained are substituted in the following expressions [29]:

$$L^* = 116\left((Y/Y_{10})^{1/3} - 0.1379\right)$$

$$a^* = 500\left((X/X_{10})^{1/3} - (Y/Y_{10})^{1/3}\right)$$

$$b^* = 200\left((Y/Y_{10})^{1/3} - (Z/Z_{10})^{1/3}\right)$$

where X_{10}, Y_{10}, and Z_{10} are the tristimulus determined for the blank with D65 illuminant.

Finally, one of the principal advantages of the CIELAB method relies on the possibility to calculate the colorimetric difference (ΔE^*) between two colors, which makes this method a useful tool

for a direct and detailed comparison of wines with similar colors submitted to different winemaking processes.

3.2.1 Analytical Procedure

1. Filter all the samples using a cellulose acetate membrane of 0.45 μm to eliminate any particle in suspension.
2. Use distilled water as a reference liquid to set the zero on the absorbance scale of the spectrophotometer.
3. Read the absorbance of each sample at the wavelength required by the CIELAB approximation used between 380 and 780 nm using an optical path (b) of 0.2 cm for red and rosé wines and 1 cm for white wines [32].
4. Use the equations described above to calculate each CIELAB parameters or a suitable computer software to apply the CIELAB method.

References

1. Fernandes A, Oliveira J, Teixeira N, Mateus N, De Freitas V (2017) A review of the current knowledge of red wine colour. Oeno One 51(1):1–15
2. Nel AP (2018) Tannins and anthocyanins: from their origin to wine analysis – a review. S Afr J Enol Vitic 39(1):1–20
3. Lorenzo C, Pardo F, Zalacain A, Alonso GL, Salinas MR (2005) Effect of red grapes co-winemaking in polyphenols and color of wines. J Agric Food Chem 53(19):7609–7616
4. Sacchi KL, Bisson LF, Adams DO (2005) A review of the effect of winemaking techniques on phenolic extraction in red wines. Am J Enol Vitic 56(3):197–206
5. Soares S, Brandão E, Mateus N, de Freitas V (2017) Sensorial properties of red wine polyphenols: astringency and bitterness. Crit Rev Food Sci Nutr 57(5):937–948
6. Flamini R, Mattivi F, De Rosso M, Arapitsas P, Bavaresco L (2013) Advanced knowledge of three important classes of grape phenolics: Anthocyanins, stilbenes and flavonols. Int J Mol Sci 14(10):19651–19669
7. Ferreira-Lima NE, Burin VM, Caliari V, Bordignon-Luiz MT (2016) Impact of pressing conditions on the phenolic composition, radical scavenging activity and glutathione content of Brazilian Vitis vinifera white wines and evolution during bottle ageing. Food Bioprocess Technol 9(6):944–957
8. Gawel R, Day M, Van Sluyter SC, Holt H, Waters EJ, Smith PA (2014) White wine taste and mouthfeel as affected by juice extraction and processing. J Agric Food Chem 62(41): 10008–10014
9. Kanavouras A, Coutelieris F, Karanika E, Kotseridis Y, Kallithraka S (2020) Colour change of bottled white wines as a quality indicator. Oeno One 54(3):543–551
10. Morozova K, Schmidt O, Schwack W (2014) Wine oxidation and the role of cork. Eur Food Res Technol 238:20–52
11. Lapidot T, Harel S, Akiri B, Granit R, Kanner J (1999) pH-dependent forms of red wine anthocyanins as antioxidants. J Agric Food Chem 47(1):67–70
12. He F, He JJ, Pan QH, Duan CQ (2010) Mass-spectrometry evidence confirming the presence of pelargonidin-3-O-glucoside in the berry skins of cabernet sauvignon and pinot noir (*Vitis vinifera L.*). Aust J Grape Wine Res 16(3):464–468
13. Monagas M, Bartolomé B (2009) Anthocyanins and anthocyanin-derived compounds. In: Moreno-Arribas MV, Carmen Polo M (eds) Wine chemistry and biochemistry. Springer, New York
14. Cheynier V, Dueñas-Paton M, Salas E, Maury C, Souquet JM, Sarni-Manchado P et al (2006) Structure and properties of wine pigments and tannins. Am J Enol Vitic 57(3): 298–305
15. Baiano A, Scrocco C, Sepielli G, Del Nobile MA (2016) Wine processing: a critical review of physical, chemical, and sensory implications of innovative vinification procedures. Crit Rev Food Sci Nutr 56(14):2391–2407

16. Fernandes A, Brás NF, Mateus N, De Freitas V (2015) A study of anthocyanin self-association by NMR spectroscopy. New J Chem 39(4): 2602–2611
17. Trouillas P, Sancho-Garcia JC, De Freitas V, Gierschner J, Otyepka M, Dangles O (2016) Stabilizing and modulating color by copigmentation: insights from theory and experiment. Chem Rev 116(9):4937–4982
18. Escribano-Bailon MT, Santos-Buelga C (2012) Anthocyanin copigmentation-evaluation, mechanisms and implications for the colour of red wines. Curr Org Chem 16(6):715–723
19. He F, Liang NN, Mu L, Pan QH, Wang J, Reeves MJ et al (2012) Anthocyanins and their variation in red wines I. Monomeric anthocyanins and their color expression. Molecules 17(2):1571–1601
20. Alcalde-Eon C, Escribano-Bailón MT, Santos-Buelga C, Rivas-Gonzalo JC (2006) Changes in the detailed pigment composition of red wine during maturity and ageing: a comprehensive study. Anal Chim Acta 563(1–2): 238–254
21. Pina F, Oliveira J, De Freitas V (2015) Anthocyanins and derivatives are more than flavylium cations. Tetrahedron 71(20):3107–3114
22. International Organization of Vine and Wine (OIV) (2009) Compendium of international methods of analysis – OIV chromatic characteristics method OIV-MA-AS2-07B: R2009. Compend Int Methods Anal 1–10
23. Bordeu E, Scarpa J (1998) Análisis químico del vino. Chile Ediciones Pontif Univ Católica Chile, Santiago
24. Pérez-Magariño S, González-Sanjosé ML (2003) Application of absorbance values used in wineries for estimating CIELAB parameters in red wines. Food Chem 81(2):301–306
25. Aleixandre-Tudo JL, Buica A, Nieuwoudt H, Aleixandre JL, Du Toit W (2017) Spectrophotometric analysis of phenolic compounds in grapes and wines. J Agric Food Chem 65(20): 4009–4026
26. Pérez-Caballero V, Ayala F, Echávarri JF, Negueruela AI (2003) Proposal for a new standard OIV method for determination of chromatic characteristics of wine. Am J Enol Vitic 54(1):59
27. Rolle L, Guidoni S (2007) Color and anthocyanin evaluation of red wine grapes by CIE L*, a*, b* parameters. J Int des Sci la Vigne du Vin 41(4):193–201
28. Negueruela AI, Echavarri JF, Perez MM (1995) A study of correlation between Enological colorimetric indexes and Cie colorimetric parameters in red wines. Am J Enol Vitic 46(3): 353–356
29. Ayala F, Echávarri JF, Negueruela AI (1997) A new simplified method for measuring the color of wines. I Red and rose wines. Am J Enol Vitic 48(3):357–363
30. de Léclarage CI (2004) CIE 15 technical report Colorimetry, Viena
31. Martínez JA, Melgosa M, Pérez MM, Hita E, Negueruela AI (2001) Visual and instrumental color evaluation in red wines. Food Sci Technol Int 7(5):439–444
32. Ayala F, Echávarri JF, Negueruela AI (1997) A new simplified method for measuring the color of wines. III White wines and brandies. Am J Enol Vitic 48(3):364–369
33. Iñiguez M, Ortega AP, Rosales A, Orive RA, Puras P (1995) Estudio de color de los vinos tintos de la DOC Rioja. Zubía 7:167–186
34. Miranda JC (2018) Clasificación automática de naranjas por tamaño y por defectos utilizando técnicas de visión por computadora. Universidad Nacional de Asunción, San Lorenzo

Chapter 6

Anthocyanin Identification and Quantitation by High Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

Maurício Bonatto Machado de Castilhos, Sergio Gómez-Alonso, and Esteban García-Romero

Abstract

Anthocyanins are considered the principal agent for wine's intense color response for the first sensory sight assessed by the consumers, the appearance. Most grapes produce five different anthocyanin classes, each presenting different chromatic attributes and oxygen predispositions since they present two phenolic rings and one heterocyclic pirano ring, determining high chemical instability. Red wines maintain their intense red color with high stability due to reactions that occur between anthocyanins (self-association), copigments, polymerization with other flavonoids, and synthesis of pyranoanthocyanins. Wines produced from *Vitis vinifera* grapes produce anthocyanin monoglucosides, and wines produced with non-*Vitis vinifera* or hybrid grapes produce mono- and diglucosidic anthocyanins. Monoglucosidic anthocyanins guarantee higher color intensity; however, diglucosidic anthocyanins provide higher chemical stability. This chapter covers analytical methods of anthocyanin identification and quantitation using spectrophotometric and high-performance liquid chromatography coupled to a diode array detector in tandem with electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS/MS).

Key words Phenolic compounds, Anthocyanin, Liquid chromatography, Mass spectrometry, Spectrophotometry, Wines, Grapes

1 Introduction

Anthocyanins are considered the principal source of a red wine's color and most grapes produce five different classes of anthocyanins each presenting different chromatic attributes and oxygen predispositions. The principal anthocyanidins found in wines are cyanidin, peonidin, delphinidin, petunidin, and malvidin, and the differences among them are determined by the different radicals linked to the aglycon (Table 1), which is composed of two phenolic rings A and B and a heterocyclic pyran ring C (Fig. 1). The color of

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Table 1
Principal anthocyanidin found in grapes and wines

Anthocyanidin	Chemical Substituents
Cyanidin	$R_1 = R_2 = H$
Peonidin	$R_1 = OH; R_2 = H$
Delphinidin	$R_1 = R_2 = OH$
Petunidin	$R_1 = OH; R_2 = OCH_3$
Malvidin	$R_1 = OCH_3; R_2 = OCH_3$

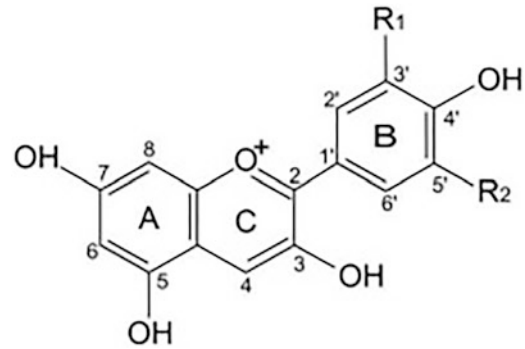


Fig. 1 Chemical structure of anthocyanin aglycone. (AROUND HERE) [2]

wine primarily depends on the grape anthocyanin composition regarding their different types and concentration [1].

Free anthocyanins have no chemical stability; however, their self-association, combination with copigments, polymerization with other flavonoids or their reaction with other wine components, and the formation of pyranoanthocyanins give them chemical stability and relative color stability for wine [1]. Other sources of wine pigmentation can be formed by oxidation and polymerization between grape and oak-derived flavonoids and the relative role of each one of these pigments is still controversial since there are analytical difficulties in quantitating these multiple forms synthesized by the reaction of several color compounds [3].

The differences noticed regarding the anthocyanin profile in grapes and wines are primarily explained by the yeast strain used in the alcoholic fermentation process [4], viticultural practices and vine management [5], clarification and stabilization procedures [6], different species of *Vitis* [7], and the winemaking process [8, 9]. Anthocyanins are also classified regarding the number of sugar molecules attached to the aglycone, that is, both mono- and diglucosidic anthocyanin can occur (Fig. 2). In summary, *Vitis vinifera* species produce only monoglucosidic anthocyanins since the gene responsible for synthesizing the diglucosidic form is

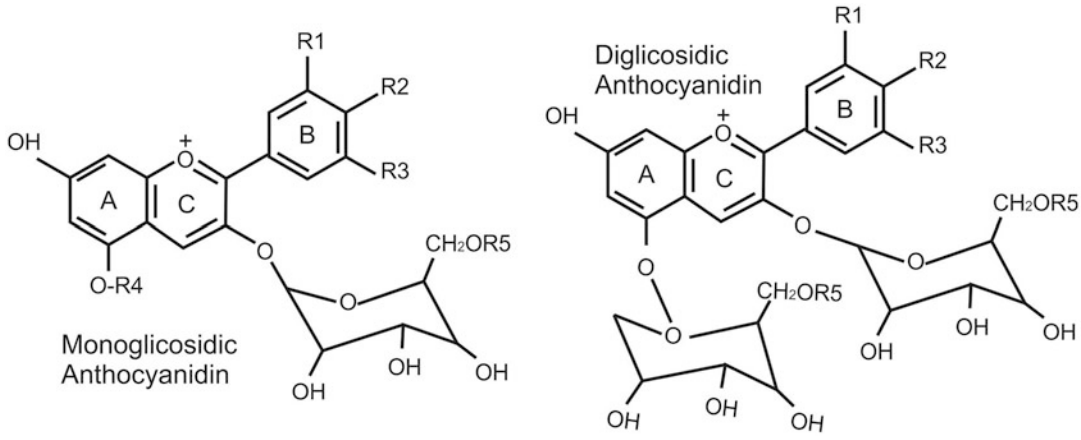


Fig. 2 Mono- and diglucosidic anthocyanidin chemical structures. (AROUND HERE)

defective [1, 10]. *Vitis rotundifolia* produces only diglucosidic anthocyanins [11] and *Vitis labrusca* can produce mono- and diglucosidic anthocyanins, the latter also present in the anthocyanin profile of hybrid grapes. These hybrid grapes are usually denoted by a genetic cross between *Vitis vinifera* and *Vitis labrusca* grapes and they produce several combinations of mono- and diglucosidic anthocyanins [1]. Monoglucoside anthocyanins present higher color indexes when compared with diglucosidic anthocyanins; however, the latter present higher chemical stability and are more susceptible to browning [12].

Several methods have been developed to identify and quantitate anthocyanins in grape extracts and wines. Conventional methods, using specific chemical substances that react with anthocyanins using spectrophotometric approach, and advanced methods using high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS^n) were useful for anthocyanin identification and quantitation; however, the conventional methods are less accurate and precise than the advanced ones. This chapter aims at describing a conventional spectrophotometric method for anthocyanin quantitation and an advanced method using HPLC-MS^n for anthocyanin identification and quantitation.

2 Total and Non-bleaching Anthocyanins Using Spectrophotometry

This method determines the total and non-bleaching anthocyanins using the decolorization method by sodium bisulfite. This method is an adaptation of the method described by Ribéreau-Gayon et al. (2006) [13, 14]. The anthocyanins quantitation is performed by the spectrophotometric method based on simple absorbance measurements at the appropriate wavelength (520 nm). The analysis takes place in an acidic medium to ensure that all anthocyanins are in the flavylium cation structural form.

2.1 Chemicals

1. HCl solution 0.1% in ethanol (*see* **Note 1**).
2. HCl solution 2% in water (*see* **Note 2**).
3. Sodium bisulfite solution 15% in water (*see* **Note 3**).

2.2 Apparatus

1. Semi-analytical balance.
2. UV-vis spectrophotometer.
3. 10 mM cuvettes.
4. Vortex for reagents and samples homogenization.
5. Volumetric flask 1000 mL and 250 mL.
6. Test tubes.
7. Automatic pipette in the range of 100–1000 μL.

2.3 Method

1. First, an initial mixture containing 0.5 mL of wine (juice or grape or fruit extract), 0.5 mL of 0.1% HCl in ethanol, and 10 mL of 2% HCl must be made. This mixture should be homogenized using a vortex and then used for both total anthocyanins (TA) and non-bleaching anthocyanins (NBA) analyses.
2. Pipette 2.5 mL of the previous mixture into separate test tubes identified for the TA and NBA analyses, performed in triplicate.
3. Add 4.5 mL of water to the test tubes identified for TA analysis.
4. Add 1 mL of bisulfite solution and 3.5 mL of distilled water to the test tubes identified for NBA analysis.
5. For blank preparation, use a mixture of 2.5 mL of 2% HCl and 4.5 mL of distilled water.
6. The absorbances of the samples in the tubes will be measured against the blank at a wavelength of 520 nM in glass cuvettes of a 10 mM optical path.
7. For the standard curve construction, a chemical standard corresponding to the predominant anthocyanin in the sample under study is used. In grape extracts and wines produced from *Vitis vinifera* grapes, it is common to use malvidin-3-glucoside; when non-*Vitis vinifera* or hybrid grapes are used, it is common to use malvidin-3,5-diglucoside. A stock solution of 1000 mg/L must be prepared, and then four dilutions must be made to obtain the curve points. These dilutions will be used as the sample, and the same procedure described above may be performed. The absorbance data from the standard dilutions should be used to construct the curve. An example of a standard curve is given in Fig. 3 (*see* **Note 4**).

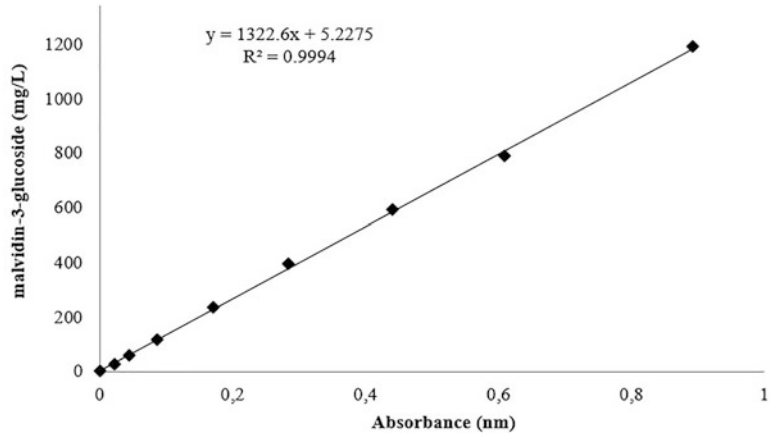


Fig. 3 Standard curve for malvidin-3-glucoside for spectrophotometric analysis

2.4 Calculation

The absorbance obtained for the sample (measured in triplicate) will be entered into the analytical expression obtained after building the standard curve (in mg equivalents of malvidin-3-glucoside per liter of the sample). Using the standard curve of malvidin-3-glucoside, we have the following equations, where *A1* and *A2* are the respective absorbances obtained for *TA* and *NBA* for each replicate:

– Total anthocyanins (mg/L of malvidin-3-glcucoside equivalents):

$$TA = (A1.1322.6) + 5.2275$$

– Non-bleaching anthocyanins (mg/L malvidin-3-glcucoside equivalent):

$$NBA = ((A1 - A2).1322.6) + 5.2275$$

3 Anthocyanin Identification and Quantitation Using HPLC-DAD-ESI-MS/MS

This method identifies and quantitates the anthocyanins present in wine and grape extracts using liquid chromatography coupled with mass spectrometry. The method is based on Castillo-Muñoz et al. (2009) [15]. Perform all procedures at room temperature unless otherwise specified. Solutions must be disposed of in accordance with all applicable waste disposal regulations.

3.1 Chemicals

1. Acetonitrile (CH_3CN), HPLC-MS grade used only for HPLC mobile phases.
2. Formic acid (HCOOH), HPLC-MS grade used only for HPLC mobile phases.

3. Milli-Q water.
4. Commercial standards of anthocyanins: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-glucuronide, peonidin 3-galactoside, peonidin 3-glucoside, peonidin 3-glucuronide, delphinidin 3-galactoside, delphinidin 3-glucoside, delphinidin 3-glucuronide, petunidin 3-galactoside, petunidin 3-glucoside, petunidin glucuronide, malvidin 3-galactoside, malvidin 3-glucoside, malvidin 3-glucuronide, cyanidin-3,5-diglucoside, peonidin-3,5-diglucoside, delphinidin-3,5-diglucoside, petunidin-3,5-diglucoside, malvidin-3,5-diglucoside.

3.2 Solutions and Solvents

1. Solvent A: 88.5% Milli-Q water, 3% Acetonitrile, 8.5% Formic acid.
2. Solvent B: 41.5% Milli-Q water, 50% Acetonitrile, 8.5% Formic acid.
3. HCl 0.1 N for dilution.

3.3 Apparatus

1. High performance liquid chromatography system couple to photodiode array detector and electrospray-ion trap mass spectrometer (HPLC-DAD-ESI-MS).
2. HPLC vials, 2 mL.
3. Reversed-phase column C18, 2.1 × 150 mM, 3.5 μM particle.
4. Syringes, 5 mL.
5. Syringe filters, polyester membrane with 0.20 μM pore size.
6. Ultrasonic bath.
7. Ultrasonic homogenizer.

3.4 Sample Preparation

1. Dilute the wine sample with HCl 0.1 N to proceed with analysis (*see* **Note 5**).
2. Filter the sample using a syringe filter with a 0.20 μM pore size and polyester membrane.
3. Put filtered samples in an HPLC vial.
4. Store samples at −20 °C until HPLC analysis.

3.5 Analysis of Anthocyanins by High Performed Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

1. Remove gas from solvents A and B with an ultrasonic bath for 1 min before using them as mobile phases of the HPLC system.
2. Inject a volume of 10 μL of wine sample on a reversed-phase column C18 2.1 × 150 mM, 3.5 μm particle at 40 °C with a flow rate of 0.19 mL/min, connected to the guard column, when the HPLC system is equilibrated, and the stable baseline is achieved. Perform a blank run to ensure proper equilibration of the column.

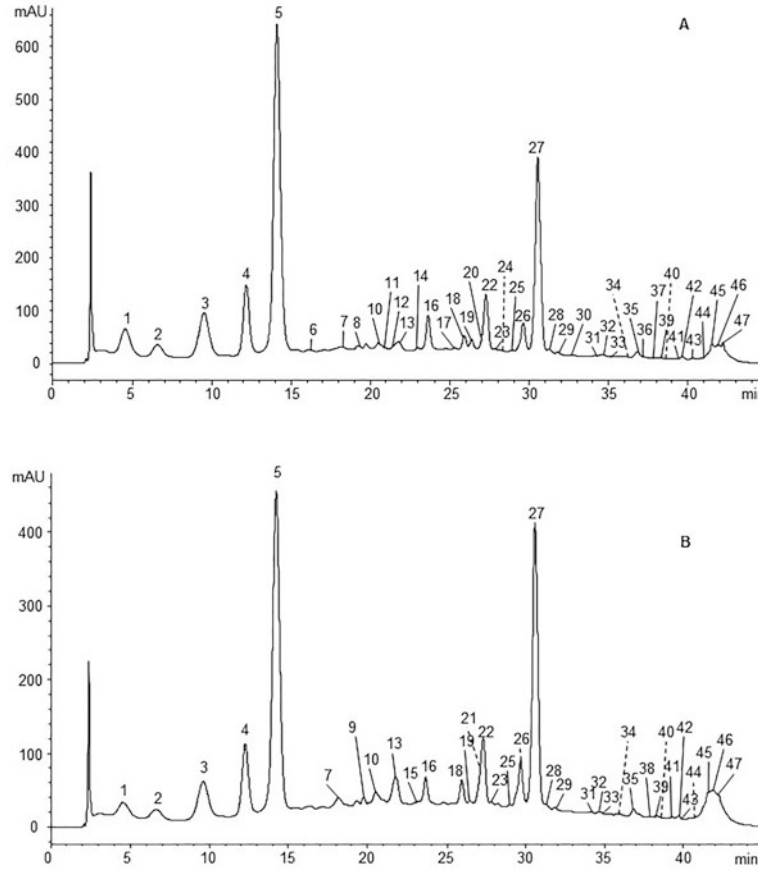


Fig. 4 HPLC–DAD chromatogram (detection at 520 nM) corresponding to anthocyanins identified in red wines. For peak assignment see Table 2. (**a**) Bordô wine; (**b**) BRS Carmem wine [9]

3. Use a linear solvent gradient for anthocyanin analysis under the following conditions: zero min (97% solvent A, 3% solvent B), 20 min (72% solvent A, 28% solvent B), 34 min (57% solvent A and 43% solvent B), 36 min (0% solvent A and 100% solvent B), 42 min (0% solvent A and 100% solvent B), 44 min (97% solvent A and 3% solvent B).
4. Set the detection wavelength at 520 nM and record UV-vis spectra at 250–650 nM.
5. Set the following parameters for the electrospray-ion trap mass spectrometer working in MS/MS mode: positive ionization mode, dry gas (N_2, 8 L/min), drying temperature (325 °C), nebulizer, (N_2, 50 psi), capillary (3500 V), skimmer 1 (−20 V), skimmer 2 (−60 V), scan range (100–1000 m/z). These parameters have been optimized for an ion trap with electrospray ionization model G2445C VL (Agilent) and should be optimized for other mass spectrometry detectors.

Table 2
Chromatographic and spectroscopic data of anthocyanins identified in grapes and wine [9]

Anthocyanins and Pyranoanthocyanins	Peak[a]	R_t (min)	Molecular Ion; Product Ions (m/z)
Anthocyanins			
Dp-3,5diglc	1	4.5	627;465,303
Cy-3,5diglc	2	6.5	611;449,287
Pt-3,5diglc	3	9.5	641;479,317
Pn-3,5diglc	4	12.1	625;463,301
Mv-3,5diglc	5	14.0	655;493,331
Pn-3glc	6	16.1	463;301
Pt-3acglc-5glc	7	18.2	683;521,479,317
Mv-3glc	8	19.2	493;331
Pn-3acglc-5glc	10	20.4	667;505,463,301
Mv-3acglc-5glc	13	21.7	697;535,493,331
cis-Pt-3cmglc-5glc	14	22.9	787;625,479,317
Dp-3cmglc-5glc	16	23.6	773;611,465,303
cis-Pn-3cmglc-5glc	17	25.3	771;609,463,301
Cy-3cmglc-5glc	18	25.8	757;595,449,287
cis-Mv-3cmglc-5glc	19	26.3	801;639,493,331
Mv-3cfglc-5glc	21	27.2	817;655,493,331
trans-Pt-3cmglc-5glc	22	27.4	787;625,479,317
trans-Pn-3cmglc-5glc	26	29.6	771;609,463,301
trans-Mv-3cmglc-5glc	27	30.5	801;639,493,331
Mv-3cmglc	34	35.9	639;331
Pyranoanthocyanins			
10H-pyrpt-3glc	9	19.8	503;341
10HP-pyrcy-3cfglc	11	20.9	727;565,403
10-carboxy-pyrmv-3glc (vitisin A)	12	21.3	561;399
10-carboxy-pyrmv-3acglc (ac-vitisin A)	15	23.0	603;399
10-methyl pyrdp-3glc	20	26.8	503;341
10-carboxy-pymv-3cmglc (cm-vitisin A)	23	27.7	707;399
10-methyl-pyrmv-3glc	24	28.3	531;369
10HP-pyrdp-3glc	25	28.9	581;419
10DHP-pyrdp-3cmglc	28	31.2	743;435

(continued)

Table 2
(continued)

Anthocyanins and Pyranoanthocyanins	Peak[a]	R_t (min)	Molecular Ion; Product Ions (m/z)
10DHP-pyrpt-3glc	29	31.7	611;449
10HP-pyrcy-3glc	30	32.8	565;403
10DHP-pyrpt-3acglc	31	34.1	653;449
10HP-pyrpt-3glc	32	34.6	595;433
10HP-pyrdp-3cmglc	33	34.8	727;419
10DHP-pyrmv-3glc	35	36.7	625;463
10DHP-pyrpt-3cmglc	36	37.5	757;449
10DPH-pyrpn-3glc	37	37.7	595;433
10HP-pyrpt-3acglc	38	38.0	637;433
10HP-pyrpn-3glc	39	38.2	579;417
10HP-pyrcy-3cmglc	40	38.6	711;403
10DHP-pyrmv-3acglc	41	39.5	667;463
10HP-pyrmv-3glc	42	39.6	609;447
10HP-pyrpt-3cmglc	43	40.3	741;433
10HP-pyrpn-3acglc	44	41.1	621;417
10DHP-pyrmv-3cmglc	45	41.5	771;463
10HP-pyrpn-3cmglc and 10HP-pyrmv-3acglc	46	41.9	725/651;417/447
10HP-pyrmv-3cmglc	47	42.2	755;447

Abbreviations: *Dp* delphinidin, *Cy* cyanidin, *Pt* petunidin, *Pn* peonidin, *Mv* malvidin, *3,5-diglc* 3,5-diglucosides, *3-acglc-5-glc* 3-(6″-acetyl)-glucoside-5-glucoside, *3-cmglc-5-glc* 3-(6″-*p*-coumaroyl)-glucoside-5-glucoside, *3-glc* 3-glucoside, *3-acglc* 3-(6″-acetyl)-glucoside, *3-cmglc* 3-(6″-*p*-coumaroyl)-glucoside, *10-HP* 10-(3″-hydroxyphenyl), *10-DHP* 10-(3′,4″-dihydroxyphenyl), *pyrdp* pyranodelphinidin, *pyrcy* pyranocyanidin, *pyrpt* pyranopetunidin, *pyrpn* pyranopeonidin, *pyrmv* pyranomalvidin, *ac* acetyl, *cm* coumaroyl
[a]Peak numbers used in Fig. 4. *Rt* retention time

6. Identify anthocyanins based on spectroscopic data obtained from commercial standards and reported in the literature (Fig. 4) (Table 2) [9, 16].
7. Quantitate these compounds using DAD-chromatograms extracted at 520 nM. Prepare the calibration curve for each compound from the commercial standards in concentrations ranging from 1 to 300 mg/L using HCl solution 0.1 N (*see* **Note 6**).

4 Notes

1. It should be remembered that hydrochloric acid (HCl) is commonly available in 37%. For example, for the preparation of 250 mL of solution, 0.68 mL of HCl is required to be made up in a 250 mL volumetric flask with ethanol.
2. Using 37% HCl, to prepare 1000 mL of solution, 54.1 mL of HCl is required to complete in a 1000 mL volumetric flask with distilled water.
3. Dissolve 15 g of sodium bisulfite in distilled water and complete in a 100 mL volumetric flask.
4. The ideal situation is running a new standard curve with malvidin-3-glucoside for every new analysis. Figure 3 is an example for better understanding.
5. The dilution factor depends on the wine anthocyanin concentration and usually it can vary from 1:2 (1 part of wine and 1 part of HCl 0.1 N) to 1:5 (1 part of wine and 4 parts of HCl 0.1 N).
6. Prepare solutions for the different points of the calibration curves by successive dilutions from a single stock solution using HCl 0.1 N. In some cases, it is necessary to add a variable percentage of methanol/ethanol to increase the anthocyanin solubilization.

References

1. Jackson RS (2020) Wine science: principles and applications. Academic Press, San Diego
2. De Castilhos MBM, Del Bianchi VL (2021) Vinhos. In: Moraes IO (ed) Biotecnologia Industrial: Biotecnologia na Produção de Alimentos. Blucher, São Paulo
3. Versari A, Boulton RB, Parpinello GP (2008) A comparison of analytical methods for measuring the color components of red wines. Food Chem 106:397–402
4. Božič JT, Butinar L, Albreht A, Vovk I, Korte D, Vodopivec BM (2020) The impact of saccharomyces and non-saccharomyces yeasts on wine colour: a laboratory study of vinylphenolic pyranoathocyanin formation and anthocyanin cell wall adsorption. LWT 123:109072
5. Cebrián-Tarancón C, Fernández-Roldán F, Sánchez-Gómez R, Alonso GL, Salinas MR (2022) Pruned vine-shoots as a new enological additive to differentiate the chemical profile in wines. Food Res Int 156:111195
6. Diblan S, Özkan M (2021) Effect of various clarification treatments on anthocyanins, color, phenolics and antioxidant activity of red grape juice. Food Chem 352:129321
7. Ju Y, Yang L, Yue X, Li Y, He R, Deng S, Yang X, Fang Y (2021) Anthocyanin profiles and color properties of red wines made from *Vitis davidii* and *Vitis vinifera* grapes. Food Sci Hum Wellness 10(3):335–344
8. De Castilhos MBM, Del Bianchi VL, Gómez Alonso S, García-Romero E, Hermosín-Gutiérrez I (2019) Sensory descriptive and comprehensive GC-MS as suitable tools to characterize the effects of alternative winemaking procedures on wine aroma. Part I: BRS Carmem and BRS Violeta. Food Chem 272: 462–470
9. De Castilhos MBM, Corrêa OLS, Zanus MC, Garcia Maia JD, Gómez-Alonso S, García-Romero E, Del Bianchi VL, Hermosín-Gutiérrez I (2015) Pre-drying and submerged cap winemaking: effects on polyphenolic compounds and sensory descriptors. Part II: BRS

Carmem and Bordô (*Vitis labrusca L.*). Food Res Int 76:697–708

10. Acevedo A, Hilbert G, Rivière C, Mengin V, Ollat N, Bordenave L et al (2012) Anthocyanin identification and composition of wild *Vitis* spp. accessions by LC-MS and LC-NMR. Anal Chim Acta 732:145–152
11. Zhu L, Zhang Y, Deng J, Li H, Lu J (2012) Phenolic concentrations and antioxidant properties of wines made from North American grapes grown in China. Molecules 17:3304–3323
12. Robinson WB, Weirs LD, Bertino JJ, Mattick LR (1966) The relation of anthocyanin composition to color stability of New York state wines. Am J Enol Vitic 17:178–184
13. Ribéreau-Gayon P, Glories Y, Maujean A, Dubourdieu D (2006) Handbook of enology: the chemistry of wine – stabilization and treatments. Wiley, New York
14. Ribéreau-Gayon P, Stonestreet E (1965) Determination of anthocyanins in red wine. Bull Soc Chim Fr 9:2649–2652
15. Castillo-Muñoz N, Fernández-González M, Gómez-Alonso S, García-Romero E, Hermosín-Gutiérrez I (2009) Red-color related phenolic composition of Garnacha Tintonera (Vitis vinifera L.) grapes and red wines. J Agric Food Chem 57:7883–7891
16. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2019) Phenolic compounds profile of different berry parts from novel Vitis vinifera L. red grape genotypes and Tempranillo using HPLC-DAD-ESI-MS/MS: a varietal differentiation tool. Food Chem 295: 350–360

Chapter 7

Flavonol Identification and Quantitation by High Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

Sergio Gómez-Alonso, Tania Paniagua-Martínez, and José Pérez-Navarro

Abstract

Flavonols are a class of phenolic compounds derived from plant secondary metabolism. In wine, these flavonoid compounds do not receive enough attention as other polyphenols such as anthocyanins and tannins; however, as its properties and structures are better understood, its importance for wine quality becomes more apparent. These compounds are involved in the copigmentation phenomena, enhancing the color intensity of younger red wines, and may also contribute to wine astringency. In addition, flavonols possess antioxidant activity and positive health benefits. High performance liquid chromatography, particularly tandem mass spectrometry, is a significant tool in the structural elucidation and quantitation of flavonoid derivatives. Thus, here we describe the methodology to be used for carrying out the flavanol analysis from grapes and wine by high performance liquid chromatography coupled to a diode array detector in tandem with electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS/MS).

Key words Phenolic compounds, Flavonol, Solid-phase extraction, Liquid chromatography, Mass spectrometry, Wine, Grapes

1 Introduction

Flavonols are a type of phenolic compound from the flavonoid group found in a wide range of plant foods, including grapes, where they are predominantly synthesized in berry skin. This class of compounds is present in glycoside form in grapes [1]. Therefore, the glycosylated structures can be found in wines with the corresponding free aglycones produced by acid and/or enzymatic hydrolysis during winemaking and aging.

In grapes and wines, the types of flavonoids are defined by differences in the oxidation state and the substitution of the C-ring. Similar to other flavonoids, the chemical backbone of flavonols is a three-ring system, that is, two benzene cycles (A and

Maurício Bonatto Machado de Castilhos (ed.), *Basic Protocols in Enology and Winemaking*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3088-4_7,

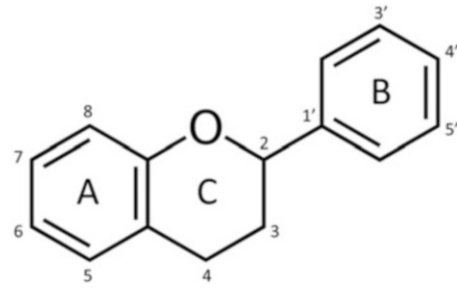
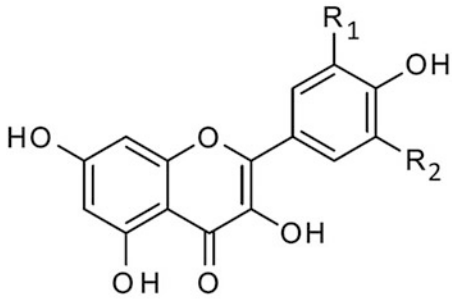


Fig. 1 Core ring system of flavonoidsFlavonoids. (AROUND HERE)



	R_1	R_2
Kaempferol	H	H
Quercetin	OH	H
Isorhamnetin	OCH_3	H
Myricetin	OH	OH
Laricitrin	OCH_3	OH
Syringetin	OCH_3	OCH_3

Fig. 2 Chemical structure of flavonolFlavonols aglycones

B-rings) on the right and left side of the central oxygen-containing cycle (C-ring) (Fig. 1) [2].

Flavonols have a keto group at position 4 and a hydroxyl group at position 3 of the C-ring, with the presence of a double bond between positions 2 and 3. The structure of these compounds changes depending on the number and type of substitutions on the B-ring. Only six flavonol aglycones are known in grapes and wine: myricetin, quercetin, kaempferol, laricitrin, isorhamnetin, and syringetin (Fig. 2); however, there is an immense diversity of their glycoside forms. Different sugars can be linked to the aglycone structure of the flavonol, for producing galactosides, glucuronides, glucosides, etc., and those derivatives containing acylated sugars [3, 4]. The flavonoids myricetin, laricitrin, and syringetin are missing in white grape varieties and, consequently, in white wines due to unexpressed enzyme flavonoid 3′,5′-hydroxylase in this type of grapes [5].

Flavonols are characterized by remarkable health-promoting properties. These bioactive compounds possess antioxidant activity, protect from oxidative stress, and ameliorate several chronic diseases. In addition, this ability allows for an increase in the stability and shelf life of foods such as wine and makes it possible to use flavonols as preservatives [6, 7]. The amount of flavonols is considered a quality marker for grapes and wine since these compounds are correlated with sun exposure, which affects the quality parameters [8]. This class of flavonoids is often forgotten; however, it

has a critical role in the protection of grapes during ripening, being involved in UV screening [9], and for the color stabilization of young red wines, providing a copigmentation effect with the flavylium form of anthocyanins [10]. Moreover, the role of flavonols in the sensory perception of wine has been also reported, increasing the astringency and bitterness [11].

Because of the great diversity of phenolic compounds from grape berries, musts, and wines, their analysis is considered a complicated task. Several techniques are used to determine the total concentration of phenolic compounds, for example, spectrophotometric analysis using the Folin–Denis and Folin–Ciocalteu methods [12, 13]. However, the employment of advanced analytical procedures is necessary, such as chromatographic techniques, to identify and quantitate individual phenolic compounds [14]. High performance liquid chromatography (HPLC) is the most applied method to determine phenolic compounds in food, specifically, in grapes and wine [15–17].

The first step to analyze phenolics is an extraction procedure that is influenced by the nature of the sample and chemical compound, particle size, solvents, and type of extraction technique employed. Liquid–liquid extraction or solid phase extraction (SPE) is generally required to separate and purify from their matrix different phenolic compounds before HPLC analysis, using solvents with different pH or polarity [18, 19]. Photodiode array and mass spectrometric detectors attached to high performance liquid chromatography are important tools in the identification of phenolic compounds, also giving valuable information regarding the structural elucidation and confirmation of different phenolic classes, for example, flavonols [20–22]. This chapter provides a robust protocol for the extraction, purification, and analysis of flavonols in grapes and wine, based on SPE and high performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS).

2 Materials

2.1 Chemicals

1. Acetonitrile (CH_3CN), HPLC-MS grade used only for HPLC mobile phases.
2. Ammonium hydroxide (NH_4OH), 20%, analytical reagent grade.
3. Commercial standards of flavonols: myricetin, myricetin 3-galactoside, myricetin 3-glucoside, myricetin 3-glucuronide, quercetin, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-glucuronide, quercetin 3-rutinoside, kaempferol 3-galactoside, laricitrin, laricitrin 3-glucoside, kaempferol 3-glucoside, kaempferol 3-glucuronide, kaempferol

3-rutinoside, isorhamnetin, isorhamnetin 3-galactoside, isorhamnetin 3-glucoside, isorhamnetin glucuronide, syringetin, syringetin 3-galactoside, syringetin 3-glucoside.

4. Ethanol (CH_3CH_2OH), 96% (v/v), analytical reagent grade.
5. Formic acid (HCOOH), 99%, analytical reagent grade.
6. Formic acid (HCOOH), HPLC-MS grade used only for HPLC mobile phases.
7. Hydrochloric acid (HCl), 37%, analytical reagent grade.
8. Methanol (CH_3OH), analytical reagent grade.
9. Methanol (CH_3OH), HPLC-MS grade used only for HPLC mobile phases.
10. Milli-Q water.

2.2 Solutions and Solvents

1. Solution A: 50% CH_3OH, 48.5% Milli-Q water, 1.5% HCOOH (v/v/v).
2. Solution B: 80% Milli-Q water, 20% CH_3OH (v/v).
3. Solution C: 80% CH_3OH, 20% Milli-Q water, containing 2% HCl (v/v).
4. Solution D: 80% CH_3OH, 20% Milli-Q water, containing 2% NH_4OH (v/v).
5. Solvent A: 3% CH_3CN, 88.5% Milli-Q water, 8.5% HCOOH (v/v/v).
6. Solvent B: 50% CH_3CN, 41.5% Milli-Q water, 8.5% HCOOH (v/v/v).
7. Solvent C: 90% methanol, 1.5% Milli-Q water, 8.5% HCOOH (v/v/v).

2.3 Equipment

1. Analytical balance.
2. C18 cartridges, silica-based bonded phase (500 mg).
3. C18 column guard.
4. Conical centrifuge tubes, 50 mL.
5. Freeze dryer.
6. Grinding mill.
7. Centrifuge.
8. High performance liquid chromatography system couple to photodiode array detector and electrospray-ion trap mass spectrometer (HPLC-DAD-ESI-MS).
9. HPLC vials, 2 mL.
10. Water bath.

11. Polymeric cation-exchange resin SPE cartridges (500 mg), which combines cation exchange with reverse phase properties and retains all phenolic compounds from grapes and wine.
12. Reversed-phase column C18, 2.1 × 150 mm, 3.5 μm particle.
13. Rotary evaporator.
14. Syringes, 5 mL.
15. Syringe filters, polyester membrane with 0.20 μm pore size.
16. Ultrasonic bath.
17. Ultrasonic homogenizer.
18. Vacuum manifold.

3 Methods

Perform all procedures at room temperature unless otherwise specified. Solutions must be disposed of in accordance with all applicable waste disposal regulations.

3.1 Phenolic Compound Extraction from Grapes

1. Select 100 healthy grapes and manually separate the different parts of berries (pulp, seeds, and skin), selecting the grape skins because flavonols are primarily found in this grape part.
2. Wash the selected skins in water and softly dry them by patting them between sheets of filter paper.
3. Store grape skins immediately at −20 °C for freeze-drying.
4. Freeze-dry the frozen grape skins for 24 h.
5. Crush the freeze-dried skins into a fine powder using a mill at 25,000 rpm for 10 s.
6. Weigh 1 g of grape skin powder into a conical centrifuge tube using an analytical balance.
7. Add 25 mL of solution A to the conical centrifuge tube with the fine powder.
8. Sonicate the prepared solution using an ultrasonic homogenizer at 4 °C for 3 min, with a duty cycle of 15 s turn on and 5 s off, 80% of output amplitude (*see* **Note 1**).
9. Centrifugate the obtained extract at 5000 rpm for 5 min.
10. Separate and filter the supernatant using glass wood placed in a funnel (*see* **Note 2**).
11. Re-extract the pellet one more time, following the procedure described above (*see* **Note 3**).
12. Join the clarified supernatants that constitute the phenolic compound extract and store it at −20 °C until analysis.

3.2 Flavonol Isolation

To isolate flavonols and obtain the anthocyanin-free fraction from grape skin extract of red grapes and red wine (*see* **Note 4**), use the following solid phase extraction procedure:

1. Reduce 3 mL of phenolic compound extract from grape skin to 1.5 mL in a rotary evaporator at 35 °C.
2. Dilute the concentrated extract with 1.5 mL of HCl 0.1 M.
3. Condition the polymeric cation-exchange resin with 5 mL of CH_3OH and 5 mL of Milli-Q water in the vacuum manifold (*see* **Note 5**).
4. Pass 3 mL of diluted extract slowly through SPE cartridges (*see* **Note 5**).
5. Wash the resin with 5 mL of HCl 0.1 M and 5 mL of Milli-Q water consecutively. Dry the resin at the end of this step (*see* **Note 6**).
6. Elute and collect the flavonol fraction with 6 mL of CH_3CH_2OH 96% by slowly passing, drop by drop (*see* **Note 7**).
7. In the case of red wines, dilute 3 mL of wine with 3 mL of HCl 0.1 M and follow the same procedure from **Step 3** to **Step 6**.
8. Regenerate the resin with 2 × 5 mL of solution D and 3 × 5 mL of solution C. Subsequent conditioning of the cartridge with Milli-Q water allows its reuse at least five more times.

In the case of skin extracts from white grapes, it is not necessary to remove anthocyanins from the extract, but some sugars or other unwanted substances may interfere with flavonol analysis. To fix this, the white grape skin extract is subjected to C18 SPE as follows:

1. Reduce 3 mL of phenolic compound extract from grape to a volume of 1.5 mL using a rotary evaporator at 35 °C.
2. Condition the C18 cartridge with 5 mL of CH_3OH and 5 mL of Milli-Q water consecutively (*see* **Note 5**).
3. Pass the concentrated extract slowly through the SPE cartridge (*see* **Note 5**).
4. Wash the C18 cartridge with 5 mL of Milli-Q water, drying the resin at the end of this step (*see* **Note 6**).
5. Elute and collect the sugar-free fraction of flavonols with 6 mL of CH_3CH_2OH 96% (*see* **Note 7**).

3.3 Sample Preparation

1. Dry the flavonol eluate in a rotary evaporator at 35 °C and redissolve in 1.5 mL of solution B.
2. For white wines, dry 2 mL in a rotary evaporator at 35 °C and reconstitute in 1 mL of solution B.

3. Filter the reconstituted sample using a syringe filter with a 0.20 μm pore size and polyester membrane.
4. Put filtered samples in HPLC vial.
5. Store samples at −20 °C until HPLC analysis.

3.4 Analysis of Flavonols by High Performed Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

1. Remove gas from solvents A, B, and C with an ultrasonic bath for 1 min before using them as mobile phases of the HPLC system.
2. Inject a volume of 20 μL for grape skin (red and white) and red wine samples, and 40 μL for white wine samples on a reversed-phase column C18 at 40 °C with a flow rate of 0.19 mL/min, connected to the guard column, when the HPLC system is equilibrated and the stable baseline is achieved. Perform a blank run to ensure proper equilibration of the column.
3. Use a linear solvent gradient for flavonol analysis, under the following conditions: zero min (96% solvent A, 4% solvent B), 8 min (96% solvent A, 4% solvent B), 37 min (70% solvent A, 17% solvent B, and 13% solvent C), 51 min (50% solvent A, 30% solvent B, and 20% solvent C), 51.5 min (30% solvent A, 40% solvent B, and 30% solvent C), 56 min (50% solvent B and 50% solvent C), 57 min (50% solvent B and 50% solvent C), and 64 min (96% solvent A and 4% solvent B).
4. Set the detection wavelength at 360 nm and record UV-vis spectra at 200–600 nm.
5. Set the following parameters for the electrospray-ion trap mass spectrometer working in MS/MS mode: negative ionization mode, dry gas (N_2, 8 L/min), drying temperature (350 °C), nebulizer, (N_2, 40 psi), capillary (3500 V), skimmer 1 (−20 V), skimmer 2 (−60 V), scan range (100–1000 m/z). These parameters have been optimized for an ion trap with electrospray ionization model G2445C VL (Agilent) and should be optimized for other mass spectrometry detectors.
6. Identify flavonols based on spectroscopic data obtained from commercial standards and reported in the literature (Figs. 3 and 4) (Table 1) [16, 23–25].
7. Quantitate these compounds using DAD-chromatograms extracted at 360 nm. Prepare the calibration curve for each compound from the commercial standards in concentrations ranging from 0.1 to 100 mg/L (*see* **Note 8**).

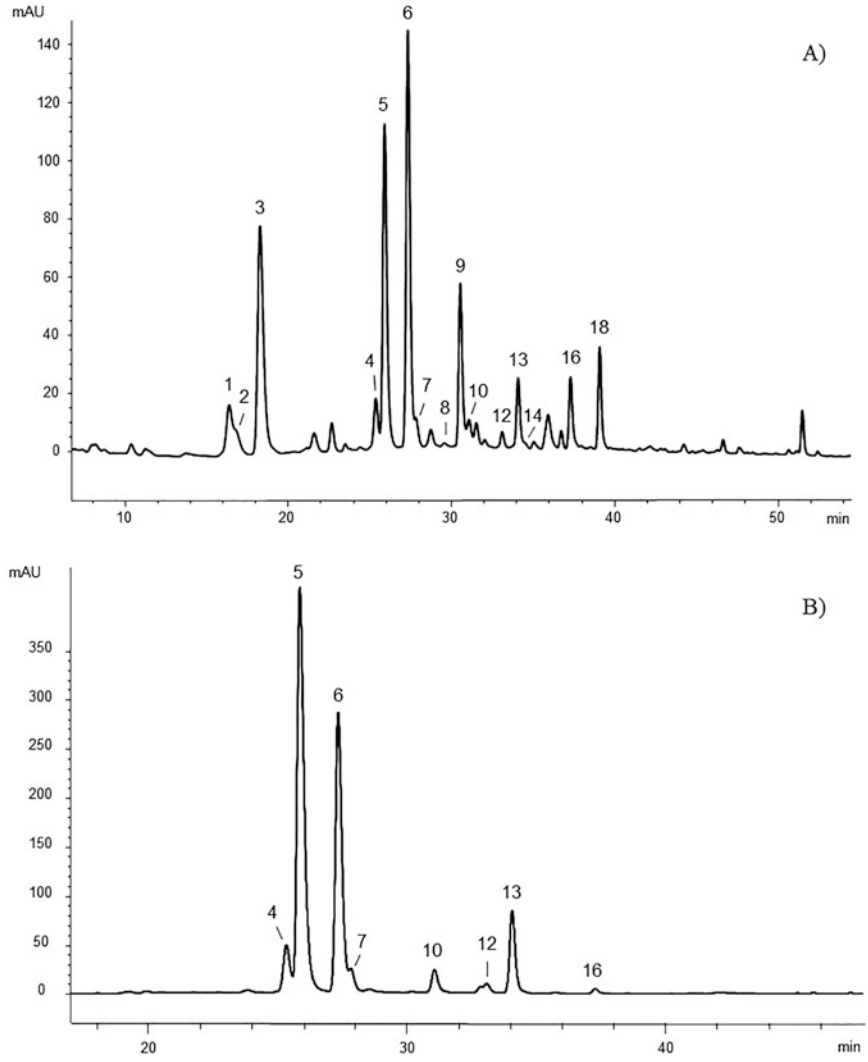


Fig. 3 HPLC-DAD chromatogram (detection at 360 nm) corresponding to flavonols identified in red (**a**) and white (**b**) grape skins. For peak assignment, see Table 1

4 Notes

1. Sample temperature increases significantly during ultrasound extraction of phenolic compounds. Thus, skins with the extraction solution must be kept in a water bath at 4 °C during this step to reduce the phenolic compound degradation by temperature.
2. To obtain a clear extract, the phenolic compound extract is filtered through a glass wood ball loosely stuffed in the narrow part of a funnel.

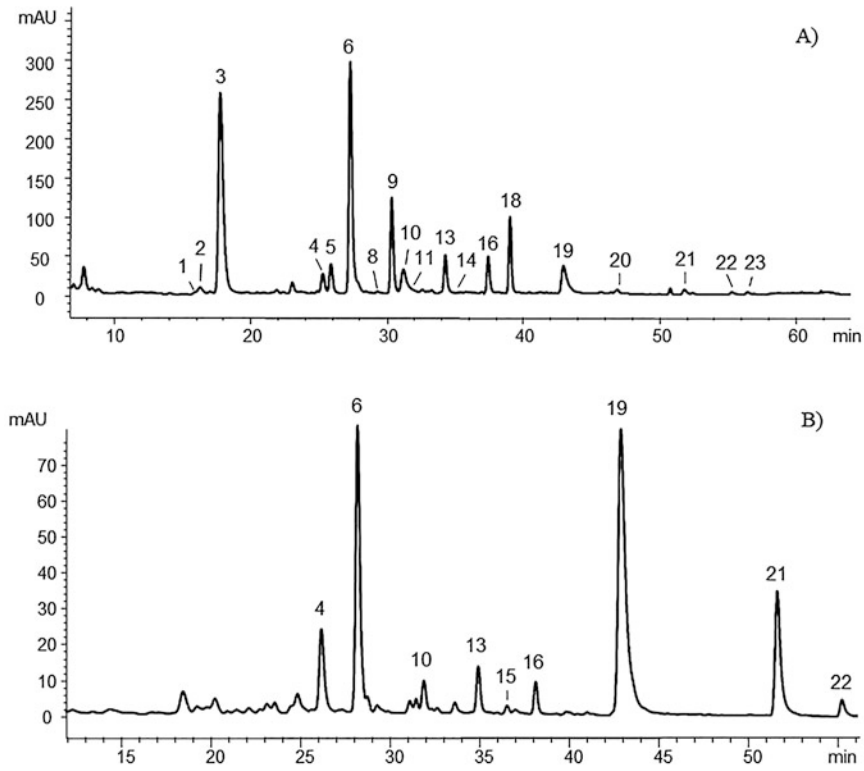


Fig. 4 HPLC-DAD chromatogram (detection at 360 nm) corresponding to flavonols identified in red (**a**) and white (**b**) wines. For peak assignment, see Table 1

3. A second extraction of skin pellets yields nearly 99% of grape phenolic compounds, as confirmed by spectrophotometry.
4. This isolation step is not necessary for white wines because these samples lack anthocyanins, which in red grapes and wines affect the identification and quantitation of the compounds of interest.
5. Do not let the resin dry at any time, only before the flavonol elution.
6. If there is any water left in the resin, it can hinder the drying process at the rotary evaporator.
7. Use CH_3CH_2OH instead of CH_3OH to elute the flavonol fraction and avoid the interference of susceptibility artifacts from the interaction of flavonols with CH_3OH. The same result is obtained using CH_3CH_2OH 96% or absolute.
8. Prepare solutions for the different points of the calibration curves by successive dilutions from a single stock solution. Use a mix of Milli-Q water and CH_3OH to prepare the stock solution by dissolving a properly standard reagent and making dilutions with Milli-Q water.

Table 1
Chromatographic and spectroscopic data of flavonols identified in grapes and wine

Peak[a]	R_t (min)	Compound	Molecular and Product Ions (m/z), Negative Ionization
1	16.32	Myricetin 3-glucuronide	493, 317
2	16.79	Myricetin 3-galactoside	479, 317
3	18.24	Myricetin 3-glucoside	479, 317
4	25.32	Quercetin 3-galactoside	463, 301
5	25.87	Quercetin 3-glucuronide	477, 301
6	27.32	Quercetin 3-glucoside	463, 301
7	27.82	Quercetin 3-rutinoside	609, 301
8	29.55	Laricitrin 3-galactoside	493, 331
9	30.51	Laricitrin 3-glucoside	493, 331
10	31.06	Kaempferol 3-galactoside	447, 285
11	31.30	Myricetin	317
12	33.10	Kaempferol 3-glucuronide	461, 285
13	34.09	Kaempferol 3-glucoside	447, 285
14	34.55	Kaempferol 3-rutinoside	593, 285
15	35.63	Isorhamnetin 3-galactoside	477, 315
16	37.28	Isorhamnetin 3-glucoside	477, 315
17	37.83	Syringetin 3-galactoside	507, 345
18	39.05	Syringetin 3-glucoside	507, 345
19	42.82	Quercetin	301
20	47.11	Laricitrin	331
21	51.71	Kaempferol	285
22	55.17	Isorhamnetin	315
23	56.28	Syringetin	345

Rt retention time
[a]Peak numbers used in Figs. 3 and 4

References

1. Price SF, Breen PJ, Valladao M et al (1995) Cluster sun exposure and quercetin in Pinot noir grapes and wine. Am J Enol Vitic 46: 187–194
2. Garrido J, Borges F (2013) Wine and grape polyphenols – a chemical perspective. Food Res Int 54:1844–1858
3. Castillo-Muñoz N, Gómez-Alonso S, García-Romero E et al (2007) Flavonol profiles of Vitis vinifera red grapes and their single-cultivar wines. J Agric Food Chem 55:992–1002
4. Favre G, González-Neves G, Piccardo D et al (2018) New acylated flavonols identified in Vitis vinifera grapes and wines. Food Res Int 112:98–107
5. Mattivi F, Guzzon R, Vrhovsek U et al (2006) Metabolite profiling of grape: flavonols

and anthocyanins. J Agric Food Chem 54: 7692–7702

6. De Beer D, Joubert E, Gelderblom WCA et al (2005) Antioxidant activity of South African red and white cultivar wines and selected phenolic compounds: in vitro inhibition of microsomal lipid peroxidation. Food Chem 90:569–577
7. Shahidi F, Ambigaipalan P (2015) Phenolics and polyphenolics in foods, beverages and spices: antioxidant activity and health effects – a review. J Funct Foods 18:820–897
8. Ritchey JG, Waterhouse AL (1999) A standard red wine: monomeric phenolic analysis of commercial cabernet sauvignon wines. Am J Enol Vitic 50:91–100
9. Haselgrove L, Botting D, van Heeswijck R et al (2000) Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of Vitis vinifera L cv. Shiraz grape berries. Aust J Grape Wine Res 6:141–149
10. Trouillas P, Sancho-Garcia JC, De Freitas V et al (2016) Stabilizing and modulating color by copigmentation: insights from theory and experiment. Chem Rev 116:4937–4982
11. Ferrer-Gallego R, Brás NF, García-Estévez I et al (2016) Effect of flavonols on wine astringency and their interaction with human saliva. Food Chem 209:358–364
12. Naczk M, Shahidi F (2006) Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. J Pharm Biomed Anal 41: 1523–1542
13. Everette JD, Bryant QM, Green AM et al (2010) Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. J Agric Food Chem 58:8139–8144
14. Khoddami A, Wilkes MA, Roberts TH (2013) Techniques for analysis of plant phenolic compounds. Molecules 18:2328–2375
15. Naczk M, Shahidi F (2004) Extraction and analysis of phenolics in food. J Chromatogr A 1054:95–111
16. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2019) Phenolic compounds profile of different berry parts from novel Vitis vinifera L. red grape genotypes and Tempranillo using HPLC-DAD-ESI-MS/MS: a varietal differentiation tool. Food Chem 295: 350–360
17. Perestrelo R, Bordiga M, Locatelli M et al (2020) Polyphenols, biogenic amines and amino acids patterns in Verdelho wines according to vintage. Microchem J 153:104383
18. Gómez-Plaza E, Gil-Muñoz R, Lopez-Roca JM et al (2001) Phenolic compounds and color stability of red wines: effect of skin maceration time. Am J Enol Vitic 52:266–270
19. Ivanova-Petropulos V, Hermosín-Gutiérrez I, Boros B et al (2015) Phenolic compounds and antioxidant activity of Macedonian red wines. J Food Compos Anal 41:1–14
20. Fabre N, Rustan I, De Hoffmann E et al (2001) Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. J Am Soc Mass Spectrom 12: 707–715
21. La Barbera G, Capriotti AL, Cavaliere C et al (2017) Liquid chromatography-high resolution mass spectrometry for the analysis of phytochemicals in vegetal-derived food and beverages. Food Res Int 100:28–52
22. Parejo I, Jáuregui O, Viladomat F et al (2004) Characterization of acylated flavonoid-O-glycosides and methoxylated flavonoids from Tagetes maxima by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 18:2801–2810
23. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2021) Genotypic variation in phenolic composition of novel white grape genotypes (Vitis vinifera L.). J Food Compos Anal 102:103987
24. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2020) Comprehensive chemical and sensory assessment of wines made from white grapes of Vitis vinifera cultivars Albillo Dorado and Montonera del Casar: a comparative study with Airén. Foods 9:1282
25. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2019) First chemical and sensory characterization of Moribel and Tinto Fragoso wines using HPLC-DAD-ESI-MS/MS, GC-MS, and Napping® techniques: comparison with Tempranillo. J Sci Food Agric 99: 2108–2123

Chapter 8

Flavan-3-ol (Flavanol) Identification and Quantitation by High Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

Juliane Barreto de Oliveira, Celso Guarani Ruiz de Oliveira, and Giuliano Elias Pereira

Abstract

Flavan-3-ols are widely distributed in higher plants, such as grapes, located in the skins and seeds, being transferred to the wine during winemaking. They are responsible for specific sensory properties such as astringency, and bitterness, acting on the stability of the wine color, and taking part in the antioxidant compounds. The interest in identifying flavan-3-ols has grown in the last decades since the development of new instruments allowing a better separation and characterization, both qualitative and quantitative. New isolation, separation, and identification techniques allowed an increase in the phenolic compounds database with new structures, providing a better understanding of the mechanisms involving phenolic metabolism in grapes, wines, and other fruits and vegetables. High-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) is one of the most relevant and used analytical techniques for the non-volatile and/or thermally unstable compounds determination. This method has shown to be valuable and robust for investigating the polyphenols (flavan-3-ols or proanthocyanidins) in grapes, wines, and derivates, in several domains, such as evaluating the effect of climate, soil, vine management, cultivars, rootstocks, protocols of elaboration, and the quality control. This chapter aims to present variations of LC-MS techniques used to identify these compounds in recent years.

Key words *Vitis vinifera* L., Grape, Wine, Phenolic compounds, Secondary metabolites

1 Introduction

Grape and phenolic wine compounds represent a large family of molecules with a high diversity of chemical structures and degrees of complexity. The term "polyphenols" or "phenolics" is used to define a group of secondary metabolites in plants that present one or more hydroxyl (–OH) groups attached to one or more benzene rings [1]. The polyphenolic composition of grapes is highly affected by different factors, such as environmental conditions (soil, climate), viticulture practices, and pathogen attacks, as well as the

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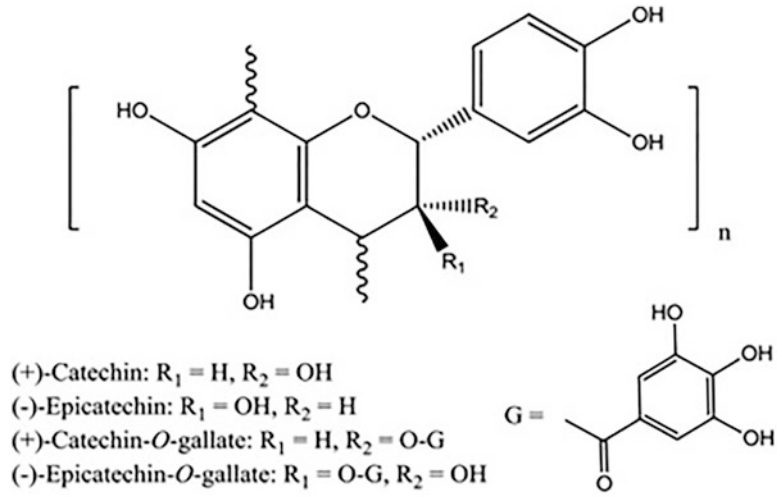

Fig. 1 Subunits structures of flavan-3-ols from grape seed. (Source: Ma et al. (2016) [12])

human factor, both in the field and the winery, with different protocols of winemaking [2]. Although one of the most crucial factors is undoubtedly the varietal or genetic variations in each specific environment [3].

Flavan-3-ol is present in grapes and consequently in wines in monomeric, oligomeric, and polymeric forms, with more or less repetitive units forming procyanidins, main monomeric flavanols of (+)catechin and its isomer (−)epicatechin. Catechin derivatives, namely, gallocatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate (Fig. 1), have also been identified in grapes and wines [4, 5]. Proanthocyanidins or tannins are oligomers and polymers of flavan-3-ol units [6]. The procyanidins from skins and seeds vary in their relative amount, length, subunit composition, and sensory properties. The tannins of the skins are reported to be lower than seeds [7–9]. Seed tannins present a lower mean degree of polymerization (mDP), while skin tannins are generally larger with a higher mDP [9–11].

Over the years, analytical methods used for determining the phenolic compounds in grapes and wines have been studied. Regarding the quantitation of tannins, several studies have been and are being evaluated. Among the methods are some considered for global quantitation, such as analyzes based on the selective precipitation of tannins with protein and other reagents, followed by UV/Vis reading [13, 14], infrared (IR) spectroscopy. It is considered a fast, accurate technique and an alternative to conventional chemical analyses [15–17], in addition to analysis by liquid chromatography (LC) [18], used for individual identification and quantitation.

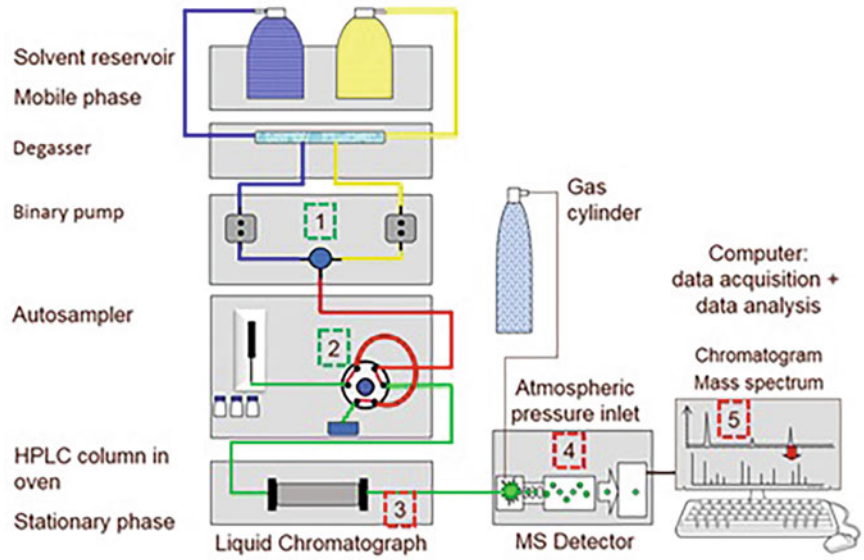

Fig. 2 A simplified diagram of a liquid chromatograph hyphenated to a mass spectrometer (LC-MS) showing: (1) binary pump for mobile phase, (2) autosampler 6-port valve and injector loop, (3) column heater with column, (4) mass spectrometer detector, (5) PC. Credit: Anthias Consulting

Liquid chromatography with mass spectrometry (LC-MS) is a powerful analytical technique that combines the separating power of liquid chromatography with the highly sensitive and selective mass analysis capability of mass spectrometry. In liquid chromatography, the components of a complex mixture are separated into two phases: the first one is a fixed phase with a large surface area called the stationary phase, and the second one is a fluid that interacts with the fixed phase, called the mobile phase.

Mass spectrometry is a technique for analysis at the trace level; however, the analytes must be previously ionized. This analyzer has an ionization source, analyzer, detector, and data system (Fig. 2). When they have two analyzers, with a collision cell between them, they are called Tandem, where the first analyzer identifies the precursor ion, and the second analyzer the product ions (LC-MS-MS). The strength of this technique (LC-MS) lies in the LC separation power for a wide range of compounds, combined with the MS ability to quantitate compounds with a high degree of sensitivity and selectivity based on unique mass/charge transitions (m/z) of each compound of interest.

The primary advantages of this technique are sensitivity, specificity, and accuracy since the analysis is carried out at the molecular level. Ion analyses contain structural information on the analyte, which can be used to determine the mass of the analytes, their elemental and isotopic composition for elucidating the chemical structure of the sample, and/or to confirm identification.

The LC-MS is an effective analytical tool for studying phenolic compounds because it offers a higher sensitivity, selectivity, and specificity compared to LC-DAD and it provides structural information [19, 20]. The MS detection has the advantage of resolving peaks that co-elute in the chromatographic dimension, presuming that the molecular masses differ sufficiently. This is a crucial consideration in the highly complex families of phenolic compounds analysis present in wines [21].

Some factors can affect the ongoing performance of LC-MS systems, and it is relevant to have protocols in place to detect deviations from regular performance. Monitoring the absolute response, peak shape, and retention time of internal standards is a simple way of checking the sensitivity of the mass spectrometer and the integrity of the LC system. Checking the internal standard response of each sample within a batch is also a valuable way of picking up problems with individual samples. The purity of reagents and solvents can also have a significant impact on the quality of results and should be evaluated during method optimization and when different sources are used [22].

Mass spectrometry has a very essential role in research and quality control in the viticulture and enology fields. Several methods have been published analyzing different compounds in grapes, juices, and wines, such as sugars [23–26], organic acids [26, 27], amino acids, volatiles amines [28], and phenolics [29], contaminating substances [30, 31] in addition to polyphenols [32, 34, 35], which form the group of the most analyzed substances.

The analytical method consists of several stages such as sampling, sample preparation, separation, detection, and data analysis. Sampling and sample preparation (extraction, preconcentration, fractionation, and isolation) are the steps that normally require the most time for the analytical procedure [36, 37].

Sample preparation is, undoubtedly, a very important step in a metabolite profiling study. The quantitation of flavan-3-ols in grapes involves some difficulties, among them is adjusting the most effective method of extracting these compounds from husks and seeds, due to their location and extractability. Some authors have evaluated the use of different solvents and techniques to obtain more representative extracts (Table 1). The efficiency of different solvents or solvent combinations in solubilizing metabolites, thus extracting them from the initial solid specimen, plays a dominant role in the comprehensiveness and the representativeness of the metabolite profile obtained. The choice of the extraction medium is not simple, as the metabolites have different natures, physicochemical properties, and concentration ranges. A suitable extraction solvent for one chemical class may be unsuitable for another [38]. The highest accuracy, fastness, and sensitivity are sought and also minimize the costs and the solvents used [37].

Table 1
Extraction methods of flavan-3-ols from grapes

Solvents	Application	Procedure	References
MeOH/HCL 0.5 N (95:5 v/v)	Skins	30 min sonication and 12 h of maceration at −20 °C	[39]
C_3H_6O/H_2O (75:25 v/v)	Skins and seeds	Acetone was removed and n-hexane was used to eliminate lipophilic material in the extract.	[40]
Ethanol 96%	Skins and seeds	Extraction at 5 °C, under stirring for 1 h, followed by centrifuging for 10 min.	[41]
C_3H_6O/H2O (7:3 v/v)	Skins	Leave overnight under N_2, with a mechanical mixer, dry and dissolve in H2O, store at −80 °C.	[33]
MeOH/HCL 0.1%	Skins	Extract using ultrasound for 60 min.	[42]
$MeOH/H_2O$ (70:30 v/v)	Seeds	Extract using ultrasound for 60 min.	[42]

2 Flavan-3-ol in Grapes and Wines by Liquid Chromatography Coupled with Mass Spectrometry

Since the first studies using LC-MS equipment, many methods have been developed and improved over the years for the phenolic compounds' detection and quantitation in grapes and wines. Most of these studies use C8 or C18 columns (reverse phase mode), with a gradient run program. Binary solvents (which generally consist of aqueous and organic phases) are most suitable. Furthermore, to control the pH, and consequently control the charge of the molecule, acids such as formic [35, 43], acetic [47, 48], or phosphoric [44] are usually incorporated in low percentages, in the aqueous phase, or even in both phases. The selection of flow rates and injection volume usually vary depending on the chosen column. For polyphenols identification by LC-MS or MS/MS, the flow rate normally ranges between 0.2 and 0.8 mL min^{-1} and the injection volume is from 2 to 40 μL [35, 46].

When the detection mode is used in combination with HPLC to quantitate flavan-3ols, the electrospray ionization (ESI) is usually used in negative ion mode, but it is also effective in the positive mode when analyzing wine samples [45]. In Table 2, some methods used for the flavan-3ols identification and quantitation in grapes, wines, and juices are described.

Table 2
Methods LC/ESI-MS for analysis of flavan-3-ols or proanthocyanidins, most used in recent years

Method 1 [45]
Sample: Grapes Silica column (250 × 2.0 mM, 5 μM) Mobile phase: (A) dichloromethane/methanol/H_2O/HAc 82:14:2:2 (v/v/v/v), (B) MeOH/H_2O/HAc 96:2:2 (v/v/v). Elution linear gradient of B into A: From 0 to 18% B in 30 min, 18 → 31% B in 15 min, 31 → 88% B in 5 min (flow rate 0.2 mL min^{-1}). LC/ESI-MS conditions: Negative mode, ionization reagent ammonium acetate 10 mM in the eluent stream at flow rate of 30 μL min^{-1}, capillary voltage 3.2 kV, cone voltage 30 V, source temperature 150 °C, desolvation gas temperature 300 °C.
Method 2 [47]
Sample: Seed grapes Nova-Pak column C18 (300 mM × 3.9 mM, 4 μM) Mobile phase: (A) distilled water and (B) water/acetic acid, 90/10, v/v. the gradient was applied at a flow (0.7 mL min^{-1}). LC/ESI-MS conditions: Was operated in negative mode, scanning from m/z 100 to 3000 using the following fragmentation program: From m/z 0 to 200 (100 V) and from m/z 200 to 3000 (200 V). The drying gas was N_2, with flow of 10 L min^{-1} and temperature 340 °C; nebulizer pressure was 40 psi; and capillary voltage (4000 V).
Method 3 [48]
Sample: Grapes C18 column (50 mM × 4.6 mM, 3 μM) Mobile phase: Solvents A (1% acetic acid in water) and B (1% acetic acid in MeOH). The flow rate was set at 0.2 mL min^{-1} and injection volume was 10 μL. LC/ESI-MS conditions: Negative ion mode (ESI-), high-purity nitrogen (99.99%) was used as dry gas at a flow rate of 5 mL min^{-1}, and the capillary temperature was 325 °C. nitrogen also was used as nebulizer at 15 psi. The samples were scanned from m/z 50 to 800. ESI was conducted by using needle voltages of 4.5 kV (negative).
Method 4 [49]
Sample: Grapes Finnigan Hypersil gold column (150 × 4.6 mM, 5 μM). Mobile phase: Solvent A (0.1% v/v of formic acid in H_2O) and solvent B (100% v/v methanol). The flow rate was 0.20 mL min^{-1}, and the gradient method started with a linear gradient ranging from 90% A to 60% A in 90 min, then reaching 100% B in 5 min, and a final isocratic gradient of 100% B during 5 min. LC/ESI-MS conditions: Was operated in the negative-ion mode with source, with a capillary temperature of 275 °C and capillary voltages of 4.5 kV. The mass spectra were recorded between 250 and 2000 m/z.
Method 5 [35]
Sample: grape juice C18 column, (50 × 2.1 mM, 5 μM) and protected with a guard column of the same material (50 × 2.1 mM, 5 μM) Mobile phase: Solvents A (formic acid and water, 2:98 v/v) and B (MeOH, formic acid and water, 90:2:8 v/v). A linear gradient was used, with flow rate de 0.45 mL min^{-1}, and injection volume was 5 μl. LC/ESI-MS conditions: The mass spectrometer was operated in the negative-ion mode (ESI-), with a capillary temperature of 600 °C and capillary voltages of −0.8 kV.

(continued)

Table 2
(continued)

Method 6 [43]
Sample: Grapes Synergi 4u MAX-RP 80A (250 × 2.0 mM, 4 μM) Mobile phase: (A) acetonitrile: Formic acid 96.99:3:0.01 (v/v/v/v), (B) acetonitrile: Water formic acid 50:49.99:0.01 (v/v/v). A linear elution gradient was applied at a flow rate of 0.3 mL min^{-1}. LC/ESI-MS conditions: Negative mode, the capillary temperature was 275 °C, source voltage was 3.50 kV, and nitrogen gas flow was 35 arb and sweep gas flow of 10 arbs. The collision energy for MS2 scans was 60%. Chromatograms were recorded at 200–800 nM.
Method 7 [49]
Sample: Wines An Acquity HSS-T3 RP18 column (150 × 2.1 mM; 1.8 μM particle size) Mobile phase: (A) water/formic acid (97/3; v/v) and (B) acetonitrile/formic acid (97/3; v/v). The gradient was applied at a flow (0.5 mL min^{-1}). LC/ESI-MS conditions: Was operated in negative mode, scanning from m/z 120 to 1500. The capillary was set at 325 °C with a voltage of −44 V. the source voltage was maintained at 4 kV, at a current of 100 μA. The tube lens was adjusted to −105 V. for quantitation, specific m/z values of polyphenolic compounds were recorded in single ion monitoring measurements using one scan event.

3 Electrospray Source Ionization (ESI)

Electrospray ionization (ESI) is a technique used to produce ions, in which a high voltage is applied. It uses electrical energy to help transfer ions from the solution to the gaseous phase before being subjected to mass spectrometric analysis. The principal advantage of using ESI for quantitative LC-MS is the formation of protonated or deprotonated molecules with little fragmentation, ideal for the ion precursors selection and to maximizing sensitivity. Electrospray ionization mass spectrometry (ESI-MS) has proven to be a very powerful tool for the characterization of flavan-3-ols and proanthocyanidins [50–52]. The advantage of ESI is that it allows the detection of the molecular ion, but it does not cause the fragmentation of the molecule as it occurs in other types of ionization, such as chemical ionization at atmospheric pressure, for example [53].

4 Types of LC-MS Instrument That Can Be Used for Quantitation (Mass Analyzer)

Novices in the field of MS are often confused by the wide variety of different ionization, mass analysis, and detection methods that exist. Although ionization methods determine the classes of substances available for measurement, it is a combination of the mass analyzer and detector that determines the quality and reliability of the analysis. Depending on the physics of mass analysis, analyzers can belong to generic types, such as quadrupole, magnetic sector, ion trap, time of flight (TOF), or Fourier transform (FT). They

could be combined to allow the analysis of both analytes and their fragments (MS/MS), the most popular combinations being triple quadrupole and quadrupole/time of flight hybrids. Alternatively, the same analyzer can perform MS and MS/MS (MS^2) analysis, sometimes for a high MS^n stage, such as a radio frequency ion trap (Paul trap) or a static electromagnetic trap (penning trap) [54].

(a) **Quadrupole mass analyzer** consists of a set of four conducting rods arranged in parallel, with a space in the middle; the opposing pairs of rods are electrically connected. This type of mass analyzer separates ions based on the stability of their flight trajectories through an oscillating electric field in the quadrupole. The quadrupole is the most popular mass analyzer at the moment mainly due to its simplicity, relatively low price, good linearity in quantitative analyses, ease of understanding, and operation. Although it is usually operated at low resolution (typically R = 1000), it can be increased under favorable conditions to values greater than 4000. Its mass accuracy is generally between 0.1 and 0.2 atomic mass units (a.m.u. or Dalton), and the mass range is usually between 10 and 4000 a.m.u. [55].

(b) **Ion traps trap and store ions** in an orbital motion within the ion trap and eject ions for detection. Storage is performed by collecting ions in potential energy comparing to quadrupole mass analyzers, which provide continuous transmission of ions. Together with the linear quadrupole, the "ion trap" is one of the most popular ion analyzers at the moment due to its relatively low cost (comparable to the quadrupole), small size, and can be used to obtain analyzers that take up little space. Its resolution is similar to the linear (unitary) quadrupole, and it can be increased using slower scans in a smaller mass range. Under these conditions, resolutions close to 5000 can be obtained. Typical applications of this analyzer are similar to those of the quadrupole [55].

(c) **A TOF mass analyzer** separates ions based on their velocity as they travel through a flight region, often called the flight tube. The measurement is similar to a race: a group of ions is accelerated by an extractor (start of the race), which causes them to drift through the flight tube (the race course) toward a detector (the finish line) [55].

These devices have high resolution (in linear mode the resolution is limited), good sensitivity, very fast scan speed (important for narrow chromatographic peaks), and accuracy. However, they require very sophisticated electronics, good control of time and initial energy, and spatial distribution of the ions. Its application is quite wide, especially when high resolution is required. The range of masses it analyzes is wide (theoretically unlimited, but in practice

very high masses—well over 500,000 Da—are difficult to determine with good precision and accuracy). Although there are other mass analyzers for MS, the three described so far (quadrupole, "ion-trap," and TOF) are by far the most used in LC-MS coupling. While the first two are compact and have great simplicity, low cost, and operational ease, their main limitation is low resolution. TOF, on the other hand, loses to both analyzers in these aspects, but it has a much higher resolution, especially in the OF mode, which may be necessary for analyzes that require high resolution [56].

The development of commercial LC-MS systems has led to a wide range of instruments being made available to the end user. Over recent years, new types of mass analyzers have been introduced regularly and have been a great help in the development of science (Table 3) [56].

5 Flavan-3-Ols Concentrations in Grapes and Wines by Liquid Chromatography Associated with Mass Spectrometry

The combination of liquid chromatography techniques coupled with mass spectrometry helped researches on the identification and quantitation of flavan-3-ols and procyanidins in grapes and wines (Table 4).

6 Conclusion

With recent advances and new developments in chromatography and MS, it is evident that there is a great improvement in the sensitivity, selectivity, and accuracy offered by the combination of these two analytical techniques, which have provided significant contributions to the determination of several metabolites.

LC-MS has taken the current leading position in the knowledge of the metabolites present in grapes and wines, as it routinely allows the determination of many specific compounds through molecular formulas and molecular fragmentation, using collision-induced dissociation in single-stage or multi-stage mass systems, with quick precision and safety.

Mass spectrometry has a very relevant role in research in the field of viticulture and enology, specially for evaluating the enological potential of grapes and wines, their quality, and typicality from different trials in the vineyards and the winery during processing. It also allows to track many compounds from different chemical groups, with ample precision and capacity for quality control and detecting any fraud in the wine and food industries.

Table 3
Types of LC-MS instrument that can be used for quantitation together with their features/benefits and disadvantages

Mass Spectrometer Type	Features and Benefits	Disadvantages
Single quadrupole	Good scan function sensitivity Good selectivity/sensitivity via SIM scanning High duty cycle with SIM Good dynamic range (3–4 orders) Fast positive and/or negative ionization	Limited mass range (generally, up to 3000 m/z) SIM functionality can be prone to matrix interferences thus limit detection limits Low resolution (1500 Full Width at Half Maximum (FWHM), or 0.7 Da)
Triple quadrupole	Good scan function sensitivity and SIM function Excellent selectivity with MRM, even with matrix Ability to run multiple analytes simultaneously with MRM High dynamic range (4–5 orders) Fast positive and/or negative ionization	Low resolution generally (1500 FWHM or 0.7 Da) Limited mass range (up to 3000 m/z generally)
Ion trap (high resolution)	High full scan sensitivity in MS, MS/MS and MSn mode Good dynamic range (3 orders) High resolution (>100,000 FWHM) Good selectivity using exact mass measurement	Resolution can be affected by scan speed (lower the resolution) Orbital trapping devices can have a limited dynamic range and be affected by matrix Limited mass range (up to 4000 m/z typically)
TOF (high resolution)	Good scan functionality and sensitivity High resolution (up to 40,000 FWHM) provides high selectivity through exact mass measurement Good dynamic range (with newer ADC based detection systems, typically 3–4 orders) Ability to get quantitation on multiple analytes in a single acquisition Mass range in excess of 20,000 m/z	No MS/MS functionality or other scan functions Generally, lower sensitivity when compared to a triple quadrupole running MRM Sensitivity can be affected by scan speed
Q-TOF (high resolution)	Good full scan sensitivity Good MS/MS scan functions High resolution (>40,000 FWHM) Good dynamic range with newer ADC based detection systems (3–4 orders) Ability to get quantitation on multiple analytes during a single run Mass range in excess of 20,000 m/z Resolution not affected by increased scan speed	Generally, lower sensitivity when compared to a triple quadrupole running MRM Sensitivity can be affected by scan speed

Table 4
Flavan-3-ols and procyanidins concentrations detected by different authors using LC-MS in grapes and wines

Compound	MS (m/z)	Formula	Concentration	Sample	References
(+)-catechin	289	$C_{15}H_{13}O_6^-$	11–123 $mgkg^{-1}$ 52–150 mgL^{-1} 60–72 $mgkg^{-1}$ 106 $mg100g^{-1}$	Grapes Wines Skins Seeds	[57] [58] [59] [60]
(−)-epicatechin	289	$C_{15}H_{13}O_6^-$	0.7–27 $mgkg^{-1}$ 26–79 mgL^{-1} 9–17 $mgkg^{-1}$	Grapes Wines Skins	[57] [58] [59]
(−)-epicatechin gallate	441	$C_{22}H_{17}O_{10}^-$	0.4–2.6 $mgkg^{-1}$ 34–54 μgg^{-1} 2–12 $mgkg^{-1}$ 12–40 mgL^{-1} 76 $mg100g^{-1}$	Grapes Grapes Seeds Wines Seeds	[57] [43] [59] [61] [60]
(−)-epigallocatechin	457	$C_{15}H_{14}O_7^-$	1.1–1.4 $mgkg^{-1}$ 1.9–2.7 mgL^{-1}	Grapes Wines	[57] [49]
Procyanidin B1	577	$C_{30}H_{26}O_{12}$	30–83 mgL^{-1} 20–26 $mgkg^{-1}$ 76–79 $mgkg^{-1}$	Wines Seeds Skins	[58] [59]
Procyanidin B2	577	$C_{30}H_{26}O_{12}$	3.3 ppm 35 mgL^{-1} 100–450 ugg^{-1} 14–51 $mgkg^{-1}$	Grapes Grapes Seeds Grapes	[42] [62] [61]
Procyanidin B4	579	$C_{30}H_{26}O_{12}$	8–58 $mg100g^{-1}$	Skins and seeds	[60]
Trimer C1	866	$C_{45}H_{38}O_{18}$	6–44 $mgkg^{-1}$ 13 $mg100g^{-1}$	Grapes Seeds	[63] [60]

References

1. Vermerris W, Nicholson R (2006) Families of phenolic compounds and means of classification. In: phenolic compound biochemistry. In: Vermerris W, Nicholson R (eds) Phenolic compound biochemistry. Springer, London
2. Downey MO, Dokoozlian NK, Krstic MP (2006) Cultural practice and environmental impacts on the flavonoid composition of grapes and wine: a review of recent research. Am J Enol Vitic 57:257–268
3. Pinasseau L, Vallverdu Queralt A, Verbaere A, Roques M, Meudec E, Le Cunff L et al (2017) Cultivar diversity of grape skin polyphenol composition and changes in response to drought investigated by LC-MS based metabolomics. Front Plant Sci 8:24
4. Decendit A, Waffo-Teguo P, Richard T, Krisa S, Vercauteren J, Monti JP et al (2002) Galloylated catechins and stilbene diglucosides in Vitis vinifera cell suspensions cultures. Phytochemistry 60(8):795–798
5. Mattivi F, Vrhovsek U, Masuero D, Trainotti D (2009) Differences in the amount and structure of extractable skin and seed tannins amongst red grape varieties. Aust J Grape Wine Res 15(1):27–35
6. Downey MO, Harvey JS, Robinson SP (2003) Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. Aust J Grape Wine Res 9:15–27
7. Kyraleou M, Kallithraka S, Theodorou N, Teissedre PL, Kotseridis Y, Koundouras S (2017) Changes in tannin composition of Syrah grape

skins and seeds during fruit ripening under contrasting water conditions. Molecules 22: 1453

8. Chira K, Schmauch G, Saucier C, Fabre S, Teissedre PL (2009) Grape variety effect on proanthocyanidin composition and sensory perception of skin and seed tannin extracts from Bordeaux wine grapes (cabernet sauvignon and merlot) for two consecutive vintages (2006 and 2007). J Agric Food Chem 57:545–553
9. Ćurko N, Kovačević Ganić K, Gracin L, Đapić M, Jourde M, Teissedre PL (2014) Characterization of seed and skin polyphenolic extracts of two red grape cultivars grown in Croatia and their sensory perception in a wine model medium. Food Chem 145:15–22
10. Bordiga M, Travaglia F, Locatelli M, Coisson JD, Arlorio M (2011) Characterization of polymeric skin and seed proanthocyanidins during ripening in six Vitis vinifera L. cv. Food Chem 127:180–187
11. Oliveira JB, Egipto R, Laurean O, de Castro R, Pereira G, Ricardo-da-Silva JM (2019) Chemical characteristics of grapes cv. Syrah (Vitis vinifera L.) grown in the tropical semiarid region of Brazil (Pernambuco state): influence of rootstock and harvest season. J Sci Food Agric 99:5050–5063
12. Ma W, Waffo-Teguo P, Jourdes M, Li H, Teissedre PL (2016) Chemical affinity between tannin size and salivary protein binding abilities: implications for wine astringency. PLoS One 11(8):e0161095
13. Sarneckis CJ, Dambergs RG, Jones P, Mercurio M, Herderich MJ, Smith PA (2006) Quantification of condensed tannins by precipitation with methyl cellulose: development and validation of an optimized tool for grape and wine analysis. Aust J Grape Wine Res 12:39–49
14. Sun BS, Pinto T, Leandro MC, Ricardo-Da-Silva JM, Spranger MI (1999) Transfer of catechins and proanthocyanidins from solid parts of the grape cluster into wine. Am J Enol Vitic 50:179–184
15. Cozzolino D, Kwiatkowski MJ, Parker M, Cynkar WU, Dambergs RG, Gishen M, Herderich MJ (2004) Prediction of phenolic compounds in red wine fermentations by visible and near infrared spectroscopy. Anal Chim Acta 513:73–80
16. Gishen M, Dambergs RG, Cozzolino D (2005) Grape and wine analysis - enhancing the power of spectroscopy with chemometrics. A review of some applications in the Australian wine industry. Aust J Grape Wine Res 11:296–305
17. Di Egidio V, Sinelli N, Giovanelli G, Moles A, Casiraghi E (2010) NIR and MIR spectroscopy as rapid methods to monitor red wine fermentation. Eur Food Res Technol 230:947–955
18. Silva MA, Ky I, Jourdes M, Teissedre PL (2012) Rapid and simple method for the quantification of flavan-3-ols in wine. Eur Food Res Technol 234:361–365
19. Abad-García B, Berrueta LA, Garmón-Lobato S, Gallo B, Vicente F (2009) A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry. J Chromatogr A 1216:5398–5415
20. Berente B, De la Calle GD, Reichenbächer M, Danzer K (2000) Method development for the determination of anthocyanins in red wines by high-performance liquid chromatography and classification of German red wines by means of multivariate statistical methods. J Chromatogr A 871(1–2):95–103
21. Pérez-Magariño S, Revilla I, González-SanJosé ML, Beltrán S (1999) Various applications of liquid chromatography-mass spectrometry to the analysis of phenolic compounds. J Chromatogr A 847(1–2):75–81
22. Pitt JJ (2009) Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30(1):9–34
23. Salman M, Alghamdi MT, Bazaid SA, Abdel-Hameed ES (2011) Determination of fructose, glucose and sucrose in Taif grape using high performance liquid chromatography and analysis of mineral salts. Arch Appl Sci Res 3(6): 488–496
24. Chidan Kumar CS, Mythily R, Chandraju S (2012) Advanced chromatographic technique for analysis of sugars extracted from black grape (Vitis vinifera L.) skins. Asian J Chem 24(5): 2170–2172
25. Han J, Lin K, Sequria C, Yang JC, Borchers CH (2016) Quantitation of low molecular weight sugars by chemical derivatization-liquid chromatography multiple reaction monitoring mass spectrometry. Electrophoresis 37:1851–1860
26. Ehling S, Cole S (2011) Analysis of organic acids in fruit juices by liquid chromatography mass spectrometry: an enhanced tool for authenticity testing. J Agric Food Chem 59(6):2229–2234
27. Silva FLN, Schmidt EM, Messias CL, Eberlin MN, Sawaya ACHF (2015) Quantitation of organic acids in wine and grapes by direct

infusion electrospray ionization mass spectrometry. Anal Methods 7:53–62

28. Malec PA, Oteri M, Inferrera V, Cacciola F, Mondello L, Kennedy RT (2017) Determination of amines and phenolic acids in wine with benzoyl chloride derivatization and liquid chromatography–mass spectrometry. J Chromatogr A 1523:248–256
29. Vujovic D, Pejin Jelena B, Djordjevic P, Velickovic M, Tesevic V (2016) Phenolic natural products of the wines obtained from three new merlot clone candidates. Nat Prod Res 8(30):987–990
30. Li J, Liu X, Han S et al (2012) Analysis of Ochratoxin A in wine by high-resolution UHPLC-MS. Food Anal Methods 5:1506–1513
31. Souza D, Souza E, Borges EM (2016) Determination of pesticides in grape juices by quechers and liquid chromatography-tandem mass spectrometry. J Braz Chem Soc 27(9): 1626–1635
32. Mazzuca P, Ferranti P, Picariello G, Chianese L, Addeo F (2005) Mass spectrometry in the study of anthocyanins and their derivatives: differentiation of Vitis vinifera and hybrid grapes by liquid chromatography/electrospray ionization mass spectrometry and tandem mass spectrometry. J Mass Spectrom 40: 83–90
33. Zerbib M, Cazals G, Enjalbal C, Saucier C (2018) Identification and quantification of flavanol glycosides in vitis vinifera grape seeds and skins during ripening. Molecules 23(11):2745
34. Trikas ED, Papi RM, Kyriakidis DA, Zachariadis GA (2016) A sensitive LC-MS method for anthocyanins and comparison of subproducts and equivalent wine content. Separations 3(18):1–12
35. Canedo-Reis NAP, Guerra CC, da Silva LF et al (2021) Fast quantitative determination of phenolic compounds in grape juice by UPLC-MS: method validation and characterization of juices produced with different grape varieties. Food Measure 15:1044–1056
36. Maslamani N, Manandhar E, Geremia DK, Logue BA (2016) ICE concentration linked with extractive stirrer (ICECLES). Anal Chim Acta 941:41–48
37. Román SM, Rubio-Bretón P, Pérez-Álvarez EP, Garde-Cerdán T (2020) Advancement in analytical techniques for the extraction of grape and wine volatile compounds. Food Res Int 137:109712
38. Theodoridis G, Gika H, Franceschi P et al (2012) LC-MS based global metabolite profiling of grapes: solvent extraction protocol optimisation. Metabolomics 8:175–185
39. García-Estévez I, Alcalde-Eon C, Escribano-Bailón MT (2017) Flavanol quantification of grapes via multiple reaction monitoring mass spectrometry. Application to differentiation among clones of Vitis vinifera L. cv. Rufete grapes. J Agric Food Chem 65(31): 6359–6368
40. Ferrer-Gallego R, Quijada-Morín N, Bras NF, Gomes P, de Freitas V, Rivas-Gonzalo JC, Escribano-Bailón MT (2015) Characterization of sensory properties of flavanols a molecular dynamic approach. Chem Senses 40:381–390
41. Pereira GE, Padhi EMT, Girardello RC, Medina-Plaza C, Tseng D, Bruce RC, Erdmann JN, Kurtural SK, Slupsky CM, Oberholster A (2020) Trunk girdling increased stomatal conductance in cabernet sauvignon grapevines, reduced glutamine, and increased Malvidin-3-glucoside and Quercetin-3-glucoside concentrations in skins and pulp at harvest. Front Plant Sci 11:707
42. Šuković D, Knežević B, Gašić U, Sredojević M, Ćirić I, Todić S, Mutić J, Tešić Ž (2020) Phenolic profiles of leaves, grapes and wine of grapevine variety Vranac (Vitis vinifera L.) from Montenegro. Foods 9(2):138
43. Colombo F, Di Lorenzo C, Regazzoni L, Fumagalli M, Sangiovanni E, Sousa LP, Bavaresco L, Tomasi D, Bosso A, Aldini G, Restani P, Dell'Agli M (2019) Phenolic profiles and anti-inflammatory activities of sixteen table grape (Vitis vinifera L.) varieties. Food Funct 10:1797–1807
44. Pires FB, Dolwitsch CB, Dal Prá V, Faccin H, Monego DL, de Carvalho LM, Viana C, Lameira O, Lima FO, Bressan L, et al (2017) Qualitative and quantitative analysis of the phenolic content of Connarus var. angustifolius, Cecropia obtusa, Cecropia palmata and Mansoa alliacea based on HPLC-DAD and UHPLC-ESI-MS/MS
45. Núñez V, Gómez-Cordovés C, Bartolomé B, Hong YJ, Mitchell AE (2006) Non-galloylated and galloylated proanthocyanidin oligomers in grape seeds from Vitis vinifera, L. cv. Graciano, Tempranillo and Cabernet Sauvignon. J Sci Food Agric 86(6):915–921
46. Monagas M, Suárez R, Gómez-Cordovés C, Bartolomé B (2005) Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC-DAD/ESI-MS. Am J Enol Vitic 56:139–147
47. Ivanova V, Stefova M, Vojnoski B, Dörnyei A, Márk L, Dimovska V, Stafilov T, Kilár F (2011)

Identification of polyphenolic compounds in red and white grape varieties grown in R. Macedonia and changes of their content during ripening. Food Res Int 44(9): 2851–2860

48. Teixeira N, Azevedo J, Mateus N, de Freitas V (2016) Proanthocyanidin screening by LC-ESI-MS of Portuguese red wines made with teinturier grapes. Food Chem 1(190):300–307
49. Weilack I, Schmitz C, Harbertson JF, Weber F (2021) Effect of structural transformations on precipitability and polarity of red wine phenolic polymers. Am J Enol Vitic 72:230–239
50. Hayasaka Y, Waters EJ, Cheynier V, Herderich MJ, Vidal S (2003) Characterization of proanthocyanidins in grape seeds using electrospray mass spectrometry. Rapid Commun Mass Spectrom 17:9–16
51. Sun BS, Fernandes TA, Spranger MI (2010) A new class of anthocyanin-procyanidin condensation products detected in red wine by electrospray ionization multi-stage mass spectrometry analysis. Rapid Commun Mass Spectrom 24:254–260
52. Li L, Sun B (2019) Grape and wine polymeric polyphenols: their importance in enology. Crit Rev Food Sci Nutr 59(4):563–579
53. De Pascual-Teresa S, Rivas-Gonzalo JC (2003) Application of LC-MS for the identification of polyphenols. In: Santos-Buelga C, Williamson G (eds) Methods in polyphenol analysis. Athenaeum Press
54. Zubarev RA, Makarov A (2013) Orbitrap is the newest addition to the family of high-resolution mass spectrometry analyzers. With its revolutionarily new, miniature design, Orbitrap combines high speed with excellent quantification properties, ranking favorably in many analytical applications. Anal Chem 85(11): 5288–5296
55. Lanças FM (2009) A Cromatografia Líquida Moderna e a Espectrometria de Massas: finalmente "compatíveis?". Sci Chromat 2(1): 35–61
56. Sargent M (2013) Guide to achieving reliable quantitative LC-MS measurements. RSC Analytical Methods Committee
57. Pantelić M, Zagorac DD, Natić M, Gašić U, Jović S, Vujović D, Djordjević JP (2006) Impact of clonal variability on phenolics and radical scavenging activity of grapes and wines: a study on the recently developed merlot and cabernet franc clones (Vitis vinifera L.). PLoS One 11(10):1–15
58. Serni E, Pedri U, Valls J, Sanoll C, Dordevic N, Überegger E, Robatscher P (2020) Chemical description and organoleptic evaluation of Pinot noir wines from different parts of Italy: a three years investigation. OENO One 54(2): 393–410
59. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A, Gascueña JM, Chacón-Vozmediano JL, García-Romero E, Hermosín-Gutiérrez I, Gómez-Alonso S (2019) Phenolic compounds profile of different berry parts from novel Vitis vinifera L. red grape genotypes and Tempranillo using HPLC-DAD-ESI-MS/MS: a varietal differentiation tool. Food Chem 295:350–360
60. Di Lecce G, Arranz S, Jáuregui O, Tresserra-Rimbau A, Quifer-Rada P, Lamuela-Raventós RM (2014) Phenolic profiling of the skin, pulp and seeds of Albariño grapes using hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry. Food Chem 145:874–882
61. Banc R, Loghin F, Miere D, Ranga F, Socaciu C (2020) Phenolic composition and antioxidant activity of red, rosé and white wines originating from Romanian grape cultivars. Not Bot Horti Agrobot Cluj Napoca 48(2):716–734
62. Sen K, Sonmezdag AS (2020) Elucidation of phenolic profiling of cv. Antep Karasi grapes using LC-DAD-ESI-MS/MS. J Raw Mater Process Foods 1:1–6
63. Savalekar K, Ahammed Shabeer TP, Khan Z et al (2019) Targeted phenolic profiling of sauvignon blanc and shiraz grapes grown in two regions of India by liquid chromatography-tandem mass spectrometry. J Food Sci Technol 56:3300–3312

Chapter 9

Hydroxybenzoic and Hydroxycinnamic Acid Derivatives (HCAD) Identification and Quantitation by High Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

Sergio Gómez-Alonso, Tania Paniagua-Martínez, and José Pérez-Navarro

Abstract

Phenolic acids emerge with an interesting potential as bioactive compounds from natural sources. These include hydroxycinnamic acids and their derivatives, which are naturally present in grapes and wine as tartrate esters, and hydroxybenzoic acids, which are often presented in a glycosylated form and joined to small organic acids. These compounds display antioxidant, anti-collagenase, anti-inflammatory, antimicrobial and anti-tyrosinase activities, as well as ultraviolet protective effects. Moreover, its influence on the color, taste, and flavor profile of wine has been tested besides the enhancement of its preservative impact on sulfur dioxide, protecting wine from the oxygen action. However, hydroxycinnamic acids can be converted into volatile flavor-affecting phenols by *Brettanomyces/Dekkera*, providing undesirable smoke flavor in affected wines. Several analytical methods have been reported in literature describing the determination of phenolic acids in food and derived products. This chapter describes a high performance liquid chromatography combined with mass spectrometry (HPLC-MS^n) method for the determination of hydroxybenzoic and hydroxycinnamic acids from grapes and wine.

Key words Phenolic compounds, Phenolic acids, Solid-phase extraction, Liquid chromatography, Mass spectrometry, Wine, Grapes

1 Introduction

Phenolic compounds are bioactive secondary metabolites in plants generally involved in plant response to abiotic stress and tolerance [1]. These compounds possess different chemical structures characterized by at least one phenol unit and can be classified in different ways, such as flavonoids and non-flavonoids. Phenolic acids are a type of non-flavonoids generally having a simpler structure than flavonoids, with a carboxyl group linked to a benzene ring. The number and position of the hydroxyl substitutions on the aromatic ring are responsible for the chemical diversity of these compounds,

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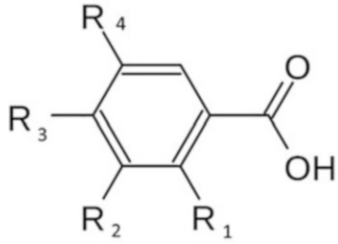
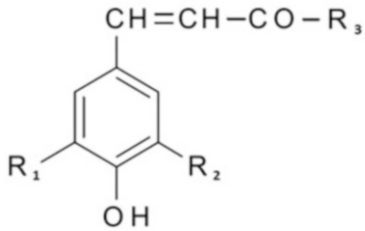


	R_1	R_2	R_3	R_4
Protocatechuic acid	H	OH	OH	H
Gallic acid	H	OH	OH	OH

Fig. 1 Chemical structure of hydroxybenzoic acids in grapes and wine



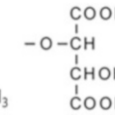
	R_1	R_2	R_3
p-Coumaric acid	H	H	OH
Caffeic acid	OH	H	OH
Ferulic acid	OCH_3	H	OH
Coutaric acid	H	H	
Caftaric acid	OH	H	
Fertaric acid	OCH_3	OCH_3	

Fig. 2 Hydroxycinnamic acids and derivatives in grapes and wine

in addition to the union of other molecules such as small organic molecules, structural components of plant cells, or large phenolic compounds [2]. In nature, these compounds occur with two separated constitutive carbon frameworks that are used to classify them into two classes: hydroxybenzoic (derivatives of benzoic acid) and hydroxycinnamic acids (derivatives of cinnamic acid).

Hydroxybenzoic acids are characterized by a C6-C1 structure directly obtained from benzoic acid. Structural changes of these compounds lie in the methylation and hydroxylation of the aromatic ring (Fig. 1). Gallic acid is the most common derivative found in wine [3]. The concentration of hydroxybenzoic acids in this fermented alcoholic beverage depends on the grape variety, growing conditions, and the winemaking process [4, 5].

Hydroxycinnamic acids have a simple chemical structure that contains a C6-C3 skeleton. Caffeic, *p*-coumaric, and ferulic acids are commonly found in grapes as tartaric acid esters (cinnamate esters) and glycosylated forms in grape pulp and skin and consequently in wines (Fig. 2) [6]. They can also be liberated from tartaric acid by hydrolysis during maturation and storage [7]. In nature, hydroxycinnamic acids are often present as a *trans* form, but isomerization to the *cis* one is induced by light exposure [8]. The concentration of these compounds varies widely with the conditions of grape growing [9].

Phenolic acids have been reported to exert antioxidant activity, inhibit oxidative damage diseases, and have an antibacterial effect [10, 11]. In addition, these compounds are involved in numerous reactions that occur during winemaking and aging. Hydroxycinnamic acids influence sensory wine properties such as color, taste, and flavor [8]. They play a crucial role in the copigmentation phenomenon, and anthocyanin-derived pigments synthesis, stabilizing the color of wine [12, 13]. The contribution of hydroxycinnamic acids to the browning of white wines has also been reported [14]. The grape reaction product (2-S-glutathionylcaftaric acid) is a reaction product of glutathione with oxidized caftaric acid that provides information on wine oxidation during winemaking and aging, avoiding the browning of wine [15]. Considering punctual conditions, *Brettanomyces/Dekkera* can produce the ethyl phenols from the hydroxycinnamic acids present in wine, generating unpleasant "Brett" aromas [16]. These non-flavonoids can also protect wine from oxygen action since they enhance the preservative effect of sulfur dioxide [17].

The most common solvents used to extract and determine phenolic acids from plant matrices and derived products are methanol, aqueous methanol, ethyl acetate, or diethyl ether [18], using solid phase extraction for isolating these compounds from other phenolic compounds in the case of grapes and wines [19, 20]. This technique is economical and rapid, and different sorbent cartridges can be used [21, 22]. High performance liquid chromatography (HPLC), particularly in reverse-phase mode, occupies a leading position in the analysis of phenolic acids, separating the molecules based on their hydrophobicity [23]. Organic and aqueous solvents with acids are employed as mobile phases. HPLC is most frequently coupled with a photodiode array detector (DAD) and mass spectrometry (MS) to identify and quantitate phenolic compounds [24, 25]. This chapter describes a methodology that can be used for the extraction, isolation, and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS^n) analysis of hydroxybenzoic and hydroxycinnamic acids in grapes and wine.

2 Materials

2.1 Chemicals

1. Acetonitrile (CH_3CN), HPLC-MS grade used only for HPLC mobile phases.
2. Ammonium hydroxide (NH_4OH), 20%, analytical reagent grade.
3. Commercial standards for hydroxybenzoic and hydroxycinnamic acids: gallic acid, protocatechuic acid, caffeic acid, caftaric acid, *p*-coumaric acid, coutaric acid, ferulic acid, fertaric acid.

4. Ethanol (CH_3CH_2OH), 96% (v/v), analytical reagent grade.
5. Formic acid (HCOOH), 99%, analytical reagent grade.
6. Formic acid (HCOOH), HPLC-MS grade used only for HPLC mobile phases.
7. Hydrochloric acid (HCl), 37%, analytical reagent grade.
8. Methanol (CH_3OH), analytical reagent grade.
9. Methanol (CH_3OH), HPLC-MS grade used only for HPLC mobile phases.
10. Milli-Q water.

2.2 Solutions and Solvents

1. Solution A: 97% CH_3OH, 3% HCOOH (v/v).
2. Solution B: 50% CH_3OH, 48.5% Milli-Q water, 1.5% HCOOH (v/v/v).
3. Solution C: 80% Milli-Q water, 20% CH_3OH (v/v).
4. Solution D: 80% CH_3OH, 20% Milli-Q water, containing 2% HCl (v/v).
5. Solution E: 80% CH_3OH, 20% Milli-Q water, containing 2% NH_4OH (v/v).
6. Solvent A: 3% CH_3CN, 88.5% Milli-Q water, 8.5% HCOOH (v/v/v).
7. Solvent B: 50% CH_3CN, 41.5% Milli-Q water, 8.5% HCOOH (v/v/v).
8. Solvent C: 90% methanol, 1.5% Milli-Q water, 8.5% HCOOH (v/v/v).

2.3 Equipment

1. C18 SPE cartridges, silica-based bonded phase (500 mg).
2. C18 column guard.
3. Centrifuge.
4. High performance liquid chromatography system coupled to photodiode array detector and electrospray-ion trap mass spectrometer (HPLC-DAD-ESI-MS).
5. HPLC vials, 2 mL.
6. Ice bath.
7. Polymeric cation-exchange resin SPE cartridges (500 mg), which combines cation exchange with reverse phase properties and retains all wine phenolic compounds.
8. Reversed-phase column C18, 2.1 × 150 mm, 3.5 μm particle.
9. Rotary evaporator.
10. Syringes, 5 mL.

11. Syringe filters, polyester membrane with 0.20 μm pore size.
12. Ultrasonic bath.
13. Homogenizer.
14. Vacuum manifold.

3 Methods

Perform all procedures at room temperature unless otherwise specified.

3.1 Phenolic Compound Extraction from Grapes

1. Select 50 healthy grapes just after sampling in the vineyard and process them immediately in the laboratory (*see* **Note 1**).
2. Immerse selected grapes in 50 mL of solution A and subject them to a homogenizer for 5 min at 15,000 rpm. Keep the sample into an ice bath during this step (*see* **Note 2**).
3. Centrifuge the phenolic extract obtained at 5,000 rpm for 5 min.
4. Filter and separate the supernatant with glass wood, placed in a funnel *(see* **Note 3**).
5. Repeat a second extraction of the resulting pellets using the same procedure but with 50 mL of solution B rather than solution A (*see* **Note 4**).
6. Join the two aliquots of extract and store it at −20 °C until analysis.

3.2 Phenolic Acid Isolation

Follow the solid phase extraction (SPE) procedure described below to isolate phenolic acids from anthocyanins in red grapes and wines (*see* **Note 5**):

1. Reduce 3 mL of phenolic compound extract from the grape to half regarding its original value using a rotary evaporator at 35 °C.
2. Dilute the concentrated extract with 1.5 mL of HCl 0.1 M. In the case of wine samples, dilute 3 mL of wine in 3 mL of HCl 0.1 M.
3. In the vacuum manifold, condition the polymeric cation-exchange resin with 5 mL of CH_3OH and 5 mL of Milli-Q water consecutively (*see* **Note 6**).
4. Pass the sample prepared in **Step 2** (3 mL of diluted extract or 6 mL of diluted wine) slowly through the SPE cartridge (*see* **Note 6**).
5. Wash the polymeric cation-exchange resin with 5 mL of HCl 0.1 M and 5 mL of Milli-Q water, consecutively. Dry the resin at the end of this step (*see* **Note 7**).

6. Elute and collect the phenolic acid fraction with 6 mL of CH_3CH_2OH 96%, passing drop by drop. Anthocyanins will remain retained in the resin due to their cationic characteristics at acidic pH, but all other phenolics will elute.
7. Regenerate the polymeric cation-exchange resin with 2 × 5 mL of solution D and 3 × 5 mL of solution E. Then, wash the cartridge with 5 mL of Milli-Q water to reuse it five more times at least (*see* **Note 6**).

In the case of white grapes, use the following SPE procedure to remove sugars that may interfere with the analysis of phenolic acids:

1. Reduce 3 mL of phenolic compound extract from grape to 1.5 mL in a rotary evaporator at 35 °C.
2. Condition the C18 cartridge with 5 mL of CH_3OH and 5 mL of Milli-Q water consecutively (*see* **Note 6**).
3. Pass the concentrated extract slowly through the SPE cartridge (*see* **Note 6**).
4. Wash the C18 cartridge with 5 mL of Milli-Q water and dry the resin at the end of this step (*see* **Note 7**).
5. Elute and collect the sugar-free fraction of phenolic acids with 6 mL of CH_3CH_2OH 96%.

3.3 Sample Preparation

1. Dry the obtained eluate (anthocyanin-free and sugar-free fractions isolated) under a vacuum in a rotary evaporator at 35 °C and redissolve in 1.5 mL of solution C.
2. Filter the reconstituted sample slowly and gently using syringe filters (polyester membrane with 0.20 μm pore size) and put it in an HPLC vial of 2 mL.
3. For white wines, evaporate 2 mL of wine to dryness in a rotary evaporator at 35 °C and reconstitute in 1 mL of solution C.
4. Keep samples at −20 °C until analysis.

3.4 Analysis of Hydroxybenzoic and Hydroxycinnamic Acid Derivatives by High Performed Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS^n)

1. Degas the solvents used as HPLC mobile phases (solvents A, B, and C) in an ultrasonic bath for 1 min.
2. Inject 20 μL of sample on a reversed-phase column C18 at 40 °C, connected to the guard, when the HPLC system is equilibrated and the stable baseline is obtained, with a flow rate of 0.19 mL/min. It is important to perform a blank run to ensure proper equilibration of the column.
3. Use a linear solvent gradient for hydroxybenzoic and hydroxycinnamic acid analysis, under the following conditions: zero min (96% solvent A, 4% solvent B), 8 min (96% solvent A, 4% solvent B), 37 min (70% solvent A, 17% solvent B, and 13% solvent C), 51 min (50% solvent A, 30% solvent B, and 20% solvent C), 51.5 min (30% solvent A, 40% solvent B, and 30%

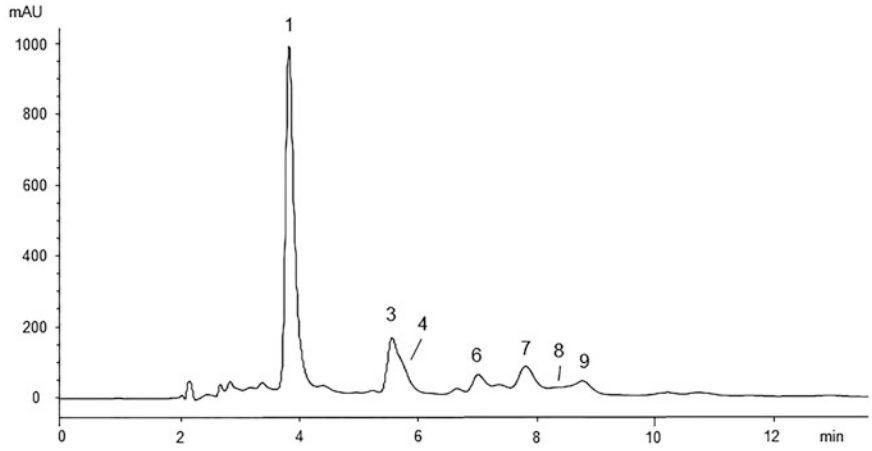


Fig. 3 HPLC–DAD chromatogram (detection at 320 nm) corresponding to hydroxycinnamic acid derivatives identified in grapes. For peak assignment, see Table 1

solvent C), 56 min (50% solvent B and 50% solvent C), 57 min (50% solvent B and 50% solvent C), and 64 min (96% solvent A and 4% solvent B).

4. Set the detection wavelengths at 280 and 320 nm and record UV-vis spectra at 200–600 nm.
5. Set the following parameters for the electrospray-ion trap mass spectrometer working in MS/MS mode: negative ionization mode, dry gas (N_2, 8 L/min), drying temperature (350 °C), nebulizer, (N_2, 40 psi), capillary (3500 V), skimmer 1 (−20 V), skimmer 2 (−60 V), scan range (100–1000 m/z). These parameters have been optimized for an ion trap with electrospray ionization model G2445C VL (Agilent) and should be optimized for other mass spectrometry detectors.
6. Identify hydroxybenzoic and hydroxycinnamic acids based on spectroscopic data obtained from commercial standards and reported in the literature (Figs. 3 and 4) (Table 1) [19, 20, 26, 27].
7. Quantitate hydroxycinnamic acids using DAD-chromatograms acquired at 320 nm and 280 nm for hydroxybenzoic acids. For each compound, prepare a calibration curve from the commercial standards in concentrations ranging from 0.1 to 100 mg/L (*see* **Note 8**).

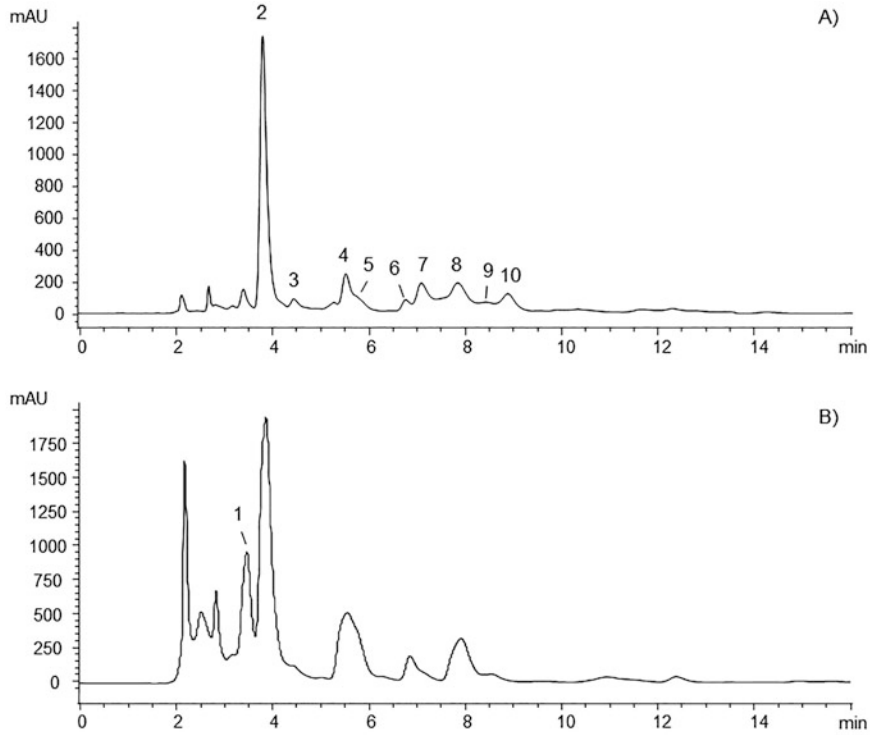


Fig. 4 HPLC–DAD chromatogram (detection at 320 nm) corresponding to hydroxycinnamic acid derivatives identified in red (**a**) and white (**b**) wines. For peak assignment, see Table 1

Table 1
Chromatographic and spectroscopic data of phenolic acids identified in grapes and wine

Peak[a]	R_t (min)	Compound	Molecular and Product Ions (m/z), Negative Ionization
	2.63	Gallic acid	169, 125
	3.23	Protocatechuic acid	153, 109
1	3.46	2-S-Glutathionylcaftaric acid	616, 484, 440, 272
2	3.80	*trans*-Caftaric acid	311, 179, 149
3	4.45	Caffeoyl-glucose	341, 179, 161
4	5.51	*trans*-Coutaric acid	295, 163, 149
5	5.92	*cis*-Coutaric acid	295, 163, 149
6	6.75	*p*-Coumaroyl-glucose 1	325, 163, 145
7	7.08	*p*-Coumaroyl-glucose 2	325, 163, 145
8	7.88	*trans*-Fertaric acid	325, 193, 149
9	8.41	*cis*-Fertaric acid	325, 193, 149
10	8.88	*p*-Coumaroyl-glucose 3	325, 163, 145

Rt retention time
[a]Peak numbers used in Figs. 3 and 4

4 Notes

1. Hydroxycinnamic acids are quickly degraded by oxygen action. Therefore, avoid damage to grapes during harvesting and transport.
2. Sample temperature increases significantly during homogenization. Due to this, grapes with the extraction solution must be kept in an ice bath during this process to reduce the phenolic compound degradation by temperature.
3. Filter the phenolic compound extract through a glass wood ball loosely stuffed in the narrow part of a funnel, to obtain a clear extract.
4. A second extraction of berry pellets yield nearly 99% of the phenolic content in grapes, as confirmed by spectrophotometry.
5. White grape extract and wine can be analyzed directly by the HPLC system, without using a previous isolation step, because these samples lack anthocyanins, that in red grapes and wines affect the identification and quantitation of the compounds of interest.
6. Do not let the resin dry at any time, only before the elution of phenolic acids.
7. If there is any water left in the resin, it can hinder the drying process at the rotary evaporator.
8. For the different points of the calibration curves, prepare solutions by successive dilutions from a single stock solution. Use a mix of CH_3OH and Milli-Q water to prepare the stock solution by dissolving a properly standard reagent and making dilutions with Milli-Q water.

References

1. Šamec D, Karalija E, Šola I et al (2021) The role of polyphenols in abiotic stress response: the influence of molecular structure. Plan Theory 10:118
2. Goleniowski ME, Bonfill M, Cusido R et al (2013) Phenolic acids. In: Ramawat K, Mérillon JM (eds) Natural products. Springer, Berlin/Heidelberg
3. Rentzsch M, Wilkens A, Winterhalter P (2009) Non-flavonoid phenolic compounds. In: Moreno-Arribas MV, Polo MC (eds) Wine chemistry and biochemistry. Springer, New York
4. Peña-Neira A, Hernández T, García-Vallejo C et al (2000) A survey of phenolic compounds in Spanish wines of different geographical origin. Eur Food Res Technol 210:445–448
5. Pozo-Bayón MA, Hernández MT, Martín-Álvarez PJ et al (2003) Study of low molecular weight phenolic compounds during the aging of sparkling wines manufactured with red and white grape varieties. J Agric Food Chem 51: 2089–2095
6. Shahidi F, Naczk M (2004) Phenolics in food and nutraceuticals. CRC Press, Boca Raton
7. Hixson JL, Sleep NR, Capone DL et al (2012) Hydroxycinnamic acid ethyl esters as precursors to ethylphenols in wine. J Agric Food Chem 60:2293–2298
8. Waterhouse AL, Sacks GL, Jeffery DW (2016) Non-flavonoids phenolics. In: Waterhouse AL, Sacks GL, Jeffery DW (eds) Understanding wine chemistry. Wiley

9. Nagel CW, Wulf LW (1979) Changes in the anthocyanins, flavonoids and hydroxycinnamic acid esters during fermentation and aging of Merlot and cabernet sauvignon. Am J Enol Vitic 30:111–116
10. Fernandez de Simon B, Perez-Ilzarbe J, Hernandez T et al (1992) Importance of phenolic compounds for the characterization of fruit juices. J Agric Food Chem 40:1531
11. Harris CS, Mo F, Migahed L et al (2007) Plant phenolics regulate neoplastic cell growth and survival: a quantitative structure-activity and biochemical analysis. Can J Physiol Pharmacol 85:1124
12. Hernández T, Estrella I, Carlavilla D et al (2006) Phenolic compounds in red wine subjected to industrial malolactic fermentation and ageing on lees. Anal Chim Acta 563:116–125
13. Schwarz M, Wabnitz TC, Winterhalter P (2003) Pathway leading to the formation of anthocyanin−vinylphenol adducts and related pigments in red wines. J Agric Food Chem 51: 3682–3687
14. Cheynier V, Rigaud J, Souquet JM et al (1990) Must browning in relation to the behavior of phenolic compounds during oxidation. Am J Enol Vitic 41:346–349
15. Cheynier VF, Trousdale EK, Singleton VL, Salgues MJ, Wylde R (1986) Characterization of 2-S-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. J Agric Food Chem 34:217–221
16. Schopp LM, Lee J, Osborne JP et al (2013) Metabolism of nonesterified and esterified hydroxycinnamic acids in red wines by Brettanomyces bruxellensis. J Agric Food Chem 61: 11610–11617
17. Bradshaw MP, Barril C, Clark AC et al (2011) Ascorbic acid: a review of its chemistry and reactivity in relation to a wine environment. Crit Rev Food Sci Nutr 51:479–498
18. Palacio L, Cantero JJ, Cusidó R et al (2010) Effect of inoculum age on kinetic of biomass formation and phenolic accumulations in Larrea divaricata Cav. Cell suspension culture. Mol Med Chem 21:64
19. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2019) Phenolic compounds profile of different berry parts from novel Vitis vinifera L. red grape genotypes and Tempranillo using HPLC-DAD-ESI-MS/MS: a varietal differentiation tool. Food Chem 295:350–360
20. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2019) First chemical and sensory characterization of Moribel and Tinto Fragoso wines using HPLC-DAD-ESI-MS/MS, GC-MS, and Napping® techniques: comparison with Tempranillo. J Sci Food Agric 99: 2108–2123
21. Matìjiček D, Klejdus B, Mikeš O et al (2003) Application of solid-phase extraction for determination of phenolic compounds in barrique wines. Anal Bioanal Chem 377:340–345
22. Del-Álamo M, Casado L, Hernández V et al (2004) Determination of free molecular phenolics and catechins in wine by solid phase extraction on polymeric cartridges and liquid chromatography with diode array detection. J Chromatogr A 1049:97–105
23. Aguilar MI (2004) Reversed-phase high-performance liquid chromatography. In: Aguilar MI (ed) HPLC of peptides and proteins, Methods in molecular biology. Springer, Totowa
24. Zuo Y, Chen H, Deng Y (2022) Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta 57:307–316
25. Tian S, Nakamura K, Cui T et al (2005) High-performance liquid chromatographic determination of phenolic compounds in rice. J Chromatogr A 1063:121–128
26. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2021) Genotypic variation in phenolic composition of novel white grape genotypes (Vitis vinifera L.). J Food Compos Anal 102:103987
27. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A et al (2020) Comprehensive chemical and sensory assessment of wines made from white grapes of Vitis vinifera cultivars Albillo Dorado and Montonera del Casar: a comparative study with Airén. Foods 9:1282

Chapter 10

Stilbene Identification and Quantitation by High-Performance Liquid Chromatography Coupled with Mass Spectrometry (HPLC-MS)

Eduardo Boido, Romina Curbelo, Luisina Rodríguez, Danilo Davyt, Eduardo Dellacassa, and Laura Fariña

Abstract

Stilbenes belong to the group of non-flavonoid phenolic compounds. Resveratrol is the main stilbene present in grapes and wines. Many analytical methods have been reported for the determination of resveratrol in wine, which are primarily based on chromatographic techniques like high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography (GC). This chapter presents a liquid-liquid extraction method for stilbenes in grapes. The analytical methodology here presented, carried out by HPLC-MS, can be used both for grapes and wine.

Key words Stilbenes, Resveratrol, HPLC-MS, Grape, Wine

1 Introduction

Stilbenes belong to the group of non-flavonoid phenolic compounds. Their presence in plant tissues is associated with resistance to fungal diseases such as *Botrytis cinerea*, although they can also appear in response to abiotic stresses such as UV irradiation. In general, stilbenes are considered phytoalexins, and their formation in grape leaves has been linked to disease resistance [1].

Resveratrol is the main stilbene present in grapes and wines [2]. Resveratrol occurs in both trans- and cis-isomeric forms, being the *trans*-form more abundant in grapes. In addition, the glyco-conjugate forms of resveratrol isomers are known as piceid (Fig. 1). Stilbenes can also be found in their oligomeric and polymeric forms, so-called viniferins [3]. Resveratrol and its derivatives can be found in different parts of the plant such as grape canes [4, 5] and grape skin [6] but also in wine [7].

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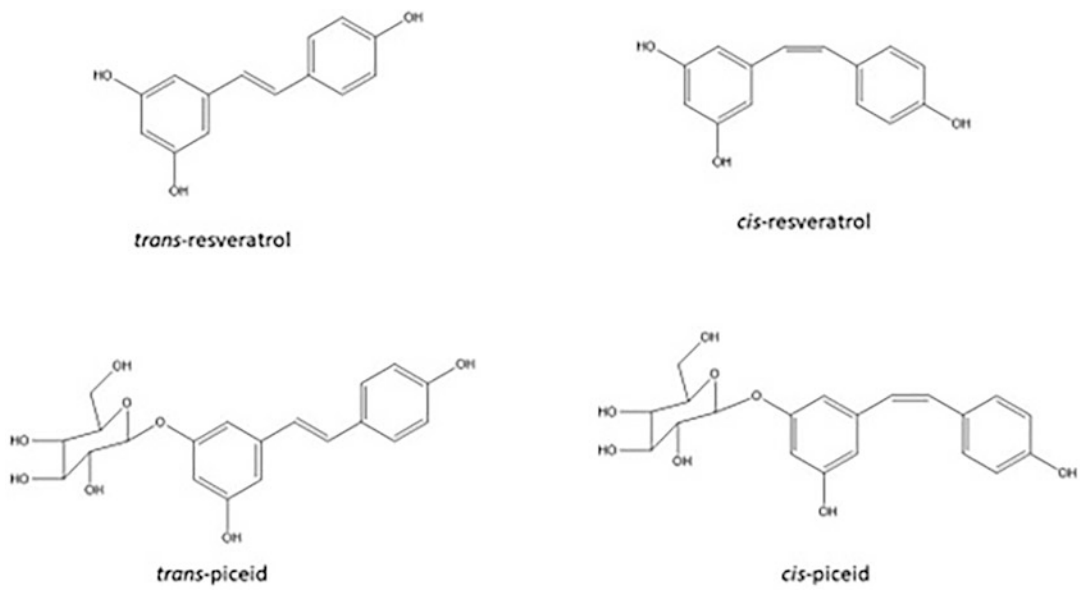

Fig. 1 Structure of the isomeric forms of resveratrol and its glycosides

Several protective and preventive effects are currently attributed to resveratrol and its derivatives, including antiaging, antioxidant, as an enhancer of NO production in endothelial cells, cardioprotection, and as a reducer of breast cancer cell invasion [8], among others. Resveratrol is rapidly adsorbed after oral administration, with maximum levels in the human body being reached in approximately 30–60 min [9].

In recent years, new stilbenoids have been identified, hence the growing interest in developing new methods and strategies for the quantitation of these compounds. Also, the new challenges posed by the need to analyze, in some cases, considerable small sample volumes gave rise to different analytical approaches and more complex instrumental techniques [8]. Moreover, the content of stilbenes is highly influenced by a set of factors such as the winemaking process, variety, and climate, among others. For this purpose, rapid screening of complex samples for the desired stilbenes is necessary, and the analytical information must be available.

Many analytical methods are reported for the determination of resveratrol in wine, mainly based on the application of chromatographic techniques like high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography (GC). Other techniques such as infrared spectroscopy, fluorimetry, and Raman spectroscopy were also used [10].

2 Materials

Solvents: methanol and ethyl acetate for HPLC (Sigma Aldrich). Acetonitrile and acetic acid for LC-MS (LiChropur, Sigma Aldrich).

Filter: syringe filter, 0.2 μm hydrophobic PTFE (Minisart® SRP15).

Column: Nucleodur 100-5 C18 (150 mm × 4.6 mm, 5 μm) (Macherey-Nagel).

Equipment: Shimadzu LCMS-8040 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Japan).

3 Method

3.1 Preparation of Grape Samples: Extraction Method

For extracting stilbenes, 1 g of berry skins or leaf tissue extracted in 10 mL methanol and ethyl acetate [50:50 (v/v)] for 24 h at 25 °C in darkness and then centrifuged at 10,000 g for 10 min [12]. The supernatants were evaporated to dryness by rotary vacuum evaporation at 40 °C. Dried residues were then dissolved in 2 mL methanol and stored at −20 °C before HPLC analysis.

3.2 Determination of Stilbenes in Grapes and Wine: HPLC-DAD Method

For HPLC analysis, wine and extracted grape samples were filtered (hydrophilic membrane filter 0.2 nm PTFE) and analyzed using a Shimadzu LCMS-8040 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Japan) consisting of a quaternary pump, degassing device, autosampler, PAD detector, and LC and LC/MS systems software. The injection volume was 20 μL. The separation of the compounds was performed on a Nucleodur 100-5 C18 column (150 mm × 4.6 mm, 5 μm) (Macherey-Nagel) thermostated at 30 °C. The chromatographic conditions of the method used were [11] mobile phase consisting of water/acetic acid (99:1 v/v) as solvent A, water/acetonitrile/acetic acid (67:32:1 v/v/v) as solvent B, and acetonitrile as solvent C, at a flow of 0.5 mL/min. The gradient program was as follows: 0 min, 80% A, 20% B; 18 min, 100% B; 28 min, 100% C; 33 min, 100% B; and 37 min, 80% A, 20% B. The compounds were detected at 200–400 nm. Figure 2 shows a chromatogram obtained from grapes observing that the maximum absorbances were 305 nm for *trans*-resveratrol and its glycosylated form (*trans*-piceid).

3.3 Stilbene Identification: HPLC-MS

The identification of stilbenes by HPLC techniques coupled with MS-MS has been described by several papers in recent years. Usually, methods perform negative-mode ionization. Table 1 shows the $[M\text{-}H]^-$ and MS/MS product ions for the main stilbenes detected in grapes and wines.

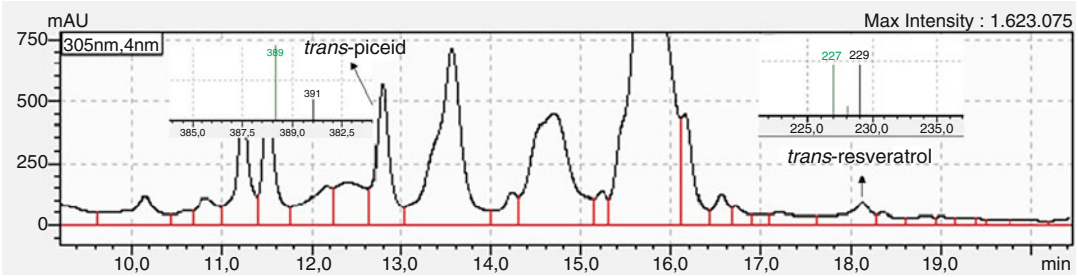

Fig. 2 Chromatogram obtained from Tannat grapes showing the retention times for trans-resveratrol and trans-piceid and their fragmentations (positive-mode ionizations m/z 391 and 229 and negative-mode ionizations m/z 389 and 227, respectively)

Table 1
Fragmentation patterns of main stilbenes present in grape and wine. Adapted from Moss et al. [7]

Compound	$[M-H]^-$	MS/MS product ions
Cis- and *trans*-resveratrol	227	185, **143**
Cis- and *trans*-piceatannol	243	201, **159**
Cis- and *trans*-piceid	389	**227**
Astringin	405	**243**, 201, 159
Cis- and *trans*-viniferin	453	435, 411, 369, **359**, **347**, 333, 225
Cis- and *trans*-viniferin	453	435, **411**, **369**, 359, 333

4 Notes

In the case of wine, only sample filtration (0.45 μm and/or by 0.22 μm) is needed previous injection.

References

1. Sun B, Ribes AM, Conceicao LM et al (2006) Stilbenes: quantitative extraction from grape skins, contribution of grape solids to wine and variation during wine maturation. Anal Chim Acta 563:382–390
2. Wilkens A, Rentzsch M, Winterhalter P (2008) Non-flavonoid phenolic compounds. In: Polo C, Moreno-Arribas MV (eds) Wine chemistry and biochemistry. Springer, New York
3. Langcake P, Pryce RJ (1977) A new class of phytoalexins from grapevines. Experientia 33: 151–152
4. Vergara C, von Baer D, Mardones C et al (2012) Stilbene levels in grape cane of different cultivars in southern Chile: determination by HPLC-DAD-MS/MS method. J Agric Food Chem 60:929–933
5. Lambert C, Richard T, Renouf E et al (2013) Comparative analyses of stilbenoids in canes of major Vitis vinifera L cultivars. J Agric Food Chem 61:11392–11399
6. Lago-Vanzela ES, Da-Silva R, Gomes E et al (2011) Phenolic composition of the edible parts (flesh and skin) of Bordô grape (Vitis labrusca) using HPLC-DAD-ESI-MS/MS. J Agric Food Chem 59:13136–13146
7. Moss R, Mao Q, Taylor D et al (2013) Investigation of monomeric and oligomeric wine stilbenoids in red wines by ultra-high-performance liquid chromatography/

electrospray ionization quadrupole time-of-flight mass spectrometry. Rapid Commun Mass Sp 27:1815–1827

8. Andrei V, Ngounou Wetie AG, Mihai J et al (2014) Detection of biomedically relevant stilbenes from wines by mass spectrometry. In: Woods AG, Darie CC (eds) Advancements of mass spectrometry in biomedical research, advances in experimental medicine and biology. Springer, Heidelberg
9. Walle T, Hsieh F, De Legge et al (2004) High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 32: 1377–1382
10. Fiod Riccio BV, Fonseca-Santos B, Colerato Ferrari P et al (2019) Characteristics, biological properties and analytical methods of trans-resveratrol: a review. Crit Rev Anal Chem 50(4):339–358
11. López-Hernández J, Rodríguez-Bernaldo de Quirós A (2016) Trans-stilbenes in commercial grape juices: quantification using HPLC approaches. Int J Mol Sci 17(10):1769
12. Liu C, Wanga L, Wang J et al (2013) Resveratrols in Vitis berry skins and leaves: their extraction and analysis by HPLC. Food Chem 136: 643–649

Chapter 11

Analysis of the Free and Bound Fraction of Volatile Compounds in Musts and Wines by GC/MS: Results Interpretation from the Sensory Point of View by OAV Technique

Pedro Miguel Izquierdo-Cañas, Sergio Gómez-Alonso, and Esteban García-Romero

Abstract

The aroma of wine is a complex equilibrium of volatile compounds originating from grapes, secondary products formed during the wine fermentation and aging. Solid-phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS) are successfully used to analyze the free and bound fraction of volatile compounds in musts and wines. The odor activity values (OAVs) from different compounds classified into seven odorant series can be calculated helping to describe the wine aroma profile (fruity, floral, green/fresh, sweet, spicy, fatty, and other odors). The total intensities for every aromatic series can be calculated as the sum of the OAV of each compound assigned to this series.

Key words Volatile compounds, Must, Wine, GC-MS, OAV, Free and bound fraction

1 Introduction

Organic volatile compounds play a relevant role in wine quality predominantly due to their influence on aroma sensory profile. It is estimated that aroma constituents of grapes and wines account for around 1,000 different compounds and comprise compounds of different chemical classes and characters, presenting concentrations ranging from ng/L to mg/L. Their contribution to the overall wine flavor is different, depending on their odor thresholds and concentrations and only a small percentage of them can impact odorants [1, 2].

The wine aroma is a complex equilibrium of volatile compounds originating from grapes (varietal and pre-fermentative aromas), secondary products formed during the wine fermentation (fermentative aromas), and aging (post-fermentative aromas).

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Volatile substances responsible for the aromatic typicity of the grape variety are reduced to a few groups of chemical compounds present in the grape as free form of monoterpenes [3], C-13-norisoprenoids [4], benzenoid compounds, C6 compounds, and other aliphatic compounds [5], which can also be found bonded to sugars forming glycosides (bound fraction) [6, 7] that can be released, at least partially, during fermentation.

From a quantitative point of view, the volatiles derived from alcoholic fermentation constitute the most significant part of the wine's aroma. As a consequence of the secondary metabolism of yeast, many compounds are produced and participate in the aromatic quality of wines: esters [1], lactones [8], fatty acids [9], alcohols, etc. The most relevant effect of the wine's contact with oak wood is the enrichment of the wine in volatile substances released, especially, volatile phenols, furan compounds, and lactones [10].

Initially, gas chromatography (GC) separations used relatively short-packed columns that separated only a few compounds in complex mixtures. For example, an early wine application separated ten fusel alcohols in wine distillates [11]. The introduction of fused-silica capillary columns increases chemical inertness and the ability to reproduce the procedure with very long and very narrow diameter columns. This fact significantly improved the efficiency and ability to separate hundreds of compounds, many of them present at trace levels [12].

After the compound separation, the detection process has also improved dramatically in the last decades, changing from Flame Ionization Detectors (FID) to Mass Spectrometry (MS). The introduction of the MS detector had significant advantages, such as the lower limits of detection and quantitation and easier and more reliable compound identification due to the availability of extensive mass spectral index databases. Contemporary MS detectors include various instrument configurations and types of mass analyzers, including transmission quadrupole, ion trap, and time-of-flight (TOF).

1.1 Wine Mayor Volatile Compounds

Major volatile compounds in wines present concentrations above 1 mg/L: acetaldehyde, ethyl acetate, methanol, n-propanol, isobutanol, 2-methyl-1-butanol, 3-methyl-1-butanol, ethyl lactate, and 3-hydroxy-2-butanone. OIV proposes the analysis of methanol (OIV-MA-AS312-03A) and major volatile compounds (OIV-MA-AS315-27) in wines using direct injection of the distillate, adding an internal standard, using gas chromatography with a Flame-Ionization Detector (FID). Detection limits can be decreased when using GC-MS systems in Single Ion Monitoring (SIM) mode, and compounds in a lower concentration, such as 2,3-butanedione or 1-butanol, can also be quantitated [13].

When concentrated aqueous samples are used for GC direct injection techniques, several problems may appear. When water is converted to steam, the volume increases dramatically: 1 μL of water becomes more than 1000 μL of steam. This is larger than the injector volume of some current gas chromatographs and it forces it to work in split mode. Moreover, polar gas chromatography liquid phases could degrade in the long run by the presence of steam, unless they are bonded to the column. The treatment with nitroterephthalic acid of polyethylene glycol (PEG) columns (named FFPA (Free Fatty Acids Phase) stationary phase) minimizes this effect [14].

1.2 Minor Volatile Compounds

The volatile wine compounds comprise a wide range of organic chemicals that possess different polarities and reactivities and usually occur in trace concentrations. Significant fragrances are present in grapes and wines at low concentrations, and many of them are characterized by a low sensory threshold. Direct injection into GC-MS in SIM mode can reach detection limits of around 0.1 mg/L, but the concentration of the most wine relevant compounds is usually below this limit, so it is essential to carry out extraction and concentration of the sample. Techniques that effectively isolate and concentrate volatile aroma compounds or their precursors from non-volatile matrix components are the critical step.

Direct solvent extraction uses solvents such as dichloromethane/pentane 2:1 (v/v) or Freon 11 using separatory funnels or commercial continuous liquid–liquid extractors followed by a concentration stage. These methods often require several hours of extraction and analysis for each wine sample, which is a disadvantage. For example, OIV validated a few methods using this technique: determination of 3-methoxypropane-1,2-diol and cyclic diglycerol previous extraction with diethyl ether (OIV-MA-AS315-15), analysis of polychlorophenols and polychloroanisols in wines, cork stoppers, wood, and bentonites by injecting a hexane extract of the wine and an ether/hexane extract of the solid samples (OIV-MA-AS315-17) or, finally, determination of phthalates in wines by extraction of the samples with isohexane (OIV-MA-AS323-10).

Micro steam distillation-extraction is another technique that allows operation with a small volume of solvent without requiring further concentration of the extract before GC analysis [15], but also requires long extraction time. The static or dynamic headspace, purge-and-trap techniques have been used in less extension. The advantage of these techniques is that they directly collect the volatile compounds from the headspace, which can then be directly related to the sample aroma. These methods are simple but have poor reproducibility, they are biased toward the extraction of highly

volatile and semi-volatile compounds, they have limitations in detecting trace analytes, and they also are often unrepresentative of the sample composition [16].

Stir Bar Sorptive Extraction (SBSE), Solid-Phase Microextraction (SPME), and Solid-Phase Extraction (SPE) are based on similar concepts of absorbing the desired compounds onto a solid phase and then desorbing the compounds either by thermal means or by displacement with a solvent.

Stir bar sorptive extraction (SBSE or Twister) has been used to analyze volatile phenols [17] or chloroanisoles [18]. This technique uses a magnetic stir bar (typically 10 mM in length) incorporated in a glass tube and coated with polydimethylsiloxane (PMDS). Upon stirring in a liquid sample matrix, the analytes are partitioned between the matrix and the PMDS phase on the stir bar. Finally, the stir bar is transferred to the thermal desorption unit coupled with a cold injection system as an injector to the GC column. The principal advantage of SBSE over SPME is that the stir bar is coated with 25–125 μL of sorptive phase compared to only approximately 0.5 μL of sorptive phase on a SPME fiber; the increased phase volume of the SBSE sorptive phase allows for a substantial increase in sensitivity. Otherwise, the SBSE coatings are currently limited regarding the sorptive phase types. Also, specialized thermal desorption and cryofocusing inlets and sampling stations are required on the GC-MS instruments.

Solid-phase microextraction of wine was developed by both headspace and liquid-phase sampling. The primary advantage of this technique is that it combines analyte extraction and preconcentration in a single step without significant sample preparation. Robinson et al. [19] exhaustively revised materials used for the extraction-concentration of aroma compounds considering the type of fiber (polydimethylsiloxane, carbowax, divinylbenzene, carboxen, polyacrylate, and different combinations of these fibers) and their affinity for the compounds of interest, and also considered the possibility of sampling automation. OIV describes various methods using SPME, that is, releasable 2,4,6-trichloroanisole in wine by cork stoppers and determination of wine alkylphenols.

In general, SPE provides high recoveries of most fermentative volatiles in wine (85–100%), but requires longer times and is quite solvent consuming. On the other hand, the principal advantage of this approach is the allowance to separate the bound fraction of the aroma, glycoside compounds that can be analyzed as aglycones after enzymatic hydrolysis [19]. This is especially relevant when you want to characterize a punctual variety, winemaking technique, or winemaking additive. The must or wine is loaded in a cartridge that contains a determined absorbent. Hydrophilic compounds are removed by water washing, free volatile compounds are removed with a non-polar organic solvent, and the fraction of glycosides is recovered with a polar organic solvent. The most widely used

absorbents have been C-18, polystyrene polymer Amberlite XAD-2, or ethylvinylbenzene-divinylbenzene copolymers as Porapak Q and Lichrolut [20]. After the solid-phase extraction step, the extract must be concentrated at least 1000-fold to perform MS analysis operating in SCAN mode to use the mass spectra libraries for compound identification.

1.3 Odor Activity Values (OAVs)

Predicting the wine aroma from the results of individual quantitation of its hundreds of volatile compounds by GC-MS is impossible in practice. The method based on the OAV has been used in the latter years, such as in the discrimination of wines obtained from different grape varieties or characterization of the varietal aroma of wines [21, 22], also on accelerated aging studies [23] and in works about the influence of different enological techniques [24].

2 Materials

1. Solvents: Milli-Q water, pentane, dichloromethane, ethyl acetate, and methanol of GC grade.
2. Filters of 0.45 μM pore size made form non-absorbent materials.
3. GC vials with 150 μL inserts, 100 mL beakers.
4. Micropipettes of 10–200 μL, 50–1000 μL, pipettes of 25 mL.
5. Glass funnel with glass wool.
6. General laboratory equipment: Analytical balance, SPE vacuum manifold with a vacuum pump, vacuum rotatory evaporator, concentration-distillation system with a 100 mL flask and a 40 cm Vigreux column, pH-meter, oven.
7. Capillary column: FFPA stationary phase, polyethylene glycol treated with nitroterephthalic acid (e.g., BP21, SGE, Ringwood, Australia) 50 m length × 0.32 mM internal diameter; 0.25 μM film thickness.
8. Gas chromatographic instrument coupled to a mass spectrometer with electron impact ionization source and quadrupole analyzer, and equipped with an autosampler.
9. SPE sorbent: LiChrolut EN (40–120 μM). It is a highly cross-linked ethylvinylbenzene-divinylbenzene copolymer.
10. Glycosidic enzyme with strong glycosidase activity, e.g., Lallzyme BETA (Lallemand).
11. Commercial standards of volatile compounds of analytical grade from different suppliers.

12. Citrate buffer 0.2 molar, pH = 5.00 (for 1 L dissolve 38.426 g of citric acid in about 950 mL of water, bring to pH = 5 with 12 N NaOH, make up to 1 L in a volumetric flask).

3 Methods

3.1 Analysis of Major Volatile Compounds

1. If the sample contains any particulates, it should be filtered by, at least, 0.45 μM pore size.
2. In a GC vial, 100 μL of wine is added to 100 μL of 4-methyl-2-pentanol (50 mg/L) as the internal standard and diluted with 1 mL of Milli-Q water.
3. The sample is injected (0.8 μL) in split mode (split ratio: 10) at 195 °C in the FFPA capillary column. Helium is used as the carrier gas with a constant flow of 1.2 mL/min.
4. The initial oven temperature is 40 °C maintained during 2 min, followed by increases of 5 °C/min up to 120 °C and 75 °C/min up to 190 °C and finally an isothermal phase of 18 min at 190 °C.
5. The Flame Ionization Detector (FID) can be used to quantitate the concentration of major volatile compounds, but some laboratories work with a mass spectrometer detector with electron impact ionization source and quadrupole analyzer in SCAN mode for major compounds and SIM mode for 1- and 2-butanol, and 2,3-butanodione.
6. The compounds analyzed are listed in Table 1 with their retention times, principal ions of mass spectra, and the m/z of the ion used for quantitation. Identification is carried out by comparing the analyte retention time and mass spectra with those of commercial standards. The quantitation was based on calibration curves made using pure standards.

3.2 Analysis of Minor Volatile Compounds

Minor volatile compounds are extracted using the method developed by Ibarz et al. (2006) [25] with some modifications.

3.2.1 Extraction

1. If the sample contains any particulates, it should be filtered by, at least, 0.45 μM pore size. In a 100 mL beaker are mixed: 25 mL of the sample of must or wine and 25 mL of Milli-Q water, adding 500 μL of 4-nonanol 0.1 g/L as internal standard.
2. SPE cartridge (musts, wines, and glycosidic extracts): a 10 mL solid phase extraction cartridge is used loaded with 0.3 g of LiChrolut EN adsorbent.
3. The cartridge is previously activated by the successive passage of 2 mL of pentane: dichloromethane (2:1, v/v), 2 mL of methanol and, in the case of musts and glycosidic extracts

Table1
Mayor volatile compounds

RT (min)	Compound	Mass spectra[a]	Quantification[b] m/z
3.12	Acetaldehyde	44/43/42/41	43
4.10	Ethyl acetate	43/70/61/88	70
4.37	Methanol	31/32	31
5.39	2,3-Butanodione (diacetyl)	43/86	86
6.25	Ethyl butyrate	71/43/88	71
6.45	Propanol	31/59/42	59
7.65	Isobutanol (2-Methyl-1-propanol)	43/41/42/74	74
7.97	Isoamyl acetate	43/55/70/61	70
9.20	1-Butanol	56/43/41	56
9.57	4-Methyl-2-pentanol (Internal Standard)	45/69	45
10.59	3-Methyl-1-butanol +2-Methyl-butan-1-ol	55/70/41/43/57	55
12.81	3-Hydroxy-2-butanone (acetoin)	45/43/88	45
14.01	Ethyl lactate	45/75	45

[a]m/z of the principal ions in the mass spectra of each compound
[b]m/z of the ion used for quantification

5 mL of water, and for extracting wines 5 mL of ethanol 6% (v/v).

4. The sample is passed through the cartridge at a rate of around 1 drop/seg.
5. The sorbent is washed with 25 mL of Milli-Q water to remove the salts, sugars, and more polar compounds.
6. The free volatile fraction elution is carried out with 15 mL of pentane-dichloromethane (2:1, v/v). This fraction is placed in a freezer so that the residual water is frozen and can be separated from the extract by filtration on glass wool.
7. Extracts are then concentrated to around 2 mL by distillation in a 40 cm length Vigreux column and finally to 100 μL under nitrogen stream and then transferred to a 150 μL chromatographic vial insert and kept at −20 °C until analysis.
8. The glycosidic fraction is eluted from the sorbent with 25 mL de ethyl acetate: methanol (9:1, v/v). The ethyl acetate extract was evaporated to dryness under vacuum at 40 °C, and then re-dissolved with 5 mL of citrate-phosphate buffer (0.2 M, pH 5).
9. Enzymatic hydrolysis of the bound fraction is carried out by adding 200 mg of Lallzyme BETA (Lallemand) and incubation at 40 °C for 18 h.

10. The solution is centrifuged, added with 500 μL of 4-nonanol 0,1 g/L and the resulting solution is passed through 0,3 g LiChrolut EN cartridge previously activated (**Step 3**) and extracted following the 4–7 steps.

3.2.2 GC-MS Analysis

The FFPA capillary column is the same used for major volatile analysis.

1. 1 μL of the SPE extract concentrated is injected in the splitless mode (splitless time: 0.3 min). The chromatographic conditions are as follows: Initial oven temperature 40 °C for 15 min, followed by increases of 2 °C/min to 100 °C, 1 °C/min to 150 °C, 4 °C/min to 210 °C, and holding this temperature for 55 min. Injector temperature, 220 °C and carrier helium gas at 1 mL/min.
2. The following detector parameters are set: mass scanning range, 40–250 amu; ion source temperature 250 °C; impact energy, 70 eV; and electron multiplier voltage, 1603 V; detector voltage, 250 V; and emission current, 150 μV.
3. The mass-spectral library and retention times of pure commercial volatile compounds allowed the volatile compound identification. When the authentic standard is unavailable, the identification is based on the comparison with the spectral data of Wiley and NIST mass-spectral libraries. The analyzed compounds are listed in Table 2 with their relative retention times vs 4-nonanol principal ions of mass spectra, and the m/z of the ion used for quantification in our laboratory.
4. The quantitation by GC-MS is done using selected m/z fragments extracted from the total ion chromatogram for each compound using the internal standard method. Results for non-available compounds are expressed in concentration units as internal standard equivalents obtained by normalizing the compound peak area to that of the internal standard and multiplying by the concentration of the internal standard.
5. The relative response areas for each volatile compound in reference to the internal standard are calculated and interpolated in the corresponding calibration graphs. Individual stock solutions are prepared for each volatile compound at 500 mg/L concentration in absolute ethanol. From these stock solutions, a initial work solution is obtained that contains each of the volatile compounds in a concentration of approximately 10 times that found for each of them in the wines. Working calibration solutions are prepared from the stock solution of the mixture at eight different concentration levels in 12% v/v

Table 2
Minor volatile compounds grouped by families

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
	Alcohols			
0,337	3-Pentanol[c]		59/41	59
0,347	1-Butanol[c]		56/41/73	56
0,574	3-Methyl-3-buten-1-ol[c]		41/68/56/86	68
0,582	1-Pentanol[c]		42/55/70	70
0,726	*c*-2-Penten-1-ol[c]		57/41/68/71/86	57
0,802	3-Methyl-2-buten-1-ol[c]		71/41/53/68/67/86	71
0,805	2-Heptanol[c]		45/55/83/70	55
0,809	3-Methyl-1-pentanol[c]		56/69/41/84	84
0,817	3-Ethoxy-1-propanol[c]		59/45/71/75/86	59
0,838	3-Octanol[c]		59/83/55/41/101	59
0,953	1-Octen-3-ol[c]		57/43/72/85	57
0,977	6-Methyl-5-hepten-2-ol[c]	Sulcatol	95/41/69/55/110	110
1,040	2-Ethyl-1-hexanol[c]		57/41/55/70/83/69	57
1,048	4-Nonanol (Internal Standard) [c]		55/73/83/101	73
1,193	L-2,3-Butanodiol[c]		45/57/75	75
1,234	1-Octanol[c]		56/55/41/43/69/70/84/83	84
1,243	Meso-2,3-butanodiol[c]		45/57/75	57
	Acetates			
0,238	Isobutyl acetate[c]		43/56/73/61	56
0,299	Isoamyl acetate[c]		43/70/55/87/61	61
0,619	Hexyl acetate[c]		43/56/61/69/84	56
0,692	*t*-3-Hexenyl acetate		43/67/82	67
0,708	*c*-3-Hexenyl acetate[c]		43/67/82	67
0,851	Octyl acetate[c]		43/57/70/41/55/56/83/112	70
1,403	Benzil acetate[c]		108/91/90/89/79/150	108
1,565	2-Phenylethyl acetate[c]		104/43/91/65	104
2,632	2-Methoxy-2-phenylethyl acetate		104/105/91/45/51	105

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
	Ketones and aldehydes			
0,675	1-Octen-3-one[c]		70/55/97/43/71/83	97
0,746	6-Methyl-5-hepten-2-one[c]	Sulcatone	43/69/55/108/58/126/93	108
0,969	2-Octenal[c]		55/41/70/83/57/97	
	Ethyl esters			
0,262	Ethyl isobutyrate[c]			
0,264	Ethyl 2-methyl-butyrate[c]		57/102/85/41/74/115	102
0,273	Ethyl 3-methyl-butyrate[c]		88/85/60/70/115/57	115
0,300	Ethyl butyrate[c]		71/43/88/60/101/116	88
0,536	Ethyl hexanoate[c]	Ethyl caproate	88/43/99/60/70	88
0,694	Ethyl pyruvate[c]	Ethyl 2-oxopropanoate	43/61/116	116
0,764	Ethyl lactate[c]	Ethyl 2-hydroxypropionate	45/75	75
0,915	Ethyl octanoate[c]	Ethyl caprilate	88/57/70/101/127	88
0,969	Ethyl-2-hydroxy-butyrate		59/41/75	59
1,057	Ethyl 3-hydroxy-butyrate[c]		43/71/60/87/117	71
1,182	Ethyl 2-hydroxy-hexanoate		69/87/43/104	87
1,196	Ethyl 4-oxo-butyrate		85/102/56/74	102
1,296	Ethyl 4-oxo-pentanoate	Ethyl levulinate	43/99/74/129/55	99
1,320	Ethyl 2-furan-carboxylate[c]	Ethyl furoate	95/112/140/39	95
1,369	Ethyl decanoate[c]	Ethyl caprate	88/60/43/73/101/115	88
1,417	Ethyl 3-hydroxy-hexanoate		117/71/89/88	117
1,445	Ethyl 9-decenoate		88/41/55/69/101/110/152	152
1,675	Ethyl salicylate[c]	Ethyl 2-hydroxybenzoate	120/92/166	120
1,695	Ethyl 4-OH-butyrate		87/88/43/60/102/74/69	87
1,729	Ethyl dodecanoate[c]	Ethyl laurate	88/101/43/70	88
2,165	Ethyl 3-hydroxydecanoate		117/43/71/88/55/127	117

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
2,361	Ethyl cinnamate[c]	Ethyl 3-phenyl-2-propenoate	131/103/77/51/176/148	131
2,453	Ethyl hexadecanoate[c]	Ethyl palmitate	88/101/43/55/70	88
2,655	Ethyl succinate	Ethyl butanedioate	101/73/55/45/128	128
2,914	Ethyl 3-hydroxycinnamate	Ethyl 3-(3-hydroxyphenyl)prop-2-enoate	147/192/119/164	192
	C6 compounds			
0,248	Hexanal[c]		44/56/57/72/82	82
0,426	*c*-3-Hexenal		41/69/55/83/80	69
0,438	*t*-3-Hexenal		41/69/55/83/80	69
0,563	*c*-2-Hexenal		41/55/69/83/98	69
0,593	*t*-2-Hexenal		41/55/69/83/98	69
0,783	1-Hexanol[c]		56/43/69/84	69
0,800	*t*-3-Hexenol[c]		41/67/82/55/69	67
0,835	*c*-3-Hexenol[c]		41/67/82/55	67
0,879	*t*-2-Hexenol[c]		57/41/82/67	57
0,896	*c*-2-Hexenol[c]		57/41/67/82	57
	Terpenoids			
0,435	Limonene [c]	1-Methyl-4-(1-methylethenyl)-cyclohexene	68/93/79/121/53/107/136	93
0,532	Eucalyptol[c]	1,8-Cineole; 1,8-Epoxy-p-menthane	43/81/108/139/154	108
0,716	*β*-Ocimene[c]	3,7-Dimethyl-1,3,6-octatriene	119/134/91/65/77	119
0,845	*t*-rose oxide[c]	Tetrahydro-4-methyl-2-(2-methylpropenyl)-2H-pyran	139/69/83/55/154	139
0,875	*c*-rose oxide[c]	Tetrahydro-4-methyl-2-(2-methylpropenyl)-2H-pyran	139/69/83/55/154	139
0,921	*t*-Furanic linalool oxide[c]	*t*-2-Methyl-2-vinyl-5-(1-hydroxy-1-methylethyl)tetrahydrofuran	59/43/94/93/68/111/137/155	94
0,970	*c*-Furanic linalool oxide[c]	*c*-2-Methyl-2-vinyl-5-(1-hydroxy-1-methylethyl)tetrahydrofuran	59/43/94/93/68/111/137/155	94
1,109	Linalool[c]	3,7-Dimethyl-1,6-octadien-3-ol	71/93/41/55/80/121	93
1,294	Hotrienol	3,7-Dimethyl-1,5,7-octatrien-3-ol,	71/82/43/67/55	71

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
1,342	Menthol[c]	2-Isopropyl-5-methylcyclohexanol	71/81/95/123/138	138
1,346	*c*-Citral[c]	3,7-Dimethyl-2,6-octadienal; geranial	41/69/84/94/109/59/53/119	109
1,370	*α*-Terpineol[c]	2-(4-Methylcyclohex-3-en-1-yl) propan-2-ol; *p*-Menth-1-en-8-ol	59/93/43/68/121/136	59
1,406	*t*-Citral[c]		69/41/84/94/53/109/123/137	123
1,529	*t*-Piranic linalool oxide	*t*-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	68/43/59/94	68
1,550	2,7-Dimethyl-4,5-octanodiol		69/43/45/87/86/57/75	69
1,561	*c*-Piranic linalool oxide	*c*-2,2,6-Trimethyl-6-vinyltetrahydro-2H-pyran-3-ol	68/43/59/94/79/121	68
1,579	Citronellol[c]	3,7-Dimethyl-6-octen-1-ol	69/41/55/95/81/109/138	95
1,597	Nerol[c]	*c*-3,7-Dimethyl-2,6-octadien-1-ol	69/41/93	93
1,647	Geraniol[c]	*t*-3,7-Dimethyl-2,6-octadien-1-ol	69/41/93/67	93
1,690	Geosmin[c]	Dimethyloctahydronaphthalen-4a (2H)-ol	112/111/125/125/149/182	112
2,041	Terpendiol I	3,7-Dimethyl-1,5-octadien-3,7-diol	82/71/43/67	82
2,084	3,7-Dimethyl-1-octen-3,7-diol		71/43/68/81/59/56/121/93	71
1,950	p-Menta-1,8-dien-7-ol[c]	4-Isopropenyl-cyclohex-1-ene-1-methanol, perillyl alcohol	68/67/93/91/79/121/134/152	152
2,361	p-Menthane-1,8-diol	*c*-4-Hydroxy-α,α,4-trimethylcyclohexanemethanol	81/96/59/43/71/139	139
2,436	Terpendiol II	3,7-Dimethyl-1,7-octadien-3,6-diol	67/71/43/82/55	67
2,514	Hydroxycitronellol	3,7-Dimethyl-1,7-octanodiol	59/43/55/70/41/83/98/123	59
2,535	3-Methyl-hepta-1,6-dien-3-ol		71/43/55	71
2,544	Limonene aldehyde	3-(4-Methyl-1-cyclohex-3-enyl) butanal	93/43/67/101/95/108/123/81/55/148	93

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
2,637	8-Hydroxylinalool	2,6-Dimethyl-2,7-octadien-1,6-diol	43/71/67/55/73/60/84/119/137	71
2,784	7-Hydroxyterpineol	p-Menth-1-en-7,8-diol	79/59/93/109/121/152	79
	Other esters			
0,597	Isoamyl butyrate[c]		71/70/43/55/89	71
0,838	Methyl octanoate[c]		74/87/59/115/127	74
1,125	Isoamyl lactate	3-Methylbutyl 2-hydroxypropanoate	45/55/70/71	70
1,162	Dimethyl malonate[c]	Dimethyl propanedioate	115/133/43/88/60/69	115
1,186	Dimethyl succinate[c]	Dimethyl butanedioate	115/55/59/87/114/84/97	115
1,252	Ethyl-methyl succinate	Ethyl-methyl butanedioate	115/55/101/87/129	115
1,266	Isoamyl octanoate		70/127/145/71	127
1,322	Diethyl succinate[c]	Diethyl butanedioate	101/129/73/55	129
1,478	Methyl salicylate[c]	Methyl 2-hydroxybenzoate	120/92/152/65/121	152
1,511	Diethyl glutarate[c]	Diethyl pentanedioate	143/91/115/97/43/45/55	143
1,627	2-Phenylethyl formate	2-Phenethyl methanoate	104/91/65/103/105	104
1,655	Ethyl-propyl succinate	Ethyl-propyl butanedioate	101/129/43/73/55/143	101
2,057	Diethyl malate[c]	Diethyl 2-hydroxybutanedioate	71/117/89/43	117
2,426	Methyl anthranilate[c]	Methyl 2-aminobenzoate	119/151/92/120/44	119
2,591	2-Phenylethyl isovalerate	2-Phenylethyl 3-methylbutanoate	104/85/57/45	104
2,611	2-Phenylethyl lactate	2-Phenylethyl 2-hydroxypropionate	104/45/105/91/77/65	104
2,605	Methyl succinate	Methyl butanedioate	101/55/45/59/73/114	101
2,881	2-Phenylethyl-ethyl succinate	2-Phenylethyl-ethyl butanedioate	104/105/101/91/77	104
	Furanic compounds			
0,703	5-Methyldihydro-3(2H)-furanone	4,5-Dihydro-5-methylfuran-3(2H)-one	42/100/58/70	100
0,743	Furfuryl formate	2-Furylmethyl formate	81/126/53/97	126

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
0,918	2-Acetylfuran[c]	1-(2-Furanyl)-ethanone	95/110/39/43/67/75	95
0,985	Furfural[c]	2-Furaldehyde	96/95/39/67/97	96
1,239	5-Methyl-2-furfural[c]	5-Methyl-2-furaldehyde	110/109/53/81/95/111	110
1,257	4-Methoxy-2,5-dimethyl-3(2H)-furanone	Berry furanone	142/68/99	142
1,349	Furfuryl alcohol[c]	Furanmethanol	98/41/81/53/69	98
1,383	2(5H)-3-Methyl-furanone		41/69/98/53/70	98
2,032	2,5-Dimethyl-4-hydroxy-3(2H)-furanone[c]	Furaneol, Strawberry furanone, Pineapple ketone	43/57/128/85/55/72	128
2,699	5-(Hydroxymethyl)furfural[c]	5-Hydroxymethyl-2-furaldehyde	97/126/41/69	97
	Norisoprenoids			
1,118	Vitispirane	2,10,10-Trimethyl-6-methylidene-1-oxaspiro[4.5]dec-7-ene	93/192/121/177/136/43/77/107/149	192
1,193	β-Damascone[c]	1-(2,6,6-Trimethyl-1-ciclohexen)-2-buten-1-one	177/69/192/41/123/81/107/135/149	177
1,554	Damascenone[c]	1-(2,6,6-Trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	69/121/41/105/190/175	190
1,740	*α*-Ionone[c]	4-(2,6,6-Trimethylcyclohex-2-en-1-yl)but-3-en-2-one	93/121/43/136/77/109/192/55	121
1,790	*β*-Ionone[c]	4-(2,6,6-Trimethylcyclohex-1-en-1-yl)but-3-en-2-one	177/43/91/135/77/121	177
2,781	3-Hydroxi-*β*-damascone	1-(3-Hydroxy-2,6,6-trimethyl-1-cyclohexen-1-yl)-2-buten-1-one	69/41/175/121/193/208	208
2,834	Dihydro-*α*-ionone[c]	4-(2,2,6-Trimethyl-5-cyclohexen-1-yl)-2-butanone	95/69/57/112/136/149	112
2,875	3-Oxo-*α*-ionol	4-(3-Hydroxybut-1-enyl)-3,5,5-trimethylcyclohex-2-en-1-one	108/43/91/135/152	108
2,884	β-Ionol[c]	4-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-3-buten-2-ol	121/119/136/161/194	121
2,945	3-Oxo-7,8-dihydro-*α*-ionol	4-(3-Hydroxybutyl)-3,5,5-trimethylcyclohex-2-en-1-one	135/93/95/43/45/69/108/150/177	135
2,990	3-Hydroxy-7,8-dihydro-*β*-ionol	4-(3-Hydroxy-1-butynyl)-3,5,5-trimethyl-2-cyclohexen-1-ol	193/208/175/131/105/91	193

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
	Pirazines			
0,895	2-Methoxy-3-methyl-pyrazine[c]		124/106/109/123/95	124
0,994	2-Methoxy-3-ethyl-pyrazine[c]		138/123/137/107/119	138
1,069	Tetramethylpyrazine[c]		136/54/92/95/108	136
1,168	2-Methoxy-3-isobutyl-pyrazine[c]		124/151/94	124
	Tiols			
1,019	2-Furanmethanethiol[c]	Furfuryl mercaptan	81/114/53	114
1,047	(3-Methylthio)propanal[c]		48/104/76/61/57	104
1,069	(2-Methylthio)ethanol[c]		61/92/45	61
1,386	(3-Methylthio)propanol[c]	Methionol	106/61/57/47/73	106
1,502	(3-Ethylthio)propanol[c]		120/42/61/63/75	120
1,740	3-Mercapto-1-hexanol[c]		55/41/57/61/100/67/82/47/83/134	61
1,827	Benzothiazol[c]	1,3-Benzothiazole	135/108/69/82	135
2,709	4-Methyl-5(2-hydroxyethyl) thiazol		112/85/143/45	143
2,809	2-[(1-Methylethyl)thio]-pentane		61/103/43/146/55/71	103
	Acid			
1,069	Acetic acid[c]		43/45/60	
1,094	Propionic acid[c]		74/73/45/57	73
1,133	Isobutyric acid[c]	2-Methyl propanoic acid	43/73/88/55	73
1,230	Butyric acid[c]		60/73/41/88	73
1,299	Isovaleryc acid[c]	3-Methyl butyric acid	60/41/43/87	87
1,426	Valeric acid[c]	Pentanoic acid	60/73/41/87	73
1,634	Hexanoic acid[c]		60/73/87	60
2,038	*t*-3-Hexenoic acid[c]		41/55/60/68/114/69/73/96	114
2,062	*t*-2-Hexenoic acid[c]		73/42/68/55/99/114	73

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
2,083	Octanoic acid[c]		60/73/43/55/110/85/115	60
2,488	Decanoic acid[c]		73/60/41/129	73
2,540	9-Decenoic acid[c]	Caproleic acid	55/69/73/60/84/110	73
2,555	Geranic acid[c]	3,7-Dimethylocta-2,6-dienoic acid	69/41/100/55/123	100
2,815	2-Phenylacetic acid	Benzenacetic acid	91/136/65	136
	Benzenic compounds			
1,061	Benzaldehyde[c]		105/106/77/51/74	105
1,256	Phenylacetaldehyde[c]		91/65/120	91
1,360	4-Methyl-benzaldehyde		119/120/91/65	119
1,470	2-Phenyl-2-propanol[c]	1-Hydroxycumene	43/121/77/51	77
1,665	2,4-Dimethylbenzaldehyde		133/134/105/77/91	133
1,698	Benzyl alcohol[c]		79/108/69	79
1,763	2-Phenylethanol[c]		91/122/65	122
1,897	4-Methyl-2,6-ditercbutyl phenol[c]		205/220/57/145/177	205
2,038	2-Phenyl-1-butanol		91/119/120/150/77/65/41	119
2,099	Phenol[c]		94/66/65/39/40/55	94
2,565	3-Hydroxy-4-phenyl-2-butanona		91/103/121/146/43/65/77/79	146
	Lactones			
1,194	γ-Valerolactone[c]	5-Methyl-dihydrofuran-2(3H)-one	56/85/41/43/57/100	85
1,224	γ-Butyrolactone[c]	Dihydrofuran-2(3H)-one	42/86/56	86
1,268	4-Hydroxy-2-hexenoic acid lactone	5-Ethyl-2(5H)-furanone	83/55/112	83
1,345	γ-Hexalactone[c]	5-Ethyldihydrofuran-2(3H)-one	85/42/57/70	85
1,499	4-Ethoxy-γ-butyrolactone	5-Ethoxydihydro-2(3H)-furanone	85/58/57/56/86	85
1,690	δ-Valerolactone[c]	Tetrahydro-2H-pyran-2-one	42/41/56/57/100/70	100

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
1,826	*t*-β-Methyl-γ-octalactone[c]	*t*-4-Methyl-5-butyldihydro-2 (3H)-furanone, *t*-Whiskey lactone	99/71/42/69/87/ 79/100	100
1,829	γ-Octalactone[c]	5-Butyldihydro-2(3H)-furanone	85/57/100/69	85
1,834	*c*-β-Methyl-γ-octalactone[c]	*c*-4-Methyl-5-butyldihydro-2 (3H)-furanone, *c*-Whiskey lactone	99/71/42/87/100	87
1,854	δ-Octalactone[c]	6-Propyltetrahydro-2H-pyran-2-one	99/42/71/55/114	114
1,984	γ-Nonalactone[c]	Dihydro-5-pentylfuran-2(3H)-one	85/41/42/84/56	85
2,018	Pantolactone[c]	3-Hydroxy-4,4-dimethyldihydrofuran-2(3H)-one	71/43/57	71
2,109	Dehydromevalonolactone	5,6-Dihydro-4-methyl-2H-pyran-2-ona	82/54/112	82
2,223	γ-Decalactone[c]	5-Hexyldihydro-2(3H)-furanone	85/41/55/128	85
2,320	δ-Decalactona[c]	Tetrahydro-6-pentyl-2H-pyran-2-one	99/71/42/55/114/ 84	99
2,393	Sotolone[c]	3-Hydroxy-4,5-dimethylfuran-2 (5H)-one	83/128/55/43/57/ 72/113	128
2,428	4-Ethoxycarbonyl-γ-butyrolactone[c]		85/86//57	85
2,443	γ-Undecalactone[c]	5-Heptyldihydrofuran-2(3H)-one	85/57/128/69/100/ 110	128
2,620	4-(1-Hydroxy-ethyl)-γ-butyrolactone		85/86/45/57	85
2,656	γ-Dodecalactone[c]	Dihydro-5-octyl-2(3H)-furanone	85/41/55/69/128	85
2,670	δ-Dodecalactone[c]	6-Heptyl-tetrahydropyran-2-one	99/71/55/42/114/ 84	114
	Methoxyphenols and volatile phenols			
1,668	Guaiacol[c]	2-Methoxyphenol	109/124/81/43/155	124
1,865	4-Metilguaiacol[c]	2-Methoxy-4-methylphenol	138/123/95/67/77/ 55	138
2,015	4-ethyl guaiacol[c]	4-Ethyl-2-Methoxyphenol	137/152/122/91/77	137
2,285	4-Propyl-guaiacol[c]	4-Propyl-2-methoxyphenol	137/166/122/70/94	137

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
2,300	Eugenol[c]	4-(2-Propenyl)-2-methoxyphenol	164/77/103/131/149/91/55137	164
2,328	4-Ethyl phenol[c]		107/122/77/91	122
2,373	4-Vinylguaiacol[c]	Ethenyl-2-methoxyphenol	150/135/77/107	150
2,463	*c*-Isoeugenol[c]	*c*-2-Methoxy-4-propenylphenol	164/77/103	164
2,488	Syringol[c]	2,6-Dimethoxyphenol	154/139/93/96/65/111	154
2,568	*t*-Isoeugenol[c]	*t*-2-Methoxy-4-propenylphenol	164/149/77/103/131/55	164
2,613	4-Vinylphenol[c]	4-Ethenyl-phenol	120/91/65/39	91
2,784	4-Ethoxymethylphenol	p-Hydroxybenzyl ethyl ether	107/152/77/95/120	107
2,784	Vanillin acetate[c]	4-Formyl-2-methoxyphenol acetate, Acetovanillin	152/151/43/123/51/79/109/194	152
2,798	Ethyl vanillin[c]	4-Hydroxy-3-ethoxybenzaldehyde	137/166/109/81/53/63	137
2,721	Methoxyeugenol[c]	2,6-Dimethoxy-4-(prop-2-en-1-yl)phenol	194/91/77/119/70/179	194
2,734	Vanillin[c]	4-Hydroxy-3-methoxybenzaldehyde	151/152/81/109/123	151
2,754	Methyl 3-(3,5-ditertbutyl-4-hydroxyphenyl) propionate		277/292/147/219/203/161	292
2,760	Methyl vanillate[c]	Methyl 4-hydroxy-3-methoxybenzoate	151/182/123/108	182
2,787	Ethyl vanillate[c]	Ethyl 4-hydroxy-3-methoxybenzoate	151/196/168/123	196
2,808	Acetovanillone[c]	1-(4-Hydroxy-3-methoxyphenyl) ethanone	151/166/123	166
2,821	Propiovanillone[c]	1-(4-Hydroxy-3-methoxyphenyl) propan-1-one	137/180/122/43/94/138	137
2,928	Vanilliyl ketone	1,3-bis(4-Hydroxy-3-methoxyphenyl)propan-2-one	151/180/123/108	180
2,973	Methyl syringol[c]	2,6-Dimethoxy-4-methylphenol	153/168/93/125	153
2,984	Methyl-3-(2-hydroxyphenyl)-propionate[c]		120/148/94/78/119/77	148

(continued)

Table 2
(continued)

RRT	Compound	Synonym	Mass spectra[a]	Q[b]. m/z
3,013	3,4-Dimethoxy-phenol[c]		154/139/111/93	154
3,033	Zingerone[c]	4-(4-Hydroxy-3-methoxyphenyl) butan-2-one, Vanillylacetone	137/194/43/91/119	194
3,067	Ethyl 3-(4-hydroxy-3-methoxyphenyl) propionate[c]		137/150/224	137
3,082	Vanillyl alcohol[c]	4-(Hydroxymethyl)-2-methoxyphenol	154/65/93/137/125/39/77	154
3,103	Homovanillyl alcohol[c]	4-(2-Hydroxyethyl)-2-methoxyphenol	137/168/122/94	137
3,110	Syringaldehyde[c]	4-Hydroxy-3,5-dimethoxybenzaldehyde	182/181/96/65/111/139/167	182
3,332	Methyl-(2-hydroxy-3-ethoxybenzyl) ether		137/182/138/65/123	137
3,353	Homovanillic acid[c]	2-(4-hydroxy-3-methoxyphenyl) acetic acid	137/138/182	138
	Miscellaneous			
1,061	2-Methyltetra hydrothiophen-3-one[c]		60/116/59/45/88	116
1,583	Tetrahydro 2-thiophene methanol		61/118/74/45/90/85	118
2,038	Maltol[c]	3-Hydroxy-2-methyl-4H-pyran-4-ona	126/71/43/55/97	126
2,394	2-Aminoacetophenone[c]	1-(2-Aminophenyl)ethanone	120/135/92/65/43	120
2,560	3-Ethyl-4-methyl-1H-pyrrol-2,5-diona	2-Methyl-3-ethylmaleimide	139/67/53/124	139
2,591	Dihydromethyl jasmonate	Methyl 3-oxo-2-pentyl-cyclopentyl-acetate	83/153/156/82/96	153
2,524	4-Methyl-5-thiazolethanol	Sulfurol	112/143/85/59	112
2,749	N(2-Phenylethyl)acetamide		104/43/91/163	104
3,163	Acetosyringone[c]	1-(4-Hydroxy-3,5-dimethoxyphenyl)ethanone	181/196/43/153/65	196

[a]m/z of the principal ions in the mass spectra of each compound
[b]m/z of the ion used for quantitation
[c]Commercial available compounds; *RRT* relative retention time (RT compound/RT P.I. (42.15 min))

ethanol with 5 g/L tartaric acid. These working calibration solutions are processed as wine samples and must be extracted by SPE (**Steps 1–7** of the extraction protocol detailed above).

3.3 The Odor Activity Value (OAV) Method

The GC-MS method described above provides quantitative information on hundreds of volatile compounds. A large amount of data can be challenging to interpret, especially when the objective is to establish the aromatic characteristics of a particular grape variety or type of wine. The Odor Activity Value (OAV) method helps us achieve this objective [26].

The contribution of each volatile compound to wine aroma is evaluated qualitatively via its associated descriptor and quantitatively via its OAVs aiming at categorizing the potential aroma impact. The OAVs are calculated using the equation $OAV = c/t$, where c is the total concentration of each compound in the wine samples, and t is their perception threshold described in the bibliography. Compounds that exhibit OAVs >1 are considered to contribute individually to the wine aroma and are designated as would-be impact odorants. The potential influence on wine aroma from volatile compounds present at sub-threshold concentrations (i.e., OAVs <1) should not be excluded due to their additive effects [24]. Nevertheless, as odor thresholds are affected by additive, synergic, and antagonistic effects of the volatile compounds in a matrix, an alternative consists to identify the most powerful odorants based only on their OAV values.

The odor descriptors are grouped in different aromatic series to estimate overall wine aroma, and every compound is assigned to one or several aromatic series based on a similar odor descriptor used. The aromatic series' total intensities are calculated as the sum of the OAV of each one of the compounds assigned to this series. The series normally use grouped compounds with similar odor descriptors representing the principal constituents of the wine aroma profile, such as fruity, floral, green/fresh, sweet, fatty, spicy, and other odors. Because of the high complexity of olfactive perceptions, some aroma compounds were included in two or more odorant series according to the findings of some authors [27, 28], as shown in Table 3. This procedure makes it possible to relate quantitative information, obtained by chemical analysis, to sensory perception providing a single aroma profile.

4 Notes

1. When using an electron impact ionization source in mass spectrometry, great care must be taken to protect the filament from overexposure caused by the solvent chromatographic band. In the case of the analysis of major volatile compounds, ethanol should be avoided, and in the case of minor volatile

Table 3
Compound, odor descriptors, odorant series, and odor threshold

	Odor descriptors	Odorant series	Odor threshold (μg/L)
Ethyl acetate	Fruit, solvent	1,7	7500 [21]
Isoamyl acetate	Banana	1	30 [28]
Hexyl acetate	Green, floral	2,3	1500 [28]
2-Phenylethyl acetate	Floral	2	250 [21]
Ethyl lactate	Acid, medicine	6	154,636 [28]
Ethyl hexanoate	Green apple	1	14 [9]
Ethyl octanoate	Sweet, fruity	1,4	5 [9]
Ethyl decanoate	Sweet, fruity	1,4	200 [28]
Isoamyl alcohols	Fusel	7	30,000 [21]
Methanol	Chemical, medicinal	7	668,000 [9]
1-Propanol	Ripe fruit, alcohol	1,7	830,000 [9]
Isobutanol	Oily, bitter, green	3,5	40,000 [9]
1-Hexanol	Flower, green, cut grass	2,3	8000 [21]
t-3-hexenol	Green, fruity	1,3	400 [9]
c-3-Hexen-1-ol	Green, cut grass	3	400 [21]
3-Methyl-thio-propanol	Cooked, vegetable	7	500 [21]
2-Phenylethyl alcohol	Floral, roses	2	10,000 [21]
Butiric acid	Rancid, cheese, sweat	6	173 [9]
Isovaleric acid	Sweet, acid, rancid	4,6	33 [28]
Hexanoic acid	Sweat	6	420 [9]
Octanoic acid	Sweat, cheese	6	500 [28]
Decanoid acid	Rancid fat	6	1000 [9]
Acetaldehyde	Pungent, ripe apple	1,7	500 [21]
Benzaldehyde	Sweet, cherry, almond	1,4	350 [28]
Citronellol	Rose citrus	2	40 [9]
α-Terpineol	Floral	2	250 [28]
Linalool	Floral	2	15 [21]
Nerol	Floral	2	300 [21]
Geraniol	Roses, geranium	2	30 [21]
Furaneol	Burnt sugar, caramel, maple	4	5 [29]
γ-Butyrolactone	Sweet, toast, caramel	4	35 [28]

(continued)

Table 3
(continued)

	Odor descriptors	Odorant series	Odor threshold (μg/L)
γ-Nonalactona	Coconut	4	30 [28]
(E)-Whiskylactone	Vanilla, spices	5	790 [30]
(Z)-Whiskylactone	Vanilla, spices	5	67 [30]
Damascenone	Sweet, fruit	1,4	0,05 [21]
4-Ethylphenol	Phenolic, bitumen	7	450 [29]
4-Vinylphenol	Almond shell	7	180 [31]
4-Vinylguaiacol	Spices/curry	6	40 [21]
Eugenol	Spices, clove, honey	4,5	6 [28]
Vanillin	Vanillin	5	60 [9]
Ethyl vanillate	Caramel, honey, vanillin	4,5	990 [9]

Odorant series: 1 = Fruity, 2 = Floral, 3 = Green, fresh, 4 = Sweet, 5 = Spicy, 6 = Fatty, 7 = Others

compounds, pentane and dichloromethane. The filament must be programmed to turn off during the time that the solvent peak is reaching the detector.

2. The detection limits of each compound depend on the intensity of the ion m/z selected for its quantitation. In some cases, it is convenient to select an ion that is not the most intense to avoid interference from compounds that elute very closely or even co-elute with the one of interests.
3. The adsorption capacity of the resins used in SPE is generally diminished by the presence in the sample of high ethanol concentrations. Due to this, the wine is diluted with water (1:1) to achieve concentrations below 7% ethanol v/v. The musts would not need to be diluted, but the decrease in their density improves their passage through the resin.
4. Tests carried out in our laboratory show that the 0,3 g sorbent cartridges can be reused up to 7 times without loss of effectiveness, if properly regenerated right after use. Regeneration is achieved by consecutively passing the following solvents, drying with air between each pass:
   - 2 times × 2 mL of 80% methanol—20% aqueous NH_4OH 2%.
   - 2 times × 2 mL of 80% methanol—20% aqueous HCl 2%.
   - 2 times × 2 mL of methanol.
   - 2 times × 2 mL of ethyl acetate.
   - 2 times × 2 mL of acetone.

References

1. Kotseridis Y, Baumes R (2000) Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. J Agric Food Chem 48(2):400–406
2. Spranger MI, Climaco MC, Sun B, Eiriz N (2004) Differentiation of red winemaking technologies by phenolic and volatile composition. Anal Chim Acta 513:151–161
3. Pérez-Navarro J, Izquierdo-Cañas PM, Mena-Morales A, Chacón-Vozmediano JL, Martínez-Gascueña J, García-Romero E, Hermosín-Gutiérrez I, Gómez-Alonso S (2020) Comprehensive chemical and sensory assessment of wines made from white grapes of Vitis vinifera cultivars Albillo dorado and Montonera del Casar: a comparative study with Airén. Foods 9(9):1282
4. Escudero A, Campo E, Farina L, Cacho J, Ferreira V (2007) Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J Agric Food Chem 55: 4501–4510
5. García-Romero E, Chacón-Vozmediano JL, Martínez-Gascueña J, Izquierdo-Cañas PM (2003) Changes in volatile compounds during ripening in grapes of Airen, Macabeo and Chardonnay white varieties grow in La Mancha region (Spain). Food Sci Technol Int 9:33–41
6. Sefton MA, Francis IL, Williams PJ (1993) The volatile composition of Chardonnay juices: a study by flavor precursor analysis. Am J Enol Vitic 44:359–370
7. Winterhalter P, Skouroumounis GK (1997) Glycoconjugated aroma compounds: occurrence, role and biotechnological transformation. In: Sheper T (ed) Advances in biochemical engineering/biotechnology. Springer, Berlin
8. Muller CJ, Kepner RE, Webb AD (1973) Lactones in wines – review. Am J Enol Vitic 24:5–9
9. Etiévant XP (1991) Wine. In: Maarse H (ed) Volatile compounds in foods and beverages. Marcel Dekker, New York
10. Chatonnet P, Boidron JN, Pons M (1990) Maturation of red wines in oak barrels: evolution of some volatile compounds and their aromatic impact. Sci Aliments 10:565–587
11. Webb AD, Kepner RE (1961) Fusel oil analysis by means of gas-liquid partition chromatography. Am J Enol Vitic 12:51–59
12. Izquierdo-Cañas PM, Ríos-Carrasco M, García-Romero E, Mena-Morales A, Heras-Manso JM, Cordero-Bueso G (2020) Co-existence of inoculated yeast and lactic acid bacteria and their impact on the aroma profile and sensory traits of Tempranillo red wine. Fermentation 6(1):17
13. Marchante L, Loarce L, Izquierdo-Cañas PM, Alañón ME, García-Romero E, Pérez-Coello MS, Díaz-Maroto MC (2019) Natural extracts from grape seed and stem by-products in combination with colloidal silver as alternative preservatives to SO_2 for white wines: effects on chemical composition and sensorial properties. Food Res Int 125:1–11
14. Blank I (1996) Gas chromatography-Olfactometry in food aroma analysis. In: Marsili R (ed) Techniques for analyzing food aroma. Marcel Dekker, New York
15. Blanch GP, Reglero G, Herraiz M (1996) Rapid extraction of wine aroma compounds using a new simultaneous distillation-solvent extraction device. Food Chem 56(4):439–444
16. Ortega-Heras M, González-San José ML, Beltrán S (2002) Aroma composition of wine studied by different extraction methods. Anal Chim Acta 458(1):85–93
17. Díez J, Domínguez C, Guillén DA, Veasa R, Barroso CG (2004) Optimisation of stir bar sorptive extraction for the analysis of volatile phenols in wines. J Chromatogr A 1025(2): 263–267
18. Hayasaka Y, MacNamara K, Baldock GA, Taylor RL, Pollnitz AP (2003) Application of stir bar sorptive extraction for wine analysis. Anal Bioanal Chem 375:948–955
19. Flamini R, Traldi P (2010) Grape aroma compounds: terpenes, C13-Norisoprenoids, benzene compounds, and 3-Alkyl-2-Methoxypyrazines. In: Desiderio DM, Nibbering NMM (eds) Mass spectrometry in grape and wine chemistry. Wiley, Hoboken
20. Ferreira V, Jarauta I, Ortega L, Cacho J (2004) Simple strategy for the optimization of solid-phase extraction procedures through the use of solid–liquid distribution coefficients: application to the determination of aliphatic lactones in wine. J Chromagr A 1025:147–156
21. Guth H (1997) Quantitation and sensory studies of character impact odorants of different white wine varieties. J Agric Food Chem 45(8):3027–3032
22. Sánchez-Palomo E, Gómez García-Carpintero E, González Viñas MA (2015) Aroma fingerprint characterisation of La Mancha red wines. S Afr J Enol Vitic 36(1): 117–125

23. Moyano L, Zea L, Moreno J, Medina M (2002) Analytical study of aromatic series in Sherry wines subjected to biological aging. J Agric Food Chem 50(25):7356–7361
24. Sánchez-Palomo E, Gómez García-Carpintero E, Alonso-Villegas R, González-Viñas MA (2010) Characterization of aroma compounds of Verdejo white wines from the La Mancha region by odour activity values. Flavour Fragr J 25:456–462
25. Ibarz MJ, Ferreira V, Hernández-Orte P, Loscos N, Cacho J (2006) Optimization and evaluation of a procedure for the gas chromatographic-mass spectrometric analysis of the aromas generated by fast acid hydrolysis of flavor precursors extracted from grapes. J Chromatogr A 1116(1–2):217–229
26. Charles M, Martin B, Ginies C, Etievant P, Coste G, Guichard E (2000) Potent aroma compounds of two red wine vinegars. J Agric Food Chem 48:70–77
27. Zea L, Moyano L, Moreno J, Medina M (2007) Aroma series as fingerprints for biological ageing in fino sherry-type wines. J Sci Food Agric 87:2319–2326
28. Ferreira V, López R, Cacho J (2000) Quantitative determination of the odorants of young red wines from different grape varieties. J Sci Food Agric 80:1659–1667
29. Lopez R, Ferreira V, Hernández P, Cacho JF (1999) Identification of impact odorants of young red wines made with merlot, cabernet sauvignon and Grenache grape varieties: a comparative study. J Sci Food Agric 79:1461–1467
30. San-Juan F, Cacho JF, Ferreira V, Escudero A (2012) 3-Methyl-2-Butene-1-thiol: identification, analysis, occurrence and sensory role of an uncommon thiol in wine. Talanta 99:225–231
31. Boido E, Lloret A, Medina K, Fariña L, Carrau F, Versini G, Dellacassa E (2003) Aroma composition of Vitis vinifera Cv. Tannat: the typical red wine from Uruguay. J Agric Food Chem 51:5408–5413

Chapter 12

Identification of Wine Compounds by Nuclear Magnetic Resonance

Werner F. Brandão, Gilson C. Santos Júnior, Fabio C. L. Almeida, Icaro P. Caruso, Gisele C. de Amorim, and Marcel M. L. da Cunha

Abstract

The main constituents of wine, such as ethanol, sugars, and organic acids, can be identified by nuclear magnetic resonance (NMR) without extensive sample preparation. Here, we describe a simple and straightforward protocol for the identification of wine compounds by NMR. Wine samples can be characterized and over 60 molecules can be identified by 2D ^{1}H-^{13}C NMR following processing and analysis with minimal sample preparation and the use of free versions or open software and web-based platforms (Topspin, Chenomx, and Colmar). Additionally, a simple protocol for 1D ^{1}H NMR was included as a starting point and beginner guide to NMR analysis of wine.

Key words Nuclear magnetic resonance, Compound identification, Topspin, Chenomx, Colmar

1 Introduction

Identification of wine compounds has several purposes. From the quality of fermentation and process characterization to adulteration detection and origin authentication [1–3].

In this chapter, we describe a protocol for the wine compounds identification by NMR without lyophilization or signal suppression. It is well known that ethanol signals might mask other compounds in the vicinity of its chemical shifts because of its high concentration in wine samples. Since the purpose of the method is to identify as many compounds as possible, including ethanol, with minimal sample manipulation or loss of volatiles, no lyophilization or signal suppression will be used.

One of the goals of this method is its simplicity. The work and time needed for sample preparation are very short, and several samples could be prepared in less than 1 hour with a few procedures

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Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3088-4_12,

and the addition of simple solutions. Also, the use of an online platform, Colmar NMR, is proposed. Colmar web-based platform is, by the time this chapter is being written, free and available. This platform allows precise identification of compounds by 2D assignment, meaning that more information from a molecule can be used for identification, ^{1}H and ^{13}C allows disambiguation of overlapped signals, indistinguishable by 1D analysis [4–6].

We expect that you, the reader, might find usefulness in this simple and straightforward method. Also, it may be of interest to those developing methods for the fast detection of important molecular markers of quality, aging, fraud, or contaminants in wine.

2 Materials

2.1 Sample Preparation

1. 2 mL of a wine sample.
2. Deuterium oxide (D_2O) 99%.
3. 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d_6) 98%: 10 mM in Milli-Q water. As a substitute, TSP-d_4 in 99% D_2O can equally be used.
4. 5 mM NMR tube.
5. Automatic pipettes and tips.

2.2 NMR Experiments

1. Bruker NMR spectrometer—400 MHz field or higher.
2. 1D ^{1}H NOESY pulse sequence. NOESY sequence was used in this chapter, but others, such as ZGPR, can equally be used [3].
3. ^{1}H-^{13}C HSQC pulse sequence.
4. ^{1}H-^{1}H TOCSY pulse sequence [4].

2.3 Software

1. TopSpin 4.3.1 (Bruker, Germany) for spectra acquisition and processing.
2. Chenomx NMR Suite (Chenomx Inc., Canada). Chenomx is used in this chapter, but others, such as MestReNova (Mestre-Lab Research, Spain) are also an option. In this chapter, we will describe the use of Chenomx only [5].
3. COLMARm web-based platform for the assignment of 2D ^{1}H-^{13}C HSQC and ^{1}H-^{1}H TOCSY spectra [6].

3 Methods

3.1 Sample Preparation

1. Centrifuge the wine sample to avoid any solid aggregates.
2. Sonicate the sample for, at least, 5 cycles of 10 min, for the complete release of dissolved CO_2 (*see* **Note 1**).

3. Measure the pH of the sample (*see* **Note 2**).
4. In the 5 mM NMR tube, add (*see* **Note 3**): 480 μL of the centrifuged and degasified wine sample (in our experiments we have used a Brazilian red wine, bottled in 2022), 60 μL of DSS 10 mM, 60 μL of D_2O.

3.2 NMR Spectra Acquisition

3.2.1 1D ^{1}H NOESY Parameters (See **Note 4**)

- SW = 20 ppm.
- TD = 64 k.
- NS = 128–512 scans (depending on sample concentration).
- D1 = 5 × T1 (use the sequence t1ir (Bruker) to calculate the T1).
- Offset = 4.7 ppm.
- Use command RGA to adjust the best RG (receiver gain) to avoid overflow..
- Temperature = 298 K.

It is crucial to acquire spectra with different reference compound concentrations to calculate the standard error of the magnet/sequence quantification.

3.2.2 2D ^{1}H-^{13}C HSQC Parameters

- SW = 12 ppm (F2) / 160 ppm (F1).
- TD = 1024 (F2) / 256 (F1).
- NS = 24.
- D1 = 2 s.
- Offset = 4.7 ppm (F2) / 75 ppm (F1).
- Temperature = 298 K.

All the experiments described below were acquired on a Bruker 500 MHz spectrometer.

3.3 1D Spectrum Processing and Analysis

3.3.1 1D Spectrum Processing

1. Open the 1D ^{1}H NOESY spectrum in TopSpin 4.3.1.
2. Type *efp* and press <ENTER> in the command line to start processing with a pre-set script.
3. Next, type *apk* and press <ENTER> in the command line to execute the auto phasing.
4. Then, type *absn* and press <ENTER> in the command line to execute the auto baseline adjustment.
5. Check the spectra phase by comparing major signals and their symmetry and baseline. Ethanol signals (1.14 and 3.6 ppm) are especially useful for this comparison.

(a) If phase correction is needed, a manual adjustment should be performed. Type *.ph* and press <ENTER> in the command line to start manual phasing.

(b) After phase correction, repeat **Step 4** for baseline adjustment.

6. For calibration of the ^{1}H spectra: Type *.cal* and press <ENTER>. Next, select the DSS singlet signal by using the cursor, and adjust its shift to 0.0 ppm (*see* **Note 5**).

3.3.2 Identification of Molecules in the Wine Sample by 1D ^{1}H Peak Assignment by Chenomx

Chenomx might be used as the software of choice for manual identification. It is performed by comparing the acquired spectra to data from open databases such as HMDB (The Human Metabolome Database, http://hmdb.ca/).

1. In Chenomx, import spectra after Topspin processing (Subheading 3.3.1) as ".1r" file for assignment.
2. In the import window (Fig. 1), under calibration, enter the reference (DSS) concentration (chemical shape indicator) and the sample pH range.
3. Skip processing and press <OK>. Spectrum processing was done in Topspin according to Subheading 3.3.1.

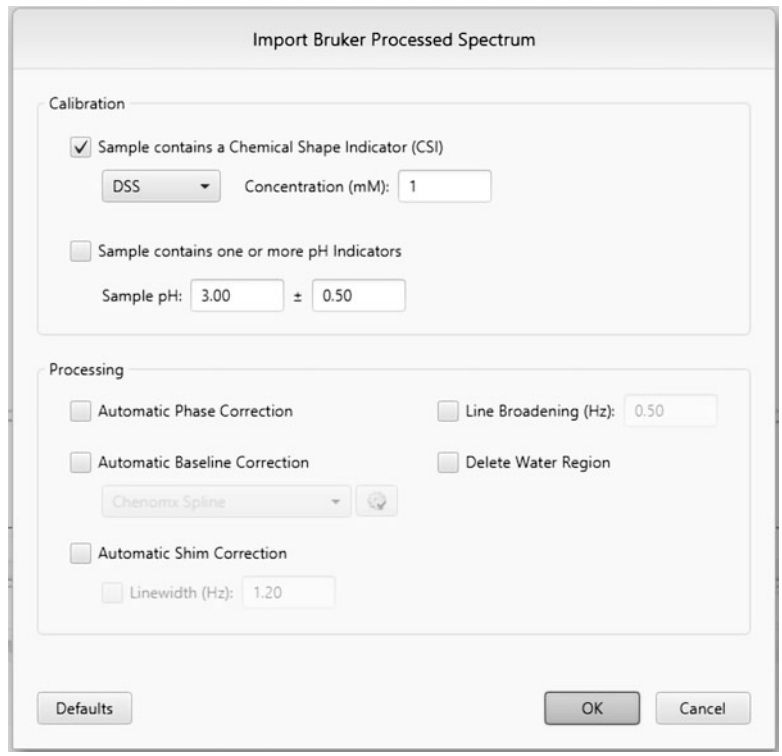

Fig. 1 Import window on Chenomx software. (AROUND HERE)

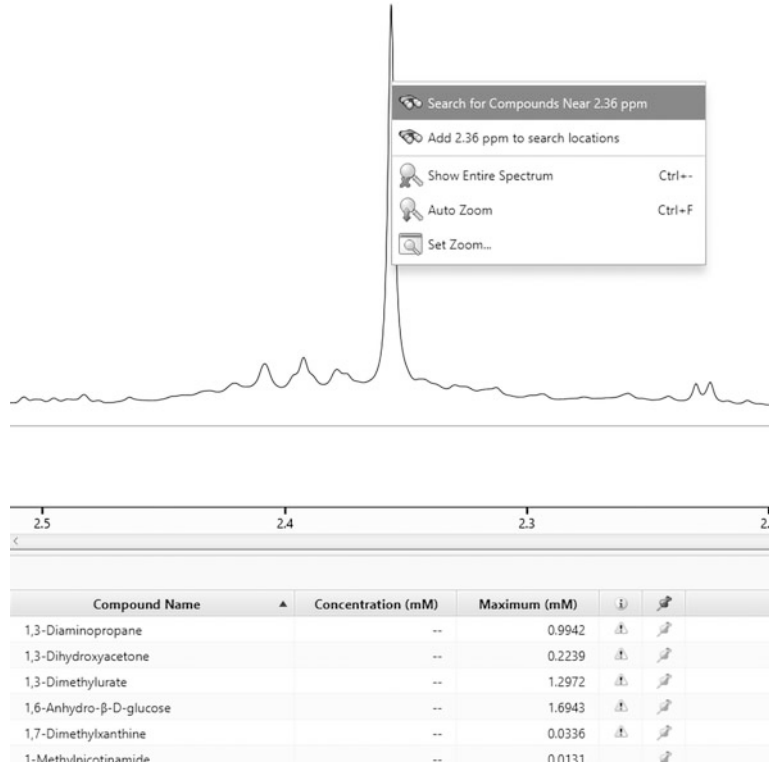

Fig. 2 Right-click menu is displayed. Search (highlighted) retrieves a list of compounds whose chemical shifts are close to the selected peak. (AROUND HERE)

4. Spectrum is loaded on the screen and a list of compounds is shown. Selecting a molecule overlaps its spectrum on your data.
5. To perform the identification based on a signal, right-click on the desired signal and choose "Search for compounds." Chenomx will search for compounds with chemical shifts near the chosen peak. All compounds are shown with an exclamation point (Fig. 2). After entering a pH value far from neutral (Fig. 1), Chenomx alerts that pH may interfere with compound identification. For more precise identification, the steps below should be considered.
6. Check if the compound's signal is within the tolerance of chemical shift for the sample's pH (Fig. 3). By clicking on a compound from the list, blue arrows over the chemical shift axis define the tolerance range. If the compound is within the tolerance range, the identification is correct.
7. Check if the relative intensity of compound peaks is compatible with the sample's signal (Fig. 4).
8. The identified compounds should be registered in a text file or spreadsheet.

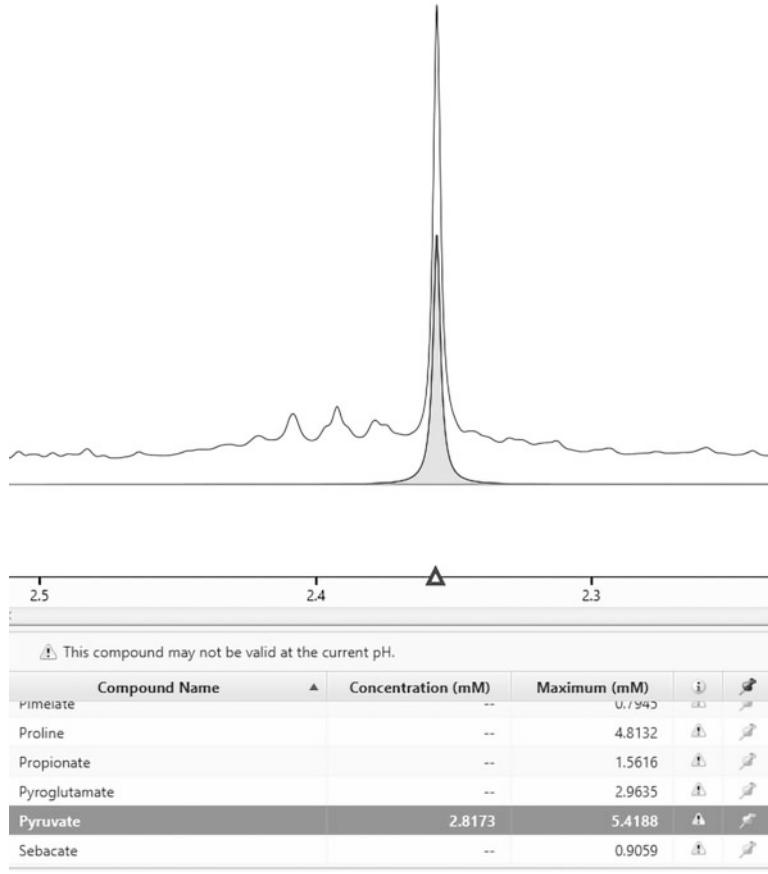

Fig. 3 In this example, pyruvate signal is within the chemical shift range tolerated at sample's pH. As supplementary information, concentration of the compound on the sample is calculated in comparison to sample's internal reference (DSS)

3.3.3 1D ^{1}H NOESY Spectra Assignment Using HMDB

1. Access Human Metabolome Database (http://hmdb.ca). Click on "Search" and then "NMR Search" to search for specific metabolites.
2. On the page Spectra Search NMR Spectrum, fill in the fields accordingly (Fig. 5):
   - Chemical peaks: enter peaks found on TopSpin.
   - Intensities: optional (for this protocol, it was left blank).
   - Search type: mixture.
   - Spectra library: 1H NMR.
   - Tolerance ± (ppm): optional (for this protocol, it was left blank).
   - Frequency (MHz): optional (for this protocol, it was left blank).

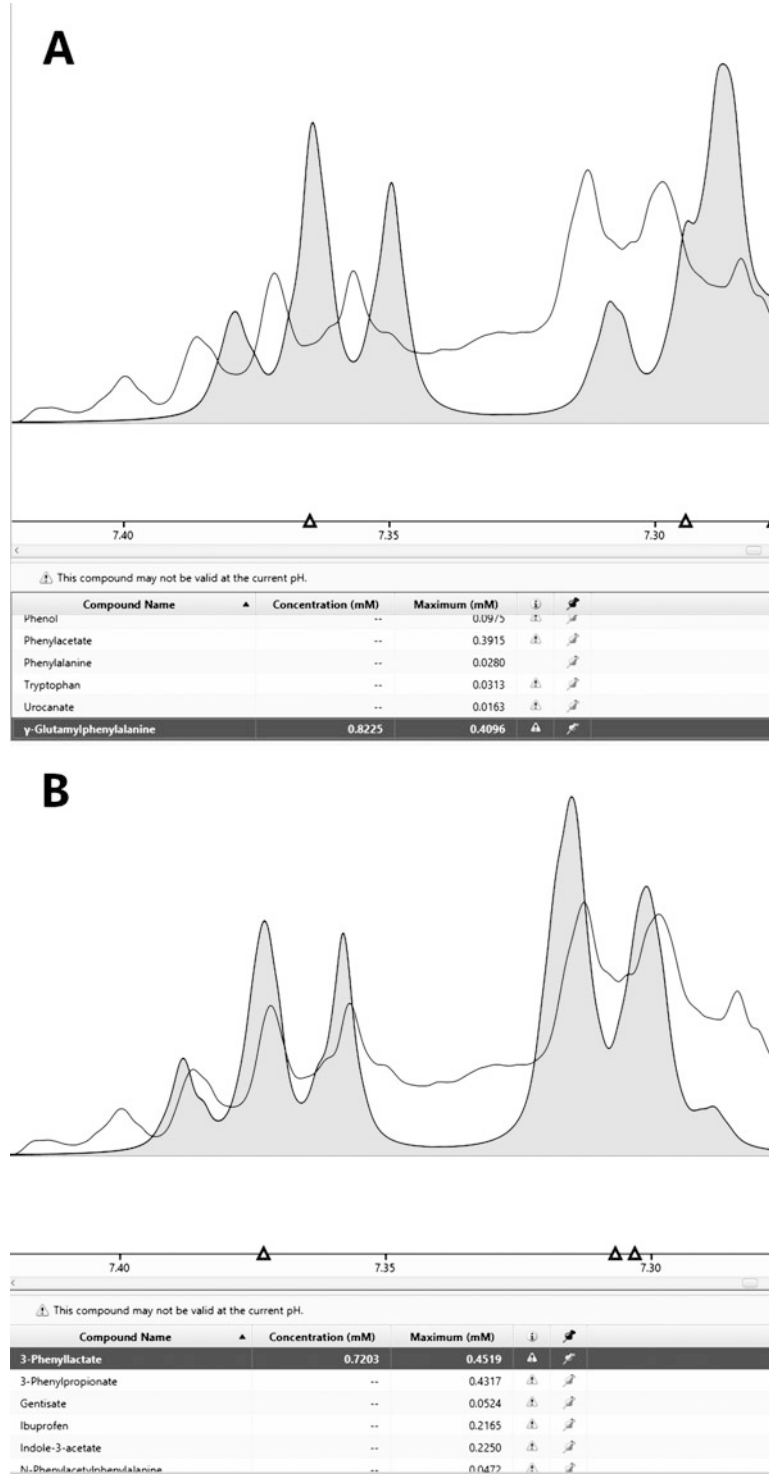

Fig. 4 (**a**, **b**) show sample data (white spectra) overlapped by selected compound (tinted spectra) (**a**) Incorrect assignment: γ-glutamylphenylalanine presents similar peaks to those in the sample, but with differences in intensity distribution within 7.30–7.35 ppm. (**b**) Correct assignment: 3-Phenyllactate presents similar peaks and peak intensities

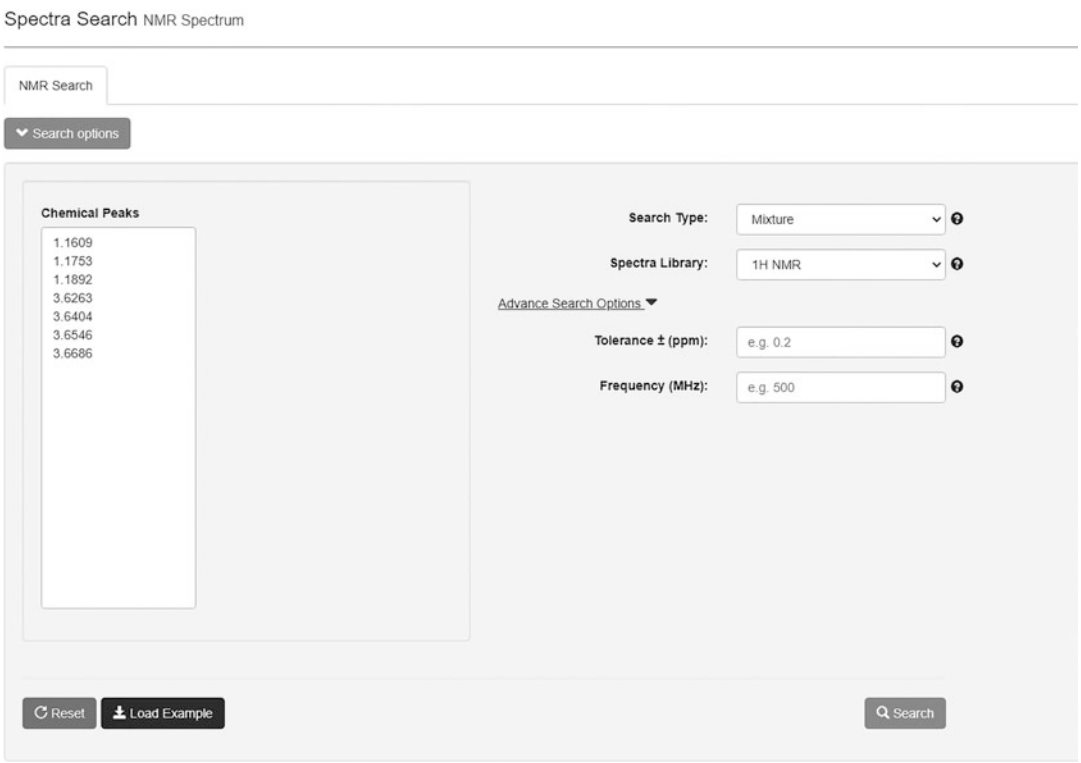

Fig. 5 NMR Search page on HMDB website. Fields should be filled accordingly to allow a working search

3. As result, a list of compounds with the same chemical shift, chemical structure, and formula will be shown. Also, a score value is given and a value of 1.0 means an exact match.

For more precise identification, the more chemical peaks of a given compound feed in the platform, the better. The example of Fig. 6 shows the precise identification of ethanol. For its identification, data from the triplet of its methyl region (1.560, 1.1707 e 1.1849 ppm) and the quartet of methylene (3.6243, 3.6384, 3.6528 e 3.6669) were entered into the platform.

4. A comparison between the spectrum from experimental data and the result is encouraged to confirm the match. For this, on the "Search results" list, click on the button HMDB*number* to display compound information (Fig. 7).
5. Click "Spectra" on the top of the page (Fig. 8).
6. On the list "NMR Spectra" (Fig. 9), click on "View Spectrum" next to the option corresponding to the experimental conditions used (Fig. 10).

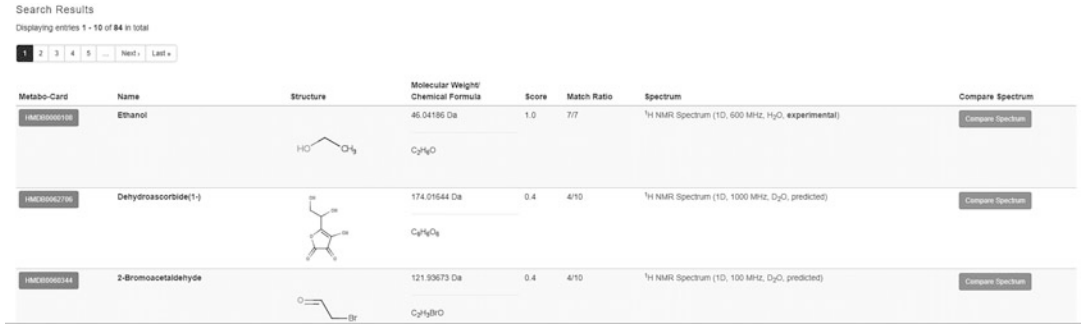

Fig. 6 Search results on HMDB website. Several information of the compound is displayed, along with search score and match ratio

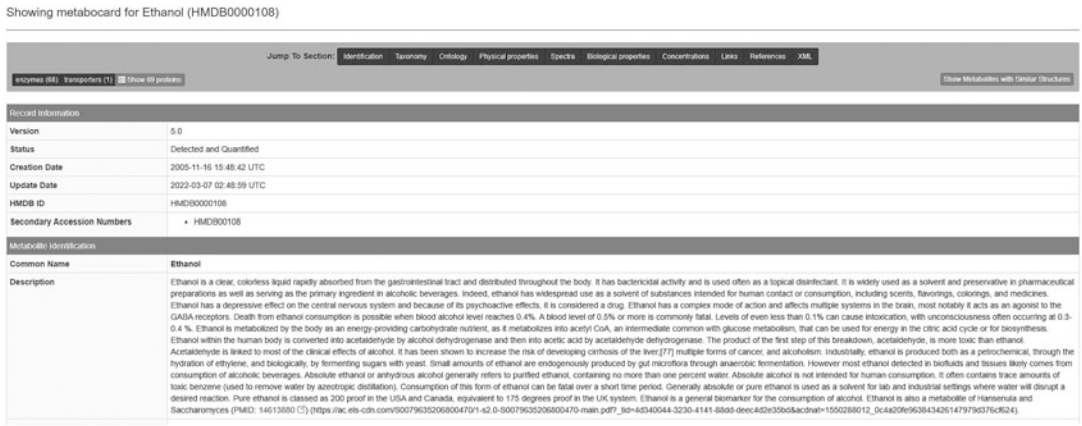

Fig. 7 Compound information "Metabocard" on HMDB website. All information available on HMDB regarding a specific compound is displayed in this webpage

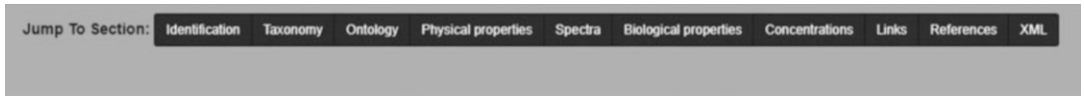

Fig. 8 Menu on webpage "Metabocard," HMDB website

7. Compare the reference spectrum and the experimental data for signal multiplicity, chemical shift, and intensity proportion to confirm the identification.

Supplemental information is also available on the same HMDB spectrum page. Such information might find usefulness in further identification and analysis of data in external identification software such as Bruker's topspin.

NMR Spectra

Spectrum Type	Description	Deposition Date	Source	View
Experimental 1D NMR	^{1}H NMR Spectrum (1D, 500 MHz, H_2O, experimental)	2012-12-04	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 100 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 100 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 1000 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 1000 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 200 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 200 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 300 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 300 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 400 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 400 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 500 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 500 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 600 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 600 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 700 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{13}C NMR Spectrum (1D, 700 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Predicted 1D NMR	^{1}H NMR Spectrum (1D, 800 MHz, D_2O, predicted)	2021-09-29	Wishart Lab	View Spectrum
Experimental 2D NMR	[^{1}H, ^{13}C]-HSQC NMR Spectrum (2D, 600 MHz, H_2O, experimental)	2012-12-05	Wishart Lab	View Spectrum

Close

Fig. 9 NMR Spectra download section on HMDB website. By clicking on the link "View Spectrum," corresponding data of spectrum is shown (Fig. 10)

Experimental Conditions

Sample Concentration: 81.04 mM
Solvent: Water
Sample Assessment: Excellent
Spectrum Assessment: Excellent
Instrument Type: Varian
Nucleus: ^{1}H
Frequency: 500 MHz
Sample pH: 7.00
Sample Temperature: 25.0 Celsius
Chemical Shift Reference: DSS

Documentation

Document Description	Download	File Size
List of chemical shift values for the spectrum (TXT)	Download file	1.53 KB
Peak Assignments (PNG)	Download file	1.53 KB
Spectra Image with Peak Assignments (PNG)	Download file	1.53 KB
Raw Spectrum Image	Not Available	Not Available
nmrML File (NMRML)	Download file	1.53 KB
JCAMP-DX File (JDX)	Download file	1.53 KB
Raw Free Induction Decay (FID) File for Spectral Processing (Varian) (FID20121204-87231-1O5EI2O)	Download file	1.53 KB
Validation Report (TAR)	Download file	1.53 KB

References

1. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazyrova A, Shaykhutdinov R, Li L, Vogel HJ, Forsythe I: HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009 Jan;37(Database issue):D603-10. doi: 10.1093/nar/gkn810. Epub 2008 Oct 25. [PubMed: 18953024]
Error displaying reference

Fig. 10 Data on the spectrum and experimental conditions of its acquisition retrieved from HMDB

3.4 2D Spectrum Processing and Analysis

3.4.1 2D Spectrum Processing

1. Open the 2D ^{1}H-^{13}C HSQC spectrum in TopSpin 4.3.1.
2. Execute a manual phase correction. Type *.ph* and press <ENTER> in the command line to start manual phasing. It is relevant to select more than two peaks for phase correction. Peaks are selected by opening a right click menu over the peak and clicking "ADD." Peaks out of phase appear off-centered. Phase is corrected by clicking on "R" or "C" on the menu for F1 or F2 adjustments, respectively, and by using the zero- and first-order phase buttons.

3. Type *abs1;abs2* and press <ENTER> to execute the auto baseline adjustment.
4. In TopSpin, click on the PROCPARS (processing parameters) tab. Increase the number of SI points to, at least, 4096 on the F2 dimension. Next, type *.xfb* and press <ENTER>.
5. Type *.cal* and press <ENTER>. Next, select the DSS singlet signal, by using the cursor, and adjust its shift to 0.0 ppm.
6. Remove the spectra zoom.
7. To save the spectra as a text file, type *totxt*.

3.4.2 Identification of Molecules in the Wine Sample by 2D ^{1}H-^{13}C Peak Assignment by COLMARm

After processing the adjustments as described in **step 2**, spectra data as a text file should be used for upload and identification on Colmar. Colmar is a public web-based identification platform for NMR data hosted at The Ohio State University (USA, https://spin.ccic.osu.edu/index.php/colmar). This platform uses chemical shifts retrieved from open access databases, such as HMDB, BMRB, NMRShiftDB, and KEGG. Several 2D spectra are suitable for the Colmar NMR platform, such as ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and TOCSY-HSQC. In this protocol, HSQC spectra only will be considered. To perform a 2D ^{1}H-^{13}C HSQC spectra identification on the Colmar NMR platform:

1. In a web browser, go to https://spin.ccic.osu.edu/index.php/colmarm/index2 and click on COLMARm.
2. Follow the steps according to the website workflow. On STEP 1 (Fig. 11), create a username and enter your institution and upload an HSQC text file as described in item.
3. An HSQC spectrum is mandatory. TOCSY and TOCSY-HSQC could be uploaded as supplemental data to increase the accuracy of identification and also increase up to a few days the time needed for analysis.
4. For peak picking on STEP 2 (Fig. 12), default values should be used. In our experience, such values worked nicely for wine and other beverages.
5. Leave STEP 3 (Fig. 13) with default values and skip to STEP 4.
6. On STEP 4 (Fig. 14), enter values for tolerance for both ^{1}H and ^{13}C chemical shifts. Those will be used for matching signals from samples to those compounds on the database. For this protocol, default values were used (^{1}H chemical shift cutoff (ppm) of 0.04, ^{13}C chemical shift cutoff (ppm) of 0.3, and matching ratio cutoff of 0.6). Also, select "Hydrophilic metabolite database in water solvent" and "Simple, match peak position only" as the Query algorithm.
7. Click on <Submit>.

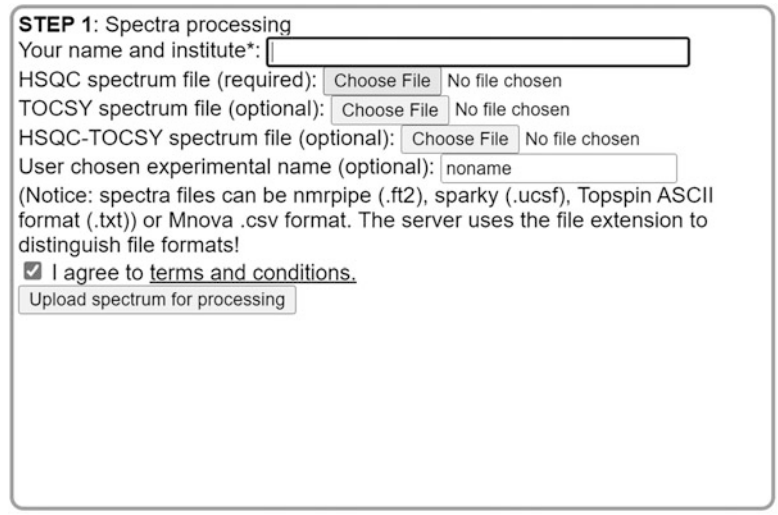

Fig. 11 Colmar is divided into defined windows, one for each step. Enter values accordingly in STEP 1, check for agreement of terms and conditions and, if so, click on the button to upload your data

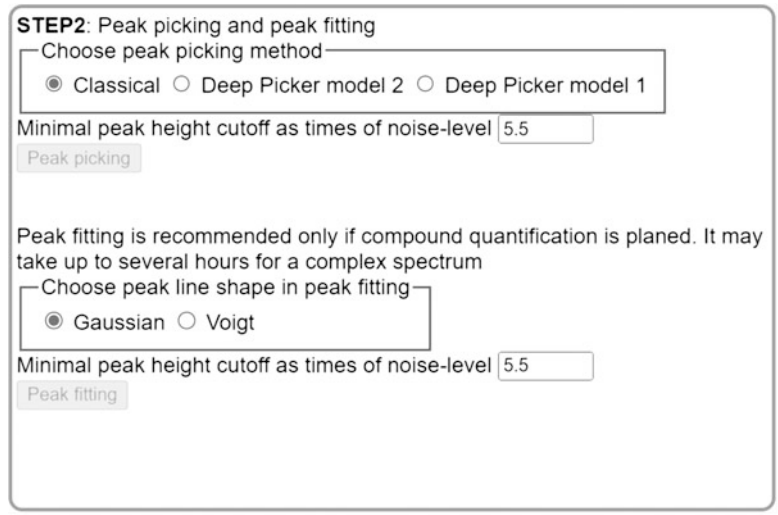

Fig. 12 STEP 2 window on Colmar page

For each spectrum query, an ID session is generated. It is important to save the session ID to retrieve the results (Fig. 15). As a result, the spectrum is shown and assigned with compounds after matches (Fig. 16). Also, a list named "Compound Reports" (Fig. 17) presents molecules identified within the sample, how precise its identification (matching ratio), root mean square deviation for ^{1}H and ^{13}C chemical shifts and compound IDs on public databases.

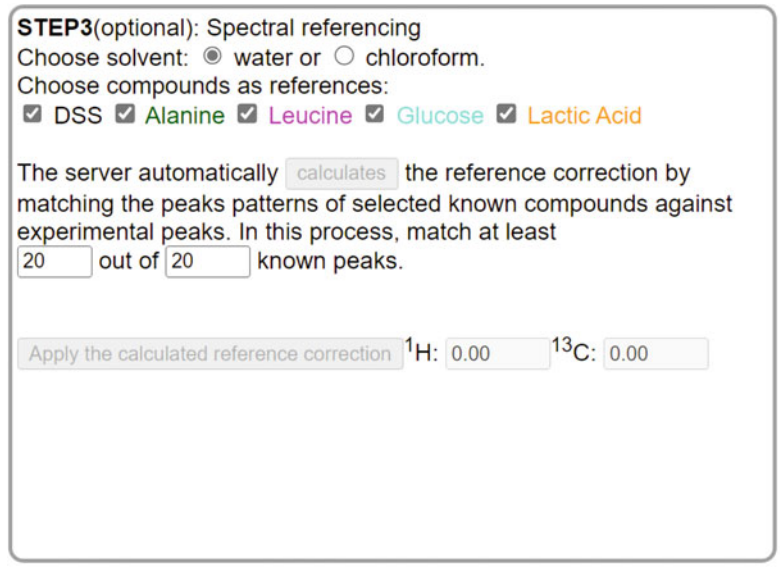

Fig. 13 STEP 3 window on Colmar page

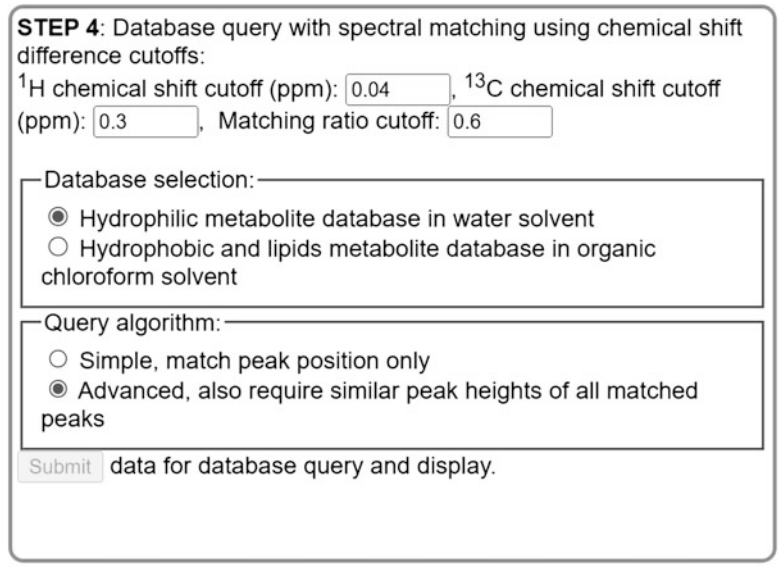

Fig. 14 STEP 4 window on Colmar page. Most default values were used in this chapter. Change "Query algorithm" to Simple

8. To generate a final list of identified compounds. Evaluate matching by selecting a compound on a dropdown box (Fig. 18) and rank it as "Good," "Fair," "Poor," or "Not sure." Such ranking is helped by the interpretation of the spectra at the bottom of the page (Fig. 19) and the comparison of experimental data (green circles) and database peaks (red

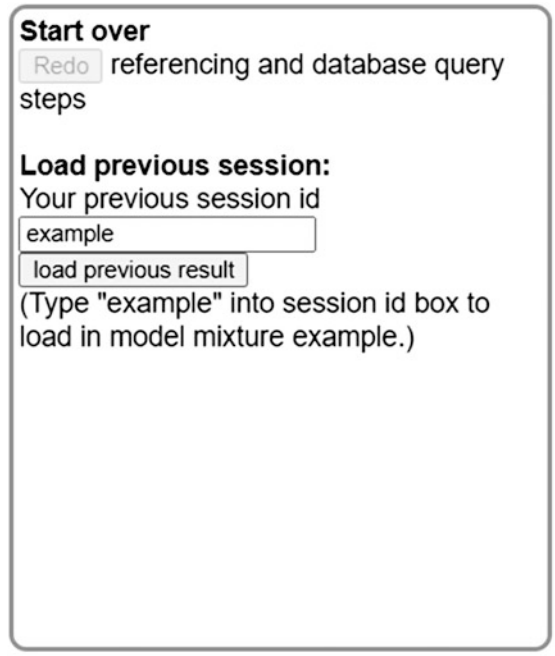

Fig. 15 Last window on Colmar page. This allows to start over the analysis or load a previously saved session

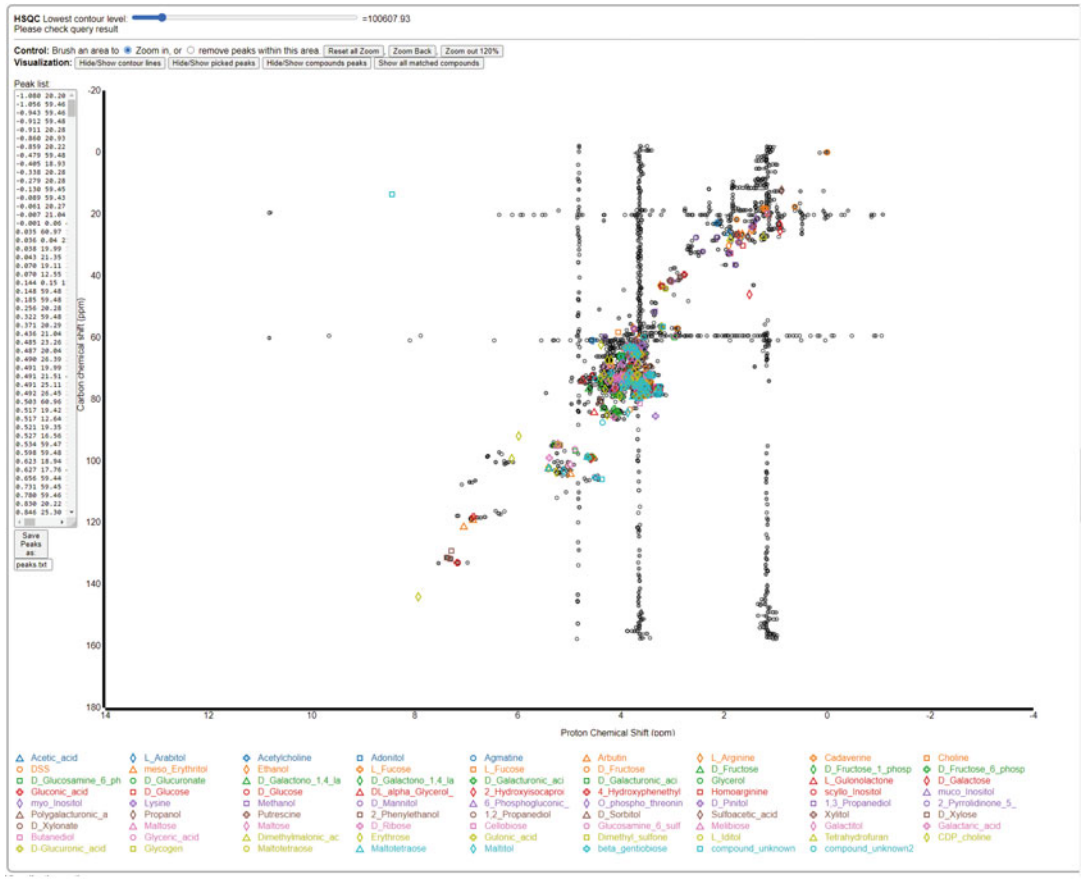

Fig. 16 Colmar windows showing assigned compounds

ID	Name	Matching_ratio	13C_RMSD	1H_RMSD	Uniqueness	origin	pubchemID
1	Acetic_acid	1.00	0.16	35	0/1	bmse000191	176
2	L_Arabitol	1.00	0.18	25	4/5	HMDB01851	439255
3	Acetylcholine	1.00	0.19	19	1/4	bmse000168	187
4	Adonitol	0.75	0.15	22	3/3	HMDB00508	439155
5	Agmatine	0.75	0.10	20	3/3	HMDB01432	199
6	Arbutin	0.78	0.22	21	5/7	HMDB29943	346
7	L_Arginine	0.60	0.14	27	2/3	HMDB00517	6322
8	Cadaverine	0.67	0.20	21	2/2	bmse000072	273
9	Choline	0.67	0.19	16	1/2	bmse000285	305
10	DSS	1.00	0.05	7	0/4	bmse000795	5167273
11	meso_Erythritol	1.00	0.17	3	3/3	HMDB02994	222285
12	Ethanol	1.00	0.07	2	1/2	bmse000297	702
13	L_Fucose	0.67	0.20	24	2/4	HMDB00174	17106
14	L_Fucose	0.67	0.23	24	4/4	HMDB00174	17106
15	D_Fructose	0.67	0.14	26	3/4	HMDB00660	439709
16	D_Fructose	0.67	0.12	14	2/4	HMDB00660	439709
17	D_Fructose_1_phosphate	1.00	0.16	18	5/6	bmse000254	439394
18	D_Fructose_6_phosphate	0.60	0.19	24	1/3	bmse000012	439160
19	D_Glucosamine_6_phosphate	0.67	0.13	14	3/4	bmse000189	439217
20	D_Glucuronate	0.60	0.11	13	3/3	bmse000140	94715
21	D_Galactono_1,4_lactone	1.00	0.15	19	2/5	HMDB00565	128869
22	D_Galactono_1,4_lactone	1.00	0.11	28	5/5	HMDB00565	128869
23	D_Galacturonic_acid	0.60	0.24	15	3/3	bmse000216	445929
24	D_Galacturonic_acid	1.00	0.12	20	2/5	bmse000216	445929
25	Glycerol	1.00	0.06	5	3/3	bmse000184	753
26	L_Gulonolactone	0.83	0.17	8	2/5	HMDB03466	439373
27	D_Galactose	0.67	0.16	15	3/4	HMDB00143	439357
28	Gluconic_acid	0.83	0.18	21	4/5	HMDB00150	7027
29	D_Glucose	0.86	0.13	18	6/6	HMDB03345	79025
30	D_Glucose	0.86	0.10	20	6/6	HMDB03345	79025
31	DL_alpha_Glycerol_phosphate	1.00	0.22	26	3/4	bmse000193	754
32	2_Hydroxyisocaproic_acid	0.60	0.18	24	2/3	bmse000338	92779
33	4_Hydroxyphenethyl_alcohol	0.75	0.12	13	1/3	bmse000173	10393
34	Homoarginine	0.60	0.15	20	3/3	bmse000745	9085
35	scyllo_Inositol	1.00	0.04	4	0/1	bmse000113	892
36	muco_Inositol	1.00	0.13	9	2/2	bmse000102	892
37	myo_Inositol	1.00	0.14	9	3/4	HMDB00211	6021
38	Lysine	0.80	0.11	20	4/4	HMDB03405	57449
39	Methanol	1.00	0.11	6	0/1	bmse000294	887
40	D_Mannitol	0.75	0.22	22	2/3	bmse000099	6251
41	6_Phosphogluconic_acid	0.60	0.19	7	0/3	HMDB01316	91493
42	O_phospho_threonine	0.67	0.25	17	0/2	bmse000631	1016
43	D_Pinitol	0.71	0.09	20	4/5	bmse000740	439990
44	1,3_Propanediol	1.00	0.07	21	0/2	bmse000303	10442
45	2_Pyrrolidinone_5_carboxylate	1.00	0.06	10	0/4	bmse000483	439685
46	Polygalacturonic_acid	0.80	0.15	20	2/4	bmse000228	445929
47	Propanol	1.00	0.14	8	0/3	bmse000446	1031
48	Putrescine	1.00	0.11	6	2/2	bmse000109	1045
49	2_Phenylethanol	0.80	0.15	6	1/4	bmse000659	6054
50	1,2_Propanediol	0.75	0.08	9	0/3	bmse000302	1030
51	D_Sorbitol	0.75	0.16	17	6/6	HMDB00247	5780
52	Sulfoacetic_acid	1.00	0.02	6	0/1	bmse000632	31257
53	Xylitol	0.75	0.14	21	2/3	bmse000129	6912
54	D_Xylose	1.00	0.16	17	5/5	HMDB00098	135191
55	D_Xylonate	0.80	0.18	16	2/4	HMDB60256	6971043
56	Maltose	0.86	0.16	20	11/12	HMDB00163	10991489
57	Maltose	0.71	0.17	26	10/10	HMDB00163	10991489
58	D_Ribose	0.83	0.18	20	1/5	HMDB00283	5779
59	Cellobiose	0.62	0.19	18	6/8	HMDB00055	10712
60	Glucosamine_6_sulfate	0.86	0.11	17	2/6	HMDB00592	72361
61	Melibiose	0.62	0.16	14	7/8	bmse000233	11458
62	Galactitol	1.00	0.11	18	3/3	HMDB00107	11850
63	Galactaric_acid	1.00	0.05	21	2/2	HMDB00639	3037582
64	Butanediol	1.00	0.15	14	2/2	HMDB03156	262
65	Glyceric_acid	0.67	0.20	19	0/2	HMDB00139	439194
66	Dimethylmalonic_acid	1.00	0.06	21	0/1	HMDB02001	11686
67	Erythrose	0.60	0.16	22	1/3	HMDB02649	439574
68	Gulonic_acid	0.80	0.13	18	3/4	HMDB03290	152304
69	Dimethyl_sulfone	1.00	0.01	8	0/1	HMDB04983	6213
70	L_Iditol	0.75	0.18	25	3/3	HMDB11632	5460044
71	Tetrahydrofuran	1.00	0.07	16	1/2	HMDB00246	8028
72	CDP_choline	0.64	0.07	20	3/7	HMDB01413	13804
73	D-Glucuronic_acid	0.80	0.11	12	4/4	HMDB00127	444791
74	Glycogen	0.79	0.13	19	22/22	bmse000232	439177
75	Maltotetraose	0.75	0.16	19	18/18	HMDB01296	439639
76	Maltotetraose	0.79	0.17	17	19/19	HMDB01296	439639
77	Maltitol	0.64	0.20	19	6/9	HMDB02928	493591
78	beta_gentiobiose	0.79	0.14	12	6/11	bmse000313	5460026
79	compound_unknown	0.67	0.09	18	2/4	none	-1
80	compound_unknown2	0.67	0.15	28	1/2	none	-1

Fig. 17 List of compounds as displayed by Colmar. Over 80 wine compounds were identified

Show detail of one matched spin-system: Show previous compound | Ethanol | Show next compound | Good | Fair | Poor | Not sure

For compound Ethanol, matching ratio is 1.00 ,carbon rmsd is 0.07 proton rmsd is 0.002 User_selected is not processed yet

Index	database 1H	database 13C	exp 1H	exp 13C	Sharing	intensity	
0	1.17	19.49	1.17	19.57	64	8.655e+8	8.655e+8
1	3.64	60.13	3.64	60.19		1.399e+9	1.399e+9

Fig. 18 Interface for match evaluation

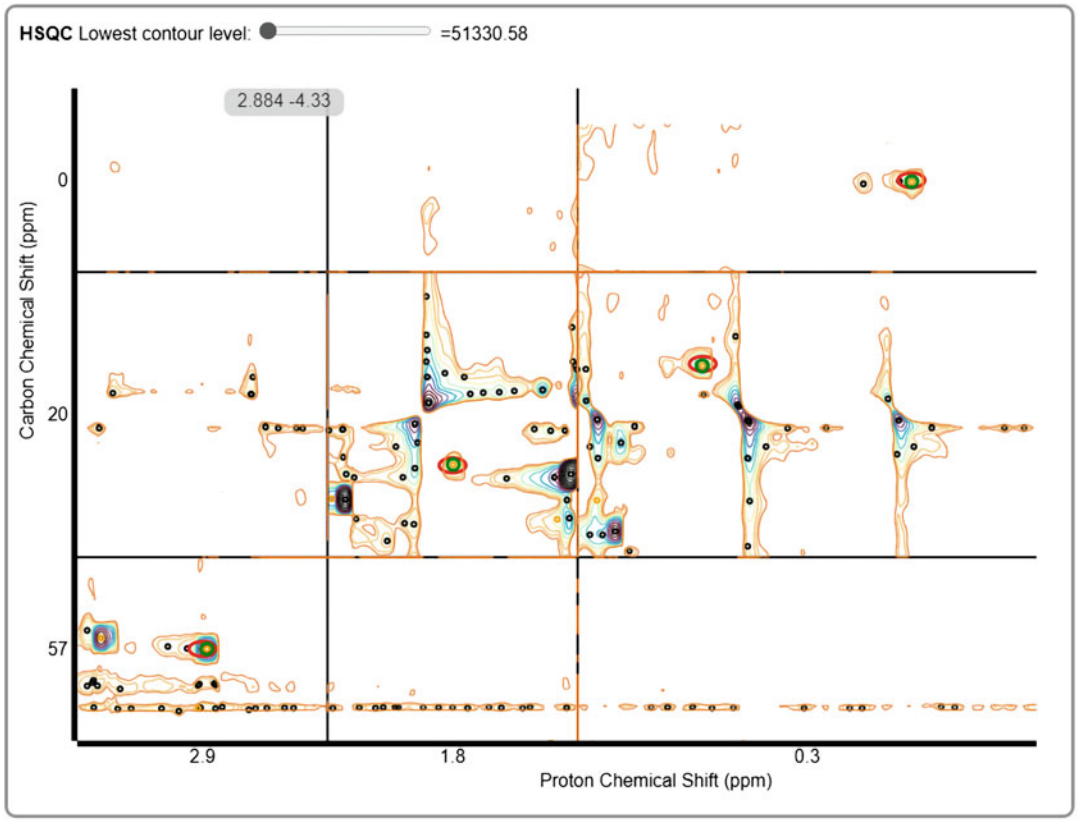

Fig. 19 Spectra on the region of interest for the compound being evaluated. This is automatically shown after selecting the compound on the interface displayed on Fig. 17. Red circles from database data should match the green circles defined by experimental data for a valid identification. (AROUND HERE)

circles). Note that some compounds will have values in the column "Sharing" (Fig. 18). This means that such a signal was identified in other compounds. User criteria, experience, and prior knowledge about the sample are needed to define if a match is valid or not.

In this box (see Fig. 20), you can save the file name, the table column delimiter, the individual images in vectorized format, and the tag revision for the session ID. The Compound report will save as a .txt file, the list of molecules identified with the same structure as the identification table generated on the platform with additional

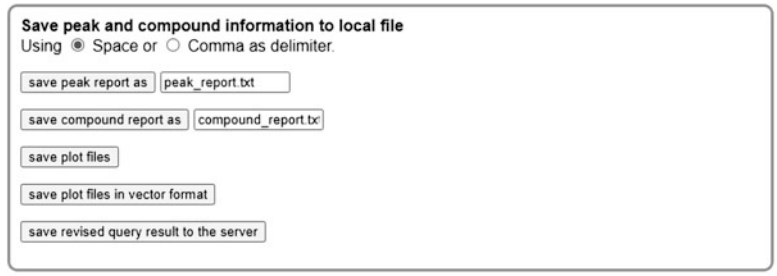

Fig. 20 Last box after identification for file and plot export

information on the signaling efficiency for each compound. The Peak report will save the list of peaks and intensity of each one of them in .txt and will relate to each molecule that was identified by the Colmar platform.

4 Notes

1. Sparkling wines have CO_2, which can impair NMR analysis. If there are noticeable air bubbles, meaning CO_2 is present in the wine, this step is mandatory. Otherwise, skip to **step 3**.
2. Chenomx and other software will require the pH value for chemical shift referencing.
3. Any variation of the sample volume should respect the ratio 8:1:1 (sample:DSS:D_2O). DSS final concentration in solution should be 1.0 mM.
4. The acquisition and operation of an NMR spectrometer should be performed by a trained professional. Since the operation of an NMR spectrometer by a non-technician would be a rare event, details of its operation are omitted in this chapter.
5. This procedure automatically updates the ".1r" file. Such a file will be used in Chenomx software for identification, as described in Subheading 3.4.1.

Acknowledgement

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References

1. Solovyev PA, Fauhl-Hassek C, Riedl J, Esslinger S, Bontempo L, Camin F (2021) NMR spectroscopy in wine authentication: an official control perspective. Compr Rev Food Sci Food Saf 20:2040–2062
2. Herbert-Pucheta JE, Lozada-Ramírez JD, Ortega-Regules AE, Hernández LR, de Parrodi CA (2021) Nuclear magnetic resonance metabolomics with double pulsed-field-gradient echo and automatized solvent suppression

spectroscopy for multivariate data matrix applied in novel wine and juice discriminant analysis. Molecules 26:4146

3. Macedo EHBC, Santos GC, Santana MN, Jesus EFO, de Araújo UB, Anjos MJ, Pinheiro AS, Carneiro CS, Rodrigues IA (2021) Unveiling the physicochemical properties and chemical profile of artisanal jabuticaba wines by bromatological and NMR-based metabolomics approaches. LWT 146:111371
4. Li DW, Wang C, Brüschweiler R (2017) Maximal clique method for the automated analysis of NMR TOCSY spectra of complex mixtures. J Biomol NMR 68:195–202
5. Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM (2006) Targeted profiling: quantitative analysis of 1H NMR metabolomics data. Anal Chem 78:4430–4442
6. Bingol K, Li DW, Bruschweiler-Li L, Cabrera OA, Megraw T, Zhang F, Brüschweiler R (2015) Unified and isomer-specific NMR metabolomics database for the accurate analysis of 13C-1H HSQC spectra. ACS Chem Biol 10: 452–459

Chapter 13

Ethanol Suppression on Wine Analysis Using Nuclear Magnetic Resonance (NMR)

Icaro P. Caruso, Fabio C. L. Almeida, Isabella O. Lourenço, Marcel M. L. da Cunha, Werner F. Brandão, and Gisele C. Amorim

Abstract

Nuclear magnetic resonance (NMR) spectroscopy has gained an outstanding role in the process of detecting, identifying, and quantitating the components of wine. However, ethanol signal suppression, a major component of wine, is an issue that needs to be addressed for better applicability of the NMR technique. Here we describe a strategy to overcome this problem by designing shaped pulses for selective suppression of ethanol signals, together with water signals, in 1D ^{1}H and ^{1}H–^{1}H, and ^{1}H–^{13}C 2D NMR experiments.

Key words NMR, Ethanol suppression, Shaped pulse, 1D ^{1}H spectrum, ^{1}H–^{1}H/^{13}C 2D spectra

1 Introduction

Wine is a relevant food commodity worldwide, with an estimated consumption of about 236 million hectoliters and a global export value of 34.3 billion euros in 2021 [1]. This alcoholic beverage is a complex mixture of several hundred compounds present at different concentrations, many of which contribute to the color, mouthfeel, or wine aromatic properties [2, 3]. The chemical composition of wine is mostly water, with 9–15% (v/v) of ethanol, glycerol, sugars, amino acids, organic acids and bases, and inorganic ions [3]. The authenticity assessment of wine depends on the vine variety, geographical origin, and vintage [4], and in this context, the knowledge of its components provides an adequate check of the quality and origin of each kind of wine [5].

Nuclear magnetic resonance (NMR) spectroscopy has gained a remarkable role in the process of detecting, identifying, and quantitating the components of wine; however, this includes limitations, since this beverage is a complex mixture [6, 7]. A crucial limitation rests on the fact that wine comprises mainly two protonated com-

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pounds, water, and ethanol, whose signals dominate the standard ^{1}H NMR spectra and need to be suppressed to allow observation of all lower abundance compounds in the sample [6]. The ^{1}H NMR spectrum of ethanol consists of a triplet and a quartet (representing the $-CH_3$ and $-CH_2$ groups, respectively), alongside a residual hydroxyl signal that could appear separately from the water signal [6]. In this chapter, a strategy to overcome this limitation is presented. Designing shaped pulses for selective suppression of ethanol signals, together with water signals, in 1D ^{1}H and ^{1}H–^{1}H, and ^{1}H–^{13}C 2D NMR experiments, it is possible to expose signals on a wine sample.

2 Materials

Prepare all solutions using Milli-Q water (18.2 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature.

2.1 Sample Preparation

1. 5 mM NMR tube.
2. Deuterium oxide (D_2O) 99%.
3. 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt (DSS-d_6) 98%: 200 mM in water.
4. Sample of wine: 500 μL.
5. Centrifuge and rotor for 1.5 mL microtubes.

2.2 Acquisition and Processing of Spectrum

1. NMR Bruker spectrometer (*see* **Note 1**).
2. Bruker TopSpin Software version 3.6 or higher.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Sample Preparation

1. Centrifuge 500 μL of wine for 10 min at 25 °C. Collect the supernatant and mix with 60 μL of D_2O and 3 μL of DSS-d_6. Adjust the final volume to 600 μL with water. Load the sample into the 5 mM NMR tube.

3.2 1D Spectrum Acquisition and Shaped Pulse Design

1. This preliminary experiment is used to identify the water resonance frequency and to calibrate the 90° ^{1}H "hard" pulse using presaturation for water suppression (ZGPR pulse sequence). The 90° ^{1}H pulse is calibrated using a 360° pulse in a single scan experiment, searching for a null in the wine signal (*see* **Note 2**).

2. The second experiment is used to determine the chemical shift of the CH_2 and CH_3 signals of ^{12}C isotopomers of ethanol. Use ZGESGP pulse sequence for water suppression with excitation sculpting [8]. A square-shaped pulse (Squa100.1000) of 2 ms 180° ^1H pulse centered in water is employed. The shaped pulse Squa100.1000 power can be calibrated by searching for a null in the water signal (*see* **Note 3**).
3. In the acquired 1D spectrum, identify the chemical shift values in hertz and ppm for the water and ethanol resonances (–CH_2 and –CH_3) (*see* **Note 4**). Keep notes of the exact values in hertz and ppm for the three resonances (*see* **Note 5**). Make sure that the solvent reference (SR) is set to zero for these calibrations.
4. Open the ShapeTool package of Bruker Topspin program. In the Acquisition tab of the ZGESGP experiment, click on "Edit shaped pulse file" (symbol E, on the right side of the SPNAM1 parameter) to edit the shaped pulse Squa100.1000. There are alternate ways to get to the ShapeTool.
5. In ShapeTool, click on "new excitation region" to select the regions containing the water and ethanol (–CH_2 and –CH_3) signals, and a spectral region selector shows up. By clicking and dragging, place the center of the selector on the chemical shift of the desired signal. Adjust the width of the selector to contain only the signal of interest. This process must be done for the water and ethanol signals (–CH_2 and –CH_3).
6. After selecting the excitation regions, click on "edit all excitation regions" to numerically edit the limits of the regions by setting the same excitation width. In the window "Edit regions," pick the options "use advanced mode" and "use the same shape for all regions," select the shape as "Rectangle" and type of rotation Inversion ($I_Z \rightarrow -I_Z$), and edit the left and right limits (in hertz or ppm) by setting the same values of the excitation widths, for example, 30 Hz (*see* **Note 6**). Then apply the adjusted parameters. Below the spectrum will appear the new shape with its amplitude and phase (Fig. 1).
7. Details about the three rectangle contributions of the combined shape will appear on the left side of the spectrum. In "General parameters," set the number of points (size [pt]) with the same value and the offset (offset [pt]) equal to zero for each contribution. Finally, save the new shape. The path reserved for saving new shapes is ~/topspinXX/exp./stan/nmr/lists/wave/user.
8. Insert the new shape into a ZGESGP pulse sequence by replacing Squa100.1000 ("Select shaped pulse file" three dots on the right of the SPNAM1 parameter). Set the 90° ^1H "hard" pulse previously calibrated and the pulse length (μs) of the new shape that appears in "General parameters" for the combined shape in ShapeTool.

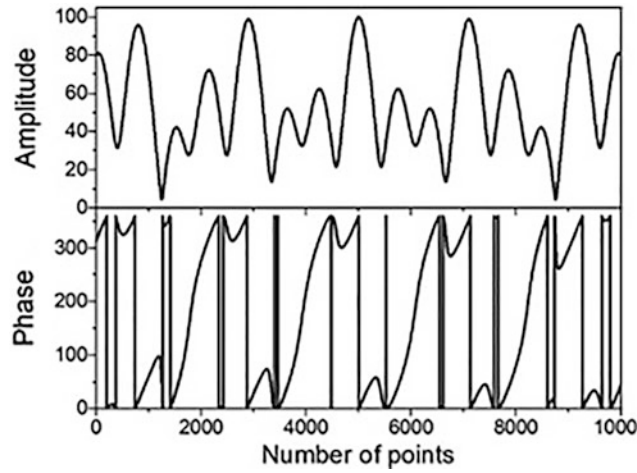

Fig. 1 Example of a shaped pulse (amplitude and phase) built to suppress water and ethanol signals using an excitation width of 30 Hz for each selected spectral region

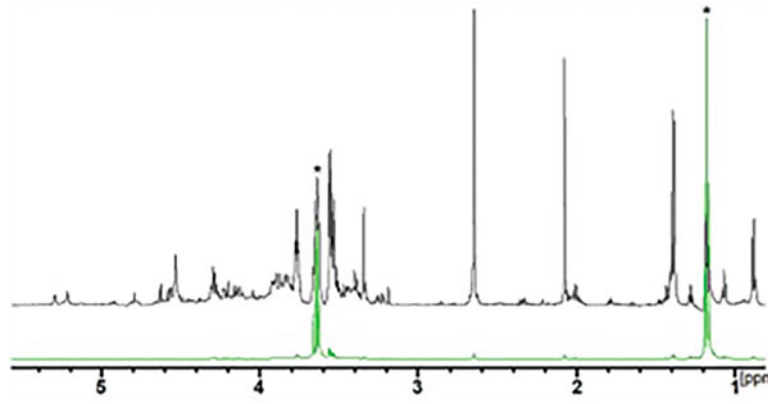

Fig. 2 Selected region of a 1D ^{1}H spectrum of a wine sample acquired using the ZGESGP pulse sequence with (black) and without (green) ethanol signal suppression. These spectra were acquired at NMR Bruker spectrometer Avance III HD 600 MHz at 25 °C. The ethanol $-CH_3$ and $-CH_2$ signals are indicated with asterisks

9. The power level of the new shape can be calculated using the "Analyze Waveform" tool (symbol of a magnifying glass), which is on the left side of the "General parameters." Choose the option "Integrate Shape" and click on "Set parameters" in "Integration." A window will appear showing the pulse power (dB) in "Set Power (Global Shape)." The spectrum acquisition with ethanol suppression is ready to be done. Figure 2 shows an example of a 1D ^{1}H spectrum with and without ethanol suppression.

3.3 2D ^{1}H–^{1}H Spectrum Acquisition with Ethanol Suppression

1. In the 2D ^{1}H–^{1}H NMR experiments with water suppression using excitation sculpting, set the 90° ^{1}H pulse and replace Squa100.1000 with the new shape for ethanol suppression, along with its pulse length and power, which were determined in Subheading 3.2. Figure 3 shows an example of a 2D ^{1}H–^{1}H TOCSY spectrum with and without ethanol suppression. The Bruker standard pulse sequences with excitation sculpting are: NOESY (NOESYESGPPH), TOCSY (MLEVESGPPH, for MLEV spin lock, or DISPSI2ESGPPH, for DIPSI2 spin lock), and double quantum filtered COSY (COSYDFESGPPH). There are variations, such as the inclusion of water flip-back pulses, among others.

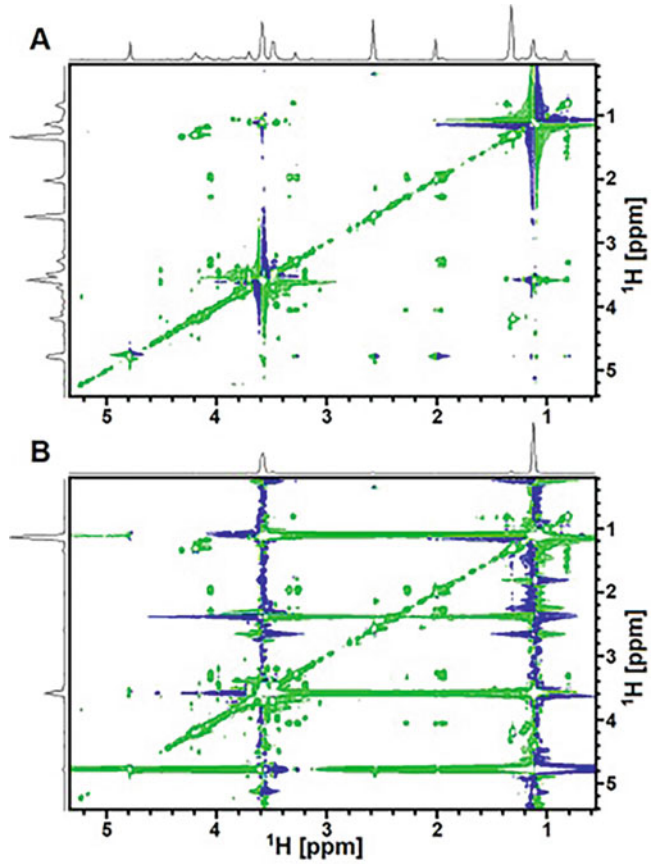

Fig. 3 Selected region of a 2D ^{1}H–^{1}H TOCSY spectrum of a wine sample acquired using the MLEVESGPPH pulse sequence with (**a**) and without (**b**) ethanol signal suppression. These spectra were acquired at NMR Bruker spectrometer Avance III HD 600 MHz at 25 °C. Both spectra have the same base level for the level curves

3.4 ^{1}H–^{13}C HSQC Pulse Sequence Modification for Ethanol Suppression

1. Use a modified version of the HSQCETGPSI pulse sequence, a ^{1}H–^{13}C HSQC with gradient selection and sensitivity improvement (*see* **Note 7**). The modified version includes a presaturation in the ^{1}H and ^{13}C resonances during the relaxation delay. For presaturation, two equally sized (P18 = P32, parameters in pulse sequence) 180° shaped pulses (^{1}H and ^{13}C) were built using the ShapeTool package. The 180° ^{1}H shaped pulse was designed to perform a triple inversion (water, $–CH_2$, and $–CH_3$ resonances), as described in Subheading 3.2, and the 180° ^{13}C shaped pulse, a double inversion ($–^{13}CH_2$ and $–^{13}CH_3$ resonances).
2. Calibrate 90° ^{1}H and ^{13}C "hard" pulse using standard methods.
3. For the ^{1}H shaped pulse, use the new shape built in Subheading 3.2.
4. For the ^{13}C shaped pulse, first set up the ^{13}C direct detection ZG30 pulse sequence and collect the spectrum (*see* **Note 8**).
5. After acquiring the 1D ^{13}C spectrum of the wine sample, identify the exact chemical shift values in hertz and ppm for the ethanol resonances ($–^{13}CH_2$ and $–^{13}CH_3$) (*see* **Note 9**).
6. At the window of the ^{13}C spectrum, open the ShapeTool package by clicking in the Topspin menu as follows: Acquire, More, and ShapeTool. Follow **Steps 5, 6,** and **7** in Subheading 3.2 for building the new shape for ^{13}C. It will look like the one of ^{1}H in Fig. 1. Save the new shape.
7. Load the new shapes for ^{1}H and ^{13}C into SPW6 and SPW7 parameters by clicking on "Select shaped pulse file" (three dots on the right of each parameter) in the HSQCETGPSI modified pulse sequence (2D ^{1}H–^{13}C HSQC spectrum). Set the 90° ^{1}H and ^{13}C "hard" pulse previously calibrated and the length (μs) of the newly shaped pulses for ^{1}H and ^{13}C (P18 and P32, respectively) that appears in "General parameters" for the combined shape in ShapeTool.
8. The calibration of the power level of the newly shaped pulses for ^{1}H and ^{13}C can be performed as in **Step 9** in Subheading 3.2. Then, ^{1}H–^{13}C HSQC acquisition with ethanol signal suppression will be ready to run. Figure 4 shows an example of the 2D ^{1}H–^{13}C HSQC spectrum with and without ethanol suppression.

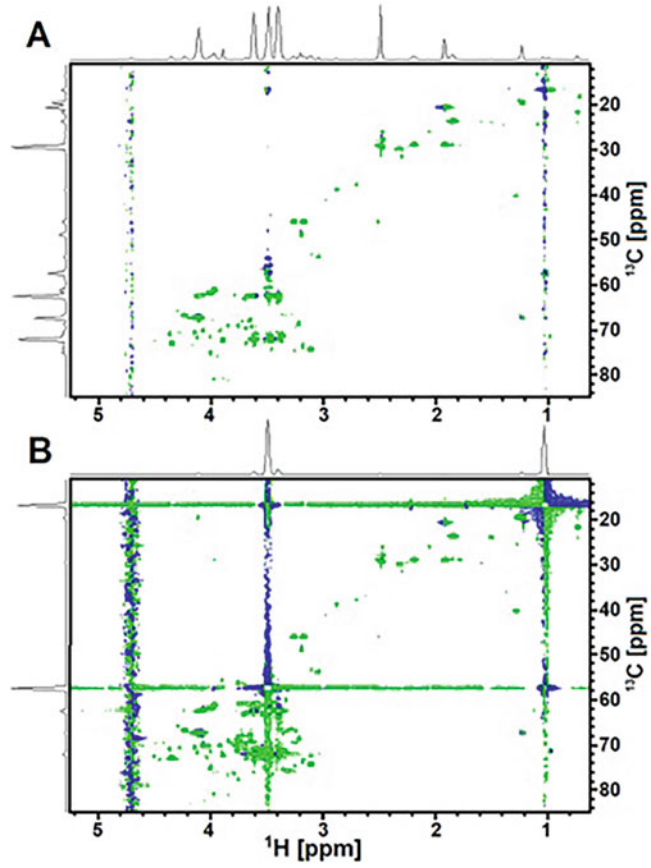

Fig. 4 2D ^{1}H–^{13}C HSQC spectrum of a wine sample acquired using the modified HSQCETGPSI pulse sequence (*see* **Note 7**) with (**a**) and without (**b**) ethanol signal suppression. These spectra were acquired at NMR Bruker spectrometer Avance III HD 600 MHz at 25 °C. Both spectra have the same base level for the level curves

4 Notes

1. The acquisition of NMR spectra at fields higher than 11.7 T, operating at 500 MHz (^{1}H) or above, is recommendable because of the resolution.
2. The calibration of the 90° ^{1}H pulse can also be done in an automated way using the "pulsecal" command in the Bruker TopSpin program. After running this command, a window with the calculated 90° ^{1}H pulse and its associated power level will pop up. Clicking "OK" will enter the displayed values into the current parameter set.

3. The power level of the shaped pulse Squa100.1000 (pl_2, 2 ms 180° ^{1}H pulse) can also be calculated by using the following equation:

$$pl_2 = pl_1 + 20\log\left(\frac{\tau_1^{90}}{\tau_2^{90}}\right) \quad (1)$$

where pl_1 and τ_1^{90} are the power level (dB) and length (μs) of 90° ^{1}H "hard" pulse, respectively, and τ_2^{90} is the pulse length for Squa100.1000 corresponding to a 90° ^{1}H pulse of 1 ms.

4. For an NMR spectrometer operating at 600 MHz, the chemical shift values are approximately 2820 Hz (~4.69 ppm) for water and 2190 (~3.64 ppm) and 702 Hz (~1.16 ppm) for $-CH_3$ and $-CH_2$ ethanol signals at 25 °C, respectively.
5. It is useful to note down the chemical shift values in hertz and ppm for water and CH_2 and CH_3 ethanol groups, as older versions of the Bruker TopSpin program use these values in hertz in the ShapeTool package, while newer versions use them in ppm.
6. The excitation width must completely cover each of the water and ethanol signals. However, it is relevant to note that the greater the excitation width, the greater the pulse length suggested by ShapeTool for the newly shaped pulse. On the other hand, if the excitation width is too short, the water signal suppression is impaired. Therefore, the choice of the excitation width value must take these issues into account.
7. Modified HSQCETGPSI Bruker pulse sequence for ethanol suppression in the ^{1}H–^{13}C HSQC spectrum (our modifications are in bold):

;hsqcetgpsi

;avance-version (12/01/11)

;HSQC

;2D H-1/X correlation via *double inept transfer*

; using sensitivity improvement

;phase sensitive using Echo/Antiecho-TPPI gradient selection

;with decoupling during acquisition

;using trim pulses in inept transfer

;modified by Fabio C. L. Almeida and Icaro P. Caruso

;A.G. Palmer III, J. Cavanagh, P.E. Wright & M. Rance, J. Magn.

; Reson. 93, 151-170 (1991)

;L.E. Kay, P. Keifer & T. Saarinen, J. Am. Chem. Soc. 114,

; 10663-5 (1992)

```
;J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt,
    O. Schedletzky,
; S.J. Glaser, O.W. Sorensen & C. Griesinger, J. Biomol. NMR 4,
; 301-306 (1994)
;$CLASS=HighRes
;$DIM=2D
;$TYPE=
;$SUBTYPE=
;$COMMENT=
#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>
"p2=p1*2"
"p4=p3*2"
"d4=1s/(cnst2*4)"
"d11=30m"
"l6=d1/p18"
# ifdef LABEL_CN
"p22=p21*2"
# else
# endif /*LABEL_CN*/
"d0=3u"
"in0=inf1/2"
"DELTA1=p16+d16-p1*0.78+de+8u"
# ifdef LABEL_CN
"DELTA=p16+d16+50u+larger(p2,p22)+d0*2"
# else
"DELTA=p16+d16+50u+p2+d0*2"
# endif /*LABEL_CN*/
"acqt0=0"
baseopt_echo
1 ze
d11 pl12:f2
2 d11 do:f2
d12 pl21:f2 pl9:f1
; jump to 13C chemical shift of solvent (methyl)
; 4u fq=cnst21(bf ppm):f2
```

```
; apply offreonance presat on 1H resonance of solvent
; 4u cw:f2
3 (center (p18:sp6 ph29):f1 (p32:sp7 ph29):f2 )
4u
lo to 3 times l6
; loop for presat time
; 4u do:f2
; jump back to center of carbon spectrum
; 4u fq=0:f2
4u pl1:f1
4 (p1 ph1)
d4 pl2:f2
(center (p2 ph1) (p4 ph6):f2 )
d4
p28 ph1
4u
(p1 ph2) (p3 ph3):f2
d0
# ifdef LABEL_CN
(center (p2 ph7) (p22 ph1):f3 )
# else
(p2 ph7)
# endif /*LABEL_CN*/
d0
50u UNBLKGRAD
p16:gp1*EA
d16
(p4 ph4):f2
DELTA
(center (p1 ph1) (p3 ph4):f2 )
d24
(center (p2 ph1) (p4 ph1):f2 )
d24
(center (p1 ph2) (p3 ph5):f2 )
d4
(center (p2 ph1) (p4 ph1):f2 )
d4
```

```
(p1 ph1)
DELTA1
(p2 ph1)
4u
p16:gp2
d16 pl12:f2
4u BLKGRAD
go=2 ph31 cpd2:f2
d11 do:f2 mc #0 to 2
F1EA(calgrad(EA) & calph(ph5, +180), caldel(d0, +in0) &
    calph(ph3, +180) & calph(ph6, +180) & calph(ph31, +180))
exit
ph1=0
ph2=1
ph3=0 2
ph4=0 0 2 2
ph5=1 1 3 3
ph6=0
ph7=0 0 2 2
ph29=0
ph31=0 2 2 0
;pl1 : f1 channel - power level for pulse (default)
;pl2 : f2 channel - power level for pulse (default)
;pl3 : f3 channel - power level for pulse (default)
;pl12: f2 channel - power level for CPD/BB decoupling
;p1 : f1 channel - 90 degree high power pulse
;p2 : f1 channel - 180 degree high power pulse
;p3 : f2 channel - 90 degree high power pulse
;p4 : f2 channel - 180 degree high power pulse
;p16: homospoil/gradient pulse
;p22: f3 channel - 180 degree high power pulse
;p28: f1 channel - trim pulse
;d0 : incremented delay (2D)   [3 usec]
;d1 : relaxation delay; 1-5 * T1
;d4 : 1/(4J)XH
;d11: delay for disk I/O   [30 msec]
;d16: delay for homospoil/gradient recovery
```

;d24: 1/(8J)XH for all multiplicities
; 1/(4J)XH for XH
;cnst2: = J(XH)
*;inf1: 1/SW(X) = 2 * DW(X)*
*;in0: 1/(2 * SW(X)) = DW(X)*
;nd0: 2
*;ns: 1 * n*
;ds: >= 16
;td1: number of experiments
;FnMODE: echo-antiecho
;cpd2: decoupling according to sequence defined by cpdprg2
;pcpd2: f2 channel - 90 degree pulse for decoupling sequence
;use gradient ratio: gp 1 : gp 2
; 80 : 20.1 for C-13
; 80 : 8.1 for N-15
;for z-only gradients:
;gpz1: 80%
;gpz2: 20.1% for C-13, 8.1% for N-15
;use gradient files:
;gpnam1: SMSQ10.100
;gpnam2: SMSQ10.100
; preprocessor-flags-start
;LABEL_CN: for C-13 and N-15 labeled samples start experiment with
option -DLABEL_CN (eda: ZGOPTNS)
; preprocessor-flags-end
;$Id: hsqcetgpsi,v 1.6.4.1.4.1 2012/01/31 17:56:32 ber Exp $

8. This is straightforward, even in NMR equipment with an indirect probe, 128 scans should be enough to acquire a good 1D ^{13}C spectrum of the wine sample in which it is possible to see the ethanol signals ($-^{13}CH_2$ and $-^{13}CH_3$).
9. For an NMR spectrometer operating at 600 MHz, the chemical shift values are approximately 8663 (~57.41 ppm) and 2530 Hz (~16.76 ppm) for ethanol $-^{13}CH_2$ and $-^{13}CH_3$ signals at 25 °C, respectively.

References

1. International Organization of Vine and Wine. State of the word vine and wine sector 2021 (2022). https://www.oiv.int/sites/default/files/documents/eng-state-of-the-world-vine-and-wine-sector-april-2022-v6_0.pdf. Accessed 30 Nov 2022
2. Sumby KM, Grbin PR, Jiranek V (2010) Microbial modulation of aromatic esters in wine: current knowledge and future prospects. Food Chem 121:1–16
3. Bakker J, Clarke RJ (2012) Wine flavour chemistry. Wiley Blackwell, Hoboken
4. Viggiani L, Morelli MAC (2008) Characterization of wines by nuclear magnetic resonance: a work study on wines from the Basilicata region in Italy. J Agric Food Chem 56:8273–8279
5. Gil AM, Duarte IF, Godejohann M et al (2003) Characterization of the aromatic composition of some liquid foods by nuclear magnetic resonance spectrometry and liquid chromatography with nuclear magnetic resonance and mass spectrometric detection. Anal Chim Acta 488:35–51
6. Kew W, Bell NGA, Goodall I, Uhrín D (2017) Advanced solvent signal suppression for the acquisition of 1D and 2D NMR spectra of Scotch Whisky. Magn Reson Chem 55:785–796
7. Alberti E, Belton PS, Gil AM (2002) Applications of NMR to food science. Annu Rep NMR Spectrosc 47:109–148. https://doi.org/10.1016/S0066-4103(02)47039-1
8. Hwang TL, Shaka AJ (1995) Water suppression that works. Excitation sculpting using arbitrary wave-forms and pulsed-field gradients. J Magn Reson Ser A 112:275–279

Chapter 14

Methods to Determine Biogenic Amines in Wine by RP-HPLC

Fernanda Cosme, Juliana Milheiro, Leonor C. Ferreira, Luís Filipe-Ribeiro, and Fernando M. Nunes

Abstract

Biogenic amines can naturally be present in grapes or appear during the winemaking and/or aging processes, mainly due to the activity of microorganisms, such as lactic acid bacteria. Determining biogenic amines in wines is primarily performed by liquid chromatography with reversed-phase (RP) separation by C18 columns, using derivatisation reagents to promote its separation and detection. Nowadays, developing faster and inexpensive techniques or methodologies to apply in the wine industry is still challenging. Thus, the most used HPLC derivatisation methods to determine biogenic amines are presented, but also a simple dispersive solid-phase extraction clean-up/concentration method for selective and sensitive quantitation of biogenic amines in wines using benzoyl chloride derivatisation.

Key words Biogenic amines, Food safety, Wine, Chromatographic methods, Sample preparation, Derivatisation

1 Introduction

Biogenic amines (BAs) are low molecular weight organic bases that have unwanted physiological effects on humans when absorbed at too high a level [1, 2]. In wine, more than two dozen BAs with aliphatic (putrescine, cadaverine, spermine, and spermidine), aromatic (tyramine and phenylethylamine), and heterocyclic structures (histamine and tryptamine) were identified [3–5]. Of all BAs found in wines, histamine and tyramine have been the most studied by food safety concerns related to their highest toxicity.

The total content of BAs in red wines has been described as a few nanograms per litre to about 50 mg/L [6]. According to the literature, total BAs ranged from non-detected to 12.8 mg/L in white wines. Red wines are often described as containing a higher quantity of BAs than white wines due to their lower acidity, higher pH, and malolactic fermentation, which is much less common in white wines [7]. Lafon-Lafourcade [8] refers to total BAs levels in

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Bordeaux wines, from non-detected to 0.8 mg/L in white wines and from non-detected to 8.9 mg/L in rosé and red wines. Mayer [9], in a total of 395 Swiss wines, indicates only 2 cases with contents above 12 mg/L, with average values of 1.1 mg/L for white wines and 3.5 mg/L for red wines. In a set of 79 Portuguese red wines produced at an industrial scale, their total BAs ranged from 19.6 to 331 mg/L [10]. Usually, they are two different sources of BAs in wine: grapes and the fermentation processes. It has been shown that some amines occur in grapes, such as histamine and tyramine, as well as several polyamines and volatile amines.

According to Plumas [11] and Schneyder [12], among other authors, histamine formation by histidine decarboxylation occurs simultaneously with malic acid decarboxylation into lactic acid. Therefore, it is supposed that the primary source of these BAs in wine seems to be the decarboxylation of the precursor amino acids, histidine or tyrosine, to histamine and tyramine, respectively. The usual biological source of decarboxylation is *Lactobacillus* sp., as some lactic acid bacteria possess enzymes that decarboxylate amino acids to form the corresponding amines and carbon dioxide. The histamine content of wines can reach values of 30 mg/L, but white wines rarely present values higher than 1 mg/L. Concentrations for histamine were detected in Portugal at up to 23.1 mg/L [13], in Italy at up to 10.8 mg/L [14], in France at up to 14.1 mg/L [15], and in different EU countries (Italy, France, Germany, Switzerland, Austria, and Spain) of 16.2 mg/L [16].

As the knowledge of the presence of BAs in wines is essential for the wine trade sector, developing methods for their precise and accurate determination is necessary. Indeed, BAs identification and determination remains a challenge from the analytical point of view, as BAs separation and detection is not straightforward due to their strong polar characteristics, the complexity of the matrix, low content, the existence of possible interfering compounds, and the existence of several BAs at the same time. The developed analytical methods are based on BAs extraction and derivatisation to overcome some of these issues, followed by chromatographic separation and quantitation using suitable detectors [17].

Their separation and determination (after extraction and derivatisation) are often carried out by the use of chromatographic methods such as RP-HPLC using C18 columns, with detection of the derivatised analytes by UV spectrophotometry, or more often by the more sensitive fluorometry [4]; however, gas chromatography (GC) and capillary electrophoresis (CE) can also be used. Sample preparation is usually performed by solvent extraction to remove compounds that may interfere with the analysis. Other strategies have also been used for example, Loukou and Zotou [18] used poly(vinylpyrrolidone) (PVP) to remove phenolic interferences before derivatisation from the sample and then clean up with solid phase extraction (SPE) after derivatisation.

As most BAs do not have enough absorption in the UV-Vis wavelength ranges or have natural fluorescence, the derivatisation process preferentially uses pre-column derivatisation. However, post-column derivatisation has also been used. The derivatisation process also enhances the retention and separation in the reversed-phase (RP) columns by reducing the polarity of the compounds [17]. The most used derivatisation agents are dabsyl chloride (Dabs-Cl) [19, 20], dansyl chloride (Dns-Cl) [18, 21–25], benzoyl chloride (Bnz-Cl) [26], and *ortho*-phthalaldehyde [3, 27–29], but others, such as naphthaquinone-4-sulfonate (NQS) [28] and 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) [30, 31], are also used.

Even, though OPA (*ortho*-phthalaldehyde) reacts fast with BAs, it only reacts with primary amines, and the formed derivatives are unstable [32]. On the other hand, Dns-Cl is light-sensitive and has limited stability [33]. Benzoyl chloride presents advantages as BAs derivatisation reagent comprising short elution time, yielding BAs derivatives not sensitive to light, and reacting with both primary and secondary amines forming stable derivatives [33, 34]. Nevertheless, the two most common derivatisation procedures used in wine BAs analysis employ the Dns-Cl and OPA. Dansyl chloride (DNS-Cl) derivatives can be detected using diode-array detector (DAD), fluorescence detector (FLD), and mass spectrometry (MS), however, the reaction is time-consuming (10–60 min) requires the application of external temperature (40–70 °C).

On the other hand, *ortho*-phthalaldehyde (OPA) is less stable, and the process can be performed at room temperature in a short time and can be used without using any preliminary separation or clean-up and, it can also be used for post-column derivatisation [17]. Normally, pre-column derivatisation with dansyl chloride is used, using RP-HPLC chromatographic separation of the derivatised analytes being carried out by gradient elution with acetonitrile-water and diode array UV detection [35]. In this method, the detection limits were 0.05 mg/L for cadaverine, histamine, and spermidine, 0.1 mg/L for tyramine, and 0.25 mg/L for spermine. Huang et al. [36] used fluorescence 2,6-dimethyl-4-quinoline carboxylic acid N-hydroxysuccinimide ester for the pre-column derivatisation of BAs. The derivatives were extracted from the medium using ultrasound-assisted liquid–liquid microextraction and were analyzed by standard RP-HPLC using methanol: water (60:40, v: v) as mobile phase and fluorescence detection at 326/412 nm. The limits of detection were in the range of 0.02–5 ng/mL.

1.1 Method of Biogenic Amines Determination in Wine by High-Performance Liquid Chromatography with Photodiode Array Detection (Adapted from Resolution of the OIV-OENO 457/2014 [37])

This method is applied to the analysis of BAs in wines according to the Resolution of the OIV-OENO 457/2014 [37]. The amines analysed by this method are histamine (0.500–20 mg/L), methylamine (0.250–20 mg/L), ethylamine (0.450–20 mg/L), tyramine (0.235–20 mg/L), putrescine (0.098–20 mg/L), cadaverine (0.480–20 mg/L), phenethylamine (0.096–20 mg/L), and isoamylamine (0.020–20 mg/L).

In this method, the diethyl 2-(ethoxymethylene)malonate (DEEMM) is used as a derivatisation agent and the BAs are detected by diode array detection [38]. Quantitation is performed by the internal standard method using 2,4,6-Trimethylphenethylamine hydrochloride (2,4,6 TPA) as an internal standard.

1.1.1 Reagents, Materials, and Equipment

1. Biogenic amines (histamine, methylamine, ethylamine, tyramine, putrescine, cadaverine, phenethylamine, isoamylamine).
2. Boric acid.
3. Sodium hydroxide.
4. Sodium azide.
5. 2,4,6-Trimethylphenethylaminehydrochloride.
6. DEEMM (Diethyl 2-(ethoxymethylene)malonate).
7. Glacial acetic acid.
8. Methanol HPLC grade.
9. Acetonitrile HPLC grade.
10. Hydrochloric acid.
11. Ultrapure water.
12. Beakers 25 mL, 250 mL, and 2000 mL.
13. Volumetric flasks 10 mL, 25 mL, 50 mL, 100 mL, 250 mL, 1000 mL, and 2000 mL.
14. Graduated cylinders 100 mL, 500 mL, 1000 mL, and 2000 mL.
15. Automatic pipettes 200 μL, 1 mL, 5 mL, and 10 mL.
16. Tips for 200 μL, 1 mL, 5 mL, and 10 mL automatic pipette.
17. Pasteur pipette.
18. 2-litre cap bottles.
19. Pyrex 10 mL hydrolysis tubes with a screw cap.
20. 2 mL screw cap vials adapted to the auto-sampler.
21. Scales for weighing from 0 to 205 g.
22. Magnetic stirrer.
23. Octadecyl-type chromatographic column (e.g., HP® C18 – HL, 250 mm × 4.6 mm, 5 μm).
24. Ultrasonic bath.

25. High-performance liquid chromatography (HPLC).
26. DAD (diode array detector).

HCL Solution 0.1 M

1. Add around 500 mL of ultrapure water into a 1 L volumetric flask.
2. Add 100 mL of 1 M HCl into the volumetric flask.
3. Adjust the volume to the mark with ultrapure water and mix thoroughly.
4. Storage at room temperature.

Internal Standard Solution (2 g/L Solution)

1. 20 mg of 2,4,6-Trimethylphenethylamine hydrochloride.
2. Dissolve in 10 mL of 0.1 M HCl.
3. Storage at room temperature.

Borate Buffer 1 M (100 mL)

1. Weight 6.183 g of boric acid in a beaker.
2. Dissolve by the addition of 80 mL of ultrapure water.
3. Adjust the pH to 9 with a 4 N NaOH solution.
4. Transfer to a 100 mL volumetric flask and adjust the volume to the mark.
5. For a good dissolution of the boric acid crystals they should be dissolved at a low pH ; NaOH should be added in small quantities (by 10 drops from a Pasteur pipette) over a period of 3 h.
6. Storage at room temperature.

HPLC Mobile Phases

Mobile phase A: 25 mM acetate buffer +0.02% of sodium azide pH 5.8:

1. Add 2.86 mL of glacial acetic acid in a beaker containing 1 L of ultrapure water.
2. Add 0.4 g of sodium azide and stir with a magnetic stirrer.
3. Adjust the pH to 5.80 with 4 M NaOH solution using a Pasteur pipette.
4. Transfer to a 2 L volumetric flask and adjust the volume to the mark with ultrapure water.

Mobile phase B: Acetonitrile/methanol (80/20):

1. Add 400 mL methanol into a 2 L bottle and add in the same bottle 1600 mL of acetonitrile.
2. Storage at room temperature.

Biogenic Amines Standard

Solutions A:

Stock solution A at 500 mg/L

1. Weight 50 mg of histamine, methylamine, ethylamine, tyramine, and putrescine.
2. Dissolve them with 0.1 M HCl and transfer to the same 100 mL volumetric flask.

Surrogate solution A at 50 mg/L

1. Measure 25 mL of solution A at 500 mg/L and transfer it into a 250 mL volumetric flask.
2. Adjust the volume to the mark with 0.1 M HCl.

Surrogate solution A at 40 mg/L

1. Measure 50 mL of 0.1 M HCl to a 250 mL volumetric flask.
2. Adjust the volume to the mark with the surrogate solution A at 50 mg/L.

Solutions B.

Stock solution B at 500 mg/L

1. Weight 50 mg of cadaverine, phenethylamine, and isoamylamine.
2. Dissolve them with 0.1 M HCl and transfer to the same 100 mL volumetric flask.

Surrogate solution B at 50 mg/L

1. Measure 25 mL of solution B to 500 mg/L and transfer it into a 250 mL volumetric flask.
2. Adjust the volume to the mark with 0.1 M HCl.

Surrogate solution B at 10 mg/L

1. Measure 50 mL of surrogate solution B at 50 mg/L and transfer it into a 250 mL volumetric flask.
2. Adjust the volume to the mark with 0.1 M HCl.

Combination of Solutions A and B—Standard Curve

1. Measure 50 mL of solution A at 40 mg/L into a 100 mL volumetric flask.

Table 1
Preparation of the different standards with varying concentrations for obtaining the BAs calibration curves

Initial solution concentrations	Initial sample solution (mL)	Adjust to 100 mL with 0.1 M	Final solution concentrations
20(A)/5 (B) mg/L	50	50	10(A)/2.5 (B) mg/L
10(A)/2.5 (B) mg/L	50	50	5(A)/1.25 (B) mg/L
5(A)/1.25 (B) mg/L	20	80	1(A)/0.25 (B) mg/L

2. Adjust the volume to the mark with solution B at 10 mg/L (the final solution will have the following concentrations 20(A)/5 (B) mg/L). Table 1 shows the procedure for obtaining the other calibration points.
3. Four concentrations of BAs present in solution A (20, 10, 5, and 1 mg/L).
4. Four concentrations of BAs present in solution B (5, 2.5, 1.25, and 0.25 mg/L).
5. Storage at −20 °C.

1.1.2 Sample Preparation

1. In a 10 mL hydrolysis tube with a screw cap add:
   - 1.75 mL of borate buffer.
   - 750 μL of methanol.
   - 1 mL of the sample to be derivatised.
   - 40 μL of the internal standard (2,4,6 TPA to 2 g/L).
   - 30 μL of DEEMM.
2. Close the tube and shake it manually.
3. Turn on the dry bath to 70 °C.
4. Place the tube in the ultrasonic bath for 30 min (2 times 15 min, stirring every 5 min).
5. Use always a plastic rack as the derivatisation is unsatisfactory if the metal rack is used.
6. Heat the reaction mixture (70 °C for 1 h) in the dry bath to degrade the surplus DEEMM.
7. Turn off the dry bath.
8. After the reaction mixture has returned to room temperature, fill the 2 mL vial.
9. Shake the tubes manually before sampling.

Note:

Table 2
Elution gradient

Time (min)	%A	%B
0	90	10
5	90	10
10	83	17
35	60	40
43	28	72
48	18	82
52	0	100
57	0	100

1. The derivatisation reaction with DEEMM is recommended on receipt of the sample because the histamine concentration in wine may reduce over time.
2. Because of the toxicity of certain reagents, the manipulation during the preparation must be done in a fume hood.
3. Heat it to 50 °C while stirring if the buffer contains borate crystals.

1.1.3 Operating Conditions (as an Example)

Mobile phase:

– A: 25 mM acetate buffer +0.02% of sodium azide pH 5.80.
– B: Acetonitrile/methanol (80/20). The elution gradient is presented in Table 2.

1. Column temperature 15 °C.
2. Detection wavelength 280 nm.
3. Flow rate 0.9 mL/min.
4. Injection volume 50 μL.
5. Analysis time 57 min.

1.1.4 Biogenic Amines Identification

Each BAs should be analyzed individually to determine its retention time (Tr) in the system used and the identification of the BAs in the wine sample is performed by comparison of their retention time to those of the standards injected in the same conditions (Table 3).

1.1.5 Calculation

1. The concentration of BAs is calculated based on the slope value of the standard curve of the corresponding BAs.

Table 3
Typical retention time of the BAs

Biogenic Amines	Average retention time (min)
Histamine	25.46
Methylamine	33.11
Ethylamine	39.00
Tyramine	41.50
Putrescine	46.00
Cadaverine	48.00
Phenethylamine	48.75
Isoamylamine	50.25
Internal standard (2,4,6-TPA)	54.75

2. For each series of analyses, a standard curve should be calculated by derivatisation and injection of each standard solution.
3. The final results are expressed in mg/L to one figure after the decimal point.

1.2 Dispersive Solid Phase Extraction (dSPE) Clean-up/ Concentration Method for Biogenic Amine Determination in Wines Using Benzoyl Chloride Derivatisation—Adapted from Milheiro et al. [39]

This method developed by Milheiro et al. [39] can determine 12 BAs, namely, ethylamine, propylamine, butylamine, putrescine, cadaverine, tryptamine, β-phenylethylamine, amylamine, spermidine, hexylamine, spermine, and histamine. The dispersive solid phase extraction (dSPE) method for BAs analysis in wines can be used with the various derivatisation reagents as it efficiently recovers BAs and reduces/eliminates interfering compounds present in the sample for derivatisation.

1.2.1 Reagents, Materials, and Equipment

1. Methanol.
2. Dowex® 50 W X8.
3. Diethyl ether.
4. Hydrochloric acid.
5. BAs: ethylamine, propylamine, butylamine, putrescine, cadaverine, tryptamine, β-phenylethylamine, amylamine, spermidine, hexylamine, spermine, histamine, and diethylamine. Ethylamine, putrescine, cadaverine, and histamine were obtained as their hydrochloride salts.
6. Acetonitrile.

7. Sodium hydroxide.
8. Di-sodium tetraborate decahydrate.
9. Benzoyl chloride.
10. Ultrapure water.
11. Erlenmeyer flasks.
12. Beakers.
13. Volumetric flasks.
14. Bottles of 1 L and 2 L.
15. Screw cap vials of 2 mL.
16. Magnetic stirrer.
17. Centrifuge.
18. Shaker.
19. Vacuum Centrifuge Evaporator.
20. HPLC equipment: Ultimate 3000 Dionex HPLC equipped with a PDA-100 photodiode array detector and an Ultimate 3000 Dionex pump.

1.2.2 Sample Preparation

Dispersive Solid Phase Extraction (dSPE) Procedure to Clean Up and Concentrate Biogenic Amines from Wine Samples

1. Cation exchange polymeric Dowex® 50 W X8 adsorbent, previously cleaned (25 g of Dowex® 50 W X8 washed with 50 mL of NaOH 3 M, filtrated, and washed again with 50 mL of HCl 3 M twice).
2. For dSPE 0.25 g of cleaned Dowex® 50 W X8 was added to a 10 mL tube and 5 mL of wine sample was added and the tube was shaken for 1 min to adsorb the BAs from the wine.
3. The suspension was centrifuged at 4000 rpm for 2 min, and the supernatant was removed.
4. Add 5 mL of 0.1 M HCl to clean the residual wine from the adsorbent. The mixture is shaken for 1 min, centrifuged for 2 min, and the supernatant discarded.
5. Add 3 mL of 1 M NaOH, followed by 100 μL of diethylamine solution at 500 mg/L (internal standard), shake for 1 min, centrifuge for 2 min, and the supernatant was collected. This procedure is used to elute the bound BAs.

1.2.3 Derivatisation Step (According to Özdestan and Üren [26] with Some Modifications)

1. In each test tube containing the resulting solution from the dSPE, add 2 mL of NaOH 2 M, 1.5 mL of acetonitrile, and 100 μL of benzoyl chloride, and shake on a vortex for 1 min.
2. Incubated the mixture at 30 °C for 30 min.
3. Add 0.5 g of NaCl.

4. Add 2 mL of diethyl ether and shake horizontally for 5 min, centrifuge at 4000 rpm for 3 min (this extraction is repeated three times).
5. Recover the organic phases and remove the solvent in vacuum using a vacuum centrifuge evaporator at 35 °C, and re-suspend in 1 mL of methanol/water (50:50) and analyze by RP-HPLC-DAD.

1.2.4 HPLC Analysis and Quantitation

1. Separation is carried out with a reversed phase column (C18, 250 mm × 4.6 mm, 5 μm particle size).
2. Flow rate of 1 mL/min at 20 °C.
3. Injection volume was 100 μL.
4. Detection from 200 to 600 nm.

Eluents

1. Disodium tetraborate 5 mM at pH 8 (A).
2. Methanol (B).

Elution Gradient

Start with 40% B from zero to 4 min followed by a linear gradient up to 70% B until 57 min and raised to 100% B from 67 to 72 min and down to 40% B maintained until 77.5 min.

Quantitation

By the internal standard method (diethylamine as internal standard) using calibration curves of standards of BAs: ethylamine, propylamine, cadaverine, putrescine, histamine, phenylethylamine, hexylamine, amylamine, spermidine, spermine, tryptamine, and butylamine.

1.3 Analysis of Biogenic Amines in Musts and Wines Using HPLC: Orto-*Phthalaldehyde (OPA) Derivatisation (Adapted from Resolution OIV-Oeno 346/2009 [37])*

This method (Resolution OIV-Oeno 346/2009) [37] can analyse BAs in musts and wines such as ethanolamine (up to 20 mg/L), histamine (up to 15 mg/L), methylamine (up to 10 mg/L), serotonin (up to 20 mg/L), ethylamine (up to 20 mg/L), tyramine (up to 20 mg/L), isopropylamine (up to 20 mg/L), propylamine (normally absent), isobutylamine (up to 15 mg/L), butylamine (up to 10 mg/L), tryptamine (up to 20 mg/L), phenylethylamine (up to 20 mg/L), putrescine (up to 40 mg/L), 2-methylbutylamine (up to 20 mg/L), 3-methylbutylamine (up to 20 mg/L), cadaverine (up to 20 mg/L), and hexylamine (up to 10 mg/L).

In this method, the BAs are directly determined by HPLC using a C18 column after *orto*-phthalaldehyde (OPA) derivatisation and fluorimetric detection. The derivatisation procedure can be adapted to be automatically performed in autosamplers with that function, and this increases the precision of the analysis due to the perfect timing between derivatisation and injection, as the OPA derivatives are unstable.

1.3.1 Reagents, Materials, and Equipment

1. Ultrapure water.
2. Disodium hydrogenophosphate dihydrate.
3. Acetonitrile.
4. *orto*-phthalaldehyde (OPA).
5. 2-Mercaptoethanol.
6. Disodium tetraborate decahydrate.
7. Methanol.
8. Hydrochloric acid.
9. Sodium hydroxide pellets.
10. BAs—ethanolamine, histamine dichlorhydrate, ethylamine chlorhydrate, serotonin, methylamine chlorhydrate, tyramine chlorhydrate, isopropylamine, butylamine, tryptamine chlorhydrate, phenylethylamine, putrescine dichlorhydrate, methylbutylamine, methylbutylamine, cadaverine dichlorhydrate, 1–6-diaminohexane, and hexylamine.
11. Nitrogen.
12. Helium.
13. Erlenmeyer flasks of 25 mL, 250 mL.
14. Beakers of 25 mL, 50 mL, 100 mL, 150 mL.
15. Volumetric flasks of 100 mL, 250 mL, 2 L.
16. Bottles of 1 L and 2 L.
17. Screw cap containers 2 mL.
18. Syringe 50 µL.
19. Needle.
20. Filter holder and 0.45 µm, 0.8 µm, 1.2 µm, and 5 µm cellulose membrane.
21. Cellulose pre-filter.
22. pH meter.
23. Magnetic stirrer.
24. HPLC equipment.
25. 5 µm C_{18} column, 250 mm × 4 mm.
26. Fluorimetric detector.

Phosphate Solution A

1. Weight 11.12 g of di-basic sodium phosphate in a 50 mL beaker and dissolve.
2. Transfer to a 2 L volumetric flask and adjust the volume to the mark with ultrapure water.
3. Homogenise using a magnetic stirrer and filter through a 0.45 µm membrane.

Solution B

Acetonitrile is used directly.

OPA Solution

1. Weight 20 mg of OPA in a 50 mL flask.
2. Make up to 50 mL with methanol.
3. Homogenise.

Note: this solution should be prepared daily.

Borate Buffer

1. Weight 3.81 g of disodium tetraborate decahydrate into a 25 mL beaker and dissolve.
2. Transfer to a 100 mL volumetric flask and adjust the volume to the mark with ultrapure water.
3. Homogenise with a magnetic stirrer.
4. Transfer to a 150 mL beaker and adjust to pH 10.5 with 10 M sodium hydroxide.

Note: this solution should be prepared weekly.

Hydrochloric Acid Solution 0.1 M

1. Add a small volume of ultrapure water into a 2 L volumetric flask.
2. Add 20 mL of hydrochloric acid and adjust the volume to the mark with ultrapure water.

Calibration Solution in 0.1 M Hydrochloric Acid

Indicative final concentration in the calibration mix (mg/L): Ethanolamine [5], Histamine [5], Methylamine [1], Serotonin [20], Ethylamine [2], Tyramine [13], Isopropylamine [4], Propylamine (2.5), Isobutylamine [5], Butylamine [5], Tryptamine [16], Phenylethylamine [2], Putrescine [7], 2- Methylbutylamine [5], 3- Methylbutylamine [10], Cadaverine [8], 1.6 Diaminohexane [14], and Hexylamine [5].

Internal Standard Solution

1. Weight 119 mg of 1,6 diaminohexane in a 25 mL Erlenmeyer flask and dissolve with 0.1 M HCl solution.
2. Transfer to a 100 mL volumetric flask and adjust the volume to the mark with 0.1 M HCl solution.

1.3.2 Sample Preparation

Filtering

1. Filter approximately 120 mL of the wine sample through a membrane of 0.45 μm. For non-clarified wine filter using sequentially 5, 1.2, 0.8, and 0.45 μm filters.

Sample Preparation

1. Add 100 mL of the wine sample into a 100 mL volumetric flask.

Table 4
Elution gradient

Time (min)	%A	%B
0	80	20
15	70	30
23	60	40
42	50	50
55	35	65
60	35	65
70	80	20
95	80	20

2. Add 0.5 mL of 1–6-diaminohexane at 119 mg/100 mL.
3. Remove 5 mL of the wine sample and transfer it into a 25 mL Erlenmeyer flask.
4. Add 5 mL of methanol.
5. Stir to homogenize.

1.3.3 Derivatisation

1. In a borosilicic glass tube.
2. Add 2 mL of OPA solution.
3. Add 2 mL of borate buffer.
4. Add 0.6 mL of 2-mercaptoethanol.
5. Close and mix.
6. Open and add 0.4 mL of wine sample previously prepared.
7. Close and mix.
8. Inject immediately, as the derivative is not stable. Rinse the recipient immediately after injection due to odor.

1.3.4 Mobile Phase

A: Phosphate buffer.

B: Acetonitrile. The typical elution gradient used is described in Table 4.

Flow rate: 1 mL/min.
Column temperature: 35 °C.
Detector: Exc = 356 nm, Em = 445 nm.

1.3.5 Internal Calibration

A calibration solution is injected for each series.

Calibration is performed by the internal standard method using response factors.

Calculation of response factors (RF):

$RF = CIS \times Area\ i / Area\ IS \times Ci.$

Ci = concentration of the component in the calibration solution.

CIS = concentration of the internal standard in the calibration solution (1–6-diaminohexane).

$Area\ i$ = area of the product peak present in the wine sample.

$Area\ IS$ = area of the internal standard peak in the wine sample.

1.3.6 Calculation of Concentrations

$Ci = (XF \times Area\ i)/ (Area\ IS \times RF).$

$Area\ i$ = area of the product peak present in the wine sample.

$Area\ IS$ = area of the internal standard peak present in the wine sample.

XF = quantity of internal standard added to wine samples for analysis.

$XF = 119 \times 0.5/100 = 5.95.$

Results are expressed in mg/L with one significant digit after the decimal point.

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References

1. Arena ME, Manca de Nadra MC (2001) Biogenic amine production by lactobacillus. J Appl Microbiol 90:158–162
2. Lonvaud-Funel A (2001) Biogenic amines in wine: role of lactic acid bacteria. FEMS Microbiol Lett 199:9–13
3. Kelly MT, Blaise A, Larroque M (2010) Rapid automated high performance liquid chromatography method for simultaneous determination of amino acids and biogenic amines in wine, fruit and honey. J Chromatogr A 1217: 7385–7392
4. Önal A (2007) A review: current analytical methods for the determination of biogenic amines in foods. Food Chem 103:1475–1486
5. Smit AY, du Toit WJ, du Toit M (2008) Biogenic amines in wine: understanding the headache. S Afr J Enol 29:109–127
6. Beneduce L, Romano A, Capozzi V, Lucas P, Barmavon L, Bach B, Vuchot P, Grieco F, Spano G (2010) Biogenic amines in wine. Ann Microbiol 60:573–578
7. Anli RE, Bayram M (2008) Biogenic amines in wines. Food Rev Int 25:86–102
8. Lafon-Lafourcade S (1975) L'histamine des vins. Conn Vigne et Vin 2:103–115
9. Mayer K (1974) Teneurs des vins suisses en histamine. Feuillets verts de l'OIV 466
10. Filipe-Ribeiro L, Milheiro J, Ferreira LC, Correia E, Cosme F, Nunes FM (2019) Biogenic amines and polyamines in wines: does *Dekkera/Brettanomyces* red wine spoilage increases the risk of intake by consumers? LWT 115:108488
11. Plumas B, Sautier C (1972) Methode de dosage simultane de l'histidine et de l'histamine dans les liquides biologiques. Application aux vins Ann Fals Exp Chim 65(703):322
12. Schneyder J (1973) Histamine et substances similaires dans les vins. Bull OIV 46(511):821
13. Ramos RM, Valente IM, Rodrigues JA (2014) Analysis of biogenic amines in wines by salting-out assisted liquid-liquid extraction and high-performance liquid chromatography with fluorimetric detection. Talanta 124:146–151
14. Martuscelli M, Arfelli G, Manetta AC, Suzzi G (2013) Biogenic amines content as a measure

of the quality of wines of Abruzzo (Italy). Food Chem 140:590–597

15. Bach B, Le Quere S, Vuchot P, Grinbaum M, Barnavon L (2012) Validation of a method for the analysis of biogenic amines: histamine instability during wine sample storage. Anal Chim Acta 732:114–119
16. Comuzzo P, Rauhut D, Werner M, Lagazio C, Zironi R (2013) A survey on wine from organic viticulture from different European countries. Food Control 34:274–282
17. Płotka-Wasylka J, Namieśnik J, Papageorgiou M, Kłodzińska E, Morrison C, Lambropoulou D (2018) Literature update of analytical methods for biogenic amines determination in food and beverages. Trac-Trend Anal Chem 98:128–142
18. Loukou Z, Zotou A (2003) Determination of biogenic amines as dansyl derivatives in alcoholic beverages by high-performance liquid chromatography with fluorometric detection and characterization of the dansylated amines by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr A 996:103–113
19. Romero RD, Gazquez M, Bagur G, Sáanchez-Viñas M (2000) Optimization of chromatographic parameters for the determination of biogenic amines in wines by reversed-phase high-performance liquid chromatography. J Chromatogr A 871:75–83
20. Hernández-Cassou S, Saurina J (2011) Derivatization strategies for the determination of biogenic amines in wines by chromatographic and electrophoretic techniques. J Chromatogr B 879:1270–1281
21. Anli RE, Vural N, Yılmaz S, Vural YH (2004) The determination of biogenic amines in Turkish red wines. J Food Comp Anal 17:53–62
22. Konakovsky V, Focke M, Hoffmann-Sommergruber K, Schmid R, Scheiner O, Moser P, Jarisch R, Hemmer W (2011) Levels of histamine and other biogenic amines in high-quality red wines. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28:408–416
23. Manetta AC, Di Giuseppe L, Tofalo R, Martuscelli M, Schirone M, Giammarco M, Suzzi G (2016) Evaluation of biogenic amines in wine: determination by an improved HPLC-PDA method. Food Control 62:351–356
24. Soufleros EH, Bouloumpasi E, Zotou A, Loukou Z (2007) Determination of biogenic amines in Greek wines by HPLC and ultraviolet detection after dansylation and examination of factors affecting their presence and concentration. Food Chem 101:704–716
25. Zotou A, Loukou Z, Soufleros E, Stratis I (2003) Determination of biogenic amines in wines and beers by high performance liquid chromatography with pre-column dansylation and ultraviolet detection. Chromatographia 57:429–439
26. Özdestan Ö, Üren A (2009) A method for benzoyl chloride derivatization of biogenic amines for high performance liquid chromatography. Talanta 78:1321–1326
27. Landete JM, Ferrer S, Polo L, Pardo I (2005) Biogenic amines in wines from three Spanish regions. J Agric Food Chem 53:1119–1124
28. Busto O, Miracle M, Guasch J, Borrull F (1997) Determination of biogenic amines in wines by high-performance liquid chromatography with on-column fluorescence derivatization. J Chromatogr A 757:311–318
29. Hlabangana L, Hernández-Cassou S, Hlabangana JS (2006) Determination of biogenic amines in wines by ion-pair liquid chromatography and post-column derivatization with 1,2-naphthoquinone-4-sulphonate. J Chromatogr A 1130:130–136
30. Hernández-Orte P, Lapeña AC, Peña-Gallego A, Astrain J, Baron C, Pardo I, Polo L, Ferrer S, Cacho J, Ferreira V (2008) Biogenic amine determination in wine fermented in oak barrels: factors affecting formation. Food Res Int 41:697–706
31. Hernández-Orte P, Peña-Gallego A, Ibarz MJ, Cacho J, Ferreira V (2006) Determination of the biogenic amines in musts and wines before and after malolactic fermentation using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate as the derivatizing agent. J Chromatogr A 1129:160–164
32. Alberto MR, Arena ME, Manca de Nadra MC (2002) A comparative survey of two analytical methods for identification and quantification of biogenic amines. Food Control 13:125–129
33. Karovicová J, Kohajdová Z (2005) Biogenic Amines in food Chemical Paper 59:70–79
34. Hornero-Méndez D, Garrido-Fernández A (1997) Rapid high-performance liquid chromatography analysis of biogenic amines in fermented vegetable brines. J Food Prot 60:414–419
35. Yongmei L, Xin L, Xiaohong C, Mei J, Chao L, Mingsheng D (2007) A survey of biogenic amines in Chinese rice wines. Food Chem 100:1424–1428
36. Huang KJ, Wei CY, Liu W-L, Xie W-Z, Zhang J-F, Wang W (2009) Ultrasound-assisted dispersive liquid–liquid microextraction combined with high-performance liquid chromatography-fluorescence detection for

sensitive determination of biogenic amines in rice wine samples. J Chromatogr A 1216: 6636–6641

37. OIV (2020) Compendium of international methods of wine and must analysis. International Organisation of Vine and Wine, Paris
38. Gómez-Alonzo S, Hermosín-Gutierrez I, García-Romero E (2007) Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as Aminoenone derivatives in wine and beer samples. J Agric Food Chem 55: 608–613
39. Milheiro J, Ferreira LC, Filipe-Ribeiro L, Cosme F, Nunes FM (2019) Dispersive solid phase extraction for sample clean-up and pre-concentration of biogenic amines in wines. Food Chem 274:110–117

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