Chapter 7

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

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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

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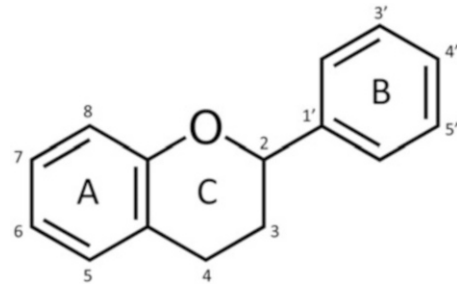
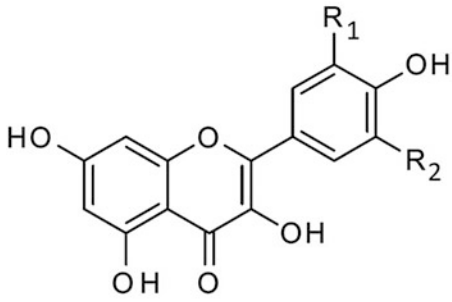


Fig. 1 Core ring system of flavonoidsFlavonoids. (AROUND HERE)



	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**).

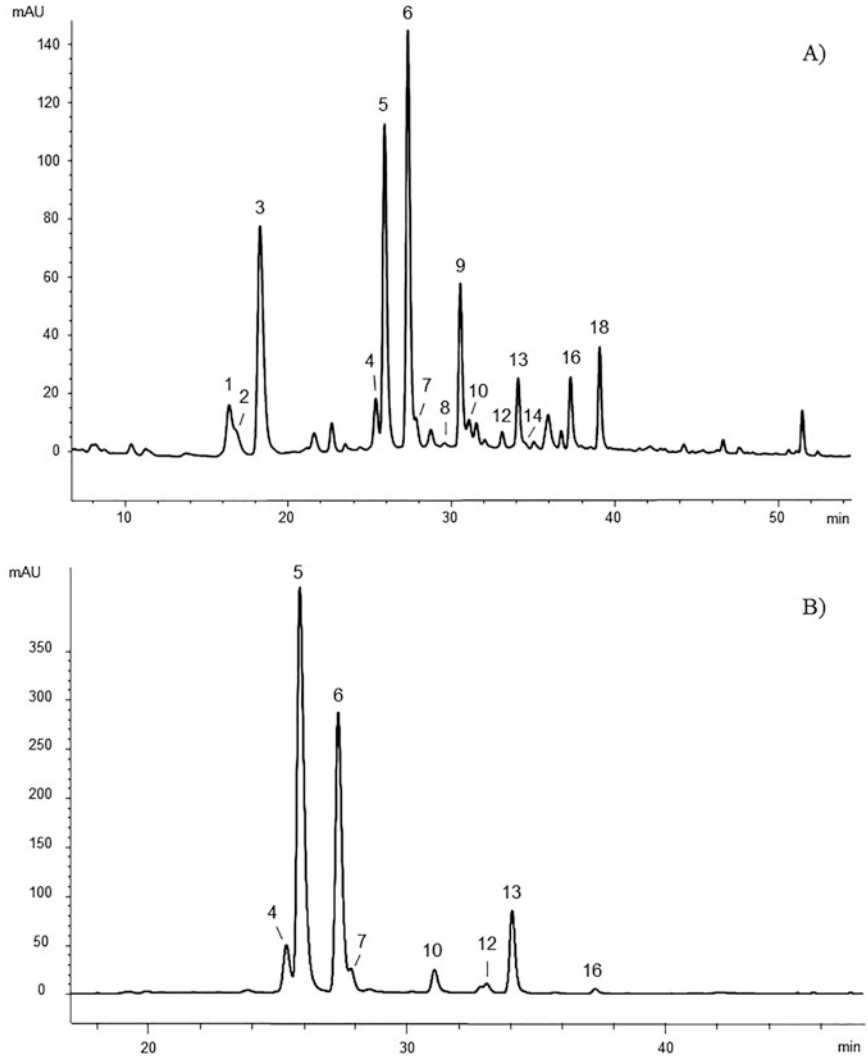


Fig. 3 HPLC-DAD chromatogram (detection at 360 nm) corresponding to flavonols identified in red (**a**) and white (**b**) grape skins. For peak assignment, see Table 1

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.

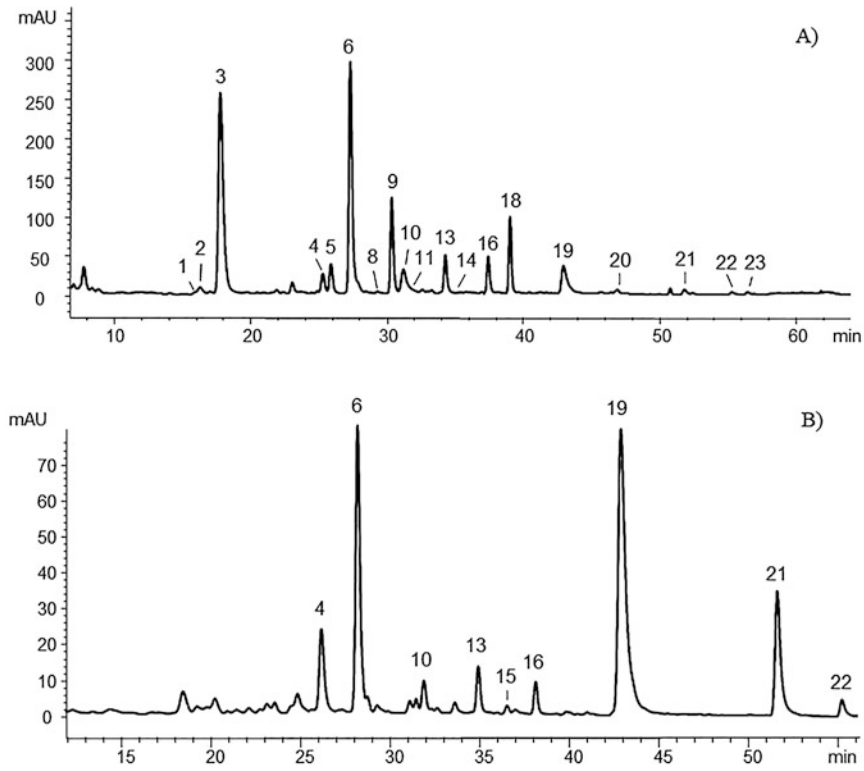


Fig. 4 HPLC-DAD chromatogram (detection at 360 nm) corresponding to flavonols identified in red (**a**) and white (**b**) wines. For peak assignment, see Table 1

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

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