Chapter 8

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

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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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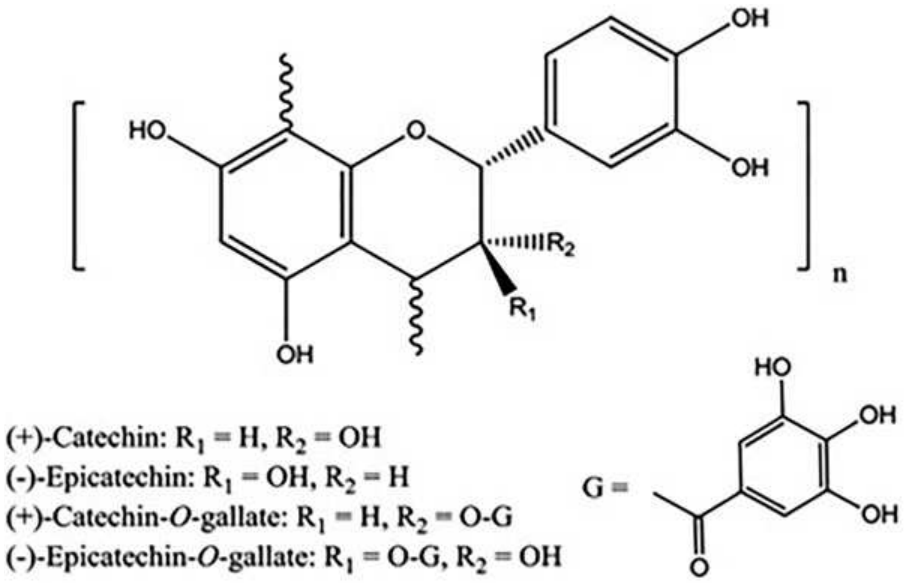

Fig. 1 Subunits structures of flavan-3-ols from grape seed. (Source: Ma et al. (2016) [12])

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.

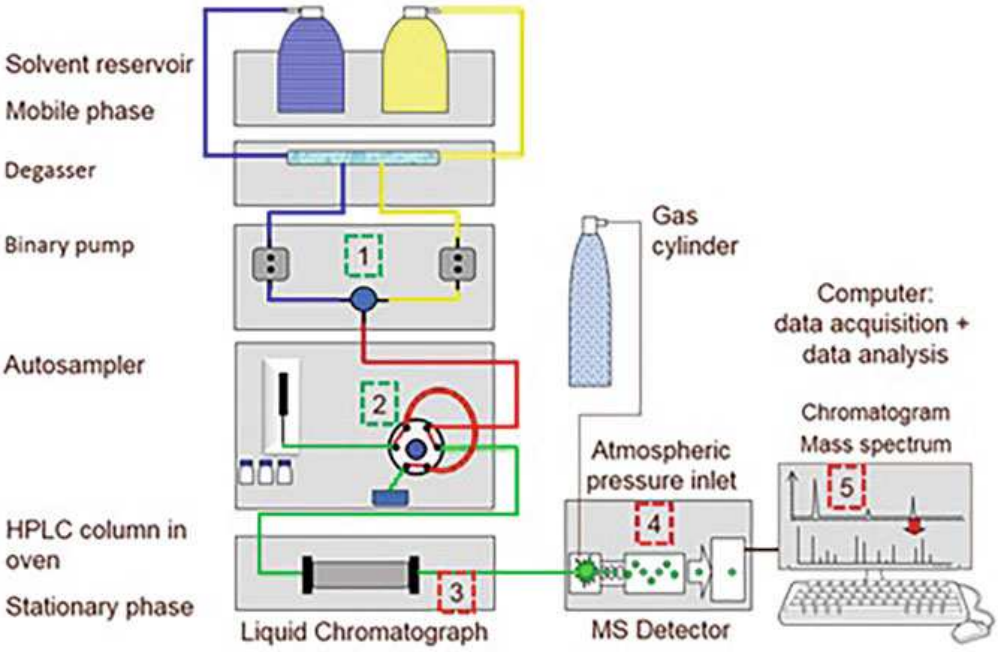

Fig. 2 A simplified diagram of a liquid chromatograph hyphenated to a mass spectrometer (LC-MS) showing: (1) binary pump for mobile phase, (2) autosampler 6-port valve and injector loop, (3) column heater with column, (4) mass spectrometer detector, (5) PC. Credit: Anthias Consulting

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]

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