SPECIAL GUEST EDITOR SECTION

Anthocyanin Profiling Using UV-Vis Spectroscopy and Liquid Chromatography Mass Spectrometry

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Background: **As a powerful antioxidant and natural colorant, anthocyanins are being used increasingly as a component of food supplements and nutraceutical products. Hence, its characterization is a prerequisite for further exploration of its nutraceutical potential. UV-Vis and MS are the two important techniques, which have been largely employed for the qualitative and quantitative determination of anthocyanins. However, a comprehensive review of the applications of these techniques in literature is scarce.** ***Objective:*** **This paper aims to review the utilization of UV-Vis spectral data as well as mass spectral data for characterization and putative identification of anthocyanins with approaches of quantification.** ***Methods:*** **The techniques described in literature have been thoroughly reviewed and comparatively evaluated. The complementary approaches of UV-Vis and MS spectra have been discussed for identification and quantification of these compounds.** ***Results:*** **Valuable information about the chemical composition and structure of anthocyanins can be predicted from the UV-Vis spectral data, such as number and type of glycosylation as well as absence or presence of acylation, to name a few. It is also pointed out that for their structural confirmation, selectivity of mass detectors with unit and high-resolution analysis could be effective.** ***Conclusions:*** **The combination of LC-MS with UV-Vis spectroscopy provides complementary information on structural details of anthocyanins. In case the analytical reference standards are available, a triple quadrupole mass spectrometer provides selectivity and quantitative sensitivity in analysis. On the other hand, high-resolution MS analysis provides valuable information for tentative identification during nontarget screening of compounds when the reference standard is not available.** ***Highlights:*** **This paper reviews the applications of UV-Vis spectroscopy and LC-MS for qualitative and quantitative analysis of anthocyanins.**

Anthocyanins are the most widely consumed flavonoids in daily diet (1). Originating from the Greek words "anthos," meaning flower, and "kiano," meaning blue, they represent a group of phenolic (flavonoids) compounds that impart the characteristic red, purple, and blue color to plants. About 700 anthocyanin compounds have been reported in the existing literature as having potential health benefits as well as being sources of natural colorants (1). The anthocyanin-rich plants include red grape, red apple, red currant, red onion, red radish, strawberry, grapefruit, peach, pear, and plum, to name a few.

Numerous in vitro and in vivo studies have demonstrated anthocyanins to be potential antioxidants with multiple health benefit properties, such as anticancerous, antidiabetic, anti-inflammatory, antimicrobial, and antiobesity.

According to the U.S. dietary consumption data, the permissible daily intake of anthocyanins is approximately 180–225 mg (2, 3). At the cellular level, they are generally accumulated in the vacuolar solution, except in certain species, in which they appear as anthocyanoplasts. Their content varies in the range of 0.1–1.0% across different plant organs.

Anthocyanins are known to have antioxidant activity, and that is why they possess anticancer, antidiabetic, anti-inflammatory, antimicrobial, and antiobesity properties. These compounds have also been associated with prevention of cardiovascular diseases and improvement of visual and neurological health benefits (4–7).

Anthocyanins can be characterized by different chromatographic and spectroscopic techniques. Among these, UV-Vis spectral data have provided substantial information about the structural features and identity of such molecules. On the other hand, MS has been another powerful tool that revealed in depth structural details, enabling unique identification and characterization of these compounds.

The present article aims to discuss the existing knowledge on the applications of UV-Vis and MS spectra for the identification and characterization of anthocyanins. It also attempts to highlight the importance of these techniques for quantification of anthocyanins.

Anthocyanin: Chemistry

"Anthocyanidins" (aglycone), when bonded to a sugar moiety, produce anthocyanins. An aglycone is composed of three ring structures, in which an aromatic ring bonds with a heterocyclic ring (containing oxygen), which is also tied with a carbon–carbon bond to the third aromatic ring (8). Anthocyanins generally contain one, two, or three monosaccharide units, which are either hexose (glucose, galactose, rhamnose, and glucuronic acid) or

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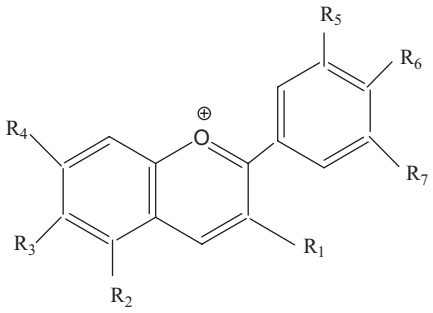
pentose (arabinose, xylose) sugars. Covalent bonding generally takes place through an O-linkage to the aglycone at the position 3-, and less commonly, at 5-, 7-, 3′-, 4′-, 5′-hydroxyl group. The commonly found sugar substituents in the anthocyanin structure include glucose, rhamnose, xylose, galactose, and arabinose. On the other hand, the predominant disaccharides comprise rutinose, sambubiose, lathyrose, and sophorose. Complex saccharide moieties have also been reported to be present in anthocyanins (Table 1).

The structural variations in anthocyanins are determined by the type, number, and position of sugar moiety; the number and position of hydroxyl and methoxy groups in the B ring; and the presence/absence of acylation (Figure 1). Chemically, the anthocyanins may differ from one another in terms of the number of hydroxyl groups, type, number of sugar moieties attached to the anthocyanidin and the position of this attachment, presence/absence of acylation, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule. These structural variations in anthocyanins are reported in Figure 1 and Table 1.

Although 31 different monomeric anthocyanidins have been reported so far (including 3-deoxyanthocyanidins, pyrano-anthocyanidins, and sphagnorubins), 90% of those compounds are based on six anthocyanidin structures (30% cyanidin, 22% delphinidin, 18% pelargonidin, and 20% combined peonidin, malvidin, and petunidin; 9). Out of these, cyanidin, delphinidin, and pelargonidin are the nonmethylated anthocyanidins. There are four classes of anthocyanins reported in nature, which include 3-monosides, 3-biosides, 3,5-diglycosides, and 3,7-diglycosides. Among these, 3-monosides are most prevalent in nature. In

Table 1. Anthocyanidins and their structural variations



Anthocyanidin with abbreviations	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Hydroxyl substitution							
Apigeninidin (Ap)	H	OH	H	OH	H	OH	H
Aurantinidin (Au)	OH	OH	OH	OH	H	OH	H
Cyanidin (Cy)	OH	OH	H	OH	OH	OH	H
Delphinidin (Dp)	OH	OH	H	OH	OH	OH	OH
6-Hydroxy cyanidin (6OHCy)	OH	OH	OH	OH	OH	OH	H
6-Hydroxy delphinidin (6OHDp)	OH	OH	OH	OH	OH	OH	OH
6-Hydroxy pelargonidin (6OHPg)	OH	OH	OH	OH	H	OH	H
Luteolin (Lt)	H	OH	H	OH	OH	OH	H
Pelargonidin (Pg)	OH	OH	H	OH	H	OH	H
Riccionidin (RiA)	OH	H	OH	OH	H	OH	H
Tricetinidin (Tr)	H	OH	H	OH	OH	OH	OH
Both methoxy and hydroxyl substitution							
Arrabidin (Ab)	H	H	OH	OH	H	OH	OMe
Capensinidin (Cp)	OH	OMe	H	OH	OMe	OH	OMe
Carajurin (Cj)	H	H	OH	OH	H	OMe	OMe
Europinidin (Eu)	OH	OMe	H	OH	OMe	OH	OH
Hirsutidin (Hs)	OH	OH	H	OMe	OMe	OH	OMe
3′-hydroxy Arrabidin (3′OHAb)	H	H	OH	OH	OH	OH	OMe
Malvidin (Mv)	OH	OH	H	OH	OMe	OH	OMe
5-Methyl Cyanidin (5MCy)	OH	OMe	H	OH	OH	OH	H
Peonidin (Pn)	OH	OH	H	OH	OMe	OH	H
Petunidin (Pt)	OH	OH	H	OH	OMe	OH	OH
Pulchelidin (Pl)	OH	OMe	H	OH	OH	OH	OH
Rosinidin (Rs)	OH	OH	H	OMe	OMe	OH	H

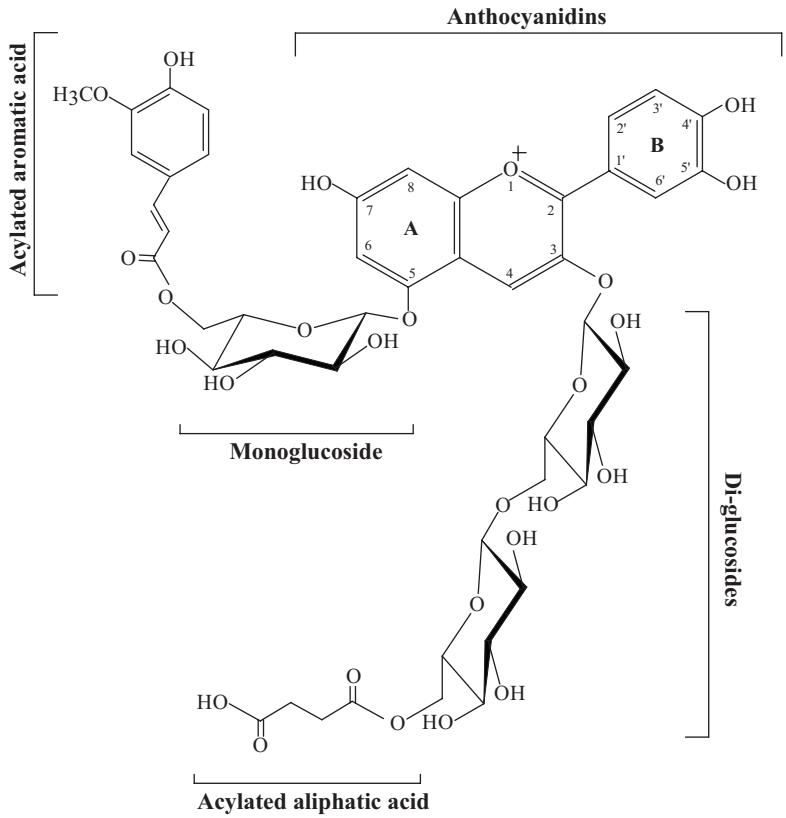


Figure 1. General structure of anthocyanins.

general, anthocyanins are present in glycosylated forms but with only a few exceptions that include 3-deoxyanthocyanidins, reported in black tea, red-skinned bananas, and sorghum in their free forms (10).

Anthocyanins mainly include two types: acylated and nonacylated. When sugars, attached with the anthocyanidin moiety, are bonded with an aliphatic/aromatic acid, it is called acylated anthocyanin; when sugar is not further bonded, it is called nonacylated. Sugar moiety, which is a constitutive part of anthocyanins, may be esterified with aliphatic and aromatic acids. The predominant aliphatic acids associated with anthocyanins include acetic, malonic, succinic, oxalic, tartaric, and malic acids, whereas the aromatic acids include coumaric, ferulic, caffeic, gallic, sinapic, cinnamic, and p-hydroxybenzoic acids (11). More than 60% of the reported anthocyanins appear in nature in their acylated forms, which have more stability than their nonacylated counterparts.

UV-Vis Spectral Analysis

Researchers have characterized anthocyanins based upon UV-Vis spectral data. The characteristic color of the molecule was first reported by Pauling in 1939 (12), who proposed the resonant structure of flavylium cation (13). Absorption spectroscopy, especially UV-Vis spectroscopy, has been extensively utilized for the identification of anthocyanins (14–17). When deciphered critically, the spectrum can provide useful information about the structural composition of anthocyanins. However, for confirmation of structure, it is important to use other complementary spectroscopic techniques. Although a mass spectrometer is more frequently used for structural elucidation of anthocyanins, the UV-Vis data are still useful (18–20) to characterize unsaturation and functional groups in various components of the anthocyanin structure.

In general, the UV-Vis spectrum of anthocyanins shows a typical absorption pattern (Figure 2). The absorption maxima (λ_{max}) in the visible region is generally recorded at around 510–520 nm, followed by a hump in the range of 400–450 nm. Furthermore, a peak is observed in the range of 310–340 nm depending upon the type of anthocyanin and its nature of substitution. The difference in the UV-Vis spectra of acylated and nonacylated anthocyanins can be observed in Figure 3.

A typical UV-Vis spectrum of an anthocyanin shows two basic clusters of absorbance, the first one at a wavelength region of 260–280 nm (UV region) and the other one at 490–550 nm (visible region). Apart from them, an additional peak is observed in the wavelength range of 310–340 nm whenever the sugar moiety is acylated. This peak is almost absent (or appears as a small hump) in nonacylated anthocyanins. Furthermore, a hump at 400–450 nm is also recorded, the size of which depends on the number of sugar moieties attached to the anthocyanidin moiety. In general, the anthocyanin structure includes a fully delocalized π-conjugated system, which provides stability to it.

In general, the nature of substitution in the B ring of an anthocyanidin molecule determines the color of the acylated and monoacylated anthocyanins (21). An increased hydroxyl substitution leads to a bathochromic shift of the absorption

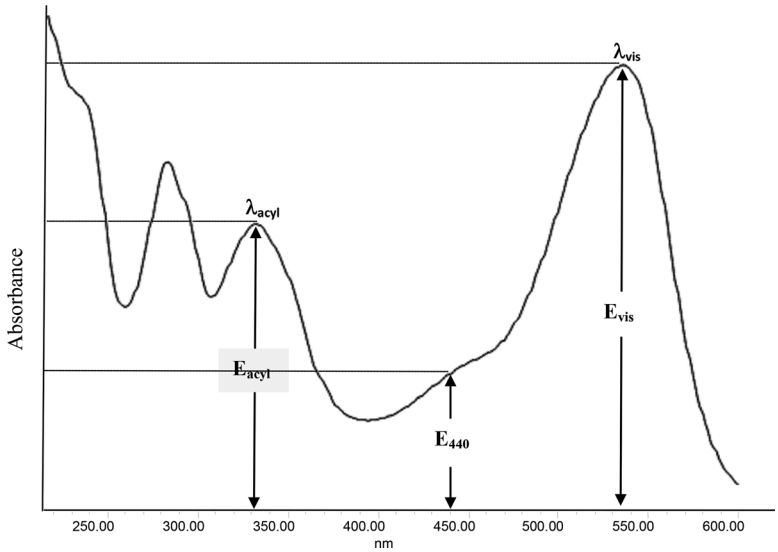


Figure 2. UV-Vis spectrum of an acylated anthocyanin. acyl, acylated.; vis, visible.

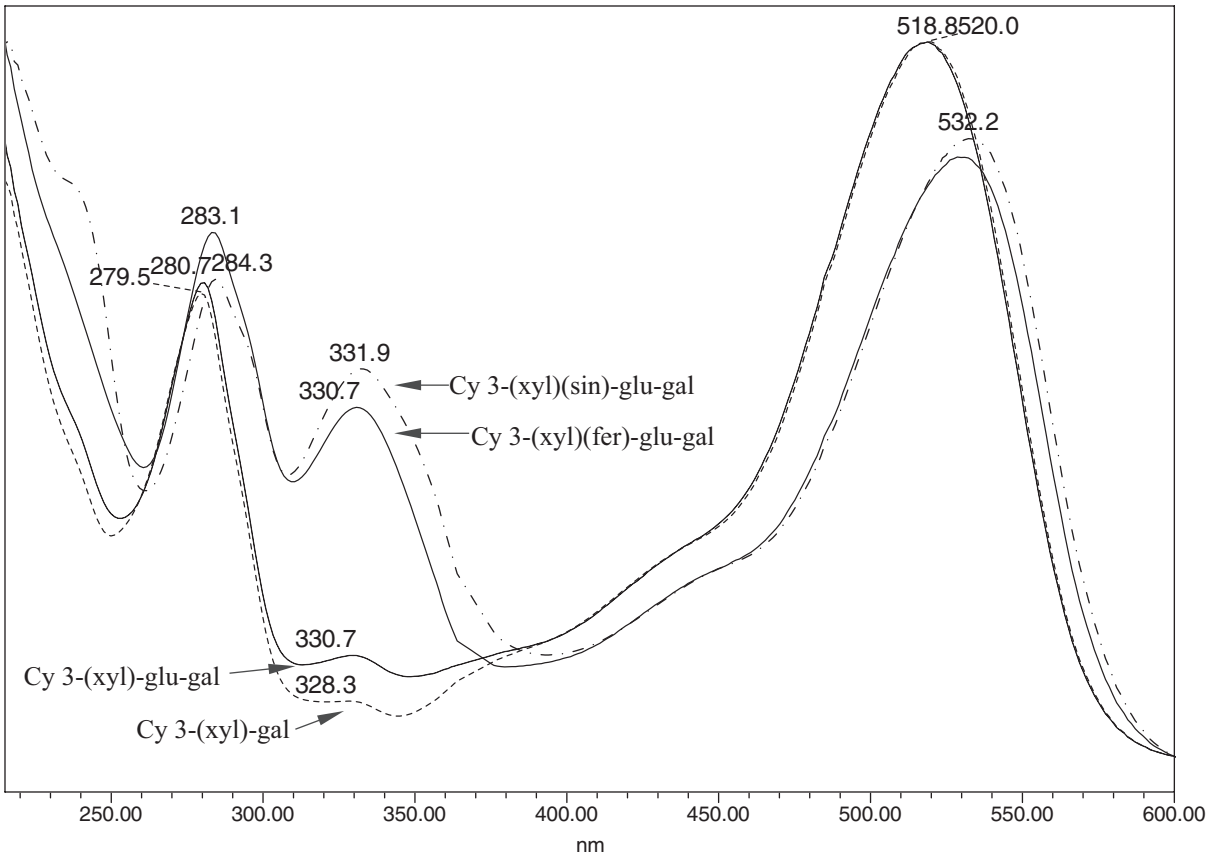


Figure 3. UV spectrum of acylated versus nonacylated anthocyanins. Cy, cyanidin; fer, ferulic acid; gal, galactose; glu, glucose; xyl, xylose.

maxima. Previous studies have shown that pelargonidin with no substitution in the B ring provided orange color with λ_{max} of 494 nm, whereas cyanidin with one hydroxyl group at 3′ position in the B ring yielded λ_{max} of 506 nm. Similarly, malvidin provided a bluish red coloration (21) due to its two methoxy substitutions. Methylation of anthocyanidin, on the other hand, is associated with a hypsochromic shift (22). In view of the above, it could be inferred that an increasing number of hydroxyl groups (pelargonidin→cyanidin→delphi nidin) might be responsible for a bathochromic shift, whereas more of methylation (cyanidin→peonidin→malvidin) could result in a hypsochromic shift.

Molar Absorptivity and Glycosylation Ratio

As a unique characteristic feature, the UV spectra provide information about the nature of sugar attached with the aglycone moiety of anthocyanins. Molar absorptivity of an anthocyanin molecule is the indicator of the presence of a sugar moiety. In general, E_{440}/E_{vis} absorptivity ratio of 29–35 predicts a monoside, whereas a lower value of 15–24 is observed for a bioside. The attachment of sugar moieties at 3 and 5 positions provides characteristic features in anthocyanin spectra (15, 23, 24). The absorbance ratio at 440 nm to the absorbance maxima (λ_{max}) helps to predict the position and number of substitutions (Figure 2) in an anthocyanin. The ratio is two times higher when the glycosidic substitution occurs at the position 3 against the substitutions at position 5 or both 3 and 5 (15, 16, 20). When glucose and sophorose were attached to pelargonidin at 3-position, the ratio ranged between 0.38 and 0.46 (Table 2); however, in case of 5-substituted and 3,5-disubstituted anthocyanins, it ranged between 0.18–0.22 (20). Thus, according to the literature, the UV-Vis spectroscopic data remained quite effective for the preliminary identification of the anthocyanins for different parts of a plant, including flowers, buds, leaves, and fruits (25–28).

In recent reports, UV spectrum, more specifically $A_{440}/A_{vis\text{-}max}$, has been used for preliminary identification of anthocyanin types (29). For example, Kim et al. (30) identified Cyanidin-3-O-sambubioside as the major anthocyanin, with the help of this characteristic ratio (0.31). Information regarding the 3- and 5- substitutions in the anthocyanidin moiety are well documented. A few examples are also available regarding substitutions, in addition to 3 and 5 positions, e.g., 4-substituted aglycone (31). Two 4-substituted anthocyanins, i.e., 5-carboxypyranopelargonidin-3-O-β-glucopyranoside and 5-carboxypyranocyanidin-3-O-*β*-glucopyranoside, were reported with the ratio, $A_{440}/A_{vis\text{-}max}$, of 0.51 and 0.38, as compared with 0.43 for pelargonidin-3-O-β-glucopyranoside. In the case of 4- and 7-substituted anthocyanins extracted from red onion, the ratio was 0.35 for all the anthocyanins, as also previously noted (28). The 4-substituted monoglycosylated cyanidin derivative provided the ratio of

Table 2. Glycosylation ratio used for the detection of sugar moiety

Source	Anthocyanin	$A_{440}/A_{vis\text{-}Max}$	Ref.
Raphanus sativus	Cy-3(sin)(fer)glc-glc-5-glc[a,b]	0.17	(25)
	Cy-3(fer)(sin)glc-glc-5-glc-(mal)glc[c]	0.23	
	Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc	0.18	
	Cy-3(fer)(2-fer)glc-glc-5-glc-(mal)glc	0.16	
Lonicera japonica bud	Cy-3-glc	0.31	(26)
	Cy-3-gal	0.31	
	Cy-3-rut	0.33	
	Cy-3(acetyl)glc	0.30	
	Cy-3,5-di-glc	0.17	
	Cy-3-rut-5-glc	0.18	
	Cy-3(cou)rut-5-glc[d]	0.16	
	Cy-3-di-glc-5-glc	0.18	
Hemigraphis colorata leaves	Me- Cy-3-di-glc	0.23	(27)
	Me-Cy-3-glc	0.23	
Plumeria rubra flowers	Cy-3-glc-gal	0.33	(88)
	Cy-3-gal	0.36	
Chinese purple yam	Cy-3-hex with hydroxycinnamic acid	0.27	(89)
	Cy-3-gly with hydroxycinnamic acid	0.29	
	Cy-3-gly with hydroxycinnamic acid	0.31	
	Pn-3-gly with hydroxycinnamic acid	0.28	
Siberian dogwood, *Cornus alba*	Cy-aglycon	0.30	(90)
	Cy-3-gal	0.28	
	Cy-3-gal-3′-glc	0.42	
	Dp-3-gal-3′-glc	0.35	
	Dp-3-gal-3′,5′-di-glc	0.45	
Red cabbage	Cy-biosides (24)	0.16–0.23	(36)
Red onion, *Allium cepa*	Cy-4′-glc	0.50	(28)
	Pn-3(mal) glc	0.28	
	Cy-3,4′-di-glc	0.45	
	Pn-3(mal)glc-5-glc	0.18	
	Cy-3-glc-(mal)glc-4′-glc	0.50	
	Cy-7-glc-(mal)glc-4′-glc	0.50	

Table 2. (*continued*)

Source	Anthocyanin	$A_{440}/A_{vis\text{-}Max}$	Ref.
Norwegian potato	Pt-3(caf)rham-glc-5-glc[e]	0.14	(28)
	Pt-3(coum)rham-glc-5-glc	0.15	
	Pn-3(caff)rham-glc-5-glc	0.13	
	Pn-3(coum)rham-glc-5-glc	0.15	
Radish, strawberry, red-fleshed potato	Pg-aglycon	0.38	(20)
	Pg-3-glc	0.45	
	Pg-5-glc	0.18	
	Pg-3-soph	0.46	
	Pg-3-glc-5-glc	0.20	
	Pg-3-soph-5-glc	0.22	
Red cabbage	Cy-3-soph-5-glu	0.22	(37)
	Cy-3-glu-5-glu	0.22	
	Cy-3(fer)soph-5-glu	0.22	
	Cy-3(sin)soph-5-glu	0.21	
	Cy-3(cou)(sin)soph-5-glu	0.23	
	Cy-3(fer)(sin)soph-5-glu	0.22	
	Cy-3(sin)(sin)soph-5-glu	0.22	
Baguacu (*Eugenia umbelliflora* Berg)	Dp-3-glu	0.20	(91)
	Cy-3-glu	0.23	
	Pt-3-glu	0.20	
	Pg-3-glu	0.23	
	Pn-3-glu	0.26	
	Mv-3-glu	0.19	
Red radish	Pg-3(fer)-glu-glu-5-glu	0.23	(38)
	Cy-3(caf)(fer)-glu-glu-5-glu	0.28	
	Pg-3(caf)-glu-glu-5-glu	0.21	
	Pg-3(caf)(caf)-glu-glu-5-glu	0.23	
	Pg-3(caf)(fer)-glu-glu-5-glu	0.23	
	Pg-3(cou)-glu-glu-5-glu	0.22	
	Pg-3(cou(caf)-glu-glu-5-glu	0.23	
	Pg-3(fer)-glu-glu-5-glu	0.21	
	Pg-3(fer)(caf)-glu-glu-5-glu	0.23	
	Pg-3(cou)(fer)-glu-glu-5-glu	0.23	
	Pg-3(fer)(fer)-glu-glu-5-glu[f]	0.22	
	Pg-3(fer)(fer)-glu-glu-5-glu[g]	0.24	
Ipomoea asarifolia flowers	Cy-3-(caf)(dihydroxycin)(caf)glu-glu-glu-5-glu	0.11	(41)
	Cy-3-(cou)(cou)(caf)glu-glu-glu-5-glu	0.12	
Raphanus sativus cv. Sango sprouts	Cy-3-digly-5-gly	0.20	(92)
	Pn-3-digly-5-gly	0.20	
	Cy-3(sin)-digly-5-gly	0.18	
	Cy-3-digly-5(suc)[h]-gly	0.19	
	Cy-3(sin)-digly-5-gly	0.16	
	Cy-3(sin)(sin)-digly-5-gly	0.15	
	Cy-3(sin)(fer)-digly-5-gly	0.18	
	Cy-3(cou)-digly-5(suc)-gly	0.17	
	Cy-3(fer)-digly-5(suc)-gly	0.18	
	Cy-3(fer)(fer)-digly-5(suc)-digly	0.18	
	Cy-3(sin)(fer)-digly-5(suc)-digly	0.17	

Table 2. (*continued*)

Source	Anthocyanin	$A_{440}/A_{vis\text{-}Max}$	Ref.
	Cy-3(fer)(fer)-digly-5(suc)-digly	0.17	
	Cy-3(fer)(cou)-digly-5(suc)-digly	0.17	
Hyacinthus orientalis	Dp-3(cou)-glu-5-glu	0.10	(40)
	Dp-3(caf)-glu-5(mal)-glu	0.11	
	Dp-3(cou)-glu-5(mal)-glu	0.10	
	Dp-3(cou)-glu-5(mal)-glu	0.09	
	Pt-3(cou)-glu-5(mal)-glu	0.11	
	Cy-3(cou)-glu-5(mal)-glu	0.11	
	Pg-3(cou)-glu-5(mal)-glu	0.19	
Synadenium grantii	Cy-3-xyl-5-glu	0.17	(93)
	Cy-3-xyl	0.30	
	Cy-3(cou)api-xyl-5-glu	0.14	
	Cy-3(caf)api-xyl	0.30	
	Cy-3(cou)api-xyl	0.27	
	Cy-3(fer)api-xyl	0.30	
Purple sweet potato	Cy-3-soph-5-glu	0.18	(49)
	Pg-3-soph-5-glu	0.25	
	Pn-3-soph-5-glu	0.18	
	Cy-3(hydroxyben)-soph-5-glu	0.17	
	Pn-3(fer)(cou)-soph-5-glu	0.15	
Ipomea nil	Pn-3(caf)diglu-5-glu	0.15	(94)
	Pn-3(caf)diglu	0.26	
	Pn-3-glu-(caf)glu-(caf)glu-(caf)glu-glu-5-glu	0.14	
	Pn-3-(caf)(fer)glu-(caf)glu-(caf)glu-glu-5-glu	0.14	
	Pn-3-glu-(caf)glu-glu-(caf)glu-glu	0.24	
	Pn-3-(caf)glu-(caf)glu-glu-(caf)glu-glu	0.25	
Purple sweet potato	Cy-3(caf)glu-glu-5-glu	0.14	(95)
	Pn-3(caf)glu-glu-5-glu	0.14	
Oxalis triangularis	Mv-3-rham-glu-5-glu	0.14	(96)
	Mv-3(mal)-rham-glu-5-glu	0.11	
	Mv-3-rham-glu-5(mal)-glu	0.13	
	Mv-3(mal)-rham-glu-5(mal)-glu	0.11	
	Mv-3(mal)-rham-glu	0.24	
	Mv-3(cou)-glu-5-glu	0.14	
	Mv-3(cou)-glu-5-glu	0.13	

[a] sin = Sinapic acid.

[b] fer = Ferulic acid.

[c] mal = Malonic acid.

[d] cou = Coumaric acid.

[e] caf = Caffeic acid.

[f] Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-(6-(E)-feruloyl-β-d-glucopyranosyl)-(1-2)-β-d-glucopyranoside]- 5-O-(β -d-glucopyranoside).

[g] Pelargonidin 3-O-[6-O-(E)-feruloyl-2-O-(2-(E)-feruloyl-β -d-glucopyranosyl)-(1-2)-β-d-glucopyranoside]- 5-O-(β-d-glucopyranoside).

[h] suc = Succinic acid.

0.50, whereas 4-substituted bi-glycosylated cyanidin derivative resulted in the ratio of 0.45. Cyanidin, substituted with sugar moiety at 3- and 4- position and 7- and 4- position provided A_{440}/A_{max} of 0.5. In general, substitution at 4- position resulted in a higher absorbance at 440 nm. In another novel anthocyanin (5-methylcyanidin derivative), the ratio (A_{440}/A_{max}) was 0.23, which was in between anthocyanin-3,5-diglucoside (>0.20) and anthocyanin-3-glucoside (≤0.31; 27).

Determination of Acylation Ratio

UV spectra of anthocyanins not only provide information on glycosylation but also furnish the extent of acylation in the sugar molecule. Substantial information can be derived from the UV-Vis spectrum, especially for acylated anthocyanins, providing typical fingerprint in the UV-Vis spectrum (15, 16). The step of acylation mostly involves aromatic or aliphatic acids. The aromatic acids include *p*-coumaric, sinapic, caffeic, gallic, ferulic, and *p*-hydroxybenzoic acid, whereas aliphatic acids include malic, acetic, malonic, oxalic, and succinic acid. Anthocyanin is rarely acylated with inorganic acid (e.g., sulfuric acid; 32). The ratio of absorbance maxima in the wavelength of 310–360 nm to the λ_{max}, observed in the visible range, allowed estimation of the number of aromatic acylating groups (Table 3). The ratio is commonly called the "acylation ratio." Acylation with aliphatic acids does not provide any absorbance peak at 310–360 nm range. Acylation is commonly found at C3 sugar, esterified to the 6- or 4-hydroxyl group, although esterification of the 4-hydroxyl group is less common. Uncommon acylation has also been reported earlier (33–35). In two anthocyanins reported in the stems of *Allium victorialis*, acylation with malonic acid was noted at the third and sixth position of the sugar unit (34).

In general, acylated anthocyanins provide an additional peak at around 300–320 nm in the UV spectrum of the compound (Figure 3). The presence of acylation in a compound can be determined by measuring the ratio of acylation maximum (λ_{acyl}) to visible maximum (λ_{max}). The acylation maxima peak generally lies between 310 and 320 nm. It has been further demonstrated that if the ratio of acylation to visible maxima value lies between 0.5 and 0.7, it represents the presence of one acylated group. On the other hand, when the value lies between 0.8 and 1.1, it indicates the presence of two acylations.

Several researchers have used these characteristic features of UV spectrum for characterization of anthocyanins. For the identification of acylated anthocyanins, and detection of the number of acylations in various agricultural and food commodities, the ratio was found to be within the postulated values between 0.5 and 0.7 for single acylation and 0.8 and 1.1 for two acylations (1, 36–41).

Variations of Acylation Ratio in Geometric Isomers

Acylated moiety in anthocyanins imparts the phenomenon of stereoisomerism. For example, in eggplant, both *cis*- and *trans*-coumaric acid derivatives of delphinidin are reported (42). The same is also true for the flowers of water hyacinth (40, 43). Similarly, acylated cyanidin and pelargonidin also presented stereoisomerism (44, 45).

More specifically, the molar ratio of the acyl moiety and anthocyanin has been found to be higher in *trans* isomer (0.80) than *cis* isomer (0.52) of Delphinidin-3-coumaroyl rutinoside-5-glucoside (46). Similar earlier findings were reported in *Petunia* sp. by Ando et al. (47) and Tatsuzawa et al. (48). Another compound, Petunidin-3-*cis*-p-coumaroylrutinoside-5-glucoside, contained $\lambda_{acyl}/\lambda_{max}$ of 0.95, whereas its *trans* isomer showed the molar ratio of 1.06. The E_{acyl}/E_{vis} absorptivity ratio of 53–69 and 98–128 predicted monoacylation and diacylation, respectively (37, 49). Similarly, Delphinidin-3-O-(6-O-*trans*-p-coumaroyl-β-D-glucoside)-5-O-(6-O-malonyl-β -D-glucoside) and its *cis* isomer were differentiated by their acylation ratio [0.53 for *cis* isomer (λ_{acyl}: 305; λ_{max}: 544) and 0.59 for *trans* isomer (λ_{acyl}: 308; λ_{max}: 541)] (40).

In spite of the possibilities discussed above, the UV-Vis spectroscopic method on its own may not be efficient enough in identifying an anthocyanin molecule without ambiguity. Identification of a compound exclusively based on UV-Vis spectral data might suffer from chances of false detections due to the fact that two or more anthocyanin molecules could generate similar spectra. The situation gets further complicated if the reference standards are not available to match and the target compounds are not chromatographically separated. To overcome such analytical limitations, MS is preferred as a complementary approach. Because anthocyanins are heat labile in nature, an HPLC is connected to the MS to enable chromatographic separation followed by molecular mass and characteristic fragment-based confirmations.

LC-MS Analysis

For more than a decade now, LC-MS has proved to be a powerful analytical tool for the identification of flavonoid glycosides, especially the anthocyanins (50–57). Hyphenation of LC to MS with various ion sources has become very useful for the structural confirmation of anthocyanins because it couples chromatographic separation with sensitive detection of the compound-specific precursor *m/z* as well as the characteristic fragment *m/z*. Hence, it leads to selective identification and sensitive quantification. In the recent past, ionization methods have been developed for natural compounds, which are either nonvolatile or thermodynamically unstable. In these techniques, ionization takes place in such a manner that volatilization of the sample is not required, and the ions in the gaseous phase are formed after applying appropriate energy. The soft ionization techniques in LC-MS provide molecular weight information apart from its structural details based on the characteristic fragments (Table 4). The ionization methods, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) hyphenated to mass analyzers, namely, ion trap, triple quadrupole, orbitrap, time-of-flight (TOF), and quadrupole time-of-flight (QTOF), are often used for the characterization of anthocyanins.

Among all the techniques available, application of ESI is most common owing to its numerous advantages. The application of ESI for the analysis of flavonoid glycosides, especially anthocyanins, applauds the success of several acclaimed research groups, including Fenn et al. (58–62). Similarly, Giusti et al. (20) successfully used the ESI-MS technique for the characterization of a number of anthocyanins from different sources including radish, red-fleshed potatoes, grape, Hibiscus

Table 3. Acylation ratio of different anthocyanin

Source	Acylation	λ_{Acyl}, nm	λ_{Vis}, nm	E_{Acyl}/E_{Vis} Acylation ratio	E_{440}/E_{Vis} Glycosylation ratio	λ_{Acyl} indication	Ref.
Red cabbage	M	272–300	522–530	55–63			(97)
	D	296–330	523–538	92–130	16–24		
Red cabbage	M	328–333	518–523	79–91			(37)
	D	316–333	517–528	108–150	21–23		
Baguacu (*Eugenia umbelliflora* Berg)	—[a]	—	518–544	10–25	19–26	Monoside	(91)
Red radish	M	316–331	505–508	52–82	21–23	Bioside	(38)
	D	319–331	507–513	100–231	22–28	Bioside	
Red cabbage	M	321–330	521–527	53–69	—		(39)
	D	314–332	521–536	93–119	—		
Ipomoea asarifolia flowers	T	324	531	136	11	Tetra glucosylated	(41)
	T	312	532	160	12	Tetra glucosylated	
Raphanus sativus cv. Sango sprouts	M	332–338	518–531	53–109	—		(92)
	D	327–329	522–534	104–111	15–20		
	T	326–331	531–535	103–140	16–18		
Raphanus sativus cv. Sango sprouts	M	327–334	522–527	59–63	18		(25)
		328–330	530–535	98–128	17–23		
Hyacinthus orientalis flower	M	309	541	76	10		(43)
	D	304–316	509–544	53–100	9–11		
Lycium ruthenicum Murray	M	301–331	536–539	43.9–92.0	–		(98)
Synadenium grantii	M	317, 319, 320, 333	529–531	63–82	14–30		(93)
Zebrina pendula, *Rhoeo spathacea*, and *Setcreasea purpurea*	Tr	334	530	157	—		(99)
		327	534	174			
	Te	329	532	207	—		
		338	537	210			
Purple Sweet Potato Cell Line	M	—	505–520	16–69	—		(49)
	D	—	522–532	55–125	15–25		
Ipomoea nil	M	329	524	78–129	15		(94)
	Tr	316–324	526–533	140–193	14–26		
	Te	318	533	197	24		
Purple sweet potato	M	330	525–529	52–64	14		(95)
Clitoria ternatea	Tr		546–547	202–211	33–35		(100)
	Te		547–549	232–250	29–33		
Oxalis triangularis	M	—	535–538	—	51–172		(96)
Saintpaulia sp.	M	—	509–537	—	16–22	Tri-glucosylated	(101)
Strawberries, radishes, red-fleshed potatoes	M		528				(102)
			536				
			498				
		D	512				
			528–530				
		T	536–538				

[a] — = Non acylated/not mentioned.

sp., red cabbage, and chokeberry (*Aronia* sp.). The technique was proved to be a confirmatory tool for the structural identification of anthocyanins. For example, Wu and Prior (18) reported a systematic identification of anthocyanins in 25 different fruits consumed in the United States using the ESI-MS technique with the complementary support of UV spectrum. In a similar way, Lopes-da-Silva et al. (62) described the use of LC-ESI-MS for the identification of anthocyanins in strawberry (Table 5).

Atmospheric pressure chemical ionization is another interface, which has been found useful in characterization of anthocyanins in purple-fleshed sweet potatoes, adzuki bean, and others (63–65). Similarly, applications of fast ion

Table 4. Common anthocyanins: their chemical formulas and monoisotopic molecular masses

	Name	Molecular formula	Accurate mass, Da	Ref.
	Cyanidin glycosides			
Nonacylated	Cyanidin-3-O-arabinoside	C20H19O10	419.0978	(103)
	Cyanidin-3-O-glucoside	C21H21O11	449.1078	(103)
	Cyanidin-3-O-galactoside	C21H21O11	449.1084	(103)
	Cyanidin-3-O-sambubioside	C22H29O15	581.1501	(103)
	Cyanidin-3-O-rutinoside	C27H31O15	595.1657	(103)
	Cyanidin-3,5-O-diglucoside	C27H31O16	611.1607	(103)
	Cyanidin 3-laminaribioside	C27H31O16	611.1612	(106)
	Cyanidin-3-O-(2″-O-xylosyl)rutinoside	C32H39O19	727.2080	(103)
	Cyanidin-3-O-(2″-O-glucosyl)rutinoside	C33H41O20	757.2186	(103)
	Cyanidin-3-(diglucosyl)rhamnoside	C33H41O20	757.2191	(104)
	Cyanidin-3-diglucoside-5-glucoside	C33H41O21	773.2140	(104)
Acylated	Cyanidin-3-(6-acetoyl)galactoside	C23H23O12	491.1184	(105)
	Cyanidin-3-(6-acetoyl)glucoside	C23H23O12	491.1184	(105)
	Cyanidin 3-(3″-malonoyl)glucoside	C24H23O14	535.1088	(106)
	Cyanidin-3-(p-coumaroyl) glucoside	C30H27O13	595.1452	(104)
	Cyanidin 3-(malonoyl)- glucoside-5-glucoside	C30H33O19	697.1616	(106)
	Cyanidin-3-(sinapoyl) glucoside-5-glucoside	C38H41O20	817.2191	(104)
	Cyanidin-3-(p-coumaroyl) diglucoside-5-glucoside	C42H47O23	919.2508	(104)
	Cyanidin-3-(caffeoyl) diglucoside-5-glucoside	C42H47O24	935.2457	(104)
	Cyanidin-3-(feruloyl) diglucoside-5-glucoside	C43H49O24	949.2614	(104)
	Cyanidin-3-(sinapoyl) diglucoside-5-glucoside	C44H51O25	979.2719	(104)
	Cyanidin-3-(p-coumaroyl) triglucoside-5-glucoside	C48H57O28	1081.3036	(104)
	Cyanidin-3-(feruloyl) triglucoside-5-glucoside	C49H59O29	1111.3142	(104)
	Cyanidin-3-(feruloyl)(feruloyl)diglucoside-5-glucoside	C53H57O27	1125.3087	(104)
	Cyanidin-3-(sinapoyl)sophoroside-5-diglucoside	C50H61O30	1141.3234	(104)
	Cyanidin-3-(sinapoyl)triglucoside-5-glucoside	C50H61O30	1141.3248	(104)
	Cyanidin-3-(sinapoyl)(feruloyl)diglucoside-5-glucoside	C54H59O28	1155.3193	(104)
	Cyanidin-3-(sinapoyl)(sinapoyl)diglucoside-5-glucoside	C55H61O29	1185.3299	(104)
	Cyanidin-3-(sinapoyl) (p-coumaroyl) triglucoside-5-glucoside	C59H67O32	1287.3615	(104)
	Cyanidin-3-(p-coumaroyl) diglucoside-5-glucoside	C60H69O33	1317.3721	(104)
	Cyanidin-3-(sinapoyl) (sinapoyl)triglucoside-5-glucoside	C61H71O34	1347.3827	(104)
	Delphinidin glycosides			
Nonacylated	Delphinidin-3-O-arabinoside	C20H19O11	435.0927	(103)
	Delphinidin-3-O-glucoside	C21H21O12	465.1033	(103)
	Delphinidin-3-galactoside	C21H21O12	465.1028	(105)
	Delphinidin-3-O-rutinoside-5-O-glucoside	C33H41O21	773.2135	(107)
Acylated	Delphinidin-3-(6-acetoyl)glucoside	C23H23O13	507.1133	(105)
	Delphinidin-3-(p-coumaroyl)glucoside	C30H27O14	611.1401	(104)
	Delphinidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	C42H47O23	919.2503	(107)
	Delphinidin-3-O-(caffeoyl)rutinoside-5-O-glucoside	C42H47O24	935.2452	(107)
	Pelargonidin glycosides			
Nonacylated	Pelargonidin-3-O-glucoside	C21H21O10	433.1129	(103)
	Pelargonidin-3-O-rutinoside	C27H31O14	579.1708	(103)
	Pelargonidin-3-O-sambubioside	C26H29O14	565.1552	(103)
	Pelaragonin-3-glucoside-5-glucoside	C27H31O15	595.1663	(103)
	Pelaragonin-3-diglucoside-5-glucoside	C33H41O20	757.2191	(103)
	Pelargonidin-3-O-(2″-O-glucosyl)rutinoside	C33H39O19	741.2237	(103)

Table 4. (*continued*)

	Name	Molecular formula	Accurate mass, Da	Ref.
Acylated	Pelaragonin-3-diglucoside-5-(malonoyl)glucoside	C36H43O23	843.2195	(104)
	Pelaragonin-3-(p-coumaroyl)diglucoside-5-glucoside	C42H47O22	903.2559	(104)
	Pelaragonin-3-(feruloyl) diglucoside-5-glucoside	C43H49O23	933.2665	(104)
	Pelaragonin-3-(p-coumaroyl)diglucoside-5-(malonoyl)glucoside	C45H49O25	989.2563	(104)
	Pelaragonin-3-(caffeoyl)diglucoside-5-(malonoyl) glucoside	C45H49O26	1005.2512	(104)
	Pelaragonin-3-(feruloyl) diglucoside-5-(malonoyl) glucoside	C46H51O26	1019.2669	(104)
	Pelaragonin-3-(p-coumaroyl)triglucoside-5-(malonoyl)glucoside	C51H59O30	1151.3091	(104)
	Pelaragonin-3-(p-coumaroyl)(feruloyl)diglucoside-5-(malonoyl)glucoside	C55H57O28	1165.3036	(104)
	Pelaragonin-3-(feruloyl) triglucoside-5-(malonoyl) glucoside	C52H61O31	1181.3197	(104)
	Pelaragonin-3-(feruloyl)(feruloyl)diglucoside-5-(malonoyl)glucoside	C56H59O29	1195.3142	(104)
	Malvidin glycosides			
Nonacylated	Malvidin-3-O-arabinoside	C22H23O11	463.1240	(103)
	Malvidin-3-O-glucoside	C23H25O12	493.1341	(103)
	Malvidin-3-galactoside	C23H25O12	493.1341	(105)
	Malvidin-3-O-rutinoside-5-O-glucoside	C35H45O21	801.2448	(105)
Acylated	Malvidin-3-O-(6″-O-acetyl)glucoside	C25H27O13	535.1452	(103)
	Malvidin-3-(6-acetoyl)galactoside	C25H27O13	535.1446	(105)
	Malvidin-3-(p-coumaroyl) glucoside	C32H31O14	639.1714	(104)
	Malvidin-3-(caffeoyl) glucoside	C32H31O15	655.1663	(104)
	Malvidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	C44H51O23	947.2816	(107)
	Petunidin glycosides			
Nonacylated	Petunidin-3-O-arabinoside	C21H21O10	449.1077	(103)
	Petunidin-3-O-glucoside	C22H23O12	479.1190	(103)
	Petunidin-3-galactoside	C22H23O12	479.1184	(105)
	Petunidin-3-O-rutinoside-5-O-glucoside	C34H43O21	787.2291	(107)
Acylated	Petunidin-3-(6-acetoyl)galactoside	C24H25O13	521.1289	(105)
	Petunidin-3-(6-acetoyl)glucoside	C24H25O13	521.1289	(105)
	Petunidin-3-(p-coumaroyl) glucoside	C31H29O14	625.1557	(104)
	Petunidin-3-(caffeoyl) glucoside	C31H29O15	641.1506	(104)
	Petunidin-3-O-(p-coumaroyl)rutinoside-5-O-glucoside	C43H49O23	933.2659	(107)
	Petunidin-3-(caffeoyl)rutinoside-5-O-glucoside	C43H49O24	949.2608	(107)
	Peonidin glycosides			
Nonacylated	Peonidin-3-O-arabinoside	C21H21O10	433.1135	(103)
	Peonidin-3-O-glucoside	C22H23O11	463.1240	(103)
	Peonidin-3-O-galactoside	C22H23O11	463.1235	(105)
Acylated	Peonidin-3-O-(6″-O-acetyl)glucoside	C24H25O12	505.1346	(103)
	Peonidin-3-(6-acetoyl)galactoside	C24H25O12	505.1341	(105)
	Peonidin-3-(p-coumaroyl) glucoside	C31H29O13	609.1608	(104)
	Peonidin-3-(caffeoyl) glucoside	C31H29O14	625.1557	(104)

bombardment-MS have also been reported for the identification of anthocyanins (25, 47, 66–71).

Recently, applications of MALDI-TOF-MS have been reported for the identification of anthocyanins (71–74) in blueberry (75), rose flowers (76), blackberry (77), cranberry (78), red wine (72, 74), and red grape (73). Desorption electrospray ionization MS is another functional technique for analysis of biomolecules in food, pharmaceutical, and forensic analysis (79), and it was successfully applied for quantitative analysis of anthocyanins in slices of wine grapes, chokeberries, and elderberries (80).

LC-high-resolution MS (HRMS) has been the most powerful tool so far to characterize the anthocyanins. Therefore, it has been increasingly used in recent years because of its complementary advantages of chromatographic separation and accurate mass-based selectivity in resolving the complexity of natural matrices for accurate characterization. Mass analyzers suitable for high mass accuracy determination include TOF and Orbitrap instruments. Even in the absence of reference standards, tentative identification of the compounds is possible based on the presence of precursor and fragment ions with high mass accuracy (mass error <5 ppm). In such cases in which the

Table 5. Anthocyanins analyzed by different MS techniques

MS mode	Major anthocyanins	Ref.
APCI[a]	3-gal (Dp, Cy), 3-glc (Dp, Cy, Pt, Pn, Mv), 3-ara (Dp, Cy), aglycons (Dp, Cy, Pt,Pn, Mv), Pn-3-sop-5-glc, Pn/Cy-3-(fer)sop-5-glc	(63, 64, 108, 109)
FAB[b]	Acylated anthocyanins (based on mal), anthocyanins 3-glc (Dp, Cy) and 3,5–di-glc, 3-rut (Dp, Cy), Dp-3-xyl-(Cis Coum/Trans Coum/Caf/Fer)glc-5(mal)glc, Dp-3(rham) glc-3′glc, Cy/Pn-(caf)(fer)sop-5-glc, Cy-3,5-di-(mal)glc, Cy-3-(fer)xyl-6(fer)glc-5-(mal) glc, Cy-3-Xyl-6-(fer)glc-(coum)glc-5(mal)glc, Cy-3-Xyl-6-(fer)glc-(coum)glc-5(mal) glc,Cy-3-Xyl-6-(fer)glc-(coum)glc-5(mal)glc,Cy-3-(fer)Xyl-6-glc-(fer)glc-5(mal)glc, Cy-3-(fer)xyl-6-(fer)glc-(coum)glc-5(mal)glc,	(47, 65–70)
ESI	Cy-3(sin)(fer)glc-glc-5-glc, Cy-3(fer)(sin)glc-glc-5-glc-(mal)glc, Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc, Cy-3(fer)(2-fer)glc-glc-5-glc-(mal)glc, 3-(sin)sop-5-di-glc, 3-(sin)(cou) sop-5-di-glc,3-(caf)(hydroxyfer)sop-5-di-glc,3-(sin)(caf)sop-5-di-glc, 3-(sin)(cou)sop-5-di-glc, Cy-3-sam, Cy-3-xyl-rut, Dp-3(cou)gal, Cy-3(cou)gal, 7-O-MethCy-3-gal, (mal) rut, (Dp) rut, (Caf) rut, (Pt, Caf, p-caoumaroyl) rut-glc, Dp, (Mal, p-caoumaroyl) rut-glc, Cy 3-(Z)-p-coumaroylsam-5-glc and Cy 3-(E)-pcoumaroylsam-5-glc, Cy 3-(2G glucosylrutinoside), Cy 3-sambubiose, cy 3-xylosylrutinoside, Cy 3-caffeoylsam bubioside-5-glc, Cy- 3-caffeoylsambubioside-5-glc, Cy 3-p-hydroxybenzoyl sop-5-glc, Cy 3-(6000-caffeoyl sop)-5-peo 3-p-hydroxybenzoyl sop-5-glc, Peo 3-(6000-caffeoyl sop)-5-glc, Cy 3-fersop-5-glc, Peo 3-fersop-5-glc, Cy 3-caffeoyl sop-5-glc, Cy 3-sop-5-glc, Cy 3-dicaffeoyl sop-5-glc, Cy 3-caffeoyl-p-gydroxybenzoyl sop-5-glc, Peo-3-caffeoyl sop-5-glc, Cy 3-caffeoyl-fersop-5-glc, Peo 3-dicaffeoyl sop-5-glc, Peo 3-caffeoyl-p-hydroxybenzoyl sop-5-glc, Peo 3-caffeoyl-fersop-5-glc, Peo 3-caffeoyl-p-coumaryl sop-5-glc, Cy-3-O-glucosylrutinoside, Cy-3-O-sam, Cy-3-O-xylosylrutinoside, Qu-methylpentoside-dihexoside, Qu-fer-hexoside, Qu-3-dihexoside, Cy-3(fer)(sin)glc-glc-5-glc-(mal)glc, Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc, Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc, Cy-3(fer)(2-fer)glc-glc-5-glc-(mal)glc, Cy-3-(fer)xyl-6-(fer)glc-(coum)glc-5(mal)glc	(18, 25, 110–128)
MALDI	3,5-diglc (Cy, Pn), 3-glc (Dp, Cy, Pt, Pn, Mv), 3-glc-cum (Dp, Cy, Pt, Pn, Mv), 3-glc-cum-5-glc (Dp, Cy, Pt, Pn, Mv)	(129, 130)
MALDI-TOF	3,5-diglc (Dp, Cy, Pt, Pn, Mv), 3-glc (Dp, Cy, Pt, Pn, Mv),Dp-3(ace)glc, 3-(cou)glc-5-glc (DP), 3-(cou)glc (Cy, Dp, Pn, Mv)	(131–135)
Nano-DESI[c]	3-glc (Pn, Pt, Mv), Dp-3-rut	(82, 83)
APPI-QqTOF MS[d]	3-glc (Dp, Cy, Pt, Pn, Mv), Mv-3-(Coum)glc,3-(ace)glc (Dp, Cy, Pt, Pn, Mv), Dp-3-O-glc, Pt-3-O-glc, Peo-3-O-glc, Mal-3-O-glc, Dp-pentose, Dp-pentose, Cy3- O-glc, Pt-pentose, Pt-pentose, Peo 3-O-glc	(84, 136)
UHPLC-ESI-MS[n]	Cy 3-(6″-malonyl) glc (cya 3-(6 M)-glu) and Cy 3-(6″-dioxalyl) glc (cya 3-(6D)-glu), Del 3-glu, Peo 3-glu), del 3-(6 M)-glu, Peo 3-(6 M)-glu, Pet 3-glu, Pet 3-(6 M)-glu	(137)
UHPLC-ESI-Orbitrap-MS[e]	3,5-diglc (Dp, Cy, Pt, Pn, Mv), 3-glc (Dp, Cy, Pt, Pn, Mv), Dp-3(ace)glc, 3-(cou)glc-5-glc (DP), 3-(cou)glc (Cy, Dp, Pn, Mv)	(138)
2DLC/MS[f]	Cy-3-O-diglc-5-O-glc, Cy-3-(p-coumaroyl)-O-diglc-5-O-glc, Cy-3-(fer)-O-diglc-5-O-glc, Cy-3-(sinapoyl)-O-diglc-5-O-glc, Cy-3-(fer)(fer)-O-diglc-5-O-glc, Cy-3-(fer)(sinapoyl)-O-diglc-5-O-glc, Cy-3-(sinapoyl)sinapoylglc	(139)
UHPLC-DAD-ESI-QTOF/MS[g]	Qu-methylpentoside-dihexoside, Qu-fer-hexoside, Qu-3-dihexoside, Cy-3(fer)(sin) glc-glc-5-glc-(mal)glc, Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc, Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc, Cy-3(fer)(2-fer)glc-glc-5-glc-(mal)glc, Cy-3-(fer)xyl-6-(fer)glc-(coum) glc-5(mal)glc	(140)
Tandem MS, (HRMS)[h]	Cy-3(sin)(fer)glc-glc-5-glc, Cy-3(fer)(sin)glc-glc-5-glc-(mal)glc, Cy-3(fer)(6-fer)glc-glc-5-glc-(mal)glc, Cy-3(fer)(2-fer)glc-glc-5-glc-(mal)glc, 3-(sin)sop-5-di-glc, 3-(sin)(cou)sop-5-di-glc,3-(caf)(hydroxyfer)sop-5-di-glc,3-(sin)(caf)sop-5-di-glc, 3-(sin)(cou)sop-5-di-glc, Cy-3-sam, Cy-3-xyl-rut, Dp-3(cou)gal, Cy-3(cou)gal, 7-O-MethCy-3-gal, (mal) rut, (Dp) rut, (Caf) rut, (Pt, Caf, p-caoumaroyl) rut-glc, Dp, (Mal, p-coumaroyl) rut-glc	(141)

[a] APCI = Atmospheric pressure chemical ionization.

[b] FAB = Fast atom bombardment.

[c] DESI = Desorption electrospray ionization.

[d] APPI-QqTOF MS = Atmospheric pressure photoionization quadrupole time-of-flight mass spectrometry.

[e] UHPLC = Ultra-high-performance LC.

[f] 2DLC/MS = Two dimensional liquid chromatography mass spectrometry.

[g] UHPLC-DAD-ESI-QTOF/MS = Ultra-high performance liquid chromatography diode array detector electrospray ionization quadrupole time-of-flight mass spectrometry.

[h] HRMS = High resolution mass spectrometry.

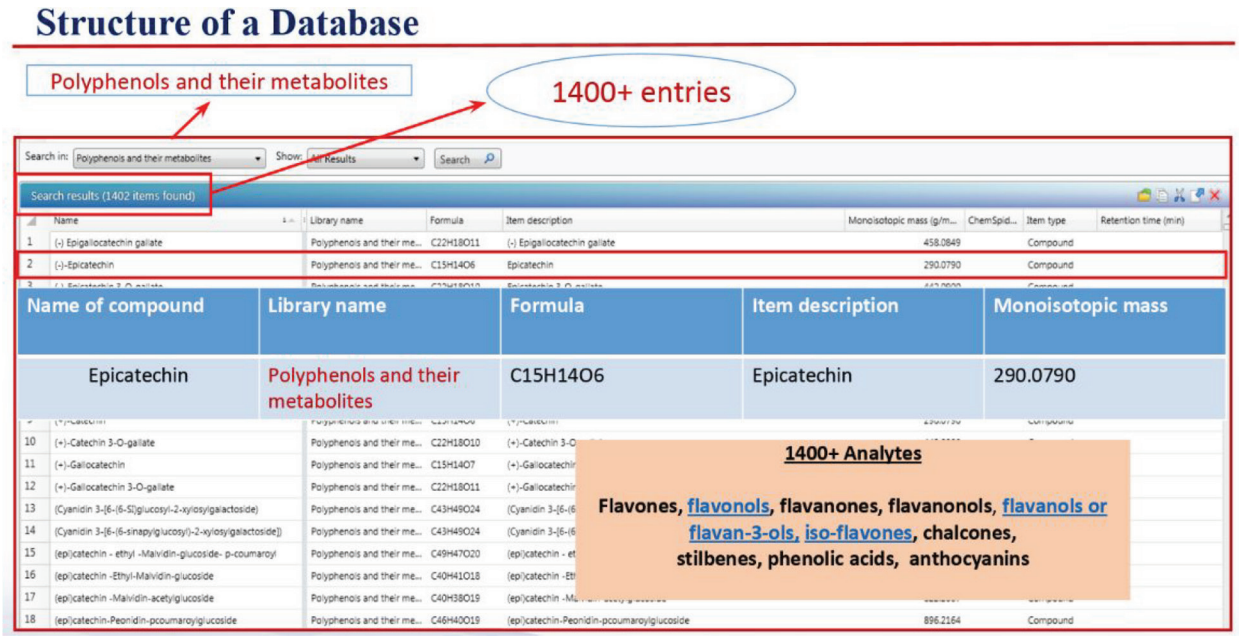


Figure 4. Representation of the structure of a database in UNIFI 1.8.

individual parameters related to ionization of the compounds are not known, a range of values is set for collision energy and cone voltage (or similar). In order to comply with the standard method performance requirements, the SANTE/11813/2017 guideline (81) may be referred to in order to evaluate performance of the LC-MS based analysis in unit resolution or high-resolution modes. Although the aforementioned guideline exists for pesticide residue analysis, its quality conforming parameters might also be useful in analytical quality control of anthocyanin analysis.

It is usually observed that the compounds with a higher intensity of precursor ion are measured with a lower mass error. For putative identification of compounds, the molecular formula, isotopic pattern, and accurate mass of the compounds are matched with an in-house (or commercially available) developed database of polyphenols. A snapshot of the structure of an example database (in the UNIFI software, version 1.8, Waters Corporation) is given in Figure 4, which includes information including monoisotopic mass, chemical formula, and mol file (structure of the compound). In another example, the identification parameters in the database of TraceFinder software (version 3.3, Thermo Scientific) are represented in Figure 5. The resulting high-energy fragment ions were compared with the fragments present in the database (e.g., TraceFinder), generated through in silico fragmentation in the software (e.g., UNIFI), or matched with previous literature reports or publicly available databases including METLIN, whichever is applicable. While reporting the putatively identified compounds, the guidelines of Metabolomics Standard Initiatives (82) are useful. In the absence of pure analytical standards, the identification is considered putative and not absolute. The threshold criteria, especially detector count (e.g., >500), and mass error (<5 ppm), are set in such a way so that false detections can be avoided. Whenever a reference standard is available, its retention time information could be used to add another confirmation criterion, with deviations within ±0.1 min. For example, Koley et al. (55, 83) described the high-resolution LC-MS profiling

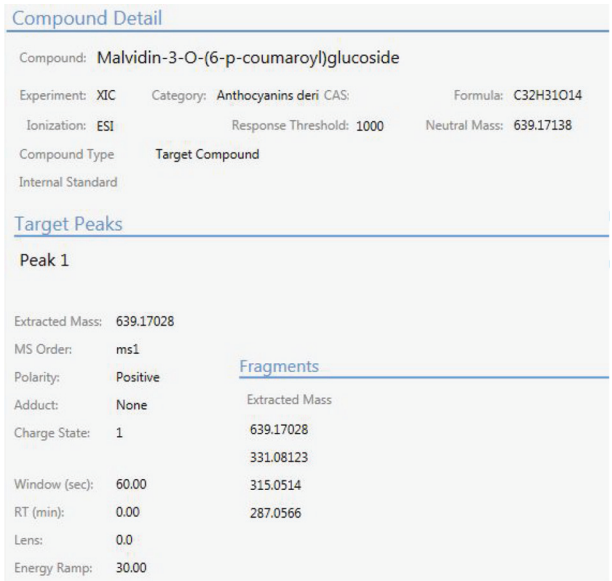


Figure 5. Representation of the structure of a database in Tracefinder 3.3.

of phenolic compounds in purple radish and Indian black carrot, respectively. The parameters on the basis of which these compounds were reported are described in Figure 6 for peonidin-3-O-acetylglucoside and in Figure 7 for malvidin-3-O-6-p-acetylglucoside. During nontarget screening of such compounds (known unknowns), some might appear at multiple retention times (RTs) as they exist as stereoisomers because of the presence of one or more chiral centers and unsaturations. For the purpose of semiquantitative analysis of such compounds, individual peaks may be separately integrated, and their peak areas may be summed up to determine their relative proportion.

A recently published article by Mayr et al. (84) used Q-TOFMS for accurate mass-based identification of secondary

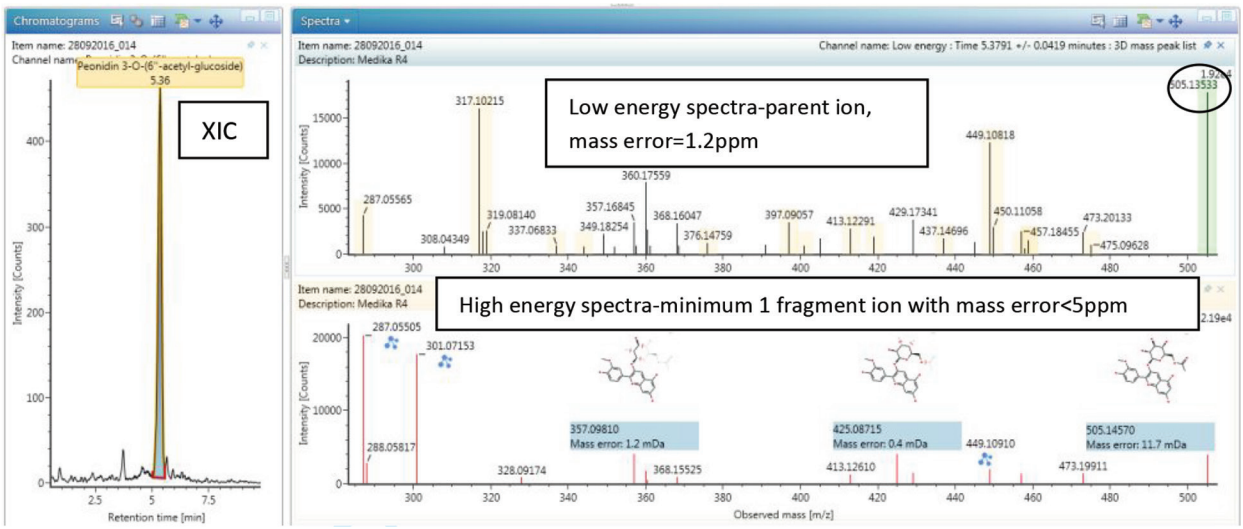


Figure 6. LC-HRMS identification of Peonidin-3-O-6-acteylglucoside (RT = 5.3 min).

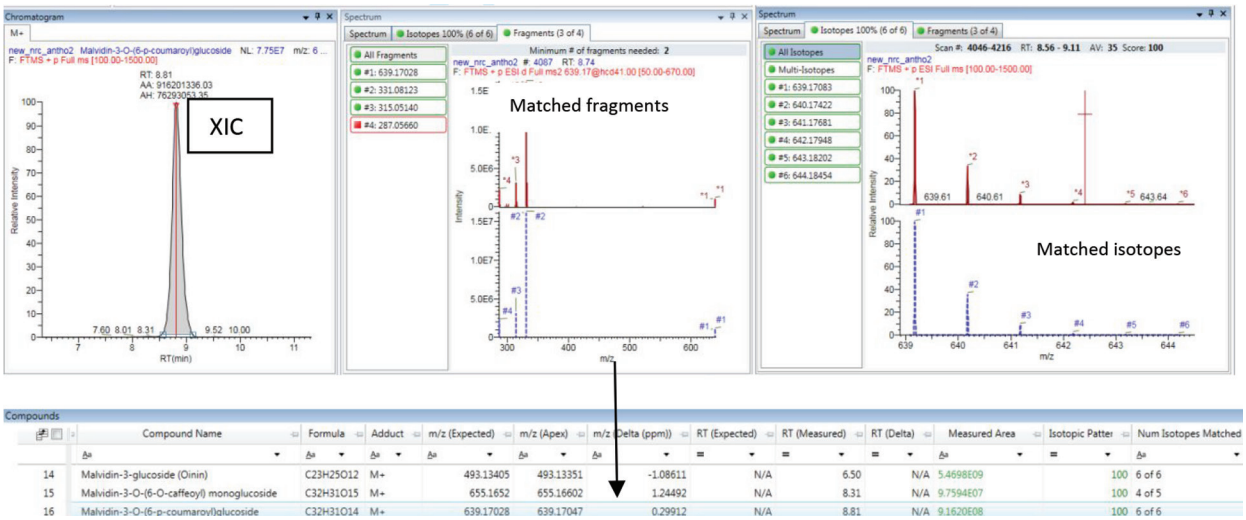


Figure 7. LC-HRMS identification of Malvidin-3-O-6-p-coumarylglucoside (RT = 8.8 min).

metabolites in four red grape varieties, which could be potentially useful as traceability markers of wines. They identified a total of 16 anthocyanins and calculated the statistical variation of the studied varieties using anthocyanins as variables. Recently, characterization of anthocyanins by HRMS has been reported with mass errors ranging between –0.107 and –2 ppm for a number of anthocyanins (85). An article by Van der Hooft et al. (86) showed the capability of tandem mass spectrometry (MS/MS) and MS^n fragmentation tools in the annotation of known metabolites, as well as identification of unknown metabolites based on in-depth fragmentation approaches, providing structural information of the molecule. Although this work was carried out on flavonoids, the principle could also be used for the identification of anthocyanins. Lin et al. in 2011 (53) described the HRMS/MS^n analysis of anthocyanins, flavonol glycosides, and hydroxycinnamic acid derivatives in red mustard greens. Another recently published study (87), albeit on triple quadrupole LC-MS analysis, performed the differentiation of two grape varieties based on targeted phenolic profiling, in which 33 compounds including anthocyanins were identified based on compound-dependent mass spectrometric parameters such as RT, adducts, quantitative and qualitative selected reaction monitoring transitions, cone voltage, and collision energy.

Conclusions

The main aim of this paper is to help researchers and students to extract important information from the UV-Vis spectrum and mass spectrum of anthocyanins. Whereas the number and type of glycosylation and acylation present in the anthocyanin can be predicted from the UV-Vis data, characterization of anthocyanin can be done with mass spectrometric techniques, especially in high-resolution mode. Integration of UV-Vis and LC-MS/MS techniques offers possibilities of authentication and identification of food fraud or adulteration especially in characterization and quantification of anthocyanins.

Based on this review, it is understood that for future work, a standardized set of analytical methodologies is clearly desirable to generate complementary information. To achieve unambiguous

identifications and facilitate productive comparisons among different studies, it could be necessary to integrate UV-Vis and MS with other spectroscopic techniques.

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