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Identification, Quantification, and Method Validation of Anthocyanins †

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Abstract: Nowadays, anthocyanins have gained scientific and industrial attention due to their biological activities and coloring properties. In this regard, anthocyanins have been proposed for use in the development of new nutraceutical foods to replace synthetic additives as well as to be value-added ingredients. The aim of this study was to evaluate current data on identification and quantification techniques and the validation process of such methods. Our results showed that anthocyanins have been identified by different methods, including nuclear magnetic resonance and chromatography-based techniques. Although problems have been described in this validation, most of the reports showed positive results on the validation parameters, suggesting that the current analytical technology offers a satisfactory identification and quantification of anthocyanins.

Keywords: anthocyanins; plant; extraction; validation

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1. Introduction

Anthocyanins are soluble glycosides linked by an O-glucosidic bond between an aglycone and a sugar molecule. It is a group belonging to the flavonoids that are found naturally in various plant sources, including fruits such as berries or grapes and flowers such as hibiscus, forming part of the secondary metabolites of plants [1]. Therefore, the extraction of anthocyanins is usually carried out from plant matrices. In the last few years, these compounds have attracted great interest due to their diverse beneficial properties. Specifically, anthocyanins present a high antioxidant capacity, which is attributed to the presence of phenolic hydroxyl groups in their chemical structure [2] (Figure 1). Furthermore, several studies have reported that a daily intake of this compound has a preventive and protective effect against cardiovascular diseases, diabetes, and heart disease [3–5]. These compounds also have coloring properties, covering ranges from salmon pink to red and from violet to dark blue. Thus, they are considered as an interesting source of natural colorants. To date, more than 20 structures are known, among which are orange pelargonidin, orange red cyanidin and peonidin, bluish-red delphinidin, and bluish-red malvidin and petunidin (Figure 1).

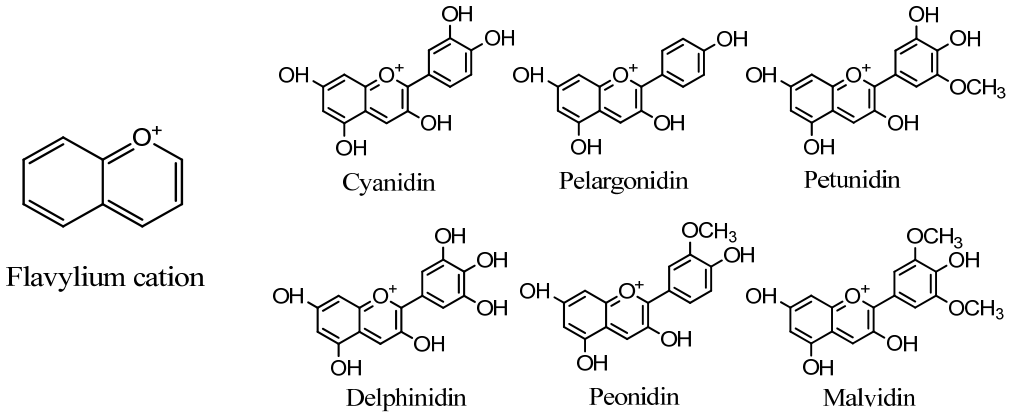

Figure 1. (**Left**) Base group of anthocyanins' structure. (**Right**) Most common anthocyanins in the nature.

Natural dyes have several advantages, such as non-toxicity and eco-friendliness, as their acquisition has a low environmental impact. Therefore, different industries have a great interest in identifying new and economically viable sources of anthocyanins to use them as new functional ingredients and/or colorants in food [6]. As the range of application of these compounds increases, it is necessary to design efficient extraction methods with better yields and to develop suitable analytical methods for the identification and quantification of anthocyanins. Therefore, the main objective of the study was to evaluate the current data on the identification and quantification techniques used and the validation process of these methods.

2. Identification and Quantification Techniques

The search for new ingredients of natural origin and the study of their bioactivities has been going on for some decades due to the growing demand for natural products with beneficial health properties, including the group of anthocyanins. Various techniques have been used for the identification and characterization of anthocyanins in different matrices, including mass spectrometry (MS), nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC). In Table 1, several examples of studies employing these techniques have been collected.

Table 1. Examples of studies employing different methods to identify and quantify anthocyanins.

Identification Technique	Source	Compounds	Ref.
MS; EI-MS; FAB-MS	Black rice, orchids, bilberries	DEL, CYA, PET, and MAL derivatives	[7–10]
NMR	Maqui berries, grapes, sumac, black currant, blue flowers, sweet potato, chokeberry	DEL, CYA, PET, MAL, and PEO derivatives	[11–15]
HPLC; HPLC-DAD; HPLC-MS/MS; HPLC-ESI/MS	Blueberries, hibiscus, red cabbage, cranberry, strawberry, grapefruits, grape skin, *Euterpe oleracea*	DEL, CYA, PET, PEO, and MAL derivatives	[6,16–22]

Abbreviation: delphinidin: DEL; cyanidin: CYA; petunidin: PET; malvidin: MAL; peonidin: PEO.

Regarding the application of MS-based technologies, several studies have obtained suitable results. For example, a work employed electron impact (EI)-MS to evaluate the degradation products of cyanidin-3-*O*-glucoside when the compound was subjected to oxidation radicals. Up to six derived products were detected, and two new molecules, 2-(3,4-dihydroxyphenyl)-4,6-dihydroxybenzofuran-3-carboxylic acid) and 2-*O*-(3,4-dihydroxybenzoyl)-2,4 glucose esters, 6-trihydroxyphenylacetic acid glucose ester, were identified. When analyzing samples of black rice stored for long periods, these two new compounds were identified, being useful to distinguish fresh rice from that stored for a long time [7]. Similarly, another EI-MS study investigated the degradation products of anthocyanin glycosides when they were exposed to the microflora of

pig intestine. According to the results, anthocyanin glycosides underwent significant changes, suggesting that the antioxidant or anticancer activities observed for anthocyanin glycosides are due to these degradation products [9]. Fast atom bombardment (FAB)-MS has been also used in the field of anthocyanins to evaluate different structural modifications. For instance, the antioxidant effect of several anthocyanin fractions isolated from bilberry extracts against pyridinium bisretinoid A2E (a prooxidant compound) was determined by this technology. According to the results, the majority of the anthocyanins were delphinidin 3-galactoside, cyanidin 3-galactoside, delphinidin 3-glucoside, and cyanidin 3-glucoside [10]. In orchid flowers, FAB-MS successfully identified eight pigments, of which two were new structures: 3-*O*-[6-*O*-(malonyl)-β-glucopyranoside]-7,3′-di-[6-*O*-(trans-synapoyl)-β-glucopyranoside] and its demalonyl derivative [8].

Regarding NMR techniques, they have been used to identify anthocyanins and study their exact structural characteristics, to establish their mechanisms of action, which can lead to a better application of these compounds as functional ingredients [12]. For example, the structure of two major acylated peonidin derivatives obtained from sweet potato were studied using NMR [14]. Another study evaluated the anthocyanins composition of chokeberry showing the presence of the structure of cyanidin galactoside and cyanidin arabinoside along the second stage of the fruit ripening [15].

Finally, high-performance liquid chromatography (HPLC) is the most widely used method for the identification and quantification of anthocyanins. In general, the analytical parameters used in the literature show very uniform conditions for the identification of anthocyanins. The most used column is C_{18}, while mobile phase composition mainly corresponds to water, acetonitrile, and methanol with acid modifiers, such as formic acid. The acid presence in mobile phases ensures that anthocyanin compounds are going to be mobilized in their cationic flavylium form, which has been described as possessing its highest absorbance, around 520 nm [23]. During the HPLC analysis of anthocyanins, as well as other compounds, the retention times and peak areas can be strongly influenced by the column temperature, mobile phase composition, or the complexity of the matrix in which they are embedded. The detection of anthocyanins is often performed by diode array detectors (DAD), mass-spectrometry detectors (both MS or MS/MS) which are, most of the time, coupled to an electrospray ionization source (ESI) [6,16]. These methodologies have been shown to provide satisfactory results for the identification and quantification of anthocyanins. Nevertheless, the use of ultra-high pressure liquid chromatography (UPLC) provided better resolution, shorter elution times, and lower consumption of mobile phases than conventional HPLC methodologies. UPLC also presents a high performance in the efficiency of the peaks of identification [23]. The anthocyanin profile of diverse vegetal samples has been evaluated by HPLC-DAD. For example, in grape, glucoside derivates of delphinidin, cyanidin, pelargonidin, peonidin, petunidin, and malvidin were identified [20]. Similarly, in grape skin samples, petunidin-3-*O*-glucoside and malvidin-3-*O*-glucoside were the major compounds [21]. HPLC-DAD can be also coupled to MS, which provides a more accurate identification, since mass information is considered in the analysis and data processing. HPLC-DAD-MS has been employed with different matrixes, such as strawberries, where cyanidin-3-*O*-glucoside, pelargonidin 3-glucoside, pelargonidin-3-*O*-rutinoside (tentative), pelargonidin-3-*O*-succinyl-glucoside, or pelargonidin-3-*O*-arabinoside were identified [22]. Regarding HPLC coupled to MS, this approach has been employed to analyze the anthocyanin composition of different samples. For example, cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, and perlagonidin-3-*O*-glucoside have been identified in *Euterpe edulis* extracts [24]. In strawberry, glucoside derivates of cyanidin, delphinidin, pelargonidin, and malvidin were identified. In muscadine grapes, 3,5-di-*O*-glucoside of cyanidin, delphinidin, and petunidin were identified as the major anthocyanins [25].

3. Validation of Methods

There are different approaches to develop a validation plan, depending on the type of technique used, the field of application of the method, and the type of samples analyzed. The scientific literature shows many examples of the development and optimization of methods for anthocyanins detection. However, when these techniques are validated, just few of them indicated the guideline used to perform this complex process.

3.1. Selectivity

To achieve a selective method, analytes are first isolated from another family of analytes or matrix interferences. Pre-treatment of anthocyanin samples includes the use of different techniques such as ultrasound or microwave-assisted extraction (UAE or MAE) or the use of solid phase extraction (SPE) cartridges. When methods for detecting anthocyanins were validated, no selectivity/specificity issues were found; the most selective instrument was ultra-high-performance liquid chromatography (UPLC) coupled to a photodiode array detector (PAD) or to mass spectrometry (UPLC-MS) against spectroscopic ones, according to the literature [6,26].

When analyzing anthocyanins together with non-anthocyanin compounds under similar conditions, resolution issues have been reported. In general, the most common option when using HPLC techniques is the selection of C_{18} columns and the modification of the mobile phase's acidity, by increasing the percentage of acid or by changing the type of acid [27]. However, other authors have also increased the resolution peak between anthocyanins and non-anthocyanin compounds by performing two different injections using C_{18} columns with different conditions [28]. The last option described is the use of a fluorinated C_{18} column, which has been demonstrated to provide better results in terms of peak separation, symmetry, and short analysis time [26].

3.2. Linearity, Limit of Detection (LOD), and Quantification (LOQ)

For anthocyanins, it has been scientifically demonstrated that they can be detected with calibration curves with ranges from 0.01 to 800 μg/mL, using different techniques. Validation studies in which calibration curves have been carried out with concentrations within these ranges have shown high linearity with an $R^2 \geq 0.99$. For example, Grace et al. (2019) validated an LC-MS method. All calibration curves showed good linearity in the range of 0.04–40 μg/mL, with a regression coefficient (r^2) $\geq$ 0.99 [6]. Fibigr et al. (2017) developed an UHPL-UV method, also achieving similar results [26]. There are few exceptions with low anthocyanin concentrations [6,26,29] or in some specific cases. For example, the quantification of malvidin-3-*O*-glucoside by a spectrophotodensitometry method required a polynomial adjustment instead of a linear one [30].

As mentioned before, anthocyanins have been extensively analyzed using different techniques such as HPLC-DAD, UPLC-DAD, UPLC-UV, HPLC-MS, or capillary zone electrophoresis (CZE), among others, but most of the validation studies have been performed using chromatographic techniques. According to the literature, the ranges of the limit of detection and limit of quantification that have been reported were 0.01–3.7 and 0.03–8.5 μg/mL (ppm), respectively, when using chromatographic-based methods, coupled to diverse detectors [6,26,31]. In general, chromatographic methods achieved good performance results as observed in the studies of Grace et al. (2019) or Fibigr et al. (2017). In the first one, the limits of detection and quantification were 0.06–0.40 μg/mL and 0.12–1.20 μg/mL, respectively [6]. On the second example, quite similar results were reported, with limits of detection and quantification being 0.11–0.14 μg/mL and 0.36–0.47 μg/mL, respectively [26].

3.3. Accuracy and Precision

In most of the validated methods carried out for anthocyanins, both accuracy and precision are determined by adding known amounts of standard solutions to the samples or by using commercial standards. In general, the results of validation studies for accuracy

were very good and the relative standard deviation for repeatability, and intermediate precision ranged from <1% to <10%, meaning that the methods are acceptable for possible routine use. Most of the validation studies employ chromatographic methods [6,26,29]. To cite an example, an LC-MS method has been recently validated. The results showed a good precision for all analytes tested; the relative standard deviation in intra and inter-day was less than 10% in both cases, while the reproducibility of all analytes was under 5% [6]. Thus, this method allowed characterizing simultaneously and selectively different anthocyanins and non-anthocyanidins, being a promising method for the analysis of these compounds.

3.4. Stability and Robustness

Regarding stability, several factors affect the stability of anthocyanins, such as their chemical structure, pH, light, or storage temperature and time, among others [23,32]. Minimal variations have been shown in studies of anthocyanin stability when these compounds are stored at low temperatures for long periods of time. However, rapid degradation processes were observed when matrixes or purified anthocyanins were stored at room temperature, as reported in the study of Gras et al., who employed UHPLC-PDA to evaluate anthocyanins from black carrot. In the study, significant losses of 8 to 14% were observed when standards (9 μg/L) were stored at room temperature for 24 h, showing that standard solution should be evaluated as soon as possible to avoid inaccurate results [23].

Regarding robustness, to our knowledge, few validation studies have evaluated these parameters. Nevertheless, studies in which it has been carried out displayed no significant differences in the total amount of anthocyanins extracted when the method was exposed to experimental variations such as changes in the pH, temperature, source, age and concentration of samples, variable standards, or solvents. This consistency in the results indicated that validated methods are robust and may be applied for the routine detection of anthocyanins [32,33]. For example, in the study of Canuto et al. (2016), the robustness of the developed reverse phase LC method was estimated introducing small variations in the mobile phase pH, column temperature, and mobile phase flow rate. According to their results, no significant results were detected in the determination of Cyn-3-glu and Plg-3-glu, demonstrating the robustness of the method [32].

4. Conclusions

There is a wide variety of identification and detection methods that have been demonstrated to be efficient for the analysis of anthocyanins. However, there is a lack of papers in which different methodologies are compared by using the same kind of samples. Therefore, it makes it difficult to conclude which method is more advantageous over others. On the one hand, the literature reviewed suggest that LC-MS represents a quick and efficient technique. However, it may present difficulties regarding the complexity of the sample matrix, which can cause ion suppression. Another issue is the determination of the best anthocyanin source. The variability of the experimental conditions, in terms of extraction protocols, analyzing parameters, and quantification, hinders the selection of the most appropriate matrix for obtaining the most efficient recovery of anthocyanins. Therefore, the standardization of extraction and analytical protocols may be critical to permit the real comparison of these experimental results.

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