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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-glycosidic bond between an aglycone and a sugar molecule. It is a group belonging to the flavonoids that occur naturally in various plant sources, such as fruits like berries or grapes and flowers like hibiscus, forming part of the secondary metabolites of plants. [1]. Therefore, the extraction of anthocyanins is usually carried out from plant matrices. These compounds possess certain beneficial properties such as a high antioxidant capacity due to the presence of phenolic hydroxyl groups [2,3]. In addition, a daily intake of this compound has a preventive and protective effect against cardiovascular diseases, diabetes and heart disease [4–7]. These compounds also possess coloring properties, being interesting natural colorants. So far, more than 20 structures are known, among which pelargonidin with an orange color, cyanidin and peonidin with orange-red colors, delphinidin with a blue-red color and malvidin and petunidin with a blue-red color are of greatest interest.

Natural dyes have several advantages, such non-toxicity and their acquisition has a low environmental impact. Hence, different industries have a great interest to identify new and economically viable sources of anthocyanins, to use them as new functional in-

gredients in food or as colorants [8]. As the range of application of these compounds increases, it is necessary to design efficient extraction methods with better yields, and also 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 also 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 ongoing for some time. This is due to the increasing human demand for natural products with health-promoting properties. Few techniques for identification and characterization of anthocyanins in different matrix have been employed. The most accurate analytical methods found can be seen in **Table 1**, including mass spectrometry (MS), nuclear magnetic resonance (NMR) and high-performance liquid chromatography (HPLC). Regarding MS, different methods have been employed to transform molecules into cations, like Electron Impact Mass Spectrometry (EI) and Fast Atom Bombardment (FAB)-MS. NMR techniques are used to identify isolated anthocyanins and to know their exact structural characteristics. This can be of great importance for understanding their stability in future food applications [9]. Finally, different HPLC-based techniques have been employed, such as HPLC- diode array detectors (HPLC-DAD-MS), HPLC-MS/MS and HPLC-ESI/MS [10,11]. All the methodologies have been demonstrated to provide satisfactory results for identification and quantification of anthocyanins, but, in general, HPLC-based techniques are the most employed along the literature.

Table 1. Different anthocyanins found in several plant matrices with different techniques

Identification technique	Source	Compounds	Ref.
MS; ESI-MS; FAB- MS	Black rice, orchids, bilberries	DEL, CYA, PET and MAL derivatives	[12,13]
NMR	Maqui berries, grapes, sumac, black currant, blue flowers	DEL, CYA, PET, MAL and PEO derivatives	[9,14,15]
HPLC; HPLC- DAD; HPLC-MS/MS; HPLC-ESI/MS	Blueberries, hibiscus, red cabbage	DEL, CYA, PET, PEO and MAL derivatives	[10,11]

Abbreviation: Delphinidin: DEL; Cyanidin: CYA; Petunidin: PET; malvidin: MAL; peonidin: PEO.

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.

3.1. Selectivity

Selectivity is the ability of a method to determine a specific target analyte(s) in a complex mixture without interference from other components present [16–18]. To achieve a selective method, analytes are first isolated from another family of analytes or matrix interferences [19]. In general, different techniques are applied to firstly remove matrix interferences and secondly separate the different classes of analytes. Pre-treatment of anthocyanin samples includes the use of different techniques such as ultrasound or microwave assisted extraction (UAE or MAE), the use of solid phase extraction (SPE) cartridges. The most selective instrument has been showed to be ultra-high performance liquid chromatography (UHPLC) coupled to a photodiode array detector (PAD) or to mass spectrometry (UHPLC-MS) against spectroscopic ones [10,20,21].

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' acidity, by increasing the percentage of acid or by changing the type of acid (S. Chen et al., 2013). 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 (Gonçalves et al., 2017). The last option described is the use of fluorinated C_{18} column, which has been demonstrated to provide better results in terms of peak separation, symmetry and short analysis time (Fibigr et al., 2017).

3.2. Linearity

This parameter involves other concepts directly related such as calibration curve and calibration or working range. Calibration curves are the basis for quantification methods. 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 $R^2 \geq 0.99$. There are few exceptions with low anthocyanin concentrations [10,20–25] or in some specific cases, for example malvidin-3-*O*-glucoside equired a polynomial adjust instead of linear one [26].

3.3. Limit of detection (LOD) and quantification (LOQ)

These terms are defined as the upper and lower limits between which it is reliable to determine the amount of analyte. As mentioned before, anthocyanins have been extensively analyzed using different techniques such as HPLC-DAD, UHPLC-DAD, UHPLC-UV, LC-MS or capillary zone electrophoresis (CZE), among others. When applying these methods, the ranges of LOD and LOQ values found in the validation studies for anthocyanins were 0.01-3.7 and 0.03-8.5 μg/mL (ppm), respectively [10,20,22,24,25,27].

3.4. Accuracy and precision

Accuracy is defined as the closeness between the actual value and the result obtained by the analytical test [28]. Precision is defined as the closeness of agreement between different results obtained under specific conditions [28,29]. In most of the validated method, both accuracy and precision are determined by adding known amounts of anthocyanin standards to the samples or by using commercial standards. The results for accuracy were very good and the relative standard deviation for repeatability and intermediate precision ranged from <1% to <10%. This means that the methods are acceptable for possible routine use [10,20–25,30,31].

3.5 Stability

The stability is the ability of a matrix to maintain its physicochemical properties, especially the analyte concentration, during different process such transport or storage [28,29]. Several factors affect the stability of anthocyanins, such as their chemical structure, pH, light or storage temperature and time, among others [21,30]. Minimal variations have been shown in studies of anthocyanin stability when these compounds are stored at low temperatures for long periods of time, but rapid degradation at room temperature occurs in the results when stored at low temperature [20,23,24,30,31].

3.6 Robustness

This term indicates to what extent a method is affected by the potential source of variation, such as the pH, temperature, source, age and concentration of samples, standards or solvents [28,29]. When robustness studies were performed, no significant differ-

ences in the total amount of anthocyanins extracted were not recorded when small variations of the method were introduced (for example, variations in the pH, temperature, source, age and concentration of samples, standards or solvents), indicating that the validated methods are robust and could be applied in routine-use [23,30].

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 comparing different methodologies for the same sample, which makes difficult to conclude which method has more advantages over others, as they are different type of samples, with different amounts of anthocyanins in the starting material.

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