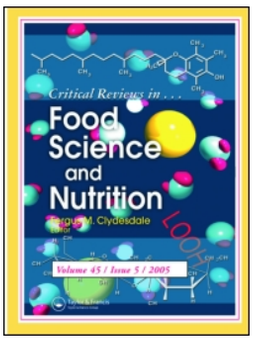

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Anthocyanin extraction from plant tissues: A review

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ABSTRACT

Anthocyanins have gathered the attention of the scientific community mostly due to their vast range of possible applications. They have been the center point of the research in many different fields, among which is food development, where their innate coloring, antioxidant capacity, and biological potential open interesting venues to the development of new food additives and functional foodstuffs. As the range of application grows, so does the necessity to obtain these compounds, and since they are naturally occurring, the most common way to obtain anthocyanins is to extract them from different plant sources, such as fruits and flowers. Several efforts have been made to develop methods that allow for better extraction yields and higher purification rates therefore this review aims to compile the information regarding extraction and purification procedures in a comprehensive manner.

KEYWORDS

Tissue pretreatment; solid–liquid extraction; modern extraction methods; anthocyanin purification

Introduction

Anthocyanins are naturally occurring, water soluble, flavonoid pigments, structurally composed by the α- or β-linkage of an anthocyanidin to a sugar moiety (Castañeda-Ovando et al., 2009; Vermerris and Nicholson, 2007). In later years, they have gathered the attention of both the scientific and industrial communities due to their vast range of possible applications. Reports have been made about their potential for the development of better industrial colorants, health promoting foods, supplements and also on the improvement/development of solar-based, renewable energies (Buchweitz et al., 2013; Chien and Hsu, 2013; Norberto et al., 2013; Stintzing et al., 2006; Wallace and Giusti, 2013; Wu et al., 2011). As anthocyanins are frequently found as secondary plant metabolites, it stands to reason that one of the main approaches used in their production is through extraction and isolation from plant tissues. However, the innate characteristics of these matrixes (e.g. uneven compound distribution and high enzymatic activity) may hinder the extraction process as they can difficult the diffusion of compounds, allow for simultaneous extraction of other compounds and even lead to artifactual changes in compounds. As such, the selection of an adequate extraction method gains particular relevance (Antolovich et al., 2000).

Solid–liquid extraction (SLE) is the classic approach used to recover anthocyanins from plant tissues as their polarity facilitates dissolution into polar solvents, e.g. acidified methanol/ethanol and acetone (Antolovich et al., 2000; Barnes et al., 2009; Burdulis et al., 2009; Castañeda-Ovando et al., 2009; Galván D'Alessandro et al.; Naczk et al., 2006; Wallace and Giusti, 2013). With the advent of the green revolution and the increase in the demand for these compounds new, technologically advanced approaches are being pursued to better extract anthocyanins. Some of these emergent technologies include supercritical extraction, pulsed electric field (PEF)-assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, and pressurized liquid extraction (PLE) (Armenta et al., 2008; Galván D'Alessandro et al., 2014; Garofulić et al., 2013; Ghafoor et al., 2010; Golmohamadi et al., 2013; Huie, 2002; Junior et al., 2010; Liazid et al., 2011; Paes et al., 2014; Paula et al., 2014; Petersson et al., 2010; Puértolas et al.; Seabra et al., 2010; Segovia et al.; Truong et al., 2012; Vatai et al., 2009). However, it is difficult to compare the extraction yields of different methods since compound variation from matrix to matrix hampers comparisons. With that in mind, the present review seeks to analytically compile the available information in an effort to facilitate a better understanding regarding each methodology's potential.

Anthocyanins in plants

Anthocyanins are found deposited inside the vacuole of cells in several different tissues such as leaves, petals, fruits, bulbs, rhizomes, or stems (Gould et al., 2008; Stintzing and Carle, 2004). However, when looking at their presence within a given tissue, the accumulation of anthocyanins may vary. For instance, while some leaves are all red (fully colored by anthocyanins) others exhibit this color in restricted sections such as the margins distribution in a given organ. Similarly, in some plants leaves are red only when they are growing, stressed or senescent, while in others they are red throughout their lives. These variations (time, tissue, placement, and inducibility) difficult the establishment of a unified explanation for the presence of anthocyanins in tissues (Gould et al., 2008).

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Several authors have proposed different roles for anthocyanins. In general, anthocyanin accumulation has been found to be upregulated in the presence of biotic and abiotic stressors such as high/low temperatures, strong light and UV-B radiation, drought, nutrient deficiencies, bacterial or fungal infections, etc. (Chalker-Scott, 1999; Feild et al., 2001; Gould et al., 2008; Neill et al., 2002; Oberbaueri and Starr, 2002; Steyn et al., 2002). Therefore, anthocyanins have been regarded as both a symptom and a coping mechanism of plants, when under stressful environments, though some authors also report their involvement in the photosynthetic process (Gould et al., 2008).

Anthocyanins and food

Nowadays, with the rise of consumer awareness regarding the need for healthy eating habits, the industry has strived to find alternative, natural, sources of additives that, while being completely safe, may also bring some sort of health benefits. Considering this, anthocyanins may be an interesting group of compounds. They can be found in several fruits and vegetables (berries, beetroot, red cabbage, pomegranate, grape, and black carrot among others) therefore are safe to consume. Several authors have reported on the antioxidant and antimicrobial potential of anthocyanins, two properties that may aid in the stabilization of foodstuffs and in increasing their shelf life. In addition, given their coloring, they are an interesting natural alternative to red/purple food colorants. Furthermore, they may also bring some health benefits as several authors reported on the potential biological benefits of anthocyanins (Table 1).

Anthocyanin basic chemistry

One of the main issues with the manipulation of anthocyanins is that they are highly susceptible to degradation, particularly when isolated. Several physicochemical factors are known to interfere with anthocyanin stability, therefore knowledge of basic anthocyanin chemistry is required in order to better understand the limitations of a specific extraction protocol (Table 2) (Adams, 1973; Cabrita et al., 2000; Fossen et al., 1998;

Table 1. Biological properties associated with anthocyanins.

Biological activity	References
Protection against oxidative damage	(Carvalho et al., 2015; Ghiselli et al., 1998; Kong et al., 2003; Tedesco et al., 2001)
Anti-edema activity	(Kong et al., 2003)
Anti-inflammatory activity	(Carvalho et al., 2015; Decendit et al., 2013; Kong et al., 2003)
Improved platelet function	(Alvarez-Suarez et al., 2014; Kong et al., 2003)
Antitumoral activity	(Chen et al., 2006; Kamei et al., 1998; Kang et al., 2003; Koide et al., 1997; Kong et al., 2003);
Antimutagenic activity	(Kong et al., 2003; Yoshimoto et al., 1999)
Stabilization of blood sugar levels	(Kong et al., 2003; Törrönen et al., 2012)
Reduction of liver lesions	(Chen et al., 2015a; Kong et al., 2003; Morrison et al., 2015; Obi et al., 1998; Wang et al., 2000)
Inhibition of nitric oxide radicals	(Cristoni and Magistretti, 1987; Kong et al., 2003; Wang and Mazza, 2002)
Antiulcer activity	(Cristoni and Magistretti, 1987; Kong et al., 2003)
Reduction of insulin resistance	(Tsuda et al., 2003)
Attenuation of lipid accumulation	(Hwang et al., 2011; Tsuda et al., 2006)
Antimicrobial activity	(Burdulis et al., 2009; Lacombe et al., 2012; Silva et al., 2015)

Table 2. Main advantages and disadvantages of the different extraction methods.

	Advantages	Disadvantages
Solid-Liquid Extraction (SLE)	Simple protocol No need for specific equipment Can be food grade (Freedman, 1980a; Mussinan and Keelan, 1994)	Relatively long extraction times (Cacace and Mazza, 2002, 2003a, b) High solvent consumption (Garcia-Viguera et al., 1998) Acid, air and light exposure may cause anthocyanin degradation (Vatai et al., 2009)
Supercritical Fluid Extraction (SFE)	Allows the removal of nonpolar interferents (Seabra et al., 2010; Vatai et al., 2009)	High associated costs (Junior et al., 2010)
	Reduced exposure to light and air (Vatai et al., 2009)	Mandatory use of a polar solvent (Ghafoor et al., 2010; Seabra et al., 2010)
	Extracts are Generally Recognized as Safe (GRAS) (Ghafoor et al., 2010)	In some cases, similar yields may be achieved using SLE (Vatai et al., 2009)
	Inhibits enzymes that cause anthocyanin degradation (Seabra et al., 2010)	Requires CO_2 (Seabra et al., 2010; Vatai et al., 2009)
Ultrasound-Assisted Extraction (UAE)	Low solvent consumption (Carabias-Martínez et al., 2005; Golmohamadi et al., 2013; Huie, 2002)	High associated costs (Vieira et al., 2013)
	Low energy consumption (Golmohamadi et al., 2013; Huie, 2002)	Energy dissipation through heat may cause anthocyanin degradation (Golmohamadi et al., 2013; Tiwari et al., 2008)
	Easy to scale up (Galván D'Alessandro et al., 2014; Golmohamadi et al., 2013; Vieira et al., 2013)	
	Safe for consumption (Galván D'Alessandro et al., 2014; Golmohamadi et al., 2013; Vieira et al., 2013)	
Pressurized Liquid Extraction (PLE)	Low solvent consumption (Carabias-Martínez et al., 2005; Paes et al., 2014; Petersson et al., 2010; Santos et al., 2012)	High temperatures used may cause anthocyanin degradation (Petersson et al., 2010)
	Low extraction time (Carabias-Martínez et al., 2005; Santos et al., 2012)	
	Automation (Carabias-Martínez et al., 2005)	
Microwave-Assisted Extraction (MAE)	Low extraction time (Armenta et al., 2008; Garofulić et al., 2013)	Temperature may cause anthocyanin degradation (Armenta et al., 2008; Garofulić et al., 2013; Liazid et al., 2011)
	Low solvent consumption (Armenta et al., 2008; Garofulić et al., 2013)	
	Good reproducibility (Armenta et al., 2008; Garofulić et al., 2013)	

Furtado et al., 1993; Kader et al., 1998; Mori et al., 2007; Seeram et al., 2001; Tanchev and Joncheva, 1973; Wesche-Ebeling and Montgomery, 1990; Zhang et al., 2007).

Though several factors that affect anthocyanins' chemical stability could be mentioned, pH and temperature stand as the most referred. Typically, anthocyanins are more stable under acidic conditions with pH values above 7 leading to their degradation (Castañeda-Ovando et al., 2009; Fleschhut et al., 2006; Seeram et al., 2001). This explains why most extraction protocols require the presence of an acidified environment, though strong acidic media may promote the hydrolysis of the glycoside bonds. Therefore, pH control stands as a relevant extraction variable with a considerable impact upon the quality of the extracted anthocyanins. Temperature, another determinant factor for anthocyanin stability, is frequently considered an extraction factor; therefore this factor is discussed more in depth in the classic methods section.

Though relatively unstable when in solutions, anthocyanins may be stabilized through the addition of some compounds such as co-pigments, metallic ions or even other antioxidants. The protection granted by antioxidant compounds is the easiest to grasp; antioxidants that are more susceptible to oxidation than anthocyanins will be oxidized before them. Co-pigments (systems rich in π electrons) and metallic ions such as Al, Fe, Mg, Mo and Sn will interact directly with the anthocyanins hampering nucleophilic attack and the oxidation of the quinoidal base, respectively (Castañeda-Ovando et al., 2009; Shaked-Sachray et al., 2002). The decision to add any compound, despite their potential as stabilizers, must be made with its expected application in mind.

Pretreatment of tissues

Anthocyanins are frequently contained within intracellular organelles; therefore their accessibility is dependent on the solvents' capacity to enter these structures and their integrity. Therefore pretreating the tissues in order to facilitate extraction could be an interesting approach to increase extraction efficiency, though any procedure must be made considering the limitations imposed by the chemical nature of the anthocyanins themselves.

Reducing particle size

Turning the samples into a powder/pulp is a common approach, as the reduction of particle size and consequent increase in contact area promotes the diffusion from the solid particles into the solvent, furthermore the sheer stress may induce some damage to the organelles that will allow for better solvent permeability (Dutta, 2007). Despite the fact that this is a relatively inert way to enhance the extraction yield, in a powder or in a pulp anthocyanins are significantly more exposed to oxidizing agents and therefore more prone to degradation. Thus, to avoid oxidation, several authors prefer to directly either homogenize the tissue with the solvent in the beginning of the extraction process or freeze the fruits (sometimes with liquid nitrogen, for avoid tissue decomposition) before turning them into a pulp (Antolovich et al., 2000; Barnes et al., 2009; Burdulis et al., 2009; Cacace and Mazza, 2002, 2003a, b; Vrhovsek et al., 2012). As freezing induces some cellular damage, the addition of a freezing step may further aid in the extraction of the compounds (Pearce, 2001).

Pulsed electric field pre-treatment

Pulsed electric field (PEF) has gained increasing interest as a pretreatment of samples, as it improves mass transfer without using high temperature (Segovia et al.). In its place, the matrix is exposed to external, moderate (1 to 10 kV/cm and 10 kJ/kg), pulsed (generally 5 to 50 pulses) electric fields that induce the electroporation of cell membranes, thus increasing membrane permeability and facilitating the extraction process, with authors describing yields up to 2.12 times higher for anthocyanins (Corrales et al., 2009; Gachovska et al., 2010; Puértolas et al.; Segovia et al.). However, despite the potential benefits of this approach (non-thermal treatment that is food safe), the pulsed field induces some degradation of anthocyanins, to chalcone and other pseudobases though, to the best of our knowledge, the reactions behind these transformations are not described (Odriozola-Serrano et al., 2009; Zhang et al., 2007). Zhang et al. (2007) reported that, for cyanidin-3-glucoside, higher intensity fields induced faster degradation rates than lower intensity fields and caused a significant reduction in the half lives and D values of the compound when compared to exposure to 45°C, thus demonstrating that the intensity of the electric field is a key factor to control when utilizing this approach.

Though several solvents can be used in the subsequent extraction, the most common is water, as this allows for the reduction of the cost and environmental issues associated with the usage of other solvents. This, combined with PEF's capacity to increases anthocyanin stability, enhances the appeal of this method (Puértolas et al.; Segovia et al.; Zhang et al., 2007).

Enzymatic-assisted extraction

In plants, anthocyanins can be found inside the cellular vacuole (Gould et al., 2008). This implies that, in order to extract the pigments the solvents must be able to transpose the cell wall, membrane, cytoplasm and the vacuolar membrane before reaching the anthocyanins. Therefore, the use of enzymatic cocktails that disrupt the cell wall network should facilitate the removal of anthocyanins from vegetable cells, particularly from matrixes that have thicker cell walls and higher amounts of pectin (Buchert et al., 2005). There are several, commercially available, blends of enzymes that can be used in the extraction of anthocyanins, most products are comprised of several enzymes, such as pectinases and cellulases, in various ratios (Buchert et al., 2005; Landbo and Meyer, 2001). Buchert et al. (2005) reported a significant increase in anthocyanin yield (13–41% in blueberry juice and 18–29% in black currant juice) when *Pectinex Ultra SP-L, Pectinex Smach, Pectinex BE 3-L* and *Biopectinase CCM* were used to produce juice. However, when using *Econase CE*, in blueberry juice, these authors reported a significant decrease in anthocyanin yield. Similar results have been reported by Landbo and Meyer (2001), who reported that the use of several enzymatic blends to extract anthocyanins from black currant residues either had no impact (*Pectinex BE* and *Novozym 89*) or caused a significant reduction (*Macer8 FJ* and *Macer8 R*) in anthocyanin yield. A possible explanation for this phenomena has been hypothesized by several authors; the

enzymes may be hydrolyzing the anthocyanins to their aglycone counterpart (Buchert et al., 2005; Wightman and Wrolstad, 1995; Wrolstad et al., 1994). This is supported by the results of Buchert et al. (2005), who found that, when using enzyme blends with high galactosidase activity, there is a considerable reduction in the amount of galactosides found when in comparison to the untreated counterpart (Buchert et al., 2005).

Classic approach to anthocyanin extraction

The classic approach used to extract anthocyanins from plant tissues is the same one that is used for other phenolics, i.e. the tissues (treated or not) are soaked with subsequent solvent extraction, in a process known as SLE. Several parameters have been reported to affect SLE yield, the most commonly reported in literature are solvent type and temperature, though several others may be mentioned such as extraction time, particle size, solvent/mass ratio (Castañeda-Ovando et al., 2009; Vrhovsek et al., 2012). These last parameters vary mostly regarding general mass transfer principles and their manipulation yield is rarely associated with chemical changes. The smaller the particle the easier it will be for a solvent to be able to permeate it and extract the desired compounds (Dutta, 2007). With the increase of the contact time between solvent and tissue, the more complete shall be the diffusion from solid particle to liquid until partition equilibria is reached (Dutta, 2007). The time frame in which equilibria is reached varies according to several other conditions (e.g. temperature, solvent), therefore the extraction time should be determined for each extraction as the other extraction conditions are defined (Cacace and Mazza, 2002, 2003a, b). As for the solvent/mass ratio the foremost thing that must be taken into account is the solubility of the desired compounds, ideally the amount of solvent added should be just enough to dissolve the desired compounds (Rostagno and Prado, 2013). However, as other compounds are extracted, frequently a higher amount of solvent is frequently necessary.

Another approach used by some authors to increase anthocyanin yield is to employ multiple extraction steps for a given tissue. Theoretically, the use of several extraction steps should allow for a more complete extraction though that is not always so (Revilla et al., 1998). Revilla et al. (1998) studied the effectivity of several extraction processes among which were four that considered several sequential extractions having found that, multiple extractions did not necessarily mean higher extraction yields.

Solvent

Most of the common solvents used in SLE are polar, employing methanol, ethanol, acetone and even water acidified with a vast array of both organic and inorganic acids (Barnes et al., 2009; Metivier et al., 1980; Vatai et al., 2009). In the 80's, Metivier et al. (Metivier et al., 1980) studied the effect of different solvent (methanol, ethanol and water) and acid (HCl, citric, acetic, propionic, tartaric and formic acid) combinations upon the extraction of anthocyanins from wine pomace having found that, on its own, methanol was the best performing solvent (extracting 20 and 73% more anthocyanins than ethanol and water, respectively). Though the type of acid used affected each solvent differently, methanol combined with citric acid appeared to be the best combination for extraction. Barnes et al. (Barnes et al., 2009), when studying the extraction of anthocyanins from blueberry samples observed a somewhat different behavior with nonacidified ethanol and acetone proving to be significantly more effective than methanol, isopropanol and acetonitrile in the extraction of anthocyanins. Overall, the acidification of the solvent led to the improvement of the extraction yield though the effect was, once again, acid dependent, the addition of trifluoroacetic acid (TFA) led to a significant increase of the anthocyanin yield for all solvents except acetone (Barnes et al., 2009). The ratio water:organic solvent is also an interesting variable to note as it varies with the solvent and the composition of the extraction tissue. For instance, in cabernet grapes decreasing the acetone:water ratio lead to lower yields of anthocyanins, while doing the same with ethanol had the opposite effect. On the other hand, for merlot grapes there was no apparent relationship between the water:solvent ratio and the extraction yield (Vatai et al., 2009). The differences observed between the efficacy of a given solvent/acid combination may be explained by the differences in anthocyanin composition in each source. Regardless, it is interesting to note that, while the most widely used extraction protocol uses methanol acidified with HCl, to the best of our knowledge, when different acids were tested, the combination methanol-HCl was not the best extractant (Barnes et al., 2009; Metivier et al., 1980).

Although acid addition appears to help the extraction process, the use of strong acids may cause the hydrolysis of the glycosidic bond, yielding an anthocyanidin and a sugar moiety (Castañeda-Ovando et al., 2009; Kapasakalidis et al., 2006). This was observed by Kapasakalidis et al. (Kapasakalidis et al., 2006) when, in an attempt to improve the phenolic content of black currant extracts, they employed an acid hydrolysis (using HCl 2M), and instead of obtaining some of the anthocyanins that were found in the original extracts, they found only their anthocyanidin counterpart. Besides the extraction protocol itself, the presence of strong acids may also pose a problem for the further processing of extracts. This is particularly true if a concentration step is required as, while the anthocyanin concentration in a solution may rise from the removal of the extraction solvent, so does the concentration of acid and thus the hydrolysis of anthocyanins may take place or be extended (Garcia-Viguera et al., 1998).

Given the possible acid mediated hydrolysis, some authors prefer to work with sulfured solutions using compounds such as SO_2 as extractants (Cacace and Mazza, 2002, 2003a; Castañeda-Ovando et al., 2009). In fact, this approach can even be more effective than the traditional alcohol/acid mix. Cacace and Mazza (Cacace and Mazza, 2002, 2003a, b) reported that a concentration of 1000-1200 ppm of SO_2 allowed for the maximum yield of total phenolics and anthocyanins and that, at low temperatures, the extraction rates for anthocyanins are significantly higher than those observed for an ethanolic solution. However, despite the advantages presented this solution also poses some limitations that must be considered. For instance, in the production of edible extracts, the presence of SO_2 while not be critical from a food safety standpoint as it is a known preservative, it can induce adverse reactions in hypersensitive

individuals (Freedman, 1980a, b; Li and Zhao, 2006; Mussinan and Keelan, 1994). In addition, if seeking to produce antimicrobial extracts the activity observed may not be due only to the extracted compounds as sulfured compounds have been known to exhibit some antimicrobial activity (Kyung and Fleming, 1997).

Temperature

Heat sensitivity is an ubiquitous characteristic of anthocyanins. Therefore, when contemplating an extraction protocol this condition must be taken into account. Wang and Xu (Wang and Xu, 2007) and Aurélio et al. (Aurelio et al., 2008), when studying the impact of heat upon blackberry and hibiscus pulps, found that heating to 90°C lead to ca. 80% reduction of the half-life of anthocyanins thus yielding significantly lower levels of anthocyanins after a six-hour period (Cacace and Mazza, 2002, 2003a, b; Castañeda-Ovando et al., 2009; Vrhovsek et al., 2012). The sensitivity of anthocyanins increases when they are present in extracts. Sadilova et al. (Sadilova et al., 2007) found anthocyanin extracts to be much more susceptible to degradation as they detected no anthocyanins in purified strawberry and elderberry extracts after a six-hour exposure to 95°C. This difference between extract and tissue stability is also evident in the work of Cacace and Mazza (Cacace and Mazza, 2002, 2003a, b), who reported that, for blackcurrants, 30–35°C appeared to be the best extraction temperature for anthocyanins, with higher temperatures resulting in lower anthocyanin contents, a likely consequence of heat induced degradation. In addition, Vatai et al. (Vatai et al., 2008) reported that aqueous acetone solutions extraction procedures carried out at 20°C yielded significantly higher results than those carried out at 60°C, the exception being for pure acetone but, as 60°C is above the boiling point for this solvent it is possible that some artifacts occurred.

Modern technologies applied to anthocyanin extraction

Given the widespread interest in plant metabolites, there has been an effort to modernize the extraction protocols. With improvements from the classical approach ranging from a reduction of the amount of organic solvents used and less exposure to reducing agents to a decrease of the need for purification and concentration steps, overall improvement of the extraction yield, selectivity, and/or kinetics (Huie, 2002). Several new methodologies have emerged and, though their potential benefits cannot be overlooked, it is important not to disregard the target compounds' characteristics in lieu of new technologies.

Supercritical fluid extraction

From a simplistic standpoint, supercritical fluid extraction (SFE) is a process in which supercritical fluids (at vapor-liquid critical point) are used to extract the components of interest from a solid or even liquid matrix. Several advantages can be listed in order to reason the usage of this approach: (i) the pretreatment of samples with supercritical CO_2 ($scCO_2$) removes nonpolar components reducing the amount of interferents though, contrary to what happens for polar polyphenolics, it's usage doesn't increase the availability of anthocyanins (Seabra et al., 2010; Vatai et al., 2009); (ii) the absence of atmospheric O_2 and light during the extraction process reduces anthocyanin oxidation (Vatai et al., 2009); (iii) SFE extracts are generally recognized as safe (GRAS) and therefore their considered safe to use as food additives (Ghafoor et al., 2010); (iv) the use of $scCO_2$ and pressure, inhibits native enzymes that degrade anthocyanins (Seabra et al., 2010). The major drawback of this approach is related with its production costs. Currently, this method is associated mainly with the production of extracts that comply with strict environmental regulations or with high value products (Junior et al., 2010).

As traditional SFE is performed using only $scCO_2$, which extracts nonpolar compounds, to extract anthocyanins (polar molecules) a polar solvent must also be used. In fact, the choice of the solvent may be a key factor for the success of the extraction procedure. The most common approach uses a mix of CO_2, ethanol and in some cases water, with proportions varying according to the tissue and/or plant material (Ghafoor et al., 2010; Seabra et al., 2010). The ethanol/water fraction must be in proportions where they are soluble in $scCO_2$, at the pressure and temperature used, so the mixture can be considered supercritical. However, higher amounts may be used. In those cases two phases coexist (liquid and supercritical) but their presence may affect the yield and recovery of target compounds. (Paes et al., 2014). It is interesting to note that, contrary to what happens in the SLE, the acidification of the solvents has been described as having no significant effect upon the extraction efficiency in SFE (Paes et al., 2014). As the water contained within the extraction matrix, when in contact with CO_2, results in the formation of carbonic acid (which lowers the pH) the addition of acid is, somewhat redundant (Junior et al., 2010; Paes et al., 2014).

Ghafoor et al. (2010) studied the extraction of anthocyanins from grape peel using SFE. They reported that, for this matrix, the optimal SFE conditions were 45°C and 16 MPa in the presence of 6–7% ethanol. These conditions were similar to those reported by Seabra et al. (2010) for the extraction of anthocyanins from elderberry pomace, and those reported by Paes et al. (2014) for blueberry residues, 40°C and 20–21 MPa using aqueous ethanol as a co-solvent. As anthocyanins are sensitive to relatively high temperatures, it is interesting to note that most authors that focus on these compounds do not employ temperatures above 40°C, though higher temperatures have been used in the extraction of other phenolic compounds (Ghafoor et al., 2010; Huie, 2002; Paes et al., 2014; Paula et al., 2014; Seabra et al., 2010; Vatai et al., 2009)

Vatai et al. (2009), compared the efficacy of conventional SLE against SFE both with and without a pretreatment using supercritical CO_2 (to remove nonpolar compounds and purify the sample). They found that for Refosk and Cabernet grape pomace, similar results may be obtained when comparing the results of SLE using acetone or ethanol against SFE performed using ethanol as a co-solvent at a pressure of 15 or 30 MPa and a temperature of 40°C.

Ultrasound-assisted extraction

In ultrasound-assisted extraction (UAE), the ultrasound frequencies are capable of facilitating the hydration of plant materials which leads to the enlargement of cell wall pores and

occasionally, cause the rupture of the cell wall. This will promote mass transfer, therefore allowing for the increase of the extraction yield (Golmohamadi et al., 2013; Huie, 2002). The ultrasound-assisted extraction has several advantages. It requires reduced amounts of solvents, it does not require CO_2 and has a relatively low energy consumption (Galván D'Alessandro et al., 2014; Vieira et al., 2013). The fact that it is a green approach, that it is relatively easy to scale up and that it is safe for Human intake, makes it a particularly interesting technique for the food industry (Galván D'Alessandro et al., 2014; Golmohamadi et al., 2013; Vieira et al., 2013). However, the production costs are relatively high, with other alternatives allowing for similar yields with lower costs (Vieira et al., 2013).

Ultrasound frequencies, a major extraction factor, can be divided into two bands, low power ultrasound (low amplitude and high frequency, 100–1000 kHz) or high power ultrasound (high amplitude and low frequency, 20–100 kHz) (Galván D'Alessandro et al., 2014; Golmohamadi et al., 2013). The last is used in food processing and cleaning processes as it allows for the formation of cavitation bubbles, whose vibration creates fluid currents and disruptive forces on nearby cells and particles (Golmohamadi et al., 2013). However, the cavitational phenomena when coupled with the microjets of fluid and the asymmetrical collapse of air bubbles near the surface of the cell wall (all caused by the ultrasound) cause an increase in temperature that, in cooled reactors, may reach up to 70°C (Golmohamadi et al., 2013). This may compromise anthocyanin stability as shown by the work of Tiwari et al. (2008), who demonstrated that exposure to an intermittent frequency of 20 kHz for a 10 min period reduced the anthocyanin content of strawberry juice by 3.2%. (Aurelio et al., 2008; Golmohamadi et al., 2013; Tiwari et al., 2008; Wang and Xu, 2007). As vibrational energy is dissipated as heat during the extraction procedure and anthocyanins are notorious for their sensitivity to heat, it stands to reason that the extraction time is a particularly important variable (Galván D'Alessandro et al., 2014; Golmohamadi et al., 2013). Golmohamadi et al. (2013) reported that, for 20 kHz, extraction times above 10 minutes caused a reduction of the total anthocyanin content in raspberry puree. However, Vieira et al. (2013) reported a steady increase of total monomeric anthocyanins throughout a 180 min period when extracting anthocyanins from jussara pulp at 40 kHz. This demonstrates the need for matrix contextualization and the development of specific protocols for each matrix.

Pressurized liquid extraction

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), allows for the fast extraction of compounds with little solvent consumption (Carabias-Martínez et al., 2005; Paes et al., 2014; Petersson et al., 2010; Santos et al., 2012). In this methodology, high pressure is used to maintain the solvents liquid at higher temperatures (frequently temperatures above solvent boiling point), this allows for the improvement of the solubility of the compounds, sample wetting and matrix penetration (Huie, 2002; Petersson et al., 2010). Immediately, the use of this technique for the extraction of anthocyanins raises concerns. In fact, as illustrated by the work of Petersson et al. (2010), degradation begins almost as the extraction itself begins. For it to be beneficial, a positive balance must be struck between the degradation and extraction kinetics. The sample composition, particularly anthocyanin profile, will be very important in the decision of whether this methodology is of interest, particularly as the possibility of process automation makes this technique particularly appealing for industrial application (Carabias-Martínez et al., 2005; Castañeda-Ovando et al., 2009; Petersson et al., 2010). Despite its limitation regarding anthocyanin extraction, this technique has been used in several food matrixes such as blueberries, red onion and sweet potato, though to the best of our knowledge, few articles contemplate the degradation kinetics (Paes et al., 2014; Petersson et al., 2010; Truong et al., 2012) has been released.

Microwave-assisted extraction

Microwave-assisted extraction (MAE) is a process that uses microwaves to quickly, efficiently and evenly heat both solvent and target tissue. In hydrated tissues, the water present absorbs the energy with the resulting heat causing cell disruption (Garofulić et al., 2013). This technique allows for lower extraction times, requires less solvents and exhibits a good reproducibility (Armenta et al., 2008; Garofulić et al., 2013). On the other hand, the enhancement of compound diffusion form matrix to solvent, while potentially increasing the extraction yield, also facilitates the extraction of nontargeted compounds (Armenta et al., 2008; Garofulić et al., 2013; Liazid et al., 2011). In addition, the usage of temperature in anthocyanins, as argued before, has significant limitations. Generally, and also to avoid overheating, low to moderate powers (coupled with longer extraction times) are used (Garofulić et al., 2013). However, this does not necessarily prevent anthocyanin degradation. Garofulić et al. (2013) reported that as temperature and/or irradiation time increases, the amount of anthocyanins detected on cherry extracts decreased significantly. While, for grape skin peels, Liazid et al. (2011) reported that, up to 100°C, there was a significant increase in the amount of anthocyanins found in the extracts, with the values reducing only for temperatures above 100°C. These authors report results that are somewhat contrary, but, in both cases, the need to find a suitable extraction temperature (where the increase in extraction kinetics compensates the degradation reactions) is clear, as is the importance of contextualizing it with the tissue that constitutes the matrix (Liazid et al., 2011; Pap et al., 2013).

Ohmic heating-assisted extraction

Ohmic heating-assisted extraction (OHM), also known as electroconductive heating, uses the inherent electrical resistance of a given foodstuff in order to turn electrical energy into thermal energy, which will increase membrane permeability (Loypimai et al., 2015). Though not extensively used for anthocyanin extraction, some work has been reported (Loypimai et al., 2015). Though, the rapid heating process has been described as allowing for less thermal damage, Sarkis et al. (2013) reported that ohmic heating caused a similar degradation as conventional heating. As such, more work is needed to better ascertain whether ohmic heating is interesting when considering anthocyanin extraction (Sarkis et al., 2013).

Purification approaches

The extraction methodologies used to extract anthocyanins from plant tissues are not selective. In fact, the final product is, frequently, a solution that has large amounts of other compounds (e.g. sugars and organic acids) that, besides introducing a bias in functionality studies, can also be detrimental for anthocyanin stability (Castañeda-Ovando et al., 2009; He and Giusti, 2011; Jampani et al., 2014). Sugars are a prime example of this, as free sugars and their degradation products may lead to Maillard reactions (Jampani et al., 2014; Tsai et al., 2005). Therefore, after contemplating the extraction process, the removal of interferents stands as an important step, particularly in the study of anthocyanin properties as the high cost of standards usually are detrimental for their usage (Castañeda-Ovando et al., 2009; He and Giusti, 2011). Several techniques have been proposed to achieve anthocyanin separation from solid phase extraction (SPE) to sophisticated chromatographic techniques (Castañeda-Ovando et al., 2009).

Precipitation of anthocyanins

The use of bivalent lead (Pb) to precipitate anthocyanins in aqueous solutions is one of the oldest approaches for anthocyanin purification. However, anthocyanins are not the only group of compounds that form low solubility lead salts, in fact other compounds with a carboxyl or other nucleophilic group (e.g. amino, phenolic, fatty and organic acids, tannins, flavonoids, etc.) are also precipitated by Pb^{2+}(Fuleki and Francis, 1968; Maekaji et al., 1963; Mattick et al., 1969). Therefore, this method stands more as a preliminary purification step, particularly as the most plentiful impurities, sugars, are not precipitated in this approach (Fuleki and Francis, 1968).

Given the amphoteric nature of the pigments, the reaction between anthocyanin and Pb^{2+} is pH dependent, with neutral and alkaline environments exhibiting better precipitation activity than their acidic counterparts (Fuleki and Francis, 1968; Mattick et al., 1969). Care must be taken when considering the pH value to be used as different anthocyanins exhibit different optimal precipitating pH and, therefore, some may remain in the original solution

Anthocyanin lead salts can be dissociated using an alcoholic solution (with either HCl or H_2SO_4) that will cause the precipitation of a lead salt that can be removed, yielding an alcoholic suspension of anthocyanins (Fuleki and Francis, 1968; Maekaji et al., 1963).

Solid phase extraction

Solid phase extraction is a separation process in which, dissolved compounds are separated according to their physicochemical characteristics. Traditional SPE uses adsorbent sorbents, such as C18, Sephadex, or Amberlite adsorption resins that establish bonds through their hydroxyl groups or hydrophobic bonds with the aromatic rings coupled with solvents with varying polarity (Buran et al., 2014; Castañeda-Ovando et al., 2009). Chandrasekhar et al. (2012) studied the efficacy of six different sorbent resins; nonpolar silica gel, weak acidic anion exchanger Amberlite IRC 80, weak acidic cation exchanger Amberlite IR 120, strong acidic cation exchanger Dowex 50WX8, nonionic acrylic ester resins with moderate polarity Amberlite XAD4 and XAD7. Results showed that nonionic acrylic ester resins showed the highest adsorption rates and highest elution capacity (ca. 93% recovery), while the silica nonpolar resin exhibited no detected adsorption of anthocyanins (Chandrasekhar et al., 2012).

This method is relatively cheap, easy and reproducible, thus explaining the preference it has been conceded. However, as nonselective interactions are established it is possible that using this approach, contaminants remain (Buran et al., 2014; Denev et al., 2010; He and Giusti, 2011; Socaciu, 2007). This is shown in the work of Buran et al. (2014) where, after purification of blueberry extract with an Amberlite resin, traces of chlorogenic acid and other flavonols were still found. As an alternative, He and Giusti (2011) developed an SPE system that takes advantage of the different charges that anthocyanins have at different pH values. The sorbent used in this new approach combines reversed-phase interactions with cation exchange interactions. This means that, if the anthocyanins are introduced into the system at a pH value of 2 (where they are in their flavylium cation form) they will interact with the negatively charged sorbent and the other compounds can be removed using solvents with various polarities (given that their pH values do not rise significantly) (He and Giusti, 2011). To remove the anthocyanins an alkaline eluent may be used, since it will shift the anthocyanins into a negatively charged particle (quinoidal base) that will no longer interact with the resin and can, therefore, be collected (He and Giusti, 2011). He and Giusti (2011) found that this new approach was superior to the other commonly used SPE methods in regards of anthocyanin purity and recovery, sorbent capacity, cost, simplicity and high throughput. A similarly effective approach has been reported by Castañeda-Ovando et al. (2014). These authors exploited the tendency of the *o*-dihydroxy arrangement of anthocyanins to for metallic complexes with Fe^{3+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, and $Mg^{2+}$and the fact that these complexes, while stable under alkaline conditions, break when exposed to an acidic environment (Castañeda-Ovando et al., 2014; Yoshida et al., 2006). This approach, while allowing good purification rates and a relatively low cost must be first contextualized with the matrix as different anthocyanins exhibit different behaviors in response to the pH values and metallic (de)complexation, possibly leading to artifactual shifts in anthocyanin composition (Castañeda-Ovando et al., 2014).

Countercurrent chromatography

Countercurrent chromatography (CCC) is a chromatographic technique that uses two immiscible liquid phases, under gentle conditions, to separate relatively large amounts of sample (up to several hundred milligrams in a single run) (Degenhardt et al., 2000; Schwarz et al., 2003). High-speed CCC (HSCCC) has been demonstrated to be a potential mechanism to achieve large scale isolation of anthocyanins from various natural sources such as blackberries or elderberries. In HSCCC, the solvents are placed in a Teflon tube wound around a coil (frequently, connected in a series) that is then rotated. The movement causes mixing and settling zones between the two phases, inducing a continuous distribution of the sample (Degenhardt et al., 2000; Kostadinovik et al., 2013; Schwarz et al., 2003). The fact that this approach doesn't require expensive columns, uses gentle operating conditions and relatively cheap solvents (e.g.

Table 3. Summary of the most common extraction methods used for anthocyanin extraction for different matrixes.

	Matrix	References
Solid–Liquid Extraction (SLE)	Black Carrot	(Türker and Erdoğdu, 2006)
	Blackcurrant	(Cacace and Mazza, 2002, 2003a, b; Denev et al., 2010)
	Blackberry	(Denev et al., 2010; Oancea et al., 2013)
	Blueberry	(Ballinger et al., 1970; Barnes et al., 2009; Denev et al., 2010)
	Chokeberry	(Denev et al., 2010; Galván D'Alessandro et al., 2014)
	Elderberry	(Denev et al., 2010; Vatai et al., 2009)
	Grape	(Vatai et al., 2009)
	Hibiscus	(Cissé et al., 2012)
	Jamum	(Jampani et al., 2014)
	Jussara	(Borges et al., 2011)
	Purple Sweet Potato	(Fan et al., 2008)
	Red Cabbage	(Chandrasekhar et al., 2012)
	Red Radish	(Patil et al., 2009)
	Strawberry	(Garcia-Viguera et al., 1998)
	Sweet Cherry	(Oancea et al., 2013)
	Wine Pomace	(Metivier et al., 1980)
Supercritical Fluid Extraction (SFE)	Blueberry	(Paes et al., 2014)
	Cricket Vine	(Paula et al., 2013; Paula et al., 2014)
	Elderberry	(Seabra et al., 2010; Vatai et al., 2009)
	Grape	(Ghafoor et al., 2010; Vatai et al., 2009)
	Jamobolan	(Maran et al., 2014)
Ultrasound-Assisted Extraction (UAE)	Blackberry	(Oancea et al., 2013)
	Chokeberry	(Galván D'Alessandro et al., 2014)
	Grape	(Corrales et al., 2008; Ghafoor et al., 2009)
	Mangosteen	(Cheok et al., 2013)
	Sugar Beet Molass	(Chen et al., 2015b)
	Sweet Cherry	(Oancea et al., 2013)
Pressurized Liquid Extraction (PLE)	Blueberry	(Paes et al., 2014)
	Grape	(Corrales et al., 2009; Corrales et al., 2008; Ju and Howard, 2003; Liazid et al., 2011)
	Jabuticaba	(Santos et al., 2012)
	Purple Fleshed Sweet Potato	(Truong et al., 2012)
	Red Cabbage	(Arapitsas and Turner, 2008)
	Red Onion	(Petersson et al., 2010)
Microwave-Assisted Extraction (MAE)	Blackcurrant	(Pap et al., 2013)
	Blueberry	(Zheng et al., 2013)
	Grape	(Li et al., 2012)
	Mulberry	(Zou et al., 2012)
	Pomegranate	(Sinha et al., 2012)
	Red Raspberry	(Sun et al., 2007; Teng et al., 2013)
	Sour Cherry	(Garofulić et al., 2013)
Ohmic Heating-Assisted Extraction (OHM)	Black Rice Bran	(Loypimai et al., 2015)
SLE with Pulsed Electric Field Pre-treatment	Grape	(Corrales et al., 2008)
	Purple Fleshed Potato	(Puértolas et al.)
	Red Cabbage	(Gachovska et al., 2010)
	Strawberry	(Odriozola-Serrano et al., 2009)

methyl tert-butyl ether (MTBE), *n*-butanol, acetonitrile, water, TFA) increases its interest from an economic standpoint, thus making it a good candidate for industrial production (Degenhardt et al., 2000; Kostadinovik et al., 2013; Schwarz et al., 2003). A system comprised of *tert*-butyl methyl ether, *n*-butanol, acetonitrile and water acidified with TFA (2:2:1:5) has been used by several authors to purify anthocyanins from a range of sources such as wine, blueberries or red cabbage (Kostadinovik et al., 2013; Socaciu, 2007).

Conclusion

Plant phenolics have definitively gathered the interest of the scientific community, with anthocyanins belonging to one of the groups that show the most biological and industrial potential. However, the pigments' complex chemistry raises several issues when contemplating the extraction protocol to use. From relatively simplistic approaches to more technological ones, the range from which to choose is vast, and given the lack of straightforward papers that compare all methodologies for a given sample, it is hard to conclude which method is better given the extraction yield alone (different samples, have different amounts of anthocyanins to begin with, therefore variations in yield from paper to paper also reflect sample variation). Therefore, a clear understanding of the underling goal of the extraction, the potential use of the final extract and the sample itself plays an important role in the selection of the extraction/purification approach to be used (Table 3). Despite that, some overall guidelines can be perceived. Methods that imply the used of high temperatures may induce degradation while promoting the extraction therefore are less suitable for samples richer in methylated anthocyanins (more susceptible to degradation) (Sarkis et al., 2013). Similarly, methods that improve the extraction yield while using mostly water as a solvent (MAE, UAE, PEF), present interesting economic and ecological advantages though the reduction of production and waste management costs must be compared with the equipment cost. If pure extracts are needed, SFE is an interesting approach as it allows for the removal of nonpolar solvents, though, from a cost perspective a simpler (less expensive) extraction approach may be of interest if followed by an appropriate purification protocol (anthocyanin precipitation for lower purity levels, CCC or adsorbent and ion-exchange SPE resins for purer extracts).

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