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Extraction methods used for extraction of anthocyanin: A review

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Abstract
The use of anthocyanin compounds in different commercial sectors such as pharmaceutical, food and chemical industries signifies the need of the most appropriate and standard method to extract these active components like anthocyanin from fruit, flower and leaf. Along with conventional methods, numerous new methods have been established but till now no single method is regarded as standard for extracting anthocyanin from plants. The efficiencies of conventional and non- conventional extraction methods mostly depend on the critical input parameters; understanding the nature of plant. This review is aimed to discuss different extraction techniques along with their basic mechanism for extracting bioactive compounds from fruits, flower and leaf.

Keywords: Extraction methods, extraction, anthocyanin, pharmaceutical

Introduction

Extraction

Extraction and purification of bioactive compounds from natural sources have become very important for the utilization of phytochemicals in the preparation of dietary supplements or neutraceuticals, functional food ingredients, food additives, pharmaceutical and cosmetic products. The polar character of anthocyanins makes them soluble in several types of polar solvents such as methanol, ethanol, acetone and water. Solvent extraction of anthocyanins is the initial step in the determination of total and individual anthocyanins prior to quantification, purification, separation, and characterization.

The extraction of anthocyanins is commonly carried out under cold conditions using methanol or ethanol containing a small amount of acid in order to obtain the flavylium cation form, which is red and stable in a highly acid medium (Du and Francis, 1973; Amor and Allaf, 2009).

Anthocyanin

Anthocyanins are one of the six subgroups of large and widespread group of plant constituents known as flavonoids as shown in fig. 1. These are responsible for the bright and attractive orange, red, purple, and blue colors of most fruits, vegetables, flowers and some cereal grains. More than 600 structurally distinct anthocyanins have been identified in nature. Earlier, anthocyanins were only known for their coloring properties but now interest in anthocyanin pigments has intensified because of their possible health benefits as dietary antioxidants, which help to prevent neuronal diseases, cardiovascular illnesses, cancer, diabetes, inflammation, and many such others diseases. Ability of anthocyanins to counter oxidants makes them atherosclerosis fighters. Therefore, anthocyanin-rich foods may help to boost overall health by offering an array of nutrients. However, the incorporation of anthocyanins into food and medical products is a challenging task due to their low stability toward environmental conditions during processing and storage.

Anthocyanin, as a water-soluble natural food colouring, can be dissolved by polar solvents, such as methyl alcohol and ethyl alcohol, as well as affected by el-ements, such as sunlight and temperature. Moreover, it has higher safety compared to the synthetic pigment. Because of the antioxidant function of anthocyanin, which is 50 times that of vitamin E, it can induce proper crosslinking of collagen, eliminate free radicals, and protect the skin.5 In addition, it can reduce serum cholesterol, triglyceride, and high-density lipoprotein, enhance low density lipoprotein, inhibit atherosclerosis, regulate blood fat, and prevent cardiovascular diseases and

high blood pressure. They play an important role in the color quality of flowers, berries, cereals, vegetables and fruits having red, purple or blue colors.

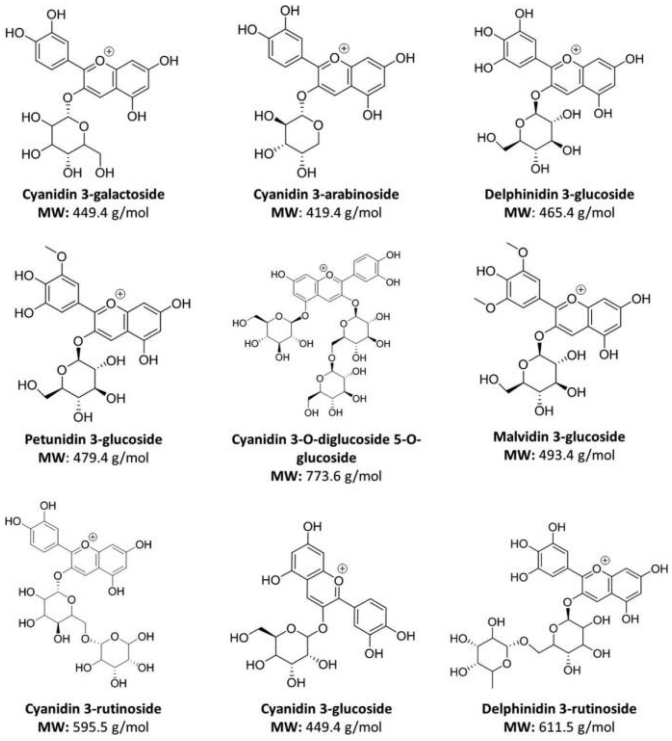

Fig 1: Example of anthocyanin found in different food

Anthocyanins are approved as food colorants in the USA under the category of fruit (21 CFR 73.250) or vegetable (21 CFR 73.260) juice colour. The EU classifies anthocyanins as 'natural colorants' under classification number E163. The use of anthocaynins may show benefits over that of synthetic colours. A potential use for kokum extract may include production of fruit juice, drinks and jam.

Preperation of samples for extraction of anthocyanins

Both fresh and dried sample is used in medicinal plants studies. In most cases, dried sample is preferred considering the time needed for experimental design. (Sulaiman *et al* 2011) limit the interval between harvest and experimental work at the maximum period of 3 hours to maintain freshness of samples, as fresh samples are fragile and tend to deteriorate faster than dried samples. Comparison between fresh and dried *Moringa oleifera* leaves showed no significant effect in total phenolics but with higher flavonoids content in dried sample [Vongsak *et al*., 2013] [1].

Lowering particle size increases surface contact between samples and extraction solvents. Grinding resulted in coarse smaller samples; meanwhile, powdered samples have a more homogenized and smaller particle, leading to better surface contact with extraction solvents. This particular pre-preparation is important, as for efficient extraction to occur, the solvent must make contact with the target analytes and particle size smaller than 0.5 mm is ideal for efficient extraction [Anonymous, 2013] [2].

This particular size of particle was mentioned in Sulaiman *et al.*, preparing vegetable samples that was ground to 400 µm (0.4 mm) in size. Conventional mortar and pestle or electric blenders and mills are commonly used to reduce particle size of sample. Investigation of nanoparticles powder of *Centellaasiatica* produced by Planetary Ball Mill (PBM) showed 82.09% higher yield compared to micro powder using maceration technique in 90% methanol for 3 days [Borhan *et al*., 2013] [3]. Particle size was a major factor when using enzyme-assisted extraction. Use of pectinolytic and cell wall polysaccharide degrading enzyme in sample preparation was influenced majorly by the particle size as smaller particle enhances enzyme action.

Extraction methods

Extraction is the separation of medicinally active portions of plant using selective solvents through standard procedures [Handa *et al*., 2008] [4]. The purpose of all extraction is to separate the soluble plant metabolites, leaving behind the insoluble cellular marc (residue).The initial crude extracts using these methods contain complex mixture of many plant metabolites, such as alkaloids, glycosides, phenolics, terpenoids and flavonoids.

Soxhlet extraction

In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is place, is in thimble chamber of the Soxhlet apparatus. Extraction solvents is heated in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drip back. When the liquid content reaches the siphon arm, the liquid contents emptied into the bottom flask again and the process is continued.

This method requires a smaller quantity of solvent. However, the Soxhlet extraction Solvents used in the extraction system need to be of high-purity that might add to cost. This procedure is considered not environmental friendly and may contribute to pollution problem compared to advance extraction method such as supercritical fluid extraction (SFE) [Naudé *et al*., 1998] [5].

The ideal sample for Soxhlet extraction is also limited to a dry and finely divided solid [Anonymous, 2013] [2] and many factors such as temperature, solvent- sample ratio and agitation speed need to be considered for this method [Amid *et al*., 2010] [6].

Microwave assisted extraction (MAE)

MAE utilizes microwave energy to facilitate partition of analytes from the sample matrix into the solvent. Microwave radiation interacts with dipoles of polar and polarizable materials (e.g. solvents and sample) causes heating near the surface of the materials and heat is transferred by conduction. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; enhancing the migration of dissolved ions and promotes solvent penetration into the matrix. In non- polar solvents, poor heating occurs as the energy is transferred by dielectric absorption only (Hande *et al*., 2008) Some of the natural product through MAE is shown in table 1.

Table 1: Application of MAE to natural product extraction

Compounds	Matrix	System	Extraction Conditions	References
Vicine, convincine	Faba beans	Domastic oven	Methanol: water(1:1`) irradiation 30s with an intermediate cooling step	Ganzler *et al*.,1986
Gossypol	Cotton seeds	Domastic oven	Three cycles of irradiation with a cooling step in between	Ganzler *et al*.,1986
Sparteine	Lupine seeds	Domastic oven	Four cycles of irradiation with a cooling step in between	Ganzler *et al*., 1990
Essential oils	Rosemary and peppermint leaves	Domastic oven	Hexane, carbon tetrachloride, tolune	Chen and spiro., 1994
Volatile oils	Mentha piperita	Modified domastic ovens	Hexane, alkanes (transperent solvents)	Pare., 1994
Carotenoids	Paprika powder	Closed vessels	Hexane high cooking level<60 s	Csiktunaid kiss., *et al*., 2000

Ultrasound-assisted extraction (UAE) or sonication extraction

UAE involves the use of ultrasound ranging from 20 kHz to 2000 kHz. The mechanic effect of acoustic cavitation from the ultrasound increases the surface contact between solvents and samples and permeability of cell walls. Physical and chemical properties of the materials subjected to ultrasound are altered and disrupt the plant cell wall; facilitating release of compounds and enhancing mass transport of the solvents into the plant cells [Dhanani *et al*., 2013] [7]. The procedure is simple and relatively low cost technology that can be used in both small and larger scale of phytochemical extraction.

Accelerated solvent extraction (ASE)

ASE is an efficient form of liquid solvent extraction compared to maceration and Soxhlet extraction as the method use minimal amount of solvent. Sample is packed with inert material such as sand in the stainless steel extraction cell (Figure 1e-1g) to prevent sample from aggregating and block the system tubing [Rahmalia *et al*., 2015] [8].

Packed ASE cell includes layers of sand-sample mixture in between cellulose filter paper and sand layers. This automated extraction technology is able to control temperature and pressure for each individual samples and requires less than an hour for extraction. Similar to other solvent technique, ASE also critically depend on the solvent types. Cyclohexaneacetone solution at the ratio of 6:4 v/v with 5 minute heating (50 °C) showed to yield highest bixin from *Bixaorellana* with 68.16% purity [Rahmalia *et al*., 2015] [8]. High recoveries (~94%) of flavonoids from *Rheum palmatun* were observed using 80% aqueous methanol by ASE, suggesting the suitability of this method for quality control evaluation.

Acidified Ethanol Extraction

The extraction was done according to method suggested by Abdulrahman *et al.,* (2004). Fifty grammes of the homogenate was filled in the glass bottle containing solvent (20% ethanol + 0.5% citric acid) equal to thrice the quantity of homogenate and it was kept for extraction by percolation method for about 72 hours in dark condition at room temperature by covering with aluminium foil and by occasional shaking. After extraction, the pigment was filtered with Whatman filter paper and stored in glass bottles at refrigerated condition. All the extracts were made up to 250ml volume in a standard flask. This method is used for the extraction of antioxidant of Spirulina platens is microalga.

Maceration, infusion, percolation and decoction:

Maceration is a technique use in wine making and has been adopted and widely used in medicinal plants research. Maceration involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation [Handa *et al*., 2008] [4].

The processed intended to soften and break the plant's cell wall to release the soluble phytochemicals. After 3 days, the mixture is pressed or strained by filtration. In this conventional method, heat is transferred through convection and conduction and the choice of solvents will determine the type of compound extracted from the samples. Infusion and decoction uses the same principle as maceration; both are soaked in cold or boiled water. However, the maceration period for infusion is shorter and the sample is boiled in specified volume of water (eg. 1:4 or 1:16) for a defined time for decoction [Handa *et al*., 2008] [4].

Decoction is only suitable for extracting heat-stable compounds, hard plants materials (e.g. roots and barks) and usually resulted in more oil-soluble compounds compared to maceration and infusion. Unique equipment called percolator (Figure 1c and 1d) is used in percolation, another method that shares similar fundamental principle. Dried powdered samples are packed in the percolator, added with boiling water and macerated for 2 hours. The percolation process is usually done at moderate rate (e.g. 6 drops /min) until the extraction is completed before evaporation to get a concentrated extracts. Used for extraction and pre phytochemical screening strategies for herbal drug.

Pulsed-electric field extraction (PEF)

The pulsed electric field (PEF) treatment was recognized as useful for improving the pressing, drying, extraction, and diffusion processes during the last decade (Barsotti and Cheftel, 1998; Angersbach *et al*., 2000; Vorobiev *et al*., 2005; Vorobiev and Lebovka, 2006) [10-13]. The principle of PEF is to destroy cell membrane structure for increasing extraction. During suspension of a living cell in electric field, an electric potential passes through the membrane of that cell. Based on the dipole nature of membrane molecules, electric potential separates molecules according to their charge in the cell membrane. Usually, a simple circuit with exponential decay pulses is used for PEF treatment of plant materials. It has a treatment chamber consisting of two electrodes where plant materials are placed. Depending on the design of treatment chamber PEF process can operate in either continuous or

batch mode (Puertolas *et al*., 2010) [15]. The effectiveness of PEF treatment strictly depends on the process parameters, including field strength, specific energy input, pulse number, treatment temperature and properties of the materials to be treated (Heinz *et al*., 2003) [16].

PEF treatment (at 1 kV/cm with low energy consumption of 7 kJ/kg) in a solid liquid extraction process for extraction of betanin from beetroots showed highest degree of extraction compared with freezing and mechanical pressing (Fincan *et al*., 2004) [17]. Guderjan *et al*. (2005) [18] showed that the recovery of phytosterols from maize increased by 32.4% and isoflavonoids (genisteinanddaidzein) from soybeans increased by 20–21% when PEF was used as pretreatment process. Corralesa *et al*. (2008) [38] extracted bioactive compound such as anthocyanins from grape by-product using various techniques and found better extraction of anthocyanin monoglucosides by PEF.

Enzyme-assisted extraction (EAE)

There are two approaches for enzyme-assisted extraction: (1) enzyme-assisted aqueous extraction (EAAE) and (2) enzyme-assisted cold pressing (EACP) (Latif and Anwar, 2009) [25]. Usually, EAAE methods have been developed mainly for the extraction of oils from various seeds (Hanmoungjai *et al*., 2001; Rosenthal *et al*., 1996, 2001; Sharma *et al*., 2002) [20, 52, 26]. In EACP technique, enzymes is used to hydrolyze the seed cell wall, because in this system polysaccharide-protein colloid is not available, which is obvious in EAAE (Concha *et al*., 2004) [37].

Various factors including enzyme composition and concentration, particle size of plant materials, solid to water ratio, and hydrolysis time are recognized as key factors for extraction (Niranjan and Hanmoungjai, 2004) [50]. Dominguez *et al*. (1995) [19] reported that the moisture content of plant materials is also an important factor for enzymatic hydrolysis. Bhattacharjee *et al*. (2006) [35] described EACP as an ideal alternate for extracting bioactive components from oilseed, because of its nontoxic and noninflammable properties.

The oil extracted by enzyme- assisted methods was found to contain higher amount of free fatty acids and phosphorus contents than traditional hexane extracted oil (Dominguez *et al*., 1995) [19]. The EAE is recognized as eco-friendly technology for extraction of bioactive compounds and oil because it uses water as solvent instead of organic chemicals (Puri *et al*., 2012) [26].

Microwave assisted extraction (MAE)

The microwave-assisted extraction is also considered as a novel method for extracting soluble products into a fluid from a wide range of materials using microwave energy. Microwaves are electromagnetic fields in the frequency range from 300 MHz to 300 GHz. They are made up of two oscillating fields that are perpendicular such as electric field and magnetic field. The principle of heating using microwave is based upon its direct impacts on polar materials (Letellier and Budzinski, 1999) [46]. Electromagnetic energy is converted to heat following ionic conduction and dipole rotation mechanisms (Jain, 2009) [43].

During ionic conduction mechanism heat is generated because of the resistance of medium to flow ion. On the other hand, ions keep their direction along field signs which change frequently. This frequent change of directions results in collision between molecules and consequently generates heat. The extraction mechanism of microwave assisted extraction is supposed to involve three sequential steps described by Alupului (2012) [33]: first, separation of solutes from active sites of sample matrix under increased temperature and pressure; second, diffusion of solvent across sample matrix; third, release of solutes from sample matrix to solvent. Several advantages of MAE have been described by Cravottoa *et al*. (2008) [39] such as quicker heating for the extraction of bioactive substances from plant materials; reduced thermal gradients; reduced equipment size and increased extract yield. MAE can extract bioactive compounds more rapidly and a better recovery is possible than conventional extraction processes.

It is a selective technique to extract organic and organometallic compounds that are more intact. MAE is also recognized as a green technology because it reduces the use of organic solvent (Alupului, 2012) [33]. For polyphenols and caffeine extraction from green tea leaves, MAE achieved higher extraction yield at 4 min than any extraction methods at room temperature for 20 h (Pan *et al*., 2003) [27]. Ginsenosides extraction yield from ginseng root obtained by 15 min using focused MAE technique was better than conventional solvent extraction for 10 h (Shu *et al*., 2003) [30]. Dhobi *et al*. (2009) [29] showed increased extraction efficiency of MAE by extracting a flavolignin, silybinin from Silybum marianum compared with the conventional extraction techniques like Soxhlet, maceration. Asghari *et al*. (2011) [34] extracted some bioactive compounds (E- and Z-guggolsterone, cinnamaldehyde and tannin) from various plants under optimum conditions and showed that, MAE is faster and easier method in comparison to conventional extraction processes.

Pressurized liquid extraction (PLE)

PLE technique requires small amounts of solvents because of the combination of high pressure and temperatures which provides faster extraction. The higher extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate and, also decrease the viscosity and surface tension of solvents, thus improving extraction rate (Ibanez *et al*., 2012) [23]. In comparison to the traditional soxhlet extraction PLE was found to dramatically decrease time consumption and solvent use (Richter *et al*., 1996). Now a days, for extraction of polar compounds, PLE is also considered as a potential alternative technique to supercritical fluid extraction (Kaufmann and Christen, 2002) [24]. PLE is also useful for the extraction of organic pollutants from environmental matrices those are stable at high temperatures (Wang and Weller, 2006) [57].

PLE has also been used for the extraction of bioactive compounds from marine sponges (Ibanez *et al*., 2012) [23]. Applications of PLE technique for obtaining natural products are frequently available in literature (Kaufmann and Christen, 2002) [24]. Additionally, due to small amount organic solvent use PLE gets broad reorganization as a green extraction technique (Ibanez *et al*., 2012) [23].

Supercritical fluid extraction (SFE)

The application of supercritical fluid for extraction purposes started with its discovery by Hannay and Hogarth (1879) [41] but the credit should also be given to Zosel who presented a patent for decaffeination of coffee using SFE (Zosel, 1964) [32]. Since this beginning, supercritical fluid technique has attracted wide scientific interest and it was successfully used in environmental, pharmaceutical and polymer applications and food analysis (Zougagh *et al*., 2004) [58].

Several industries have been using this technique for many years, especially, decaffeinated coffee preparation industries (Ndiomu and Simpson, 1988) [48]. Every earthly substance has three basic states namely; Solid, Liquid and Gas. Supercritical state is a distinctive state and can only be attained if a substance is subjected to temperature and pressure beyond its critical point. Critical point is defined as the characteristic temperature (Tc) and pressure (Pc) above which distinctive gas and liquid phases do not exist. In supercritical state, the specific properties of gas and/or liquid become vanish, which means supercritical fluid cannot be liquefied by modifying temperature and pressure. Supercritical fluid possesses gas- like properties of diffusion, viscosity, and surface tension, and liquid-like density and solvation power.

These properties make it suitable for extracting compounds in a short time with higher yields (Sihvonen *et al*., 1999) [55]. A basic SFE system consists of the following parts: a tank of mobile phase, usually CO2, a pump to pressurize the gas, co-solvent vessel and pump, an oven that contains the extraction vessel, a controller to maintain the high pressure inside the system and a trapping vessel. Usually different type of meters like flow meter, dry/wet gas meter could be attached to the system.

Carbon dioxide is considered as an ideal solvent for SFE. The critical temperature of CO2 (31_C) is close to room temperature, and the low critical pressure (74 bars) offers the possibility to operate at moderate pressures, generally between 100 and 450 bar (Temelli and Guclu-Ustundag, 2005) [31].

The successful extraction of bioactive compounds from plant materials rely upon several parameter of SFE and most importantly these parameter are tunable (Raverchon and Marco, 2006) [28]. These parameter need to be precisely controlled for maximizing benefits from this technique. The major variables influencing the extraction efficiency are temperature, pressure, particle size and moisture content of feed material, time of extraction, flow rate of CO2, and solvent-to-feed-ratio (Temelli and Guclu-Ustundag, 2005; Ibanez *et al*., 2012) [31, 23].

Conclusion

The ever growing demand for extraction of bioactive compounds encourages continuous search for convenient extraction methods. The chromatography advancement and awareness about environment are two important factors for the development of most non-conventional extraction processes. However, understanding of every aspect of non-conventional extraction process is vital as most of these methods are based on different mechanism and extraction enhancement is resulted from different process. Incorporation and development of hybrid methods should also be investigated considering plant material characteristics and choice of compounds. Sufficient experimental data is still lacking in some of the existing methods. Proper choice of standard methods also influences the measurement of extraction efficiency.

On the other hand, the increasing economic significance of bioactive compounds and commodities rich in these bioactive compounds may lead to find out more sophisticated extraction methods in future.

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