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ANTHOCYANINS IN PURPLE MAIZE

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Abstract

Natural dyes potential increases continuously as the production of synthetic dyes requires strong acidic and alkaline obtaining conditions. Natural dyes are less toxic, easily available and biodegradable and have a low negative impact on the environment. The main dyes extracted from purple maize are anthocyanins, but in some cultivars were also found low concentration of carotenoids. The extraction of natural dyes can be performed either through traditional methods (aqueous, acid, alkaline, solvent, Soxhlet), either through modern methods (enzymatic, microbial, microwave or ultrasound assisted, supercritical fluids). This paper is also focused on comparing the most used methods for identification and quantification of anthocyanins in purple maize, methods that can be spectrophotometric or chromatographic. The multiple benefits of anthocyanins (antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial, neuroprotective etc.) support their use in various applications such as: food, textile, pharmaceutic, medical etc.

Key words: *anthocyanins, chromatography, dyes, maize, spectrophotometry.*

INTRODUCTION

Since ancient times, people have used dyes from various natural sources such as plants, shells, insects or others. But since the synthetization of dyes in laboratories at mid-18th century, these natural dyes were replaced by synthetic ones, as the latter produced stronger colours, were more resistant to washing and were able to imprint the colour much faster. However, due to the carcinogenic action of synthetic dyes, trends are directed more to the use of natural dyes.

Natural dyes have become lately more appealing for various biotechnological applications due to their numerous benefits. In this sense, they have a low negative impact on the environment, are less toxic, easily available and they do not determine allergic reactions such as synthetic dyes. Furthermore, natural dyes can have various beneficial properties such as: antibacterial, antifungal, antioxidant and others.

These colorants may be obtained from various sources: minerals, plants, animals or even microorganisms.

Mineral dyes are obtained through purification of natural compounds such as iron buff, chrome yellow, Prussian blue or manganese brown (Mansour, 2018).

Plants are often used for extraction of natural dyes such as carotenoids, flavonoids, anthraquinones, indigoids etc. Various parts of the plants are abundant in dyes such as: seeds, stem, bark, root, fruits or even flowers (Irimescu et al., 2019). Moreover, some plants can contain more than one type of dye.

The animal sources of dyes are generally represented by insects such as *Dactylopius coccus* (cochineal dye), lac insects (lac dye), *Murex* snail (Tyrian purple dye), octopus/cuttlefish (Sepia brown dye), shellfish (purple dye) etc. (Wood, 1986; Wisniak, 2004).

Microbial dyes can be extracted using microorganisms belonging to the families of: *Bacillus, Micrococcus, Monascus, Rhodotorula, Sarcina, Achromobacter* or *Phaffia* (Heer & Sharma, 2017). The main dyes produced by microorganisms are generally carotenoids.

There are many criteria used for classification of dyes: the colour they impart, chemical structure or even their methods of application.

Based upon the colour, natural dyes can be: yellow, orange, red, blue, green or even black and brown. However, the main classification of natural dyes is based upon the chemical structure: anthraquinones, carotenoids, flavonoids, indigoids or dihydropyrans.

MAIZE PIGMENTS

Purple maize/corn (*Zea mays* L.), a plant with the deepest shade of purple is a widely used cultivar in low valleys from South America or China. Nowadays, the cultivation of this maize variety is extended to other regions throughout the world.
The natural dyes content of purple maize is mainly based on anthocyanins, although some cultivars had also low contents of carotenoids.
Anthocyanins are the main flavonoid dyes found in plants. They are a class of water-soluble compounds with phenolic structures that imprint various colours to fruits and vegetables: orange, red, blue and purple.
Anthocyanins are mainly found in the skin, but can also be detected in the flesh of some fruits such as strawberries or cherries (Martín et al., 2017).
Regarding the chemical structure of anthocyanins, they are glycosylated polymethoxy or polyhydroxy derivatives of 2-phenylbenzopyrilium, with two benzyl rings (noted with A and B) as seen in Figure 1.

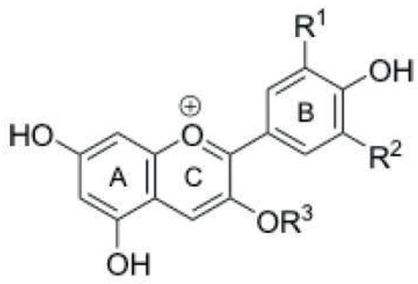

Figure 1. Chemical structure of anthocyanins-3-O-glucoside (Martín et al., 2017)

Usually, anthocyanins contain one glucoside unit, but they can also have more units attached at different positions (Martín et al., 2017). Also, the colour imprinted and its intensity is correlated with the number of methoxyl (red) and hydroxyl (blue) groups (Martín et al., 2017).
The main anthocyanins glycosides are delphinidin, peonidin, pelargonidin, cyanidin, malvidin and petunidin (Mansour, 2018).
The anthocyanins from purple maize are represented by: pelargonidin-3-glucoside cyanidin-3-glucoside, peonidin-3-glucoside and their malonated counterparts (Yang et al., 2009).
The anthocyanins content in purple maize is usually around 16.4 mg/g fresh matter (Lao & Giusti, 2016), higher than other plants known for their anthocyanins content such as blueberry, red and black currants, cherries, pomegranate, cabbage, grape, eggplant, fig, or chokeberry (Georgieva, 2020; Coarfă and Popa, 2019; Hoxha and Kongoli, 2019; Albu et al., 2019; Raducu et al., 2019).
The low cost of cultivating purple maize and the high content of anthocyanins make purple maize an important material for extracting natural dyes (Lao & Giusti, 2016), that could be used in many applications due to their potential health benefits.

EXTRACTION OF NATURAL PIGMENTS FROM MAIZE

The high interest for natural dyes extraction is due to their many benefits such as: cheap substrates, wide range of shades of the extract obtained, the extract contains besides the dyes also colour fixating compounds and not the least the health benefits they provide (Kasiri & Safapour, 2015).
Since one of the disadvantages of natural dyes is their low to moderate content in plant sources, an important step in their valorization is the extraction method used. Therefore, the method chosen for the extraction of natural dyes should be centred on the plant matrix and dye characteristics (Mansour, 2018).
The extraction methods can be traditional (conventional) or modern ones, according to Figure 2.

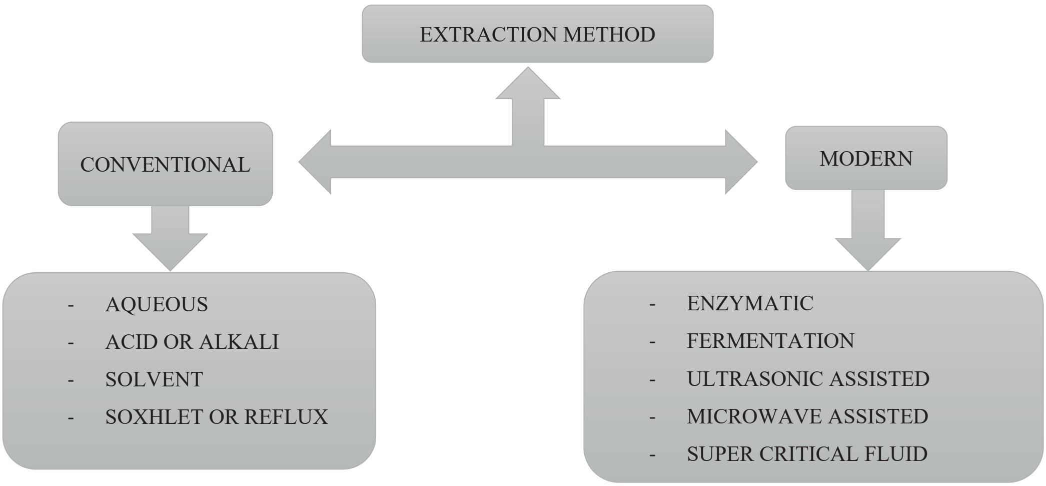

Figure 2. Classification of extraction methods

Aqueous extraction is considered to be the first extraction method used since ancient times. The first step in this method is the disintegration of the material, in this case maize kernels, into smaller particles or even powder. The next step is to soak the material with water for a prolonged time in order to affect the cell membrane. Later, the mixture is boiled, centrifuged and filtered, a process that usually is repeated for a better extraction yield. The advantages of using this extraction method are considered to be: low cost, simplicity and the fact that the solution obtained is compatible with many applications that usually require aqueous media. However, the disadvantages are considered too important to ignore such as: high requirements of water, long extraction time or high temperature necessary. Moreover, the extraction yield is low due to the extraction of only water-soluble components of dyes.

Acid or alkaline extraction leads to higher yield than aqueous extraction. Acid extraction is mainly used for flavone type dyes, while alkaline extraction is best suited for the rest of phenolic type dyes. A major disadvantage of this chemical extraction is that due to harsh process conditions, some dyes components are destroyed, according to their sensibility to pH. Also, due to the fact that most natural dyes are mainly a mixture of different compounds with different chemical structures, by modifying the pH, only a few of those compounds can be extracted. Generally, the anthocyanins obtained through an acid extraction protocol are not used in food applications, due to their high toxicity as suggested by Lao & Giusti (2018).

Solvent extraction seems to be one of the best traditional extraction methods. The solvents used are either organic ones (methanol, ethanol, acetone, chloroform) either a mixture between them, or a mixture of them with water. The best combination is considered to be an alcohol-water media, that can achieve the extraction of both water-insoluble and water-soluble components of dyes (Mansour, 2018). An improved solvent extraction is the one that uses an aqueous solvent with acid/alkali.

This method has the advantage of an easier purification procedure performed by solvent distillation. The disadvantages of this process consist in the fact that the dye obtained is not ready to use and the toxicity of the solvents and their impact on the environment (Mansour, 2018).

A research study found that through statistical optimization the best extraction of anthocyanins from purple maize is obtained with 95% ethanol acidified with HCl 1.5N at a solid-liquid ratio of 1:25, at 70°C for cca 70 min (Yang et al., 2009).

Soxhlet or reflux extraction is considered to be an easy method to perform, doesn't require filtering after the extraction and has also the benefit of retrieving the solvents used. However, this process is time consuming,

requires high volumes of solvents, has a negative impact on the environment and can also thermodegrade sensitive dyes.
The Soxhlet extraction of anthocyanins from purple maize is a technique used industrially due to low cost of the extraction unit. However, the many disadvantages it presents as described before may be a reason to select a modern method to extract anthocyanins (Viganó and Martinez, 2015). Another downside of using this method is that there is a higher risk for degradation of labile compounds (Cristianini and Guillén Sánchez, 2020).
Enzymatic extraction implies the use of enzymes to degrade other plant components that can interfere with dye extraction, such as starch, cellulose, xylan or pectin. By using commercial enzymes like amylase, cellulase, xylanase or pectinase to breakdown several binding components, the material left can be subjected to dye extraction in milder conditions (Mansour, 2018; Fernandez-Aulis et al., 2019). This method is mainly promoted for plant materials such as roots or bark.
A research study (Fernandez-Aulis et al., 2019) concluded that even though the anthocyanins yields weren't the highest obtained, the enzymatic extraction with xylanase, cellulase and β-glucanase resulted in higher content of cyanidin-3-(6"malonyl) glucoside.
Extraction through fermentation is a process that uses enzymes, produced by microorganisms present in natural environment, usually close to the material subjected to extraction. The fermentation conditions are selected based upon the microbial strain used, conditions that benefit both the growth of the microorganism and the extraction of natural dyes. The method is similar to aqueous extraction, but without high temperature requirements (usually the fermentation is conducted at 30°C ± 2). Some disadvantages of this method include: long periods of extraction, unpleasant smell of the used biomass or the requirements of performing the extraction must be conducted immediately after harvesting (Mansour, 2018). Additionally, another major disadvantage is the fact that by using microorganisms, the dyes obtained can be considered unsafe for some applications when the microorganisms are not labelled as GRAS (Generally Recognized as Safe).
Ultrasonic assisted extraction is similar to the microwave assisted extraction. The efficiency of this method is increased by the action of ultrasounds, that in return help reduce the necessary quantity of solvents or lower the extraction periods. Also, this method can be performed in milder conditions such as lower temperature (Mansour, 2018), which can improve the extraction of dyes components that are sensitive to high temperature. In conclusion, this method is considered to be a "green process" (Mansour, 2018) due to its many benefits: quick, efficient and cheap in regards to energy consumption (Kasiri & Safapour, 2015; Oancea et al., 2021).
A study that compared several extraction methods of anthocyanins from purple maize (Fernandez-Aulis et al., 2019), concluded that the most efficient protocol was the one that was performed with 20 min 100 W ultrasound, preceded by an acidifed (lactic acid) aqueous ethanol (80%) treatment.
Microwave assisted extraction is considered to be a process with high extraction yields of natural dyes, due to the efficiency provided by the action of microwaves, that are able to heat faster the material subjected to extraction in comparison with other classic methods. Therefore, there is a lower extraction period and a lower energy consumption. Also, this method doesn't negatively impact the environment. Some studies also found that this process led to higher extraction yields than other methods such as ultrasound assisted or reflux (Dabiri et al., 2005). What was similar to the ultrasonic assisted extraction, was that this method also requires low volumes of solvents and has lower extractions periods. Another benefit of using this extraction process is that by irradiation, the dyes obtained had a higher adsorption capacity upon acrylic fibers, as discussed in the research led by Sinha et al. (2012). The downside of this method is the high cost of the microwave system required at an industrial level.
A study concluded through statistical optimization that the best microwave assisted extraction of anthocyanins from purple maize is achieved with a microwave irradiation of 555W for 19 min, using a solid-liquid ratio of 1: 20 (Yang and Zhai, 2010). In comparison with the conventional solvent extraction, this protocol

had a high efficiency and was quick in extracting the major types of anthocyanins.

Supercritical fluid extraction is a modern method used for dyes extraction. Due to the specific characteristics of a supercritical fluid, this process creates a better interaction between the solvent and the material subjected to extraction, and also enhances the solubility of numerous components in any solvent. This method is considered to be a better alternative for the conventional solvent extraction, due to its many benefits: low cost of reagents, low toxicity, easily available, and less residues traces (Mansour, 2018). This process is mainly used for biotechnological applications in areas such as pharmaceutic or food. The disadvantages of this method are mainly the high cost of the equipment and low yields of extraction of polar dyes (the supercritical fluid used is CO_2, a nonpolar molecule).

Nowadays, the most used extraction methods for natural dyes such as anthocyanins are microwave or ultrasound assisted ones, both being considered eco-friendly and very efficient.

METHODS OF IDENTIFICATION AND QUANTIFICATION OF MAIZE PIGMENTS

Since purple maize is a rich source for natural dyes such as anthocyanins, an accurate identification and quantification is required in order to properly evaluate the actual content of this material.

In comparison with quantification methods of anthocyanins in berries, where the results seemed to be consistent with a high correlation (Lao & Giusti, 2016), the methods used for purple maize provide different results even when the materials used are from the same variety or species.

The identification and quantification of anthocyanins in purple maize can be performed either spectrophotometrically, either chromate-graphically.

An easy and quick spectrophotometric method is considered to be the analysis of the total anthocyanins, where the purple maize powder is immersed in an acidic alcoholic solution (usually 95% ethanol acidified with HCl 1.5N or formic acid), the quantification being evaluated without further purification. After the extraction at 4°C overnight, the mixture was filtered and analysed at an UV-Visible spectro-photometer at 535 nm. The results were calculated based on the average extinction coefficient for anthocyanins found in cranberries (Lao & Giusti, 2016; Yang et al., 2009).

Another spectrophotometric method is the pH differential one used often for the quantify-cation of dark coloured maize pigments. This method requires an additional step before quantification which is usually an acetone extraction. The protocol requires that the purple maize powder is homogenised with 70% acetone acidified with 0.01% HCl 6N. The mixture is filtered, the filtrate is homogenised with chloroform and evaporated in a rotary evaporator. The final extract is added to a an HCl acidified water and subjected to pH differential method. The quantification method is performed by diluting the dye containing extracts with buffer of different pH: 1.0 and 4.5 and analysed with an UV-Visible spectropho-tometer at 700 nm (Lao and Giusti, 2016).

The HPLC (High Performance Liquid Chromatography) chromatographic method is the most used for identification and quantification of anthocyanins. With this method, the components of the anthocyanin group are identified in different matrix. Another advantage of this method is that it can provide the most precise content of each anthocyanin detected (Lao & Giusti, 2016). However, this method is more complicated than the spectrophotometric ones, requires chemical standards for each anthocyanin compound and is more time consuming.

A simple HPLC method is the one that uses intact anthocyanins, where the extract obtained from purple maize with acetone extraction (as described previously) are passed through a C18 Sep-Pak cartridge that will remove unnecessary components such as, non-phenolic compounds, sugars or acids. After that, the methanol is evaporated, the material is mixed with acidified water and filtered before being analysed through the HPLC-MS system (Lao & Giusti, 2016).

Another HPLC method is the one that analysed the anthocyanins by using the extract obtained through acetone extraction and subjecting it to an acid hydrolysis (HCl), at high temperature. The rest of the method was conducted similarly as described previously, mainly purification with a C18 Sep-Pak cartridge and addition of acidified water before analysis (Lao & Giusti, 2016). The main benefit of this method is the obtaining a clearer chromatogram, eliminating the possibility of anthocyanin degradation during hydrolysis.

In comparison, the total anthocyanins and HPLC with acid hydrolysis methods provides an inaccurate content of anthocyanins since the first measures all the compounds that imprint a reddish colour and the latter has the risk of pigment degradation during acid hydrolysis. Therefore, the pH differential and the HPLC intact anthocyanins methods seemed to give more accurate results due to the fact that they only measure the monomeric anthocyanins.

POTENTIAL APPLICATIONS OF ANTHOCYANINS CORRELATED WITH HEALTH BENEFITS

According to several market reports, the annual sales of natural dyes has come closely to 600 million $ (Lao & Giusti, 2016) and the market is still growing. This suggests the evolving uses of natural dyes in various industrial applications, such as: food, textiles, artisanal products, medical, art, leather processing, histology, pharmaceutic and cosmetic (Mansour, 2018).

Many natural dyes have certain characteristics that make them suited for industrial applications such as: antimicrobial, antioxidant, anti-inflammatory or even anticancer.

Anthocyanins are known for their health benefits, due to their wide range of actions: antibacterial, antifungal, antioxidant, anti-inflammatory, anti-mutagenic, neuroprotective, anti-obesity, anti-allergic, anti-carcinogenic Also, they can: promote eye health, prevent LDL oxidation, support collagen, improve capillary stability, ameliorate hyperglycemia, protect against UV irradiation or even increase intercellular levels of vitamin C (Mansour, 2018; Yang et al., 2009; Martín et al., 2017; Kasiri & Safapour, 2015).

When used as **textile colorants**, anthocyanins can provide a wide range of colours for fabrics such as silk or cotton.

Food industry

Due to rigorous legislation and trends in food industry, natural additives are preferable to synthetic ones. Therefore, there's a growing interest for natural dyes as food additives.

Anthocyanins (E163) are mainly used as dyes in food for the colour they imprint: red (acid pH), purple (neutral pH) or blue (alkaline pH). These dyes are used in various food products such as: dairy products, fruit fillings, snack bars, dry-mix beverages or confectionery products (Solymosi et al., 2015).

In addition to their colouring action, anthocyanins are also able to stimulate cell defence mechanisms neutralising free radicals, being therefore important for vital processes (He and Giusti, 2010).

Another benefit for using anthocyanins in food products is the fact that they can act as markers for food quality, participate in lowering the risk of coronary heart disease, stroke or even cancer and improve the nutritional quality (Lao & Giusti, 2016; Mansour, 2018). Anthocyanins are also used as a marker for wine classification upon their grape variety (Martín et al., 2017).

Cosmetic industry

Due to its action as a protective agent against UV irradiation (Martín et al., 2017), purple maize anthocyanins can be used in skincare formulations for lotion or creams that are marketed as sun protectants. Some studies revealed that efficacy of anthocyanins regarding its ability of absorption of UV irradiation was about 46% (Rojo et al., 2013).

Pharmaceutical or medical applications

Due to their wide range of actions mentioned before, anthocyanins are perfect candidates for medication for prevention or treatments for cancer, diabetes mellitus or cardiovascular diseases (Martín et al., 2017). They often are prescribed as dietary supplements or nutraceuticals.

Amongst other health benefits are worth mentioning: anti-obesity (suppressed body weight gain, fat tissue gain and other metabolic disorders), visual health (improved visual functions in tension glaucoma, increased ocular blood flows, decreased lens opacity, prevented retinal degeneration), or antimicrobial (Khoo et al., 2017; Tsuda et al., 2003; Shim et al., 2012; Thiraphatthanavong et al., 2014; Paik et al., 2012; Ohguro et al., 2012).

Other applications

New promising researches studied the use of anthocyanins as sensitizer in dye-sensitized solar cells that simulate photosynthesis in plants, but in low light conditions. Although the efficiency of this type of solar cells was low, the experiments were considered somewhat a success due to the fact that they were easy to obtain and had a low cost (Mansour, 2018), in comparison with classic dye-sensitized solar cells that use ruthenium-based dyes.

Another application is centred in the horticulture domain, anthocyanins being used as dyes in a genetic engineering protocol in order to create flowers with new colours such as blue roses (Martín et al., 2017).

CONCLUSIONS

In comparison with synthetic dyes, natural dyes are less toxic and biodegradable, therefore having a low negative impact on the environment.

The main dyes extracted from purple maize are anthocyanins, but in some cultivars were also found low concentration of carotenoids. The main anthocyanins found in purple maize are represented by: pelargonidin-3-glucoside cyanidin-3-glucoside, peonidin-3-glucoside and their malonated counterparts.

The extraction of natural dyes can be conducted either through conventional methods, either through modern methods, the most used being microwave or ultrasound assisted ones, both being considered eco-friendly and very efficient.

The best methods regarding accuracy for identification and quantification of anthocyanins from purple maize are the pH differential and the HPLC intact anthocyanins methods.

The multiple benefits of anthocyanins (antioxidant, anti-obesity, anticarcinogenic, anti-inflammatory, antimicrobial, neuroprotective etc.) support their use in various applications such as: food, textile, pharmaceutic, cosmetic or medical.

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