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Development of a natural food colorant from *Passiflora edulis* Sims epicarp

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Equations

Abbreviations

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ADHD	Attention Deficit Hyperactivity Disorder
ADI	Acceptable Daily Intake
ATCC	American Type Culture Collection
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BOD	Biological Oxygen Demand
CAC	International Codex Alimentarius Commission
CEC	European Commission Scientific Committee
CCCD	Circumscribed Central Composite Design
DAD	Diode Array Detector
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
EC	European Commission
EC_{50}	Extract concentration corresponding to 50% of antioxidant activity
EDTA	Ethylenediaminetetraacetic
EEC	European Economic Community
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FSC	Food Supply Chain
HAE	Heat Assisted Extraction
HBSS	Hank’s balanced salt solution
HHP	High Hydrostatic Pressure
HPLC	High-Performance Liquid Chromatography
INT	p-Iodonitrotetrazolium Chloride
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LPS	Lipopolysaccharide
MA	Malt extract
MBC	Minimal Bactericial Concentration
MDA	Malodialdehyde
MFC	Minimum Fungicidal Concentration
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentration
MS	Mass Spectrometry
NED	N- (1-naphthyl) ethylenediamine hydrochloride
NO	Nitric oxide
NCTC	National Collection of Type Cultures

OxHLIA	oxidative hemolysis inhibition assay
PBS	Phosphate-buffered saline
PEF	Pulsed Electric Field
RPMI	Roswell Park Memorial Institute
RSM	Response Surface Methodology
Rt	Rentention time
S	Solvent
SE	Soxhlet Extraction
SRB	Sulforhodamine B
S/L	Solid-Liquid ratio
T	Tempreture
t	time
TAC	Total anthocyanin Content
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Species
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic acid
TSB	Tryptic Soy Broth
UAE	Ultrasonic Assisted Extraction
UE	Ultrasonic Extraction
USA	The United States of America
WFD	the European Waste Framework Directive
WHO	The World Health Organization

Abstract

Food industry is dedicated in the continuous improvement of products safety and quality, satisfying the consumer's requirements. Thus, this sector has invested in the development of new solutions to decrease the amount of used artificial additives and whenever possible, replacing them by more innocuous natural counterparts. Colorants are one of the most important additives in terms of marketing, since their presence influences consumer's perceptions, choices and preferences, interfering in market success or failure of food products. In this context, there are many fruits in which their bio-residue can be used to extract colorant ingredientes.

Passiflora edulis, known as passion fruit, is native from Brazil and much appreciated by the cosumers. The purple passion fruit variety (*Passiflora edulis* Sims) is the most common variety and is cultivated for juice and pulp production. However, the largest portion of the fruit is constituted by the epicarp, resulting in a high quantity of bio-waste production. This part of the fruit has a dark purple colour, being a rich source of pigmented compounds, namely anthocyanins, which could be used as a source of natural ingredients with colourant potential.

In this work, the extraction methodology of anthocyanins from passion fruit epicarp was optimized through a heat assisted extraction (HAE), by applying a response surface methodology combining different independent variables of the process. Furthermore, the bioactive properties (antioxidant, antimicrobial and cytotoxic activities) of the optimized extract and also of the extract obtained by a conventional extraction method (maceration using 80:20 v/v of acidified ethanol/water at room temperature) were compared. The anthocyanins profile was determined by HPLC-DAD-ESI/MS, using 520 nm as the preference wavelength and operating in positive mode. The antioxidant activity was evaluated by means of TBARS and OxHLIA methodologies; the antimicrobial activity was determined using the microdilution method in Gram-positive and Gram-negative bacteria, and in fungi; and finally, the cytotoxic properties were studied in four human tumor cell lines: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (lung carcinoma); and in a non-tumor cell line: PLP2 (primary culture of pig liver cells).

The HAE optimal conditions were established as t= 37.5 min, T= 20 ºC, S= 0 % (ethanol) using 50 g/L of solid/liquid ratio, conducting to an extraction yield of 37%, with a total anthocyanins' content of 9.02 mg of anthocyanins (A) per g of extract (dried extracted residue-basis) and 3.35 mg of A per g of dried epicarp basis. Regarding the bioactive properties, the optimized extract

rich in anthocyanin compounds was highlighted with higher antioxidant, antimicrobial and cytotoxic potential in comparison to the extract obtained by the conventional method.

Overall, this study has a great interest in the food sector, because it highlights the colorant and bioactive potential of this natural ingredient, while enhancing the valorization of a specific bio-residue, widely produced by the food sector.

Keywords: *Passiflora edulis* Sims, Extraction optimization, Response surface methodology (RSM), Anthocyanins, Bioactivities.

Resumo

A indústria alimentar tem se dedicado à melhoria contínua da segurança e qualidade dos produtos, satisfazendo os requisitos do consumidor. Assim, este setor tem investido no desenvolvimento de novas soluções para diminuir a quantidade de aditivos artificiais usados e, sempre que possível, substituí-los por equivalentes naturais mais inócuos. Os corantes são um dos aditivos mais importantes em termos de marketing, uma vez que a sua presença influencia as percepções, escolhas e preferências do consumidor, interferindo no sucesso ou no fracasso dos produtos alimetares no mercado. Neste contexto, existem muitos frutos cujos seus subprodutos podem ser utilizados para extrair ingredientes corantes.

Passiflora edulis, conhecida como maracujá, é nativa do Brasil e muito apreciada pelo cosumidor. A variedade de maracujá roxo (*Passiflora edulis* Sims) é a mais comum e é cultivada para produção de sumo e polpa. Contudo, a maior parte da fruta é constituída pelo epicarpo, resultando numa elevada quantidade de produção de bio-resíduos. Esta parte do fruto tem uma cor púrpura escura, sendo uma fonte rica em compostos corantes, nomeadamente antocianinas, sendo uma fonte de ingredientes com potencial corante.

Neste trabalho, a metodologia de extração de antocianinas a partir do epicarpo de maracujá foi otimizada através da extração assistida por calor (HAE), aplicando uma metodologia de superfície de resposta, combinando diferentes variáveis independentes do processo. Além disso, foram comparadas as propriedades bioativas (atividades antioxidante, antimicrobiana e citotóxicas) do extrato otimizado e também do extrato obtido pelo método de extração convencional (maceração usando 80:20 *v/v* de etanol/água acidificada, à temperatura ambiente). O perfil de antocianinas foi determinado por HPLC-DAD-ESI/MS, usando 520 nm como o comprimento de onda preferencial e operando em modo positivo. A atividade antioxidante foi avaliada através das metodologias TBARS e OxHLIA; a atividade antimicrobiana foi determinada usando o método de microdiluição em bactérias Gram-positivas e Gram-negativas, e em fungos; e, finalmente, a citotoxicidade foi estudada utilizando quatro linhas celulares tumorais humanas: HeLa (adenocarcinoma cervical), HepG2 (carcinoma hepatocelular), MCF-7 (adenocarcinoma de mama) e NCI-H460 (carcinoma de pulmão); e numa cultura de células não tumorais: PLP2 (cultura primária de células de fígado de porco).

As condições ótimas de HAE foram estabelecidas com t = 37,5 min, T = 20 ºC, S = 0% (etanol) usando 50 g/L de razão sólido/líquido, conduzindo a um rendimento de extração de 37%, com um teor total de antocianinas de 9,02 mg de antocianinas (A) por g de extracto (resíduo-base de extrato seco) e 3,35 mg de A por g de resíduo (base de epicarpo seco). Quanto às propriedades bioativas, o extrato otimizado rico em compostos antociânicos destacou-se com alto potencial antioxidante, antimicrobiano e citotóxico, comparativamente com extrato obtido pelo método convencional.

No geral, este estudo tem um grande interesse na área alimentar, pois destaca o potencial corante e bioativo de um ingrediente natural, ao mesmo tempo reforçando a valorização de um subproduto específico, amplamente produzido pelo setor alimentar.

Palavras-chave: *Passiflora edulis* Sims, Otimização de extração, Metodologia de superficie de resposta (RSM), Antocianinas, Bioatividades.

Biblioghraphic review

1 INTRODUCTION

1.1 FOOD ADDITIVES

The use of food additives comes from ancestral times, especially the use of colorants. Their use has been reported in several studies, in which their applications draw back to the 2600 BC in China. Also, in Europe the use of these compounds is quite old, reporting to the Bronze age. Moreover, and according to literature, around 1500 BC in Egyptian cities, some manufacturers used these additives to improve the appearance of various food products (Lakshmi, 2014).

The World Health Organization (WHO), the International Codex Alimentarius Commission (CAC), and the Food and Agriculture Organization (FAO) defines food additivesas "*any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods*". The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities ("Codex Alimentarius Commission - Procedural Manual").

Additives can be applied in numerous processed foodstuffs, namely, in non-perishable food, such as, chips, ketchup, sauces, chocolates, puddings, colored candies, powdered drink mix, processed meat, dairy products, canned and fermented products, and instant soups (FDA, 2010). The application of these compounds by the industry sector has several objectives, especially to ensure the quality characteristics of processed foods, to increase the shelf-life of food (preservatives and antioxidants) and to improve the presentation of compound feeds (colorants and texture agents) (Lakshmi, 2014).

The Codex Alimentarius Commission (CAC), an international association made up with 190 countries, presently groups food additives into 27 classes (such as, anti-caking agents, antioxidants, colorings, enzymes, mineral salts, vitamins and others), based on their functionalities ("Codex Alimentarius Commission - Procedural Manual").

In Europe, together with the nutritional information, these compounds are also listed on product labels, being mentioned with the “E” letter, followed by the number corresponding to its category. Thus, in the case of coloring agents the identifications are made using numbers between 100 and 180, the preservatives are identified with numbers between 200 to 285, the antioxidants are identified using numbers between 300 to 321 and for texturing agents, the numeric scale range between 322 to 495. The numerical scale of 500 to 1520 comprises acids, alkalis, flavour enhancers and sweeteners, as well as additives with various other functions (Janiszewska-Turak et al., 2016).

The use of all food additives is controlled by legislation, which is harmonized across the European Union. The legislation, in each member state, is based on the various additive directives incorporated into the appropriate national legislation.

The framework Directive on Additives (89/107/ EEC) provides the “umbrella legislation”, under which the individual additives directives are developed. It includes a definition of a food additive, exclusions from the scope of the definition and a list of food additive categories, one of which is “colour”. The three major detailed directives on additives are “colours” (94/36/EC), “sweeteners” (94/35/EC) and “additives” other than “colours and sweeteners” (95/2/EC), the latter usually being referred as “The Miscellaneous Additives Directive”. Commission Directive 95/45/EC deals with purity criteria for colours, i.e. specifications (Downham and Collins, 2000).

Aiming the application of food additives by the industry, studies must be carried out which demonstrat not only their purpose, but also the daily dose of consumption, so as not to endanger the health of the consumer. In European Union, the EU's Scientific Committee for Food (CEC) is the entity responsible for evaluating all food additives (Pisanello, 2014). The evaluations performed by this organization are based on reviews of all available toxicological data, including observations in humans and in animal models, making studies in lifetime feeding and multigeneration in experimental animals. Thus, the maximum consumption level of an additive cannot demonstrate toxic effects in humans (European Parliament and Council., 1994).

1.1.1 The relevance of food additives

Food additives are used in food products following a regulatory framework to control health risks. In fact, the use of these compounds is defined by two factors: safety and technological need ("Codex Alimentarius Commission - Procedural Manual").

Regarding the safety, all food additives are tested and evaluated with an appropriate assessment, considering some parameters, such as, any cumulative effects, synergistic or strengthening of their functions. The additives should not represent health risk to consumers, at proposed levels of application. All food additives are kept under a permanent and reassessed observation, in order to know the variations in the conditions of the proposed use levels and new scientific data must always be in accordance with the approved specifications and purity ("Codex Alimentarius Commission - Procedural Manual").

Concerning the technological needs, these compounds are applied for several reasons, such as to maintain the food quality and nutritional features, by introducing the necessary constituents in foodstuff (beneficiating certain group of consumers with special dietary needs); and for keeping quality or stability of a food products and, improving the organoleptic properties (this application cannot change the nature, substance or quality of the food product). These changes in food products can provide benefits in the manufacture process, preparation, treatment, packing, transport or shelf life of foodstuff ("Codex Alimentarius Commission - Procedural Manual").

In addition, the approval of a food additive is limited as much as possible to a specific food, for specific purposes, and under specific conditions (Janiszewska-Turak et al., 2016). Food additives could be classified according to its application (Sezgin and Ayy, 2017), such as:

i) Extending the shelf life by protecting quality - preservatives: antimicrobial substances (nitrite, nitrate, benzoic acid, propionic acid, sorbic acid) and antioxidant (BHA, BHT, gallates) substances;
ii) Improving food structure, preparation and cooking: pH regulators, anti-caking agent (silicate, magnesium oxide, magnesium carbonate), emulsifiers (lecithin, mono and diglycerides), stabilizers, thickeners, sweeteners, fermentation agents, moisture regulators, maturing agents, bleaches, fillers, foam conditioners, and polishers;
iii) Improving colour and flavour: flavour enhancers, condiments (flavour substances), colorants (tartrazine and indigotine), protecting and improving nutritional value

(nutritional elements), replacing missed nutrients during processing (B1, B2, and niacin), adding nutritional elements that might be lacking in the diet (vitamins A and D).

1.1.2 Application of colorants in the food industry

According to some authors a colorant "*is any dye, pigment or substance which when added or applied to a food, drug or cosmetic, or to the human body, is capable (alone or through reactions with other substances) of imparting colour*" (Kumar et al., 2017). In particular, colorants play an important role in consumer preferences, because when applied the turn products much more attractive, making then catch attention. Thus, colour is often a significant factor that will define the food choice and eating desires, being extremely important in the selection of competitive products (Ruumpol, 2014).

Although artificial colours began to dominate the market for paints and textiles, in the nineteenth century these pigments started to be highly used in the food industry to improve the appearance of certain foods (López et al., 2018). The first artificial colorant (mauveine) was developed in 1856, by the researcher Sir William Henry Perkin. Thus, the beginning of that century was marked for the major production and recovery of artificial colours obtained from petroleum-derived products, such as aniline. These products were called 'coal-tar' colours because the starting materials were obtained from coal (Lakshmi, 2014).

The colorants can be divided into 3 groups, such as: i) colorants that their ADI (acceptable daily intake) values are determined and allowed for use; ii) colorants permitted to be applied only for special purposes (such as surface finishing, $CaCO_3$, aluminum, silver, and gold), and iii) colorants that are only allowed to be uses in certain food products (titanium dioxide, vegetable carbon, and red beet) (Sezgin and Ayy, 2017).

Moreover, colour additives can also be divided regarding their origin, into natural and artificial additives. Natural additives are molecules extracted from plants (indigo, saffron, passiflora), insects (cochineal beetles, lac scale insects), animals (some species of mollusks, shellfish), and minerals (ferrous sulfate, ochre, clay), having importance not only as coloring agent, but also due to their medicinal properties (antibacterial and anti-fungal, antioxidant, anti-inflammatory, and anti-cancer properties) (Alagusundaram and Chetty, 2010). However, unfortunately these compounds have a low stability, because of several factors like low resistance to a higher temperature, light, oxygenation, and pH change (Cortez et al., 2017).

On the other hand, artificial additives are not extracted, but are a result of a chemical or enzymatic reaction. They are either completely identical to a natural equivalent, or pure creations which do not exist in a natural state (Janiszewska-Turak et al., 2016). They are synthesized from raw materials obtained from coal tar or petroleum by-products. Artificial colours, namely brilliant blue (E133) are used in dairy products, sweets and drinks. It can cause hyperactivity, skin rashes, bronchoconstriction (especially when combined with other artificial colours). Indigo carmine (E132), fast green (E143), erythrosine (E127), and Allura red (E129), have been linked to behavioural changes, especially in children (Turner et al., 2012). Unfortunately, these reactions and their potential connection to artificial food additives have been largely dismissed by governmental and the food industry. In 2004, an analysis of fifteen studies found evidence that artificial colours worsen the behaviour of children with attention deficit hyperactivity disorder (ADHD), which is a diagnosis given to children that exhibit symptoms of hyperactivity, impulsivity, and distractibility (Eugene et al., 2012). Other symptoms include fidgeting, squirming, inability to listen, forgetfulness, and lack of response to discipline. According to the Centers for Disease Control, approximately 5% of children have ADHD, although other studies have put the estimate between 8-10% of school-aged children (Stevens, 2014).

The several norms attached to the EU regulations (94/36/EC) set out a list of the permitted colours, having been identified 43 colours (17 artificial and 26 natural pigments). In addition, a list of basic foodstuffs to which colours must not be added, a list of foodstuffs in which only a limited number of colours may be used, a list of colours which have restricted application and a list of colours generally permitted and colour with maximum inclusion levels for particular food categories is present (Downham and Collins, 2000).

Additives are added in several stages of food production with two main purposes, one is to make food safe by inhibiting bacterial growth, oxidation formation, and other chemical changes. The second reason is that addiditves are attracting the consumer, improving by-products organoleptic properties, such as colour, appearance, flavour and smell (Lakshmi, 2014).

1.1.3 Advantages of the use of natural colorant additives

Colour is spread widely in nature in fruit, vegetables, seeds and roots. In our daily diets, we consume large quantities of many natural pigments, especially anthocyanins, carotenoids,

chlorophylls, and betacyanins (Martins et al., 2016). Significant development has occurred with natural colorants since their wider commercialization around 25 years ago. The growth in the use of natural colours comes from increasing the consumer preference for natural products with clean labels (Meghwal and Goyal, 2016). Natural pigments have a surprising number of colours, which indicate a degree of sweetness, ripeness or deterioration, type of flavour and visual information about phytochemical properties, namely the presence of natural pigments, such as anthocyanins, curcumin, betalains, and others (Lakshmi, 2014).

Regarding the advantages of the use of natural compounds, the risk of toxicity is analysed by several processes, in which the structure/activity relationship of the constituent's present is analysed. The same tests do not apply to the artificial compounds, since the active principles of natural matrices vary according to several factors: the plant classification family, conditions imposed on its growth, the plant part from which the compound is isolated, being also important to mention that a particular compound can be isolated from different species (Sharma, 2014; Burdock and Wang, 2017). The food additives (namely natural colorants) differ from each other according to several properties, such as chemical structures, sources and use purposes. Some examples of natural food colorants and their health benefits are presented in **Table 1** (Alagusundaram, 2010).

Table 1: Some pigmented molecules, possible sources, health benefits and apparent colours.

Molecule	Source	Health benefits	Colour	Reference
Anthocyanins	Grape, elderberry, blackcurrant, red beets, black carrots, purple passion fruit.	Anti-inflammatory, antiviral, antimicrobial and anti-cancer benefits and the prevention of chronic diseases.	Red to blue.	Khoo, 2017
β-carotene	Carrots, pumpkin, sweet potato, winter squash.	Skin protection and cell growth.	Orange, red and yellow.	Burri, 1997
Curcumin	*Curcuma longa*.	Prevents cancer formation and progression, increases the activity of certain enzymes responsible for digestion, and promotes detoxification of liver which acts as an antibacterial agent.	Yellow.	Soriano, 2012
Lycopene	Tomatoes, watermelon, grapefruit, papaya, sweet red peppers, persimmon, asparagus, red cabbage, and mangos.	Reducing all types of cancer, especially the risk of breast, prostate and cervical cancer.	Orange-red.	Story et al., 2010

Other carotenoids	Melon, papaya, orange, mango.	Antioxidant activity, pre-substance of vitamin A, also protects against oxidative damage and is evaluated positively for health.	Golden yellow to orange.	Fiedor and Burda, 1997

Thus, the importance of these molecules makes it necessary to develop new techniques and processes that allow not only the extraction of substances from natural matrices, but also, to stabilize substances already discovered. It is also important to study new species that are the source of these substances, taking advantage of all available resources in nature (Martins et al., 2016).

1.1.4 Anthocyanins and their natural sources

Anthocyanins are the most important and broad group of natural pigments, after chlorophyll, visible to the human eye. These molecules are characterized as polyphenols and are responsible for several colours of many fruits and vegetables, varying from a wide range of colours between red and blue (Khoo, 2017).

This group of compounds have been widely used as food additives or dietary supplements, because of their multiple health promoting effects, including anti-inflammatory, anti-proliferative and antioxidant roles. Therefore, the extraction of anthocyanins from natural resources has become an important research topic (Mei et al., 2018).

Anthocyanin is the glycosidic form, while anthocyanidin is known as the aglycone and are grouped into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and *O*-methylated anthocyanidins (Khoo, 2017). This pigment belongs to the flavonoid group, although it has a positive charge in the oxygen atom of the C-ring of the basic flavonoid structure. It is also called flavylium (2-phenylchromenylium) ion (Garcia-Alonso et al., 2009).

The empirical formula for the flavylium ion of anthocyanin is $C_{15}H_{11}O^{+}$, with a molecular weight of 207.24724 g/mol. The conjugated bonds of anthocyanins result in red, blue, and purple-colored plants (Taylor et al., 2014). The general molecular structure of anthocyanin is shown in **Figure 1** (Khoo, 2017).

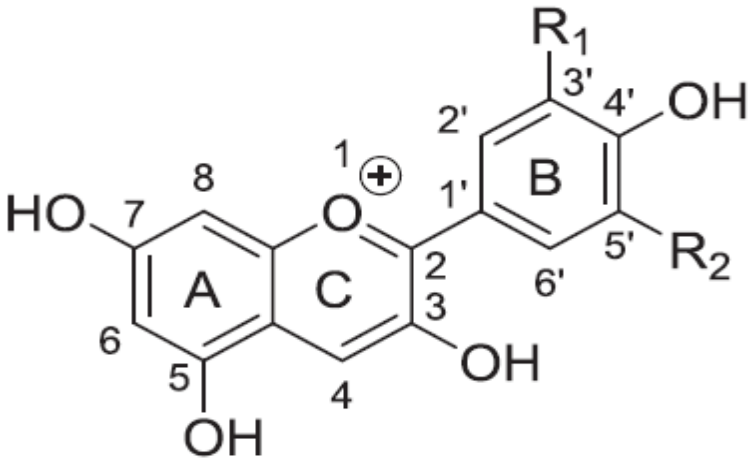


Figure 1: The general anthocyanin chemical structure.

The most common types of anthocyanins are cyanidin, delphinidin, pelargonidin, peonidin, malvidin and petunidin (**Table 2**) and they are largely distributed in fruits, vegetables and flowers (Castañeda-Ovando et al., 2009).

Table 2: Structure of common anthocyanins found in nature (Castañeda-Ovando et al., 2009).

Anthocyanins	R1	R2	Main colour
Cyanidin	OH	H	Orange-red
Delphinidin	OH	OH	Bluish-red
Pelargonidin	H	H	Orange
Peonidin	OCH_3	H	Orange-red
Malvidin	OCH_3	OCH_3	Bluish-red
Petunidin	OCH_3	OH	Bluish-red

In nature, cyanidin is a reddish-purple (magenta) pigment, and it is the major pigment in berries and other red-colored vegetables, such as red sweet potato and purple corn (Cevallos-Casals and Cisneros-Zevallos, 2003).

On the other hand, delphinidin has a chemical characteristic similar to most of the anthocyanidins. It appears as a blue-reddish or purple pigment in the plants. The blue hue of flowers is due to the delphinidin pigment (Katsumoto et al., 2007). Pelargonidin differs from most of the anthocyanins, and in nature, it appears as red-colored pigment and it gives an orange hue to flowers and red colour to some of the fruits and berries (Jaakola, 2013).

The methylated anthocyanidin, such as peonidin is another type of anthocyanin abundantly found in plants, it has visible colour magenta. Peonidin is abundantly found in berries, grapes, and red wines (Khoo, 2017). Malvidin is another *O*-methylated anthocyanidin. It has a purple

colour, and is abundant in blue colored flowers, especially Summer Wave Blue. Besides, it is the major red pigment in red wine; it appears in a darker dusty red colour in matured red wines (Barnard et al., 2011). Petunidin is a methylated anthocyanidin, its darker red or purple pigment is soluble in water and it has been detected in blackcurrants and purple petals of flower (Yabuya et al., 1997).

Anthocyanins extracted and isolated from natural matrices can be used in different industries, being most sought by the food industry, where they are used as colorants in order to enhance colour in food products (Carocho et al., 2015).

These are considered not only as a coloring matter, but also as a class of colorants because more than 600 types of anthocyanins have been found and characterized in nature, all of which have shades ranging from blue to red (Wrolstad and Culver, 2012; Carocho et al., 2015). The most common sources of anthocyanins, the pigment, the kind of industrial application and health benefits are present in **Table 3**.

Table 3: Sources of anthocyanins, health benefits and industrial applications.

Source	Colour	Health benefits	Industrial applications	Reference
Blueberries, Chokeberries, Black raspberries,	Red/purple	Anti-inflammatory	Juice industry	El-ella D.M.A. and, Bishayee. A. (2019).
Rubired Grape	Red/purple	Antioxidant	Wine and juice industry	
Black Carrot	Red/orange	Antioxidant	Natural colorant in yoghurt	Bilek, 2016
Purple sweet potato	Magenta	Antioxidants, Anticarcinogenic, Antidiabetic	Sweeteners, beverage, noodle production, industrial alcohol, and derived products as maltose	Jaffer and Moothandassery, 2012
Purple passion fruit	Purple	Anti-inflammatory, Antimicrobial Anticarcinogenic	Juice industry	Fang, 2015

1.2 OBTAINING FOOD COLORANTS RICH IN ANTHOCYANINS

Anthocyanins present a high potential to be used as colorants in the industrial sector, due to the attractive orange, red, blue and purple colors, and in particular in food industry due to the easy solubility in water, that allows their incorporation into aqueous food systems (Mazza and Miniati, 1993). The FAO/WHO Expert Committee on Food Additives (JECFA), concluded that extracts rich in anthocyanin compounds have a very low toxicity (Bkowska-Barczak, 2005),

indicating a safe consumption, even at high doses, compared to artificial colorants. However, anthocyanins have not only colorant potential, but also health benefit effects (Khoo, 2005). According to some authors, the health benefits are associated with the increase of sight acuteness, anti-carcinogenic activity, antioxidant capacity, anti-ulcer activity and the maintenance of normal vascular permeability (vitamin C_2). Also, the isolated and purified anthocyanins from fruit and vegetables may be useful in the treatment of chronic diseases, particularly, type 2-diabetes. However, the most significant function of anthocyanin extracts is their antioxidant activity (Bkowska-Barczak, 2005).

All these bioactive properties, give anthocyanins a higher value in the industrial sector, especially for the food and nutraceutical industry. However, the extraction of these compounds involves complex processes, which require the adjustment of various parameters, such as the extraction technique to be applied, solvent type, extraction time, temperature and all the sample handling (Pereira and Meireles, 2012). For this, it is necessary to develop and optimize extraction methodologies that allow not only the extraction of these molecules, but also to guarantee a high yield and quality (Santana et al., 2009).

1.2.1 Anthocyanins extraction techniques

A huge array of solid-liquid extraction procedures are available to recover compounds of interest from natural matrices (Chemat et al., 2017; Zhu et al., 2017). Concisely, the solid-liquid extraction consists in keeping the solid sample (usually in powder form) in direct contact with a solvent for a specific time, and by applying a certain level of energy (conventional heat, ultrasound or microwave radiation, pressure, etc.) (Fattore et al., 2016; Zhu et al., 2016). The common solid-liquid procedures comprise the conventional methods, such as Soxhlet and maceration extractions. These methods are easy to apply and relatively inexpensive; nevertheless several authors have pointed out some disadvantages, which are mainly associated with their application at industrial level, i.e. the use of large amounts of solvent and long extraction times (Azmir et al., 2013). Thus, the application of different extraction techniques may increase the extraction speed and yield, meaning, that it is possible to choose a more environmentally friendly extraction system, with a better performance than its conventional extraction. However, conventional methods are still important at industrial level, mainly due to the lack of comparative results showing the advantages of the alternative modern techniques.

The following points will describe the conventional and non-conventional methods applied to extract natural colorants, such as maceration, soxhlet extraction (SE), high hydrostatic pressure (HHP), pulsed electric field (PEF), and ultrasonic extraction (UE) (Lakshmi, 2014).

1.2.1.1 Maceration extraction

Maceration extraction (**Figure 2**) has been the most common used method for the extraction of a diverse number of compounds present in plants, including phenolic compounds. These molecules have been extracted from different dry plant parts powders, or only by soaking fresh fruits and plants with subsequent solvents (Castañeda-Ovando et al., 2009).

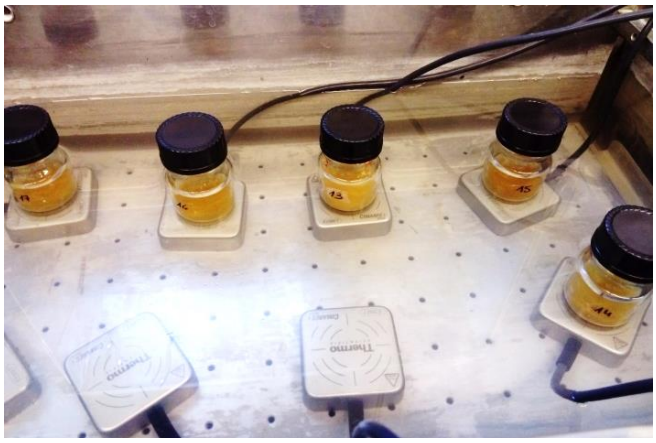


Figure 2: Maceration extraction of anthocyanin compounds

This technique, in addition to being the simplest system, effectively promotes the extraction of active compounds using organic solvents, or water/alcohol mixtures, and can be performed with or without temperature and agitation (Albuquerque et al., 2016).

Anthocyanin compounds are commonly extracted from flowers, berries, black currant, purple-colored fruits and vegetables. In spite of water to be the typical extraction solvent for isolation of these pigments, some food processing industries use alcoholic solutions (such as ethanol, methanol, acetone or mixed solvents) to perform the extraction of these compounds. This can be justified because anthocyanins are soluble in water and in polar organic solvents (Kahkonen et al., 2001).

However, one of the disadvantages of these compounds is the absence of solubility in the nonpolar organic solvent and the instability in alkaline or neutral solutions (Khoo, 2005).

The extraction methods using acidified methanol or ethanol are the most applied and according to some studies, the extraction with methanol is the most efficient, achieving higher yields in

comparison to ethanol (Ongkowijoyo et al., 2018; Blackhall et al., 2018), concluded that the extraction with methanol is more effective than with ethanol in the anthocyanin extractions. However, the food industry prefers the use of ethanol in this process, due to the toxicity underlying to methanol (Castañeda-Ovando et al., 2009).

1.2.1.2 Soxhlet extraction (SE)

The Soxhlet extractor was invented in 1879 by Franz von Soxhlet and the equipment can be seen schematically in **Figure 3**. The extraction solvent is located in a heated flask and on top of that is the main chamber, containing the solid material (usually placed in a filter paper). Solvent vapour migrates up a distillation arm and condenses into the sample chamber, which is filled with warm solvent. The extraction temperature cannot be changed and is a few degrees lower than the boiling point of the solvent. Some of the desired compounds will dissolve and when the chamber is filled with solvent, a siphon side arm will automatically empty the extract down to the heated flask again.

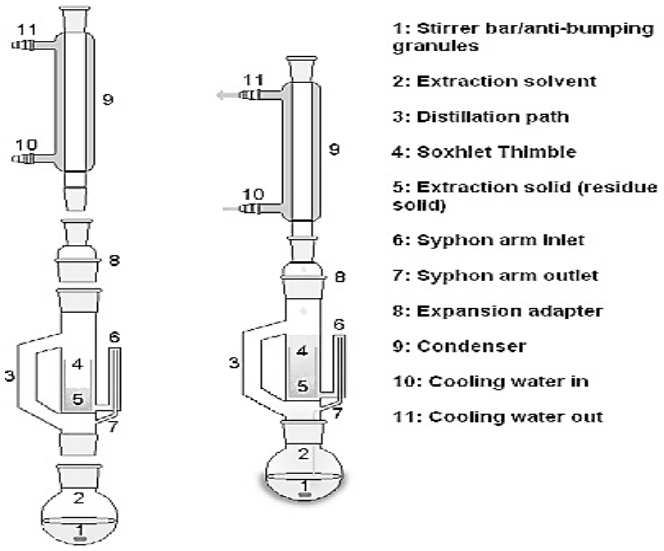


Figure 3: Schematic representation of a Soxhlet extractor system

The process restarts, and the desired compound are by this process concentrated at the bottom flask. The cycle can be repeated many times, over hours or days. After extraction, the solvent is removed, typically through a rotary evaporator, yielding the extracted compound (Petersson, 2009). Due to the high temperatures used, in combination with long extraction times, soxhlet extraction may not be suitable for thermolabile compounds. Soxhlet extraction has been used for extraction of acrylamide from foods (Saini and Keum, 2018).

There are many factors that influence the Soxhlet extraction process, such as matrix characteristics, particle size of samples, and boiling temperature. Temperature does not only affect the yield and quality of final products but also reduces the visual colour of the extracted compounds. It can be slightly solved by using membrane separation or vacuum (Petersson, 2009; Di Khanh, 2015; Saini and Keum, 2018). This extraction methodology has been widely used for industrial applications with high efficiency and reproductivity. Nevertheless, it is an old model and requires longer time and more solvents for the operation, compared with other novel fast extraction techniques, such as microwave-assisted and ultrasound-assisted extractions. Thus, this technique is mostly applied for the extraction of oil and other phenolic compounds, instead of the extraction of anthocyanins from fruits and vegetable (Di Khanh, 2015).

1.2.1.3 High hydrostatic pressure (HHP) and Pulsed electric field (PEF)

High hydrostatic pressure (HHP) and pulsed electric fields (PEF) belong to an environment friendly category and energy efficient technologies. These methods enhance the mass transfer processes within plant or animal cellular tissues, as the permeability of cytoplasmic membranes can be increased, which in turn enhances extraction of valuable cell components (Di Khanh, 2015).

In addition, the decrease in the dielectric constant of water under HHP combined with temperature, leads to a decrease in the polarity of the media, contributing to the higher yield of total phenolics and other antioxidants. PEF is reported to enhance mass transfer rates by electroporation of plant cell membranes, improving tissue softness and thus influencing the textural properties. PEF is reported to be an ideal method to enhance juice production, and increase the extraction of valuable components (Lakshmi, 2014).

1.2.1.4 Ultrasonic assisted extraction (UAE)

One of the extraction techniques that have the potential for speeding up and simplifying sample treatment is the ultrasonic assisted extraction (UAE). It can improve the recovery of bioactive components, mainly the ones that are sensitive to heat at prolonged extraction times, by keeping these variables at low levels (**Figure 4**). Ultrasonic energy, when imposed into a solution,

causes acoustic cavitation – which means bubble formation and subsequent implosion (Renata Vardanega et al., 2014).

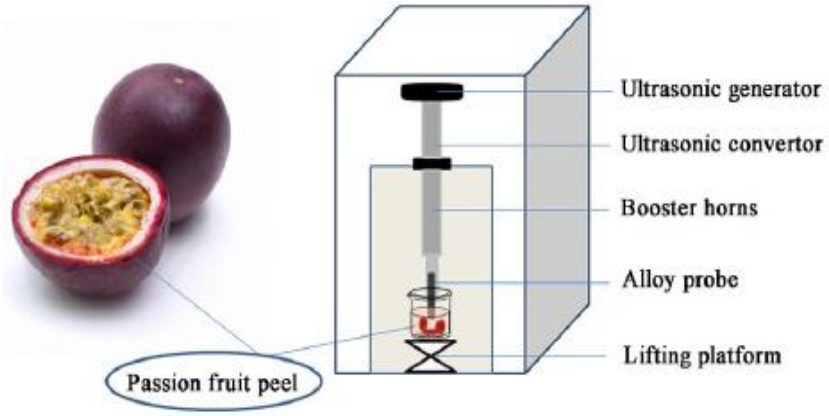


Figure 4: Representative diagram of an ultrasonic cell disruptor.

The collapse of bubbles created by the sonication of solutions results in the generation of extremely high local temperature and pressure gradients referred to as localized "hot spots". These hot spots contain effective temperatures of around 5000 K, pressures of about 1000 atm, and heating and cooling rates above 10^{10} K/sec. UAE is an effective extraction technique, in comparison to conventional methods, because the ultrasound radiation is able to disrupt cellular walls allowing better penetration of solvents in the matrix material, thus improving mass transfer and increasing cell content release (Bonfigli et al., 2017; Chemat et al., 2017). Ultrasonic extraction has been used for the extraction of anthocyanins from foods (Petersson, 2009).

1.2.2 Stability of natural extracts rich in anthocyanins

The replacement of artificial colorants by natural pigments is a challenge, due to the higher stability of artificial colorants with respect to some parameters, such as light, oxygen, temperature and pH, among others (Bukowska-Barczak, 2005).

The chemical stabilization of anthocyanins is the main focus and preoccupation of recent studies, due to their abundant and potential applications, the beneficial effects and their use as alternatives to the artificial counterparts (Cortez et al., 2016).

However, the stability of this natural pigment is determined by numerous factors, including the structure and concentration of the pigment, pH, temperature, light intensity, the presence of

copigments, metal ions, enzymes, oxygen, ascorbic acid, sugars, their degradation products, and sulfur dioxide (Bkowska-Barczak, 2005).

Regarding the molecular structure, the stability of anthocyanins is different from other natural compounds. The anthocyanin radical is more stable than other radicals generated in the human body, so the duration of this radical is longer (Joshi and Preema, 2017). The stability of this molecule is also influenced by the B-ring in the structure of this compound, and the presence of hydroxyl or methoxy groups. Hydroxyl or methoxy groups are known to decrease anthocyanin stability in solution (Khoo, 2017). So, the stability of anthocyanin is based on 3 principal factors, such pH, co-pigmentation and temperature.

1.2.2.1 Stability of the anthocyanins' colour based on pH

The stability of anthocyanins and their colour is affected by different pH conditions. This can be explained because the molecular structure of anthocyanins has an ionic nature (Cortez et al., 2016). In a strongly acidic condition (pH=1), the red-colored flavylium cation is the predominant species. Acylated anthocyanins are only stable at pH values where the flavylium cation dominates. Between pH values of 2 and 4, the uncharged blue quinonoid unstable species prevails, and if the pH increases, the ionization of the hydroxyl groups forms the anionic blue quinonoid unstable species. At pH 5 and 6, acylated anthocyanins are unstable and decolorize quickly by hydration at the 2-position of the anthocyanidin skeleton (carbinol pseudobase and chalcone structures are formed). Evidence has been provided that the chroma of some pelargonidin derivatives increased when the pH was further increased to neutral conditions showed that colorants rich in acylated anthocyanins, such as sweet potato and purple carrot, were more resistant to the pH solution than colorants rich in non-acylated anthocyanins such as red grape. The other researchers confirmed the unusual stability of acylated anthocyanins at pH over 5.0 (Khoo, 2017).

1.2.2.2 Stability of the anthocyanin's colour based on co-pigmentation

The co-pigmentation is a phenomenon in which the pigments and other colorless organic compounds, or metallic ions, form molecular or complex associations, generating a change or an increment in the colour intensity (Castañeda-Ovando et al., 2009).

In food science, this phenomenon is considered as a fascinating natural process based on noncovalent (supramolecular) complexation. It has been shown to be the main mechanism by which certain colours, particularly blue, violet, and red, are stabilized and modulated in flowers, vegetables, and fruit (in particular, berries), as well as food products derived from them. This is very relevant because colour is one of the main quality factors crucial in product acceptance (Trouillas et al., 2016).

The term "*co-pigmentation*" is commonly reserved for anthocyanins, due to their extended π-conjugated systems. This phenomenon is known to influence the colour change of anthocyanins in a solution in addition to different pH conditions. Co-pigmentation of the anthocyanin aglycone is referred to as a phenomenon where anthocyanins are reinforced by metallic ions or other flavonoids, helping to stabilize the colour of the leaves, flowers, and fruits of the plant. Also, the colour changes of anthocyanins in flower are due to the co-pigmentation of these molecules with other flavonoids and phenolic acids, increasing the colour intensity of flowers. In addition, glycosylation and acylation increase the colour strength of anthocyanin (Trouillas et al., 2016).

Recent research has shown that anthocyanins with acylating substituents are more stable during processing and storage than other natural pigments, so the full colour stabilization is best achieved when the anthocyanins are bear aromatic than aliphatic ones (Khoo, 2017).

1.2.2.3 Stability of the anthocyanin's colour based on temperature

In addition to the pH and co-pigmentation stability, the temperature is another significant parameter that influences the colour of anthocyanin compounds. These molecules are less stable at higher solution temperatures. A study performed by West and Mauer (2013) reports that heat treatment at a maximum temperature of 35 °C reduces the total anthocyanin content in 50% in grapes, comparison to the control berries at 25°C. On the other hand, it was also reported by the same authors that up to 40 °C, the anthocyanin colour changes from red to orange, although having a low the pH in the solution.

In addition, mild heat treatment of the extract to up to 50 °C has been shown to inactivate the enzymatic reaction (Patras et al., 2010). Therefore, mild heat treatment of raw materials, such as blanching, in the food processing industry can prevent oxidation of anthocyanins by polyphenol oxidase (Khoo, 2017).

1.2.3 Application of anthocyanin colorants in food products

Anthocyanin pigments are mainly applied with the purpose to give colour to the products which do not have colour, or that have lost colour during processing. Due to this, food sensory attributes can be improved, resulting in the satisfaction of a majority of consumers, which play a key role in the influence of the industrial sector (Thackston, 2013).

The application of this pigment aims to emphasize the colour of products; however, it is important to note that a pigment should not be added to hide the symptoms of food deterioration or worsen the quality of food (Janiszewska-Turak et al., 2016).

The global food coloring market has grown rapidly in recent years and it is expected to continue growing by 10% to 15% annually (Carle and Schweiggert, 2016). As demonstrated in other studies involving natural colorant extracts with the aim of replacing artificial additives (López et al., 2019; Fernandes et al., 2019), the key lies down behind the preservation of the beneficial properties of the extracts without altering the organoleptic characteristics of the original product.

The choice of an appropriate colour to food is very challenging and a wrong selection of colour, may lead to many problems, like the lack of consumer appeal and the failure of the success of the product in the commercial market. Colour suppliers are facing challenges to make an effective cost production, higher stability, easy to handle productions, and higher technology in the production of colours (Janiszewska-Turak et al., 2016; López et al., 2019; Fernandes et al., 2019). In this attempt, new technologies/methodologies are welcome in the food industry in order to improve and benefit over existing technologies (Petersson, 2009).

1.3 THE USE OF INDUSTRIAL AGRI-FOOD WASTES

The food industry is, maybe, one of the most regulated sectors in Europe. Hygiene and safety are the principal objectives, which take priority to reduce environmental impacts. In terms of wastes, the European Waste Framework Directive (WFD) defines bio-waste as "*biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises and comparable waste from food processing plants*" (Pap and Myllykoski, 2014). The WFD prescribes that member states shall take measures to encourage the separate collection of bio-waste, with a view to the composting and digestion of bio-waste and their

treatment, in a way that protect the environment and the use of environmentally safe materials, produced from bio-waste (Pap and Myllykoski, 2014).

The wastes resulting from the food processing are solid and liquid. In the fruit case, the amount of the obtained by-product during the processing is usually 30-50%, depending on the fruit. In this sector, two groups of by-products can be distinguished, such as pre-processing by-products that include stems, stalks and rotten fruits from sorting processes, and by-products that occur during processing such as seeds, pulp, pomace, and peels (Djilas, 2009).

Many companies dump the waste near to their installations, since these wastes have high nutrient levels, high water content and can support bacterial growth and fermentation, which may cause odours and other environmental problems (Van Dyk et al., 2013). Typically, the disposal of fruit solid waste may be achieved by incineration or utilization as animal feeds and fertilizers. Only in some cases, fruit wastes are used as raw material to produce secondary products in industrial scale. For example, in the case of a grape, seeds have long been known for their oil-rich characteristics (Waste et al., 2002). Apart from traditional uses as feeds and fertilizers, in some developing countries, those wastes may be simply discarded on the outskirts of the cities, causing major pollution to the environment, or disposed of in local landfills. The disposal of fruit wastes incurs a very high cost to the industry. In the USA, the disposal fee of apple pomace has been estimated to be higher than USD 10 million annually (Sawasdee and Stathopoulos, 2017).

Phenolic compounds are found in many fruits and vegetables and have several properties, which make them useful as antioxidants, antimicrobials and anticancer compounds, as well as having cardio-vascular protective properties. Phenolic compounds may differ according to the source of fruit and can be present at different concentrations. Therefore, these compounds could be extracted from different waste products, in order to add a higher value to these products (Van Dyk et al., 2013).

1.3.1 The waste problematic in the food industry

According to a report published by the Food and Agriculture Organization, the 2007 production volume of fruits and vegetables worldwide was 1,650 million tones, of which approximately 12% (or 198 million tons) was wasted at processing stage (**Figure 5**). Geographically, high percentages of fruit and vegetable manufacturing wastes (20-25%) were generated in Sub-

Saharan Africa, North Africa, West, and Central Asia, South and Southeast Asia, and Latin America, while those percentages in Europe, North America and Oceania, and in industrialized Asia were smaller (2%) (Sawasdee and Stathopoulos, 2017).

The food loss means the decrease in edible food mass, throughout the food chain. This occurs in production, postharvest and processing stages in the Food Supply Chain (FSC). Based on this definition, food losses do not include the parts of the merchandise not intended for human consumption, such as peels or seeds of fruits and bones of animal origin products. These losses can be avoided by a correct action, by maintaining the cold supply chain or ensuring correct storage conditions for products. Based on this definition, food loss also occurs if the product that was originally intended for human consumption is recovered in the form of feed, fertilizer or energy. On the other hand, the term "food waste" is ampler and includes all resources that are lost in the different sectors of the food supply chain (Pap and Myllykoski, 2014).

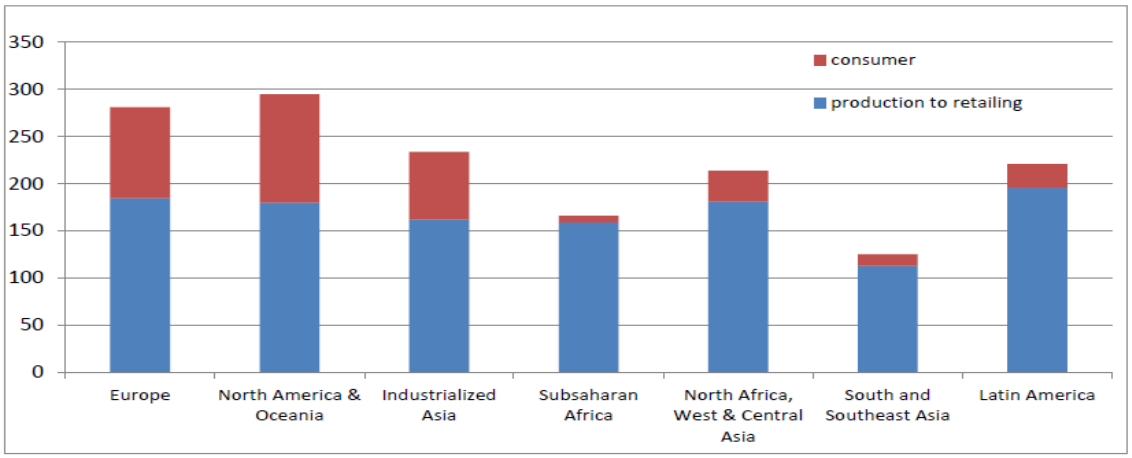


Figure 5: Per capita food losses and waste, at consumption and pre-consumptions stages in different regions (Cederberg, 2015).

With a high volume of production, the beverage industry inevitably generates a large quantity of waste. The waste streamed from the fruit juice processing are produced in solid and liquid forms. The liquid waste streams are mainly discharge of cleaning water and process water which have low-to-medium biological oxygen demand (BOD) values and can be treated by aerobic or anaerobic systems. On the other hand, the solid waste is highly polluted and more difficult to treat (Allobergenova, 2006). Conventionally these wastes are conducted for using as animal feeds or fertilizers. Although they are discarded from the process, because they cannot be further used, these solid wastes retain high concentrations of bioactive compounds. It is very relevant to refer, that peels of several fruits (for example apple, peach, pomegranate) contain

higher amounts of bioactive compounds than the edible parts (Sawasdee and Stathopoulos, 2017).

Substantial evidence revealed that all parts of fruit solid wastes are rich in health-benefit phytochemicals. Rather than using them conventionally for feeds and fertilizers, an alternative valorisation of these materials is to create higher value-added products. This alternative has attracted great interest among researchers and the industrial sector, in the last few decades (Sawasdee and Stathopoulos, 2017).

The food and beverage industry is one of the most important industrial sectors in the European Union. In Finland, food industry ranks fourth, after the metal, forest and chemical industries, in terms of the value of its output. The food supply chain is also a major employer, the entire chain provides work for some 300,000 wage and salary earners, 40,000 of whom are employed by the food industry (Pap and Myllykoski, 2014). At the same time, they reported that close to one-third of the food produced globally would virtually become waste, totalling 1.3 billion tons per year. This is clearly unsustainable, since food waste will have serious social, environmental and economic impacts. Food wastage also entails the waste of resources used to produce food, such as water, energy, land for agricultural production, and other inputs (Wunderlich and Martinez, 2018). In addition, the environmental impacts of the food chain would have been meaningless if the produced goods became waste. It is for this reason that the waste minimization and utilization become a desirable strategy, because the waste streams from the food industry are a good source of organic content, usually rich in valuable compounds, such as oils, sugars, and others compounds (Pap and Myllykoski, 2014).

The losses in industrialized countries are so high as in developing countries, however, in developing countries, more than 40% of the food losses occur at postharvest and processing levels, while in industrialized countries, more than 40% of the food losses occur at retail and in consumption. The food waste by the consumer in industrialized countries (222 million tonnes) is almost as high, as the total net food production in sub-Saharan Africa (230 million tonnes) (Pap and Myllykoski, 2014).

The environmental legislation has been very important, contributing significantly, to the introduction of sustainable waste management practices throughout the European Union. The primary aim of waste legislation is the prevention of waste generation. The Waste Framework Directive 2008/98/EC defines waste prevention as "*measures taken before a substance,*

material or product has become waste, that reduces the quantity of waste, including re-use or extension of life-span, the adverse impacts of the generated waste and the content of harmful substances in materials and products. Once waste is formed, it should be recycled or recovered for better environmental and economic performance" (Dialogue, 2010).

1.3.2 The particular case of passion fruit bio-residues

The exotic fruit juice manufacturing is another segment that generates a considerable quantity of waste. Pineapple, mango, and passion fruit are among the most important fruits for juice industry and have high percentages of inedible/unusable parts (Sawasdee and Stathopoulos, 2017).

Passion fruit *(Passiflora edulis* Sims) (**Figure 6**) is one of the most economically important crops of *Passiflora* L. genus which due to the quality of the fruit. It is widely cultivated in tropical and semi tropical regions all over the world, and it is mainly used for the production of concentrated juice and frozen pulp (Chóez-guaranda et al., 2017).

Figure 6: Passion fruit pulp and epicarp.

In the first months of 2015, Ecuador exported 6,034 tons of juice, only to Netherlands. It can be inferred that approximately 4,369 tons of residues that contains epicarp and seeds were produced in this period (Chóez-guaranda et al., 2017). One passion fruit has approximately 58% of juice and 42% of residues altogether, which correspond to around 34% of epicarp and 8% of seeds. However, this fruit residue could be as high as 75% of the raw material, as it has a thick rind. Although passion fruit seeds are edible, they are not a part of the final product and are removed as waste (Sawasdee and Stathopoulos, 2017).

Regarding the bio-residues, the major part of the passion fruit bio-residues is the epicarp, accounting in more than 50% of the fruit weight, which is obtained in the beverage production, being the main destination of the harvested fruits. Brazil is the largest producer of passion fruit in the world, being responsible for 85% of the marketed passion fruit in 2012 worldwide (Kelly et al., 2014). The passion fruit epicarp (peel) is constituted by the epicarp (purple part) and mesocarp (white part), being rich in soluble and insoluble fibers, between other compounds (Kelly et al., 2014).

1.3.3 Chemical compounds in passion fruit epicarps with emphasizes in pigments

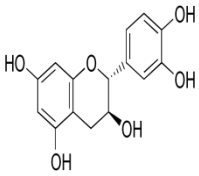
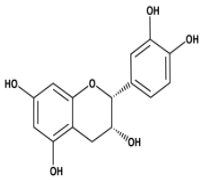
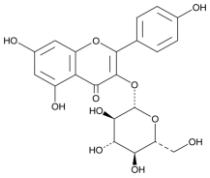
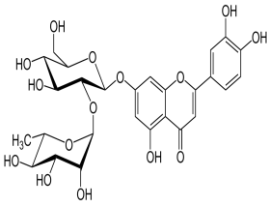
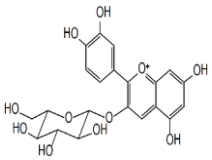
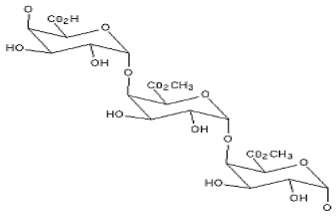
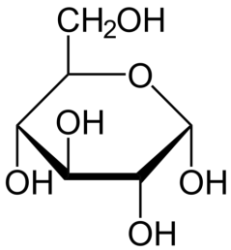
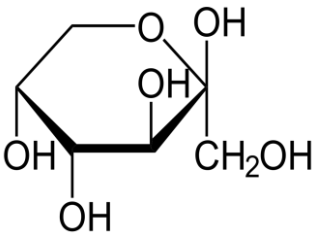
The purple passion fruit (*Passiflora edulis* Sims) is a delicious fruit with multiple nutritional and medicinal benefits. Passion fruit juice and juice concentrate are one of the most popular processed fruit products in the world market (Tripathi, 2016). During the processing of this fruit, the epicarp is usually disposed, as a solid residue that constitutes approximately, half of the fruit mass. The disposal would cause a substantial worry on the environment, and thus, it is imperative to convert the waste that is generated in processing into valuable products (Lazarevic, 2010). Previous studies, have mainly focused on pectin extraction from the epicarp, thus there is limited research about the extraction of other important constituents. Qualitative tests revealed that purple passion fruit epicarp have been reported to be rich in bioactive constituents, such as flavonoids, phenolic acids and pigments like anthocyanins (Mei et al, 2018).

The edible part and by-products of Passiflora fruits have shown high antioxidant capacity (Figueiredo et al., 2016). In general, the antioxidant capacity of passion fruits and their by-products has been attributed to their content in phenolic compounds (Sasikala et al., 2011). The extraction of phenolic compounds from Passiflora species has mostly been performed by solid-liquid extraction using different extraction solvents such as water, methanol, ethanol, and mixtures of these solvents sometimes acidified with trifluoroacetic acid and HCl (Betim Cazarin et al., 2016; Simirgiotis et al., 2013; Zeraik and Yariwake, 2010).

Phenolic compounds (**Table 4**) have scarcely been studied in *Passiflora* epicarp. Flavanols (catechin or epicatechin), flavonols (kaempferol-3-*O*-glucoside), flavones (luteolin-8-*C*-neohesperidoside) and anthocyanins (cyanidin-3-*O*-glucoside) have been identified in *P. edulis* through HPLC-DAD (Zibadi et al., 2007), as well as, isoorientin and isovitexin in P. edulis flavicarpa ericarp extracts (López-Vargas et al., 2013). Additionally, flavones have been identified by HPLC-DAD-ESI-MS/MS in *P. mollisima* and *P. edulis* epicarp extracts such as

isoorientin, orientin, isovitexin, vitexin, schaftoside and vicenin-2 (Simirgiotis et al., 2013; Zucolotto et al., 2012).

Table 4: Some chemical compounds in the Passion fruit epicarps

Phenolic compound				
Flavanoids				**Polyphenols**
Flavanols		**Flavonols**	**Flavones**	**Anthocyanins**
catechin	epicatechin	kaempferol-3-*O* glucoside	luteolin-8-*C*-neohesperidoside	cyanidin-3-*O*-glucoside
				
Sugars				
Pectin		Glucose	Fructose	
				

2 OBJECTIVES AND WORK PLAN

Approximately one-third of all food produced globally is wasted every year, throughout the whole value chain from farmers to consumers. According to the Food and Agriculture Organization (FAO), this value represents around 1.3 billion tons per year.

These wastes can be reused for other processes; they are a potential source of bioactive compounds, such as betalains, betacyanins, polyphenols (including anthocyanins), and carotenoids, among others. The bioactive compounds extracted from natural matrices have a potential application as natural ingredients for the industry, particularly as functional and colorant agents. These molecules are known for their healthy properties, especially, antioxidant and antimicrobial activity, as well as, for their potential use as natural food colorants.

The main objective of this study is to characterize the phenolic profile in anthocyanin compounds of epicarp of the purple passion fruit (*Passiflora edulis* Sims), provided by the company KiwiCoop, Portugal. Moreover, the extraction optimization will also be explored, to obtain extracts rich in anthocyanins, in order to be used as natural ingredients with colorant and bioactive properties.

Thus, the specific objectives were:

- Evaluation the colour parameters in epicarp of purple passion fruit, before and after freez drying by lyophilization process;
- Characterization of the phenolic profile in anthocyanin compounds of purple passion fruit epicarp by conventional method and using chromatographic techniques, specifically HPLC-DAD-ESI/MS;
- Optimization of the anthocyanins' extraction from purple passion fruit epicarp, using a heat-assisted extraction (HAE) system using the surface response methodology (RSM).
- Evaluation of the bioactive potential of the optimal extract rich in anthocyanins and that obtained by the conventional method, thought antioxidant, cytotoxic, anti-inflammatory and antimicrobial (antifungal and antibacterial) assays.

Fig. 7 shows the adapted methodology plan in this investigation.

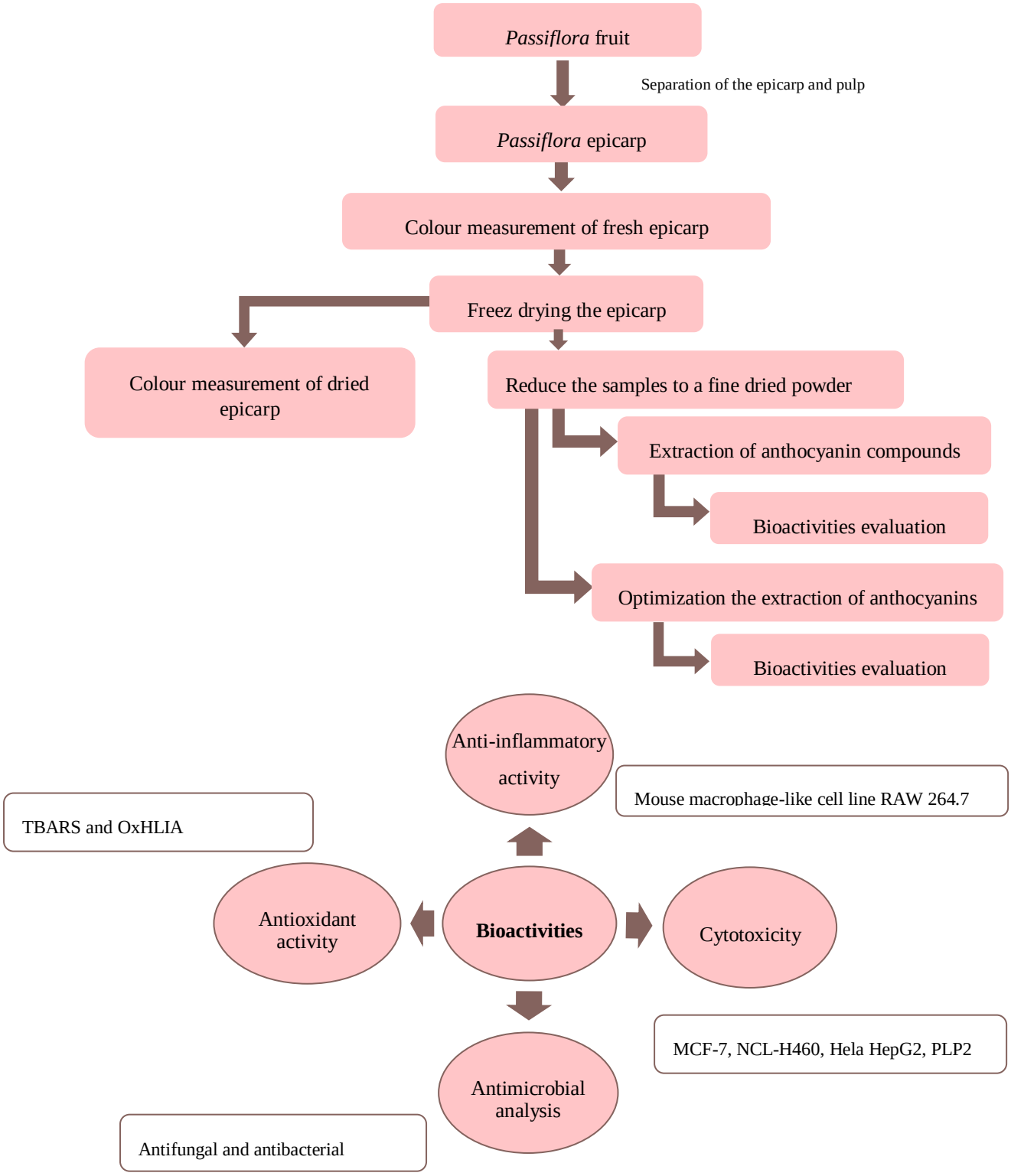


Figure 7: Representative scheme of the adopted metholdology plan.

Material and Methods

3 MATERIAL AND METHODS

3.1 STANDARDS AND REAGENTS

Acetonitrile 99,9% and ethanol of HPLC grade quality was purchased from Fisher Scientific (Lisbon, Portugal). The cyanidin-3-*O*-glucoside standard was bought from Polyphenols AS (Sandnes, Noruega). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). The agar Mueller-Hinton (MH) and the agarmalt extract (MA) were obtained from the Institute of Immunology and Virology of Torlak (Belgrade, Serbia), and the *p*-iodonitrotetrazolium chloride (INT) was acquired by Panreac Applichem (Barcelona, Spain). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Aldrich. Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma Aldrich and Tween 80 from Panreac. All other solvents and reagents were purchased from scientific retailers.

3.2 SAMPLE PREPARATION

The purple passion fruit (*Passiflora edulis* Sims) was provided by a company named KiwiCoop, of Oliveira do Bairro, Portugal (**Figure 8**). After receiving the samples, the epicarp and pulp were separated, and then the epicarp was frozen, lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) and reduced to a fine dried powder (~20 mesh). The homogeneous sample obtained was stored in dark place keeped from light and temperature, until further analysis.

Figure 8: The passion fruit morphology.

3.3 DETERMINATION OF COLOUR IN FRESH AND DRIED PASSION FRUIT EPICARP

The colour parameters in the purple passion fruit epicarp were evaluated as previously described by (Pereira et al., 2015). The colour was measured by a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) with an adapter for granular materials (model CR-A50). The measurements were made in the CIE *L*a*b** colour space (*L** values represent the brightness; *a** values represent the range colour of red/green and *b** values represent the range colour of blue/yellow), using the illuminate C and a diaphragm aperture of 8 mm (**Figure 9**). Data was processed with the "Spectra Magic Nx" (version CM-S100W 2.03.0006) software, from Konica Minolta.

Before starting measurements, the instrument was calibrated against a standard white tile. A sampling of 3 epicarps was selected and 3 measurements were performed on each sample.

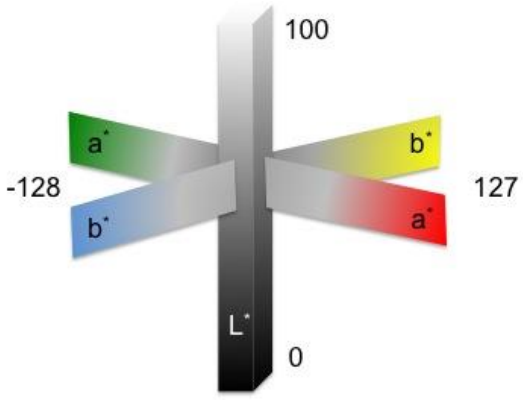


Figure 9: CIE *L* a* b** model.

(*L** = 0 yields black and *L** = 100 indicates diffuse white; specular white may be higher; *a**, negative values indicate green while positive values indicate magenta; *b**, negative values indicate blue and positive values indicate yellow).

3.4 DETERMINATION OF ANTHOCYANIN COMPOUNDS IN THE EXTRACT OF PASSION FRUIT EPICARP

3.4.1 Extraction procedure

The extraction of anthocyanin compounds by conventional methodology (**Figure 10**) is made by maceration. A quantity of 1 g of the dry epicarp was extracted with 20 mL of ethanol/water (80:20 *v/v*; acidified with 0.05% of citric acid), during 1h at room temperature (25 °C). After the maceration process the supernatant was filtered (Whatman Nº4 paper) and the plant residue

was re-extracted with the same solvent. The ethanolic fraction of the solvent was evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) and then the aqueous fraction was lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The obtained lyophilized extract was dissolved in a solution of ethanol/water (20:80 *v/v*) in a concentration of 5 mg/mL and filtered (0.2 μm nylon filters) to a vial (1.5 mL) for subsequent HPLC injection (Jabeur et al., 2017).

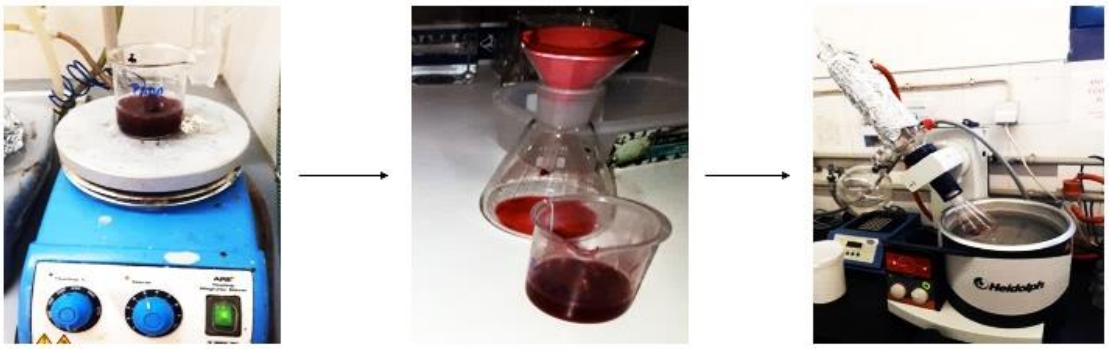


Figure 10: Extraction procedure of anthocyanins from passion fruit epicarp.

3.4.2 Analytical analysis

3.4.2.1 HPLC analysis

The chromatographic analysis (HPLC-DAD-ESI/MSn) will be performed using a HPLC Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) system, equipped with a quaternary pump, an automatic injector (at 5 ºC), a degasser and a column compartment with automated thermostat. Detection of the compounds will be performed with a diode array detector (DAD), using wavelength of 520 nm, coupled to a mass spectrometry detector (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source, working in positive mode (Bessada et al., 2016).

Chromatographic separation was performed using a column AQUA® (150 mm x 4.6 mm i.d., 5 μm, Phenomenex, Torrance, California, EUA) of reverse phase. The mobile phase was 0.1% TFA in water (A) and 100% acetonitrile (B). The elution gradient was 10% B up to 3 min, 10 to 15% B more 12 min, isocratic 15% B more 5 min, 15 to 18% B more 5 min, 18 to 30% B more 20 min and 30 to 35% more 5 min. The column was rebalanced for 10 min and the flow rate used was 0.5 mL/min.

3.4.2.2 Mass spectrometry analysis

The MS detection was performed by a mass spectrometer Ion Trap Linear LTQ XL (Thermo Finnigan), equipped with an electrospray ionization source (ESI). The nitrogen (50 psi) was the drag gas used; the system used a spray voltage of 4,8 kV, at an initial temperature of 320 °C and capillary tension of 14 V. The voltage of the tube lens offset was maintained at 75 V. The spectra were recorded in positive ion mode between 100 and 1500 m/z. The collision energy used was 20 (arbitrary units). The data were collected and analyzed using the program Xcalibur® (Thermo Finnigan).

The anthocyanins were characterized according to their UV–Vis and mass spectra, and quantification was performed through a calibration curve performed using cyanidin-3-*O*-glucoside standard ($y=243287x - 1^E6$; $R^2=0.995$). The results were expressed as mg/g of dry weight.

3.5 OPTIMIZATION OF THE NATURAL COLORANT EXTRACTION PROCESS OF PASSION FRUIT EPICARPS USING THE RESPONSE SURFACE METHODOLOGY

3.5.1 A heat assisted extraction (HAE)

Heat-assisted extraction (HAE) consists of extracting molecules from a powdered sample using an acidified solvent under defined conditions of temperature, time and agitation (Wang et al., 2016). This technique excels by the ease of employability in the industrial sector, since it is characterized by the simplicity and the reduced number of equipment necessary for its execution (Roriz et al., 2017; Backes et al., 2018; López et al., 2018).

The extraction procedure (**Figure 11**) was carried out in a water bath using a magnetic stirrer (CimarecTM, Thermo Scientific) under a fixed speed (5000 rpm) and using closed bottles in order to avoid evaporation of the solvent. For the extraction 20 mL of solvant (ethanol/water) acidified with 0.05% of citric acid (pH ≈ 3) were added to 1 g of powdered sample (epicarp of passion fruit). This procedure was performed following the extraction conditions predefined by the established RSM model (time (t or X1, 5 to 69 min), temperature (T or X2, 20 a 90 ºC) and ethanol content (S or X3, 0 to 100%). The solid-liquid ratio (S/L or X4) was maintained at 50 g/L.

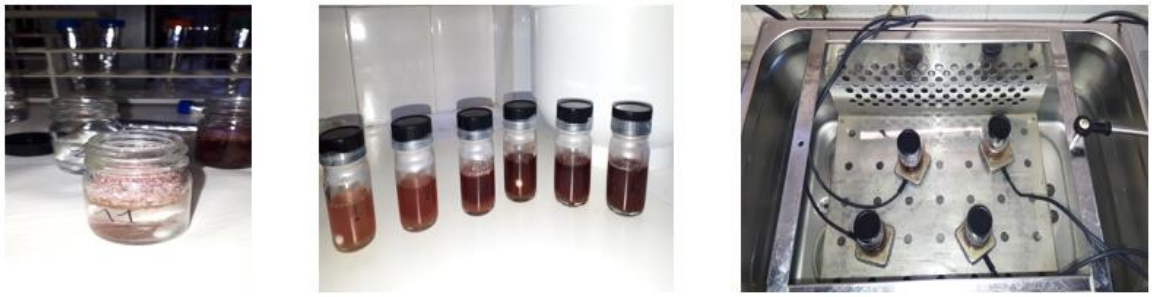


Figure 11: HAE-extraction process of the anthocyanins from passion fruit epicarp.

3.5.2 Preparation of extracts obtained by HAE

Subsequent to the extraction process, the extract solutions were centrifuged (5000 rpm, for 10 minutes at 10 ºC) and filtered through filter paper (Whatman n°4), in order to remove suspended solids. The supernatant was collected and divided into two fractions: one for HPLC-DAD analysis (**Figure 12**) and the second for determination of extraction yield. The separate fraction for HPLC analysis (1.5 mL) was filtered through LC syringe filter (0,22 µm) and then injected. The second fraction was collected to determine the extraction yield (5 mL), followed by a drying process at a temperature of 105 ºC for 48 hours, for subsequent weighing of the solids.

Figure 12: T The obtained exctact

3.5.3 Extraction Yield

The extraction yields (%) were calculated based on the dry weight (crude extract) obtained after evaporation, and then 5 ml of acidified water (0.05% citric acid) was added to the extract to become dissolvent. The obtained solution was filtrated by a LC filter disk (0.22 µm).

In all cases, the filtrates were concentrated at 35 °C in a rotary evaporator (Büchi R-210, Flawil, Switzerland) under reduced pressure and the aqueous phase was then freez dried to obtain a dried extract.

3.5.4 Identification and quantification of anthocyanin compounds through an HPLC-DAD-ESI/MS system

The analysis of the colourant extracts rich in anthocyanin compounds, obtained by the HAE method, was performed by a Dionex Ultimate 3000 HPLC system (Thermo Scientific) coupled to a DAD and mass spectrometry detector, previously described in ***section 3.4.2***.

3.5.5 Experimental Design, Modelling and Optimization

3.5.5.1 Experimental Design (RSM)

An RSM of five-level circumscribed central composite design (*CCCD*) of 20 runs with 6 replicated values at centre points was applied to optimize the HAE conditions for the extraction of anthocyanin compounds. Coded and natural values of the independent variables *X1* (processing time (t), min), *X2* (temperature (T), °C) and *X3* (solvent (S), % of ethanol, *v/v*) are presented in **Table** .

Table 5: Experimental domain and codification of independent variables in the CCCD factorial design with 5 range levels.

CODED VALUES	NATURAL VALUES		
	t (min)	*T (ºC)*	*S (%)*
-1.68	5	20	0
-1	21.2	34.2	20.3
0	45	55	50
+1	68.8	75.8	79.7
+1.68	85	90	100

3.5.5.2 Response used for analytical purposes

Three response value formats were used as response. The anthocyanin content (in terms of A) in the epicarps (P) dry weight material (Y_1, mg A/g P dw), the anthocyanin content in extracted residue (R) material (Y_2, mg A/g R) and extraction yield (Y_1/Y_2, g R/g P dw). Y_1 evaluates the

total content in the dry material, Y_2 evaluates the A purity in the extract and Y_1/Y_2 evaluates the yield of the extraction.

3.5.5.3 Mathematical Modelling

The response surface models were fitted by means of least-squares calculation using the following second-order polynomial equation with interactive terms (**Equation 1**). In this equation, *Y* represents the dependent variable (response variable) to be modelled, X_i and X_j are the independent variables, b_0 is the constant coefficient, bi is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} is the coefficient of quadratic effect, and n is the number of variables. The extraction yield and the individual and grouped anthocyanin compounds, 13 individual compounds plus the total anthocyanin content (*TAC*), were used as dependent variables.

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2$$

Equation 1: Second order polynomial equation.

3.5.5.4 Maximization of the Responses

For the optimization of the prediction model a simplex method was used, which solved non-linear problems, maximizing the extraction yield and the recovery of anthocyanins (Vieira et al., 2017). To avoid variables with unnatural and unrealistic physical conditions, some limitations were imposed on the coded variables (namely $t \geq 0$).

3.5.5.5 Dose-response analysis of the solid-liquid ratio

The solid-liquid ratio standard is an important parameter to determine, in order to obtain a process with greater productivity and sustainability. Thus, after the optimization of the experimental conditions (previously defined as *X1*, *X2* and *X3*), the solid-liquid ratio S/L (or X4, expressed in g/L) was studied. For the representation of the response effect, as a function of the solid-liquid relationship, a linear equation was used with ordinate at the origin, since the points follow a linear distribution as the *S/L* increases. The parametric slope value (m) was used for dose response evaluation. In this evaluation, positive values indicate an increase in the

extraction responses, while, negative values indicate a decrease in extraction efficiency, both resulting from an increase in S/L.

3.5.5.6 Numerical methods, statistical analysis and graphic illustrations

Adjustment procedures, coefficient estimates and statistical calculations of the experimental results were performed according to a procedure previously described by Prieto and Vázquez (2014). So:

a) For the determination of the coefficients, the non-linear quasi- Newton algorithm (least squares) was used, through the macro 'Solver' introduced in Microsoft Excel, to minimize the differences between the values obtained and the values predicted by the model;
b) The significance of the coefficients was evaluated through the macro 'SolverAid', in order to determine their intervals ($\alpha = 0.05$);
c) The consistency of the model was verified through several statistical criteria applied: i) Fisher's *F*-test ($\alpha = 0.05$) was used to evaluate the fit of the model to the observed data; ii) the 'SolverStat' macro was applied to determine the uncertainties in the prediction of parameters and models (Murado and Prieto, 2013)*;* iii) R^2 was interpreted as the ratio of variability of the dependent variable explained by the model.

3.6 PREPARATION OF THE EXTRACT RICH IN ANTHOCYANIN COMPOUNDS OBTAINED UNDER OPTIMUM CONDITIONS FROM THE PASSION FRUIT EPICARP

In order to obtain extract rich in anthocyanin compounds, particularly cyanidin derivatives, an extraction was performed from the purple of passion fruit epicarp, following the procedure previously optimized and described. The sample (1g) was placed together with 20 mL of 100% water acidified with 0.05% of citric acid (until obtaining pH = 3), in a glass jar with lid. The extraction established at temperature conditions (*T*=20 ºC) and time (37.5 minutes Then, the samples were centrifuged (Centurion K24OR, West Sussex, United Kigdom) at 5000 rpm during 10 min at 10 ºC. Then, they were filtered through syringe filters (previously mentioned), for removal of suspended solids. The separated supernatant, the ethanolic fraction was eliminated at a temperature of 35 °C, in order to avoid compounds degradation. Finally, the obtained aqueous fraction was frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City,

MO, EUA), obtaining an extract rich in anthocyanin compounds. The lyophilized extract was stored under darck conditions to avoid light exposure for the further bioactivities analysis.

3.7 EVALUATION OF THE BIOACTIVE POTENTIAL OF OPTIMAL EXTRACT RICH IN ANTHOCYANIN COMPOUNDS AND THE EXTRACT OBTAINED BY CONVENTIONAL METHOD, FROM PASSION FRUIT EPICARP

3.7.1 Preparation of the hydroethanolic extract

The extraction was performed as described previously in ***section 3.4.1*** and ***3.5.1***. The lyophilized extracts were re-dissolved at a concentration of 5 mg/mL in ethanol/water (80:20, *v/v*) for the antioxidant activity assays; in DMSO (10 mg/mL) for analysis of antimicrobial activity; and in distilled water at a concentration of 8 mg/mL for cytotoxic evaluation, hepatotoxicity and anti-inflammatory activities.

3.7.2 Antioxidant activity

3.7.2.1 TBARS

Lipid peroxidation can be determined by the products of the oxidation that react with thiobarbituric acid (TBA) giving rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS). One of the products commonly used as a biomarker of lipid peroxidation is malodialdehyde (MDA) that associated with TBA in the presence of H^+ ions to form a chromogen (MDA-TBA) according to the reaction shown in **Figure 13**. In this methodology, the oxidation of a lipid-rich preparation is induced by addition of a metallic ion (iron or copper), and the extension of the reaction with thiobarbituric acid is determined by the ability of the antioxidants present in the sample to stop the oxidation process, thus inhibiting the formation of the chromogen (less pink) (Gutteridge, 1995; Ng et al., 2000).

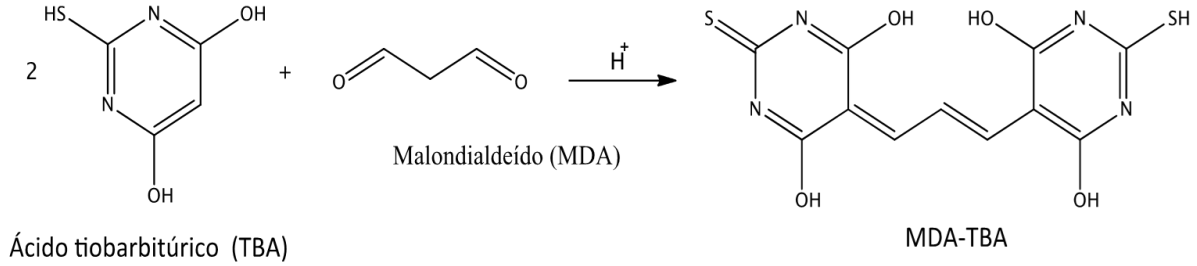


Figure 13: Formation of the complex MDA-TBA.

Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenised with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate that was centrifuged at 3000 *g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 µM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w/v*, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000 *g* for 10 min to remove the precipitated protein, the colour intensity of the MDA-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the **Equation 2**.

$$\text{Inhibition ratio (\%)} = \frac{(A-B)}{A} \times 100$$

Equation 2: Equation of the inhibition ratio determination in TBARS assay.

Where: A: absorbance of the control and B: absorbance of the compound solution.

3.7.2.2 OxHLIA

For the accomplishment of this method, a blood of sheep was harvested. The blood sample was centrifuged (Multifuge X1R, Thermo Fisher Scientific; 2900 rpm, 5 min, 10°C) and the supernatant was discarded in order to recover only the erythrocytes.These were subjected to a first wash with NaCl (150 mM) followed by three washes with phosphate-saline buffer (PBS; pH 7.4), with centrifugation and removal of the supernatant at each wash (Evans et al., 2013). A solution of erythrocytes at 2.8% was prepared (*v/v*), resuspending in PBS. In 48-well microplate was added 200µL of the erythrocyte solution at 400 µL of PBS (control), of water (complete hemolysis) or extract of the passion fruit epicarp dissolved in PBS (20 at 0.625 mg/mL). The microplates were preincubated with shaking (37°C, 10 min) for further addition of 200 µL of dihydrochloride of 2,2'-azobis(2-amidinopropan) (AAPH; 160 mM). After measuring the optical density at 690 nm, the microplates were incubated again under the same conditions, with measurements every 10 min (Takebayashi et al., 2012). The percentage of the erythrocyte population that remained intact (PE) was calculated as follows in the **Equation 3**.

$$PE\ (\%) = (S_t - CH_0 / S_0 - CH_0) \times 100$$

Equation 3: The percentage of the erythrocyte population (PE) in the OXHLIA assys.

S_t and S_0 correspond to the optical density of the sample at t and 0 min, respectively, and CH_0 is the optical density of complete hemolysis at 0 min. The results were expressed as hemolysis delay time (Δt), which was calculated as follows in the **Equation 4**.

$$\Delta t\ (min) = Ht_{50}\ (sample) - Ht_{50}\ (control)$$

Equation 4: The hemolysis delay time

Ht_{50} is the time corresponding to 50% hemolysis (min) obtained graphically from the hemolysis curve for each concentration of antioxidant sample. Subsequently, linear correlations were established between the values of Δt and the different sample concentrations (Lockowandt et al., 2019). From these, the concentration capable of delaying hemolysis was calculated in 60 min ($EC_{50\ (60\ min)}$, mg/mL) and 120 min ($EC_{50\ (120\ min)}$, mg/mL).

3.7.3 Antimicrobial activity

3.7.3.1 Antibacterial activity

The following Gram-negative bacteria were used: *Escherichia coli* (ATCC (American type culture collection) 35210), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538) and *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973). These microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Siniša Stanković" at the University of Belgrade in Serbia. Fresh overnight culture of bacteria was adjusted with a spectrophotometer to a concentration of 1×10^5 CFU/mL.

The requested colony forming units (CFU)/mL corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. The sample solutions were pipetted into the wells containing 100 µL of Tryptic Soy Broth (TSB), with 10 µL of inoculum being added to all the wells.

The microplates were incubated for 24 h at 37 °C. The MIC (minimal inhibitory concentration) of the samples was determined by adding 40 µL of iodonitrotetrazolium chloride (INT) (0.2

mg/mL) and incubation at 37 °C for 30 min. The lowest concentration that produced a significant inhibition (around 50%) of the growth of the bacteria in comparison with the positive control was identified as the MIC. The MICs obtained from the susceptibility testing of various bacteria to tested samples were also determined by a colorimetric microbial viability assay based on reduction of INT colour and compared with a positive control for each bacterial strain (CLSI, 2009). MBC (minimal bactericidal concentration) was determined by serial sub-cultivation of 10 μL into microplates containing 100 μL of TSB. The lowest concentration that showed no growth after this sub-culturing was regarded as the MBC. Streptomycin and ampicillin were used as positive controls, while 5% dimethyl sulfoxide (DMSO) was used as negative control (Soković et al., 2010). The results of MIC and MBC were expressed in mg per mL.

3.7.3.2 Antifungal activity

The following microfungi were used: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Trichoderma viride* (IAM 5061). These organisms were also obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" at the University of Belgrade in Serbia. The micromycetes were maintained on malt agar (MA) and the cultures were stored at 4 °C and sub-cultured once a month. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (*v/v*). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×105 in a final volume of 100 μL/well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. The MICs determination was performed by a serial dilution technique using 96-well microplates. The sample solutions were added to broth malt medium with the fungal inoculum. The microplates were incubated for 72 h at 28 °C.

The lowest concentrations without visible growth (using a binocular microscope) were defined as the MIC. The minimum fungicidal concentrations (MFC) were determined by serial sub-cultivation of 2 μL in microtiter plates containing 100 μL of malt broth per well and further incubation for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum. 5% DMSO was used as a negative

control, while bionazole and ketoconazole were used as positive controls (Soković et al., 2006). The results of MIC and MFC were expressed in mg per mL.

3.7.4 Evaluation of cytotoxicity in tumor cell lines

Four human tumor cell lines were used: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines was plated in a 96-well plate, at an appropriate density (7.5 × 103 cells/well for MCF-7 and NCI-H460, and 1.0 × 104 cells/well for HeLa and HepG2) and allowed to attach for 24 h. The cells were then incubated in the presence of different extract concentrations during 48 h.

Afterwards, cold trichloroacetic acid (TCA 10%, 100 μL) was added in order to bind the adherent cells and further incubated for 60 min at 4 ºC. After the incubation period, the plates were washed with deionised water and dried, and sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 μL) was incorporated to each plate well and incubated for 30 min at room temperature.

The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 μL) and the absorbance was recorded at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Guimarães et al., 2013). **Figure 14** shows an example of a microplate ready for citotoxicty measurement.

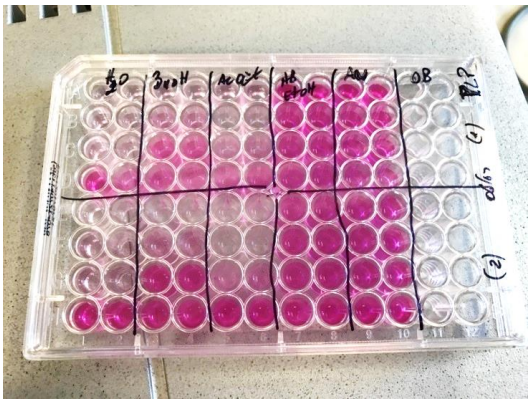


Figure 14: Representative image of microplate for cytotoxicity measurement

3.7.5 Evaluation of hepatotoxicity in non-tumor cells

A freshly harvested porcine liver, obtained from a local slaughter house, was used in order to obtain the cell culture, designated as PLP2. The liver tissues were rinsed in Hank's balanced salt solution containing penicillin (100 U/mL) and streptomycin (100 µg/mL) and divided into 1×1 mm^2 explants. A few of these explants were transferred to tissue flasks (25 cm^2) containing DMEM medium supplemented with fetal bovine serum (FBS, 10%), nonessential amino acids (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL), and incubated at 37 ºC with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cell cultivation was continuously monitored using a phase contrast microscope. When confluence was reached, the cells were sub-cultured and plated in 96-well plate (density of 1.0×104 cells/well) containing DMEM medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Guimarães et al., 2013). The growth inhibition was evaluated using the SRB assay described in the previous section.

3.8 EVALUATION OF ANTI-INFLAMMATORY ACTIVITY

The macrophages mouse cells RAW 264.7 were used to evaluate the anti-inflammatory activity according to the procedure of Jabeur et al. (2016). Cell culture was performed in DMEM medium supplemented with 10% heat inactivated bovine serum and L-glutamine at 37 ºC with 5% CO_2 in humidified air. Cells with active growth were released with a cell scavenger, the experimental density of the cells was established at 5×10^5 cells/mL and the proportion of dead cells was less than 1%, according to the Trypan Blue exclusion test. The cells were then dispensed into a 96-well plate (150,000 cells/well) and allowed to adhere to the microplate overnight. Afterwards, the cells were treated with different concentrations of the passion fruit hydroethanolic extract for 1 hour, followed by lipopolysaccharide (LPS) (1 µg/ml) stimulation over 18 hours. Controls were prepared without the addition of LPS in order to observe whether they induced changes in the basal levels of nitric oxide (NO).

The presence of nitric oxide was determined using a Griess Reagent Kit (Promega) containing sulfanilamide, N- (1-naphthyl) ethylenediamine hydrochloride (NED) and nitrated solutions. The cell supernatant (100 µL) was transferred to the plate and mixed with sulfanilamide and NED solution, 5 to 10 minutes each, at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (ELX800 Biotek microplate reader) and compared to the calibration curve (Jabeur et al., 2016).

3.9 STATISTICAL ANALYSIS

The described assays were performed in triplicate and the results were expressed as mean ± standard deviation (SD). The data for the evaluation of the epicarp of purple passion fruit epicarp (fresh and dehydrated), in the several assays, were analyzed through the *t-student* test, with the purpose of determining the significant differences between 2 samples, with *p-value* = 0,05 (SPSS v. 23.0; IBM Corp., Armonk, Nova Iorque, EUA).

Fitting procedures, coefficient estimates and statistical calculations were achieved as previously described by other authors (Prieto and Vázquez, 2014). In brief, a) the parameters determination was accomplished using the quasi-Newton algorithm (least-square) by running the integrated macro '*Solver*' in Microsoft Excel minimizing the differences between observed and predicted values; b) the coefficient significance was evaluated using the '*SolverAid*' macro to determine their intervals (α= 0.05); and c) the model consistency was proved by means of several statistical criteria: i) the Fisher *F*-test (α= 0.05) was used to assess the adequacy of the models to describe the observed data; ii) the '*SolverStat*' macro was used for the assessment of parameter and model prediction uncertainties (Murado and Prieto, 2013); and iii) the R^2 was interpreted as the proportion of variability of the dependent variable explained by the model.

Results and Discussion

4 RESULTS AND DISCUSSION

4.1 DETERMINATION OF COLOUR IN FRESH AND DRIED EPICARP OF PASSION FRUIT

The colour measurement in food products is an evaluation of great interest in the food industry and can be made by visual (for sensorial analysis) or instrumental analysis (Appelhagen et al., 2018). The results of the chromatic analysis in the colour space CIE *L*a*b** of the purple passion fruit epicarp are presents in **Table 6**. The luminosity scale (*L**) range between 0 and 100, and the *a** (from green to red) and *b** (from blue to yellow) parameters range between -120 and 120 (Xu et al., 2016). The colour of the fresh epicarp for the *L** parameter revealed, on average, a value of 34.2 ± 0.9 and to *a** and *b** parameters showed values of 6.9 ± 0.3 and 5.1 ± 0.2, respetively. Otherwise, in dried epicarp the *L** parameter evidenced a value of 34.3 ± 1.5, but in the *a** and *b** parametrs some differences were noticed, showing values of 16.0 ± 0.6 and 2.8 ± 0.1, respectively.

According to the obtained results, the influence of dehydration by lyophilization on the epicarp colour was evident, showing a statistically significant difference (*p-value*< 0.05) in fresh and dried samples. The *a** parameter evidenced a statistically significant increase after lyophilization. Otherwise the *b** parameter showed a statistically significant decrease, which translates into an approximation of the blue tonality. The *L** parameter did not show significant changes between the two types of passion fruit epicarp. These changes in the coordinates indicate that, after freez drying, the passion fruit epicarp acquired a shade closer to purple. For better understanding the colour shape, the values obtained were converted to RGB values through a program (http://www.easyrgb.com/en/convert.php) and the two types of colouring are present in **Table 6**.

Table 6: The colour measurement of the passion fruit epicarp samples.

Samples	*L**	*a**	*b**	RGB colour
Fresh epicarp	34.2 ± 0.9	6.9 ± 0.3	5.1 ± 0.2	
Dried epicarp	34.3 ± 1.5	16.0 ± 0.6	2.8 ± 0.1	
p-value	0.761	<0.01	<0.01	

In relation to this fruit, another study was carried out, namely in the determination of the colour of the epicarp, using a Konica Minolta colorimeter model CR-400 (Osaka, Japan) operating on the CIELAB scale (*L**, *a** and *b**).The evaluation was done in the different states of passion fruit maturation (fresh samples) and the values obtained varied between 8.7 and 51.7 for the parameter *L**, -13.0 and 6.2 for the coordinate *a**, and the value of *b** ranged between 4.5 and

23.1 (Mercante de Souza et al., 2016). Thus, it is possible to verify that the present study presents values of the fresh sample concordant with the values presented by these authors.

4.2 DETERMINATION OF ANTHOCYANIN COMPOUNDS IN THE EXTRACT OBTAINED FROM THE PASSION FRUIT EPICARP

The results for the anthocyanic profile of the hydroethanolic extracts of passion fruit epicarp were determined and the results are described in **Table**. Identification of these compounds was obtained according to the retention times (Rt), the UV-Vis spectrum and the mass fragmentation patterns.

Table 7: Retention time (Rt), wavelength of maximum absorption in the visible region (λmax), data of mass spectra, identification and quantification of anthocyanin compounds of passion fruit (mean ± SD).

Peak	Rt (min)	λmax (nm)	Tentaive identification	[M+H]+	ESI- MSn [intensity (%)]	Quantification (mg/g dry weight)
1	10.5	520	Cyanidin-3-*O*-glucoside	449	287(100)	8.3 ± 0.1

The analysis revealed the presence of one anthocyanin compound, being positively identified with a commercial standard (cyanidin-3-*O*-glucoside). The identified compound ($[M+H]^+$ a *m/z* 449) presented one fragment in MS^2 with a *m/z* 287, being identified as cyanidin-3-*O*-glucoside (**peak 1**) (**Figure 15**), taking into account the commercial standard charateristics. This extraction performed by the conventional method presented a yield of 33.99%, and the detected compound (**peak 1**; Figure 15) revealed a concentration of 8.3 ± 0.1 mg per g of R, i.e., 2.82 mg per g of DW epicarp.

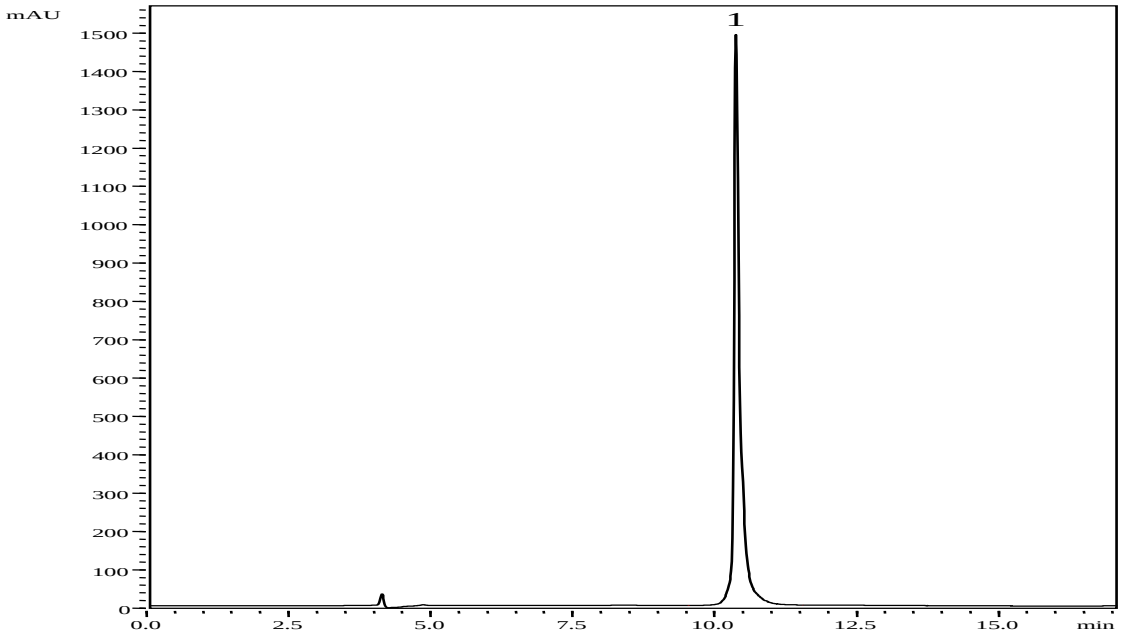


Figure 15: Chromatogram of the anthocyanin compoundcyanidin-3-*O*-glucoside (1) found in passion fruit obtained at 520 (B) nm. The numbers of the identified peak correspond to that identified in **Table**.

Other authors also carried out several studies with the purpose of determining the anthocyanin profile of passion fruit.

KIDØY et al., (1997) studied the anthocyanin pigments in the *Passiflora edulis* fruit (extracted with methanol acidified with 2% trifluoroacetic), using combinations of chromatographic and spectroscopic techniques. In this study, in addition to cyanidin-3-*O*-glucoside (97%), small amounts of cyanidin-3-*O*-(6-malonylglucoside) (2%) and pelargonidin-3-*O*-glucoside (1%) were found in the rind of the passion fruit.

In another way, Jiménez et al., (2011) also studied the presence of anthocyanins in *Passiflora Sims edulis* peels, through the extraction by maceration, using as solvent methanol acidified with acetic acid (19:1, *v/v*). The presence of anthocyanin cyanidin-3-*O*-*β*-*D*- glucopyranoside was confirmed.

The presence of anthocyanins in the pulp and in by-products of *Passiflora edulis* Sims fruit was also determined by Ribeiro da Silva et al., (2014). The evaluation was made using a refrigeration extraction in the dark and with anextraction solution of 1.5 N HCl in 85% ethanol.The authors verified the presence of anthocyanin compounds in a concentration of 3.48 ± 0.26 and 3.70 ± 0.39 mg/100 g dry basis for pulp and by-products, respectively.

Ramos dos Reis et al., (2018) were other authors that evaluated the presence of anthocyanin compounds in pulp, peels and seeds of *Passiflora edulis* Sims through extraction by homogenization in an Ultra-Turrax (T25, IKA, China) with acidified methanol (HCl 1%). The results showed the presence of seven anthocyanin compounds in peel, namely kaempferol (74.70 ± 1.44 µg/100g dry weight), cyanin (1477.47 ± 20.85 µg/100g dry weight), delphinidin-3,5-*O*-glucoside (8679.60 ± 341.32 µg/100g dry weight), cyanidin-3-*O*-glucoside (2852.92 ± 177.93 µg/100g dry weight), pelargonidin-3-*O*-glucoside (1551.94 ± 239.03 µg/100g dry weight), aglycone delphinidin (90998.72 ± 5218.53 µg/100g dry weight) and aglycone cyanidin (103686.48 ± 542.11 µg/100g dry weight).

The different extraction solvent and different methodologies used can explain this discrepancy. For example, in the case of solvent choice, several authors report that acidified methanol has been described as one of the most efficient solvents for the conventional extraction of anthocyanin (Jiao and Pour, 2018). However, the present study opted for the use of a hydroethanolic solvent acidified with citric acid because it intended to use a friendly and food-compatibal solvent that is not considered toxic either in the food industry or in clinical practice (sectors for which this study is directed).

For the extraction of these compounds, the solvent must be sufficiently acidified so that the anthocyanins do not undergo partial hydrolysis of the acyl moieties and retain their flavylium cation form (red colour), which is the most stable form of anthocyanins (Flores et al., 2016; Teng et al., 2017).

4.3 OPTIMIZATION OF THE PROCESS OF OBTAINING A COLOURANT EXTRACT RICH IN ANTHOCYANINS FROM THE PASSION FRUIT EPICARP

The industrial production process of natural based colouring extracts has been established for years and consists mainly in conventional heat assisted extractions (HAE, or maceration) using mixtures of solvents in water followed by several additional steps. This conventional process, although used for large-scale production, is known for requiring high-energy consumption and long extraction times (Wange et al., 2016; Wang et al., 2013; Zhu et al., 2016).

Therefore, to simplify and reduce the operational extraction processes costs, solvent loss and time process, an experimental design using response surface methodology (RSM) criteria, devoted to shorten the treatment time, decrease the energy requirements, and reduce the solvent consumption is developed (Roriz et al., 2017; Zhu et al., 2016).

To obtain anthocyanin extracts it is crucial to consider the factors affecting these compounds, among which, their structure and concentration, pH, temperature, light exposure, oxygen levels, and extraction solvents (Rodriguez-Amaya., 2016). Thus, the conditions of the different extraction variables, are essential to guarantee maximum recovery efficiency (Jiménez et al., 2018). Additionally, the efficiency is also strongly affected by the discrepancies observed among different matrices (Montesano et al., 2008), and therefore, optimizing these variables in order to achieve higher yields and lower costsis mandatory.

Through RSM it is possible to optimize the factors simultaneously, by obtaining polynomial models capable of describing within the tested experimental interval, the ideal conditions that maximize the used response criteria (Roriz et al., 2017). The response variables applied in the development of mathematical models, describing the extraction process (namely individual and grouped anthocyanic compounds) were obtained by high-performance liquid chromatography coupled to mass spectrometry (HPLC-DAD-ESI/MS).

4.3.1 Response criteria for RSM analysis

Although there are abundant studies on the extraction of anthocyanins from *Passiflora edulis Sims* epicarp, a small number are available detailing with finding optimal conditions for maximizing their extraction. In addition, the compositional diversity of anthocyanins in natural sources does not allow to directly extrapolate the extraction conditions of these pigments from previously studied sources (Pinela et al., 2019). Therefore, the first approach is to optimize the efficiency of the HAE to recover anthocyanins from purple *Passiflora edulis Sims* epicarp consisted of the application of the RSM technique.

To accomplish these objectives, there are several experimental designs available, each of them with different advantages and disadvantages, but in general, its application reduces significantly the number of experimental runs needed.

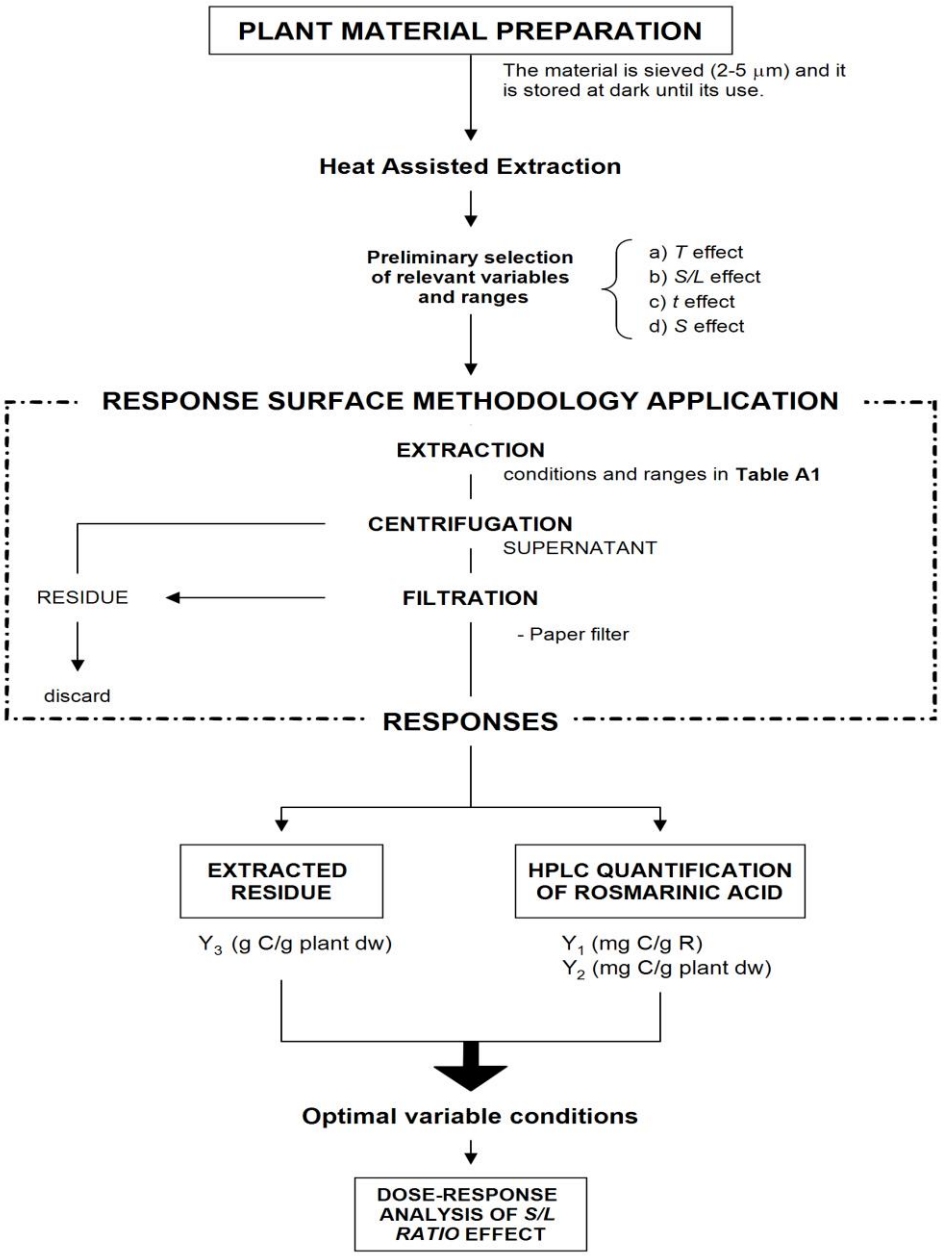


Figure 16: Diagram of the different steps carried out for optimizing the conditions that maximize the extraction responses.

In this regard, the *CCCD* with five levels per factor (**Table**) is a popular experimental design for RSM and has been applied by a number of researchers for optimization of various food processing methods (Oludemi et al., 2018; Pinela et al., 2016). Therefore, for optimization purposes the RSM experimental design of *CCCD* with five levels of variation for the three independent variables was used for optimizaing the extraction conditions of anthocyanin using the following variable and ranges: *t* (5-85 min), *T* (20-90 ºC) and *S* (0-100%).

A detailed description of the coded and natural values of the selected variables for each extraction method in the *CCCD* design is presented in **Table** . Additionally, **Figure 16** shows

a comprehensive summary of the different steps carried out in the optimization recovery of anthocyanin compounds.

4.3.2 RSM analysis, statistical verification and effect of the extraction variables on the target responses

The response results used to optimize the anthocyanin extraction were the anthocyanin content and the extraction yield according to the *CCCD* are shown in **Table** .

Table 8: Experimental RSM results of the *CCCD* for the HAE optimization of the three main variables involved (X_1, X_2,and X_3). Variables, natural values and ranges are described. The experimental results of the extraction of antocyanin compound (A) from the epicarps (P) comprise three response value formats (Y_1, mg A/g P dw; Y_2, mg A/g R dw; and Y_1/Y_2 g R/g P dw).

	CODED VALUES			NATURAL VALUES			EXPERIMENTAL RESPONSES		
	X_1	X_2	X_3	X_1: t min	X_2: T ºC	X_3: S %	Y_1 mg A/g P dw	Y_2 mg A/g R	Y_1 / Y_2 g R/g P dw
1	-1	-1	-1	21.2	34.2	20.3	2.58	6.54	0.395
2	-1	-1	1	21.2	34.2	79.7	1.96	9.34	0.210
3	-1	1	-1	21.2	75.8	20.3	1.48	5.41	0.274
4	-1	1	1	21.2	75.8	79.7	1.06	3.11	0.342
5	1	-1	-1	68.8	34.2	20.3	0.74	2.84	0.262
6	1	-1	1	68.8	34.2	79.7	1.25	7.51	0.166
7	1	1	-1	68.8	75.8	20.3	1.13	7.88	0.143
8	1	1	1	68.8	75.8	79.7	1.55	8.39	0.185
9	1.68	0	0	85	55	50	0.87	4.98	0.174
10	-1.68	0	0	5	55	50	0.88	7.28	0.121
11	0	-1.68	0	45	20	50	2.57	9.12	0.282
12	0	1.68	0	45	90	50	2.17	7.84	0.277
13	0	0	-1.68	45	55	0	1.95	4.36	0.446
14	0	0	1.68	45	55	100	0.93	5.74	0.162
15	0	0	0	45	55	50	2.14	8.56	0.251
16	0	0	0	45	55	50	2.27	8.38	0.271
17	0	0	0	45	55	50	2.06	7.93	0.260
18	0	0	0	45	55	50	2.27	8.27	0.274
19	0	0	0	45	55	50	2.27	8.46	0.268
20	0	0	0	45	55	50	2.06	8.55	0.241

The parametric values of the second-order polynomial model of **Equation 1**obtained after fitting the extraction response format values and the corresponding statistical information (α= 0.05) are presented in part A and B of **Table 9**. The fitting procedure of **Equation 1** applied to the experimental responses was performed using nonlinear least-squares estimations and those that were non-significant (*ns*) values were excluded.

Table 9: *Part A* shows the parametric results after fitting the second-order polynomial equation of **Equation 1** to the responses used to optimize the HAE according to the *CCCD* with 5 range levels (**Table**). Responses comprise the three response value formats (Y_1, mg A/g P dw; Y_2, mg A/g R dw; and Y_1/Y_2 g R/g P dw). Analysis of significance of the parameters (α= 0.05) are presented in coded values. *Part B* shows a brief statistical information of the fitting procedure to the model is presented. *Part C* shows the variable conditions in natural values that lead to optimal response values for RSM for the responses used.

		Y_1	Y_2	Y_2 / Y_1
A) Parametric information				
Intercept	b_0	2.18±0.06	8.38±0.17	0.26±0.01
Linear effect	b_1	*ns*	0.70±0.14	-0.02±0.01
	b_2	-0.15±0.04	-0.27±0.14	*ns*
	b_3	-0.30±0.04	0.33±0.14	-0.07±0.01
Quadratic effect	b_{11}	-0.47±0.04	-0.80±0.13	-0.04±0.01
	b_{22}	0.06±0.04	*ns*	*ns*
	b_{33}	-0.27±0.04	-1.19±0.13	0.02±0.01
Interactive effect	b_{12}	*ns*	-1.16±0.18	0.05±0.01
	b_{13}	0.25±0.05	0.58±0.18	*ns*
	b_{23}	0.34±0.05	1.66±0.18	*ns*
B) Statistical information				
R^2		0.9677	0.9546	0.9542
R^2 *adjusted*		0.962	0.951	0.951
C) Optimal variable conditions for response maximization				
INDIVIDUAL	*Time (min)*	34.57±1.61	78.14±8.20	64.23±7.71
	*Temperature (*ºC*)*	19.99±4.34	19.99±3.59	90.01±3.14
	Solvent (%)	0.00±2.13	29.42±3.99	0.00±0.91
	Response	3.36±0.57	11.52±1.53	0.29±0.04
GLOBAL	*Time (min)*	37.51±3.60		
	*Temperature (*ºC*)*	20.00±2.14		
	Solvent (%)	0.00±2.16		
	Response	3.35±0.52	9.02±1.14	0.37±0.05

Once the models are validated by statistical analysis (**Table 9** part A and B), it is possible to determine the absolute/relative optimal values of the variable conditions to maximize the responses individually and globally in order to obtain the most efficient extraction. **Table 9** part C shows the HAE individual and global optimal response values and the corresponding conditions for the responses assessed.

Although the parametric values show the responses and can be used to understand the patterns of the responses, the best way to express the effects of any independent variable on the extraction of any type of response, is to generate 3D surface plots, varying two variables in the experimental range under investigation and holding the other two variables at their fixed level. In this regard, **Figure 17** and **Figure 18** show the 3D surface plots parameters on the extraction

behaviour. The plots enable to visualize the influence and interaction between the variables. Visual analysis of 3D surface and curve plots are in accordance with parametric values derived from the multiple regression analysis described in **Table 9**.

The extraction results of HAE as function of the combination of the three main variables involved ($X_{1\text{-}3}$: *t*, *T*, and *S*) can be observed in **Figure 17** and **Figure 18**. In a more detailed form, **Figure 17 part A** shows the graphical analysis by net surfaces that represents the 3D response surface predicted with the second order polynomial of **Equation 1**. The binary actions between variables are presented when the excluded variable is positioned at the individual optimum (**Table 9**). The experimental design and results are described in **Table** . These graphical illustrations are helpful to visualize the tendencies of each response and guide the selection of the most favourable conditions, considering simultaneously all responses.

Additionally, part B of **Figure 17** illustrates the capability to predict the obtained results and the residual distribution as a function of each of the considered variables. Regarding statistical terms, the distribution of residues (**Figure 17**) presents, for the majority, more than 90% of reliability. This result is showing good agreement between experimental and predictive values. This is also verified by the high values of R^2 (**Table 9**).

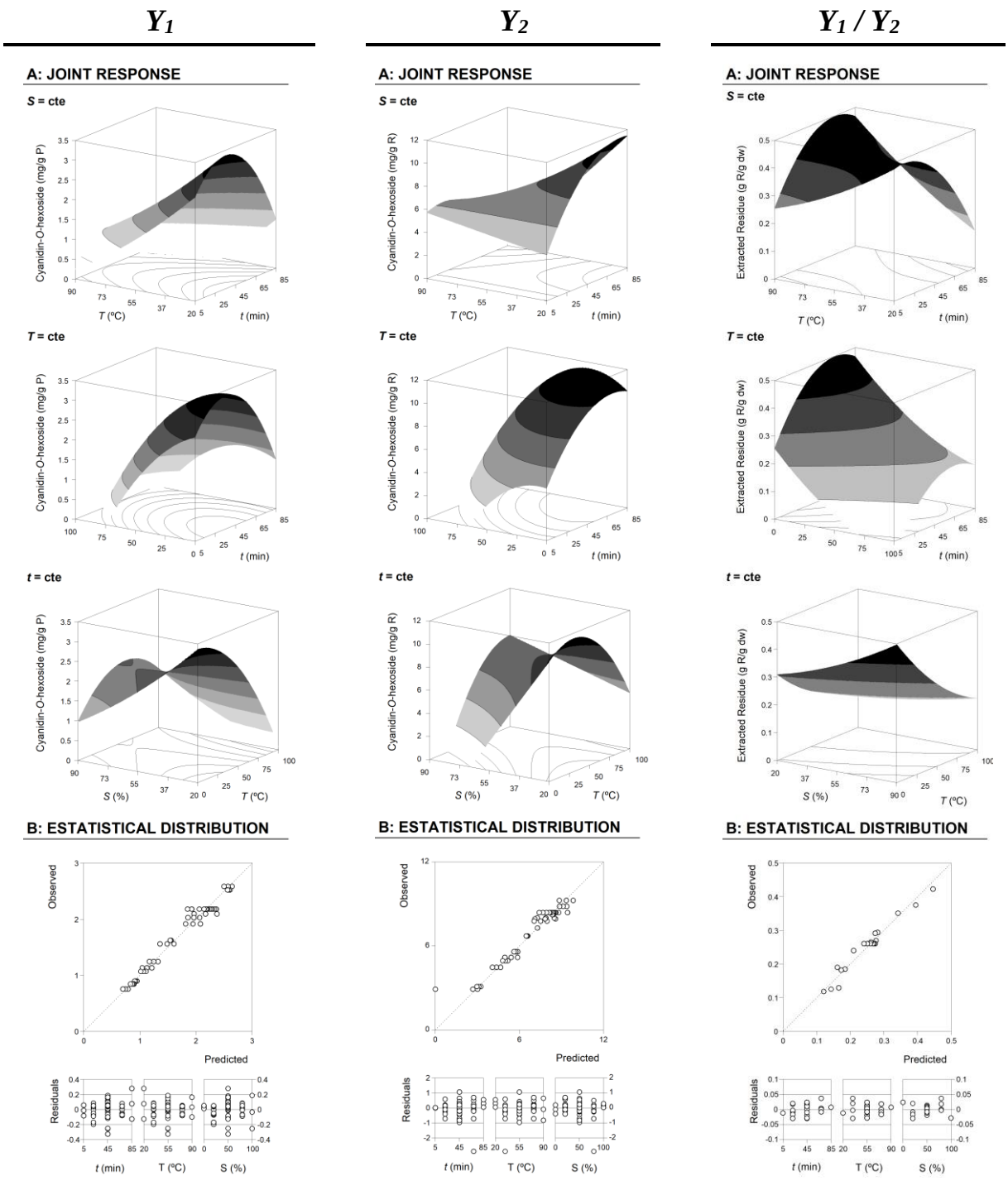


Figure 17: Shows the HAE graphical results for the extraction yield of the three response value formats (Y_1, mg A/g P dw; Y_2, mg A/g R dw; and Y_1/Y_2 g R/g P dw). Each figure is divided in two parts. *Part A*: Shows the graphical analysis by net surfaces that represents the 3D response surface predicted with the second order polynomial of **Equation 1**. The binary actions between variables are presented when the excluded variable is positioned at the individual optimum (**Table 9**). The experimental design and results are described in **Table** . *Part B*: To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables.

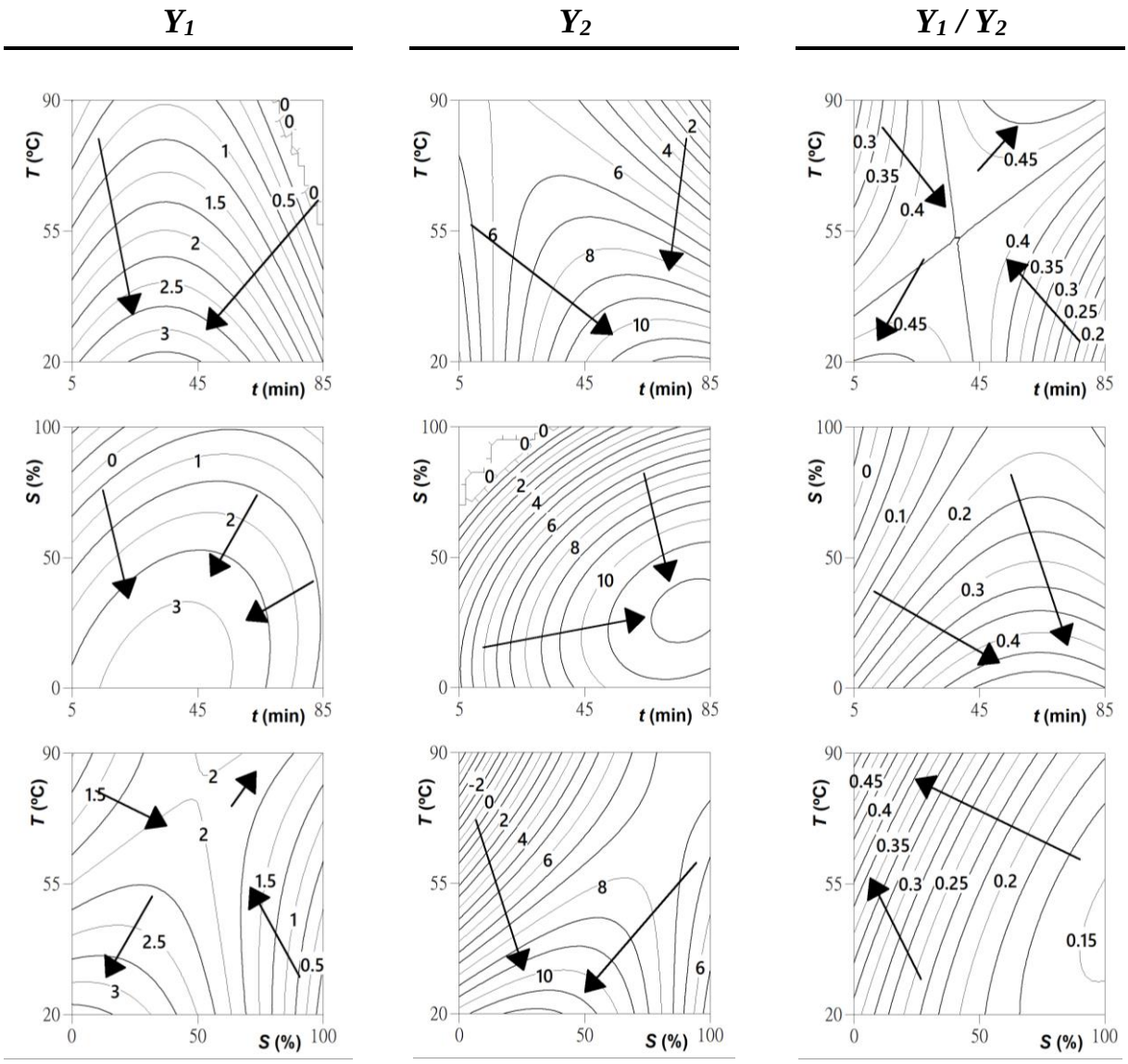


Figure 18: Shows the optimized isolines projections for the combination of the three main variables involved (X_1, $X_2$and X_3) in the HAE of the three response value formats (Y_1, mg A/g P dw; Y_2, mg A/g R dw; and Y_1/Y_2 g R/g P dw) to describe visually the tendencies of each response and guide the selection of the most favorable conditions, taken into account simultaneously all responses. Each of the contour graphs represents the projection in XY plane of the theoretical three-dimensional response surface predicted with the second order polynomial of **Equation 1**. The binary actions between variables are presented when the excluded variable is positioned at the individual optimum of the experimental domain.

4.3.3 Optimum numerical conditions that maximize experimental extraction and verification of predictive models

Based on the experimental results and statistical analysis, numerical optimizations have been conducted in order to establish the optimum level of the independent variables with desirable response levels. In order to verify the predictive mathematical model of the investigated process, the experimental confirmation was performed on the estimated optimal conditions. The predicted results matched well with the experimental results obtained at optimal extraction conditions, which were validated by the RSM model with good correlation. The values of the

variable conditions that lead to optimal response values for RSM using a *CCCD* for the extracting technique assessed are shown in **Table 9**. The HAE global optimal conditions were established as ***t*= 37.5 min**, ***T*= 20 ºC**, ***S*= 0 % (ethanol).** These values were obtained combining the information produced by all the responses assessed. This table shows the individual and global optimal variable conditions for the HAE extraction technique and the respective amounts of the extracted anthocyanin content.

4.3.4 Dose-response analysis of the effect of solid-liquid ratio under optimum conditions

The study of *S/L* was performed in the optimal conditions predicted by the RSM models obtained for each response factor (**Table 9**). The individual *S/L* study was designed to verify the behaviour between 5 to 100 g/L. The maximum value of 100 g/L was used as limit condition due to the impossibility of producing a homogenised reaction when higher values were introduced.The dose responses of the *S/L* obtained were consistent with the results obtained in the RSM analysis, and could be described by a simple linear relationship (data not showed). All experimental points are distributed around the equation with only one independent variable and, consequently, the dose response is explained by the slope (*m*) of the linear relation and intercept (*b*). None of the cases showed positive values of *m* (the extraction efficiency increases as the *S/L* increases), being in all the other cases the parametric value of *m* negative (the efficiency decreases as the *S/L* increases). Therefore, intercept (*b*) of the linear equation represents the maximum extraction achievable at the lowest *S/L* possible.

Although, at the initial *S/L* values the results obtained conducted to similar results, these values decreased as the *S/L* increased. Negative *m* values show that the *S/L* increase leads to a decrease in the extraction ability, obtaining a maximum value of extraction at 5 g/L and a minimum at 100 g/L. However, the observed decrease is strong, which means that the increase of 1 g/L implies the loss of important mg of antocyanin per g of extract. Such values produce losses at the maximum tested experimental value (100 g/L) of ~45%, comparatively with the one extracted at 5 g/L. Nevertheless, the economic advantages of working at 100 g/L are much superior than the possible benefits of extracting at the optimal *S/L* value. However, from the point of view of optimization, the optimal *S/L* value will be approximately 50 g/L.

4.4 EVALUATION OF THE BIOACTIVITIES IN THE OPTIMAL EXTRACT RICH IN ANTHOCYANIN COMPOUNDS AND THE EXTRACT OBTAINED THROUGH THE CONVENTIONAL METHOD, FROM THE PASSION FRUIT EPICARP

4.4.1 Antioxidant activity

The antioxidant activity of the optimal extract rich in anthocyanins (optimal extract) and the extract obtained by the conventional method from passion fruits epicarp was evaluated using two colorimetric *in vitro* assays (inhibition of lipid peroxidation – TBARS and oxidative hemolysis inhibition assay - OxHLIA) results are showen in **Table** .

The results showed that all studied extracts (optimal and normal extract) revealed antioxidant potential, presenting statistically significant differences (*p-value* < 0.01) between both extracts in all assays.

In TBARS assay the EC_{50} values ranging between115 ± 3 and 136 ± 4 µg/mL to the optimal extract and to the normal extract, respectively. Theselower EC_{50}values translate the highest antioxidant potential in the optimal extract (115 ± 5 µg/mL), since less amount of extract corresponds to 50% of antioxidant activity.

Likewise, also in the OxHLIA assay, the optimal extract revealed better antioxidant capacity (EC_{50} = 78 ± 3 µg/mL), compared to the extract obtained by the conventional method (EC_{50} = 144 ± 4 µg/mL).

This can be explained by the greater extractability of anthocyanic compounds using the optimal conditions, the obtention of a higher concentration of cyanidin-3-*O*-glucoside.

Table 10: Antioxidant activity of the optimal extract rich in anthocyanins (optimal extract) and the extract obtained by the conventional method (normal extract) from passion fruit epicarp (mean ± SD).

Antioxidant Activity	Optimal Extract	Normal Extract	*t*-Students test *p*-value
TBARS (EC_{50} values, µg/mL)	115 ± 3	136 ± 4	< 0.01
OXHLIA (Δt = 60 min) (EC_{50} values, µg/mL)	78 ± 3	144 ± 4	< 0.01

EC_{50} values: Extract concentration corresponding to 50% of antioxidant activity. EC_{50}values 85 µg/mL. Trolox (positive control) EC_{50} values: 23 µg/mL (TBARS inhibition) and 85 µg/mL (OXHLIA).

Several studies were carried out with the purpose of studying the antioxidant potential (through *in vitro* assays) of different parts of passion fruit, and the obtained results are in agreement with the present study.

In a study carried out by Rotta et al., (2019), the antioxidant activity of different varieties of passion fruit, namely, the pulp of *P. edulis, P. alata* and *P. ligularis*. For this, *in vitro* assays such us DPPH radical and ABTS cation radical were applied, and the results showed a great antioxidant potential in all studied varieties.

Nascimento et al., (2016) also evaluate the antioxidant potential of ethanolic extracts obtained from fresh and dried samples of passion fruit peel (Colombian origin). The antioxidant capacity was evaluated using the Ferric-Reducing Ability Power (FRAP) assay and the results revealed a great antioxidant potential; both in fresh and dehydrated passion fruit peel.

Janzantti et al., (2012) investigated the influence of the cultivation system on the volatile composition of the passion fruit and in the total antioxidant activity. For this, the antioxidant potential was determined using the ABTS radical reaction. The organic passion fruit showed higher levels of total phenolic compounds and total antioxidant activity than the conventional fruit, suggesting that the cultivation system influenced the production of antioxidant bioactive compounds.

Other authors, (Martínez et al., 2012) determine the chemical, technological and *in vitro* antioxidant properties (for ABTS, DPPH and FRAP assays) of the ethanolic extract of passion fruit. The obtained results indicate a good correlation between total phenol content and antioxidant capacity of the fruit extracts, being a good source of natural compounds.

4.4.2 Antimicrobial activity

The results of the antibacterial and antifungal activities of the hydroethanolic extracts obtained from the passion fruit epicarp are presented in **Table** and **Table ,** respectively.

The samples were tested against a set of five bacterial strains and six fungal strains, specifically selected on the basis of their importance for public health. For the evaluation of the antibacterial potential the Gram (+) bacteria: *Staphylococcus aureus* (ATCC 11632), *Listeria monocytogenes* (NCTC 7973) and Gram (-) bacteria *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030) and *Salmonella* Typhimurium (ATCC 13311) were used.

Likewise, for the determination of the antifungal potential the *Aspergillus fumigatus* (ATCC 9197), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), *Trichodermaviride* (IAM 5061) strains were used.

Regarding the antibacterial potential (**Table**) of the tested extracts (optimal and normal extract) the inhibitory and bactericidal capacity were evident in all the bacterial strains used.

For the optimal extract, the MIC values varied between 4.00 and 8.00 mg/mL, exhibiting better inhibitory potential in *Listeria monocytogenes* (MIC = 4.00 mg/mL) and *Escherichia coli* (MIC = 4.00 mg/mL) strains. The Minimum Bactericidal Concentration (MBC) presenting values of 8.00 mg/mL in all tested strains, with no emphasis on any of them.

The extract obtained by the conventional method (normal extract) also showed efficiency in the inhibitory capacity of all tested strains (MIC = 8.00 mg/mL), as well as in the bactericidal potential (MBC ≥ 8.00 mg/mL). However, in general, the optimal extract rich in anthocyanins revealed better inhibitory and bactericidal activity, compared to the extract obtained by the conventional method.

Table 5: Antibacterial activity (MIC and MBC, mg/mL) of the hydroethanolic extracts (optimal and normal extract) obtained from apicarp of passion fruit.

		Staphylococcus aureus	***Listeria monocytogenes***	***Escherichia coli***	***Enterobacter cloacae***	***Salmonella Typhimurium***
Optimal extract	**MIC**	8.00	4.00	4.00	8.00	8.00
	MBC	8.00	8.00	8.00	8.00	8.00
Normal extract	**MIC**	8.00	8.00	8.00	8.00	8.00
	MBC	>8.00	>8.00	8.00	>8.00	>8.00
Ampicillin (control)	**MIC**	0.012	0.40	0.40	0.006	0.75
	MBC	0.025	0.50	0.50	0.012	1.20

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration.

In antifungal activity (**Table**), the evaluated extracts (optimal and normal extract) demonstrated inhibitory and fungicidal efficiency in all tested strains.

For the optimal extract rich in anthocyanins (optimal extract) the MIC values ranging between 1.00 and 8.00 mg/mL, exhibiting better inhibitory potential in *Penicillium ochrochloron* (MIC = 1.00 mg/mL) strain, following the *Trichoderma viride* (MIC = 4.00 mg/mL) and *Aspergillus niger* (MIC = 4.00 mg/mL) strains. The remaining tested strains presented MIC values of 8.00 mg/mL.

Regarding the Minimum fungicidal Concentration (MFC), the values oscillated between 1.00 and ≥ 8.00 mg/mL, highlighting the *Penicillium ochrochloron* strain with a better result (MFC = 1.00 mg/mL).

Taking into account the extract obtained by the conventional method (normal extract), the results revealed inhibition in all tested strains (MIC ≥ 8.00 mg/mL), as well as in the bactericidal potential (MFC ≥ 8.00 mg/mL). Such as in the antimicrobial activity, also in the antifungal activity, the optimal extract rich in anthocyanins revealed better efficiency compared to the extract obtained by the conventional method.

Table 6: Antifungal activity (MIC and MFC, mg/mL) of the hydroethanolic extracts (optimal and normal extract) obtained from epicarp of passion fruit.

		Aspergillus fumigatus	***Aspergillus versicolor***	***Aspergillus niger***	***Penicillium funiculosum***	***Penicillium ochrochloron***	***Trichoderma viride***
Optimal Extract	***MIC***	8.00	8.00	4.00	8.00	1.00	4.00
	MFC	>8.00	8.00	8.00	8.00	1.00	8.00
Normal Extract	***MIC***	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
	MFC	>8.00	>8.00	>8.00	>8.00	>8.00	>8.00
Ketoconazol (control)	***MIC***	0.20	0.20	0.20	0.20	0.20	0.20
	MFC	0.50	0.50	0.50	0.50	0.50	0.30

MIC: Minimal Inhibitory Concentration; MFC: Minimal Fungicidal Concentration.

Calderon et al., (2019) evaluate the antimicrobial activity of the compounds in the *Passiflora mollissima* (Tumbo) fruit and leaves included also on cultured strains of the microorganisms *Streptococcus mutans, Streptococcus oralis, Streptococcus sanguinis* and *Candida albicans*. This potential was analyzed by the disk diffusion method and evaluated in terms of their zones of inhibition, and the results demonstrated the antimicrobial activity of ethanolic *Passiflora mollissima* extract against the cultured strains of *Streptococcus mutans, Streptococcus oralis* and *Streptococcus sanguinis* with zones of inhibition after the incubation period.

Also, Bandara et al., (2018) investigated the antimicrobial capacity of several extracts (hexane, chloroform, methanol and water) of *Passiflora suberosa.* L. The antibacterial activity and minimum inhibition concentrations were evaluated using three Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecium*) and three Gram-negative bacteria (*Pseudumonas aeruginosa, Salmonella typhimuriam* and *Escherichia coli*). The results indicated that only the methanol extract of *P. suberosa* exhibited antibacterial activities against all the strains of Gram-negative and Gram-positive bacterial with stronger activity against Gram-negative bacteria.

4.4.3 Cytotoxic, hepatotoxic and anti-inflammatory activity

The results of *in vitro* cytotoxicity, hepatotoxicity and anti-inflammatiry assays, of the optimal extract and the extract obtained by the conventional method from the epicarp of passion fruit are presented in the **Table** .

For the cytotoxic evaluation the NCI H460, MCF7, HepG2 and HeLa tumor cell lines was used. Taking into account the obtained results, it was evident that only in the HepG2 cell line it was found inhibitory capacity, presenting GI_{50} values of 363 ± 15 µg/mL. Otherwise, no inhibitory potential was observed in the other tumor cell lines tested, with values of GI_{50}> 400 µg/mL.

Regarding the evaluation of the hepatotoxicity of the two obtained extracts (optimal and normal extract), evaluated through the primary non-tumor cell culture PLP2, the absence of toxicity was evident (GI_{50}> 400 µg/mL). In relation to the anti-inflammatory potential, in both studied extracts no activity was observed.

Table 7: Cytotoxic, hepatotoxic and anti-inflammatory activity of the hydroethanolic extract, obtained by the conventional methodology, from the *P. edulis* fruits.

	Optimal Extract	**Normal extract**
Tumor cell lines (GI_{50} values; µg/mL)		
NCI H460	>400	>400
MCF7	>400	>400
HepG2	363 ± 15	>400
HeLa	>400	>400
Non-tumor cell lines (GI_{50} values; µg/mL)		
PLP2	>400	>400
Anti-inflammatory (GI_{50} values; µg/mL)		
RAW264,7	>400	>400

GI_{50} – concentration that inhibited 50% of cell growth. GI_{50} values of Ellipticin (positive control): 1.21 µg/mL (MCF-7), 1.03 µg/mL (NCI-H460), 0.91 µg/mL (HeLa), 1.10 µg/mL (HepG2) and 2.29 µg/mL (PLP2). GI_{50}>400 µg/mL- does not have activity.

Several studies have been done to evaluate several properties of passion fruits, however, in the literature no studies reported the evaluation of the cytotoxic potential, as well as, hepatotoxicity activity.

In relation to the anti-inflammatory activity, Cavalcanti de Albuquerque et al., (2019) determine this capacity in four different water extracts of fruit by-products (peels and seeds, namely from

passion fruit). For this assay the RAW 264.7 macrophage cell line was used, and the results showed anti-inflammatory potential by decreasing the highest nitric oxide levels. However, of all fruits evaluated in this study passion fruit was the one with the lowest anti-inflammatory potential.

Conclusions & Perspectives

5 CONCLUSIONS

Colourants are one of the most important additives in terms of marketing, since their presence in food products influences the perceptions, choices and preferences of consumers.

This study describes the extraction of anthocyanins from epicarp of purple passion fruit by applying a maceration methodology, valorizing a bio-residue produced in large scale by the food industry.

The colour evaluated in fresh and dried epicarp revealed the positive influence of dehydration by lyophilization process, changing the *a** and *b** parameter and, consequently, the epicarp tone to a purple colour.

In the optimization process different time, temperatures and solvent ratios (ethanol/water; *v/v*) were tested and the extraction optimization was measured by using a response surface methodology (RSM). The optimal parameters obtained (t= 37.5 min, T= 20 ºC, S= 0 % (ethanol) using 50 g/L of solid/liquid ratio) conducting to an extraction yield of 37%, with a total anthocyanins' content of 3.35 mg of A per g of dried epicarp and 9.02 mg of A per g of extract (dried extracted residue-basis).

It was also possible to verify the bioactive potential of the extracts (optimal extract rich in anthocyanic compounds and extract obtained through the conventional method) obtained throughout the experimental work, namely through the antioxidant activity, which revealed promising EC_{50} values, principally in the extract rich in anthocyanin compounds. It was also evident that both extracts present antibacterial and antifungal efficiency, with MIC, MBC and MFC values between 1.00 and 8.00 mg/mL, highlighting, once again, the optimal extract.

In the evaluation of the cytotoxic and hepatotoxic potential only the optimal extract revealed inhibitory potential in the HeLa tumor cell line, being clear the absence of toxicity of both extracts tested in the primary non-tumor cell culture PLP2. In contrast, both extracts had no effect in the anti-inflammatory activity.

Based on the obtained results, it is possible to conclude that the epicarp of purple passion fruit is a great choice for the extraction of anthocyanin compounds and can be used as an alternative to obtain a natural coloring ingredient.

Besides of the colourant potential, the studied sample also exhibited some bioactive properties, with potential to be incorporated in the food and nutraceutical industry.

All these results show that waste discarded from the food industry can have a beneficial purpose for man and the environment, and the possibility of having high added value when processed and transformed.

In order to continue this experimental work, as future prospects, it would be interesting to study different techniques of incorporation of the optimal extract rich in anthocyanins in food products. To that end, the use of encapsulation techniques such as microencapsulation, liposomes, or nanoemulsions could be a next line of investigation in assessing their stability and incorporation of these anthocyanin compounds as well as the influence of the anthocyanin compound detected (cyanidin-3-*O*-glucoside) in food products.

It would also be pertinent to carry out market studies with the objective of realizing the feasibility and advantages of using this bio-waste at an industrial level.

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