UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA

TESIS DOCTORAL

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New natural sources of betacyanins : optimization of the extraction process, stability studies and incorporation as a colorant in different foods

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Dra. Lillian Bouçada de Barros

To my father

Jose Miguel Magalhães de Freitas Roriz
(07/01/1953 – 18/03/2015)

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a*	Greenness-redness
AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
Abs	Absorbance
ADI	Admissible daily intake
ANOVA	Analysis of variance
AOAC	Association of analytical communities
API	Atmospheric pressure ionization
ATCC	American type culture collection
b*	Blueness-yellowness
BHT	Butylated hydroxytoluene
C	Control samples
CA	Codex alimentarius
CCCD	Circumscribed central composite design
CFU	Colony forming units
DAD	Diode array detector
DM	Dynamic maceration
DMEM	Dubelco's modified eagle medium
DMSO	Dimethyl sulfoxide
EC_{50}	Concentration with 50% of antioxidant activity
EFSA	European food safety authority
EMM	Estimated marginal means
ESI	Electrospray ionization
Et	Ethanol percentage
EU	European Union
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum
FDA	Food and drug administration of the United States of America
FID	Flame ionization detector
FTIR	Fourier-transformed infrared spectroscopy
FW	Fresh weight
GAE	Gallic acid equivalents
GC	Gas chromatography
GI_{50}	Concentration that inhibits 50% of cell growth
GLM	General linear model
GRAS	Generally regarded as safe
HBSS	Hank's balanced salt solution
HeLa	Cervical carcinoma
HepG2	Hepatocellular carcinoma
HP	Hewlett-Packard
HPLC	High performance liquid chromatography
Ht_{50}	50% haemolytic time (min)
IC_{50}	Extract concentration required to keep 50% of the erythrocyte population intact
INT	Iodonitrotetrazolium
IS	Internal standard
L*	Lightness
LBEF	Liquid biphasic electric flotation
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDA	Linear discriminant analysis
MA	Malt agar
MAE	Microwave assisted extraction
MBC	Minimum bactericidal concentration
MCF-7	Breast adenocarcinoma
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
mM	Milimoles
MS	Mass spectrometry
MUFA	Monounsaturated fatty acids
NAC	N-acetylcysteine
NCTC	National collection of type cultures
nd	Not detected

nm	Nanometer
PBS	Phosphate-buffered saline
PDA	Photo-diode array detector
PEF	pulsed electric fields
PLP2	Porcine liver primary cell line
PUFA	Polyunsaturated fatty acids
RGB	Red-green-blue colour system
RI	Refraction index
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
RSM	Response surface methodology
Rt	Retention time
S/L	Solid-liquid ratio
SD	Standard deviation
SFA	Saturated fatty acids
SPSS	Statistic package for social sciences
SRB	Sulforhodamine B
ST	Storage time
t	Time
T	Temperature
TAC	Total available carbohydrates
TCA	Trichloroacetic acid
Tris	tris(hydroxymethyl) aminomethane
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid
TSB	Tryptic soy broth
UAE	Ultrasound assisted extraction
UFLC	Ultra-fast liquid chromatography
UK	United Kingdom
USA	United States of America
UV	Ultra violet
v.	Version
Δt	Delayed time of haemolysis
λmax	Wavelength of maximum absorption
EC	European Committee
ADHD	Attention deficit hyperactivity disorder
EAU	Extratción assistida por ultrasonidos
cDNA	Complementary deoxyribonucleic acid
APAP	Acetominophen
NF-κB	Nuclear factor kappa- light-chain-enhancer of activated B cells
SFE-CO_2	CO_2Supercritical fluid extraction
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
dw	Dry weight
CCC	Countercurrent cromatography
HHP	High-hydrostatic pressure
Vis	Visible
IS	Internal Standard
P	Power
MHB	Mueller Hinten broth
TSB	Tryptic soy broth
EDTA	Ethylenediamine tetraacetic acid
RPMI-1640	Mammalian Cell Culture Media
DMEM	Dulbecco's Modified Eagle Medium
NCI-H460	Non-small cell lung cancer
UHT	Ultra-high-temperature processing
CIE	International Commission on Illumination
BRESA	Herbarium of Escola Superior Agrária
OxHLIA	Oxidative haemolysis assay
MRSA	Methicillin-resistant *Staphylococcus aureus*
R^2	Coefficient of determination
R^2_{adj}	Adjusted coefficient of determination

DPPH	2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazin-1-yl
FRAP	Ferric Antioxidant Power
AEAC	Ascorbic acid Equivalent Antioxidant
TE	Trolox equivalents
TEAC	Trolox Equivalent antioxidant capacity
AAE	Ascorbic acid equivalent
TFA	Trifluoroacetic acid
MSE	Mean squared error
RMSE	Root means squared errors
MAPE	Mean absolute percentage error
DW	Durbin-Watson coefficient
ns	Non-significant
IF	Ice cream formulations
CI	Control ice cream
BSI	Betalain standard ice cream
GGI	*G. globosa* extract ice cream
BVI	*B. vulgaris* extract ice cream
EMM	Estimated marginal means
PCA	Plate count agar
VRBLA	Violet red bile lactose agar
DRBC	Dicloran Rose-Bengal Cloranfenicol Aga
MYP	Mannitol egg Yolk Polymyxin agar
CT	Colourant type
TOF	Time of flight
H	Hydrophilic
L	Lipophilic
HAE	Heated assisted extraction
ME	Maceration extraction
HSCCC	High-speed counter-current cromatography
CF	Cookie formulation

INDEX

ABSTRACT

"New natural sources of betacyanins: optimization of the extraction process, stability studies and incorporation as a colorant in different foods"

Nature can be an inexhaustible source of natural additives. Looking at the colouring diversity present in a huge range of natural matrices, easily these matrices turned into target raw materials to obtain these molecules. Another interesting point is the tons of industrial bioresidues that result from the food industry. These residues are usually discarded and have no economically viable destination, representing a global and environmental issue. This work intends to contribute to the valorisation of industries bio-residues, by exploiting these materials as sources of high valuable molecules, targeting colouring compounds in the range of pink, the betacyanins, for application in different foodstuff. To that end, three plant species were explored, mainly *Gomphrena globosa* L., *Amaranthus caudatus* L. and *Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose. These three plants are characterized for the intense colour displayed by their flowers (*G. globosa*, and *A. caudatus*), and fruits (*H. costaricensis*), indicating the presence of colouring compounds, known as betacyanins. *A. caudatus* for instance, are explored for seeds production, due to their very interesting nutritional profile, and the deseeded bloomed flowers are considered bioresiduos. *H. costaricensis* fruits are used for various purposes, such as juice making, but the peels are discarded, originating tons of bioresidues. In both cases the crude mater discarded is filed with colouring compounds and can be exploited to recover high value molecules.

These natural compounds with high colouring potential, have been described has unstable to some specific conditions, namely temperature, pH and light, just to mention a few, to overcome that problem, recently several stabilization techniques have been developed and applied to ensure stabilization, making possible for these valuable compounds, namely betacyanins to resist those hazardous conditions, enhancing their applicability range.

Betacyanins are also described as molecules presenting biological activity, due to their chemical structure, and in some specific cases, increased by the presence of acyl groups.

Bearing this in mind, the main objective of this work was to explore *G. globosa*, *A. caudatus* and *H. costaricensis* as natural alternative sources of betacyanins. These studied samples where firstly submitted to a chemical characterization, resorting to different chromatographic methodologies. Then, different extraction procedures were applied, namely dynamic maceration, microwave and ultrasound assisted extraction, supported by the application of mathematical models in order to optimize the extraction procedure and achieve a betacyanin extract composed by the higher amount of colouring compounds possible. Afterwards, stabilization techniques were also applied, when needed to stabilize the final extracts (Freeze dry and spray drying). As an extra benefit, the final extracts were also evaluated with the expectative to achieve some biological activity, thus providing colouring capacity and bioactive properties.

The developed extracts showing high colouring capacity and stability were incorporated in chosen matrices (ice cream and cookies) to analyse their stability and viability after incorporation processes and their resistance to the storage conditions of each food matrices. The matrices were chosen regarding the children's preferences for colourful foodstuff, since colour is a determinant factor for children food options, thus the use of natural additives in these products is mandatory and needed.

To complete the work, the final products were evaluated for their nutritional, physicochemical and microbial load to guarantee the organoleptic integrity, and food safety.

According to the results, all the studied plants presented a very different chemical profile. The flowers of *G. globosa* were the sample with highest concentration in organic acids and presented six betacyanin compounds such as gomphrenin/isogomphrenin II and III, and 17-descarboxy-amaranthin. *H. costaricensis* sample presented the higher concentration in terms of tocopherols, presenting the four isoforms, in this specie were identified four betacyanin compounds betanin/isobetanin and phylocactin/isophylocactin. *A. caudatus* also presented four betacyanin compounds, namely amarabthine/isoamaranthine and betanin/isobetanin, and when compared with the *H. costaricensis* sample, it presented the better results for the antihaemolytic and antimicrobial activity.

For recuperation of the colouring compounds, the extraction procedure with better results was the ultrasound assisted extraction (UAE) an emergent extraction procedure. The optimization procedure was carried out supported by the response surface methodology (RSM), a mathematical tool that allows the evaluation of several factors that affect the

extractability and stability of the target molecules. For *G. globosa*, when applied this time saving procedure the obtained extract presented a betacyanin concentration of 45.5 g betacyanins/kg plant material, *A. caudatus* presented a result of 77.6 g betacyanins/kg plant material, and *H. costaricensis* 36 g betacyanins/kg plant material.

The enriched extract recovered from the flowers of *G. globosa* was incorporated in ice cream and cookies to assess the extract colour capacity and the stability.

The results obtained for the ice cream incorporated with the enriched extract of *G. globosa* flowers, were compared with different ice cream formulations in different storage times, and the interactions among both factors was also assessed. Regarding the nutritional composition the interaction between ice cream formulations and storage time influenced all the parameters studied. In the colour parameter, the storage time did not significantly affect this parameter, enhancing the good stability of these compounds. The fatty acid profile of the ice cream formulations showed no significant differences, being affected only by storage time.

Following the same line, the cookies incorporated with the extract of *G. globosa* were also compared with different cookies formulations in different storage times, as well as assessing the interaction among both factors. The chemical composition of the cookies was not significantly altered by the incorporation process, as well as the physical studied parameters. The colour of the cookies incorporated with the *G. globosa* extract presented a deep pink colouration, even after the cooking process, and since the colour intensity did not undergo drastic changes along the storage time, demonstrating once more a good stability of these compounds, even when subjected to high temperatures.

The findings of this work support the exploration of this family of compounds as natural colourants, as well as the three studied plants as alternative sources for the recovery of these same compounds. In this way, the industry has at its disposal a greater options range of natural origin colourants, being able to offer consumers healthier alternative products, thus meeting their requirements

RESUMEN

"Nuevas fuentes naturales de betacianinas: optimización del proceso de extracción, estudios de estabilidad e incorporación como colorante en diferentes alimentos"

La naturaleza puede ser una fuente inagotable de aditivos naturales. Tanto es así que, debido a diversidad de colores presente en la misma, está puede fácilmente ser en fuente de materias primas para la obtención de colorantes. Otro punto a tener en cuenta como posible fuente de colorantes alimentarios son los subproductos derivados del procesado de vegetales, hoy en día infrautilizados y sin un destino económicamente viable, lo que representa un problema global y ambiental.

Este trabajo pretende contribuir a la revalorización de ciertos subproductos vegetales, como fuentes de compuestos bioactivos, concretamente de betacianinas (compuestos colorantes en la gama del rosa), para su aplicación en diferentes alimentos. Para ello, se evaluaron tres especies vegetales distintas, *Gomphrena globosa* L., *Amaranthus caudatus* L. e *Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose. que se caracterizan porque sus flores (*G. globosa* y *A. caudatus*) y frutos (*H. costaricensis*) presentan un intenso color rosa debido a la presencia de betacianinas y suelen descartarse como subproductos derivados del procesado industrial de los mismos, como ocurre con las flores florecidas sin semillas de *A. caudatus*, y las pieles del fruto *H. costaricensis*, en ambos casos dando lugar a toneladas de subproductos sin un uso definido pudiéndose emplear como fuente de compuestos bioactivos y colorantes alimentarios.

Estos compuestos naturales con alto potencial colorante, han sido descritos como inestables a algunas condiciones del medio, como la temperatura, pH y luz, etc. Para poder solventar este problema, recientemente se han desarrollado y aplicado varias técnicas de estabilización, de modo las betacianinas resistan esas condiciones incialmente adversas mejorando su rango de aplicabilidad.

Las betacianinas también se han descrito como moléculas que presentan actividad biológica, debido a su estructura química, y en algunos casos específicos, incrementada por la presencia de grupos acilo.

Teniendo todo esto en cuenta, el objetivo principal de este trabajo fue explorar *G. globosa*, *A. caudatus* y *H. costaricensis* como fuentes alternativas naturales de betacianinas. Estas especies fueron caracterizadas químicamente mediante diferentes metodologías cromatográficas, para posteriormente optimizar el procedimiento de extracción, a través de maceración dinámica, extracción asistida por microondas y ultrasonidos, apoyados en la aplicación de modelos matemáticos con el fin de mejorar el procedimiento de extracción y lograr un extracto rico en betacianinas. A continuación, también se aplicaron técnicas de estabilización (Liofilización y Spray drying) para garantizar la estabilidad de los extractos finales. Como beneficio adicional, estos extractos también fueron evaluados con la expectativa de lograr alguna actividad biológica, proporcionando así capacidad colorante y potenciales propiedades bioactivas. Los extractos optimizados fueron incorporados como colorantes en diferentes alimentos ampliamente consumidos por niños como son los helados y galletas, evaluando estabilidad y viabilidad del colorante durante las condiciones de almacenamiento de cada uno de ellos.

Para completar este trabajo, se analizó la composición nutricional, propiedades fisicoquímicas y carga microbiana de los alimentos formulados con los extractos ricos en betacianinas para garantizar las propiedades organoléptica y la seguridad alimentaria de los mismos.

Según los resultados obtenidos, todas las plantas estudiadas presentaron un perfil químico muy diferente. Las flores de *G. globosa* fueron la muestra que mayor concentración en ácidos orgánicos presentaron, destacando por su contenido en ácido málido, además de caracterizarse seis betacianinas distintas como gomfrenina / isogomfrenina II y III, y 17-descarboxi-amarantina. La muestra de *H. costaricensis* presentó la mayor concentración de tocoferoles totales, identificándose los cuatro vitámeros correspondientes, además de caracterizarse cuatro betacianinas, como son la betanina/isobetanina y filocactina/ isofilocactina. Por último, en *A. caudatus* se identificaron amarantina/isoamarantina y betanina/isobetanina, y cuando se comparó con la muestra de *H. costaricensis*, se observó que presentó mejores resultados de actividad antihemolítica y antimicrobiana.

En cuanto a la optimización del proceso de extracción de las betacianas, el procedimiento que mejores resultados demostró fue la extracción asistida por ultrasonido (EAU), un procedimiento de extracción emergente y actualmente poco utilizado. La optimización se llevó a cabo con el apoyo de la metodología de superficie de respuesta (RSM), una herramienta matemática que permite evaluar varios factores que afectan la extractabilidad

y estabilidad de las moléculas diana. Tras aplicar el RSM, obtuvimos extractos ricos en betacianinas, con rendimientos de 45,5 g de betacianinas/kg de material vegetal para *G. globosa,* 77,6 g de betacianinas/kg de material vegetal para *A. caudatus* y 36 g de betacianinas/kg de material vegetal para *H. costaricensis*.

Tal y como se ha mencionado anteriormente, el extracto enriquecido recuperado de las flores de *G. globosa* se incorporó en helados y galletas para evaluar la capacidad colorante del extracto y su estabilidad. Los resultados obtenidos para el helado formulado con el extracto de *G. globosa*, se compararon con diferentes formulaciones de helado en diferentes tiempos de almacenamiento, y también se evaluaron las interacciones entre ambos factores. En cuanto a la composición nutricional, la interacción entre las formulaciones de helado y el tiempo de almacenamiento influyó en todos los parámetros estudiados, excepto en el caso del perfil de ácidos grasos que no mostró diferencias significativas entre formulaciones, siendo afectado únicamente por el tiempo de almacenamiento. Por otro lado, el tiempo de almacenamiento no afectó significativamente al parámetro de color, corroborando la buena estabilidad de estos compuestos.

Siguiendo la misma línea, también se compararon las galletas incorporadas con el extracto de G. globosa con diferentes formulaciones de galletas en diferentes tiempos de almacenamiento, evaluándose la interacción entre ambos factores. La composición química de las galletas no se vio alterada significativamente por el proceso de incorporación, así como los parámetros físicos estudiados. El color de las galletas incorporadas con el extracto de *G. globosa* presentó una coloración rosa intensa, incluso después del proceso de cocción, demostró la buena estabilidad de estos compuestos, incluso cuando se incorporan a alimentos tratados a altas temperaturas.

Los hallazgos de este trabajo apoyan la potencial aplicación de esta familia de pigmentos como colorantes naturales, así como que las tres plantas estudiadas pueden ser una fuente interesante de obtención de estos compuestos. De esta forma, la industria alimentaria tiene a su disposición una mayor gama de opciones de colorantes de origen natural, pudiendo ofrecer al consumidor productos alternativos potencialmente más saludables.

Part I. INTRODUCTION

1 A brief overview on food additives

Food industry has exerted a large and fascinating impact on the science evolution, but also on health and nutrition assurance together with consumers' taste and appearance satisfaction. An increasing delivery of more and more specific products with different formats, colours, tastes, smells, textures and so on, are available in supermarkets, mostly created to ensure consumers' expectations (Ayala-Zavala et al., 2011; Carocho, Barreiro, Morales, & Ferreira, 2014; Dias, Ferreira, & Barreiro, 2015). There are no doubts about the real impact that consumers' perception, opinions and desires exert on food industries. Therefore, more appealing and delightful products have been produced and offered (Carocho et al., 2014; Francisco Delgado-Vargas & Paredes-López, 2002). Among the food constituents responsible for a considerable improvement of the organoleptic characteristics, food additives revealed to be pivotal. According to the European Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives defined "*Food additives as any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods"*. Moreover, according to this regulation, substances should not be considered as food additives when they are used for the purpose of imparting flavour and/or taste or for nutritional purposes, such as salt replacers, vitamins and minerals. Moreover, substances considered as foods which may be used for a technological function, such as sodium chloride or saffron for colouring and food enzymes should also not fall within the scope of this Regulation. However, preparations obtained from foods and other natural source material that are intended to have a technological effect in the final food and which are obtained by selective extraction of constituents (e.g. pigments) relative to the nutritive or aromatic constituents, should be considered additives within the meaning of this Regulation.

Additives can be used for various purposes. Annex I of European Regulation (EC) No 1333/2008 defines 27 "technological purposes", according to the role they play in food they can be classified as: sweeteners, colourants, preservatives, antioxidants, carriers,

acids, acidity regulators, anti-cacking agents, anti-foaming agents, bulking agents, emulsifiers, emulsifying salts, firming agents, flavour enhancers, foaming agents, gelling agents, glazing agents, humectants, modified starches, packaging gases, propellants, raising agents, sequestrants, stabilizers, thickeners, flour treatment agents, and contrast enhancers. This classification and distinction, with respect to food additives, is quite different in the USA, for the regulatory authority, the FDA food ingredients are divided into 18 classes, which are: preservatives, sweeteners, colour additives, flavour and spices, flavour enhancers, fat replacers (and components of formulations used to replace fats), nutrients, emulsifiers, stabilizers (and thickeners, binders, texturizers), pH control agents and acidulants, leavening agents, anti-cacking agents, humectants, yeast nutrients, dough straightener's and conditioners, firming agents. enzyme preparations, and gases. Despite the different classes of additives, and the different classifications used, food additives can be roughly divided into four fundamental groups, considering their origin and manufacture. So, we can talk about food additives of natural origin (obtained directly from animals or plants), food additives similar to the natural ones (produced synthetically but imitating the natural ones), modified natural food additives (chemically modified natural additives) and finally food additives artificial (synthetic compounds).

Among other things, food additives are used as Colours (they are used to add or restore colour in a food), Preservatives (these are added to prolong the shelf-life of foods by protecting them against micro-organisms), Antioxidants (substances which prolong the shelf-life of foods by protecting them against oxidation (i.e. fat rancidity and colour changes) and Flour treatment agents (added to flour or to dough to improve its baking quality. In fact, they exert truly important benefits on the shelf life, microbiological quality and security of numerous foodstuffs. Not least important to highlight is their sensorial attractiveness, namely the visual perception, colour and smell, and in particular food colourants revealed to be of the utmost importance in this field (Carocho et al., 2014; Ray, Raychaudhuri, & Chakraborty, 2016; Shim et al., 2011).

European Food and Safety Authority (EFSA) and Food and Drug Administration (FDA) represent the most important regulatory organizations empowered to ensure the quality and security of food products, as also to protect and promote the human health (Amchova, Kotolova, & Ruda-Kucerova, 2015; Carocho et al., 2014). Several food additives that were used over decades are no longer currently allowed, due to the real evidences of their side effects, toxicity at medium- and long-terms and high frequency of health disturbance incidents. More important to emphasize is that not only synthetic but also commercial

plant-animal derived additives have been consecutively suspended by those authorities (Amchova et al., 2015; Rodriguez-Amaya, 2016; Tumolo & Lanfer-Marquez, 2012). The European Union, in order to standardize the nomenclature of food additives, has adopted a nomenclature consisting of the letter "E" (representing Europe) followed by a specific number. This nomenclature has been extended to the Codex Alimentarius Commission to easily identify food additives worldwide, and thus make it easier to create a database of legal additives in the EU, accompanied by some information such as their acceptable daily intake (ADI). The FDA in the USA adopted an acronym "GRAS" meaning Generally Recognized As Safe, to identify food additives, however, for an additive to be considered fit to incorporate this list, it goes through several toxicological assays, if for some reason it does not comply with any parameter it may not enter, or be removed from the list.

In recent years, more precisely since 2009, EFSA undergo in a major revision of the safety of all food additives approved for use in the EU, and never stop since. Some of the initially authorized additives have seen new data become available, and therefore, needed to be reassessed. The first class pointed for review was the class of food colours, more precisely the synthetically produced, and later the ones obtained from natural sources. This reevaluation is a continuous procedure, since the science in this field is always evolving, and the colourants on the approved list are always subjected to evaluations according to the increasing knowledge. For instance, some food colourants were pointed as possible to cause behavioral effects in children, and for that, an EFSA's panel of experts was gather in order to evaluate the available scientific evidence, in order to reach to a conscious and knowledge base conclusion. Another very important subject is the evaluation of the food additives individually, rather than assess the safety of the mixtures of chemicals. The number of possible combination of additives and other substances naturally present in the human diet it is nearly infinite, it is necessary to take into account the food composition, choices made by the consumer and dietary patterns, and all these very different parameters make this assessment difficult and almost impossible.

Despite the increasingly specific and regulatory legislation, an intense investigation on the field of food industry have reached a prestigious level (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009; Shahid, Shahid-Ul-Islam, & Mohammad, 2013).

2 Colour food additives

Colour may be considered one of the most impressive and delightful attributes of foodstuffs, which directly influences preference, selection and eating desires of the consumers (Francisco Delgado-Vargas & Paredes-López, 2002; Shim et al., 2011). However, despite natural food products have their own colour intensity, storage conditions, manufacturing and processing practices/methods have a pronounced influence on their final colouration; thus, food additives may be considered as promising means to mask their unpleasant characteristics.

Food colourants present a long history of use. In the broadest sense, according to FDA, a food colourant 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"* (FDA, 2016). Moreover, according to the European Regulation (EC) No 1333/2008, colours *"are substances which add or restore colour in a food, and include natural constituents of foods and natural sources which are normally not consumed as foods as such and not normally used as characteristic ingredients of food. Preparations obtained from foods and other edible natural source materials obtained by physical and/or chemical extraction resulting in a selective extraction of the pigments relative to the nutritive or aromatic constituents are colours within the meaning of this Regulation*".

Apart from their direct use, i.e. application to colouring foods, they might be also used to contribute to the flavourful assurance, safety, quality and organoleptic characteristics of foodstuffs, and not least important to warrant the consumers satisfaction. Thus, food colourants are mainly applied to offset and overcome those unpleasant characteristics, as also to homogenize the colour of foodstuffs, through correction of colour variations and/or enhancement of the naturally occurring food colour, and even making available colourless products. The final result arising from this intervention is the appearance of specifically targeted and requested products by consumers, and the commonly named "fun foods", that significantly improves their attractiveness and consequent worldwide demand (M. González, Gallego, & Valcárcel, 2002a; D. M. Jiménez-Aguilar et al., 2011).

A progressive empowerment of food industry has been observed in the last decades, mainly incited by the increasing demands by consumers (Agócs & Deli, 2011; Francisco Delgado-Vargas & Paredes-López, 2002; Shahid et al., 2013). More delightful, nutritive, attractive, healthy and high sensorial quality products are already available, and therefore

more specific and applied methods/techniques need to be developed and then implemented to achieve the industrial goals and consumer desires. (Cejudo-Bastante, Hurtado, Mosquera, & Heredia, 2014a; Sagdic et al., 2013; Türker & Erdog ˇdu, 2006).

Food colourants are among the most interesting features at industrial and scientific level. In fact, due to increasing demands by consumers, pronounced advances and opportunities have been achieved in food industry (Carocho et al., 2015; Shahid et al., 2013).

Food colourants can be classified according to their colour or their origin. Firstly, depending on their colour, food colourant can be classified in yellow (E-100 to E-109), red (E-120 to E-129), blue (E-131 to E-133), green (E-140 to E-142), just to mention a few. Moreover, can be also classified according to their origin in synthetic or natural food colourants. Apart from the ancient use of food additives, natural food colourants have received a particular attention, not only for their potent ability to colour foods, but also for providing some healthy benefits (Francisco Delgado-Vargas & Paredes-López, 2002; A. Gengatharan, Dykes, & Choo, 2015a; Shim et al., 2011). The use of more natural ingredients in the foodstuffs formulation constitutes the main focus of food industries, since a pivotal interest by consumers on natural pigments for colouring foods is doubtlessly expressed. Both food manufacturing industries as also consumers have shown a growing interest for natural colourants in line with a consequent replacement of synthetic food additives (Carocho, Morales, & Ferreira, 2015; Rodriguez-Amaya, 2016; Shahid et al., 2013).

With the growing and continuous search, numerous synthetic food colourants were developed to be added to improve food products quality and organoleptic characteristics, however, over time, most of them were banned due to the clearly evident side effects, signals of toxicity at short and long terms, as also health impairment abilities, including their possible carcinogenic effects (Amchova et al., 2015; Carocho et al., 2014). Thus, consumer expectations were largely affected but not changed, and requested the addition of natural pigments to foodstuffs in favour of the synthetic ones (Masone & Chanforan, 2015; Wissgott & Bortlik, 1996).

Natural food colourants revealed to be as much effective as those derived from chemical synthesis, with the subsequent benefits of: being more safe, providing health benefits besides conferring organoleptic features, exerting two or more benefits as food ingredients (in fact several food additives exerting colourant effects also act as antioxidants and even preservatives), and lastly contributing functional properties to food products (Carocho et al., 2014; Francisco Delgado-Vargas & Paredes-López, 2002;

Rodriguez-Amaya, 2016). In fact, proper regulatory practices and guidelines are still being developed and improved towards legislation and reassurance of consumers safety and life quality, and at the same time to contribute to adequate foodstuffs labelling information (Bagchi, 2006; Jauho & Niva, 2013; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2011).

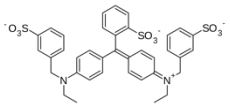
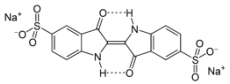
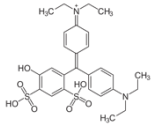
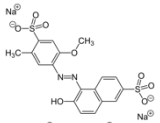
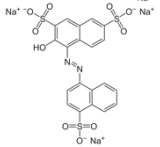
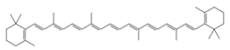
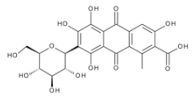
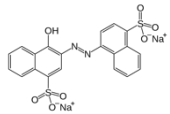
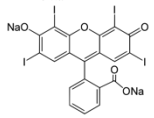
2.1 Synthetic food colourants

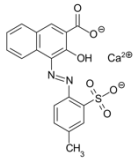
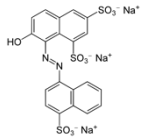
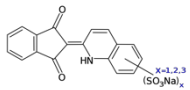
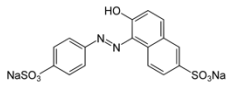
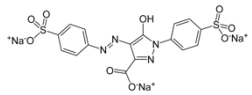
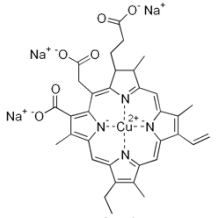
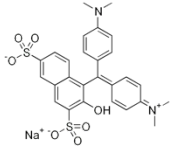
Synthetic food colourants are widely used to improve the attractiveness of numerous foodstuffs. In spite a considerable amount of them have been increasingly removed and even prohibited in the food industry, blue, red to orange, yellow and green and white synthetic food colourants are among the most commonly used and studied in terms of security, side effects, toxicity at short, medium and long terms, as well as health impact. Their application in food products is currently allowed by FDA and EFSA, with already established acceptable daily intake (ADI) doses.

Based on the ADI values, blue, followed by yellow and lastly green and white colourants seem to be the less dangerous, even at the higher ADI doses. Interestingly, titanium dioxide (E171) has not an established ADI, being thus currently used in confectionary, baked goods, cheeses, icings and toppings with permitted maximum levels not defined. Some red to orange colourants present the lower ADI doses, namely erythrosine – E127 (0.1 mg/kg b.w.), red 2G – E128 (0.1 mg/kg b.w.) and amaranth – E123 (0.8 mg/kg b.w.), which means that the occurrence of side effects and related toxicity is possible. Not least important to highlight is the occurrence of cumulative effects related with their daily intake. In fact, the majority of food products in which these colourants are applied are also daily consumed, i.e. beverages, cocktails, alcoholic drinks, fish and meat products, and candied cherries (widely consumed by children and teenagers). Apart from allergic reactions, several reports have inclusively reported that artificial food colourants highly affect children's behavior (Gostner, Becker, Ueberall, & Fuchs, 2015a; D. M. Jiménez-Aguilar et al., 2011; Masone & Chanforan, 2015). Attention deficit hyperactivity disorder (ADHD) is the most common, with six synthetic food colourants being currently indicated as having negative effects on the concentration activity (Council Regulation (EC) 1333/2008), namely tartrazine (E102), quinolone yellow (E104), sunset yellow FCF (E110), carmoisine/ azorubine (E122), Ponceau 4R (E124), Allura Red AC (E129).

Thus, it is feasible to infer that the risk of these products.

Table 1 Examples of synthetic food colorants. Adapted from Martins et al., 2016

Colorant	E code	ADI	Chemical structure	Uses	References
Brilliant blue FCF	E133	10 mg/kg b.w.		Dairy powders, color beverages, jellies, candies, condiments, icings, syrups, extracts	(Bonan et al., 2013; Chen et al., 1998; EFSA, 2010c; Huang et al., 2003, 2002; Kapadia et al., 1998; Kong et al., 2015; F.-J. Liu, Liu, Li, & Tang, 2015; K. Ma, Yang, Jiang, Zhao, & Cai, 2012; Medeiros, Lourencao, Rocha-Filho, & Fatibello-Filho, 2012b, 2012a; Minioti, Sakellariou, & Thomaidis, 2007; Ni, Wang, & Kokot, 2009; Pan, Ushio, & Ohshima, 2005; Qi et al., 2011; Vidotti, Costa, & Oliveira, 2006)
Indigo carmine (syn.: indigotine)	E132	5 mg/kg b.w.		Ice cream, sweets, baked goods, confectionery, and biscuits	(Berzas, Flores, Llerena, & Fariñas, 1999; Bonan et al., 2013; Chen et al., 1998; EFSA, 2014; Huang et al., 2003, 2002; Kapadia et al., 1998; Kong et al., 2015; Minioti et al., 2007; Pan et al., 2005; Qi et al., 2011; Yuzhen Wang et al., 2009)
Patent blue V	E131	15 mg/kg b.w.		Scotch eggs and jelly sweets	(Berzas et al., 1999; Bonan et al., 2013; EFSA, 2013; Minioti et al., 2007)
Allura Red AC	E129	7 mg/kg b.w.		Soft drinks and processed meats	(Bonan et al., 2013; Chen et al., 1998; EFSA, 2009a; El-Sheikh & Al-Degs, 2013; Huang et al., 2003, 2002; Kapadia et al., 1998; Karanikolopoulos et al., 2015; Kong et al., 2015; K. Ma et al., 2012; Masone & Chanforan, 2015; Minioti et al., 2007; Obón, Castellar, Cascales, & Fernández-López, 2005; Pan et al., 2005; Qi et al., 2011; Xie et al., 2012; T. Zou et al., 2013)
Amaranth	E123	0.8 mg/kg b.w.		Beverages, alcoholic drinks and fish roe	(Basu & Kumar, 2015b; Bonan et al., 2013; Chen et al., 1998; Daoud, Mesmoudi, & Ghalem, 2013; EFSA, 2010a; Karanikolopoulos et al., 2015; Kong et al., 2015; K. Ma et al., 2012; M. Ma, Luo, Chen, Su, & Yao, 2006; Minioti et al., 2007; Mpountoukas et al., 2010; Ni et al., 2009; Obón et al., 2005; Pan et al., 2005; Qi et al., 2011; Ryvolová, Táborský, Vrábel, Krásenský, & Preisler, 2007; Yuzhen Wang et al., 2009; Xie et al., 2012; Xing et al., 2012)
β-carotene	E160a	5 mg/kg b.w.		Sauces, milk, spice blends, marinades, beverages, coatings, fruit juices, margarines	(Campardelli, Adami, & Reverchon, 2012; EFSA, 2012a; Fernandez et al., 2009; Paz, Martín, Bartolomé, Largo, & Cocero, 2014)
Carminic acid	E120	5 mg/kg b.w.		Cakes, cookies, beverages, jam, jelly, ice cream, sausages, pies, dried fish, yogurt, gelatins, cider, tomato, dairy products, cherries, non-carbonated drinks, chewing gum, pills and cough drops	(EFSA, 2015b; Kapadia et al., 1998; F.-J. Liu et al., 2015)
Carmoisine (syn.: azorubine)	E122	4 mg/kg b.w.		Blancmange, Swiss rolls, jams, jellies, yoghurts, bread-crumbs, mouthwash and cheesecakes	(Basu & Kumar, 2014, 2015a; Bonan et al., 2013; Datta, Mahapatra, & Halder, 2013; EFSA, 2009b; Karanikolopoulos et al., 2015; Masone & Chanforan, 2015; Minioti et al., 2007; Obón et al., 2005; Ryvolová et al., 2007)
Erythrosine	E127	0.1 mg/kg b.w.		Cocktails and candied cherries	(Chequer, Venâncio, Bianchi, & Antunes, 2012; EFSA, 2011b; Ganesan, Margolles-Clark, Song, & Buchwald, 2011; Kapadia et al., 1998; Karanikolopoulos et al., 2015; Kong et al., 2015; K. Ma et al., 2012; Minioti et al., 2007; Mpountoukas et al., 2010; Obón et al., 2005; Pan et al., 2005; Qi et al., 2011; Ryvolová et al., 2007; Xie et al., 2012; T. Zou et al., 2013)

Lithol Rubin BK		E180	1.5 mg/kg b.w.		Cheese rind	(EFSA, 2010g; Kapadia et al., 1998)
Ponceau 4R		E124	4 mg/kg b.w.		Non-alcoholic drinks, sweets, jellies	(Bonan et al., 2013; Capitán-Vallvey, Fernández, Orbe, & Avidad, 1998; Chen et al., 1998; Daoud et al., 2013; EFSA, 2009c; Huang et al., 2003, 2002; Karanikolopoulos et al., 2015; Kong et al., 2015; K. Ma et al., 2012; M. Ma et al., 2006; Minioti et al., 2007; Ni et al., 2009; Obón et al., 2005; Pan et al., 2005; Qi et al., 2011; Ryvolová et al., 2007; Xie et al., 2012; Xing et al., 2012; T. Zou et al., 2013)
Quinoline yellow		E104	10 mg/kg b.w.		Fruit and vegetables juice	(Bonan et al., 2013; EFSA, 2009d; Kapadia et al., 1998; Masone & Chanforan, 2015; Minioti et al., 2007; Shahabadi & Maghsudi, 2013; Xing et al., 2012)
Sunset yellow FCF		E110	2.5 mg/kg b.w.		Non-alcoholic drinks, sweets, jellies	(Bonan et al., 2013; Capitán-Vallvey et al., 1998; Chen et al., 1998; Daoud et al., 2013; EFSA, 2009e; El-Sheikh & Al-Degs, 2013; Huang et al., 2003, 2002; Kapadia et al., 1998; Karanikolopoulos et al., 2015; Kong et al., 2015; F.-J. Liu et al., 2015; X. P. Liu, Fan, Bai, Li, & Liao, 2009; K. Ma et al., 2012; M. Ma et al., 2006; Masone & Chanforan, 2015; Medeiros et al., 2012a, 2012b; Minioti et al., 2007; Ni et al., 2009; Pan et al., 2005; Qi et al., 2011; Vidotti et al., 2006; Yuzhen Wang et al., 2009; Xie et al., 2012; Xing et al., 2012; T. Zou et al., 2013)
Tartrazine		E102	7.5 mg/kg b.w.		Non-alcoholic drinks, sweets, jellies	(Berzas et al., 1999; Bonan et al., 2013; Capitán-Vallvey et al., 1998; Chen et al., 1998; Daoud et al., 2013; EFSA, 2009f; El-Sheikh & Al-Degs, 2013; Huang et al., 2003, 2002; F.-J. Liu et al., 2015; K. Ma et al., 2012; M. Ma et al., 2006; Masone & Chanforan, 2015; Medeiros et al., 2012a, 2012b; Minioti et al., 2007; Mpountoukas et al., 2010; Ni et al., 2009; Oancea & Meltzer, 2013; Pan et al., 2005; Qi et al., 2011; Vidotti et al., 2006; Xing et al., 2012)
Copper chlorophyllin-complexes		E141ii	7.5 mg/kg b.w.		Green table fresh olives	(EFSA, 2015a; Gandul-Rojas, Roca, & Gallardo-Guerrero, 2012; Mortensen & Geppel, 2007)
Green S		E142	5 mg/kg b.w.		Canned peas, cake mixes, mint jelly and sauce	(EFSA, 2010f; Minioti et al., 2007)
Titanium dioxide		E171	Not specified		Confectionary, baked goods, cheeses, icings, toppings	(EFSA, 2006; Gu et al., 2015)

2.2 Natural food colourants

With the increasing demand by consumers for naturally-derived and safer food ingredients, in general with less toxic effects and even health promoters, numerous experiments have been carried out to provide more effective and selective food colourants. As an example, brown to black food colourants still continue to be highly explored, both derived from synthesis as also from natural sources; their food applications, chemical stability, side effects and related toxicity are among the main parameters exploited, towards determining the most effective and safer ADI. Thus, for these currently approved food colourants, E codes were approved and ADI were established, namely Curcumin (E100), which is one of the most important and widely known curcuminoids derived from *Curcuma longa* L. rhizomes is also widely used as food colourant for multiple purposes. The yellow to red-orange natural food colourant, carminic acid (E120) already exists from synthetic origin, with an established ADI of 5 mg/kg b.w., being largely used for several purposes, such as in cakes, cookies, beverages, jam, jelly, ice creams, sausages, pies, dried fish, yogurt, gelatines, cider, tomato, dairy products, cherries, non-carbonated drinks, chewing gums, pills and cough drops (Bibi, Galvis, Grasselli, & Fernández-Lahore, 2012; Huang, Chiu, Sue, & Cheng, 2003; Huang, Shih, & Chen, 2002; Masone & Chanforan, 2015).

Other were establish and approved food colourants are caramel – E150 (160-200 mg/kg b.w.), authorized to be used in sauces, biscuits, crisps, pickles and several alcoholic and non-alcoholic beverages (EFSA, 2011); brilliant black – E151 (1 mg/kg b.w.), used in several cheeses, wine, sauces, and drinks (EFSA, 2010a); vegetable carbon – E153 (not established), used in jam and jelly crystals (EFSA, 2012); brown FK – E154 (0.15 mg/kg b.w.), authorized in smoked and cured fish, meat and crisps (EFSA, 2010b); and brown HT – E155 (1.5 mg/kg b.w.), used in several biscuits, chocolate and cakes (EFSA, 2010c).

Carotenoids (E-160) are another group of naturally-derived food colourants with a renowned impact and demand, mainly due to their prominent colouring attributes and bioactive properties, among other health benefits. Their antioxidative potential is widely known and scientifically recognized, being used in large scale by the food industry also as natural preservatives (M. G. Dias, Camões, & Oliveira, 2009; Rodriguez-Amaya, 2016). These pigments have been also increasingly used for cosmetic, pharmaceutical and nutraceutical purposes, with available supplements containing both individual and

mixtures of these ingredients (Martín, Mattea, Gutiérrez, Miguel, & Cocero, 2007; Rodriguez-Amaya, 2016). However, their food colourant attributes are also highly appreciated. Carotenoids are particularly selected by food industries for inclusion in foodstuffs with high fatty acid content. In fact, butter and margarines, cakes, milk products and soft drinks possess, respectively, a high, moderate and low percentage of lipids, and therefore possess different susceptibilities to oxidation process. Not only vegetable sources, such as plant roots, flowers, leaves and even the whole matrix are used as raw material to extract carotenoid pigments, but also algae/microalgae, fungus/yeasts and aquatic animals (Danesi, Rangel-Yagui, De Carvalho, & Sato, 2002; Grewe, Menge, & Griehl, 2007; Hong, Suo, Han, & Li, 2009; K. Nabae et al., 2005). For example, astaxanthin (E161j) is isolated from animal sources, while β-carotene (E160a) may be both extracted from the roots of *Daucus carota* L. and even from fungus (*Blakeslea trispora* Thaxter). The most common colour attributes of carotenoids are yellow to orange and even red colour. As previously highlighted, lutein (E161b) and astaxanthin (E161j) are the carotenoids most commonly used for pharmaceutical and nutraceutical purposes, being used not only to confer bioactive and functional properties but also colourant attributes (Carocho et al., 2015; Devasagayam et al., 2004).

Regarding red-purple colourants derived from beets, betacyanins and betalains are the most commonly studied and were already approved (E162) to be safely used. Interestingly, not only *Beta vulgaris* L. root is a source of these natural colourants, but also fruit of *Hylocereus polyrhizus* (Weber) Britton & Rose (Florian C. Stintzing, Schieber, & Carle, 2002), *Opuntia ficus-indica* [L.] Miller (Cassano, Conidi, & Drioli, 2010; Otálora, Carriazo, Iturriaga, Nazareno, & Osorio, 2015), *Opuntia stricta* (Haw.) Haw. (Obón, Castellar, Alacid, & Fernández-López, 2009) and *Rivina humilis* L. (Khan & Giridhar, 2014a) are also rich in these ingredients, widely used in burgers, desserts, ice creams, jams, jellies, soups, sauces, sweets, drinks, dairy products and yogurts.

Anthocyanins (E-163) are the most widely studied natural food colourants, being obtained from flowers, fruits, leaves and even whole plants. Commercial anthocyanins, namely cyanidin 3-glucoside, pelargonidin 3-glucoside and peonidin 3-glucoside have been also used, and their effectiveness has been increasingly assessed. It is really important to highlight that external interferences highly affect the anthocyanin pigment colours, namely pH, temperature, humidity, salinity, stress conditions and even storage conditions. Thus, the anthocyanins colour may vary from red to purple and blue colour

(Cabrita, Fossen, & Andersen, 2000; D. M. Jiménez-Aguilar et al., 2011; Nontasan, Moongngarm, & Deeseenthum, 2012; Türker & Erdog ˇdu, 2006). As a particular example, (Cabrita et al., 2000)evaluated the effects of pH, and temperature during storage on the anthocyanins stability and colour, and described that in strong acidic medium reddish colour is the most prominent, while at relative neutral conditions bluish colour dominates. Furthermore, for the anthocyanins 3-glucosides a maximum level of stability was obtained at pH values 8-9, while for other ones a pronounced stability was obtained at pH values ranging from 5 to 7 (Cabrita et al., 2000).

Other natural colourants that are not still approved in Europe as food additives are yeast-derived natural pigments; one of the latest studied is monascin, a secondary yellow natural pigment produced by the genus *Monascus*. Appart from their interesting food colouring attributes, several biological activities, such as anti-cancer, anti-inflammatory, anti-diabetic, and anticholesterolemic effects has been also reported (Patakova, 2013; C. Wang et al., 2015). But, like does not exist a general consensus about their safe use, no E code was stablished, whereas their use considered illegal in Germany and in Asian countries is largely applied in food products (Wild, 2000). Thus, and considering these aspects, further investigation on this field should be carry out.

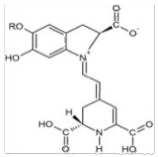
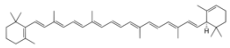
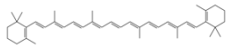
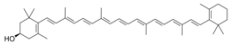
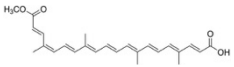
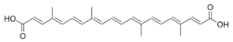
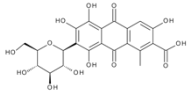
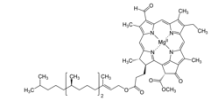
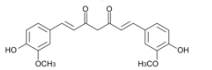
Also, another promising class of natural food colourants is phenolic compounds. Flavanones (naringin), flavones (4',5,7-trihydroxyflavones and apigenin) and flavonols (fisetin, myricetin, myricitin, quercetin and rutin) have been the most widely investigated on the colour ranges of orange and yellow, but so far commercial products are the most commonly used. Only myricetin and myricitrin were from plant origin, namely isolated from *Myrica cerifera* L. roots. The colourant attributes of many other phenolic compounds have been also studied but their safety, stability and spectrum of activity still remains unclear (Carocho & Ferreira, 2013; Grotewold, 2006; Robbins, 2003). In fact, phenolic compounds do not possess approved E code and ADI, and despite their wide recognition as prominent antioxidants and health-promoters and functional ingredients, the use of this large group of bioactive molecules as food additives continues to be poorly investigated. Lastly, it is of the utmost importance to refer that apart from their biological attributes, phenolic compounds are among the main responsible agents for the wide variety of naturally-occurred food colours (D. M. Jiménez-Aguilar et al., 2011; Shahid et al., 2013).

Despite synthetic food colourants have presented a large utilization and lower-associated costs than those derived from natural origin, they have been progressively substituted by

naturally-derived food colourants, which are safer, specific, absent of side effects and related toxicity, and are also able to confer health-improving effects and functional benefits (Carocho et al., 2014; Dias et al., 2015).

There are other food colourants under investigation, such as c-phycocyanin, a blue pigment isolated from *Arthrospira platensis* (cyanobacteria) (Martelli, Folli, Visai, Daglia, & Ferrari, 2014); c-phycoerythrin, a red-orange pigment from blue-green algae (Mishra, Shrivastav, Pancha, Jain, & Mishra, 2010); hot-air and freeze dried aerial parts of *Crithmum maritimum* L., which provides, respectively, a very interesting grey and green colour when added to pasta, sauces, rice, fish and meat (Renna & Gonnella, 2012). Genipin, a blue pigment derived both from fruits of *Gardenia jasminoides* Ellis and *Genipa americana* L. also revealed prominent attractive potentialities when added to beverages, juices, nectars, desserts and gels (Gao, Zhang, Cui, & Yan, 2014; Hou, Tsai, Lai, Chen, & Chao, 2008; Ramos-De-La-Peña et al., 2014). The red pigment madder colour, isolated from *Rubia tinctorum* L. roots also revealed to improve the general acceptability of hams, sausages, boiled fish, paste, beverages and even some confectionaries products, as well as the violet pigment violacein isolated from the bacteria *Chromobacterium violaceum* UTM 5 when added to yogurt and jelly (Venil et al., 2015). Other naturally-occurring pigments, but from commercial origin, have been also studied, such as geniposide, monascorubrin and purple corn colour (Kyoko Nabae et al., 2008; Ozaki et al., 2002; Wada et al., 2007). In fact, for the majority of naturally-occurring food pigments, studies using natural pigments and also those from commercial sources, have been carried out. These procedures are considered very important mainly for two reasons: the first one is to compare both the efficiency and efficacy between pigments according to their origin, and the second one to determine if the safety and probability of side effects, and related toxicity are similar. Overall, and despite the current worldwide advances in the food industry, particularly on the field of food colourants, it is still necessary to further the knowledge on this expansive area.

Table 2 Examples of natural food colorants. Adapted from Martins et al., 2016

Colorant	E code	Pigment color	Source	Chemical structure	Uses	Reference
Betacyanins  	E 163	red-purple	*Hylocereus polyrhizus* (Weber) Britton & Rose fruits		Burgers, desserts, ice cream, jams, jellies, soups, sauces, sweets, drinks, dairy products, yogurts	(Stintzing et al., 2002)
			Beta vulgaris L. roots			(Ravichandran et al., 2013)
			Opuntia ficus-indica [L.] Miller fruits			(Cassano et al., 2010)
			Opuntia stricta (Haw.) Haw. fruits			(Obón et al., 2009)
			Rivina humilis L. fruits			(Khan & Giridhar, 2014)
α-Carotene	E160a	Red-orange	*Daucus carota* L. roots		Butter and margarines, cakes, milk products and soft drinks	(Sun & Temelli, 2006)
β-Carotene 	E160a	Red-orange	*Blakeslea trispora* (fungus)			(K Nabae et al., 2005)
			Commercial			(Kohno et al., 2014; Martín et al., 2007)
			Daucus carota L. roots			(Sun & Temelli, 2006)
trans-β-Carotene	E160a	Red-orange	Commercial		Butter and margarines, cakes, milk products and soft drinks	(Kapadia et al., 1997)
Lutein (or xanthophylls) 	E161b	Yellow-orange	Commercial		Dairy products, soft drinks, sugar confectionary, salads	(Martín et al., 2007; Sobral et al., 2016)
			Daucus carota L. roots			(Sun & Temelli, 2006)
			Tagetes erecta L. flowers			(Mejía, Loarca-Piña, & Ramos-Gómez, 1997)
			Tagetes spp. flowers			(Khalil et al., 2012)
Annato (bixin and/or norbixin) 	E160b	Yellow-red	*Bixa orellana* L. seeds	 Bixin	Cakes, biscuits, rice, flour, soft drinks, smoked fish, sausages, meat products, dairy products, snack food, ice creams	(A. R. Agner, Barbisan, Scolastici, & Salvadori, 2004; Aniele R. Agner, Bazo, Ribeiro, & Salvadori, 2005; Anantharaman et al., 2015; Bautista, Moreira, Batista, Miranda, & Gomes, 2004)
			Commercial			(Kohno et al., 2014; Lima, Azevedo, Ribeiro, & Salvadori, 2003; Sobral et al., 2016)
				 Norbixin		
Carminic acid 	E 120	Yellow to red-orange	Commercial		Cakes, cookies, beverages, jam, jelly, ice cream, sausages, pies, dried fish, yogurt, gelatins, cider, tomato, dairy products, cherries, non-carbonated drinks, chewing gum, pills and cough drops.	(Bibi et al., 2012; Huang et al., 2003, 2002; Masone & Chanforan, 2015)
Chlorophyll 	E140	Green	*Spinacea oleracea* L. leaves		Beverages, fruit juices, pasta, dairy products, soups, sweeter preparations	(Fernandes, Gomes, & Lanfer-Marquez, 2007)
			Spirulina pratensis (algae)			(Danesi et al., 2002)
Curcumin 		Yellow-orange	*Curcuma longa* L. rhizomes		Fish and baked products, dairy products, ice cream, yoghurts, yellow cakes, biscuits, sweets, cereals, sauces, gelatines	(Gómez-Estaca, Gavara, & Hernández-Muñoz, 2015; Silva et al., 2005)
			Commercial			(Han & Yang, 2005; Kapadia et al., 1997; Maier et al., 2010; Martins et al., 2013; Masone & Chanforan, 2015; Yu Wang, Lu, Wu, & Lv, 2009)

2.3 Current desires and futures perspectives of natural food colourants

Nowadays, the consumer satisfaction is not only related with the taste, appearance, smell and attractiveness of foodstuffs, but also with their health impact, improvement of life quality and longevity (Giusti & Wrolstad, 2003; Kammerer et al., 2014; Xi et al., 2007). However, colour appreciation comprises one of the earliest aesthetic parameters considered by consumers during foodstuffs selection, being a direct predictive parameter that ensures good quality (Sagdic et al., 2013; Todaro et al., 2009). Visual cues present a doubtless influence on food preference, acceptability and lastly food choice. Therefore, it is clearly evident that food industries aim to provide increasingly uniform, attractive and pleasant coloured foodstuffs using colour food additives, to fully satisfy consumers' expectations and current needs (Cai, Sun, & Corke, 2005; Junqueira-Goncalves et al., 2011; Otálora et al., 2015; Sagdic et al., 2013).

Not least important to highlight is the progressive and increasingly strict regulatory legislation on food additives, and particularly on food colourants, clearly distinguishing permitted from prohibited and safe from harmful food colourants (Carocho et al., 2014; Shahid et al., 2013; Zou, He, Yasen, & Li, 2013). Lists of permitted and prohibited food colourants have been progressively updated (Council Regulation (EC) 1129/2011; Council Regulation (EC) 1333/2008), but prominent discrepancies are observed among different countries of European Union and even other countries (Chemical Engineering, 2002; Europe Environment, 2004; Official Journal of the European Communities Legislation, 2009). Therefore a wide variety of food colourants currently applied in food industry has been highly scrutinized, both from regulatory agencies and subsequent updated legislation as also scientific researchers, that exert a meticulous and increasingly detailed study of food colourants, including a wide variety of evaluation criteria (Konczak et al., 2005; Shahid et al., 2013; Venil, Zakaria, & Ahmad, 2013). The observed side effects, related toxicity, at medium and long terms, as well as the cumulative effects of their ingestion were more than evident factors that led to their suspension. However, and since regulatory legislation varies from different countries, an increasingly strict evaluation has become increasingly necessary in order to ensure consumers safety and security (Bonan et al., 2013; Kammerer et al., 2014; Shahid et al., 2013; Zou et al., 2013). Consumer demands and their preferences for naturally-derived colourants have increased exponentially, widely associated with the image of healthy, safe and good quality

products, which constitutes a great challenge to food industries and related food science research institutions (Carocho et al., 2014; Dias et al., 2015; Shahid et al., 2013). There are no doubts that nature is highly rich in colour pigments and that the majority of plants has not yet been exploited for their colouring properties/abilities. In line with this, a progressive substitution of synthetic with natural food colourants has been intensified, up to a point where numerous used natural pigments still continue to be poorly studied and their real functions are unknown. Although some natural pigments have already been used as substitutes of their related synthetic ones, the wide benefits and related use/application of many others has not been tested yet. Therefore, gaining knowledge on this field could be considered a promissory advance in order to develop more specific and functional foods/products.

2.4 Strengths and weaknesses of Natural food colourants

As previously mentioned, world consumers are more exigent in relation to the quality, safety, credibility and scientific assurance of commercialized foodstuffs and their chemical additives, even requesting "clean labels" (Carocho et al., 2014, 2015; A. Gengatharan et al., 2015a), in this sense, food industry is currently trying to ensure consumers' expectations developing novel foodstuffs formulation in the in which a progressive substitution of synthetic with natural food additives was performed, making an special effort to replace synthetic food colourants.

Furthermore, the scientific advances go in the same direction, clearly evidencing that natural products are associated with a consequent promotion of quality of life and health improvement while synthetic dyes are critically assessed, being even some of them no longer currently available, in spite to the notable transnational disagreements (Official Journal of the European Communities Legislation, 2009; Rodriguez-Amaya, 2016).

On the other hand, the use of natural colourant in food products, implies an extra effort since several reports have confirmed the real interference that some external factors exert on the food colour stability (D. M. Jiménez-Aguilar et al., 2011; Lemos, Aliyu, & Hungerford, 2012; Zhu et al., 2015). Anthocyanin pigments are a good example of natural food colourants highly affected by those external agents; in fact, they are very unstable compounds and highly susceptible to degradation (Assous, Abdel-Hady, & Medany, 2014; D. M. Jiménez-Aguilar et al., 2011; Sagdic et al., 2013; Zhu et al., 2015). Therefore, their final colouration will vary significantly, at the same time that various degrees of

susceptibility to external factors may be also observed among different species belonging to the same plant family (Sagdic et al., 2013). For example, Sagdic et al. (2013) observed that red tulip anthocyanins are less sensitive to temperature than violet tulip anthocyanins that exhibited a lower chemical stability. Jiménez-Aguilar et al. (2011) evaluated the colour stability of spray-dried blueberry, a very important source of anthocyanins, and observed a progressive increase of air outlet temperature together with a loss of total phenolics and anthocyanins and a decrease in the antioxidant activity. On the other hand, Tan et al. (2014) aiming to assess both the effects of temperature (under refrigerated and room temperature conditions) and pH (from 1.0 to 11.5) on a purple-red anthocyanin-derived extract of *Rhoeo spathacea* (Swartz) Stearn leaves, concluded that it exhibited a phenomenal colour stability under a range of pH values and temperatures. Over a period of 60 days, the authors observed a remarkable stability of anthocyanin-derived extract in acidic pH, and a complete stability when solid food (jelly) and liquid food (barley water) were used (J. B. L. Tan, Lim, & Lee, 2014). Furthermore, Munawar & Jamil (2014), aiming to access the colour changes, anthocyanin stability and antioxidant activity of several spray dried plant pigments, observed that the most prominent antioxidant potential was obtained to those possess higher anthocyanin content, being again emphasized the negative impact of storage temperature on the final chemical stability and acceptability of products. But, even so, the authors highlighted the safety and healthy abilities of the studied natural colourant, may even be upcoming used as functional ingredient in food products (Munawar & Jamil, 2014).

Many other examples related with natural pigments stability might be highlighted but the most interesting feature that indeed deserves a particular attention is that highly specific and reliable procedures, and industrial techniques have been effectively used to overcome the problem of poor stability of numerous food pigments, as also to identify and quantify their relative abundance in numerous foodstuffs.

In order to improve the general stability and the attractiveness of numerous foodstuffs, some techniques have been applied. More interestingly is that some of them are also used to neutralize and/or to remove several unpleasant characteristics of foodstuffs and even food colourants. Silva et al. (2005) investigating the influence of different hydrodistillation procedures on the turmeric deodorization observed that distillation under high vacuum and using a rotatory evaporator were not efficient. On the other hand, distillation of medium size grated turmeric using a Clevenger apparatus led to a pronounced decrease of turmeric flavour. Then, the authors assessed the general

acceptability of both turmeric-derived colourants in gelatine, and the gelatine prepared with deodorized turmeric was preferred (L. V. Silva, Nelson, Drummond, Dufossé, & Glória, 2005). In a similar way, Laokuldilok et al. (2016) evaluated the odour masking ability and encapsulation efficiency of turmeric extract prepared by a binary blend of wall materials. They concluded that the optimal formulation was the microcapsules consisting of 5% of core loading in addition with 20 g/L of β-cyclodextrin, once that a high amount of curcuminoids was encapsulated and corresponded to the formulation with low volatile release (Laokuldilok et al., 2016). Furthermore, Martins et al. (2013) evaluating the spray drying effects on curcuminoid and curcumin content as well as the related solubility of microparticles containing curcuma extract, concluded that the solubility of curcuminoids was markedly improved 100-fold by using microparticles. In fact, encapsulation by drying technology and microencapsulation techniques have gained progressively a particular reliability up to a point that are used both in food industries, but also for pharmaceutical and nutraceutical purposes (Dias et al., 2015; Fernandez et al., 2009; Otálora et al., 2015; Ray et al., 2016). As an example, Venil et al. (2015) aiming to assess the spray drying effect on the stability of an encapsulated violet pigment produced from *Chromobacterium violaceum* UTM 5, described that the high stability of this pigment was achieved at pH 7, temperature 25-60 ºC and under dark conditions, during entire storage period (Venil et al., 2015). Khalil et al. (2012) also evaluated the lutein stability and bioavailability under food processing conditions and application of spray drying, and concluded that the emulsification of medium-chain triacylglycerols oil markedly improved the stability and bioavailability of lutein esters (Khalil et al., 2012).

On the other hand, thermal treatments have been also revealed to significantly influence colour and chemical stability of some food colourants and their related enriched foodstuffs. Khan and Giridhar (2014) aiming to evaluate the stability of betalains derived from *Rivina humilis* L. berry juice observed that at 90 ºC for 36 min and at 25 ºC for 48 days, up to 95% and 96% of betacyanins were degradated, respectively, while at 5 ºC for 90 days only 15% of them were destroyed (Khan & Giridhar, 2014a). Jiménez-Aguilar et al. (2011) carried out a deepen evaluation of the colour variation and concentration of chemical compounds (responsible for the final colouration) on spray-dried powders derived from blueberry extracts, with added mequite gum. The authors observed that the lowest losses of bioactive colourant chemical compounds and minor colour variations

were found in samples dried at 140 ºC and 9.1 mL/min, followed by microencapsulates stored at 4 ºC for 4 weeks in the absence of light.

Lastly, gamma irradiation techniques have been also increasingly investigated, as well as upcoming procedures that improve the shelf life of numerous foodstuffs, at the same time ensure the relative stability of numerous food additives (among them food colourants). For example, Komolprasert et al. (2006) assessing the effects of 10- and 20-kGy gamma irradiation in a polystyrene polymer containing either yellow or blue colourant, concluded that both yellow and blue colourants are relatively stable under these gamma irradiation doses (Komolprasert et al., 2006).

Overall, and despite the prominent advances, it still continues to be imperative to deepen knowledge on this field, towards providing more feasible, secure and highly specific procedures to contribute to the consumers' satisfaction, security and long-term health security, at the same time that food industry achieves its strategic goals and produce increasingly valuable foodstuffs.

3 Betalains as natural food colourants

3.1 Occurrence in nature

Plants produce several molecules, on which they depend for the maintenance of a basic cellular function, in addition to a myriad of other chemical compounds, including betalains. These pigmented compounds occur naturally in some plant families and in some higher order fungi.

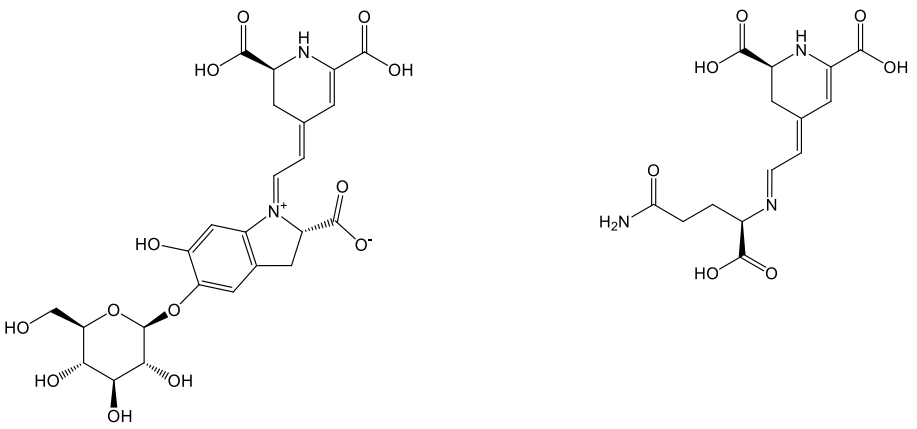
Betalains are water-soluble compounds, which have nitrogen in their composition, presenting a core structure (protonated system 1,2,4,7,7-pentasubstituted 1,7-diazaheptametine) known as betalamic acid [4- (2-oxoethylidene) - 1,2, 3,4-tetrahydropyridine-2,6-dicarboxylic acid]. Depending on the condensation of betalamic acid with cyclo-DOPA (L-3,4-dihydroxyphenylalanine) or its glucosyl derivatives, or amino acids or its derivatives, two different categories of betalains can be formed, violet betacyanins and yellow betaxanthines, respectively (Khan, 2016a).

Research carried out so far have allowed identification of about 75 betalains derived from 17 families of different plants (Khan & Giridhar, 2015). With the report of tentatively identified new compounds on a large scale due to advances in analytical technologies and

the appearance of better methodologies in the area of liquid chromatography and mass spectrometry are allowing the continuous increase in the number of reported betalains (Sekiguchi, Ozeki, & Sasaki, 2012).

This family of compounds was found in different parts of the plant such as roots, fruits, flowers, leaves and stems (Strack et al., 2003). We can check in **Table 3.** a summary of the species that contain these compounds in their composition, the order of the caryophilales, namely the family of amarantaceas and cactaceas holding the majority of the representations. As examples *Beta vulgaris* L. ssp. *vulgaris* (red and yellow beetroot), *Beta vulgaris* L. ssp. *cicla* (coloured Swiss chard), fruits of *Opuntia* and *Hylocereus* genera, and *Amaranthus* sp. (leafy or grainy amaranth) are the main edible sources (Gandía-Herrero, Escribano, & García-Carmona, 2016; Stintzing, Schieber, & Carle, 2002; Vaillant, Perez, Davila, Dornier, & Reynes, 2005; Wang, Chen, & Wang, 2007), but there are also less known edible sources, such as the tubers of *Ullucus tuberosus* L., fruits of *Eulychnia* cacti, or berries of *Rivina humilis* L. and other plants like *Phytolacca americana* L. (Masson, Angélica Salvatierra, Robert, Encina, & Camilo, n.d.; Schliemann et al., 1996; Svenson, Smallfield, Joyce, Sansom, & Perry, 2008). This family of compounds can also be found in higher fungi of the genus *Amanita* and *Hygrocybe* (Babos et al., 2011; Musso, 1979; Stintzing & Schliemann, 2007).

Betanin (**Figure 1A**), isobetanin, betanidin and isobetanidin are the major betacyanins (red-violet pigments) presented in red beetroor varieties Bonel', 'Nero', 'Favorit', 'Rubin', and 'Detroit', whereas the major betaxanthines (yellow pigments) found were vulgaxanthin I (**Figure 21B**) and vulgaxanthin II. Some varieties presenting higher levels of these compounds them others (Gasztonyi, Daood, Hájos, & Biacs, 2001). Beetroot are mainly explored for the red-violet compounds, and when compared with cactus fruits, these present a broader colour spectrum. Hylocereus cacti fruits are known for their higher levels of betacyanis, acylated and non-acylated, and also present betaxanthynes, contrarily to beetroot (Vaillant et al., 2005; Wybraniec et al., 2001). Cactus fruits presents a variety of combinations of betacyanin/betaxanthin ratios, at different concentration, resulting in a broad possibility of colours, and as a good example of these we found the cactus pears, which, curiously, do not have fruits containing exclusively betaxanthins (Stintzing & Carle, 2006). The exclusive presence of betacyanins it was also detected in *Opuntia stricta* (Haw.) Haw., containing a higher amount of these compounds than *Opuntia ficus-indica* (L.) Mill. and *Opuntia undulata* Griffiths, this last one also presenting betaxanthins (Castellar, Obón, Alacid, & Fernández-López, 2003).



A B

Figure 1 Chemical structures of betanin (A) and vulgaxanthin I (B).

Under code 73.40 in the 21 CFR section of the Food and Drug Administration (FDA) in the USA (FDA, 2020) and under code E-162 in the European Union (EFSA, 2015), the powder obtained by dehydration of beetroot is the only natural betalain-based colorant approved and used in food industry. It was for a long time considered the main food additive for the purpose of coloring foods, respecting the good manufacturing practices of this class of compounds, however, due to the emerging studies and patents on the production and stabilization of this class of compounds in *Hylocereus* sp. (Patent No. WO 2010/090508 Al, 2017) and *Opuntia* sp. (Patent No. EP 2 057 994 A1, 2009) new alternatives appear as very promising.

Table 3 Plant species containing betalains

Botanic family	Scientific name	Common/local name
Amaranthaceae	*Amaranthus cruentus* L.	Red amaranth
	Amaranthus gangeticus L., syn. *Amaranthus tricolor* L.	Edible amaranth
	Beta vulgaris L.	Beetroot
	Gomphrena globosa L.	Globe amaranth
	Iresine herbstii Hook. ex Lindl.	-
	Iresine lindenii Van Houtte	-
Basellaceae	*Basella rubra* L.	Malabar spinach
	Ullucus tuberosus Loz.	Ulluco
Cactaceae	*Escontria chiotilla* (Weber) Britton & Rose	Jiotilla
	Hylocereus megalanthus (K. Schumann ex Vaupel) Ralf Bauer	Yellow pitaya
	Hylocereus polyrhizus (Weber) Britton & Rose	Red pitaya
	Hylocereus undatus (Haw.) Britton & Rose	White-fleshed red pitaya
	Myrtillocactus geometrizans (Mart.) Console	Bilberry cactus
	Opuntia dillenii (Ker Gawl) Haw	Cactus pear
	Opuntia engelmannii Salm-Dyck ex Engelm.	Prickly pear
	Opuntia ficus-indica (L.) Mill.	Cactus pear
	Opuntia joconostle F.A.C. Weber ex Diguet	Prickly pear
	Opuntia littoralis (Engelm.) Cockerell	Prickly pear
	Opuntia oligacantha C.F. Först	Xoconostle
	Opuntia stricta (Haw.) Haw.	Prickly pear
	Stenocereus pruinosus (Otto) Buxbaum	Cactus
	Stenocereus queretaroensis (F.A.C. Weber) Buxbaum	Cactus
Nyctaginaceae	*Bougainvillea glabra* Choisy	Paperflower
Phytolaccaceae	*Rivina humilis* L.	Pigeon berry

Despite a lot have been done in these field, mainly on the biotechnological production of betalains, this expensive technology cannot compete with the abundant and inexpensive crop of beetroot. Jiménez-Aparicio and Gutiérrez-López (1999), pointed some important aspects detrimental to the economic viability of this technology, such as, detrimental to the economic viability of this technology, such as the low productivity of existing bioreactor systems, and the high process costs. However, the obtaining of pure betalains continues to be explored, either by semi-synthesis methods, which requires the presence of betalamic acid obtained by the degradation of betalains, or by its production in plant cell cultures (Gandía-Herrero, García-Carmona, & Escribano, 2006; Guadarrama-Flores, Rodriguez-Monroy, Cruz-Sosa, Garcia-Carmona, & Gandia-Herrero, 2015; Henarejos-Escudero et al., 2018; Milech et al., 2017). A novel scalable bioprocess is been

investigated to produce these pigments in a fast and simple system, carried out in a bioreactor controlled by the heterologous expression of 4,5-DOPA-extradioldioxygenase of bacterial origin and superior activity. The possibility of using water in this procedure was also investigated, in an attempt to lower the costs of large-scale biological production and make it easier to purify recovered compounds, however the dramatic reduction in betaxanthin recovery points to the need for technology improvement (Guerrero-Rubio, López-Llorca, Henarejos-Escudero, García-Carmona, & Gandía-Herrero, 2019).

3.2 Accumulation and functions in plants

Betalains are similar in colour to anthocyanis, both are secondary metabolites and possess similar function in plants, but are biochemically different. For a long time, betalains were mistakenly classified as nitrogen-containing anthocyanin variants, and called as 'nitrogenous anthocyanins' (Mabry, 1964). In fact, the functions that both play in the plant are analogous, such as attracting pollinating agents, protecting against UV light, herbivores to pathogens and giving resistance to stress (Jackman & Smith, 1996; Stafford, 1994; Strack, Vogt, & Schliemann, 2003).

The colour of these compounds in the plant plays an important role in attracting animals, whether they are pollinating agents or animals that feed on fruits and then disperse their seeds (Piattelli, 1981), on the other hand the colour can also act as a repellent signal present in the spines of certain cactus to deter herbivores (Lev-Yadun, 2001).

The amount of this compounds is also related to the response of some different stresses, and have been observed in some betalain- producing Caryophyllales, where wounded leaves of red beet, pass 48 hours, showed a seven-folder increase of these compounds concentration (Sepúlveda-Jiménez, Rueda-Benítez, Porta, & Rocha-Sosa, 2004) The photoprotection effect of betalain compounds is based on the fact that when subjected to excessive light/UV radiation exposure, can lead to accumulation of reactive oxygen species (ROS), which lead to numerous disorders, and may in extreme cases result in death, plants reveal a common protection mechanism, increase the production of pigments like betalains, carotenoids or anthocyanins acting as antioxidants compounds (Ibdah et al., 2002; Kishima, Shimaya, & Adachi, 1995; Tariq, Ali, & Abbasi, 2014; Vogt et al., 1999a). Some experiments showed reduced damage in red pigments leaves, when compared with green ones, assuming in this way a high photosynthetic capacity of these

pigments against excessive light conditions (Jain & Gould, 2015a; Jain, Schwinn, & Gould, 2015; Nakashima, Araki, & Ueno, 2011).

These compounds also seem to act against drought and saline stress conditions, because when under these conditions, plants increase the induction of expression of betalains (Casique-Arroyo, Martínez-Gallardo, González de la Vara, & Délano-Frier, 2014; Hayakawa & Agarie, 2010; Jain & Gould, 2015a; Jain et al., 2015; Nakashima et al., 2011). Something that is well evidenced in the order of Caryophyllales, which reflect abiotic stress resistance, due to their dominance in arid and semi-arid regions, and in saline and alkaline soils. *Aizoaceae* (ice-plant family), *Portulacaceae* (purslane family), and *Cactaceae* (cacti family) are examples of plants adapted to arid or saline regions. In a previous study on leaves of *Disphyma australe* (Sol. ex Aiton) J.M.Black, it was observed the increase in betalains production to confer photoprotection to chloroplasts under salinity stress. When exposed to salinity stress halophyte *Salicornia fruticosa* (L.) L. decreased the clrorophyl production accompanied by a four-fold increase in betalain concentration (Duarte, Santos, Marques, & Caçador, 2013). However, under similar conditions *Portulaca oleracea* L. showed an higher amount of proline content and betalain pigmentation (Sdouga et al., 2019), a very similar response to the one observed in *Opuntia* species, indicating to a consequence of their adaptation to semi-arid climates. In this species, interestingly, the compound with higher expression is indicaxanthin, leading to the belief that betaxanthines can act as osmoregulators by the modulation of the amino acid pool through betaxanthin cleavage or synthesis (Piattelli, Minale, & Prota, 1964; F.C. Stintzing, Schieber, & Carle, 1999; F.C. Stintzing et al., 2002; Florian C. Stintzing, Schieber, & Carle, 2003).

Despite little information, it is believed that betalains are also capable of acting against pathogens (Brockington, Walker, Glover, Soltis, & Soltis, 2011). In a study carry out by Polturak et al. (Polturak et al., 2017) in transgenic betalain-producing tobacco plants it was noticiable an increased resistance to leaf infection by *Botrytis cinerea*, with delayed cell death and necrotrophic fungus proliferation. Moreover, ROS also induced betalain synthesis in red beet leaves due to bacterial infiltration with *Agrobacterium tumefaciens* or *Pseudomonas syringae* (Sepúlveda-Jiménez et al., 2004).

To the best of our knowledge, despite all these findings on the functions exerted by betalains in plants, their role in fungi remains unknown.

3.3 Biosynthesis pathway

It is from the tyrosine hydroxylation that the betalains synthesis process begins, resulting in two precursor molecules of the L-5,6-dihydroxyphenylalanine (L-DOPA) (Piattelli, 1981). One of the molecules is transformed into DOPA-quinone and spontaneously converted to *cyclo*-DOPA, while the other undergoes a 4,5-extradiol oxidative cleavage, resulting in 4,5-*seco*-DOPA being used in the production of betalamic acid (Tanaka, Sasaki, & Ohmiya, 2008), presenting two distinct fates that lead to the formation of betacyanins or betaxanthins. To obtain betacyanins, condensation occurs with an amino group of cyclo-DOPA and produce betanidin, which is subsequently glycosylated and forms betanin, a step that can occur before the condensation process with betalamic acid in *cyclo*-DOPA, or after formation of betanidin. betalains can further acylate and form 1-*O*-acylglucosides (F. Delgado-Vargas, Jiménez, Paredes-López, & Francis, 2000). On the other hand, to form betaxanthins, betalamic acid can condense with the imino or amino group of amino acids (Tanaka et al., 2008), as shown in the **Figure 2**.

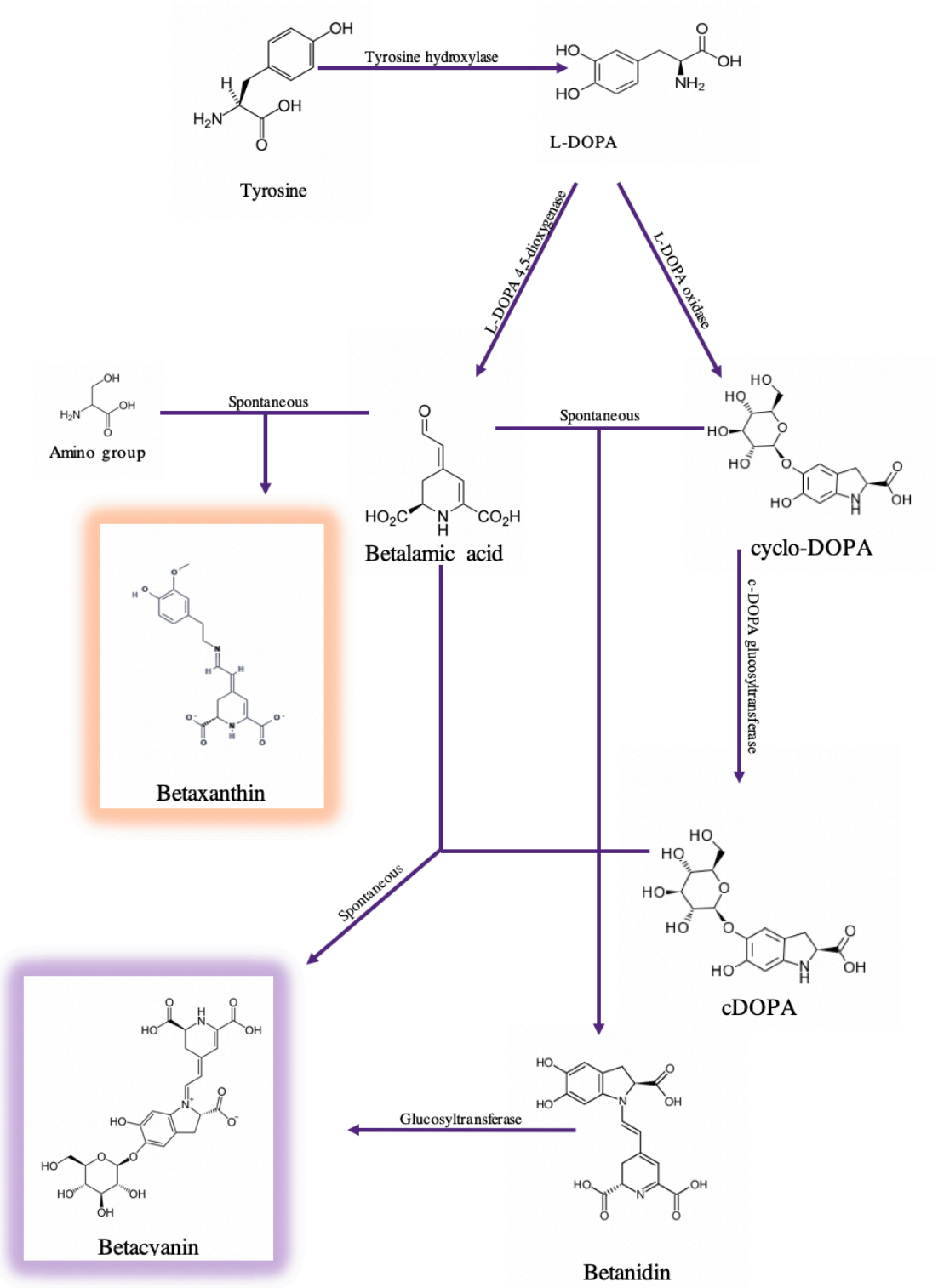


Figure 2 Betalain biosynthesis pathway scheme. Adapted from Timoneda et al., (2019)

The main enzymes involved in these reaction processes are tyrosinase or phenol-oxidase complex and DOPA-4,5-dioxygenase (DOD), being the first one responsible for the catalysing the conversion of tyrosine into L-DOPA and dehydrogenation to *O*-quinone

DOD is responsible of enhancing the conversion of L-DOPA into betalamic acid. It was from the *Amanita muscaria* basidiomycete that tyrosine was isolated first, followed by Caryophyllid taxa *Portulaca grandiflora* Hook. (Portulacaceae) and *Beta vulgaris* L. (Amaranthaceae). In order to produce *cyclo*-DOPA via the intermediate *O*-quinone is required monophenol monooxygenase and *O*-diphenol oxidase activity, provided by these enzymes (F. Delgado-Vargas, Jiménez, Paredes-López, et al., 2000; Steiner, Schliemann, Böhm, & Strack, 1999; Steiner, Schliemann, & Strack, 1996). *Phytolacca americana* L. (Phytolaccaceae) provided two cDNA clones encoding polyphenol oxidase, that in fruits with betalains increase their levels of transcription in their ripening process (Joy, Sugiyama, Fukuda, & Komamine, 1995). The DOD protein purification and gene cloning were also achieved in fungi, through particle bombardment technique, a clone encoding the fungal DOD was expressed in white *P. grandiflora* petals, confirmed by the appearance of red-violet and yellow cells, explained by the presence of betacyanins and betaxanthins, respectively, which proved that the recombinant protein maintained its specificity for 4,5- and 2,3-cleavage in the plant cell environment (Mueller, Hinz, Uzé, Sautter, & Zryd, 1997). In *P. grandiflora* through cDNA subtraction the enzyme was found, being its action confirmed, where transient expression of DOD cDNA by particle bombardment generated red spots of betacyanin in the white cultivar petals (Christinet, Burdet, Zaiko, Hinz, & Zrÿd, 2004). In *Hygrocybe conica* and *A. muscaria*, 2,3- and 4,5-*seco*-DOPA revealed to occur naturally being identified by direct HPLC comparison with authentic standards generated enzymatically from DOPA and confirmed by their conversion to their cyclized successors, muscaflavin and betalamic acid (Terradas & Wyler, 1991).

Other enzymes mostly act at late reactions catalysing like glucosyltransferase, hydroxycinnamoyltransferase, and malonyltransferase. Despite being rarer, decarboxylation and methylation reactions have also been considered enzymatic steps (Strack et al., 2003).

Although a large part of the reactions is enzymatically catalysed, there are intermediate steps that are believed to occur spontaneously, as is the case of cyclisations and aldimine formation. These spontaneous reactions include the formation of *cyclo*-DOPA via dopaquinone, betalamic acid via 4,5-*seco*-DOPA, muscaflavin via 2,3-*seco*-DOPA, and the condensations of betalamic acid with *cyclo*-DOPA or amino acids/amines, in betanidin and betaxanthin formation, respectively (Strack et al., 2003).

It appears that the formation of betanidin is also possible via betaxanthines without the presence of *cyclo*-DOPA. in this case betanidin is glucosylated by betanidin-5-*O*-glucosyltransferase to produce betanin, whereas β-glucosidase may revert it to betanidin (Zakharova & Petrova, 2000). Although this process of betanidin formation has only been proposed in beetroot, it is likely that it will happen in other matrices given the co-presence of DOPA, tyrosine, and oxidising enzymes, and can related to the accumulation of various derivatives like betanidin-5-*O*-glucosides and betanidin-6-*O*-glucosides in ulluco tubers (Svenson et al., 2008) and *Gomphrena globosa* L. petals (Kugler, Stintzing, & Carle, 2007a; Svenson et al., 2008). It's also believed that betanin could also result by condensation of betalamic acid and *cyclo*-DOPA-5-*O*-glucoside, due to the detection of *cyclo*-DOPA-5-*O*-glucosyltransferase enzyme. Regarding the route by which the formation of betalain will take place, which route will be favoured there is no evidence to allow this type of conclusion to be made.

3.4 Mutual exclusiveness of betalains and anthocyanins

Like anthocyanins, betalains have the particularity of appearing mutually exclusive in the plant kingdom. With a very different structure from flavonoids and anthocyanins, betalains suggest at least the occurrence of two phases of evolution, one within a fungal lineage (Basidiomycetes), and another within the order of flowering plants (Caryophyllales) (Mabry, 1964). Plants of this order are more likely to produce this class of compounds, with the exception of Caryophyllaceae and Molluginaceae, which preferentially produce anthocyanins, according to chemosystematic marker studies (Brockington et al., 2011). Since there is a difference in the promoter regions of the genes for dihydroflavonol-4-reductase and anthocyanidin synthase in anthocyanin producing plants, the authors point out that this is the most likely explanation for the presence of betalains in most Caryophyllales species (Shimada, Otsuki, & Sakuta, 2007). Nonetheless, through combination of genetic modification and subtract feeding, anthocyanin-producing plants, presented the possibility of expressing betalains (both betacyanins and betaxanthins), namely *Solanum tuberosum* L. (potato) and *Antirrhinum majus* L. (Harris et al., 2012). As well as transformed and stable strains of *Arabidopsis thaliana* (L.) Heynh. (arabidopsis) showed the capacity of bethaxanthins production, suggested by the authors that this possibility was related to the presence of an enzyme,

possibly tyrosinase, responsible for the conversion of L-DOPA to *cyclo*-DOPA (or dopaxanthin to betacyanin) (Harris et al., 2012).

Despite the mutual occurrence of this compounds, anthocyanins and betalains, is still unknown, there are some theories developed that try to explain this fact. In one theory, the evolution of betalains is due to their fungicidal properties, more than their pigmentation characteristics (Piattelli, 1981). Therefore, the two pigments could have co-existed, complementing their most evident capabilities, however this theory remains incomplete and unproven due to the fact that no taxon has shown co-existence (Brockington et al., 2011). Some theories are based on the fact of the metabolic costs, but the true metabolic cost in the synthesis of these compounds and the lack of knowledge of their physiological role, do not allow a concrete conclusion. It is known that a lower concentration of betalains is needed to absorb the same amount of visible light, when compared to the amount of anthocyanins needed to play the same role, and this relative absorptivity efficiency may be the cause of the prevalence of betalain compounds in certain plants (Clement & Mabry, 1996). According to this point of view betalains are most cost-effective in terms of visible light, but its known that these compounds showed ineffectiveness at absorbing in the UV spectrum, and in fact pollinators vision possesses UV light receptors (Menzel & Blakers, 1976).

This non established possible advantage of betalains biosynthesis over anthocyanins is not yet clarified, as well as the absence of these pigments in anthocyanin-producing species, however, it is believed that this mutual exclusivity is an indicator that pigmentation by betalains replaces that of anthocyanins and vice versa (Mabry, 1964).

3.5 Betalains bioactivities and health benefits

In this section, it will address one of the most promising subjects regarding betalains, concerning their bioactive potential. With the introduction and application of these molecules in increasingly diverse fields of study, it is essential to understand all the biological mechanisms of action associated with this type of compounds. Rahimi, Abedimanesh, Mesbah-Namin, & Ostadrahimi (2019) made an in-depth review about the effects of acute and chronic consumption of betalains, as also the *in vivo* and *in vitro* tests regarding the bioavailability and bioaccessibility of betanin and indicaxanthin.

The biological capacity of betalains relies mainly on the antiradical effects of betalamic acid (base structural unit), however further studies with purified compounds are needed

(Gandía-Herrero et al., 2016), as also a comprehensive study on the biochemical properties, biosynthesis process and roles of betalains in response to environmental stress, as performed by Li, Meng, Zhu, and Li (2019).

Inside the class of betalains, the compound indicaxanthin is one of the most promising one, with well-kwon therapeutically effects, summarized by Allegra et al. (2019), such as anti-inflammatory, neuro-modulatory, and antitumor. Biochemical and molecular modelling investigations are also being conducted specifically for indicaxanthin, to identify the pharmacological target, such as the proteins binding site (Allegra et al., 2019; Tutone, Virzì, & Almerico, 2018).

Although there is no EFSA official scientific opinion about betalains bioactive properties, in the health area there are several patents that provide the industry with a natural and eco-sustainable response for the treatment, prevention, regulation and control of hepatopathies (Radillo et al., 2014), treatment of osteoarthritis, acne, allergic conditions, sinusitis, and contact dermatitis (Pietrzkowski, 2015), treatment against pre-diabetes and other type of diabetes (Patent No. WO 2013/058643 Al, 2013), and for improving hyperlipidemia and hypercholesterolemia (Patent No. US 8,455,017 B2, 2013) with betalain-enriched natural extract.

Given the fact that there is so much information compiled in review articles in which they make an in-depth study on the bioactivities of betalains, only the last 10 years were aproach as described in **Table 4.**

Table 4 Summary of the main bioactivity studies in betalains.

Extract/Isolated compound	Plant	Part of the plant studied	Origin	Type of extraction/preparation	Bioactivity	Main results	Reference
Betalains rich extract	*B. vulgaris*	Root	Tunisia	Methanol (100%) and juice	Antioxidant, anticoagulant and genotoxic activities	Did not present genotoxicity or cytotoxic effects	(Edziri et al., 2019)
	B. vulgaris and *O. stricta*	Pulp	Iran	Betalains-rich supplement of red beetroot and a betacyanins-rich supplement of *O. stricta*	Anti-inflammatory	Elevation in SIRT1 levels and decrease in the expression levels of LOX1 and plasma concentration of high sensitive C Reactive Protein (hs-CRP) in both supplementations.	(Rahimi, Mesbah-Namin, Ostadrahimi, Separham, & Asghari Jafarabadi, 2019)
	B. vulgaris ssp. esculenta var. rubra	Root	Hungary	Vegetable table beet	Anti-cancer	Influences redox homeostasis and transmethylation in tumorous anaemia	(Blázovics & Sárdi, 2018)
	B. vulgaris subsp. vulgaris	Roots (peel and pulp)	Poland	15% methanol with 0.05% formic acid and non-fermented juices	Antihypertensive	The peels and the whole root presented the highest ACE inhibitory activity	(Sawicki et al., 2019)
	H. costaricensis, *H. undatus*, and *H. megalanthus*	Pulp	Israel	100% Methanol and 100% water	Antioxidant and cytotoxic	Samples with higher content of betalains presented also higher antioxidant and cytotoxic effects.	(Paško et al., 2021)
	H. polyrhizus	Seedlings, leaves, inflorescences, and stem	Hong Kong	Water (100%)	Diet-induced obesity, liver steatosis and insulin resistance	Reduced HFD-induced body weight gain and visceral obesity and improved hepatic steatosis, adipose hypertrophy, and insulin resistance in mice	(Song et al., 2016)
	H. polyrhizus	Fruits (peel and pulp)	Brazil	Hexane (100% for non-polar compounds) + ethanol/water solution (70:30, *v/v*, for polar compounds)	Anxiolytic-like effect	Significant anxiolytic activity, similar to the effect of diazepam.	(Lira et al., 2020)
	M. geometrizans	Peel, pulp, and whole fruits	Mexico	Methanol:water (1:1, v:v)	Antidiabetic and anti-inflammatory	Anti-hyperglycemic activity was higher in peel and pulp tissues. An 83% inhibition of hyaluronidase showed high anti-inflammatory activity.	(Montiel-Sánchez, García-Cayuela, Gómez-Maqueo, García, & Cano, 2020)
	O. dillenii	Fruit (peel and pulp)	Taiwan	Methanol (100%)	Antioxidant	Induced low-density lipoprotein peroxidation	(Chang, Hsieh, & Yen, 2008)
	O. ficus-indica	Purple-skinned pears	Mexico	High hydrostatic pressure extraction and re-dissolved in methanol:water (1:1; *v/v*)	Anti-inflammatory and antioxidant	The method of extraction used increased the extractability of phenolic and betalain compounds, leading to the increasing of the *in vitro* antioxidant and anti-inflammatory activities.	(Gómez-Maqueo, García-Cayuela, Welti-Chanes, & Cano, 2019)

	O. littoralis	Cladodes and fruits	Egypt	Organic extracts (benzene, diethyl ether, chloroform, ethyl acetate, ethyl alcohol 96%, ethyl alcohol 70%, and water) and juice preparations	Antidiabetic, antimicrobial and cytotoxic	Antimicrobial activity against some strains of bacteria and fungi. The ethanolic extract of the fruit presented strong Alpha-glucosidase inhibition and anti-diabetic activities.	(Abd El-Moaty, 2020)
	O. oligacantha var. Ulapa	Pear	Mexico	Encapsulated extract within double emulsions (water-in-oil-in-water)	Antioxidant and antidiabetic	The inhibitory activity of α-amylase and α-glucosidase was higher in microencapsulated extracts.	(Medina-Pérez et al., 2020)
Betanin			Japan	Betanin-loaded nanoliposomes	Anti-diabetic	Liposomal encapsulation improved significantly the stability of betanin and its antioxidant activity	(Amjadi, Mesgari Abbasi, Shokouhi, Ghorbani, & Hamishehkar, 2019)
			China	Dextrin (100%)	Prevention of platelet associated thromboembolic CVDs.	Inhibition thrombin-induced platelet aggregation and granule secretion without affecting mice tail bleeding time.	(Song et al., 2019)
			Japan	Dissolved in water (100%)	Anti-inflammatory and antioxidant	Protective effect against paraquat-induced acute kidney damage.	(D. Tan, Wang, Bai, Yang, & Han, 2015)
	O. robusta and *O. streptacantha*	Fruits	Mexico	Juice	Acetaminophen-induced acute liver failure	The higher concentration of betacyanins in *O. robusta* led to a higher effectiveness. Opuntia extracts were at least as potent as N-acetylcysteine (NAC) in the protection against APAP-induced hepatotoxicity.	(González-Ponce et al., 2020)
Betacyanins and betaxanthins individualy purified extracts	*Rivina humilis* L.	Berries (red variety)	India	Water, Methanol, and Methanol:water (acidified with 50 mmol/L ascorbic acid)	Antioxidant and cytotoxic	Purified betacyanins and betaxanthins revealed higher antioxidant capacity than the controls. Only betaxanthins presented cytotoxic effects	(Khan, Sri Harsha, Giridhar, & Ravishankar, 2012a)
Betanin and dopaxanthin, and synthetic analagues	*B. vulgaris*/*Lampranthus productus*	Roots/Flowers	Spain	Water (100%)	Anti-inflammatory	Phenethylamine-betaxanthinpresented the highest inactivation effect of cyclooxygenase (32% reduction), while the natural betanidin and a betalain analogue presented the highest capacity to inactivate lipoxygenase	(Vidal, López-Nicolás, Gandía-Herrero, & García-Carmona, 2014)
Bioactive extracts, gomphrenins II and III (and cis-isomers), and sinapoyl-gomphrenin I (and its diastereomers)	*G. globosa*	Flowers		20% aqueous solution of acetone (v/v), saturated ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and 10% (w/w) of ascorbic acid.	Antimicrobial	Acylated betacyanins presented higher antimicrobial activity than non-acylated gomphrenin I/isogomphrenin I.	(Spórna-Kucab et al., 2018)

Indicaxanthin	*O. ficus-indica*	Fruits (yellow cultivar)	Italy	Methanol (100%)	Anti-inflammatory	Proved to inhibits the proliferation highly metastatic and invasive A375 cells, apoptosis induction, and cell invasiveness reduction. Also, reduced tumor development when orally administered to mice.	(Allegra et al., 2018)

As it is possible to observe the majority of the studies is performed with betalains-enriched extracts, which in an industrial point a view allows a faster and economical process as it does not depend on purification steps. Regarding this, Gómez-Maqueo, García-Cayuela, Welti-Chanes, and Cano (2019), studied the influence of high hydrostatic pressure extraction methodology in the anti-inflammatory and antioxidant capacity of *O. ficus-indica* purple-skinned pears, reaching to the conclusion that a higher bioaccessibility of the bioactive compounds (betalains enriched extract) is achieved after processing, and consequently it was observed an increasing of the *in vitro* bioactivities.

Also, stabilization procedures of microencapsulation (subject thoroughly discussed in sub-section 11.5) are also being used to increase the bioactive potential of enriched betalain extracts, as performed by Medina-Pérez et al. (2020) with encapsulated extract of *Opuntia oligacantha* var. Ulapa pears within double emulsions (water-in-oil-in-water), in which 60-80% of the extract maintains is viability during longer periods of time, and consequently is antioxidant and antidiabetic activities.

Betalains-enriched extracts obtained from *H. polyrhizus* fruit peel and pulp are also being studied for its anxiolytic-like effects, presenting similar results to diazepam (a powerful chemical anti-anxiolytic), which opens the possibility for the medical community to have a plant based-alternative for anxiolytic therapy (Lira et al., 2020). Along with this, a human clinical trial was performed by inserting in the daily diet of patients undergoing chemotherapy, a vegetable table containing betalain-enriched extracts from the roots of *Beta vulgaris* ssp. *esculenta* var. *rubra*, iron, and folic acid, verifying that this supplement influences redox homeostasis and transmethylation in tumorous anaemia (Blázovics & Sárdi, 2018).

Apart from the betalain-enriched extract, many studies are also being performed with isolated or purified compounds, with the view to understand the biochemical mechanisms underlying the therapeutic effects observed. Commercial isolated betanin is one of the most common compounds studied for the past 10 years, having been proved that betanin-loaded nanoliposomes can improve the stability of the betanin and increase the therapeutic effect of positively regulation of hyperglycemia, hyperlipidemia and oxidative stress, with lesser damages to kidney, liver and pancreas, and thus with higher anti-diabetic activity (Amjadi et al., 2019). The works performed by Song et al. (2019) and Tan, Wang, Bai, Yang, & Han, (2015) with commercial purified betanin, have also proven the capacity to inhibit thrombin-induced platelet aggregation as also the protective effect against paraquat-induced acute kidney damage, respectively. The work performed

by González-Ponce et al. (2020) with purified betanin from the fruit juice of *O. robusta* and *O. streptacantha*, proved that a higher concentration of this compound led a higher biological effectiveness in reducing the biochemical, molecular and histological markers of liver (*in vivo*) and hepatocyte (*in vitro*) injury, showing similar results as N-acetylcysteine (NAC) in the protection against APAP-induced hepatotoxicity, and thus proving its potential for the prevention of acetaminophen-induced acute liver failure.

The studies using isolated or purified compounds encompass a wide variety of biological studies (**Table 4**), highlighting the work performed by Spórna-Kucab et al. (2018) with *G. globosa* flowers, from where were extracted and purified the compounds gomphrenins II and III (and cis-isomers), and the compounds sinapoyl-gomphrenin I (and its diastereomers). This study, besides proving the high antimicrobial effect of these compounds, it also proved the existence of a synergetic effect between the acylated moieties and the pigment chromophoric, resulting in a higher antimicrobial activity in acylated betacyanins with phenolic compounds. The synergistic effects between betalain and other compounds is still an area to be explored and developed.

Indicaxanthin is another betalain compound with high therapeutic value, as previously stated in the review papers by Allegra et al. (2019) and Tutone, Virzì, and Almerico (2018). The anti-inflammatory effect of this compound raises a great deal of attention in academia, since it has been proved to inhibit the proliferation of highly metastatic and invasive A375 cells, apoptosis induction, and cell invasiveness reduction, but also reduced tumour development in mice (Allegra et al., 2018). The biochemical mechanism involved is the inhibition of NF-κB pathway as predominant, and considering the resistance of melanoma to the current artificially-created chemical therapeutic approach, indicaxanthin represents a novel, safer, and effective natural substitute for the control development of human melanoma cell proliferation *in vitro* and for the impair of tumour progression *in vivo* (Allegra et al., 2018).

4 An overview of extraction, separation and processing methods of betalains

Among the most commonly used analytical techniques to identify and/or quantify food colourants, are spectrometry, thin-layer chromatography, adsorptive voltammetry and differential pulse chromatography, which have been progressively substituted by capillary electrophoresis and ion chromatography, once revealed to be less time-consuming to pretreat samples and applicable to complex colourant mixtures (Chen, Mou,

Hou, Riviello, & Ni, 1998; Huang et al., 2002; Karanikolopoulos, Gerakis, Papadopoulou, & Mastrantoni, 2015). But, with the progressive use of those techniques sensitivity problems are still frequent and the robustness of these methods is still significantly affected and limited. Thus, more robust and high resolute, selective and sensitive analytical techniques are increasingly preferred, namely high-performance reversed-phase liquid chromatography and ion-pair liquid chromatography coupled with UV or diode-array detectors (Karanikolopoulos et al., 2015; Reinholds, Bartkevics, Silvis, van Ruth, & Esslinger, 2015).

4.1 Betalains extraction methods

More efficient techniques have been applied by food industries to optimize the extraction of naturally-derived pigments, to ensure their stability, to avoid colour loses of those pigments and the appearance of unpleasant characteristics (Cerón, Higuita, & Cardona, 2012; Dias et al., 2015; Ray et al., 2016; D. T. Santos, Albuquerque, & Meireles, 2011; Sivakumar, Vijaeeswarri, & Anna, 2011; Z. Yang & Zhai, 2010). In addition, modern food processing and packaging techniques have been also developed, not only to improve the shelf life of natural products and to reduce the subsequent addition of synthetic additives, but also to contribute for the production of safer products, without affecting their attractiveness by consumers and at the same time to improve their subsequent demands (Buchweitz, Brauch, Carle, & Kammerer, 2013; Chung, Rojanasasithara, Mutilangi, & McClements, 2015; Fernandez, Torres-Giner, & Lagaron, 2009; Komolprasert, Diel, & Sadler, 2006).

Like most compounds of natural origin, betalains can be recovered by simple solid-liquid extraction techniques. However, in the last few years a lot has been done to optimize the recovery of these pigments from different matrices using different methodologies, increasing their yield in a more efficient and sustainable way. In the **Table 5** we can see the multiplicity of natural matrices and extraction technologies that can be used to recover these pigments, Some authors like Fathordoobady et al. (2016) assessed the suitability of dynamic maceration, as well as supercritical fluid extraction in red pitaya (*H. polyrhizus*) peel and fresh (common waste of juice manufacturing). Its well known the high amount of this compounds in the flesh of these fruits, but the peels also revealed very interesting amounts of these pigments. The compounds were efficiently recovered by SFE-CO_2 with 10% ethanol as co-solvent, in a process carried out at 25 MPa for 90 min at 50 ºC.

However, the DM resulted in higher betacyanin yields from both peel and flesh samples using 50% and 70% ethanol for 20 min processing, respectively, in a process carried out at room temperature. Whem used 100% water as the extraction solvent, the results in betacyanin contents were very satisfying, but the problem of the mucilaginous effect of the soluble pectins caused problems of the samples in the filtration process. In the flesh samples, different water concentration of water gave different filtrations times and it was noticeable a decreased on the yield recovered. The recovered extracts showed no significant colour differences, and as for the compounds, identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS), Betanin, isobetanin, phyllocactin, butyrylbetanin, isophyllocactin, and isobutyrylbetanin were the main betacyanins identified.

Table 5 Extraction conditions used in the recovery of betalains from different plant materials.

Plant material	Extraction system and conditions						Betalain yield	Reference
	System		*T* (°C)	*t* (min)	*S/L* (g/L)	Solvent		
Red pitaya (*H. polyrhizus*) peel	DM	300 rpm	rt	20	100	50% Ethanol	0.284 g betacyanins/L	(Fathordoobady et al., 2016a)
Red pitaya (*H. polyrhizus*) peel	DM	200 rpm	50	120	18.2	Water	0.187 g betacyanins/kg extract	(Ramli, Ismail, & Rahmat, 2014)
Red pitaya (*H. polyrhizus*) peel	MAE	100 W	35	8	-	Water	0.009 g betacyanins/L	(Thirugnanasambandham & Sivakumar, 2017)
Red pitaya (*H. polyrhizus*) peel	UAE	50 kHz	25	30	18.2	Water	0.176 g betacyanins/kg extract	(Ramli et al., 2014)
Red pitaya (*H. polyrhizus*) peel	SFE	25 MPa	50	90	100	CO_2, 10% ethanol as co-solvent	0.246 g betacyanins/L	(Fathordoobady et al., 2016a)
Red pitaya (*H. polyrhizus*) flesh	DM	300 rpm	rt	20	100	70% Ethanol	1.203 g betacyanins/L	(Fathordoobady et al., 2016a)
Red pitaya (*H. polyrhizus*) flesh	DM	200 rpm	50	120	40	Water	0.828 g betacyanins/kg extract	(Ramli et al., 2014)
Red pitaya (*H. polyrhizus*) flesh	UAE	50 kHz	25	30	40	Water	0.713 g betacyanins/kg extract	(Ramli et al., 2014)
Red pitaya (*H. polyrhizus*) flesh	SFE	25 MPa	50	90	100	CO_2, 10% ethanol as co-solvent	0.913 g betacyanins/L	(Fathordoobady et al., 2016a)
Red-purple pitaya (*H. polyrhizus*) peel	LBEF	3 V	25	15	100	Ethanol (pre-mixture with K_2HPO_4)	0.081 g betacyanins/kg extract	(Leong et al., 2019)
Red-purple pitaya (*H. polyrhizus*) flesh	LBEF	3 V	25	15	100	Ethanol (pre-mixture with K_2HPO_4)	0.157 g betacyanins/kg extract	(Leong et al., 2019)
White-fleshed red pitaya (*H. undatus*) peel	MAE	600 W	49	5	6.7	Water	1.51 g betacyanins/kg extract	(Ferreres et al., 2017)
White-fleshed red pitaya (*H. undatus*) peel	UAE	100 W	60	20	40	60% Ethanol	1.60 g betacyanins/kg extract	(Bhagya Raj & Dash, 2020)
Yellow pitaya (*H. megalanthus*) peel	MAE	600 W	49	5	6.7	Water	-	(Ferreres et al., 2017)
Cactus pear (*O. ficus-indica*) fruit	M	-	42	115	24	Water, pH 6.9 adjusted with citric acid	0.415 g betalains/kg extract	(Prakash Maran, Manikandan, & Mekala, 2013)
Cactus pear (*O. ficus-indica*) fruit	M	-	40	115	28.8	Water	0.134 g betacyanins/kg extract 0.243 g betaxanthins/kg extract	(Prakash Maran & Manikandan, 2012)
Cactus pear (*O. ficus-indica* var. gialla) peel	DM	150 rpm	25	60+60	40	80% ethanol	1.25 g betacyanins/kg extract	(Melgar et al., 2017a)
Cactus pear (*O. ficus-indica* var. sanguigna) peel	DM	150 rpm	25	60+60	40	80% ethanol	3.97 g betacyanins/kg extract	(Melgar et al., 2017a)
Prickly pear (*O. engelmannii*) peel	DM	150 rpm	25	60+60	40	80% ethanol	19.4 g betacyanins/kg extract	(Melgar et al., 2017a)
Prickly pear (*O. engelmannii*) peel	MAE	400 W	36.6	1.4	5	Water, pH 7 adjusted with McIlvaine buffer solution	132.9 g betalains/kg extract	(Melgar et al., 2019a)
Prickly pear (*O. engelmannii*) peel	UAE	2 kHz, 200 rpm	30	2.5	5	34.6% methanol, pH 7 adjusted with McIlvaine buffer solution	201.6 g betalains/kg extract	(Melgar et al., 2019a)

Prickly pear (*O. joconostle*) pulp	M	-	15	10	20	20% Methanol, pH 5 adjusted with 1% citric acid	0.92 g/kg fw	(Sanchez-Gonzalez, Jaime-Fonseca, San Martin-Martinez, & Zepeda, 2013)
Beetroot (*B. vulgaris*)	M	-	60	84	30	Water	0.099 g betalamic acid/kg fw 0.163 g betaxanthin/kg fw 0.309 g betacyanin/kg fw	(Swamy, Sangamithra, & Chandrasekar, 2014)
Beetroot (*B. vulgaris*)	MAE	400 W	23	2.3-2.7	4	50% Ethanol	1.25 g betaxanthins/kg dw	(Cardoso-Ugarte, Sosa-Morales, Ballard, Liceaga, & San Martín-González, 2014a)
Beetroot (*B. vulgaris*)	MAE	400 W	23	1.5-2	4	50% Ethanol acidified with ascorbic acid (0.040 mol/L)	1.88 g betanines/kg dw	(Cardoso-Ugarte et al., 2014a)
Beetroot (*B. vulgaris*) pomace	DM	Stirring	50	10	66.7	Water, pH 2.5	0.018 g betacyanins/L 0.015 g betaxanthins/L	(Kushwaha, Kumar, Vyas, & Kaur, 2018)
Beetroot (*B. vulgaris*) leaves	M	60 rpm	61	40	100	80% ethanol, pH 6 adjusted with HCL	0.78 g betacyanins/kg dw	(Bengardino, Fernandez, Nutter, Jagus, & Agüero, 2019)
B. glabra flower	UAE	88 W (20 kHz)	55	37	58.8	Water	1.72 g betacyanins/kg 5.78 g betaxanthins/kg	(Prakash Maran, Priya, & Nivetha, 2015)
Red amaranth (*A. cruentus*) leafy part	DM	100 rpm	70	5	62.5	Water	47.6 g betacyanins/kg dw 13.2 g betaxanthin/kg dw 13.4 g betalamic acid/kg dw	(Ahmed, Ramachandraiah, Jiang, & Eun, 2020a)
Red amaranth (*A. cruentus*) leafy part	UAE	35 kHz	70	5	62.5	Water	53.3 g betacyanins/kg dw 15.2 g betaxanthin/kg dw 17.9 g betalamic acid/kg dw	(Ahmed et al., 2020a)
G. globosa L.	DM	500 rpm	25	165	5	Water	45.0 g betacyanins/kg extract	(Roriz, Barros, Prieto, Morales, & Ferreira, 2017a)
G. globosa L.	MAE	600 rpm	60	8	5	Water	39.6 g betacyanins/kg extract	(Roriz, Barros, Prieto, Barreiro, et al., 2017a)
G. globosa L.	UAE	500 W (20 kHz)	rt	22	5	Water	46.9 g betacyanins/kg extract	(Roriz, Barros, Prieto, Barreiro, et al., 2017a)

T: temperature; *t*: time; *S*/*L*: solid/liquid ratio; rt: room temperature; DM: dynamic maceration; MAE: microwave-assisted extraction; UAE: ultrasound-assisted extraction; SFE: supercritical fluid extraction; LBEF: liquid biphasic electric flotation; M: maceration; fw: fresh weight; dw: dry weight.

Being a water-soluble compound, some studies reported water as the most suitable solvent for extracting them. In their study Thirugnanasambandham and Sivakumar (2017) resorted to a response surface methodology (RSM) applied to a microwave assisted extraction to optimized the recovering of betalain compounds from peels of pitaya, accordingly, the increase in temperature and processing time promoted the extraction. In another study, Ramli et al. (2014) concluded that DM leads to higher betacyanin levels than UAE from red pitaya peel and flesh, although this first method required a longer extraction time (120 min vs. 30 min). It is also interesting to note that a greater solvent volume was applied in both extractions made with the peel. The highest levels of these pigments were measured in the red pitaya fresh, according to the results of Fathordoobady et al. (2016). Leong et al. (2019) applied a liquid biphasic electric flotation (LBEF) system in the extraction of betacyanins from red-purple pitaya (*H. polyrhizus*) peel and flesh by-products. The extraction was promoted as the processing time increased from 5 to 15 min (this being the optimum time value), and then decreased for times up to 25 min. The MAE of betacyanins from white-fleshed red pitaya (*H. undatus*) and yellow pitaya (*H. megalanthus*) peel was optimized by Ferreres et al. (2017). The authors implement a three-level Box-Behnken design combining the effects of the independent variables time (5–65 min), temperature (25–75 ºC), and solid/liquid ratio (6.7–20 g/L). Some problems were also described by these authors regarding the difficulty in filtrating the recover extracts due to the presence of pectins. By the HPLC-DAD-ESI/MS analysis it was possible to identify in these extracts Betanin, isobetanin, phyllocactin, and isophyllocactin. Bhagya Raj & Dash (2020) and Ferreres et al. (2017), for the same extraction technique, namely MAE, with different scales on the parameters, achieved comparable results. Another example of an interesting source of betaxanthins and batalains are Prickly pears or cactus pears (*Opuntia* spp.). it is possible to observe the conditions applied by some authors in different extraction methodologies on. For Prakash Maran et al. (2013) time, temperature, and solid/liquid ratio have a significant effect on the extraction of betalain from cactus pear (*O. ficus-indica*) fruit. Similar results were obtained by Prakash Maran and Manikandan (2012). The pigments were better extracted with the consequent increase in the extraction time and temperature, especially betaxanthins. Melgar et al. (2017) submitted lyophilized orange-red (*O. ficus-indica* var. gialla) and red-violet (*O. ficus-indica* var. sanguigna) cactus pear peels and prickly pear (*Opuntia engelmannii*) fruit peel to a conventional ethanol/water extraction (80:20, *v/v*) for 1 h under continuous stirring at room temperature, followed by plus 1 h re-extraction.

The HPLC-DAD–ESI/MS^n analysis allowed identifying two betaxanthins in both *O. ficus-indica* varieties, namely indicaxanthin isomer I and II, as also five betacyanins, betanidin-5-*O*-β-sophoroside, betanidin-5-*O*-β-glucoside (betanin), isobetanin, gomphrenin I, and betanidin, which were quantified in higher amounts in the *O. engelmannii* peel extract.

Since the conventional extraction method used in this characterization study was time consuming (120 min) and used 80% ethanol, the authors carried out a further study (Melgar et al., 2019a) to optimize the extraction of betalains from the most promising matrix (i.e., *O. engelmannii*). The effects of the extraction time, temperature, methanol concentration, and solid/liquid ratio were investigated in a five-level central composite design implemented for both MAE (400 W) and UAE (2 kHz, 200 rpm) using response surface metrology (RSM). Overall, both processes were highly effective in terms of processing time (up to 2.5 min).

Sanchez-Gonzalez et al. (2013) tested two alcohol/water systems to extract betalains from the vacuum-dried prickly pear (*O. joconostle*) pulp, in a process combining different extraction times (10–30 min) and temperatures (5–30 ºC). The HPLC analysis allowed identifying betalain, betanidin, and isobetalain in the extracts, and the chromatographic method was pointed out a very useful tool for separation and analysis of betacyanins and betaxanthins, due to their similar characteristics. The extraction method was also used to recover pigment from beetroot and cactus pear (*O. ficus-indica*) fruit pulp to compare the pigments stability (evaluated by the UV–Vis spectra). In general, the compounds were affected by an increase in temperature (up to 80 ºC) and heating time (um to 60 min), with *O. joconostle* pigments being more stable than those extracted from beetroot, manly at pH 5. This difference was justified by the higher betaxanthins content in beetroot, which are more heat sensitive than betacyanins. In fact, betalains can lose their colour at high temperatures, and also at pH values above 6, due to the hydrolysis of the aldimine bonds, leading to the formation of the bright yellow betalamic acid and the colourless cyclo-Dopa-*O*-β-glucoside (Schwartz & von Elbe, 1983).

As shown in **Table 5**, there are several studies focusing on the extraction of betalains from beet root and leaves. Swamy et al. (2014) optimized an aqueous maceration to maximize the recovery of betalains from peeled beetroot using RSM, considering different levels of temperature (40–70 ºC), processing time (30–90 min), and solid/liquid ratio (10–30 g/L). The total contents of betalamic acid, betaxanthins, and betacyanins were strongly affected by the extraction variables. In the same year, the MAE was used

to extract betalains from diced beetroot (Cardoso-Ugarte et al., 2014a) in a process involving different levels of extraction time, power, and duty cycle. A difference between the times at which the higher levels of betanines and betaxanthins was reported. It was also found that the solvent acidification with ascorbic acid (0.040 mol/L) and the implementation of a two-stage MAE process with a cooling period in-between and after processing can led to an improvement in the extraction yield. Overall, MAE had a remarkable effect in the extraction when compared to conventional extractions made by the authors. Microwave radiation in known to promote the diffusion of solutes into the solvent during extraction; however, these can be degraded due a faster heating rate and pigment release during MAE as compared to conventional methods.

The suitability of pulsed electric fields (PEF) as a non-thermal pre-treatment for extracting betalains from beetroot was investigated by Nowacka et al. (2019). Different electric field strengths (4.38 and 6.25 kV/cm), pulse number (10–30), and energy input (0–12.5 kJ/kg) were tested. The highest betanin and vulgaxanthin yields were obtained when beetroot cylinders were pre-treated with 20 pulses of electric field at a low intensity (4.38 kV/cm). Beetroot pomace is a by-product with no commercial value resulting from the juice extraction that needs to be economically valorised. Kushwaha et al. (2018) showed that this waste material still contains valuable pigments, whose recovery can be maximized by processing the sample for 10 min at 50 ºC, using 66.7 g/L of distilled water at pH 2.5. Beet leaves constitute another by-product underexploited by the industry. Despite being edible, these are generally discarded as a waste. Bengardino et al. (2019) demonstrated that 78 mg betacyanins/100 g dw can be recovered when shaking the samples at 60 rpm in a thermostatic bath for 40 min at 61 ºC, with 80% ethanol at pH 6. The authors also reported that processing times over 100 min lead to pigment degradation.

The UAE of natural pigments from *B. glabra* flowers has also been the target of research using an ultrasonic probe system. The optimization carried out by Prakash Maran et al. (2015) revealed that the pigments recovery from this plant matrix is affected by different factors, with 37 min processing at 55 ºC, with 58.8 g/L water and 88 W (20 kHz) of ultrasonic power being the optimal conditions.

Red amaranth (*A. cruentus*) is an underutilized plant, Ahmed et al. (2020) reported that the recovery of betacyanins, betaxanthin, and betalamic acid from red amaranth leafy parts is more effective when using UAE in ultrasonic bath that a DM in a shaking water bath. For UAE, the highest yields were achieved at 70 ºC (the highest tested temperature), while for DM the temperature had no major effects. In addition, a Fourier-transformed

infrared spectroscopy (FTIR) analysis showed that both methods induced a similar effect on the extracts structure. A correlation between betalains and antioxidant activity through $ABTS^{+}$ scavenging activity was also found.

Globe amaranth (*Gomphrena globosa* L.) is another underexplored source of betalains. Roriz, Barros, Prieto, Morales, et al. (2017) identified gomphrenin and isogomphrenin II and III in globe amaranth flowers, and ~24 mg/g dw of betacyanins were recovered when processing the sample for 165 min at 25 ºC and at a solid/liquid ratio of 5 g/L water (**Table 5**). In order to replace this conventional maceration with a more sustainable and efficient method, MAE and UAE processes were implemented in a subsequent study (Roriz, Barros, Prieto, Barreiro, et al., 2017a). These non-conventional techniques were highlighted as time-saving approaches, and UAE (22 min sonication at 500 W) was pointed out as the best method to maximize the aqueous extraction up to 46.9 mg betacyanins/g extract. For MAE (8 min irradiation at 60 °C), the betacyanins yield (39.6 mg/g extract) was lower than those of UAE and conventional maceration.

4.2 Novel separation methods for betalains

The free sugars present in natural extracts can lead to fermentation and caramelization phenomena during food processing at high temperatures, and accelerate the degradation of betalains. Therefore, the removal of these hydrophilic molecules is desired to facilitate the application of betalain-rich extract in food formulations, and can be achieved using simple aqueous two-phase extraction systems (**Table 6**). Chethana et al. (2007) were the first authors to apply this partition method in the separation of betalains from beetroot extract. Using this system, betalains (70–75%) and sugars (80–90%) were partitioned in the upper and bottom phases, respectively. After removing polyethylene glycol from the aqueous system by means of organic-aqueous extraction, a 3.4-fold increase in betalain concentration was observed. Years later, Sandate-Flores et al. (2020) produced a low-sugar betaxanthin ingredient from a crude yellow pitaya (*S. pruinosus*) extract using a novel aqueous two-phase system. Polyethylene glycol–phosphates were reported to be more suitable than UCON (a copolymer with 50% ethylene oxide and 50% propylene oxide)-salts for fractionation the pigments. The upper phase of the biphasic system yielded up to 52.3% betaxanthins and up to 2.8% sugars. Moreover, since polyethylene glycol is a food-grade solvent, the resulting colouring ingredients can be applied in food and cosmetic products.

Table 6 Novel methods used in the separation/isolation of betalains from plant matrices.

Betalain source	Separation method	Main achievements of the applied separation method	Reference
Beetroot extract	Aqueous two-phase system (polyethylene glycol 6000 and ammonium sulphate were mixed with the extract, followed by phase separation for 4-5 h)	Betalains (70–75%) and sugars (80–90%) were partitioned in the upper and bottom phases of the biphasic system, respectively. A 3.4-fold increase in betalain levels was obtained.	(Chethana et al., 2007)
Yellow pitaya (*S. pruinosus*)	Aqueous two-phase system (polyethylene glycol–phosphates were mixed with the extract for 10 min, followed by phase separation for 10 min)	The upper phase of the biphasic system yielded up to 52.3% betaxanthins and up to 2.8% sugars.	(Sandate-Flores et al., 2020)
Microfiltered cooked beetroot juice	Membrane separation (microfiltration using loose reverse osmosis and reverse osmosis polymeric membranes of 1 kD, at 25 bar and 35 ºC)	The process retained most of the pigments (~98%) in the extract and was efficient in removing up to 96% salt and 47% other dissolved solids. The betalains content was increased in up to 46% and, after three diafiltrations, more than 95% of nitrates were removed.	(Mereddy, Chan, Fanning, Nirmal, & Sultanbawa, 2017)
Beetroot juice	Membrane separation (microfiltration using a hydrophilic mixed cellulose ester membrane of 0.45 μm pore size at 0.1, 0.5 and 1 bar at 4.4 and 6 mL/s)	Microfiltration reduced the beetroot juice turbidity, colour, and betacyanin and betaxanthin contents. Standard blocking and cake resistance were the main technical hitches reported.	(Amirasgari & Mirsaeedghazi, 2015)
Beetroot stalks	Membrane separation (tubular ceramic membranes of 0.05 mm nominal pore size for microfiltration and of 20 kDa molecular weight cut-off for ultrafiltration)	The final permeates reached 99.5% reduction in peroxidase activity and more than 99.9% reduction in turbidity, while its colour was intense and luminous. The betanin content in the final permeate was significantly lower. Some technical hitches were reported.	(C. D. Dos Santos, Scherer, Cassini, Marczak, & Tessaro, 2016)
Cactus pear (*O. ficus-indica fruits*) juice	Membrane separation (after filtration in a three-stage column evaporator, the juice was microfiltered through a 0.2 μm ceramic membrane)	The process retention 71–83% of the pigments and preserved the initial colour of the juices after reconstitution. Non-enzymatic browning and formation of 5-hydroxymethylfurfural were found after concentration at the pilot plant-scale.	(Moßhammer, Stintzing, & Carle, 2006)
Cactus pear (*O. dillenii*) juice	Membrane separation (integrated process employing microfiltration ceramic membranes (0.1–0.2 μm, 1.8–3.3 bar) and ultra/nanofiltration organic membranes (0.2–4.0 kDa, 5–30 bar)	Insoluble solids were removed by microfiltration after enzymatic liquefaction. Then, ultra/nanofiltrations were applied to concentrate and purify betacyanins, allowing either the concentration of all betacyanins, their separation from the soluble solids, or even some fractionation between them.	(Tamba, Servent, Mertz, Cissé, & Dornier, 2019)
Processed beetroot juice	Countercurrent chromatography (high performance CCC)	Highly polar solvent systems (containing ammonium sulphate salt and ion-pair solvent systems) were useful for the separation of two mixtures of decarboxylated and dehydrogenated betacyanins. Ion-pair solvent systems with heptafluorobutyric acid were more effective than those with trifluoroacetic acid.	(Spórna-Kucab, Ignatova, Garrard, & Wybraniec, 2013)
I. herbstii leaf extract	Countercurrent chromatography (high-speed CCC)	The effective separation of very polar and somewhat unstable betacyanins was achieved, as well as the identification of low-concentrated betacyanins.	(Spórna-Kucab, Wróbel, Kumorkiewicz-Jamro, & Wybraniec, 2020)
I. lindenii leaf extract	Countercurrent chromatography (ion-pair high-speed CCC)	The affinity of betacyanins to the organic stationary phase was improved when heptafluorobutyric acid used as the ion-pair forming additive. The fractionation of the most polar compounds resulted in their good resolution.	(Jerz et al., 2014a)

Membrane technology has the potential for selective separation of betalains in an efficient and acceptable way, being considered an adequate alternative to common thermal preservation treatments for the retention of thermolabile compounds. Mereddy et al. (2017) successfully separated and concentrated betalains from beetroot juice (a quite complex food matrix) using this cold sterilisation method. A 1 kD loose reverse osmosis spiral-wound membrane retained most of the pigments (~98%) in the extract and was very efficient in removing up to 96% salt and 47% other dissolved solids. In another study, Amirasgari and Mirsaeedghazi (2015) described that the membrane processing efficiency is better at higher transmembrane pressure and feed flow rate. Applying the microfiltration reduced the beetroot juice turbidity, colour, and betacyanin and betaxanthin contents, as well as the levels of total phenolics and total soluble solids and the antioxidant activity.

Beetroot stalks constitute an agro-industrial by-product and also a source of betalains, and dos Santos et al. (2016) implemented a combined microfiltration and ultrafiltration process, the final permeate obtained with this process reached 99.5% reduction in peroxidase enzyme activity and more than 99.9% reduction in turbidity, while its colour was more intense and luminous than that of the initial extract. However, the betanin content in the final permeate was lower.

Regarding cactus pear (*O. ficus-indica*) juice, Moßhammer et al. (2006) applied cross-flow microfiltration for the non-thermal preservation of the juice, as an alternative for high-temperature short-time pasteurisation. The viability of this process was pointed out based on the pigment retention of 71–83% and the preservation of the original colour after the reconstitution of the semi-concentrated and concentrated juices. However, non-enzymatic browning and formation of 5-hydroxymethylfurfural (at levels below 2 mg/L) occurred after concentration at the pilot plant-scale. Later, Tamba et al. (2019) implemented an integrated process coupling crossflow micro and ultra or nanofiltration to separate betacyanins in cactus pear (*O. dillenii*) juice at low temperature. Ultra and nanofiltrations were then applied to concentrate and purify the betacyanins. Countercurrent chromatography (CCC) has been reported as a useful technique for the preparative isolation of natural compounds from plant extracts. Since preparative isolation of unstable betalains is often problematic due to the catalytic action of the solid stationary phase, CCC emerged as an important possibility of obtaining pure compounds from complex matrices. This technique is also useful for recovering betalain molecules normally detected at low concentrations, which would otherwise make their isolation

difficult and time consuming. Spórna-Kucab et al. (2013) described for the first time that highly polar solvent systems (containing ammonium sulphate salt and ion-pair solvent systems) are very useful for the separation of two mixtures of decarboxylated and dehydrogenated betacyanins from processed beetroot juice. The authors found that the ion-pair solvent systems with heptafluorobutyric acid were far more efficient than those with trifluoroacetic acid, as betalains created more hydrophobic structures. Some years later, the same authors (Spórna-Kucab et al., 2020) effectively separated and pre-concentrated betalains from *I. herbstii* leaf extract by high-speed CCC, using a polar solvent system composed of propan-1-ol/acetonitrile/ammonium sulphate/water (1.0:0.5:1.2:1.0, *v/v/v/v*), which was a very useful tool for further LC-MS/MS analysis of low-concentrated and somewhat unstable betacyanins. Thus, it was possible to identify 22 betacyanins, 18 of which were described for the first time in the leaves of this species and 4 (sinapoyl-gomphrenin and coumaroyl-gomphrenin and their epimers) were new for the *Iresine* genus. In another study, Jerz et al. (2014) fractionated betacyanins by ion-pair high-speed CCC from *I. lindenii* leaf extract, and also reported that the affinity of betacyanins to the organic stationary phase was strongly improved when heptafluorobutyric acid was used as the ion-pair forming additive to the solvent system. It was possible to identify 18 betacyanins/isobetacyanins of different polarities in this Amaranthaceae species.

4.3 Influence of Processing methods on betalain stability

Over the years, most accurate and reliable methods, and even increasingly specific analytical procedures have been developed and applied for a wide variety of purposes by food industries. Detection of undeclared substances, illegal ingredients and the abundance of many other additives, both allowed and prohibited, have deserved a particular interest (Bonan, Fedrizzi, Menotta, & Elisabetta, 2013; Xing et al., 2012). Furthermore, another important aspect, which has also received special attention, is the real stability of food colourants and respective stored foodstuffs.

Colour appreciation comprises one of the earliest aesthetic parameters considered by consumers during foodstuffs selection, being a direct predictive parameter that ensures good quality (Sagdic et al., 2013; Todaro et al., 2009). Visual cues present a doubtless influence on food preference, acceptability and lastly food choice. Therefore, it is clearly evident that food industries aim to provide increasingly uniform, attractive and pleasant

coloured foodstuffs, to fully satisfy consumers' expectations and current needs (Cai, Sun, & Corke, 2005; Junqueira-Goncalves et al., 2011; Otálora et al., 2015; Sagdic et al., 2013).

Several reports have confirmed the real interference that some external factors exert on the food colour stability (D. M. Jiménez-Aguilar et al., 2011; Lemos, Aliyu, & Hungerford, 2012; Zhu et al., 2015). Anthocyanin pigments are a good example of natural food colourants highly affected by those external agents; in fact, they are very unstable compounds and highly susceptible to degradation (Assous, Abdel-Hady, & Medany, 2014; D. M. Jiménez-Aguilar et al., 2011; Sagdic et al., 2013; Zhu et al., 2015). Therefore, their final colouration will vary significantly, at the same time that various degrees of susceptibility to external factors may be also observed among different species belonging to the same plant family (Sagdic et al., 2013). For example, Sagdic et al. (2013) observed that red tulip anthocyanins are less sensitive to temperature than violet tulip anthocyanins that exhibited a lower chemical stability. Jiménez-Aguilar et al. (2011) evaluated the colour stability of spray-dried blueberry, a very important source of anthocyanins, and observed a progressive increase of air outlet temperature together with a loss of total phenolics and anthocyanins and a decrease in the antioxidant activity. On the other hand, Tan et al. (2014) aiming to assess both the effects of temperature (under refrigerated and room temperature conditions) and pH (from 1.0 to 11.5) on a purple-red anthocyanin-derived extract of *Rhoeo spathacea* (Swartz) Stearn leaves, concluded that it exhibited a phenomenal colour stability under a range of pH values and temperatures. Over a period of 60 days, the authors observed a remarkable stability of anthocyanin-derived extract in acidic pH, and a complete stability when solid food (jelly) and liquid food (barley water) were used (J. B. L. Tan, Lim, & Lee, 2014). Furthermore, Munawar & Jamil. (2014), aiming to access the colour changes, anthocyanin stability and antioxidant activity of several spray dried plant pigments, observed that the most prominent antioxidant potential was obtained to those possess higher anthocyanin content, being again emphasized the negative impact of storage temperature on the final chemical stability and acceptability of products. But, even so, the authors highlighted the safety and healthy abilities of the studied natural colourant, may even be upcoming used as functional ingredient in food products (Munawar & Jamil, 2014).

Many other examples related with natural pigments stability might be highlighted but the most interesting feature that indeed deserves a particular attention is that highly specific and reliable procedures, and industrial techniques have been effectively used to overcome

the problem of poor stability of numerous food pigments, as also to identify and quantify their relative abundance in numerous foodstuffs.

As happened with other pigments, chemical stability of betalains may be largely affected by numerous factors, being this the main issue related to the application of betalains as natural colourants. The most prominent are those that directly affect its structural and chromatic features. As shown in **Figure 3**, structural and chromatic modifications on betalains are considered the main determinant factors. But it is of the utmost importance to highlight that there are not a clear division between them, because in a direct or indirect manner all of them interfere with the chemical stability of betalains. Therefore, these factors or "hurdles" must be present or applied in an intensity that does not negatively impact the product quality; otherwise, its colour will be affected, as well as its bioactivity or other attributes.

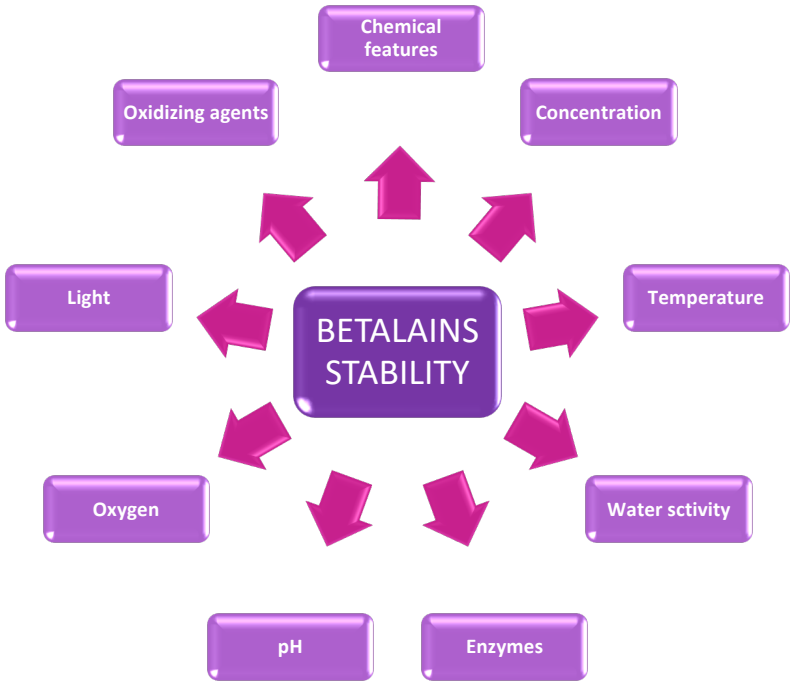


Figure 3 Factors affecting the stability of betalains

With regard to this theme, some studies already carried out demonstrate how the processing methods affect the content of betalains in food products is summarized in **Table 7**. Being the only natural betalain based colorant approved for utilization in food industry, beetroot powder was investigated by Gokhale and Lele (2014), relatively to the impact of hot air (50–120 ºC) convective drying and concluded that 50–65 ºC is the best range of temperatures for betalain retention. Additionally, a 5-month shelf-life analysis

evidenced up to 50% pigment degradation and a slight increase in antioxidant activity. In his study, Mocanu et al. (2020) compared the effects of convective and infrared drying methods on beetroot purées with lactic acid bacteria, and concluded that Infrared processing was a suitable mild drying method.

Table 7 Impact of different food processing methods on betalain contents.

Processed product	Processing method and conditions	Impact on betalain content	Reference
Beetroot powder	Hot ait (50–120 ºC) convective drying. Stage for 150 d at 27 ºC and 60% relative humidity	Lower temperatures (50–65 ºC) were better for betalain retention. A 5-month shelf-life analysis evidenced up to 50% pigment degradation.	(Gokhale & Lele, 2014)
Beetroot (fresh-cut)	Minimal processing	The greatest loss occurred in the initial stages of peeling and sanitation.	(Preczenhak, Tessmer, Berno, de Abreu Vieira, & Kluge, 2018)
Beetroot (fresh-cut)	Addition of cysteine (2, 4, 8 and 16 mM)	Cysteine doses above 4 mM maintained the betalains levels. It increased the betalain contents up to 6 d of cold storage.	(Preczenhak et al., 2019)
Beetroot juice	Thermal processing (85 ºC for 8 h)	The red-purple colour was changed to yellow-orange due to the generation of neobetanin and betanin degradation products.	(Herbach, Stintzing, & Carle, 2004)
Beetroot in cow milk	Thermal processing (70–90 ºC for up to 140 min)	The thermal degradation betalains increased with increasing processing time and temperature.	(Güneşer, 2016)
Beetroot slices	Thermal (90 ºC for 7 min) and high hydrostatic pressure (HHP; 650 MPa for 3–30 min) processing	HHP resulted in a higher betanin content, but the longer the HHP processing, the greater the degradation.	(Paciulli, Medina-Meza, Chiavaro, & Barbosa-Cánovas, 2016)
Beetroot	Boiling (80 ºC for 60, 120, 180 s), microwaving (450, 900 and 1800 W for 10, 20, and 30 s), roasting (60, 120, 180 s), and vacuuming (94% and high 99%)	Boiling and roasting reduced the betalain contents, which were higher in vacuum and microwave processed samples.	(Ravichandran et al., 2013b)
Beetroot	Boiling (for 60 min) and fermentation (23 °C for 7 days)	The betalain content was reduced in 51–61% and 61–88% by boiling and fermentation, respectively.	(Sawicki & Wiczkowski, 2018)
Solid and liquid beetroot products	Boiling (for 45 min), fermentation (7 days), and microwave-vacuuming (under industrial conditions)	A 42–70% reduction was observed in the food products. While the highest betalain contents were detected in solid samples (whole fresh roots > crunchy slices > boiled roots > fermented roots), fermented juice showed the lowest levels.	(Sawicki et al., 2019)
Beetroot juice supplemented with highbush blueberries–waste sucrose osmotic syrup	Fermentation (lactic acid)	The highest betacyanin levels were found in juices fermented by *L. brevis* ŁOCK 0944. For other strains, betacyanins decreased with the increasing amounts of osmotic syrup added to juice, which caused a dilution.	(Chwastek, Klewicka, Klewicki, & Sójka, 2016)
Red pitaya (*H. polyrhizus*) flesh extract and milk formulations (1% of 50 mg/mL betacyanins)	Thermal treatment (30–100 ºC for 5 min) and storage (10 weeks at 4 °C). Milk pasteurization (63 ºC for 30 min) and storage (7 d at 4 °C)	Treatments at 50 and 85 ºC caused a greater betalain reduction in E-162 (beetroot colorant) than in red pitaya. Cold storage induced colour changes in both red pitaya and E-162. In milk, red pitaya suffered less degradation and colour change than E-162. Both natural colorants improved the microbial stability of the formulated milk.	(Ashwini Gengatharan, Dykes, & Choo, 2016)
Red pitaya (*H. polyrhizus*) juice	Thermosonication (20–25 kHz at 190–570 W with a 3 s interval off for 5–40 min at 10–70 °C), ultrasound (475 W for 20 min at 10 ºC), and pasteurization (83 ºC for 1.5 min and 50 ºC for 20 min) treatments	The inactivation of polyphenol oxidase and the retention of polyphenols (>93%) in thermosonicated juice was observed. The pigments decompositions in thermosonicated juice was related to the degradation and isomerisation of betanin and phyllocactin, and to Maillard reaction. Temperature was a major factor in the betalains stability.	(H. Liao, Zhu, Zhong, & Liu, 2020)
Ulluco (*U. tuberosus*) peel extract	Thermal (4, 20 and 80 ºC) and pH (4, 5 and 6) treatments over time (0 and 5 h, and up to 12 d)	Both pH and temperature induced changes in colour and betalain contents. In highly-acidic foods, the colour intensity (conferred by the extract) would be lower, but more stable during storage.	(Cejudo-Bastante, Hurtado, Mosquera, & Heredia, 2014b)
Cactus pear (*O. dillenii*) juice	Pasteurization (60–90 ºC) and storage (4, 25, 30, 35 and 45 ºC for up to 50 d)	At 100 ºC, 0.6 min was the pasteurization time necessary to ensure the juice quality and safety, while it was 180 min for 62 ºC. To ensure the juice stability for at least 2 months, storage temperatures below 20 ºC were recommended.	(Bassama et al., 2020)
Cactus pear (*O. ficus-indica*) beverages with 10% peel and 90% pulp, with or without addition of acids and antimicrobials	High pressure processing (400 and 550 MPa, room temperature, 0–16 min) and heat sterilization (2 s at 131 ºC)	HHP at 550 MPa for at least 2 min can retains, and even increase, the betaxanthins (6–8%) and betacyanin (4–7%) contents in beverages incorporating acids and antimicrobials. Heat sterilization induced significant losses of betalains (7–45%).	(Dulce María Jiménez-Aguilar et al., 2015)

As is common knowledge, processing can lead to the destruction of most of the compounds that make up food, and Preczenhak et al. (2018) reported that the initial stages of minimal processing of beetroot result in significant loss of betalains. In another study, Preczenhak et al. (2019) applied cysteine as additive for the preservation of minimally processed beetroot. This antioxidant amino acid increased the betalain contents up to 6 d of cold storage. In fact, it appeared to promote the accumulation of betalains and polyphenols. Furthermore, the contribution of betalains to the antioxidant activity was higher than that of the phenolic compounds.

Studies reporting the impact of heat stability has been described as the most relevant technological parameter for betalain degradation. Herbach et al. (2004) observed that the typical red-purple colour of beetroot juice is changed to yellow-orange upon heating at 85 ºC for 8 h due to the generation of yellow neobetanin and orange-red betanin degradation products. Güneşer (2016) also showed that thermal degradation of beetroot betalains in cow milk increases with increasing temperature (70–90 ºC) and time (up to 140 min). In another study, Paciulli et al. (2016) applied high-hydrostatic pressure (HHP) at 650 MPa for 3–30 min as an alternative to blanching pre-treatment (90 ºC for 7 min) of beetroot slices. HHP processing resulted in a significant increase in the betanin content compared to unprocessed samples, the longer the pressurization time, the greater the pigment (and ascorbic acid) degradation. Overall, the cold-processed beetroot slices better retained the original texture than the thermal treated ones, and revealed an improved nutritional quality.

Ravichandran et al. (2013) compared the impact of different thermal processing methods (boiling, microwaving, roasting, and vacuuming) on the beetroot betalains. While boiling for 180 s caused an up to 51% and 33% reduction in betacyanin and betaxanthin levels, respectively, In another study, Sawicki et al. (2019) compared the effects of boiling, microwave-vacuuming, and spontaneous fermentation treatments on the beetroot betalains profile, and reported a 42–70% reduction in the obtained food products. The most interesting profile was characterized in the fermented root, where eleven betalains were identified. The negative impact of the boiling and fermentation processes on beetroot betalains was also reported by Sawicki and Wiczkowski (2018), more specifically a 51–61% and 61–88% reduction, respectively. Regarding lactic acid fermentations, Chwastek et al. (2016) fermented beetroot juice supplemented with highbush blueberries– sucrose osmotic syrup to produce a new probiotic beverage. Juices fermented by *Lactobacillus brevis* ŁOCK 0944 contained the highest levels of

betacyanins. Regarding other food matrices, Gengatharan et al. (2016) investigated the stability of red pitaya (*H. polyrhizus*) flesh betacyanins during thermal processing (30–100 ºC for 5 min) and storage for 10 weeks at 4 °C, as well as their potential to be used as a functional colorant to mimic strawberry colour in cow milk. Treatments at 50 and 85 ºC caused a greater betalain reduction in the beetroot colorant E-162 (used as control) than in the red pitaya extract. The cold storage induced colour changes in both red pitaya and E-162. When evaluating the potential of red pitaya betalains as a natural colorant in milk (pasteurised at 63 ºC for 30 min), these ingredients suffered less degradation and colour change than E-162, and also had better colour acceptability. In another study with red pitaya (*H. polyrhizus*), Liao et al. (2020) tested the suitability of thermosonication processing for preserving the juice colour since it has been associated with consumer appeal and potential bioactive and health-promoting effects. Longer treatments and higher temperatures caused a change in the red pitaya juice colour. However, the colour of the thermosonicated juice changed less than that of the pasteurized juice. The decompositions of betacyanins in this processed juice was primarily attributed to the degradation and isomerisation of betanin and phyllocactin, but Maillard reaction was also another cause of colour change. In general, the applied temperature was a major factor in the betalains decomposition and, therefore, in colour stability.

Ulluco (*U. tuberosus*) is one of the most widely cultivated and economically important root crops in the Andean region of South America, with potential application if the food and cosmetic sectors for being a source of natural bioactive pigments. Cejudo-Bastante et al. (2014) prepared *U. tuberosus* peel extracts (by maceration with 60% methanol for 24 h at 10 ºC) and studied its stability at different temperatures and pH values. Both parameters induced changes in colour and betalains contents. The authors stated that if the vivid red coloured extract were added to highly-acidic foods (such as fruits and vegetables), the colour intensity would be lower (with a tendency to yellowish tones), but more stable during storage.

Regarding studies with cactus pears, Bassama et al. (2020) found that *O. dillenii* juice betacyanins resist typical heat treatments, with a 10% loss at temperatures above 80 ºC. At 100 ºC, 0.6 min was the pasteurization time necessary to ensure the quality and safety of the food product, while it was 180 min for a temperature of 62 ºC. During storage, the degradation of betacyanins was correlated with temperature and accompanied by the appearance of a brown shade. Thus, to ensure the juice stability for at least 2 months, temperatures below 20 ºC were recommended. Concerning cold pasteurization, Jiménez-

Aguilar et al. (2015) showed that HHP treatments at 550 MPa for at least 2 min can retains, and even increase, the betaxanthins (6–8%) and betacyanin (4–7%) contents in prickly pear (*O. ficus-indica*) beverages incorporating acids and antimicrobials, when compared to untreated samples, thus originating a food product of improved nutraceutical quality. In turn, heat sterilization induced significant losses of vitamin C (46–76%), total phenolics (27–52%), betalains (7–45%) and antioxidant activity (16–45%).

5 Stabilization of Bioactive Molecules:

5.1 Case study of betalains

As it is well known, natural products have a stability problem when compared to products obtained by chemical synthesis. And for enable their use by the industry new approaches have been done to overcome this problem (N. Martins, Roriz, Morales, Barros, & Ferreira, 2016). Different factors can affect the molecular stability of this natural products, because, in order to obtain this molecules it is necessary to remove them from their original surroundings, something that leaves them unprotected and susceptible to the factors that may destabilize them, resulting in a loss of properties (Sarker & Nahar, 2012). The simplest and easiest way to stabilize a molecule is to convert it into its solid conformation, removing the extracting solvent, since a greater physical and chemical stability is achieved in the solid state (Teixeira et al., 2017).

Betalain application in foodstuffs has been limited due to the low betalain stability during extraction, processing, and storage, which represents a limitation compared to the more stable artificial colouring additives. In fact, when these natural pigments are exposed to both extrinsic and intrinsic factors in a certain intensity (**Figure 3**), betalains may undergo isomerisation, deglycosylation, or decarboxylation depending on their structural features (Manchali, Chidambara Murthy, Nagaraju, & Neelwarne, 2012).

Betacyanins are usually accompanied by their respective isobetacyanins, but acidic or alkaline media, as well as thermal treatments, can cause their isomerization (Manchali et al., 2012). In the particular case of betanin, it is mainly hydrolysed into betalamic acid and cyclo-Dopa-*O*-β-glucoside, but its stability can be promoted by esterification with aliphatic acids (Schwartz & von Elbe, 1983). Glucosylation at C-6 of betanidin appears to increase the pigment chromatic strength owing to a more solid conformation.

The use of flavan-3-ols such as catechin has also been proposed for betalains stabilization (Patent No. US 2010/0330239 A1, 2009). The use of stabilizers, such as ascorbic and citric acids, have also been used due to the ability to remove oxygen from the solution and reduce the polarity at the N-1 position (Khan, 2016b). Therefore, novel methodologies have been used to stabilize betalains in different media in order to boost and widespread its industrial application as natural colorants. **Table 8** summarizes stabilization techniques applied to betalains, such as complex formation, encapsulation, and copigmentation.

Table 8 Novel methods used in the stabilization of betalains.

Processed product	Stabilization methods	Impact on betalain content	Reference
Beetroot betalain solution	Complex formation with soy protein isolate fibrils	The betalain–soy protein isolate fibrils complex was a small, amorphous aggregate, decreased colour loss and increased the thermal retention of betalains from 55.3% to 75.9%.	(Zhao, Ma, & Jing, 2020)
Pigeon berry (*R. humilis*) juice	Complex formation with metals (Se^{+}, Zn^{2+}, and Cu^{2+}), at 10 and 40 mg metal/mL with or without ascorbic acid (0.25 and 0.5 g/100 mL). Storage in the dark for 30 d at 25 ºC after blanching at 90 ºC for 3 min	The betalains stability was achieved with ascorbic acid (0.25 g/100 mL) and improved with the addition of Se (40 mg/mL); this synergistic mixture better regenerated the pigments after thermal processing.	(Khan & Giridhar, 2014b)
Red-purple pitaya (*H. polyrhizus*) peel and flesh extracts	Food additives supplementation (ascorbic acid and guar gum)	The extracts supplementation with 0.5% (*w/v*) of ascorbic acid was a good option to preserve betacyanins during storage at 4ºC.	(Leong et al., 2018a)
Beetroot juice	Microencapsulation (spray-drying using low-crystallised maltodextrin, Arabic gum, and a mixture of both (1:1) as carriers)	The highest pigment content was found in Arabic gum powders, which were more stable due to less hygroscopicity.	(Janiszewska, 2014)
Beetroot juice	Microencapsulation (spray-drying using gum Arabic as carrier)	The microcapsules stability was influenced by water activity of the storing medium.	(Pitalua, Jimenez, Vernon-Carter, & Beristain, 2010)
Beetroot extract	Microencapsulation (spray-drying using maltodextrin, inulin, and whey protein isolate as carriers)	The combined use of inulin and whey protein isolate as carriers resulted in a good powder stability.	(Carmo et al., 2018a)
Pitaya juice	Microencapsulation (spray-drying with maltodextrin and resistant maltodextrin)	Maltodextrin was more suitable than resistant maltodextrin and more effective during 3-month storage. Despite resistant maltodextrin was more effective for betanin retention after incorporation into sugar confection, after 3-month storage, the stability was higher in candies with maltodextrin.	(Shaaruddin, Ghazali, Hamed Mirhosseini, & Muhammad, 2017)
Jiotilla (*E. chiotilla*) and cactus (*S. queretaroensis*) fruit extracts	Microencapsulation (spray-drying using cactus pear (*O. ficus-indica*) mucilage as carrier)	Acidified cactus pear mucilage was used in the extraction of betalains from the peels and the resulting solutions were spray-dried, originating smooth and spherical microcapsules capable of retaining more than 90% of betalains after 3 months of storage. The microcapsules of both samples gave similar colours.	(Delia et al., 2019)
Amaranthus gangeticus juice	Microencapsulation (spry-dying using β-cyclodextrin and maltodextrin as carriers)	The β-cyclodextrin concentration had a positive effect on droplet size, which was positively influenced by inlet temperature.	(Chong et al., 2014)
Malabar spinach (*B. rubra*) fruit extract	Encapsulation (co-crystallization with sucrose and gum acacia)	Co-crystals with betacyanins were characterized by an entrapment efficiency of 65% and good storage and handling properties, had low water activity and hygroscopicity, and improved storage stability.	(Karangutkar & Ananthanarayan, 2020)
Malabar spinach (*B. rubra*) fruit juice	Encapsulation (soybean lecithin nanoliposomes prepared using a thin-film hydration-sonication technique)	A 76% improvement in betalain colour and stability was obtained. Betalain nanoliposomes colour was heat stable up to 121 ºC. In gummy candies, provided good colour, betalain retention, and antioxidant activity for 28 d at 5 ºC.	(Sravan Kumar, Singh Chauhan, & Giridhar, 2020)

Regarding complex formation, Zhao et al. (2020) verified that the interaction of beetroot betalain with soy protein isolate fibrils decreases colour loss and increases thermal retention of betalains from 5.3% to 75.9%. In a different study, Khan and Giridhar (2014) explored betalain complexes with metals (Se^{+}, Zn^{2+}, and Cu^{2+}), with or without ascorbic acid. The betalains stability and colour intensity (hyperchromaticity) were enhanced with the presence of both ascorbic acid (0.25 g/100 mL) and Se (40 mg/mL). These same authors reported that betacyanins juice suffered a degradation of up to ~95% with a treatment at 90 ºC for 36 min and 48 days storage at 25 ºC, while only 15% of the pigments were degraded at 5 ºC for 90 days. The synergistic mixture of betalains and metals, has the ability to better regenerated the pigments after the thermal treatment. The protective effect of ascorbic acid was also reported by Leong et al. (2018), who verified that the supplementation of red-purple pitaya (*H. polyrhizus*) peel and flesh extracts with 0.5% (*w/v*) of ascorbic acid is a good option to preserve betacyanins under storage at 4 ºC. In addition, there was no need to adjust the pH.

Spray-drying is one of the most widely used microencapsulation techniques (will be extensively review in section 5.2), and there are studies with beetroot pigments employing different encapsulating agents. Janiszewska (2014) obtained the highest pigment content in microparticles based on Arabic gum, while a contrary result was obtained for maltodextrin powders. The Arabic gum microcapsules were more stable because of their lower hygroscopicity in comparison to those of maltodextrin. Despite this, beetroot formulations with maltodextrin were suggested as preferable food ingredients. Pitalua et al. (2010) also verified that the betalain powder stability depends on the water activity of the storing medium. In turn, Carmo et al. (2018) concluded that the simultaneous use of inulin and whey protein isolate as carriers results in beetroot powders with good stability. Regarding betanin in pitaya powder, Shaaruddin et al. (2017) described maltodextrin as a more suitable encapsulating agent than resistant maltodextrin, and also more effective during 3-month storage. Although resistant maltodextrin was more effective for betanin retention after incorporation into sugar confection, the stability was higher in candies incorporated with maltodextrin after 3-month storage.

Delia et al. (2019) investigated the potential of cactus pear (*O. ficus-indica*) mucilage as encapsulating matrix for the spray-drying of jiotilla (*E. chiotilla*) and cactus (*S. queretaroensis*) pulp and peel betalains. The microcapsules made from both plant by-products originated a similar colour when redissolved in water.

Co-crystallization is a novel encapsulation technique that can be used in the stabilization of bioactive compounds. Recently, Karangutkar and Ananthanarayan (2020) prepared co-crystals containing betacyanins from Malabar spinach (*B. rubra*) fruit extracts, which were characterized by an entrapment efficiency of 65% and good handling properties. The co-crystals containing sucrose and gum acacia had low water activity and hygroscopicity, and improved storage stability. The stability of betalains has also been promoted through nanoliposomes, an encapsulation system suitable for entrapping hydrophobic or hydrophilic compounds. Using lecithin nanoliposomes, Sravan Kumar et al. (2020) reach a 76% improvement of betalain colour and stability.

5.2 Spray drying

Spray drying is an ancient technique, which has adapted and evolved and is being applied on different fronts of scientific research.

It's the most reliable technique of encapsulation for the food and pharmaceutical industry, as its capable to stabilize the active molecules to a successful application into the final formulations. It is also a very versatile technique, and offers the ability to optimize the operating conditions, the use of carrier agents, the drying gases, all of them having a direct impact in the final powders/particles according to the final objective.

5.2.1 Spray-Drying - What and Why?

This methodology can be explained as the transformation of a fluid to a powder by spraying the particles through a hot drying medium (Anandharamakrishan & Ishwarya, 2015). It was first applied by Samuel Percy who filed a patent to the U.S. Patent Office named "Improvement in Drying and Concentrating Liquid Substances by Atomizing" (Patent No. US125406A) in 1870's. But since then, it has undergone several changes, technological improvements and has been eliminated by various industries ranging from production or transformation of foods, pharmaceuticals, soaps, fertilizers, clays, ceramics, polymers and many other products (McHugh, 2018).

For several industries, the removal of solvent extractor, with the possibility of encapsulation offered by the spray drying technique, has made this technique preferential, as it is simple, effective and relatively inexpensive (Fang & Bhandari, 2010). Thanks to the external protective barrier that forms around the molecules of interest, the factors that impair their molecular stability lose their expression, and all of this takes place in an

extremely fast process where the barrier is created and the solvent is extracted almost simultaneously. This technique can be used to overcome some problems, such as rubbery texture of dried fruits, simply adding carrier agents (F. González, García-Martínez, Camacho, & Martínez-Navarrete, 2019). These carriers depending on the proportion in which it is added, may act as encapsulating agent, where the carrier becomes the actual wall material.

The spray drying process can be applied to a huge range of products for the food, pharmaceutical and other industries (Álvarez-Henao et al., 2018; Dadi, Emire, Hagos, & Eun, 2020; He et al., 2016; Hoyos-Leyva, Chavez-Salazar, Castellanos-Galeano, Bello-Perez, & Alvarez-Ramirez, 2018; Salminen et al., 2019; Sarabandi & Jafari, 2020; Sarkar, Arfsten, Golay, Acquistapace, & Heinrich, 2016). Considering the food industry, spray-drying has been used in dairy products, essential oils, aromas, colouring compounds, phenolic compounds, probiotics and others (Arepally, Reddy, & Goswami, 2020; Cortés-Rojas, Souza, & Oliveira, 2016; Martínez et al., 2015; Neves, Desobry-Banon, Perrone, Desobry, & Petit, 2019; Pellicer et al., 2019; Vincenzetti et al., 2018).

Spray drying is a highly inclusive technique regarding the type of samples that can be processed. It is possible to perform drying or encapsulation by spray drying on natural compounds, bioactive molecules, colour-holding molecules, probiotics, essential oils, molecules with pharmacological activity, among others (Arepally et al., 2020; Carmo et al., 2018b; Cortés-Rojas et al., 2016; Herbrink, Vromans, Schellens, Beijnen, & Nuijen, 2018; Martínez et al., 2015). Carrier molecules can be luteulin, maltodextrins, gum arabic, modified starches and simple sugars such as trehalose and inulin. (Álvarez-Henao et al., 2018; Ding et al., 2020). Oils are also an example of carrier agents (Başyiğit, Sağlam, Kandemir, Karaaslan, & Karaaslan, 2020; El-Messery, Altuntas, Altin, & Özçelik, 2020; Encina et al., 2018; Lee, Tan, Sulaiman, Hee, & Chong, 2020; Martínez et al., 2015; Miranda-Linares, Quintanar-Guerrero, Del Real, & Zambrano-Zaragoza, 2020; Premi & Sharma, 2017; Salminen et al., 2019; Sarkar et al., 2016; Shi et al., 2020; Taktak et al., 2019; S. Zhang et al., 2018). In the case of oils, the technique of spray drying is of great importance, because, considering their composition, they are very susceptible to oxidative decomposition, and have a volatile portion that besides having interesting biological activities and can be protected. Encapsulation in the case of oils helps to provide a barrier against deterioration processes, increasing stability, and can suppress unpleasant aromas. The spray drying technique is capable of much more than protecting molecules against deterioration processes and camouflaging unwanted aromas/flavours.

The added value of this technique is to increase the stability of the molecules of interest, and thus, enable different industries to resort to new alternatives to incorporate in their products. It allows to make products more natural and to develop new products for the food industry, since it offers a whole new panoply of substances, for example with regard to molecules to be explored as colourants of natural origin (Álvarez-Henao et al., 2018; Carmo et al., 2018b; Ferreira Nogueira, Matta Fakhouri, & de Oliveira, 2019; F. González et al., 2019; Kaimainen, Laaksonen, Järvenpää, Sandell, & Huopalahti, 2015; Righi da Rosa et al., 2019; Souza et al., 2018; R. Zhang et al., 2020) probiotics and bioactives (Arepally et al., 2020; Barbosa et al., 2015; Cortés-Rojas et al., 2016; L. K. Liao et al., 2017; Moreno, Cocero, & Rodríguez-Rojo, 2018; Parlindungan, Dekiwadia, May, & Jones, 2019).

In the pharmaceutical industry, this technique is used to increase molecular stability, producing small-sized particles, which are easier to transport and thus allow the use and approach of new therapies. With spray drying, it is possible to encapsulate active ingredients that have some difficulty in expressing their beneficial effect, such as solubility problems. The pharmaceutical industry takes advantage of all the possibilities that the spray drying technique offers, such as encapsulating the active ingredients, increasing its stability and altering its bioavailability in the organism. Through this technique it is possible to obtain particles with a gradual and controlled release of the active principle. This is of great importance as it allows a gradual dosing of the same and that it remains constant during therapy (Shetty et al., 2018; J. Wu et al., 2019; S. Zhang et al., 2018).

Mainly due to these characteristics, the spray drying technique is progressively a viable resource for different industries, which is constantly being improved, either by varying the molecules, the carriers, and the operating conditions, allowing to explore a whole new range of molecules and products. This technique allows to explore and develop the industries, more precisely the food and pharmaceutical industry.

5.2.2 Featured Advantages and Disadvantages

As previously mentioned, the food industry is the biggest user of this technology, followed by the pharmaceutical industry. All due to the considerable advantages of spray drying when compared to other transformative technologies, being the factor of complete automation and continuous work, with the minimum of human intervention, which

reduces the contamination of products, whether food or pharmaceuticals. It is a technique that has very short residence times, and despite operating at temperatures considered high, these do not affect the most thermostensic molecules, adjusting the different parameters, allows the application of specific conditions for different products, with different needs. Encapsulation by this technique is widely used, resulting in a homogenous product, resistant to thermal degradation and allowing a controlled release, being especially important for the pharma industry, and to encapsulate bioactive substances for foods (Sosnik & Seremeta, 2015; McHugh, 2018). Like any other technology, this one also has disadvantages, and the most important one is related to the price of the spray-drier equipment, as well as the considerable maintenance issues. Some more technological drawbacks, are possible the yield of the particles, as they tend to get stuck to the walls of the drying chamber, resulting in a 20% loss, and if the particle size is under 2 μm can usually pass into the exhaust air and be removed. Another disadvantage, found especially for microencapsulation, limited types of wall materials, with good solubility in water to be used (Gharsallaoui et al., 2007; Sosnik & Seremeta, 2015).

5.2.3 Spray-drying Operating Processes

The spray drying technology is processed in four steps, namely the atomization, followed by the droplet-to-particle stage, the moisture evaporation and finally the particle collection (**Figure 4**). One of the most crucial steps being the atomization one, since is this step were the liquid is divided into small particles, atomized, and continuous to the other steps. The atomization step can be adjusted depending on the fluid being atomized, or the final product characteristics. For instance, rotary atomizers are used in low viscosity fluids, two fluid nozzle atomizers use kinetic energy, allowing the production of particles with a relatively greater size. Hydraulic atomizers discharge the fluid under pressure through an orifice of variable sizes. The ultrasonic atomizers force the liquid through two piezoelectric disks that vibrate at high frequencies and ensure the vibration of the atoms of the droplets, reducing the surface tension. This type of atomization is intended for low-viscosity Newtonian fluids. And finally, electrohydrodynamic atomizers pass an electric current through the fluid enabling the production of droplets of narrow particle sizes. in terms of collectors, the there are four types of collectors, namely the cyclone separators that uses a centrifugal force to separate the solid particles from the carrier gas. Bag filters separate the particles by retaining them inside the bag and allowing

the carrier gas through the bag material, while the electrostatic precipitators retain the particles by using electrostatic forces to ionize the air and making the particles cling to collecting plates (Anandharamakrishan & Ishwarya, 2015; Santos et al., 2017).

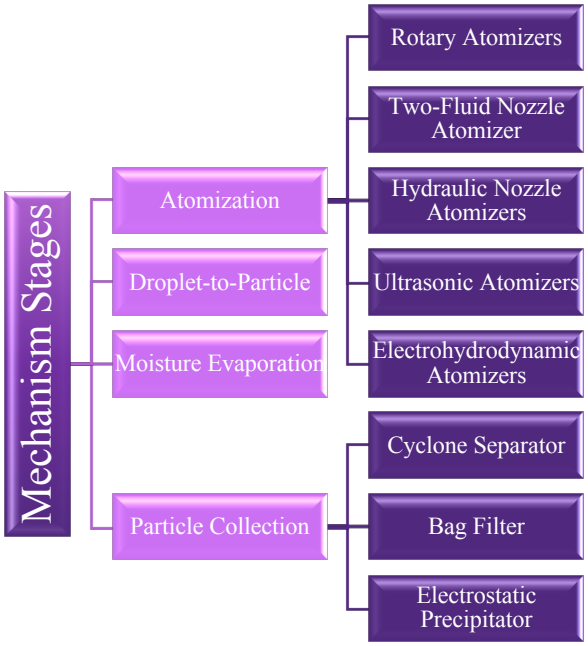


Figure 4 Stages of the spray-drying procedure and different variations of the technology. Adapted from Anandharamakrishan & Ishwarya, (2015) and Santos et al. (2017).

The spray-drying operating conditions must be chosen considering the chemical features of the material that will be spray-dried. This technology also allows the encapsulation of bioactive compounds, leading to the increase in their solubility, their affinity with the destination matrix or to allow a controlled release (Su et al. 2008). The secret to a successful spray-drying operation is the choice of the operating conditions, namely: inlet/outlet temperature, drying airflow rate, feedflow rate, speed of the atomizer, carrier agent and respective concentration (Murali, Kar, Mohapatra, &Kalia, 2015; Phisut, 2012), regarding the chemical features of the material to be spray dried, as well as the final desirable particles are crucial to establish the most efficient operating conditions.

Temperature

The perfect balance in the temperature is of utmost importance, since this technology needs high temperatures, possible to cause degradation of the target molecules, the only advantage is that the contact of the material subjected to the spray drying with high temperatures happens in a matter of seconds. The inlet temperature ranges from 150-220 ºC and the outlet from 50-80 ºC. as a matter of fact, the inlet tempearute when higher, due to a fast evaporating capacity of the solvent at that specific temperature, causes the

production of spheres without the ideal shrinkage, thus producing larger particles (Daza et al., 2016). Other parameter that is affected by the inlet temperature is the solubility Daza et al. (2016) described that an increase in the inlet temperature from 120 to 160ºC improved the solubility of samples. For instance, the outlet temperature is a crucial parameter, and must be controlled to assure that this temperature is lower than the thermal degradation temperature of the constituents, to avoid the powder degradation by high temperatures (Singh and Van den Mooter, 2016; Shishir and Chen, 2017). Some studies supported that the outlet temperature is very relevant to control the droplet temperature or droplet drying speed. High outlet temperatures cause the reduction in moisture contents, increasing the process yields, while low outlet temperatures improve the sphericity of particles, increasing the retention of some compounds, a key factor on the physicochemical properties of the final powders (Ziaee et al., 2019). The outlet temperature cannot have very low values, as it can lead to the accumulation of water, which affects the quality of the final product. Directe contact of the material with the inlet temperature affects the particle formation, according to Singh et al., (2016) this contact leads to a to a pressure gradient inside, but also outside the droplet, causing morphologic alterations in the final powder, namely surface roughness.

Thus, a thermal equilibrium must be found in order to maintain the particles stability during the process, without compromising the final stability.

Carrier agents

The addition of these molecules will help to overcome some problems of this technique. Samples with a high concentration in sugars, such as fruit juices, for instance, can not be spray-dried without carrier agents (Shishir and Chen, 2017). The use of carrier agents decreases the stickiness of samples and their hygroscopy allowing the dried powders formation.

There is a multitude of carrier agents available such as arabic gum, maltodextrins, starches, pectin, alginates, and combinations of them (Igual et al., 2014), used for their high solubility, low viscosity, high molecular weight, capacity to decrease stickiness, protect the material from external factors such as heat, oxygen, humidity, pH, among others (Sishir and Chen, 2017). With regard to the concentration to which they should be added, they must be applied accordingly to each different sample, because even in the literature for the same sample different amounts are used by different authors.

Feed Concentration and Rate

The concentration of the feed solutions will vary the feed flow rate and, consequently, the atomizer speed. Thus, high concentrations, imply high solids content, which implies a lesser amount of solvent, resulting in short evaporation times and the formation of agglomerates consisting of porous particles with low density. (Suhag et al., 2016).

Another important factor is the feed rate, if is high, the systems will need more energy to evaporate the solvent from the droplets not allowing an ideal interaction between the feed droplets and the hot air, and leading to a less effective heat and mass transfer, corresponding to high moisture contents in the final particles, and low processed yields (Tonon et al. 2008; Ziaee et al., 2019).

According to the available literature, high feed rates lead to lower yields in the spray-drying process, and it increase the particle size and bulk density (Banat et al., 2002).

Atomization Parameters and Drying Gases

The atomization step is probably the most important step in all spray-drying procedure, particles size, density, velocity, among other important characteristics of the final powders, are related to this step. Therefore, pick the ideal atomizer is crucial (Tee et al., 2012), the most commonly used are the rotary atomizer, pneumatic, ultrasonic and hydraulic nozzles. When comparing the efficiency of these different atomizers, the literature describes that rotary atomizers create larger particles when comparing with nozzle atomization, and two-fluid nozzle atomizer usually produces the smaller particles (Sishir and Chen, 2019). The pressure is an important parameter of the atomization conditions, higher pressures in the range of 1-2.5 bar, creates smaller particles and larger surface areas, increases the total solid percentage and bulk density, increasing the drying process efficiency (Jumah, Tashtoush, Shaker, and Zraiy 2000; Sishir and Chen, 2019). Tee et al., (2012) reported that increasing the atomizer pressure from 80 to 100% produced smaller particles and decreased the moisture content, while also increasing the process yield and hygroscopicity, however excessive pressures also leads to an enormous energy consumption without bringing additional benefits regarding the particle size and yields of the process. The speed of the atomization is another parameter that directly influences the final product characteristics. As the increase in the atomization speed results in the increase in the flow rate, creating tiny particles, resulting in a higher area of contact that allows for a faster drying procedure, and an increase the yields (Souza et al., 2009; Sishir and Chen, 2019).

The most common gases used in the spray-drying process are compressed air, CO_2 and N_2, these gases and their properties also represent key factors to the success of the spray-drying products. For instance, the use of low-density gases such as nitrogen, an inert gas, that is commonly used in solutions with high concentrations of organic solvents and in solutions with easily oxidable compounds, produces smaller particles different surface morphologies (Singh and Van den Mooter, 2016). On the other hand, CO_2, that presents higher density properties produces larger particles. Several authors reported the effect of the atomization gas type and concluded that the crystallinity of the final particles is directly influenced by the type of gas, describing that N_2 allows the production of higher crystallin particles than CO_2 and compressed air (Islam and Langrish, 2010).

As stated above, all the spray-drying parameters are strongly related with the final particle's characteristics, namely in terms of particle distribution, moisture, yields, particle size and morphology. According to the final applications, the operating conditions can be adjusted and optimized to target different morphologies, yields, particle sizes and distribution.

Furthermore, these parameters can be optimized using mathematical models such as the response surface methodology that can predict the ideal operating conditions to the desirable target particle characteristics, a technology based on reduced experimental data that is already applied to the spray-drying processes, namely by the pharmaceutical industry to increase particle yields (Krishnaiah et al., 2012; Sishir and Chen, 2019).

Overall, the contribution of spray-dryers to the food and pharmaceutical industries is undeniable. From a simple method of drying and encapsulating food during the end of the XX century to a technology that allows controlled release of ingredients and active compounds in drugs, spray-drying is an essential technology in the XXI century. With the previewed enhancements to the technique, longer shelf-lives are expected in foods, better efficacy in drugs, nano spray driers and an overall reduction in the cost of the equipment which will democratize its use, leading to a broader use.

6 Betalains potential food application

The research of betalains has been limited in comparison to other plant pigments, such as anthocyanins and carotenoids. In fact, the elucidation of the biosynthetic routes involving the formation of these compounds has only been clarified in the past few years, which, in

a certain way, has limited the exploitation of the real potential of these pigments (Polturak & Aharoni, 2018).

Considering the structural aspects of betalains, these compounds are more stable than any other plant pigment, regarding temperature (betacyanins are more stable than betaxanthins) and pH ranges (from 3–7), as also higher solubility in water and tinctorial strength (Azeredo, 2009; Stintzing & Carle, 2007). These characteristics allows the application of betalain compounds in food matrices with very distinct chemical features; except for the liposoluble matrices, were usually a stabilization step of the betalain compounds is required.

Food applications are without a doubt the most common studies performed to explore the colouring capacity of betalains, such as the work made by Otálora, de Jesús Barbosa, Perilla, Osorio, and Nazareno (2019) with a betalain-rich extract from *O. ficus-indica* fruit encapsulated by ionic gelation with calcium alginate, and further applied in gummy candies. The authors found no significant variation in the colour parameters of the betanin-enriched gummies during storage at 4 °C for 30 days, maintaining the desired vivid red-purple colour. In another study, betalains from reed beet, anthocyanins from grape wines, and their mixtures, where incorporated in white currant juice. The authors observed a faster degradation of betalains promoted by the coexistence with anthocyanins, as also a decrease in colour intensity from pink to yellow during storage time (W. Yang et al., 2021). Dairy products are among the most daily consumed products worldwide, due to its nutritional and organoleptic characteristic, and as such become one of the most interesting matrices to be incorporated with enriched-natural colorant extracts, to provide the consumer a new and very appealing product. Betalains enriched extracts from beet root were added to petit Suisse cheeses and storage at 6 ºC during 40 days, presenting a half-life time and percentage colour retention values adequate for the shelf life of this product, which means that the desired colour for the final product was obtained (Prudencio, Prudêncio, Gris, Tomazi, & Bordignon-Luiz, 2008).

The application of betalains in the food industry does not rely only in the food product itself, but also these extraordinary compounds could be used for the development of the so called “intelligent packages”. Hu, Yao, Qin, Yong, and Liu (2020) proved the antioxidant and antimicrobial capacity of a multifunctional food packaging for shrimp conservation by incorporation of a betalains-rich extract from amaranth (*A. tricolor*) extract into a quaternary ammonium chitosan/fish gelatine blend film. Not only the amaranth extract contributed to an antimicrobial activity against four foodborne

pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium,* and *Listeria monocytogenes*) and a higher ammonia sensitivity, but also proved to have physical effects in the developed film, significantly increasing the thickness, UV–Vis light barrier ability, and the elongation at break.

One of the main concerns of betalain application into food products is their sensitivity to thermal degradation, which could limit their use in food products that required thermal processing steps during preparation, pasteurization, and confection (Zhao et al., 2020). The interaction of betalain and soy protein isolate fibrils proved to be effective against colour degradation and increased the thermal retention of betalain, after heat treatment, producing small amorphous aggregates (Zhao et al., 2020). Sravan Kumar, Singh Chauhan, and Giridhar (2020), also showed an improvement of betalain colour and stability after incorporation in lecithin nanoliposomes, presenting very promising results in betalain retention, colour, texture, antioxidant activity, and shelf-life of gummy candies incorporated with the microencapsulated betalain after storage (5 °C, 28 days) (Kumar et al., 2020). These two studies from Zhao et al., (2020) and Kumar et al. (2020) proved to overcome the thermal instability of betalain pigments, further encouraging its use in the food sector, where at least one solution for the obtaining of a powder enriched-betalain extract from purple-flesh (red-violet) dragon fruit (*H. polyrhizus*) with increased stability, miscibility, dispensability, and bioavailability was achieved (Patent No. WO 2010/090508 Al, 2009).

As further stated, the knowledge regarding the potentiality of betalains, besides the colouring capacity, in comparison to other plant pigments such as anthocyanins and carotenoids is very scarce and restricted to the food sector. However, several research and review papers positioned themselves against this trend and opened up a range of possibilities for the application of betalains in other areas of study, such as health and health linked to food with the development of, for example, functional foods (Gengatharan et al., 2015).

Another type of less conventional applications for betalains has also been described and reported by some authors, mainly based on the fluorescence capacity of these compounds. Pioli, Mattioli, Esteves, Dochev, & Bastos (2020), developed semi-synthetized *N*-methyl phenylbetalain and *N*-aryl phenylbetalain dyes, with faster hydrolysis, lower anodic potentials and increased fluorescence quantum yields, which leads to the development of new hydrolytically stable betalain dyes with characteristic and tunable redox and optical properties to be applied in several industrial fields (Pioli et al., 2020). Zhang et al. (2008)

developed a new dye-sensitized solar cell based on natural betalain pigments from red beet roots, in which the yellow betaxanthin pigments were removed to obtain an enriched betanin solution, a water soluble pigment with strong visible light absorption. A similar research has been conducted by other research institutions and the validation of the potentiality of betalain compounds to be used, has already been published and made available for the industrial sector (Patent No. US 2012/0125439 A1, 2014).

The fluorescence capacity of betalain has also been explored for the detection of the presence of spores from *Bacillus* by the interaction between betalain and metal complexes which causes fluorescence response changes, and therefore allows the detection of spores below the infective dose for anthrax (Guerrero-Rubio, Martínez-Zapata, Henarejos-Escudero, García-Carmona, & Gandía-Herrero, 2020).

Part II. SCOPE AND OBJECTIVES

Colour is often the first sensory quality by which we judge all the things that surround us, and food is no different. Today, most consumers prefer foods with natural additives in place of the synthetic. The demand for colourants without adverse effects has become a topic of great importance, leading to the growing interest of natural colourants.
Betalains are a group of chromoalkaloids pigments synthesized from tyrosine (Junqueira-Goncalves et al., 2011). Are similar compounds to anthocyanins regarding colour features, but not so exhaustively studied, display three-fold more colouring strength than anthocyanins. The only betalain legislated for use is derived from beetroot (E-162), but there are some other matrices underexploited. Although quite promising, natural additives still face some drawbacks and limitations. A specific problem with certain natural colouring agents is their chemical instability that may be largely affected by numerous factors (*e.g.* pH value, co-presence of compounds, water activity, temperature, oxygen) that are considered the main structure and chromatic modifiers of betalains. Therefore, studies considering all these factors are welcome to overcome this problematic.

The **main objective** of the present Thesis was "*to develop and characterized a powerful natural colouring extract based on betacyanins content, from different natural sources and improve their stability for practical application in different food matrix*".

The **specific objectives** will include:

1. First approach to chemical characterization of plant matrix (flowers of *Gomphrena globosa* L., *Amaranthus caudatus* L. and fruit peels *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose fruit peels) in terms of bioactive compounds.
2. Optimization of extraction and purification methodologies to achieve the highest yield of Betalains as natural colouring agent in lowest time in different natural matrix, namely *Gomphrena globosa* L., *Amaranthus caudatus* L. flowers and *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose fruit peels. Using traditional (e.g., maceration) and alternative (e.g., microwave and ultrasound assisted extraction) methodologies will be tested, based on green and scalable approaches.
3. Stability studies of betacyanins extract according with the most critical factors for application in food matrices (e.g., pH, temperature, storage time, light and oxygen).
4. Stabilization of the betacyanins extract, if necessary, in order to overcome degradation problems, by using encapsulation techniques.

5. Evaluation of the safety of the betacyanins extract, by performing toxicity assays in liver cells.
6. Proof-of-concept by developing case studies of practical application of the obtained natural colouring agent (betacyanins extract): incorporation into different food products (ice cream and cookies).

The majority of the working plan was carried out in Centro de Investigação de Montanha (CIMO) in Instituto Politécnico de Bragança. Some of the analyses where also carried out in Departamento de Ciencias Quimicas, Faculdade de Farmácia, Universidade do Porto, during the international predoctural research stay, of three monts, form 1of septembre to 30 of novembre, under direct supervision of Professor Doctor Maria Beatriz Prior Pinto Oliveira, the the work focused the evaluation of bioactivities (antioxidant and antimicrobial properties) of the extracts recovered from the flowers of *Amaranthus caudatus* L., and the peel of *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose.

Part III. WORKING PLAN

To achieve the goals and objectives of the study, the following working plan was followed.

Task 1. Plant selection and characterization

In order to fulfil the proposed objectives, in the first phase, the samples under study were selected. A total of 3 different natural matrices were selected, two flowers from the amarantacea family (*Gomphrena globosa* L., *Amaranthus caudatus* L.) and peels of one hylocereus spp. fruits (*Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose). The selection was made based on the knowledge of presence of specific colouring compounds, betacyanins. An exhaustive chemical characterization was carried out for the different matrices, supported by different chromatographic techniques in order to describe their profiles in phenolic compounds, betacyanins, organic acids, tocopherols and soluble sugars. For the different samples, their bioactivities (antioxidant, antimicrobial, and antitumor potential) were also tested. For this purpose, aqueous extractions were performed by dynamic macerations, and the recovered extracts were then subjected to the aforementioned evaluations.

Task 2. Optimization of the extraction procedure

After characterization of the compounds present in the samples, an optimization of the extraction procedure was applied. Therefore, in order to obtain the highest yield of the colouring compounds (betacyanins), three solid-liquid extraction systems were optimized: maceration, microwave and ultrasound assisted extractions.

Variables common to each extraction system were explored, such as time, solid-liquid ratio, while variables concerning each extraction system are: temperature (maceration), ultrasonic power (ultrasound) and radiation power (microwave).

The study was approached from a mathematical and statistical perspective using experimental design tools, based on surface response combinations, thus reducing experimental conditions.

The responses for each system used were evaluated based on the extraction yield (dry weight) and by monitoring the extract colouring agents by HPLC coupled to different detectors (DAD and MS) and the colouring capacity measured by UV/Vis spectroscopy

and colourimetry. The responses generated by the surface analysis, allowed to estimate the optimal operating conditions for each extraction system, maximizing the colourant potential and optimizing these parameters in an industrial perspective, decreasing production costs and operating time, and increasing efficiency.

Task 3. Stabilization methodologies

For the extracts recovered in the optimal operating conditions, were evaluated supported by HPLC quantification of betacyanins, before and after exposure to the factor capable of affect their stability. Colour changes were also be monitored by UV/Vis spectroscopy, and by measuring colour with the colourimeter (Sant'Anna, Gurak, Ferreira Marczak, & Tessaro, 2013) then, the CIElab values were converted to RGB values.
If problems of stability were identified, a stabilization methodology was applied in order to maintain the colouring capacity of the natural ingredient, without compromising the final product quality, namely spray drying.

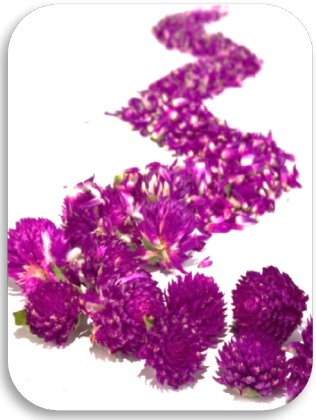
Task 4. Proof-of-concept by incorporating the natural colouring agents in food products

The developed natural colourants based on betacyanins were incorporated directly or after stabilization in different food products, namely ice cream and cookies.
The content to be incorporated was established according with the intended final colour intensity. Physico-chemical and nutritional characteristics of the final food products were evaluated along the shelf life. Results were compared with reference formulations (products incorporating beetroot and synthetic colourants). Colour and texture were measured using specific methodologies. Proximate composition was determined following official methods, and the individual profiles were determined using methodologies optimized by the BioChemCore group: fatty acids (GC-FID), sugars (HPLC-RI), tocopherols (HPLC-Fluorescence), organic acids (including ascorbic acid; HPLC-DAD) and betacyanin compounds (HPLC-DAD).
The stability of the colouring agent (free or protected forms) was evaluated along the shelf-life of the food products, considering all the parameters mentioned.

Task 1. Plant selection and characterization

Plant selected according to the presence of betacyanin

Gomphrena globosa L.



Hylocereus costaricensis (F.A.C.Weber) Britton & Rose

Amaranthus caudatus L.

Chemical study previously done during the master degree thesis

Dried samples subjected to different extractions according to the different assays for prior identification of:

Betacyanins
Organic Acids
Tocopherols

Antioxidant activity; Antimicrobial activity; Antitumor activity

Task 2. Optimization of the extraction procedure

Dynamic Maceration

Ultrasound Assisted Extraction

Microwave Assisted Extraction

Response surface Methodology

Independent Variables
Time;
Solid/liquid ratio
Temperature
Ultrasonic power
Radiation power

Betacyanin content → Chromatographic techniques

Colour → Spectroscopy, Colourimetry

Task 3. Stabilization Methodologies

Spray drying

Colour → Spectroscopy, Colourimetry

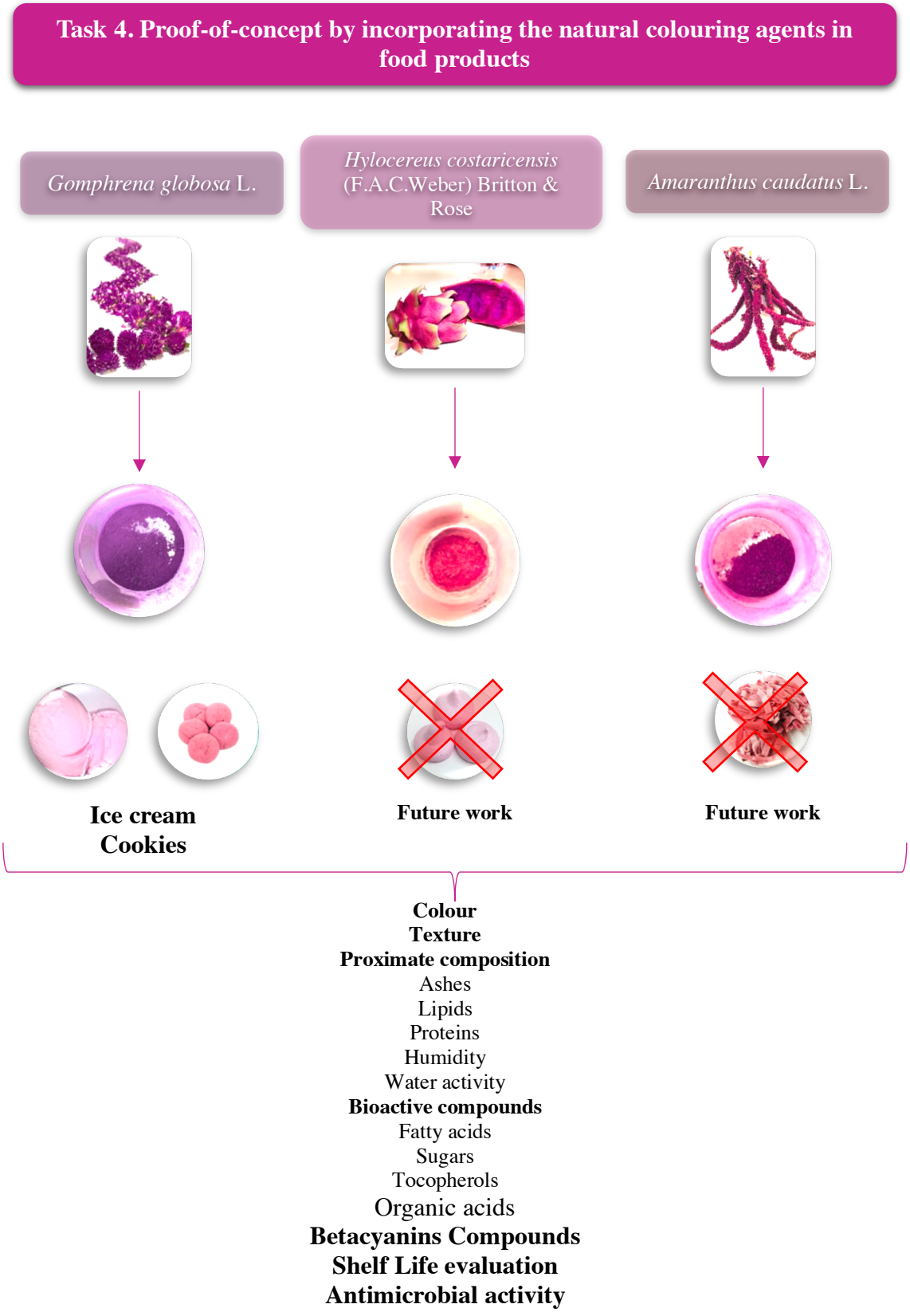


Figure 5 Depiction of the different tasks

Part IV. MATERIALS AND METHODS

1 Betacyanins natural colourants extract development and characterization

In the first phase of the work, in order to get a deeper knowledge of the samples, when arrived a dynamic maceration was done, and the result extract was subjected to a chemical characterization, and evaluation of its bioactivities. The betacyanins natural colourants were obtained, in a second phase, from optimization extraction procedures of three extraction techniques, dynamic maceration, ultrasound assisted extraction, and microwave assisted extraction. After the obtained results, de extract presented the best results, was the one used in the incorporations.

1.1 Plant samples selection

The plant samples of *Gomphrena globosa* L. and *Amaranthus caudatus* L. were purchased in the company "Cantinho das Aromáticas", a company dedicated to the commercialization of dried aromatic plants, based in Vila Nova de Gaia, Portugal. The peel samples of *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose were kindly provided by Ángel Ferrero, from "Pitayas de Galicia", a research project on the acclimatization and cultivation of this fruit in the region.

1.1.1 *Gomphrena globosa* L.

Scientific classification
Kingdom: Plantae
Clade: Tracheophytes
Clade: Angiosperms
Clade: Eudicots
Order: Caryophyllales
Family: Amaranthaceae
Genus: Gomphrena
Species: *G. globosa*
Binomial name: *Gomphrena globosa* L.

Figure 6 *Gomphrena globosa* L flower. Author Custódio Lobo Roriz

Gomphrena globosa is an eudicotyledonous angiosperm that belongs to the Order Caryophyllales, family Amaranthaceae and Genus Gomphrena (Ilyas et al., 2013), is characterized by being an annual herbaceous plant, non-cespitous with a fibrous root, and

can reach between 20-60 centimetres and height. The stem is robust, branched, with slightly quadrangular branches, slightly swollen in the nodes, with rigid greyish hair. The petiole has a grey vein, the limbus is oblong or oval-oblong, with a narrow base, wavy margin, and an acute or obtuse apex.

Inflorescence in terminal chapter usually purple, sometimes pink or white. Opposite bracts, green, oval or chordate, at the apex the bracts are white or purple ovals, purple bracts, triangular lanceolates longer than the bracts. Tepals covered with a white woolly substance and acuminated apex. Bright brown kidney-shaped seeds (Zhengyi et al., 2004).

Gomphrena globosa is a species of the Amaranthaceae family native to Latin America spread widely to Asia and is commonly known as Bachelor's button / Globe Amaranth / kenop flower. In many parts of the globe, has been acknowledged a variety of medicinal properties led to the consumption of its inflorescences for the treatment of diseases of the respiratory system (Cai et al., 2006), of various respiratory inflammatory conditions, such as bronchial asthma, acute and chronic bronchitis or cough whooping (Silva et al., 2012); the infusion of flowers is used to treat oliguria and indigestion and also as an expectorant (Rahman & Gulshana, 2014). It is also used in traditional medicine for diabetes, jaundice, hypertension, urinary incontinence, kidney and prostate problems (Dinda et al., 2006; Lans, 2007; Ferreres et al., 2011). All parts of *G. globosa* plant can be used as medicine against various diseases. *G. globosa* leaves and flower used as a folk remedy, food colorant, oliguria, heat, hypertension, antioxidant, antimicrobial, cough, diabetes, kidney problems, hoarseness, bronchitis, jaundice and high cholesterol.

1.1.2 *Amaranthus caudatus* L.

Scientific classification

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Eudicots

Order: Caryophyllales

Family: Amaranthaceae

Genus: Amaranthus

Species: *A. caudatus*

Binomial name: *Amaranthus caudatus* L.

Figure 7 *Amaranthus caudatus* L flower. Author Custódio Lobo Roriz

Amaranthus caudatus is an eudicotyledonous angiosperm that belongs to the Order Caryophyllales, family Amaranthaceae and Genus *Amaranthus*. The plant of this genus height varies from 0.3 m to 5 m among the various species. Leaves generally presents shapes from oblong to elliptical with the colour going from light to dark green with some expressing red pigment throughout the genus. The inflorescence is very prominent, colourful, terminal and contains one male flower per glomerule of nearly 100–250 flowers. The pollen grains are spherical with golf ball like aperture in monoecious form. Seeds are small and lenticular averaging 1–1.5 mm in diameter with 1000 seeds weight ranged from 0.6 to 1.2 g, with colour varying from pale ivory to black (Martinez-Lopez, Millan-Linares, Rodriguez-Martin, Millan, & Montserrat-de la Paz, 2020).

Amaranthus spp., (amaranthus meaning immortal in Greek), is native of tropical America, and was a staple crop in the Aztec, Mayan, Incan civilizations. Currently it is widely cultivated and consumed throughout India, Nepal, China, Indonesia, Malaysia, Phillipines; whole of Central America, Mexico; Southern and Eastern Africa. Genus Amaranthus, includes branched annual herbs with about 70 different species, 17 of which are edible, such as *A. caudatus* (Peter & Gandhi, 2017). Extracts of Amaranthus have been used to treat several ailments since ancient times. It is typically rich in bioactive compounds such as phenolic acids, lycopene, polyphenols, unsaturated fatty acids, glucosinolates, proteins, soluble peptides, flavonoids, among others. Its described in literature for the treatment of various ailments such as Diabetes mellitus, cancer, malaria,

hypercholesterolemia, atherosclerosis, helminthic and bacterial infections, inflammation, hepatic diseases and cardiovascular complications (Jimoh, Afolayan, & Lewu, 2019).

1.1.3 *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose

Scientific classification
Kingdom: Plantae
Clade: Tracheophytes
Clade: Angiosperms
Clade: Eudicots
Order: Caryophyllales
Family: Cactaceae
Subfamily: Cactoideae
Genus: Hylocereus
Species: *H. costaricensis*
Binomial name: *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose

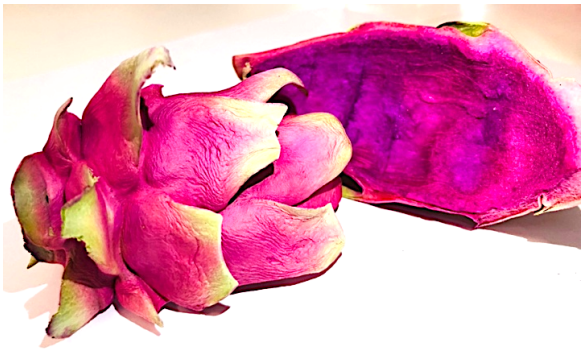


Figure 8 *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose, peel Author Custódio Lobo Roriz

H. costaricensis is an eudicotyledonous angiosperm that belongs to the Order Caryophyllales, family Cactaceae and Genus Hylocereus. *H. costaricensis* is characterized by vigorous vines, perhaps the most robust of this genus. Stems are waxy white and flowers are margined; the outer perianth sediments are reddish, especially at the tips; and stigma lobes are rather short and yellowish. Its scarlet fruit is ovoid and covered with scales that vary in size; it has a red purple flesh with many small black seeds, pleasant texture and good taste (Le Bellec & Vaillant, 2011).

Regular consumption of these fruits will have a positive impact in fighting against cough and asthma. Also, due to high amount of vitamin C in it, it helps for healing wounds and cuts quickly. It is also capable to enhance immune system and stimulate the activity of other antioxidants in the body. It is a fruit very rich in flavonoids that act against cardio-related diseases. Dragon fruit also presents a high amount of B vitamin group (B1, B2, and B3) holding an important role in health benefit, such as increment of carbohydrate metabolism and energy production (B1); helps to recover and increase appetite loss (B2); lowering bad cholesterol levels, provides and moisturizes smooth skin, improves eyesight and prevents hypertension (B3) (Hussain, Sadiq, & Zia-Ul-Haq, 2018).

1.2 Preparation of the Samples

After receiving the samples in the laboratory, they were subjected to some mechanical treatments to separate the pigmented parts of the plant. This step is of enormous importance, due to the main focus of this work. Therefore, after obtaining the pigmented parts of the plant. The *Gomphrena globosa* L. sample was previously studied by the author, therefore the chemical characterization and bioactive properties were already assessed, only the *Amaranthus caudatus* L. and *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose samples were subjected to different assays in order to analyse all the compounds and bioactivities outlined in the work plan. The results from that previous study will be used only in terms of comparison between samples.

1.3 Plant chemical characterization

1.3.1 Betacyanin compounds

1.3.1.1 Method principles

Betacyanin compounds are the molecules responsible for the intense colour on the flowers, and fruit peels of the studied samples. Their determination and quantification can provide an idea to the best methodology and natural sources to recover the highest content of these compounds, in order to have an enrich extracts to be used as natural food colourant. The detection of the betacyanin compounds was carried out by HPLC coupled to a diode array detector (DAD) with electron spray ionization (ESI) and mass spectrometry (MS) detection, following the procedure previously reported by Roriz et al., (2017). The analysis of the betacyanin compounds was carried out for all the samples studied.

1.3.1.2 Solvents, reagents and materials

Acetonitrile was acquired from fisher scientific (Lisbon, Portugal), formic acid was acquired from Panreac (Barcelona, Spain) and the standard (betalain) was acquired from Sigma (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). All other chemicals and solvents were of analytical grade and purchased from common suppliers.

1.3.1.3 Methodology

Betacyanin enrich extract using dynamic maceration was performed following the procedure previously reported by Roriz et al., (2017), in brief 30 mL of ethanol/water (20/80) was used to extract 1 g of each sample. The extraction was performed twice in a magnetic stirrer plate (25 ºC, 150 rpm, 1 h), them the obtained combined extracts for each sample, were filtered (Whatman No. 4 paper) and vacuum dried in a rotary evaporator (Büchi R-210, Flawil, Switzerland) until the solvent complete removal. The obtained aqueous extracts were frozen (-20 ºC) and freeze dried under controlled conditions.

10 mg of each extract was added to 1 mL of ethanol:water (80:20) mixture

They were then dissolved and filtered through a Watman (0.2) filter, into an HPLC vial.

For betacyanin determination and for the followed chromatographic methodology all the conditions are described in **Table 9.** Double online detection was carried out with a PDA using 530 nm as the preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in positive mode, using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source.

Spectra were recorded in a full scan cover the mass range from *m/z* 100 to 1500. Betacyanin compounds were characterized according to their UV-vis, mass spectra, retention times, and compared with standards when available. For the quantitative analyses, peak areas were calculated according to baseline projection mode in a baseline to valley integration. For quantification, calibration curves were generated by injection of a standard compound Gomphrenin III ($y = 14670x - 19725$, $R^2 = 0.9997$): results were expressed as mg per g of plant dry weight (dw).

Table 9 Chromatographic conditions for betacyanin compounds

HPLC-DAD-ESI/MS operating conditions for Betacyanin compounds	
System	Dionex Ultimate 3000 UPLC instrument (Thermo Scientific, San Jose, CA, USA)
Column	Waters Spherisorb S3 ODS-2 C18, (3 μm, 4.6 mm× 150 mm, Waters, Milford, MA, USA)
Detectors	DAD coupled to MS equipped with as ESI source; using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA)
Mobile phase/Gradient	(A) 0.1% trifluoracetic acid (TFA) in water and (B) acetonitrile. The gradient elution performed using a flow rate of 0.5 mL/min, was as follows: 10% B for 3 min, from 10 to 15% B for 12 min, 15% B for 5 min, from 15 to 18% B for 5 min, from 18 to 30% B for 20 min, from 30 to 35% B for 5 min, and from 35 to 10% B for 10 min, resulting in a total run time of 60 min, plus 10 min for column reconditioning.
Flux	0.5 mL/min
Wavelength	530 nm
Temperature	35°C
Software	Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA)

1.3.2 Organic Acids

1.3.2.1 Method Principles

These bioactive compounds, are considered weak acids, soluble in water and organic solvents. Organic acids are characterized by a carboxyl group (-COOH), that when it dissociates in the proton and its conjugated base, they manifest their acidic properties. Its well known that this compounds contribute largely on the organoleptic properties fresh fruit (Baccichet et al., 2021), and some of them play important roles in the Krebs cycle, making them essential for humans, and plants. Organic acid determination was carried out using a UFLC (ultra-fast liquid chromatography) coupled to a DAD detector. This analysis was carried out for all the studied plants following the procedure previously reported by Perreira et al., (2013).

1.3.2.2 Solvents, reagents and materials

Metaphosphoric and sulfuric acid were acquired from Fisher Scientific (Lisbon, Portugal), the standards of organic acids, namely L(+)-ascorbic acid, citric acid, malic acid, oxalic acid, shikimic acid and fumaric acid were purchased from Sigma (St Louis, Missouri, USA), the other reagents and solvents were acquired from scientific retailers, water was treated using the previously mention purification system.

1.3.2.3 Methodology

In brief, 25 mL of metaphosphoric acid at 4.5% was used to extract 1 g of each sample. The extraction was performed in a magnetic stirrer plate (25 ºC, 150 rpm, 1 h), protected from light, them the obtained extracts for each sample, were filtered (Whatman No. 4 paper). The recovered filtrate was again filtered through Whatman HPLC filter (0.2 μm) filter into HPLC vials.

The chromatographic methodology followed for these compounds was previously described by Perreira et al., (2013). And the operating conditions are expressed in **Table 10.** The analysis was performed on a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan) coupled to a PDA detector (Shimadzu), using as preferred wavelengths 215 nm and 245 nm (for ascorbic acid). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm × 4.6 mm i.d.) thermostated at 35 ºC. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. The organic acids found were quantified by

comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound: oxalic acid, ($y=9\times10^{6}x$; $R^2=0.99$); malic acid ($y=912441x$; $R^2=0.99$); citric acid ($y=1\times10^{6}x$; $R^2=0.99$); shikimic acid ($y=9E+07x$; $R^2=0.99$); fumaric acid ($y=148083x$; $R^2=0.99$); Ascorbic acid ($y=4\times10^{7}x+1\times10^{6}$; $R^2=0.99$). The results were expressed in g per 100 g of plant dry weight (dw).

Table 10 Chromatographic conditions for organic acids detection

UFLC-PDA operating conditions for organic acids	
System	Shimadzu 20 A series UFLC (Shimadzu Corporation, Kyoto, Japan)
Column	SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d.)
Detectors	PDA Shimadzu detector
Mobile phase/Gradient	3.6 mM sulphuric acid
Flux	0.8 mL/min
Wavelength	215 and 245 nm (ascorbic acid)
Temperature	35°C
Software	LabSolutions, LCsolutions Version 1.25

1.3.3 Tocopherols

1.3.3.1 Method principles

Vitamin E is composed by 8 different vitamers (4 tocopherols and 4 tocotrienols), namely α-, β-, γ-, δ-tocopherol/tocotrienols. These compounds due to their capacity to stop lipid peroxidation by donating hydrogen to unstable molecules, are very important in the antioxidant capacity of plants and extracts. Other major asset it is the capacity to establish a synergic bound with ascorbic acid, by regenerating them to continue scavenging free radicals (Carocho & Ferreira, 2013). Tocopherols were analyzed through HPLC coupled to a fluorescent detector using the internal standard (IS) method, following the procedure reported by Barros et al. (2013). This analysis was carried out for all the studied plants.

1.3.3.2 Solvents and reagents

Methanol, hexane (HPLC grade) and ethyl acetate (HPLC grade) were acquired from Fisher Scientific (Lisbon, Portugal), butylated hydroxytoluene (BHT) from Panreac (Barcelona, Spain) and Tocol (IS) from Matreya (State College, Pennsylvania, USA), the other reagents and solvents were acquired from scientific retailers, water was treated using the previously mention purification system.

1.3.3.3 Methodology

Given the high sensibility of these compounds to light and temperature, all the methodology was carried out with the least light possible and with an ice bath to maintain the extracts cold. In brief, 500 mg of the samples were added to 400 μL of tocol (IS) at 50 μg/mL, 100 μL of BHT (antioxidant acting as tocopherol protector); 4 mL of methanol and hexane, and 2 mL of aqueous solution of NaCl were added sequentially, followed by a vortex shake for 30 seconds after every addition; the solution was centrifuged (Centurion, K2Or-2003, West Sussex, UK) at 4,000 g for 5 min at 10 °C; the top phase was removed to a vial covered with aluminium foil, another 4 mL of hexane were added and all the process was performed twice removing the top phase into the same vial once again; two small spoons of anhydrous sodium sulphate were added to the vial to remove any reminiscent aqueous phase; the hexane was evaporated using nitrogen current; the remaining residue was re-dissolved in 2 mL of hexane (HPLC grade), filtered through Whatman HPLC filter (0.2 μm) into HPLC vials for further analysis.

The chromatographic methodology followed was previously described by Barros et al. (2011), and the system described on **Table 11.** The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), autosampler (Jasco AS-2057, Easton, MD, USA), and a fluorescence detector (Jasco FP- 2020) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters (Dinslaken, Germany) operating at 30°C (7971 R Grace oven). The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on calibration curves obtained from commercial standards of each compound using the internal standard (IS) methodology: α-tocopherol ($Y=1.52801X$; $R^2=0.99$); β-tocopherol ($Y=0.4573X$; $R^2=0.99$); γ-tocopherol ($Y=0.40211X$; $R^2=0.99$); δ-tocopherol ($Y=0.553X$; $R^2=0.99$): racemic tocol was used as IS. The results were expressed in μg per g of plant dry weight.

Table 11 Chromatographic conditions for tocopherols detection

HPLC-Fluorescence operating conditions for tocopherols	
System	Pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), autosampler (Jasco AS-2057, Easton, MD, USA)
Column	Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters (Dinslaken, Germany)
Detectors	Fluorescence detector (Jasco FP- 2020, Easton, Maryland USA)
Mobile phase/Gradient	Hexane and ethyl acetate (70:30, v/v)
Flux	1 mL/min
Wavelength	Excitation at 290 nm and emission at 330 nm
Temperature	30°C
Software	DataApex Clarity, Version 2.4.1.43

2 Betacyanins colouring compounds extraction optimization

2.1 Optimization of the extraction by RSM

The extraction procedure depends on several process variables whose values cannot be generalized for all matrices due to their specificity in terms of composition and target compounds. Thus, the optimization of the variables involved in the process is needed to select the best conditions to ensure a maximum yield, minimum time, energy and solvent consumption, squeezing the utmost from the extraction system. Traditionally, optimization is achieved by monitoring the influence of one factor at a time. However, by using the response surface methodology (RSM), optimization is done simultaneously and in a more precise manner obtaining polynomial models able to describe within the experimental range tested the optimal conditions that maximize the response criteria used (Bezerra et al., 2008; Ferreira et al., 2007; Kalil & Maugeri, 2000).

2.1.1 Dynamic Maceration

The powdered samples of the selected pigmented parts were extracted at different time (t), temperature (T), water-ethanol proportion (Et) and solid/liquid ratio (S/L) ranging as defined by the RSM design (**Table 12**). The solvent volume was fixed at 20 mL and samples were stirred using a CIMAREC i Magnetic Stirrer with a fixed agitation speed (500 rpm, Thermo Scientific, San Jose, CA, USA). After that, the mixture was filtered and centrifuged at 14,000 rpm for 10 min. The pellet was discarded and the supernatant was carefully collected and divided in two parts. One part was used to quantify the powder yield extract and its light intensity. The second part of the supernatant was employed to quantify the total betacyanin compounds content by: 1) reading the extracts absorbance

in a spectrophotometer (AnalytikJena, Jena, Germany) at 530 nm (subtracting the turbidity absorbance obtained at 650 nm); and 2) HPLC-PDA-MS/ESI analysis identifying the individual betacyanin compounds.

2.1.2 Ultrasound assisted extraction

The UAE was carried out using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA), equipped with a digital timer, and working in the range of 100–500 W, at a frequency of 20 kHz. The used solvent volume was settled at 50 mL. The samples were extracted according to different conditions regarding t, power (P), Et and S/L, as defined by the RSM design (**Table 12**). After the microwave and ultrasonic extractions, the mixtures were centrifuged at 14000 rpm for 10 min. The solid was discarded and the supernatant divided into two parts. One part was used to quantify the extraction yield and measure the light intensity using a colorimeter (Minolta spectrophotometer, Konica Minolta Sensing, Inc., Chroma Meter CR-400, Japan). The second part was carefully collected into a glass vial, filtered through 0.2 μm nylon filters (Whatman), and used directly to quantify the total betacyanin content by HPLC-DAD analysis.

2.1.3 Microwave assisted extraction

The MAE process was performed in a Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) in closed vessels of high-precision glass. The solvent volume, an ethanol-water mixture, was fixed at 20 mL. The samples were extracted according to different conditions regarding time (t), temperature (T), ethanol content (Et) and solid/liquid ratio (S/L), defined as the independent variables by the RSM design (**Table 12**). During processing, samples were stirred at 600 rpm using a magnetic stirring bar. After applying the established microwave power and time, the mixture in the extraction vessel was quickly cooled in the processing chamber.

Table 12 Experimental domain of the response surface experimental plan for the optimization of independent variables of time (*t*), temperature (*T*)/power (P), ethanol content (*Et*) and solid-liquid ratio (*S/L*) of the DM, MAE, UAE for the extraction of betacyanins.

				DM CONDITIONS				MAE CONDITIONS				UAE CONDITIONS			
	X_2	X_3	X_4	X_1 *t* min	X_2 *T* ºC	X_3 *Et* %	X_4 *S/L* g/L	X_1 *t* min	X_2 *T* ºC	X_3 *Et* %	X_4 *S/L* g/L	X_1 *t* min	X_2 *P* w	X_3 *Et* %	X_4 *S/L* g/L
1	-1	-1	-1	45	40	25	15	5	90	25	15	7	55	25	15
2	-1	-1	-1	125	40	25	15	15	90	25	15	17	55	25	15
3	1	-1	-1	45	70	25	15	5	150	25	15	7	85	25	15
4	1	-1	-1	125	70	25	15	15	150	25	15	17	85	25	15
5	-1	1	-1	45	40	75	15	5	90	75	15	7	55	75	15
6	-1	1	-1	125	40	75	15	15	90	75	15	17	55	75	15
7	1	1	-1	45	70	75	15	5	150	75	15	7	85	75	15
8	1	1	-1	125	70	75	15	15	150	75	15	17	85	75	15
9	-1	-1	1	45	40	25	35	5	90	25	35	7	55	25	35
10	-1	-1	1	125	40	25	35	15	90	25	35	17	55	25	35
11	1	-1	1	45	70	25	35	5	150	25	35	7	85	25	35
12	1	-1	1	125	70	25	35	15	150	25	35	17	85	25	35
13	-1	1	1	45	40	75	35	5	90	75	35	7	55	75	35
14	-1	1	1	125	40	75	35	15	90	75	35	17	55	75	35
15	1	1	1	45	70	75	35	5	150	75	35	7	85	75	35
16	1	1	1	125	70	75	35	15	150	75	35	17	85	75	35
17	0	0	0	5	55	50	25	0	120	50	25	2	70	50	25
18	0	0	0	165	55	50	25	20	120	50	25	22	70	50	25
19	-2	0	0	85	25	50	25	10	60	50	25	12	40	50	25
20	2	0	0	85	85	50	25	10	180	50	25	12	100	50	25
21	0	-2	0	85	55	0	25	10	120	0	25	12	70	0	25
22	0	2	0	85	55	100	25	10	120	100	25	12	70	100	25
23	0	0	-2	85	55	50	5	10	120	50	5	12	70	50	5
24	0	0	2	85	55	50	45	10	120	50	45	12	70	50	45
25	0	0	0	85	55	50	25	10	120	50	25	12	70	50	25
26	0	0	0	85	55	50	25	10	120	50	25	12	70	50	25
27	0	0	0	85	55	50	25	10	120	50	25	12	70	50	25
28	0	0	0	85	55	50	25	10	120	50	25	12	70	50	25

2.2 Betacyanins extract Bioactive properties

Betacyanins have been described as possessing many desirable properties, such as antioxidant, antimicrobial activity (antibacterial and antifungal), and the ability to inhibit cell growth and induce ultra-structural changes and cell fragmentation on carcinogenic cells. These red-purple pigments constitute an optimal example of natural additives used as food colorants, displaying noticeable coloring attributes, as well as various in vitro and in vivo biological properties.

2.2.1 Oxidative haemolysis inhibition assay (OxHLIA).

2.2.1.1 Solvent reagents and materials

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid), tris(hydroxymethyl) aminomethane (Tris) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.1.2 Methodology

Accordingly, with Lockowandt et al., (2019), sheep blood samples were collected from healthy animals and centrifuged at 1,000 g for 5 min at 10 °C. Plasma and buffy coats were discarded and erythrocytes were first washed once with NaCl (150 mM) and three times with phosphate-buffered saline (PBS, pH 7.4). The erythrocyte pellet was then resuspended in PBS at 2.8% (v/v). Using a flat bottom 48-well microplate, 200 µL of erythrocyte solution was mixed with 400 µL of either PBS solution (control), antioxidant sample dissolved in PBS, or water (for complete haemolysis). Trolox was used as positive control. After pre-incubation at 37 °C for 10 min with shaking, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 160 mM in PBS, 200 µL) was added and the optical density was measured at 690 nm. After that, the microplate was incubated under the same conditions and the optical density was measured every 10 min at the same wavelength for approximately 400 min. The percentage of the erythrocyte population that remained intact (P) was calculated according to the equation, where St 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 the complete haemolysis at 0 min:

$$P(\%) = \left(\frac{St - CHO}{SO - CHO}\right) \times 100$$

The results were expressed as delayed time of haemolysis (Δt), which was calculated according to the following equation, where Ht_{50} is the 50% haemolytic time (min) graphically obtained from the haemolysis curve of each antioxidant sample concentration.

$$\Delta t\ (\text{min}) = \text{Ht50 (sample)} - \text{Ht50 (control)}$$

The Δt values were then correlated to antioxidant sample concentrations and, from the correlation obtained, the extract concentration able to promote a Δt haemolysis delay was calculated. The results were given as IC_{50} values (µg/mL) at Δt 60 and 120 min, i.e., extract concentration required to keep 50% of the erythrocyte population intact for 60 and 120 min.

2.2.2 Antimicrobial activity

2.2.2.1 Solvents, reagents and materials

Dimethylsulfoxide (DMSO) (Merck KGaA, Germany) was used as a solvent in antimicrobial assays. The culture media Mueller Hinton broth (MHB) and tryptic soy broth (TSB) were purchased from Biomerieux (Marcy l'Etoile, France). The dye p-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St Louis, MO, USA) to be used as a microbial growth indicator.

2.2.2.2 Antibacterial activity methodology

The following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13311), *Morganella morganii* (ATCC XXXX), *Proteus mirabilis* (ATCC XXXX), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Enterococcus faecalis* (ATCC xxxx), and *Listeria monocytogenes* (NCTC 7973) were used. The microorganisms were obtained from the Mycological laboratory, Department of Plant Physiology, Institute for biological research "Siniša Stanković", University of Belgrade, Serbia. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method. Each fresh overnight culture of bacteria was adjusted spectrophotometrically to a concentration of 1×10^5 CFU/mL. The requested CFU corresponded to a bacterial suspension determined in a spectrophotometer at 625 nm (OD625). Dilutions of inocula were cultured on solid medium to verify the absence of contamination and check the validity of each inoculum. Different solvent dilutions of the extracts were added to the wells containing 100 µL of Tryptic Soy Broth (TSB) and afterwards, 10 µL of inoculum was added to all wells. The microplates were incubated for 24 h at 37 °C. The MIC of the samples was detected following the addition of 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 minimum inhibitory concentrations (MICs) obtained from the susceptibility testing of various bacteria to the tested extracts were determined also by a colorimetric microbial viability assay based on the reduction of the INT colour and compared with a positive control for each bacterial strain. MBC 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 read as the MBC. Standard drugs, namely streptomycin and ampicillin, were used as positive controls. 5% dimethyl sulfoxide was used as the negative control. Samples were tested in duplicate and experiments were repeated three times.

2.2.2.3 Antifungal activity Methodology

For the antifungal bioassays, the following microfungi were used: *Aspergillus flavus* (ATCC 1022), *Aspergillus niger* (ATCC 6275), *Penicillium ochrochloron* (ATCC 9112), and *Penicillium verrucosum var. cyclopium* (food isolate). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, 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 1.0×10^5 in a final volume of 100 µL per well. The inocula were stored at 4 °C for further use. Dilutions of each inoculum were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtitre plates. The investigated extract was dissolved in a 5% solution of dimethyl sulfoxide and added to broth malt medium with a fungal inoculum. The microplates were incubated for 72 h at 28 °C. The lowest concentrations without visible growth (as assessed using a binocular microscope) were defined as the MICs. The minimum fungicidal concentrations (MFCs) 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% dimethyl sulfoxide was used as a negative control, while bifonazole and ketoconazole were used as positive controls. Samples were tested in duplicate and experiments were repeated three times.

2.2.3 Antiproliferative activity

2.2.3.1 Solvents, reagents and materials

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 purchased from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Griess Reagent System Kit was purchased from Promega (Madison, WI, USA). Human tumor cell lines tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma) from DSMZ (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH).

2.2.3.2 Methodology

Following the procedure previously described by Vaz et al. (2010). The lyophilized extracts were re-dissolved in water to obtain stock solutions of 4 mg/mL, and then submitted to further dilutions. Four human tumor cell lines were tested: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma), and HepG2 (hepatocellular carcinoma). Sulforhodamine B assay was performed according to a procedure previously described by Barros, Pereira et al. (2013). Each of the cell lines were plated in a 96-well plate, at an appropriate density (1.0 × 104 cells/well) 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 deionized water and dried. 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 solubilized with Tris (10 mM, 200 μL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA).

2.2.4 Hepatotoxicity

2.2.4.1 Solvents, reagents and material

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 purchased from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma-

Aldrich (St. Louis, MO, USA). The Griess Reagent System Kit was purchased from Promega (Madison, WI, USA).

2.2.4.2 Methodology

Accordingly, with Perreira et al., (2013). A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse. It was designated as PLP2. The liver tissues were rinsed in Hank's balanced salt solution containing 100 U m/L penicillin and 100 μg/mL streptomycin and divided into 1 × 1 mm3 explants; some of these explants were placed in 25 cm3 tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids, 100 U m/L penicillin and 100 μg/mL streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2; The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2–3 days using a phase contrast microscope; Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U mL penicillin and 100 μg mL streptomycin. Cells were treated for 48 h with the different diluted sample solutions and the same procedure described in the previous section for the SRB assay was followed. The results were expressed as GI_{50} values (sample concentrations that inhibited 50% of the net cell growth). Ellipticine was used as a positive control.

3 Spray drying encapsulation

According to Molina et al., (2019), for spray-drying, the samples were dried using 20% of maltodextrin (w/w), a drying adjuvant. The percentage of maltodextrin (20%, w/w) was relative to the total solids content of the extract sample to be spray-dried, established in the aforementioned study. The solutions containing the betacyanins and maltodextrin were prepared immediately before atomization. Briefly, the extracts were mixed with maltodextrin and further homogenized by stirring for 10 min at room temperature. The used spray-drying equipment was a Mini Spray Dryer B-290 Büchi (Flawil, Switzerland) programmed in normal operation mode (nozzle diameter: 0.7 mm; atomized volume: 200 mL, solids content < 33%). The used operation conditions were established according to previous works using this drying co-adjuvant (inlet temperature 140 °C, outlet temperature 72 °C, aspiration 90% and pump 20% (6 mL/min)). The collected dry samples were kept in sterile flasks protected from light (4 °C) until further analysis. The

overall yield was estimated as the ratio between the weight of recovered powder (dry basis) and the weight of the initial solids in the atomized solution (dry basis).

4 Incorporation of the natural colourant extract in food matrices

The incorporation of the extracts in the different foods was carried out manually, during the process all the hygienic precautions were considered to avoid contamination.
After incorporation, the novel developed food products were fully characterized.

4.1 Incorporation in Ice cream

4.1.1 Recipe and manufacture

Ice cream was prepared from a base recipe: 240 g of sugar were mixed with 500 mL of milk (UHT entire milk, Continente, Portugal), in order to dissolve the sugar; Meanwhile, 1000 mL of double cream (UHT cream, Continente, Portugal) were used to obtain whipped cream, which was previously added to the mixture; The final batter was left to stand for 12 h, further divided in four batches, each placed in an ice- cream machine equipment (Ice cream Maker SECN 12 A1, SilverCrest, Hamburg, Germany).

4.1.2 Details of the incorporated concentrations

The ice cream batches were identified as:

i) control (ice cream without colouring agents);
ii) ice cream with betalain standard (200 mg, i.e. ≈46 mg/100 g ice cream);
iii) ice cream with *G. globosa* extract (670 mg, i.e. ≈154 mg/100 g ice cream);
iv) ice cream with *B. vulgaris* extract (670 mg, i.e. ≈154 mg/100 g ice cream).

The amount of each ingredient was added in order to obtain the desired colour. Moreover, the added quantities were different because commercial betalain (Sigma-Aldrich, St. Louis, MO, USA) is an isolated compound, obviously with a higher degree of purity in comparison with the extracts from *G. globosa* and *B. vulgaris* extract was obtained by grinding the sample with 10% of water, followed by a filtration step and freeze prior to its lyophilization (FreeZone 4.5, Labconco, Kansas City, MO, USA).

4.1.3 Preparatory procedures

The samples were analysed immediately after preparation and after: 15, 30, 45 and 60 days (maximum desired storage limit for this type of ice cream) of storage at –22 °C.

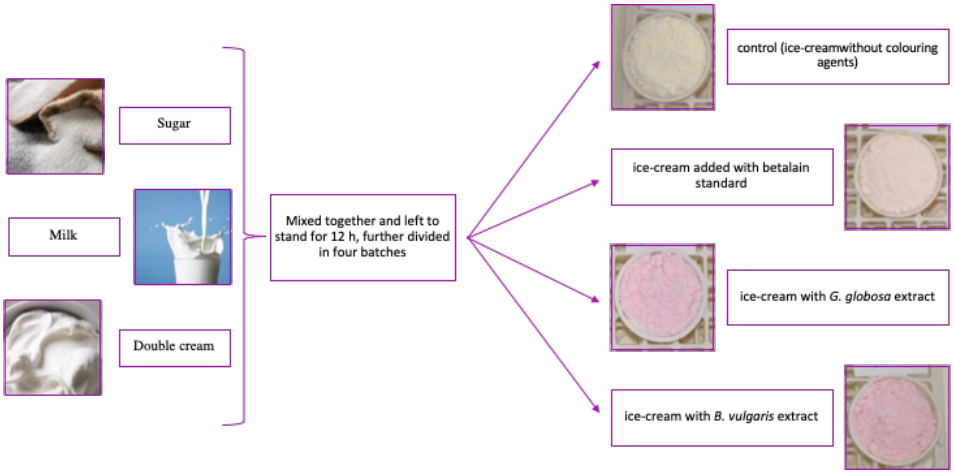


Figure 9 Illustrative scheme of the manufacture of ice cream. Author Custódio Lobo Roriz

4.1.4 Recipe and manufacture

Cookie dough was prepared following a eggless recipe: 450 g of butter (pasteurized butter, Continente®, Portugal) was mixed with 540 g of sugar (granulated white sugar, Continente, Portugal); Then, 840 g of flour (self-raising wheat flour, Continente, Portugal), 20 g of yeast powder (yeast powder, Continente®, Portugal), 50 g of water and 100 g of vegetable oil (cooking oil, Continente®, Portugal) mixed with a stand mixer (Food Processor SKM 550 A1, SilverCrest, Hamburg, Germany), further divided in four equivalent batches

4.1.5 Details of the incorporated concentrations

The cookie dough equivalent batches were identified as:

i) control (cookie dough without colouring agents);

ii) cookie dough coloured with E162 (commercial natural food colourant; 2 g, i.e. ≈4 mg/100 g cookie dough);

iii) cookie dough coloured with lyophilized *G. globosa* extract (1 g, i.e. ≈2 mg/100 g cookie dough);

iv) cookie dough coloured with spray- dried *G. globosa* extract (1 g, i.e. ≈2 mg/100 g cookie dough).

For the batches where the colourant was added, the dough was continuously kneaded until the dough colour presented was homogeneous. For each batch, the cookie dough was divided into approximately 12 g balls, on a tray lined with parchment paper and baked for 25 min at ≈ 140 °C.

4.1.6 Preparatory procedures

After baking, the cookies were separated to be analysed through at four different storage times, using 10 cookies per time. Prior to analysis, all cookie samples were lyophilized, finely crushed and analysed (in triplicate), immediately after preparation, and at three more sampling times (7; 15; and 30 days of storage). Cookies were stored at room temperature and packed in a sealed plastic bag (zip lock bag, ≅ 0,1mm of thickness) covered with aluminium paper.

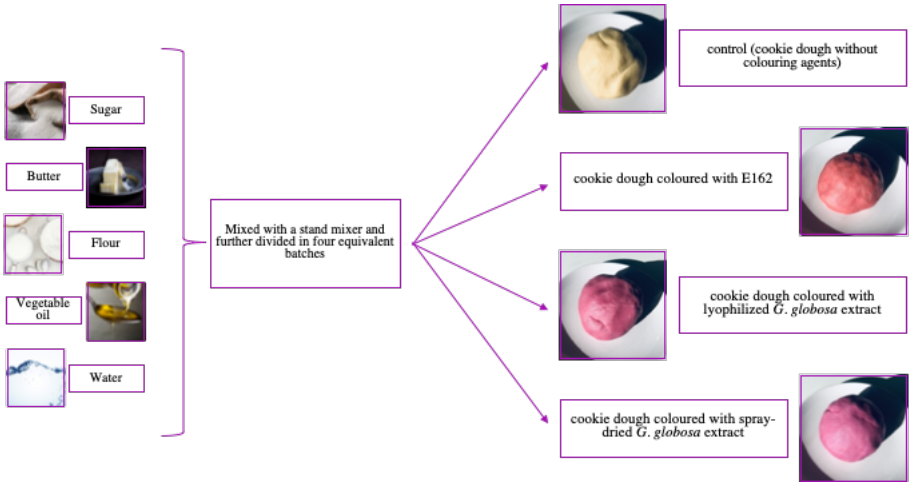


Figure 10 Illustrative scheme of the manufacture of cookies. Author Custódio Lobo Roriz

4.2 Novel developed foodstuffs characterization

4.2.1 Colour measurement

Ice cream

For the incorporation in the ice cream, the colour was determined on the ice cream for the various storage times, and the lyophilisate resulting from each of them. The CIE colour parameters were measured in three different points for each sample using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The illuminant C and a

diaphragm aperture of 8 mm were selected. The Hunter colour L*(lightness), a*(redness) and b*(yellowness) values were registered using the data software "Spectra Magic Nx" (version CM-S100W 2.03.0006, Konica Minolta Company, Japan). The instrument was calibrated to standard white tiles before analysis (Spectra Magic NX Instruction Manual, Konica Minolta Sensing, Inc. (ver. 2.0), 2009, Japan)

Cookies

The external colour was measured in three different points of the cookies for the various storage times. Colour was measured on the exterior and interior of the cookie, as well as on the powder of ground cookies. This assay was performed with a portable CR400 colourimeter from Konica Minolta (Chiyoda, Tokyo, Japan) with the D65 illuminant, a standard illuminant defined by the International Commission on Illumination (CIE) which represents the midday light in Europe (daylight illuminant). The CIE L* a* b* colour space of 1976 was used, with L* representing lightness, a* representing redness (red- green), and b* representing yellowness (yellow-blue), with a 10° observation angle and 8 mm aperture.

4.2.2 Determination of nutritional parameters

4.2.2.1 Moisture

In order to measure the moisture present in the food samples, 2 g were put in a metal plate and placed in a moisture analyzer (Adam Equipment, PMB 163). This equipment increases the temperature gradually to 105 °C to force moisture to evaporate from the food sample. When the weight achieves a constant value, i.e. no evaporation is detected, the sample is weighed a second time. The results were obtained using the following equation:

$$\% \text{ Moisture} = (mi-mf)/mi \times 100.$$

Where mi is the initial weight and mf is the weight after reaching a constant weight.

4.2.2.2 Salt

The salt determination was achieved using the Mohr's method as described by (Osaili et al., 2014). Initially, 1 g of the sample was homogenized in 20 mL of distillated water and filtered through a Whatman No.4 paper. The powder was then homogenized with an

additional portion of 20 mL and this procedure was repeated a total of five times. Then the pH of the aqueous solution was adjusted to 8.5 with sodium hydroxide and 1 mL of potassium chromate solution (5%) was added. The mixture was titrated against $AgNO_3$ (0.05 mol/L until the appearance of the first reddish color (Ag_2CrO_4 precipitate). The salt concentration was calculated using the following equation:

Salt content% = $[(Vtitrated\ of\ AgNO3\ x\ 0.00292)]\ /\ [(m\ sample)]\ x\ 100$

where 1 mL of $AgNO_3$ corresponds to 0.00292 g of NaCl. The results were expressed in g/100 g of fresh weight

4.2.2.3 Crude Protein

The Kjeldahl method was applied to assess the protein content on both foodstuff samples. The nitrogen conversion factor for the ice cream was 6.38, while for the cookies it was 5.70, according to AOAC (2010). According to this method, the amount of nitrogen present in the sample can indicate the amount of crude proteins. It relies on the destruction of all organic matter by adding strong acid (sulphuric acid) and its capacity to retain the nitrogen present under the form of $(NH_4)_2SO_4$, subsequently the addition of NaOH will release the NH_3 (steam distillation) collected by H_2SO_4 N/10, and after a titration with NaOH N/10 using methyl red as indicator, a colour shift is observed. The assay was carried out in an automatic distillation and titration unit (model Pro-Nitro M Kjeldahl Steam Distillation System, Barcelona, Spain). The crude protein was expressed as g/100g FW.

4.2.2.4 Crude fat and fatty acids profile

Crude fat was assessed by different methodologies for the different foodstuff samples. In the ice cream crude fat was determined by Rose-Gottlieb method (AOAC 905.02, AOAC, 2016); in this method the sample is pretreated with ammonia, in order to dissolve the protein content, and ethyl alcohol to help precipitate them. Fat content is extracted with the help of diethyl ether and petroleum ether, subsequently evaporated and the remain residue, fat content, is weighed.

For the cookies the crude fat content was assessed by a classical methodology, the Soxhlet extraction procedure, which relies on the non-polarity of petroleum ether to extract the fat content from the sample, where the extractant is recirculated through the sample (30 circles) until extraction completed. The petroleum ether is evaporated and the remain residue, fat content, is weighed.

For both methodologies, the fat percentage is calculated according to the following equation:

$$Fat\ percentage(w/w) = \frac{Weight\ of\ fat}{Weight\ of\ sample} \times 100$$

The crude fat was expressed as g/100g of FW

Fatty acids were characterized according to the procedure previously reported by Barros et al. (2013). Due to its chemical characteristics, fatty acids are a reliable source of information to monitoring changes during the storage period, therefore individual fatty acids profile determination was carried out in both foodstuff by gas chromatography (GC), coupled to flame ionization detector (FID), with the condition described on **Table 13**.

4.2.2.4.1 Solvent reagents and material

Methanol and sulphuric acid were acquired from Fisher Scientific (Lisbon, Portugal), toluene and ethyl ether from Sigma (St. Louis, Missouri, USA) and sodium sulphate from Carlo Erba Reagents (Peypin, France). The fatty acids methyl esters (FAME) refernce standard mixture 37 (standard 47885-U) and other individual fatty acid isomers were purchased from Sigma (St. Louis, Missouri, USA), the other reagents and solvents were acquired from scientific retailers, water was treated using the previously mention purification system.

4.2.2.4.2 Methodology

Fatty acids (obtained after the crude fat extraction methods) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (v:v:v), during at least 12 h in a bath at 50 °C and 160 rpm; then 3 mL of deionized water and 3mL of ethyl ether were added, to obtain phase separation; the upper phase was recovered to a vial containing sodium sulphate anhydrous in order to eliminate the water; before injection the sample was filtered with 0.2 μm nylon filter from Whatman to a HPLC vial. The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey- Nagel (Düren, Germany) column (50% cyanopropyl- methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm df). The oven temperature programme was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C

and held for 15 min. The carrier gas (hydrogen) flow rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex, Podohradska, Czech Republic) and expressed in relative percentage of each fatty acid.

Table 13 Chromatographic conditions for fatty acids detection

GC-FID operating conditions for fatty acids	
System	DANI model GC 1000
Column	Macherey- Nagel (Düren, Germany) column (50% cyanopropyl- methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 μm df)
Detectors	Flame ionization detector
Carrier gas	Hydrogen
Flow rate	4 mL/min
Temperature	Variable
Software	DataApex Clarity, Version 2.4.4.43

4.2.2.5 Soluble sugars

4.2.2.5.1 Method principles

Since soluble sugars naturally occurred in plants, when a full chemical characterization is carried out, it's also important to detected them. For all this, but also due to the extraction solvent that is used in the recovery of betacyanin compounds, which is water, also manifests a great affinity for sugar molecules, and in this way, it may end up extracting this class of compounds together with the compounds of interest. Besides some soluble sugars, such as mannitol, sucrose and raffinose are responsible for antioxidant activity in plants. Soluble sugars determination was carried out by HPLC-RI for all the studied plants following the described method reported by Barros et al., (2013).

4.2.2.5.2 Solvents Reagents and materials

Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Lisbon, Portugal), the sugar standards (D(-)-fructose, D(+)-glucose anhydrous, D(+)-sucrose, trehalose and melezitose) were acquired from Sigma Chemical Co. (Saint Louis Missouri, USA), the other reagents and solvents were acquired from scientific retailers, water was treated using the previously mention purification system.

4.2.2.5.3 Methodology

40 mL of 80% aqueous ethanol was used to extract 1 g of each sample spiked with melezitose (IS, 5 mg/mL). The extraction was performed at 80 for 90 min, them the obtained suspension for each sample was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000 g for 10 min. The supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL and filtered through Whatman HPLC filter (0.2 μm) filter into HPLC vials.

Barros et al. (2010) previously describe the chromatographic methodology followed, and in **Table 14** showed the operating conditions. Soluble sugars were determined by HPLC coupled to a refraction index detector (RI). Analysis was performed by HPLC (equipment described above) using an RI detector (Knauer Smartline 2300, Berlin, Germany). Data were analyzed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer, Berlin, Germany) operating at 30 ºC. The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards: fructose (y=0.98177x; R^2=0.999); glucose (y=1.00778x; R^2=0.999); sucrose (y=0.95604x; R^2=0.999); trehalose (y=0.90995x; R^2=0.999). Quantification was performed using the internal standard method; melezitose was used as IS. The results were expressed in mg per g of dry weight.

Table 14 Chromatographic conditions for sugars detection

HPLC-RI operating conditions for sugars	
System	Pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), autosampler (Jasco AS-2057, Easton, MD, USA)
Column	Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer, Berlin, Germany)
Detectors	Refraction index Smartline 2300 (Knauer, Berlin, Germany)
Mobile phase/Gradient	Acetonitrile/deionized water, 70:30 (v/v)
Flux	1 mL/min
Wavelength	-
Temperature	30°C
Software	DataApex Clarity, Version 2.4.1.43

4.2.2.6 Ash

Ash content was analyzed on both samples by incineration in a muffle. This method relies on the destruction of all the organic matter by means of high temperature. The ash percentage was calculated according to the following equation:

$$Ash\ percentage(w/w) = \frac{Weight\ of\ ash}{Weight\ of\ sample} \times 100$$

The ash was expressed as g/100g of FW.

4.2.2.7 Total carbohydrates

In both foodstuff samples, the total carbohydrates were calculated by difference, according to the following equation:

$$Total\ carbohydrates = 100 - (fat + protein + ash)$$

The total carbohydrates where expressed as g/100g of FW.

4.2.2.8 Energy

Based on the European Parliament and Council Regulation, Nº.1169/2011. Energy value in both foodstuff samples was calculated according to the following equation:

$$Energy\ (kcal) = 4 \times (g\ protein + g\ carbohydrate) + 9 \times (g\ crude\ fat)$$

The energy was expressed in kcal/100g fw of ice cream or cookies.

5 Statistical Tools

All statistical tests were performed with IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA) considering a 5% significance level. Data were expressed as mean ± standard deviation (the number of significant numbers was maintained according to the standard deviation magnitude). For each ice cream formulation, three different samples were used.

Each of the samples were analysed in triplicate. A 2-way analysis of variance (ANOVA) with type III sums, using the general linear model (GLM) procedure was applied to compare all parameters among different formulations or storage times. Besides evaluating the effect of each factor, their interaction was also assessed. When no statistically significant interaction was found, the means were compared using Tukey's multiple comparison test, with a previous assessment of the equality of variances through a Levene's test. Otherwise, differences were analysed in the estimated marginal means plots obtained for all levels of each factor. In addition, a linear discriminant analysis (LDA)

was used to compare the effect of formulation and storage time over all parameters simultaneously. A stepwise technique was applied, based on the Wilks' ʎ test with the usual probabilities of F (3.84 to enter and 2.71 to be removed) for variable selection. Only variables with a statistically significant classification performance ($p < 0.005$) were maintained by the statistical model. This statistical classification tool was performed to estimate the relationship between single categorical dependent variables (ice cream formulations) and the quantitative independent variables (results obtained in laboratorial assays). A leaving-one-out cross validation procedure was carried out to assess the model performance.

Part V. RESULTS AND DISCUSSION

Article 1: Chemical and bioactive features of *Amaranthus caudatus* L. flowers and optimized ultrasound-assisted extraction of betalains

Reference: Roriz, C. L., Xavier, V., Heleno, S. A., Pinela, J., Dias, M. I., Calhelha, R. C., Morales, P., Ferreira, I. C. F. R. & Barros, L. (2021). *Foods*, 10(4), 779. DOI: https://doi.org/10.3390/foods10040779

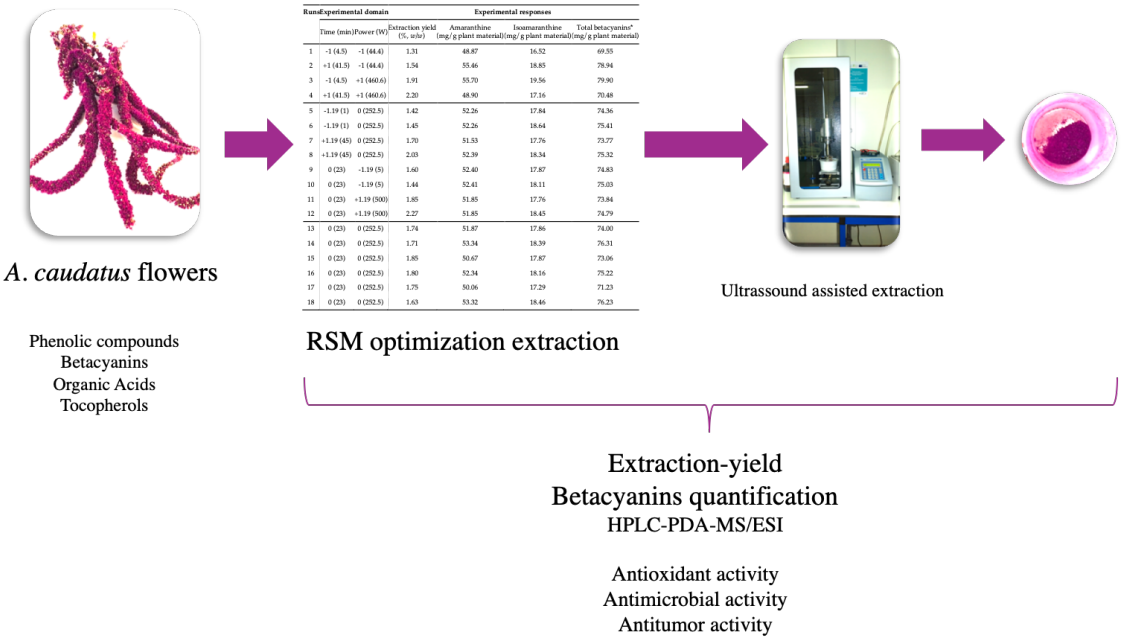


Figure 11 Graphical Abstract Article 1

Open Access Article

Chemical and Bioactive Features of *Amaranthus caudatus* L. Flowers and Optimized Ultrasound-Assisted Extraction of Betalains

by Custódio Lobo Roriz [1,2], Virginie Xavier [1], Sandrina A. Heleno [1,*], José Pinela [1], Maria Inês Dias [1], Ricardo C. Calhelha [1], Patricia Morales [2], Isabel C. F. R. Ferreira [1] and Lillian Barros [1,*]

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(This article belongs to the Special Issue Nutraceutical Components of Plants and Plant Seeds)

Resumen

Los colores vibrantes de muchas plantas se deben a metabolitos secundarios, como los compuestos que contienen nitrógeno, donde se incluyen las betacianinas. Estos compuestos se pueden encontrar en plantas como *Amaranthus caudatus* L., que, debido a su potencial nutricional, se ha sobre producido, lo que conduce a la acumulación de grandes cantidades de subproductos. Entre estos subproductos se encuentran las flores que tienen un color rosa muy intenso y no presentan actualmente valor económico ni otro destino alternativo, pero si podrían ser utilizadas como fuentes de colorantes naturales (betacianinas). Este trabajo tuvo como objetivo caracterizar el extracto de la flor de *A. caudatus* en términos de compuestos bioactivas como tocoferoles, ácidos orgánicos, pero fundamentalmente en términos de betacianinas, para obtener un colorante natural. Para la extracción, se obtuvieron condiciones ideales de extracción asistida por ultrasonido (EAU) utilizando la Metodología de Superficie de Respuesta (RSM), permitiendo obtener un extracto enriquecido en betacianinas con altos rendimientos y pureza. Los extractos obtenidos se analizaron por su potencial bioactivo, es decir, propiedades antioxidantes, antimicrobianas y citotóxicas. De los resultados obtenidos se detectaron tres vitameros de tocoferoles, siendo el β -tocoferol (0.884 mg/100g sss) el más abundante. En cuanto a los ácidos orgánicos, se caracterizó el oxálico (2.48 mg/100g sss), shikímico (0.17 mg/100g sss) y trazas de ácido fumárico. Además, se identificaron y cuantificaron cuatro betacianinas, a saber: amarantina (171mg/g de extracto), isomarantina (38 mg/g de extracto), betanina (1.6 mg/g de extracto) e isobetanina (1.3 mg/g de extracto). El extracto desarrollado también presentó actividad antioxidante con valores de CI_{50} de 29.0 μg/mL y 114 μg/mL para Δt de 60 min y 120 min, respectivamente en el ensayo OxHLIA. Este extracto también presentó una interesante actividad antibacteriana con concentraciones inhibitorias mínimas que oscilan entre 5-20 mg/mL frente a bacterias patógenas; y no mostró toxicidad para las células normales.

Abstract

The vibrant colours of many plants are due to secondary metabolites, such as nitrogen-containing compounds, where betacyanins are included. These compounds can be found in plants such as *Amaranthus caudatus* L., that due to its high nutritional benefits, has been overproduced, which leads to the accumulation of large amounts of bio-residues. Among these bio-residues, the flowers which have a very intense pink colour and present no economic value or subsequent destination, can be exploited as sources of natural colouring agents (betacyanins). This work aimed at characterizing the flower´s extract in terms of bioactive molecules such as tocopherols, organic acids, but essentially in terms of betacyanins, in order to obtain a natural colouring agent. For the extraction, ultrasound-assisted extraction (UAE) ideal conditions were obtained using the Response Surface Methodology (RSM), allowing to obtain an enriched extract in betacyanins in high yields and purity. The obtained extracts were analysed for their bioactive potential, namely antioxidant, antimicrobial and cytotoxic properties. From the obtained results, three isoforms of tocopherols were detected, being β-tocopherol (0.884± 0.003 mg/100g dw) de most abundant one. Regarding the organic acids, oxalic (2.48±0.05 mg/100g dw), shikimic (0.170± 0.003 mg/100g dw) and traces of fumaric acid were found. Four betacyanins were identified and quantified, namely: amaranthine (171±1 mg/g extract), isomaranthine (38±1 mg/g), betanin (1.6 ± 0.1 mg/g), and isobetanin (1.3 ± 0.1 mg/g). The obtained extract also presented antioxidant activity with IC50 values of 29.0±0.4 μg/mL and 114±4 μg/mL for Δt of 60 min and 120 min, respectively in the OxHLIA assay. The obtained extract also presented an interesting antibacterial activity with minimum inhibitory concentrations ranging from 5-20 mg/mL against pathogenic bacteria; and revealed no toxicity for normal cells.

Article 2. Red pitaya (*Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose.) peel as a source of valuable molecules: Extraction optimization to recover natural colouring agents

Custódio Lobo Roriz, Sandrina A. Heleno, Maria José Alves, M. Beatriz P.P. Oliveira, José Pinela, Maria Inês Dias, Ricardo C. Calhelha, Patricia Morales, Isabel C.F.R. Ferreira, & Lillian Barros.

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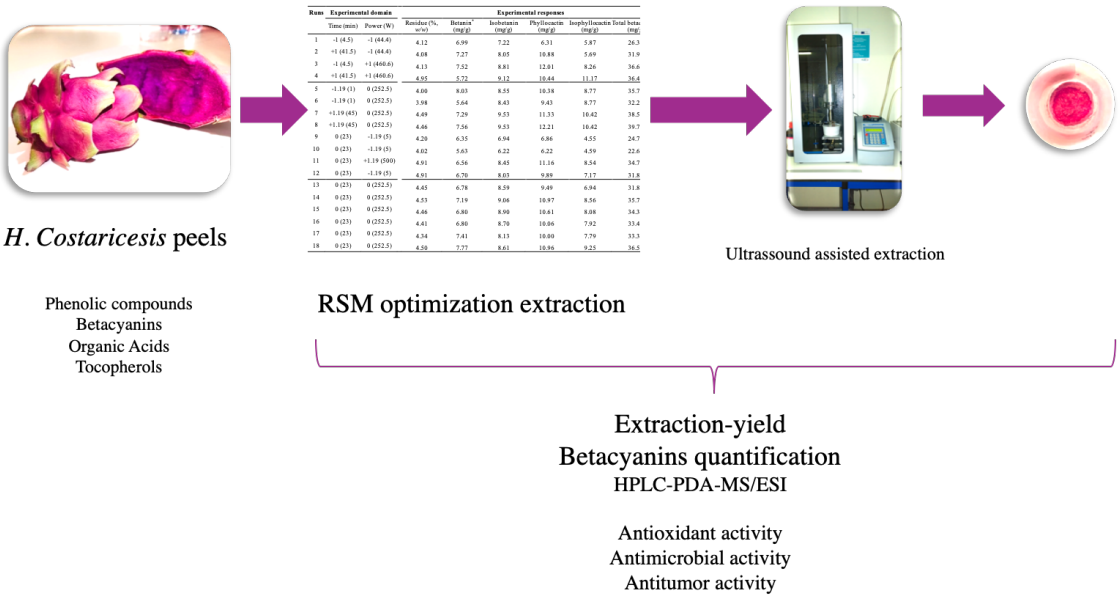

Figure 12 Graphical Abstract Article 2

Resumen:

Debido a su apariencia atractiva y visualmente impactante, las frutas de *Hylocereus* spp. se han consumido mucho en todo el mundo. Su fuerte color se debe a la presencia de betalaínas, más precisamente betacianinas, presentes en la piel y/o pulpa de estos frutos. Las cáscaras se descartan habitualmente y presentan una alta cantidad de estos compuestos, que pueden ser utilizados como fuente alternativa de colorantes naturales, revalorizando así una materia prima sin valor. El objetivo del presente trabajo fue, en primer lugar, caracterizar las cáscaras en términos de tocoferoles y ácidos orgánicos, y también optimizar las condiciones ideales para obtener un extracto enriquecido en betacianina mediante la extracción asistida por ultrasonidos y aplicando la metodología de superficie de respuesta. Además, como beneficio extra, se evaluó el extracto obtenido por sus propiedades bioactivas. De acuerdo con los resultados obtenidos, se verificó la

presencia de cantidades muy similares de ácidos oxálico y málico, y trazas de ácido fumárico. En cuanto a los tocoferoles, las cuatro vitámeros se encontraron en las cáscaras, siendo el γ- tocoferol el principal vitámero, seguido de los α, δ y finalmente los β-tocoferoles. Fue posible identificar y cuantificar cuatro betacianinas, a saber: filocactina, isobetanina, isofilocactina y betanina. En cuanto a las propiedades bioactivas, para la actividad antioxidante, el extracto presentó valores de IC^{50} de 255 μg/mL y 381 μg/mL para Δt de 60 y 120 min para el método de OxHLIA, y también presentó una interesante actividad antibacteriana con concentraciones inhibitorias mínimas que oscilan entre 5-20 mg/mL contra bacterias patógenas; no revelando toxicidad para las células normales.

Abstract

Due to its appealing and visually striking appearance, *Hylocereus* spp. fruits have been highly consumed worldwide. Their strong colour results from the presence of betalains, more precisely betacyanins, present on the peels and/or pulp of this fruits. The peels are usually discarded and present a high amount of these compounds, which can be exploited as an alternative source of natural colouring agents, thus valorising a non-valuable raw material. The aim of the present work was, firstly to characterize the peels in terms of tocopherols and organic acids, and also to optimize the ideal conditions to obtain a betacyanin enriched extract through the ultrasound assisted extraction and applying the response surface methodology. Furthermore, as an extra benefit the obtained extract was evaluated for its bioactive properties. According to the obtained results, the presence of very proximate amounts of oxalic and malic acids, and traces of fumaric acid was verified. Regarding tocopherols all the four isoforms were found in the peels, being γ-tocopherol the major vitamer, followed by the α, δ and finally β-tocopherols. It was possible to identify and quantify four betacyanins, namely: phyllocactin, isobetanin, isophyllocactin, and betanin. Concerning the bioactivities, for the antioxidant activity, the extract presented IC_{50} values of 255 μg/mL and 381 μg/mL for Δt of 60 and 120 min, and also presented an interesting antibacterial activity with minimum inhibitory concentrations ranging from 5-20 mg/mL against pathogenic bacteria; revealing no toxicity for normal cells.

Article 3: Floral parts of Gomphrena globosa L. as a novel alternative source of betacyanins: Optimization of the extraction using response surface methodology

Reference: Roriz, C. L., Barros, L., Prieto, M. A., Morales, P., & Ferreira, I. C. (2017). *Food chemistry*, 229, 223-234.

DOI: https://doi.org/10.1016/j.foodchem.2017.02.073

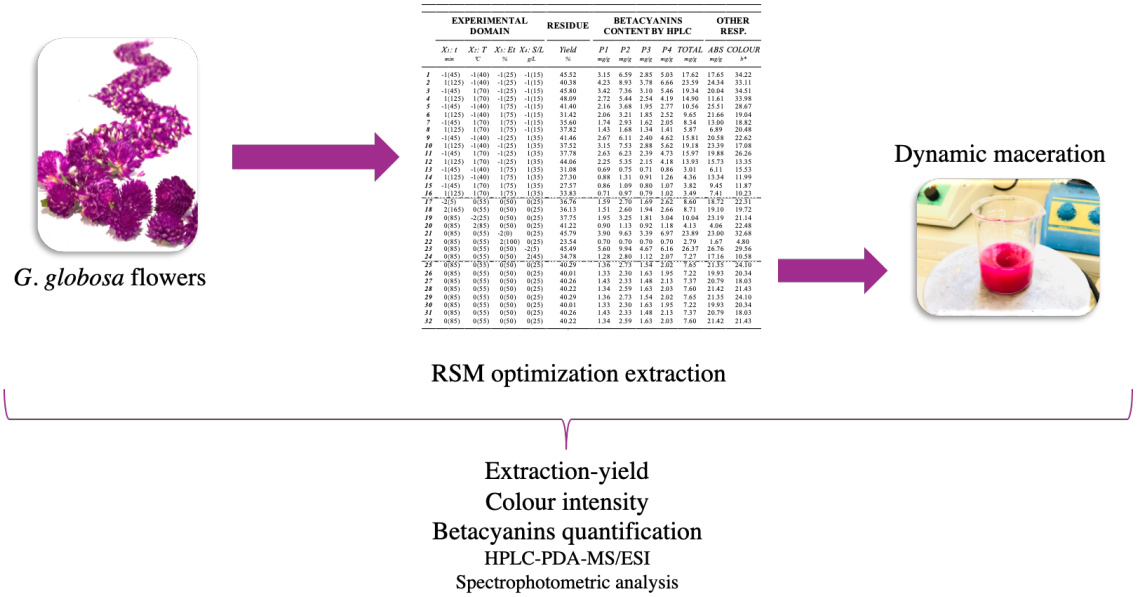

Figure 14 Graphical Abstract Article 3

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Food Chemistry

Volume 229, 15 August 2017, Pages 223-234

Floral parts of *Gomphrena globosa* L. as a novel alternative source of betacyanins: Optimization of the extraction using response surface methodology

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Resumen

El presente estudio describe un novedoso proceso de extracción por maceración de betacianinas a partir de las partes pigmentadas de *Gomphrena globosa* L. con la ayuda de la metodología de superficie de respuesta (RSM) optimizando las condiciones que maximizan la extracción de estos compuestos. Para ello se diseñó un modelo matemático permitía seleccionar las variables de extracción (tiempo, temperatura, proporción etanol-agua y relación sólido-líquido) en base a los resultados de cuantificación de betacianinas (mediante HPLC-PDA-MS/ESI y análisis espectrofotométrico), el rendimiento de extracción y la intensidad del color del polvo producido. Las betacianinas identificadas fueron gomphrenina e isogomphrenina II y III. El contenido más elevadode betacianinas (45 mg / g) se observó a 165 min, 25 ° C, 0% de etanol y 5 g / L de proporción sólido-líquido. El contenido de betacianinas de las partes florales de *G. globosa* es superior a los que se encuentran normalmente en otras fuentes destacando su aplicación industrial.

Abstract

The present study describes a novel mechanical process for the pigmented parts of *Gomphrena globosa* L. The effects of the variables of the maceration extraction of betacyanins have not been properly described. Therefore, this study also aims to optimize the conditions that maximize betacyanins extraction from *G. globosa* as an alternative source. Assisted by response surface methodology, an experimental design was developed for testing the extraction variables (time, temperature, ethanol-water proportion and solid-liquid ratio). The responses used were betacyanins quantification (by HPLC-PDA-MS/ESI and spectrophotometric analysis), the extraction-yield and the colour intensity of the produced powder. The betacyanins identified were gomphrenin and isogomphrenin II and III. The highest betacyanins content (~45 mg/g) was obtained by 165 min, 25 °C, 0% of ethanol and 5 g/L of solid-liquid ratio. The betacyanins content from the floral parts of *G. globosa* is higher than those normally found in other sources highlighting its industrial application.

Article 4. Modern extraction techniques optimized to extract betacyanins from *Gomphrena globosa* L.

Reference: Roriz, C. L., Barros, L., Prieto, M. A., Barreiro, M. F., Morales, P., & Ferreira, I. C. (2017). *Industrial Crops and Products*, 105, 29-40

DOI: https://doi.org/10.1016/j.indcrop.2017.05.008

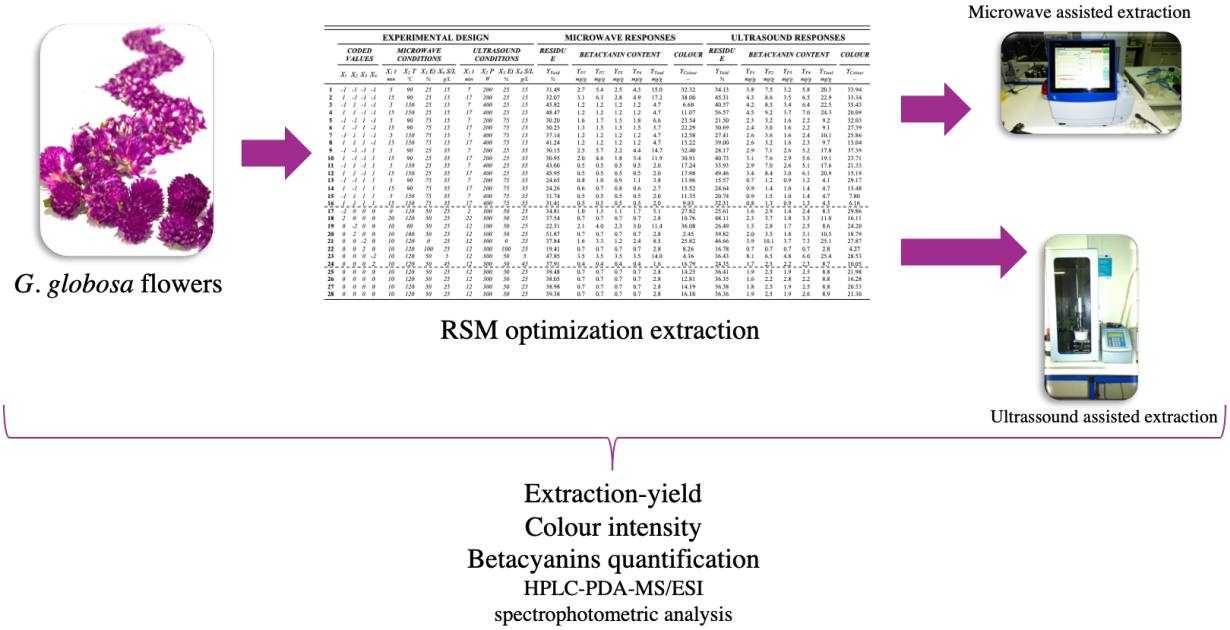

Figure 15 Graphical Abstract Article 4

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Industrial Crops and Products
Volume 105, 15 October 2017, Pages 29-40

Modern extraction techniques optimized to extract betacyanins from *Gomphrena globosa* L.

Custódio Lobo Roriz [a, b], Lillian Barros [a, c], M.A. Prieto [a, d], Maria Filomena Barreiro [c], Patricia Morales [b], Isabel C.F.R. Ferreira [a]

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Resumen

Gomphrena globosa L. es fuente de betacianinas con elevado potencial comercial debido al poder colorante de estos compuestos. Con el fin de reemplazar las técnicas de extracción convencionales, se optimizó el proceso de extracción mediado por microondas (MAE) y ultrasonidos (EAU) utilizando el modelo matemático de metodología de superficie de respuesta (RSM) para maximizar la recuperación de betacianinas de *G. globosa*. Para ello se implementó un diseño factorial completo de cinco niveles, con 24 combinaciones y 4 réplicas para MAE y UAE. Las condiciones óptimas de procesamiento para MAE (t = 8 min; T = 60 ° C; Et = 0%; y S / L = 5 g / L) condujeron a un rendimiento de extracción de 39.6 ± 1.8 mg / g de peso seco, mientras que para EAU (t = 22 min; P = 500%; Et = 0%; y S / L = 5 g / L) se logró un valor de 46,9 ± 4,8 mg / g, validando la extracción mediada por ultrasonidos (EAU) como la más adecuada para obtener estos compuestos diana. Esta técanic puede proporcionar extractos ricos en betacianinas con alto potencial para ser utilizados como colorantes naturales.

Abstract

Gomphrena globosa L. is a source of a betacyanin molecules with high commercial value due to its colorant power. To replace common conventional extraction technics, a microwave- and ultrasound assisted extraction process (MAE and UAE, respectively) were optimized, by means of response surface methodology (RSM), to maximize the recovery of betacyanins from *G. globosa*. A five-level full factorial design of 24 combinations and four replicates at the laboratory was successfully implemented for MAE and UAE optimization, in which the processing time (*t*), temperature (*T*), or power (*P*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) were relevant independent variables. The proposed model was validated based on the high values of the adjusted coefficient of determination and on the non-significant differences between experimental and predicted values. The general optimum processing conditions for MAE (*t* = 8 min; *T* = 60 °C; *Et* = 0%; and *S/L* = 5 g/L) revealed an optimum response of 39.6±1.8 mg/g, while for UAE (*t* = 22 min; *P* = 500%; *Et* = 0%; and *S/L* = 5 g/L) it was at 46.9±4.8 mg/g (expressed as plant dry weight), validating the UAE as the ideal extraction technic for obtaining the compounds of interest. This technic can provide extracts rich in colorant properties with high potential to be used as natural colorant additives and highlights *G. globosa* as a source of these colorant compounds.

Article 5. Enhancing the antimicrobial and antifungal activities of a coloring extract agent rich in betacyanins obtained from *Gomphrena globosa* L. flowers

Reference: Roriz, C. L., Barros, L., Prieto, M. A., Ćirić, A., Soković, M., Morales, P., & Ferreira, I. C. (2018). *Food & function*, 9(12), 6205-6217.
DOI: https://doi.org/10.1039/C8FO01829D

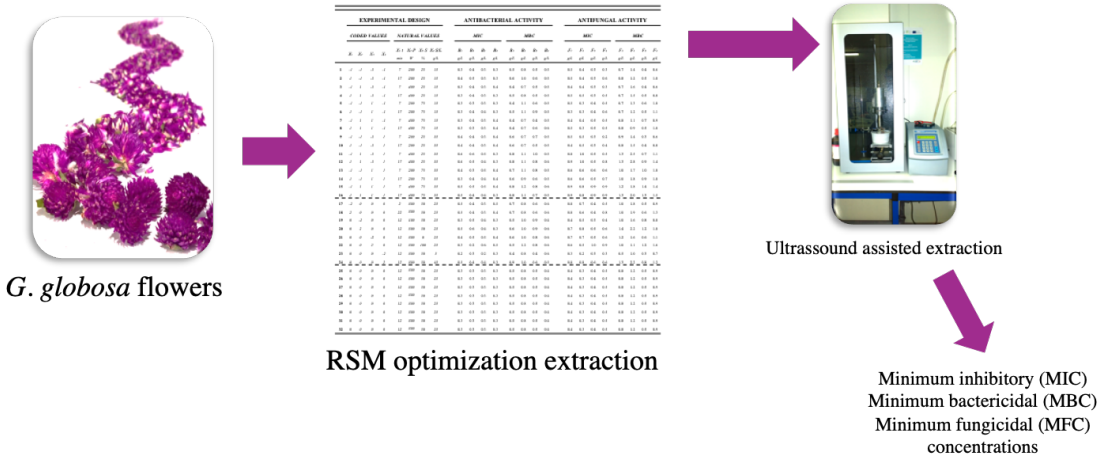

Figure 16 Graphical Abstract Article 4

From the journal:
Food & Function

Enhancing the antimicrobial and antifungal activities of a coloring extract agent rich in betacyanins obtained from *Gomphrena globosa* L. flowers†

Check for updates

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Resumen

Aunque menos exploradas que la remolacha (*Beta vulgaris* L.), las flores de *Gomphrena globosa* L. son una fuente muy adecuada de betacianinas con fuertes propiedades colorantes, junto con muchas otras propiedades bioactivas deseables. Así, el objetivo de este estudio fue desarrollar un extracto a partir de las flores de *G. globosa* que además de poder colorantes presentara los mejores resultados de actividad antimicrobiana y antifúngica mediante la extracción asistida por ultrasonido (EAU). El procedimiento se apoyó con la aplicación de la metodología de superficie de respuesta, de modo que nos permitió estudiar de manera conjunta los efectos de varias variables y respuestas. Para potenciar las actividades antimicrobianas (frente a *Bacillus cereus, Listeria monocytogenes, Escherichia coli y Salmonella typhimurium)* y antifúngicas (frente a *Aspergillus flavus, Aspergillus niger, Penicillium ochrochloron y Penicillium verrucosum*), se estudiaron los resultados en términos de Concentraciones minima bactericidas (MBC) y concentración mínima antifungicidas (MFC). Se encontró´ que las condiciones optimas de los EAU fueron 10,8 min, 410,5 W, 57,8% de etanol y 5 g/L de la relación sólido-líquido, proporcionando los siguientes valores de respuesta: (1) de las especies de bacterias estudiadas, la CMI osciló entre 0,15 y 0,35 g/L y los rangos de MBC fueron ~0,30 a 0,65 g/L; y (2) de las especies de hongos estudiadas, la CMI varió de ~0,20 a 0,30 g/L y los rangos de MFC fueron de ~0,40 a 0,65 g/L. Los niveles de dosis de actividad antibacteriana fueron más bajos que los antifúngicos. En conclusión, los resultados obtenidos en este estudio destacan los extractos de flores de *G. globosa* como fuentes naturales de betacianinas con aplicación como colorantes alimentarios pero con importantes actividades antimicrobianas y antifúngicas.

Abstract

Although less explored than beetroot (*Beta vulgaris* L.), the flowers of *Gomphrena globosa* L. are a very suitable source of betacyanins with strong pigmentation features, together with many other desirable bioactive properties. Thus, the aim of this study was to enhance the antimicrobial and antifungal activity of a pigmented extract obtained from *G. globosa* flowers by ultrasound assisted extraction (UAE). The procedure was supported with the application of response surface methodology, a robust optimization technique that allows to study jointly the effects of several variables and responses. To enhance the antimicrobial (*Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*) and antifungal (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium ochrochloron* and *Penicillium verrucosum*) activities, the responses were evaluated in terms of the concentrations needed to obtain minimum inhibitory (MIC), minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations. It was found that the optimal UAE conditions were 10.8 min, 410.5 W, 57.8% of ethanol, and 5 g/L of solid-liquid ratio providing the following response values: 1) from the studied species of bacteria, MIC ranged from ~0.15 to 0.35 g/L and MBC ranges were ~0.30 to 0.65 g/L; and 2) from the studied fungus species, MIC ranged from ~0.20 to 0.30 g/L and MFC ranges were ~0.40 to 0.65 g/L. The antibacterial activity dose-levels were lower than the antifungal ones. In conclusion, the results obtained in this study highlights extracts from *G. globosa* flowers as natural sources of betacyanins with application as food colorants with important antimicrobial and antifungal activities.

Article 6. *Gomphrena globosa* L. as a novel source of food-grade betacyanins: Incorporation in ice cream and comparison with beet-root extracts and commercial betalains

Reference: Roriz, C. L., Barreira, J. C., Morales, P., Barros, L., & Ferreira, I. C. (2018). *Lwt*, 92, 101-107.

DOI: https://doi.org/10.1016/j.lwt.2018.02.009

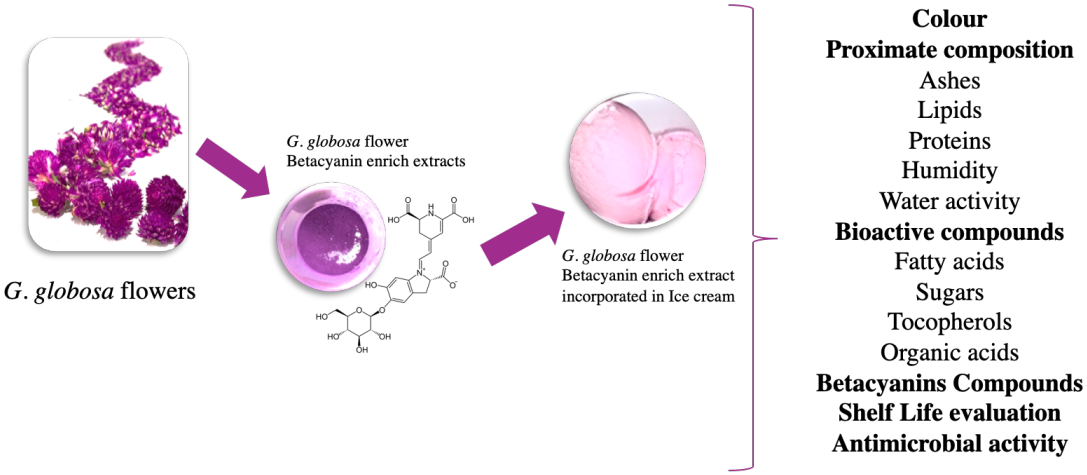

Figure 17 Graphical Abstract Article 6

ELSEVIER

LWT

Volume 92, June 2018, Pages 101-107

Gomphrena globosa L. as a novel source of food-grade betacyanins: Incorporation in ice-cream and comparison with beet-root extracts and commercial betalains

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Resumen

Actualmente, existen algunos ejemplos de colorantes naturales con uso comercial. Sin embargo, estos colorantes suelen estar infrautilizados, además de obtenerse de un número reducido de especies de plantas o algas. En consecuencia, proponemos el uso de betalaínas obtenidas de una especie vegetal alternativa, *Gomphrena globosa*, que tienen una potente actividad colorante además actividad antioxidantes, como colorante para ser incorporado en helados. Con fines comparativos, se prepararon varias formulaciones de helado, a saber, sin colorantes añadidos, con betalaína comercial, con extracto de *Beta vulgaris* y con colorante obtenido a partir de *G. globosa*. Además de evaluar los parámetros de color L *, a * y b *, también se estudió la composición nutricional, contenido azúcares solubles y ácidos grasos. Estos parámetros se evaluaron a lo largo del periodo de almacenamiento (máximo de 60 días) en congelación a -22 ° C. También se realizó la cuantificación del contenido en betacianina de cada formulación para determinar su estabilidad durante el almacenamiento. En general, los helados preparados con *G. globosa* presentaron resultados similares (considerando la composición nutricional, de color, azúcares solubles y ácidos grasos) comparándolos con el helado preparado con el extracto de *B. vulgaris*, validando así´ la idoneidad de esta planta alternativa como fuente de colorantes alimentarios, particularmente como colorantes para helados. Además, se mantuvo el color estable durante el tiempo de almacenamiento, como lo indica la distribución de marcadores en el análisis discriminante lineal.

Abstract

The colour of food products is a key factor for its acceptability. Likewise, incorporating natural ingredients with validated bioactive properties into novel food formulations is essential to fulfil current consumers' demands. Currently, there are some examples of natural colourants with commercial use. However, these colourants are usually under-exploited, besides being obtained from a reduced number of plant or algal species. Accordingly, we propose using the betalains obtained from an alternative plant species, *Gomphrena globosa* L., which have a powerful colouring activity besides being strong antioxidants, as a novel ice cream colourant. For comparison purposes, other ice cream formulations were prepared, namely without colourants, added with commercial betalain or with *Beta vulgaris* extract. Besides evaluating the colour parameters *L**, *a** and *b**, the nutritional parameters, individual sugars and fatty acids profiles were also studied. To obtained reliable outcomes, these parameters were evaluated throughout time, up to a maximum of 60 days of freeze (-22 ºC) storage. Betacyanin quantification of each formulation was also performed to determine its maintenance along storage. In general, ice creams prepared with *G. globosa* were similar (considering nutritional, colour, individual sugars and fatty acids profiles) to those including *B. vulgaris* extract, thereby validating the suitability of this alternative plant as a source of food colourants, particularly as ice cream colourants. Furthermore, the positive effects induced by the addition of this natural colourant were maintained throughout storage time, as indicated by the markers distribution in the linear discriminant analysis.

Article 7. Betacyanins from *Gomphrena globosa* L. flowers: incorporation in cookies as natural colouring agents

Reference: Roriz, C. L., Heleno, S. A., Carocho, M., Rodrigues, P., Pinela, J., Dias, M. I., Fernandes, I. P., Barreiro, M. F., Morales, P., Barros, L. & Ferreira, I. C. F. R. (2020). Food Chemistry, 329, 127178.
DOI: https://doi.org/10.1016/j.foodchem.2020.127178

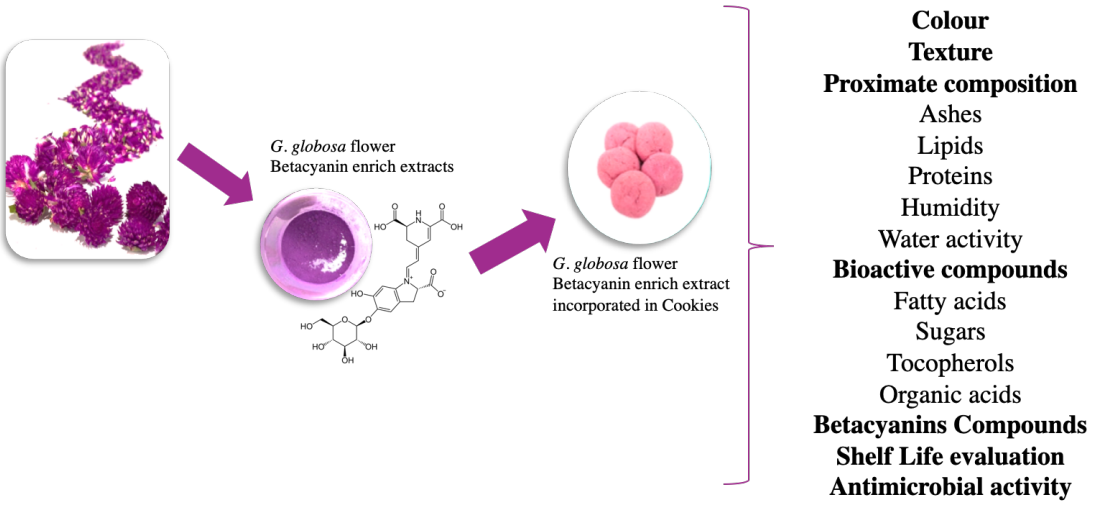

Figure 18 Graphical Abstract Article 7

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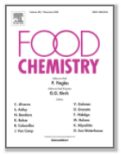
Food Chemistry
Volume 329, 1 November 2020, 127178



Betacyanins from *Gomphrena globosa* L. flowers: Incorporation in cookies as natural colouring agents

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Resumen

Se obtuvo un extracto rico en betacianina a parir de las flores de *Gomphrena globosa* L. mediante extracción asistida por ultrasonido y posterior liofilización o secado por aspersión para ser incorporado como colorante natural en galletas. y se comparó con un colorante comercial. Previamente a su incorporación, los extractos se caracterizaron en términos de contenido de betacianina y potencial antioxidante. Los efectos de la incorporación de colorantes (comercial y colorante desarrollado a partir de las flores de *G. globosa*) en las galletas se evaluaron a través de la composición nutricional, azúcares solubles, ácidos grasos, color, textura y carga microbiana, durante una vida útil de 30 días. Considerando todos los ensayos y analizando los resultados a través de un análisis de varianza de 2 factores, las galletas elaboradas con el colorante secado por atomización mostraron una coloración rosa más intensa mientras que las galletas elaboradas con el extracto liofilizado perdieron menos intensidad de color con el tiempo. Demostrando el potencial colorante de estos extractos de betacianina para ser incorporados en estas matrices alimentarias.

Abstract

A betacyanin enriched extract was obtained from the flowers of *Gomphrena globosa* L. by ultrasound-assisted extraction and dried either by lyophilization or spray-drying, to be applied as a natural colourant. The extracts were characterized in terms of betacyanin content and antioxidant potential for further incorporation in cookies. The incorporation effect was evaluated by analysing the proximate composition, individual profiles in soluble sugars, fatty acids, physical parameters and microbial growth of the final cookies, over a shelf life of 30 days. From the obtained results, the cookies with spray-dried extract showed the most promising stability, maintaining a pink colour after cooking, and during the 30 days of analysis; without altering the chemical composition of the cookies. The observed changes in the texture analysis, resulted from a significant interaction between the type of colourants and storage time. Thus, betacyanin extracts have potential as natural colourants in the food industry.

Part VI. INTEGRATIVE DISCUSSION

1 Plant species as source of potential natural colourants

1.1 Comparative study of *Gomphrena globosa*, *Amarantus caudatus* and *Hylocereus costaricensis* chemical characterization

During this PhD thesis, *Gomphrena globosa, Amarantus caudatus* and *Hylocereus costaricensis* were studied. These samples were subjected to a full chemical characterization and bioactive evaluation, although some data regarding *G. globosa* characterization and bioactivity was already performed during my master's degree (Roriz, 2014). Thus, *G. globosa* was maintained to perform the PhD work plan, given its richness in colouring compounds. All the three samples were used as natural colourants and some incorporated in food matrices. This integrated discussion will provide an overview of the main differences as well as the main similarities between the analysed samples.
The data regarding the characterization of these samples correspond to **articles 1 and 2.**

1.1.1 Organic acids and tocopherols in *Gomphrena globosa*, *Amarantus caudatus* and *Hylocereus costaricensis*.

After interpreting the results shown in **table 20**, it is possible to verify that with regard to the composition of the samples in organic acids, the plant that has a greater diversity and, therefore, the greater concentration of these compounds is the flower of *G. globosa*. In this sample it is possible to verify the presence of oxalic, malic, citric, and fumaric acids, presenting a total of 25.65 g/100g dw. The samples of *H. costaricensis* presented oxalic and malic acids with a total of 3.33 g/100g dw, and for *A. caudatus* sample i it is possible to verify the presence of oxalic and shikimic acids, with a total of 2.65 g/100g dw. This difference can be attributed to several factors such as different sources, from different years, and as it is known, the different ecological conditions involved in the development of plants, end up influencing their chemical composition. Also, they are plants that despite belonging to the same order, are of different species, which in itself already implies a different profile of their chemical composition. Although all samples have been subjected to previous studies, for some of them, little information was provided regarding the organic acid's composition. For *A. caudatus* sample, for instance, as far as the authors know, there is no studies describing the organic acid profile for the flowers of this species. For *H. costaricensis*, the profile in organic acids has been performed in some fruits of

Hylocereus spp, presenting some differences when compared to the profile achieved in the present work, something that can be due to the different fruit species, and the fact that all fruits derived from different parts of the globe, with very distinct edaphoclimatic conditions.

Table 20 Oganic acids (g/100g dw) and tocopherols (mg/100g dw) composition of *A. caudatus* flowers, *H. costaricencis* peels and *G. globosa* flowers.

	G. globosa	*A. caudatus*	*H. costaricensis*
Organic acids (g/100 g dw)			
Oxalic acid	10.64±0.04^{a}	2.48±0.05^{b}	1.68±0.01^{c}
Malic acid	12.33±0.55^{a}	nd	1.65±0.10^{b}
Citric acid	2.40±0.01	nd	nd
Shikimic acid	nd	0.170±0.003	nd
Fumaric acid	0.28±0.01	tr	tr
Total organic acids	25.65±0.51^{a}	2.65±0.03^{b}	3.33±0.96^{b}
Tocopherols (mg/100 g dw)			
α-Tocopherol	0.38±0.04^{c}	0.47±0.01^{b}	3.099 ± 0.059^{a}
β-Tocopherol	nd	0.884±0.003a	0.164 ± 0.023b
γ-Tocopherol	3.02±0.08^{b}	nd	11.828 ± 0,222^{a}
δ-Tocopherol	5.20±0.01^{a}	0.60±0.06^{c}	0.842 ± 0.066^{b}
Total tocopherols	8.60±0.10^{b}	1.95±0.06^{c}	15.932 ±0.074^{a}

tr: traces; nd: not detected, In each row different letters mean significant differences (p<0.05).

Regarding the composition in tocopherols, it is also possible to verify in **Table 20**, that the sample that presented a greater quantity of these compounds, was the peels of *H. costaricensis*, where it was possible to characterized four of the eight possible vitamin E vitamers, namely all tocopherol isoforms, presenting a total of 15.932 mg/100g dw. On the other hand, *G. globosa* flowers showed a total of 8.60 mg/100g dw, with the presence of three tocopherol isoforms, namely α-, γ- and δ-tocopherol. Finally, *A. caudatus* flowers revealed itself as the one with the lowest content in these compounds, corresponding to 1.95 mg/100g dw, which presents α, β- and δ- tocopherol isoforms. Once again, significant differences are present between the sample's contents in tocopherols, attributed to the factors mentioned for organic acids. For the tocopherols profile, in the present work, when searching in literature for data comparison, the same problem has arisen and no information was found. The data available in literature, for tocopherol composition, in both species, *A. caudatus* and *H costaricensis*, concerns the profile of these compounds in the seeds. A comparison can be done, but it will not be accurate since

seeds have a high composition in oils, as it is commonly known, tocopherols are liposoluble compounds. Therefore, the amount of these compounds in seeds will be higher than the amount presented in flowers (*A. caudatus*) and peels (*H. costaricensis*).

1.1.2 Betacyanins in *Gomphrena globosa*, *Amarantus caudatus* and *Hylocereus costaricensis*.

Betacyanin's tentative identification and quantification data corresponding to the three plant species are presented in **Table 21**. Upon observing the betacyanin composition, it is possible to see that in the *G. globosa* flowers, six compounds were tentatively identify, presenting gomphrenins/isogomphrenins II and III and 17- descarboxybetanin. The major compound was Gomphrenin III (50.21 %) followed by isogomphrenin III (17.21%) , and the minor compound observed in this sample was 17- descarboxybetanin (4.10%) . For *A. caudatus* flowers, and *H. costaricensis* peels only four betacyanin compounds were identified in each sample. In the *A. caudatus* sample it was possible to identify amaranthine, isoamaranthine, betanin and isobetanin, being amaranthine the major compound (171 mg/g extract), and isobetanin the minor one (1.3 mg/g extract). Concerning the *H. costricensis* sample, the four compounds identified were betanin, isobetanin, phyllocactin and isophylocactin. The major compound in this sample was phyllocactin (14.8 mg/g extract), and the minor one was the betanin (10.1 mg/g extract) compound.

Table 21 Betacyanins of *G. globosa* flowers, *A. caudatus*, and *H. costaricensis* extracts. Chromatographic identification (retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), and mass spectral data) and quantification (g/kg extract, for *A. caudatus* and *H. costaricensis*, and relative percentage %, for *G. globosa*). (Mean±SD)

Peak	Rt (min)	λ_{max} (nm)	$[H]^+$ (*m/z*)	MS^2	Tentative identification	Quantification (g/kg extract)
				G. globosa flowers		
1	28.79	550	697	551(2),389(22)	Gomphrenin II	7.66 ± 0.56
2	31.91	550	697	551(3),389(39)	Gomphrenin II	12.99±0.41
3	32.48	550	727	551(4),389(41)	Gomphrenin III	50.21±0.94
4	34.27	550	697	551(2),389(21)	Isogomphrenin II	6.13±0.11
5	35.40	546	727	551(4),389(38)	Isogomphrenin III	17.23±0.24
6	36.65	500	683	507(2),345(22)	17-Descarboxy-amaranthin	4.10±0.11
				A. caudatus flowers		
1	18.68	536	727	551(100),389(40)	Amaranthine	171±1
2	20.02	536	727	551(100),389(27)	Isoamaranthine	38±1
3	22.04	536	551	389(100)	Betanin	1.6±0.1
4	23.23	536	551	389(100)	Isobetanin	1.3±0.1
					Total	212±1
				H. costaricensis peels		
1	19.40	534	551	551(100), 389(40)	Betanin	10.1±0.2
2[c]	20.86	533	551	551(100), 389(27)	Isobetanin	11.8±0.6
3[c]	22.74	535	637	619(12),593(15),551(35),389(100)	Phyllocactin	14.8±0.3
4[c]	23.76	533	637	619(12),593(15),551(35),389(100)	Isophyllocactin	11.7±0.2
					Total	48.3±0.8

Analysing **Table 21**, it is possible to verify the presence of different classes of this family of compounds. It is noticeable that the evaluated species present very different compounds among each other, being betanin and isobetanin the only common compounds in the samples of *A. caudatus* and *H. costaricensis*. These different classes, as well as the quantities in which they appear in the different sources, are responsible for the intensity, hue and tone that the different samples present.

For each studied sample, the compounds identified are characteristic of that species, and their names derive from the scientific name of the species where they were found. Something quite notorious in the majority compounds found in the different samples studied. Considering the concentration of these colouring compounds in the recovered extract, in the extract of *G. globosa* they represent ~98% of the value of recovered extract, ~ 21% in the extract of *A. caudatus* and, ~ 4% *H. costaricensis*. This difference in the

concentration of coloring compounds in the recovered extract is also visible in the color intensity of the extracts, since the strongest shade is present in the *G. globosa* extract, followed by the extract of *A. caudatus* and finally the extract *H. costaricensis*

Regarding the profile of the three studied samples, and their concentration in betacyanin compounds, when compared with the species that today is considered the main source of betacyanin compounds (the beet root (*Beta vulgaris*)), the authors Sawicki et al. (2016) used the HPLC–TOF-MS/MS method and identified in different varieties of beet root a total of thirty betalains, eighteen betacyanins and twelve betaxanthins. Among the identified betalains, betanin and isobetanin (betacyanins) and vulgaxanthin I (betaxanthins) were predominant. In another study, Koss-Mikołajczyk et al. (2019), analyzed a sample of red beet root identifying nine compounds, with a concentration of (10.65 g/kg dw). The compounds identified in that sample were Vulgaxanthin I, Dopamine-betaxanthin, Phenylalanine-betaxanthin, Tyrosine-betaxanthin, Tryptophan-betaxanthin, 2-Decarboxy-neobetanin, 17-Decarboxy-neobetanin, Betanin, and Isobetanin. Comparing the profiles, it can be seen that some of the compounds identified by these authors, also appear in the samples studied in the present work, but the majority are different. In fact, it is known that the betacyanin family is quite huge, and that the presence of these compounds are unique in each specie, resulting in very different colour intensity, hue and shad, exhibited by the flowers, stems, roots and bulbs where these compounds are found.

2 Optimization extraction procedures of natural bioactive compunds

The central point of this PhD study is the extraction of colouring compounds from natural origin matrices. To achieve this goal, the extraction optimization procedures were divided in two parts. In the first part, a well-known and previously studied plant, was subjected to three different extraction techniques, that were applied to *G. globosa*, and the results obtained were evaluated in order to determine the ideal technique for recovering the maximum content in betalains, the molecules responsible for the colouring power. After that, in a second part of the work, the best extraction technique was applied on two different natural matrices, to achieve the same end. The enriched extracts recovered by the different natural matrices, were then used as natural food colourants, incorporated in foodstuffs. These food products were evaluated for their final colour, to assess the natural colourant colouring power, along with the nutritional profile and chemical composition

of the foodstuff developed. The extraction procedures of the different techniques and different samples, depicted in this section corresponded to **articles 1, 2, 3 and 4.**

With the main purpose of optimizing the extraction of colouring compounds from different sources, in this work, a technique that allows to easily and quickly measure the contribution of different variables in a given process was applied: the RSM.

This tool can be applied to a multitude of assays for different purposes. It is possible to use it in different areas, as we can see in the study carried out by Pinto et al., (2020) where RSM was used for developing polyols from abundant and renewable biomass resources, in the polymer synthesis area. Several authors use this methodology to recover biologically active molecules for application in different industries. For instance, Vieira et al. (2018) used this tool for the extraction of phenolic compounds from walnut leaves (*Juglans regia* L.) optimizing the heat-assisted extraction and deep eutectic solvents based on choline chloride and carboxylic acids, also explored this tool for a comparison of maceration (ME) and microwave assisted (MAE) extractions aiming to maximize the extraction of valuable compounds from *Juglans regia* L. leaves (Vieira et al., (2017)). Another author resorted to this technique to optimize the extraction of phenolic compounds by High hydrostatic pressure (HHP) from watercress (*Nasturtium officinale* W.T.Aiton) (Pinela et al. (2018)). The same authors some years earlier have resource to the same tool applied to microwave-assisted extraction (MAE) to maximize the recovery of phenolic acids and flavonoids and obtain antioxidant ingredients from tomato (Pinela et al., (2016a; 2016b)), and also from tomato fruit wastes using a MAE, and the same for recover bioactive ingredients from tomato rich in hydrophilic (H) and lipophilic (L) antioxidants.

Silva et al., (2020) used RSM to a conventional heated assisted extraction in bio-residues of *Pleurotus ostreatus* (Jacq. ex Fr.) P.Kumm., 1871, *Agrocybe cylindracea* (V. Brig.) Vizzini 2014, and *Pleurotus eryingii* (DC.) Quél., 1872 as sustainable sources of ergosterol. For the same family of compounds, Taofiq et al., (2019) applied RSM to optimize the recovery of ergosterol from an agro-industrial residue of *Agaricus blazei* Murrill, by comparing conventional (HAE) and more sustainable non-conventional approaches (UAE and MAE). Still regarding ergosterol compound Rudke et al., (2019) resorted to RSM to optimize the encapsulation by coacervation of an ergosterol enriched extract from *Agaricus bisporu*s L.. Heleno et al., (2016a; 2016b) used the RSM tool to optimized the recover ergosterol from *Agaricus bisporus* L. by-products in a MAE, and UAE. In order to optimize the obtaining of bioactive phenolic extracts from *Pleurotus*

sajor-caju Finimundi et al., (2018) resorted to RSM tool coupled to a heated assisted extraction technique. Caleja et al., (2017) compare the extraction of rosmarinic acid from *Melissa officinalis* L. using three techniques (heat-, microwave- and ultrasound-assisted extraction) applying the RSM o obtain the conditions that maximize the rosmarinic acid extraction. However, this technique is widely explored for the recovery of colouring compounds from the most diverse sources. RSM is widely applied to UAE to recover betalain compounds, for example, to achieve this end, Melgar et al., (2019) explored prickly pear peel (*O. engelmannii)*, Ahmed et al., (2020), explored the Red amaranth (*A. cruentus*) leafy part, Ramli et al., (2014) used peels of a different species of dragon fruit (*H. polyrhizus*), Bhagya Raj & Dash, (2020) used the peel of white-fleshed red pitaya (*H. undatus*), and Prakash Maran et al., (2015) in *B. glabra* flowers, among others.
Only referring to some cases of successful implementation of RSM technique, and thus to demonstrate and diversity of possible applications of the same.

2.1 Optimization extraction procedures for betacyanins content

2.1.1 Assessment of the best extraction procedure for colour in *G. globosa* flowers

Regarding the optimization of the extraction procedures, the three plant samples were studied separately. *G. globosa* flowers was the first plant under analysis, thus the three extraction methods were applied, namely dynamic maceration (DM), microwave assisted extraction (MAE), and ultrasound assisted extraction (UAE).
The samples preparation for the extraction process was as follows: after separating the pigmented parts of the sample, the extraction of the pigments was carried out. There were several factors that may interfere, such as time, temperature, power type of solvent and solid-liquid ratio. The basic concept of the preliminary experiments, for DM, MAE and UAE systems, ensuring that the experimental domain of the operating conditions, for the optimization procedure, were properly defined in order to maximize the responses. Therefore, for the optimization extraction procedures time (*t*), temperature (*T*, for DM and MAE) power (*P*, for UAE), type and proportion of solvent (*Et*) and solid-liquid ratio (S/L) were considered as variables.

Table 22 Operating conditions that maximize the extraction of betacyanins from *G. globosa* and optimal response values for the parametric response criteria. For the different extraction techniques

	OPTIMAL EXTRACTION CONDITIONS				RESPONSE OPTIMUM	
	X.: t (min)	*X.: T (ºC) Or P(W)*	*X.: Et (%)*	*X.: S/L (g/L)*		
Global Optimal responses for DM						
Yield	132.1	28.5	0.0	5.0	45.64	%
Gomphrenin II					7.73	*g/kg*
Gomphrenin III					15.77	*g/kg*
Isogomphrenin II					6.19	*g/kg*
Isogomphrenin III					11.35	*g/kg*
Total					39.86	*g/kg*
ABS					19.95	*g/kg*
COLOUR					48.11	*a* values*
Global Optimal responses for MAE						
Yield	20.0	60.0	0.0	5.0	8.0	%
Gomphrenin II					4.9	*g/kg*
Gomphrenin III					12.4	*g/kg*
Isogomphrenin II					4.2	*g/kg*
Isogomphrenin III					9.2	*g/kg*
Total					39.6	*g/kg*
COLOUR					68.7	*a* values*
Global Optimal responses for UAE						
Yield	22.0	257.8	0.0	5.0	55.2	%
Gomphrenin II					9.3	*g/kg*
Gomphrenin III					17.5	*g/kg*
Isogomphrenin II					3.6	*g/kg*
Isogomphrenin III					12.1	*g/kg*
Total					45.5	*g/kg*
COLOUR					37.6	*a* values*

ABS. absorbance

Evaluating the achieved results for the different techniques, displayed on **Table 22** it is possible to see that the extraction presenting the worst results for the amount of the betacyanin compounds, when applied the global optimal operating extraction conditions, is the MAE. Despite displaying a total of 39.6 g betacyanins/kg of extract, which is a very interesting amount, the yield presented was only of 8.0%. Another downfall of this technique was the degradation, very visible, of the colouring compounds. MAE is a very interesting extraction technique, but the operating conditions of this type of extraction are sometimes extreme, being able to cause the degradation/destruction of some more sensitive compounds, such as betacyanins. DM presented a total of betacyanin compounds of 39.86 g betacyanins/kg of extract, and a yield of 45.64%, very interesting values, but the operating conditions, mainly the operating time of 132.1 min makes the process time consuming, considering the quantity of the recovered compounds. Therefore, the extraction procedure presenting the best global results, was the UAE. With this extraction technique it was possible to recover 45.5 g betacyanins/kg of extract

applying the extraction conditions of 22 min, 257.8 W, using water as the extraction solvent and 5 g/L as the solid/liquid ratio.

In this way, and with the knowledge acquired at this stage of the work, the two other plants (*A. caudatus* flowers and *H. costericencis* peels) were subjected to this extraction technique by applying an RSM, in order to explore new natural matrices as alternative sources of colouring agents for application as natural origin food colourants. Upon reaching this conclusion, from this point on, only UAE was applied to obtain extracts enriched in betacyanin compounds.

2.1.2 *Amaranthus caudatus* and *Hylocerus costaricensis* UAE optimization process

As stated earlier, the purpose of this work is to explore natural matrices as alternative sources of natural origin colouring compounds. All this to offer to food industry, which is increasingly in need these compounds to meet the demands of consumers who are progressively attentive to the composition of processed foods. For that, the optimization of the extraction process of the betacyanin compounds of different matrices (*A. caudatus* and *H. costaricensis*) was achieved, with the support of the RSM tool and the results are presented in **Table 23**. For the three species studied, *G. globosa*, *A. caudatus* and *H. costaricensis*, after application of RSM to a UAE system, values of 45.5 g betacyanins/kg extract, for *G. globosa* were obtained, applying room temperature (~25ºC) for 22 min with water as the extracting solvent, ultrasonic power of 257.8 *W* and a solid-liquid ratio of 5 g/L (**Table 22**). For the sample of *A. caudatus*, the result obtained was 77.6 g betacyanins/kg of plant material in an extraction in which the parameters are the same as those used for *G. globosa*, with the exception of the extraction time, which was 13.3 min, and ultrasonic power 500 *W*. For the *H. costaricensis* peels, the extraction was carried out at room temperature, 5 g/L of solid-liquid ratio, extraction time 38 min, ultrasonic power of 487 *W* and the solvent used was a 90:10 solution (water: methanol, *v:v*), due to problems with the mucilage present in this matrix, which hinder the filtering process. As a result, a value of 36 g betacyanins/kg plant material was achieved.

Table 23 Optimal processing conditions that maximize the extraction of betacyanins from *A. caudatus H. costaricensis* and model-predicted response optimum.

	Optimal processing conditions		**Response optimum**
	Time (min)	Power (W)	Content
Global conditions considering all response variables for A.caudatus			
Extraction yield	13.3	500	1.92%
Amaranthine			54.1 mg/g
Isoamaranthine			19.0 mg/g
Total betacyanins			77.6 mg/g
Global conditions considering all response variables for H.costaricensis			
Residue	38	487	5.05 %
Betanin			6.8 mg/g
Isobetanin			8.9 mg/g
Phylocactin			10.2 mg/g
4'-Malonyl-betanin			10.1 mg/g
Total betacyanins			36 mg/g

These values are quite auspicious and promising, making a comparison with works carried out in this family of compounds, using its recovery by this extraction technique (UAE), it is possible to verify that values higher than those obtained in this work, were obtained by Melgar et al., (2019), that used Prickly pear peel (*O. engelmannii*), for extraction in UAE at 30 °C for 2.5 min with a solid-liquid ratio of 5 g/L, and an extraction solvent composed of 34.6% methanol, pH 7 adjusted with McIlvaine buffer solution. These authors reached a recovery of betalain compounds of 201.6 g betalains/kg extract. In the same ranges of the present work, Ahmed et al., (2020), explored the Red amaranth (*A. cruentus*) leafy part, performing an UAE for 5 min at 70 °C with water as the extraction solvent and a solid-liquid ratio of 62.5 g/L, obtained an amount of 53.3 g betacyanins/kg dw. It should be noted that the solid-liquid ratio used by this author is of a much higher dimension when compared to the one used in the present study. Other authors have explored this technique of extraction but the results achieved are of a much lower order than those achieved in this work. In the work developed by Ramli et al., (2014) that used peels of a different species of dragon fruit (*H. polyrhizus*), and applied an UAE extraction time of 30 min at 25 °C and water as a solvent for extraction, and with the variation of only one of the extraction parameters, namely the solid-liquid ratio, which went from 18.2 g/L to 40 g/L, obtained an increase of 0.5 g betacyanins/kg extract. Bhagya Raj & Dash, (2020) used the peel of white-fleshed red pitaya (*H. undatus*), processed at 60 °C for 20 min at a solid-liquid ratio of 40g/L with a 60% ethanol as the solvent, obtaining 1.60 g betacyanins/kg extract. Prakash Maran et al., (2015) resorted to

an UAE to recover the pigments present in *B. glabra* flowers, processing the sample for 37 min at 55 °C with a solid liquid ratio of 58.8 g/L, and obtaining a concentration of 1.72 g betacyanins/kg. When observing the data obtained in the samples explored in the present work, and in the ones found in literature, it can be concluded that in all the works, the recovered content in colouring compounds is not very high, as stated above. Nevertheless, a particular feature of this family of colouring compounds, betacyanins, is its high colouring power. In this way, a small amount of colourant is able to produce a very intense and strong shade.

2.2 Optimization of the compound's extraction by RSM for enhancing antimicrobial activity

In addition of optimizing the extraction of betacyanin compounds to achieve the best results in the colour parameters in the recovered extract and considering that betacyanins are described as having antimicrobial potential, a new extraction optimization was carried out to enhance the antimicrobial potential of these extracts. However, the purpose of this new optimization process was the recovering of an extract rich in betacyanin compounds, and with the strongest antimicrobial capacity. Since in order to reduce losses associated with microbial contamination, at an industrial level, antimicrobial dose-levels activity of the extracts is one of the most reliable techniques to preserve the phytochemical composition and bioactive properties, resulting in the obtaining of high-quality products in several industrial sectors. Based on these optimized processing parameters it is possible to produce a food colorant ingredient with antimicrobial properties and thus give add value to *G. globosa* extracts.

For that and resorting to the RSM, a very diverse tool, since it can be applied and modulated to achieve the desired outcome. Above are the results obtained for the recovery of an enriched extract in betacyanins, but, in order to demonstrate the wide range of this tool and evaluate the possibility of obtaining extracts rich in betacyanins with enhanced antimicrobial activity using the flowers of *G. globosa*, another optimization process was performed. To achieve this goal, the extracts were obtained by ultrasound assisted extraction (UAE) in order to maximize the antimicrobial activity, with the support of RSM, and the results are presented in **article 5**. In a study performed by Spórna-Kucab et al., (2018), after a purification process combining High-speed counter-current chromatography (HSCCC) and prep HPLC techniques, these authors obtained isomers of

betacyanins from *G. globosa* flowers, namely gomphrenin derivatives, and proceeded to assess its antimicrobial efficiency. The isolated compounds demonstrated a very wide microbial spectrum, varying between 0.19-1.5 mg/mL, demonstrating a mild, moderate and good activity towards the tested Gram-positive bacteria (*S. aureus* ATCC 6538, *S. aureus* ATCC 43300, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *B. cereus* ATCC 10876, *B. subtilis* ATCC 6633, *M. luteus* ATCC 10240) and mild activity towards Gram-negative bacyeria (*E. coli* ATCC 35218, *E. coli* ATCC 25922, *E. cloaceae*, *P. aeruginosa* ATCC 27853, *P. mirabilis* ATCC 12453, *K. pneumoniae* ATCC 13883, *S. typhimurium* ATCC 14028, *B. bronchiseptica* ATCC 4617, *S. sonnei*). They stated that the acylated betacyanins showed a higher antimicrobial activity than the non-acylated ones, concluding that the presence of the acyl group leads to an increase in the activity of betacyanins against fungi and bacteria, which may be due to the synergistic effect between the acyl group and the chromophore system of the pigment.

All the extracts obtain from UAE were tested and the responses include the antibacterial concentration (MIC and MBC) for the bacteria strains (*Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*), graphically represented in **Figure 19**, and antifungal concentrations (MIC and MFC) of fungal strains (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium ochrochloron* and *Penicillium verrucosum* var. *cyclopium*), graphically represented in **Figure 20**.

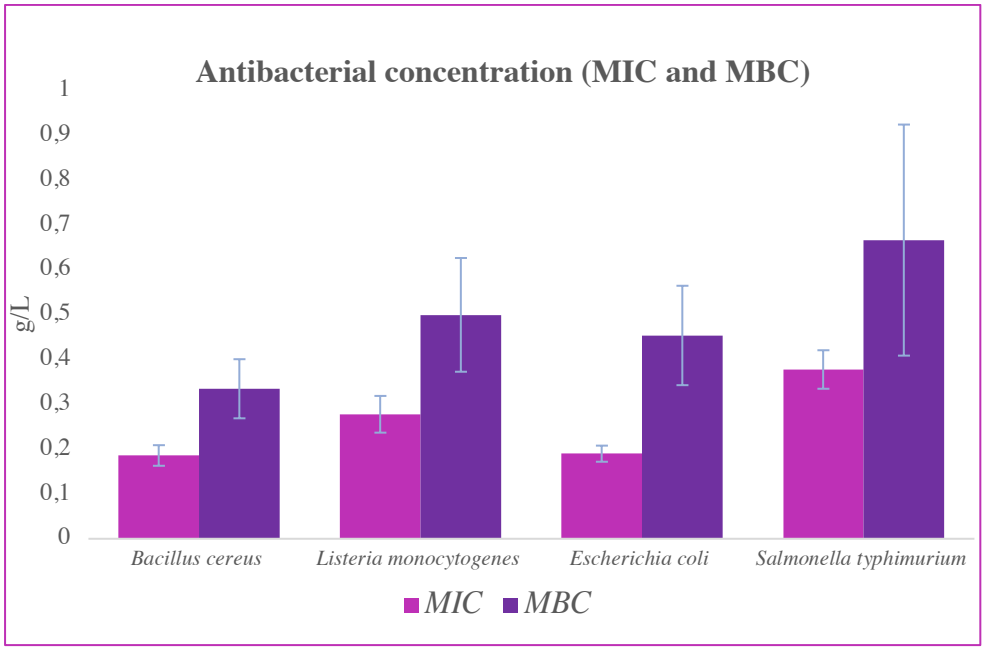

Figure 19 Antibacterial concentrations (MIC and MBC)

The UAE global optimal conditions results were 10.8 min, 410.5 W, 57.8% of ethanol and 5 g/L of solid-liquid ratio providing response values from the studied bacteria species, where the MIC ranged from ~0.15 to 0.35 g/L and regarding its MBC the ranges were from ~0.30 to 0.65 g/L; and from the studied fungi species, the MIC ranged from ~0.20 to 0.30 g/L and regarding its MFC the ranges were from ~0.40 to 0.65 g/L. The antibacterial active dose-levels were lower than the antifungal ones.

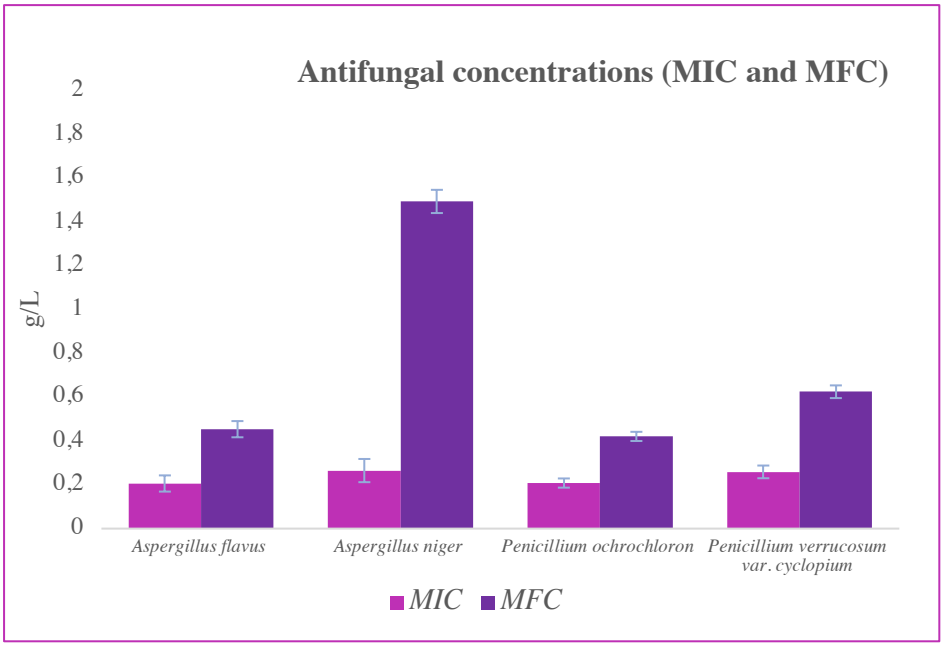

Figure 20 Antifungal concentrations (MIC and MFC)

2.2.1 Evaluation of bioactive properties in *G. globosa*, *A. caudatus* and *H. costaricensis* UAE extracts

Regarding the bioactive properties of these three samples, only the results referring to *A. caudatus* and *H. costaricensis* will be presented plotted, since for the sample of *G. globosa* only was assess for Δt of 60 min. Regarding the antimicrobial activities, in the sample of *G. globosa* flowers, this was evaluated in a different way because it was the result of an extraction optimization study using a RSM response surface methodology.

According to the obtained results for the Antihaemolytic activity, graphically presented on **Figure 21**, for the *A. caudatus* sample an extract mean concentration of 29 µg/mL and 111 µg/mL were required to protect half the erythrocyte population from the oxidative haemolytic induced by AAPH, a temperature-dependent free radical initiator, for Δt of 60 and 120 min, respectively. Concerning *H. costaricensis* sample, an extract mean

concentration of 255 μg/mL and 381 μg/mL was needed to induce the same results in the same periods of time. As previously stated, for the *G. globosa* sample only the 60 min ⊿t was tested, presenting a result of 80 μg/mL, and for that ⊿t, showed higher values than *A. caudatus*, but lower then *H. costaricensis*. Trolox, the used positive control, was more effective in protecting the erythrocyte membranes than the extracts, with IC_{50} values of 16.6 μg/mL and 44 μg/mL for ⊿t of 60 and 120 min, respectively. The strongest activity exhibited by Trolox is due to being a pure molecule whereas the *A. caudatus*, *G. globosa* and *H. costaricensis* extracts are a complex mixture of compounds and some of its components may not present biological activities or even present antagonistic effects. But between the two extracts analysed, it is possible to observe that the *A. caudatus* flower extract is more efficient than the one obtained from the *G. globosa* flowers, and *H. costaricensis* peel, since a lower extract concentration was needed to avoid oxidation.

The form and methodologies in which the antioxidant activity can be tested are so diverse that it is sometimes difficult to make a comparison between the data obtained for this bioactivity by the different authors. For the samples studied in this work, the methodology chosen to test its antioxidant activity was the Antihaemolytic activity test, and as far as the author knowledge, there are no reports concerning the antioxidant potential of these species using the same methodology. However, there are several authors who present results of antioxidant activity for these species, applying different methodologies. For instance, Jo et al., (2015) evaluated the radical scavenging activity of *A. caudatus* flower with DPPH and ABTS (+) analysis and reported it as a promising source of antioxidants, due to the promising results achieved, for the ABTS (+) assay the RC_{50} values obtained where 321.72 μg/mL, and for the DPPH assay the flowers of *A. caudatus* presented RC_{50}values >1.000 μg/ml. For the *Hylocereus spp*. For instance, de Mello et al. (2015) evaluated the antioxidant activity of *H. undatus* extract by DPPH and FRAP assays, presenting values of 177.14 μmol AEAC/100 g for DPPH and 109.29 μmol AEAC/100 g for the FRAP assay. Tenore at al. (2012) applied the same assays in *H. undatus* peel and flesh, peels fraction presented 805±1 μmol TE/100 g fw, and 478 ± 2 μmol TE/100 g fw for the DPPH and FRAP assays, respectively, and for flesh 999.8 ± 1.4 μmol TE/100g fw and 437 ± 2 μmol TE/100 g fw for DPPH and FRAP assays, respectively. Esquivel et al. (2007) applied the TEAC (Trolox equivalent antioxidant capacity) assay to different Hylocereus genotypes and the values obtained by this author ranged from 24.5 ± 0.8 to 36 ± 3 mg/100 mL AAE (ascorbid acid equivalents).

However, and due to the different mechanisms of action of the performed antioxidant assays, and as mentioned above, the results obtained are so different, the comparison of the achieved values in the different studies is quite challenging.

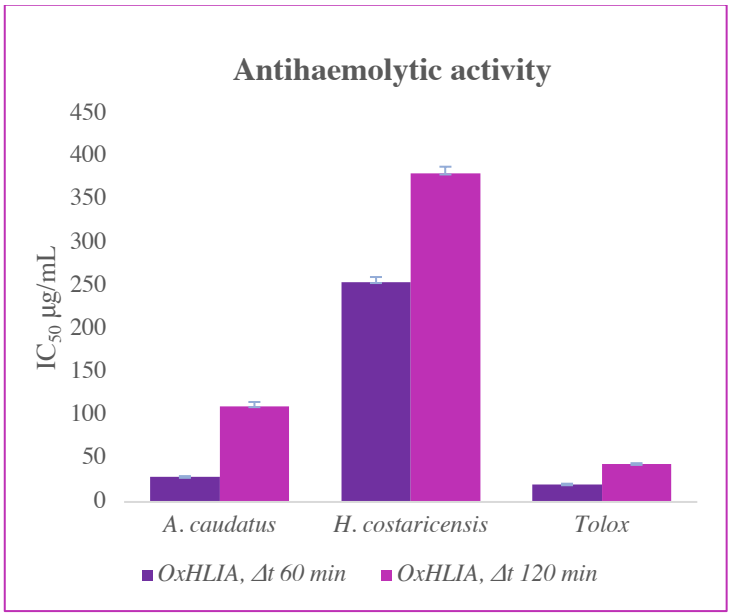

Figure 21 Antihaemolytic activity of *A. caudatus* and *H. costaricencis*.

Relatively to the results obtained for antimicrobial activity, the minimum inhibitory concentrations (MIC) were obtained, and are presented graphically in **Figure 22**. It can be observed that the extract of *A. caudatus* flowers is significantly more efficient than the extract recovered from *H. costaricensis* peels. The difference verified between the two samples is quite visible in the MIC result obtained for *Morganella morganii*, *Kleibsiella pneumoniae*, *Proteus mirabilis* and MRSA. In most of these examples, the difference presented by these two extracts is quite noticeable. For instance, *A. caudatus* extract presented a better result against the strains of *Kleibsiela pneumonae*, *Morganella morganii* and MRSA with a MIC for all of them of 5 mg/mL, while *H. costaricensis* for those same bacteria showed values of 20, 10 and 20 mg/mL respectively.

It should also be noted that the extract of *A. caudatus* present itself as more efficient against strains of gram-negative bacteria.

The *G. globosa* results for the antimicrobial activity resulted from an optimization procedure, tested in different condition with some different bacteria strains, and the results will be presented later. However, two strain were similar, namely *Listeria monocytogenes*, *Escherichia coli*, and the results presented MIC values ~0.2 g/L, therefore, *G. globosa* is the sample presenting the best results in this activity.

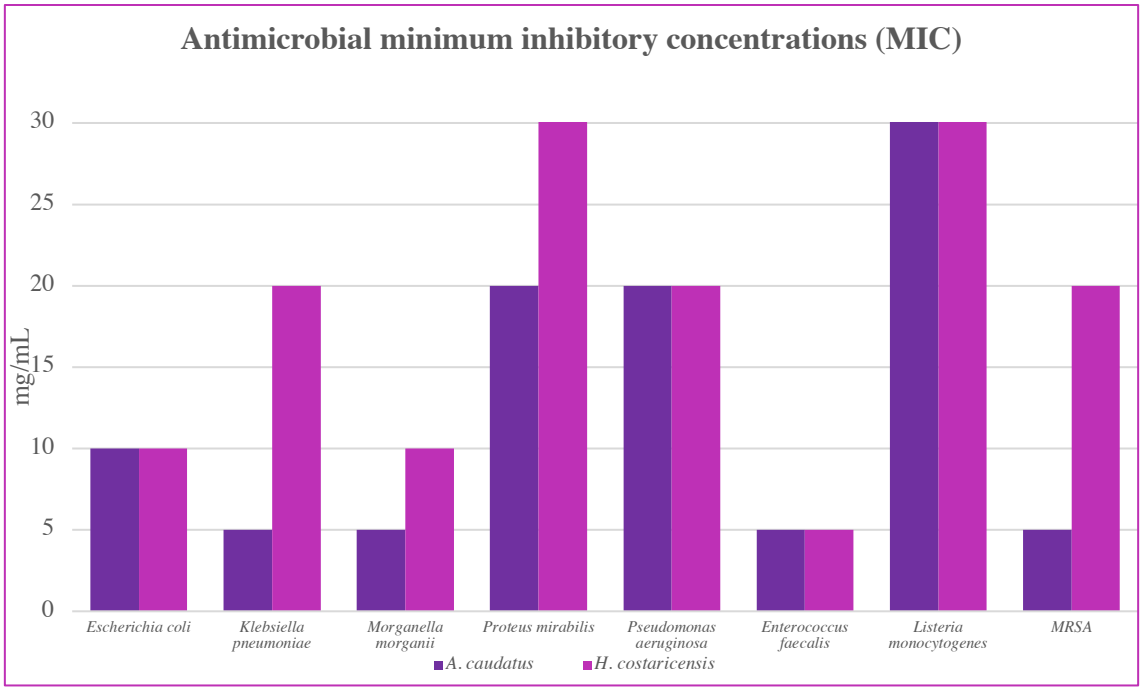

Figure 22 Minimum inhibitory concentrations (MIC) of *A. caudatus* and *H. costaricensis*

Regarding the MBC for the two extracts, the results obtained for all the tested bacteria were higher than 20 mg/mL.

3 Betacyanins extract stabilization techniques

The substitution of artificial additives, namely colourants, with natural alternatives, faces several challenges, since their removal from the natural matrices where they are kept and protected, ends up interfering with their molecular stability. In order to overcome this issue, it becomes necessary to find alternatives that protect, and or manage to confer some stability to the molecules. This is very important, so that during the entire process of extraction, storage and subsequent incorporation, the molecules, in this specific case, betacyanins responsible for the color, remain stable and unchanged. For this purpose, in the present PhD work, during the recovery of the betacyanin compounds of the flower of *G. globosa*, in a very early stage of the work, after the extraction and recovery of these compounds, a removal of the extractor solvent was carried out. The extraction solvent, water, was removed using the freeze-drying technique, in the expectation of obtaining a loose powder with a high concentration of betacyanins. This process alone was already a simple stabilization process, as the solid state of matter is more stable than the liquid state, but this does not happen. Due to their specific characteristics, the betacyanins present in the flower of *G. globosa*, after the freeze-drying process did not appear as a loose powder,

but had a "gelatinous" texture. To overcome this setback, and in order to provide greater stability to the extract enriched in recovered betacyanins, the spray drying technique was explored and compared with the freeze-drying technique, and the results are presented in a section 2.2. on **article 7.**

Two different techniques were used to dry the betacyanin extracts, freeze drying and spray drying. The spray drying process had a yield of 41% due to inherent equipment losses. In the freeze-drying process, a non-powdered and heterogeneous sample was obtained due to the presence of interfering molecules such as sugars. With the spray drying technique, a homogeneous, and shiny powder without agglomeration was produced due to the presence of maltodextrin that inactivates the sugars. Even having a lower yield, the spray drying technique provided an extract with better appearance and homogeneity. The main objective of using these two techniques was to evaluate which of them allows higher stability to the betacyanins and enables the obtaining of a homogeneous extract. In **Figure 23** its possible to see the betacyanin compounds present in the two processed extracts of *G. globosa*, and the amount in which they presented. As it can be observed, the amount of betacyanins present in the samples of globe amaranth (174.1 and 188.8 mg/g of extract in the freeze and spray dried sample, respectively), thus revealing that these extracts are excellent sources of these type of compounds. The large quantity obtained in the globe amaranth samples is mainly due to the presence of gomphrenin III (61 mg/g of extract) in the freeze-dried extract and amaranthin (48.8 mg/g of extract) in the spray dried sample. Between the two processed extracts (freeze and spray drying), it can also be observed that there are significant differences, with the spray dried one revealing the highest amount of total betacyanins.

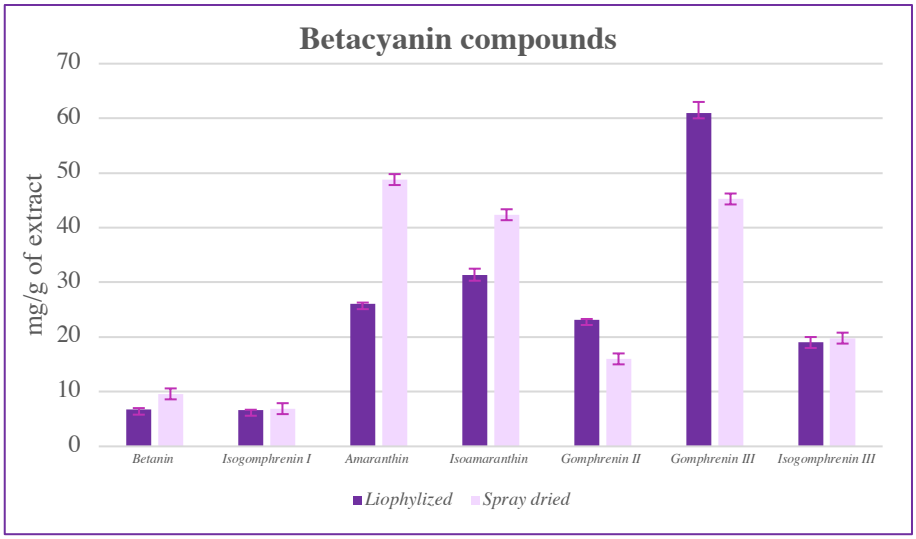

Figure 23 Betacyanin compounds present in the freeze and spray dried extract of *G. globosa* flower

Both freeze and spray dried samples were further analysed for their antioxidant potential, to verify their capacity, besides the colouring capacity. Once the use of spray drying technique allowed to obtain a more homogenous powder, this activity was performed aiming at increasing the antioxidant potential. The results of the antihaemolytic potential measured by the OxHLIA assay are graphically presented in **Figure 24**. The best result was sought for the spray dried extract of *G. globosa*, with an IC_{50} of 80 ± 2 µg/mL, followed by the spray dried extract, with an IC_{50} of 176 ± 11 µg/mL. Trolox, the used positive control, was more efficient in protecting the erythrocyte membranes than the tested extracts, with an IC_{50} value of 19 ± 1 µg/mL.

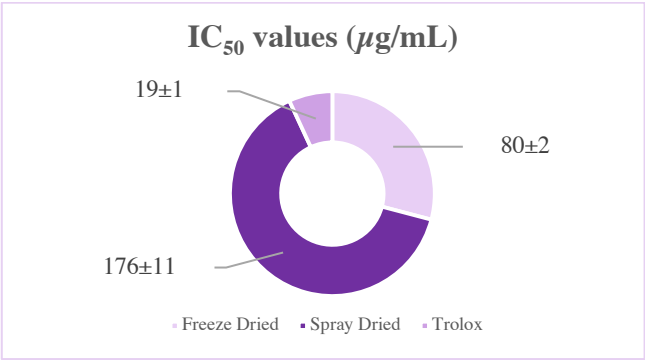

Figure 24 Antihaemolytic activity in the freeze and spray dried extract of *G. globosa* flower extract

As it can be observed, the spray drying process did not enhance the antioxidant potential, and even decreased this capacity. This fact may be due to the inactivation of sugars that in the freeze-drying process are available to exert this activity.

In the continuity of the work, and when exploring the remaining matrices subjected to the betacyanin compounds recovery process, the spray drying process was not carried out for two reasons: i) due to the different compounds present in the different samples, the freeze drying process revealed to be quite effective in recovering a homogeneous powder without agglomerations, and ii) the molecules present in the extracts recovered from the different samples, although different from each other, belong to the betacyanins family, which are compounds in which, in relation to their colour, present a very robust stability. Therefore, the spray drying process was carried out only on the extract recovered from *G. globosa*.

4 Proof of concept: Incorporation of the colouring extract in food samples

In the last few decades, food additives have been kept under a magnifying glass, due to some associated health issues. The advance of science in the investigation field of food additives and their interactions and causes, upon ingestion, allowed a more in-depth knowledge, which led consumers to postpone the additives of artificial origin, for healthier alternatives. Due to the different biological activities that some natural molecules present, they can be explored as natural alternatives in the different classes of natural additives, mainly in the class of preservatives and colourants. Carocho (2016) developed natural preservative ingredients from *Castanea sativa* Mill. flowers, *Ocimum basillicum* L. and *Melissa officinalis* L.. And tested its effectiveness as natural preservatives in bakery and cheese products. Caleja et al. (2015) used *Matricaria recutita* L. to assess the preservative effect of this specie in the development of a dairy food. Takwa et al. (2017) explored the *Arbutus unedo* L. and *Ocimum basillicum* L. as sources of natural preservatives for food industry, tested in loaf brad.

Although all classes of food additives are subject to this change, the class of food colourants is under great pressure to become a reality, as the preferred consumers of foods displaying intense and vibrant colours, are children. Therefore, companies are increasingly looking for alternative natural food colourants, to mitigate these effects, responding to the consumer´s demands for foods with health benefits besides conferring organoleptic and colouring features.

This path has already been done by some researchers, who focused their research theme on the search for natural matrices, such as fruits, flowers, bulbs and roots, among others, as alternative sources for the recovery of natural food colourants.

For instance, Sampaio et al. (2020), explored anthocyanin-rich extracts from purple and red *Solanum tuberosum* L. species as natural colourants. Albuquerque et al. (2020) obtained an anthocyanin-rich extract from *Plinia cauliflora* (Mart.) Kausel epicarp to use as a natural food colorant. Pires et al. (2017) incorporated natural colorants obtained from edible plants in yogurts. The family of compounds displaying colour in Nature are massive, and the colour present by them will depend on what compounds they present. It is possible to find in nature molecules responsible for several colours: a) chlorophylls – green hue; b) carotenes – orange-red; c) curcumin – yellow-orange; and d) anthocyanin and betacyanins – red-pink. This last family of compounds are very interesting, namely in the intensity of colour, as well as the stability displayed by the extracts recovered from the plants where they appear. These red-purple colourants are ususallly obtained from beets, betacyanins and betalains are the most commonly studied and were already approved (E162) to be safely used. Interestingly, *Beta vulgaris* L. root is not the only source of these natural colourants. The fruit of *Hylocereus polyrhizus* (Weber) Britton & Rose (Stintzing, Schieber, & Carle, 2002), *Opuntia ficus-indica* [L.] Miller (Cassano, Conidi, & Drioli, 2010; Otálora, Carriazo, Iturriaga, Nazareno, & Osorio, 2015), *Opuntia stricta* (Haw.) Haw. (Obón, Castellar, Alacid, & Fernández-López, 2009) and *Rivina humilis* L. (Khan & Giridhar, 2014) are also rich in these ingredients, widely used in burgers, desserts, ice creams, jams, jellies, soups, sauces, sweets, drinks, dairy products and yogurts. For instance, Caldas-Cueva et al., (2016) used the natural colorant recovered from Ayrampo (*Opuntia soehrensii*) and Gengatharan, Dykes, & Choo, (2017) recovered natural colourant from *H. polirhyzus* as a natural food colorants in yogurts, comparing their performance against the commercial natural food colorant recovered from beet root (E162) . (Kaimainen, Laaksonen, Järvenpää, Sandell, & Huopalahti, 2015) used a spray dried beet root powder as natural colourant in model juices

In the present PhD work, after the whole process of extraction optimization, drying and stabilization of betacyanin compounds, recovered from the flowers of *G. globosa*, it was then passed to the proof of concept, where the natural origin colourants were incorporated in food matrices. The food matrices tested were ice cream and cookies. This selection of foods was not carried out at random. It is known know that the target audience for food with vibrant and intense colours are children. In this way, two foods whose target audience is the smallest ones were selected, to try this way, substituting the artificial dyes, and provide a less harmful alternative. The foods are also of completely different lines,

in order to test the performance of the natural colourants obtained from the flowers of *G. globose* when subjected to different conditions and in different foods. The result of the incorporation in different foods, as well as all the parameters evaluated for the different foods correspond to **articles 6 and 7.**

4.1 Incorporation of the betacyanin enrich extract in Ice cream

Ice cream is a product widely consumed all over the world and presents itself as a poor foodstuff in what concern the presence of bioactive compounds. It often displays vibrant colours, due to the addition of artificial colourants, therefore, it would certainly benefit from the incorporation of bioactive natural substances, particularly if these substances could simultaneously improve its appearance, such as natural food colourants.

In the present work, some natural food colourants were tested: one already commercialized (E 162), and one obtained from the flowers of *G. globosa* as well as the betalain standard acquired from Sigma-Aldrich. Different parameters were evaluated as well as the chemical profiling and colour stability during its storage period of 60 days. The food industry is in desperate need to obtain new sources of natural food colourants, to meet the consumers demands, and therefore it becomes very important to test the real behaviour of the natural alternative developed in the present work, asses their performance in some real foodstuff incorporation.

To this end, in the present work four different ice cream formulations were evaluated, namely control ice cream (CI, ice cream with no added colourants), ice cream added with betalain standard (BSI), ice cream with *G. globosa* extract (GGI), and ice cream with *Beta vulgaris* extract (BVI).

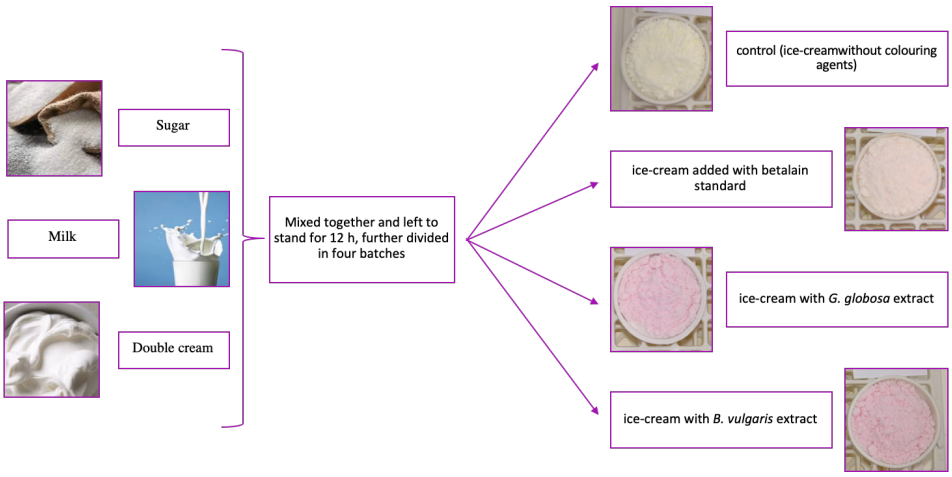

Figure 25 *G. globosa* flower extract, scheme of the incorporation in ice cream.

Table 24 Amount of added colourant and its concentration on the ice cream.

	Amount of added colourant	Concertation in the ice cream
Betalain standard	200 mg	0.5 mg/g of ice cream
G. globosa extract	670 mg	1.6 mg/g of ice cream
B. vulgaris extract	670 mg	1.6 mg/g of ice cream

This comparative study was extended to different storage periods, namely in the day of preparation and after 15, 30, 45 and 60 days at -22ºC. Storage time (ST) effect could depend on the ice cream formulation (IF), and *vice-versa*, therefore, the interaction among both factors was studied, in addition to evaluating the significance of each individual factor.

The nutritional and proximate composition (water, proteins, ash, fat and fatty acids profile, carbohydrates and soluble sugars, as well as energy value) was fully characterized in all ice cream formulation for all the different storage times (results were reported in **article 6**).

4.2 Incorporation of betacyanin enriched extract in Cookies

Cookies are products of high commercial interest, since they present a good acceptability, particularly by children. Therefore, the need to improve this type of product, either by changing some of its ingredients, by using healthier alternatives, such as flour (Bassinello et al., 2011; Cheng & Bhat, 2016; Kaur, Singh, & Kaur, 2017), sugars (Aggarwal, Sabikhi, & Sathish Kumar, 2016), fibre supplementing (Baumgartner, Özkaya, Saka, & Özkaya, 2018; Galla, Pamidighantam, Karakala, Gurusiddaiah, & Akula, 2017; Mudgil, Barak, & Khatkar, 2017), or bioactive compounds (Infante et al., 2017) is of great importance.

In this work, the possibility of introducing natural origin colourants in a formulation of cookies was tested, and evaluate the different parameters, chemical and physical, as well as the colour stability during its storage period. Although there are already some natural origin food colourants on the market, these are not enough to meet the needs of the industry, so the search for alternative natural sources becomes of great importance, as well as testing the behaviour of natural colourants in their actual application.

To this end, four different cookies formulations were evaluated namely control (cookies with no added colourants), Commercial (cookies added with commercial beet root colourant), freeze dried (cookies with *G. globosa* freeze dried extract), and spray dried

(cookies with *G. globosa* spray dried extract). Furthermore, this comparative study was extended to different storage periods, namely in the day of preparation and after 7, 15, and 30 days at room temperature.

The nutritional and proximate composition (water, proteins, ash, fat and fatty acids profile, carbohydrates and soluble sugars, as well as energy value) physical parameters (hardness, adhesiveness, springiness, cohesiveness, chewiness, and resilience) was fully essessed in all cookie formulations for all the different storage times (results were reported in **article 7**).

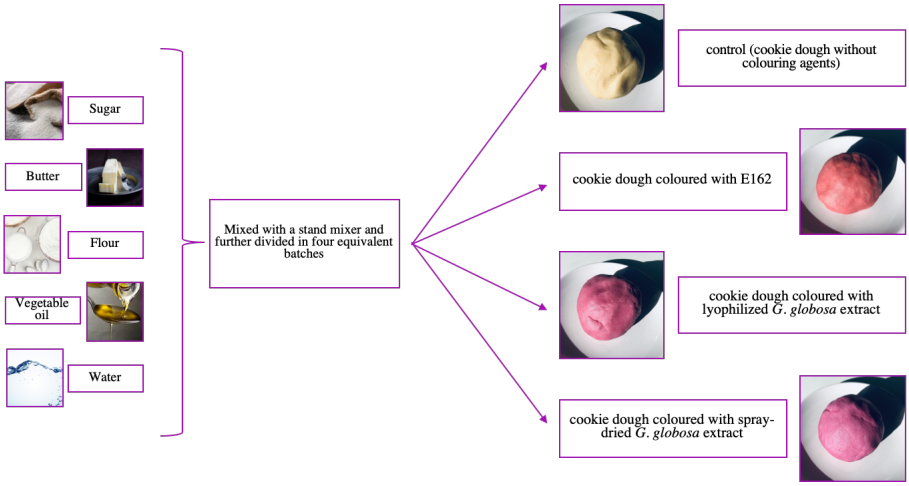

Figure 26 *G. globosa* flower extract, scheme of the incorporation in cookies.

Table 25 Amount of added colourant and its concentration on the cookie dough.

	Amount of added colourant	**Concertation in the cookie dough**
E162	2 g	4 mg/g of cookie dough
Freeze dried *G. globosa extract*	1 g	2 mg/g of cookie dough
Spray dried *G. globosa extract*	1 g	2 mg/g of cookie dough

4.3 Colour stability in ice cream and cookies formulated with betacyanins extracts

The colour analysis is of crucial importance, and the resistance of colourants to oven cooking and storage time is of utmost importance for their success in the industry. Color analysis in both proof-of-concept novel food formulations (ice creams and cookies) was performed using CIELab colour space, that measures coordinates of L* (lightness, variation between -100 and +100, black to white), a* (greenness and redness, variation between -100 and +100, red to green), and b* (yellowness/blueness, variation between -100 and +100, yellow to blue). In the case of ice cream formulated with betacyanins extracts, considering that the main purpose of this work was finding an alternative natural colouring agent for ice cream, the parameters lightness (*L**), redness (*a**) and blueness

(*b**) were evaluated in the fresh and freeze-dried forms of all IF throughout ST (**Table 26**). In line with the observed in nutritional composition, the studied factors, IF and ST, had a cooperative (p<0.001) effect over colour parameters. However, the effect of ST was not significant in most cases, except *L** value in fresh samples (p=0.030), while differences among different IF (as it might have been anticipated) were significant in all cases. In general, *L** and *b** presented the highest values in CI samples, fresh and freeze dried, which on the other hand showed the lowest *a** values, owing to the absence of any colouring agent. Concerning IF added with colourants, BSI presented higher *L** and *b** values, while GGI and BVI were characterized as having the lowest.

Table 26 Colour parameters measured in different ice cream formulations (IF). Results are presented as mean±standard deviation.

		Fresh ice cream				Freeze dried ice cream			
		*L**	*a**	*b**	*Colour*	*L**	*a**	*b**	*Colour*
IF	Control	94±1	-2.5±0.2	13±1		95±1	-2.3±0.2	11±1	
	Betalain standard	93±1	2.0±0.2	8.1±0.4		94±1	1.7±0.3	8±1	
	G. globosa extract	86±3	8±1	2.4±0.3		87±2	8±1	2.5±0.3	
	Beta vulgaris extract	85±2	10±2	3.0±0.4		87±1	10±1	3.4±0.4	
	ANOVA *p*-value (n = 45)[c]	<0.001	<0.001	<0.001		<0.001	<0.001	<0.001	

Lightness (*L**), redness (*a**) and blueness (*b**)

On the other hand, **Figure 27** shows the colour of the three different colourants used in cookies by converting the L*, a* and b* coordinates to RGB colours. As can be seen the commercial sample and freeze-dried extract present a darker colour very similar, while the freeze-dried extract is lighter and shows a violet colour. The overall colour of the cookies during the 30 days of storage is also presented in **Figure 27**. The external colour of the commercial samples showed a shift to orange, while the cookies with the *G. globos*a extracts (freeze and spray dried) was more consistent with a pink. The spray dried extract of *G. globosa* presents a deeper pink colour observed for all cookie sections, especially in the inner part. The orange colour found for the commercial sample does not fit the desired pink colouration intended for the enhancement of the cookies appearance, thus representing an opportunity for these natural colourants to fill a void in the food industry in terms of food colouring.

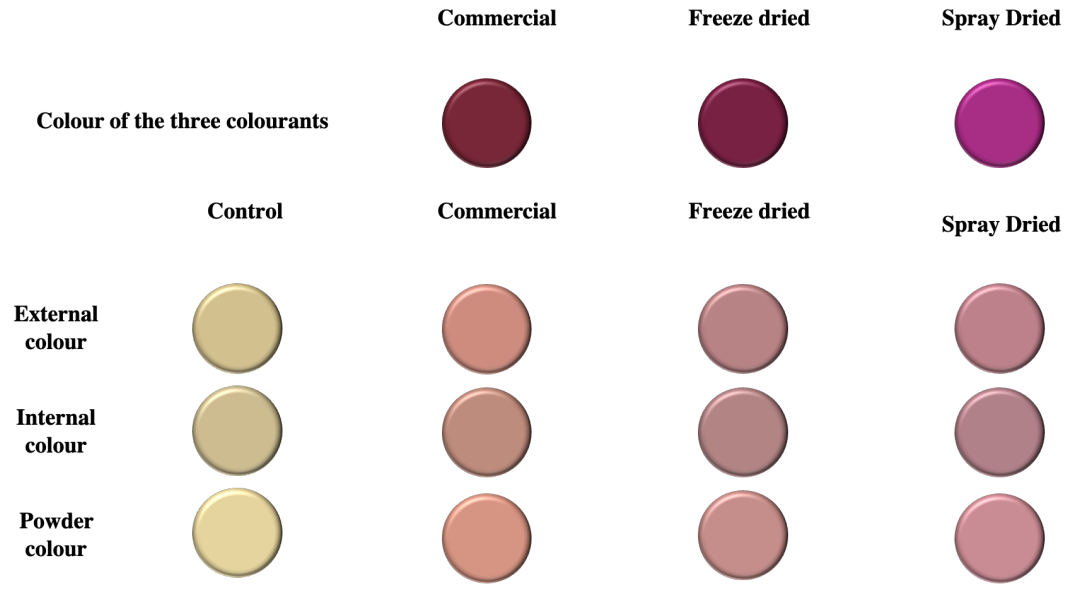

Figure 27 Colours of the cookies, obtained from the L*, a*, b*, considering an average of the colours during all storage times

Although they are completely different foodstuffs, both were subjected to a process of incorporating an extract of colouring compounds of natural origin, recovered from *G. globosa* flowers. Thus, a comparison, although very simple, of the behaviour of the colouring compound in the different foods, with respect to CIELab color space, in the coordinates of L * (lightness,), a * (redness), and b * (blueness), were be carried out, in order to assess the behaviour of the colouring extract, when subjected to different manufacturing conditions, and their stability along the storage time, in two distinct foodstuffs. Therefore, the ice cream incorporated with *G. globosa* freeze dried extract presented the CIElab coordinate values of: L * (86), a * (8), and b *(2.4), while the cookies incorporated with the same freeze-dried extract presented CIElab coordinate values of: L * (56.3), a * (22.1), and b *(8). First of all, and despite the incorporated amount of the colouring extract was not the same, due to the colour intensity pretended in the different foodstuff, on the L* (lightness value) parameters, it is noticeable the difference between both foodstuffs. In the ice cream, the value presented is higher, a fact that may be due to the higher amount of water in this foodstuff, since it is in the solid form, and frozen water has a very shiny surface. The a* value is also very different, being higher in the cookie formulation, may be due to the amount of colouring extract added in this foodstuff. The pink shade pretended in the cookie was stronger, and therefore, the redness value displayed in the cookie was higher, as observed. Concerning the b* parameter, it was higher in the cookie, once more, due to the stronger hue pretended on

this foodstuff, achieving a deep pink colour. The blueness ends up interfering in a significant way so that this tonality is reached. Regarding the stability of the colour along the storage time, for ice cream, the CIElab coordinate values presented in the storage time of 0 days where: L * (91), a * (4), and b *(7) while for the storage time of 60 days: L * (89), a * (4), and b *(6). For the cookie storage time of 0 days, the CIElab coordinate values presented were: L * (62), a * (18), and b *(15), while for the storage time of 30 days where: L * (62), a * (17), and b *(16). These values, for both food samples show a very minimal difference, meaning that the colour was very stable along the different storage times. For ice cream, since it was stored at -22°C, the below zero temperature may be one reason for the stability of the colouring compounds present in the natural colouring extract, recovered from *G. globosa* flowers, being in accordance with what was stated by Gengatharan, Dykes & Choo (2021), in a study on ice cream incorporated with *H. polyrhizys* natural food colourant. But this tendency, is also observed, in the difference between the presented values of cookies, for storage time of 0 days to 30 days. The difference is once more minimal, demonstrating once again the colour stability in a different foodstuff, that was subjected to cooking process, and stored at room temperature. The low quantity of water presented in the cookies, may explain the high stability of the colour displayed, but as already stated, the betacyanin compounds, are a class of natural colouring compounds presenting a good stability, when compared with some others natural colouring compounds. Therefore, the betacyanin enrich extract recovered from flowers of *G. globosa*, is a promising candidate to be used as natural food colourants.

4.4 Proposed food labelling information of the new developed food products formulated with *G. globosa* betacyanins extract.

As already stated, the main focus of this work was to find alternative sources of natural colourants rich in betacyanins compounds, objective that was achieved, and corroborated with the results presented above. After that, and in order to obtain a better understanding of the behaviour of the colourant obtained from flowers of *G. globosa*, the process of incorporation into different foods was carried out. The detailed description of the nutritional profile and proximate composition of ice cream and cookies, the chosen food products for the incorporation process, during storage time was detailed discussed in **article 3 and 5**, respectively. In this section the information will be summarized in order

to present the labelling information of each developed food product, ice cream and cookies, as a future prospect of its commercialization, as healthier foods (free from artificial food colourants).

In terms of nutritional composition, the interaction among ice cream formulation (IF) and storage time (ST) (**Figure 28**) both significantly influenced all the evaluated parameters. This indicates that the effect of ST over the water, fat, protein, ash, carbohydrates contents, and energy values varied according to the IF. This tendency is also verified in in the majority of the effect of each factor *per se*, with the exception of protein along ST and ash among different IF. As can be seen in **Figure 28**, the variation over the ST, for the different parameters of nutritional value, is very small. Also represented in **Figure 28** is the mean value for each parameter that will be used as reference value on the proposed label for the ice cream.

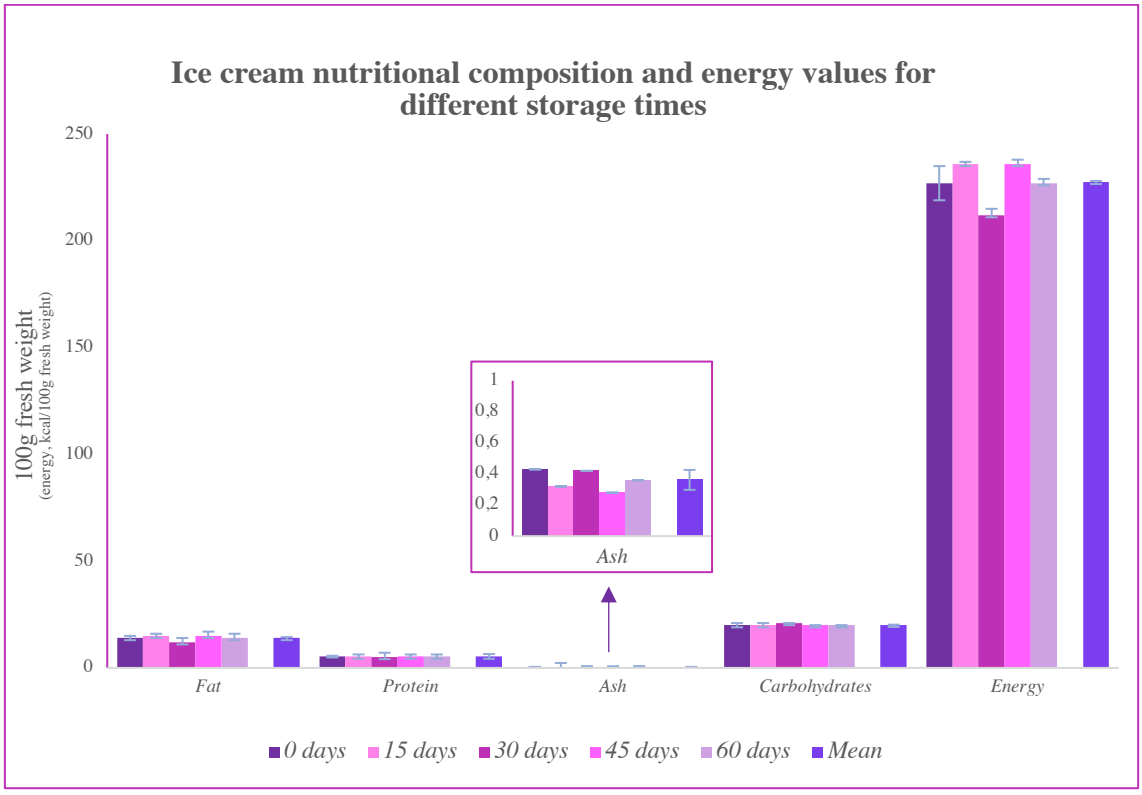

Figure 28 Ice cream nutritional composition and energy values for different storage times

With the exception of the protein contents, that presented values higher than the most commonly reported values (2.5–4.5 g/100 g) in ice cream (Erkaya, Dağdemir, & Sengül, 2012; Senaka Ranadheera, Evans, Adams, & Baines, 2013; P. D. L. da Silva, Bezerra, Santos, & Correia, 2015), the nutritional profile is in agreement with the reported in

typical ice cream formulations (dos Santos Cruxen et al., 2017). The protein is crucial for texture in the ice cream, whereas the sucrose and glucose are essential for flavour and aroma retention.

Figure 29 is the graphical representation of the fatty acids composition in the different IF and ST. The fatty acids profile where especially sensitive to ST, for all cases, with the exception of C18:1 and MUFA, where a significant effect was induced. This is something only noticeable due to the statistical analysis applied, since by only observing the graphical representation, that is not something easily noticeable; being only possible to observe that the individual effect of ST and IF was not significant. According to the global profile of these compounds is noticeable that myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids, were the major saturated fatty acids present in all IF, and ST, contributing to the large amount of SFA present in the different IF, for all ST. One monounsaturated fatty acid, the oleic acid (C18:1n9) presents itself as the second major compound present in all IF, for all ST. In most cases, with very few exceptions, either in the different IF or in the different ST, the variations that occur are minimal or none. This behavior was specifiable in the fatty acid profile, since it is a food product that is stored at low temperatures, hindering the fatty acid degradation process, resulting in small or no changes in the profile of these compounds in the different IF, and different ST.

Figure 29 Ice cream fatty acid composition for different storage times

For the ice cream, and according to the Regulation (EU) No 1169/2011 the proposed label is presented in **Table 27**.

Table 27 Proposed label example for the ice cream incorporated with *G. globosa* colourant

Nutritional composition	Per 100 g	Per porcion (50 g)
Energy	227.6 kcal / 952,92 kJ	113.8 kcal / 476.46 kJ
Total Fat	14	7
Of which Saturated	9.72	4.86
Carbohydrates	20.2	10.1
Of which sugars	20.2	10.1
Protein	5.26	2.63
Salt	0.57	0.28
INGREDIENTS: Double cream (57.78%), Milk (28.78%), Sugar (13.33%), *G. globosa* colourant (0.04%)		

In the case of the novel development of cookies. The nutritional composition along the full storage time**, Figure 30** presents the centesimal profile, the interaction of the different cookies along the 30 days of storage time. The fat, protein, ash and carbohydrate contents (calculated by difference) and energy show that the nutrients detected in the highest quantity were the carbohydrates (although they are comprised of fibres present in the flour, and also the added sucrose), followed by fat and proteins. The cookie formulation (CF)×storage time (ST) is lower than 0.05, which indicates a significant interaction between the storage time and cookie formulation, being not possible to assess the individual contribution of each parameter. This may be due to the low amount of water in the cookies (about 5%). Likewise, to what happened with the ice cream sample incorporated with natural colourant obtained from *G. globosa* flowers, the differences that observed in the nutritional parameters throughout the different ST are also quite small. Figure 20 also shows the average value for each of the parameters that will be used as a reference value in the proposed label for the cookies.

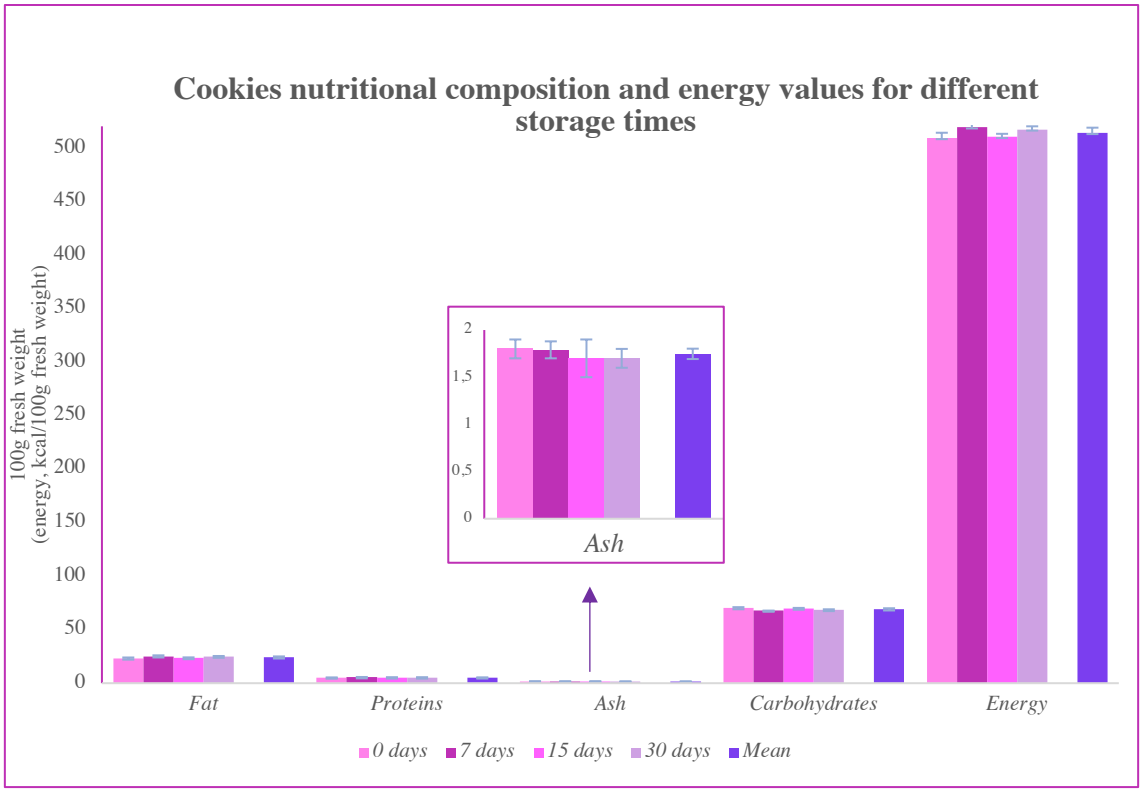

Figure 30 Cookies nutritional composition and energy values for different storage times

Figure 31 represents the monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA), along the cookie's storage time of 30 days, where is possible to observe that SFA are the ones presenting the higher relative percentage, with some variation in the different cookie's formulations, and storage time.

Although a higher number of individual fatty acids were detected, only the SFA, MUFA, and PUFA are present in the graphical representation of **Figure 31**. The most abundant individual fatty acid was palmitic acid (C16:0), followed by oleic acid (C18:1) and linoleic acid (C18:2), contributing for the profile observed in **Figure 31**. This profile is consistent with the used ingredients, being mainly composed by butter, sunflower oil and flour

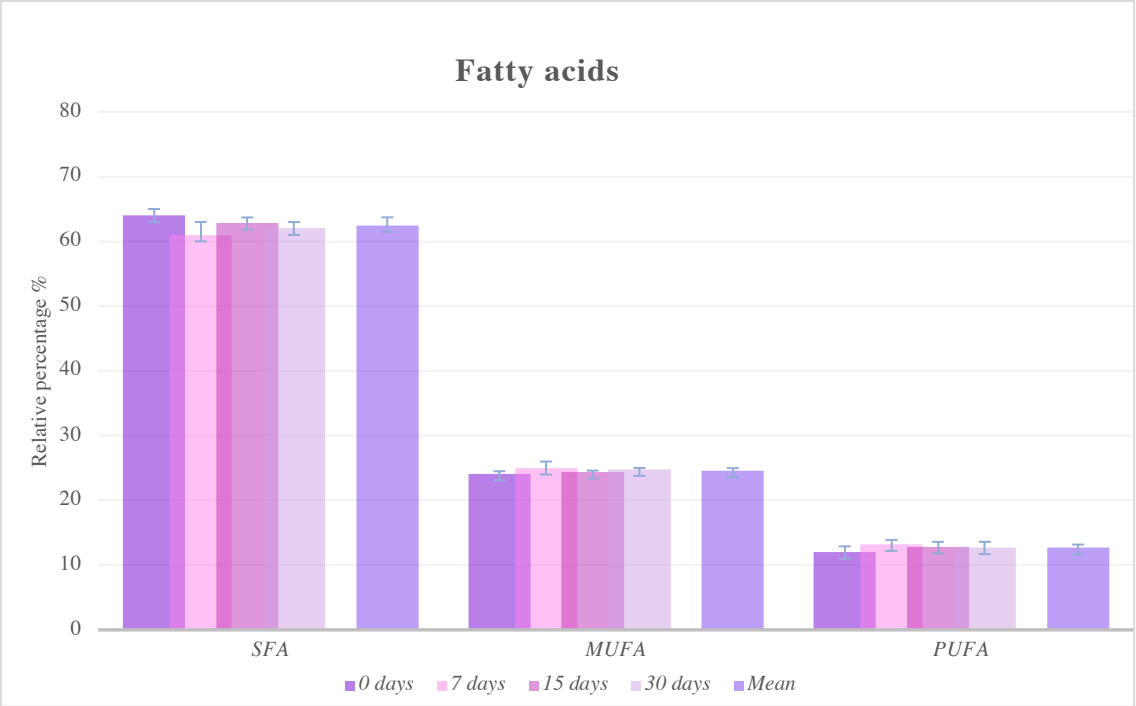

Figure 31 Cookies fatty acid composition for different storage times

For the cookie developed with the addition of the natural colouring extract from G. globose flowers, according to the regulation Regulation (EU) No 1169/2011 the proposed label is presented in **Table 28.**

Table 28 Proposed label example for the cookie incorporated with *G. globosa* colourant

Nutritional composition	Per 100 g	Per portion (20 g)
Energy	513.75 kcal/ 2150.97 kJ	102.75 kcal/ 430.194 kJ
Total Fat	24.02	4.804
Of which Saturated	15.00	3
Carbohydrates	68.90	13.78
Of which sugars	68.90	13.78
Protein	5.30	1.06
Salt	0.73	0.15
INGREDIENTS (flavoured cookies): Flour (42%), Sugar (27%), Butter (22.5%), Vegetable oil (5%), Yeast powder (1%), *G. globosa* colourant (0.05%)		

Part VII. CONCLUSIONS

Conclusions

The main objective of this work was to assess the chemical composition and optimize the extraction of the compounds with colouring capacity from three species of plants, as possible colouring ingredients of natural origin in different foodstuff. This work was designed with the purpose of respond to industry and consumers demands all over the world, for food ingredients/additives of natural origin. This work was designed considering sustainability and ecological vision. Therefore, it aims to use surplus, or bio residues resulting from the production of *G. globosa*, *H. costaricencis* and *A. caudatus* as raw material. Food colorants of natural origin are a theme with increasing expression due to some harmful effects observed in some food colourants of artificial origin. With the use of these natural food colourants, it is intended to reduce or even eradicate this problem, and if possible, take advantage of the well-known bioactive capacity of plants, making certain foods healthier and safer for consumers as also enriching them with biological benefits. As food matrices selected for incorporating the obtained food colorants was ice cream and cookies, thus these are foods highly consumed all over the world, specially by children, those in which the harmful effects of artificial food colourants are felt in a higher proportion.

Partial conclusions

1 Studies plants and their chemical and bioactive characterization

After analysing the chemical composition of the studied plants, it is possible to assess some conclusions in terms of their molecular composition, as well as their bioactivities

i) *G. globosa* flower is the sample with the higher amount of organic acids, being the malic acid the major compound present in this sample.

ii) *H. costaricensis* has the richer profile in terms of tocopherols, presenting the 4 vitamers, being γ-tocopherol the major one present in this sample.

iii) For the betacyanin profiling, *G. globosa* presented 6 betacyanins, gomphrenin/isogomphrenin II III, and 17-descarboxy-amaranthin, being gomprenin III, the major compound.

iv) *A. caudatus* sample presented 4 betacyanin compounds, amarabthine/isoamaranthine and betanin/isobetanin, and the major one detected compound was Amaranthine.

v) For *H. costaricensis*, it was possible to identify 4 betacyanins, namely betanin/isobetanin and phylocactin/isophylocactin, and the compound most abundant in this sample was phylocactin.

vi) Between *A. caudatus* and *H. costaricensis,* the sample presenting the better results for the antihaemolytic and antimicrobial activity was the *A. caudatus* sample.

2 Studied plants and their betacyanin optimization extraction procedure

After analysing the selected plants, and further application of an RSM for betacyanin compounds extraction optimizing procedure, different conclusions may be drawn.

i) The most suitable extraction procedure, of the three tested, considering all the parameters studied, was UAE since this procedure achieve the higher amount of betacyanin compounds, and the strongest colour displayed by the extract.

ii) For the three studied samples, after applying the UAE optimized procedure, it was possible to obtain three betacyanin enriched extracts, very different in terms of betacyanin concentration, texture and hue, for further application as natural food colourants.

iii) For the *G. globosa* sample, after applying an extraction for 22 min, with a power of 257.8 W, with water as the extraction solvent in a solid liquid ratio of 5 g/L, it is possible to obtain an extract presenting a betacyanin concentration of 45.5 g betacyanins/kg plant material.

iv) *A. caudatus*, presented a result of 77.6 g betacyanins/kg plant material in an extraction performed at room temperature, during 13.3 min, with water as extraction solvent, at 500 W of power, and 5 g/L as solid-liquid ratio.

v) For the *H. costaricensis* peels, the extraction was carried out at room temperature, applying 487 W of power, for 38 min and the solvent used was a 90:10 solution (water: methanol *v:v*), due to problems with the mucilage present in this matrix, which hinder the filtering process. Thus, obtaining a value of 36 g betacyanins/kg plant material.

3 Application in ice-cream

The colour capacity of the betacyanin enriched extract recovered from *G. globosa* flowers, by optimized UAE, was evaluated by incorporation in ice-cream, and comparing the results with different ice-cream formulations in different storage periods.

i) In terms of nutritional composition, the interaction among ice-cream formulations and storage time influenced significantly all the evaluated parameters.

ii) Colour parameters showed significant differences among the different ice-cream formulations, as expected, since the colourants used where not the same. However, the storage time did not significantly affect the colour, demonstrating a good stability of these compounds after the incorporation.

iii) The fatty acids profile showed no significant differences among ice-cream formulations, being mainly altered by storage time.

iv) Overall, the effects of ice-cream formulations were more pronounced than those induced by storage time, as evidenced by LDA outputs, where markers were only clustered according to the levels of each factor in the case pf ice-cream formulations.

4 Application in cookies

Cookies were another foodstuff where the colour capacity of *G. globosa* betacyanin enriched extracts (lyophilized and spray dried) were evaluated, also comparing with different cookies formulations and different storage periods.

i) The incorporation did not significantly alter the chemical composition of the cookies.

ii) The physical parameters did not undergo drastic changes and the ones registered were a product of the interaction of the storage time and colourant type, showing a consistency along the storage time.

iii) There was a significative interaction between storage time and colourant type.

iv) Cookies with the spray-dried extract showed a deeper pink colouration, after the cooking process, which can be related to the protective effect of maltodextrin, coadjutant of the spray-drying process.

v) Cookies with the lyophilized extract and the commercial colourant did not achieve this protective effect conferred by maltodextrin, and thus, the colour did not maintain the intended deep pink, since these cookies lost some colour capacity after the cooking process.

vi) Both drying techniques provided a higher stability over the analysed time, since cookies incorporated with these extracts, beyond presenting no degradation, shown no microbial growth.

Concluding remarks and future perspectives

Resorting to plants, fruit stems or roots to obtain natural colouring compounds, able to compete with the artificial colourants available on the market is an increasingly common practice. It is also an enormous need nowadays, since consumers demand it, and the food industry is under increasing pressure to do so. The use of foods bio-residues is also an important goal of these work, since it is based on an integrated strategy to valorise this raw material, by exploiting it as a source of high value compounds with strong application by the industry, thus promoting sustainability, circular economy and decreasing food waste.

In both ice cream and cookies, the incorporation of the natural colourant recovered from *G. globosa* flowers did not alter the nutritional profile and physicochemical characteristics of the foodstuff. Due to the small amount of natural colouring extract needed to exert the colouring purpose, no bioactive potential was observed and as it was not the main objective, these activities were not deeply explored. Nevertheless, the developed extracts revealed themselves as secure for food application, by revealing no toxicity in the screening of toxicity for non-tumour cell lines. The colour of the food remained stable throughout the storage period tested, and in the case of cookies it resisted the baking process at higher temperatures. In both cases, the tested extract was able to colour the food in the desired hue, thus making the food more appealing and meeting the wishes of consumers of natural food colourants.

Analysing data of the incorporation extracts recovered from *A. caudatus* flowers and *H. costaricensis* peels, the next step, will be the treatment of these data, since despite this task was performed, due to some restrictions employed in the last year caused by the pandemic situation, it was not possible to present these data in the present work.

The flowers of *G. globosa*, *A. caudatus* and peels of *H. costaricensis* have an immense visual impact in nature, due to their strong colour and different aesthetic, and proved that nature is a good source of alternative sources of natural origin colouring compounds, meaning that nature provides all the necessary raw material; we just have to look around.

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ANNEX1. PUBLISHED WORK FROM THIS RESEARCH

Portuguese National Patente

Sandrina A. Heleno; Isabel Cristina Fernandes Rodrigues Ferreira; Lillian Bouçada de Barros; Miguel Angel Prieto; Custódio Miguel Lobo Roriz; Barreiro, Maria Filomena. 2019. "*Gomphrena globosa* L.: uma fonte alternativa e promissora para a obtenção de corantes naturais". Portugal.

(PATENTE DE INVENÇÃO NACIONAL No 109174; Concedida/Emitida)

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ANNEXO2. FIGURE CAPTIONS

ANNEXO3. TABLE CAPTIONS

ANNEXO4. ARTICLES SUPPLEMENTAL MATERIAL

Article 2. Red pitaya (Hylocereus costaricensis (F.A.C. Weber) Britton & Rose.) peel as a source of valuable molecules: Extraction optimization to recover natural colouring agents

Table S1 Natural and codded values of the independent variables used in the five-level central composite design (CCD) implemented to optimize the extraction of betacyanins from H. costaricensis peel.

Coded values	Natural values	
	Time (min)	Power (W)
-1.19	1	5
-1	4.5	44.4
0	23	252.5
+1	41.5	460.6
+1.19	45	500

Table S2 Parametric values of the polynomial Eq. (1) and statistical information of the models fitting procedures.

		Residue	Isobetanin	Phyllocactin	Isophyllocactin	Total betacyanins
Intercept	b_0	4.47±0.03	8.6±0.1	10.2±0.3	7.9±0.2	33.6±0.8
Linear effect	b_1	0.20±0.03	0.37±0.09	0.8±0.2	0.7±0.2	1.8±0.6
	b_2	0.20±0.03	0.68±0.09	1.5±0.2	1.6±0.2	3.7±0.6
Quadratic effect	b_{11}	-0.16±0.03	0.3±0.1	0.6±0.2	1.2±0.2	2.4±0.7
	b_{22}	ns	-0.8±0.1	-1.0±0.2	-1.2±0.2	-3.7±7
Interactive effect	b_{12}	0.22±0.04	ns	-1.5±0.3	0.8±0.3	ns
Statistics	Model F-value	59.45	30.74	27.90	38.00	23.18
	Lack of Fit	ns	ns	ns	ns	ns
	R^2	0.9520	0.9111	0.9269	0.9453	0.8854
	R^2_{adj}	0.9360	0.8814	0.8937	0.9204	0.8472
	Ad. Precision	23.06	17.21	17.42	20.88	15.15
	C.V. (%)	1.85	3.66	5.98	6.87	5.59

Parametric superscripted 1 and 2 stand for the independent variables time and ultrasonic power, respectively. R^2: coefficient of determination; R^2ajd: adjusted coefficient of determination.

Table S3 Optimal processing conditions that maximize the extraction of betacyanins from H. costaricensis peel and model-predicted response optimum

	Optimal processing conditions		**Response optimum**
	Time (min)	Power (W)	Content
Individual conditions for each response variable			
Residue	37	500	5.70±0.06 %
Isobetanin	44	337	9.6±0.2 mg/g
Phyllocactin	45	229	11.9±0.3 mg/g
Isophyllocactin	45	377	11.4±0.4 mg/g
Total betacyanins	34	346	40±1 mg/g
Global conditions considering all response variables			
Residue	38	487	5.05±0.06 %
Betanin			6.8±0.2 mg/g
Isobetanin			8.9±0.2 mg/g
Phyllocactin			10.2±0.4 mg/g
Isophyllocactin			10.1±0.4 mg/g
Total betacyanins			36±1 mg/g

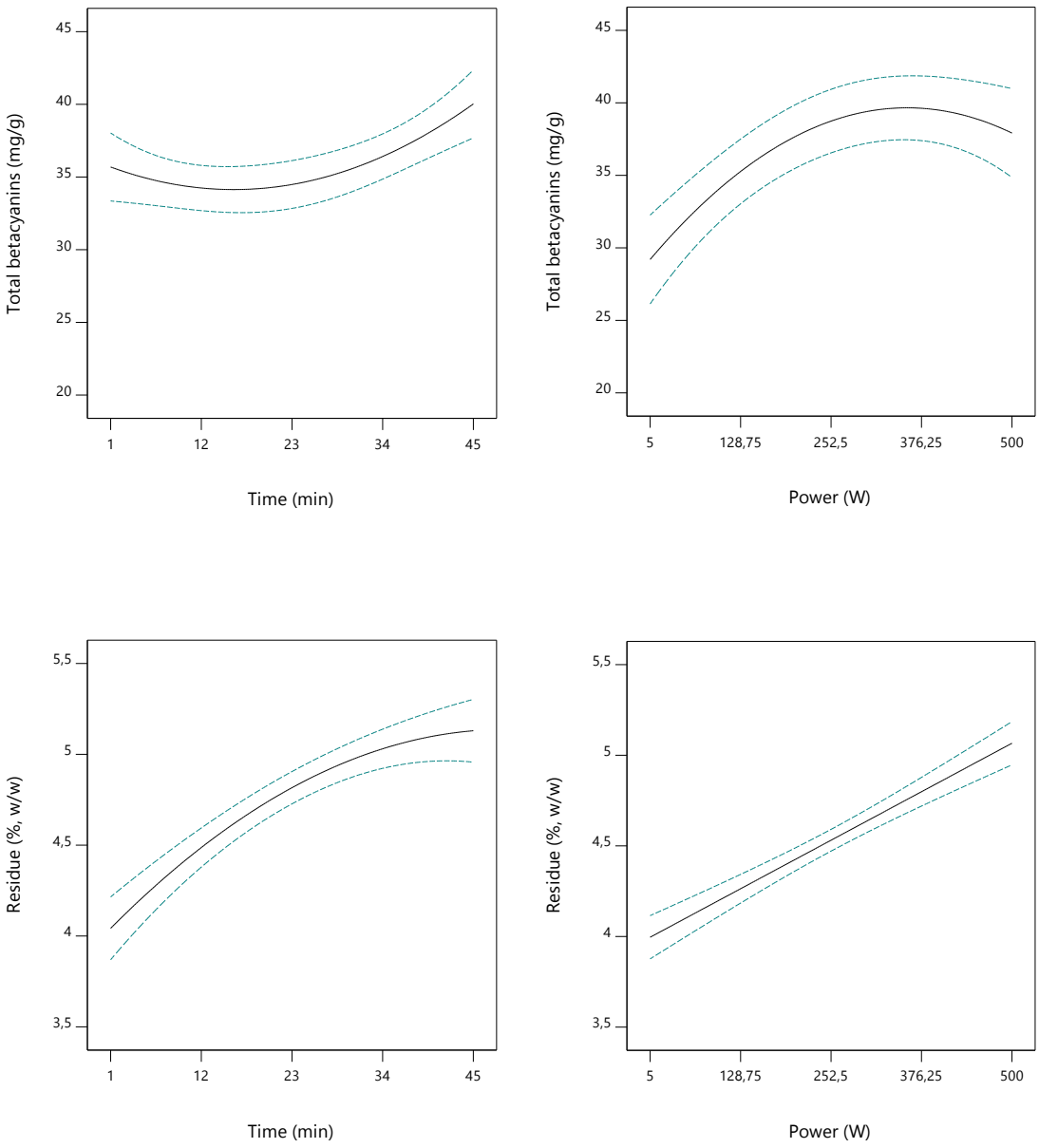

Figure S1 2D response graphs for the effects of the independent variables time and ultrasonic power on the residue and total betacyanins content recovered from H. costaricensis peel. In each graph, the excluded variable was fixed at its optimal value.