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## Antioxidant Capacity of Anthocyanin Pigments

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Additional information is available at the end of the chapter

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### Abstract

Anthocyanins are a family of natural pigments classified into the group of flavonoids, considered to be responsible for the color and taste of many fruits and vegetables, i.e. berries. Anthocyanins are common components of the human diet. Besides their interest as colorant because of their coloring properties, the study of anthocyanin compounds stems from their wide applicability in the prevention and even in the treatment of various human diseases. However, various aspects of the pharmacological roles of anthocyanins remain in the dark, having still several obstacles to the development of robust diets or prescribing lines on consumption of anthocyanins. The chemical structure of anthocyanins determines in large measure its capacity and efficacy as an antioxidant agent. In this study, the following aspects are reviewed: the antioxidant effect of anthocyanin pigments; the oxidative stress, the bioavailability after intake and biological aspects of anthocyanins, the method for measuring the antioxidant activity of anthocyanins, the relationship between structure and activity; and the influence of the anthocyanins in the antioxidant activity of wines. Finally an overview of some potential uses in food industry is attempted mainly focusing in the anthocyanin encapsulation topic. Attention has been paid to the more recent publications in the field.

**Keywords:** anthocyanins, antioxidant, biological properties, wines, encapsulation

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### 1. Introduction

Fruits and vegetables supply a number of micronutrients, such as minerals, fibres and vitamins, as well as a whole series of compounds called phytochemicals, among which are the secondary metabolites of a phenolic nature, called polyphenols [1–3]. Phenolic compounds have attracted the attention of researchers for decades [4–7]. This was initially due to their physiological importance to plants, mainly relating to pigmentation and flavour [8, 9] and,

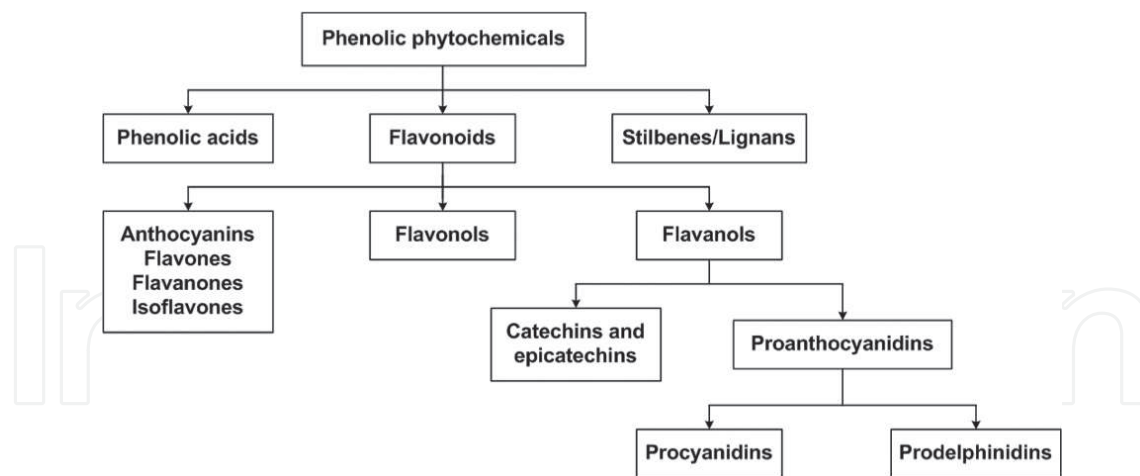
more recently, because of their free radical scavenging capacity, which, among other biological effects, increases antioxidant activity and prevents cellular oxidation [10, 11].

The flavonoids (**Figure 1**) constitute the largest group of phenols and are considered to be responsible for the colour and taste of many fruits and vegetables. More than 9000 flavonoid structures have been described, with formula, references and biological information [12, 13]. These include more than 600 different anthocyanins that are widely distributed among at least 27 families, 73 genera and innumerable species. It has been shown that, of the flavonoids studied, around 5000 have antioxidant activity [4, 5, 14].

Anthocyanins, the largest group of phenolic pigments, are found in red wine, some cereals, root vegetables and red fruits. The red, blue and purple colours (**Figure 2**) of most fruits, flowers and leaves are due to anthocyanins. They are glycosides (water-soluble molecules) of aglycons called anthocyanidins and effective donors of hydrogen. A wide variety of anthocyanins are produced by the higher plants via modification of the six common anthocyanin aglycons (cyanidin, delphinidin, pelargonidin, malvidin, peonidin and petunidin) present in nature. A summary of previous history with references to the pioneers in this field of work has been given [5, 14]. Apart from their physiological role in plants, anthocyanins are regarded as important components in human nutrition [5, 14–16]. It has been stated that the consumption of the anthocyanins is of the order of 200 mg/day, a high amount if compares with the intake of other dietary flavonoids [5]. A possible association between consumption of anthocyanins and quality of the diet is admitted [17], although there are currently no recommendations regarding their dietary intake. A glass of red wine provides around 115 mg of polyphenols, contributing towards a total intake of phenolic compounds of 1171 mg/person/day [18, 19]. The antioxidant activity of anthocyanins is depending to a large extent with their chemical structure: number and position of the hydroxyl groups and the conjugated double bonds, as well as on the presence of electron donors in the structural ring [5, 20].



**Figure 1.** Some selected samples containing anthocyanins.



**Figure 2.** Type of phytochemicals [5].

Numerous epidemiological studies have confirmed the influence of the consumption of antioxidants contained in fruits, vegetables and grains [21–24]. Some beverages [25, 26], such as wine, tea and coffee, have received considerable attention due to their protective effects against the oxidative damage related to various chronic diseases, including cancer, reducing the risk of contracting these diseases by 30–50% [27]. The principal cause of death in the Western world is related to chronic diseases such as coronary heart disease or heart attacks. Low plasmatic levels of vitamin E and vitamin C have been shown to increase the risk of angina pectoris among the population of Scotland [28, 29]. This is attributed, to a great extent, to the low consumption of foodstuffs rich in micronutrients, vitamins and antioxidants, combined with the general lifestyle.

In agreement with the *French Paradox* [30, 31] and other studies [32, 33] undertaken about the European population (WHO Project MONICA, MOLI-SANS, FLORA and ATHEAN EU Projects), the components of the Mediterranean diet [34–38], especially vitamins and polyphenols, are the factors responsible for the low incidence of coronary heart disease in these populations [39–41]. The moderate consumption of red wine [42–46] is another factor closely linked to this low incidence, as the phenolic compounds have a cumulative effect. A diet rich in fruits and vegetables increases by itself the antioxidant capacity of the plasma and the level of plasmatic polyphenols [45]. These factors are increased when supplemented by the intake of red wine. Consumption of wine in moderate amounts has also proved to be beneficial [47] to the skeletal system lowering the risk of loss of mass and fractures. What is clear is that a high consumption of fruits or vegetables rich in antioxidants is related to a decrease in cardiovascular diseases and cancer [41].

Anthocyanins have an antioxidant potential twice that other known antioxidants, such as (+)-catechin and other compounds like vitamin E, synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), compounds widely used in food technology [13, 48–51] that have undesirable effects on the enzymes of the human body. The apparent capacity of the strongly polarized anthocyanins to regenerate lipophilic antioxidants like vitamin E could be because they have similar properties to vitamin C, such as protecting the biomembranes from peroxidation, by effectively trapping the peroxy radicals.

Using the oxygen radical absorbance capacity (ORAC) method, Wang and Goodman [52] evaluated the antioxidant capacity of 14 anthocyanins and obtained values more than 3.5 times greater than those for trolox (a synthetic antioxidant similar to vitamin E). Kuskoski et al. [51], using the ABTS method in purified and isolated patterns of anthocyanins, found an activity twice that of trolox and also confirmed the influence of the structure or the combination of anthocyanins on the antioxidant capacity.

Sources rich in anthocyanins are very interesting options as functional foods [53–58]. Here, the oxidative process, the antioxidant effect and the biological properties of the anthocyanin pigments, described in last years, are reviewed. Furthermore, the most commonly used chemical methods to determine the antioxidant capacity of the anthocyanins are outlined. An overview of the bioavailability of anthocyanins, the metabolism after their intake and their presence and influence in red wine is also given. Finally, an overview of some potential uses in food industry is attempted mainly focusing in the anthocyanin encapsulation topic.

The fertility field of flavonoids antioxidants (e.g. anthocyanins) has grown exponentially in recent decades in such a way that a number of areas are involved such as nutrition, food processing, physiology, biochemistry, pharmacology and analytical chemistry affecting foods and health. Emphasis in this contribution is given in most recent reviews and references. Some 150 journals are cited from the fields of food science and technology, nutrition, chemistry (analytical) and biochemistry, engineering, agriculture, medicine, pharmacy, biology, physiology and clinic. Taking into account that thousands of references are available, the authors apologize for those they may have overlooked or inadvertently omitted. For older references please consult, for example, some reviews [4–7] published on 2012 and the excellent monograph of Andersen and Markhan [59].

## 2. Oxidative process

The process of oxidation has been studied for many years [60], because of the importance it has both for the organisms and the foodstuffs. In live organisms, the oxidative metabolism is essential for the survival of the cells. Oxidation is related to the production of energy associated with the degradation of glucans, lipids and proteins, to the detoxification of many xenobiotics and to the immune response through some of the free radicals (FR) generated [61].

Oxygen is associated with the conditions for aerobic life and is the motive force for the maintenance of the metabolism and cellular viability, but, at the same time, it is responsible for the formation of partially reduced mediators with high reactivity, known as reactive oxygen species (ROS). The majority of ROS are FR, that is, active molecular species with a separated electron at a higher energy level, which, therefore have paramagnetic properties, providing them with high reactivity [20, 62].

The systems of antioxidant protection have to act on the substrates susceptible to oxidation in a controlled way to maintain the physiological equilibrium of the organism. The protective effect of some enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, may start when an excess of FR is produced. If this excess cannot be neutralized,



oxidation of the lipidic membrane, the low-density lipoproteins (LDLs), the protein cellular components, DNA and enzymes can occur, thereby destroying them [63, 64].

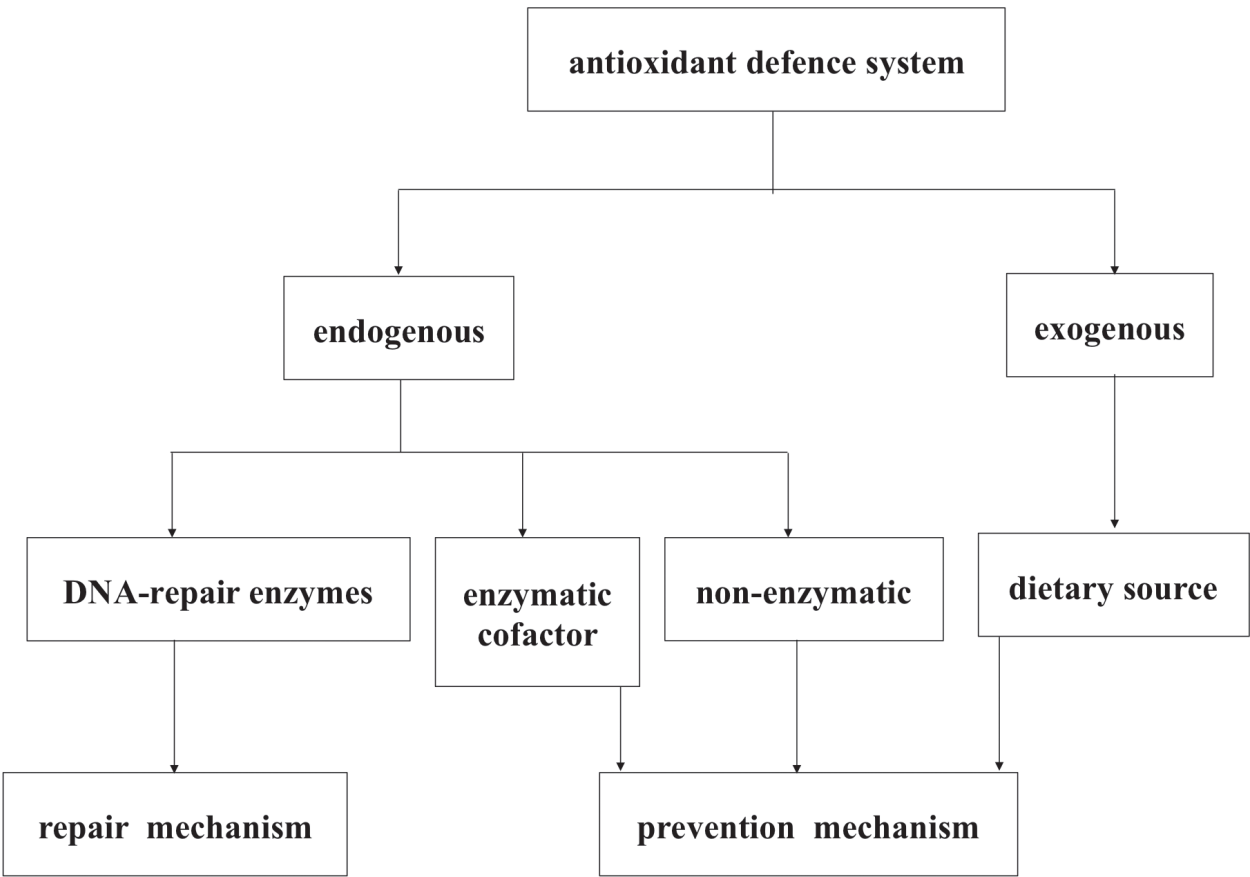
It is worth emphasizing that arteriosclerosis is currently defined as chronic inflammation of the vascular system, triggered by a specific inflammatory agent, the oxidized LDL. The LDLs are very small particles made up by lipids, cholesterol and proteins, with the function of transporting cholesterol and lipids from the blood to the adipose and muscular tissue and, in general, to all cells of the body [65, 66]. However, the LDLs can be oxidized by the FR, affecting, consequently, the molecules of cholesterol and fatty acids that constitute each LDL. The oxidized LDLs are involved in the pathogenesis of coronary heart diseases [67, 68].

Environmental, dietary or physiological factors can provoke an imbalance in favour of oxidation, causing what is known as oxidative stress [69–71]. Whether the oxidation or the oxidative stress, in particular, is either a primary cause or a side effect of many chronic diseases and of the phenomenon of ageing itself has been a scientific debate prompted over the last few decades. Therefore, many efforts and resources have been devoted to finding out the role oxidants play in hindering oxidation, thus resulting in either the prevention or the retardation of the oxidative stress [72].

An excessive production of ROS, particularly hydroxyl radicals, can easily initiate the process of oxidation of the LDLs. In turn, they contribute to a greater or lesser degree to the onset of coronary heart diseases, rheumatoid arthritis, inflammatory diseases, cancer, renal diseases, pancreatitis, multiple sclerosis, Parkinson's disease, cataracts, diabetes, pulmonary disorders and all diseases related to cellular ageing [73]. The intake of dietary antioxidants, that is exogenous antioxidants (**Figure 3**), is very important [74], and some compounds of this family, that is vitamin E,  $\beta$ -carotene and phenolic compounds, are only synthesized by plants [27, 31, 34, 35]. Therefore, it is important to maintain a balance between oxidants and antioxidants. It is worth bearing in mind that over a lifetime, as the individual ages, this balance tilts in favour of the oxidants [75].

In foods, oxidation can be one of the main causes of alterations leading to rancidity, deterioration and loss of nutritional, commercial and organoleptic quality (colour, taste, smell and texture), besides being a possible health risk to the consumer. For this reason, the food industry, by improving the preparation of the products and by using antioxidants, is trying to prevent and slow down the process of deterioration, in order to offer the consumer a safer deadline for use, which guarantees the quality of the food product [76–80].

However, according to studies carried out in vivo during the last two decades, FR and ROS are no longer seen only as [71] destructive factors but also (and perhaps first of all) as messengers involved in intracellular signalling. So, there has been a substantial change [10] in the conception of these processes in both normal and pathological conditions. Ideas about the role of FR in the functioning of cells and organisms have been revised, resulting in a new concept of redox equilibrium. Oxidative stress is then viewed as [72] a modulation of thiol redox reactions, involved mainly in signalling pathways. On this way, nonradical oxidants (enzymatically generated hydrogen peroxide, other peroxides, quinones, etc.) play a basic role [10] in the oxidation of thiols for the sake of signalling, the formation of free radical intermediates being not necessary. The common conviction of the beneficial effect that the phenolic plants exert on the improvement of health is being revised [64].



**Figure 3.** Summary of antioxidant defence system [74].

The potential health benefits of natural antioxidants, while interesting, seem to escape our basic understanding of biological oxidation processes. Oxidation balances both very beneficial, even crucial, outcomes with decidedly negative impacts. This suggests that moderation in the use of some antioxidants may be advisable. Note that a large measure of the biological oxidation occurring in the body is essential for extracting energy from food and is highly adaptive, depending on health status. Apart from energetics, oxidation supports immunological integrity. While the bulk of epidemiological evidence supports the nutritional/health value of fruits and vegetables [4, 11, 12, 19, 22, 33, 39, 40, 50], the doses of individual components they contain, such as specific antioxidants that may contribute to improved health and reduced risk of certain diseases, remain uncertain.

### 3. Bioavailability and metabolism of the anthocyanins

The existing knowledge concerning with the absorption, distribution, metabolism and excretion (ADME) of anthocyanin compounds (including their decomposition within the gastrointestinal lumen) has been the subjects of several recent reviews [81–95]. In general, few

comparative studies have been undertaken about their metabolism, physiological availability or biotransformation after intake in comparison with the number of studies devoted to absorption and distribution. Little information is also available on the effects of food matrix on anthocyanin bioavailability, particularly food matrices of the usual diet [92].

In general, anthocyanins are considered to have a remarkably low bioavailability (relatively low as well in comparison with that of other flavonoids), on the basis of the levels detected in human blood after ingestion [81]. This fact contrasts with the health-promoting properties [81, 83, 84, 90] of anthocyanins, suggesting bioavailability and their interaction with other components present. Anthocyanins appear to be rapidly absorbed in the stomach and small intestine [89] and removed, being in the plasma and urine where reach low maximal concentrations [90]. After oral administration, anthocyanins follow a particular pattern different from other flavonoids [84]. A 20–25% of intact anthocyanins were detected in plasma few minutes after intake [86]. Kinetic studies have shown that anthocyanins have a rapid distribution and appearance in blood that is compatible with a tricompartamental model. Elimination takes place mainly through bile. Anthocyanins could be absorbed from the stomach as well as intestines where they undergo decomposition catalysed by microbiota. Bacterial action is capable of hydrolysing anthocyanins or aglucons into simpler phenolic compounds, which can be absorbed and still maintain free phenolic groups, retaining part of the reducing capacity of the original molecule. Active transport may play a role in the absorption of anthocyanins from the stomach as well as in their transfer within the kidney or liver [84]. The metabolic destination of the anthocyanins can differ depending on their aglucon structure, as well as on the tissue where they are metabolized (intestine or liver).

However, the persistence of anthocyanin metabolites, phenolic acid breakdown products (which could be responsible for the health benefits associated with anthocyanins) suggests enterohepatic recycling, leading to prolonged residence time, and supports the notion that anthocyanins are far more bioavailable than previously suggested [81, 88, 92]. However, the compounds as well as the molecular mechanisms involving all those biological events [83] still remain underexplored. The ability to cross membranes, pH effect, digestive enzymes, microbiota, biliary acids and food matrix are critical factors, which may contribute to this apparent paradox [86]. There are many doubts if the effect is due to the native compounds or other forms, their mechanism or which factors have crucial impact on bioavailability [86]. To clear the access both native and metabolized forms *in vivo* and to distinguish their different biological roles have been a very challenging task. Accumulative evidence, which is emerging, suggests multiple roles [92] explaining the apparent incongruity (poor absorption). Compared with other flavonoids, much remains to be discovered [94, 95] about details and mechanisms of anthocyanin absorption and transport. The activity of anthocyanins could be associated with the ability to elicit cell adaptive responses involving the transcription factor Brf2 by affecting the “nucleophilic one” of the organism [89]. Recent studies on the bioavailability topic are summarized in **Table 1** [82, 96–108].



Comments	References
Pharmacokinetic trial to evaluate the bioavailability of anthocyanins and colonic polyphenol metabolites after consumption of aronia berry extract in plasma and urine	[96]
Pharmacokinetic characterization and bioavailability of strawberry anthocyanins relative to meal intake	[97]
Bioavailability studies and anticancer properties of malvidin-based anthocyanins, pyranoanthocyanins and nonoxonium derivatives	[98]
Effect of red cabbage fermentation on anthocyanin bioavailability and plasma antioxidant capacity in humans	[99]
Bioavailability of red raspberry anthocyanins and ellagitannins: new insights	[82]
Bioavailability and uptake of anthocyanins and their metabolites from grape/blueberry juice and smoothie in vivo and in vitro	[100]
Tissue bioavailability and intake of tart cherry anthocyanins	[101]
Confirmation and identification of tart cherry anthocyanins in several target tissues of healthy rats	[102]
Bioactive anthocyanins in ‘Queen Garnet’ plum: maturity and bioavailability	[103]
Use of anthocyanins as bioactive colourants in lipstick formulations	[104]
Application of the developed flavonoid-poor menu meals to the study of the bioavailability of bilberry anthocyanins as model flavonoids	[105]
Anthocyanin stability, mucus binding, and uptake into epithelial cells in healthy individuals that retained red grape or chokeberry juice in the mouth	[106]
Absorption and bioavailability of anthocyanins across the gastrointestinal mucosa	[107]
Effects of processing sour cherry fresh fruit to the final juice product on the content of anthocyanins and other related polyphenols	[108]

**Table 1.** Bioavailability of anthocyanins.

**4. Biological activity of the anthocyanins**

Establishing the biological activities of phytochemicals, flavonoids and polyphenol is dependent on the complete understanding of their intake, absorption, metabolism and excretion; however, to date, this had only realized for a limited few structures [109]. The increasing evidence of potential therapeutic effects that present anthocyanin compounds has boosted the interest in the knowledge of their biochemistry and biological effects during the last two decades [95, 110–112]. Biological properties of anthocyanins depend on their bioavailability. The chemical structure of anthocyanins [113] determines their rate and extent of intestinal absorption and nature of the metabolites in the plasma. The growing and current interest in the study of anthocyanin compounds [114, 115] stems from their wide applicability in the prevention and even in the treatment of various human diseases. They could also be used in the control of the viruses that cause immunodeficiency, such as the causal agent of AIDS, and they have a strong activity against the influence A and B viruses as well as against the herpes virus [116]. Though many articles have been devoted to varying biological effects of anthoxyanins, only a limited number of studies deal with their antimicrobial activity [117].

The favourable effects of anthocyanins on improvement of vision in humans (increase in visual acuity), one of the first reported, were described in 1966, which prompted their introduction into ophthalmology [56, 57]. It continues to be an interesting field of study due to the prevalence of myopia in today's society [118]. Although these effects are not completely understood [119], it has been confirmed that cyanidin helps regeneration of rhodopsin. Anthocyanins have been associated with substances that strengthen the capillaries, reinforce the action of vitamin C and favour the accumulation of this vitamin in the liver and in the suprarenal glands. Blackcurrant anthocyanins inhibit transient myopia, reduce eye fatigue, improve dark adaptation and enhance retinal blood flow with glaucoma [56]. Anthocyanin-rich bilberry extract has a protective effect on visual function during retinal inflammation [116].

Anthocyanins have been shown to be effective in the prevention of arteriosclerosis and cardiovascular diseases [25, 40, 41, 72]. Commercial extracts of *Vaccinium myrtillus* (bilberry) [120, 121] contain glucosides of delphinidin and cyanidin and, since 1977, have been used to inhibit platelet aggregation [122] because of their preventive effect in the initial stage of the formation of thrombi, in the treatment of some diseases related to poor microcirculation resulting from capillary fragility, and also to prevent the oxidation of the LDLs [123–125].

Moreover, it has been demonstrated that these preparations accelerate the spontaneous process of cicatrization and that they have a preventive and curative activity against gastroduodenal ulcers induced in rats. These effects are probably due to their influence on the biosynthesis of mucopolysaccharides [126], which improves the efficacy of the gastric mucous layer and increases the base substance of the connective tissue and of the capillaries.

Another described effect is the inhibition in vitro that certain anthocyanins have on the porcine pancreatic elastase [127]. This enzyme attacks fibres and collagen, playing an important role in some pathologies, such as arteriosclerosis, emphysema and rheumatoid arthritis. Beneficial effects have also been described in experiments with diabetes, with a substantial reduction observed in the sugar concentration in urine and plasma of rats treated with the anthocyanin pigments of grapes [128]. It is suggested that anthocyanins act by reducing the biosynthesis of collagen, lipoproteins and glycoproteins, as well as reducing the activity of elastase and adenosine deaminase, which are both known to be high in diabetic patients.

Anthocyanins are recognized for their various [67, 129] pharmacological and medicinal properties. They are antimutagenic, anti-inflammatory and vasotonic [111, 112, 130, 131]. They protect against radiation, are chemoprotective against the toxicity of platinum, are used in therapy against cancer and are hepatoprotective against carbon tetrachloride. They also have other effects due to several actions of a variety of enzymes and metabolic processes.

There are various patented pharmaceutical preparations [132] containing flavylium salts and anthocyanins for the treatment of wounds, gastroduodenal ulcers, inflammation of the mouth and throat, vascular diseases and other diseases linked with the lipidic and the glyceric acid metabolisms. More recently, they have been used in the treatment of circulatory diseases.

Some studies specify the anticarcinogenic effect of anthocyanins [12, 13, 19, 111, 112, 133–135]. They inhibit the growth of carcinogenic cells that provoke colon cancer, induce the apoptosis

effect, have the capacity to inhibit in vitro the growth of cells that cause tumours in humans and are even able to act as modulators of the macrophages in the immune response [89, 112].

Anthocyanins are effective against cytotoxicity, lipidic peroxidation, and as protectors of DNA, by forming co-pigments of DNA-anthocyanins. Moreover, anthocyanins have cellular antioxidant mechanisms comparable to or greater than other micronutrients, such as vitamin E. The capacity of the anthocyanins for stabilizing triple-helical complexes of DNA [136] by forming complexes of anthocyanins-DNA [137] is well established.

Pharmacokinetics of anthocyanins has recently reviewed [85, 113, 138, 139]. The most recent papers published on the subject are summarized in **Table 2** [96, 101, 140–151]. Anthocyanins are metabolized to a structurally diverse range of metabolites that exhibit dynamic kinetic profiles. A multicompartamental (theoretical physiologically based) pharmacokinetic (PBMK) model has been proposed [138] in order to describe the anthocyanins fate in vivo. Understanding the elimination kinetics of these metabolites is key to the design of future studies [152] concerning with their utility in dietary intervention or as therapeutics for disease risk reduction.

Comments	References
Pharmacokinetic trial to evaluate the bioavailability of anthocyanins and colonic polyphenol metabolites after consumption of aronia berry extract in plasma and urine	[96]
Evaluation of the protective effects of protocatechuic acid	[140]
Effects of black raspberry extract and protocatechuic acid on DNA adduct formation and mutagenesis in rat oral fibroblasts	[141]
Influence of ethanol on the bioavailability and pharmacokinetics of blackberry anthocyanins	[142]
Pharmacokinetic trial to evaluate the of nanoencapsulation of a phenol extract from grape pomace on human plasma	[143]
Pharmacokinetic characterization of anthocyanins in overweight adults on the basis of meal timing	[97]
Determination of cyanidin 3-glucoside in rat brain, liver and kidneys: a short-term pharmacokinetic study	[144]
Pharmacokinetics, bioavailability and regional brain distribution of polyphenols from apple-grape seed extract mixture and bilberry extract	[145]
Evaluation of changes in metabolic parameters, and in cardiovascular and liver structure and function in rat due to administration of either cyanidin 3-glucoside or Queen Garnet plum juice	[146]
Bioavailability and uptake of anthocyanins and their metabolites from an anthocyanins-rich grape/blueberry juice and smoothie in vivo and in vitro	[101]
Effects of anthocyanins and their corresponding anthocyanidins on the expression levels of organic anion transporting polypeptides in primary human hepatocytes	[147]
Effect of flavan-3-ols and anthocyanins against inflammatory-related diseases	[148]
Anthocyanin pharmacokinetics and dose-dependent plasma antioxidant pharmacodynamics by intake of Montmorency tart cherries in healthy humans	[149]
Pharmacokinetics of the metabolites of cyanidin-3-glucoside	[150]
Abundance and persistence of metabolites of anthocyanins in human urine	[151]

**Table 2.** Selected papers on pharmacokinetics of anthocyanins in the 2014–2016 period.

In words of Kay [152], 'These studies on (flavonoid) metabolism and biological activity of metabolites mark a new beginning in phytochemical research and, in this respect, this work is in its infancy'. Phenol-Explorer web database gathers polyphenol metabolites [153] identified in human and animal biofluids, from 221 publications.

## 5. Methods for measuring the antioxidant activity of anthocyanins

Although a plethora of biological actions has been ascribed to flavonoids, their antioxidant activity, in particular, has recently attracted much attention. Anthocyanins behave as antioxidants by a variety of ways, including direct trapping of ROS, inhibition of enzymes responsible for superoxide anion production, chelation of transition metals involved in processes forming radicals and prevention of the peroxidation process by reducing alkoxy and peroxy radicals.

There are a variety of methods for measuring antioxidant activity, either *in vitro* or *in vivo* (greater complexity involved) or a combination of both. The number of reviews published on the matter reflects the transcendence of this hot topic and its richness. Selected reviews found in the literature from 2000 up to the present time are summarized in **Table 3** [154–204]. The most common chemical methods used for measurement *in vitro* of antioxidant activity of polyphenolic compounds (e.g. anthocyanins) are shown in **Table 4** [197–241]. Methodological contributions are preferably cited in **Table 4** instead of specific practical applications. Both conceptual and technical problems limiting the use and validity of three commonly used [119] assays TEAC/ABTS<sup>+</sup>, DPPH and ORAC have been subject of recent revision. Some reviews dealing with the DPPH [208, 212, 214], ORAC [223] and CUPRAC [239, 240] assays have also been the subject of recent treatments. However, the aspects concerning with the assay chemistry, standardization and report of the antioxidants determination have not been solved after 25 years of intense study [199].

Antioxidant activity is always measured in an indirect way as a response (of the antioxidants present in the sample) to induced oxidation [192, 160, 173]. For foodstuffs, there is a range of methods for determining antioxidant activity. These can vary from those that evaluate the inhibition of lipidic peroxidation by the antioxidants and quantify the products as peroxides, hydroperoxides and products resulting from decomposition measured by the thiobarbituric acid reactive substances (TBARS) assay [171], to methods that determine the content of free fatty acids, polymer content, viscosity, absorptivity at 232 and 268 nm, colour and physiological measurements *in vivo*, such as measuring the products from oxidation of the LDLs, or indirect indicators of lipidic oxidation. Alternatively, antioxidant activity can be evaluated by measuring the immunological response to antigens (the products of lipidic oxidation). Though solvent effect is a vital parameter [203] exerting an influence on the chemical behaviour of antioxidant compounds, the information concerning about its role on the antioxidant capacity is relatively scarce.

There are some drawbacks to assays *in vivo*. The interpretation of changes in the antioxidant activity of the plasma can be complicated because of the possibility of producing adaptability in response to an increase in oxidative stress. However, assays *in vitro* can also have their drawbacks, such as the interactions between samples and reagents.

Content	References
Antioxidant activity/capacity measurement: classification, physicochemical principles, mechanisms and electron transfer-based assays	[154]
Antioxidant activity/capacity measurement: hydrogen atom transfer-based, mixed-mode and lipid peroxidation assays	[155]
Antioxidant activity/capacity measurement: reactive oxygen and nitrogen species scavenging assays, oxidative stress biomarkers and chromatographic/chemometric assays	[156]
Recent applications for in vitro antioxidant activity assay	[157]
Evaluation of procedures for assessing anti- and pro-oxidants in plant samples	[158]
Capacity of antioxidants to scavenge multiple reactive oxidants and to inhibit plasma lipid oxidation induced by different biological oxidants	[159]
Analytical methods applied to antioxidant and antioxidant capacity assessment in plant-derived products	[160]
Advantages and limitations of common testing methods for antioxidants	[161]
A comprehensive overview on the biology behind some reactive molecules and the means for their detection	[162]
Potentiometric study of antioxidant activity: development and prospects	[163]
Methods for determining the efficacy of radical-trapping antioxidants	[164]
Electrochemical methods for total antioxidant capacity	[165]
The role of consumption of dietary bioactives on the prevention of adverse health	[166]
Synthetic and natural phenolic antioxidants: mode of action, health effects, degradation products and toxicology	[167]
Up-to-date overview of methods available for measuring antioxidant activity	[168]
Use of metallic nanoparticles and quantum dots as novel tools for reliable assessment of antioxidant activity in food and biological samples	[169]
Review on in vivo and in vitro methods evaluation of antioxidant activity	[170]
IUPAC technical report: methods of measurement and evaluation of natural antioxidant capacity/activity	[171]
Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays	[172]
Application of free radical diphenylpicrylhydrazyl to estimate the antioxidant capacity of food samples	[173]
Application of both stationary and flow electrochemical methods for analysis of antioxidant properties of plant and clinical samples	[174]
Phenol-based antioxidants and the in vitro methods used for their assessment	[175]
Main components in the foodstuffs and beverages: antioxidant methods, chemical and kinetic basis	[176]
Estimation of antiradical properties of antioxidants using DPPH assay	[177]
Evaluation of antioxidants: scope, limitations and relevance of assays	[178]
A comprehensive review of cupric reducing antioxidant capacity methodology	[179]
Overview of the importance and mechanism of action of antioxidants, as well as of the methods of assessment of the antioxidant capacity	[180]
Methods for evaluating the potency and efficacy of antioxidants	[181]
A comprehensive review of chemical methods to evaluate antioxidant ability	[182]
Assessment of antioxidant capacity in vitro and in vivo	[183]



Content	References
Direct measurement of the total antioxidant capacity of foods: the 'QUENCHER' approach	[184]
Flow injection-based methods for fast screening of antioxidant capacity	[185]
Flow injection-based systems for determination of scavenging capacity against biologically relevant reactive species of oxygen and nitrogen	[186]
Antioxidant assays for plant and food components	[187]
Oxygen radical antioxidant capacity and trolox equivalent antioxidant capacity assays comparison to estimate the total antioxidant capacity of food products	[188]
How to standardize the multiplicity of methods to evaluate natural antioxidants	[189]
The use of total antioxidant capacity, total antioxidant capacity test, as a biomarker of disease in biochemistry, medicine, food and nutritional sciences	[190]
Critical review of the most commonly used methods for in vitro determination of antioxidant capacity	[191]
Updated methodology to determine antioxidant capacity in plant foods, oils and beverages	[192]
Methods to measure the antioxidant defence system	[193]
Popular methods commonly used for testing antioxidant activity in vitro: reliability, efficiency, accessibility and biological relevance	[194]
Methods for measuring antioxidant activity and assays for measuring overall reducing capacity	[195]
Model systems of the evaluation of antioxidants in three types of foods: bulk oil, oil-in-water emulsions, and muscle foods	[196]
The multifaceted aspects of antioxidants and the basic kinetic models of inhibited autoxidation	[197]
Application of various chemical methods to determine antioxidant activity in fruit pulp	[198]
Overview of cell culture models for antioxidant research	[199]
Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements	[200]
Review of methods to determine chain-breaking antioxidant activity in food	[201]
Methods of measuring antioxidant activity particularly as they relate to lipid oxidation	[202]
Methods used to evaluate the free radical scavenging activity in foods and biological systems	[203]
Methodologies for evaluation of total antioxidant activities in complex mixtures	[204]

**Table 3.** Published reviews on used methods for measurement of antioxidant activity.

The *in vivo* antioxidant potential of anthocyanins can be measured by reducing the serum concentration of the reactive substance to thiobarbituric acid (TBARS assay) or by increasing the resistance to oxidation in the plasma of the lipidic peroxidation caused by 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) or by  $\text{Cu}^{2+}$ .

Most *in vitro* measurements of the antioxidant activity of anthocyanins involve the following factors: calculating the rate and range of the decrease of the substance in assay or the oxygen consumption, the formation of products from oxidation and the formation or decline of the number of FR. Detection can be carried out by inhibition of fluorescence, chemoluminescence, oxygen consumption or absorbance, the evolution of which is related to the end product.

Method	Detection	Measurement/oxidant	References
Radical ABTS**	Reduction of absorbance of the radical cation in an aqueous medium at 414 nm (or 645, 734 or 815 nm)	TEAC value, antioxidant activity equivalent to trolox (μmol/g)	[197–205]
Radical DMPD**	Reduction of absorbance to 505 nm	Expressed in μmol equivalent to trolox (TEAC) by g of sample	[206, 207]
Radical DPPH**	Reduction of absorbance to 517 nm	Expressed in EC <sub>50</sub> (quantity of antioxidant required to reduce to 50% of the initial concentration of DPPH) or in TEAC	[208–219]
FRAP	Increase of the absorbance to 593 nm	Expressed in μmol of equivalents of reduced ferric ion (Fe <sup>2+</sup> ) by g of sample or a value equivalent to a pattern	[203, 204, 220]
ORAC-PE	Reduction of fluorescence (β-phycoerythrin)	μmol equivalent to trolox (TEAC) by g of sample	
ORAC-FL	Reduction of fluorescence (fluorescein)	μmol equivalent to trolox (TEAC) by g of sample	[221–236]
ORAC-PGR	Reducon of fluorescence (pyrogallol red)	μmol equivalent to trolox (TEAC) by g of sample	
CUPRAC	Absorbance measurement of the Cu(I)-neocuproine chelate	μmol equivalent to trolox (TEAC) by g of sample	[199, 237–241]

*Abbreviations:* ABTS (2,2'-azino-bis(3-ethylbenzothiazolinine-6-sulfonic acid)); DMPD (N,N-dimethyl-p-phenylenediamine dihydrochloride); DPPH (2,2-diphenyl-1-picrylhydrazyl); FRAP (ferric reducing ability of plasma); ORAC-PE (oxygen radical absorbance capacity) with β-phycoerythrin; ORAC-FL (oxygen radical absorbance capacity) using fluorescein (3'6'-dihydroxy-9-oxo-9H-xanthene-2,7-dione); ORAC-PGR (oxygen radical absorbance capacity) with pyrogallol red (pyrogallol sulphone phthalein); TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

**Table 4.** Commonly used methods for measurement in vitro of antioxidant activity.

FR can be generated by various chromogenic compounds, such as azo ABTS (2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid), DMPD (N,N-dimethyl-p-phenylenediamine dihydro-chloride), DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing ability of plasma) and DMPO (5,5-dimethyl-1-pyrroline N-oxide). Inhibition of oxidation can be measured by the reduction in fluorescence by the ORAC method or by the TRAP (total radical-trapping antioxidant parameter) assay.

Currently, ABTS is one of the methods most frequently used for assays of coloured compounds, like anthocyanins [197], as the radical generated has a maximum absorption at a wavelength of 734 nm, reducing the possibilities of interference of antioxidants that absorb in the red colour zone. The radical ABTS<sup>•+</sup> can be generated by enzymes (peroxidase, myoglobin) or chemically (manganese dioxide, potassium persulphate or ABAP (2,2'-azobis-2-amidino-propane hydrochloride). The radical, once generated, displays new characteristics with maximums of absorption at 414, 645, 734 and 815 nm.

Kuskoski et al. [51] found a maximum absorption of around 754 nm in an alcoholic medium, and this wavelength was used to determine the antioxidant activity of fruit extracts of baguaçu (*Eugenia umbelliflora* Berg) that are rich in anthocyanin pigments [242].

If compared with other methods of formation of FR, such as DPPH, DMPD and others, the capture reaction time of the radical ABTS<sup>•+</sup> is fairly rapid, it can range from 1 to 7 min, although according to Re et al. 4 min is sufficient to complete the reaction. Antioxidant data based on ABTS assay are dependent on reaction time because the applied standard compounds (trolox) present a scavenging kinetic profile [200] different from that of polyphenol-rich foods. Studies have been carried out on the effects of molecular structure (molecular weight, number of –OH groups, redox potential) on kinetics and dynamics of [201] the trolox equivalent antioxidant capacity assay with ABTS. Attempt has been made to standardize the method [202] by extrapolating to zero sample concentration.

The chromatic properties of the stable radical cation DPPH were first described [171] by Blois in 1958, who used the radical to measure the antioxidant activity of several natural compounds. Only much later did Brand-Williams et al. develop a technique based on the reduction of the absorbance of the radical DPPH<sup>•</sup> at 517 nm. This technique has also been applied by other authors with modifications and measurement of absorbance at 515 nm. Results are expressed as IC<sub>50</sub> [213], that is, the quantity of antioxidant required to reduce the initial concentration of DPPH to 50%, or as the percentage of interacted DPPH % DDPH = [(Absreferencia – Abse xtracto)/(Absreferencia)] × 100. DPPH assay on food additives and foods and beverages has been subject to interlaboratory study [211, 215]. The DMPH reaction has been revisited and re-evaluated [216–218] and simplified in order to characterize samples of wine origin [210].

The influences of reaction time, DPPH concentration inference and kinetics parameters of bioactive molecules and plant extracts [209] in the reaction with the DPPH radical have been evaluated. A collaborative study on the DDPH assay [215] has been promoted as well as a kinetic-matching approach to express antioxidant capacity in a more standardized way.

The spectrophotometric DMPD method, described by [171, 206, 207] Fogliano et al. in 1999, is similar to the ABTS method. In the presence of an adequate oxidant solution, the radical cation DMPD<sup>•+</sup> generated has the ability to link hydrogen atoms, causing the discolouration of the solution, producing a reduction of absorbance measured at 515 nm. DMPD cannot be used with hydrophobic antioxidants, as it is only water soluble [171, 206]. DMPD method is not considered suitable for assays of coloured compounds, as interference can occur in the measurements, because they absorb in the same region of the spectrum.

The ferric reducing ability of plasma (FRAP) assay measure the ability of antioxidant to reduce the ferric [Fe<sup>3+</sup>–(TPTZ)<sub>2</sub>]<sup>3+</sup> complex to the ferrous [Fe<sup>2+</sup>–(TPTZ)<sub>2</sub>]<sup>2+</sup> complex (blue coloured) in acidic medium. It is a simple, reproducible method that can not only be applied to the study of the antioxidant activity of plasma, or in foods and beverages, but also to the study of the antioxidant efficacy of pure compounds with results that are comparable to those of more complex methodologies. It is widely used to determine the antioxidant activity of anthocyanins in different samples. However, the FRAP assay is carried out at a very low pH (3.6), far from the pH found in biological fluids. Nevertheless, this method has the advantage of determining the activity of the

antioxidant directly in plasma; it does not depend on an enzymatic or a nonenzymatic method for generating FR and evaluates the antiradical efficacy of plasma. It also does not need the isolation of plasma fragments as is required in LDL.

The assay by fluorescence spectrophotometry known as ORAC was first set up [25, 29, 171] by Cao et al. in 1993 and later modified by Cao et al. in 1995. The ORAC method is based on measuring the decrease of the fluorescence of the proteins  $\beta$ -phycoerythrin and R-phycoerythrin (PE). These proteins have a high fluorescence in the presence of peroxy radicals generated by the thermic decomposition of the 2,2'-azobis (2-amidinopropan) dihydrochloride (AAPH); the decrease is recorded in the presence of antioxidants. It is considered to be a very sensitive method that evaluates the oxidation process from its beginning, although it has the drawbacks of being expensive and time-consuming [194].

However,  $\beta$ -phycoerythrin [219] is photo-unstable and it forms complexes with polyphenols giving, therefore low values of ORAC-PE. For this reason, it is substituted [234–236] by fluorescein (ORAC-FL), which, in contrast, is much more stable, and does not react with polyphenols, making it a much more precise and more economic method. Two alternative solutions have been proposed to decrease a systematic error related to AAPH addition in the fluorescence-based ORAC assay [221].

A simple mathematical model for conversion of ORAC values to mass units [229] has been proposed. ORAC standardization [227] and validation [230] have been attempted. The use of pyrogallol red as a probe [233, 225] for competitive antioxidant assay is a significant improvement. Pyrogallol reacts faster than fluorescein with  $\text{RCOO}^*$  radicals, and its consumption does not present induction times, even in the presence of very reactive oxidants, with the exception of ascorbic acid. First action ORAC assay has been reported both with fluorescein [226] (dextracts from tea, blueberry and grape skins) and pyrogallol red [228] (red wine, fruit juices and iced teas).

A proportional measurement of antioxidant activity is obtained using the ORAC assay, which is currently one of the most commonly used methods for measuring the antioxidant activity of the anthocyanins [218].

Cupric reducing antioxidant capacity (CUPRAC) test is conceptually similar to the FRAP test, but is based on the reduction of  $\text{Cu}^{2+}$  ions in the presence of neocuproine (2,9-dimethyl-1,10-phenantroline) at pH 7, which involves faster kinetics. The ammonium acetate buffer solution account for the liberated protons in reaction with polyphenols.

The total radical-trapping antioxidant parameter (TRAP) method [171] proposed by Wayner et al. (1985) is based on the measurement of oxygen consumption during a peroxidation reaction of lipids controlled and induced by the thermic decomposition of some substances, such as ABAP or AAPH, which produces a flow of peroxy radicals at a constant rate that is temperature dependent. These peroxy radicals initiate a chain of lipoperoxidations. The method has some problems, including being sensitive to temperature and to changes in pH. The storage conditions of the samples are also important due to the liability of some antioxidants; therefore, their immediate analysis is recommended. When this is not possible, it is advisable to rapidly collect plasma for blood samples, to store them at  $-80^\circ\text{C}$  and to measure them within 3 days. The concentration of proteins or uric acid, because of their high antioxidant power, should be taken into account when describing the results.

It is interesting to mention the fact that electrochemical [243–249] and ESR [250] methods are increasingly being applied to the determination of antioxidant capacity. The kind of technology and free radical generator or oxidant influences the antioxidant capacity measurement. A key factor that helps researchers to choose a given method and to understand the results obtained is the comparison of different analytical methods. In order to gather comprehensible information about the total antioxidant capacity of a food [184], at least two of these tests, and preferably all, should be combined, if possible, taking into account both the arguments for and against, and its applicability. **Table 5** shows selected articles [20, 190, 198, 204, 231, 251–260] in which more than one criterion has been applied to real samples with practical purposes. Advantages and limitations of the most common chemical methods of determination of the antioxidant capacity are compiled in **Table 6** [160, 161, 167, 171–173, 176, 184, 231, 261–263].

Samples	Methods	References
Eight anthocyanidins, seven anthocyanins and two synthesized 4'-hydroxy flaviliums	DDPH, ABTS, hydroxyl radical scavenging activity, FRAP	[20]
Dried fruits and juices from chokeberry	FRAP, ABTS	[251]
Protocatechuates	DPPH, ORAC, CAT	[252]
Six deoxyanthocyanidins and cyaniding-3-glucoside	DPPH, FRAP	[253]
Plant foods	CUPRAC, ABTS	[254]
Anthocyanins from different varieties blueberries	Inhibiting activity on lipid peroxidation, hydroxyl radical scavenging, superoxide anion radical, DPPH	[255]
Plan extracts	DPPH, ABTS, AAPH	[198]
Commercial beverages (wines, beers, soft drinks and waters)	TRAP, TEAC, FRAP	[256]
Popular antioxidants-rich US foods	ABTS, DPPH	[257]
Commercially available vegetable juices (23)	FRAP, DPPH, ABTS	[258]
Anthocyanidins, anthocyanidin-3-glucosides and portisins	DPPH, FRAP	[259]
Plants extracts of industrial interest (30)	DDPH, ABTS, FRAP, FRAP, SOD, ORAC	[260]
Food products	ORAC, TEAC	[231]
Selected small fruits	ABTS, FRAP, DPPH	[204]
Pulps of frozen fruits	ABTS, DPPH, DMPD	[190]
CAT (conjugate autoxidizable assay); SOD (superoxide dismutase assay).		

**Table 5.** Antioxidant capacity of selected samples evaluated using more than one criterion.



<i>Mixed hydrogen atom transfer (HAT) and single electron transfer (SET)</i>		
ABTS	<ul style="list-style-type: none"><li>• Inexpensive and easy to use</li><li>• Applicability in lipid and aqueous phase</li><li>• Stable to pH</li><li>• Fast reaction</li><li>• Can be automated and adapted for use with microplates</li></ul>	<ul style="list-style-type: none"><li>• Complex mechanism of reaction</li><li>• Extra step to generate free radical</li><li>• Free radical not stable for long periods of time</li><li>• Not standardised</li></ul>
DPPH	<ul style="list-style-type: none"><li>• Simple and highly sensitive</li><li>• Can be automated</li><li>• It just needs a UV-vis spectrophotometer to perform</li><li>• No sample separation is needed</li></ul>	<ul style="list-style-type: none"><li>• High price of ABTS reagent</li><li>• Complex mechanism of reaction</li><li>• DPPH colour can be lost</li><li>• Steric accessibility influences the reaction</li><li>• Sensitive to acidic pH</li></ul>
<i>Single electron transfer (SET)</i>		
DMPD	<ul style="list-style-type: none"><li>• Simpler, more productive and less expensive and compared ABTS test</li></ul>	<ul style="list-style-type: none"><li>• No data of its stoichiometry with anti-oxidant standard and radical stability are available</li><li>• DMPD is only soluble in water</li></ul>
FRAP	<ul style="list-style-type: none"><li>• Simplicity, speed and robustness</li><li>• It does not require specialized equipment</li><li>• It can be performed using automated, semi-automated, or manual methods</li></ul>	<ul style="list-style-type: none"><li>• It is nonspecific</li><li>• Not all antioxidants reduce Fe<sup>3+</sup> at a rate fast enough to allows its measurement</li><li>• Compounds that absorbs at the wave-length of the determination may interfere</li><li>• Requiring an acidic pH</li></ul>
CUPRAC	<ul style="list-style-type: none"><li>• Rapid way to study plant extract profiles</li><li>• Fast enough to oxidize thiol-type antioxidants</li><li>• Selective</li><li>• Stable and accessible reagents</li><li>• Applicable to both hydrophilic and lipophilic antioxidants It is carried out at nearly physiological pH values</li></ul>	<ul style="list-style-type: none"><li>• FRAP and CUPRAC depend on the reaction time</li><li>• The antioxidant which reduce metal ions may exert pro-oxidant effect under certain conditions</li><li>• Low correlation between the capacity measured by FRAP or CUPRAC method with that for radical scavenging measured by competition method such as ORAC</li></ul>
<i>Hydrogen atom transfer (HAT)</i>		
ORAC	<ul style="list-style-type: none"><li>• Uses biologically relevant free radicals</li><li>• Simple and standardised</li><li>• Integrates both degree and time of antioxidant reaction</li><li>• Determine the capacity of hydrophilic and hydrophobic samples simply</li><li>• May be performed in thermostated microplates</li></ul>	<ul style="list-style-type: none"><li>• Expensive equipment</li><li>• Data variability can be large across equipment</li><li>• pH sensitive</li><li>• Requires long times to quantifies results</li></ul>

**Table 6.** Advantages and disadvantages of the most commonly chemical methods used for testing the antioxidant activity [160, 161, 167, 171–173, 176, 184, 231, 261–263].

Mechanisms involved in the corresponding chemical reactions are also shown in the table: hydrogen atom transfer, HAT, ability of an antioxidant to reduce radicals by hydrogen donation for ORAC and TRAP assays; single electron transfer, SET, ability of an antioxidant to transfer one electron to reduce any compounds, including metals, carbonyl and radicals for DMPD and FRAP assays. HAT and SET mechanisms may occur together as in ABTS and DPPH assays. The DPPH method is one of the oldest and most frequently used for determining the antioxidant activity of food extracts and single compounds. In comparison with DPPH assay, the ABTS assay estimates more accurately [183, 254] the antioxidant capacity of foods, especially for those contain lycophilic, lipophilic and highly pigmented compounds. However, it has been stated that methods using HAT reactions will be preferred to those with SET reactions because the peroxy radicals used in the first are the main FR found in lipid oxidation and biological systems [259]. ORAC is the most commonly used total radical-trapping antioxidant assay and the most widely used essay for evaluating antioxidant [172] both in the industry and in the academic institutions. The evaluation of total antioxidant capacity is preferable than the individual antioxidant measurements [74] due to the complexity of food composition and the possibility of synergic interactions among the antioxidant compounds.

## 6. Antioxidant activity of the anthocyanins

The capacity of phenolic compounds to trap FR depends upon their structure, in particular, of the hydrogen atoms of the aromatic group that can be transferred to the FR [5, 10, 20, 24, 63, 113] and of the capacity of the aromatic compound to cope with the uncoupling of electrons as a result of the surrounding displacement of the electrons- $\pi$  system. As compared to other antioxidants, research on their health effects started more recently. This late interest in polyphenols is largely explained by the complexity [264] of their chemical structures. The anthocyanin and anthocyanidin health properties are due to their peculiar chemical structure, as they are very reactive towards ROS because of their electron deficiency [265–269]. The antioxidative properties of anthocyanidins have been recently explored; most of the widely distributed anthocyanidins and anthocyanins show more scavenging activity than that of the well-known strong antioxidants trolox and catechol [20]. The physicochemical characteristics of anthocyanins [83, 90, 91], that is structure and size of the molecules (number and position of hydroxyl and methoxyl groups), water solubility and acidity constants, can control their ability to cross biological barriers. Results of antioxidant activity of foods are commonly expressed in TEAC (mmol or  $\mu\text{mol/g}$  sample), a capacity equivalent to trolox (a hydrosoluble synthetic antioxidant similar to vitamin E). However, some authors suggest [270] that the results should be expressed in vitamin C equivalent antioxidant capacity (VCEAC in  $\text{mg/100 g}$ ), given that vitamin C is found naturally in some foods, whereas trolox is a synthetic compound.

Quantum chemical computations have recently been performed to study [265] the antioxidative properties of anthocyanidins, quantitative structure activity relationships (QSAR) and mechanisms of action involved such as HAT, SET and SPLET (sequential proton loss electron transfer). Construction and evaluation of QSAR for predicting anthocyanin activity radical scavenging using quantum chemical descriptor have been developed [271] with good prediction efficiency.

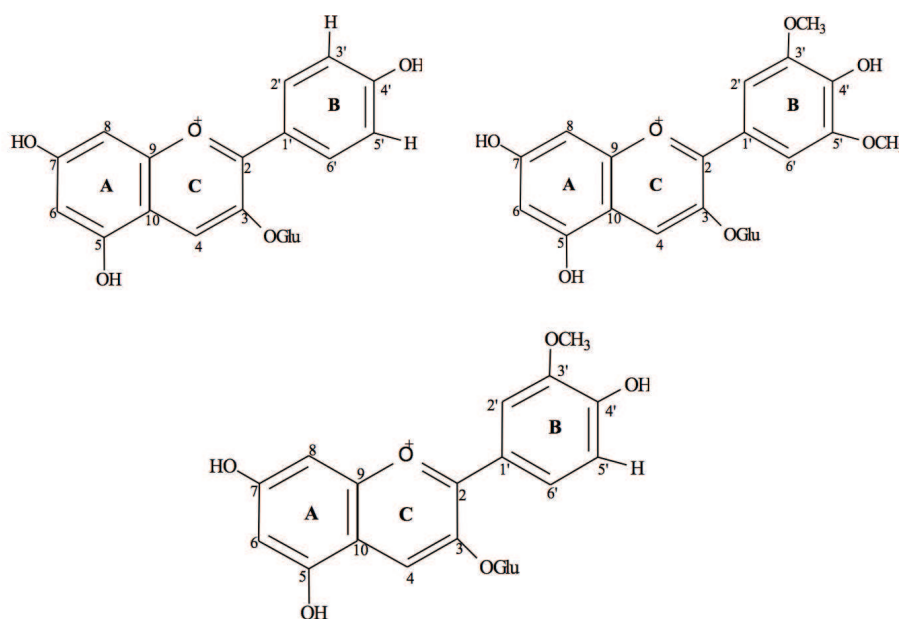
3D-QSAR models from 21 anthocyanins based on their ORAC values have been used [272] with prediction (eggplant and radish) purposes. 3D-QASR models have also been developed in a series [273] of anthocyanin derivatives of CYP3A4 inhibitors (cytochrome P450).

## 7. Structure of the anthocyanins

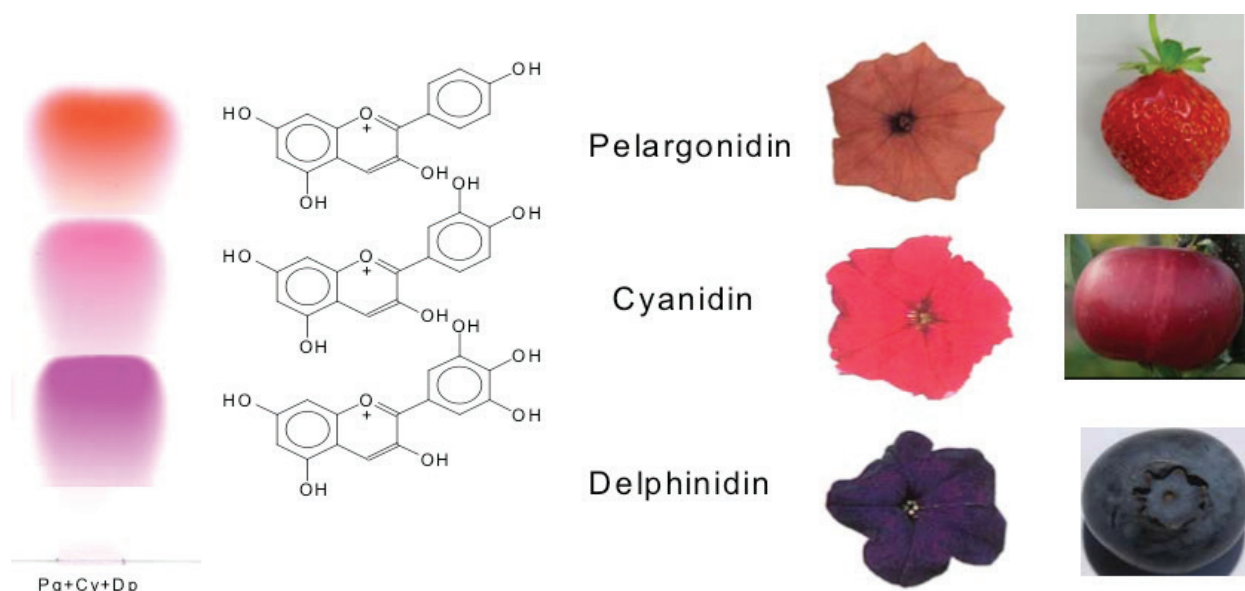
The chemical structure of anthocyanins is appropriate for acting as antioxidants, as they can donate hydrogen or electrons to the FR or trap them and delocalize them in their aromatic structure [5, 10, 20, 265–269]. The structural differences among anthocyanins are related [5, 14, 274, 275] to the number of hydroxyl or methoxyl groups in the anthocyanidin skeleton, the position and the number of bonded sugar residues as well as by the aliphatic or aromatic carboxylates bonded to them. The hydroxylation pattern influences [276, 277] physiological properties such as light absorption and antioxidative activity, which is the base for many beneficial health effects of flavonoids. The hydroxyl groups in positions 3' and 4' provide a high stability to the radical formed by trapping FR and displacing the electrons in ring B, as well as the free hydroxyl groups in position 3 of ring C, and in position 5 of ring A, together with the carbonyl group in position 4 (**Figure 4**).

There are three important structural criteria for evaluating the antiradical effectiveness of a compound: (1) the presence of neighbouring hydroxyl groups, that is, in the position of ring B; (2) double bonds at conjugation 4-oxo of ring C; and (3) hydroxyl groups in positions 3 and 5 of ring A.

The aglucons with identical hydroxylation in rings A and C, and a single OH group in ring B (4'-OH), including pelargonidin, malvidin and peonidin (**Figure 4**), have lower antioxidant activity when compared to compounds with groups 3', 4' di-OH substituted (e.g. cyanidin) (**Figure 5**).



**Figure 4.** Chemical structure of pelargonidin (top left), malvidin (top right) and peonidin (bottom).



**Figure 5.** Chemical structures of pelargonidin, cyaniding and delphinidin, their spots on TLC and the colour of plant tissues [276].

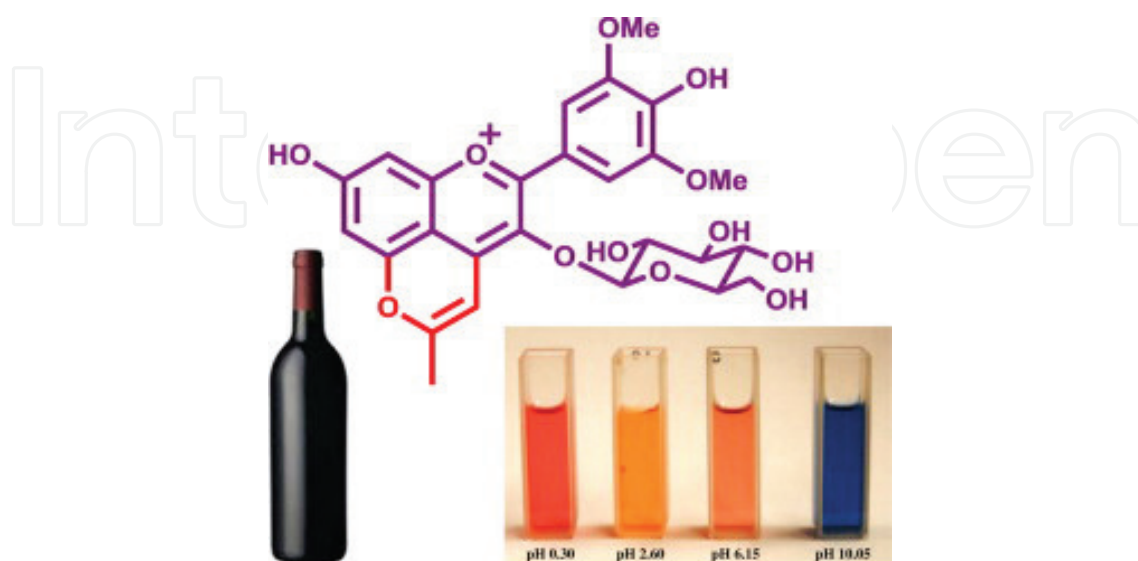
Apparently, the OH groups in position 3' and 4' of ring B (catechol) are determinants of the antioxidant activity of saturated flavonoids. However, delphinidin is an exception to this principle as it has groups 3' and 4' di-OH substituted (**Figure 5**) and still has a low antioxidant activity. The importance of the hydroxyl groups in position 3' and 4' of ring B contributes to the high antioxidant capacity also found for flavones [276, 277].

Most flavonoids are found naturally in a glycosylate form, and glycosylation changes the antioxidant activity [5, 278]: for cyanidin, there is an increase; for malvidin, a decrease; and for pelargonidin, no significant effect was shown [137]. Different sugars can have distinct effects on antioxidant activity. For example, in ORAC assays for cyanidin, glycosylation in position 3 of ring C with glucose or rhamnose increases the antioxidant activity, but with galactose, it declines.

The glycosylation (site, type and number of the glycosyl, glycosidic bond type) generally enhances [269] the stability, results in the hypsochromic effect and blueing, decreases the bioavailability and anticancer activity, and decreases, increases, or does not change the antioxidant activity of the anthocyanidins or anthocyanins. Note the diverse and complex chemistry of acyl groups and that their stabilizing effect exerted may be either independent or synergic. However, the acylation decreases the polarity of anthocyanins and creates steric hindrance effects (changing molecular size and spatial structure) to decrease the sensitivity of the anthocyanins to nucleophilic attack [274] and increasing the *in vitro* and *in vivo* chemical stability (though it lowers their apparent absorption) [113]. Nonacylated monoglycosylated anthocyanins have a greater inhibitory effect on human colorectal adenocarcinoma (HT29) cell proliferation [279]; anthocyanins with pelargonidin, triglycoside and/or acylation with cinnamic acid have a lesser effect.

Anthocyanins are more than flavylium cations [280]. In aqueous solutions, equilibrium of at least four other species determined by pH (and temperature) exists [281–285]. Above about

pH 2.5, the coloured flavylum cation (only stable at  $\text{pH} \leq 1$ , rare in natural environments) form typically hydrates (pH 4–5) to form the colourless hemiacetal (carbinol pseudo-base), followed by ring-opening tautomerization to the light yellow (E)-chalcone, which can isomerize to the (Z)-chalcone. At pH values of 7–8, blue-purple quinoidal anions (which fades in several minutes) are formed. **Figure 6** shows a sample of wine (moderately acid pH 3.5–4.0) at different pH values and corresponds to the graphical abstracts of reference [280]. The state of ionization of the anthocyanins can be an important factor in relation to their antiradical activity. This is corroborated by the fact that the pseudo-base and the quinoidal base of malvidin 3-glucoside, generated at pH 4.0 and pH 7.0, respectively, have differences in antioxidant activity. It is possible play with the colour of anthocyanins [286, 287], for example, complexation with metal ions or with colourless organic molecules (co-pigments) such as hydroxylated benzoic or cinnamic acids. Experiments undertaken with synthetic colourants (Ponceau 4R) have shown that they do not have antioxidant activity, whereas anthocyanin pigments confer an antioxidant activity far greater than that of the synthetic colourants available on the market. This shows that natural pigments besides providing a good source of colour have considerable antioxidant potential. Public concern about synthetic food dyes (suspected to cause adverse effect on health) has increased recently. For this reason, consumers and food manufacturers (i.e. beverage industry) increasingly demand “cleaner” colourants from natural sources [13, 48, 49, 54, 57, 79]. **Table 7** compares [288] the characteristics of both synthetic and natural colourants. Interesting alternatives in food systems to synthetic colourants are acylated anthocyanins [289–293]. A huge variety of hues can be achieved as a function of anthocyanin structure and pH of food matrix. The increasing interest in foods that help to prevent diseases has boosted the market for nutraceutical and/or medicinal food [294]. The term functional food appeared in Japan in the 1980s associated with processed food containing ingredients that affect physiological functions. Identification of health effects provoked by anthocyanins will increase their demanding what would open new perspectives [295] for their use in the food market.



**Figure 6.** Red wine at various pH values: graphical abstracts of Ref. [285].



Synthetic antioxidants	Natural antioxidants
Inexpensive	Expensive
Widely applied	Usage of some products restricted
Medium to high antioxidant activity	Wide ranging antioxidant activity
Increasing safety concern	Perceived as innocuous substances
Usage of some of them banned	Increasing usage and expanding applications
Low water solubility	Broad range of solubilities
Decreasing interest	Increasing interest
Some of them stored in adipose tissue	Completely metabolized

**Table 7.** Advantages and disadvantages of natural and synthetic antioxidants commonly used for food protections [288].

## 8. Influence of the anthocyanins in the antioxidant activity of wines

Anthocyanins are the most abundant polyphenolic compounds in red wines. Red wine is probably the foodstuff that presents the highest diversity of these polyphenolic pigments in their original form and in other derivative structures. Various studies *in vitro* and *in vivo* have confirmed that wine has antioxidant properties, mainly attributable to its composition rich in phenolic compounds [296–298], which vary from 1200 to 2400 mg/L [299–303].

Wines, particularly red wines, inhibit platelet aggregation, increase antioxidant capacity in humans and reduce the susceptibility to lipidic peroxidation in plasma [45, 304–306]. Anthocyanins are the pigments responsible for the attractive colour of red wine and are one of the main flavonolic compounds with antioxidant activity, which is why red wine has a greater antioxidant activity than white wine [307–310]. Its antioxidant capacity can be up to 10 times stronger than that of white wine [205].

Nevertheless, alcohol itself has a protective effect as, to some extent, it is a mediator of the increase (close to 50%) of the level of high-density lipoproteins (HDLs) and of the decrease (of around 18%) of low-density lipoproteins (LDLs), such as cholesterol [311, 312]. However, various studies have correlated the effect of the consumption of red wine with a reduction in coronary heart disease, which is more significant than that for beer or other alcoholic drinks [18, 45]. Therefore, this reduction can be attributed to nonalcoholic components present in red wine [304, 307].

The nonalcoholic components of wine, mainly phenolic compounds, are considered to be the primary factor responsible for this protective effect. There is a significant concentration of flavonoids in red wines (>500 mg/L) and a very low one in white wines (<60 mg/L) [313–315]. In a study by Frankel et al. [300], the relative antioxidant activity of 20 Californian wines was mainly correlated with the presence of cyanidin and malvidin 3-glucoside. Similar results were obtained by Aguirre et al. [316] and Rivero-Pérez et al. [317, 318] in Chilean and Spanish red wines, respectively. According to Fernández-Pachón et al. [319], in the ranking of activity, the most active is the anthocyanins and flavan-3-ol, followed by the phenolic and flavonol acids.

Ghiselli et al. [320] studied three polyphenolic subfractions of red wine, evaluating the capacity to trap hydroxyl and peroxy radicals, the inhibition *in vitro* of the oxidation of LDLs and platelet aggregation. The fraction containing the anthocyanins proved to be the most effective in its capacity to trap ROS and to inhibit the oxidation of LDLs and platelet aggregation. Anthocyanins are quantitatively the most abundant phenolic subclass in red wine [321, 322]. The other two fractions, containing the phenolic acids and quercetin 3-glucuronide, and procyanidins, catechins and quercetin 3-glucoside, are less active.

Some authors still attribute the antioxidant activity of red wines to all polyphenolic compounds [323–325], not discarding the hypothesis that the different classes of polyphenolic compounds can be more effective and act in a synergistic way. However, according to Fernández-Pachón et al. [319], no synergistic effects were observed among the isolated fractions of red wines (anthocyanins, flavonols and phenolic acids). Galanakis et al. [326] characterized the phenolic content and antioxidant capacity of Cypriot wines. The higher concentrations of phenols did not always reflect higher antioxidant capacity of wines, probably due to the observed antagonistic effect between hydroxycinnamic acid derivatives, flavonols and anthocyanins.

## 9. Encapsulation of anthocyanins

As it has been mentioned throughout the chapter, anthocyanins are potentially used in food and pharmaceutical industries since their practical applications as natural colourants [13, 49, 58, 76, 79, 327] as well as their potential health benefits to humans [13, 56, 90, 110, 114]. Nevertheless, the incorporation of anthocyanins into food and medical products is a challenging task due to their low stability and susceptibility to degradation [292] towards environmental conditions during processing and storage. In order to prevent these limitations, delivery systems have been developed, and among them, encapsulation [328–334] would appear to be an interesting option.

Encapsulation, developed approximately 60 years ago [335], is a rapidly expanding technology to entrap one substance (active agent, solid, liquid or gas) within another substance (a matrix or a polymeric wall) in the form of micro- and nanoparticles to protect the 'actives' from environmental conditions and their interactions with other components or to control their release [331, 332]. In addition, once encapsulated bioactive compounds are easier to transportation and handling, masking of undesirable flavour and compartmentalization of two or more reactive species [328, 335].

In general, the three-stage process during encapsulation is [335] as follows: (i) formation of the wall around the material; (ii) ensuring that undesired leakage does not occur; and (iii) ensuring that undesired materials are kept out. For that end, different techniques have been studied and applied to encapsulate active agents, some of them successfully applied for anthocyanins including spray-drying, emulsification, ionotropic gelation or coacervation, and thermal gelation [328, 330].

Among the most important factors to take into account when choosing the microencapsulation technique are particle size, physicochemical properties of the core, the process cost and the selection of wall materials. According to the literature, encapsulation by spray-drying is the most economical, simplest and the most applied method (80–90% of encapsulates are spray-

dried) for preservation of anthocyanin pigments [332], being maltodextrins as the most used coating material. The use of other techniques than spray-drying [331] still remains an unexplored area. This fact could be explained by the hydrophilic nature of anthocyanins [332, 336], so it is, therefore, a promising area of research.

In order to increase the efficiency and stability by spray-drying, different biopolymers are used [328, 333], most common are natural gums (gum arabic, alginates, etc.), proteins (dairy proteins, soy proteins, gelatine, etc.), carbohydrates (maltodextrins and cellulose derivatives) and/or lipids (waxes, emulsifiers). Some authors [330, 332] have revealed that a combination of other wall materials and other modifiers (as oxygen scavengers, antioxidants, chelating agents, and surfactants) increases the encapsulation efficiencies.

**Table 8** [131, 328–342] summarizes some selected reviews on anthocyanin, polyphenol and bioactive compounds encapsulation. In last years, the use of biodegradable polymeric nanoparticles has attracted the interest of researches [337] due to their good biocompatibility, easy design and preparation, structure variations and interesting biomimetic characters.

Content	References
Anthocyanins	
Overview of the most recent studies and patents aimed at enhancing anthocyanin stability in food systems	[328]
Anthocyanin extraction, microencapsulation and release properties during in vitro digestion	[329]
Study on colour stability and microencapsulation from Jamun of anthocyanin pigment using spray-drying	[330]
Health benefits of anthocyanins and their extraction, characterization, encapsulation and delivery	[331]
Encapsulation of anthocyanins from berry-type fruit species as a technology for improving the stability and/or bioavailability of anthocyanins	[332]
Microencapsulation of anthocyanins with different biopolymers through spray-drying	[333]
Nonthermal stabilization mechanisms of anthocyanins in model and food systems	[334]
Stabilization of cranberry anthocyanins in nutraceutical capsules	[335]
Polyphenols	
Relevant recent studies on biopolymer nanoparticles and natural nanocarriers for nanoencapsulation of phenolic compounds	[338]
The encapsulation methods in plants using protein matrices	[339]
Phenolic-enriched foods: sources and processing for enhanced health benefits	[131]
Using nanoparticles to enhance absorption and bioavailability of phenolic phytochemicals	[340]
Overview on encapsulation of natural polyphenolic compounds	[341]
Overview of encapsulation of widely used polyphenols: effectiveness, variations, developments and trends	[336]
Bioactive substances	
Development of food applications containing micro-encapsulated coffee antioxidants	[342]
Encapsulation of active compounds used in food products by drying technology	[337]

**Table 8.** Selected reviews on encapsulations of anthocyanins, polyphenols and bioactive substances.

## 10. Final comments

Anthocyanins [13, 14, 17, 293, 343–346] are members of the flavonoid group of phytochemicals, a group predominant in fruits and vegetables, especially in berries. Recent research raised awareness of the importance [347, 348] of anthocyanins in the diet. Anthocyanin identification is critical in adulteration and profiling [349, 350] studies and in evaluating the quality of crude and processed food. The design of plant products with a high added value allows increasing the synthesis [351] of plant-derived food antioxidants and in particular anthocyanins. In an effort to expand the palette of natural organic colourants (colour additives of food and beverage products), the food industry has launched a search for new products, for example blue colourants [352, 353]. Food, pharmaceutical and nutraceutical industries are interested in [354] clean recovery of valuable compounds. Thus, exploration of more efficient, cost-effective and eco-friendly techniques of polyphenol extraction, that is anthocyanins, from food matrices and waste plant food processing residues (grape fruit, fruits by-products, winery waste materials, by-products) is a challenge [355–360]. In any case, in order to ascertain the nutraceutical potential of bioactive compounds, quantification [359, 361] is required, thus obtaining vital information for future food industrial applications.

Apart from their well-known potential for their practical applications as natural colourants [13, 48, 49, 58, 76, 79, 281, 328], anthocyanins show antioxidant activity and a wide variety of health-promoting properties for human health [12, 56, 81, 85, 90, 111, 112, 120, 130, 264, 343], ranging from cytoprotective, antimicrobial and antitumor activities to neuroprotective, anti-obesity and lipidomic potential. Moreover, epidemiological evidence suggests [12, 111, 112, 362] a direct correlation between anthocyanin intake and a lower incidence of chronic and degenerative diseases.

However, the issue of food antioxidants although important is a controversial topic [11, 64, 72, 363–365]. The plethora of published studies on mechanisms [132] that may mediate therapeutic or chemical chemopreventive effects of dietary constituents contrasts sharply with a scarcity of information on their pharmaceutical and clinical-pharmacological properties. Most of the evidence supporting a therapeutic effect of anthocyanins is *in vitro* or mechanical in nature, although the number of studies on bioavailability in humans has increased significantly over the past two decades. Anthocyanins show a complex biochemical (more than other compounds of flavonoids type), and there is still much to discover [94, 95, 366] about the biochemical activity and clinical pharmacology of these compounds (stability, bioavailability and formulation of dietary constituents), which constitutes an obstacle [367] to understand their health benefits. As evidence of their therapeutic effects accumulates, it is important to understand the nature [81, 85, 87, 89, 139] of the absorption and metabolism “*in vivo*” and that such knowledge will enable the development of new food products, both fresh and manufactured with greater therapeutic efficacy [95, 366]. Progress in this field requires a multidisciplinary research carried out by a wide range of professionals: food science and technology scientists, chemists (analytical chemists), nutritionist, physiologists, pharmacist, pharmacologists, engineers, physicians, biologists, genetics, clinics, etc., being a field in which promising progress will be undoubtedly made in the future.

More complete details of the basics of polyphenols and anthocyanins can be seen in previous reviews [4–7, 346, 368] by the authors.

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