Int. J. Mol. Sci. **2007**, *8*, 797-809

International Journal of
Molecular Sciences
ISSN 1422-0067

www.mdpi.org/ijms

Full Research Paper

Measurement of Antioxidant Activity of Wine Catechins, Procyanidins, Anthocyanins and Pyranoanthocyanins

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Received: 13 June 2007; in revised form: 7 August 2007 / Accepted: 9 August 2007 / Published: 14 August 2007

Abstract: Nowadays, there is considerable interest in finding out about antioxidants that are consumed in the habitual diet. It is known that polyphenols are involved in reducing the risk of diseases associated with oxidative stress. The *in vitro* antioxidant activity of the principal wine polyphenolic compounds (catechins, procyanidins, anthocyanins and pyranoanthocyanins) was studied in this work. Four distinct methods were used to assess the antioxidant capacity of the tested compounds: inhibition of peroxynitrite mediated tyrosine nitration, TEAC (Trolox equivalent antioxidant capacity assay), FRAP (Ferric reducing/antioxidant power assay) and TBARS (thiobarbituric acid reactive substances) methods. In general, it could be concluded that procyanidins were, among the *in vitro* tested groups, the ones which showed more antioxidant capacity using the four different methods, followed by anthocyanins and pyranoanthocyanins. On the basis of the simple regression testing, there was a statistically significant relationship between these different methods used in aqueous phase ($r > 0.92$). However, no correlation was found between the results obtained in lipid media with the TBARS method and those obtained in the aqueous media (peroxynitrite scavenging activity, TEAC and FRAP methods).

Keywords: Antioxidant activity, TEAC, TBARS, FRAP, peroxynitrite, procyanidins, anthocyanins, pyranoanthocyanins.

1. Introduction

Reactive nitrogen and oxygen species (RNS, ROS) have been intensively studied in recent years with regard to their relevant physiological and pathological importance connected also with oxidative stress. Antioxidant compounds are able to neutralize the excess of ROS or RNS and, as a consequence of this activity, it has been suggested that they play an important role in prevention of many diseases, e.g. atherosclerosis, cardiovascular and neurological diseases and cancer [1–3]. The interest in searching for antioxidants that can be consumed in the habitual diet has increased considerably in the last years. Polyphenolic compounds have shown strong antioxidant effects [4–6] and so it has been suggested that they are responsible for reducing the risk of diseases associated with oxidative stress. Flavonoids, a family of polyphenolic compounds, are an important group of antioxidants present in vegetables and fruits and other products made from these, such as wine, beer or juice. Catechins, anthocyanins and procyanidins are an interesting class of flavonoids ubiquitously found in food. Catechins and procyanidins (Figure 1), found in some fruits, such as plum and apple, as well as in tea and red wine [7], have versatile biological effects such as anticancer, antiallergy and antioxidant activities [8]. The main dietary sources of anthocyanin (Figure 1), pigments responsible for the red-blue color, include red-colored fruits, vegetables and red wine. They have shown ability to prevent lipid oxidation and scavenging activity against free radicals [9]. During red wine ageing, there is a loss of anthocyanins and, it appears, other pigments, anthocyanin-derived pigments, which are denominated pyranoanthocyanins. Some of these pigments are formed through the interaction of the original anthocyanins with pyruvic acid, and their structures (Figure 1) are based on the anthocyanin-3-glucosides with additional C_3O_2 between position C4 and the 5-hydroxyl group of the molecule [10]. The biological properties of these compounds have been little studied.

It has been proposed that regular consumption of red wine in moderate amounts reduces the risk of coronary heart disease via protection of LDL against oxidative damage and via inhibition of platelet aggregation [11].

There are different methods to evaluate the *in vitro* antioxidant capacity of isolated compounds, mixtures of compounds, biological fluids and tissues which involve different mechanisms of determination of antioxidant activity, for example: chemical methods based on scavenging of ROS or RNS such as peroxynitrite [12], the hydroxyl radical and superoxide [13]. Other methods measure the disappearance of free radicals using spectrophotometry, such as $ABTS^{\bullet+}$ (2,2′-azinobis-(3-ethyl-benzo-thiazoline-6-sulphonate) cation radical) [14] or DPPH (2,2-diphenyl-1-picrylhydrazyl) [15]. Other assays to determine the total antioxidant power include techniques such as the ferric reducing/antioxidant power method [16] or use the in situ electrochemically generated bromine [17]. The results in the measurement of antioxidant capacity depend on the method used. This is because a single method can not give a comprehensive prediction of antioxidant efficacy of the different compounds [18].

The purpose of the present study was 2-fold: 1) to evaluate the *in vitro* antioxidant activity of catechins, procyanidins, anthocyanins and pyranoanthocyanins from wine by four methods based on different mechanisms 2) to investigate statistically the inter-relationship between the *in vitro* methods used.

2. Results and Discussion

Four *in vitro* methods based on different mechanisms of determination of the antioxidant capacity were used to test polyphenolic compounds. The inter-relationships between these methods were examined for all the tested compounds. The first method, peroxynitrite scavenging activity, was based on automated measurement of the inhibition of peroxynitrite mediated tyrosine nitration by the tested natural compounds. The next, the TEAC assay, was used for measuring the capacity of tested compounds to scavenge the stable cation radical $ABTS^{\bullet+}$ compared to Trolox C, a water soluble analogue of vitamin E. The FRAP assay, based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, colored form was used to assess the total reducing power of antioxidants. The last, TBARS, was based on determination of the inhibition of peroxidation of the phosphatidylcholine substrate induced by the ascorbate/iron complex in lipid phase system.

The remarkable antioxidant activities of catechins, procyanidins, anthocyanins and pyranoanthocyanins in these different systems were analyzed and the results are presented in Table 1. TBARS data for anthocyanins and pyranoanthocyanins are not presented in this table. IC50's could not be determined since they present a maximum of absorbance around 520 nm, very close to the wavelength fixed for the measurement in this antioxidant method. It was, therefore, necessary to find a blank for these color compounds to avoid the interference caused by the color of the tested compounds. The blank should represent 100% inhibition of the sample in the assay, defined as baseline peroxidation of phosphatidylcholine without added iron/ascorbate. In these cases, due to the chemical properties of the studied pigments, it was impossible to find an adequate blank. These compounds in the acid mixture of the reaction, without Fe^{III} and ascorbate, present a red-pink color. Nevertheless, when Fe^{III} was added it resulted in unstable products which presented even less color than the blank. It is known that anthocyanins in acid medium are in a flavylium form and develop red-blue color and that in the presence of metal ions (such as Fe^{III}) they are able to form chelates and this could be the cause of the change in color.

The aqueous phase antioxidant activity of the studied catechins and procyanidins increased from monomer to trimer (Figure 2). The most active of all the compounds tested in the aqueous phase systems were the trimer (Ec-Ec-cat). The TEAC value of catechin was approximately doubled in dimer and threefold in trimer. Procyanidin dimers differ in their catechin moieties and the kind of interflavan linkages between them. We analyzed five different dimers, all of them belonging to the B series, linked 4-8 or 4-6. The antioxidant activities of catechin and epicatechin were not significantly different in the three aqueous methods however the activity of dimer Ec-Ec was significantly higher than the activity of dimer cat-cat. A similar effect was observed for cat-Ec and Ec-cat dimers, which showed higher activity than dimer cat-cat in these three aqueous methods. The linkage between positions 4 and 8 in procyanidins (Ec 4-8 cat) significantly increase antioxidant efficiency in aqueous phase assays with respect to 4 and 6 linkage (Ec 4-6 cat). In the lipid phase system the trend of decreasing antioxidant activity with polymerization was found in contrast to the antioxidant activity studied in aqueous phase,