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Profiling ellagic acid content: The importance of form and ascorbic acid levels

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ABSTRACT

As the importance of plant-based antioxidants to human health becomes clearer there is a rapidly expanding search for rich sources of these compounds. Much attention is currently focussed on the antioxidant potential of ellagic acid (EA). Making assessment difficult is that EA occurs in different forms: free EA, EA glycosides and polymeric ellagitannins. The overall structure of these forms has a pronounced effect on their antioxidant efficiency and is responsible for widely differing reactivity, solubility and hence bioavailability properties. Often associated with EA is vitamin C which also contributes to the plant foods total antioxidant activity. Previous studies have suggested that ascorbic acid may have protective effects on the polyphenol content of plants. With a view to gaining evidence that the bioactive forms of vitamin C influence EA content, several fruits with a range of EA and vitamin C contents were examined. To facilitate a more detailed assessment of the selected fruits antioxidant potential the relative proportions of EA forms were also determined. In strawberries and boysenberries EA content was predominantly in the polymeric form (21% and 12% free EA plus EA glycoside vs total EA levels for strawberry and boysenberry respectively), while in Kakadu plum it was mainly in the free form (70% of total EA). An increasing percentage of dehydroascorbic acid (9 to 14% of total vitamin C) indicating enhanced transformation of ascorbic acid to its oxidative degradation product together with stable free EA levels (≈950 mg/100 g DW) over the 4 month frozen storage period for the Kakadu plum samples are consistent with a possible protective effect of EA by ascorbic acid.

1. Introduction

Consumption of fruits and vegetables is known to lower the risk of several diseases, including cardiovascular diseases, cancer and stroke (Willett, 2002). Such health benefits are mainly attributed to the content of antioxidant compounds most notably vitamin C and polyphenols including gallic and ellagic acids as well as related compounds (reviewed by Crozier, Jaganath, & Clifford, 2009). As the importance of these antioxidants to human health becomes clearer there is a rapidly expanding search for rich plant sources of these compounds with much attention focussing on the antioxidant potential of ellagic acid (EA). Ellagic acid together with the bioactive forms of vitamin C (ascorbic, AA and dehydroascorbic acids, DHAA) (Fig. 1), possess double bonds with an associated electron deficiency which is highly reactive to free radicals from molecular oxygen (Atkinson, Nestby, Ford & Dodds, 2005).

Ellagic acid occurs in plants in different forms: as free EA (Fig. 1a), glycosylated via its hydroxyl groups or, most commonly, as complex polymers esterified with a sugar known as ellagitannins (ETs) (Clifford & Scalbert, 2000). Investigations that focus on identifying EA forms in fruit and other plant materials are made more difficult by the fact that these forms differ widely in solubility and reactivity. Hydrolysis of EA glycosides or water-soluble ETs with acids or bases yields an unstable intermediate which spontaneously rearranges to the water-insoluble EA. This reaction has been utilised for the detection and quantification of total EA, with content being expressed as EA equivalents after acid hydrolysis (Rommel & Wrolstad, 1993). The complete disappearance on acid hydrolysis but not alkaline hydrolysis provides a means of identifying and quantifying EA glycosides (Aaby, Skrede, & Wrolstad, 2005). To determine free EA content separately, the measurement is simply performed before the hydrolysis step. The final analytic for measuring all forms is free EA (Fig. 1a).

Few studies have reported a thorough EA characterisation of fruit with most focussing on total EA content (Atkinson et al., 2005; Maas, Wang, & Galletta, 1991; Rommel & Wrolstad, 1993) or free EA levels (Amakura, Okada, Tsuji, & Tonogai, 2000; Konczak, Maillot, & Dalar, 2014). Data obtained from several studies that investigated more fully the EA forms present led to the conclusion that most plant EA was

Abbreviations: AA, ascorbic acid; DHAA, dehydroascorbic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EA, ellagic acid; ET, ellagitannins; FRAP, ferric reducing ability of plasma; ORAC, oxygen radical absorbance capacity.

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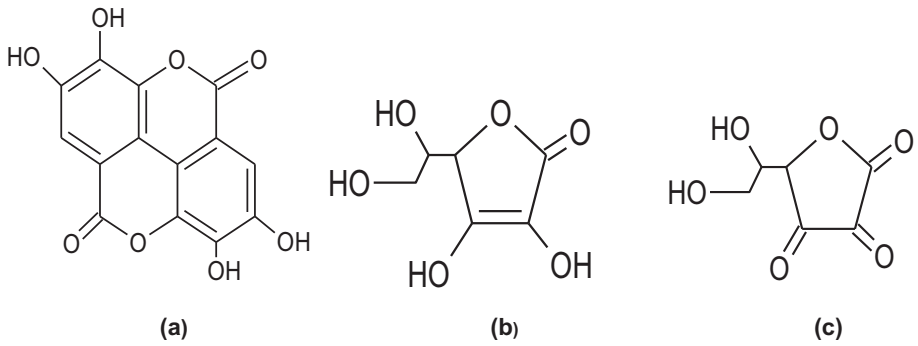

Fig. 1. Structure of (a) ellagic acid, (b) ascorbic and (c) dehydroascorbic acids.

present in the ET form with only a small proportion, if any, occurring in the free form or as EA glycosides (Aaby et al., 2005; da Silva Pinto, Lajolo, & Genovese, 2008; Zafrilla, Ferreres, & Tomas-Barberan, 2001) Other reports found the proportion of the free form to be highly variable with some fruit exceeding 50% of the total EA content (Rommel & Wrolstad, 1993; Wada & Ou, 2002).

The antioxidant efficiency of the EA forms is believed to be correlated with their degree of hydroxylation (Pfundstein et al., 2010). Zafrilla et al. (2001) reported that in red raspberries EA had the highest antioxidant activity among isolated EA glycosides, whereas an unidentified polymeric derivative showed an antioxidant capacity double that of EA. The authors stated this was expected as this latter compound possesses a far larger number of phenolic hydroxyls per molecule. Confirmation of the presence of two major ETs (sum represented 81% of the total ETs in raspberries) was provided by Gasperotti, Masuero, Vrhovsek, Guella & Mattivi (2010). There are also differences in bioavailability between EA forms, although these differences are the focus of numerous current investigations (reviewed in Crozier et al., 2009). Variations in bioavailability of the EA forms could explain the discrepancies observed between *in vivo* and *in vitro* experiments, with the *in vitro* results often failing to match the findings of the *in vivo* studies (see Tomás-Barberán & Andrés-Lacueva, 2009; Tomás-Barberán, Somoza, & Finley, 2009). The form in which EA occurs may be as important to its antioxidant activity and bioavailability as the actual content of EA. Accurate quantification of free EA, EA glycosides and ETs present in fruit, fruit-based beverages and supplements is a necessary pre-requisite for any study that evaluates their antioxidant properties and their subsequent effect on human health.

A possible link between EA and AA was recognised by Atkinson et al. (2005) when they increased these natural antioxidants in strawberries by applying enhanced radiation during growth. To date, literature reports that focus on whether the levels of endogenous bioactive forms of vitamin C (AA, and DHAA) may influence EA content in plant foods are very rare. This seems surprising as several studies showed that added AA prevented the oxidation of polyphenols even during alkaline hydrolysis (Nardini et al., 2002). A recent study by Oszmianski, Wojdyło, and Kolniak (2009) demonstrated that AA was a very useful EA oxidation protector during the storage and thawing of frozen strawberries. As AA is easily oxidisable and is very often considered a terminal oxidant (Halliwell, 1994), EA may well be protected by AA in plants.

Although several studies have measured free EA content and even more have quantified the bioactive forms of vitamin C in plant foods, there are, to the best of our knowledge, no studies have been carried out to gain direct evidence that AA has a protective effect on EA levels. To achieve this aim as well as gaining knowledge about the importance of structure (form) in defining its antioxidant efficiency and bioavailability we measured the free, EA glycoside and total EA content together with bioactive vitamin C levels in three fruit types. The determination of pH, titratable acidity and moisture of the fruit extracts completed the necessary characterisation. Samples of Kakadu plum fruit known to contain high levels of free EA and AA were selected for examination whilst undergoing frozen storage (Konczak et al., 2009, 2014). This approach should add to our understanding of the complex mechanisms associated with the different EA forms and their respective contribution to overall antioxidant properties and bioavailability.

2. Materials and methods

2.1. Reagents

EA, DL-homocysteine, AA and DHAA were purchased from Sigma-Aldrich Inc. (Sydney, NSW, Australia). The HPLC-grade methanol, formic acid, 2-propanol and acetonitrile were purchased from Thermo Fisher Scientific (Melbourne, Victoria, Australia). All other chemicals were of analytical grade and purchased from Thermo Fisher Scientific.

2.2. Plant materials and preparation

Recently harvested (Spring, September 2013) strawberries (*Fragaria ananassa* cv. Camarosa) were obtained from a commercial grower located to the south of Brisbane, Qld, Australia, frozen and stored in polyethylene bags at −20 °C.

Frozen packs (1 kg) of New Zealand grown boysenberries (*Rubus ursinus x idaeus*) were obtained from Harvestime Qld. (Yatala, Qld, Australia). Samples were kept frozen (−20 °C) until processing and analysis.

Commercially available whole Kakadu plum fruit (*Terminalia ferdinandiana*) were obtained from Australian Produce Company (Brisbane, Qld,Australia). Individual whole fruit (harvested in late Autumn, May 2012) were placed in polyethylene bags, vacuum sealed and blast frozen then stored at −20 °C.

Sub-samples (comprising ≈ 20 individual fruit for all three fruit types) for EA and vitamin C analysis were freeze-dried, finely ground (including the seed for the Kakadu plum whole fruit samples) in a Retsch MM301 cryomill (Retsch GmbH, Haan, Germany) and subsequently stored at −20 °C.

For the storage trial samples of Kakadu plum were stored whole at −20 °C. After 0, 1, 4, 5 and 8 months storage samples (comprising ≈ 10 individual fruit for each individual sampling date) were manually de-seeded, freeze-dried and finely ground prior to determining moisture, free EA and vitamin C content.

2.3. Moisture content of the freeze-dried fruit powders

The moisture content of the freeze-dried fruit powders was determined according to AOAC (1995), official method 964.22. Briefly, in triplicate, each sample (1 g) was dried for approximately 16 h to a constant weight at 70 °C in a vacuum oven (W. C. Heraeus GmbH, Hanau, Germany). The difference between initial weight and constant weight