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Increasing the antioxidant power of tea extracts by biotransformation of polyphenols

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ABSTRACT

Green tea (*Camellia sinensis*) and yerba mate (*Ilex paraguariensis*) are rich in polyphenolic compounds, which are thought to contribute to the health benefits of tea. The aim of this study was to evaluate the potential antioxidant properties of green tea and yerba mate extracts before and after the enzymatic biotransformation reaction catalysed by the *Paecilomyces variotii* tannase. The antiradical properties of the tea extracts, as well as the standards of chlorogenic acid and EGCG, were assessed using the ORAC and DPPH assays before and after the tannase biotransformation. The antioxidant power of enzyme-treated green tea and yerba mate increased by 55% and 43%, respectively, compared with that of untreated teas. The antioxidant power of the standards was also highly increased by enzyme treatment. These results provide relevant data about the potential of the tannase application on various polyphenol sources and to increase the antioxidant power of two widely consumed beverages.

1. Introduction

Tea is the second most widely-consumed beverage worldwide (after water) and is rich in polyphenolic compounds, known as tea flavonoids. Green tea contains several tea polyphenols, including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) (Suganuma et al., 1999). These flavonoids (also known as catechins) possess strong antioxidant properties (Majchrzak, Mitter, & Elmadfa, 2004). Catechins have been proven to have antioxidant, antimutagenic, and anticarcinogenic properties, and they can also prevent cardiovascular diseases (Cao & Ito, 2004).

Yerba mate (*Ilex paraguariensis*) is a plant originally from the subtropical region of South America and is present in the south of Brazil, the north of Argentina, Paraguay and Uruguay. Mate beverages have been widely consumed for hundreds of years as infusions popularly known as chimarrão, tererê (both from green dried mate leaves) and mate tea (roasted mate leaves). Mate beverages are rich in polyphenolic compounds, which are mainly caffeoyl derivatives, such as dicaffeoylquinic and chlorogenic acids, saponins and purine alkaloids (Martins et al., 2009).

The considerable antioxidant potential of green tea and yerba mate has long been recognised and is dependent on many factors involved in tea preparation. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, singlet-oxygen quenchers and metallic-ion chelators (Atoui, Mansouri, Boskou, & Kefalas, 2005).

Despite the proven antioxidant capacity of tea polyphenols, many clinical studies and animal models have shown that these compounds, especially the polymers, esters, and glycosides, are abundant, but are not always absorbed by oral administration. The functional effect of the compound depends not only on the amount ingested, but on its bioavailability (Holst & Williamson, 2008). Therefore, the enzymatic hydrolysis of polyphenols from food is a subject worth investigating.

Tannin acylhydrolases, commonly referred to as tannases (E.C. 3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria. Tannases have mostly been characterised by their activity on complex polyphenolics, and are able to hydrolyse the "ester" bond (galloyl ester of an alcohol moiety), as well as the "depside" bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, and chlorogenic acid (Fig. 1) (Garcia-Conesa, Ostergaard, Kauppinen, & Williamson, 2001).

In this paper, the activity of tannase on the extracts of green tea and yerba mate was investigated. The aim of this work was to study the potential antioxidant properties of extracts of green tea and yerba mate before and after an enzymatic reaction, catalysed by the tannase, produced by *Paecilomyces variotii* (Battestin, Pastore, & Macedo, 2005). The antiradical properties of these samples were assessed using the oxygen radical-absorbance capacity (ORAC) (Cao, Sofic, & Prior, 1996) and 2,2-diphenyl-1-picrylhydrazyl

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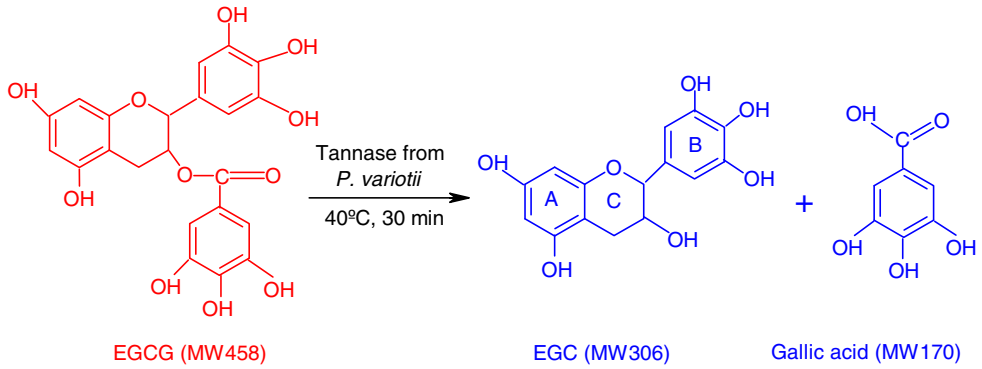

Fig. 1. Degalloylation of EGCG by the tannase from *Paeciloyces variotii*.

(DPPH) assays (Benzie & Strain, 1996; Bondet, Brand-Williams, & Berset, 1997). To date, the ORAC assay has been largely applied to the assessment of the free-radical scavenging capacity of human plasma, proteins, DNA, pure antioxidant compounds and antioxidant plant/food extracts (Dávalos, Goméz-Cordovés, & Bartolomé, 2004).

2. Material and methods

2.1. Reagents

Epigallocatechin gallate (EGCG, 95%), epigallocatechin (EGC, 98%), 2,2′- azobis (2-methylpropionamidine) (97%), and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma–Aldrich (Steinheim, Germany). All other chemicals were purchased in the grade commercially available. The fluorescein was from ECIBRA, and the Trolox® (97%) was from ACROS Organics.

2.2. Enzyme

The tannase from *Paecilomyces variotii* was obtained according to a previously published procedure (Battestin & Macedo, 2007). A 250 ml conical flask containing 5 g of wheat bran, 5 g of coffee husk, 10 ml of distilled water and 10% tannic acid (w/w) (Ajinomoto OmniChem Division, Wetteren, Belgium) was used for the fermentation process. The culture medium (pH 5.7) was sterilised at 120 °C for 20 min. After sterilization, the flasks were inoculated with 2.5 ml (5.0×10^7 spores/ml) of the pre-inoculum suspension and incubated at 30 °C for 120 h. After fermentation, 80 ml of 20 mM acetate buffer at pH 5.0 was added and shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 9650*g* for 30 min at 4 °C (Beckman J2–21 centrifuge, Beckman-Coulter, Inc. Fullerton, CA, USA). The supernatant was then treated with solid ammonium sulphate (80% saturation) and stood overnight at 4 °C. The precipitate was collected by centrifugation (9650*g* for 30 min), resuspended in distilled water and dialysed against distilled water. The dialysed preparation was freeze-dried and used as crude tannase.

2.3. Sample preparation

2.3.1. Preparation of tea extracts for LC–MS analysis

The extraction of green tea (*Camellia sinensis*) and yerba mate (*Ilex paraguariensis*) (1 g) were performed with 20 ml of ethanol/water (50% v/v) and 20 ml of chloroform using a blender (Ultra-Turrax) for 5 min, according to the procedure described by De Freitas, Carvalho, and Mateus (2003). The 50%-ethanol upper aqueous layer was separated from the chloroform layer containing the chlorophylls, lipids and other undesirable compounds. The ethanol was removed using a rotary evaporator, before the resulting aqueous solution, containing catechins, was dissolved in acetate buffer (pH 6.0, 0.2 M) for identification of the compounds present.

2.3.2. Preparation of tea extracts for antioxidant assays

Fifty milliliter of distilled water and 250 mg of each sample of tea were combined in 125 ml Erlenmeyer flasks. The extraction of compounds from green tea and yerba mate was performed in a water bath at 100 °C for 30 min. After being filtered on filter paper, the extracts were freeze-dried. The resulting powder was called dried tea extract and used for antioxidant assays (Cao et al., 1996).

2.3.3. Polyphenol commercial standards

As an identified representative polyphenol from green tea, the commercial standard epigallocatechin gallate (EGCG, 95%) was used as a control sample, as was the chlorogenic acid (95%) from yerba mate tea. These samples were tested for antioxidant power (by DPPH and ORAC assays) and treated with tannase, using the same procedures that were employed on the tea extracts.

2.4. Enzymatic biotransformation

The extracts obtained from the green tea, yerba mate and the commercial control samples were used as substrates for enzymatic hydrolysis by tannase isolated from *Paecilomyces variotii* (Battestin, Macedo, & Freitas, 2008). The dried tea extract (5 mg) was dissolved in 1 ml of phosphate buffer (pH 7.4, 75 mM) and incubated with 5 mg of tannase at 40 °C for 30 min. The hydrolysis process was stopped by placing the reaction in an ice bath for 15 min. The biotransformed tea was used for the antioxidant assay after suitable dilution with the same phosphate buffer (pH 7.4, 75 mM) for ORAC and with a 70% methanol solution for DPPH.

2.5. LC–MS analysis

A Finnigan Surveyor-series liquid chromatograph, equipped with a 150 × 4.6 mm i.d., 5 μm LicroCART® (Merck, Darmstadt, Germany), reversed-phase C18 column maintained at 25 °C by a thermostat, was used. Mass detection was carried out using a Finnigan LCQ DECA XP MAX (Finnigan Corp., San José, CA, USA) mass detector with an API (atmospheric pressure ionisation) source of ionisation and an ESI (ElectroSpray ionisation) interface. The solvents used were formic acid in H_2O (1%, v/v) and acetonitrile. The capillary voltage was 4 V and the capillary temperature was 275 °C. The spectra were recorded in the positive-ion mode between 120 and 1500 *m/z*. The mass spectrometer was programmed to carry out a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS–MS of the most intense ion using relative collision energy of 30 and 60.

2.6. ORAC assay

The ORAC method used, with fluorescein (FL) as the "fluorescent probe", was described by Ou, Huang, Hampsch-Woodill, Flanagan, and Deemer (2002) and modified by Dávalos et al. (2004). The automated ORAC assay was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37 °C as the reaction was started by thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to pH. The solution of FL (70 nM) in phosphate buffer (PBS) (75 mM, pH 7.4) was prepared daily and stored in complete darkness. The reference standard was a 75 μM Trolox® solution, prepared daily in PBS, and diluted to 1500–1.5 μmol/ml solutions for the preparation of the Trolox® standard curve. In each well, 120 μl of FL solution were mixed with either 20 μl of sample, blank (PBS), or standard (Trolox® solutions), before 60 μl of AAPH (12 mM) was added. The fluorescence was measured immediately after the addition of AAPH and measurements were then taken every 6 min for 87 min. The measurements were taken in triplicate. ORAC values were calculated using the difference between the area under the FL decay curve and the blank (net AUC). Regression equations between net AUC and antioxidant concentration were calculated for all of the samples. A control for the tannase was performed as a regular sample, where the ORAC value obtained was subtracted from the samples treated with the enzyme. ORAC-FL values were expressed as μMol of Trolox equivalent/mg of tea extract (Cao et al., 1996).

2.7. DPPH assay

The potential antioxidant activity of a tea extract was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, according to Peschel et al. (2006) with modifications. Various concentrations (0.1–0.01 mg/ml in 70% (v/v) methanol) of test samples were prepared. The reaction mixtures, consisting of 50 μl of test samples and 150 μl of 0.2 mM DPPH in methanol, were mixed in 96-well plates (BMG Labtech 96), before the reaction was carried out on a NovoStar Microplate reader (BMG LABTECH, Germany) with absorbance filters for an excitation wavelength of 520 nm. The decolourising process was recorded after 90 min of reaction and compared with a blank control; for the coloured samples and tannase treated samples, an additional blind control was performed which contained the extract solution (or tannase solution) and pure methanol, instead of DPPH. The solutions were freshly prepared and stored in darkness. The measurement was performed in triplicate. Antiradical activity was calculated from the equation determined from the linear regression after plotting known solutions of Trolox with various concentrations. Antiradical activity was expressed as μMol of Trolox equivalent/mg of tea extract (Faria et al., 2005).

2.8. Calculations and statistics

Values are expressed as the arithmetic mean. Statistical significance of the differences between the groups was analysed by the Tukey test. Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. Detection and identification of the compounds extracted from green tea and yerba mate

The extracts of green tea and yerba mate containing polyphenolic compounds were analysed by HPLC/DAD-MS. The use of mass spectrometry, coupled with high-performance liquid chromatography, allowed the identification of EGCG, EGC (Fig. 2) and

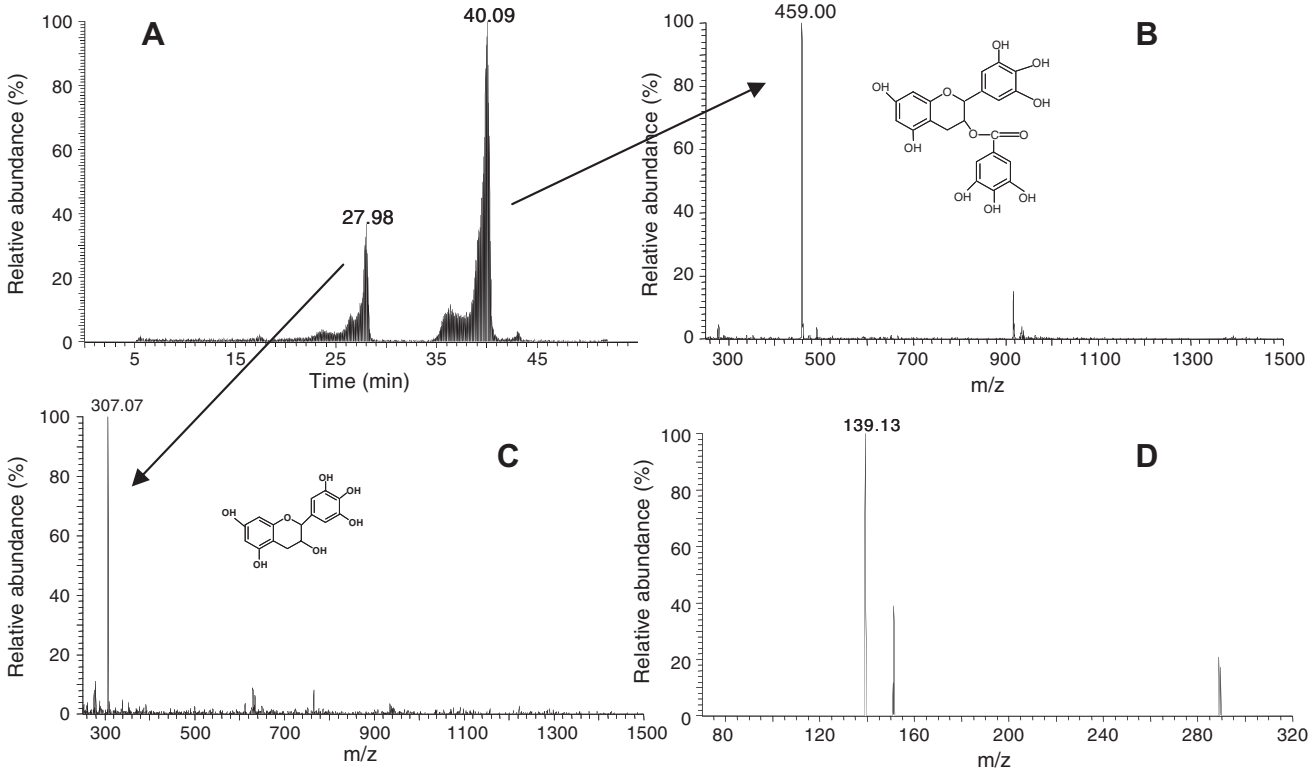

Fig. 2. LC–ESI mass spectrometry analysis of green tea extract (positive-ion mode): (A) total ion chromatogram; (B and C) MS spectrum; (D) MS^2 spectrum of the ion at m/z 307.

chlorogenic acid (Fig. 3). The mass spectrum showed an ion mass consistent with the structure of epigallocatechin (MW: 306 g/mol). The mass spectrum of this compound revealed a $[M^+]$ molecular ion at m/z 307 and a major fragment ion $[M\text{-}168]^+$ at m/z 139, which correspond to a retro-Diels–Alder of the catechin moiety (Freitas, Souza, Silva, Santos-Buelga, & Mateus, 2004). The HPLC/DAD-MS analysis exhibited a significant peak with the same retention time (40 min) as the EGCG in the UV–Vis spectrum. Furthermore, the mass spectrum indicated an ion mass $[M^+]$ at m/z 459, consistent with the structure of EGCG (Fig. 2).

Analysis of the extract of yerba mate identified only those compounds related to the chromatographic peaks, detected at 9.61, 14:14 and 14.93 min, corresponding to the compound chlorogenic acid (MW: 354 g/mol) (Fig. 3). The MS, MS^2 and MS^3 mass spectra obtained for this compound are shown in Fig. 4.

Analysis by LC/MS of the mate extract revealed the presence of chlorogenic acid. It was found that the chromatographic peaks detected at 9.61, 14.14 and 14.93 min had a molecular-ion mass ($[M^+]$, $m/z = 355$; Fig. 4A (I, II, III)) corresponding to the mass of chlorogenic acid (MW: 354 g/mol). MS^2 fragmentation of the extract's chromatographic peaks ($[M\text{-}192]^+$) (Fig. 4B (I, II, III) presents a fragment derived from cinnamic acid ester by severing the link. MS^3 fragmentation ($[M\text{-}192\text{-}18]^+$) (Fig. 4C (I, II, III)) of the previous fragment indicates the output of a water molecule. Further identification of other compounds in the extract of yerba mate was not possible in this sample, probably because it had many impurities.

Above all, this analysis successfully confirmed the significant presence of two potential substrates for the biotransformation catalysed by the tannase: the EGCG in the green tea extract, and the chlorogenic acid in the yerba mate extract.

3.2. ORAC and DPPH analysis of the biotransformed teas extracts

Various methods have been developed to characterise the total antioxidant capacity of biological fluids and natural products. One such method, the semiautomated ORAC protocol, developed by Cao et al. (1996), has received extensive coverage and utilisation in the field of antioxidant and oxidative stress. The ORAC assay measures free-radical damage to a fluorescent probe, causing a change in its fluorescence intensity. The change of fluorescence intensity is an index of the degree of free-radical damage. The capacity of antioxidants to inhibit free-radical damage is measured as the degree of protection against the change of probe fluorescence in the ORAC assay (Huang, Ou, & Hampsch-Woodi, 2002).

Table 1 describes the antioxidant capacities of the various samples (chlorogenic acid, yerba mate extract, EGCG and green tea extract), before (as control) and after tannase treatment, as determined by the ORAC-FL method. The linearity between the net AUC and the sample concentrations was determined for all compounds (Table 1). For each sample, the solutions with concentrations within the linearity range gave the same ORAC-FL value.

The results in Table 1 indicate that the antioxidant capacity of tea increased after tannase treatment. The tannase was probably able to hydrolyse the substrates contained in these teas, and the products of hydrolysis apparently contributed to the increase in the teas' antioxidant capacity. The antioxidant capacity of green tea increased by 55% after enzymatic treatment. Tannase also exhibited high activity on epigallocatechin gallate, the commercial standard substrate from the green tea, increasing its antioxidant activity by 46%. These results indicate that the tannase from *P. variotii* was able to hydrolyse the ester bonds from natural substrates. Epigallocatechin and gallic acid can be formed by the degalloylation of the gallate (epigallocatechin gallate) present in the tea extract (Fig. 1). According to Battestin et al. (2008), tannase can completely hydrolyse the epigallocatechin gallate in green tea to epigallocatechin and gallic acid by increasing the antioxidant activity of tea.

For yerba tea, the increase of the antioxidant activity after enzymatic hydrolysis was 43%, which was a significant result. According to Miranda et al. (2008), the yerba mate is rich in several bioactive compounds that can act as free-radical scavengers. The activity of the tannase in increasing the antioxidant power of chlorogenic acid by 58% (Table 1), suggests that the enzyme was able to act on the chlorogenic acid by yerba mate extract, and that the products of this reaction contributed to the increase of the antioxidant power of this beverage. These data are consistent with the results presented in Fig. 3, in which the chlorogenic acid was found in large quantities in the yerba mate extract. Similar results have been demonstrated by Roy et al. (2010). The antioxidant activity of green tea and yerba mate infusions has long been attributed to the polyphenolic content of green tea and yerba mate.

Table 2 describes the antioxidant capacity of the various samples (chlorogenic acid, yerba mate extract, EGCG and green tea extract), before (as control) and after tannase treatment, as determined by the DPPH method.

In the DPPH method, the substances tested were reacted with the DPPH, which is a stable free radical, where a decrease in the absorbance measured at 515 nm was induced, suggesting the scavenging potential of the extracts. The results in Table 2 indicate that there is a trend for increasing radical-scavenging capacity after enzymatic hydrolysis. This trend was similar to the one observed in the ORAC results, which supports the results obtained by enzymatic treatment of the extracts.

Catechins (including epicatechins) with three hydroxyl groups in the B ring are known as the gallocatechins, whereas those esterified to gallic acid at the 3-OH group in the C ring are known as the catechin gallates. With antioxidant activity governed broadly by the rule that the structures with the most hydroxyl groups exert the greatest antioxidant activity, the catechin-gallate esters reflect the contribution from gallic acid (Rice-Evans, Miller, & Paganga,

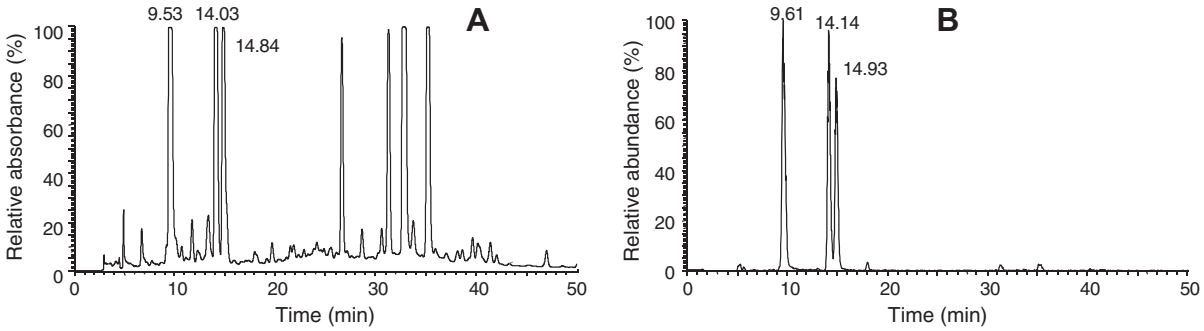

Fig. 3. LC/MS chromatogram of the extract of yerba mate with UV detection (A), and MS detection after extraction of the mass of the ion corresponding to chlorogenic acid ($[M^+]$, $m/z = 355$) (B).

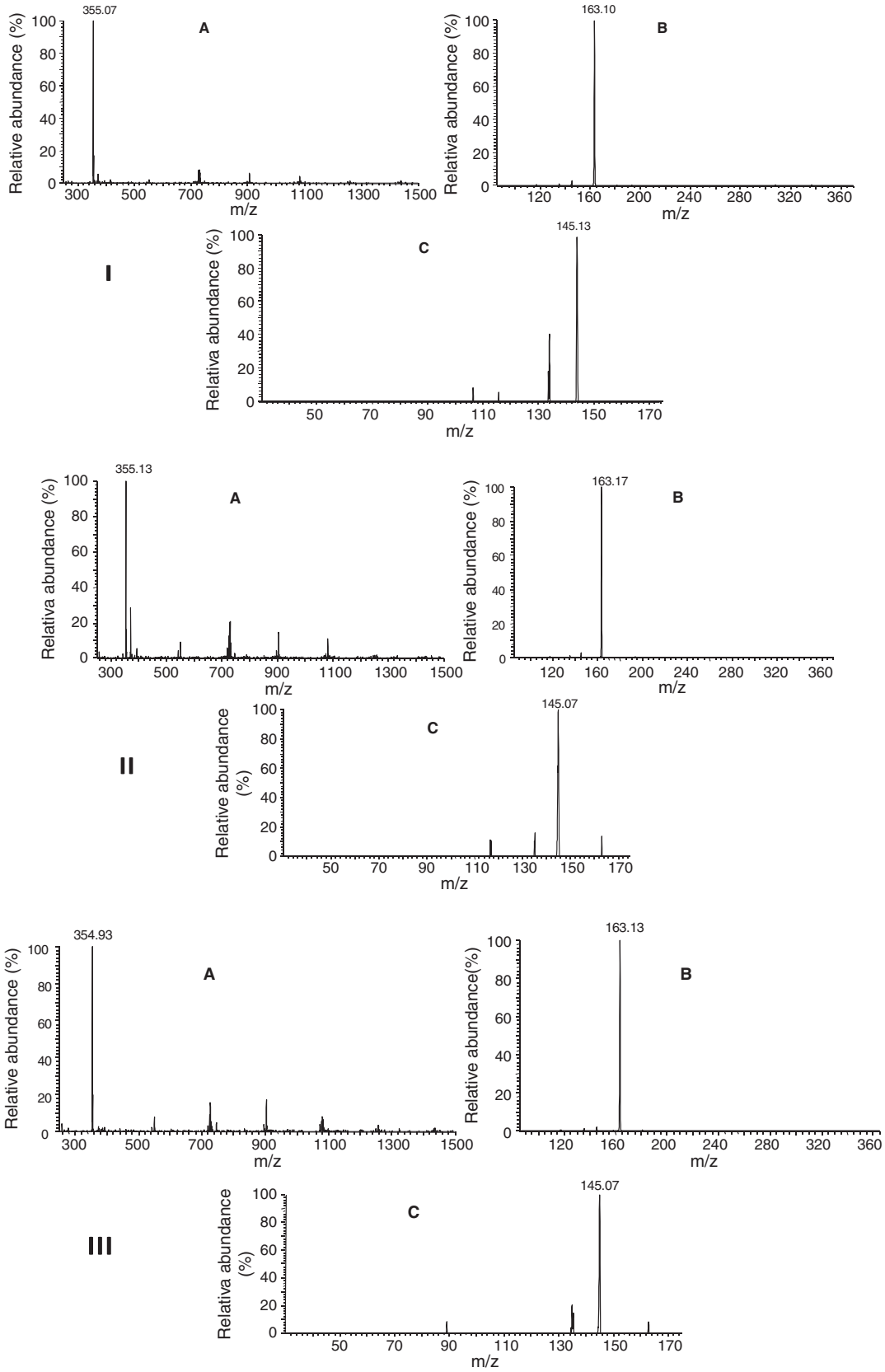

Fig. 4. MS (A), MS^2 (B) and MS^3 (C) mass spectra of chlorogenic acid, referring to the chromatographic peaks detected to 9.61 (I), 14.14 (II) and 14.93 (III) min, respectively, obtained by LC/MS.

1996). Concerning any structure–activity relationships, the *o*-dihydroxy groups in the B-ring and the hydroxyl group in the C-ring are associated with the antioxidant properties of the flavonoids (Faria et al., 2005).

Table 1
Trolox equivalents and linearity ranges for the ORAC assay (net AUC *vs.* concentration) performed on the control and biotransformed samples.

Sample	Trolox equivalents (μmol/g dry sample)	Sample concentration range (mg/ml)	Slope	Intercept	r^2
Tea extracts samples					
Yerba mate (without tannase)	5600 ± 156[a]	0.2–0.02	180.60	4.76	0.99
Yerba mate biotransformed	7989 ± 245[b]	0.2–0.06	252.01	9.10	0.99
Green tea (without tannase)	4704 ± 138[a]	0.2–0.06	146.16	5.62	0.99
Green tea biotransformed	7356 ± 616[b]	0.2–0.06	265.35	5.38	0.99
Commercial standard samples					
Chlorogenic acid (without tannase)	15,345 ± 1003[a]	0.1–0.02	206.0	6.10	0.98
Chlorogenic acid biotransformed	24,206 ± 3508[b]	0.1–0.01	475.59	3.56	0.99
Epigallocatechin gallate (without tannase)	11,735 ± 886[a]	0.2–0.02	242.18	7.48	0.96
Epigallocatechin gallate biotransformed	17,182 ± 514[b]	0.2–0.01	260.86	7.18	0.97

[a,b] Results are presented as the mean ($n = 3$) ± SD, and those with different letters are significantly different, with $p < 0.05$.

Table 2
Trolox equivalents of the control and biotransformed samples by DPPH.

Sample	Trolox equivalents (μmol/g dry sample)
Tea extracts samples	
Yerba mate (without tannase)	2879 ± 29[a]
Yerba mate biotransformed	3029 ± 35[b]
Green tea (without tannase)	2682 ± 08[a]
Green tea biotransformed	3302 ± 58[b]
Commercial standard samples	
Chlorogenic acid (without tannase)	2529 ± 57[a]
Chlorogenic acid biotransformed	4130 ± 01[b]
Epigallocatechin gallate (without tannase)	1551 ± 32[a]
Epigallocatechin gallate biotransformed	1869 ± 09[b]

[a,b] Results are presented as the mean ($n = 3$) ± SD, and those with different letters are significantly different, with $p < 0.05$.

When comparing the antioxidant activity of the commercial standard samples (control and biotransformed) with those of the samples of green tea and yerba mate, the antioxidant activity of the standards was observed to be much higher. This was expected because the green tea and yerba mate samples are more diluted than the commercial standard samples, largely due to the extraction process used. The commercial standard samples showed a high degree of purity, which raised the antioxidant power of these samples (Tables 1 and 2).

Few studies have investigated the use of enzymes in extracts of teas. Interestingly, the data from this study reveal important information about the increase in antioxidant capacity of these drinks after treatment with tannase. This result was confirmed by analysis of ORAC and DPPH.

4. Conclusions

This study demonstrated that tea treated with tannase exhibits greatly increased antioxidant capacity *in vitro*. The tannase may be able to hydrolyse the substrates contained in these teas, and the products of hydrolysis may significantly increase the antioxidant capacity of these drinks. This study yielded the identification of an important polyphenol in each tea extract (chlorogenic acid from yerba mate and epigallocatechin gallate for green tea) and the finding that treatment of the extracts with tannase increased their antioxidant power. These results demonstrate the ability of tannase to catalyse hydrolysis on several different substrates from the tea extracts tested and confirm that the reaction results in higher antioxidant capacities for those polyphenols.

The increase in antioxidant capacity of tea extracts and commercial standards following tannase treatment was ascertained using the ORAC and DPPH assays, which, in both analyses, confirmed the result of increased antioxidant capacity of all biotransformed samples. The ORAC assay provides a novel and efficacious method for evaluating the potential antioxidant activities of various compounds and biological samples. Further studies are needed to determine the mechanism and potential applications of tannase in order to increase the antioxidant capacity of green tea and yerba mate.

Acknowledgments

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