

Effect of particle size on antioxidant activity and catechin content of green tea powders

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Abstract This work evaluated the effect of grinding and sieving process of green tea leaves on particle size distribution and antioxidant activity. Granulometric classes ranging from 20 μm to 500 μm were studied. Hydroalcoholic extracts of green tea powders (GTPs) were analysed for total phenolic, total flavonoid, and catechin contents. The fraction of 100–180 μm ground at 6000 rpm showed the maximum catechin content with 33.5 mg/g dry matter (DM) and, the best antioxidant activity with IC₅₀ values of 0.28 $\mu\text{g/mL}$ and 0.13 $\mu\text{g/mL}$ using DPPH and ABTS radical scavenging assays, respectively. However, antioxidant properties and catechin content decreased for particle of sizes less than 50 μm . Catechin content was higher for those ground at 6000 rpm as compared to at 18,000 rpm. The best grinding conditions and particle size were 6000 rpm and 100–180 μm .

Keywords Green tea powders · Fine grinding · Antioxidant activity · Catechins · LC-MS

Introduction

Tea is viewed as an important member of the antioxidant food group, a source of pharmacologically active molecules, and a functional food endowed with beneficial health properties (Dufresne and Farnworth 2001). Indeed, several studies have reported that green tea extract has antioxidant, antibacterial, antiviral, anticarcinogenic, antimutagenic functions (Higdon and Frei 2003), and also diuretic properties (Jouad et al. 2001). Epidemiological reports show that the percentage of cardiovascular diseases decreases due to green tea consumption (Kuriyama 2008). The antioxidant properties of flavonoids, compounds significantly present in green tea, have been shown by Davis et al. (2009). Moreover, these compounds have anti-inflammatory, vasodilator, antithrombotic, antiatherogenic, antipyretic and analgesic properties (Cook and Samman 1996).

Among the bioactive constituents of green tea, polyphenols account for 25–35 % on dry matter. Important characteristics of tea polyphenols are provided by flavan-3-ols, or catechins, with 14–25 % content of dry tea (Yao et al. 2006). Catechins are the most important functional components of green tea. They present a wide range of functional activities, especially a strong antioxidant activity (Pastore and Fratellone 2006). Epigallocatechin gallate has been described by Murase et al. (2002) as the predominant flavan-3-ol in green tea. Catechin and epicatechin are also present in onion, endives, cruciferous vegetables, black grapes, red wine, grapefruits, apples, cherries and berries (Dufresne and Farnworth 2001). Catechins of green tea showed no toxic effects in animals (Chengelis et al. 2008) and can thus be used in various drinks and foods (Kurita et al. 2004; Lu et al. 2010).

Industrial extraction of polyphenolic compounds from tea is mainly based on maceration combined with agitation,

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circulation, ultrasonic, microwave or enzymatic treatment (Lee and Lee 2008). A way to enhance compound extraction could be to grind plants. Indeed, fine grinding reduces particle size, thickens particle size distribution, and increases specific surface area, which lead to the improvement of material functionalities (Zhao et al. 2009; Zhang et al. 2012; Barnwal et al. 2015). However, grinding conditions have to be chosen carefully. Indeed, during grinding, temperature increases biochemical alterations of food materials and may result in modifications of their physicochemical properties (Maaroufi et al. 2000) like color alteration (Singh and Goswami 1999), which lowers their organoleptic properties (Murthy et al. 1999) and may cause bioactivity losses (Pesek et al. 1985).

To that purpose, a differential split method based on a recent patent (Baudelaire E. 2013) using grinding and sieving processes was used to increase the content of some bioactive compounds in powder extracts. Green tea powders (GTPs) were classified according to the following particle sizes: <20 μm , 20–50 μm , 50–100 μm , 100–180 μm , 180–315 μm , and 315–500 μm . Powders were produced at two grinding speeds, 6000 and 18,000 rpm. Total phenolic content, total flavonoid content, and antioxidant activity were assessed for the different granulometric classes and non-ground tea leaves (NGT). The main flavan-3-ols contained in green tea were also quantified by LC-MS. This article aims at evaluating the effect of particle size and grinding speed on the antioxidant activity and catechin content of green tea extracts.

Materials and methods

Plant material and GTP production

Green tea leaves (*Camellia sinensis* (L.) O. Kuntze) were provided by Gunpowder tea (Zhejiang Province, China). About 1.5 kg of leaves were slowly ground (50 g plant for each batch) with the Ultra Centrifugal Mill ZM 200 (Retsch France), operating by impact and shearing effects. Grinding was performed at room temperature (approximately 20 °C) using a 1 mm sieve with trapezoid holes. The grinding time was fixed to 2 min. Indeed, Hu et al. (2012) showed that concentrations of polyphenols, flavonoids and the antioxidant properties decrease when the grinding time increases. Two speeds were used for milling: 6000 rpm and 18,000 rpm. After grinding, plant powders were sieved with the vibratory sieve shaker Analysette 3 Spartan (Fritsch, Idar-Oberstein, Germany) at 0.5 mm vibration amplitude for 10 min. 20 mm diameter sieves (Fritsch) were employed so as to obtain the following granulometric fractions: <20 μm ; 50–100 μm ; 100–180 μm ; 315–500 μm ; >500 μm . Powders were then stored under vacuum in sealed bags at 10 °C.

Chemicals

Chemicals Folin & Ciocalteu's phenol reagent, gallic acid, ABTS as diammonium salt, 2,2'-Azobis(2-amidino-propane) dihydrochloride (AAPH), standards of (+)-catechin (C), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin gallate (EGCG) were acquired from Sigma-Aldrich Corporation (Saint Quentin Fallavier, France). HPLC-grade acetonitrile, water, methanol and formic acid were purchased from Sigma Chemicals Co. (Saint Louis, MO). Sodium carbonate (Na_2CO_3), aluminum chloride (AlCl_3), sodium nitrite (NaNO_2) and sodium hydroxide (NaOH) from Acros Organics (Geel, Belgium). Each standard (C, EC, EGC, ECG, and EGCG) was prepared in a methanol-water solvent 70–30 (v/v) at a concentration of 0.5 mg/mL.

Preparation of extracts

Extraction of polyphenols from GTPs were carried out according to the method of Kim et al. (2002), with some modifications. 2 g of dried leaves and 2 g of powdered dried leaves were macerated during 24 h under stirring, in 20 mL methanol/water 70/30 (v/v). Then, the methanol/water extract was centrifuged at 20,000 rpm for 20 min. The supernatant layer was filtered, brought to 15 mL and stored at 4 °C before analysis.

Total phenolic content

Total phenolic compounds (TPC) were determined according to the protocol used by Muanda et al. (2011). 40 μL of samples was added to test tubes containing 3.16 mL of distilled water followed by an addition of 200 μL of Folin-Ciocalteu reagent (1 N) and 600 μL of sodium carbonate (Na_2CO_3 , 20 %). Samples and blank were thoroughly mixed and vortexed. After 40 min of incubation at room temperature, the absorbance was measured at 725 nm. For the calibration curves, diluted solutions of gallic acid (solutions at 1 mg/mL, 1.5 mg/mL, 2 mg/mL, 2.5 mg/mL and 3 mg/mL) were used and total phenolic content was expressed in terms of equivalent amounts of gallic acid per 100 g of dry matter (GAE/100 g DM). Absorbance measurements were carried out with UV-visible spectrophotometer Cary 50 Scan (Agilent, Santa Clara, California, USA).

Total flavonoid content

Total flavonoid compounds (TFC) were dosed by a colorimetric assay described by Chen and Chen (2011). 250 μL of sample were added to a volumetric flask containing 1 mL of distilled water. At 0 min, 75 μL of sodium nitrite (NaNO_2 , 5 %) were added to the flask. After 5 min, 75 μL of aluminum

chloride (AlCl_3 , 10 %) were added. After 6 min, 500 μL of sodium hydroxide (NaOH , 1 N) were added to the mixture. At this time the mixture was diluted with 2.5 mL of distilled water, then the vortexed samples were kept at room temperature. The absorbance was directly measured at 510 nm. For use as calibration curve, diluted solutions of catechin were used and total flavonoids were expressed as mg of catechin equivalent per 100 g of dry matter (mg CE/100 g DM). As for TPCs, absorbance measurements were performed with UV–visible spectrophotometer Cary 50 Scan (Agilent, Santa Clara, California, USA).

Antioxidant activity

ABTS tests

The method developed by Muanda et al. (2010) was used in this experiment; 1 mM of AAPH solution was mixed with 2.5 mM ABTS as diammonium salt in phosphate buffered saline (PBS) solution (100 mM, pH 7.4) containing 150 mM NaCl. After incubation in a water bath at 68 °C for 20 min, the resulting ABTS solution was diluted with the methanol to get blue-green coloration with an absorbance of 0.70 ± 0.02 at 734 nm, then 2.94 mL of ABTS radical anion solution was added. The decrease of absorbance was measured at 734 nm. The radical stock was prepared daily and all the tests were triplicated. The half maximal inhibitory concentration (IC_{50}), which refers to the smallest concentration of antioxidants required to scavenge 50 % of the ABTS radical cation, was calculated.

DPPH tests

The DPPH radical scavenging activity was evaluated according to the method described by Sharififar et al. (2009). 1 mL of 100 μM DPPH solution in methanol was mixed with 1 mL of plant extract. The reaction mixture was incubated in the dark for 20 min, and the optical density was recorded at 517 nm against the blank. For the control, 1 mL of DPPH solution in methanol was mixed with 1 mL of methanol, and optical density of solution was recorded after 20 min. IC_{50} values related to the DPPH radical scavenging activity were calculated.

LC-MS analyses

Analysis parameters

LC-MS analyses were performed on a LC-MS 2020 system (Shimadzu, Tokyo, Japan) associated to an electrospray ionization source (ESI). Separation was carried on a 150×4.6 mm i.d. C18 reverse phase Gemini column (Phenomenex, Torrance, CA, USA) with particle size of 3 μm and pore size of 130 Å. The column oven was fixed at 30 °C. The mobile phase consisted of (A) 0.5 % formic acid in water and (B) acetonitrile.

The injection volume was 20 μL and the flow rate was 0.6 mL/min. The 40 min gradient used was as follows: 0–5 min, 90:10 (A:B); 5–7 min from 90:10 to 86:14 (linear gradient); 7–17 min held at 86:14; 17–19 min from 86:14 to 75:25 (linear gradient); 19–24 min held at 75:25; 24–25 min from 75:25 to 10:90 (linear gradient); 25–31 min held at 10:90; 31–32 min from 10:90 to 90:10, 32–40 min held at 90:10 (re-equilibration step). The electrospray ionization source was operated in negative mode. The nebulization gas flow was set to 1.5 L/min, drying gas flow at 20 L/min, the heat block temperature was fixed at 350 °C, and the desolvation line (DL) temperature at 250 °C. The probe voltage was set to -4500 V.

Calibration method

For the determination of calibration curves, standards of (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, (–)-epicatechin gallate, (–)-epigallocatechin gallate were prepared in 70/30 methanol/water (v/v) at concentrations comprised between 0.3 mg/mL and 2 mg/mL. Dosages of catechins were performed using the selected ion monitoring (SIM) mode to increase sensitivity compared to the full scan mode.

LOD and LOQ

Solutions of standard compounds were diluted at different concentrations to perform calibration curves in order to dose the main catechins present in GTP extracts. The detector response was linear over a range from 0.005 mg/mL to 0.5 mg/mL for the catechin standards (C, EC, EGC, ECG and EGCG). The method sensitivity was assessed by determining the limit of detection (LOD) and the limit of quantification (LOQ) defined as the concentrations with a signal-to-noise (S/N) of 3 and 10, respectively. Data are expressed in mg of standard per gram of dried matter.

Statistical analyses

Each experiment and measurement was performed in triplicate. Data are expressed as means \pm standard deviation. Statistical analysis was conducted using SAS software (v8.2, SAS Institute Inc., Cary, NC, USA) with analysis of variance (ANOVA). Means for significant differences were compared by using Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Total phenolic content of GTPs

To evaluate polyphenol content in GTPs, Folin & Ciocalteu test is used, results are displayed in Fig. 1. The most important

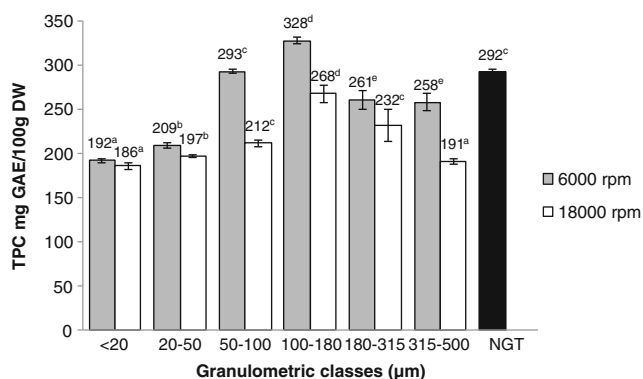


Fig. 1 Total phenolic content of green tea powders at 6000 and 18,000 rpm; *DM* dry matter, *GAE* gallic acid equivalent, *NGT* non-grinded tea leaves. All values are expressed as means \pm SD ($n = 3$). For a same grinding speed, values followed by different letters were statistically different ($p < 0.05$)

total phenolic content in GTPs was found in the fraction 100–180 μm at 6000 rpm, 328 mg GAE/100 g DM. Values of TPC increased when granulometry decreased from 500 μm to 50 μm . However, between 20 μm and 50 μm , TPCs decreased dramatically to 192 mg GAE/100 g DM (6000 rpm). The non-ground tea extract showed a high TPC, 305 mg GAE/100 g DM, which is superior than every GTPs obtained at 18,000 rpm. This can be explained by the speed increase from 6000 rpm to 18,000 rpm which leads to a temperature rise causing a loss of catechin content, as reported by Chen et al. (2001). Optimal parameters to obtain the highest TPC were the 100–180 μm sieve at 6000 rpm. The use of a faster grinding speed led to lower TPC values.

Total flavonoid content of GTPs

This test was performed to assay more specifically flavan-3-ols, compounds predominantly present in green tea (Oliveira 2012). Total flavonoid contents are reported in Fig. 2. At 6000 rpm,

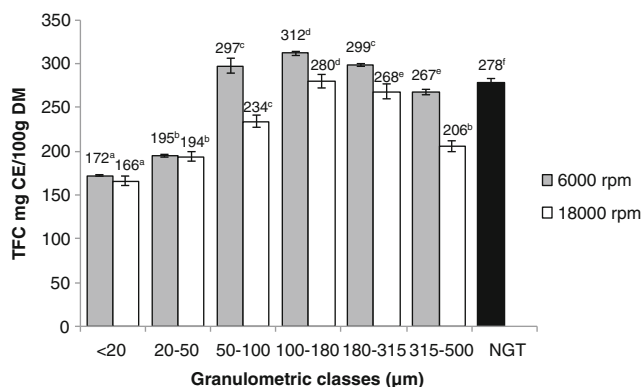


Fig. 2 Total flavonoid content of green tea powders at 6000 and 18,000 rpm; *DM* dry matter, *CE* catechin equivalent, *NGT* non-grinded tea leaves. All values are expressed as means \pm SD ($n = 3$). For a same grinding speed, values followed by different letters were statistically different ($p < 0.05$)

TFCs tended to increase when particle size decreased to 50 μm , then, for 20 μm at 6000 rpm, the amount of flavonoids fell down to 172 mg CE/100 g DM. Here also, the highest TFC is reached with the 100–180 μm fraction at 6000 rpm with 312 mg CE/100 g DM. As for total phenolic content, the non-ground tea TFC was higher than GTPs at 18,000 rpm with 278 mg CE/100 g DM, confirming the hypothesis previously raised of catechin degradation during grinding at higher speed.

Antioxidant activity of GTPs

DPPH radical scavenging activity

Antioxidant activity is defined as the ability of a compound to inhibit oxidative degradation (Roginsky and Lissi 2005). The DPPH method has been extensively used to measure the radical scavenging abilities of various antioxidant substances. The antioxidant activity of GTPs and NGT assessed by DPPH is reported in Table 1. Values are expressed as mean \pm standard deviation from triplicate. The IC₅₀ is the concentration of antioxidant required to reduce the original amount of radical by 50 %.

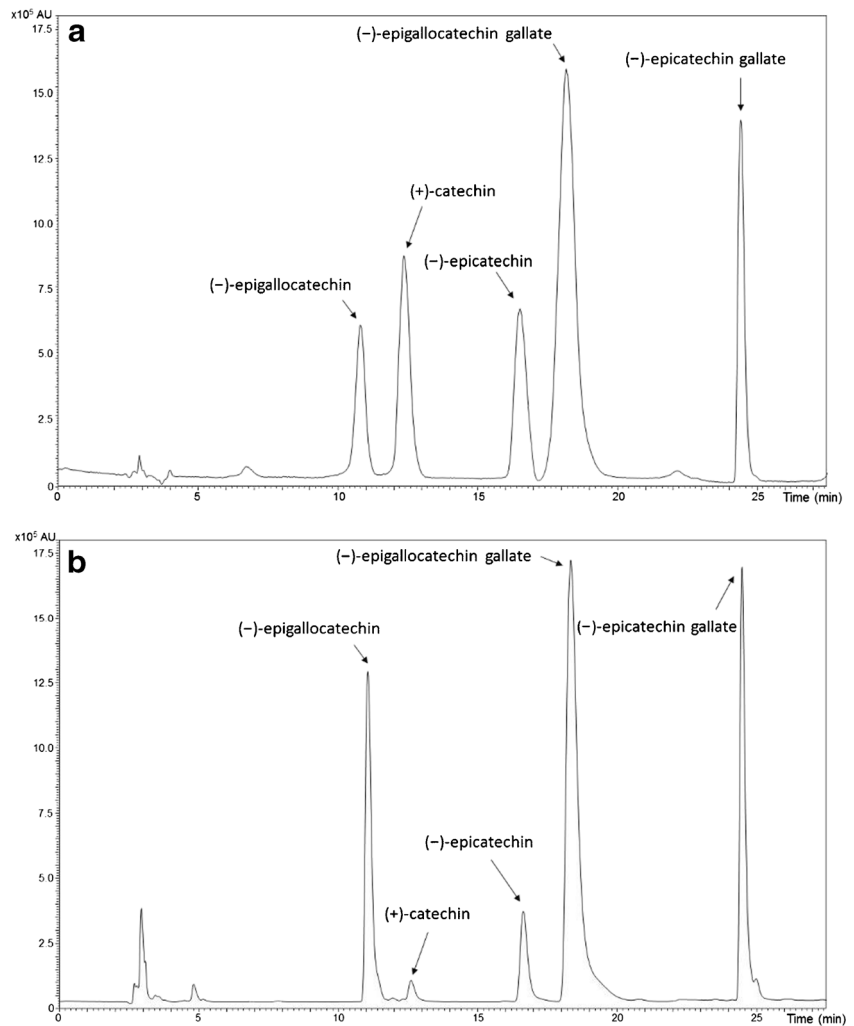
At both grinding speed, IC₅₀ values were not significantly different among 50–100 μm , 100–180 μm and 180–315 μm fractions ($p > 0.05$). These granulometric classes showed thus the highest DPPH scavenging activity with a maximum IC₅₀ of 0.28 $\mu\text{g}/\text{mL}$ for the 100–180 μm GTP. In comparison, the IC₅₀ value of the non-ground tea was evaluated at 0.39 $\mu\text{g}/\text{mL}$ which constitutes a significant difference ($p < 0.05$). Similar variations were observed at 18,000 rpm: intermediate fractions led to the highest activity. However, as for the total phenolic content and total flavonoid content, the best results

Table 1 Evaluation of the antioxidant activities of extracts from green tea powders with different particle sizes obtained at grinding speeds of 6000 rpm and 18,000 rpm, and non-grinded tea leaves (NGT) as determined by DPPH radical scavenging effect and ABTS radical scavenging effect

GTPs (μm)	IC ₅₀ values of radical scavenging activity ($\mu\text{g}/\text{mL}$)			
	DPPH test		ABTS test	
	6000 rpm	18,000 rpm	6000 rpm	18,000 rpm
<math><20</math>	0.53 \pm 0.02 ^a	0.70 \pm 0.02 ^a	0.24 \pm 0.02 ^a	0.54 \pm 0.02 ^a
20–50	0.44 \pm 0.03 ^b	0.61 \pm 0.03 ^b	0.22 \pm 0.02 ^a	0.44 \pm 0.04 ^b
50–100	0.31 \pm 0.01 ^c	0.51 \pm 0.02 ^c	0.16 \pm 0.03 ^b	0.32 \pm 0.03 ^c
100–180	0.28 \pm 0.01 ^d	0.48 \pm 0.01 ^c	0.13 \pm 0.02 ^b	0.28 \pm 0.04 ^d
180–315	0.31 \pm 0.02 ^d	0.53 \pm 0.01 ^c	0.14 \pm 0.03 ^b	0.33 \pm 0.03 ^c
315–500	0.44 \pm 0.02 ^b	0.64 \pm 0.03 ^b	0.21 \pm 0.02 ^a	0.46 \pm 0.05 ^b
NGT	0.39 \pm 0.02 ^d	-	0.35 \pm 0.03 ^c	-

The data were mean \pm standard deviation of triplicate ($n = 3$). Means in the same column followed by different letters were statistically different ($p < 0.05$)

Fig. 3 LC-MS profiles of **a** a mixture of standard compounds (+)-catechin (C), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin gallate (EGCG) prepared at a concentration 0.015 g/L and **b** green tea extract



were obtained at 6000 rpm. A positive correlation was also observed between the TPC ($r^2 = 0.801$) and TFC ($r^2 = 0.840$) of the GTP extracts and their antioxidant activity assessed by the DPPH test. This shows the relation between antioxidant activity and polyphenols content, as described by Burin et al. (2014).

ABTS radical scavenging activity

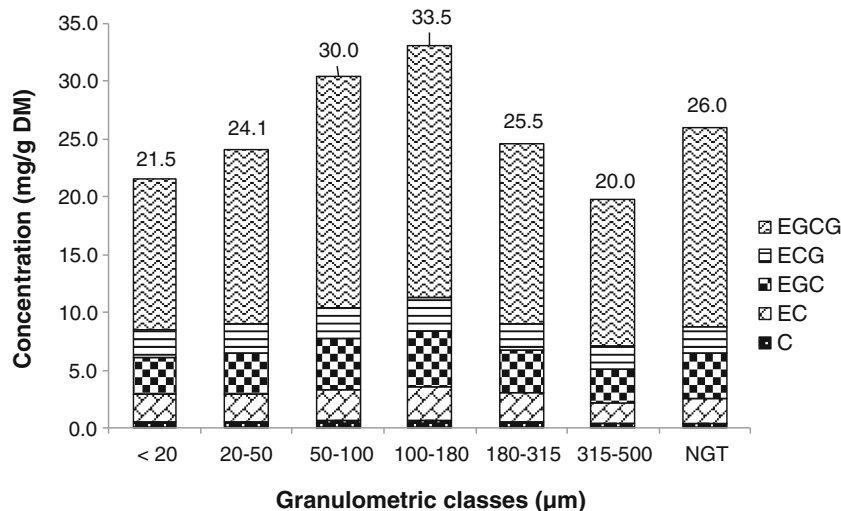
ABTS radical scavenging assay can also be applied to test food and natural water-soluble phenolics. The antioxidant

activity of GTPs and NGT assessed by ABTS are reported in Table 1. Results showed that the difference between samples come from different GTPs. IC50 values were significantly different among 100–180 μm and 315–500 μm ($p < 0.05$). The best activity was obtained for the fraction between 100 and 180 μm at 6000 rpm, with an IC50 of 0.13 $\mu\text{g}/\text{mL}$. The scavenging capacity of <20 μm on ABTS decreased significantly with 0.24 $\mu\text{g}/\text{mL}$ at 6000 rpm. Antioxidant activity were systematically lower for GTPs ground at 18,000 rpm. The trends were in agreement with those exhibited by the results of DPPH radical scavenging test, confirming that

Table 2 Retention times, m/z ratios, validation (r^2) and LOD-LOQ values for flavan-3-ols quantified

Compound	m/z	Retention time (min)	LOD (ppm)	LOQ (ppm)	Coefficient of determination (r^2)
(+)-catechin (C)	289	12.4	2	8	0.998
(–)-epicatechin (EC)	289	16.5	2	6	0.999
(–)-epigallocatechin (EGC)	305	10.8	4	13	0.999
(–)-epicatechin gallate (ECG)	441	24.4	5	18	0.996
(–)-epigallocatechin gallate (EGCG)	457	18.2	3	10	0.997

Fig. 4 LC-MS dosages of flavan-3-ols: (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) from tea powder extracts and the unground leaves extract at 6000 rpm. Total content of the catechins quantified are displayed above each bar in mg/g of dried matter. Variations between granulometric classes were statistically significant ($p < 0.05$)



particle size has an important influence on extract antioxidant activity. It can be also noticed that IC_{50} values obtained with ABTS assay were lower than DPPH assay, this difference between these two tests is consistent with the study of Cyzowska et al. (2015).

LC-MS

Analysis of catechin standards and GTP extracts

The LC-MS profile of standard compound analysis is displayed in Fig. 3a. This initial experiment allows us to know the retention times of each catechin. Retention times and m/z ratios of the selected catechins are given in Table 2. The same settings were applied for the analysis of GTP and NGT extracts. Before their analysis, extracts were filtrated and diluted ten times in order to prevent the electrospray source from contamination. The LC-MS profile of this experiment is presented in Fig. 3b. The chromatographic peaks found in tea extracts showed the same retention times and m/z ratios than

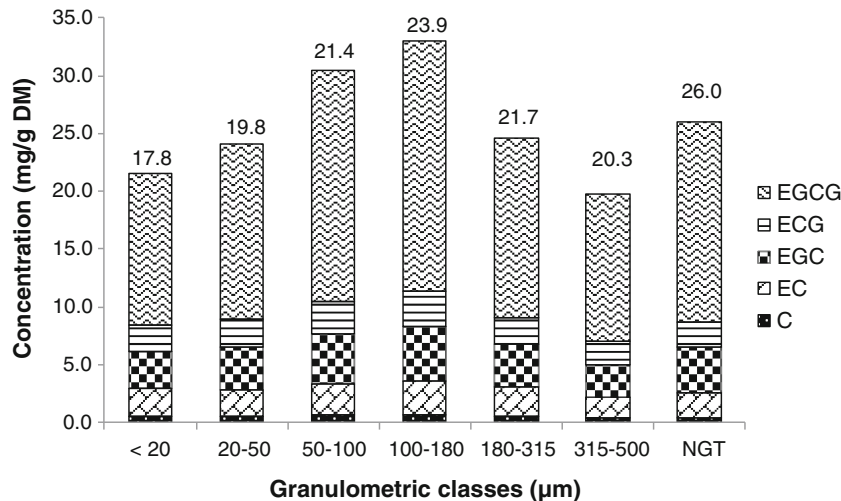
standard compounds, enabling to identify the main catechins in green tea in the following elution order: C, EC, ECG, EGC, and EGCG. The major signals were peaks at 18.2 and 24.4 min, which correspond to the (-)-epigallocatechin gallate and the (-)-epicatechin gallate, respectively (Fig. 3b).

Quantification of catechins

Coefficients of determination (r^2) from the calibration curves obtained with the external method are displayed in the Table 2. The catechin signals (S/N ratios) at the studied concentrations were equal or 10 times higher than the limits of the quantification (LOQs). These calibration curves were used to quantify catechins in GTP extracts. The catechin peaks in chromatograms obtained from extracts were quantified in terms of equivalents of external standards. Figures 4 and 5 display catechin contents calculated for GTPs at 6000 rpm and 18,000 rpm, respectively, and NGT.

Concentrations of EGCG were much higher than other catechins, from five to twenty times higher according to the

Fig. 5 LC-MS dosages of flavan-3-ols (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) from tea powder extracts and the unground leaves extract at 18,000 rpm. Total content of the catechins quantified are displayed above each bar in mg/g of dried matter. Variations between granulometric classes were statistically significant ($p < 0.05$)



compound. The order of relative concentrations of catechins (EGCG > EGC > ECG > EC > C) is similar to the one described by Suzuki et al. (2013). Nevertheless, another article showed a different ranking (Khokhar and Magnusdottir 2002). This may depend on the green tea variety. Variations of concentration of the five catechins followed the same trend according to particle size. For all catechins, maximum concentrations were found for granulometric classes between 100 and 180 μm . The lowest catechin contents were obtained for the <20 μm , 20–50 μm and 315–500 μm granulometric classes. The concentration for the non-ground leaf extract (26.0 mg/g DM) was lower than 100–180 μm (33.5 mg/g DM) at 6000 rpm. A positive correlation was calculated between antioxidant activity and catechin content ($r^2 = 0.893$).

Even if variations of catechin concentrations were similar at both speeds, NGT extract led to a higher catechin content at 18,000 rpm (Figs. 5). Indeed, as Hu et al. (2012) mentioned, it is crucial to avoid excessive heat during grinding, and operating at 18,000 rpm may increase heat. Catechin contents increased when particle size decreased except for smaller particle size, <20 μm and 20–50 μm . In that case, the larger surface area in finer GTPs could increase the contact surface between O_2 and particles, thus leading to the oxidation of catechins (Neilson et al. 2007).

Conclusion

This study showed the advantage of grinding and sieving green tea leaves to increase content of polyphenols. Our results revealed that antioxidant activity was dependent on particle size. However, it is important to control grinding speed and particle size to avoid catechin degradation by heat and oxidation. The highest antioxidant activity and catechin content were obtained with the particle size of 100–180 μm at 6000 rpm, which then determine the best conditions for the grinding of green tea leaves. This procedure may be extended to other food applications, for example to improve nutritive values.

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