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Catechin content and free radical scavenging activity of *Camellia sinensis* twig extracts

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Abstract

The catechin content and antioxidant properties of various *Camellia sinensis* twig extracts, including a water extract (WE), 10% ethanol extract (10% EE), 50% ethanol extract (50% EE), and 95% ethanol extract (95% EE) were investigated. The 50% EE had the highest total phenolic content (161.3 ± 8.5 mg gallic acid equivalents/L) and total flavonoid content (278.9 ± 12.2 mg quercetin equivalents/L). High-performance liquid chromatography analysis suggested that epigallocatechin gallate and epigallocatechin were the predominant catechins in the twig extracts. The relative concentrations of six catechins isolated from the extracts were: 50% EE > 10% EE > WE > 95% EE. The 50% EE showed free radical-scavenging activity. The concentration of dry matter of 50% EE required to scavenge 50% of ABTS radicals was 102.8 ± 4.2 µg/mL. These results suggest that 50% EE can potentially be used as a source of catechins.

Introduction

Tea is a popular beverage with unique flavour and potential health-promoting effects. According to various fermentation degrees, tea can mainly be classified into oolong tea (semi-fermented), black tea (fully fermented), and green tea (non-fermented) (Zhang and Ku, 2019). Approximately 3.5 million tons of dried tea are globally produced every year with 20% produced as green tea (Wang *et al.,* 2012). To produce green tea, the fresh *C. sinensis* leaves are heated to inactivate the polyphenol oxidase. Because green tea is non-fermented, it is presumed to contain high total levels of phenolic and flavonoid compounds, and high antioxidant activity (Chacko *et al.,* 2010). Green tea contains polyphenols, and most of the green tea polyphenols are catechins. Kilmartin and Hsu (2003) reported that polyphenols are secondary plant metabolites involved in a wide range of specialised physiological functions. The major polyphenols present in the tea leaves are catechins (Del Rio *et al.,* 2004). Catechins have been shown to have antioxidant activity, and may be beneficial for human health. These health benefits have resulted in considerable interest in catechins as food additives to improve the antioxidant properties of foods (Oze *et al.,* 2014; Rashidinejad *et al.,* 2016).

Tea catechins are comprised of a central 3-carbon unit, which is connected to two phenolic nuclei with several hydroxyl groups. These molecules are primarily divided into non-epistructured catechins and epistructured catechins (Xu *et al.,* 2018). The non-epistructured group includes gallocatechin gallate (GCG), catechin (C), catechin gallate (CG), and gallocatechin (GC). The epistructured catechin group includes epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and epicatechin (EC) (Saklar *et al.,* 2015; Musial *et al.,* 2020). Green tea is an excellent source of catechins (Balentine *et al.,* 1997). In green tea, catechins account for 80 to 90% of total flavonoids; EGCG is the most abundant catechin (48 - 55%) followed by ECG (9 - 12%), EGC (9 - 12%), and EC (5 - 7%) (Babu and Liu, 2008). The structural features of catechins that enable scavenging of DPPH radicals are an *ortho*-trihydroxyl group in the B ring, and a galloyl moiety at the 3-position. These catechins have been widely used as food antioxidants (Ayyildiz *et al.,* 2018).

Extraction is a major step in obtaining catechins from green tea. Catechins are soluble in polar solvents, such as water and ethanol. Catechins have commonly been extracted from green tea using 50% ethanol (Jun *et al.,* 2010; Banerjee and Chatterjee, 2015). High-performance liquid chromatography (HPLC) is the widely used method for the analysis of catechins in green tea (Novak *et al.,* 2010). El-Shahawi *et al.* (2012) analysed the contents of EGCG, EGC, EC, and ECG using 29 commercial green tea samples. The average concentrations of EGCG, C, EGC, EC, and ECG were found to be in the range of 1.0 - 43.3, 0.1 - 24.9, 0.5 - 10.2, and 0.2 - 13.9 mg/g, respectively.

The green tea twig is a low-quality by-product

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of tea processing that is disposed as a waste after processing. Tea twigs may contain the same catechins as green tea leaves. Although catechins have been isolated from green tea leaves, the catechin contents in tea twig extracts have not been reported. Liang *et al.* (2007) reported that 50 and 75% ethanol were suitable for the commercial extraction of catechins from dry and fresh tea leaves, respectively. In the present work, we assessed various ethanol concentrations (0, 10, 50, and 95%) for the extraction of catechins from *C. sinensis* twigs, and evaluated their free radical-scavenging performance. The catechin contents including GC, CG, EGC, ECG, EC, and EGCG in the twig extracts were also analysed.

Materials and methods

Preparation of tea twig extracts

Tea twig (*C. sinensis*) was obtained from Formosa Black Tea Co. (Hsinchu, Taiwan). The preparation of the tea twig extracts was performed using a modified method of Vuong *et al.* (2011) as follows. Tea twig samples (10 g) were extracted with 250 mL of distilled water, 10% ethanol, 50% ethanol, and 95% ethanol at 80°C for 20 min. The extracts were filtered through a filter paper, and the samples (250 mL) were collected to yield four extracts, including the water extract (WE), 10% ethanol extract (10% EE), 50% ethanol extract (50% EE), and 95% ethanol extract (95% EE). These twig extracts were freeze-dried to yield the dry matter of WE, 10% EE, 50% EE, and 95% EE.

Determination of the total phenolic content in twig extracts

The total phenolic content of the samples, including the WE, 10% EE, 50% EE, and 95% EE was determined based on the method of Kao and Chen (2006). Briefly, the sample (30 µL) was mixed with 10% Folin-Ciocalteu reagent (120 µL), and incubated at 30°C for 5 min. Then, 15% Na_2CO_3 (600 µL, w/v) was added. The absorbance at 750 nm was measured using a spectrophotometer. The total phenolic content was expressed as gallic acid equivalents (GAE) per litre of twig extract (mg GAE/L). Each sample was analysed in triplicate.

Determination of the total flavonoid content in twig extracts

The total flavonoid content of the WE, 10% EE, 50% EE, and 95% EE samples was determined based on the method of Kao and Chen (2006). Sample (500 µL) was mixed with 5% sodium nitrite (30 µL). After 5 min incubation at 30°C, the mixture was mixed with 10% aluminium chloride (60 µL). The samples were then mixed with 1 mM sodium hydroxide (300 µL) and chloroform (200 µL). The absorbance at 510 nm was measured using a spectrophotometer. The total flavonoid content was expressed as quercetin equivalents (QE) per litre of twig extract (mg QE/L). Each sample was analysed in triplicate.

HPLC analysis and identification of the catechins

The catechin content of the WE, 10% EE, 50% EE, and 95% EE samples were determined based on the method of Hadad *et al.* (2014). The WE, 10% EE, 50% EE, and 95% EE were subjected to column chromatography on an HPLC system and a Mightysil RP-18 GP Cica-Reagent column (250 × 4.6 mm; Kanto Chemical Co., Inc. Japan). The mobile phase was solvent A (ultrapure water) and solvent B (100% acetonitrile). The HPLC elution conditions were as follows: 88 - 85% solvent A to solvent B for 0 - 3 min, and 85 - 80% solvent A to solvent B for 3-22 min at a flow rate of 1.0 mL/min. Signal was detected at a wavelength of 245 nm. The GC, EGC, EGCG, and CG were obtained from Santa Cruz Biotechnology (USA), and EC and ECG were obtained from Sigma Chemical Co. (USA). Six catechins were identified using ultraviolet spectra and LC-MS using a Dionex™ Ultimate 3000 XRS system coupled to a TSQ Quantiva triple quadrupole mass spectrometer.

ABTS radical scavenging activity

ABTS radical scavenging activity of the samples, including the dry matter of WE, 10% EE, 50% EE, and 95% EE was measured at various concentrations (0 - 500 µg/mL) based on the method of Rusak *et al.* (2008). ABTS reagent was prepared by mixing 5 mL of ABTS aqueous stock solution (7 mM) with 88 µL of potassium persulfate (140 mM). The samples (180 µL) were dispensed into a 96-well plate, and ABTS reagent (20 µL) was added. The plates were placed into a spectrophotometer for detection of absorbance at 734 nm (A_{734}). The method described by Mensor et al. (2001) was used to determine the sample concentration required to scavenge 50% of the ABTS radicals (EC_{50}). Each sample was analysed in triplicate.

Statistical analysis

The data were analysed using the Statistical Analysis System (SAS software release 9.4 for Windows, version 13.2, SAS Institute, Inc., Cary, NC, USA). The statistically significant differences between the treatments were determined by one-way ANOVA, and the significance level was set at $p < 0.05$.

Results and discussion

Total phenolic and flavonoid contents in the WE, 10% EE, 50% EE, and 95% EE

Tea twig samples (10 g) were extracted with distilled water, 10% ethanol, 50% ethanol, and 95% ethanol to yield 250 mL of WE, 10% EE, 50% EE, and 95% EE, respectively. These extracts contained 75.7, 75.0, 92.2, and 46.1 mg of dry matter, respectively. Rusak *et al.* (2008) reported that the total phenolic and flavonoid contents in green tea ranged from 759 to 2377 mg GAE/L and 431 to 1768 mg GAE/L, respectively. Therefore, the total phenolic and flavonoid contents in the WE, 10% EE, 50% EE, and 95% EE were evaluated. As shown in Figure 1, the total phenolic contents in the WE, 10% EE, 50% EE, and 95% EE were 121.2 ± 3.9, 153.3 ± 6.0, 161.3 ± 8.5, and 126.9 ± 3.8 mg GAE/L, respectively. The total flavonoid contents in the WE, 10% EE, 50% EE, and 95% EE were 218.4 ± 4.0, 269.5 ± 14.8, 278.9 ± 12.2, and 271.1 ± 28.3 mg QE/L, respectively. Do *et al.* (2014) reported that extraction yield depends on the polarity index of the solvent. In the present work, the extracts were obtained from green tea twigs using water and various concentrations of ethanol (10, 50, and 95%). The dry tea twig extracts with the highest total phenolic and flavonoid content were obtained using 50% ethanol, indicating an inverse relationship between the extraction efficiency of flavonoids/phenolic compounds and solvent polarity. Very high concentrations of ethanol (95%) reduced the extraction of flavonoids and phenolic compounds. Overall, these results are in good agreement with those reported by Liang *et al.* (2007). These authors indicated that the highest level of total catechins from dried tea leaves was extracted with 50% ethanol. Fadhel and Amran (2002) reported that epicatechin and catechins are the main flavonoids in green tea. Therefore, 50% ethanol was a better solvent for the extraction of phenolics and flavonoids than the other tested solvents. These results suggest that the 50% EE can potentially be used as a readily accessible source of natural antioxidants. These extracts can be used to delay oxidation in a variety of food products.

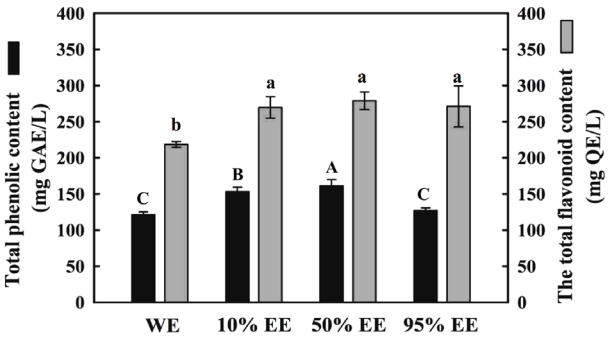

Figure 1. Total phenolic and flavonoid contents of the WE, 10% EE, 50% EE, and 95% EE. The total phenolic content is expressed as gallic acid equivalents (GAE) per litre of twig extracts (mg GAE/L). The total flavonoid content is expressed as quercetin equivalents (QE) per litre of twig extracts (mg QE/L).

HPLC analysis of catechins in the WE, 10% EE, 50% EE, and 95% EE

Li *et al.* (2008) reported that the catechins extracted from green tea are potent antioxidants. The analysis of catechins is performed using HPLC coupled to a mass spectrometer and a spectrophotometric detector (Jin *et al.,* 2014). Therefore, catechins in the WE, 10% EE, 50% EE, and 95% EE were detected by HPLC and mass spectrometry (Figure 2). As shown in Figure 2A, HPLC was used to analyse six catechin standards (arrows, peaks 1 - 6), including GC, EGC, EC, EGCG, ECG, and CG. These catechins were isolated from the WE by HPLC (Figure 2B), and purified catechins were identified by direct comparison with the catechin standards. These catechins were identified in the 10% EE (Figure 2C), 50% EE (Figure 2D), and 95% EE (Figure 2E) by HPLC. The spectral data of the identified catechins obtained by ESI-MS and UV were as follows:

i) Peak 1 (Gallocatechin, GC): ESI-MS (m/z) of 307.06 $[M+H]^+$ (calculated for $C_{15}H_{14}O_7$) and UV (50% ethanol) λ_{max} 210 and 270 nm.
ii) Peak 2 (Epigallocatechin, EGC): ESI-MS (m/z) of 307.07 $[M+H]^+$ (calculated for $C_{15}H_{14}O_7$) and UV (50% ethanol) λ_{max} 210 and 270 nm.
iii) Peak 3 (Epicatechin, EC): ESI-MS (m/z) of 291.07 $[M+H]^+$ (calculated for $C_{15}H_{14}O_6$) and UV (50% ethanol) λ_{max} 210 and 275 nm.
iv) Peak 4 (Epigallocatechin gallate, EGCG): ESI-MS (m/z) of 459.13 $[M+H]^+$ (calculated for $C_{22}H_{18}O_{11}$) and UV (50% ethanol) λ_{max} 225 and 275 nm.
v) Peak 5 (Epicatechin gallate, ECG): ESI-MS (m/z) of 443.14 $[M+H]^+$ (calculated for $C_{22}H_{18}O_{10}$) and UV (50% ethanol) λ_{max} 278 nm.
vi) Peak 6 (Catechin gallate, CG): ESI-MS (m/z) of 443.13 $[M+H]^+$ (calculated for $C_{22}H_{18}O_{10}$) and UV (50% ethanol) λ_{max} 278 nm.

Catechin concentrations in the WE, 10% EE, 50% EE, and 95% EE

The concentrations of six catechins in the WE were determined by HPLC (Table 1). According

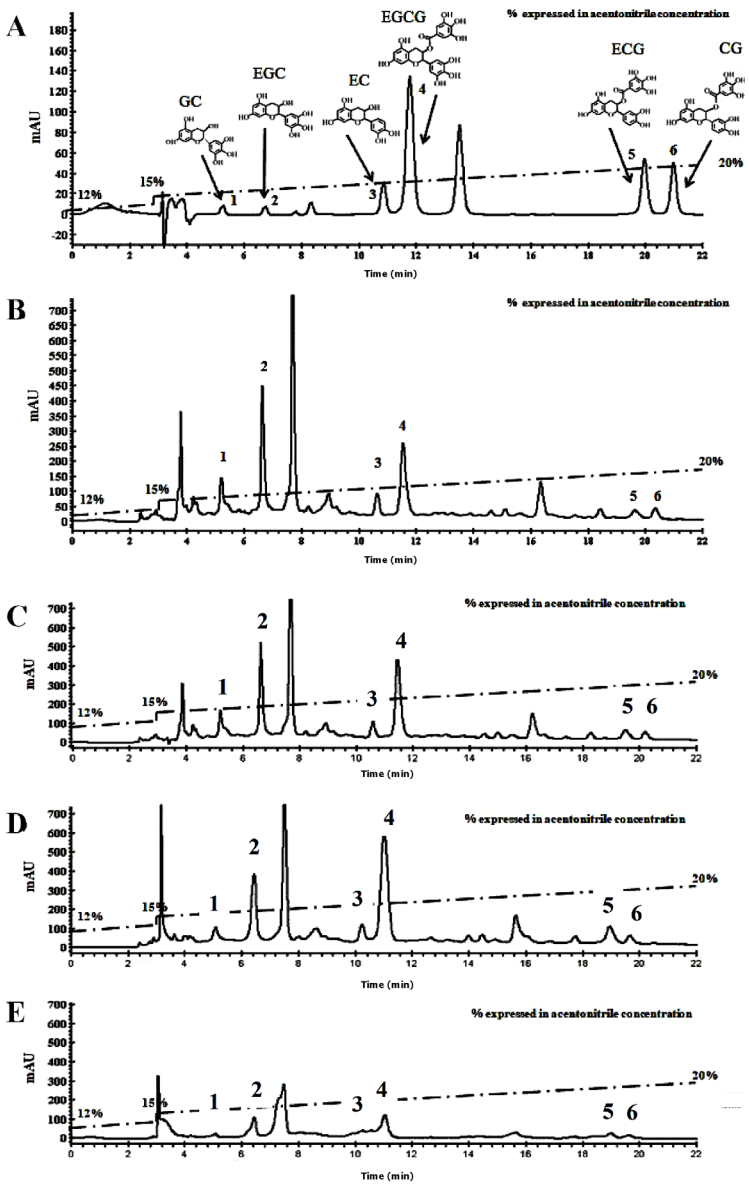

Figure 2. HPLC chromatograms of catechins extracted from the twig extracts: (A) catechin standards, (B) WE, (C) 10% EE, (D) 50% EE, and (E) 95% EE. Chemical structures of catechins: (1) GC, (2) EGC, (3) EC, (4) EGCG, (5) ECG, and (6) CG.

Table 1. Catechin concentrations in the WE, 10% EE, 50% EE, and 95% EE.

	GC (mg/mL)	EGC (mg/mL)	EC (mg/mL)	EGCG (mg/mL)	ECG (mg/mL)	CG (mg/mL)
WE	0.02 ± 0.00^{bc}	1.25 ± 0.06^{c}	0.36 ± 0.09^{ab}	1.54 ± 0.17^{c}	0.19 ± 0.03^{c}	0.38 ± 0.03^{b}
10% EE	0.08 ± 0.00^{b}	2.05 ± 0.25^{b}	0.14 ± 0.13^{a}	2.32 ± 0.10^{b}	0.31 ± 0.03^{b}	0.43 ± 0.05^{ab}
50% EE	0.20 ± 0.08^{a}	2.47 ± 0.16^{a}	0.50 ± 0.15^{a}	4.33 ± 0.29^{a}	0.53 ± 0.07^{a}	0.49 ± 0.06^{a}
95% EE	0.01 ± 0.00^{c}	0.61 ± 0.06^{d}	0.18 ± 0.02^{b}	0.94 ± 0.03^{d}	0.18 ± 0.01^{c}	0.28 ± 0.01^{c}

Data are expressed as the mean ± standard deviation of triplicate experiments ($n = 3$). Means with different letters within the same row are significantly different at $p < 0.05$.

to our results, the relative concentrations of the six catechins in the WE were EGCG > EGC > CG > EC > ECG > GC. EGC and EGCG were the predominant catechins in the WE. These data are similar to the results published by El-Shahawi *et al.* (2012) who reported the relative concentrations of catechins in water extracts from green tea as follows: EGCG > EGC > ECG > EC. The average concentrations of

EC, ECG, EGC, and EGCG in green tea were in the ranges of 0.5 - 10.2, 0.2 - 13.9, 0.2 - 24.9, and 1.0 - 43.3 mg/g, respectively. The concentrations of catechins in tea twig were lower than that in tea leaves. The relative concentrations of the six catechins were 50% EE > 10% EE > WE > 95% EE. This phenomenon was also observed in a previous study (Wang *et al.,* 2012) which reported that the extraction efficiency of the main catechin, EGCG, increased concomitantly with increasing ethanol concentrations from 0 to 60%. However, the extraction efficiency decreased when the ethanol concentration was higher than 60%.

The concentrations of catechins in the 50% EE were significantly higher than those in the WE, 10% EE, and 95% EE ($p < 0.05$). The quantities of catechins extracted from the 50% EE were GC (0.20 ± 0.08 mg/mL), EGC (2.47 ± 0.16 mg/mL), EC (0.50 ± 0.15 mg/mL), EGCG (4.33 ± 0.29 mg/mL), ECG (0.53 ± 0.07 mg/mL), and CG (0.49 ±0.06 mg/mL). Zuo *et al.* (2002) reported that the concentrations of EGCG, EGC, ECG, and EC in green tea samples extracted with 80% methanol were 1.75, 1.17, 0.76, and 0.39 mg/mL, respectively. Toschi *et al.* (2000) reported that the antioxidant activity of green tea is higher in tea that contains higher levels of EGCG and EGC. We noticed that the levels of EGCG (4.33 ± 0.29 mg/mL) and EGC (2.47 ± 0.16 mg/mL) in the 50% EE were higher than those in the WE, 10% EE, and 90% EE. EGCG has considerable health benefits in humans. EGCG contributes to the beneficial therapeutic effects of green tea and functions as an anti-inflammatory agent (Šilarová *et al.,* 2017; Khalatbary and Khademi, 2020; Lin *et al.,* 2020). Therefore, our results suggest that EGCG and EGC are the most abundant catechins in the 50% EE.

Free radical scavenging activities of dry matter of WE, 10% EE, 50% EE, and 95% EE

As previously described, tea twig samples (10 g) were extracted with distilled water, 10% ethanol, 50% ethanol, and 95% ethanol to yield 250 mL of the WE, 10% EE, 50% EE, and 95% EE, respectively. These extracts contained 75.7, 75.0, 92.2, and 46.1 mg of dry matter, respectively. Pan *et al.* (2003) reported that dried green tea leaves contain 10 - 30% polyphenols, which are soluble in water and ethanol. Kilmartin and Hsu (2003) reported that polyphenols are secondary plant metabolites involved in a wide range of specialised physiological functions. The major polyphenols present in tea leaves are catechins (Del Rio *et al.,* 2004).

The ABTS radical scavenging activities of the dry matter of WE, 10% EE, 50% EE, and 95% EE were measured. As shown in Figure 3, the dry matter of WE, 10% EE, 50% EE, and 95% EE showed ABTS radical scavenging activities. When 100.0 μg/mL of the dry matter of WE, 10% EE, 50% EE, and 95% EE were added, the ABTS radical scavenging effect (%) values were 31.7 ± 2.7, 39.4 ± 0.9, 48.4 ± 1.7, and 53.5 ± 0.8%, respectively. The concentrations of the dry matter of WE, 10% EE, 50% EE, 95% EE, and ascorbic acid required to scavenge 50% of ABTS radicals (EC_{50}) were 195.0 ± 14.8, 145.3 ± 2.1, 102.8 ± 4.2, 90.6 ± 2.0, and 35.1 ± 0.5 μg/mL, respectively. Tea polyphenols have a scavenging effect on active oxygen radicals (Pan *et al.,* 2003). Rusak *et al.* (2008) reported that the antioxidant capacity of the tea extracts correlates with the phenolic content. Komes *et al.* (2010) indicated that the order of contribution to the antioxidant effectiveness in green tea was EGC ≈ EGCG » ECG ≈ EC > C.

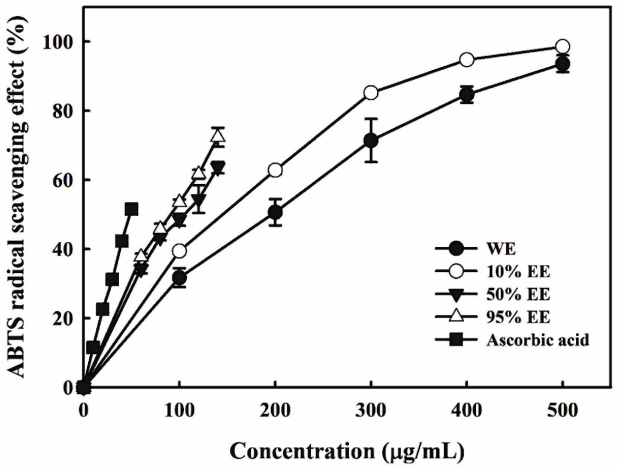

Figure 3. ABTS radical scavenging activities of the dry matter of WE, 10% EE, 50% EE, and 95% EE were measured at various concentrations.

Conclusion

Our results suggest that the highest level of total catechins was extracted in the 50% EE, which had the highest total phenol and flavonoid contents. The major catechins in 50% EE were GC, EGC, EC, EGCG, ECG, and CG. The relative catechin concentrations were EGCG > EGC > ECG > EC > CG > GC. These results suggest that EGCG and EGC are predominant catechins in 50% EE. Moreover, the dry matter of 50% EE required to scavenge 50% of ABTS radicals (EC_{50}) was 102.8 ± 4.2 μg/mL. Therefore, the 50% EE can be potentially used as a readily accessible source of catechins. These catechins can be used as antioxidant additives in the food industry.

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