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Journal of Chromatography B, 810 (2004) 187–195
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction

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Received 24 February 2004; accepted 19 July 2004

Abstract

Catechins levels in organ tissues, particularly liver, determined by published methods are unexpectedly low, probably due to the release of oxidative enzymes, metal ions and reactive metabolites from tissue cells during homogenization and to the pro-oxidant effects of ascorbic acid during sample processing in the presence of metal ions. We describe a new method for simultaneous analysis of eight catechins in tissue: (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (−)-gallocatechin gallate (GCG) and (−)-epigallocatechin gallate (EGCG) (Fig. 1). The new extraction procedure utilized a methanol/ethylacetate/dithionite (2:1:3) mixture during homogenization for simultaneous enzyme precipitation and antioxidant protection. Selective solid phase extraction was used to remove most interfering bio-matrices. Reversed phase HPLC with CoulArray detection was used to determine the eight catechins simultaneously within 25 min. Good linearity (>0.9922) was obtained in the range 20–4000 ng/g. The coefficients of variance (CV) were less than 5%. Absolute recovery ranged from 62 to 96%, accuracy 92.5 ± 4.5 to 104.9 ± 6%. The detection limit was 5 ng/g. This method is capable for determining catechins in rat tissues of liver, brain, spleen, and kidney. The method is robust, reproducible, with high recovery, and has been validated for both in vitro and in vivo sample analysis.

Keyword: Catechin

1. Introduction

Green tea has highly reputed chemotherapeutic effects and is one of the most widely investigated herbs. Since it has been imbibed in China, Korea and Japan for thousands of years, its long-term safety is well established. Its wide safety margin makes it one of the safest herbal medicines available [1]. The main constituents of green tea extract are catechins: (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (−)-gallocatechin gallate (GCG) and (−)-epigallocatechin gallate (EGCG). Green tea extract has well known antimicrobial properties, inhibiting the growth of *Staphylococcus aureus*, *Salmonella typhi* and *Vibro cholerae*. EGCG and ECG act against some *Streptococcus* strains and may help to prevent dental caries by inhibiting formation of insoluble glycans. EGCG has also been reported to inhibit rotaviruses and enterovirus [1]. Green tea catechins can relieve inflammation by inhibiting COX1 and COX2 enzymes and lowering prostaglandin production [2–4]. Meanwhile, the most important property of catechins is their antioxidative ability to scavenge free radicals from damaging biomolecules, chelate catalytic metal ions from free radical formation and quench singlet oxygen from activating organic molecules to form peroxides

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doi:10.1016/j.jchromb.2004.07.031

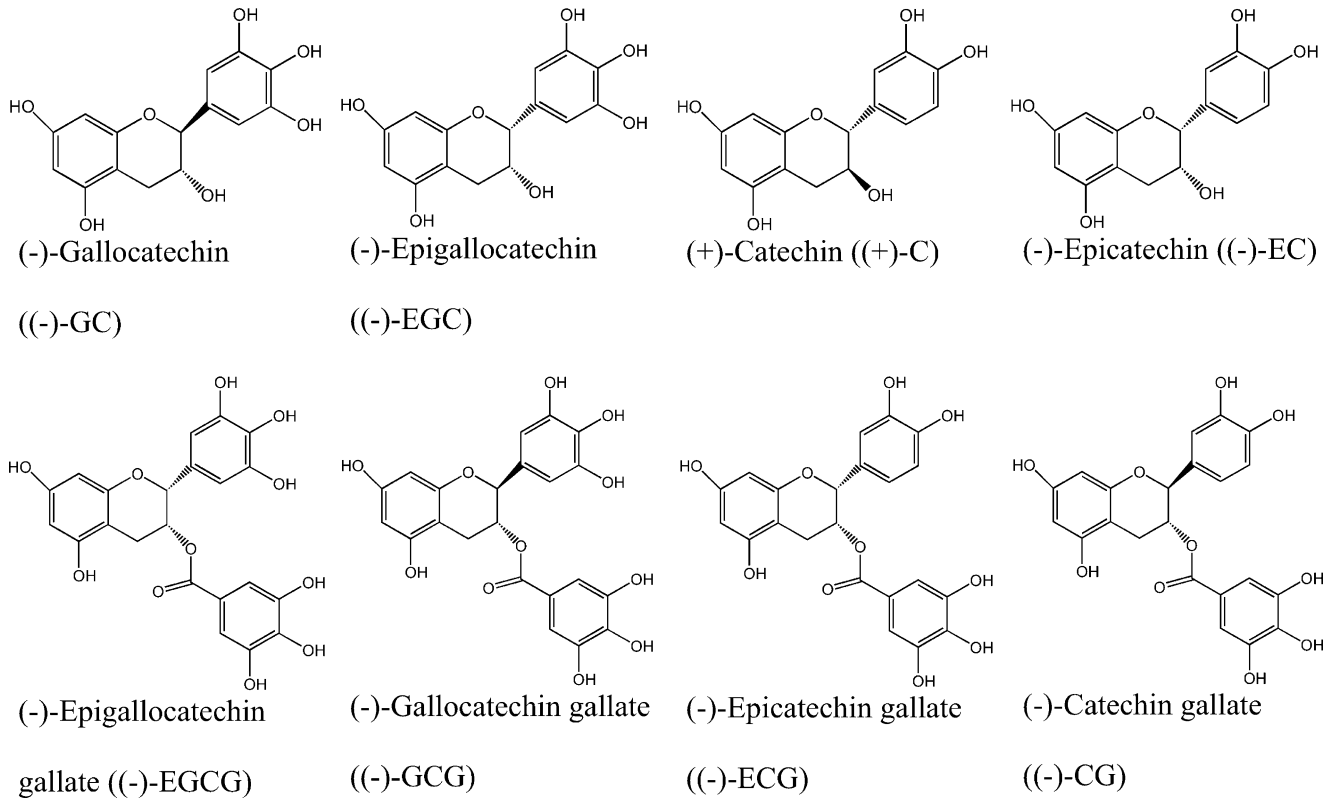

Fig. 1. Structures of the eight catechins.

and free radicals [5–7]. Such properties prevent DNA damages by reactive oxygen species. Catechins are therefore both anti-mutagenic and anticarcinogenic [1]. Catechins have also been shown to protect neurones and hepatic cells against damages from free radicals generated during ischemia-reperfusion [8–11], enhance resistance of red blood cells to oxidative stress [12], retard progression of lens cataracts [2,13–14], inhibit ultra-violet radiation-induced oxidative stress on skin [15], and reduce cholesterol level by protecting low density lipoproteins from oxidation [2] (Fig. 1).

Many methods have been reported to measure catechins in tissues. They involve essentially the same technical approaches, using ascorbic acid during the homogenization process and digestive enzymes to release catechins from conjugation. The homogenate is precipitated by water-soluble solvents, such as acetonitrile or ethanol, and extracted by water immiscible solvents, like ethyl acetate, with or without methylene chloride clean-up. After evaporation of the solvents, the residues are subsequently dissolved and analyzed by HPLC with chemiluminescence or coulometric array detection [16–19].

Pharmacokinetic studies utilizing these methods to assess the distribution of catechins in various tissues have found surprisingly low levels in the liver and spleen when compared with other organs [17–19]. Following absorption, catechins enter the liver where metabolism occurs. The liver and spleen are organs for blood storage, and the spleen is also where white blood cells reside. During the analysis procedure, even though the blood in the tissue specimens has been removed by perfusion and washing prior to homogenization, the levels of catechins obtained should not be too low compared to other organ tissues [18,19]. There could be losses during the homogenization and extraction processes. The liver contains high levels of metabolic enzymes, such as microsomal oxidase, metal ions and free radical metabolites. They are released from the cellular compartments during homogenesis and may lead to oxidization of catechins. Although ascorbic acid itself is an antioxidant, it also acts as a strong pro-oxidant in the presence of oxygen and catalytic ions such as Fe^{2+} and Cu^{+} [20]. It can cause catechin degradation. Similarly, the spleen contains numerous macrophage and neutrophil cells that contain high levels of reactive metabolites and oxidative enzymes. A similar situation may therefore occur that lead to degradation of catechins when the spleen tissue is homogenized.

In this study, we attempted to find an alternative antioxidant and to develop an analytical methodology to improve the performance of catechin determination in tissues. We wish to achieve good recovery, high sensitivity, and low detection limit to enable reliable catechin analysis in fetal and ocular tissues which have very small sample size that we intend to study. By modifying our reported catechin analysis in biological fluids [21], we also attempted to simultaneously determine eight catechins in tissue specimens of liver, brain, spleen and kidney.

2. Experimental

2.1. Materials

C, EC, GC, EGC, CG, ECG, GCG, EGCG, β-D-glucuronidase (G-0251), sulfatase (S-9754), ascorbic acid, uric acid and reduced form glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium dithionite was obtained from RDH (Wunstorfer Street, Seelze). Waters Oasis HLB 30 mg columns were from Waters (Milford, MA). Other reagents and HPLC grade solvents were obtained in their highest grade available from BDH (Poole, England). A standard stock mixture of C, EC, GC, EGC, CG, ECG, GCG, EGCG at 100 μg/ml was prepared in 0.2% ascorbic acid-EDTA (0.2% ascorbic/0.1% EDTA in 0.4 M phosphate buffer, pH 3.6) solution and stored in small aliquots at −80 °C until use. The stock solutions remained stable for at least 6 months.

2.2. Samples

Samples were analysed by the Waters 600S HPLC system, consisting of a 626 pump, 717 autosampler, AF In-line Degasser, linked with an eight channel ESA CoulArray Detector (Model 5600A) and an ESA column heater. A Heto Maxi-Dry Lyo Vacuum evaporator was used. The operation system controlling the pump and autosampler was a Millennium Version 3.2. CoulArray for Windows Version 1.04 was used for data acquisition. The analytical column was a Waters Spherisorb ODS-2 (150 mm × 4.6 mm, 3 μm), linked with a Waters spherisorb guard column ODS-2 (7.5 mm × 4.6 mm, 3 μm) from Alltech.

2.3. Experimental animals

Female Sprague-Dawley rats, weight at 200–220 g and at 9 weeks of age from the Animal Center of the Chinese University of Hong Kong, were housed at 25 °C with a 12 h light and dark cycle with free access to commercial chow and water for a week. The rats were starved for 24 h and weighed. Three groups of 6 rats were force fed by stomach tube with green tea extract powder suspension (Green tea extract tablets DCF-1 from Taiyo Kagaku Co. Ltd. (Yokkaichi, Japan)) at a dose of 166.25 mg (equivalent to 53.7 mg EGCG). After 0, 0.5, 1, 2, 3, 5 h of administration, the rats were anesthetized with 0.5 ml katamine 10% and xylazine 2% (9:1) mixture i.m. Liver, brain, spleen and kidney were perfused in situ, excised, and washed with ice cold 0.15 M saline.

2.4. Tissue sample preparation

Tissue specimen of 100 mg was homogenized in 0.25 ml methanol/ethyl acetate (2:1) and 0.25 ml 0.3 M sodium dithionite/0.1 % (w/v) Na_2EDTA in a 1.5 ml tube in ice. The homogenized sample was centrifuged at 10,000 g at 4 °C. Organic solvents in the collected supernatant were purged off by nitrogen to about 0.2 ml. 0.25 ml 0.4 M phosphate buffer (pH 6.8) and 20 μl of a mixture of β-D-glucuronidase (2500 U) and sulfatase (1 U) were then added for digestion of conjugated catechins. After purging with nitrogen, the sample was incubated at 37 °C for 45 min. The reaction was stopped by slap cold in ice.

2.4.1. Extraction of catechins

Samples of blood, urine or tissue homogenate were diluted with 1 ml 0.05 M phosphate buffer (pH 7.0) and applied to a 30 mg Waters HLB column conditioned by methanol water. After washing with 2 ml water, 1.0 ml 0.05 M phosphate buffer (pH 3.0), 1.0 ml 0.05 M phosphate buffer (pH 7.0) and 1 ml 5% methanol, the column was vacuum dried. Ethyl acetate, 10 ml, was added to remove lipophilic matrix. After vacuum drying, 10 ml methanol/ethyl acetate (2:1) at 35 °C was used to elute catechins into a tube containing 20 μl 2% ascorbic acid-EDTA. The eluate was vacuum evaporated and blown dry by nitrogen. The residues were dissolved in a mixture containing 10% acetonitrile and 0.06 % (v/v) trifluoroacetic acid (TFA) in 0.05 M phosphate pH 3.0, and filtered through a 0.2 μm pore size PTFE membrane filter. 20 μl was injected into HPLC for analysis.

2.4.2. Conventional procedure for sample preparation

Many studies utilized the same procedure with slight modifications [16–19]. We used a typical conventional method, described by Lee et al. [16], to compare with the new method established in the present study as described above. Briefly, 0.6 g tissue sample was homogenized with 3.0 ml 20% ascorbic acid-EDTA. Five samples of 0.45 ml homogenates were spiked with standard catechin solution at 10, 40 or 160 ng. One 0.45 ml homogenate sample was spiked only with 10 μl 0.2% ascorbic acid-EDTA as blank. The samples were buffered by 0.45 ml 0.4 M phosphate (pH 7.4). A 20 μl mixture of β-D-glucuronidase (2500 U) and sulfatase (1 U) was added to each homogenate for digestion at 37 °C for 45 min. The reaction was stopped by precipitation with 200 μl ethanol. After centrifugation, the supernatant was mixed with 500 μl methylene chloride. The aqueous layer was transferred to 400 μl water. The aqueous layers were combined and extracted by 1 ml and 750 μl ethyl acetate successively. The combined ethyl acetate layers were blown dry by nitrogen, and dissolved in a mixture containing 10% acetonitrile, 0.06 % (v/v) TFA in 0.05 M phosphate buffer pH 3.0. The solution was filtered through a 0.2 μm pore size PTFE membrane filter. 20 μl solution was injected into HPLC.

2.4.3. Selection of antioxidants for catechins extraction and tissue homogenization in the conventional method

Five solvent compositions were tested to find out the optimum conditions for catechin extraction from tissue: (a) 20% ascorbic acid-EDTA, (b) methanol/ethylacetate/20% ascorbic acid-EDTA (2:1:3) mixture, (c) methanol/ethylacetate/0.1 M sodium dithionite-EDTA (2:1:3), (d)

methanol/ethylacetate/0.3 M GSH mixture (2:1:3), and (e) methanol/ethylacetate/saturated uric acid (2:1:3).

Specimens of liver tissue were homogenized in each of the solvents following the procedures by Lee et al. [11]. In each of the five 0.45 ml blank tissue homogenate samples, 40 ng catechin mixtures were spiked, and 10 μl of 0.2% ascorbic acid-EDTA was added to one homogenate as blank.

2.5. Standard preparation

Standards solutions were prepared by dissolving the 8 standard catechins at a final concentration of 4 μg/ml in 0.2% ascorbic acid-EDTA solution (0.4 M NaH_2PO_4 buffer containing 0.2 % (w/v) ascorbic acid and 0.1% Na_2EDTA, pH 3.6). Standard calibration solutions were prepared at concentrations of 20, 80, 200, 500, 1000, 2000 and 4000 ng/ml.

2.6. Chromatographic conditions

Binary gradient elution was used. Mobile phase A contained acetonitrile, water, and TFA 80:920:0.6 (v/v). Mobile phase B contained methanol, acetonitrile, water, and TFA 30:270:700:0.6 (v/v). Both solutions were adjusted to pH 2.5. The gradient program: Between the 0th and 10th min mobile phase A was linearly decreased from 100 to 55.9% and mobile phase B increased from 0 to 44.1% at a flow rate of 0.8 ml/min. Between the 10th and 20th min, 55.9% mobile phase A and 44.1% mobile phase B were maintained. Mobile phase A was then linearly decreased to 20% and mobile phase B linearly increased to 80% between the 20th and 21st min, with the flow rate linearly increased to 1 ml/min. Between the 21st and 25th min, 20% mobile phase A, 80% mobile phase B, and flow rate 1 ml/min were maintained. From the 25th and 26th min, mobile phase A was increased to 100%, mobile phase B decreased to 0%, and the flow rate decreased to 0.8 ml/min linearly. After the 26th min, 100% mobile phase A was maintained at 0.8 ml/min for next injection (Table 1). A multi-electrode electrochemical detector with 8 channels, ESA CoulArray detector (Model 5600A), was used in this study. The channel potentials were set to −90, −10, 70, 150, 230, 310, 390, and 470 mV. The oven temperature was maintained at 33 °C.

Table 1
Chromatographic conditions of the analysis

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (ml/min)
0	100	0	0.8
10	55.9	44.1	0.8
20	55.9	44.1	0.8
21	20	80	1.0
25	20	80	1.0
26	100	0	0.8

Mobile phase A contained acetonitrile, water, and TFA 80:920:0.6 (v/v). Mobile phase B contained methanol, acetonitrile, water, and TFA 30:270:700:0.6 (v/v). Both solutions were adjusted to pH 2.5. Linear gradient was used in the chromatography. Temperature: 33 °C; channel voltages: −90, −10, 70, 150, 230, 310, 390, 470 mV.

2.7. Validation of protocols

2.7.1. Linearity

Standard mixture solutions containing the eight catechins were spiked into 0.1 g blank tissue samples to final concentrations of 20, 80, 200, 500, 1000, 2000, 4000 ng/g for homogenization and processing as described. The linearity, r^2, was calculated by plotting the peak area of each concentration against the respective spiked concentration. The selected concentrations covered physiological ranges of catechins in tissue [17–19].

2.7.2. Reproducibility

Catechin standards of 10, 40, and 160 ng, were spiked into 5 samples of 0.1 g blank liver tissue for homogenization, extraction and analysis as described. The intra-batch coefficient of variance (CV) was obtained from the standard deviation of measured concentrations in spiked samples (minus the blank) from a batch divided by the mean measured concentration (minus the blank). The inter-batch CV was obtained from the standard deviation of the mean measured concentration (minus the blank) of four batches divided by the overall mean measured concentration.

2.7.3. Absolute recovery

Catechin standards of 10, 40, and 160 ng, were spiked into 5 samples of 0.1 g blank liver tissue for homogenization, extraction and analysis as described. The recoveries were obtained by dividing the mean of measured concentrations (minus the blank) by the actual spiked concentration.

2.7.4. Accuracy

Standard calibration curves were constructed by analysis of catechin standards at final concentrations of 20, 80, 200, 500, 1000, 2000 and 4000 ng/g for liver tissue. Catechins were spiked into five 0.1 g blank liver tissue samples at 10, 40, 160 ng. Accuracy was obtained from the measured concentration calculated from the calibration curve divided by the spiked concentration and multiplied by 100%.

2.7.5. Detection limit

Catechin standards spiked into tissue samples to concentrations of 1, 5, 10, 15, and 20 ng/g were analyzed to obtain a plot of signal:noise ratio against concentrations. The catechins concentration that gave a ratio of 3:1 was the detection limit.

3. Results

We utilized both our newly developed method and the conventional method for analysis of catechins in tissues. Major differences in the two methods were in the extraction and homogenization procedures (Table 2). Using our new method, we obtained clean chromatogram for determination of all eight catechins in rat tissues of liver, spleen, brain, and

Table 2
Comparison of the new and conventional methods

	New method	Conventional method
In methodology		
Homogenization	Tissue homogenization in 0.3 M sodium dithionite/MeOH/ethyl acetate (3:2:1)	Tissue homogenization in 20% ascorbic acid-EDTA
Digestion	After centrifugation, the supernatant were buffered and treated by β-glucuronidase and sulfatase	After centrifugation, the supernatant were neutralized and treated by β-glucuronidase and sulfatase
Extraction	The digested mixture were cooled and extracted by selective SPE column, washed by buffers, 5% MeOH, ethyl acetate and eluted by MeOH/ethyl acetate (2:1)	The enzyme reaction was stopped by ethanol precipitation. After centrifugation the supernatant was washed by dichloromethane and back washed by water. The aqueous phase was extracted twice by ethyl acetate
Chromatography	Eight catechins were analyzed within 25 min	Four catechins were analyzed in more than 1 h
In performance		
Homogenization	Sodium dithionite/MeOH/ethyl acetate in the homogenization mixture precipitated out oxidative enzymes and did not promote free radical generation during the extraction process	Homogenization in ascorbic acid-EDTA released oxidative enzymes which degraded catechins. Ascorbic acid further enhanced catechin degradation by generating free radicals in the presence of O_2 and free Fe^{2+}
	Digestion process under nitrogen atmosphere prevented oxygen induced generation of free radicals	No nitrogen purge had been mentioned. Free radicals degraded catechins
	The homogenates were selectively extracted and washed in the solid phase extraction procedure. Better purified catechin mixture was obtained for chromatography	The homogenates were non-selectively washed and extracted by organic solvents. There were interferences to catechin analysis. The many liquid/liquid extraction steps promoted oxygen induced generation of free radicals
Chromatography	Tidy chromatograms were obtained with eight catechins simultaneously determined within 25 min	Only four catechins were determined in more than 1 h

kidney (Fig. 2). In fact, unlike the conventional method, our new method produced a very clean background for the blank samples. Low levels of residues were still found in the blank sample after overnight fast. Usually CG, ECG, GCG were not found, while EC and EGCG could be detected at 0.2–3 ng/g.

Improvements in analytical performance over the conventional method were obtained in analytical recoveries and reproducibility (Table 3). In general, higher recovery and better reproducibility was found in the present method. The performance of the new and conventional methods were further compared by assaying rat liver from animals sacrificed at 0, 0.5, 1, 2, 3, 5 h after being given green tea extract (Table 4). Higher levels were obtained with the new method with the exception of GCG. But GCG determination by the convention method was not reliable due to interference with non-specific peaks in the chromatogram.

The new method has been validated: the inter-batch and intra-batch coefficients of variations ($n = 5$) were within 5% (Table 5). The linearity from 20–4000 ng/g was higher than 0.9922. The absolute recovery ($n = 5$) was from 62–96% (Table 6) and the accuracy ($n = 5$) from 92.5 ± 4.5 to 104.9 ± 6% (Table 6). The detection limit was 5 ng/g.

4. Discussion

We have developed a new method for determination of catechins in tissues with performance superior to the typical conventional method (Table 2). Conventionally most methods for catechin determination used ascorbic acid-EDTA as preservative with no protein precipitation during homogenization. This resulted in a high level of soluble proteins remaining in the supernatant and could cause column blockage if solid phase extraction is subsequently used, as in our new method. Using mixture of organic solvents should facilitate protein precipitation. But it caused variation in catechin recoveries. Among the reducing reagents that we have been tried, sodium dithionite and GSH gave promising results. However, near saturation of GSH was used and there was no room for further optimization. Also, GSH could compete with catechins for the absorption sites in the column. Therefore, sodium dithionite instead of GSH was used. High dithionite concentration could also compete with catechins for column absorption sites. But at the optimal concentration of 0.3 M sodium dithionite, high recovery rates and clean chromatogram were obtained (Fig. 2).

The liver is the major site of metabolism in the body. It contains many enzymes that generate highly reactive free radical intermediates and reactive oxygen species. The cells also contain many protein binding ions such as Fe^{2+} in haemoprotein and Cu^{+} in cytochrome oxidase for catalytic purposes. In vivo, these enzymes are compartmentalized in microsomes and the endoplasmic reticulum. But once the liver tissue has been homogenized, they are released and exhibit their enzymic actions against sample molecules such as catechins. The performance for in vivo assay of the new and the conventional methods were compared by assaying liver catechins of rats sacrificed at 0, 0.5, 1, 2, 3, 5 h after being given green tea extract. As seen in Table 3, the amount of catechin assayed by the new solid phase extraction method gave higher level if the concentration was simply calculated from the absolute recovery as many studies [18,19]. The absolute recovery of GCG appeared to be higher using the conventional liquid/liquid extraction method. This, however, was an

Table 3
Comparison of the analytical performance of the newly established method in this study and the old conventional method for catechins in different tissues spiked at 40 ng into 0.1 g tissue homogenates

	Liver		Spleen		Brain		Kidney	
	New method	Old method	New method	Old method	New method	Old method	New method	Old method
(A) Recovery (%)								
GC	82.6**	51.9	82.7**	47.7	78.7**	26.4	85.3**	50.6
EGC	76.7**	60.0	80.1**	27.1	76.0**	29.4	78.9**	26.4
C	95.7**	65.9	87.2**	35.2	94.4*	44.1	78.0**	40.5
EC	87.2	86.7	78.3**	27.2	81.1**	31.7	83.6**	27.6
EGCG	62.2**	38.3	67.8**	49.6	82.5*	53.7	78.3**	50.8
GCG	63.9*	58.3	81.3**	56.7	91.0**	53.2	84.3**	56.0
ECG	69.3	76.6	76.9**	27.5	86.0**	33.9	80.1**	30.3
CG	74.9	74.1	87.7**	48.6	90.2**	51.9	88.4**	47.9
(B) CV (%)								
GC	2.2	6.6	1.3	6.9	3.9	81	0.8	14
EGC	4.3	6.7	1.5	3.2	1.9	40	1.1	16
C	1.7	9.7	0.4	3.9	3.1	55	4	22
EC	1.8	7.4	1	3.2	4	42	3.5	20
EGCG	4	13	0.7	6.3	2.8	29	3.8	12
GCG	2.6	7.9	0.1	2.4	2.2	17	5.8	5.7
ECG	4.2	9.2	1.5	4.7	4.4	37	3	14
CG	4.2	3.4	0.1	1.1	2.6	6.4	4.3	3.6

(A) Absolute recovery (%); (B) reproducibility, CV, $n = 5$(%).
* Significant difference ($P = 0.05$) analyzed by *t*-test assuming unequal variances.
** Significant difference ($P = 0.01$) analyzed by *t*-test assuming unequal variances.

Table 4
Comparison of the pharmacokinetic parameters of catechins obtained by the new method and the convention method

	New method			Convention as method		
	Tmax (h)	Cmax (ng/g)	AUC (ng h/g)	Tmax (h)	Cmax (ng/g)	AUC (ng h/g)
GC	1	92.3	193.3	0.5	56.0	156.6
EGC	0.5	699.5	841.5	0.5	188.3	308.3
C	0.5	78.2	128.9	0.5	13.7	16.2
EC	0.5	878.4	1767.8	0.5	315.8	623.8
EGCG	1	1022.2	1273.6	1	213.7	302.0
GCG	0.5	33.4	23.2	1	44.3	86.7
ECG	1	634.1	779.1	1	132.9	134.5
CG	1	23.2	41.8	0.5	9.8	21.9

GC, EGC, C, EC, EGCG, GCG, ECG and CG in rat livers were determined after a single oral administration of 166.25 mg green tea extract. The area under the curve (AUC) indicates the amount of catechins absorbed by the tissue.

Table 5
Reproducibility of catechins analyzed by the present method with liver tissue blank spiked at (A) 10 ng, (B) 40 ng, (C) 160 ng

(A)

	Intra-variation of 10 ng spiked				Inter-variation
	CV of batch 1 ($n = 5$)	CV of batch 2 ($n = 5$)	CV of batch 3 ($n = 5$)	CV of batch 4 ($n = 5$)	CV of the four batches ($n = 4$)
GC	6.3	6.0	5.3	6.2	5.4
EGC	3.7	4.0	3.9	4.3	3.3
C	4.1	4.6	3.7	3.8	3.7
EC	6.3	6.1	6.6	5.9	5.8
EGCG	8.6	7.4	7.0	7.3	7.1
GCG	7.4	8.0	7.2	7.3	6.7
ECG	6.0	5.1	6.3	5.3	5.1
CG	5.8	5.2	5.4	5.2	5.1

(B)

	Intra-variation of 40 ng spiked				Inter-variation
GC	3.6	1.4	3.1	0.8	1.8
EGC	4.4	0.5	5.5	1.1	2.4
C	2.4	0.8	4.9	4.0	2.7
EC	2.3	1.5	2.1	3.5	2.1
EGCG	3.1	3.0	4.7	3.8	3.1
GCG	3.5	1.8	3.9	5.8	3.2
ECG	2.0	1.9	5.4	3.0	2.7
CG	4.2	1.3	3.2	4.3	2.9

(C)

	Intra-variation of 160 ng spiked				Inter-variation
GC	0.4	0.6	0.3	0.5	0.3
EGC	1.2	0.8	0.7	1.1	0.7
C	0.3	0.5	0.2	0.4	0.3
EC	0.3	0.5	0.6	0.6	0.4
EGCG	1.2	1.5	1.1	0.9	1.0
GCG	0.9	0.7	0.5	0.5	0.5
ECG	0.9	0.6	0.7	0.7	0.6
CG	0.7	0.4	0.5	0.7	0.4

Five samples were processed in each batch. Inter-batch coefficient of variation (CV) was calculated by the mean of the four batches.

Table 6
Recoveries and accuracy of catechins processed by the present method with liver tissue blanks spiked at different concentrations 10, 40, and 160 ng

(A)

	Recovery ± S.D. (%)		
	Spike at 10 ng	Spike at 40 ng	Spike at 160 ng
GC	83.9 ± 4.8	82.6 ± 2.0	93.1 ± 2.9
EGC	81.6 ± 1.8	76.7 ± 3.6	85.0 ± 5.0
C	73.8 ± 2.7	95.7 ± 1.8	90.5 ± 2.1
EC	71.9 ± 4.1	87.2 ± 1.8	96.5 ± 2.2
EGCG	70.1 ± 5.4	62.2 ± 2.7	72.5 ± 5.9
GCG	62.9 ± 303	63.9 ± 1.8	75.2 ± 4.6
ECG	64.2 ± 3.5	69.3 ± 3.2	74.4 ± 4.9
CG	70.7 ± 2.9	74.9 ± 3.4	81.3 ± 4.1

(B)

	Accuracy		
GC	92.6 ± 4.9	96.8 ± 2.9	96.9 ± 2.8
EGC	95.4 ± 3.0	96.3 ± 5.7	97.4 ± 5.5
C	97.6 ± 4.9	96.3 ± 5.1	99.0 ± 2.3
EC	95.5 ± 2.6	103.4 ± 1.8	101.1 ± 2.4
EGCG	94.1 ± 4.7	92.3 ± 3.0	92.5 ± 5.7
GCG	93.5 ± 5.7	98.2 ± 3.9	94.1 ± 4.5
ECG	97.0 ± 3.3	104.9 ± 6.0	94.9 ± 5.0
CG	98.8 ± 3.4	97.3 ± 6.8	96.0 ± 4.2

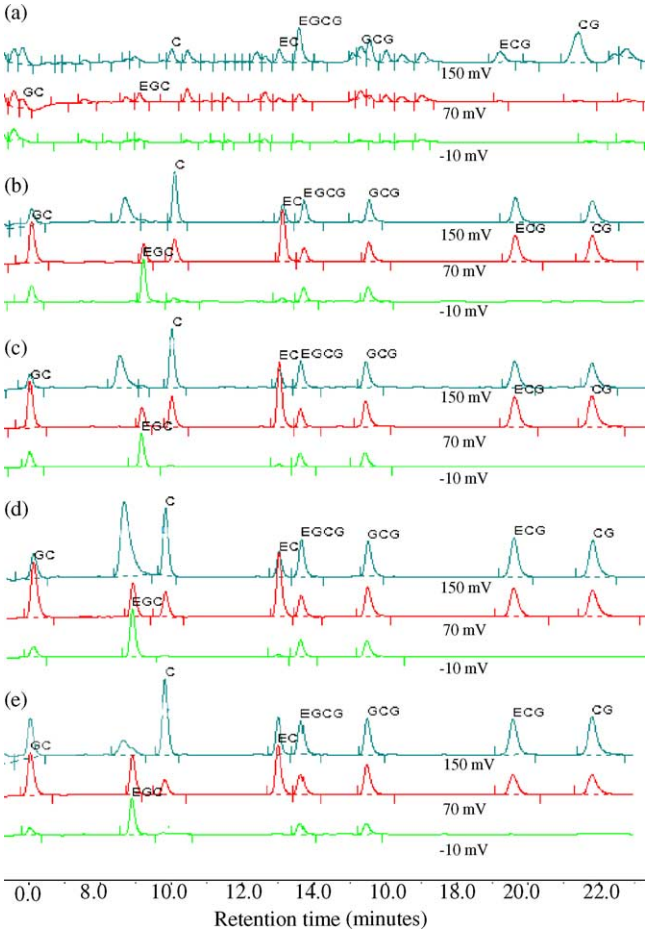

Fig. 2. Typical chromatograms of catechins spiked at 40 ng in (a) liver homogenate with ascorbic acid-EDTA and processed by conventional method; and in (b) liver, (c) spleen, (d) brain and (e) kidney homogenates with 0.3 M sodium dithionite/methanol/ethylacetate and processed by new method. The chromatograms are tidier and better resolved with the new method.

artifact due to overlap of the GCG peak by matrix peaks. Interference was also found in other peaks processed by the conventional method, GC in particular.

Ascorbic acid is a powerful anti-oxidant that can protect many biomolecules in blood and tissues against oxidation. It can also regenerate other antioxidants such as tocopherol and GSH from their oxidized state [22], protecting the lipophilic parts of tissue where ascorbic acid itself cannot gain access. However, ascorbic acid is also a strong pro-oxidant when it encounters oxygen and catalytic ions such as Fe^{2+} and Cu^{+} [20] though the Fenton and iron catalyzed Haber–Weiss reactions.

$$AH^{-} + Fe^{2+} \rightleftarrows [AH^{-}Fe^{2+}]$$

$$[AH^{-}Fe^{2+}] + O_2 \rightleftarrows [AH^{-}Fe^{2+}O_2]$$

$$[AH^{-}Fe^{2+}O_2] \rightleftarrows [AH^{-}Fe^{3+}O_2^{\bullet -}] \rightleftarrows A^{\bullet -} + Fe^{2+} + HO_2^{\bullet}$$

$$A^{\bullet -} + O_2 \rightarrow A + O_2^{\bullet -}$$

Many pharmacokinetics studies on catechins have been carried out to correlate their levels to various biological effects using the conventional methods of analysis [16–19]. Tissue samples were homogenized in the presence of ascorbic acid. After enzymatic digestion to release free catechins, the homogenate is washed with methylene chloride and extracted by ethyl acetate prior to evaporation and re-constitution for HPLC analysis. During homogenization, oxidases, free radical metabolites and catalytic metal ions are released from cellular compartments into ascorbic acid-EDTA. In the homogenate, vigorous oxidation reactions may occur, not only because of enzymatic oxidative activities, but also from the combined pro-oxidant effects of ascorbic acid, oxygen and metal ions [23]. These would accelerate the decomposition of catechins in the tissue homogenate leading to low recoveries, especially in liver where there are plenty metabolic enzymes. We develop a new method to overcome these problems with performance superior to the conventional method (Table 2). Liver contains high concentrations of metabolic enzymes and reactive metabolites, but other tissues also possess such activities and affect catechin determination in the homogenates. If the enzymatic activities can be inhibited and the reactive oxygen metabolites kept to low concentrations, better recovery of catechins would be obtained. We tested alternative antioxidants to replace ascorbic acid as a protective agent during homogenization. Sodium dithionite, uric acid and reduced glutathione were used because they are not known to possess pro-oxidant activity in the presence of oxygen and metal ions. Moreover, sodium dithionite is an inorganic antioxidant and is highly water-soluble. GSH is another water soluble antioxidant in vivo. Uric acid, besides being an antioxidant, is also a metal chelator. But its low solubility limited its use. Methanol/ethyl acetate (2:1) had been used to precipitate oxidized enzymes and extract catechins that have a strong affinity to protein [24].

By comparing different antioxidant mixtures in a commonly used liquid/liquid extraction method for catechins, we found that ascorbic acid-EDTA was associated with lower recovery than dithionate and produced a highly matrix interfered chromatogram and larger variation (data not shown). The interference was worse after precipitation by methanol/ethyl acetate (2:1). There might have been generation of free radicals that degraded catechins during sample processing. The noisy chromatogram suggested many intermediate compounds with similar physico-chemical properties were present. The new method, however, has fewer manipulation steps. It allows a shorter oxygen contacting time for generation of reactive intermediates and minimal pro-oxidative effect. Catechins recoveries were higher. It is noted that even using solid phase extraction and nitrogen purge, larger variation was still found when ascorbic acid was used.

5. Conclusion

In summary, ascorbic acid-EDTA was found unsuitable for assaying catechins in tissue because of its pro-oxidant effects. But it is still useful for studying body fluids such as plasma

and urine. A mixture of 0.3 M sodium dithionite/MeOH/ethyl acetate offers better protection during tissue homogenization. A new solid phase extraction procedure has been validated with adequate performance. Good recovery rates and little interference were obtained for determination of catechins in liver, brain, spleen and kidney. Pharmacokinetic study of rat liver tissue following feeding with green tea extract showed significantly higher amounts of catechins when assayed by the new method against the commonly used conventional liquid/liquid extraction method. This newly established method therefore is capable for reliable catechin analysis in fetal and ocular tissues which have very small sample size.

Acknowledgements

The study was supported by Hong Kong Special Administration Region Research Grant Council for the grant entitled “Evaluation of Green Tea Polyphenols in Pregnancy”, CUHK4077/01M. We thank Taiyo Kagaku Co. Ltd. for their generous donation of green tea extract tablets, Sunphenon DCF-1.

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