

**UNIVERSITY OF LEEDS**

This is a repository copy of *High performance liquid chromatography tandem mass spectrometry dual extraction method for identification of green tea catechin metabolites excreted in human urine*.

White Rose Research Online URL for this paper:
<http://eprints.whiterose.ac.uk/81459/>

Article:

Clarke, KA, Dew, TP, Watson, REB et al. (5 more authors) (2014) High performance liquid chromatography tandem mass spectrometry dual extraction method for identification of green tea catechin metabolites excreted in human urine. *Journal of Chromatography B*, 972. 29 - 37. ISSN 1570-0232

<https://doi.org/10.1016/j.jchromb.2014.09.035>

Reuse

Unless indicated otherwise, fulltext items are protected by copyright with all rights reserved. The copyright exception in section 29 of the Copyright, Designs and Patents Act 1988 allows the making of a single copy solely for the purpose of non-commercial research or private study within the limits of fair dealing. The publisher or other rights-holder may allow further reproduction and re-use of this version - refer to the White Rose Research Online record for this item. Where records identify the publisher as the copyright holder, users can verify any specific terms of use on the publisher's website.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.



eprints@whiterose.ac.uk
<https://eprints.whiterose.ac.uk/>

1 **High performance liquid chromatography tandem mass spectrometry** dual extraction method for
2 identification of green tea catechin metabolites excreted in human urine

3
4 Kayleigh A. Clarke^a, Tristan P. Dew^{a,b}, Rachel E. B. Watson^c, Mark D. Farrar^c, Susan Bennett^c, Anna
5 Nicolaou^{b,d}, Lesley E Rhodes^c, Gary Williamson^{a*}

6 *Corresponding author (g.williamson@leeds.ac.uk, 0113 3438380)

7
8 ^aSchool of Food Science and Nutrition, University of Leeds, Leeds LS2 9JT, UK

9 ^bSchool of Pharmacy, School of Life Sciences, University of Bradford, Bradford BD7 1DP, UK

10 (present address for Tristan Dew)

11 ^cPhotobiology Unit, Dermatology Centre, Institute of Inflammation and Repair, University of
12 Manchester, Manchester Academic Health Science Centre, Salford Royal NHS Foundation Hospital,
13 Manchester M6 8HD, UK

14 ^dManchester Pharmacy School, Faculty of Medical and Human Sciences, The University of
15 Manchester, Manchester, M13 9PT, UK (present address for Anna Nicolaou)

16
17 Key words: green tea catechins, metabolites, isolation, urine, liquid-chromatography mass
18 spectrometry, human intervention study, free-form, conjugates, extraction

19
20 Acknowledgements: This study was supported by a grant from the Biotechnology and Biological
21 Sciences Research Council, Diet and Health Research Industry Club (grant no. BB/G005575/1).

22
23 The authors declare that they have no conflict of interest.

24
25 **For Journal of Chromatography B**

1 **Abstract**

2 The simultaneous analysis of free-form and conjugate flavonoids in the same sample is difficult but
3 necessary to properly estimate their bioavailability. A method was developed to optimise the
4 extraction of both free and conjugated forms of catechins and metabolites in a biological sample
5 following the consumption of green tea. A double-blind randomised controlled trial was performed in
6 which 26 volunteers consumed daily green tea and vitamin C supplements and 24 consumed a placebo
7 for 3 months and urine was collected for 24 hours at 4 separate time points (pre- and post-
8 consumption) to confirm compliance to the supplementation and to distinguish between placebo and
9 supplementation consumption. The urine was assessed for both free and conjugated metabolites of
10 green tea using LC-MS² analysis, after a combination extraction method, which involved an ethyl
11 acetate extraction followed by an acetonitrile protein precipitation. The combination method resulted
12 in a good recovery of EC-O-sulphate (91±7 %), EGC-O-glucuronide (94±6 %), EC (95±6 %), EGC
13 (111±5 %) and ethyl gallate (74±3 %). A potential total of 55 catechin metabolites were investigated,
14 and of these, 26 conjugated (with methyl, glucuronide or sulphate groups) and 3 free-form
15 (unconjugated) compounds were identified in urine following green tea consumption. The majority of
16 EC and EGC conjugates significantly increased post-consumption of green tea in comparison to
17 baseline (pre-supplementation) samples. The conjugated metabolites associated with the highest peak
18 areas were O-methyl-EC-O-sulphate and the valerolactones M6/M6'-O-sulphate. In line with previous
19 studies, EC and EGC were only identified as conjugated derivatives, and EGCG and ECG were not
20 found as mono-conjugated or free-forms. In summary, the method reported here provides a good
21 recovery of catechin compounds and is appropriate for use in the assessment of flavonoid
22 bioavailability, particularly for biological tissues that may contain endogenous deconjugating
23 enzymes.

1. Introduction

Green tea is the second most popular tea beverage, with the majority of consumption in Asian countries and North Africa, and with a recent increase in popularity in Western countries [1]. Green tea consists of catechins (or flavan-3-ols), which are a sub-group of the flavonoids. In comparison to other teas, green tea has the highest flavanol content (35-50 %) [2], including catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) (Fig. 1).

Daily consumption of green tea has been highly associated with beneficial health effects, studied both in vivo and in vitro [3-7]. Epidemiological studies have linked green tea consumption with reducing the incidence of cancer onset [8], factors associated with cardiovascular disease [9-11] and factors related to the onset of diabetes [1,12]. Research has become more focused on the presence of conjugated metabolites in biological fluids following green tea interventions [13-15]. However, analysis of flavonoids in biological samples presents several challenges. The free and aglycone forms of flavonoids are most soluble in lipophilic solutions, but are unstable during analysis [16-18]. Above pH 7 and 37 °C, the stability of free-form catechins is reduced due to degradation and epimerisation, and therefore, most free-form analysis utilises a low pH and low temperature environment with the addition of an antioxidant, such as ascorbic acid, to improve the stability [19-21]. As most of the conjugated catechins are commercially unavailable, enzyme deconjugation of green tea catechin metabolites present within biological samples [22-25] allows for the assessment of the metabolites relative to the free-form standards. However, the employment of this method has been questioned due to inefficient and incomplete hydrolysis [26], and as a result the more stable conjugated catechin forms have been assessed by monitoring the fragmentation patterns of the compounds using liquid chromatography mass spectrometry (LC-MS²) analysis [27-31]. Due to the hydrophilic nature of the conjugated catechins, the majority of the methods that omit deconjugation utilise a protein precipitation of urine with acetonitrile prior to LC-MS² analysis [28,30]. A direct injection of the filtered urine sample following the consumption of green tea can also be employed, however, this can lead to ion suppression [32,33].

1 The extraction of free-form catechin metabolites in acetonitrile is associated with a poor
2 recovery [28], and therefore, these molecules cannot be efficiently monitored in biological samples
3 using extraction techniques that are suitable for the hydrophilic conjugated forms. In the present
4 study, a combination of using an initial ethyl acetate extraction to isolate free-form metabolites
5 followed by protein precipitation of the remaining urine sample with acetonitrile, which allows the
6 identification of green tea conjugated metabolites, was utilised for the first time. This method is
7 applicable for future human intervention studies in which both the free and conjugated forms can be
8 monitored in biological samples following consumption of green tea, particularly for samples where
9 β -glucuronidase and sulphatase are already present such as tissue extracts.

10

2. Experimental

2.1 Materials

Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), catechin (C) and taxifolin were purchased from Extrasynthèse (Genay, France). Ethyl gallate and 3-methyl gallic acid were obtained from Apin Chemicals Ltd (Oxfordshire, UK), hippuric acid, benzoic acid and 3-hydroxybenzoic acid were purchased from Fluka (Dorset, UK), ascorbic acid (AA) was from Sigma Aldrich (Dorset, UK) and syringic acid and gallic acid were purchased from Alfa Aesar (Lancashire, UK). All standards were of a HPLC purity (>90 %). A Millipore Q water purifying system (Millipore, Hertfordshire, UK) was used to provide ultrapure water ($\geq 18.2 \text{ M}\Omega \text{ cm}$ at $25 \text{ }^\circ\text{C}$) for LC-MS² analysis. EC-O-sulphate and EGC-O-glucuronide were synthesised using the method by Wong et al (2010) [34].

2.2 Human intervention

In total, 50 volunteers enrolled on the 3-month double-blind randomised controlled trial conducted in the Photobiology Unit, Dermatology Centre, Salford Royal NHS Foundation Hospital (Manchester, UK). Ethical approval was received from the North Manchester Research Ethics Committee (reference 08/H1006/79) and the study conformed to the Declaration of Helsinki principles. Inclusion criteria for the study included white Caucasian adults (aged 18-65 years) with sun-reactive skin type I-II. Volunteers were excluded from the study if they had a history of skin cancer or photosensitivity, underwent sunbed use or sunbathing in the 3 months prior to the study, consumed photoactive medication or nutritional supplements, consumed >2 cups of tea per day or were pregnant or breastfeeding. The volunteers were allocated either placebo or green tea supplements that were the equivalent of 5 cups of green tea per day (5.25 mg C, 31.25 mg EC, 123.25 mg EGC, 65 mg ECG and 181.5 mg EGCG), along with 50 mg vitamin C to improve the gastrointestinal stability of the green tea supplements. The placebo and supplement were encapsulated and coded to maintain the blind aspect of the study. Urine was collected for 24 hours at four time points during the intervention: baseline (pre-supplementation), day one (post-supplementation), week 6 and week 12. Urine was excreted into HCl-washed flasks containing AA (approximately 1 g/L) and

1 was stored at -20°C prior to analysis. Compliance to the study was tested through monitoring the
2 presence of EC-O-sulphate and EGC-O-glucuronide excreted into urine during the trial at four time
3 points, and by counting residual supplements.

4 5 **2.3 Urine analysis**

6 Urine was defrosted and 50 µL AA (6 mM in water, 1 mM final concentration in urine) and
7 50 µL ethyl gallate (internal standard; 4 µg/mL in water) were added to 200 µL urine (technical
8 duplicates from one biological sample) in a 1.5 mL microcentrifuge tube (Eppendorf UK Ltd.,
9 Stevenage, UK) on ice. Samples were vortexed for 10 sec before the addition of 500 µL ice cold ethyl
10 acetate. Samples were then vortexed for 30 sec and placed on ice for 2 min before centrifugation at
11 17,000 g (4 °C) for 2 min. The extract (supernatant) was placed in a new microcentrifuge tube on ice.
12 The process was repeated again and the extracts were pooled and dried down under nitrogen flow on
13 ice. The dried down sample was then placed at -80 °C.

14 The urine sample (still on ice) remaining after the ethyl acetate extraction was then mixed
15 with 800 µL ice cold acetonitrile and vortexed for 2 min before centrifuging at 17,000 g (4 °C) for 10
16 min. The supernatant was placed into a pre-weighed microcentrifuge tube and the organic solvent was
17 removed by centrifugal evaporation (settings: HPLC fraction, lamp off; Genevac, EZ-2 plus model;
18 Genevac, Suffolk, UK). The microcentrifuge tube was re-weighed to calculate the remaining aqueous
19 portion of the sample, using 1 g = 1 mL. Samples were placed at -80 °C until required. For LC-MS²
20 analysis, 90 µL of the aqueous sample was added to the dried down ethyl acetate fraction and 10 µL
21 taxifolin (to monitor any changes to samples during HPLC-MS² analysis); 20 µg/mL in 50 %
22 acetonitrile and 1 % AA, final concentration of 5 % and 0.1 %, respectively) was also added prior to
23 vortexing for 1 min and 5 min of sonication. The sample then underwent centrifugation for 5 min at
24 17,000 g (4 °C) and was placed into an amber HPLC vial.

25 A 5 µL sample aliquot was injected onto a Kinetex C18 column (2.6 µm, 150 x 2.1 mm;
26 Phenomenex, Cheshire, UK) connected to an Agilent 1200 SL system (column oven 35 °C; Agilent
27 Technologies UK Ltd., Berkshire, UK) and an Agilent 6410a triple quadrupole LC-MS² (negative

1 mode, multiple reaction monitoring (transitions monitored are shown in Table 1), gas temperature 350
2 °C, a 30 psi nebulizer pressure, nitrogen flowing at 11L/min and tuned using ESI tuning mix).

3 A binary gradient of 95 % LC-MS grade water (solvent A: 5 % acetonitrile, 0.1% formic
4 acid) vs. 95 % acetonitrile (solvent B: 5 % water, 0.1% formic acid) was run at 0.3 ml/min. The
5 gradient began at 0 % solvent B and was held at 0 % for 6 min (5 column volumes), increasing to 100
6 % over 15 column volumes (by 24 min), remaining at 100 % for 3.6 min (3 column volumes) before
7 returning to 0 % over 1.2 min (1 column volumes) and finally re-equilibrating over 4.8 min (4 column
8 volumes). The total method length was 33.6 min.

9

10

11

1 Results

2 3.1 Identification of green tea catechin metabolites

3 LC-MS² was used to identify the green tea catechin metabolites present within the urine
4 samples throughout the intervention study. Examples of the fragmentation patterns monitored are
5 displayed in Fig. 2. The conjugated metabolites were measured as peak area relative to the peak area
6 of the internal standard, ethyl gallate. Identification of EC-O-sulphate and EGC-O-glucuronide in the
7 urine at significantly higher concentrations than baseline urine (approximately 9x higher for
8 EC-O-sulphate, with EGC-O-glucuronide only found in a few baseline samples of volunteers)
9 confirmed compliance with the daily supplementation and also identified volunteers consuming the
10 placebo. For the placebo volunteers, EC-O-sulphate was also identified, however, there was no
11 significant increase between pre- and post-supplement urine samples and therefore the urine samples
12 for the placebo volunteers were not assessed further for other green tea catechin metabolites. In total,
13 24 volunteers consumed the placebo and 26 consumed the green tea and vitamin C supplements daily
14 for 3 months, with 21 volunteers compliant with the green tea supplementation. Following the
15 confirmation of compliance, the urine of the 21 volunteers was analysed for the total green tea
16 catechin metabolite content.

17 Without reference standards the identification of green tea catechin metabolites can be
18 achieved by monitoring the loss of conjugated moieties upon fragmentation. The loss of 176 atomic
19 mass units (amu) represents cleavage of a glucuronyl unit, 80 amu is the removal of a sulphate moiety
20 and 14 amu is the cleavage of a methyl group. Other changes in transitions and feasible
21 fragmentation patterns of green tea catechin metabolites are displayed in Fig. 2 and Table 1. The
22 collision energies were optimised by repeat analysis of each green tea catechin metabolite by using
23 week 12 urine sample from one of the volunteers. The chromatograms recorded, corresponding to
24 each green tea catechin metabolite within urine samples, are shown in Fig. 3. Without reference
25 standards, the chiral form of particular catechins and their corresponding metabolites cannot be
26 determined between e.g. conjugated forms of (-)-epicatechin and (+)-catechin, and M6 and M6'.

28 3.2 Quantification of green tea catechin metabolites

1 Standard curves were created by spiking known amounts of free-form catechin reference
2 standards into baseline urine samples (range, 0.2-0.0002 mg/mL; R^2 range, 0.90-0.99), and the limit of
3 detection (LOD) and limit of quantification (LOQ) were identified for each free-form compounds
4 (average LOD, 110 ± 50 ; range, 4-650 pmol; and average LOQ 170 ± 60 ; range 4-820 pmol,
5 respectively). The precision of the method was assessed by repeat injection of EC and EGC standards

6 over the standard curve range, and the relative standard deviation was 3% for EGC and 14% for EC.

7 A total amount (μmol) excreted in urine was determined for each conjugated metabolite as an
8 equivalent to its associated free-form, relative to the internal standard (ethyl gallate). This can result in
9 over-quantification of metabolites due to differences in response factors for each compound [35].

10 Recoveries (performed by analysing three concentrations in duplicate) were determined for
11 EC-O-sulphate (91 ± 7 %), EGC-O-glucuronide (94 ± 6 %), EC (95 ± 6 %), EGC (111 ± 5 %) and ethyl
12 gallate (74 ± 3 %) by spiking known amounts of the compounds into blank urine and relating the peak
13 area from the spiked urine sample to the peak area from the direct analysis of an equivalent volume by
14 LC-MS. When analysing the recoveries of the free-form catechins using acetonitrile only, the
15 recovery is clearly reduced to 3 ± 2 % for EC and 13 ± 6 % for EGC, which is consistent with another
16 study [28], and highlights the importance of using a method that is suitable for extracting the free-
17 form catechins as well as the conjugated forms. The most common method for assessing free-form
18 catechins following consumption of green tea is using enzyme deconjugation of the biological sample
19 followed by an ethyl acetate extraction. This results in a good recovery of EC by 91 ± 5 % and EGC by
20 88 ± 9 %. Therefore, using a combination of ethyl acetate and acetonitrile allows for a good recovery
21 and assessment of both free-form and conjugated catechins. The green tea catechin conjugated
22 metabolites were quantified as equivalents to their corresponding commercially available free-form
23 standards, relative to the internal standard.

24 Of the 55 conjugated and free-form metabolites investigated, 29 were detected in the urine
25 samples collected for 24 h at 4 separate time points (Table 2). The majority of compounds were
26 present in the conjugated form, with only three compounds identified in the free-form; 3-O-me-gallic
27 acid, 3-hydroxybenzoic acid, and hippuric acid. Of the catechins, EGCG and ECG were not identified
28 in any form as they were either not present within the samples, or because they were below the limit

1 of detection. Hippuric acid had the largest corresponding peak area, followed by O-me-EC-O-sulphate
2 and M6/M6'-O-sulphate. For the first time, glucuronide and sulphate derivatives of 3-hydroxybenzoic
3 acid, benzoic acid, hippuric acid and syringic acid were identified in urine following the consumption
4 of green tea. Through using quantification by free-form equivalents due to the lack of validated
5 metabolite standards, it is unknown whether large quantities of the metabolites were excreted or
6 whether the result is due to more efficient ionisation. Although there is a large inter-individual
7 variability for the green tea catechin metabolites, there are statistically significant differences between
8 the pre and post-supplementation samples (Table 2).

9 10 **4. Discussion**

11 The optimisation of an analytical method that allows the determination of the bioavailability
12 and biotransformation of the green tea catechins is important as this will allow for future identification
13 of compounds that may be related to the putative health benefits associated with green tea
14 consumption. This may also lead to the tailoring of studies to understand the mechanisms of the health
15 benefits at a molecular level through use of the identified metabolites.

16 The present study confirmed that a combination method utilising both an ethyl acetate
17 extraction for the more non-polar compounds and acetonitrile to precipitate the protein content of the
18 urine, results in a good recovery of the compounds assessed ($93\pm 13\%$). As sulphate and glucuronide
19 conjugates of green tea catechins are not commercially available, previous studies have reported the
20 identification of urinary green tea catechin metabolites as either deconjugated free-forms (using
21 sulphatase and β -glucuronidase) [22,36] or through monitoring the loss of the conjugate through
22 fragmentation during LC-MS² analysis [27-31]. More recently, studies have focused on the latter as
23 the information obtained is more realistic of the compounds actually present post-consumption of
24 green tea. Problems with using sulphatase have also been highlighted recently as it has been shown
25 that the use of the enzyme results in underestimation of the bioavailability of green tea catechins due
26 to incomplete hydrolysis and also because of the instability of the resulting free-forms during analysis
27 [26].

1 The majority of these studies assess biological samples obtained following green tea
2 consumption with a protocol that utilises acetonitrile to precipitate the protein from the biological
3 sample [28,30], but other studies have used direct injection following filtration onto the LC-MS
4 [27,32]. The free-form catechins have been reported previously to have a poor recovery following
5 isolation using acetonitrile [28], which is also supported within this study. Direct injection allows for
6 identification of both free-form and conjugated green tea catechins without further processing.
7 However, direct injection without sample preparation can lead to filtration problems throughout the
8 LC-MS system and also suppressed ionisation due to matrix effects [33].

9 Throughout the 3-month human intervention study, which was a double-blind randomised
10 controlled trial of green tea supplements or placebo simultaneously with ascorbic acid to improve the
11 gastrointestinal stability of the catechins, 29 green tea catechin metabolites were identified out of the
12 55 investigated, with only 3 present in the free-form. EC and EGC were largely conjugated with
13 methyl ester, sulphate ester or glucuronic acid, and the free-forms were not detected. EGCC and ECG
14 were also not identified in any form, which is comparable to other studies. It is possible that they
15 undergo biliary excretion [37] and are therefore not present within the urine, are conjugated in forms
16 that were not targeted or were below the detection limit. The data reported are all from the
17 supplemented volunteers as confirmed following the compliance testing, and as no difference was
18 evident between the excretion of EC-O-sulphate and EGC-O-glucuronide in the urine samples from
19 the placebo volunteer, the other metabolites were not assessed further.

20 In previous studies the conjugated forms have also been reported, with only minimal
21 identification of the free-forms. EGCG and ECG are not present in any form in the present study,
22 which is in line with previous reports [24,25,28,38]. The method developed in the present study may
23 be utilised for the assessment of other biological samples (e.g. organ tissues) that have sulphatase and
24 β -glucuronidase present within cells, and therefore may possibly have free-form compounds present.

25 The large inter-individual variability for the green tea catechin metabolites has been reported
26 previously [39]. This is due to individual differences in gut microbiota leading to a variation in
27 metabolism [40]. Hippuric acid had the largest corresponding peak area relative to the peak area of
28 ethyl gallate, followed by O-methyl-EC-O-sulphate and M6/M6'-O-sulphate. Without the validated

1 metabolite standards, it is unknown whether large quantities of the metabolites were excreted or
2 whether the result is due to more efficient ionisation, which is the case for sulphated metabolites.
3 Hippuric acid is an endogenous metabolite and although it is also detected post-consumption of tea,
4 the only significant increase in excretion in post-consumption urine samples compared with baseline
5 was at day one. The majority of EC, EGC and valerolactone (M4 and M6/M6') conjugates excreted
6 were significantly increased when post-consumption urine samples were compared to baseline
7 samples, and after day 1, there was no significant change in the metabolite elution across the 12-week
8 supplementation period except for EC-O-glucuronide (day 1 vs. week 6; $p=0.03$) and 3-
9 hydroxybenzoic acid-O-sulphate (day 1 vs. week 12; $p=0.04$).

10 Catechin conjugates in urine have previously been quantified as aglycone equivalents [28].
11 The volunteers consumed a lower catechin dose compared to the present study (141 and 28 mg EGC
12 and EC for Stalmach et al, 2009 [28] compared with 370 and 94 mg EGC and EC, respectively),
13 however, the excretion of EGC metabolites was relatively similar (EGC-O-glucuronide, 6.5 vs. 8
14 μmol ; EGC-O-sulphate, 2.6 vs. 1.9 μmol for Stalmach et al [28] and the present study, respectively),
15 whereas the difference in quantification of EC conjugates was substantial (EC-O-glucuronide, 1.5 vs.
16 10 μmol ; EC sulphate, 6.7 vs. 35 μmol , O-methyl-EC-O-sulphate 10.9 and 500 μmol for Stalmach et
17 al (2009) and the present study, respectively). This difference may be partly explained by the tuning
18 of the LC-MS with a solution of EC in the study by Stalmach et al (2009). This maximises the
19 response of EC and related compounds, which may also result in smaller differences between the
20 response factors from EC and conjugated derivatives. The comparison of the two studies highlights
21 the importance of using validated reference standards, to enable an accurate measure of dose-response
22 factors. This problem has also been reported previously for hydroxycinnamic acid conjugates which
23 were over-estimated when expressed relative to corresponding free-form (unconjugated) derivatives
24 [35], and requires exploring further in future studies.

25 In conclusion, to the best of our knowledge, this is the first time that a combination method
26 has been utilised that allows the identification of both free-form and conjugated derivatives of green
27 tea metabolites. This method is applicable for future studies involving biological samples in which
28 endogenous deconjugation enzymes are present.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

References

- [1] Q.V. Vuong, Epidemiological Evidence Linking Tea Consumption to Human Health: A Review. *Crit Rev Food Sci Nutr.* 54 (2014) 523-536.
- [2] Y. Cai, H.H.S. Chow, Pharmacokinetics and bioavailability of green tea catechins, in: M.S. Meskin, W.R. Bidlack, A.J. Davies, D.S. Lewis, R.K. Randolph (Eds.), *Phytochemicals: mechanisms of action*, CRC Press, Boca Raton, FL, 2004, pp. 35-50.
- [3] J.D. Lambert, S. Sang, C.S. Yang, Biotransformation of green tea polyphenols and the biological activities of those metabolites. *Mol Pharm.* 4 (2007) 819-825.
- [4] C.H. Wu, C.T. Yeh, G.C. Yeh, Epigallocatechin gallate (EGCG) binds to low-density lipoproteins (LDL) and protects them from oxidation and glycation under high-glucose conditions mimicking diabetes. *Food Chem.* 121 (2010) 639-644.
- [5] P. Bogdanski, J. Suliburska, M. Szulinska, M. Stepień, D. Pupek-Musialik, A. Jablecka, Green tea extract reduces blood pressure, inflammatory biomarkers, and oxidative stress and improves parameters associated with insulin resistance in obese, hypertensive patients. *Nutr Res.* 32 (2012) 421-427.
- [6] P. Wang, W.J. Aronson, M. Huang, Y. Zhang, R.P. Lee, D. Heber, S.M. Henning, Green Tea Polyphenols and Metabolites in Prostatectomy Tissue: Implications for Cancer Prevention. *Cancer Prev Res (Phila).* 3 (2010) 985-993.
- [7] T. Singh, S.K. Katiyar, Green tea polyphenol, (-)-epigallocatechin-3-gallate, induces toxicity in human skin cancer cells by targeting beta-catenin signaling. *Toxicol Appl Pharmacol.* 273 (2013) 418-424.
- [8] J.D. Lambert, Does tea prevent cancer? Evidence from laboratory and human intervention studies. *Am J Clin Nutr.* 98 (2013) 1667S-1675S.

- 1 [9] K. Boehm, F. Borrelli, E. Ernst, G. Habacher, S.K. Hung, S. Milazzo, M. Horneber, Green tea
2 (Camellia sinensis) for the prevention of cancer. *Cochrane Database Syst Rev.* 3 (2009)
3 CD005004.
- 4 [10] X.X. Zheng, Y.L. Xu, Green tea intake lowers fasting serum total and LDL cholesterol in
5 adults: a meta-analysis of 14 randomized controlled trials. *Am J Clin Nutr.* 94 (2011) 601-
6 610.
- 7 [11] J.M. Yuan, Cancer prevention by green tea: evidence from epidemiologic studies. *Am J Clin*
8 *Nutr.* 98 (2013) 1676S-1681S.
- 9 [12] K. Liu, R. Zhou, B. Wang, K. Chen, L.Y. Shi, J.D. Zhu, M.T. Mi, Effect of green tea on
10 glucose control and insulin sensitivity: a meta-analysis of 17 randomized controlled trials. *Am*
11 *J Clin Nutr.* 98 (2013) 340-348.
- 12 [13] E. Roura, C. Andrés-Lacueva, O. Jáuregui, E. Badia, R. Estruch, M. Izquierdo-Pulido, R.M.
13 Lamuela-Raventós, Rapid liquid chromatography tandem mass spectrometry assay to
14 quantify plasma (-)-epicatechin metabolites after ingestion of a standard portion of cocoa
15 beverage in humans. *J Agric Food Chem.* 53 (2005) 6190-6194.
- 16 [14] G. Williamson, F. Dionisi, M. Renouf, Flavanols from green tea and phenolic acids from
17 coffee: Critical quantitative evaluation of the pharmacokinetic data in humans after
18 consumption of single doses of beverages. *Mol Nutr Food Res.* 55 (2011) 864-873.
- 19 [15] D. Del Rio, L. Calani, F. Scazzina, L. Jechiu, C. Cordero, F. Brighenti, Bioavailability of
20 catechins from ready-to-drink tea. *Nutrition.* 26 (2010) 528-533.
- 21 [16] Q. Zhou, H. Chiang, C. Portocarrero, Y. Zhu, S. Hill, K. Heppert, H. Jayaratna, M. Davies, E.
22 Janle, P. Kissinger, Investigating the stability of EGCg in aqueous media. *Curr Sep.* 20 (2003)
23 83-86.
- 24 [17] Q.Y. Zhu, R.R. Holt, S.A. Lazarus, J.L. Ensunsa, J.F. Hammerstone, H.H. Schmitz, C.L.
25 Keen, Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins
26 derived from cocoa. *J Agric Food Chem.* 50 (2002) 1700-1705.
- 27 [18] N. Li, L.S. Taylor, M.F. Ferruzzi, L.J. Mauer, Kinetic study of catechin stability: Effects of
28 pH, concentration, and temperature. *J Agric Food Chem.* 60 (2012) 12531-12539.

- 1 [19] Z.Y. Chen, Q.Y. Zhu, Y.F. Wong, Z.S. Zhang, H.Y. Chung, Stabilizing effect of ascorbic acid
2 on green tea catechins. *J Agric Food Chem.* 46 (1998) 2512-2516.
- 3 [20] R. Wang, W. Zhou, X. Jiang, Reaction kinetics of degradation and epimerization of
4 epigallocatechin gallate (EGCG) in aqueous system over a wide temperature range. *J Agric*
5 *Food Chem.* 56 (2008) 2694-2701.
- 6 [21] Q.Y. Zhu, A. Zhang, D. Tsang, Y. Huang, Z.Y. Chen, Stability of green tea catechins. *J Agric*
7 *Food Chem.* 45 (1997) 4624-4628.[22] M. Renouf, P. Guy, C. Marmet, K. Longet, A.L.
8 Fraering, J. Moulin, D. Barron, F. Dionisi, C. Cavin, H. Steiling, G. Williamson, Plasma
9 appearance and correlation between coffee and green tea metabolites in human subjects. *Br J*
10 *Nutr.* 104 (2010) 1635-1640.
- 11 [23] M. Renouf, K. Redeuil, K. Longet, C. Marmet, F. Dionisi, M. Kussmann, G. Williamson, K.
12 Nagy, Plasma pharmacokinetics of catechin metabolite 4'-O-Me-EGC in healthy humans. *Eur*
13 *J Nutr.* 50 (2011) 575-580.
- 14 [24] U. Ullmann, J. Haller, J.P. Decourt, N. Girault, J. Girault, A.S. Richard-Caudron, B. Pineau,
15 P. Weber, A single ascending dose study of epigallocatechin gallate in healthy volunteers. *J*
16 *Int Med Res.* 31 (2003) 88-101.
- 17 [25] H.H. Chow, I.A. Hakim, D.R. Vining, J.A. Crowell, J. Ranger-Moore, W.M. Chew, C.A.
18 Celaya, S.R. Rodney, Y. Hara, D.S. Alberts, Effects of dosing condition on the oral
19 bioavailability of green tea catechins after single-dose administration of Polyphenon E in
20 healthy individuals. *Clin Cancer Res.* 11 (2005) 4627-4633.
- 21 [26] S. Saha, W. Hollands, P.W. Needs, L.M. Ostertag, B. de Roos, G.G. Duthie, P.A. Kroon,
22 Human O-sulfated metabolites of (-)-epicatechin and methyl(-)-epicatechin are poor
23 substrates for commercial aryl-sulfatases: implications for studies concerned with quantifying
24 epicatechin bioavailability. *Pharmacol Res.* 65 (2012) 592-602.
- 25 [27] W. Mullen, G. Borges, M.E. Lean, S.A. Roberts, A. Crozier, Identification of Metabolites in
26 Human Plasma and Urine after Consumption of a Polyphenol-Rich Juice Drink. *J Agric Food*
27 *Chem.* 58 (2010) 2586-2595.

- 1 [28] A. Stalmach, S. Troufflard, M. Serafini, A. Crozier, Absorption, metabolism and excretion of
2 Choladi green tea flavan-3-ols by humans. *Mol Nutr Food Res.* 53 (2009) S44-S53.
- 3 [29] A. Stalmach, W. Mullen, H. Steiling, G. Williamson, M.E. Lean, A. Crozier, Absorption,
4 metabolism, and excretion of green tea flavan-3-ols in humans with an ileostomy. *Mol Nutr*
5 *Food Res.* 54 (2010) 323-334.
- 6 [30] S. Sang, M.J. Lee, I. Yang, B. Buckley, C.S. Yang, Human urinary metabolite profile of tea
7 polyphenols analyzed by liquid chromatography/electrospray ionization tandem mass
8 spectrometry with data-dependent acquisition. *Rapid Commun Mass Spectrom.* 22 (2008)
9 1567-1578.
- 10 [31] D. Del Rio, L. Calani, C. Cordero, S. Salvatore, N. Pellegrini, F. Brighenti, Bioavailability
11 and catabolism of green tea flavan-3-ols in humans. *Nutrition.* 26 (2010) 1110-1116.
- 12 [32] G. Borges, W. Mullen, A. Mullan, M.E. Lean, S.A. Roberts, A. Crozier, Bioavailability of
13 multiple components following acute ingestion of a polyphenol-rich juice drink. *Mol Nutr*
14 *Food Res.* 54 (2010) S268-S277.
- 15 [33] C. Müller, P. Schäfer, M. Störzel, S. Voqt, W. Weinmann, Ion suppression effects in liquid
16 chromatography–electrospray-ionisation transport-region collision induced dissociation mass
17 spectrometry with different serum extraction methods for systematic toxicological analysis
18 with mass spectra libraries. *J Chromatogr B Analyt Technol Biomed Life Sci.* 773 (2002) 47-
19 52.
- 20 [34] C.C. Wong, W. Meinl, H.R. Glatt, D. Barron, A. Stalmach, H. Steiling, A. Crozier, G.
21 Williamson, In vitro and in vivo conjugation of dietary hydroxycinnamic acids by UDP-
22 glucuronosyltransferases and sulfotransferases in humans. *J Nutr Biochem.* 21 (2010) 1060-
23 1068.
- 24 [35] T. Farrell, L. Poquet, F. Dionisi, D. Barron, G. Williamson, Characterization of
25 hydroxycinnamic acid glucuronide and sulfate conjugates by HPLC-DAD-MS(2): enhancing
26 chromatographic quantification and application in Caco-2 cell metabolism. *J Pharm Biomed*
27 *Anal.* 55 (2011) 1245-1254.

- 1 [36] M. Renouf, C. Marmet, P.A. Guy, M. Beaumont, M. Lepage, G. Williamson, F. Dionisi,
2 Dose-response plasma appearance of green tea catechins in adults. *Mol Nutr Food Res.* 57
3 (2013) 833-839.
- 4 [37] T. Kohri, N. Matsumoto, M. Yamakawa, M. Suzuki, F. Nanjo, Y. Hara, N. Oku, Metabolic
5 fate of (-)- 4-H-3 epigallocatechin gallate in rats after oral administration. *J Agric Food*
6 *Chem.* 49 (2001) 4102-4112.
- 7 [38] S.M. Henning, Y. Niu, Y. Liu, N.H. Lee, Y. Hara, G.D. Thames, R.R. Minutti, C.L.
8 Carpenter, H. Wang, D. Heber, Bioavailability and antioxidant effect of epigallocatechin
9 gallate administered in purified form versus as green tea extract in healthy individuals. *J Nutr*
10 *Biochem.* 16 (2005) 610-616.
- 11 [39] M.J. Lee, P. Maliakal, L.S. Chen, X.F. Meng, F.Y. Bondoc, S. Prabhu, G. Lambert, S. Mohr,
12 C.S. Yang, Pharmacokinetics of tea catechins after ingestion of green tea and (-)-
13 epigallocatechin-3-gallate by humans: formation of different metabolites and individual
14 variability. *Cancer Epidemiol Biomarkers Prev.* 11 (2002) 1025-1032.
- 15 [40] P.B. Eckburg, E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill,
16 K.E. Nelson, D.A. Relmon, Diversity of the human intestinal microbial flora. *Science.* 308
17 (2005) 1635-1638.