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Analysis of (+)-catechin, (−)-epicatechin and their 3′- and 4′-*O*-methylated analogs A comparison of sensitive methods

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Abstract

(+)-Catechin and (−)-epicatechin are found in many foods and may have important effects on human health. These compounds, like many other catechols, are thought to be converted to methylated metabolites after ingestion. This paper describes the synthesis of the 3′- and 4′-methyl ethers and their unambiguous identification. These products, along with catechin, epicatechin and an internal standard, (+)-taxifolin, were separated using RP-HPLC with ultraviolet, electrochemical and fluorescence detection. The trimethylsilylated derivatives of the seven compounds were also separated by GC with mass spectrometric detection. The limits of detection and selectivity of the analytical methods were compared with respect to their application in complex matrices such as human plasma.

Keywords: Catechin; Epicatechin

1. Introduction

(+)-Catechin and (−)-epicatechin are flavonoids that are widely distributed in plant derived foods including red wine, green tea, chocolate and many fruits [1,2]. Epidemiological and animal studies have correlated flavonoid consumption with reduced rates of heart disease and some cancers [3–10]. The precise role of flavonoids in human health and disease, however, is far from well understood. This is partly because quantitative information regarding their metabolism and distribution after consumption from common foods is extremely scarce. It is known, however, that both catechin and epicatechin can be absorbed after ingestion by humans because catechin itself and metabolites of both compounds have been found in urine and plasma [11–14]. Hackett et al. [11] showed that after ingestion of 2 g catechin, ≈60% of the catechin excreted in urine was methylated on the catechol ring, presumably by the enzyme, catechol-*O*-methyl-transferase (COMT). This earlier investigation, like several others, was conducted using large doses of purified catechin (1–6 g/person) whereas a serving of red wine or green tea generally contains less than 50 mg catechin or epicatechin along with many other flavonoids

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[14,15]. Recent metabolic research has focussed on epicatechin (and related compounds) from green tea [14,16,17], although methylated metabolites were not measured during these studies. This was likely due to the lack of suitable methodology and availability of the methylated compounds for use as standards.

Analytical methods with acceptable sensitivity and selectivity are crucial to quantify these flavonoids as well as their metabolites in biological samples. High-performance liquid chromatography (HPLC) has been used with electrochemical (EC) detection [14], chemiluminescence detection (CL) [16] and fluorescence (FL) detection [13,18] to quantify catechin and epicatechin in human plasma. We recently described a method for the analysis of catechin and epicatechin in human plasma by gas chromatography (GC) with mass spectrometry (MS) [19]. None of these studies, however, reported the analysis of the methylated derivatives of these compounds. The purpose of this investigation was to determine which of these methods could be expanded to include these potentially important metabolites. This paper describes the synthesis of the 3′- and 4′-methyl ethers of catechin and epicatechin and their unambiguous identification. These products, along with catechin, epicatechin and an internal standard, (+)-taxifolin, were separated using reversed-phase (RP) HPLC with UV, EC and FL detection. The trimethylsilylated (TMS) derivatives of the seven compounds were also separated by GC and detected by MS. The limits of detection (LODs) and selectivity of the analytical methods were compared with respect to their potential application to the analysis of complex matrices such as human fluids after consumption of catechin and epicatechin containing foods.

2. Experimental

2.1. Chemicals and reagents

(+)-Catechin (*trans*-3,3′,4′,5,7-pentahydroxyflavan) and (−)-epicatechin (*cis*-3,3′,4′,5,7-pentahydroxyflavan) were obtained from Fluka (Onkonkoma, NY, USA) and Sigma (St. Louis, MO, USA), respectively. (+)-Taxifolin (3,3′,4′,5,7-pentahydroxyflavanone) was purchased from Apin (Abingdon, UK). The structures of these three compounds are shown in Fig. 1. The derivatizing reagent, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), was obtained from Pierce (Rockford, IL, USA). Methyl iodide was purchased from Aldrich (Milwaukee, WI, USA). All solvents were HPLC or GC grade and other reagents were either purchased from Fisher Scientific (Pittsburgh, PA, USA) or Aldrich.

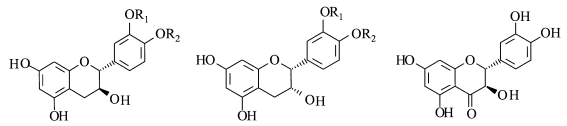

Fig. 1. Structures of (+)-catechin (1; R_1=H, R_2=H), (−)-epicatechin (2; R_1=H, R_2=H), 3′-*O*-methyl-(+)-catechin (1; R_1=CH_3, R_2=H), 4′-*O*-methyl-(+)-catechin (1; R_1=H, R_2=CH_3), 3′-*O*-methyl-(−)-epicatechin (2; R_1=CH_3, R_2=H), 4′-*O*-methyl-(−)-epicatechin (2; R_1=H, R_2=CH_3) and (+)-taxifolin (3).

2.2. Synthesis and structural elucidation of methylated derivatives

A mixture of (+)-catechin or (−)-epicatechin (250 mg), K_2CO_3 (500 mg) and methyl iodide (1 ml) in acetone (20 ml) was irradiated in an ultrasonic bath for 2.5 h. The progress of the reaction was monitored by HPLC. After approximately 25–30% conversion into products the mixture was filtered and the acetone was removed by rotary evaporation. The major products of the reactions (the 3′- and 4′-methyl ethers of catechin and epicatechin, Fig. 1) were purified by semi-preparative HPLC (described in Section 2.3). The positions of the methoxyl substituents were confirmed by one-dimensional (1D)-difference Nuclear Overhauser effect (NOE) spectroscopy which were conducted by interlaying eight scans of irradiation of the peak of interest with eight scans of irradiation off-resonance, with the receiver phase cycled 180° every eight scans to generate the NOE difference spectrum. Nuclear magnetic resonance (NMR) spectra were obtained on a GE-Omega 500 MHz instrument and chemical shifts and assignments (all in d_6 acetone) are as follows: 3′-*O*-methyl-(+)-catechin: 2.49 (1H, dd, J=8.9, 15.9 Hz, H-4 eq), 2.95 (1H, dd, J=5.9, 15.9 Hz, H-4 ax), 3.82 (3H, s, OCH_3), 3.95 (1H, bs, 3-OH), 4.01 (1H, m, H-3), 4.53 (1H, d, J=8.0 Hz,

H-2), 5.85 (1H, d, J=1.8 Hz, H-6), 6.00 (1H, d, J=1.8 Hz, H-8), 6.78 (1H, d, J=8.1 Hz, H-5′), 6.86 (1H, dd, J=1.5, 8.0 Hz, H-6′), 7.00 (1H, d, J=1.5 Hz, H-2′), 7.61 (1H, s, OH), 8.06 (1H, s, OH) and 8.27 (1H, s, OH). NOE: OMe (3.82) – 1H, d, J=2.0 Hz (7.00). 4′-*O*-methyl-(+)-catechin: 2.52 (1H, dd, J=8.9, 16.1 Hz, H-4 eq), 2.95 (1H, dd, J=5.9, 16.1 Hz, H-4 ax), 3.83 (3H, s, OCH_3), 3.99 (1H, bs, 3-OH), 4.00 (1H, m, H-3), 4.58 (1H, d, J=7.3 Hz, H-2), 5.87 (1H, d, J=2.2 Hz, H-6), 6.01 (1H, d, J=2.2 Hz, H-8), 6.84 (1H, dd, J=8.0, 1.5 Hz, H-6′), 6.89 (1H, bs, H-2′), 6.90 (1H, d, J=8.0 Hz, H-5′), 7.62 (1H, s, OH), 8.06 (1H, s, OH) and 8.27 (1H, s, OH). NOE: OMe (3.82) – 1H, d, J=8.0 Hz (6.90). 3′-*O*-Methyl-(−)-epicatechin: 2.73 (1H, dd, J=3.2, 16.6 Hz, H-4 eq), 2.86 (1H, dd, J=4.6, 16.6 Hz, H-4 ax), 3.81 (3H, s, OCH_3), 3.62 (1H, bs, 3-OH), 4.20 (1H, m, H-3), 4.90 (1H, s, H-2), 5.90 (1H, d, J=2.2 Hz, H-6), 6.00 (1H, d, J=2.2 Hz, H-8), 6.77 (1H, d, J=8.1 Hz, H-5′), 6.93 (1H, dd, J=1.5, 8.1 Hz, H-6′), 7.16 (1H, d, J=1.5 Hz, H-2′), 7.53 (1H, s, OH), 8.00 (1H, s, OH) and 8.19 (1H, s, OH). NOE: OMe (3.82) – 1H, d, J=2.0 Hz (7.00). 4′-*O*-Methyl-(−)-epicatechin: 2.73 (1H, dd, J=3.2, 16.6 Hz, H-4 eq), 2.86 (1H, dd, J=4.6, 16.6 Hz, H-4 ax), 3.81 (3H, s, OCH_3), 3.62 (1H, bs, 3-OH), 4.20 (1H, m, H-3), 4.89 (1H, s, H-2), 5.90 (1H, d, J=2.0 Hz, H-6), 5.99 (1H, d, J=2.0 Hz, H-8), 6.88 (1H, d, J=8.4 Hz, H-5′), 6.90 (1H, dd, J=1.5, 8.4 Hz, H-6′), 7.04 (1H, d, J=1.5 Hz, H-2′), 7.52 (1H, s, OH), 8.01 (1H, s, OH) and 8.17 (1H, s, OH). NOE: OMe (3.82) – 1H, d, J=8.0 Hz (6.90).

2.3. Chromatographic analysis

Prior to HPLC analysis, compounds were dissolved in 20% ethanol and filtered through 0.45-μm poly(tetrafluoroethylene) (PTFE) syringe tip filters (Gelman Sciences, Ann Arbor, MI, USA). Separations with UV and FL detection were performed on a Hewlett-Packard (Palo Alto, CA, USA) 1090 Model HPLC equipped with three solvent pumps and a LiChrosphere C_{18}, 4×250 mm, 5 μm particle size analytical column. The mobile phase was heated to 40°C, delivered at 0.5 ml/min and consisted of two solvents: solvent A=50 m*M* ammonium dihydrogen phosphate ($NH_4H_2PO_4$) at pH 2.60, solvent B=20% solvent A in acetonitrile. The multi-linear gradient began using 100% solvent A; from 5–35 min solvent B was increased to 21.5%, and from 35 to 70 min solvent B was increased to 50%. The mobile phase was then returned to 100% solvent A in preparation for the next analysis. Semi-preparative chromatography was performed under the same conditions except with a LiChrosphere C_{18}, 250×10 mm, 10 μm particle size column (Hewlett-Packard) and a flow-rate of 4 ml/min. A Hewlett-Packard diode-array detector (190–600 nm) was connected in series to a Hewlett-Packard 1046A programmable fluorescence detector. Optimal signal-to-noise ratios for UV detection were obtained at 280 nm ($\approx\lambda_{max}$ of flavan-3-ols), a sampling interval of 1.3 s without the use of a reference wavelength. Absorbance spectra were collected from 200–600 nm in 2-nm steps. Optimal signal-to-noise ratios for FL detection were excitation at 280 nm, emission at 310 nm, lamp frequency of 220 Hz, response time of 4.0 s, and photomultiplier tube (PMT) gain at 14. All data was collected and analyzed with HPChemstation software (Version 3.03; Hewlett-Packard). Electrochemical response was determined by performing an isocratic separation using 50 m*M* sodium phosphate (NaH_2PO_4) containing 7% acetonitrile at pH 3.0 with a flow-rate of 0.5 ml/min and continued recycling of the mobile phase after oxidation at 800 mV at the output of the electrochemical cell. The HPLC system consisted of a Waters Model 510 pump and 712 WISP autoinjecton system (Waters, Milford, MA, USA) with the analytical column (described above) coupled to an ESA Analytical Coulochem II electrochemical detector equipped with a Model 5010 dual voltage analytical cell (ESA Analytical, Huntingdon, UK). All data was collected and analyzed using Waters Maxima software version 3.1. For GC–MS analysis, the dried compounds were dissolved in 20 μl pyridine and the TMS derivatives were prepared with 30 μl BSTFA at 65°C for 2 h. GC–MS analyses were performed on a Hewlett-Packard 6890 GC system equipped with a 5973 quadropole mass spectrometer using a DB-23 capillary GC column (60 m×0.25 mm I. D., 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). Splitless injections of 1 μl were made, and the column temperature was programmed from 150°C (3 min hold) to 230°C at 5°C/min with a final hold at 230°C for 30 min. Helium (99.999%) was used as the carrier gas and was set at 0.7

ml/min with an average linear velocity of 23 cm/min. Quantitative analyses were performed using 70 eV electron ionization (EI) and selected ion monitoring (SIM) of the base peak fragment ions; *m/z* 368 for catechin, epicatechin and taxifolin and *m/z* 310 for the methylated compounds using a dwell time of 100 ms/channel.

3. Results and discussion

3.1. Nuclear magnetic resonance (NMR) spectroscopy

The (1D) NMR spectra of the methylated derivatives of epicatechin have not been reported. However, the (1D) NMR spectra of epicatechin, catechin, 3′-*O*-methyl catechin and 4′-*O*-methyl catechin were similar to those reported in the literature [12,20]. The correct assignment of the methoxyl groups were confirmed by (1D) NOE difference spectra. The compounds with methoxyl groups at the 3′ position showed NOEs with doublets (J=1.5–2.0 Hz, *meta* coupling) at δ 7.0 ppm upon irradiation of the methoxyl group protons (δ 3.82 ppm) whereas the 4′-methyl ethers of catechin and epicatechin showed NOEs with doublets (J=8 Hz, *ortho* coupling) at δ 6.90ppm.

3.2. HPLC separation with UV, FL and EC detection

The HPLC method described here was sufficient to separate all seven of these compounds in less than 50 min (Fig. 2). UV detection at 280 nm had a limit of detection (LOD) in the 20–23 ng range (on-column) for (+)-catechin, (−)-epicatechin and all of the methylated analogs whereas (+)-taxifolin had a LOD of 3 ng (Table 1). This translates to a LOD of ≈100 ng/ml of a fluid that was concentrated ten-fold before a 20 μl injection. UV detection is the most common detection method for the analysis of flavonoids in fruits and beverages where they often occur at >10 μg/ml or g [1]. However UV detection is not likely to be sensitive enough for the accurate measurement of flavonoids and their metabolites in human plasma from consumption of amounts commonly found in foods. Although there is one published report where UV detection was used and ≈1 μg/ml of certain flavonoids (although not epicatechin or catechin) were identified in human plasma [21], other researchers have reported that the plasma levels of flavonoids and their metabolites reach maximum levels at around 50–200 ng/ml 1–3 h after consumption of typical flavonoid containing foods such as tea [14] and onions [22]. However, one recent study reported that plasma levels of catechin can reach 800 ng/ml 12 h after ingestion of a flavonoid rich meal [13]. Interestingly, epicatechin was not identified in this plasma. In a preliminary study, our laboratory detected μg/ml quantities of catechin in human plasma using UV detection [23], but it appears that this observation was due to an interfering compound. This demonstrates the importance of a selective method that can also provide qualitative information to ensure accurate identification of chromatographic peaks. The lack of selectivity, sensitivity and qualitative information provided by UV detection make this method unsuitable for the analysis of catechin in complex clinical samples such as human fluids.

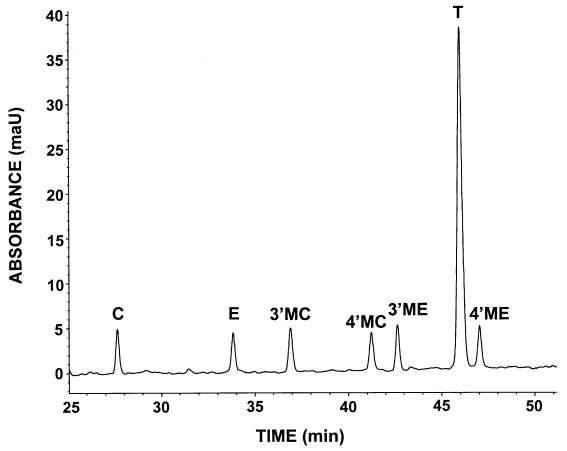

Fig. 2. High-performance liquid chromatogram of catechin (C), epicatechin (E), 3′-*O*-methyl-catechin (3′MC), 4′-*O*-methyl-catechin (4′MC), 3′-*O*-methyl-epicatechin (3′ME), 4′-*O*-methyl-epicatechin (4′ME) and taxifolin (T) with UV detection at 280 nm; 90 ng on-column.

Coulometric EC detection at 200 mV had an on-column LOD of 5 ng for catechin, epicatechin and taxifolin (Table 1). Injections of 200 ng of the methylated compounds were not detected at this potential. This was expected because when one a hydroxyl group on a catechol ring is substituted, the

Table 1
Comparison of the detection limits (S/N ratio≈3) for catechin, epicatechin, their 3′ and 4′ methylated derivatives, and taxifolin using HPLC–UV, HPLC–EC, HPLC–FL and GC–MS (reported quantities are on-column)

Compound	UV 280 nm	EC 200 mV	EC 400 mV	FL 280, 310 nm	GC–MS (SIM)[b]
Catechin	22 ng	5 ng	4 ng	3 ng	3 pg
Epicatechin	23 ng	5 ng	4 ng	3 ng	3 pg
3′-*O*-Methyl-catechin	20 ng	nd[a]	15 ng	3 ng	2 pg
3′-*O*-Methyl-epicatechin	20 ng	nd	10 ng	2 ng	2 pg
4′-*O*-Methyl-catechin	23 ng	nd	20 ng	3 ng	2 pg
4′-*O*-Methyl-epicatechin	23 ng	nd	15 ng	3 ng	2 pg
Taxifolin	3 ng	5 ng	5 ng	nd	2 pg

[a] nd: Not detected when 200 ng was injected.
[b] All LODs for GC–MS were calculated in the SIM mode. Catechin, epicatechin and taxifolin were determined at $m/z=368$, methylated derivatives at $m/z=310$.

oxidation potential is significantly increased [24,25]. At 400 mV, catechin, epicatechin and taxifolin had limits of detection of 4–5 ng, however the LODs were several times higher for the methylated compounds. At 600 mV (data not shown), the limits of detection for all of the compounds were ≈5 ng but the use of a potential this high would not be selective enough for complex matrices. Although it is possible to use a mobile phase which increases slowly in organic concentration with EC detection, in this study, an isocratic separation was performed. The separation was fairly long (last peak eluted at 75 min) and because peaks were not as sharp as possible, the LOD may have been slightly increased. A recent study reported a LOD of 0.25 ng for epicatechin in human plasma [14]. This study used a Coulochem electrode array system where eight potentials, including several reducing potentials, were monitored in series. The potential used to determine the LOD is unclear. Although no attempts were made to detect methylated metabolites of epicatechin, these derivatives are likely to have gone undetected at the lower potentials. At the two highest potentials (390 mV and 470 mV), the LODs for the methylated compounds would have been several times higher than the LOD of epicatechin.

Chemiluminescence has been used to detect low levels (≈1 ng) of a derivative of epicatechin found in tea, epigallocatechin-3-gallate (EGCG), in rat and human plasma [16]. This technique requires a post-column oxidation reaction with acetaldehyde or horseradish peroxidase and H_2O_2. Chemiluminescence detection, however useful for EGCG, was not investigated further during this study because the reaction was reported to occur only with flavonoids possessing vicinal hydroxyl groups [26], and would not be useful for the analysis of the methylated metabolites of catechin and epicatechin.

FL was the most sensitive method of detection used with HPLC. FL detection was similarly sensitive for all of the flavan-3-ols tested and the LODs were 2–3 ng for catechin, epicatechin and all of the methylated analogs (Fig. 3). Taxifolin did not fluoresce at these excitation and emission wavelengths but this compound could still be used as an internal standard if UV detection was coupled to

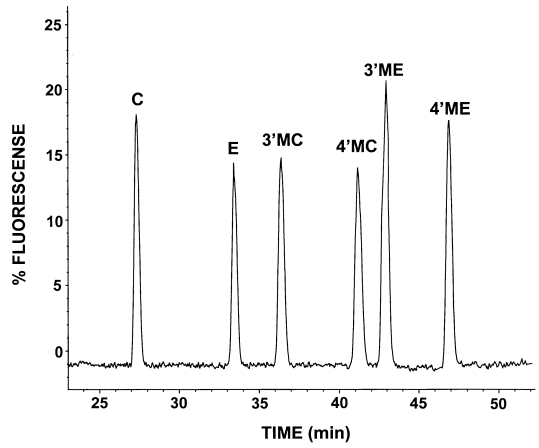

Fig. 3. High-performance liquid chromatogram of catechin (C), epicatechin (E), 3′-*O*-methyl-catechin (3′MC), 4′-*O*-methyl-catechin (4′MC), 3′-*O*-methyl-epicatechin (3′ME), 4′-*O*-methyl-epicatechin (4′ME) with FL detection, excitation at 280 nm and emission at 310 nm; 70 ng on-column.

fluorescence (as it was in this study) because the LOD of taxifolin by UV was 3 ng. Our results are similar to the previously reported LOD for catechin by fluorescence (1 ng, on-column) [18].

3.3. GC–MS

Initial experiments using DB-5, DB-WAX, DB-200 and DB-23 (30 m) columns did not provide adequate separation of the six flavan-3-ols and taxifolin. The DB-23 (60m) column provided adequate separation of all seven compounds (Fig. 4). All of the compounds had fragmentation patterns resulting from retro-Diels Alder cleavage which is characteristic of flavonoids [12]. The mass spectra of the TMS derivatives of catechin, and 3′- and 4′-*O*-methyl-catechin were similar to those reported in the literature [12]. The TMS derivatives of catechin and epicatechin had weak molecular ion peaks at *m*/*z* 650 (5–6%), base peaks with *m*/*z* 368 (B ring fragment) and major fragmentation ions with *m*/*z* 355 (30–37%) and *m*/*z* 283 (1–3%) (see Fig. 4). The methylated derivatives had molecular ion peaks of *m*/*z* 592 (5–6%), base peaks with *m*/*z* 310 (B ring fragment) and major fragmentation ion peaks of *m*/*z* 355 (23–30%), 280 (15–18%), 209 (8–12%) and 297 (1–2%) The fragmentation patterns did not differ significantly for methylation at the 3′ or 4′ positions. Taxifolin had a weak molecular ion at *m*/*z* 664 (1–2%) and major fragmentation ions at *m*/*z* 368 (base peak, B ring fragment) and 297 (A ring fragment 1–2%). The LOD of this technique, 2–3 pg, is ≈1000-fold lower than HPLC–FL. This corresponds to a LOD of ≈200 pg/ml in a fluid after a ten-fold concentration and a 1 μl injection. The one notable disadvantage of this technique is that it cannot be expanded for the direct analysis of the larger, more polar metabolites of flavonoids such as glucuronide and sulfate conjugates.

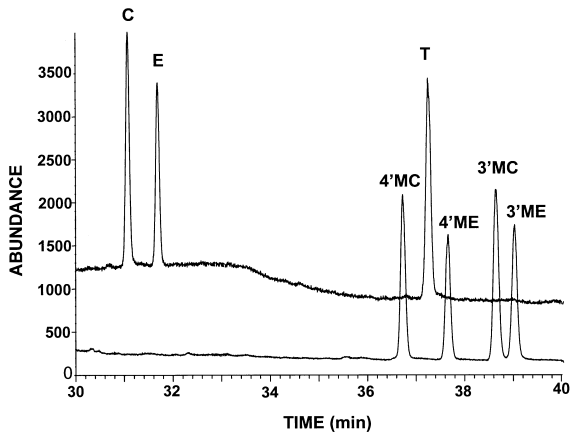

Fig. 4. Gas chromatogram of ions *m*/*z* 368 (upper trace) and *m*/*z* 310 (lower trace) of the TMS derivatives of catechin (C), epicatechin (E), 4′-*O*-methyl-catechin (4′MC), taxifolin (T), 4′-*O*-methyl-epicatechin (4′ME), 3′-*O*-methyl-catechin (3′MC) and 3′-*O*-methyl-epicatechin (3′ME); SIM mode, 66 pg on-column.

4. Conclusions

In this study we have investigated several methods for the analysis of catechin, epicatechin and their methylated metabolites, however, only two of these methods, HPLC–FL and GC–MS, are likely to be sensitive and selective enough for the analysis of small quantities of all of these compounds in complex biological matrices. If HPLC separation is performed, the described methods of detection are FL, EC and UV. The LODs for all of these compounds by UV detection are similar, however, this method was the least sensitive and perhaps the least selective of the methods studied. EC detection is quite sensitive for catechin, epicatechin and taxifolin at 200 mV. However, the methylated compounds were not detected at this potential and although higher potentials may be used with equal sensitivity, the disadvantage will be decreased selectivity which is especially important in complex matrices. FL detection was similarly sensitive to catechin, epicatechin and their methylated metabolites and was the most sensitive method of HPLC detection. The low LOD and selectivity of fluorescence give this method the potential to be expanded for the analysis of these compounds in clinical studies and this method may also have the potential to be expanded for the analysis of glucuronide and sulfate conjugates. GC–MS analysis was also equally sensitive to all of the compounds studied and ≈1000-fold more sensitive than FL detection. MS of the major fragmentation ions is not only sensitive and selective but provides further confidence in identification especially when combined with the superior separation provided by capillary GC. This method also has the

potential to be expanded for use in clinical studies and would be necessary for measuring minute quantities found in some biological samples.

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