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Analysis of theaflavins in biological fluids using liquid chromatography–electrospray mass spectrometry

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Abstract

A HPLC–MS procedure for the sensitive and specific analysis of the black tea flavonoid theaflavin in human plasma and urine was developed. Levels were measured after enzymatic deconjugation, extraction into ethyl acetate, and separation by HPLC, using tandem mass spectrometry as a detecting system. Two healthy volunteers consumed 700 mg theaflavins, equivalent to about 30 cups of black tea. The maximum concentration detected in blood plasma was 1.0 $\mu g\ l^{-1}$ in a sample collected after 2 h. The concentration in urine also peaked after 2 h at 4.2 $\mu g\ l^{-1}$. Hence, only minute amounts of theaflavins can be detected in plasma and urine samples of healthy volunteers after ingestion.

Keywords: Theaflavins

1. Introduction

Flavonoids are widely distributed in the plant kingdom and over 4000 different flavonoids have been identified [1]. Most of these compounds are strong anti-oxidants that can be found in vegetables, fruits, wine and tea. Epidemiological studies in humans indicate that a high intake of flavonoids is associated with a reduced risk of coronary hart disease [2] and stroke [3]. Although tea consists for over 99% water, it is also a very rich source of flavonoids. There is a large interest in the effect of tea flavonoids on health [2,3], but remarkably little is known about the bioavailability. The main flavonoids found in green tea are (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate. Studies with ^{14}C-labelled (+)-catechin showed that this compound is absorbed from the intestine [4]. Once absorbed, biotransformation enzymes in the gut wall and the liver extensively modify catechins [4–6]. Recently, methods for the measurement of unlabelled, native (−)-epicatechins, in blood plasma and urine were developed and the absorption of catechins in rats and humans was confirmed [7,8].

During the fermentation step in the production of black tea, most of the catechins are oxidised and condensed into theaflavins (dimers) and thearubigins (polymers?) [9]. Of the flavonoids in black tea, approximately 30% are catechins, 10% are theafl-

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avins, and 50% are thearubigins [9]. In most western countries, people drink black tea and consequently black tea accounts for approximately 80% of the world tea consumption. The bioavailability of the typical black tea flavonoids, theaflavins and thearubigins, however, has not been studied.

The identification and quantification of low levels (μmol l^{-1}–nmol l^{-1}) of flavonoids in complex matrices such as biological fluids is very difficult. Elegant analytical methods for the detection of catechins [7] and quercetin [10] in plasma and urine were developed, but these methods are more or less specific for the flavonoid studied. Because more than 4000 different flavonoids are known [1], a more generic approach for the analysis of these compounds is needed. Recently Andlauer et al. [11] showed that 19 different flavonoids, and closely related compounds, can be analysed in plant extracts using high-performance liquid chromatography (HPLC) combined with electrospray ionisation mass spectrometry (ESI-MS). The combined separation power of liquid chromatography (LC) and MS makes this type of technique a more generally applicable tool for screening all kinds of biological matrices for flavonoid content. Sägesser and Deinzer [12] took this approach one step further and identified 12 different glycosides of two flavonols present in hops by using parent-MS–MS.

MS was already used to identify catechin metabolites in 1980 [13], but after the introduction of ESI in the mid-1980s [14], ESI-MS and LC–ESI-MS became increasingly popular for the analysis of flavonoids. The recent development of LC–ESI-MS and LC–ESI-MS–MS instruments with high sensitivities, user-friendly software, and relatively low prices has boosted the popularity of these analytical techniques [15]. In this paper we report that LC–ESI-MS–MS can be used to identify and quantify very low levels of theaflavin in biological matrices.

2. Experimental

2.1. Materials

A freeze–dried preparation containing 17.7% (w/w) theaflavin, 31.8% theaflavin-3-gallate, 16.7% theaflavin-3′-gallate and 31.4% theaflavin-3,3′-digallate was a generous gift from Dr. D.A. Balentine, T.J. Lipton (Englewood Cliffs, NJ, USA). (−)-Epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, (−)-epigallocatechin gallate, β-glucuronidase with a high sulfatase activity from *Helix pomatia* (100 000 units ml^{-1} and 7500 units ml^{-1}, respectively), catechol-*O*-methyltransferase (EC 2.1.1.6, from porcine liver), *S*-adenosyl-L-methionine, glutathione, phenolphtalein glucuronide, *p*-nitrophenyl sulfate, and frozen bovine serum were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, for spectroscopy, L-ascorbic acid and Na_2EDTA were from Merck (Darmstadt, Germany). All other materials were obtained from Fisher Scientific (Loughborough, UK) and were analytical grade.

2.2. Healthy volunteers

Two healthy volunteers (one male, one female, aged 40 and 33 years, respectively) refrained from drinking tea for at least 48 h. On the morning of the experiments the volunteers consumed 700 mg mixed theaflavins (approximately 1 mmol) dissolved in 150 ml hot water and supplemented with 20–40 ml lemonade syrup to improve palatability. This amount of theaflavins is equivalent to approximately 30 cups of black tea. Except for not consuming tea for the next 24 h, no further dietary restrictions were imposed.

Blood was collected in EDTA containing vacuum tubes. Blood samples were collected before drinking theaflavins ($t=0$) and 20 min and 1, 2, 4, 7 and 24 h after consumption of theaflavins. Blood was centrifuged at 2500 *g* for 10 min at room temperature. The plasma was collected, and 10% (v/v) of an ascorbic acid solution was added. This solution consisted of 20% (w/v) ascorbic acid, 0.4 mol l^{-1} Na_2HPO_4 and 0.1% (w/v) Na_2EDTA, adjusted to pH 3.6 using 10 mol l^{-1} NaOH. After the addition of the ascorbic acid solution, the pH of the plasma samples was approximately 5.0. Urine samples were collected before drinking theaflavins ($t=0$) and 1, 2, 4, 7 and 24 h after consumption of the theaflavins. All urine samples were adjusted to a pH of 5.0 by addition of the ascorbic acid solution mentioned above. Plasma and urine samples were frozen under nitrogen gas and stored at −20°C. Preliminary

experiments had indicated that spiked theaflavins were not stable in untreated plasma. Only 25% could be recovered after overnight storage at −20°C. After addition of the ascorbic acid solution, however, recoveries of spiked theaflavin increased to 82% (data not shown).

In order to prepare a control plasma containing catechins, the male volunteers consumed 3 g of green tea extract and 2 h later a blood sample was taken and stored as described above.

2.3. Sample treatment

To 1 ml plasma or urine (supplemented with ascorbic acid, pH approximately 5.0) 100 μl β-glucuronidase/sulfatase was added and the mixture was incubated at 37°C for 1 h in closed, nitrogen gas flushed tubes. Preliminary experiments using plasma samples spiked with phenolphtalein glucuronide or *p*-nitrophenyl sulfate indicated an almost complete digestion under the conditions employed. The enzyme preparation was found to have a tannase activity that resulted in the de-gallation of the gallated catechins and theaflavins present in the plasma and urine samples.

After cooling the samples to room temperature, 500 μl acetone was added, samples were vortex-mixed, 2 ml ethyl acetate was added, samples were vortex-mixed again and centrifuged for 5 min at 2500 *g*. The upper ethyl acetate phase was collected. After a second extraction of the water phase with 2 ml ethyl acetate, the organic fractions were pooled and evaporated at 37°C under a stream of nitrogen gas. The residues were dissolved in 50 μl HPLC starting eluent supplemented with 1 mmol l^{-1} ascorbic acid. After centrifugation, 20 μl of the supernatant was injected onto the HPLC system.

Loss of the very low amounts of theaflavins in the samples during storage, deconjugation, and extraction were estimated by spiking theaflavin-free plasma with theaflavin at two levels: 1000 and 50 μg l^{-1}. These samples were stored, deconjugated, extracted and analysed together with the analytical samples. Recoveries were 70 and 50%, respectively.

In order to determine the retention time of the methylated (−)-epigallocatechin, it was prepared essentially as described by Zhu et al. [16].

2.4. HPLC

Standards, plasma extracts and urine extracts were separated on a Hewlett-Packard 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany). The Symmetry C_{18} reversed-phase column (150×2.1 mm, particle size 3.5 μm) was from Waters (Milford, MA, USA) and the Spherisorb ODS2 and Inertsil ODS2 columns (250×4.6 mm, 5 μm particle size) were obtained from Chrompack International (Middelburg, The Netherlands). Columns were operated at 30°C.

For LC–scanning-MS and LC–parent-MS–MS analyses, a gradient of mobile phase A (water–acetonitrile–acetic acid, 96:2:2, v/v) to mobile phase B (acetonitrile–acetic acid, 98:2, v/v) was used. Standards and samples were separated by a gradient from 0% B to 74% B over 50 min followed by 100% B for 6 min. For LC–single ion recording-MS (LC–SIR-MS) and LC–multiple reaction monitoring MS–MS (LC–MRM-MS–MS) analyses an isocratic elution of mobile phase A–mobile phase B (77:33) was used. The flow-rate in all experiments was 0.3 ml min^{-1}.

2.5. Mass spectrometry

The Micromass Quattro II (Micromass, Manchester, UK) triple quadrupole MS system was operated using the ESI source. All measurements were carried out using positive ESI, except for LC–SIR-MS, which was done in the negative ESI mode.

For optimisation of the MS parameters an approximately equimolar test mixture of the four (−)-epicatechins and the four theaflavins, dissolved in HPLC mobile phase A, was used. For optimisation of the LC–parent-MS–MS analyses the test mixture was separated on an LC column using the gradient HPLC system described above. Repetitive on-column injections of the test mixture (approximately 70 μmol l^{-1} $compound^{-1}$), were made at varying parameter settings. For the LC–scanning-MS and the LC–parent-MS–MS a source temperature of 120°C and a desolvation temperature of 250°C were optimal. Nitrogen was used as nebulizer gas and drying gas at flow-rates of 400 and 25 l h^{-1}, respectively. The capillary voltage was 4.0 kV and the cone voltage was ramped from 21 to 44 V over a range of

150 to 2000 u. For LC–scanning-MS the scan range was 250 to 2000 u, the scan speed 2 s scan^{-1} and the interscan time 0.03 s. For the LC–parent-MS–MS the scan range of MS1 was 250 to 2000 u, the daughter ion detected in MS2 was at m/z 139, the scan speed 2 s scan^{-1} and the interscan time 0.03 s. The collision gas was argon at a pressure of $2.3 \cdot 10^{-3}$ mbar and a collision energy of 27 eV.

For the optimisation of the LC–SIR-MS parameters repetitive on-column injections of the test mixture were made using the isocratic HPLC system described above. A source temperature of 100°C and a desolvation temperature of 300°C were optimal for the LC–SIR-MS analysis. The optimum nitrogen gas flow was 200 l h^{-1} for the drying gas and 30 l h^{-1} for the nebulizer gas. Capillary voltage was 4.0 kV and the cone voltage was 40 V. The dwell time was 0.5 s, the inter-channel delay 0.03 s and the span 0.2 u. For SIR-MS the SIR mass was 562.9 (negative ESI).

For the optimisation of the LC–MRM-MS–MS the source settings obtained in LC–SIR-MS optimisation and the collision energy and collision gas pressure obtained from the LC–parent-MS–MS optimisation were used. The settings for the parent mass and the daughter mass were 565.0 and 139.0 u, respectively.

3. Results

3.1. Choice of HPLC column

Initially, two standard C_{18} HPLC columns (Spherisorb ODS2, Inertsil ODS2) that were frequently used in flavonoid analysis were tested. When low levels of theaflavin were injected, these columns retained this flavonoid infinitely. In contrast, the Symmetry C_{18} column displayed no secondary retention effects with theaflavins, even when very low levels (nmol l^{-1} range) were injected. Therefore, this column was used in all further experiments.

3.2. Continuous flow MS experiments

A test mixture containing equimolar amounts of the four (−)-epicatechins and the four theaflavins was measured in ESI and APCI (atmospheric pressure chemical ionisation) in positive and negative modes. Several post-column additives (sodium iodide, lithium chloride, diammonium citrate, ammonium acetate, acetic acid) were tested during continuous introduction of the test mixture (20 μmol l^{-1} compound^{-1}). The best results were obtained using ESI without post-column addition. Daughter-ion-MS–MS was performed on all eight model compounds. The fragmentation mechanism of (−)-epicatechins and theaflavins as deduced from in the daughter ion analysis in positive-ESI are shown in Figs. 1 and 2, respectively.

With exception of (−)-epicatechin gallate, cleavage a, yielding fragment ion m/z 139, gave the highest relative intensity in all catechin and theaflavin daughter-MS–MS spectra (Table 1). In negative ionisation mode, m/z 125 was the fragment ion with the highest intensity, but due to the large amount of fragments formed, this ion represented only 12% of the parent ion.

3.3. LC–ESI-parent-MS–MS

Different amounts of theaflavin (0.3–30 mg l^{-1}) were injected on-column (Fig. 3). The detection limit of pure theaflavin, defined as a signal-to-noise ratio of 3 to 1, was 60 μg l^{-1} for the UV signal, and 300

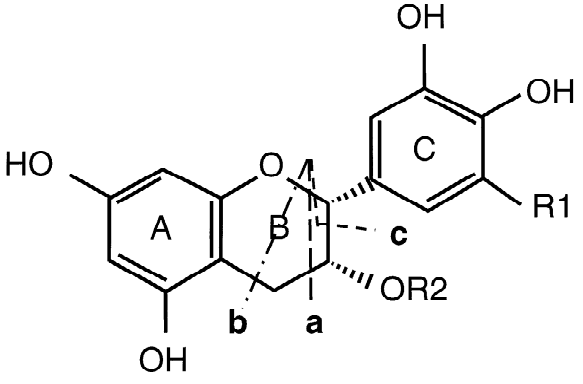

Fig. 1. Proposed fragmentation mechanism of (−)-epicatechins in positive ESI-collision induced dissociation-daughter-ion-MS–MS as adapted from Lin et al. [22]. Analysis was performed on (−)-epicatechin (R1=H, R2=H), (−)-epigallocatechin (R1=OH, R2=H), (−)-epicatechin gallate (R1=H, R2=gallic acid) and (−)-epigallocatechin gallate (R1=OH, R2=gallic acid). Gallic acid=3,4,5-trihydroxybenzoic acid. m/z values of the resulting daughter ions of the model compounds are given in Table 1.

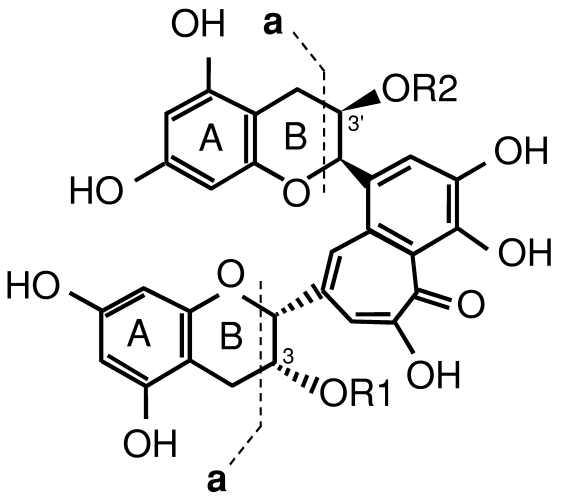

Fig. 2. Proposed fragmentation mechanism of theaflavins in positive ESI-collision induced dissociation-daughter-ion-MS–MS. Analysis was performed on theaflavin (R1=H, R2=H), theaflavin-3-gallate (R1=gallic acid, R2=H), theaflavin-3′-gallate (R1=H, R2=gallic acid) and theaflavin-3,3′-digallate (R1=R2=gallic acid). Gallic acid=3,4,5-trihydroxybenzoic acid. Line a denotes the proposed cleavages during collision induced dissociation. m/z values of the resulting daughter ions of the model compounds are given in Table 1.

$\mu g\ l^{-1}$ for the LC–parent-MS–MS signal. In theory, the detection limits for plasma and urine samples were 20-fold lower because the dried residue of the extraction of 1 ml plasma or urine was dissolved in 50 μl before injection. The background signal of the plasma and urine extracts, however, limited the sensitivity severely.

In the extract of a control sample taken after consumption of green tea, peaks of (−)-epicatechin and (−)-epigallocatechin (gallates were removed during enzymatic deconjugation) could easily be detected (Fig. 4). In addition, an ion with a parent m/z 321, a daughter of m/z 139 and a retention time of 21 min was detected. This ion was identified as methylated (−)-epigallocatechin.

A HPLC peak that produced the characteristic fragment of m/z 139 in LC–parent-MS–MS analysis was detected in the urine samples from the male volunteer collected at 1, 2, 4 and 7 h after theaflavin consumption. The $[M+H]^+$ parent ion producing the fragment had a m/z of 565. Together with the retention time of 47 min, these values indicated that the parent molecule was unmodified theaflavin. No gallated theaflavins were detected, presumably because the gallate group was removed during the enzymatic deconjugation reaction. The maximum urinary theaflavin concentration was estimated to be 10 $\mu g\ l^{-1}$, which is just below the calculated detection limit of 15 $\mu g\ l^{-1}$. Careful inspection of the traces of the urine samples of the female volunteer indicated that theaflavin might also be present. In the extracts of the plasma samples no theaflavin could be detected.

3.4. LC–SIR-MS

Theaflavin ionises in both positive and negative ESI modes with similar sensitivities. Since negative ESI is more selective, the LC–SIR-MS method was set-up in the negative mode. The dynamic range of the optimised LC–SIR-MS was at least three orders of magnitude (6–6000 $\mu g\ l^{-1}$) and the detection limit of pure theaflavin was 6 $\mu g\ l^{-1}$ on-column.

Table 1
Collision induced-positive ESI-daughter ion-MS–MS of (−)-epicatechins and theaflavins

Compound	Parent ion (m/z)	Cleavage a (m/z)	Cleavage b (m/z)	Cleavage c (m/z)	Others (m/z)
Epicatechin	291	139 (100)	165 (40)	123 (65)	151 (8)
Epicatechin gallate	443	139 (10)	165 (5)	123 (100)	151 (20), 273 (10), 291 (5)
Epigallocatechin	307	139 (100)	181 (15)	139 (100)	151 (12), 169 (5)
Epigallocatechin gallate	459	139 (100)	181 (3)	139 (100)	151 (38), 289 (20), 307 (5)
Theaflavin	565	139 (100)	–	–	151 (6), 427 (20)
Theaflavin-3-gallate	717	139 (100)	–	–	151 (9), 153 (20) 579 (17)
Theaflavin-3′-gallate	717	139 (100)	–	–	151 (8), 153 (25) 579 (22)
Theaflavin-3,3′-digallate	869	139 (100)	–	–	151 (8), 153 (38), 333 (22)

Intensities (in parentheses) are given as percentages relative to the most abundant fragment.

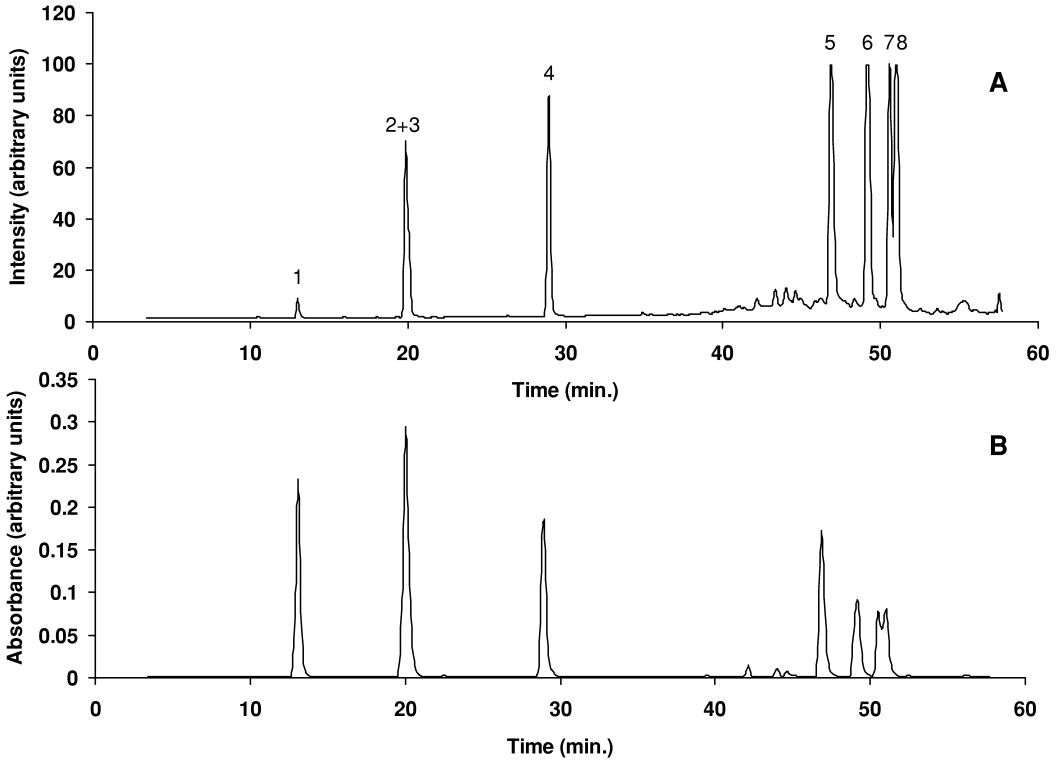

Fig. 3. Injection of an equimolar mixture of black tea flavonoids in a concentration of approximately 70 pmol μl^{-1} compound^{-1} on column. (A) Parents of m/z 139 monitored using LC–ESI-parent-MS–MS. (B) UV trace at 280 nm. Peaks in order of elution: (1) (−)-epigallocatechin; (2) (−)-epicatechin co-eluting with (3) (−)-epigallocatechin gallate; (4) (−)-epicatechin gallate; (5). theaflavin; (6) theaflavin-3-gallate; (7) theaflavin-3′-gallate; (8) theaflavin-3,3′-digallate.

However, when the sample extracts were injected, a significant, variable background signal at m/z 565 limited the detection of theaflavins severely.

3.5. LC–MRM-MS–MS

Injections of mobile phase containing different concentrations of pure theaflavin (0.6–6000 $\mu g\ l^{-1}$) were made on-column. The standard curve was linear and the dynamic range was at least three orders of magnitude (Pearson correlation coefficient 0.996). The detection limit (signal-to-noise ratio of more than 3:1) of pure theaflavin was approximately 1 $\mu g\ l^{-1}$ injected on-column. Thus, the detection limits could theoretically be as low as 0.05 $\mu g\ l^{-1}$ for plasma and urine samples.

Theaflavin could be detected in all plasma and urine samples collected 1, 2 and 4 h after consumption (Fig. 5). Maximum theaflavin levels detected in the plasma of the female and the male volunteer were 1.0 and 0.5 $\mu g\ l^{-1}$, respectively, and maximum urine levels were 0.6 and 4.2 $\mu g\ l^{-1}$, respectively.

The average relative standard deviation between the duplicates analysed was 4.4 and 17.1% for the plasma samples and the urine samples, respectively.

4. Discussion

There is a rapidly growing number of papers dealing with the application of LC–MS in the analysis of natural substances in food (see Careri et

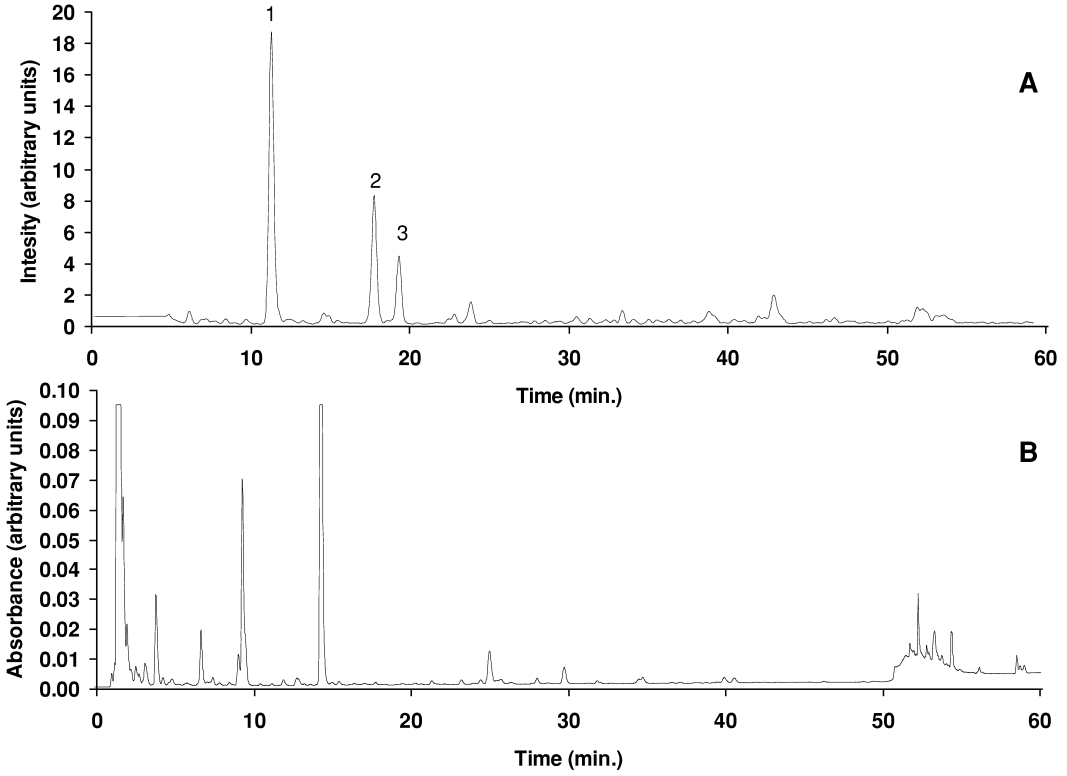

Fig. 4. Analysis of an extract from a deconjugated plasma sample obtained 2 h after the consumption of green tea. The extract separated by HPLC and analysed using UV absorption and parent-MS–MS. (A) Parents of m/z 139 as monitored using LC–ESI-parent-MS–MS. (B) UV trace at 280 nm. Peaks in order of elution: (1) (−)-epigallocatechin; (2) (−)-epicatechin; (3) 3-*O*-methyl-(−)-epigallocatechin.

al. [15] for review). Both LC–MS [17–19] and LC–MS–MS [20,21] have been applied for the analysis of catechins in tea extracts. Lin et al. [22] analysed a mixture of four catechins by thermospray LC–MS–MS. The collision-induced dissociation spectra they reported are almost identical to those obtained in the present study. A very similar fragmentation behaviour of catechins during electron-ionisation MS was reported recently by Miketova et al. [23]. The fragmentation behaviour of theaflavins, as reported by Chen et al. [24] using LC–APCI-MS, was also very similar to the pattern we proposed.

Apart from these mechanistic MS studies, LC–MS can also be used to detect the low concentrations of catechins [25] and quercetin-glycosides [26] in plasma, or isoflavones in urine [27]. These methods had detection limits of 100 mg l^{-1}, 300 μg l^{-1} and 5 μg l^{-1}, respectively. Coward et al. [28] developed an LC–MRM-MS–MS method for the measurement of isoflavones in plasma with a detection level of 0.3 μg l^{-1}. The detection limit of our LC–MRM-MS–MS was approximately 0.05 μg l^{-1} theaflavin. This high sensitivity can, at least partly, be explained by the lower background signal obtained by the use of LC–MRM-MS–MS instead of LC–SIR-MS.

The maximum theaflavin concentration detected in plasma was 1.0 μg l^{-1}. This level must be at least doubled to correct for the low recovery at these minute levels of theaflavin. Corresponding values for catechins in plasma are more than a 100-fold higher. Lee et al. [7] reported maximum plasma catechin levels of 500 μg l^{-1} after consumption of 235 mg catechins. Yang et al. [8] reported maximum plasma catechin concentrations of 300 μg l^{-1} after an oral dose of 300 mg catechins. Plasma half-life of theaflavin seemed to be in the order of 1–3 h, which

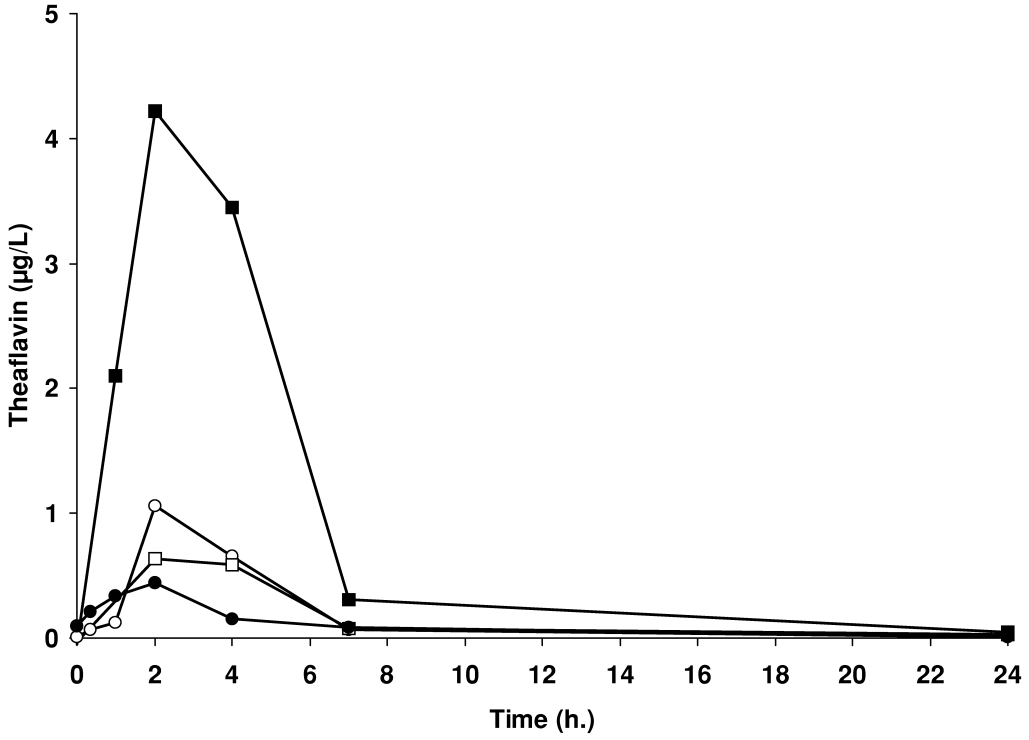

Fig. 5. Theaflavin concentrations in plasma and urine samples from two healthy volunteers who consumed 700 mg mixed theaflavins. Samples were treated with glucuronidase/sulfatase and extracted into ethyl acetate. The dried ethyl acetate extracts were re-dissolved and separated using reversed-phase HPLC. Theaflavin was detected using LC–MRM-MS–MS at parent ion m/z 565, daughter ion m/z 139. Open symbols=female volunteer, closed symbols=male volunteer. Circles=plasma, squares=urine.

is similar or slightly shorter than the values reported for catechins [8].

The maximum urinary theaflavin concentration was 4.2 μg l^{-1}. Total urinary theaflavin excretion during 24 h was calculated to amount to 4 μg theaflavin or less. This represents 0.0006% or less of the dose administered. In the study by Lee et al. [7] approximately 5 mg of the 235 mg (or 2%) of catechins ingested was recovered in 24 h urine.

A major drawback of the current study is that the assay can only detect theaflavin metabolites with an unmodified A and C ring. Metabolites, in which these ring systems were modified, escaped detection. Only uniformly carbon-labelled flavonoids will allow detection of nearly all substances that are derived from the labelled precursor. Until these labelled theaflavins are available the conclusion must be that consumption of black tea does not lead to significant levels of intact theaflavins in the blood.

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