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ORIGINAL PAPER

Chiral separation of (+)/(−)-catechin from sulfated and glucuronidated metabolites in human plasma after cocoa consumption

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Abstract Cocoa is well-known to be rich in flavan-3-ols. Previous analyses have established that alkaline treatment of cocoa beans results in epimerization of (−)-epicatechin to (−)-catechin and (+)-catechin to (+)-epicatechin. Now, the question is whether both epimers can be absorbed by the human organism. This paper describes sample preparation and an HPLC method for chiral determination of (+)/(−)-catechin from sulfated and glucuronidated metabolites in human plasma. The sample preparation includes enzymatic hydrolysis of the catechin metabolites, and solid-phase extraction (SPE). A PM-γ-cyclodextrin column is used with a coulometric electrode-array detection (CEAD) system. The recovery of catechin ranges from 89.9 to 96.8%. The limit of detection is 5.9 ng mL^{-1} for (−)-catechin and 6.8 ng mL^{-1} for (+)-catechin, and the limit of quantification is 12.8 ng mL^{-1} for (−)-catechin and 16.9 ng mL^{-1} for (+)-catechin. The relative standard deviation of the method ranges from 0.9 to 1.5%. This method was successfully applied to human plasma after consumption of a cocoa drink. In one human self-experiment, (+)-catechin and (−)-catechin were found in human plasma, but metabolism of the two enantiomers differed.

Benno F. Zimmermann was involved in the development of the SPE sample preparation.

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Introduction

Cacao (*Theobroma cacao* L.) naturally contains appreciable amounts of bioactive polyphenols, located in the cocoa beans. The main components are monomeric flavan-3-ols, called catechins (~37%), oligomeric flavan-3-ols, called procyanidins (~58%), and anthocyanins (~4%) [1]. Numerous studies reported, that these compounds elicit various physiological activities that potentially result in beneficial health effects [2–6]. Of great importance are the monomers (+)-catechin and (−)-epicatechin. It has been shown, that these monomeric flavan-3-ols pass well from the gastrointestinal tract into the bloodstream [7–9].

During the processing from the cocoa bean to cocoa powder and chocolate, chemical and enzymatic reactions occur. The alkaline treatment of the cocoa powder, known as *dutching*, has been found to catalyze the epimerization of (−)-epicatechin to (−)-catechin, and of (+)-catechin to (+)-epicatechin [10] (Fig. 1). The question arose was whether these newly formed, atypical enantiomers are as bioavailable as the naturally occurring enantiomers. A study on rats showed that the bioavailability of the epimers may be different [7, 11]. However, studies with animals cannot be transferred one-to-one to humans. So, it is indispensable to perform studies on humans.

The question of the different bioavailability of epimers can be clarified using chiral separation. The difficulty in analysis and chiral separation of catechins in biological matrices, like human plasma, is the low catechin concentration, the tendency of the compounds to bind to proteins, and the extent and nature of metabolic changes. Further-

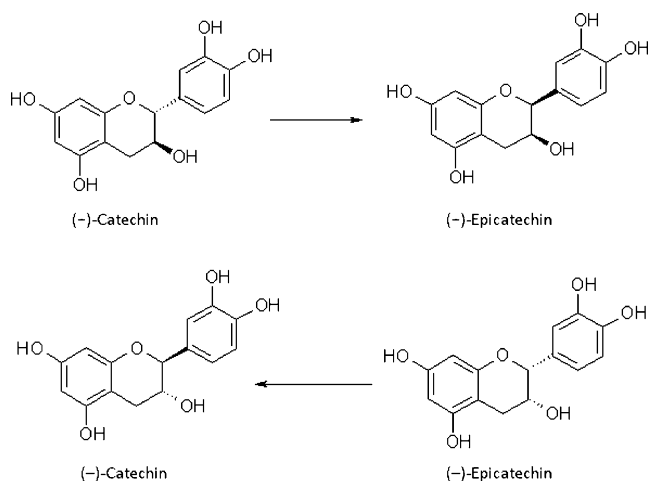

Fig. 1 Reaction of the epimerisation of the flavan-3-ols

more, catechins are susceptible to oxidation during sample collection, storage, and preparation. Oxidation can be minimized by acidic pH, and addition of ascorbic acid and ethylenediaminetetraacetic acid (EDTA) [12, 13]. Additionally, rapid sample preparation reduces exposure to oxygen.

This paper describes a HPLC method for chiral separation of (+)/(−)-catechin from glucuronidated and sulfated metabolites in human plasma after consumption of a cocoa drink.

Experimental

Materials and standards

A standard mixture of (+)/(−)-catechin of purity greater than 96% and (−)-epicatechin of purity greater than 90% were purchased from Sigma–Aldrich (Steinheim, Germany). (+)-Epicatechin was isolated from Guaranà *(Paullinia cupana var. sorbilis* KUNTH*)*. β-Glucuronidase type VII-A (EC 232-606-8) from *E. coli*, and sulfatase type VIII (EC 232-772-1) from abalone entrails were also obtained from Sigma–Aldrich. β-Glucuronidase (4.8 mg, 25,000 units) was suspended in 500 µL phosphate buffer (75 mmol L^{-1}, pH 6.8) (50 U μL^{-1}) and sulfatase (34.5 mg, 1,000 units) was suspended in 250 µL phosphate buffer (4 U μL^{-1}). The solvents methanol and acetonitrile (HPLC grade) were from Fisher Scientific (Loughborough, UK). Dimethylformamide (DMF) and sodium dihydrogen phosphate (NaH_2PO_4) were purchased from Merck (Darmstadt, Germany).

The polyamide solid-phase extraction (SPE) cartridges (Chromabond PA, 3 mL/500 mg) and the C_{18} SPE cartridges (Chromabond C18 hydrate, 3 mL/500 mg) were obtained from Macherey–Nagel (Düren, Germany).

Alkalized cocoa powder was bought in a local store. The chromatograms shown in Fig. 2 contrast the (+)/(−)-catechin and (+)/(−)-epicatechin ratio for a non-alkalized cocoa (obtained from Schokinag, Mannheim, Germany) with the ratio for the alkalized cocoa used in the self-experiment. The cocoa drink contained 46 g cocoa powder and 20 g sucrose dissolved in 250 mL full-fat milk. This is 1 g cocoa powder per kg body weight (BW).

Collection and storage of blood samples

All plasma samples were obtained in a self-experiment from a healthy female volunteer (33 years old, 46 kg BW, non smoker). First, blood was drawn after one day of a non-polyphenol diet and fasting for 12 h. Blood plasma thus obtained was found to be free of polyphenols. The volunteer then consumed a cocoa drink (the composition of which is given in the section Materials and standards) or 225 mg (+)/(−)-catechin standard dissolved in 250 mL full-fat milk. After consuming the cocoa drink or the (+)/(−)-

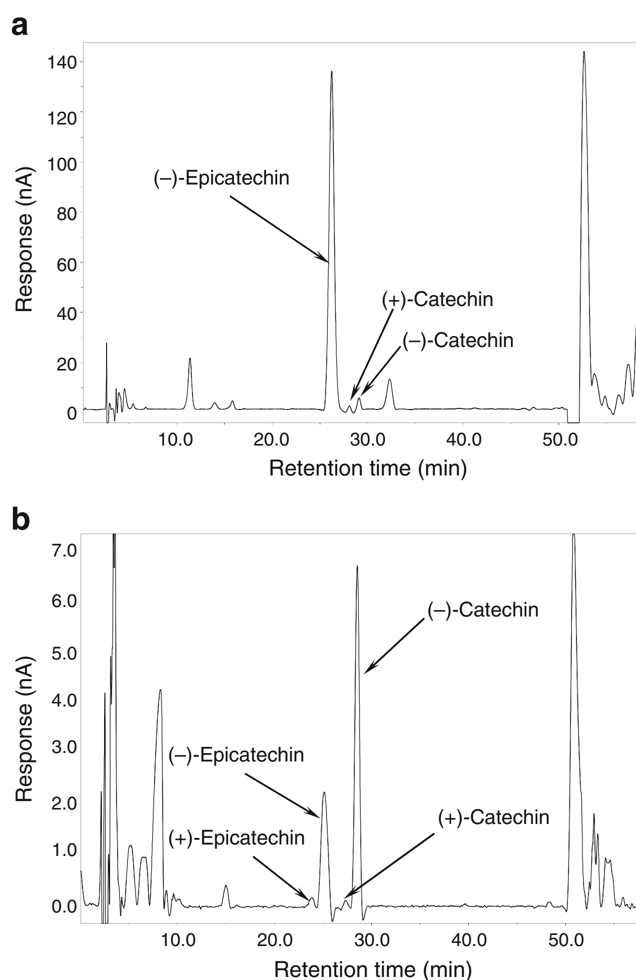

Fig. 2 Chromatograms obtained from a non-alkalized cocoa (**a**) and an alkalized cocoa (**b**)

catechin drink, no beverages or food were consumed until blood withdrawal. Two hours after consumption of the drinks, blood was withdrawn again.

Immediately after blood withdrawal into EDTA tubes, the blood samples were cooled with ice and centrifuged for 15 min at 4000 rpm. The blood plasma obtained was divided into 500-μL portions in 1.5-mL tubes (Eppendorf), stabilized with 10 μL of an antioxidative solution [14] and frozen at −80°C. Catechins have been shown to be stable for at least 6 months under these conditions [13–16].

Preparation of the blood plasma samples

For deconjugation of sulfates and glucuronides in plasma, 6250 U β-glucuronidase and 250 U sulfatase were added to 500 μL human plasma. The sample was mixed and incubated at 37°C for 45 min.

The human plasma was applied to an SPE polyamide cartridge directly after enzymatic hydrolysis, and washed with 10 mL H_2O, to remove the proteins. The flavan-3-ols were eluted with acetone–H_2O–acetic acid 70:29.8:0.2 (*v*/*v*). The solvent was reduced to 200 μL under a nitrogen stream at 40°C and 20-μL volumes were injected for HPLC analysis.

HPLC instrumentation

Chiral HPLC analysis was performed with an ESA (Chelmsford, MA, USA) HPLC system equipped with a model 5600 coulometric electrode array detector (CEAD) with four electrodes, a Degasys DG-1310 degasser (Uniflow, Tokyo, Japan), two pumps (580 solvent-delivery modules), a M800 mixer (Kontron, Neufahrn, Germany), a pulse damper (#14-0177), a model 540 autosampler with 100-μL injection loop and tray cooling (set at 4°C), and a Mistral column oven (set at 30°C).

The column was a Nucleodex PM-γ-cyclodextrin, 200 mm length, 4 mm i.d., particle size 5 μm (Macherey–Nagel) and an Aqua RP18, 4 mm, 3 mm i.d. security guard column (Phenomenex, Aschaffenburg, Germany).

The mobile phase was a gradient prepared from 20 mmol L^{-1} aqueous NaH_2PO_4 buffer at pH 3.4 (eluent A) and a 2:1 (*v*/*v*) mixture of acetonitrile and eluent A (eluent B). The gradient conditions (time: percentage of eluent B) were: 0 min: 5%; 34 min: 27%; 46 min: 33%; 47 min: 100%; 52 min: 100%; 55 min: 5%; 68 min: 5%; between the indicated points the gradient was linear. The flow rate was set at 0.6 mL min^{-1}.

The catechins were oxidized at low potentials, thus the potentials of the four electrodes were set at 0, 100, 200, and 300 mV. In this potential range, other compounds did not interfere with the flavanol peaks.

For quantification of the catechins, the sum of peak areas at 100, 200, and 300 mV was calculated. The peaks of (+)/(−)-catechin and the peak of (−)-epicatechin were identified by comparison of retention times and voltamograms with those obtained from pure reference compounds. Figure 3 shows the voltamograms obtained from (+) and (−)-catechin. Those obtained from (+) and (−)-epicatechin look the same.

Method validation

Limits of detection (LOD) and limits of quantification (LOQ) were calculated according to DIN 32645 [17]. This standard specification defines detection, identification, and quantification limits and contains formulae for their calculation.

For experimental assessment of the LOD and LOQ, blank plasma was spiked with (+)/(−)-catechin standards of different concentrations and the optimized sample preparation method was applied. Nine concentration values from 6 ng mL^{-1} to 600 ng mL^{-1} were analyzed in triplicate. The linearity, r^2, for the nine concentration values, cited above was calculated by plotting the peak area at each concentration against the respective spiked concentration.

Reproducibility of the optimized method was determined by analysis of blank plasma samples spiked with a concentration of 25 ng mL^{-1} (+)/(−)-catechin standard. The spiked samples were measured six times. The relative standard deviation was calculated.

For determination of recovery, blank plasma samples were spiked with (+)/(−)-catechin standard at 100 ng mL^{-1}. The optimized sample preparation method was applied. The analytical results were compared to those obtained from aqueous solutions of standards at the same concentration as the spiked plasma samples.

Results and discussion

Sample preparation

Solid-phase extraction

In an initial approach, the plasma proteins were precipitated with DMF mixed with trichloroacetic acid (TCA) as published by Zimmermann et al. [14]. After this sample preparation, it was not possible to separate the catechin enantiomers. This is caused by interactions between the TCA molecules and the cyclodextrins of the chiral column.

Some methods in the literature [11, 16] use liquid–liquid extraction with ethyl acetate to extract catechin from plasma. It has been shown that recovery by this kind of sample preparation is not very effective. For epicatechin, recovery is about 42% and for *O*-methyl-EC is 45% [18]. Furthermore, this method of extraction has relatively low

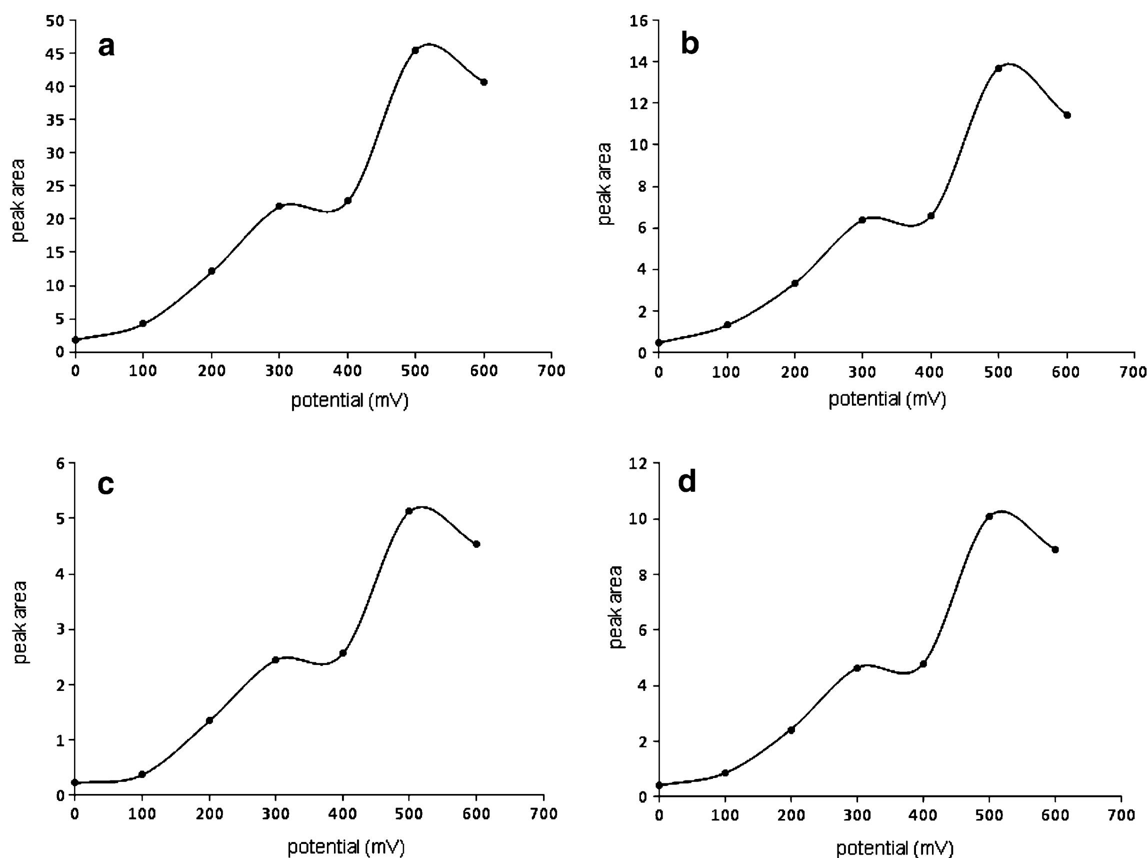

Fig. 3 Voltamograms obtained from the catechin enantiomers: (+)-catechin in a standard solution (**a**), (−)-catechin in a standard solution (**b**), (+)-catechin in human plasma after consumption of cocoa drink (**c**), and (−)-catechin after consumption of cocoa drink (**d**)

reproducibility [16, 19] and is too non-specific because of the co-extraction of polar, or non-polar matrices. Many interfering peaks co-elute with the catechins [15].

Therefore, a solid-phase extraction method was used to remove the plasma proteins and to concentrate the catechins. A C_{18} SPE cartridge was examined with 100% methanol as eluent. This solid-phase has problems similar to those of extraction with ethyl acetate in that it is also a non-specific method of extraction. Recovery is substantially better (about 72–76%) than for liquid–liquid extraction.

A polyamide cartridge with a mixture of acetone, H_2O, and acetic acid as eluent was investigated.

In comparison with extraction with ethyl acetate, the SPE method with polyamide cartridges improved both recovery (90% to 97%) and reproducibility. An additional positive aspect of SPE is the possibility of an automation using an ASPEC (automated sample preparation with extraction cartridges).

Enzymatic deconjugation

Catechin and epicatechin are metabolized completely into glucuronides, sulfates, and methylated derivates by the human organism [20–23] and cannot be found as free catechins in plasma. A number of publications [22–25] report that catechin is found only as glucuronides and methylated derivatives in plasma, but not as sulfates. To discover whether or not catechin exists as sulfates in human plasma, sulfatase was added to plasma in addition to β-glucuronidase. An increase in the catechin concentration (sum of (+)-catechin and (−)-catechin) during incubation with both enzymes was noticed. To verify this result, human plasma was treated with sulfatase only. After that, unconjugated catechin was also detected. Hence, catechin is not only glucuronidated and methylated but also sulfated in human plasma. Further investigation of the interrelation of β-glucuronidase and sulfatase is essential.

Further, the concentrations of β-glucuronidase and sulfatase were gradually increased until no further increase in catechin concentration was registered, to examine the amounts of enzymes required. The results showed, that 6250 U β-glucuronidase and 250 U sulfatase are needed for 500 μL human plasma to hydrolyze all glucuronidated and sulfated catechins. In comparison with Lee et al. [26], who used 625 U β-glucuronidase and 2.5 U sulfatase to hydrolyze tea catechins in 500 μL plasma, 10-fold more β-glucuronidase and 100-fold more sulfatase were needed. Figure 4 illustrates, using the example of β-glucuronidase, how catechin concentration increased with increasing

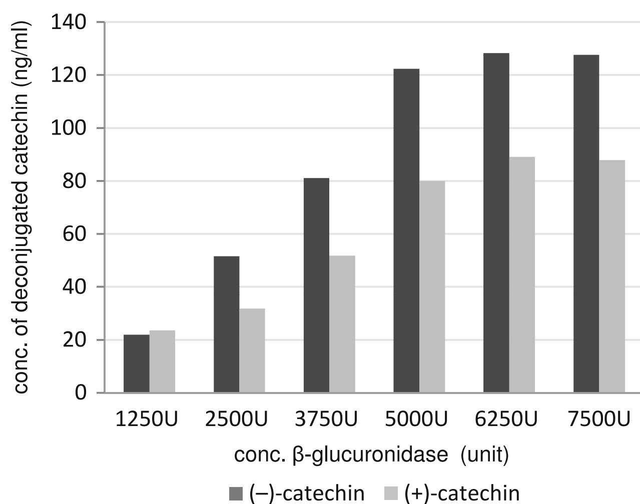

Fig. 4 The effect of β-glucuronidase concentration on the amount of deconjugated (+) and (−)-catechin. For this experiment human plasma two hours after cocoa consumption was used

quantity of the enzyme. A similar diagram was obtained for sulfatase concentration (not shown).

Summary

In brief, the optimized sample preparation for human plasma containing catechin enantiomers includes the following steps:

1. incubation of 500 µL human plasma at 37°C for 45 min with 6250 U β-glucuronidase and 250 U sulfatase;
2. SPE on polyamide cartridges and elution of the catechins with 4 mL acetone–H_2O–acetic acid 70:29.8:0.2 (*v*/*v*); and
3. solvent evaporation with a nitrogen-stream.

Chiral separation method

At the beginning of method development, pure standard solutions and human plasma free from polyphenols spiked with (+)/(−)-catechin of different concentrations were used.

In an initial approach, the usefulness of a β-cyclodextrin HPLC column (Cyclobond I-2000 RSP 250×4.6 mm; Astec, Whippany, NJ, USA) was examined with 90% mobile phase A and 10% mobile phase B and a flow rate of 1 mL min^{-1} (isocratic) (mobile phase A: 50 mmol L^{-1} NaH_2PO_4 at pH 3.0 and mobile phase B: acetonitrile/30 mmol L^{-1} NaH_2PO_4 at pH 3.0, 80:20 *v*/*v*). With this approach, chiral separation of (+)/(−)-catechin standards was possible.

However, human plasma is a complex matrix containing many different compounds. These plasma compounds cause interferences in the chromatogram. In the method mentioned above, the catechin peaks were overlapped by other compounds. This difficulty could not be remedied by using the method from Cooper et al. [27], who describe chiral HPLC separation of (+)/(−)-catechin and (+)/(−)-epicatechin in chocolate.

In a second approach, we took advantage of our experience in chiral separation of (+)/(−)-catechin and (+)/(−)-epicatechin by capillary electrophoresis. In that work a γ-cyclodextrin was used as chiral selector [10]. So, a γ-cyclodextrin HPLC column was tested. All other HPLC conditions are given in the section HPLC instrumentation.

With the method described here, (+)/(−)-catechin and (+)/(−)-epicatechin were successfully separated in a standard solution, in a cocoa extract, and in human plasma. Figure 2(b) demonstrates the chiral separation of (+)/(−)-catechin and (+)/(−)-epicatechin in a cocoa extract.

Application of the method to human plasma after cocoa consumption

The described method was applied to human plasma samples obtained after consumption of a cocoa drink. Human plasma obtained before and 2 h after drinking the cocoa drink was measured after treatment with β-glucuronidase and sulfatase. Representative chromatograms are shown in Fig. 5(a) and (b). Each of these plasma samples was also measured without enzymatic hydrolysis (Fig. 5c). Chromatograms B and C confirm already existing statements, about the metabolism of the catechins [20–23]. They are mostly metabolized and can only be found in a very small proportion as free catechins in human plasma.

The consumed cocoa contained 0.076 mg g^{-1} (+)-catechin and 0.35 mg g^{-1} (−)-catechin. Hence, the human volunteer consumed a total of 3.6 mg (+)-catechin and 16.6 mg (−)-catechin. The ratio of (+)-catechin to (−)-catechin in the cocoa drink was 0.22. After cocoa consumption, the concentrations of (+) and (−)-catechin, which result after enzymatic hydrolysis from the sulfated and glucuronidated metabolites and from the free catechins in plasma, average 22.1 ng mL^{-1} and 27.5 ng mL^{-1} for (+)-catechin and (−)-catechin, respectively. The ratio of (+)-catechin to (−)-catechin shifted from 0.22 in the drink to 0.80 in plasma.

This shift of ratio was confirmed in another experiment: the female volunteer consumed 225 mg (+)/(−)-catechin standard mixture with 250 mL milk. The consumed drink contained 103.5 mg (+)-catechin and 121.5 mg (−)-catechin (ratio 0.81). After consumption of the (+)/(−)-catechin drink, the concentrations of (+) and (−)-catechins in plasma were 255 ng mL^{-1} and 63 ng mL^{-1}, respectively. The ratio of (+)-catechin to (−)-catechin shifted from 0.81 in the drink to 4.05 in plasma.

In both experiments (consumption of cocoa or pure (+)/(−)-catechin) more glucuronidated and sulfated (+)-catechin

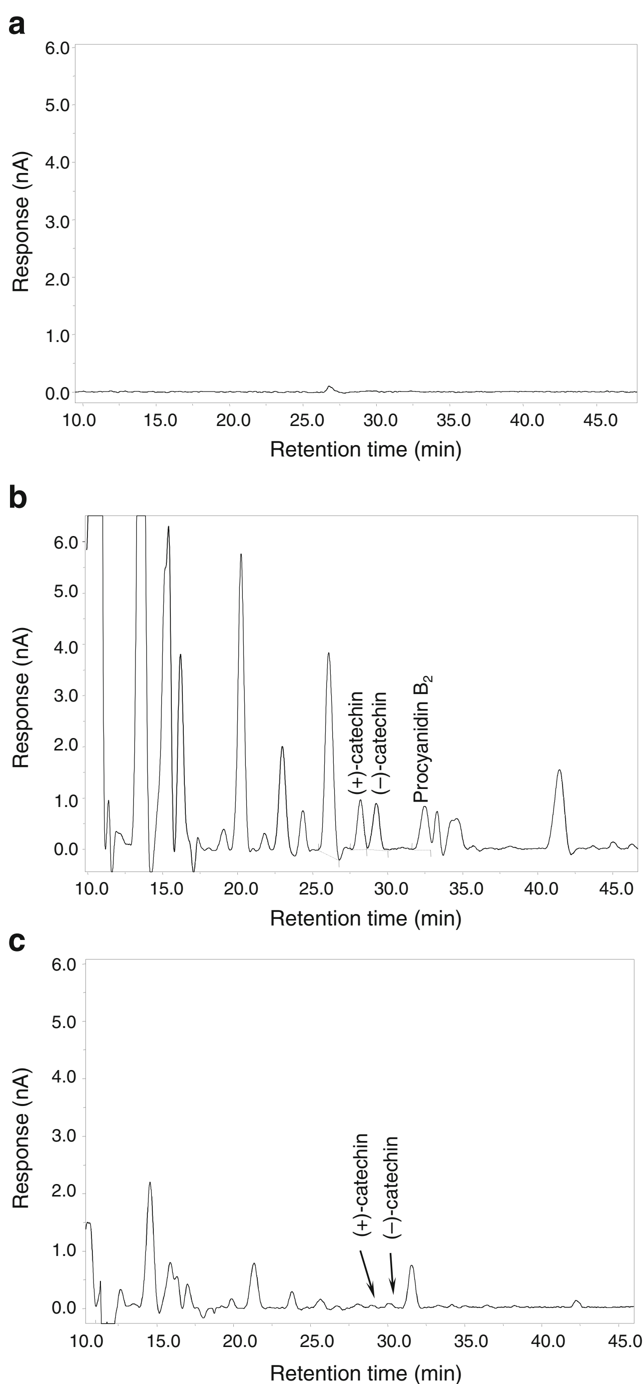

Fig. 5 HPLC–CEAD chromatograms (at 200 mV) obtained from human plasma after 12 h fasting and before drinking cocoa (**a**), from plasma 2 h after consumption of 250 mL cocoa drink and after enzyme treatment (**b**), and from plasma 2 h after consumption of 250 mL cocoa drink, but without enzymatic hydrolysis (**c**)

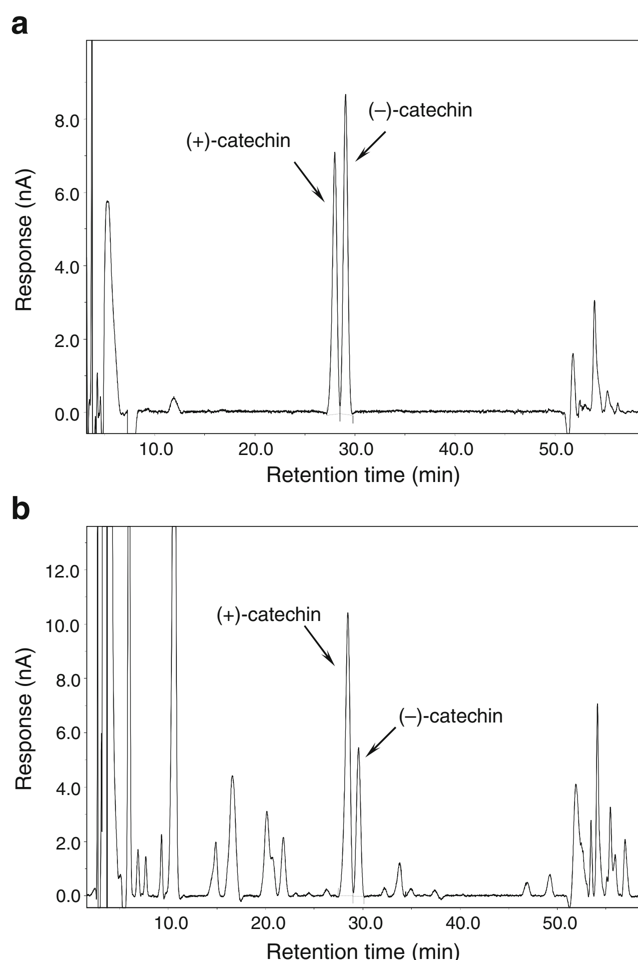

Fig. 6 HPLC–CEAD Chromatograms (at 200 mV) obtained from pure (+)/(−)-catechin standard (**a**) and from plasma 2 h after consumption of 250 mg (+)/(−)catechin standard (**b**)

than (−)-catechin was found, relative to the amounts consumed. Supposing that the enzymes used for deconjugation are not stereo-specific, the values of both experiments suggest that (−)-catechin has a lower absorption rate than (+)-catechin or another means of metabolism, e.g. another rate of methylation. We prefer the hypothesis of lower absorption, but cannot yet exclude the hypothesis of different conjugation.

Representative chromatograms obtained in these experiments are shown in Figs. 5(b) and 6. In addition to the (+)/(−)-catechin and (−)-epicatechin peaks, the chromatogram in Fig. 5(b) shows a peak of procyanidin B2. So, this finding confirms the result of Holt et al. [28], who found procyanidin B2 in human plasma after consumption of flavanol-rich cocoa.

Table 1 Limit of detection (LOD), limit of quantification (LOQ), relative standard deviation (R.S.D.), recovery (rec), and relative standard deviation of recovery (R.S.D. rec) of the developed method

	LOD (ng/ml)	LOQ (ng/ml)	R.S.D. (%)	rec. (%)	R.S.D. rec (%)
(+)-catechin	6.8	16.9	0.97	89.9	1.5
(−)-catechin	5.9	12.8	1.54	92.3	1.1
total catechin	5.9	12.7	0.89	96.8	1.4

The chromatogram obtained from human plasma after cocoa consumption (Fig. 5b) does not show (+)-epicatechin, even though the cocoa used for consumption contains (+)-epicatechin (Fig. 2b). The reason for this is the very small amount of (+)-catechin in untreated cocoa. During the epimerization only small amounts of (+)-epicatechin could arise from (+)-catechin. Because of these low concentrations, it is not possible to detect (+)-epicatechin in human plasma.

The objective of the method described here was to chirally separate the flavan-3-ol epimers from the glucuronidated and sulfated metabolites in human plasma. So it must be pointed out that the methylated catechins are not recorded by this method, because methylated catechins cannot be released by enzymatic hydrolysis. Surely, one of the unidentified peaks in the chromatogram in Fig. 5(b) could be that of methylated catechin, but exact classification of these unknown peaks is not yet possible. There are, furthermore, discrepancies with regard to the potentials which are necessary to detect methylated catechins with the coulometric electrode array detector. Because of the blockade of the hydroxyl group at the catechol ring by methylation, it is quite possible that the methylated metabolites can only be detected at potentials higher than 300 mV [29]. In contrast, Donovan et al. [11] detected 3′-*O*-methylcatechin at 285 mV.

Method validation

Recovery, reproducibility (standard deviation, S.D.), and LOD and LOQ for (+)/(−)-catechin are given in Table 1. Recovery of total catechins in our chiral separation method is marginally lower than the highest recovery in the literature, measured with non-chiral methods [30–32]. The reproducibility lies within the range of non-chiral techniques [31]. Better values for LOD and LOQ are only achieved by a few non-chiral methods or by more laborious methods [33, 34]. The linearity, r^2, for the range 6–600 ng mL^{-1} in human plasma was greater than 0.9980. Considering these validation values, it can be assumed that this chiral HPLC method is accurate, sensitive, and selective.

Conclusion

This publication describes a method for chiral separation of (+)/(−)-catechin from glucuronidated and sulfated metabolites in human plasma. Parts of method development were presented in a poster session at the Deutscher Lebensmittelchemikertag 2009 in Berlin [35]. To our knowledge, no chromatogram yet exists which shows chiral separation of the enantiomers of catechin in human plasma. Moreover, this method yields high recovery and reproducibility, and low values of LOD and LOQ. In a one human (female) self-experiment, it was shown that both (+)-catechin and (−)-catechin migrate into human plasma after consumption of cocoa drink. It was also shown that atypical enantiomers of the flavan-3-ols have another means of metabolism than the enantiomers occurring naturally in the cocoa bean.

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