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Validated application of a new high-performance liquid chromatographic method for the determination of selected flavonoids and phenolic acids in human plasma using electrochemical detection

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Abstract

We developed a sensitive method for determination of 23 flavonoids and phenolic acids, which represent phenolic acids and five subclasses of flavonoids. Plasma samples were extracted with selective solid-phase-extraction columns and separated by RP-high-performance liquid chromatography (HPLC). For detection an electrochemical detector was used. Identification and test of purity were carried out via retention times and spectra analyses. Limits of detection varied from 1.45 to 22.27 nmol/l. Recovery varied from 81% to 106%. Reproducibility for all analytes was below 10% (coefficient of variation, CV (%)) and ranged between 3.1% and 9.8%. This method can be applied to samples from interventional studies as well as observational studies.

Keywords: Flavonoids; Phenolic acids; Polyphenols; HPLC; Biomarker; Diet

1. Introduction

Flavonoids and phenolic acids are potentially bioactive polyphenols, which are commonly found in food plant. In vitro studies and animal studies indicate a notable preventive potential for inhibition of cancer and other chronic diseases [1]. There are a variety of physiological benefits, which the polyphenols are held credit for, including antioxidant activity, antithrombotic, anticarcinogenic, antibacterial, anti-inflammatory and immuno-modulating effects [2–4]. Since the estimation of supply can be afflicted with considerable methodical shortcomings, measurement of biomarkers of intake gives important and valid information needed for evaluation of postulated effects in man. Yet still, our insight on polyphenol bioavailability, metabolism and synergistic impact in man is scarce and conflicting. This lack of information is connected with the lack of sensitive, convenient methods for measuring an extended spectrum of polyphenols in plasma and body fluids, compared with the number of methods devised for their investigation in food [5–7]. Many existing studies examine flavonoids from one specific subclass, but diets may contain a mixture of several representatives of different subclasses. This study poses a new, highly sensitive and reliable method for analysing plasma concentrations of selected components from each of the subclasses catechins (catechin, gallocatechin, epicatechin, epigallocatechin, epigallocatechingallat), flavonols (quercetin, kaempferol, isorhamnetin, fisetin (internal standard)), flavanones (hesperetin, naringenin), flavones (apigenin, luteolin), isoflavones (daidzein, genistein), hydroxybenzoic acids (protocatechuic acid, gentisic acid, vanillic acid, syringic acid, salicylic acid, ellagic acid) and hydroxycinnamic acids (ferulic acid, caffeic acid, *p*-cumaric acid). To give an example for the possible use of the method, we measured polyphenol concentrations in fasting plasma

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samples obtained from 41 subjects who followed their regular diet.

2. Experimental

2.1. Chemicals and solvents

(−)-Gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epicatechin, (−)-epigallocatechingallat, vanillic acid, caffeic acid, salicylic acid, hesperetin and Sulfatase from Helix pomatia [EC 3.1.6.1] (secondary activity: beta-glucuronidase) were purchased from Sigma (Taufkirchen, Germany). Protocatechuic acid, syringic acid, *p*-cumaric acid, ferulic acid, ellagic acid, daidzein, quercetin, genistein, luteolin, kaempferol, and apigenin were obtained from Fluka (Taufkirchen, Germany). Fisetin and gentisinic acid were acquired from Aldrich (Taufkirchen, Germany). Naringenin was purchased from ICN (Ingolstadt, Germany) and Isorhamnetin was purchased from Roth (Karlsruhe, Germany). All used chemicals were of analytical or HPLC grade. Methanol was obtained from J.T. Baker (Deventer, Netherlands). Water was purified (<0.056 μS/cm) using a water-purification system from SG Wasseraufbereitung und Regeneration GmbH (Hamburg, Germany). Sodium acetate, ascorbic acid and acetic acid were acquired from Merck (Darmstadt, Germany).

2.2. Preparation of stock solution and standard curves

Stock solutions were prepared by dissolving 1.8–2.2 mg of standard substance in 10 ml methanol. Each solution was sonicated until completely dissolved and stored at −20 °C until analysis. Standard solutions were obtained by diluting each stock solution at ratios of 1/10, 1/20, 1/50, 1/100, 1/1000 and 1/10,000 in methanol/0.1% phosphoric acid at pH 2.3 (3/2, v/v).

2.3. Sample preparation and hydrolysis

To a volume of 1 ml plasma, 110 μl of sodium acetate (0.78 M) and 100 μl of ascorbic acid (0.1 M) were added.100 μl fisetin (1.93 μg/ml) was added as internal standard. After adding 232.5 U sulfatase and vortex-mixing for 1 min, the mixture was incubated for 30 min at 37 °C. Incubation was stopped by adding 20 μl of phosphoric acid (85%).

2.4. Two-dimensional solid-phase extraction

Solid-phase cartridges (Oasis HLB, Waters) were conditioned with 1 ml of methanol and equilibrated with 1 ml of water at a flow of less than 10 ml/min. The cartridge was loaded with 1 ml of previously prepared plasma at a flow of 2 ml/min. A washing step was applied, in order to cleanse the polyphenols of matrix interferences at less than 10 ml/min with washing-solution. To determine the appropriate composition of the wash-solution the following test series was set up. Two percent aqueous acetic acid was mixed with methanol. The content of methanol was increased in 5% steps. Fig. 1 shows the results in the particular methanol eluate for quercetin and salicylic acid as prominent representatives for flavonols and hydroxybenzoic acids. With 5% of methanol in the wash-solution, recovery in the methanolic extract was maximal. Best results for recovery could be obtained for the following mixture of which was, therefore, used for all further analyses: 1 ml of 5% methanol + 2% acetic acid in water. The polyphenols were eluted with 2 ml methanol at a rate of 2 ml/min and dried under vacuum. The residue was then resolved with 100 μl extract-solution methanol/H_2O (60/40; v/v) and transferred into vials (Chromacol, Welwyn Garden city, UK) for high-performance liquid chromatography (HPLC) analysis.

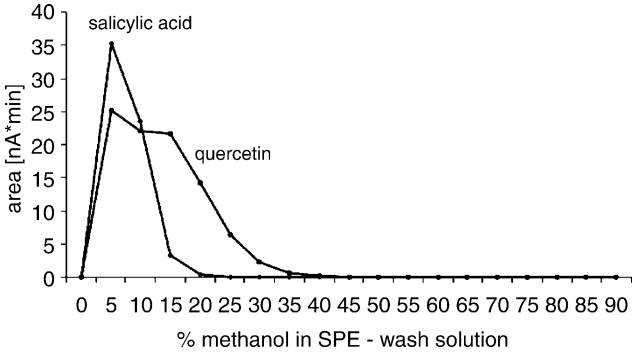

Fig. 1. Optimization of methanol concentration in washing solution within SPE-extraction (cleaning step).

2.5. HPLC parameters

A 30 μl-aliquot of the prepared extract and of a mixture of all standards (1/20 dilution) was injected by autosampler GINA50 from Gynkotek (Germering, Germany). The analytes were separated on a column-system, consisting of two analytical columns put in row. Temperature was held constantly at 60 °C by column thermostat for HPLC-STC 585 from Gynkotek (Germering, Germany). First column was Luna RP-C18, 5 μm (4.6 mm × 150 mm) column from Phenomenex (Aschaffenburg, Germany), second was Zorbax Eclipse XDB-C18, 3.5 μm (3.0 mm × 150 mm) column from Agilent Technologies (Böblingen, Germany). The columns were protected by Eclipse XDB-C8 Narrow-Bore, 5 μm (2.1 mm × 12.5 mm) Guard Column from Agilent Technologies (Böblingen, Germany). This combination of columns was necessary in order to achieve the necessary resolution for all substances. The mobile phase consisted of two solvents: A (water adjusted to pH 2.1 with phosphoric acid) and B (methanol). The gradient elution programme is given in Table 1. We used a degasser (DegaSys DG 1310) from Dionex (Idstein, Germany). The pump was a high-pressure gradient pump P580 from Dionex (Idstein, Germany). Flow rate was kept at 0.4 ml/min at all times. Chromatograms were

Table 1
Gradient elution program

Time	% B	Flow rate
0	0	0.4
15	18	
50	25	
65	40	
109	56	
110	100	
115	100	
120	0	

monitored on an electrochemical detector ED40 from Dionex (Idstein, Germany) at +1100 mV and recorded and analysed with a personal computer (Scenic Pro M5, Siemens Nixdorf). Direct-current voltage amperometric mode was used. For onward determination UV spectra were monitored using a diode array detector (DAD) UV/vis-detector UVD 340S purchased from Gynkotek (Germering, Germany). Flavonols and flavones have been monitored at 370 nm, flavanons at 287 nm, isoflavones, flavan-3-ols and phenolic acids at 225 nm.

2.6. Identification and quantification

For identification the retention times of the samples were compared to those of the standards. For confirmation of identity we compared the UV-spectra (diode array detector) of the peak in question with that of the referring standard. Quantification of polyphenols was performed by the internal standard method. Calibration and quantification were made for all compounds at +1100 mV with direct voltage amperometric electrochemical detector. Fisetin was used as internal standard, because it does not naturally occur in food commonly used for nutrition. Chromatography Data Collection has been executed with Chromeleon 6.3 software and chromatograms have been manually revised. Results were calculated with Microsoft Excel Version 9.0 and expressed as nmol/l.

2.7. Application to human blood samples

To check up on the applicability of this method for analysing selected polyphenols, human plasma was examined. Plasma samples from a subgroup of participants in the Bavarian Nutrition Survey II (BVS II) were used. The Bavarian Nutrition Survey II is a representative cross-sectional study where recruitment has been carried out by triple-stage random-route method. A validated 24 h recall was applied to get information on the dietary intake over the last 24 h. Three 24 h dietary recalls were obtained by means of standardized telephone interviews using the software EPIC-Soft. Nutrient intake was calculated by means of the German Food Composition Table BLS, Version II.3 (BgVV, Berlin, Germany). On weekdays, between 10.00 a.m. and 6.00 p.m. non-fasting venous blood was collected into EDTA tubes (S-Monovette, Sarstedt) and centrifuged at 2000 × *g* for 15 min at ambient temperature. Plasma samples were immediately cooled and stored at −80 °C until analysis. Anthropometric parameters such as weight, height and waist circumference were measured.

In addition, this method was also used to test for bioavailability of apigenin in humans after parsley intervention (manuscript submitted).

3. Results and discussion

3.1. HPLC

Example chromatograms for a standard mixture and a plasma sample spiked with the mixture of standards (20–120 nmol/l) are shown in Fig. 2. Additionally, an example for a chromatogram of a non-spiked plasma sample is included (Fig. 2c).

The entire procedure, including the stabilisation time lasts approximately 120 min. As this separation was conducted on two reversed phase columns, the most polar phenols (phenolic acids and catechins) were eluted first, followed by flavonols, isoflavones, flavanones and flavones in order of their respective polarity, which is mainly depending on hydroxyl groups at the B-ring [8]. Table 2 shows the 23 polyphenols in the order of their retention times. In some areas resolution was diminished but still all standards could be integrated. The combination of two columns resulted in accomplishing the separation in area peaks 6–9 and 17–21 (Fig. 2.), better resolution, peaks with less tailing, and more stable retention

Table 2
Retention times (mean, S.D.), linear range and limit of detection

Nr.	Name	RT		Linear range (nmol/l)	Limit of detection (nmol/l)
		(Mean)	(S.D.)		
1	Gallocatechin	18.20	0.03	6–2400	5.14
2	Protocatechuic acid	19.95	0.02	3–6700	2.59
3	Epigallocatechin	23.75	0.02	22–2500	22.27
4	Catechin	24.63	0.03	3–5100	2.97
5	Gentisinic acid	26.98	0.04	3–8700	2.56
6	Epigallocatechingallat	30.32	0.03	5–2000	3.29
7	Caffeic acid	30.94	0.03	4–10000	3.63
8	Vanillic acid	31.83	0.03	2–5300	2.05
9	Epicatechin	32.50	0.04	3–6100	1.72
10	Syringic acid	36.50	0.03	4–9600	4.18
11	*p*-Cumaric acid	42.33	0.04	2–10500	1.45
12	Ferulic acid	51.03	0.04	4–9600	4.13
13	Salicylic acid	63.19	0.06	6–15000	6.16
14	Ellagic acid	66.38	0.03	3–6100	2.53
15	Fisetin	74.26	0.03	3–7300	2.69
16	Daidzein	78.32	0.02	5–6900	1.54
17	Quercetin	82.28	0.03	3–5700	5.31
18	Naringenin	83.22	0.02	5–7100	2.85
19	Luteolin	86.24	0.03	3–7600	5.68
20	Genistein	87.43	0.03	6–7600	1.60
21	Hesperetin	88.90	0.02	2–7100	2.55
22	Kaempferol	93.70	0.03	4–6000	1.70
23	Apigenin	95.97	0.03	4–7700	2.39
24	Isorhamnetin	98.18	0.04	3–4400	2.18

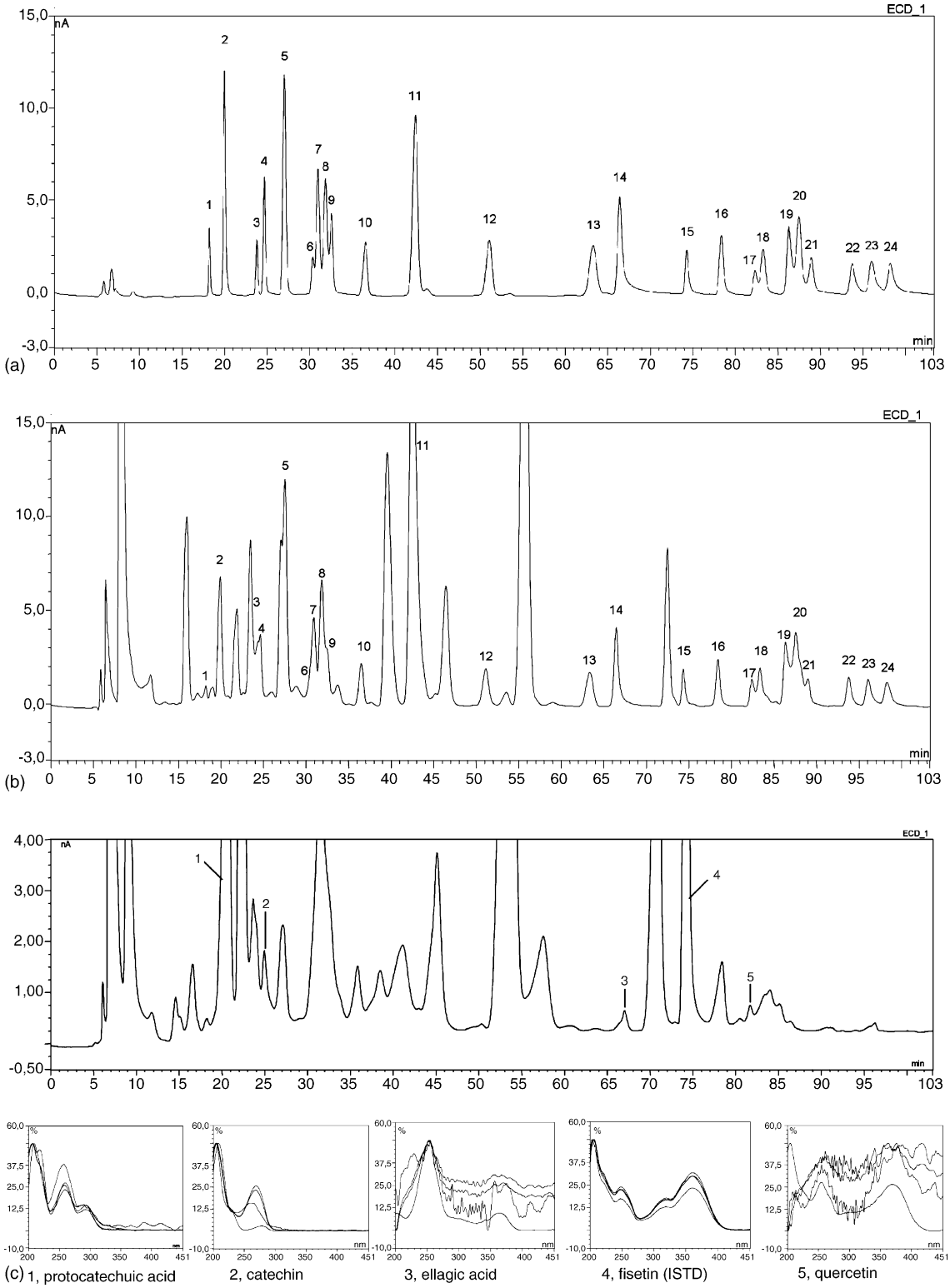

Fig. 2. (a) Typical chromatogram of a mixture of standards. (b) Chromatogram of human plasma, spiked with a mixture of standard substances: (1) gallocatechin [0.02 μmol/l], (2) protocatechuic acid (0.11 μmol/l), (3) epigallocatechin (0.06 μmol/l), (4) catechin (0.06 μmol/l), (5) gentisic acid (0.11 μmol/l), (6) epigallocatechingallat (0.02 μmol/l), (7) caffeic acid (0.10 μmol/l), (8) vanillic acid (0.10 μmol/l), (9) epicatechin (0.04 μmol/l), (10) syringic acid (0.09 μmol/l), (11) *p*-cumaric acid (0.10 μmol/l), (12) ferulic acid (0.09 μmol/l), (13) salicylic acid (0.12 μmol/l), (14) ellagic acid (0.05 μmol/l), (15) fisetin (ISTD, 0.06 μmol/l) (16) daidzein (0.06 μmol/l), (17) quercetin (0.05 μmol/l), (18) naringenin (0.06 μmol/l), (19) luteolin (0.06 μmol/l), (20) genistein (0.06 μmol/l), (21) hesperetin (0.06 μmol/l), (22) kaempferol (0.06 μmol/l), (23) apigenin (0.06 μmol/l), (24) isorhamnetin (0.05 μmol/l). (c) Chromatogram shows detected polyphenols from a fasting plasma sample. UV-spectra from detected polyphenols were measured with HPLC–DAD: 1, protocatechuic acid; 2, catechin; 3, ellagic acid; 4, fisetin (ISTD); 5, quercetin.

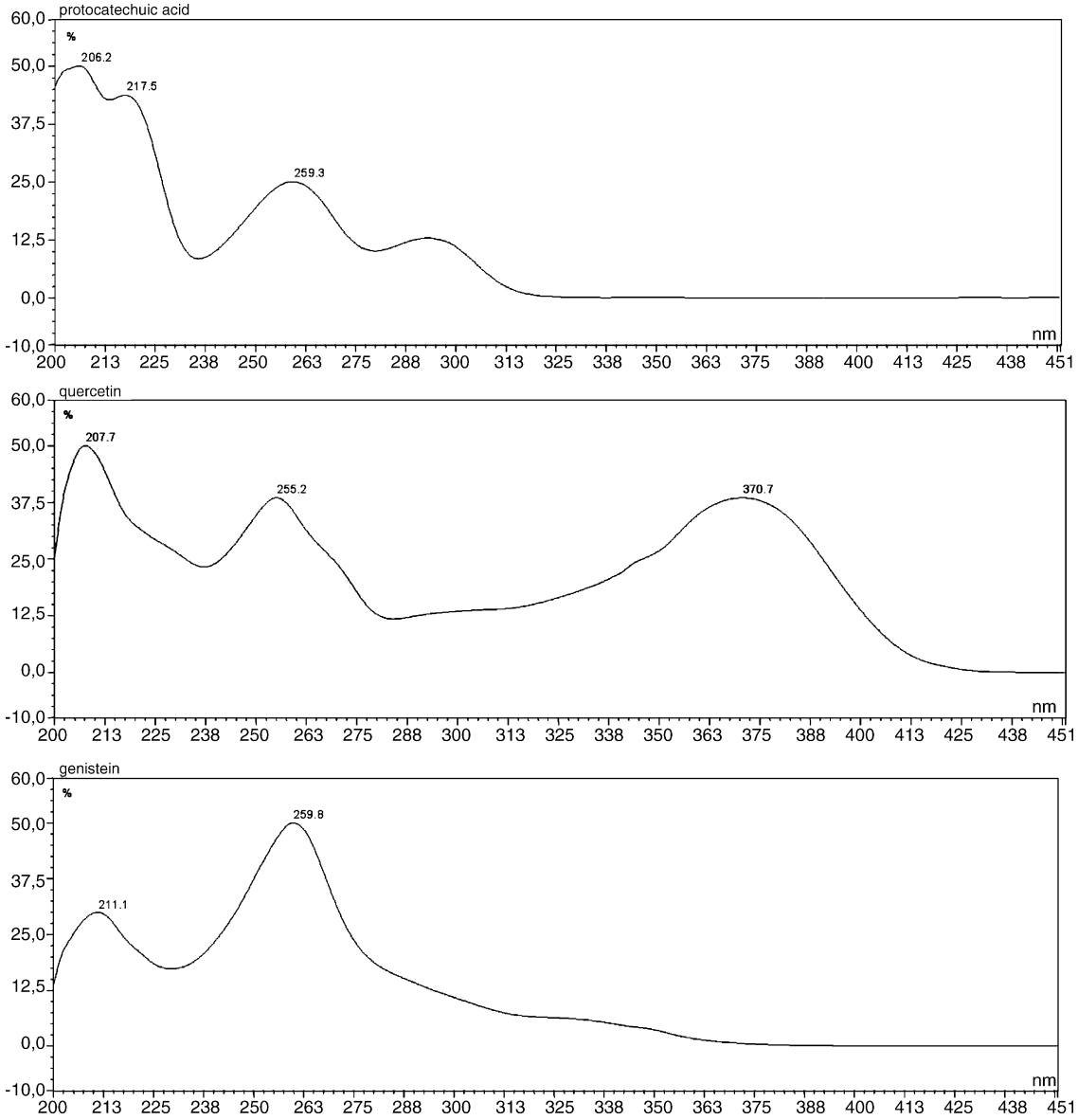

Fig. 3. UV-spectra of the standards of protocatechuic acid, quercetin and genistein, measured with diode array detector (HPLC–DAD) in methanol/water.

times. Peak identification was realised by retention times and further confirmed using UV spectra (Fig. 3). Using only Luna RP-C18 column from Phenomenex resulted in a good separation of phenolic acids from polar catechins as well as separation of some flavonoid subclasses. However, catechins (epigallocatechingallat and epicatechin) and species of flavones, flavonols, and isoflavones (quercetin, naringenin and kaempferol, apigenin) could not be separated. In previous publications it has been shown that Zorbax Eclipse XDB-C18 column (Agilent Technologies) was already capable of very useful separation characteristics [9]. To enhance performance, Zorbax Eclipse XDB-C18 column was attached to Luna RP-C18 column (Phenomenex), and by varying column temperature, gradient characteristics, flow and pressure, HPLC parameters were set to the given values. Thus, we were able to optimise the previous results, as can be seen in Fig. 2a. Large peaks appeared at retention times 6–10 min, 20–23 min, 54–58 min and 69–71 min (Fig. 2) which may be ghost peaks since they correspond to about 6 min after changing of solvent components (Table 1), probably caused by non-faradaic reaction (base line shifts). However, identification and quantification of the substances of interest was not affected.

There are a lot of publications that deal with the investigation of phenolic substances from various materials. According to a literature review RP-HPLC with UV–VIS photodiode array detection is the most widespread chromatographic technique for phenolic acids [10].

Separation systems for polyphenols in food have been oriented towards the measurement of prominent flavonoids in a single food such as e.g. grape and wine [11,12], tea [13–16], apples [17,18] or procedures for specific measurement of

flavonoid concentrations in several commonly consumed foods [5,6]. Many of these systems have been employed to investigate several aspects related to plant physiology, biosynthesis, etc. The results of this work forms the basis of food consumption tables for flavonoids and phenolic acids. However, only very few HPLC procedures have been developed that separate and measure prominent food polyphenols which are members of five flavonoid subclasses [8,19]. A great number of analytical investigations on polyphenolic substances are carried out in biological matrices, that aim at a better understanding of the purported role of polyphenols in the protection against diseases caused by oxidative damage [20,21] or their bioavailability [22,23]. Until now such studies concentrated on few specific substances like quercetin [24], apigenin [25] or subclasses like isoflavones [26,27]. The need for further proceedings at in vivo studies as well as a method to simultaneously measure the prominent five flavonoid subclasses (catechins, flavonols, flavanones, flavones, isoflavones) and both phenolic acid subclasses (hydroxycinnamic acid and hydroxybenzoic acid) has repeatedly been expressed [28,10]. Our method covers these requirements and has been developed to further those proceedings in polyphenol research and to provide a procedure that can be applied to various human studies. UV–VIS with photodiode array is the most prevalent detection technique for HPLC investigations of polyphenolic compounds. Other less common detection systems that provide increased specificity are electrochemical, fluorescence and mass selective detectors [10]. It is well known, that mass selective detection is a distinguished detection technology. At present, however, there is no HPLC/MS, LC/MS or GC/MS method known to the authors that covers the range of 24 polyphenols and that is applicable in epidemiological studies and is therefore suitable to give comparable data on the issue, although LC/MS is becoming more common [10,29]. Kammerer et al. stated that their work on polyphenol screening of pomace from red and white grape varieties by HPLC–DAD–MS/MS is the first study presenting comprehensive data on the contents of phenolic compounds comprising all polyphenolic subclasses [30]. To compare our results with HPLC–ESI-MS data, plasma samples (0.5–1.0 ml) were examined by a co-operation partner (Dr. R.W. Owen, German Cancer Research Centre, Heidelberg [31]). Except for lignan glycosides and samples high in catechins or salicylic acid (aspirin use) polyphenols could hardly be detected due to a lack of sensitivity, especially for phenolic acids. Therefore, validation of results close to the detection limit could not be done by means of a MS-based method so far. For higher concentrations, the UV (DAD) spectra could easily be used to ensure specificity of the method. As true for most methods, results which are close to the detection limit has to be interpreted with caution.

3.2. Linearity and limit of detection

Linearity of the chromatographic analysis was checked by linear regression. It was established by plotting the resulting peak areas of a series of dilutions of each polyphenol against the known concentration of the analyte in the standard solution. The regression coefficients were linear and $r^2 > 0.99$ for all compounds. Limit of detection was defined as that concentration, which generated a peak at a signal to noise ratio of three. The limits were tested by duplicate analysis and are based on eight 30 μl injections (Table 2). Concerning the sensitivity of the method, validation was conducted with pooled, low polyphenol, spiked human plasma. Content of polyphenols in pooled plasma was subtracted from total concentration. Our detection limits ranged between 1.45 and 22.27 nmol/l for the selected standards (Table 2).

Detection limits of 1.9 nmol/l for electrochemical detection (quercetin) [32], 17.2 nmol/l for fluorescence detection (catechin) [24] and 275 nmol/l for UV-detection [32] have been previously reported.

3.3. Recovery and reproducibility

Recovery of this method was determined by measuring the percentage of recovery after reagent blank and pooled human plasma have been spiked with known amounts of standard compounds. As analyte-free matrix is not possible, pooled human plasma was analysed likewise and results were subtracted from spiked human plasma concentrations. Each survey was accomplished by duplicate analysis. Comparison of the reagent blanc (dest. water) recovery with that of plasma indicates that all compounds are quantitatively extracted by two-dimensional solid phase extraction (Table 3). Reproducibility was recorded by duplicate analysis of eight spiked human plasma samples as intra-day assay and by duplicate analysis of six spiked human plasma samples over 5 days as inter-day assay (Table 4). The intra- and inter-day assay results expressed as coefficients of variation (CV (%)) were less than 10% in every case and so resemble a method of sufficient accuracy for the intended applications.

3.4. Selectivity

After introducing a second analytical column, resolution was improved by modification of the eluent composition. Previously, the issue of selectivity and reproducibility has been approached by Dolan et al. [33,34]. Since our method should not only be applied to samples from intervention studies but also from observational studies, it was important to increase the detector sensitivity. HPLC detection for quantification was effectively carried out via direct-current voltage because of shown high selectivity and sensitivity towards phenolic substances and catecholamines [35,10]. Screening for dominant potential was accomplished by variation of electrochemical potentials and recording the produced peak area (refer to Fig. 4). Quercetin and kaempferol were used as routing substance in this process due to their frequency of occurrence in common food. Guo et al. previously reported that flavonoids had high oxidation potentials [36]. Additionally, peak identity was verified with photodiode-array detector, since this

Table 3
Recovery of added standard substances to reagent blanc (dest. water) and to human plasma

Compound	Added standard concentration (μmol/l)	Recovery from reagent balance[a] (dest. water) (%)	Analytical recovery from plasma[b] (%)
Gallocatechin	0.58	92.65 ± 3.11	81.32 ± 2.55
Protocatechuic acid	1.59	94.85 ± 1.62	93.10 ± 6.87
Epigallocatechin	0.59	98.94 ± 4.38	97.01 ± 4.71
Catechin	1.22	96.43 ± 1.39	98.55 ± 8.19
Gentisinic acid	2.10	98.11 ± 3.80	102.52 ± 7.51
Epigallocatechingallat	0.45	97.20 ± 2.92	100.89 ± 7.69
Caffeic acid	2.44	91.27 ± 5.01	106.79 ± 5.63
Vanillic acid	1.27	96.22 ± 4.04	88.45 ± 7.74
Epicatechin	1.51	97.73 ± 1.79	84.12 ± 5.95
Syringic acid	2.25	98.05 ± 1.91	83.36 ± 5.55
p-Cumaric acid	2.53	95.91 ± 2.76	98.90 ± 7.26
Ferulic acid	2.35	92.98 ± 4.42	102.58 ± 9.22
Salicylic acid	3.68	98.02 ± 3.37	105.43 ± 7.32
Ellagic acid	1.47	99.26 ± 1.50	95.98 ± 9.44
Daidzein	1.65	98.49 ± 1.22	96.14 ± 6.56
Quercetin	1.39	97.36 ± 3.25	101.30 ± 5.37
Naringenin	1.68	98.92 ± 2.13	95.31 ± 9.34
Luteolin	0.82	95.61 ± 1.22	94.73 ± 7.29
Genistein	1.83	96.71 ± 2.85	98.82 ± 7.67
Hesperetin	1.59	98.84 ± 3.04	85.84 ± 6.63
Kaempferol	1.38	97.71 ± 2.06	102.79 ± 8.05
Apigenin	1.86	99.36 ± 2.06	83.09 ± 4.51
Isorhamnetin	1.07	97.61 ± 1.68	101.03 ± 3.43

[a] Six runs.
[b] Eight runs.

Table 4
Reproducibility [mean ± S.D. (μmol/l) and coefficient of variation (CV; %)] of added standard substances

Compound	Intra-day[a]		Inter-day[b]	
	Mean ± S.D.	CV (%)	Mean ± S.D.	CV (%)
Gallocatechin	1.18 ± 0.03	2.86	1.20 ± 0.05	4.26
Protocatechuic acid	2.36 ± 0.04	1.86	2.41 ± 0.04	2.06
Epigallocatechin	1.62 ± 0.10	6.19	1.46 ± 0.03	2.61
Catechin	1.46 ± 0.02	1.56	1.49 ± 0.08	5.43
Gentisinic acid	2.77 ± 0.04	1.54	2.84 ± 0.13	4.85
Epigallocatechingallat	0.85 ± 0.05	6.49	0.82 ± 0.07	9.50
Caffeic acid	1.98 ± 0.02	0.98	2.03 ± 0.19	9.54
Vanillic acid	2.42 ± 0.04	1.84	2.50 ± 0.23	9.58
Epicatechin	1.43 ± 0.03	2.00	1.45 ± 0.05	3.48
Syringic acid	2.21 ± 0.05	2.36	2.25 ± 0.11	5.02
p-Cumaric acid	2.01 ± 0.04	2.00	2.07 ± 0.12	6.24
Ferulic acid	1.86 ± 0.05	2.50	1.89 ± 0.15	8.18
Salicylic acid	3.16 ± 0.09	2.87	3.18 ± 0.12	3.82
Ellagic acid	3.86 ± 0.10	2.54	3.97 ± 0.06	1.52
Daidzein	1.48 ± 0.03	1.75	1.51 ± 0.14	9.63
Quercetin	1.31 ± 0.06	4.64	1.31 ± 0.08	6.26
Naringenin	1.33 ± 0.03	2.62	1.36 ± 0.09	7.26
Luteolin	4.58 ± 0.09	1.98	4.68 ± 0.24	5.31
Genistein	1.36 ± 0.04	2.93	1.39 ± 0.05	3.81
Hesperetin	1.41 ± 0.05	3.49	1.44 ± 0.08	6.20
Kaempferol	1.47 ± 0.03	2.26	1.48 ± 0.08	5.88
Apigenin	1.58 ± 0.03	2.05	1.61 ± 0.11	7.40
Isorhamnetin	3.98 ± 0.08	1.89	4.06 ± 0.08	2.07

[a] Eight runs, duplicate assay.
[b] Five days, mean of six samples, duplicate assays.

method has been commonly used to monitor at wavelength from 190 to 380 nm [10]. The chromatogram in Fig. 2c shows the polyphenols detected in fasting plasma (ECD). The UV-spectra of the respective peaks indicates good selectivity for the polyphenols detected in fasting plasma. To diminish the possibility of interferences, resulting in use of high potentials, solid phase extraction was accomplished by using a macro-porous, co-polymeric [poly(divinyl-benzene-co-*N*-vinylpyrrolidone)] SPE-column, offering hydrophilic and lipophilic capacity (Oasis HLB, Waters). This phase was previously used by several researchers and displayed good performance [29,37,38]. Preliminary investigations on sample hydrolysis showed that 232.5 Units sulfatase are sufficient to complete hydrolysis. Longer incubation showed no recognisable enhancement of yield or performance. For

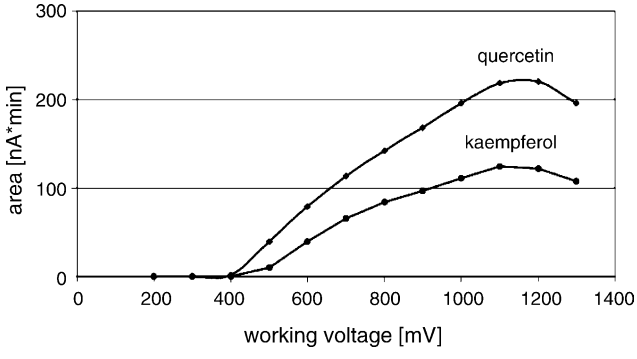

Fig. 4. Maximum detector response potential for quercetin (696.5 μmol/l) and kaempferol (687.6 μmol/l).

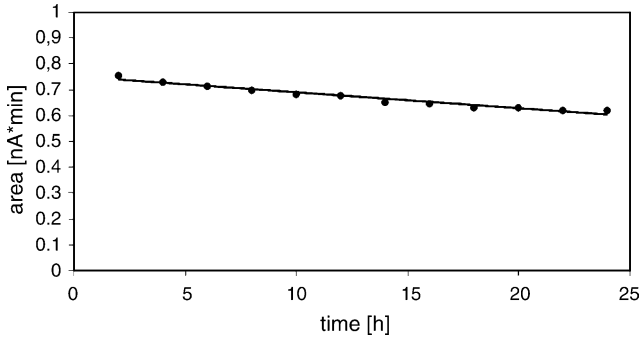

Fig. 5. Stability of epigallocatechin over 24 h, stabilized by addition of ascorbic acid (0.1 M).

enterolignans, completeness of hydrolysis with this method was confirmed by HPLC–MS technology.

3.5. Stability within work-up procedure

Considering the length of analysis there was set up a test series to determine the degree of degradation of polyphenols over 24 h at ambient temperature. Therefore, human plasma was pooled and spiked with 23-standard mixture. Spiked plasma was stored at ambient temperature and analysed over a course of 24 h. Peak areas were analysed at 12 points in time and change in detected peak area was recorded. The peak areas of the internal standard (fisetin) and the analytes changed by 9%, for both, internal standard and analytes likewise, during 24 h (see Fig. 5, shown for epigallocatechin). When no antioxidant for protection of polyphenols was applied, recovery of analytes was poor, ranging from 2% to 67% and considerably varying among substances and between days. To offer increased stability of the analytes during sample preparation and extraction we tested BHA, BHT, Trolox and ascorbic acid as antioxidants. Ascorbic acid was used for further analysis, as it proved to be most effective in protecting polyphenols from degradation.

3.6. Application to studies in human plasma samples

With plasma samples analysed under given conditions, interferences could not be entirely prevented in any case, especially at the lower concentrations. Interferences were diminished to the present degree by selection of sample preparation and system parameters as discussed before. Where interferences arouse, data acquisition was abandoned. Table 5 shows the concentrations of all analysed polyphenols in fasting plasma samples of 41 male test persons following their regular diet. The plasma samples were gained during the last three months in 2002 and, therefore, also reflect specific dietary habits before Christmas time in Germany. Every analyte could be found in plasma samples. As expected, the found concentrations cover a very far range. Lowest mean values of concentration were found for apigenin (9.3 nmol/l) and daidzein (10.1 nmol/l). Highest mean values were found for salicylic acid with 6990.04 nmol/l, including values of subjects with recent aspirin use. Due to a generally low soy intake in this population, a higher consumption of raisins (contained in bakery goods around Christmas time) can explain the higher genistein concentrations as compared to daidzein concentrations than found in other studies [27]. A

Table 5
Concentrations (nmol/l) of selected flavonoids and phenolic acids in fasting plasma samples of 41 men participating in a cross-sectional study

	Median	Mean ± S.D. (nmol/l)	Minimum	Maximum
Gallocatechin	68.6	91.2 ± 17.1	0.0[a]	561.9
Protocatechuic acid	413.0	520.8 ± 77.2	48.0	2641.9
Epigallocatechin	742.8	1031.3 ± 153.4	0.0[a]	4528.3
Catechin	82.1	107.8 ± 15.6	0.0[a]	388.1
Gentisinic acid	1357.8	2160.6 ± 275.9	0.0[a]	6849.7
Epigallocatechingallat	158.9	267.7 ± 48.6	0.0[a]	1348.7
Caffeic acid	372.4	477.3 ± 67.2	0.0[a]	2290.8
Vanillic acid	159.5	260.3 ± 42.7	0.0[a]	1121.6
Epicatechin	114.0	277.7 ± 78.5	0.0[a]	2542.9
Syringic acid	478.3	908.1 ± 203.5	0.0[a]	7103.0
p-Cumaric acid	644.6	1304.6 ± 254.3	0.0[a]	6103.4
Ferulic acid	460.6	652.1 ± 94.6	58.8	2904.4
Salicylic acid	339.1	6990.0 ± 3077.9	26.2	104468.3
Ellagic acid	53.8	140.1 ± 41.4	0.0[a]	1253.4
Daidzein	0.0[a]	10.1 ± 2.8	0.0[a]	59.9
Quercetin	78.5	108.7 ± 15.9	19.7	563.8
Naringenin	79.7	122.5 ± 21.7	0.0[a]	533.1
Luteolin	388.0	545.8 ± 80.8	0.0[a]	2555.2
Genistein	108.1	157.1 ± 24.1	0.0[a]	639.7
Hesperetin	27.6	37.2 ± 4.9	0.0[a]	151.7
Kaempferol	36.8	126.1 ± 40.4	0.0[a]	1356.8
Apigenin	5.3	9.3 ± 1.9	0.0[a]	52.5
Isorhamnetin	14.2	31.0 ± 7.3	0.0[a]	204.8

[a] Below limit of detection.

higher consumption of nuts and citrus fruits during this season also helps to explain the frequent detection of ellagic acid (from walnuts) and naringenin/hesperitin (from citrus fruits) in plasma samples of these subjects. For quercetin and ferulic acid, minimum values of 11.28 nmol/l and 36.99 nmol/l, respectively, were found (Table 5); for other substances minimum values were below detection limit. This should reflect a continuous intake of quercetin and ferulic acid by ordinary consumption of common foodstuff [39,40] in this population.

This sensitive method was also applied to plasma samples obtained from an interventional study in humans. After administration of parsley, apigenin concentrations in plasma and urine samples increased, thereby clearly confirming bioavailability of this compound from foodstuff. The results of this study are described in detail elsewhere (manuscript submitted).

4. Conclusion

This paper describes a validated RP-HPLC method for the identification and quantification of twenty-three polyphenols in form of their aglycons. Selected flavonoids and phenolic acids have been chosen to cover all five mayor subclasses of flavonoids and both phenolic acid subclasses and with regard to their sources in commonly consumed food. Special attention has been paid to the sensitivity of the method, particularly with regard to its possible implementation in epidemiological studies (observational and intervention studies) with limited sample volumes. The sensitivity and number of measurable polyphenols is distinguished and has been realised through the use of an electrochemical detector. Recovery and reproducibility mirror the accuracy and regularity of this method. This method can be used for measurement of other body fluids, like urine, with minor adjustments.

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