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Quantification of 38 dietary polyphenols in plasma by differential isotope labelling and liquid chromatography electrospray ionization tandem mass spectrometry

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ABSTRACT

Polyphenols constitute one of the most complex classes of phytochemicals in the human diet and have been suggested to play a role in the prevention of chronic diseases such as cardiovascular diseases, diabetes and cancers. However, evidence from epidemiological studies is still needed to better understand their role in disease prevention. To do so, robust methods for the accurate measurement of these molecules in large series of samples are needed. We report here the development of a highly-sensitive method based on differential isotope labelling with ^{13}C- and ^{12}C-dansyl chloride for the analysis of 38 structurally diverse polyphenols in 50 μL plasma by tandem mass spectrometry with limits of quantification varying between 0.11 to 44 nmol/L. Full validation of the method was achieved for 37 compounds out of the 38 tested. The method showed intra- and inter-batch coefficients of variations of 2.3–9.0% and 2.8–20.3% respectively depending on polyphenols when applied to 1163 plasma samples from the European Prospective Investigation on Cancer and Nutrition (EPIC) study. For the first time this method allowed to quantify with high accuracy and reproducibility a large selection of compounds representative of the main classes of dietary polyphenols in low volumes of plasma.

1. Introduction

Polyphenols form a large family of plant secondary metabolites, with more than 500 different compounds identified in foods and beverages such as coffee, tea, wine, fruits, vegetables and cereals [1,2]. They have raised considerable interest for their possible role in the prevention of major chronic diseases such as cardiovascular diseases, diabetes, cancers and neurodegenerative diseases in preclinical and clinical studies [3–5]. While a protective role against chronic diseases like diabetes and cardiovascular diseases has also been supported by a number of epidemiological studies [6,7], epidemiological evidence for cancer prevention is still limited and inconsistent [8–12]. This may be partly explained by insufficient accuracy of exposure assessments most often based on dietary questionnaires [8]. Biomarkers may constitute a good alternative to measure polyphenol exposures with improved accuracy. So far, most of the methods developed for the analyses of polyphenols in human samples were focused on a single or limited number of polyphenol biomarkers, mostly isoflavones and lignans [8]. Other polyphenol biomarkers such as alkylresorcinols and catechins have also been measured in some studies [13–15] but none of these studies did appraise the high complexity of polyphenol exposures in some large-scale epidemiological studies.

We previously described a method based on differential isotope labelling with ^{13}C- and ^{12}C-dansyl chloride which allows measuring 37 compounds from all main classes of dietary polyphenols in urine [16]. In this method, polyphenols in urine samples are successively deconjugated with enzymes, treated with ^{13}C-dansyl chloride to form ^{13}C-dansylated polyphenols, and mixed with a reference urine sample tagged with ^{12}C-dansyl chloride. Polyphenol concentrations are determined through measurement of ratios of signals for ^{13}C-labelled polyphenols and their ^{12}C-labelled analogs by tandem mass spectrometry.

We report here the development and validation of a method to measure 38 polyphenols in plasma samples, biospecimens most

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commonly available in large cohort studies and showing concentrations of polyphenols much lower than in urine. The method was applied to 1163 plasma samples from the European Prospective Investigation on Cancer and Nutrition (EPIC) study.

2. Experimental

2.1. Chemicals and reagents

Caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, quercetin, kaempferol, phloretin, (+)-catechin, (−)-epicatechin, (+)-gallocatechin, vanillic acid, enterolactone, enterodiol, isorhamnetin, hesperetin, naringenin, 3-(3,4-dihydroxyphenyl)-1-propanoic acid, 3-(3,5-dihydroxyphenyl)-1-propanoic acid, 3,5-dihydroxybenzoic acid, 3-hydroxyphenylacetic acid, dansyl chloride, type H1 β-glucuronidase from *Helix pomatia*, LC/MS grade methanol and acetonitrile and acetone for HPLC, formic acid as eluent additive for UHPLC–MS were purchased from Sigma-Aldrich (St-Louis, MO, USA). Homovanillic acid, (−)-epigallocatechin, procyanidin dimer B2 and procyanidin dimer B1 were purchased from Extrasynthese (Genay, France). Protocatechuic acid, 3- and 4-hydroxybenzoic acids, and ethyl acetate were purchased from Acros (Thermo Fischer Scientific, Geel, Belgium). Daidzein, genistein, (R,S)-equol, *m*-coumaric acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, resveratrol, apigenin, tyrosol, hydroxytyrosol and gallic acid ethyl ester were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). $^{13}C_2$-Dansyl chloride was synthesized as previously described [17].

2.2. Plasma samples

Citrated plasma samples were collected from subjects of the European Prospective Investigation on Cancer and Nutrition (EPIC) study (a large multicenter prospective cohort study designed to investigate the relation between diet, other lifestyle factors, environmental factors and cancer risk which consists of approximatively half a million participants) in 23 centres located in 10 European countries (Denmark, France, Germany, Greece, Italy, The Netherlands, Spain, Sweden, Norway and The United Kingdom) [18]. Samples analysed in this work (n = 1163) originated from 8 of the 10 countries (Denmark, France, Germany, Greece, Italy, The Netherlands, Spain and The United Kingdom) and were part of a case-control study on colorectal cancer nested in EPIC [19]. For evaluating precision, accuracy and recovery of the method, two sets of plasma samples were used: 'orphan' citrate plasma samples from the EPIC study for which any information on individuals except for the center of origin was missing, and citrate plasma samples from the Blood Donor Centre (Etablissement Français du Sang, Lyon, France).

2.3. Extraction and dansylation of polyphenols

Plasma samples were thawed at room temperature, centrifuged (5 min at 3000*g*) and treated with β-glucuronidase (EC: 3.2.1.31) type H1 from *H. pomatia (*also showing sulfatase activity). The enzyme was tested at different incubation times (1–24 h) and enzyme quantities (1500 and 3000 U), as well as for the presence of ascorbic acid.

The following conditions were finally selected: 50 μL plasma was treated with 25 μL of a solution of β-glucuronidase (1500 U) containing 10 μg ascorbic acid for 2 h at 37 °C. After hydrolysis, samples were extracted twice with ethyl acetate (400 + 200 μL) shaken at 1100 rpm for 10 min, and centrifuged for 5 min at 12,000*g*. The organic phase (540 μL) was evaporated for 30 min under vacuum at room temperature. Dried plasma extracts were dissolved in a ^{13}C-dansyl chloride solution (100 μg in 100 μL acetone) and carbonate buffer (pH = 9.5, 0.1 M, 100 μL) and kept at 60 °C for 30 min. Dansylated plasma samples were evaporated for 90 min under vacuum at 43 °C. Dried dansylated plasma extracts were redissolved in 100 μL acetonitrile/water mixture (1/1, v/v)(quenching).

For each analytical batch (50 samples), a pooled plasma sample was prepared by mixing equivolumes (10 μL) of every plasma sample analysed in the batch. This pooled sample was extracted and treated with ^{12}C-dansyl chloride in similar conditions. An aliquot (18 μL) of the dansylated pooled plasma sample was added to each dansylated plasma sample (18 μL) and the mixture directly analyzed by Ultra High Pressure Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (UHPLC-ESI–MS/MS).

2.4. Analysis of dansylated polyphenols by tandem mass spectrometry

UHPLC-ESI–MS/MS was performed on an Agilent 1290 UHPLC system coupled to a 5500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA). All dansylated plasma samples for a given batch were maintained at 10 °C in the autosampler until analysis. They were injected (10 μL) onto an Acquity CSH C18 column (Waters, 2.1 × 100 mm, 1.7 μm) maintained at 50 °C. Eluents were 0.1% formic acid in methanol/water 10/90 (v/v) (eluent A) and acetonitrile (eluent B). The gradient profile was: 0% B from 0 to 1 min, 0 to 50% B from 1 to 7 min, 50 to 60% from 7 to 9.5 min, 60 to 90% from 9.5 to 11.5 min, 90 to 100% B from 11.5 to 14 min, 100% B from 14 to 16 min, 100 to 90% B from 16 to 16.5 min, 90% B from 16.5 to 20 min, 90 to 0% B from 20 to 20.5 min. Flow rate varied as follows: 500 μL/min from 0 to 14 min, 500 to 700 μL/min from 14 to 16 min, 700 to 1300 μL/min from 16 to 16.5 min, 1300 μL/min from 16.5 to 20 min and 1300 to 500 μL/min from 20 to 20.5 min. Mass detection was carried out in scheduled MRM mode using a Turbo V-ion source operating in positive mode at 400 °C as described before [16]. The same transitions were used for each dansylated compound as described previously for urine analyses, apart from declustering potential (DP) and collision energy (CE) parameters which had been modified to voluntarily reduce sensitivity for the most highly concentrated compounds and avoid signal saturation [16]. The original DP and CE values, set as initially determined during optimization for urine analysis, have been used for these polyphenols to maximize sensitivity for analysis of plasma samples.

2.5. Quantification of dansylated polyphenols

Calibration curves were built using a mix of the 38 polyphenol standards at 11 concentration levels corresponding to expected concentrations for each compound in the plasma samples. Standards were dansylated with ^{13}C-dansyl chloride and mixed with the pooled plasma sample dansylated with ^{12}C-dansyl chloride. Quantification was carried out using the MultiQuant 3.1 software (AB Sciex, Foster City, CA). Calibration curves were established as previously reported [16]. The 1163 plasma samples from the EPIC colorectal cancer study were analyzed in 24 batches, with a calibration curve injected in single run along each batch. Three QC samples (two orphan citrate plasma samples from the EPIC study and one citrate plasma sample from Etablissement Français du Sang) were analyzed in duplicate within each batch, together with two blank samples [plasma is replaced by phosphate buffer saline solution (PBS)].

2.6. Method validation

The method was validated based on FDA guidelines [20]. Intra-batch precision was evaluated by repeated injection (n = 6) of three orphan samples from the EPIC study and three samples from

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Etablissement Francais du Sang, with different polyphenol concentrations, in a single batch. Inter-batch precision was evaluated on two orphan samples and one sample from Etablissement Francais du Sang analyzed in duplicate in each of the 24 batches over a 4-month period. Accuracy was evaluated by spiking four different plasma samples containing low polyphenol concentrations with standards at low and median concentrations along each calibration curve.

Matrix effect and recovery of extraction were determined through spiking polyphenol standards in a plasma sample before or after polyphenol extraction, with or without isotopic dilution with a reference sample of ^{12}C-dansylated polyphenols.

The matrix effect (ME) is defined as the ratio of the concentrations of a plasma sample spiked with polyphenol standards after extraction to the concentrations measured in a pure solution:

$$ME(\%) = (c\text{-}a)/d \times 100$$

where c is the concentration of polyphenols of plasma samples spiked after extraction, a is the concentration of polyphenols in the unspiked plasma sample, and d is the concentration of polyphenol standards measured in pure solution. Concentrations a, c and d were measured as peak intensities of the ^{13}C-dansylated polyphenols, and no correction with ^{12}C-labelled polyphenols is made.

The reduced matrix effect (RME) is defined as the same ratio with concentrations measured using the differential ^{12}C/^{13}C-isotope labelling method:

$$RME(\%) = (C\text{-}A)/D \times 100$$

where C is the concentration of polyphenols of plasma samples spiked after extraction, A is the concentration of polyphenols in the unspiked plasma samples, and D is the concentration of polyphenol standards in a pure solution.

Recovery of extraction (RE) is measured as the ratio of polyphenol concentrations of a plasma sample spiked before extraction and after extraction:

$$RE(\%) = (B\text{-}A)/(C\text{-}A) \times 100$$

where A and C are as defined above and B is the concentration of polyphenols in the plasma sample spiked before extraction.

Process efficiency (PE) [21] integrates recovery of extraction and matrix effects:

$$PE(\%) = RME \times RE/100 = (B\text{-}A)/D \times 100$$

Accuracy or dilution integrity was also evaluated by diluting four different plasma samples at ½ and ¼ in acetate buffer at 2 h and 24 h for hydrolysis.

LOQ was defined as the lowest point of the calibration giving an accuracy varying from 80% to 120% of the theoretical value.

3. Results

Thirty eight dietary polyphenols were selected for their widespread occurrence in foods or in human biofluids and for being representative of the main polyphenol classes (Table 1). They include phenolic acids (Fig. 1; peaks 1–17), flavonoids (peaks 18–33), one stilbene (peak 34), tyrosols (peaks 35–36) and lignans (peaks 37–38).

3.1. Polyphenol dansylation

The dansylation reaction was checked in full scan experiments (not shown) and found to be quantitative (all phenolic groups dansylated) as previously reported for the analysis of the same compounds in urine samples [16]. Most dansylated polyphenols were found to be stable for up to 13 h at 10 °C in the conditions of the reaction. However, quenching of the reaction was needed to limit their degradation beyond this period. As the analysis of a batch of 50 plasma samples together with calibration curve, QC and blank samples takes about 28 h, samples had to be quenched before injection. This was obtained by evaporation of the dansylated extracts under vacuum, followed by dissolving them in a water/acetonitrile mixture. Dansylated polyphenols were found to be stable over the 48 h following dansylation (see Supplementary data, Fig. S-1).

3.2. Quantification of dansylated polyphenols by tandem mass spectrometry

Dansylated polyphenols were quantified by tandem mass spectrometry (Fig. 1). Limit of quantification (LOQ) varied from 0.11 nM for apigenin to 44.4 nM for quercetin (Table 2). Within each analytical batch, two blank samples were added to assess the presence of possible polyphenol contaminants in the glucuronidase preparation [22]. Most of the 38 polyphenols were either absent (n = 16) in the blanks or present at concentrations lower than the lowest concentrations measured in any plasma samples (n = 17). Five polyphenols (protocatechuic acid, caffeic acid, gallic acid, *p*-coumaric acid and apigenin) were present in blanks at concentrations close to the median concentrations measured in the EPIC subjects. However, plasma dilution experiments (Table 1) showed that the contribution of the blanks was more significant at the highest level of dilution, while it was negligible for these five compounds before dilution, suggesting that blanks reflected poorly the contribution of polyphenol contaminants from the glucuronidase enzyme in real plasma samples.

Conditions of enzymatic hydrolysis were optimized. The optimal duration of hydrolysis was found to vary among the different polyphenols, and 2 h of hydrolysis was found to provide the best compromise for deconjugation of the 38 polyphenols (Fig. 2). However, in these conditions, eight phenolic acids (4-hydroxybenzoic, 3-hydroxybenzoic, protocatechuic, vanillic, gallic, *p*-coumaric, caffeic and ferulic acids) and one stilbene (resveratrol) showed suboptimal hydrolysis. Incubation time could not be increased due to the degradation of polyphenols with catechol groups for longer time of reaction, despite the addition of 10 μg ascorbic acid (Fig. 2).

Calibration curves showed linear responses for all polyphenols within the range of concentrations found in free-living subjects from the EPIC cohort (Table 2). Precision and accuracy of the method were evaluated. Very good intra-batch precision was observed, with CVs varying between 2.3% and 9% (Table 1). Inter-batch CVs varied between 4.0% and 15% for 33 of the 38 polyphenols tested. It exceeded 15% for 5 compounds including gallic acid (16.9%), hydroxytyrosol (18.2%) and enterodiol (20.3%). Particularly large interbatch CVs were observed for the two flavonols (quercetin, 23.4%; isorhamnetin, 30%). This was explained by their partial degradation at the high pH of the dansylation reaction, particularly for the solutions of standards. Quercetin was found to be relatively more stable when measured in plasma and this resulted in some overestimation in plasma analyses.

The capacity of the differential ^{12}C/^{13}C-isotope labelling method to limit matrix effects was evaluated. Matrix effect (ME) measured without correction with isotopic dilution varied widely according to polyphenols, from slight ion suppression effect (78.1% for equol) to large enhancements of the signal (431% for gallic acid) (Fig. 3). Dilution with the dansylated pool plasma sample led to a reduction of the matrix effect as seen by the RME significantly reduced for most polyphenols when compared to ME (Fig. 3). Major reductions of matrix effects were observed in particular for all compounds that showed the highest matrix effects such as gallic acid (431–183%), quercetin (402–163%), (+)-gallocatechin (331–217%) or hydroxytyrosol (352–198%).

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Table 1
Intra- and inter-batch precision, spike and dilution recovery for the measurement of 38 polyphenols in plasma.

ID	Polyphenol	Concentration (nM)[a]	Precision		Accuracy			
			Intra-batch CV (%)	Inter-batch CV (%)	Spike recovery[b] (%)		Dilution recovery (%)	
					Mean	SD	Mean	SD
1	4-Hydroxybenzoic acid	111–246	8.5	10.3	116.9	15.3	NA[c]	N/A
2	3-Hydroxybenzoic acid	6.9–75	9.0	12.2	96.4	7.3	109.2	9.6
3	Protocatechuic acid	87–128	8.6	13.1	98.0	20.2	NA[c]	N/A
4	Vanillic acid	88–275	7.6	12.5	108.1	14.4	86.9	11.3
5	3,5-Dihydroxybenzoic acid	8.3–72	3.0	6.0	85.2	5.6	104.0	6.6
6	Gallic acid	30–157	4.8	16.9	78.9	15.9	119.1	49.1
7	gallic acid ethyl ester	<LOQ –6.1	3.3	12.8	95.9	12.5	97.2	5.3
8	4-Hydroxyphenylacetic acid	156–645	4.3	6.1	76.1	13.2	99.6	10.8
9	3-Hydroxyphenylacetic acid	26–111	6.3	7.7	76.3	10.0	102.0	8.3
10	3,4-Dihydroxyphenylacetic acid	34–112	5.8	14.9	62.2	10.6	110.9	15.4
11	homovanillic acid	64–267	4.8	9.2	95.6	11.5	108.4	7.3
12	3,4-Dihydroxyphenylpropionic acid	91–221	6.2	12.6	84.4	13.4	95.5	35.1
13	3,5-Dihydroxyphenylpropionic acid	13.3–91	3.7	10.4	92.8	4.6	104.4	6.7
14	*p*-Coumaric acid	18.2–83	2.3	3.8	111.6	7.1	NA[c]	N/A
15	*m*-Coumaric acid	3.1–40	8.9	14.1	114.0	9.6	102.2	8.6
16	Caffeic acid	187–377	5.9	12.8	82.1	13.2	NA[c]	N/A
17	Ferulic acid	56–350	5.0	11.2	116.4	8.1	96.6	7.9
18	Kaempferol	52–123	4.5	12.6	100.6	8.7	77.5	22.4
19	Quercetin	148–778	6.5	23.4	155.6	45.4	115.4	25.5
20	Isorhamnetin	315–993	6.8	30	186.0	32.0	109.5	25.3
21	Apigenin	7.9–16.3	5.4	8.2	115.1	10.8	ND[d]	N/A
22	Naringenin	2.4–90	5.4	9.5	112.2	10.2	99.4	25.4
23	Hesperetin	1.3–71	5.9	13.2	123.3	17.8	100.1	8.8
24	Daidzein	2.2–158	6.3	8.8	107.2	7.3	96.0	3.5
25	Genistein	1.1–55	4.6	8.9	119.5	10.9	90.6	18.4
26	Equol	0.22–8.4	8.2	10.2	102.6	6.1	114.1	20.5
27	Phloretin	< LOQ–5.5	6.7	12.8	85.8	14.1	120.3	9.3
28	(+)-Catechin	<LOQ–135	2.9	10.5	145.8	12.9	122.1	13.9
29	(−)-Epicatechin	<LOQ–251	3.3	14.1	145.9	19.4	138.5	20.4
30	(−)-Gallocatechin	<LOQ–58	3.0	5.1	114.2	34.4	ND[d]	N/A
31	(−)-Epigallocatechin	<LOQ–435	4.4	14.4	93.3	34.4	176.8	23.5
32/33	Procyanidin dimer B1/B2[e]	<LOQ	N/A	N/A	118.3	23.9	ND[d]	N/A
34	Resveratrol	<LOQ–27.9	5.4	12.3	120.8	9.9	132.0	19.4
35	Tyrosol	3–4.9	7.1	15.2	136.0	27.6	92.0	14.8
36	Hydroxytyrosol	12.0–36	6.0	18.2	151.3	32.6	110.2	12.8
37	Enterodiol	0.5–11.0	6.9	20.3	199.8	58.4	89.9	7.8
38	Enterolactone	2.4–79	2.8	4.7	105.0	5.5	96.3	5.0

[a] Range of concentrations in plasma samples used for validation.
[b] Spike recovery = process efficiency (PE; see Experimental section for definition).
[c] NA, dilution recovery not applicable because of incomplete hydrolysis at 2 h.
[d] ND, not determined because of too low concentrations.
[e] Procyanidin dimers B1 and B2 could not be separated in chromatograms.

Recovery (RE) was evaluated independently of matrix effect (see experimental section) and found to be good for 29 polyphenols (72%–122%) and satisfactory for 9 polyphenols (43%–62%) out of the 38 polyphenols tested. (−)-Epigallocatechin showed the lowest recovery (32%). Process efficiency, the product of RE and RME, was found to vary between 76% and 116% for most polyphenols tested, except for hesperetin, isorhamnetin, quercetin, (+)-catechin, (−)-epicatechin, tyrosol, hydroxytyrosol and enterodiol, which showed higher recovery values (123–199%) (Table 1, spike recovery). Recovery after 1/2–1/4 (v/v) dilution varied between 90 and 120% for most compounds with the exception of (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and resveratrol which showed higher recoveries (122–177%) and of kaempferol which showed lower recovery (77%) (Table 1, dilution recovery).

Based on these different tests, isorhamnetin was finally excluded from further analyses out of the 38 polyphenols tested because of its high interbatch variability. Quercetin was maintained in the assay although an overestimation is anticipated.

3.3. Polyphenol concentrations in EPIC plasma samples

The method was then applied to plasma samples from 1163 subjects from the EPIC study (Table 2). Procyanidin dimers B1 and B2 could not be detected in any of the samples. (+)-Gallocatechin, (−)-epigallocatechin and gallic acid ethyl ester were detected in a limited fraction of the samples (8, 26 and 34% respectively). All other 32 compounds were detected and quantified in a large fraction (56–100%) of the samples. Median concentrations varied widely between compounds. Some were present at very low concentrations such as equol (median concentration, 0.5 nM), enterodiol (1.3 nM), tyrosol (3.1 nM), phloretin (3.2 nM), resveratrol (3.8 nM), gallic acid ethyl ester (4.1 nM), genistein (4.9 nM) and the two flavanones, hesperetin (2.5 nM) and naringenin (4.7 nM). The twelve phenolic compounds present at the highest concentrations (median concentration >50 nM) were all phenolic acids with the exception of quercetin (346 nM) known to be overestimated (see above) and kaempferol (94 nM). These phenolic acids are caffeic acid (415 nM), ferulic acid (89 nM) and several compounds of

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Table 2
Lowest, median, average and highest concentrations of polyphenols in plasma samples (n = 1163) from the EPIC study.

Polyphenol	Limit of quantification (nM)	Lowest concentration (nM)	Number of samples with concentrations <LLOQ (%)	Highest concentration (nM)	Number of samples with concentrations>ULOQ (%)	Median concentration (nM)	Average concentration (nM)	
							Mean	SD
4-Hydroxybenzoic acid	11.1	103	0	>888	0.1	192	207	67
3-Hydroxybenzoic acid	5.5	<LOQ	1.6	200	0	18.9	25	19
Protocatechuic acid	11.1	74	0	289	0	158	159	23
Vanillic acid	22.2	83	0	>1776	0.3	190	218	106
3,5-Dihydroxybenzoic acid	0.44	2.0	0	>355	0.1	19.0	37	45
Gallic acid	11.1	16	0	>222	0.1	34	41	24
Gallic acid ethyl ester	2.2	<LOQ	66	47	0	4.1	7.0	7.1
4-Hydroxyphenylacetic acid	4.4	95	0	>888	5.5	304	340	139
3-Hydroxyphenylacetic acid	4.4	<LOQ	8.1	>888	0.1	70	96	91
3,4-Dihydroxyphenylacetic acid	5.5	18	0	>222	0.2	42	46	20
Homovanillic acid	2.2	31	0	>444	0.3	88	100	47
3,4-Dihydroxyphenylpropionic acid	22.2	83	0	>444	2.2	182	196	59
3,5-Dihydroxyphenylpropionic acid	1.11	2	0	>222	2.6	31	44	39
p-Coumaric acid	0.56	9.5	0	176.6	0	18.2	21	11.2
m-Coumaric acid	4.4	<LOQ	27	>88.8	2.2	13.0	18.7	16.1
Caffeic acid	44	251	0	>888	0.1	415	428	80
Ferulic acid	11.1	37	0	>888	0.2	89	142	121
Kaempferol	22.2	49	0	341	0	94	99	29
Quercetin	44	132	0	>888	0.4	346	371	122
Isorhamnetin	44	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]
Apigenin	0.11	8	0	82.1	0	15.4	16	4.2
Naringenin	0.28	1	0	>222	1.4	4.7	15.4	29
Hesperetin	0.28	0.3	0	>222	2.3	2.5	20	38
Daidzein	0.56	1.2	0	>444	1.0	11.5	32	55
Genistein	0.22	0.8	0	>355	0.3	4.9	13.4	27
Equol	0.22	<LOQ	30	>44	0.5	0.5	1.0	2.4
Phloretin	2.2	<LOQ	43	102	0	3.2	5.1	6.6
(+)-Catechin	11.1	<LOQ	15.2	194	0	23	31	23
(−)-Epicatechin	11.1	<LOQ	25	289	0	22	38	39
(+)-Gallocatechin	22.2	<LOQ	92	82.7	0	28	31	9.2
(−)-Epigallocatechin	22.2	<LOQ	74	267	0	35	49	34
Procyanidin dimer B1/B2[a]	22.2	<LOQ	100	<LOQ	0	<LOQ	<LOQ	
Resveratrol	2.2	<LOQ	32	>89	0.1	3.8	6.8	7.2
Tyrosol	1.1	<LOQ	0.1	>89	0.2	3.1	4.2	4.5
Hydroxytyrosol	11.1	13	0	>222	0.6	26	33	21
Enterodiol	0.44	<LOQ	30	77	0	1.3	2.8	5.5
Enterolactone	0.28	<LOQ	1.6	322	0	8.5	13.4	19.8

[a] Procyanidin dimers B1 and B2 could not be separated in the chromatograms.
[b] ND, not determined.

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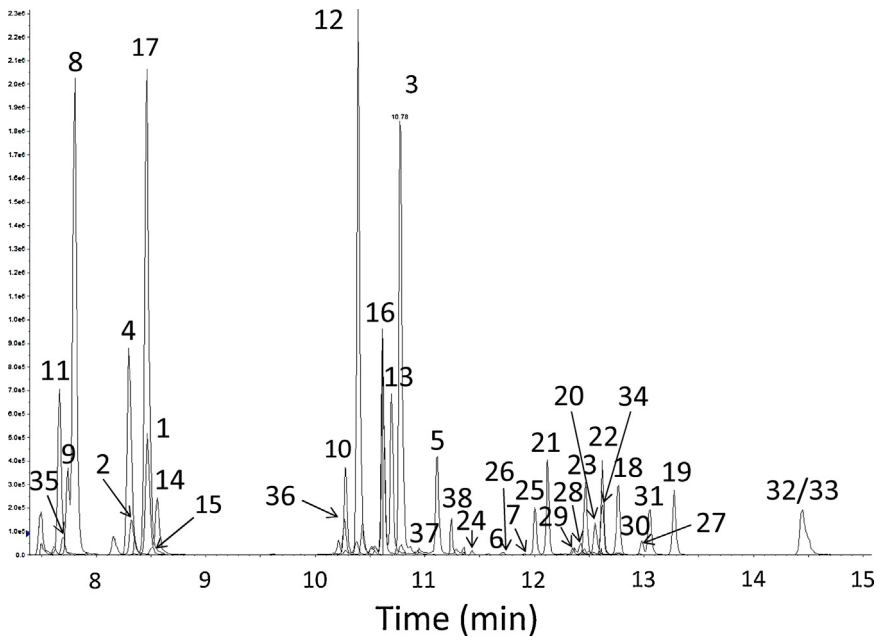

Fig. 1. LC–ESI–MS/MS chromatogram of a pool of 50 plasma samples from the EPIC study. The pooled sample was supplemented with low amounts of (−)-epigallocatechin, (+)-gallocatechin and procyanidin dimers B1 and B2, otherwise present at too low concentrations in plasma samples. Only the quantifier transition of the ^{12}C-dansylated polyphenols is shown. Run time is 20 min. See Table 1 for peak identity.

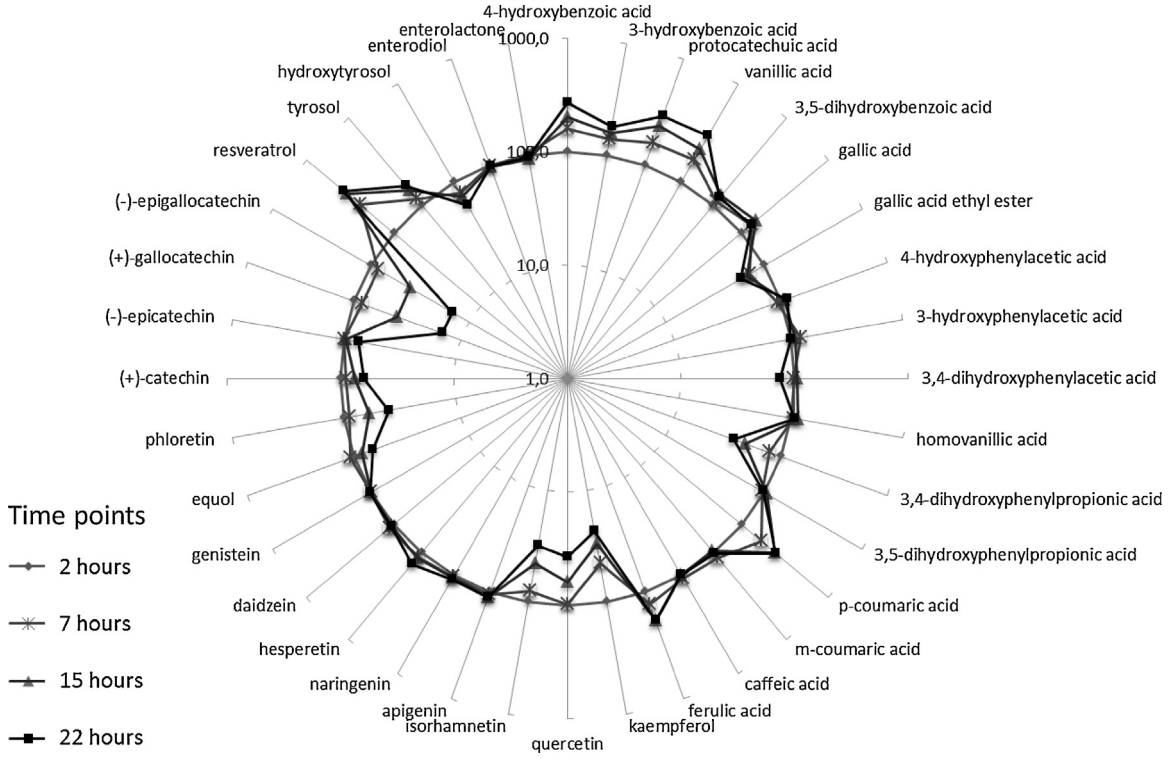

Fig. 2. Kinetics of enzymatic hydrolysis of conjugated polyphenols illustrated in the form of a radar chart. Radii show concentration of each free polyphenols measured as dansylated derivative at four different time points. Concentrations are expressed in relative intensities (%) and in log scale with time 2 h taken as reference. Lines connect concentration measurements for all polyphenols at a given time point.

microbial origin such as 4-hydroxyphenylacetic acid (304 nM) and 4-hydroxybenzoic acid (192 nM).

4. Discussion

A method was developed to quantify 38 polyphenols from 13 different classes or sub-classes in blood samples. The method uses differential ^{12}C-/^{13}C-isotope labelling of polyphenols with labelled dansyl chloride and is derived from a method previously developed for the analysis of urine samples [16]. This method presents a number of advantages including an improved retention and separation of dansylated polyphenols on the reverse phase column, a higher molecular weight of the measured products thereby increasing the signal to background ratio. The use of labelled dansyl chloride and

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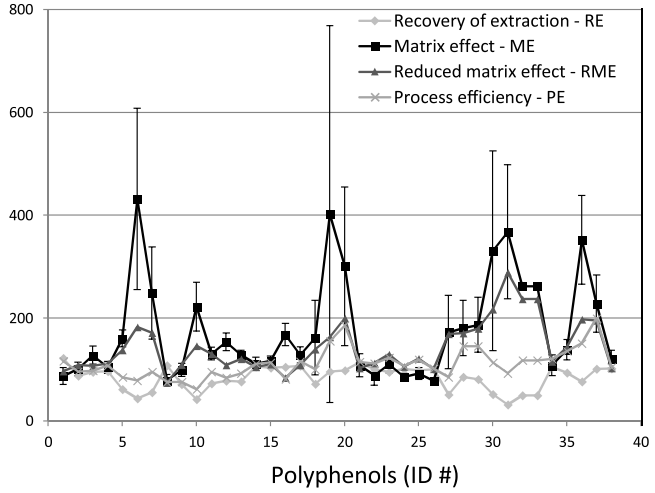

Fig. 3. Recovery, matrix effects and process efficiency for the measurement of 38 polyphenols in plasma samples.

the formation of labelled dansylated polyphenols overcome the need for costly labelled standards.

4.1. Optimization of the protocol for the analysis of plasma samples

When compared to our method for quantification of polyphenols in urine [16], several parameters were modified. Parameters of ionization and fragmentation were optimized (see experimental section), dilution factors in the protocol of extraction were reduced, and injection volume was increased. This resulted in a large gain of sensitivity evaluated by comparing LOQ values for the 37 polyphenols in plasma and in urine. This gain of sensitivity was in average 95× for the 37 polyphenols, and >600× for compounds like naringenin and hesperetin.

The conditions of enzymatic hydrolysis were optimized to achieve highest yields of unconjugated polyphenols in plasma samples. Few data on the efficiency with which the enzymes hydrolyse polyphenol glucuronides and ester sulfates are available [23]. We compared four incubation times and found that 2 h offered the best compromise with a majority of metabolites fully hydrolysed and a limited degradation of the most labile polyphenols, in particular those with oxidizable catechol groups, which could only be partially preserved by the addition of ascorbic acid [24].

4.2. Reduction of the matrix effects by isotopic dilution

One of the main assets of the present method based on dansylation and differential isotope labelling is to reduce matrix effects (Fig. 3). Nitrogen in the dansyl group of dansylated polyphenols increases proton affinity and improves their ionization. Dansylation also improves chromatographic behaviour of polyphenols [25–27]. The use of ^{13}C-dansyl chloride and isotopic dilution with the ^{12}C-dansyl pool reference sample limits matrix interference. Indeed, the ^{13}C- and ^{12}C-dansyl-polyphenol ion pairs are coeluted and subjected to the same degree of matrix effect [17]. We observed that for *ca* 75% of polyphenols, matrix effect is drastically reduced, and partially reduced for the remaining 25% of polyphenols (RME, Fig. 3).

4.3. Application to polyphenol measurement in the EPIC cohort

Thirty five out of the 37 polyphenols included in the method could be detected in plasma samples from the EPIC cohort (Table 2). Procyanidin dimer B2 and B1 could not be detected or were present at concentrations lower than the LOQ. These two dimers have been previously reported in blood after consumption of dietary sources rich in procyanidins [28,29]. However, their bioavailability was 100 times lower than that of the structurally related monomers. Most other compounds were detected at a concentration higher that the LLOQ in more than half of the plasma samples with the exception of the two gallocatechins expected to be present in tea consumers only [1].

Concentrations of the various polyphenols measured in plasma can be compared to those previously reported in the literature and curated in the Exposome-Explorer database [30]. For most polyphenols documented in the Exposome-Explorer database (kaempferol, apigenin, naringenin, hesperetin, equol, enterodiol and enterolactone), mean or median concentrations measured in the EPIC subjects are close to the ones previously measured using assays specific for one or a few polyphenols (see Supplementary data, Table S-1). For other compounds, concentration values vary between studies according to countries. Lower concentrations of 3,5-dihydroxybenzoic and 3,5-dihydroxyphenylpropionic acids, two metabolites of alkylresorcinols, were observed when compared to plasma levels previously reported in Finland, in agreement with a higher intake of wholegrain products in Northern Europe [31]. Levels of daidzein and genistein measured here are similar to those previously described in UK and USA and much lower than in Japan because of a higher consumption of soy products in this latter country. Measured levels of quercetin were higher than those previously reported in three other studies conducted in Germany or China. These differences are explained by technical issues leading to an overestimation of quercetin as discussed above.

Median concentrations measured here on plasma samples were in average over 30 times lower than those previously in urine for the same 37 polyphenols [16] with large variations between compounds. In general polyphenols showing highest concentrations in blood were also those showing highest concentrations in urine. These compounds were all phenolic acids (hydroxybenzoic, hydroxyphenylacetic, hydroxyphenylpropionic and hydroxycinnamic acids).

4.4. Comparison with methods previously published

A number of methods have been proposed earlier to quantify a variety of polyphenols in plasma or serum by LC–MS [32–37]. Most of these methods were targeted at specific polyphenol classes and did not use derivatization. They were mainly applied to dietary intervention studies and used larger sample volume (100–800 μL) than the present method (50 μL). None of these methods have been applied to our best knowledge to epidemiological studies. The present method is therefore the first that allows the measurement of main representatives for most classes of dietary polyphenols in blood. It is also unique because of the low plasma volume needed, its high sensitivity and its validation on a large number of plasma samples.

5. Conclusions

The method proposed here is the first application of dansylation to the quantification of dietary polyphenols in plasma. Thirty seven polyphenols were quantified in small volume of plasma. Sensitivity and robustness of the method make it particularly suitable for the analysis of large series of samples in epidemiological study. The method has also some limitations, like the requirement for a costly labelled reagent, although the cost is much lower than creating a set of isotope analogy standards of polyphenols, and the partial degradation of flavonols during the dansylation reaction. Nevertheless when applied to large scale epidemiological studies, it should con-

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tribute to improve our understanding of the role of polyphenols in health and disease prevention.

Conflict of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.05.017.

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