Article

Differential Isotope Labelling of 38 Dietary Polyphenols and their Quantification in Urine by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

David Achaintre, audrey Bulete, Cécile Cren-Olivé, Liang Li, Sabina Rinaldi, and Augustin Scalbert

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.5b03609 • Publication Date (Web): 26 Jan 2016

Differential Isotope Labelling of 38 Dietary Polyphenols and their Quantification in Urine by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

David Achaintre,[†] Audrey Buleté,[‡] Cécile Cren-Olivé,[‡] Liang Li,[§] Sabina Rinaldi,[†] Augustin Scalbert*[,†]

[†]Biomarkers Group, Nutrition and Metabolism Section, International Agency for Research on Cancer (IARC), 69372 Lyon Cedex 08, France

[‡]Université de Lyon, Institut des Sciences Analytiques, UMR 5280 CNRS (Equipe TRACES), Université de Lyon 1, ENS-Lyon, 5 rue de la Doua, 69100 Villeurbanne, France

[§]Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2 Canada

*To whom correspondence should be addressed

Dr. Augustin Scalbert,

Biomarkers Group

Nutrition and Metabolism Section

International Agency for Research on Cancer (IARC)

150 cours Albert Thomas

F-69372 Lyon Cedex 08

France

Tel. +33 472 73 80 95

Email: scalberta@iarc.fr

Running title: Differential isotope labelling of 38 dietary polyphenols

Word count: 4725 (title, abstract and references included, tables excluded).

Total number of figures/tables: 4

Conflict of Interest: None disclosed

ABSTRACT: A large number of polyphenols are consumed with the diet and may contribute to the prevention of chronic diseases such as cardiovascular diseases, diabetes, cancers and neurodegenerative diseases. More comprehensive methods are needed to measure exposure to this complex family of bioactive plant compounds in epidemiological studies. We report here a novel method enabling the simultaneous measurement in urine of 38 polyphenols representative of the main classes and subclasses found in the diet. This method is based on differential ^{12}C-/^{13}C-isotope labelling of polyphenols through derivatization with isotopic dansyl chloride reagents and on the analysis of the labelled polyphenols by tandem mass spectrometry. This derivatization approach overcomes the need for costly labelled standards. Different conditions for enzyme hydrolysis of polyphenol glucuronides and sulfate esters, extraction and dansylation of unconjugated aglycones were tested and optimized. Limits of quantification varied from 0.01 to 1.1 μM depending on polyphenols. Intra-batch coefficients of variation varied between 3.9% and 9.6%. Inter-batch variations were lower than 15% for 31 compounds and lower than 29% for 6 additional polyphenols out the 38 tested. Thirty seven polyphenols were validated and then analysed in 475 24-hour urine samples from the European Prospective Investigation on Cancer and Nutrition (EPIC) study. Thirty four polyphenols could be detected and successfully estimated and showed large interindividual variations of concentrations (two to three orders of magnitude depending on the compound), with median concentrations spanning from 0.01 to over 1000 μM for all 34 compounds.

Word count: 239

Keywords: Polyphenols, isotope-labelling, urine profiling, dansylation, liquid chromatography–tandem mass spectrometry

INTRODUCTION

Polyphenols are secondary plant metabolites found in a large variety of foods and beverages such as coffee, tea, wine, fruit and vegetables and cereals. Their antioxidant properties and possible role in the prevention of chronic diseases such as cardiovascular diseases, diabetes, cancers, osteoporosis, and neurodegenerative diseases have raised considerable interest.[1-4] These effects have been substantiated in a large number of preclinical and clinical studies.[5,6] However, results from epidemiologic studies are in comparison more limited and often inconsistent in findings. This is largely explained by the complexity of the polyphenol family (over 500 compounds known in foods)[7] and the diversity of their metabolites formed in the body[8] which makes the estimation of polyphenol exposures difficult.[9]

Biomarkers have been used to compare individual exposures to polyphenols and their metabolites in case-control studies but so far these studies have been largely limited to phytoestrogens (isoflavones and lignans) and a few more flavonoids.[9,10] However, the various polyphenols found in foods have highly diverse biological properties and different effects on health and diseases are expected. Analytical methods with broader polyphenol coverage are therefore clearly needed to measure a larger diversity of polyphenols. A number of methods utilizing mass spectrometry have been proposed to estimate selections of polyphenols in urine after deconjugation of glucuronides and sulfate esters.[11-15] We report here a novel method based on polyphenol derivatization with ^{13}C- and ^{12}C-dansyl chloride and the analysis of the dansylated products by mass spectrometry. Derivatization with dansyl chloride has previously been used to quantify phenolic compounds such as steroids present at very low concentrations.[16,17] A ^{13}C-dansyl chloride reagent and a differential isotope labelling method was proposed to introduce an isotope tag on amines and

phenols.[18] In this approach, samples containing amines and/or phenols tagged with ^{13}C-dansyl groups are mixed with a reference sample tagged with ^{12}C-dansyl groups and analyzed by tandem mass spectrometry. Amine and/or phenol concentrations are estimated through the calculation of ratios of labelled over non-labelled compounds. This isotope labelling approach limits matrix and ion suppression effects without a need for synthetizing costly isotope-labelled analogs. In the present work, a method is developed and optimized to estimate 38 polyphenols in urine by differential isotope labelling with dansyl chloride. It is applied to 475 urine samples from the European Prospective Investigation on Cancer and Nutrition (EPIC) study.

EXPERIMENTAL SECTION

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, 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.[18]

Urine Samples. Two sets of urine samples were used. The first set (dietary intervention study) was made of spot urine samples collected in the morning from three subjects who received a meal either poor or rich in polyphenols in the previous evening.[19] This sample set was used for evaluating precision, accuracy and recovery of the method. The second set of urine samples were 24-h urine samples collected from 475 participants of the 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 four European countries (France, Germany, Italy and Greece).[19] Subjects were female and male adults, 50-61 years old, following their regular diet. Urine samples were collected between 1995 and 1999 and stored at -20ºC with boric acid as preservative.

Extraction and Dansylation of Polyphenols. Urine samples were thawed at room temperature, centrifuged (10 min at 2000 rpm) and treated with deconjugating enzymes. Three enzyme preparations (sulfatase (EC: 3.1.6.1) type H1 from *H. pomatia* (also showing β-glucuronidase activity), a β-glucuronidase (EC: 3.2.1.31) type H1 from *H. pomatia* (also showing sulfatase activity), and a β-glucuronidase (EC: 3.2.1.31) type IX-A from *Escherichia coli*), different incubation times (1 to 24 hours) and enzyme quantities (360, 750 and 1500 U) were tested on urine samples collected in the dietary intervention study. Yields of unconjugated metabolites were evaluated on the basis of the highest reponse. The following conditions of hydrolysis were finally selected: 50 μL urine was treated with 25 μl of a solution of *H. pomatia* glucuronidase (1500 U β-glucuronidase) for 2h at 37°C. Samples were then extracted

twice for 5 min with ethyl acetate (800 μL), shaken at 1100 rpm, and centrifuged 15 min at 3000 rpm. The organic phase (1400 μL) was evaporated for 40 min under vacuum at room temperature.

Dried urine extracts were redissolved in a ^{13}C-dansyl chloride solution (100 µg in 100 µL acetone) and carbonate buffer (pH=9.0, 0.1 M, 100 µL) and kept at 60°C for 30 min. A pooled urine sample generated by mixing equivolumes (10 µL) of every urine sample analysed in the batch (n=25) was extracted and treated with ^{12}C-dansyl chloride in similar conditions. An aliquot (15 µL) of the dansylated pooled urine sample was added to each dansylated urine sample (15 µL) and the mixture directly analyzed by Ultra High Pressure Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (UHPLC-ESI-MS/MS).

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 urine samples for a given batch were maintained at 10°C in the autosampler until analysis. They were injected (2 μL) onto an Acquity CSH C18 column (Waters, 2.1 mm x 100 mm, 1.7 µm) maintained at 50°C. The following solvents were used for elution; solvent A, 0.1% formic acid in methanol/water 10/90 (v/v/v); solvent B, acetonitrile. The following gradient was used at a flow rate of 500 μL/min: 100% A for 2 min, ramped successively to 50% B in 3min, 60% in 2.5 min, 90% in 2 min, 100% B in 2.5 min. It was finally maintained at 100% B for two more min with a flow rate at 600 μL/min. Mass detection was carried out in scheduled MRM mode using a Turbo V-ion source operating in positive mode at 600ºC with a nebulizer gas pressure of 50 psi, a drying gas pressure of 60 psi, a curtain gas pressure of 30 psi, and collision activated dissociation (CAD) with medium pressure and capillary voltage of 5500 V.

Two transitions were determined for each dansylated compound and technical parameters (DP and CE) optimized to obtain the highest sensitivity (Table 1). For some of the most concentrated polyphenols the sensitivity was voluntarily reduced by modification of these parameters to avoid signal saturation (Table 1).

Quantification of Dansylated Polyphenols. Calibration curves were built using a mix of the 38 polyphenol standards at 10 concentration levels corresponding to expected concentrations in free-living subjects. Standards were dansylated with ^{13}C-dansyl chloride and mixed with the pooled urine sample (made of all samples in the batch) dansylated with ^{12}C-dansyl chloride. Quantification was carried out using the MultiQuant 2.1 software (AB Sciex, Foster City, CA). Calibration curves were established by plotting ^{13}C-dansyl (dns)-polyphenol/^{12}C-dns polyphenol peak area ratios vs. concentrations of ^{13}C-dns-polyphenols and by fitting data using a quadratic regression with 1/X weighting. R2 coefficients were above 0.999 for all compounds. The 475 urine samples from the EPIC study were analysed in 19 batches, with a ten-point calibration curve injected in duplicate along each batch. Three QC samples (two urine samples from the dietary intervention study and a pooled urine sample from the EPIC calibration study) were analyzed in duplicate within each batch, together with a blank sample (urine is replaced by water and the same procedure used for urine samples is followed to extract and analyse the blank sample), to assess the level of interference and/or contaminations.

Method Validation. The method was validated according to FDA guidelines.[20] Intra-batch reproducibility was evaluated by repeated injection (n=6) of four urine samples from the dietary intervention study (two with low polyphenol concentrations and two with high polyphenol concentrations) in a same batch. Inter-batch reproducibility was evaluated on two samples from the intervention study (high and low polyphenol

concentrations) and on the pooled urine sample from the EPIC calibration study analyzed in duplicate in each of the 19 batches. Accuracy was evaluated by spiking three different urine samples with low polyphenol concentrations (intervention study) with standards at three different concentrations representative of the concentrations found in urine from free-living subjects over a 4-month period. Recovery was determined by comparing the peak area of dansylated polyphenols in the spiked urine samples with that of pure dansylated standards. LOQ was defined as the lowest point of the calibration giving an accuracy varying from 80 to 120% of the theoretical value.

RESULTS

Polyphenol Dansylation. Thirty eight dietary polyphenols and metabolites formed in the tissues and by the microbiota were selected for their widespread occurrence in foods or in human biofluids and for being representative of the main polyphenol classes. They include phenolic acids (Table 1, peaks 1-17), flavonoids (peaks 18-32), one stilbene (peak 34), tyrosols (peaks 35-36) and lignans (peaks 37-38). Phenolic acids can be further divided into the following subclasses: hydroxybenzoic acids (Table 1, peaks 1-7), hydroxyphenylacetic acids (peaks 8-11), hydroxyphenylpropanoic acids (peaks 12-13), hydroxycinnamic acids (peaks 14-17), and flavonoids include flavonols (peaks 18-20), one flavone (peak 21), flavanones (peaks 22-23), isoflavonoids (peaks 24-26), one dihydrochalcone (peak 27) and flavanols (peaks 28-33).

The reaction of dansylation was optimized on pure compounds (ferulic, caffeic and gallic acids, daidzein and resveratrol) using different solvents (acetone or acetonitrile), and different pH (9 and 10.5). Highest yields of dansylation were observed at pH 9.0 with acetone. Different concentrations of dansyl chloride (1, 5 and

20 mg/mL) and different reaction times (15 min to 2 hours) were then compared on the 38 pure polyphenol standards. Highest yields were observed at a dansyl chloride concentration of 1 mg/mL and reaction time of 30 min. The completeness of dansylation was checked in full scan experiments (data not shown). It was found to be quantitative (all phenolic groups dansylated) with the exception of the two flavanones (hesperetin and naringenin). Their carbonyl group were also dansylated with a yield of approx. 50% and this led to the formation of two different products with 3 and 4 dansyl groups at two different retention time. The tetra-dansylated compounds were selected for quantification.

Dansylated polyphenols, prepared from the 38 standards, were also analysed at different time points after the reaction to assess their stability. Most dansylated polyphenols were found to be stable for up to 13 hours at 10°C in the conditions of the reaction. Some degradation (up to 50% at 16h and 90% at 48h) was observed for about half of the polyphenols measured (Figure 2A). However, the ratio of the ^{12}C-dansylated polyphenol (sample) to the ^{13}C-dansylated polyphenol (reference sample) was found to be stable over the 48 hours following dansylation (Figure 2B). This result was compatible with the analysis of batches of 25 samples and a duration of about 14 hours for the analysis of all samples (including samples for calibration curve, QC and blank samples) in a batch.

Quantification of Dansylated Polyphenols by Tandem Mass Spectrometry. Dansylated polyphenols were quantified by tandem mass spectrometry (Table 1, Figure 1). Multicharged ions were observed for several compounds and selected for quantification when the mass of the monocharged ions exceeded the higher mass that could be scanned by the mass spectrometer.

Limit of quantification (LOQ) varied from 0.01 µM for equol to 1.10 µM for 4-OH-phenylacetic acid (Table 2). For some of the most concentrated polyphenols (Table 2), sensitivity was voluntarily reduced by modifying the mass spectrometry operational parameters (Table 1) to avoid signal saturation. Within each analytical batch of 25 samples, a blank sample was added to assess the presence of possible polyphenol contaminants in the glucuronidase preparation.[21] Traces of apigenin, protocatechuic and caffeic acids were found in the enzyme preparation and the corresponding concentrations in the blank were subtracted from measured concentrations in samples.

Precision and accuracy of the method were evaluated. Very good intra-batch precision was observed, with CVs varying between 3.9% and 9.6% (See Table S1 of the Supporting Information for intra- and inter-batch CV values). Inter-batch CVs varied between 5% and 15% for 31 of the 38 polyphenols tested. It exceeded 15% for catechins [(+)-catechin, 17.4%; (-)-epicatechin, 17.5%; (-)-epigallocatechin, 49.8%], the two flavonols (quercetin, 26.4; isorhamnetin, 29.1%), apigenin (25.1%) and phloretin, (21.4%). Recovery of spiked samples varied between 72% and 107%, except for gallic acid, two flavonols (kaempferol and quercetin), (+)-gallocatechin, procyanidin dimer B1 and B2, and phloretin, which showed lower recovery values (25 to 63%) (See Table S1 of the Supporting Information for recovery values). Recovery after dilution 1/2 to 1/8 (v/v) varied between 84 and 120% for most compounds with the exception of 3-hydroxybenzoic acid, caffeic acid, quercetin and naringenin which showed higher recoveries (124-149%)(See Table S1 of the Supporting Information for dilution recovery values). Based on these results, 37 compounds out of the 38 tested were kept for further analyses and (-)-epigallocatechin was excluded because of a too high inter-batch variability.

Polyphenol Concentrations in EPIC Urine Samples. The 37 polyphenols were measured in urine samples from 475 subjects of the EPIC cohort (Table 2). Out of these 37 polyphenols, procyanidin dimers B1 and B2 could not be detected in any of the samples, and (+)-gallocatechin was detected in a very small number of the samples (2.5%). Thirty four compounds were detected and quantified in a large fraction of the samples; the proportion of concentration values lower than LOQ varied between 0.6% (enterolactone) and 53.7% (isorhamnetin). Median concentrations varied widely from compounds present at very low concentrations such as equol or resveratrol (median, respectively 0.03 μM and 0.05 μM) to phenolic compounds present at high concentration such as 4-hydroxyphenylacetic acid (median, 92 μM).

DISCUSSION

Direct analysis of polyphenols by liquid chromatography without chemical derivatization is the analytical method most commonly used to estimate polyphenols in human biofluids such as urine. We set up a different method based on derivatization with differential isotopic dansyl groups. The present method is applicable to small volumes of urine samples (50 μL) to estimate 34 polyphenols from 13 different classes or sub-classes, and excreted in highly variable concentrations (two to three orders of magnitude for any given compound).

Dansylation reaction. Derivatization with dansyl groups has several advantages when analysing a large panel of polyphenols and their metabolites showing large differences in molecular weight, chemical structure, and physico-chemical properties. It increases lipophilicity of the compounds and their affinity for the reverse phase column and improves chromatographic separation. It also increases the mass of the

molecule to avoid the low mass background signal interference often encountered in conventional LC-MS, thereby increasing the signal to background ratio.

The number of phenolic groups in the 38 selected polyphenols varies from one (as in some phenolic acids) to eight (as in procyanidin dimer B1). For all polyphenols tested, all phenolic groups were dansylated, resulting in derivatized products with one to eight dansyl groups (Table 1). Different parameters of the reaction (reaction time, concentration of reagent, pH) were optimized to maximize the yield of derivatization. When analysed by mass spectrometry, the formation of multicharged ions (Table 1) and some in-source fragmentation were observed for a number of dansylated polyphenols. For example, a full scan chromatogram of dansylated phloretin showed the formation of mono-, di-, tri- and tetracharged ions from tetradansylated phloretin, all having the same retention time (not shown). However, it was also found that the multiplicity of ions had limited impact on the accuracy of the measurements. The stability of the dansylated products was also assessed. Some degradation of the dansylated groups was observed for some polyphenols starting after 10 hours at 10°C (Figure 1A). However, as our method is based on the measurement of ratios of ^{13}C-dns- to ^{12}C-dns-polyphenols which degrade at a similar pace, this partial degradation of the reaction products had limited impact on the measurements (Figure 1B).

Overall, measurements of 28 polyphenols out of the 38 tested could be validated according to FDA guidelines[20], with intra- and inter-batch CVs lower than 15% (See Table S1 in Supporting Information for intra- and inter-batch CV values). Inter-batch CVs were higher than 15% for 6 other polyphenols which were considered still acceptable for epidemiological applications.[22] A total of 37 polyphenols were therefore estimated in urine samples from participants of the EPIC calibration study.

Application to polyphenol measurement in the EPIC cohort. Out of the 37 polyphenols measured, 34 were detected in all or a fraction of these subjects and 3 polyphenols could not be detected in any of the subjects (Table 2). Two major reasons may explain these results: either these polyphenols were present at concentrations below the limit of detection or major sources of these polyphenols were not actually consumed. Procyanidin dimer B2 or (+)-gallocatechin were previously detected in urine in two dietary intervention studies with respectively cocoa and tea,[23,24] and their concentrations were likely too low to be detected in the present samples. Gallic acid ethyl ester and resveratrol, characteristic of red wine consumption,[25] could not be detected in respectively 49 and 10% of the EPIC subjects (Table 2), suggesting that a large fraction of the subjects did not consume wine in the few days before urine collection. Similarly, hesperetin, mainly found in orange fruit and juice, was absent in 26% of the subjects who most likely did not consume these food items in the few days before urine collection. Interestingly, soy isoflavones (daidzein and genistein) were detected in a large fraction of the subjects (97%; Table 2). Consumption of soy is low in Europe but soy or soy extracts are widely used as food ingredients or food additives. The low LOQ for equol, a microbial metabolite formed from daidzein in the gut, also made possible its detection in 88% of the subjects. These results are in agreement with a previous work in the EPIC-Norfolk cohort in which daidzein and genistein were detected in a large fraction of the urine and serum samples tested.[26]

Overall, highest median concentrations in urine were observed for phenolic acids formed by the gut microbiota from dietary polyphenols or aromatic amino acids (phenylacetic acids, benzoic acids and phenylpropionic acids; 1.2-92 μM), for caffeic acid consumed with coffee (2.8 μM) and ferulic acid, its *O*-methylated metabolite (24

μM) (Table 2). Enterolactone, the main mammalian lignan, and hydroxytyrosol originating from olive oil and wine, are also excreted in significant amounts (median concentration, respectively 1.7 and 1.3 μM). Other polyphenols, either flavonoids, phenolic acids (gallic acid, gallic acid ethyl ester) or stilbenes (resveratrol) are compounds absorbed in the gut from various foods and are present in urine in much lower concentrations (0.03 to 0.95 μM).

Comparison with methods for polyphenol quantification previously published. Most methods previously published to measure polyphenols in human urine were targeted at a limited number of compounds from specific polyphenol classes or subclasses. Many of these methods used LC-MS as in the present work. Gasperotti et al. measured 20 phenolic acids and 3 small phenols in urine[14] with a significant overlap (10 compounds) with phenolic acids measured in this work. Nielsen et al. quantified 7 flavonoids (flavonols, flavanols and dihydrochalcone) in urine samples from a dietary intervention study.[27]

A larger diversity of polyphenols was measured in two other studies. Urpi-Sarda et al. measured 17 phenolic acids, 2 mammalian lignans, and 2 flavanols (including 16 compounds measured in the present work) in urine samples after consumption of cocoa.[12] Magiera et al. developed and fully validated a method to estimate 43 polyphenols and their metabolites in urine.[13] Starting from 2 ml urine aliquots, they were able to detect in human urine from 32 patients, 24 flavonoids and phenolic acids out the 43 polyphenols tested. With 34 polyphenols from 13 polyphenol classes or subclasses quantified in urine samples from the EPIC cohort, the number and diversity of polyphenols quantified in the present study are higher than in these two previous studies.

CONCLUSIONS

The method proposed here has several advantages: it encompasses 13 classes and sub-classes of polyphenols, and several of the most representative compounds in each of these classes or subclasses. A substantial number of polyphenol metabolites either formed by the microbiota (phenolic acids) or in the human tissues (*O*-methylated metabolites) are also measured. Derivatization with dansyl chloride improves chromatographic separation of highly diverse polyphenols. In addition, the use of a ^{13}C- and ^{12}C-reagents overcomes the need for costly labelled standards, which are essential to limit matrix and/or ion suppression effects commonly observed in complex biological matrices. Isotopic dilution has been used to estimate specific polyphenols such as isoflavones and lignans using ^{13}C-labelled standards chemically synthesized.[28] Isotopic dilution is particularly useful to quantify compounds present in biofluids at very low concentrations. However the present differential isotope labelling approach allows for the first time to apply isotopic dilution to the estimation of a large diversity of phenolic compounds. Overall the present method allows to quantify polyphenols in urine samples with concentrations spanning over five orders of magnitude, including very low concentrations without compromising precision and accuracy. This method was initially targeted at a list of polyphenols selected *a priori* and quantified by tandem mass spectrometry. However, the same method combined with the use of a high resolution mass spectrometer would also permit broad scan acquisitions to measure additional and *a priori* unknown dansylated phenols and amines as initially proposed by Guo and Li[18] and to semi-quantify these compounds for which chemical standards may not be available.

AUTHOR INFORMATION

Corresponding author

*Tel: +33 472 73 80 95. Email: scalberta@iarc.fr (A. Scalbert)

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Institut National du Cancer, Paris (INCa grant #2011-105) and the Wereld Kanker Onderzoek Fonds (WCRF NL grant #2012/604).

REFERENCES

(1) Arts, I. C.; Hollman, P. C. *Am. J. Clin. Nutr.* **2005,** *81*, 317S-325.

(2) Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. *Crit. Rev. Food Sci. Nutr.* **2005,** *45*, 287-306.

(3) Hollman, P. C.; Cassidy, A.; Comte, B.; Heinonen, M.; Richelle, M.; Richling, E.; Serafini, M.; Scalbert, A.; Sies, H.; Vidry, S. *J. Nutr.* **2011,** *141*, 989S-1009S.

(4) Rodriguez-Mateos, A.; Vauzour, D.; Krueger, C. G.; Shanmuganayagam, D.; Reed, J.; Calani, L.; Mena, P.; Del Rio, D.; Crozier, A. *Arch. Toxicol.* **2014,** *88*, 1803-53.

(5) Hooper, L.; Kroon, P. A.; Rimm, E. B.; Cohn, J. S.; Harvey, I.; Le Cornu, K. A.; Ryder, J. J.; Hall, W. L.; Cassidy, A. *Am. J. Clin. Nutr.* **2008,** *88*, 38-50.

(6) Thomasset, S. C.; Berry, D. P.; Garcea, G.; Marczylo, T.; Steward, W. P.; Gescher, A. J. *Int. J. Cancer* **2007,** *120*, 451-458.

(7) Perez-Jimenez, J.; Neveu, V.; Vos, F.; Scalbert, A. *J. Agric. Food Chem.* **2010,** *58*, 4959-4969.

(8) Rothwell, J. R.; Urpi-Sarda, M.; Boto-Ordonez, M.; Llorach, R.; Farran-Codina, A.; Barupal, D. K.; Neveu, V.; Manach, C.; Andres-Lacueva, C.; Scalbert, A. *Mol. Nutr. Food Res.* **2015**, in press.

(9) Zamora-Ros, R.; Touillaud, M.; Rothwell, J. A.; Romieu, I.; Scalbert, A. *Am. J. Clin. Nutr.* **2014,** *100*, 11-26.

(10) Perez-Jimenez, J.; Hubert, J.; Ashton, K.; Hooper, L.; Cassidy, A.; Manach, C.; Williamson, G.; Scalbert, A. *Am. J. Clin. Nutr.* **2010,** *92*, 801-9.

(11) Ito, H.; Gonthier, M.-P.; Manach, C.; Morand, C.; Mennen, L.; Rémésy, C.; Scalbert, A. *Br. J. Nutr.* **2005,** *94*, 500-509.

(12) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Llorach, R.; Ma Lamuela-Raventos, R.; Jauregui, O.; Estruch, R.; Izquierdo-Pulido, M.; Andres-Lacueva, C. *J. Chromatogr. A* **2009,** *1216*, 7258-7267.

(13) Magiera, S.; Baranowska, I.; Kusa, J. *Talanta* **2012,** *89*, 47-56.

(14) Gasperotti, M.; Masuero, D.; Guella, G.; Mattivi, F.; Vrhovsek, U. *Talanta* **2014,** *128*, 221-230.

(15) de Ferrars, R. M.; Czank, C.; Saha, S.; Needs, P. W.; Zhang, Q.; Raheem, K. S.; Botting, N. P.; Kroon, P. A.; Kay, C. D. *Anal. Chem.* **2014,** *86*, 10052-8.

(16) Li, X.; Franke, A. A. *Steroids* **2015,** *99*, 84-90.

(17) Santa, T. *Biomed. Chromatogr.* **2011,** *25*, 1-10.

(18) Guo, K.; Li, L. *Anal. Chem.* **2009,** *81*, 3919-3932.

(19) Edmands, W.; Ferrari, P.; Rothwell, J. A.; Rinaldi, S.; Slimani, N.; Barupal, D. K.; Biessy, C.; Jenab, M.; Clavel-Chapelon, F.; Fagherazzi, G.; Boutron-Ruault, M. C.; Katzke, V.; Kuhn, T.; Boeing, H.; Trichopoulou, A.; Lagiou, P.; Trichopoulos, D.; Palli, D.; Grioni, S.; Tumino, R.; Vineis, P.; Mattiello, A.; Romieu, I.; Scalbert, A. *Am. J. Clin. Nutr.* **2015**, in press.

(20) Food_and_Drug_Administration *Guidance for Industry - Bioanalytical Method Validation*; 2001. Downloaded from http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf (12 September 2015).

(21) Nakamura, T.; Tanaka, R.; Ashida, H. *Biosci. Biotechnol. Biochem.* **2011,** *75*, 1506-10.

(22) Zamora-Ros, R.; Achaintre, D.; Rothwell, J. A.; Rinaldi, S.; Assi, N.; Ferrari, P.; Leitzmann, M.; ...; Slimani, N.; Romieu, I.; Scalbert, A. **2015**, in preparation.

(23) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventos, R. M.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Permanyer, J.; Andres-Lacueva, C. *Anal. Bioanal. Chem.* **2009,** *394*, 1545-56.

(24) Yang, B.; Arai, K.; Kusu, F. *Anal. Biochem.* **2000,** *283*, 77-82.

(25) Neveu, V.; Perez-Jiménez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D.; Scalbert, A. *Database* **2010,** *2010*, bap024.

(26) Grace, P. B.; Taylor, J. I.; Low, Y. L.; Luben, R. N.; Mulligan, A. A.; Botting, N. P.; Dowsett, M.; Welch, A. A.; Khaw, K. T.; Wareham, N. J.; Day, N. E.; Bingham, S. A. *Cancer Epidemiology Biomarkers & Prevention* **2004,** *13*, 698-708.

(27) Nielsen, S. E.; Freese, R.; Cornett, C.; Dragsted, L. O. *Anal. Chem.* **2000,** *72*, 1503-1509.

(28) Grace, P. B.; Taylor, J. I.; Botting, N. P.; Fryatt, T.; Oldfield, M. F.; Al-Maharik, N.; Bingham, S. A. *Rapid Commun. Mass Spectrom.* **2003,** *17*, 1350-1357.

Table 1. UHPLC-ESI-MS/MS Parameters for Quantification of 38 Dietary Polyphenols and Polyphenol Metabolites.

ID	polyphenol	number of dns groups	number of charges	label	retention time (min)	molecular weight	precursor ion (m/z)	DP (V)	quantifier ion		qualifier ion	
									(m/z)	collision energy (V)	(m/z)	collision energy (V)
1	dns-4-hydroxybenzoic acid [a]	1	1	^{12}C	5.68	371	372.0	200	171.0	55	156.1	75
				^{13}C-labelled	5.68	373	374.0		173.0		157.1	
2	dns-3-hydroxybenzoic acid [a]	1	1	^{12}C	5.55	371	372.0	200	171.0	55	156.1	75
				^{13}C-labelled	5.55	373	374.0		173.0		157.1	
3	dns-protocatechuic acid [a]	2	2	^{12}C	7.74	620	311.0	200	171.1	40	156.0	65
				^{13}C-labelled	7.74	624	313.0		173.1		157.0	
4	dns-vanillic acid [a]	1	1	^{12}C	5.55	401	402.0	200	171.1	45	156.1	80
				^{13}C-labelled	5.55	403	404.0		173.1		157.1	
5	dns-3,5-dihydroxybenzoic acid [a]	2	2	^{12}C	8.05	620	311.1	200	170.1	45	171.0	45
				^{13}C-labelled	8.05	624	313.1		172.1		173.0	
6	dns-gallic acid	3	1	^{12}C	8.6	869	870.0	200	636.0	50	619.1	45
				^{13}C-labelled	8.6	875	876.0		640.0		623.1	
7	dns-gallic acid ethyl ester	3	1	^{12}C	8.86	897	898.0	76	644.0	39	349.1	57
				^{13}C-labelled	8.86	903	904.0		668.0		351.1	
8	dns-4-hydroxyphenylacetic acid [a]	1	1	^{12}C	5.16	385	386.0	200	171.1	55	156.0	75
				^{13}C-labelled	5.16	387	388.0		173.1		157.0	
9	dns-3-hydroxyphenylacetic acid [a]	1	1	^{12}C	5.11	385	386.0	200	171.1	55	156.0	75
				^{13}C-labelled	5.11	387	388.0		173.1		157.0	
10	dns-3,4-dihydroxyphenylacetic acid [a]	2	2	^{12}C	7.31	634	318.1	200	156.0	65	171.1	40
				^{13}C-labelled	7.31	638	320.1		157.0		173.1	
11	dns-homovanillic acid [a]	1	1	^{12}C	5.05	415	416.0	200	156.1	61	171.1	33
				^{13}C-labelled	5.05	417	418.0		157.1		173.1	
12	dns-3,4-dihydroxyphenylpropionic acid [a]	2	2	^{12}C	7.42	648	325.0	200	156.1	65	171.1	40
				^{13}C-labelled	7.42	652	327.0		157.1		173.1	
13	dns-3,5-dihydroxyphenylpropionic acid [a]	2	2	^{12}C	7.71	648	325.1	200	171.1	45	170.1	45

				^{13}C-labelled	7.71	652	327.1		173.1		172.1	
14	dns-*p*-coumaric acid[a]	1	1	^{12}C	5.77	397	398.0	200	171.1	55	156.2	80
				^{13}C-labelled	5.77	399	400.0		173.1		157.2	
15	dns-*m*-coumaric acid	1	1	^{12}C	5.72	397	398.0	200	171.1	55	170.0	50
				^{13}C-labelled	5.72	399	400.0		173.1		172.0	
16	dns-caffeic acid	2	1	^{12}C	7.62	646	647.1	86	170.1	39	396.0	25
				^{13}C-labelled	7.62	650	651.1		172.1		398.0	
17	dns-ferulic acid[a]	1	1	^{12}C	5.7	427	428.1	200	156.1	85	171.1	50
				^{13}C-labelled	5.7	429	430.1		157.1		173.1	
18	dns-kaempferol	4	3	^{12}C	9.48	1218	407.1	96	493.7	13	170.0	23
				^{13}C-labelled	9.48	1226	409.8		496.7		172.0	
19	dns-quercetin	5	3	^{12}C	10.05	1467	490.3	96 and 91	170.1	23	618.2	13
				^{13}C-labelled	10.05	1477	493.5		172.1		622.2	
20	dns-isorhamnetin	4	3	^{12}C	9.42	1248	417.2	81 and 61	508.7	13	170.0	23
				^{13}C-labelled	9.42	1256	419.8		511.7		172.0	
21	dns-apigenin	3	3	^{12}C	9.05	969	324.1	71	171.1	25	170.1	27
				^{13}C-labelled	9.05	975	326.1		173.1		172.1	
22	dns-naringenin	4	3	^{12}C	9.48	1204	402.4	200	486.6	18	170.1	40
				^{13}C-labelled	9.48	1212	405.1		489.6		172.1	
23	dns-hesperetin	4	3	^{12}C	9.34	1234	412.2	51 and 91	501.6	11	170.1	23
				^{13}C-labelled	9.34	1242	415.1		504.6		172.1	
24	dns-daidzein	2	1	^{12}C	8.43	720	721.1	126	170.1	51	171.2	51
				^{13}C-labelled	8.43	724	725.1		172.1		173.2	
25	dns-genistein	3	3	^{12}C	8.94	969	324.1	71	171.1	25	170.1	27
				^{13}C-labelled	8.94	975	326.1		173.1		172.1	
26	dns-equol	2	1	^{12}C	8.57	708	709.1	101	170.1	57	171.1	57
				^{13}C-labelled	8.57	712	713.1		172.1		173.1	
27	dns-phloretin	4	3	^{12}C	9.77	1206	403.2	91 and 76	170.1	23	487.7	15
				^{13}C-labelled	9.77	1214	405.8		172.1		490.7	

28	dns-(+)-catechin	4	3	^{12}C	9.3	1222	408.3	86	171.2	29	495.8	17
				^{13}C-labelled	9.3	1230	411.1		173.2		498.8	
29	dns-(-)-epicatechin	4	3	^{12}C	9.25	1222	408.3	86	171.2	29	495.8	17
				^{13}C-labelled	9.25	1230	411.1		173.2		498.8	
30	dns-(-)-gallocatechin	5	4	^{12}C	9.85	1471	369.1	91	171.1	25	170.0	25
				^{13}C-labelled	9.85	1481	371.6		173.1		172.0	
31	dns-(-)-epigallocatechin	5	4	^{12}C	9.79	1471	369.1	91	171.1	25	170.0	25
				^{13}C-labelled	9.79	1481	371.6		173.1		172.0	
32/33	dns-procyanidin dimer B1/B2[b]	8	4	^{12}C	11.2	2442	611.7	86	170.2	37	171.0	35
				^{13}C-labelled	11.2	2458	615.7		172.2		173.1	
34	dns-resveratrol	3	2	^{12}C	9.34	927	464.8	86	171.1	31	170.2	33
				^{13}C-labelled	9.34	933	467.8		173.1		172.2	
35	dns-tyrosol	1	1	^{12}C	5.13	371	372.1	81	171.1	33	156.1	49
				^{13}C-labelled	5.13	373	374.1		173.1		157.1	
36	dns-hydroxytyrosol	2	2	^{12}C	7.34	620	311.1	200	171.1	40	156.0	65
				^{13}C-labelled	7.34	624	313.1		173.1		157.0	
37	dns-enterodiol	2	1	^{12}C	8.02	768	769.2	176	517.1	43	170.1	53
				^{13}C-labelled	8.02	772	773.2		519.1		172.1	
38	dns-enterolactone	2	1	^{12}C	8.26	764	765.1	161	531.1	60	170.1	75
				^{13}C-labelled	8.26	768	769.1		533.1		172.1	

[a]For these compounds, sensitivity was voluntarily reduced by modifying the declustering potential and/or the collision energy.

[b]Procyanidin dimers B1 and B2 could not be distinguished in the chromatograms.

Table 2. Limit of Quantification for 37 Dietary Polyphenols and Urinary Concentrations in 475 Subjects from the EPIC Cohort.

polyphenol	limit of quantification (uM)	lowest concentration (uM)	number of samples with concentrations <LOQ (%)	highest concentration (uM)	median concentration (uM)	average concentration (uM)	
						Mean	SD
4-hydroxybenzoic acid[a]	0.09	2.3	0	59.0	11.2	12.8	7.78
3-hydroxybenzoic acid[a]	0.03	0.03	0	25.1	1.18	1.63	1.72
protocatechuic acid[a]	0.03	0.33	0	11.1	1.93	2.23	1.27
vanillic acid[a]	0.13	0.75	0	192	20.3	27.3	23.1
3,5-dihydroxybenzoic acid[a]	0.06	0.15	0	19.9	2.36	3.3	2.49
gallic acid	0.13	< LOQ	1.1	6.42	0.352	0.617	0.74
gallic acid ethyl ester	0.13	< LOQ	49.3	8.21	< LOQ	1.05	1.37
4-hydroxyphenylacetic acid[a]	1.1	16.8	0	1017	92.2	111	88.7
3-hydroxyphenylacetic acid[a]	0.27	<LOQ	2.3	182	26.1	33	24.5
3,4-dihydroxyphenylacetic acid[a]	0.23	0.34	0	33.1	2.99	3.71	2.92
homovanillic acid[a]	0.19	2.11	0	69.5	14.0	15.2	7.25
3,4-dihydroxyphenylpropionic acid[a]	0.23	0.44	0	38.0	5.17	7.32	6.42
3,5-dihydroxyphenylpropionic acid[a]	0.33	0.62	0	43.4	6.6	8.41	6.22
p-coumaric acid[a]	0.04	0.16	0	29.4	1.18	1.53	1.66
m-coumaric acid[a]	0.08	< LOQ	1.7	51.7	1.27	2.51	4.09
caffeic acid	0.11	0.46	0	14.1	2.78	3.27	2.25
ferulic acid[a]	0.19	3.76	0	92.7	24.2	27.1	16.4
kaempferol	0.04	< LOQ	7.4	> 1.83[c]	0.07	0.098	0.1
quercetin	0.11	0.11	0	2.48	0.286	0.348	0.27
isorhamnetin	0.44	< LOQ	53.7	4.32	<LOQ	0.546	0.42
apigenin	0.02	< LOQ	24.0	3.01	0.043	0.11	0.21
naringenin	0.21	< LOQ	2.3	31.7	0.951	2.34	3.68
hesperetin	0.19	< LOQ	26.1	24.8	0.545	2.46	3.59
daidzein	0.07	< LOQ	2.7	64.1	0.693	1.95	4.2
genistein	0.01	< LOQ	2.5	> 1.83[d]	0.124	0.237	0.3
equol	0.01	< LOQ	11.4	> 1.09[e]	0.03	0.059	0.12
phloretin	0.27	< LOQ	52	> 5.5[e]	< LOQ	0.525	0.51
(+)-catechin	0.04	< LOQ	34.7	> 0.92[f]	0.061	0.125	0.11
(-)-epicatechin	0.1	< LOQ	25.9	2.23	0.13	0.207	0.21
(+)-gallocatechin	0.05	< LOQ	97.5	0.136	< LOQ	0.083	0.03
procyanidin dimers B1/B2[b]	0.03	< LOQ	100	< LOQ	< LOQ	< LOQ	NA
resveratrol	0.02	< LOQ	10.9	2.85	0.05	0.156	0.28
tyrosol	0.02	0.02	0	9.42	0.458	1.06	1.46
hydroxytyrosol[a]	0.23	0.23	0	58.5	1.31	2.95	5.06
enterodiol	0.05	< LOQ	4.6	9.95	0.208	0.501	1.0
enterolactone[a]	0.05	< LOQ	0.6	30.8	1.73	3.1	4.12

[a]Sensitivity voluntarily reduced for these polyphenols.

[b]Procyanidin dimers B1 and B2 could not be distinguished in the chromatograms.

[c]One sample with concentration higher than that of the highest concentration of the calibration curve.

[d]Nine samples with concentrations higher than that of the highest concentration of the calibration curve.

[e]Two samples with concentrations higher than that of the highest concentration of the calibration curve.

[f]Three samples with concentrations higher than that of the highest concentration of the calibration curve.

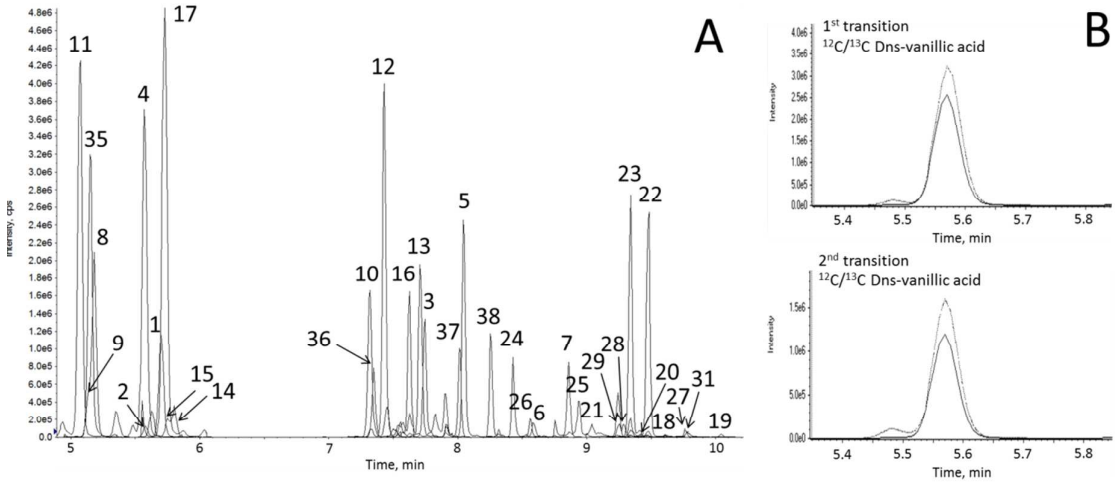


Figure 1. (A) LC-ESI-MS/MS chromatogram of a pool of 25 urine samples from the EPIC cohort. Only the quantifier transition of the ^{12}C-dansylated polyphenols is shown. Run time is 12 min. See Table 1 for peak identity. (B) Scheduled MRM chromatogram showing quantifier and qualifier mass transitions for dansylated vanillic acid.

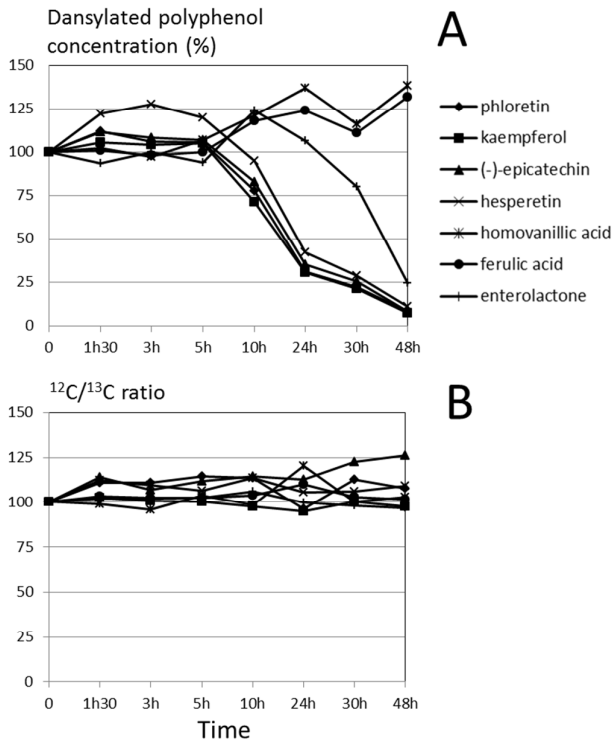
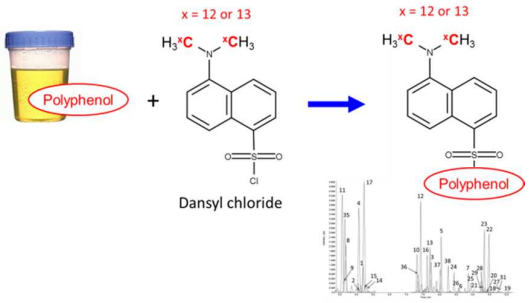


Figure 2. Kinetic of degradation of dansylated polyphenols. Dansylated polyphenol standards were maintained at 10°C in the conditions of the reaction and analysed at different time points after the end of the reaction. Seven out of the 38 polyphenols measured are shown. (A) Concentrations of dansylated polyphenols expressed in relative intensity (%) as compared to initial time (t=0 h). Initial time correspond to the immediate analysis of the urine sample after dansylation. (B) $^{12}C/^{13}C$ ratio for each dansylated polyphenol expressed in relative intensity (%) as compared to initial time (t=0 h).

For TOC only



Quantification of 38 polyphenols in human urine