Rapid Folin–Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake

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\textbf{A B S T R A C T}

Nutritional markers have several advantages for epidemiologic and clinical assays, when compared to dietary data obtained by food frequency questionnaires. Few studies have assessed whether total polyphenol (TP) compounds provide a valid biomarker for TP intake. To date, there has been almost no literature describing methods to determine TP in complex matrices such as urine, which have many interfering substances.

We report a rapid Folin–Ciocalteu method to determine TP in urine samples using Oasis® MAX 96-well plate cartridges for solid phase extraction. These plates allow analysis of a high number of samples at the same time. We performed a prospective, randomized, crossover trial and one cross-sectional study with 60 volunteers from the PREDIMED trial, seeking to evaluate whether the TP in urine were correlated with polyphenol intake and could, therefore, be considered as a marker of intake of these compounds.

The assay was optimized; the sensitivity and the polarity range of urine polyphenols were increased and the detection and quantification limits were significantly reduced. The metabolites in standards solution and urine samples were stable under the storage and handling conditions. In the clinical trial and the cross-sectional study, TP excreted in spot urine samples were positively correlated with TP intake, \( r = 0.48, P < 0.01 \) and \( r = 0.257, P = 0.04 \), respectively.

The methodology described may be used to detect TP in urine samples, employing the high throughput of 96-well microtiter plates and reader. The method is fast and simple and it allows analysis of a large number of samples at the same time.

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1. Introduction

Total dietary polyphenol levels are approximately 1 g day\textsuperscript{-1} \cite{1}, higher than all other known dietary antioxidants, around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid levels. The health effects of polyphenols depend on their respective intakes, synergism and bioavailability, which can vary greatly \cite{2}. Epidemiological studies have shown an inverse association between the risk of myocardial infarction and the consumption of food rich in phenolic compounds: tea, wine, fruit and vegetables (F&V). Clear associations have been found between cancer risk and polyphenol consumption \cite{3–7}. Nutritional markers have several advantages for epidemiologic and clinical assays, when compared to dietary data obtained by food frequency questionnaires (FFQ) \cite{8}. Biomarkers of the intake of some nutrients are more precise than self-reported information based on recalled dietary assessment and thus provide a more objective measure of specific nutrient intake rather than the subjective information obtained by an FFQ. The development of biomarkers, measured in both blood and urine, is essential for making accurate estimates of polyphenol intake. However, the relationship between dietary intake and nutritional biomarkers is often highly complex \cite{9}.
For most polyphenols, urinary excretion values have been reported to be consistent with plasma kinetics data. The Folin–Ciocalteu (F–C) assay for total phenols (TP) is affected by several interfering substances such as sulfur dioxide, ascorbic acid, sugar, aromatic amines, organic acid, Fe(II) and nonphenolic organic substances that react with the F–C reagent [10]. Roura et al. in their research on cocoa [11] studied these interferences after a single solid phase extraction (SPE) cleanup procedure and these substances were not in the eluate, so cannot react with F–C reagent. With the aim of increasing the number and recovery of a larger number of polyphenols from the diet, it was necessary to improve this methodology.

To our knowledge, there is no methodology available to analyze total phenols using microtiter 96-well plates, the use of which would allow a rapid determination for total phenols in urine samples at the same time. However, micro-well plates are used in new studies for the determination of some compounds in food [12,13] and biological sample [14–17]. The aim of the methodology was to have a fast method for total phenols in urine, which could then be used as a biomarker of phenol intake. Toward this end, we tested various solid phase extractions and selected the one with the best reproducible and recovery results in quantifying a high range of phenolic compounds from different foods. We applied Oasis® MAX 96-well plate cartridges for the first time, so that we could reduce analysis time from 11.5 to 2 h. The plate allows the analysis of a high number of samples at the same time for clinical and epidemiological studies. We conducted two trials, one prospective, randomized, crossover trial and one cross-sectional trial, which sought to evaluate whether the total polyphenols in urine are correlated with polyphenol intake and can, therefore, be considered as a marker of intake, bioavailability and accumulation of these compounds.

2. Material and methods

2.1. Standards and reagents

All samples and standards were handled with no exposure to light. Catechin, gallic acid, quercetin, caffeic acid, rutin and formic acid were purchased from Sigma®; epicatechin, F–C reagent and creatinine were obtained from Fluka. The 4-O-methylgallic acid, naringin and tyrosol from extrasyntesis; isoquercetin was obtained from Chroma Dex; the picric acid, sodium hydroxide, hydrochloric acid 35% and acetic acid 99.8% from Panreac and anhydrous sodium acetate (2 M) from Merck. Methanol, acetonitrile and formic acid were obtained from Scharlau. Ultrapure water (Milli-Q) from Millipore System (Bedford, MA). Calibration synthetic urine was prepared as described by Miro-Casas et al. [18] to avoid any possible interference generated by the matrices with F–C reagent.

2.2. The stationary phase for the sample extraction procedure

Oasis® MCX, 30 mg (mixed-mode cation-exchange and reversed-phase solvent), Oasis® MAX, 30 mg (mixed-mode anion-exchange and reversed-phase solvent) and Oasis® HLB, 30 mg (hydrophilic-lipophilic-balanced cartridges, water-wettable and reversed-phase solvent), from Waters (Milford, U.S.A.) were tested using the 96-well plate SPE with its special characteristics. Ten representative polyphenols (4-O-methylgallic acid, catechin, gallic acid, tyrosol, naringin, epicatechin, caffeic acid, rutin, quercetin and isoquercetin) with different polarity were prepared in synthetic urine (5, 10, 25 and 50 mg L⁻¹ for each standard) and 1 mL was loaded into each kind of stationary phase (n = 6). For Oasis® MAX cartridges, 1 mL of methanol 98% and 1 mL of sodium acetate 50 mM pH 7 were loaded to equilibrate the cartridges. The synthetic urine samples with standard were loaded into the cartridges separately. These were then rinsed with sodium acetate 50 mM pH 7/5% methanol. The polyphenols were eluted with 1800 μL of 2% formic acid in methanol. Oasis® MCX was previously equilibrated with 1 mL of methanol and washed with 1 mL of 2% formic acid in methanol. The standards were eluted with 1 mL sodium acetate 50 mM pH 7/5% methanol and 1 mL of methanol 98%. The results were compared those obtained with Oasis® HLB.

To quantify the standards obtained with the SPE, each of eluates was analyzed using high-performance liquid chromatography (HPLC) according to the method of Andrés-Lacueva et al. [19], with minor modifications. The linear gradient profile with the following acetonitrile properties (v/v) was applied (t (min), % acetonitrile): (0, 0.2), (2, 4), (10, 12), (14, 16), (16, 16), (18, 17.6), (22, 19.2), (30, 24), (37, 48), (40, 80). Using a Hewlett-Packard (HP) 1050 (Palo Alto, CA) liquid chromatograph with diode array detector HP 1050 at 280 nm (4-O-methylgallic acid, catechin, gallic acid, tyrosol, naringin, and epicatechin), at 306 nm (caffeic acid), at 385 nm (rutin, quercetin and isoquercetin).

2.3. Analysis of total polyphenols in urines

The F–C method described by Roura et al. [11], used for cocoa epicatechin metabolites, was improved in order to detect a high range of phenolic compounds from different foods in studies with large numbers of samples. Oasis® MAX 96-well plate SPE cartridges were selected because they provided the best recovery. Urine samples were thawed on a ice bed for 3 h; they were centrifuged for 10 min at 4°C and 1 mL of supernatants, catechin and gallic acid standards for calibrated line (1, 2, 4, 6, and 8 mg L⁻¹) were diluted with 1 mL of water Milli-Q and acidified with 34 μL of hydrochloric acid at 35%; they were used to load the Oasis® MAX 96-well plate SPE cartridges separately. The extraction procedure described above for Oasis® MAX cartridges was applied and 15 μL of the eluted fractions were mixed with 170 μL of Milli-Q water in the thermo microtiter 96-well plate (nunc™, Roskilde, Denmark), adding 12 μL of F–C reagent and 30 μL of sodium carbonate (200 g L⁻¹). The multichannel pipette minimized differences in the times (3 s) of the F–C reaction between the eight lines of the 96-well plate, ensuring a similar reaction time for all samples analyzed on the same plate. The 96-well plate permitted less reagent to be used in a more environmentally friendly test. The mixtures were incubated for 1 h at room temperature in the dark. After the reaction period, 73 μL of Milli-Q water were added with the multichannel pipette. Absorbance was measured at 765 nm in UV/VIS Thermo Multiskan Spectrum spectrophotometers (Vanta, Finland). This spectrophotometer allowed the absorbance of a 96-well plate to be read in only 10 s.

For creatinine in urine samples, Jaffé alkaline picrate method [20] was adapted to thermo microtiter 96-well plates, 3 μL of urine were mixed with 60 μL of aqueous picric acid solution (1%) and 5 μL of sodium hydroxide (10%). After shaking, the mixture was left 15 min in the dark at room temperature; 232 μL of Milli-Q water was added and the absorbance was measured at 500 nm in the UV/VIS spectrophotometers. In the absence of disease, creatinine concentrations in urine are usually very stable and can be used to estimate the urinary excretion of substances with only spot urine samples [21–23]. Total polyphenols were expressed as mg gallic acid equivalent (GAE) per g creatinine and mg catechin per g creatinine.

2.4. Folin–Ciocalteu assay validation

The method was validated with gallic acid and catechin standard to compare the results with previous results obtained by Roura et al. [11] expressed in catechin. To evaluate the linearity of the F–C assay a series of calibrators with 1, 2, 4, 6 and 8 mg L⁻¹ were prepared in synthetic urine. The absorbances were plotted against the
corresponding standard concentrations. The sensitivity of the method was evaluated determining the limits of detection (LoD) and limits of quantification (LoQ). These were calculated by measuring the analytical background response, reading 10 blanks at the maximum sensitivity allowed (765 nm) by the system. LoD was considered to be three times the standard deviation (S.D.) of 10 blank samples analyzed while LoQ was considered to be 10 times the S.D.

Accuracy was evaluated by repeatedly spiking the matrix with known levels of analyte standards at five different concentrations. The percentage deviations of added analyte concentrations recovered from a blank matrix were used to estimate accuracy. Precision was calculated by dividing the S.D. by the concentration to obtain the coefficient of variation (CV), which when expressed on a percentage basis gives the relative standard deviation (R.S.D.). It was validated according to the recommendations of the AOAC INTERNATIONAL [24] for the validation of methods.

Short-term temperature stability was evaluated using three aliquots of each concentration (1, 2, 4, 6 and 8 mg L\(^{-1}\)) of gallic acid and catechin in Milli-Q water, previously frozen at \(-20\) and \(-80\) °C. The aliquots were thawed at room temperature, kept at that temperature for 2 h (equivalent to the mean sample preparation time for a 96-well plate) and then analyzed. We also evaluated stability after successive freeze and thaw cycles. Three aliquots of each concentration were stored at \(-20\) and \(-80\) °C for 24 h, and thawed at room temperature. After being completely thawed, the samples were refrozen for 24 h, and the freeze–thaw cycle was repeated two more times. Then the aliquots were analyzed on the third cycle. Lastly, long-term stability was determined by storing three aliquots of each concentration under the same conditions as the study samples (\(-80\) °C) for a period of 2 years. The concentrations of all the stability samples were compared with the mean back-calculated values for the standards at appropriate concentrations from the first day of long-term stability testing. For sample stability the same processes were applied [25].

2.5. Study design and samples

We obtained the first urine samples from the volunteers in morning, at the same time. The morning urine samples were collected in sterilized bottles and the aliquots were immediately prepared and frozen at \(-80\) °C until analysis. For the assay, thawing is necessary over an ice bed for 3 h, to avoid degradation of the polyphenols. The samples must be transported in a polystyrene box with dry ice to maintain their temperature. Low temperature and direct sublimation to a gas makes dry ice an effective coolant, since it is colder than water or ice and leaves no moisture as it changes state. The samples must be kept frozen at \(-80\) °C in aliquots if they are required for a long period, and the same thawing procedures described above must be repeated for subsequent assays.

2.5.1. Clinical trial

This prospective, randomized, crossover trial included 12 healthy adult volunteers (8 women and 4 men; age range 24–54 years) with no previous relevant illnesses. The study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 1996. The Ethical Committee for Human Experimentation of the Barcelona University, Spain approved the study, and written informed consent was obtained from each participant.

The volunteers were randomly separated into two groups; the first group began with a high polyphenol diet (HPD) for 3 days and then changed to a low polyphenol diet (LPD) for 3 days, after a 3-day intermediate period of taking the usual diet (UD). They were instructed by a dietician to abstain from food and beverages the required polyphenols for each type of diet. The volunteers’ daily intakes were recorded using an FFQ. They received a list of recommended or restricted foods and beverages. They abstained from taking medication and vitamin supplements for 1 week before and during the study.

2.5.2. Cross-sectional study

The PREDIMED (PREvention with MEditerranean Diet) study is a large, parallel group, multicenter, controlled, randomized 4-year clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease (http://www.predimed.org). We selected a subsample of 60 volunteers from the center at the University of Navarra (School of Medicine) to analyze their total urinary polyphenols; 29 men and 31 women between 56 and 80 years old having a body mass index between 20.4 and 36.6. At baseline, participants completed a 137-item validated FFQ [26] and the validated Spanish version of the Minnesota Leisure Time Physical questionnaire [27]. Exclusion and inclusion criteria have been described previously by Estruch et al. [28]. The volunteers were divided into tertiles depending on their daily intake of total F&V: the first tertile had a consumption less than 508.03 g day\(^{-1}\) (low F&V consumers, LFVC), the second tertile (medium F&V consumers, MFVC) between 508.04 and 640.23 g day\(^{-1}\), and the third tertile from 640.24 g day\(^{-1}\) upwards (high F&V consumers, HFVC). The Institutional Review Board of the Hospital Clinic of Barcelona approved the study protocol, and written informed consent was obtained from each participant. Urine samples were collected before the FFQ, and were coded and stored at \(-80\) °C until analysis.

TP intake from plant food and beverages (mg g\(^{-1}\) fresh matter) in both studies was quantified according to Saura-Calixto and Goni [29] taking into account the FFQ. The edible portion of the daily amount consumed, per capita, for each food as eaten was divided into eight groups: cereals, vegetables, legumes, nuts, chocolate, fruit, oils and phenolic beverages (coffee, tea, wine, beer and fruit juices).

2.6. Statistical analysis

The statistical analyses were performed using the SPSS package program version 14.0. Descriptive statistical tests with the mean (S.D.) were used for the baseline characteristics of the participants. Variables were examined for normality and skewness (Kolmogorov and Levene tests) and were tested for carry-over and treatment–period interactions. The TP content in morning urine and phenol intake were not normally distributed in both studies. Because this was not normalised by the logarithmic transformation, non-parametric tests were used in the statistical analyses of the data. The Wilcoxon test was performed for related samples to compare changes in outcome variables in response to each intervention period in the clinical trial and the Mann–Whitney test for independent samples in the cross-sectional study. Spearman correlations (\(r_s\)) were performed to evaluate the association between TP content in morning urine and TP intake from selected food groups in both studies. \(P\)-values <0.05 (two-tailed) were considered to be significant. To compare groups in the cross-sectional study, we used the two-tailed t-test.

3. Results

3.1. SPE cartridge selection

Table 1 shows the recoveries for 10 polyphenol standards in the urine matrix, for Oasis® MCX, MAX and HLB. They were calculated using the mean recoveries at four different levels: 5, 10, 25 and 50 mg L\(^{-1}\) for all standards. The best recoveries were obtained with Oasis® MAX cartridges. The AOAC INTERNATIONAL [24] establishes a good recovery for biological samples of between 80% and 110%. MCX
was ruled out because some polyphenol recoveries were lower than 80%: for gallic acid and for catechin, they were 71.23% and 70.43%, respectively, and 80.92% for quercetin. For HLB, the recovery values were similar to MAX in some standards, although of the two resins MAX was selected because of the higher results obtained, being approximately 100%. The gallic acid recovery was 88.44% in HLB compared to 100.25% in MAX cartridges; the recovery for quercetin in the HLB was slightly lower than 80%.

The assay was optimized and Oasis® MAX 96-well extraction plates were selected. This significant change allowed acidic, basic and neutral compounds to be detected, confirmed and quantified in different fractions in biological fluids. The sensitivity and the polarity range of the urine polyphenols were increased and the detection and quantification limits were significantly reduced. The 96-well plate makes the method particularly well adapted to the analysis of large batches of samples for clinical and epidemiological studies.

### 3.2. Validation of the method for the analysis of total polyphenols in urine

The F–C method was linear over the working range between 1 and 8 mg L\(^{-1}\) for gallic acid and catechin. These two standards were selected as they are the ones most frequently used for F–C assays. Least-squares regression analysis gave the following result for the gallic acid calibration curve: mean (S.D.); slope, 0.082 (0.005); \(y\)-intercept, 0.030 (0.009); \(r^2 = 0.997\) (0.001) and standard deviation of residuals, 0.077. For the catechin calibration curve: mean (S.D.); slope, 0.0778 (0.002); \(y\)-intercept, 0.065 (0.009); \(r^2 = 0.995\) (0.003) and standard deviation of residuals, 0.072. The limit of detection (0.07 mg L\(^{-1}\)) and limit of quantification (0.11 mg L\(^{-1}\)) were determined for gallic acid and catechin standards.

Assay precision and accuracy were evaluated at five different concentrations (Table 2). The values obtained for precision “R.S.D. (%)” were lower than those established by AOAC INTERNATIONAL [24], which would be 7.5% at this concentration. Accuracy was measured according to the percentage of gallic acid and catechin added. The AOAC INTERNATIONAL [24] accepts values of 80–110% recovery for the values analyzed, and values were found between 82.5% and 105.7%.

### Table 1

<table>
<thead>
<tr>
<th>Cartridges</th>
<th>MCX</th>
<th>MAX</th>
<th>HLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid, mean (S.D.)</td>
<td>71.23 (3.56)</td>
<td>100.25 (3.66)</td>
<td>88.42 (6.25)</td>
</tr>
<tr>
<td>Isoquercetin, mean (S.D.)</td>
<td>107.52 (8.98)</td>
<td>105.13 (8.07)</td>
<td>96.23 (9.79)</td>
</tr>
<tr>
<td>Quercetin, mean (S.D.)</td>
<td>80.92 (3.74)</td>
<td>95.74 (7.12)</td>
<td>79.23 (6.52)</td>
</tr>
<tr>
<td>Catechin, mean (S.D.)</td>
<td>70.43 (3.97)</td>
<td>92.52 (4.43)</td>
<td>93.7 (6.6)</td>
</tr>
<tr>
<td>Epicatechin, mean (S.D.)</td>
<td>109.78 (4.51)</td>
<td>105.02 (1.90)</td>
<td>105.62 (2.10)</td>
</tr>
<tr>
<td>4-0-Methylgallic acid, mean (S.D.)</td>
<td>104.42 (0.89)</td>
<td>97.87 (1.52)</td>
<td>94.11 (8.42)</td>
</tr>
<tr>
<td>Tyrosol, mean (S.D.)</td>
<td>94.32 (3.07)</td>
<td>98.13 (2.23)</td>
<td>96.38 (5.23)</td>
</tr>
<tr>
<td>Naringin, mean (S.D.)</td>
<td>103.23 (0.42)</td>
<td>101.16 (9.45)</td>
<td>95.44 (6.48)</td>
</tr>
<tr>
<td>Caffeic acid, mean (S.D.)</td>
<td>109.50 (0.57)</td>
<td>101.07 (2.88)</td>
<td>93.85 (7.3)</td>
</tr>
<tr>
<td>Rutin, mean (S.D.)</td>
<td>104.54 (2.17)</td>
<td>97.66 (0.72)</td>
<td>93.77 (9.56)</td>
</tr>
</tbody>
</table>

\(R.S.D., \text{ relative standard deviation.}\)

### Fig. 1

Concentration of total phenolic “mg gallic acid equivalent per g creatinine” excreted in morning urine after the ingestion of the low (LPD), usual (UD) and high (HPD) polyphenol diets in the clinical trial.

The standards in Milli-Q water after freeze and thaw cycles remained stable for both temperature tested and at the different concentrations. At higher concentrations (6 and 8 mg L\(^{-1}\)) the gallic acid remained near 100%, while at smaller concentrations (1 and 2 mg L\(^{-1}\)) it was ~90%. Catechin standards frozen at ~20 °C remained between 86% and 89% at all concentrations, while at ~80 °C the standards remained at 89–95%. At room temperature (short-term stability) for 2 h, decreases in both standards (gallic acid and catechin) were observed; the amounts were reduced to ~86% when working with the lowest concentration at ~20 °C, which was very similar to ~80 °C (~89%). The standards remained higher (>96%) in the highest concentrations, and they were similar for both standards and both deep-freeze temperatures (~20 and ~80 °C). For the rest of the concentrations at the two deep-freeze temperatures, the standards remained higher than 90%. For the long-term stability of standards, the level decreased to 93% and 95% for catechin and gallic acid, respectively, after 2 years, in both deep-freeze temperatures (~20 °C and ~80 °C). We observed no statistically significant differences (\(P > 0.05\)) in total polyphenols in urine samples (\(n = 24\)), after testing freeze and thaw cycles and short-term and long-term stability. In conclusion, the phenolics in standards solution and urine samples were stable under the storage and sample handling conditions used for these assays; standards solution and urine samples should be stored at ~20 °C over a long period of time, i.e., approximately 2 years in length or more.

### 3.3. Total polyphenols measured in urine samples

#### 3.3.1. Clinical trial

The concentration of TP compounds excreted in morning urine after ingestion of different diets is shown in Fig. 1. TP excreted in the urine, expressed as mg GAE per g creatinine was 107.07 (17.01)
after the HPD. The UD consumed maintained the excretion of TP at the intermediate value of 95.44 (10.50), higher than 82.73 (13.43) in LPD. The intake of TP compounds in the HPD was 1255.02 (491.89) mg GAE per person per day; in UD and LPD, it was 759.90 (239.81) and 370.51 (121.98), respectively.

The Wilcoxon test for related samples for each intervention period showed a significant difference \( P = 0.002 \) between the urine excreted after the HPD and LPD; and a tendency to significant difference was found \( P = 0.06 \) when comparing UD with HPD and LPD. This statistical analysis was performed to compare the TP intake. The results showed a significant difference \( P < 0.01 \) between the TP intake for each intervention period. The differences between groups are shown in Table 3.

The Spearman correlation analyses (see Table 4) revealed a positive significant correlation between the TP excretion in morning urine and the consumption of TP from fruit \( P < 0.05 \), vegetables \( P < 0.01 \), F&V \( P < 0.01 \) and TP intake \( P < 0.01 \); there was also a significant negative correlation with cereals \( P < 0.01 \). No significant correlations were observed with the rest of the food groups.

### 3.3.2. Cross-sectional study

TP excreted in the urine for LFVC was 111.86 (34.74) mg GAE per g creatinine after the TP intake of 1293.38 (357.29) mg GAE per person per day. The excretion for NFVC was 141.87 (48.81) mg GAE per g creatinine and 160.73 (82.42) mg GAE per g creatinine for HFVC after the intake of 1364.01 (402.23) and 1768.87 (527.32) mg GAE per person per day, respectively. The daily F&V ingestion was 397.96 (91.44) g, 574.73 (39.57) g and 844 (202.74) g for low, intermediate and high polyphenol diets, respectively.

The Spearman correlation analyses (Fig. 2) revealed a positive significant correlation between the TP excretion in morning urine and total dietary polyphenols \( r = 0.257, P = 0.04 \) and the total F&V intake \( r = 0.339, P = 0.008 \). No correlation between the total urinary polyphenols and the other groups were found.

### 4. Discussion

In this work, we developed a method especially adapted to analyse large batches of samples, which could be used for TP and creatinine assay, the 96-well plates accommodate much larger sample volumes, with the potential for proportionally greater sensitivity. The well plates are also a less cumbersome format and better suited to automated manipulation, which proved faster than previous methods [10,11,29]. It was possible to analyze 96 urine samples in only 3 h (2 h for the sample preparation and 1 h for the reaction) by using the Oasis® MAX 96-well plate for the SPE and the thermo microtiter 96-well plate for the Folin–Ciocalteu reaction. This eliminated laborious sample preparation after the thawing period over a bed of ice (approximately 3 h) in contrast to other methods, which required \(~12.5\) h for 96 samples (1 h for the reaction and 0.5 h to read the cuvettes), and it is a methodology that is much more environmentally friendly. This permitted the analysis of a large number of samples per day, which is ideal for clinical and epidemiological studies in which the subjects ingest a large variety of polyphenols with their usual diet. It was possible to increase the recovery of a high number of polyphenols using the SPE with MAX cartridges, due to the mixed mode anion exchange and reversed phase solvent with a high selectivity and sensitivity for all polyphenols compounds tested. The SPE diminishes the interference for TP analysis in urine samples.

Mennen et al. [30] and Krogholm et al. [31] studied the correlation between the consumption of polyphenol-rich foods and beverages and the concentration of polyphenols in urine samples determined by LC–MS/MS. Their results suggested that several polyphenols measured in urine samples can be used as biomarkers of polyphenol-rich food intake. In our investigation the total polyphenol food intake was positively correlated to the total polyphenol excreted in urine samples. Most correlations observed between urinary excretion of polyphenols and food intake were expected from their contents in food and established recoveries in urine in intervention studies of specific food items [32].

The intake of TP in the clinical trial on HPD, 1255.02 (491.89) mg per person per day was slightly higher than the daily intake of TP in the Spanish diet, estimated by Saura-Calixto and Goni [29] as 1171 mg per person per day. However, in our study the volunteers were intentionally consuming a HPD while Saura-Calixto estimated

### Table 3

Relative differences between the groups’ total polyphenol intake, expressed as mg gallic acid equivalent, and between the groups’ urinary excretion of total polyphenols, expressed as mg gallic acid equivalent/g creatinine, for different diets in the clinical trial.

<table>
<thead>
<tr>
<th>Differences between</th>
<th>95% Confidence interval for the difference</th>
<th>Mean</th>
<th>Lower</th>
<th>Higher</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPI in HPD and TPI in UD</td>
<td>495.12 265.90 724.34 0.002</td>
<td>265.90</td>
<td>724.34</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>TPI in UD and TPI in LPD</td>
<td>389.39 271.95 506.84 0.002</td>
<td>271.95</td>
<td>506.84</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>TPI in HPD and TPI in LPD</td>
<td>884.51 429.74 1240.06 0.002</td>
<td>429.74</td>
<td>1240.06</td>
<td>0.002</td>
<td></td>
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<tr>
<td>F&amp;V in HPD and F&amp;V in UD</td>
<td>11.63 9.91 14.38 0.006</td>
<td>9.91</td>
<td>14.38</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>F&amp;V in HPD and F&amp;V in LPD</td>
<td>12.70 0.59 24.18 0.06</td>
<td>0.59</td>
<td>24.18</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>F&amp;V in LPD and F&amp;V in UD</td>
<td>24.34 11.81 36.87 0.002</td>
<td>11.81</td>
<td>36.87</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

TPI, total polyphenol intake; UETP, urinary excretion of total polyphenols; UD, normal polyphenol diet; HPD, high polyphenol diet; LPD, low polyphenol diet.

### Table 4

Correlation between consumption of different food groups and morning urinary polyphenols (mg gallic acid per g creatinine).

<table>
<thead>
<tr>
<th>Food group</th>
<th>( r )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits</td>
<td>0.332</td>
<td>0.048</td>
</tr>
<tr>
<td>Vegetables</td>
<td>0.504</td>
<td>0.002</td>
</tr>
<tr>
<td>Fruits and vegetables</td>
<td>0.501</td>
<td>0.002</td>
</tr>
<tr>
<td>Legumes</td>
<td>−0.106</td>
<td>0.539</td>
</tr>
<tr>
<td>Cereals</td>
<td>−0.644</td>
<td>0.001</td>
</tr>
<tr>
<td>Phenolic beverages</td>
<td>0.293</td>
<td>0.082</td>
</tr>
<tr>
<td>Oils</td>
<td>−0.094</td>
<td>0.586</td>
</tr>
<tr>
<td>Nuts</td>
<td>0.221</td>
<td>0.195</td>
</tr>
<tr>
<td>Chocolate</td>
<td>0.103</td>
<td>0.551</td>
</tr>
<tr>
<td>Total phenols</td>
<td>0.482</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Krogholm et al. [31] and Nielsen et al. [33], and it does not require of intake of polyphenols rich foods and is cheaper, more envi-
mental.


