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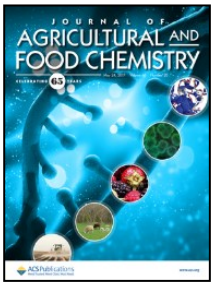

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A new method to estimate total polyphenol excretion: comparison of Fast Blue BB *vs.* Folin-Ciocalteu performance in urine

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ABSTRACT

Polyphenols are bioactive substances of vegetal origin with a significant impact on human health. The assessment of polyphenols intake and excretion is therefore important. The Folin-Ciocalteu (F-C) method is the reference assay to measure polyphenols in foods as well as their excretion in urine. However, many substances can influence the method, making it necessary to conduct a prior clean up using solid phase extraction (SPE) cartridges. In this paper we demonstrate the use of the Fast Blue BB reagent (FBBB) as a new tool to measure the excretion of polyphenols in urine. Contrary to F-C, FBBB showed no interference in urine, negating the time-consuming and costly SPE clean up. In addition, it showed excellent linearity ($r^2 = 0.9997$) with a recovery of 96.4% and a precision of 1.86-2.11%. The FBBB method was validated to measure the excretion of polyphenols in spot urine samples from Spanish children, showing a good correlation between polyphenols intake and excretion.

KEYWORDS

Polyphenols, daily intake, urinary excretion, Folin-Ciocalteu, Fast Blue BB.

INTRODUCTION

Polyphenols are a group of chemical substances found in plants characterized by the presence of more than one phenol unit or building block per molecule.[1] Fruits, vegetables, and beverages are the main sources of phenolic compounds in the human diet.[2] Western populations consume around 1–2 g/day polyphenols, mainly from fruits, vegetables and beverages such as tea, coffee, wine and fruit juices.[3] Many associations between intake of certain polyphenols and a reduction in disease risk factors have been observed in cohort studies[4-7] mainly due to their antioxidant,[8-9] anti-inflammatory,[10] cardio-protective,[11] cancer chemo-preventive[12-13] and neuroprotective properties.[14]

Polyphenol intake is most commonly estimated using dietary recalls, food-frequency questionnaires and databases on the polyphenol content of foods. These tools, particularly the development of databases on the food content of polyphenols, have improved significantly over the past few years.[15-17] In this sense, Phenol-Explorer is a searchable database that has revolutionized the estimation of dietary intake of polyphenols.[17] It contains the average polyphenol composition of more than 500 food items as well as their retention factors.[18] As a comprehensive polyphenol knowledge base, the Phenol-Explorer database has become a unique and essential tool for polyphenol scientists.

There is an urgent necessity to obtain accurate biomarkers of polyphenol intake in order to identify associations between polyphenol intake, and health and disease outcomes in cohort studies.[19] Biomarkers are objective measures since they do not depend on dietary-assessment methods, limiting potential bias and error associated with dietary assessment and inaccuracies in food-composition data.[20-21] The main biomarker of polyphenol intake is the excretion of phenol metabolites in urine[22.] This is due to the short half-life of polyphenols in plasma and the stronger expected dependence of polyphenol concentrations on sampling time in urine.[23]

The main method to quantify polyphenols in natural products is the Folin–Ciocalteu (F-C) assay, with some improvements described by Singleton & Rossi.[24] However, the F-C assay is affected by several non-phenolic interfering substances (such as sugars, aromatic amines, sulfur dioxide, ascorbic acid or organic acids) that react with the F-C reagent.[25] Roura et al.[22] studied the effect of such

interferences in urine in order to assess the intake of polyphenols coming from cocoa. These authors have proposed a method where such interfering substances are removed from urine after a single solid phase extraction (SPE) cleanup procedure, so that they were not present in the eluate and cannot react with the F–C reagent. An alternative method of analyzing polyphenols in food extracts has been developed by Medina.[26] This new assay does not suffer from interferences with the same substances as the F-C method, because of the use of the Fast Blue BB diazonium salt (FBBB). In this salt, the diazonium group specifically couples with reactive phenolic hydroxyl groups under alkaline conditions, to form stable azo complexes that can be measured at 420 nm.[27,28] The aim of this study was to study the feasibility of the Fast Blue BB method in order to assess the amount of total phenols excreted in orine, avoiding the time-consuming and constly SPE clean up used with the Folin-Ciocalteu method. We tested the application of this method in a population of children to determine the associations between polyphenol intake (assessed with a Food Frequency Questionnaire) and excretion of total phenolics (measured with the FBBB reagent).

MATERIALS AND METHODS

Chemicals. 4-Amino-2,5-diethoxybenzanilide diazotated zinc double salt (Fast Blue BB), Folin-Ciocalteu reagent, gallic acid, quercetin, luteolin, naringenin, catechins, genistein, malvidine, enterodiol, caffeic acid, resveratrol, hydroxytyrosol, picric acid, creatinine, sodium hydroxide, calcium chloride, magnesium chloride, sodium chloride, sodium sulphate, sodium citrate, dihydrogen phosphate, potassium chloride, ammonium chloride and urea were from Sigma-Aldrich (Germany). All these reagents were of analytical grade.

Human intervention. Five schools located in a similar rural environment and with the same socio-economic status were included in the study. The study involved 228 children aged 10-11 years

old, of which 112 were girls and 116 boys. The sample was randomized at school-level into two groups. The program consisted of a group which did not receive any intervention (CG; n = 114) and another group which received nutritional education sessions (IG; n = 114). The study was carried out over a six month period. The nutritional education sessions informed participants about the benefits of the Mediterranean diet (high fruit, vegetable, legumes, fish, cereals, unsaturated to saturated fat ratio, and low meat, meat products and dairy products). Parents and students were invited to attend separate sessions. Parents received six classes, each lasting approximately two hours. One session was provided each week for the first six weeks of intervention. Either one or both parents could attend the sessions. Children received two nutritional education sessions during school hours (tutorial hours), each lasting about one hour. One session was held each week for the first two weeks of intervention. It was compulsory for pupils to attend both sessions. All children were healthy and were not receiving any medical treatment. All the participants took part voluntarily in accordance with the Declaration of Helsinki regarding ethical research. The bioethical committee of the University of Granada for human research approved the study under reference SA/11/AYU/246. Informed consent was also obtained from all of the children's parents.

Polyphenol intake-excretion evaluation. Nutritional data were obtained, both at pre-test and post-test by means of a semi-quantitative 136-item Food Frequency Questionnaire[29]. Participant's parents indicated the average frequency of consumption of each food over the past 6 months. For each food item, a commonly used portion size was specified, and the parent's participants were asked how often their children had consumed that unit over the previous year. Nine options for frecuency consumption were offered: never or hardly ever, one to three times a month, once a week, two to four times a week, five to six times a week, once a day, two to three times a day, four or six times a day and more than six times a day. The evaluation of total phenols intake was performed with the data on polyphenols content retrieved from the Phenol Explorer 3.6 database. Foods were classified into eight

groups: carbohydrates/cereals, vegetables, fruits, legumes, nuts, oil, drinks and 'others' (chocolate, cacao, olives, among others). Processed foods such as pizzas, lasagnes, etc. were broken down into their principal ingredients, from which total phenols of that food in the diet was estimated. Excretion of total phenols was studied with first morning urine spot samples at the beginning and at the end of the study. The urine samples were acidified with HCl to preserve the phenolic compounds before storage at -80°C.Total phenols in urine were determined by means of the FBBB and F-C methods.

FBBB assay. Polyphenol excretion was assessed by means of the FBBB method[26] adapted to urine instead of aqueous food extracts. In summary, 25 µL of urine were diluted with 975 µL of water. Then, 100 µL FBBB reactive (prepared as an aqueous 0.1% solution by sonication over 5 minutes) and 400 µL 5% NaOH were added, vortexed for 30 s and the resulting mixture allowed to incubate for 90 min at room temperature. Absorbance was measured at 420 nm on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37°C). Quantification was carried out using a standard curve of gallic acid. Results were expressed as mg gallic acid/g creatinine.

F-C assay. F-C assay was performed according to the Folin-Ciocalteu.[24] Briefly, 30 µL of urine were mixed with 15 µL Folin-Ciocalteu reagent, 60 µL sodium carbonate solution 10% (w/v) and 190 µL of distilled deionized water. Absorbance readings were taken every 60 s for 60 min on a FLUOStar Omega microplate reader (BMG Labtech, Germany) with temperature control (37°C). Quantification was carried out using a standard curve of gallic acid. Results were expressed as mg gallic acid/g creatinine.

Creatinine assay. We used first morning urine samples instead of 24 h urine samples. Thus, it was necessary to standardize diuresis with creatinine concentration in orine.[22] In the absence of disease, creatinine concentrations in serum and urine are usually very stable and can be used to

estimate the urinary excretion of substances with only spot urine samples.[30-33] In the Jaffe method[34] a red color is formed when creatinine reacts with picric acid in an alkaline medium, being the absorbance at 500 nm proportional to creatinine concentration. Briefly, 40 μL of urine were mixed with 800 μL of aqueous picric acid solution (1%, vol/vol) and 60 μL of sodium hydroxide (0.01% w/vol). After shaking, the mixture was left in the dark for 15 min at room temperature. Then, 3.1 mL of water was added and the absorbance at 500 nm was read on a Lambda 25 spectrophomometer (Perkin Elmer, USA).

Synthetic urine. Artificial urine was prepared as blank for the different assays as previously reported by Roura et al.[22] Synthetic urine was composed of calcium chloride (0.65 g/L), magnesium chloride (0.65 g/L), sodium chloride (4.6 g/L), sodium sulphate (2.3 g/L), sodium citrate (0.65 g/L), dihydrogen phosphate (2.8 g/L), potassium chloride (1.6 g/L), ammonium chloride (1.0 g/L) urea (25 g/L) and creatinine (1.1 g/L) and adjusted to pH = 6.5. After solubilization of the different salts, the solution was filtered and stored at -80°C until use.

SPE purification. In order to check the presence of interfering compounds in urine, a solid phase extraction cleanup was also performed before the determination of polyphenols in urine.[22] Briefly, 1 mL of the participants control or acidified urine samples was applied to an activated Waters Oasis HLB 3-mL (60 mg) cartridge. The SPE cartridge was washed with 2 mL of formic acid (1.5 M) and 2 mL of water–methanol (95:5 vol/vol). The polyphenols were eluted with 1 mL of acidified methanol (1 mL formic acid/L).

Data Analysis. Normality of the data was analyzed using the Kolmogorov-Smirnov test with Lillieforts correction. Statistical significance of the data was tested using a T-test of pre- and post-test time points, followed by the Duncan test to compare the means of values showing significant variation ($P < 0.05$). Evaluation of the relationship between polyphenol intake and excretion was carried out by

144 computing the relevant correlation coefficient (Pearson linear correlation) at the $P < 0.05$ confidence
145 level. All statistical analyses were performed using the Statgraphics Centurion XVI statistical software
146 (2009).
147
148 **RESULTS AND DISCUSSION**
149 **FBBB method suitability.** We elected to use the Fast Blue BB method to assess the amount of
150 total phenols in urine since it has been proven to be a better approach than the Folin-Ciocalteu method
151 when assessing foods.[26,27,35] In addition, we avoided the time-consuming and costly SPE clean up
152 needed with the F-C method. Table 1 shows the results obtained with the FBBB and F-C methods
153 (with and without SPE purification) in control urine (a syntetic urine prepared as previously reported
154 by Roura et al., 2006), a regular sample spot urine and the same urine spiked with a known amount of
155 Gallic acid. As stated in Table 1, bot FBBB and F-C reacted with urine components, giving rise to a
156 higher absorbance in the case of the F-C reagent. The analysis of the same urine spiked with Gallic
157 acid 10 mM gave rise to a statistically significant increase ($P < 0.0.5$) in absorbance of 0.1 units in the
158 case of FBBB and 0.3 units for F-C. When a prior SPE purification was performed for the control
159 urine, the absorbance obtained with the FBBB method was statistically similar ($P > 0.05$) to that
160 obtained withour SPE. However, in the case of the F-C method, absorbance decreased to 0.329 ($P <$
161 0.05), clearly showing the presence of interfering compounds in urine as discovered previously by
162 Roura et al.[22] When urine was spiked with Gallic acid, both FBBB and F-C gave rise to the similar
163 absorbances. No statistically significant differences ($P > 0.05$) were obtained with the FBBB reagent
164 with or without SPE clarification. Therefore, results indicated that a prior SPE process is needed when
165 using the F-C method in order to avoid reactions with interfering substances, while the FBBB reagent
166 allows the direct determination of polyphenols metabolites in urine with fewer interferences.
167
168 **FBBB linearity.** A series of gallic acid calibrators with concentrations ranging from 0.1 to 50
169 mg/L were prepared in distilled water. The FBBB method showed an excellent linearity ($r^2 = 0.9997$)

with this calibration curve. When the same calibrators were applied to urine, the lineal range extended up to 40 mg/L of gallic acid added ($r^2 = 0.9994$) as the non-lineal part of the graph was reached due to very high absorbance values at this concentration.

FBBB accuracy and recovery. Recovery was assessed by analyzing a urine sample spiked with known concentrations of gallic acid (Table 2). The average recovery of polyphenols from urine was 96.4 ± 3.6% and within the range of 10-55 mg gallic acid/L. Concentrations higher than 55 mg gallic acid/L gave rise to lower recoveries (≈ 76%) because the non-lineal part of the calibration curve is reached. The same recovery experiment was performed with 10 different phenols belonging to the main polyphenol families (Table S1). Recovery ranged from 90.2 to 98.4% for enterodiol and catechins, respectively, with a mean recovery of 94.6 ± 2.8%. Therefore, the FBBB method demonstrated capability to react with different type of polyphenols present in foods, which in turn will be transformed into many different chemical species excreted through urine.

FBBB precision. Precision (repeatability) is defined as the closeness of agreement between a series of measurements obtained from multiple sampling under the same operating conditions over a short interval of time. For this evaluation, samples from four diferent children were collected and analyzed seven times using the FBBB method. As stated in Table 3, the mean intra-day precision was 1.86. Repeatability was also assessed as intermediate precision (inter-day precision) by analyzing the urine of two children during two non-consecutive days (seven times each). The mean coefficient of variation obtained was 2.11% (Table 3), showing excellent reproducibility of the analysis conditions for the F-C method with SPE purification, similar to that found by Roura et al.[22]. In addition, an interlaboratory validation of the FBBB method was performed (Table S2), including analysis of the same six urine samples used to obtain the results of Table 1. The z-scores obtained ranged from -0.36 to +1.02, with a mean z-score of 0.36. Thus, the FBBB method can be replicated and used in different labs to perform epidemiological analysis in large cohorts.

196

197 **Nutritional intervention.** Following validation of the FBBB method to measure excretion of
198 polyphenols in spot urine samples from school children, polyphenol intake and excretion were
199 measured to identify relationships among them and validate the method for a human cohort. The mean
200 daily intake of total phenols was 2079 ± 654 mg/day, which is close to that previously stated by Saura-
201 Calixto et al.,[36] who defined a range of 2590–3016 mg gallic acid /person/day (including extractable
202 and hydrolysable polyphenols) for the Spanish adult population. On the contrary, the PREDIMED
203 estimated a mean phenols consumption of 820 ± 323 mg/day for a Spanish adult cohort at high
204 cardiovascular risk.[37] These differences could be due to the inclusion of both extractable and
205 hydrolysable polyphenols in the search performed in Phenol-Explorer, while the PREDIMED study
206 did not include such data. No statistically significant differences ($P > 0.05$) were obtained according to
207 sex (mean intake of 2066 ± 642 mg/day for boys and 2092 ± 666 mg/day for girls). The contribution of
208 different food groups is shown in Table S3. The group "legumes" had the largest contribution to the
209 daily polyphenol intake (935 mg gallic acid/day), due to the high polyphenol content of lentils. The
210 other two groups with the highest contribution were "fruits" (oranges, bananas, apples and
211 strawberries) and "others" (chocolate and cocoa powder) with 372 and 332 mg gallic acid/day.

212 The effect of the nutritional intervention is shown in Figure 1. At the pre-intervention level there
213 were no statistically significant differences ($P > 0.05$) between the intervention (Figure 1A) and
214 control groups (Figure 1B) due to the homogeneity of both groups. However, after intervention there
215 was a statistically significant ($P < 0.05$) increase of 25.4% on total polyphenol intake in the
216 intervention group (Figure 1A) from 2031 ± 180 to 2448 ± 215 mg gallic acid/day due to a higher
217 consumption of fruits, vegetables and cereals (Table S3).

218 Total polyphenol excretion in the intervention and control groups was determited by the FBBB
219 method and expressed as mg gallic acid/g creatinine.[22] As in the case of polyphenol intake, there were
220 no significant differences ($P > 0.05$) between polyphenol excretion at pre-intervention stage (Figures

1C and 1D). However, there was a statistically significant increase ($P < 0.05$) in polyphenol excretion in the intervention group (Figure 1C) 180 ± 65 to 215 ± 52 mg gallic acid/g creatinine. Thus, the range of detected polyphenols in urine obtained in the present paper are in line with those reported by Hussein et al.[38] and Zamora-Ros et al.[39] for child-adoslescent and elderly populations, respectively, which in turn were obtained with the F-C method after proper SPE purification.[22] In fact, it is notworthy to mention that Hussein et al.[38] found an increase in polyphenol excretion (in a Egyptian population aged 7-14 years) from 90 ± 9 mg gallic acid/g creatinine to 287 ± 64 mg gallic acid/g creatinine after a 7-day nutritional intervention consisting of dietary supplementation with tomato juice. This increase is in the same range as that found in the present study.

Finally, the Pearson correlation between polyphenol intake and excretion was calculated at the pre-intervention (Figure 2A) and post-intervention (Figure 2B) levels. The high positive correlations found ($r = 0.9405$ and 0.9327 for pre- and post-intervention, respectivelly) support the use of the analysis of polyphenol excretion in urine (measured by the FBBB method) as a good biomarker of polyphenols intake-excretion.

In conclusion, the results of this study provide the first evidence that the Fast Blue BB method has some clear advantages over the Folin-Ciocalteu method since it is cheap, fast and repeatable. Thus, it is an easy way to assess the amount of total phenols excreted in urine, avoiding the time-consuming and costly SPE clean up used with the Folin-Ciocalteu method. In this sense, the FBBB method could be used in the future in large human cohort studies to unravel the intake, excretion and beneficial effects of polyphenols within the human diet. However, some drawbacks are still present including the differences in absorbance obtained after reaction with several phenolic standards, which could play a role in differences on polyphenol excretion when foods containing different phenol species are eaten. In addition, some other metabolites excreted in urine could slightly interfere with the FBBB reagent. In that sense, future studies should be performed with a strategy similar to that reported by Sánchez-Rangel et al.[40] in order to improve the specificity of the total phenolic determination.

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SUPPORTING INFORMATION

Table S1: Recovery of Different Polyphenols in Urine With the FBBB Method. Table S2: Inter-Laboratory Validation of the FBBB Method. Table S3: Polyphenol Intake Grouped by Food Types.

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Figure captions

Figure 1. Effects of nutrition education program in daily polyphenols intake (intervention group, 1A; control group, 1B) and daily polyphenols excretion (intervention group, 1C; control group, 1D).

Figure 2. Correlation between daily polyphenols excretion and intake pre-intervention (2A) and post-intervention (2B).

Figure 1

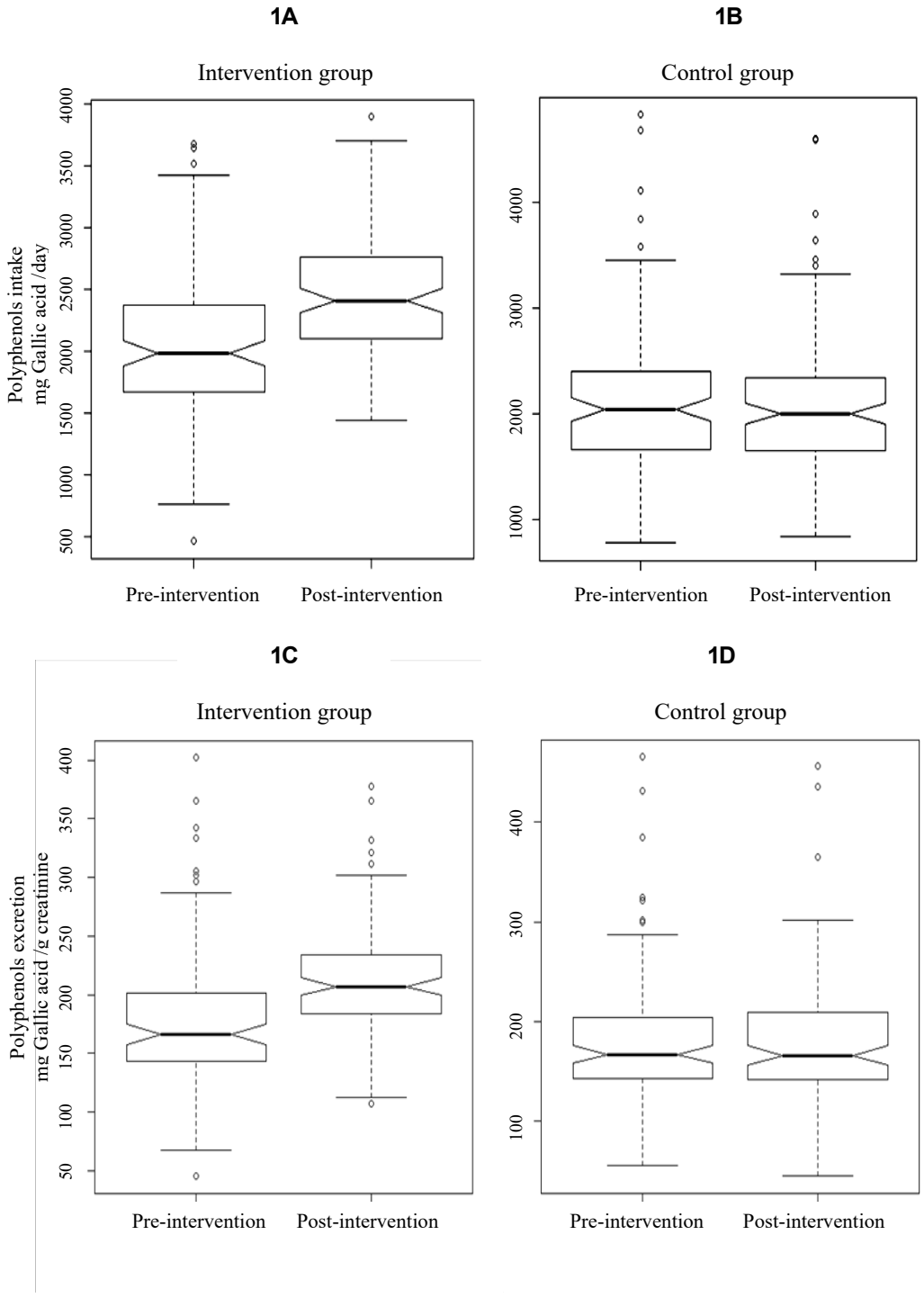
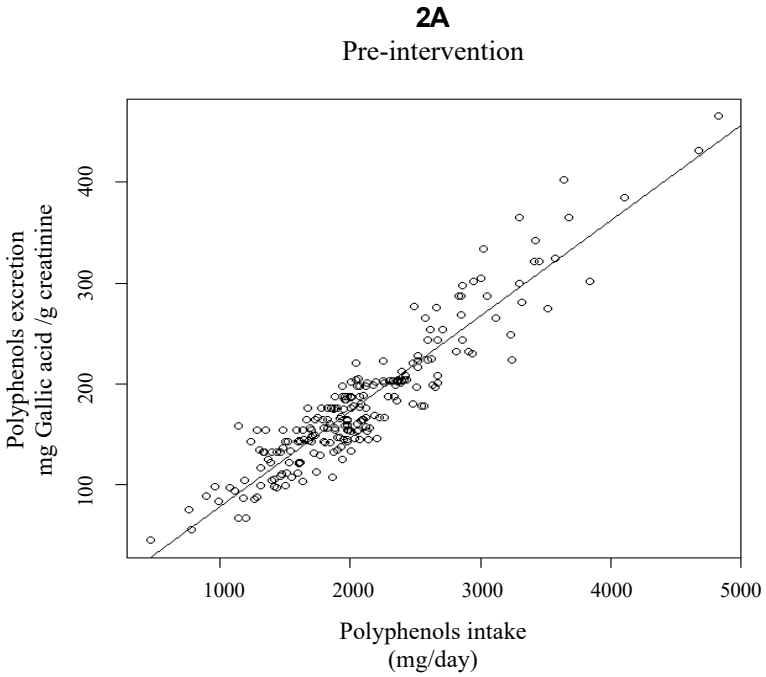

1A
Intervention group
Polyphenols intake
mg Gallic acid /day
500
1000
1500
2000
2500
3000
3500
4000
Pre-intervention
Post-intervention
1B
Control group
1000
2000
3000
4000
Pre-intervention
Post-intervention
1C
Intervention group
Polyphenols excretion
mg Gallic acid /g creatinine
50
100
150
200
250
300
350
400
Pre-intervention
Post-intervention
1D
Control group
100
200
300
400
Pre-intervention
Post-intervention

Figure 2


2A
Pre-intervention
Polyphenols excretion
mg Gallic acid /g creatinine
100
200
300
400
1000
2000
3000
4000
5000
Polyphenols intake
(mg/day)

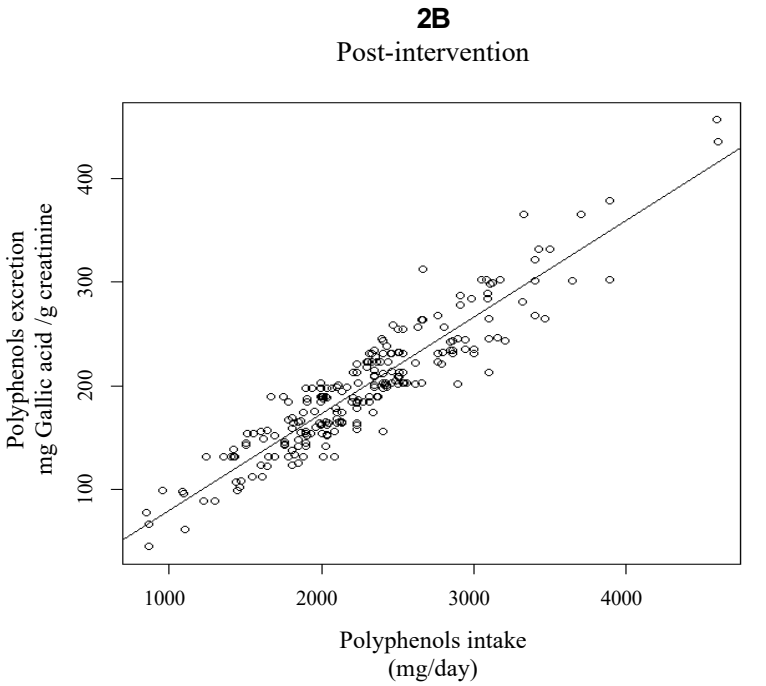

2B
Post-intervention
Polyphenols excretion
mg Gallic acid /g creatinine
100
200
300
400
1000
2000
3000
4000
Polyphenols intake
(mg/day)

Table 1. Comparison of F-C and FBBB Methods With/Without Urine SPE Purification.[a,b]

Samples	SPE Purification	F-C absorbance	FBBB absorbance
Control urine	No	0.006 ± 0.002[a]	0.002 ± 0.001[a]
Sample urine	No	1.122 ± 0.031[b]	0.354 ± 0.008[b]
Sample urine + gallic acid 10 mM	No	1.435 ± 0.042[c]	0.466 ± 0.010[c]
Control urine	Yes	0.008 ± 0.001[a]	0.001 ± 0.001[a]
Sample urine	Yes	0.349 ± 0.009[d]	0.369 ± 0.009[b]
Sample urine + gallic acid 10 mM	Yes	0.456 ± 0.0011[e]	0.477 ± 0.011[c]

[a] Different letters within the same column indicate statistically significant differences ($P < 0.05$).
[b] Each assay was repeated with the urines of six different children ($n = 6$).

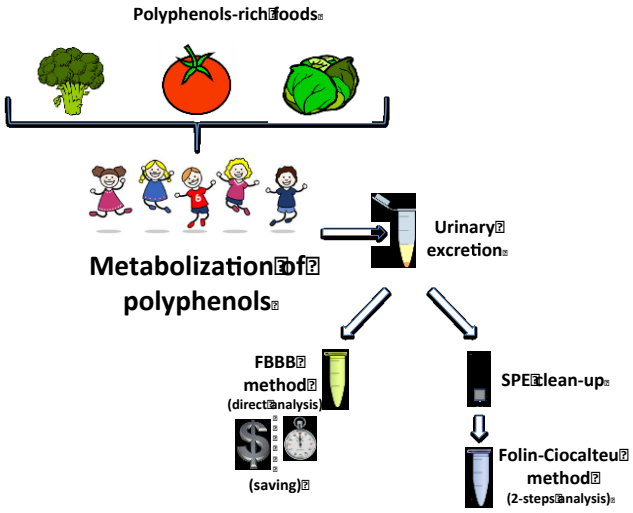
Table 2. Gallic Acid Recovery in Urine With the FBBB Method.

Sample	Water (µL)	Urine (µL)	Gallic acid (µL)	Gallic acid (µg)	Abs	Theoric Conc. (µg/mL)	Experimental Conc. (µg/mL)	Diffference	%
Urine	975	25	0	0	0.202	13.3	13.3	0.0	99.9
Urine + gallic acid	925	25	50	5	0.242	18.3	16.0	2.3	87.2
Urine + gallic acid	915	25	60	6	0.284	19.3	18.8	0.5	97.2
Urine + gallic acid	905	25	70	7	0.298	20.3	19.7	0.6	97.0
Urine + gallic acid	895	25	80	8	0.335	21.3	22.2	-0.9	104.0
Urine + gallic acid	885	25	90	9	0.36	22.3	23.8	-1.5	106.8
Urine + gallic acid	875	25	100	10	0.358	23.3	23.7	-0.4	101.7
Urine + gallic acid	775	25	200	20	0.49	33.3	32.5	0.8	97.6
Urine + gallic acid	675	25	300	30	0.658	43.3	43.7	-0.4	100.9
Urine + gallic acid	575	25	400	40	0.736	53.3	48.9	4.4	91.7
Urine + gallic acid	475	25	500	50	0.728	63.3	48.4	14.9	76.4
								Average	**96.4**
								S.D.	**3.6**

Table 3. Precision of FBBB Method in Urine.

Precision	Sample	Day	n	Average absorbance	DS precision (%)	Minimum absorbance	Maximum absorbance
Intra-day	**1**	1	7	0.184	1.46	0.181	0.187
	2	1	7	0.213	1.68	0.209	0.217
	3	1	7	0.241	1.86	0.237	0.245
	4	1	7	0.224	2.45	0.219	0.229
				Mean	**1.86**		
Inter-day	**1**	1	7	0.213	3.19	0.208	0.221
		2	7	0.217	1.74	0.215	0.222
	2	1	7	0.170	1.56	0.168	0.173
		2	7	0.165	1.93	0.161	0.167
				Mean	**2.11**		

TOC Graphic


Polyphenols-rich foods
Urinary excretion
Metabolization of polyphenols
FBBB method
(direct analysis)
(saving)
SPE clean-up
Folin-Ciocalteu method
(2-steps analysis)