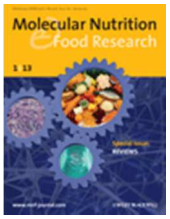

Validation of dried blood spot cards to determine apple phenolic metabolites in human blood and plasma after an acute intake of red-fleshed apple snack

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Keywords:	dried blood spot cards, plasma, phenolic metabolites, tandem MS, whole blood

Validation of dried blood spot cards to determine apple phenolic metabolites in human blood and plasma after an acute intake of red-fleshed apple snack

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Abstract

The application of dried blood spot (DBS) cards for dietary polyphenol pharmacokinetics in human blood has been poorly studied. An analytical method based on blood sampling with DBS cards combined with liquid chromatography coupled to tandem mass spectrometry has been developed and validated. To test the method validation, the phenolic metabolites were determined in human blood and plasma obtained after an acute intake of a red-fleshed apple snack in ten volunteers. Capillary blood by finger prick was compared to venous blood by venipuncture and whole blood was also compared to their corresponding venous plasma samples. Moreover, the venous plasma results using DBS cards were compared to those obtained by microElution solid-phase extraction (μSPE). The main phenolic metabolites detected in blood and plasma samples were phloretin glucuronide, dihydroxyphenylpropionic acid sulphate, (methyl) catechol sulphate, catechol glucuronide and hydroxyphenyl-γ-valerolactone glucuronide as phase II conjugated and colonic metabolites. No significant differences were observed between capillary blood, venous blood and plasma samples using DBS, and neither between plasma samples analyzed by DBS or μSPE. In conclusion, finger-prick blood sampling based on DBS appears to be a suitable alternative to the classic invasive venipuncture for the determination of circulating phenolic metabolites in nutritional postprandial studies.

Key words: dried blood spot cards, plasma, phenolic metabolites, tandem MS, whole blood

1. Introduction

Over recent years, there has been a large increase in the reporting of the use of dried blood spot (DBS) cards for therapeutic drug monitoring and quantitative biomarker assessment [1]. It has established itself as an innovative sampling technique where wet blood is spotted onto absorbent paper offering several potential benefits inherent to the technique, namely being less invasive than venous blood sampling, simplified blood sample collection and convenient sample storage [2]. The small volume of the blood sample and complexity of the matrix leads to analytical challenges in terms of sensitivity and selectivity which could have hampered the attractiveness of DBS cards. However, these limitations have been overcome by the significant improvement in the sensitivity of current liquid chromatography coupled to mass spectrometry (MS) instruments [1].

One area that has received less attention in the application of DBS cards as a blood collection technique concerns the evaluation of the pharmacokinetic parameters of food bioactive compounds, such as dietary polyphenols. Red blood cells, particularly erythrocytes, represent a significant compartment for distribution of drugs and endogenous biological metabolites and it has been suggested that these could be factored into pharmacokinetic and pharmacodynamic evaluations [3]. In fact, the binding of low concentrations of polyphenols to red blood cells has been previously reported [4-6]. Therefore, the possibility of direct blood sampling as the mainstay matrix instead of plasma might yield valuable information in in-vivo bioavailability studies of dietary polyphenols.

Moreover, as DBS sampling is based on arterial capillary blood and some amount of interstitial fluid, the concentrations of circulating metabolites could potentially be different from venous blood. As these differences are dependent on the characteristics of particular drugs or diet xenobiotics, case-to-case evaluation is necessary to validate the method [7].

The analysis of phenolic metabolites in human blood was successfully applied in our previous studies after the acute intake of strawberry tree fruit [8], and an olive oil phenolic

extract [9]. In these studies, the blood was collected with micro-capillary blood collection tubes, and a fixed volume of blood was spotted onto the filter paper with a micropipette. However, a simplified strategy to quantify the phenolic metabolites by placing blood directly onto the DBS cards has not been tested.

The aim of the present study was to develop and validate an analytical method based on the combination of DBS cards as simple sampling procedure combined with a sensitive chromatographic method (ultra-performance liquid chromatography coupled to tandem mass spectrometry, UPLC-MS/MS) to analyze the main circulating phenolic metabolites in blood and plasma after a human acute intake of a red-fleshed apple snack. The method was validated in terms of linearity, reproducibility, method detection limits, method quantification limits, accuracy and matrix effects.

Specifically, the study focuses on three issues: 1) the comparison of venous and capillary blood sampling, 2) the assessment of the differences between plasma and whole blood, and 3) the comparison of DBS cards and microElution solid-phase extraction (µSPE), as the most common method for the analysis of circulating phenolic metabolites in plasma.

2. Materials and methods

2.1. Chemicals and reagents

The commercial standard 3-(2',4'-dihydroxyphenyl)propionic acid was purchased from Fluka (Buchs, Switzerland); epicatechin was from Sigma-Aldrich (St Louis, MO, USA) and phloretin-2'-*O*-glucoside from Extrasynthese (Genay, France). Catechol-4-*O*-sulphate and 4-methyl catechol sulphate were supplied by Dr. Claudia N. Santos (IBET, Oeiras, Portugal) and synthetized according to the method reported by Pimpao *et al*. [10]. Stock solutions of individual phenolic standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000 mg/L, and storing these in dark flasks at 4ºC.

Acetonitrile (HPLC-grade) was from Romil (Tecknokroma, Barcelona, Spain). Methanol (HPLC-grade), phosphoric acid (85 %) and glacial acetic acid (99.8 %) were from Scharlau S.L. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Red-fleshed apple snack

The red-fleshed apple variety used was 'Redlove Era' and was provided by Nufri (Mollerussa, Lleida, Spain) and planted in the experimental plot at La Rasa (Soria, Spain). Immediately after the fresh apples arrived at the laboratory, they were washed, wiped with paper towels and cut into 1 cm cubes. The apple cubes were frozen in liquid nitrogen and freeze-drying was then performed with a first drying at 0.6 bar with a temperature ramp of -20 to 0°C for 25 hours, followed by a second complete vacuum drying with a temperature ramp of 0 to 20°C for 40 hours (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain). After that, the apple snack was preserved in a desiccator protected from light.

For the quantification of phenolic compounds, 0.1 g of crushed apple snack was analyzed according to our previous study [11]. The phenolic dose ingested through a portion of 80 g of red-fleshed apple snack is shown in **Table 1**, where 46% were phenolic acids (mainly chlorogenic acid), 22% were anthocyanins (mainly cyanidin-3-*O*-galactoside), 17 % dihydrochalcones (mainly phloretin glucoside derivatives), 9% flavonols (mainly quercetin derivatives), 7% flavan-3-ols (mainly epicatechin and dimer), and 0.2% flavanone (mainly eriodictyol derivatives).

2.3. Study design and blood sample collection

The protocol of the study was approved by the Ethical Committee of Human Clinical Research at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: 13/2016). Ten heathy volunteers (five females and five males, mean age 37.3 ± 8.4 years)

were recruited and exclusion criteria were age < 25 or > 50 years, body mass index <18.5 or >24.9 kg/m^2, pregnancy or lactation, any chronic medication, any antibiotic treatment during the 4 months prior to the study, cigarette smoking, alcohol intake> 80 g/day and use of dietary supplements. Subjects were asked to avoid the consumption of polyphenol-rich foods (e.g., coffee, fruit, vegetables, dark chocolate, green tea and red wine) for the 3 days prior to the study. On the day of the study, the participants were invited to eat a portion of 80 g of red-fleshed apple snack after fasting overnight. Capillary blood, venous blood and plasma samples were obtained at the baseline and at different time points after consumption of the apple snack (0 to 24 h). During this period, the participants avoided the consumption of polyphenol-rich foods. Capillary blood was obtained by finger-pricking and blood drops were directly applied to DBS cards at 0, 0.5, 1, 2, 4, 6, 12 and 24 h. Venous blood was collected by venipuncture at 0, 0.5, 1, 2, 4, 6, and 24 h in 6 mLVacutainer™ tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing ethylene-diaminetetraacetic acid (EDTA) as an anticoagulant. To obtain plasma samples, the blood tubes were centrifuged at 8784 *g* for 15 min (Hettich, Tuttlingen, Germany). The blood and plasma samples were aliquoted and stored at −80 ºC until further processing.

2.4. Blood and plasma samples pre-treatment

2.4.1. DBS cards

Capillary blood samples were obtained by pricking the volunteers' fingers with disposable lancets (Unistik®, Owen Mumford Ltd, Woodstock, UK) and collected on FTA® DMPK-A cards (DBS filter paper) (GB Healthcare, Buckinghamshire, UK). The first droplet was discarded, since the initial flow from the 'prick' can be contaminated with interstitial fluid, and good practice warrants discarding this first drop. After that, blood droplets were directly spotted on to two pre-marked circles on the filter papers until they were completely soaked, while avoiding direct contact between the finger and the card. Venous blood obtained

by venipuncture and the corresponding plasma samples were also applied to DBS cards. 30 µL of venous blood or plasma was defined as the exact volume to completely fill each pre-marked circle. The pre-treatment procedure is depicted in **Figure 1A**.

In order to dry the spotted cards, they were maintained in the dark at room temperature for 2 h. Afterwards, in order to extract and pre-concentrate the target compounds, the whole surface of two blood or plasma-soaked circles were punched out using a 3-mm diameter Harris Uni-Core punch and a Cutting Mat (Whatman Inc., Sanford, ME, USA). Different conditions were tested for extracting the analytes, and the optimal conditions appeared to be 150 µL of methanol/Milli-Q water (50:50, v/v) as the elution solvent, vortexed for 20 min and centrifuged at 8784 *g* for 10 min at room temperature. The supernatant was filtered with 0.22 µL Nylon 96-well filter microplate (Agilent technologies, Santa Clara, CA, USA), and 7.5 µL of the filtered solution was injected into the chromatographic system.

2.4.2. µSPE cartridges

Venous plasma samples were also analyzed by µElution solid-phase extraction (µSPE) based on our previous studies [12,13]. OASIS HLB µElution plates 30 µm (Waters. Milford, MA, USA) were used. Briefly, the micro-cartridges were activated with 250 µL of methanol and equilibrated with 250 µL of 0.2% acetic acid. 350 µL of venous plasma and 350 µL of 4 % phosphoric acid were centrifuged at 8784 *g* for 10 min at 4ºC, and the supernatant was loaded into the micro-cartridge. The loaded micro-cartridges were cleaned-up with 200 µL of Milli-Q water and 200 µL of 0.2% acetic acid. Then, the retained compounds were then eluted with 2 x 50 µL of methanol. 2.5 µL of the eluted solution was directly injected into the chromatographic system (**Figure 1B**).

2.5. Phenolic metabolites analysis by liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS)

The phenolic compounds and their generated metabolites were determined in blood and plasma samples by AcQuity Ultra-Performance™ liquid chromatography (UPLC) coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford). The analytical column was an AcQuity BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 µm,) equipped with a VanGuard™ Pre-Column AcQuity BEH C_{18} (2.1 × 5 mm, 1.7 µm), also from Waters. During the analysis, the column was kept at 30 °C, and the flow rate was 0.3 mL min^{-1}. The mobile phase and elution gradient were the same as those reported in our previous studies [8,11].

Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface (ESI). Ionization was achieved using the electrospray interface operating in the negative mode $[M-H]^-$ and the data were acquired through selected reaction monitoring (SRM). The ionization source parameters were the same as the ones reported in our previous studies [8,11].

Two SRM transitions were selected, the most sensitive one was used for quantification, and the second for confirmation purposes. **Supplementary Table 1** shows the SRM transition for quantification and identification, as well as the cone voltage and collision energy for each phenolic metabolite. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx 4.1 software. Due to the lack of commercial standards of phenolic metabolites, some of these compounds were tentatively quantified by using the calibration curve of their native compound or another phenolic compound with a similar structure. Phloretin glucuronide was tentatively quantified by using the calibration curve of phloretin-2'-*O*-glucoside, dihydroxyphenylpropionic acid sulphate by using the calibration curve of 3-(2',4'-dihydroxyphenyl)propionic acid, and hydroxyphenyl-γ-valerolactone by using the calibration curve of epicatechin. Catechol sulphate and methyl catechol sulphate were quantified with their own calibration curves, and catechol glucuronide by using the calibration curve of catechol-4-*O*-sulphate.

2.6. Validation procedure

The instrumental quality parameters of the developed methods, such as linearity, precision, accuracy, detection limits (LODs), and quantification limits (LOQs), as well as the extraction recovery (%R) and matrix effect (%ME) for the determination of the main phenolic metabolites in blood and plasma samples by DBS cards, and plasma samples by µSPE, were determined by spiking blank biological samples (venous blood and plasma samples obtained under fasting conditions) with known concentrations of standard phenolic compounds. Calibration curves were prepared with venous blood, since it was not possible to obtain large volumes of blank capillary blood. These instrumental quality parameters, as well as the %R and %ME, were determined as reported in our previous study [14], and the results obtained are shown in **Table 2**.

2.7. Statistical analysis

The quantitative data were analyzed by Student's *t*-test in order to determine significant differences between mean values of the concentration of the main circulating apple phenolic metabolites in: 1) the capillary and venous blood at different post-intake times, 2) the venous blood (venipuncture) and plasma samples (venipuncture) at different times, and 3) the venous plasma analysed by DBS cards and by µSPE at different times. Significant differences were considered at the level of $p < 0.05$. All the statistical analyses were carried out using STATGRAPHICS Plus 5.1 (Manugistics Inc., Rockville, MD, USA).

3. Results and Discussion

3.1. Optimization of DBS technique for phenolic metabolite analysis

This work was based on our previous studies, where phenolic metabolites were determined in plasma or blood samples after the acute intake of strawberry tree fruit [8] and olive oil phenolic extract [9]. In these previous studies, 20 µL of venous blood or plasma were spotted onto one pre-marked circle on the DBS and 7 disks of 2 mm diameter were punched out from the card using 100 µL of methanol/Milli-Q water (50/50, v/v) for the phenolic

extraction. Under these experimental conditions, only the most abundant circulating phenol metabolites were detected. In the present study, in order to enhance the method sensitivity, different conditions were tested to improve the extraction and increase the pre-concentration of the analytes. We also aimed to explore a strategy to quantify the phenolic metabolites directly by depositing blood droplets on DBS card in order to simplify the process.

The factors studied were: (i) the blood volume spotted on the pre-marked circle (from 20 to 50 µL), (ii) the number of disks punched out from the card (from 7 disks to the entire pre-marked circle) and the number of the pre-marked circles (1 or 2), (iii) the nature of the extraction solvent (methanol, methanol/Milli-Q water (50/50, v/v), and acetonitrile), and its volume (from 100 to 200 µL), and (iv) the extraction time with vortex (from 5 to 30 min).

In the present study, six different phenolic metabolites derived from phase II and microbial metabolism were detected in both the blood and plasma human samples. Specifically, the main metabolites detected were phloretin glucuronide, dihydroxyphenylpropionic acid sulphate, (methyl) catechol sulphate, catechol glucuronide, and hydroxyphenyl-γ-valerolactone glucuronide. Therefore, to optimize the method different phenol standards as catechol-4-*O*-sulphate, 4-methyl catechol sulphate, 3-(2',4'-dihydroxy-phenyl)propionic acid, epicatechin and phloretin-2'-*O*-glucoside were spiked at known concentrations into a pooled venous human blood obtained under fasting conditions.

3.1.1 Blood volume spotted on the pre-marked circle

Four different blood volumes applied to the cards were tested (20, 30, 40 and 50 µL) (data not show). In our previous study, volumes from 5 to 20 µL were tested and a greater instrumental response of the phenolic standards was reported as the blood volume increased [9]. In the present study, 30 µL was selected as the optimum volume observing that, with this volume, one pre-marked circle was entirely soaked to its edge with all volunteers' blood samples. This fact denoted that the viscosity of the blood from all volunteers was similar as were its spreading properties on the DBS cards, thus indicating a similar hematocrit.

When droplet of capillary blood is directly deposited on the filter paper, the volume of this blood cannot be precisely controlled and inaccuracies in quantification values can be obtained due to differences in hematocrit. In the literature, different strategies to overcome this drawback have been proposed, such as the use of volumetric absorptive microsampling (VAMS) [15] or the photometric measurement of the protein content [16]. In the present study, a simplified strategy to quantify the phenolic metabolites was tested, assuming a linear relation between volume of blood applied and the area of the pre-marked DBS circle. In this sense, we asked volunteers to soak the entire surface of the pre-marked circles with capillary blood, estimating that the entire surface of one pre-marked circle contained 30 µL of capillary blood, as observed when a fixed volume of the volunteers' blood was dispensed on to the DBS.

3.1.2 Spot punch surface

In the present study, the fact of punching out the maxim number of disks (eight 2-mm diameter disks) or the entire surface of the pre-marked circle was tested and the instrumental response of all the phenolic compounds studied increased slightly when the entire pre-marked circle was analyzed (data not shown). Moreover, in a previous study, it was reported that the whole spot approach can effectively avoid any hematocrit effect in the analysis of a specific drug (apixaban) in human DBS samples compared to a partial spot-center punch [17].

Moreover, the fact of analyzing two instead of one pre-marked circles with reduced elution solvent volume was also tested, and a major pre-concentration effect of the target compounds was observed.

3.1.3 Extraction solvent volume and nature

The tested elution volumes were 100, 150, and 200 µL. The use of 100 µL was discarded because this was not large enough to cover two entire pre-marked circles. Volumes of 150 and 200 µL allowed the phenolics to be extracted with good %R, but with 150 µL, a higher pre-concentration rate was obtained and the %R and % ME were not significantly affected (data

not shown). Different elution solvents were also tested in order to obtain the maximum sensitivity (peak efficiency or narrow peaks) and maximum extraction recovery (%R). Among all the elution solvents tested, methanol/Milli-Q water (50/50, v/v) was the optimum in terms of peak efficiency, and %R, and therefore this elution solvent was chosen.

3.1.4 Extraction time

Firstly, the extraction time (vortex) of the disks with the elution solvent was optimized, and 5, 10, 20 and 30 min were tested. It was observed that the extraction of the main phenolic metabolites increased with the extraction time and this fact was observed until 20 min with no differences between 20 and 30 min, so 20 min was selected as the optimum time (data not shown). So, the optimum pretreatment conditions with DBS cards were defined as: 30 µL of venous blood in each pre-marked circle, punching out the entire surface of two pre-marked circles, 150 µL of methanol/Milli-Q water (50/50, v/v) solution as the extraction solvent and 20 min as the extraction time (vortex) (**Figure 1A**). The same conditions were used for the venous plasma and capillary blood. Under these experimental conditions, the %R of all the studied phenolics was above 75% and the matrix effect (ME%) was lower than 18% (**Table 2**).

3.2. µSPE phenolic metabolite analysis

µSPE was also applied for the analysis of the main circulating phenolic metabolites in venous plasma samples as it is the most common sample pretreatment used in our previous studies, and the obtained results were compared to those achieved from plasma by using DBS cards. The methodology used is the previous reported in our studies for the determination of epicatechin [12], and dihydroxyphenylpropionic acid and (methyl) catechol sulfate [13] in plasma samples.

3.3. Analytical methods validation

The instrumental quality parameters of the developed methods using DBS cards for the analysis of the venous blood and plasma samples, and µSPE for the analysis of plasma

samples, are shown in **Table 2**. In order to carry out these studies, catechol-4-*O*-sulphate, 4-methyl catechol sulphate, 3-(2',4'-dihydroxyphenyl)propionic acid, epicatechin and phloretin-2'-*O*-glucoside were spiked into blank venous blood samples and blank plasma samples at different known concentrations. The linearity range was from 0.04 to 10 µM for venous blood, and 0.03 to 10 µM for plasma samples when DBS cards were used; and 0.0008 to 10 µM for plasma samples when µSPE was used.

The calibration curves (based on the integrated peak area) were calculated by using five points at different concentration levels, and each concentration was injected three times. The determination coefficient (R^2) of the calibration curves was higher than 0.993. The precision of the analytical method (reproducibility) were determined by the relative standard deviation (% RSD) in terms of concentration, and these were calculated at three concentration levels, these being 5, 0.1 and 0.01 µM for the analysis of venous blood and plasma samples (**Table 2**). For all the phenolic compounds the %RSDs were lower than 10% in both DBS cards and µSPE methods. The accuracy was calculated from the ratio between the concentration found for the standard phenolic compounds studied compared with the spiked concentration. This quotient was then multiplied by 100. This quality parameter was also studied at three concentration levels, the same as for the RSD%, and these ranged from 97% to 103%.

The LODs and LOQs were calculated using the signal-to-noise ratio criterion of 3 and 10, respectively. The respective values were in the 15-30 and 45-95 nM ranges for venous blood samples, and the 15-25 and 34-75 nM ranges for plasma samples when DBS cards were used. These results were 10-fold lower to our previous study where olive oil phenol metabolites were determined in blood samples using DBS cards [9]. So, the fact of punching two entire pre-marked circles and decrease the elution solvent volume, improved the sensitivity of the method.

The LODs and LOQs in the analysis of plasma samples by µSPE were 0.3-20 and 0.8-50 nM, respectively. It is important to highlight the lower LODs and LOQs in comparison with

DBS cards. This fact could be explained by the analyte pre-concentration (3.5-fold) that is performed in μSPE (350 μL of plasma are loaded and 100 μL elution solvent are used to elute). Nevertheless when DBS cards are used, the analytes are diluted (2.5-fold). So, 60 μL of biological samples are directly deposited in the circles of the DBS card (2 circles with 30 μL each one) and then 150 μL of extraction solvent are used to analyze the compounds of interest.

The %R for the analysis of the studied phenolics in venous blood and plasma samples (in both DBS cards and μSPE) were similar and these were higher than 75 % (**Table 2**), except for (methyl) catechol sulphate in μSPE, being between 59-63 %. The %ME for the analysis of the studied phenolics in these biological samples was lower than 15 %, being lower in the μSPE pre-treatment (**Table 2**).

3.4. Capillary blood *versus* venous blood in DBS cards

Figure 2 shows the time-course of the main generated phenolic metabolites determined in capillary blood, venous blood and venous plasma using DBS cards at different time points (0-24 h) after the acute intake of red-fleshed apple snack. When comparing mean values of capillary blood *versus* venous blood, no significant differences ($p > 0.05$) were observed in any metabolite or at any time point. Previous studies with drugs found concentrations about 1.7 times higher in capillary than venous blood [18], but these differences are dependent on the characteristics of particular compounds, so case-to-case evaluation is necessary. The results of the present study indicate that in the case of apple phenolic metabolite, values do not differ significantly between venous blood and capillary blood.

Regarding the time-course data, the maximum concentration of the circulating metabolites phloretin glucuronide and dihydroxyphenylpropionic acid sulphate was found between 2 and 4 h, indicating their metabolic transformation in the small intestine and liver (**Figure 2**). The other metabolites (catechol sulphate, methyl catechol sulphate, catechol glucuronide, and hydroxyphenyl-γ-valerolactone glucuronide) appeared to have their

maximum concentrations 12 h after consumption of the apple snack, indicating that they could be products of the gut microbiota catabolism in the colon.

One of the most important advantages of using DBS cards reported in the present study is the possibility of analyzing more post-intake time points by self-sampling compared to venipuncture. Particularly, the possibility of taking sample at 12 h enabled the detection and quantification of two colonic metabolites (catechol glucuronide, and hydroxyphenyl-γ-valerolactone glucuronide) (**Figure 2**). These results indicate the importance of collecting these time points to gain deeper insights into the in-vivo distribution of metabolites produced by gut microbiota.

3.5. Venous blood *versus* venous plasma samples in DBS cards

The concentration values of the phenolic compounds determined in venous blood samples after the acute intake of the red apple snack were also compared to those obtained for the analysis of their corresponding plasma samples and the results showed no significant differences in any metabolite at any time point (**Figure 2**).

Pharmacokinetic studies are commonly carried out focusing on the levels of phenolic metabolites in plasma or serum. However, in the case of some polyphenols their affinity for human blood cells has been observed. For instance, quercetin [19-20] and resveratrol [21] are known to partition into blood cells, associating with cell membranes, haemoglobin and other proteins. The binding of low concentrations of polyphenols to erythrocytes was also reported for caffeic acid, taxifolin, and ferulic acid in a human study [6] and hydroxytyrosol phase-II metabolites in a rat model [5]. In a recent study it was also reported a differential distribution of phenolic compounds between serum and blood cells depending on the characteristics of particular polyphenols [22], confirming that case-to-case evaluation is necessary.

As shown in **Figure 2**, in the case of phenolic metabolites derived mainly from phenolic acids and anthocyanins from apple, plasma levels appear to be lower than blood

concentration. However, no significant differences were found between blood and plasma, probably due to the large interindividual variability in the phenolic metabolism.

Based on our results and also considering previous studies, the application of DBS cards with blood as the mainstay matrix might yield valuable information as a practical alternative to classic plasma analysis to determine the in-vivo human absorption of phenolic metabolites.

3.6. DBS cards *versus* µSPE for venous plasma samples

µSPE is the sample pre-treatment technique most commonly used to extract phenolic metabolites from plasma samples. However, this technique requires venipuncture to obtain a sufficient volume of blood sample and subsequently of plasma sample. In order to evaluate whether DBS cards can be an alternative strategy to µSPE, venous plasma samples were analysed by using both methods and the obtained results were compared. **Table 3** shows the average concentrations from the ten healthy volunteers of the main phenolic metabolites and no significant differences were detected among both methods. Although the sensitivity (LODs and LOQs) of DBS cards technique was lower than µSPE, the average concentrations of the main phenolic metabolites detected in plasma did not significantly differ between the two sample pre-treatment techniques. Therefore, we conclude that DBS cards is a suitable strategy for the analysis of circulating phenolic metabolites in human interventional studies, and also for urine analysis as we reported previously [12].

4. Conclusions

A method based on DBS cards combined with liquid chromatography (UPLC-MS/MS) was developed and validated for the determination of phenolic metabolites in human blood and plasma samples. DBS cards has been reported as a rapid and easy blood-sampling strategy to determine the main circulating phenolic metabolites after an acute intake of red-fleshed apple snack. The comparison of capillary and venous sampling and also the

assessment of the differences between whole blood and plasma samples showed no significant differences. In addition, there were no significant differences among DBS cards and μSPE.

The self-sampling of blood by the volunteers following simple instructions, allowed more times points to be collected during postprandial period, especially those late time points (12 h) to assess the colonic metabolism of phenolic compounds, which are normally missing with venipuncture. Our study reveal that finger-prick blood sampling based on DBS cards appears to be a suitable alternative to the classic invasive venipuncture for determining phenolic metabolites, including colonic fermentation catabolites, in human polyphenol bioavailability and pharmacokinetic studies.

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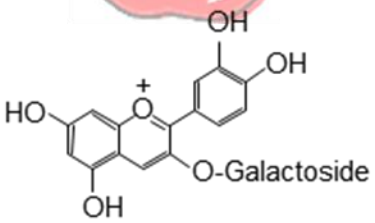
Figure Captions

Figure 1. Schematic representation of the sample pre-treatment protocol developed based on dried blood spot cards and microElution solid-phase extraction.

Figure 2. Time-course of the main phenolic metabolites determined in capillary blood, venous blood and venous plasma samples by DBS and UPLC-MS/MS after the acute intake of red-fleshed apple snack.

Human acute intake

Red-fleshed apple snack



Cyanidin

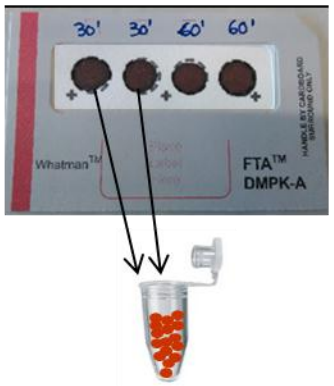
Biological samples

Capillary blood

Venous blood

Venous plasma

Sample pre-treatment by Dried Blood Spot cards



UPLC-MS/MS analysis
Phenolic metabolites pharmacokinetics

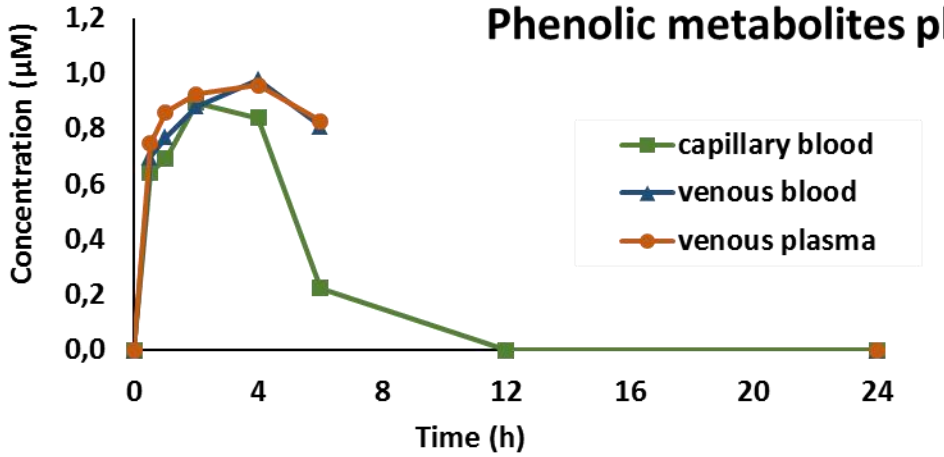


Table 1. Phenolic compounds ingested in a dose of 80 g of red-fleshed apple snack.

Phenolic compound	mg/80 g red-fleshed apple snack
Cyanidin galactoside	39.7 ± 1.0
Cyanidin arabinoside	2.60 ± 0.24
Total Anthocyanins	**42.3 ± 1.18**
Protocatechuic acid	1.71 ± 1.06
Coumaric acid hexoside	0.77 ± 0.11
Ferulic acid hexoside	2.12 ± 0.26
Vanillic acid hexoside	4.28 ± 0.11
Chlorogenic acid	79.1 ± 2.75
Total Phenolic acids	**88.03 ± 3.34**
Epicatechin	5.58 ± 0.78
Dimer	6.92 ± 0.29
Trimer	1.30 ± 0.12
Total Flavan-3-ols	**13.8 ± 1.18**
Quercetin arabinoside	3.67 ± 0.45
Quercetin rhamnoside	9.26 ± 0.94
Quercetin glucoside	4.41 ± 0.57
Total Flavonols	**17.3 ± 1.97**
Eriodictyol hexoside	0.42 ± 0.02
Total Flavanones	**0.42 ± 0.02**
Phloretin glucoside	21.7 ± 2.54
Phloretin xylosyl glucoside	11.7 ± 0.52
Hydroxyphloretin xylosil glucoside	0.32 ± 0.03
Total Dihydrochalcones	**33.7 ± 3.08**
Total Phenols	***195 ± 9.60***

Table 2. Instrumental quality parameters for the analysis by UPLC-MS/MS of the studied phenolic compounds in spiked venous blood and plasma samples by DBS cards, and venous plasma samples by µSPE.

Phenolic compound	RT (min)	%R	%ME	Linearity (µM)	%RSD (*n*=3), inter-day			Accuracy (%),	LOQ	LOD
					5 µM	0.1 µM	0.01 µM	*n*=3, (0.1 µM)	(nM)	(nM)
DBS cards										
Venous blood										
Catechol-4-*O*-sulphate	3.01	85±0.1	4.52±0.1	0.09 - 10	5.1	6.4	8.2	97	90	30
4-Methyl catechol sulphate	4.87	82±0.3	9.50±0.1	0.08 - 10	4.3	5.9	8.6	98	85	30
3-(2',4'-dihydroxyphenyl)propionic acid	5.36	75±0.2	7.91±0.1	0.08 - 10	6.2	7.5	8.5	101	85	30
Epicatechin	7.05	90±0.1	-5.83±0.2	0.09 - 10	5.9	6.8	8.8	102	95	30
Phloretin-2'-*O*-glucoside	16.4	80±0.2	-18.5±0.1	0.04 - 10	7.8	8.9	10	101	45	15
Venous plasma										
Catechol-4-*O*-sulphate	3.01	83±0.1	5.68±0.1	0.07 - 10	4.8	6.1	8.3	103	70	25
4-Methyl catechol sulphate	4.87	80±0.2	10.3±0.2	0.07 - 10	4.6	6.4	8.3	101	73	25
3-(2',4'-dihydroxyphenyl)propionic acid	5.36	80±0.1	-6.82±0.1	0.07 - 10	6.6	7.3	8.8	100	75	25
Epicatechin	7.05	88±0.2	-16.7±0.2	0.05 - 10	5.8	7.4	9.4	99	50	20
Phloretin-2'-*O*-glucoside	16.4	80±0.1	-17.2±0.1	0.03 - 10	7.8	8.9	10	101	35	15
µSPE										
Venous plasma										
Catechol-4-*O*-sulphate	3.01	63±0.1	-4.60±0.1	0.0008 - 10	5.2	5.9	8.2	102	0.8	0.3
4-Methyl catechol sulphate	4.87	59±0.1	-6.71±0.2	0.001 - 10	3.9	5.2	8.0	100	1	0.3
3-(2',4'-dihydroxyphenyl)propionic acid	5.36	95±0.2	-5.35±0.1	0.007 - 10	5.8	6.2	8.1	98	7	2
Epicatechin	7.05	98±0.2	-6.39±0.2	0.05 - 10	5.4	6.8	8.5	101	50	20
Phloretin-2'-*O*-glucoside	16.4	90±0.1	-9.99±0.1	0.003 - 10	7.0	8.0	9.7	99	3	1

Table 3. Mean concentration of the generated phenolic metabolites (µM or nM ± standard deviation) in plasma samples, analysed by DBS cards and µSPE combined with UPLC-MS/MS, after the acute intake of a red-fleshed apple snack at different time points.

	0 h	0.5 h	1 h	2 h	4 h	6 h	24 h
Phloretin glucuronide (nM)							
DBS	n.d.	26.9 ± 9.46	32.5 ± 9.42	39.4 ± 16.8	27.6 ± 11.2	9.55 ± 15.4	n.d.
µSPE	n.d.	28.2 ± 11.2	42.9 ± 8.36	53.8 ± 16.2	54.0 ± 34.6	23.6 ± 16.4	n.d.
Dihydroxyphenylpropionic acid sulphate (nM)							
DBS	n.d.	17.3 ± 14.6	12.5 ± 19.4	19.5 ± 43.2	70.3 ± 105.6	50.6 ±68.2	n.d.
µSPE	n.d.	14.4 ± 18.6	9.87 ± 10.2	10.6 ± 9.6	53.9 ± 63.1	33.0 ± 40.7	n.d.
Catechol sulphate (µM)							
DBS	0.22 ± 0.19	0.30 ± 0.28	0.27 ± 0.22	0.28 ± 0.21	0.40 ± 0.33	0.50 ± 0.23	0.20 ± 0.05
µSPE	0.42 ± 0.37	0.45 ± 0.36	0.42 ± 0.37	0.42 ± 0.29	0.55 ± 0.40	0.83 ± 0.71	0.29 ± 0.12
Methyl catechol sulphate (µM)							
DBS	0.19 ± 0.21	0.27 ± 0.25	0.28 ± 0.27	0.27 ± 0.25	0.28 ± 0.25	0.32 ± 0.31	0.42 ± 0.33
µSPE	0.41 ± 0.51	0.45 ± 0.47	0.45 ± 0.50	0.40 ± 0.46	0.35 ± 0.31	0.47 ± 0.44	0.47 ± 0.31

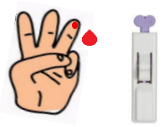
Catechol glucuronide and hydroxyphenyl-γ-valerolactone glucuronide were not detected in plasma samples at the collected time points.
n.d. not detected

Human acute intake

80 g of red-fleshed apple snack
n = 10 healthy voluntears

Biological sample collection

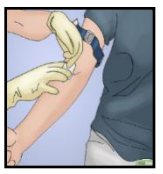
Skin puncture



Capillary blood

(0, 0.5, 1, 2, 4, 6, 12, 24h)

Venipuncture



Venous blood

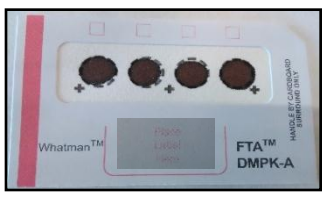
Venous plasma

(0, 0.5, 1, 2, 4, 6, 24h)

A

Dried Blood Spot cards (DBS)

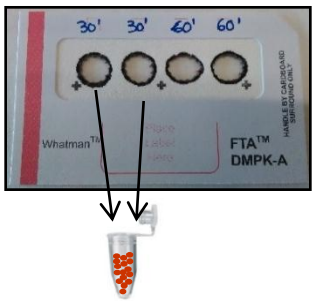
1



- ❑ Capillary blood: circles completely soaked by finger-prick
- ❑ Venous blood and venous plasma: 30 µL in each pre-marked circle by micropipette

2 Drying 2h
dark / room temperature

3

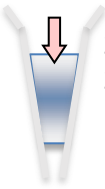


- ❑ Punch out the complete surface of two blood/plasma-soaked circles
- ❑ 150 µL methanol/Milli-Q water (50/50, v/v)
- ❑ 20 ' vortex and centrifuge

B

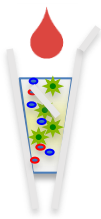
µElution solid phase extraction (µSPE)

1 Conditioning



250 µL methanol
250 µL Milli-Q water (pH2)

2 Sample loading



350 µL plasma
350 µL H_3PO_4 4%

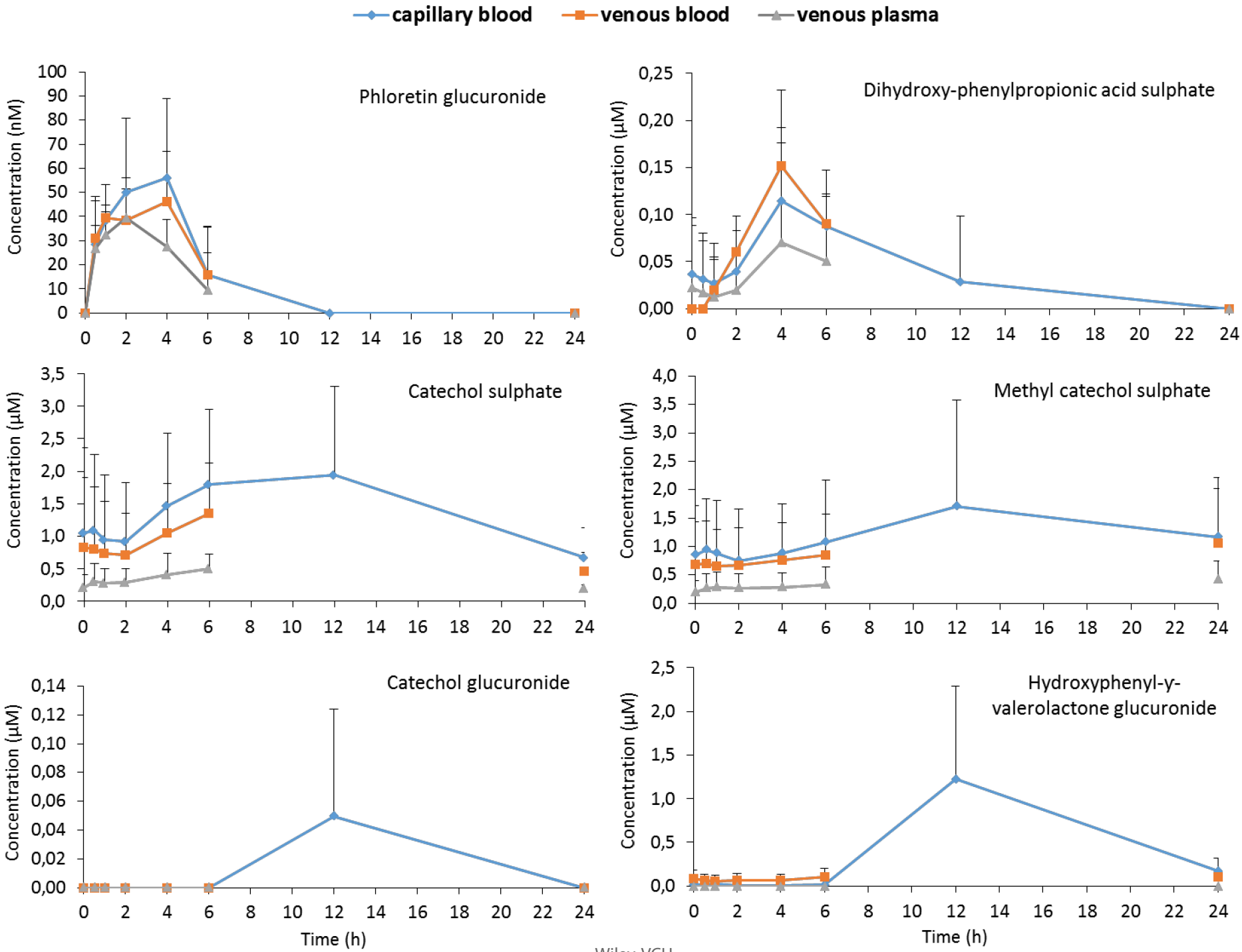
3 Clean-up

200 µL 0.2% acetic acid
200 µL Milli-Q water

4 Analyte elution

2 x 50 µL methanol

UPLC-MS/MS analysis


capillary blood
venous blood
venous plasma
Phloretin glucuronide
Concentration (nM)
Dihydroxy-phenylpropionic acid sulphate
Concentration (µM)
Catechol sulphate
Concentration (µM)
Methyl catechol sulphate
Concentration (µM)
Catechol glucuronide
Concentration (µM)
Hydroxyphenyl-γ-valerolactone glucuronide
Concentration (µM)
Time (h)
Time (h)