Journal Pre-proofs

Application of Dried Blood Spot Cards combined with liquid chromatography-tandem mass spectrometry to determine eight fat-soluble micronutrients in human blood

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PII: S1570-0232(20)30384-6
DOI: https://doi.org/10.1016/j.jchromb.2020.122247
Reference: CHROMB 122247

To appear in: *Journal of Chromatography B*

Received Date: 11 March 2020
Revised Date: 21 May 2020
Accepted Date: 11 June 2020

Please cite this article as: L. Rubió, S. Yuste, I. Ludwig, M-P. Romero, M-J. Motilva, L. Calderón, L. Pla-Pagà, J. Companys, A. Macià, Application of Dried Blood Spot Cards combined with liquid chromatography-tandem mass spectrometry to determine eight fat-soluble micronutrients in human blood, *Journal of Chromatography B* (2020), doi: https://doi.org/10.1016/j.jchromb.2020.122247

tandem mass spectrometry to determine eight fat-soluble micronutrients in human blood

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The analysis of the fat-soluble vitamins A and E and lipid micronutrients in blood, such as carotenoids, is an important parameter to monitor the micronutrient status in humans. Although the potential of dried blood spot (DBS) cards, the use of this technique for blood sampling and subsequent analysis of these fat-soluble micronutrients has been poorly or not studied. An analytical method based on DBS cards (FTA® DMPK-A) combined with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been developed and validated for the determination of carotenoids (lutein, zeaxanthin, β-cryptoxanthin and β-carotene), tocopherols (α-tocopherol, γ-tocopherol and δ-tocopherol) and all-*trans*-retinol in human blood. Under optimum DBS card extraction conditions, the extraction recoveries of the studied compounds were higher than 72%, the sample matrix effect lower than 17%, and the detection limits at hundred nM concentration levels. The developed method was applied to the analysis of human blood, and the concentration ranges obtained fell within the expected ranges previously reported in healthy adults. Moreover, the influence of hematocrit effect was investigated in a range of 25–55% in order to compare the obtained results to those reported in the literature for the analysis of plasma samples. This method represents an improvement over current techniques reported in the literature due to the use of a non-invasive blood collection method, and moreover, this methodology was for the first time 1) validated for the analysis of all-*trans*-retinol, tocopherols and carotenoids, and 2) applied for the determination of tocopherols in human blood samples.

Key words: Carotenoids - DBS cards – Retinol - Tandem MS – Tocopherols - Whole blood

Epidemiological studies suggest a lower risk of major chronic age-related diseases and an increased life expectancy for people with diets high in fruit and vegetables, and consequently higher blood concentrations of vitamins and carotenoids [1]. Some of these micronutrients exert antioxidant effects, which are responsible for some physiological functions, e.g., the anti-inflammatory properties of carotenoids in quenching singlet oxygen and peroxyl radicals [2], the membrane-stabilizing effects of vitamin E [3], or the free scavenging properties of retinol (vitamin A) [4]. Furthermore, these micronutrients are key players in cellular homeostasis and in general physiology. Both carotenoids and the fat-soluble vitamins A and E can be labeled as fat-soluble micronutrients, and an excess or lack of these has been associated with the expression of certain diseases. Consequently, over recent years, their analysis in biological samples, such as plasma [5-12], whole blood [13-17], and feces [18, 19] has increased.

These analyses have been carried out by liquid chromatography (LC) and UV or fluorescence detection [5, 8, 9, 12-16, 18-20], and tandem mass spectrometry (MS) [6, 7, 10, 11, 17], as the detection systems. Prior to the chromatographic analysis, these compounds were extracted from the biological matrix, and different sample pre-treatments have been used, such as liquid-liquid extraction (LLE) [5, 6, 8, 9, 18, 19], protein precipitation [7, 10, 12], supercritical fluid extraction (SFE) [17], and dried blood spot (DBS) cards [13-16].

DBS is a form of bio-sampling where blood samples are obtained by a finger-prick lancet and blotted on a filter paper. DBS has been suggested as a great strategy for the analysis of biological samples in large epidemiological studies, and it appears to be especially advantageous in comparison with venipuncture, since it is minimally invasive and allows self-sampling by the participants in the study. These

literature, only five studies have reported the use of DBS cards for the determination of specific fat-soluble micronutrients in whole blood samples, such as all-*trans*-retinol [13, 16, 20], and carotenoids [14, 15] (**Table 1** in **Supporting Information**). For the analysis of all-*trans*-retinol, the extraction parameters were only optimized in one study [16]. On the other hand, for the analysis of carotenoids these extraction conditions were poorly or none described [14, 15]. Regarding the instrumental quality parameters of the chromatographic method, these parameters were only determined in one study focused on the analysis of all-*trans* retinol [16]. The fact that the current analytical methodologies were only applied in these studies for restricted fat-soluble micronutrients in blood samples (all-*trans*-retinol or carotenoids) could be due to two important drawbacks: a) the abundance of functional homologues within each family of fat-soluble vitamins, and carotenoid isomers as well as their polarity range; and b) the broad concentration range in which these compounds are present in human blood, from low µM to low nM.

In order to expand the use of the DBS cards for the analysis of fat-soluble micronutrients as the health status biomarkers (micronutrients), the aim of the present study was to develop a method based on the use of DBS cards, as blood sampling, combined with liquid chromatography coupled to tandem MS for the determination of all-*trans*-retinol, tocopherols (α-, γ-, and δ-), and carotenoids (lutein, zeaxanthin, β-cryptoxanthin, and β-carotene) in human blood samples. Firstly, the extraction conditions of the studied compounds from the DBS cards were optimized; and then under the optimum DBS conditions, the instrumental quality parameters of the chromatographic method were determined. After that the method was applied for the determination of the target fat-soluble micronutrients in blood samples from ten healthy volunteers. To the best of our knowledge, this is the first time that DBS cards

carotenoids in human blood samples.

2. Materials and methods

2.1. Chemicals and reagents

The carotenoids lutein, zeaxanthin, β-cryptoxanthin, and β-carotene were from Extrasynthese (Genay, France), and α-tocopherol, γ-tocopherol, δ-tocopherol, α-tocopherol acetate (as the internal standard (IS)), all-*trans*-retinol and retinol acetate (as the IS) were from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of each compound was prepared by dissolving each standard compound in ethanol or methanol at a concentration of 1000 mg/L, and storing these in dark flasks at 4ºC. Stock dilutions were prepared daily. Methanol (HPLC-grade), and methyl-*tert*-butyl ether (MTBE) were purchased from Scharlau S.L. (Barcelona, Spain).

2.2. Blood sample collection and pre-treatment by DBS cards

Human whole blood was pre-treated by using DBS filter paper, specifically FTA® DMPK-A cards (GB Healthcare, Buckinghamshire, UK). To optimize the instrumental quality parameters of the method, a pool of venous blood obtained by venipuncture from healthy subjects under fasting conditions was used. Samples of venous blood were collected in vacuum tubes containing EDTA as an anticoagulant. 30 µL of venous blood was defined as the exact volume to completely fill a pre-marked circle.

For the application of the method, capillary blood was obtained from healthy volunteers between 25 and 50 years of age with an even number of males and females (5 females and 5 males). 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). None of the participants reported taking supplements containing vitamin A, vitamin E or carotenoids in the 3 months

compounds were all expected to fall within the normal biological range. Capillary blood was taken by pricking the volunteers' fingers with disposable lancets (Unistik®, Owen Mumford Ltd, Woodstock, UK) under fasting conditions. The first droplet was discarded, since the initial flow from the 'prick' can be contaminated with interstitial fluid. After that, blood droplets were directly spotted onto a pre-marked circle on the filter papers until this was completely soaked, while avoiding direct contact between the finger and the card (**Figure 1** shows a schematic representation of this sample pre-treatment procedure).

The cards soaked with blood were dried in the dark at room temperature for 2 h. Then, in order to extract the fat-soluble micronutrients, the whole surface of a soaked circle was punched out using a 2-mm diameter Harris Uni-Core punch and a Cutting Mat (Whatman Inc., Sanford, ME, USA). Different extraction conditions were tested in order to obtain the maximum extraction efficiency. The optimal extraction conditions for the analysis of all-*trans*-retinol and carotenoids were 150 µL of the methanol/MTBE (50:50, v/v) solution, vortexed for 20 min and centrifuged at 8784 *g* for 10 min at room temperature. Regarding the analysis of tocopherols, two extractions were needed to completely extract these micronutrients from the DBS cards. To carry out this second extraction, 150 µL of the methanol/MTBE (50:50, v/v) solution was again added to the disks, vortexed for 20 min and centrifuged. Prior to the chromatographic analysis, the extraction solutions from tocopherols were diluted 5-fold with the methanol/MTBE (50/50, v/v) extraction solution in order to decrease the matrix effect (%ME) (**Figure 1).**

In order to determine the blood hematocrit (Hct) value, a pool of the whole blood samples from all the volunteers was centrifuged at 8784 *g* for 10 min to separate the plasma from the red blood cells. Then, the plasma and the red blood cells were

(from 30% to 60%). For example, to determine the Hct value of 40%, 40 µL of red blood cells were combined with 60 µL of plasma.

2.3. UPLC-ESI-MS/MS analysis

The analysis of fat-soluble micronutrients in blood samples was performed by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) as the detection system (Waters, Milford, MA, USA). The chromatographic column was an YMC^{TM} Carotenoid C_{30} (150 mm x 4.6 mm i.d.) with a 3-µm particle size (Waters). The UPLC^{TM} system was equipped with a binary pump system, and a gradient elution was used. The mobile phase was methanol (eluent A) and MTBE (eluent B). The flow-rate was 0.8 mL/min. The gradient was performed as follows: 0-12 min, 8-40%B; 12-18 min, 40-100%B; 18-20 min, 100%B isocratic; 20-23 min, 100-8%B; and 23-25 min, 8%B isocratic. The injection volume was 10 µL.

Tandem MS (MS/MS) analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters) equipped with a Z-spray electrospray interface, except for all-*trans*-retinol which was analysed by DAD at 325 nm. The ionization source was electrospray ionization (ESI), and the tocopherols were ionized in the negative ion mode, and carotenoids in the positive ion mode. The data were acquired with the selected reaction monitoring mode (SRM). The MS/MS parameters were as follows: capillary voltage, 3 kV; source temperature, 150 ºC; cone gas flow-rate, 90 L/h and desolvation gas flow-rate, 900 L/h; desolvation temperature, 450 ºC. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. The cone voltages and collision energies were optimized for each micronutrient by injecting each standard compound into a mixture of methanol/MTBE (50:50, v/v) at a concentration of 5 mg/L. Two transitions were studied for each compound, when possible, the most abundant being used for

established for each transition was 100 ms. Data acquisition was carried out with the MassLynx v 4.1 Software. **Table 2 in Supporting Information** shows the SRM transition for quantification and identification, as well as the cone voltage and collision energy for each fat-soluble micronutrient.

2.4. Instrumental quality parameters

The instrumental quality parameters of the method developed, such as the linearity, reproducibility, accuracy, detection limits (LODs) and quantification limits (LOQs), as well as the extraction recovery (%R) and matrix effect (%ME), were determined by spiking venous blood samples obtained from volunteers under fasting conditions with known concentrations of the standard fat-soluble micronutrients studied. These instrumental quality parameters were determined as reported in our previous study [21], and according to the guiding principles of the Food and Drug Administration (FDA).

The solutions used to prepare the standards and to study the reproducibility and accuracy of the method were the same ones. The linearity range of the method was evaluated in different days by using a pool of human venous blood spiked with the studied compounds (three different days). The calibration curves were obtained by analyzing five points at different concentration levels and each standard concentration was injected three times. The calibration curves were prepared by spiking human venous blood with known concentrations of the standard compounds, and the sample pre-treatment with DBS cards reported in "*Blood sample collection and pre-treatment by DBS cards*" Section was applied.

The reproducibility of the method was determined by the relative standard deviations (%RSDs) of the concentration. The accuracy was calculated from the ratio between the concentrations of the studied compounds found compared to the spiked

calculated using the signal-to-noise ratio criterion of 3 and 10, respectively.

The extraction recovery (%R) and the matrix effect (%ME) were also studied. To determine the %R of each micronutrient, the peak areas of the spiked compounds in the pool of venous blood before and after sample pre-treatment (DBS cards), were compared. The %ME was evaluated by comparing the peak areas obtained from the pool venous blood sample spiked after sample pre-treatment (DBS cards) with those obtained from dissolving the standards with methanol/MTBE (50/50, v/v) at the same concentration level. The %R and the %ME were evaluated at three concentration levels with three replicates for each concentration. The concentration levels studied were 1, 5 and 10 µM for all-*trans*-retinol, 0.2, 5 and 10 µM for tocopherols, and 0.3, 2 and 5 µM for carotenoids. Regarding to the study of the %R and %ME, as the blood matrix already contained these micronutrients, their instrumental response was subtracted from the instrumental response of the compounds spiked in the pool of venous blood.

3. Results and Discussion

3.1. Analysis of fat-soluble micronutrients in standard solutions

The initial experiments for the analysis of fat-soluble micronutrients by LC were the reported by Nimalaratne *et al*. [22], who determined all-*trans*-retinol, tocopherols and carotenoids in infant formula and dietary supplements. These authors used an aqueous methanol solution (eluent A) and methanol/MTBE mixture (eluent B) as the mobile phase, and APCI as the ionization source. In our study, the mobile phase and gradient elution were modified, and methanol (eluent A) and MTBE (eluent B) were proposed as the mobile phase in order to increase the ionization of the compounds studied by ESI as the ionization source. The use of a highly organic mobile phase

desolvation and analyte ionization.

The elution gradient began with a low percentage of MTBE (8%) in order to retain the most polar compound, the all-*trans*-retinol. **Figure 2** shows the total ion chromatogram (TIC) obtained for the analysis of the eight fat-soluble micronutrients studied by LC-ESI-MS/MS, and PDA chromatogram at 325 nm for the analysis of all-*trans*-retinol. The concentration of the tocopherols was 0.1 mg/L, and for carotenoids and all-*trans*-retinol were 1 mg/L. The standard solutions were prepared with methanol/MTBE (50/50, v/v). Alpha-, γ-, and δ- tocopherols were analyzed in the negative mode, and carotenoids in the positive ion mode in a single run with polarity switching. In order to obtain the maximum sensitivity, the first 5 min data was acquired on negative mode for the detection of tocopherols, and was followed with positive mode for the detection of carotenoids. The elution order was all-*trans*-retinol (the most polar compound), then δ-tocopherol, γ-tocopherol, α-tocopherol, followed by the carotenoids lutein, zeaxanthin, β-cryptoxanthin, and β-carotene (the most apolar compound) (**Figure 2**).

3.2. Development of the DBS card method

To develop and validate an analytical method to determine eight fat-soluble micronutrients in blood samples, different parameters that affect their extraction from the DBS cards were studied and optimized for the first time. FTA DMPK-A cards were used, as they were successfully applied in our previous studies for the determination of phenolic metabolites in whole blood, plasma and urine samples [21, 23-25], and also to be the optimum in bioavailability studies [26].

To achieve the maximum sensitivity, the blood volume spotted to the DBS card was the amount required to soak pre-marked circle entirely to its edge. This whole

samples compared to a partial spot-center punch [27]. In our previous study, we reported that one pre-marked circle was entirely soaked to its edge with 30 µL of blood, so we assumed that the entire surface of one pre-marked circle contained 30 µL of blood [25].

After depositing 30 µL of a pool of venous human blood spiked with a known concentration of the studied standard compounds (prepared in methanol), the card was dried in the dark at room temperature for 2 h (recommended by the manufacturer). Then, several extraction parameters were studied and optimized to obtain the best extraction efficiencies from the DBS card. These were: 1) the addition of a stabilizing agent onto the paper card, 2) the extraction method, 3) the nature and volume of the extraction solvent, 4) the extraction time, and 5) the number of extractions.

3.2.1. Effect of the addition of a stabilizing agent onto the paper

Some authors have reported the addition of a stabilizing agent, such as butylated hydroxytoluene (BHT) [14], and a patented stabilizing solution (provided by an analytical laboratory (Vitas AS, Oslo, Norway)) [14, 15] onto the DBS card in order to improve the stability, and suppress the degradation of fat-soluble micronutrients in the cards. In all of these studies, Whatman 903 cards were used, and these are untreated papers that consist of pure cellulose, and are manufactured from 100% pure cotton linters [26].

In the present study, although FTA DMPK-A cards were used, the addition of the synthetic antioxidant BHT (0.1%) onto the DBS card before or after the blood deposition was also tested. Unlike Whatman 903 cards, FTA DMPK-A cards are cellulose papers impregnated with sodium dodecyl sulfate (SDS, < 5%) and tris(hydroxymethyl)aminomethane (< 5%) [26]. It was observed that the addition of

of the studied compounds when it was compared when no BHT was added (data not shown). Therefore, any stabilizing agent was not added onto the DBS card.

3.2.2. The extraction method from the DBS cards

In order to obtain the best extraction efficiency of the studied micronutrients from the DBS cards, two extraction methods were compared: LLE and organic extraction. LLE was chosen to be the strategy reported in the literature for the analysis of the studied compounds [13, 15, 16, 20], and the organic extraction to be the one used in our previous studies with DBS [21, 23-25]. Regarding LLE extraction, firstly the proteins were precipitated with ethanol and then the micronutrients all-*trans*-retinol [13, 16, 20], and carotenoids [15] were extracted with hexane and isopropanol, respectively.

On the other hand, for the extraction with organic solvents, different ones were tested, such as: 1) isopropanol, 2) *n*-hexane, 3) ethanol, and 4) methanol and MTBE mixtures (from 0 to 100 %), and 5) Milli-Q water/methanol/MTBE (1/1/1, v/v/v) solution. For all the studied micronutrients, the extraction efficiencies were considerably higher when organic extraction was used in comparison with LLE extraction (data not shown). Therefore, organic extraction was selected.

Figure 3A shows the instrumental response of the micronutrients when organic extraction with the different extraction solvents or solutions was tested. These experiments were performed by spiking blood with tocopherols at a concentration of 0.1 µM, and carotenoids and all-*trans*-retinol at 1 µM. The extraction volume was 150 µL and extraction time 20 min. As observed, the best solvents or solutions used to extract the studied fat-soluble micronutrients (instrumental response) from the DBS cards were the solutions that contained methanol and/or MTBE, being

extraction solution was also the best in terms of peak efficiency. Therefore, methanol/MTBE (50/50, v/v) was chosen to extract the studied compounds from the DBS cards. Once the elution solution was chosen, its volume, extraction time (vortex), and the number of the extractions were studied and optimized.

3.2.3. Extraction volume

Four different extraction volumes of methanol/MTBE (50/50, v/v) were tested, and these were 100, 150, 200 and 300 µL. A hundred µL was discarded because it was not enough volume to cover all the disks. It was important to select the lowest extraction volume in order to obtain the highest concentration sensitivity. When 200 and 300 µL of extraction solutions were tested, the sensitivity for the analysis of carotenoids and all-*trans*-retinol was reduced, and these micronutrients almost were not detected when 300 µL of extraction solution was used. Therefore, the volume of 150 µL was chosen to be the minimum extraction volume that allowed extract and detect the studied micronutrients (data not shown).

3.2.4. Extraction time

Different extraction times through agitation (vortex) of the disks with 150 µL of methanol/MTBE (50/50, v/v) were tested. These times were 5 min, 10 min, 20 min, 1 h, 2 h and 4 h. As it is shown in **Figure 3B**, the instrumental response of all-*trans*-retinol, γ-tocopherol and β-carotene (these were selected to represent tocopherol and carotenoid compounds, respectively) was linearly increased when the extraction time was increased from 5 to 20 min. Afterwards, when the extraction time was increased up to 4 h min, the instrumental response of the all-*trans*-retinol and carotenoids was maintained, an exception was for tocopherols, which was linearly increased.

retinol and carotenoids, the %R for these fat-soluble micronutrients was from 72% to 99%, and around 52% for tocopherols (**Table 1**). The low %R of tocopherols was due to the low extraction time to completely extract them from the disks (see **Fig. 3B**)

In order to develop a rapid and efficient analytical method for the extraction of the eight studied fat-soluble micronutrients, 20 min was selected as the optimum extraction time for all-*trans*-retinol and carotenoids. Then, in order to increase the %R for tocopherols, the fact of increasing the number of extractions of 20 min was tested and evaluated.

3.2.5. Number of extractions

In order to increase the %R of tocopherols, 2, 3 and 4 consecutive extractions of 150 µL of MeOH/MTBE (50/50, v/v) for 20 min in each one were tested. Then, the obtained results were compared to those obtained when only one extraction of 150 µL of MeOH/MTBE (50/50, v/v) and 2 h was used.

When two consecutive extractions of 150 µL of MeOH/MTBE (50/50, v/v) for 20 min each one were used (**Figure 1**), the %R of the tocopherols was noticeably improved, and these ranged from 73-86 %. When a third and fourth extractions were performed, the %R was only enhanced by 3%. So, two DBS extractions were selected for the analysis of tocopherols to completely extract these compounds from the blood disks. The obtained results were compared to those reported when one extraction for 2 h was performed, and similar %R results were observed (see **Table 3 in Supporting Information**).

Regarding the carotenoids and all-*trans*-retinol, these were extracted with only one extraction of 150 µL of MeOH/MTBE (50/50, v/v) for 20 min and not two consecutive extractions as tocopherols for two reasons: firstly, the %R of these

secondly, when more extractions were performed, the resultant extraction solution was diluted and these micronutrients were scarcely quantified. This fact is due to the different concentration range in which these fat-soluble micronutrients are found in blood samples.

In summary, for the analysis of carotenoids and all-*trans*-retinol, only one extraction for 20 min was applied; while for tocopherols, two extractions with 150 µL of methanol/MTBE (50/50, v/v) for 20 min were required (**Figure 1**).

3.3. Sample matrix effect

In addition to the %R, the %ME was also studied. Ion suppression occurs when the signal response of the compounds spiked in the matrix (after the sample pre-treatment) is lower than the signal obtained in standard solutions at the same concentration level. **Figure 1 in Supplementary Material** shows the instrumental response of all-*trans*-retinol, γ-tocopherol and lutein (these were selected to represent tocopherols and carotenoids, respectively) when these compounds were prepared in the standard solution, and spiked after the DBS sample pre-treatment, at the concentration level of 0.5 µM for all-*trans*-retinol, 0.1 µM for tocopherols, and 1 µM for carotenoids. It was observed that the instrumental response of γ-tocopherol in the standard solutions was higher than the instrumental response spiked after the DBS sample pre-treatment. Similar behavior and results were also observed for δ-tocopherol and α-tocopherol. On the other hand, the instrumental response of all-*trans*-retinol and lutein in standard solutions was similar to the instrumental response spiked after the DBS sample pre-treatment, and similar behavior and results were observed for the other carotenoids. Although the micronutrient all-*trans*-retinol was

all the linearity range studied, this behavior was similar.

In order to decrease the suppression ionization (matrix effect) of tocopherol compounds, the fact of diluting the eluted DBS sample was evaluated. It has been reported that decreasing the concentration of the analyte injected by diluting the sample can reduce the matrix effect [28]. However, this can also decrease the sensitivity of the method. Therefore, the extraction solution prepared with methanol/MTBE (50/50, v/v) solution that contained the compounds was diluted, and the most suitable dilution needed to minimize the ion suppression without affecting the sensitivity was studied. For this purpose, the standard and methanol/MTBE (50/50, v/v) solutions spiked after the DBS sample pre-treatment were diluted 2-, 5- and 10-fold. Finally, a 5-fold dilution (**Figure 1**) was selected as the optimum for the analysis of tocopherols (**Figure 1 Supplementary Material**). By using this dilution, the %ME for the three tocopherols was lower than 17% (**Table 1**). On the other hand, it was not necessary to dilute the methanol/MTBE (50/50, v/v) solution for the analysis of carotenoids and all-*trans*-retinol because the ME was low (less than 12 %). In addition, when dilution was done its sensitivity decreased significantly.

3.4. Instrumental quality parameters

The instrumental quality parameters of the developed method were studied by spiking the target compounds in a pool of venous blood at various known concentrations. These parameters were linearity range, reproducibility, accuracy, LOQ and LOD. The obtained results are shown in **Table 1**.

The linearity range for the analysis of the fat-soluble micronutrients studied was from 0.10-14 µM for all-*trans*-retinol, 0.01 to 10 µM for tocopherols, and 0.05-10 µM for carotenoids. The calibration curves (obtained based on the integrated peak area)

concentration was injected three times. The determination coefficient (R^2) of the calibration curves was higher than 0.994.

The reproducibility of the analytical method was determined by the relative standard deviation (% RSD) in terms of concentration. This was calculated at three concentration levels, 1, 5 and 10 µM for all-*trans*-retinol, 0.2, 5 and 10 µM for tocopherols, 0.3, 2 and 5 µM for carotenoids, and the %RSDs were lower than 8.6, 9.0 and 9.5%, respectively.

The accuracy was calculated from the ratio between the concentration found for the standard antioxidant compounds studied compared with the spiked concentration. This quotient was then multiplied by 100. This quality parameter was also studied at the same three concentration levels as the RSD%, and these ranged from 96 to 104%.

The LODs and LOQs were calculated using the signal-to-noise ratio criterion of 3 and 10, respectively. As can be observed, the fat-soluble micronutrients could be detected (LODs) and quantified (LOQs) at low concentration levels. The LOQs for the analysis of all-*trans*-retinol, tocopherols and carotenoids were 0.3, 0.01-0.02 and 0.05-0.07 µM, respectively. The respective values for the LODs were 0.1, 0.003-0.007 and 0.015-0.025 µM. In the literature, there is a single report based on the determination of carotenoids in blood samples that reported the LODs and LOQs [17]. These values were slightly lower for zeaxanthin and β-cryptoxanthin and lower for β-carotene than those reported in the present study. This could be due to the use of *on-line* supercritical fluid extraction, as well as supercritical fluid chromatography and APCI instrumentation.

Comparing these results with those reported in the literature for plasma samples, the LODs and LOQs were lower [6, 9] or similar [5, 6], but slightly higher

are obtained for the analysis of plasma samples and their pre-treatment included a pre-concentration step (LLE and SPE) prior to the chromatographic analysis, such as an evaporation and reconstitution. By contrast, when DBS cards are used, the blood samples are diluted 5- and 10-fold, since 30 µL of blood sample is deposited on the DBS card and all-*trans*-retinol and carotenoids are extracted with 150 µL, and tocopherols with 300 µL (2x 150 µL).

3.5. Impact of blood hematocrit value on the micronutrients quantification

To compare the results of the concentration of the studied compounds in blood samples with the concentration in plasma samples reported in other studies we studied the sample matrix effect of the hematocrit (Hct). In order to carry out this study, the accuracy and precision of fat-soluble micronutrient quantitation were determined at four Hct levels, expected for healthy adults (30%, 40%, 50% (reference), and 60%) without spiking the compounds, and also by spiking the compounds at three concentration levels (1, 2 and 5 µM for all-*trans*-retinol; 0.05, 2 and 10 µM for tocopherols; and 0.1, 0.25 and 0.5 µM for carotenoids).

The results demonstrated minimal effect of Hct within the range of 30-60% on the measured concentration of the studied compounds using DBS cards (data not shown). Each of the measured compounds displayed a difference of less than ± 10% from that measured at the middle Hct level (50%) within the range studied. So, the influence of the hematocrit on blood viscosity and its consequent effect on spot distribution over the DBS card was not relevant for the quantification of the studied compounds.

Once the LC-ESI-MS/MS and DBS conditions were optimized, and their instrumental quality parameters and stability were evaluated, the developed method was applied for the determination of the target fat-soluble micronutrients in blood samples from ten healthy volunteers. **Figure 4** shows the extracted ion chromatograms (EICs) of all the compounds analyzed in these human blood samples. **Table 2** shows the mean concentration value of each micronutrient in these 10 subjects. It also shows the concentration values obtained in plasma samples of large population surveys in order to compare the results. As observed, the acquired biological ranges for all the compounds analyzed in the present study were encompassed within the expected range [6, 15, 29-31]. Some caution must be taken when comparing DBS data with plasma data. Absolute values from DBS samples (i.e. whole blood) are expected to be about 50% of the values reported in plasma (Vitas, S.A.). This is due to the fact that whole blood includes blood cells as well as plasma. In normal adult women, hematocrit values are about 50%, and thus about half of the blood volume represents blood cells. Consequently, in the comparison between DBS and plasma analysis, all DBS values were multiplied by a factor of 2, since as it has previously evaluated the Hct value was similar in the studied range 30-60% [14, 15].

4. Conclusions

A method based on DBS cards combined with LC-DAD-ESI-MS/MS was developed, optimized and validated, and it allowed the separation and quantification of eight fat-soluble micronutrients in human blood samples with accuracy and precision. The method is sensitive enough to determine vitamin A (all-*trans*-retinol), vitamin E isoforms α-, δ-, γ-tocopherols and carotenoids lutein, zeaxanthin, β-

cryptoxanthin, and β-carotene in blood samples taken by pricking the volunteers' fingers. The method can be applied as a monitoring method for the micronutrient status in humans, one that can be particularly valuable for clinical trials in order to understand the relationship between these micronutrients and health outcomes. In conclusion, the method represents an improvement over current techniques due to the non-invasive blood collection method and analytical improvement as it allows the analysis of all-*trans*-retinol, carotenoids and tocopherols, being the last ones determined for the first time in blood samples by DBS cards.

Acknowledgments

This study was supported by the Spanish Ministry of Industry, Economy and Competitiveness through the AGL2016-76943-C2-1-R and AGL2016-76943-C2-2-R projects (co-funded by the European Social Fund, European Union); M.J. Motilva thanks to Consejo Superior de Investigaciones Científicas-CSIC for partial funding throught the "Ayudas incorporación a escalas científicas CSIC, 2018" (Reference 201870I129). I.A.L. enjoys a post-doctoral contract (2017PMF-POST2-19) from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement and from the Universitat Rovira i Virgili (URV). S.Y. was supported by a pre-doctoral grant from the University of Lleida.

Conflict of interest

The authors declare no competing financial interest.

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Figure 1 Schematic depiction of the methodology used for the determination of fat-soluble micronutrients (all-*trans*-retinol, tocopherols and carotenoids) in human blood samples by DBS cards and LC-DAD-ESI-MS/MS

Figure 2 Total ion chromatogram (TIC) obtained for the analysis of all-*trans*-retinol, tocopherols and carotenoids. The concentration of the fat-soluble micronutrients was 0.1 mg/L for tocopherols and 1 mg/L for all-*trans*-retinol and carotenoids. Peak designation: (1) all-*trans*-retinol, (2) δ-tocopherol, (3) γ-tocopherol, (4) α-tocopherol, (5) lutein, (6) zeaxanthin, (7) β-cryptoxanthin, and (8) β-carotene

Figure 3 (A) Optimization of the nature extraction solvent; and (B) vortex time (min) for the analysis of the studied fat-soluble micronutrients by DBS cards and LC-DAD-ESI-MS/MS

Figure 4 Extracted ion chromatograms (EICs) obtained for the determination of the fat-soluble micronutrients studied in whole blood samples by DBS cards and LC-DAD-ESI-MS/MS

1. Determination of 8 fat-soluble micronutrients in blood was developed and validated
2. Dried Blood Spot cards were used as the sample pre-treatment strategy
3. High recoveries, low matrix effect and detection at nanomolar level were obtained
4. Tocopherols were determined for the first time in blood samples by DBS cards
5. The method is applicable to determine the micronutrient status in clinical trials

Declaration of interest: None

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1 Instrumental quality parameters for the determination of fat-soluble micronutrients all-*trans*-retinol, tocopherols, and carotenoids by DBS cards and LC-DAD-ESI-MS/MS in human blood samples

Fat-soluble micronutrients	Extraction recovery (%R) (0.2	Matrix effect (%ME)	Linearity (µM)	Reproducibility (%) (0.2 µM), *n*=3	Accuracy (%) (0.2 µM), *n*=3	LOQ (µM)	LOD (µM)

***n*=3**

Retinol							
All-*trans*-retinol	81 *	1.5 *	0.10-14	1.5 *	99 *	0.3	0.1
Tocopherols							
δ-tocopherol	78	17	0.01-10	7.8	104	0.01	0.003
γ-tocopherol	73	12	0.01-10	8.1	97	0.01	0.003
α-tocopherol	86	-15	0.02-10	8.3	101	0.02	0.007
Carotenoids							
Lutein	89 **	-5 **	0.07-7	8.0 **	98 **	0.07	0.025
Zeaxanthin	99 **	-12 **	0.05-5	8.2 **	96 **	0.05	0.015
β-cryptoxanthin	75 **	6 **	0.07-7	8.4 **	99 **	0.07	0.025
β-carotene	72 **	-12 **	0.06-10	8.6 **	101 **	0.06	0.02

* 1 µM
** 0.3 µM

Abstract

The analysis of the fat-soluble vitamins A and E and lipid micronutrients in blood, such as carotenoids, is an important parameter to monitor the micronutrient status in humans. Although the potential of dried blood spot (DBS) cards, the use of this technique for blood sampling and subsequent analysis of these fat-soluble micronutrients has been poorly or not studied. An analytical method based on DBS cards (FTA® DMPK-A) combined with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been developed and validated for the determination of carotenoids (lutein, zeaxanthin, β-cryptoxanthin and β-carotene), tocopherols (α-tocopherol, γ-tocopherol and δ-tocopherol) and all-*trans*-retinol in human blood. Under optimum DBS card extraction conditions, the extraction recoveries of the studied compounds were higher than 72%, the sample matrix effect lower than 17%, and the detection limits at hundred nM concentration levels. The developed method was applied to the analysis of human blood, and the concentration ranges obtained fell within the expected ranges previously reported in healthy adults. Moreover, the influence of hematocrit effect was investigated in a range of 25–55% in order to compare the obtained results to those reported in the literature for the analysis of plasma samples. This method represents an improvement over current

method, and moreover, this methodology was for the first time 1) validated for the analysis of all-*trans*-retinol, tocopherols and carotenoids, and 2) applied for the determination of tocopherols in human blood samples.

Table 2 Concentration (µM) of fat-soluble micronutrients in blood and plasma from 10 healthy volunteers by DBS cards and LC-DAD-ESI-MS/MS compared to other plasma samples from large population surveys. Data is expressed as mean ± standard deviation (*Hematocrit values are about 50%, and thus about half of the blood volume represents blood cells. Consequently, DBS values were multiplied with a factor of 2)

Fat-soluble micronutrinets	**Present work**		**EPIC study Spain (*n*=591)** *(Al-Delaimyl et al., 2004)*	**Norway study (*n*=361)** *(Markussen et al., 2015)*	**Catalan study (*n*=343)** *(Garcia-Closas et al., 2002)*
	Whole blood (*n*=10)	**Plasma***			
Retinols					
All-*trans*-retinol	2.56 ± 1.50	5.12	n.a.	n.a.	1.81
Tocopherols			n.a.	n.a.	n.a.
δ-Tocopherol	0.13 ± 0.12	0.26	n.a.	n.a.	n.a.
γ-Tocopherol	0.14 ± 0.16	0.28	n.a.	n.a.	n.a.
α-Tocopherol	8.34 ± 3.08	16.7	n.a.	n.a.	31.8
Carotenoids					
Lutein	0.22 ± 0.09	0.44	0.35-0.42	0.23 ± 0.13	n.a.
Zeaxanthin	0.08 ± 0.03	0.16	0.10-0.12	0.05 ± 0.02	n.a.
β-Cryptoxanthin	0.13 ± 0.05	0.26	0.30-0.52	0.16 ± 0.11	n.a.
β-Carotene	0.14 ± 0.05	0.28	0.21-0.38	0.44 ± 0.28	0.45

n.a.: not analyzed