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Robust measurement of vitamin A status in plasma and blood dried on paper

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Abstract

Vitamin A deficiency is the leading cause of preventable blindness in children and increases the risk of disease and death from severe infections. In addition, fat soluble vitamin A and associated retinoids directly regulate the expression of genes involved in fatty acid metabolism. Conventional methods for measuring vitamin A involve venipuncture, centrifugation and refrigeration all of which make measuring vitamin A in nutritional surveys expensive. We aimed to develop a simple and robust system for measurement of retinol (biomarker for vitamin A) using dried blood spot (DBS) samples. Low recoveries and inconsistent results reported by others were found to be due to poor extraction efficiency rather than retinol instability. Maintaining acid conditions during extraction resulted in recoveries >95% with <6.5% of coefficient of variation. Using isocratic high performance liquid chromatography, separation was achieved in <3.5 mins. Detector response was linear (R^2=0.9939) within a range of 0.05 – 2 μg/mL, with a limit of quantification of 0.05 μg/mL. Retinol in DBS was shown to be stable (>95%) at room temperature for up to 10 weeks. DBS values for retinol were highly correlated with venous blood samples from 24 healthy subjects (*r*=0.9724) and were consistent with results from a commercial laboratory. This simple and reliable method for the determination of vitamin A status should prove particularly valuable for population studies and large clinical trials.

Keywords:

Dried Blood Spot, vitamin A, retinol, HPLC

1. Introduction

Vitamin A is a lipid soluble vitamin that plays important roles in human health including being an essential component of the photoreceptor rhodopsin, and influences immune function, reproduction, and bone health. Vitamin A directly regulates the expression of genes involved in fatty acid metabolism in liver cells, adipocytes, and skeletal muscle [1]. Vitamin A deficiency can change membrane lipid composition and the activity and gene expression of the regulatory enzymes associated with fatty acid oxidation in mitochondria [2].

Vitamin A can only be acquired from the diet as either preformed vitamin A (retinol and retinyl esters) or provitamin A (some carotenoids). Vitamin A deficiency is rare in affluent countries; however, severe vitamin A deficiency occurs in many developing and undeveloped countries due to poor nutrition. This is responsible for half a million or more instances of unnecessary death or blindness each year [3]. On the other hand, the risk of excess vitamin A intake is higher in developed countries as a result of fortified foods and supplements [4] and concerns have been expressed about the potential for long term mild vitamin A over consumption which may result in health problems [5].

Despite the active form being retinoic acids, all-*trans* retinol is usually used as biomarker to indicate vitamin A status in blood. Conventional measurement of vitamin A status involves venipuncture to obtain sufficient blood volume but this creates difficulties for infants, the elderly and some cultures. Furthermore, most methods require separation of blood cells by centrifugation and refrigerated transport and storage of samples. This adds substantial cost to nutritional surveys of populations and large scale clinical trials. Finger prick blood collection methods using dried blood spots (DBS) have been popularised in many fields including metabolomics [6]. However, retinol was reported to be unstable in DBS for long term storage [7, 8]. It was hypothesised that oxidation was responsible for the loss, however pre-treating the paper medium with antioxidants did not solve the issue [9, 10].

The aim of the present study was to develop a simple and robust DBS assay of retinol levels which would allow samples to be stable at room temperature for over 2 months.

2. Materials and Methods

2.1. Solvents and Chemicals

Methanol, HPLC grade, Sigma Aldrich, USA; hexane, ACS reagent, Sigma-Aldrich, USA; ethanol, HPLC grade, Scharlau, Spain; retinol, Sigma-Aldrich, USA; tocol (internal standard), Abcam, Cambridge, UK; butylated hydroxytoluene (BHT), Sigma Aldrich, USA; ethylenediamine-tetraacetic acid disodium salt dehydrate (EDTA) reagent grade, Sigma Aldrich, USA; NaCl, analytical reagent, Chem-supply, South Australia, Australia; glacial acetic acid, HPLC grade, APS Ajax Finechem, NSW, Australia.

2.2. Subjects and sampling

The methods for the collection of blood for the study were approved by the Human Research Ethics Committee, The University of Adelaide, H-2012-087. Participants were given an

information sheet explaining the nature of the study before signing the Consent Forms. Initial method development was conducted using blood samples from a single healthy donor with vitamin A status in the normal range. Twenty-four healthy volunteers were later recruited for a method validation study. Blood samples from each volunteer included a finger capillary blood dried (capillary DBS) on 15x30mm 903® filter paper strips (Whatman, Buckingham, UK), and a venous blood draw from antecubital veins and collected in vacuum EDTA tubes (K3EDTA, VACUETTE®, Greiner Bio One, Australia). To compare with capillary DBS, 40 µL of the venous blood was spotted on Whatman 903® filter paper (venous DBS) and dried for 4 h at room temperature. The DBS was sampled using an 8.5 mm punch that corresponded to a known amount of blood [7]. The remainder of the venous blood sample was centrifuged and plasma stored at -20°C for later analysis. Dried plasma spots (DPS) were prepared by pipetting 40 µL aliquots of plasma on to 903® filter paper, drying at room temperature (≈ 25 °C) for 4 h.

2.3. Estimation of blood volume in DBS

Whatman 903® filter paper is widely used in dried blood spot technology because its porosity allows for an even distribution of blood on the paper. We found a direct relationship (R^2=0.9988) between the volume of whole blood applied to the 8.5 mm circle 903® paper and dried blood spot weight. This allowed us to calculate that a punch size of 8.5 mm is gravimetrically equivalent to a whole blood volume of 29.2 ±1.2 µL.

2.4. DBS Method development

The method was initially developed using pooled sample of human plasma that was stored at -20 °C. Whole blood was subsequently used for method validation.

2.4.1. Effect of pre-treatment of paper with protectant

Experiments were conducted to exclude factors that enhance oxidation including light, moisture, and oxygen by placing DPS samples in foil bags together with desiccants, and extracted in amber vials. While this contributed to consistent and reproducible results, retinol recovery was only 40-60% of that applied (recoveries based on standard methodology [11]). Attempts were then made to block oxidation by pre-treating Whatman 903® filter paper with different protectants such as phenolic antioxidants and chelating reagents because they have been reported to protect unsaturated fatty acids in DBS from oxidation [12]. Thus the antioxidant BHT (1, 5, 10 mg/mL) and the chelating reagents, EDTA (0.5, 1, 5, 10 mg/mL), and L-ascorbic acid (1.25, 2.5, 5, 10 mg/mL), were tested individually and in combination. Plasma samples were spotted onto the pre-treated paper and dried at room temperature (≈ 25 °C) in darkness. Untreated DPS was used as a control. Liquid plasma samples from the same origin were extracted immediately after thawing and this retinol value was used as the reference. All samples were processed in triplicate.

2.4.2. Effect of acid on the extraction of retinol from dried plasma spots

To optimise the best extraction procedure, different organic acid solutions were tested during retinol extraction from DPS. L-ascorbic acid (1.25, 2.5, 5, 10 mg/mL), oxalic acid (0.1, 0.5, 1, 5, 10 mg/mL), citric acid (0.1, 0.5, 1, 5, 10 mg/mL), and acetic acid (0.06, 0.12, 0.3, 0.6, 3 mg/mL) were tested. Untreated DPS was used as a control. Liquid plasma samples from the same origin were extracted immediately after thawing and used as the reference. All samples were processed in triplicate.

2.5. Extraction protocols for plasma and blood samples

Dried plasma spot samples were obtained by applying 40 µL of plasma on to Whatman 903® paper discs and dried. The disc was placed into a 5 mL amber vial (Phenomenex, Australia), followed by 0.04 µg internal standard (tocol). The paper disc was submerged in 1 mL methanol (+/- 2 mM acetic acid) for 10 mins and subjected to intermittent vigorous vortexing and the solvent collected. The extraction was repeated and the total supernatant methanol solution was transferred into a fresh amber vial and then dried under nitrogen and finally reconstituted in 100 µL mobile phase for HPLC analysis. Liquid plasma samples from the same origin were extracted immediately after thawing and were used as the reference to calculate recovery.

Dried blood spot samples (≈ 8.5 mm discs) were punched from DBS papers yielding a known volume (equivalent to 29.2±1.2 µL). The disc was placed in a 5 mL amber vial, with 200 µL 14 mM ascorbic acid aqueous solution and left for 30 mins with intermittent vortexing. Internal standard (tocol, 0.04 µg) and 400 µL of ethanol were then added. After vortexing, 750 µL of hexane was added. The two phase mixture was vortexed for 5-10 seconds every 5 mins for 15 mins. A 600 µL aliquot of saturated NaCl aqueous solution was added to facilitate the phase partition, which was also vortexed vigorously for 5-10 seconds at least 2 times before being centrifuged (3000 rpm, 5 mins). The upper phase was collected into a fresh amber vial. The hexane extraction was repeated and the combined hexane layers were dried under nitrogen and reconstituted in 100 µL mobile phase for HPLC analysis. Liquid whole blood samples from the same origin were extracted immediately and used as the reference.

Plasma/whole blood samples were extracted using a modification of published methods [11, 13] whereby 40 µL of plasma/whole blood samples were deproteinised by addition of 400 µL ethanol and internal standard in amber vials. After vortexing, 750 µL of hexane was added. The mixture was vortexed for 5-10 seconds every 5 mins for 15 mins. A 600 µL aliquot of saturated NaCl solution was then added. The mixture was vortexed vigorously for 5-10 seconds at least 2 times before being centrifuged (3000 rpm, 5 mins). The top phase was collected into a fresh amber vial. The hexane extraction was repeated and the upper organic phases were combined and dried under nitrogen, the residue was reconstituted in 100 µL mobile phase for HPLC analysis. The concentration of retinol in whole blood was calculated and normalised as per plasma volume using average haematocrit (male 45% erythrocytes, female 40%) values [14].

2.6. Stability of retinol in DBS

Replicate DBS samples were extracted using our optimised method as well as by a previously published DBS method [15]. Extractions were performed on day 0, 12, 19 and 33 after spotting. Recoveries of retinol were directly compared between the two methods over time. Triplicates were used at each time point.

In a more detailed experiment, DBS samples (+/- antioxidant BHT) were stored at room temperature (≈ 25 °C) in foil bags with desiccants. The DBS samples were extracted on day 0, 3, 7, 14, 28, 42, 56 and 70 to evaluate the stability of retinol in DBS. Triplicates were used at each time point.

2.7. HPLC conditions

An Agilent HPLC 1260 system with isocratic mobile phase of methanol with 0.05% acetic acid (HAc) at flow rate 1.5 mL/min, 30 °C, and ZORBAX Eclipse XDB-C18; 4.6 x 150 mm, 5μm column was used. Twenty μL of sample was injected. Retinol and the internal standard tocol were measured using a diode array detector (DAD) at 325 nm and 292 nm, respectively. The total run time was 6.5 mins, with retinol and tocol eluting at 1.9 and 3.3 mins, respectively.

Tocol was used as the internal standard because we found that retinyl acetate, the widely used internal standard for retinol analysis, was unstable when undergoing extraction. The instability of retinyl acetate was also reported by Yuan [16].

2.8. HPLC method comparison

In order to test the accuracy of values acquired from our HPLC quantification, we compared our values to values obtained in another laboratory (SA Pathology) which offers commercial services for vitamin analyses, also using an HPLC based method. Retinol concentrations of plasma from 24 subjects were measured. The linear correlation coefficient showed good agreement (r=0.9385, $p<0.0001$) between the two methods. Using a Bland-Altman test, we found that the differences from the two laboratories were within 96% CI. However, the mean bias in the test was -7.68%, indicating that our result is systematically slightly higher than the result from SA Pathology.

2.9. Method validation

A linearity test was conducted by spiking a series of concentrations of retinol standard solution (0.02, 0.05, 0.1, 0.5, 1, 2 μg/mL) onto 8.5 mm DBS discs and dried with nitrogen in amber vials for 10 mins prior to extraction. The spiked spots then underwent extraction as per Section 2.5. Total retinol concentrations were measured and plotted against the corresponding concentrations of spiked retinol using Microsoft Excel 2010. Additionally, the different concentrations of retinol standard solutions were also injected directly through HPLC to test the accuracy of the assay.

For quality control purposes, a set of aliquots of one plasma sample were stored at -20 °C and measured on different days. Intra-day errors were determined by analysing 6 aliquots of the sample and inter-day errors were determined by measuring 3 aliquots on 13 different days.

The clinical validation was conducted using blood samples from 24 healthy volunteers as per Section 2.2. Results from DBS or liquid whole blood were converted to retinol content per plasma sample using the average haematocrit value (erythrocyte percentage: male 45%, female 40%) for the purposes of comparison. Scatter plots were drawn to determine correlations using Microsoft Excel 2010.

2.10. Statistical analyses

Unless indicated otherwise, all data reported are the mean of triplicate determinations with standard deviation. Treatment group comparisons were made using one-way ANOVA by IBM SPSS Statistics 21. The least significant p value was set at <0.05. In method comparison, Pearson correlation coefficient was calculated with significance p value, and additional Bland-Altman test was conducted to check the bias using GraphPad Prism 6 v007.

3. Results and Discussion

3.1. Effect of paper pre-treatment with antioxidants

We initially evaluated the retinol stability by using samples of a stock plasma sample dried on Whatman 903® filter paper. We included the antioxidant BHT as well as the chelating reagents EDTA and ascorbic acid to test if they improved the recovery of retinol from the paper. Compared to the reference value, adding the antioxidant BHT resulted in no increase in retinol recovery (Fig. 1). On the other hand, the chelating agent EDTA gave higher recovery but still resulted in around 20% losses. However, in the presence of ascorbic acid, retinol recovery increased markedly. This improved recovery of retinol was not influenced by the additional presence of BHT (Fig 1). We then tested different concentrations of ascorbic acid in order to optimise the ratio of ascorbic acid to retinol. The result showed that concentrations between 1.25 mg/mL and 5 mg/mL ascorbic acid were sufficient to prevent losses in retinol recovery (Fig. 2). Levels of ascorbic acid above 10 mg/mL resulted in lower retinol recovery. Although ascorbic acid was added in a method reported previously [15] the high dose of ascorbic acid used (10 mg/mL) might have inhibited retinol extraction.

3.2. Effect of acid on the extraction of retinol from dried plasma spot

We addressed the question of whether the effect of ascorbic acid was due to the fact that it was a weak acid and whether other weak acids might have a similar effect by facilitating a higher retinol recovery. Some other organic acids tested (oxalic acid, citric acid) were less effective (≈ 80% recovery) than ascorbic acid. However, when we introduced 2mM (0.12 mg/mL) acetic acid into the extraction solvent it resulted in close to 100% recovery (Fig. 3). The effect of acetic acid was unaffected by the presence of BHT or ascorbic acid. We concluded that acidic conditions increased the extraction of retinol from the paper since it was unlikely that acetic acid could have any antioxidant activity.

It is unclear why acidic conditions during extraction give close to 100% retinol recovery. We hypothesise that reasons could involve the dissociation of retinol from its binding protein in acidic conditions which increases retinol abundance during extraction. This is supported by

the findings of Guy et al [17] who reported that urinary retinol binding protein is pH dependent, and is most unstable at pH 5-7.

3.3. Stability of retinol in DBS

A comparison between the recovery of retinol from DBS using our method and a previously published method [15] over time is shown in Fig. 4. In our hands the published method resulted in a retinol loss of 18.3% (Fig. 4), which is similar to their reported percentage loss (23%) [15]. In contrast, our method showed a consistent recovery of 93.2% even after day 33. The results confirmed that using acidic extraction, retinol can be extracted from whole blood dried on 903® filter paper with minimum loss.

We conducted a more detailed time course stability experiment using DBS at room temperature (≈ 25 °C) in foil bags with desiccants. As shown in the Table, with our acidic extraction method, retinol in DBS was stable (<5% relative loss to conventional 2-phase extraction of liquid blood) for up to 10 weeks. In contrast to previous reports [9, 10], we found no difference in stability between samples with and without BHT. This further indicates that, if stored in dark, dry conditions with minimum exposure to air, retinol in DBS can remain stable at room temperature for up to 10 weeks.

3.4. Linearity and precision

Within the range of 0.05 – 2 μg/mL the retinol standard curve was linear (Fig. 5). The slope (1.02) and intercept (-0.01) were close to the mathematical model y=x, which indicates that retinol concentrations measured are identical to standards that were injected directly into HPLC. The correlation between total retinol concentrations in DBS containing spiked standards against concentrations by direct injection was R^2=0.9939. The intercept showed the concentration of endogenous retinol in the DBS samples was 0.85 μg/mL (1.83 μM). The total retinol concentrations in DBS with spiked standards were positively and linearly proportional to the concentration of direct retinol injection. The extraction recovery was calculated by the ratio of retinol to original added amount and was 86% - 116%.

To test the precision of the assay, aliquots of the same plasma sample were measured both within a day (n=6) or between different days of a month (n=13). Intra-day coefficient of variation was 1.7% with a mean of 2.02 ± 0.03 μg/mL (mean ± SD), and inter-day variation was 6.2% with a mean of 2.13 ± 0.13 μg/mL (mean ± SD).

The limit of quantification was defined as the value obtained when the retinol peak height was 10 times higher than baseline. With this definition, the cut-off value for LOQ was 0.05 μg/mL (0.175 μM), which is satisfactory for biological samples even with vitamin A deficiency (usually considered as lower than 0.2 μg/mL, or 0.7 μM [18]).

3.5. Clinical validation of the DBS method

Blood samples collected three ways (finger capillary blood dried on paper, venous blood dried on paper, and venous plasma extracted with 2-phase solvent extraction) from 24 subjects were compared.

Fig. 6 shows that there are positive linear correlations between capillary blood, venous blood and venous plasma retinol values (r_A= 0.9724, r_B= 0.9538, r_C= 0.9398). This is in agreement with other dried blood methods showing minimal differences in retinol levels between finger blood and venous blood/plasma [19].

We acknowledge that between individuals the haematocrit values may vary as a result of biological variation and dehydration levels. Also the precision of relating punch size to blood volume in practical situations can be dependent on the quality of DBS collected. However with the proper training of blood collection staff, and the average haematocrit value, our DBS method is satisfactory for large-scale population level studies. It also proved to be practical when values are compared with the matched plasma samples (Fig. 6).

Complex and expensive steps including venipuncture, centrifugation and refrigerated transport of samples [20] add to the expense of screening large numbers of people for vitamin A deficiency. Because the DBS system does not require trained phlebotomists, and no centrifugation or refrigeration is required, the system is ideally suited for large scale population studies regardless of location. Even though our DBS method requires that the DBS sample must softened before extraction and the solvent acidified, these factors are more than offset by the convenience of being able to take, transport and store samples without refrigeration.

4. Conclusions:

Endogenous retinol in both plasma and whole blood dried on Whatman 903® filter paper is stable (under dark and dry conditions) for periods up to 10 weeks without refrigeration. Acidified methanol is required to maximise retinol extraction efficiency during sample processing. This simple and reliable method for the determination of vitamin A status is particularly valuable for population studies and large clinical trials.

Conflicts of Interest Statement

The authors have no conflicts of interest in this study.

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Table 1. Stability of retinol on Dried Blood Spot (DBS) over 10 weeks.

Conc. (μg/mL) \ Days	0	3	7	14	28	42	56	70
Blank[#]	0.48 ± 0.01	0.48 ± 0.01	0.49 ± 0.01	0.51 ± 0.02	0.50 ± 0.02	0.51 ± 0.03	0.48 ± 0.02	0.48 ± 0.02
BHT[*]	0.48 ± 0.03	0.48 ± 0.02	0.50 ± 0.02	0.50 ± 0.02	0.51 ± 0.03	0.49 ± 0.01	0.48 ± 0.01	0.52 ± 0.02

No pre-treatment was applied on 903® paper.

* BHT (Butylated hydroxytoluene) was pre-treated on 903® filter paper, the amount being 0.35 mg.

Concentrations are shown as mean ± SD (n=3). No significant differences were detected (one-way ANOVA, sig. level set at $p<0.05$).

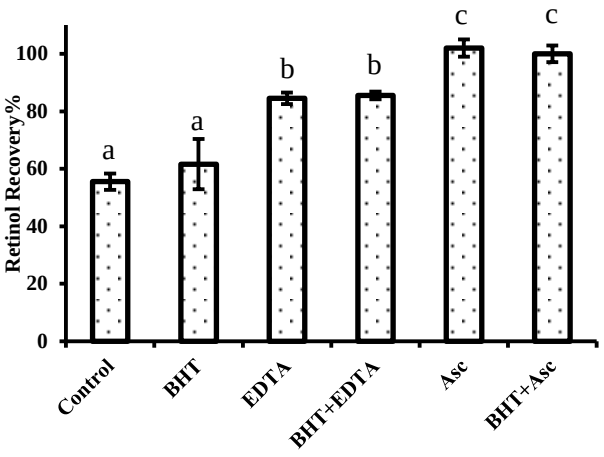


Fig. 1. Recovery of retinol (mean ± SD; n=3) from dried plasma spots pre-treated with various protectants after 4 hours drying at room temperature in darkness. The values of retinol recovery% are referring to plasma 2-phase extraction. **Control**: 50 µL plasma spotted on blank 903® paper; **BHT** (Butylated hydroxytoluene): 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg BHT; **EDTA** (Ethylenediaminetetraacetic acid): 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg EDTA; **BHT+EDTA**: 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg BHT and 0.25 mg EDTA; **Asc** (Ascorbic acid): 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg ascorbic acid; **BHT+ Asc**: 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg BHT and 0.25 mg ascorbic acid. . Different letters indicate significant differences ($p<0.05$) using one-way ANOVA and post-hoc Duncan test.

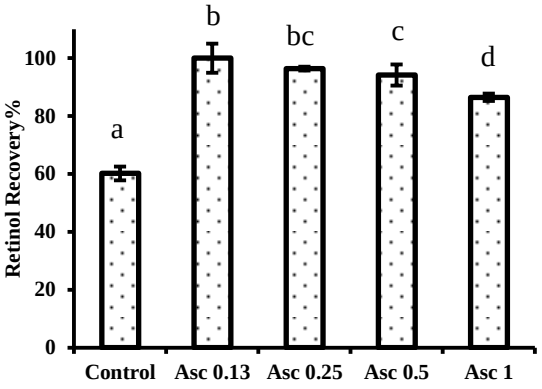


Fig. 2. Retinol stability (mean ± SD; n=3) from dried plasma spot with different doses of ascorbic acid (**Asc**) stored in aluminium foil bags with desiccants. The values of retinol recovery% are referring to plasma 2-phase extraction. **Control**: 50 µL plasma spotted on blank 903® paper; **Asc 0.13**: 50 µL plasma spotted on 903® paper pre-treated with 0.13 mg ascorbic acid; **Asc 0.25**: 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg ascorbic acid; **Asc 0.5**: 50 µL plasma spotted on 903® paper pre-treated with 0.5 mg ascorbic acid; **Asc 1**: 50 µL plasma spotted on 903® paper pre-treated with 1 mg ascorbic acid. Triplicates were included for each group. Different letters over the bars indicate significant differences ($p<0.05$) using one-way ANOVA post-hoc Duncan test.

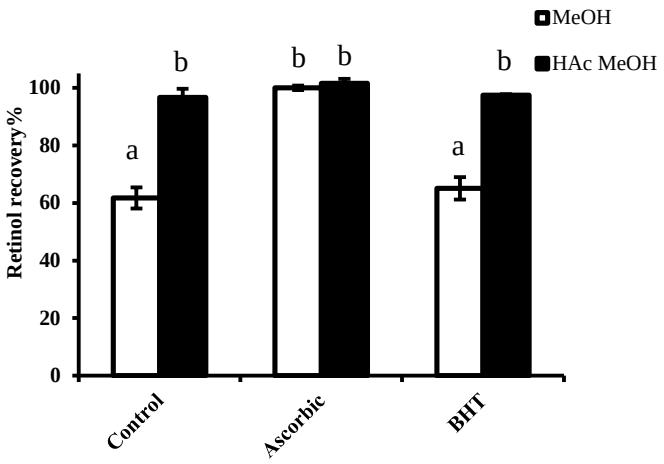


Fig. 3. Retinol recovery (mean ± SD; n=3) from dried plasma spots using different extraction conditions with or without acetic acid (HAc). HAc concentrations: 2mM. Dried plasma spots were extracted after 4 h drying; the percentages were related to plasma 2-phase extraction. **Control**: 50 µL plasma spotted on 903® filter paper without pre-treatment. **Ascorbic**: 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg ascorbic acid. **BHT** (Butylated hydroxytoluene): 50 µL plasma spotted on 903® paper pre-treated with 0.25 mg BHT. Different letters indicate significant differences ($p<0.05$) using one-way ANOVA and post-hoc Duncan test.

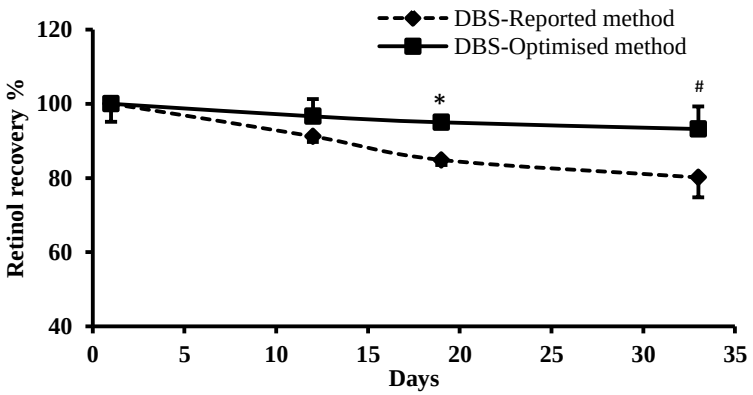
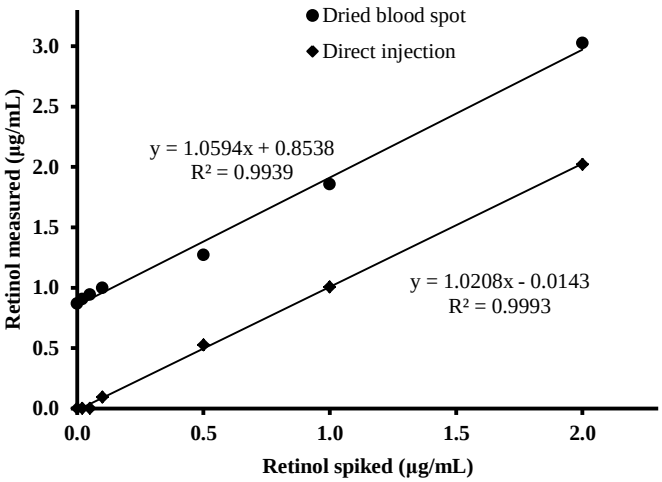


Fig. 4. Stability of retinol on dried blood spots (DBS) stored at room temperature over 33 days with two extraction methods. **DBS-Reported method**: Referring to the method published in literature [15]; **DBS-Optimised method**: method used in this study. Triplicates were included for each group. Statistical analyses were conducted using one-way ANOVA and post-hoc Duncan test. Significant differences were found between the two methods on day 19 (* $p=0.010$) and day 33 ($^{\#}$ $p=0.013$).



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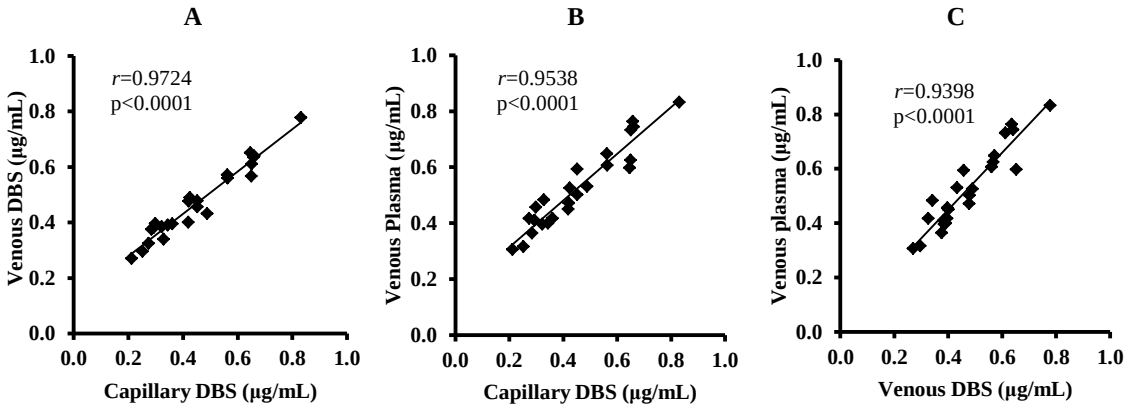
402 **Fig. 5.** Linearity of DBS spiked with serially diluted retinol standard solutions extracted with our
403 modified method compared with direct injection of standard solutions. The intercept shows the
404 concentration of endogenous retinol in the DBS samples being 0.85 µg/mL (1.83 µM).

405



406

407 **Fig. 6.** Linear correlation of retinol concentration between dried blood spots from finger (capillary
408 DBS), venous blood and venous plasma from 24 healthy subjects. **A**, Finger DBS against Venous
409 DBS; **B**, Finger DBS against Venous Plasma; **C**, Venous DBS against Venous Plasma. Extraction of
410 DBS was conducted using our acidified DBS extraction method. Retinol concentrations were
411 converted to the equivalent amount in plasma according to average haematocrit (male 45%
412 erythrocytes, female 40%). Pearson correlation coefficients were calculated for each plot with p (2-
413 tailed) <0.0001.

414