UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

Department of Biological and Biochemical Sciences

Lenka Laštovičková

The Application of Separation Techniques for the Screening of Metabolism Using Dried Blood Spot Sample

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Author: **Mgr. Lenka Laštovičková**

Supervisor: **prof. Mgr. Roman Kanďár, Ph.D.**

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Abstract

The thesis of dissertation focuses on dried blood spot as a simple and non-invasive sample collection technique than can be used as an alternative sample for the screening of metabolism in humans since changes in the levels of some fatty acids, amino acids, and keto acids can be associated with metabolic disorders (for example diabetes mellitus). The samples in this study were obtained from 60 volunteers by a finger prick. In total 3 different methods were optimized, 20 amino acids, 5 keto acids, and 24, respectively 18 fatty acids (percentage content, respectively concentration) were quantified. The data were statistically evaluated. All presented methods are sensitive enough for the determination of above-mentioned analytes from a small volume of a biological material in dried blood spot samples.

Abstrakt

Predložená práca sa zameriava na techniku suchej kvapky krvi ako na jednoduchú a neinvazívnu metódou odberu vzorky. Keďže zmeny hladín niektorých aminokyselín, ketokyselín či mastných kyselín môžu byť spojené s rozvojom metabolických porúch (napríklad *diabetes mellitus*), môže byť suchá kvapka krvi použitá ako alternatívna vzorka pri skríningovom vyšetrení stavu metabolizmu. Pre túto štúdiu boli vzorky suchej kvapky krvi získané punkciou prstu 60 dobrovoľníkov. Celkovo boli optimalizované 3 rozličné metódy a vo vzorkách bolo stanovených 20 aminokyselín, 5 ketokyselín a 24, respektíve 18, mastných kyselín (percentuálne zastúpenie, resp. koncentrácia). Výsledky boli štatisticky spracované. Použité metódy sú dostatočne citlivé pre kvantifikáciu spomínaných analytov z veľmi malého množstva biologického materiálu obsiahnutého vo vzorke suchej kvapky krvi.

Keywords
amino acids, chromatography, dried blood spot, fatty acids, keto acids, metabolomics

Kľúčové slová
aminokyseliny, chromatografia, ketokyseliny, mastné kyseliny, metabolomika, suchá kvapka krvi

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List of abbreviations

2-AB	2-aminobutyric acid
2-OG	2-oxoglutarate
AA	amino acids
ACN	acetonitrile
BHT	butylated hydroxytoluene
DBS	dried blood spot
DDB	1,2-diamino-4,5-dimethoxybenzene
DMB	1,2-diamino-4,5-methylenedioxybenzene
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FA	fatty acids
FAME	fatty acid methyl ester
FID	flame-ionization detector
FLD	fluorescence detector
GC	gas chromatography
HPLC	high-performance liquid chromatography
KA	keto acids
KIC	α-ketoisocaproate
KIV	α-ketoisovalerate
KMV	α-keto-β-methylvalerate
MS	mass spectrometry
NDA	naphthalene-2,3-Dicarboxaldehyde
OPA	*o*-phthalaldehyde
OPD	*o*-phenylenediamine
PDA	photodiode array
PYR	pyruvate

1 Introduction

The protection of an environment and minimization of environmental burden is currently an upward trend. This approach spreads in several areas of our life including analytical chemistry. The smaller size of analytical instruments and their higher sensitivity enables an analysis of small amounts of a biological material with a very low consumption of chemicals, what is economically and environmentally beneficial. There is also a great interest in simple, fast, patient friendly and minimal invasive sample collection technique. The dried blood spot (DBS) sampling standardly used in the newborn screening of congenital metabolic disorders may be an option. There is no need for qualified medical personnel for blood collection and fast transport of samples to laboratories at low temperatures is possible. Thus, DBS shows great potential as an alternative to a conventional whole blood sampling in metabolic screening or in monitoring of treatment response. Although in some cases this type of sampling is still only in the research phase, it can overall simplify the sample collection and the transportation process.

1.1 Dried blood spot sample

The dried blood spot (DBS), a new sampling technique, was first introduced by Ivar Christian Bang in 1913 for the determination of blood sugar levels in rabbits (Schmidt, 1986). Even though several other scientists used this type of sample collection in their studies, it became popular only in 1963, thanks to Robert Guthrie and his neonatal screening of phenylketonuria (Guthrie and Susi, 1963). The biggest advantages of this sample collection technique are possibility of self-collection by patient himself/herself or by any adult at home, no need of the presence of trained medical personnel, minimal invasiveness of sample collection, higher stability of analytes in dried form, disruption of virus envelopes while drying, thus lower biohazard, simplicity of transport and storage. On the contrary, the biggest disadvantage is the variety of haematocrit level which can affect quantitative results, and, in some cases, also low amount of material taken for analysis (thus this type of sample is not appropriate for all analytes) and need to establish reference values for this type of sample.
In general, dried samples are very popular in laboratory analysis last days. Besides DBS, there is evidence also about dried urine spot (Menkovic *et al.*, 2019; Newman *et al.*, 2020), dried saliva spot (Numako *et al.*, 2016; Zheng *et al.*, 2016), dried sweat spot (Kanďár *et al.*, 2016) or even dried milk spot (Jackson *et al.*, 2016; Olagunju *et al.*, 2015). Also, articles about dried plasma/serum spots (Parker *et al.*, 2015; Suzuki *et al.*, 2017) can be found in the literature, but this type of sample is not compatible with non-invasive self-collection.
The blood for DBS sample preparation is usually taken from a middle finger of non-dominant hand, but it can be taken also from ear lobe or heel (newborns). The skin must be first disinfected with alcohol and then allowed to air-dry. Then the skin is pricked by a sterile disposable lancet. The very first drop is usually wiped by a piece of paper. The other drop of whole blood can be applied to a sampling filter card within the marked area. This area should be homogenously filled with preferably one drop of blood and should show the same red colour from both sides of filter paper. Then samples are left to air dry at horizontal position without direct sunlight (or any source of heat) for at least

2-3 hours. Contact with the marked area of a filter paper before or after blood application should be avoided (Li and Tse, 2010; Mei *et al.*, 2001; Deep *et al.*, 2012).

1.2 Determination of selected analytes in dried blood spot sample

Most of methods commonly used for the determination of amino acids (AA), keto acids (KA) or fatty acids (FA) from biological material can be applied also for DBS samples, but low quantity of analytes in the sample must be considered. The direct analysis without previous derivatization is the most favourable because of speeding up the whole procedure of sample preparation and elimination of a problem with reaction by-products, but this type of analysis is possible only when electrophoretic or chromatographic techniques are connected to MS detector. Amino acids from DBS are most often determined using HPLC with different types of detection – HPLC-FLD (Kanďár, 2009), HPLC-PDA (Haghighi *et al.*, 2014), HPLC-ESI-MS/MS (Giordano *et al.*, 2019), eventually using MS/MS without previous chromatographic separation (Wang *et al.*, 2016). The methods for AA determination from biological materials are summarized in review article (Song *et al.*, 2018). Methods for determination of KA from DBS samples are quite rare, but as an example HPLC-FLD (Kanďár, 2009) can be mentioned. Generally, KA in biological material can be determined after derivatization via RP-HPLC connected to FLD (Fujiwara *et al.*, 2020) or MS detector (Zhang *et al.*, 2018). When analysing KA with GC, addition of another derivatization step is necessary (Nguyen *et al.*, 2013). Regarding FA determination from DBS sample, the most frequently used method is GC connected either to FID (Gunash *et al.*, 2019) or MS detector (Ingels *et al.*, 2011). The direct analysis of FA is difficult, but there are some articles about LC-MS/MS detection of underivatized FA in biological material (Serafim *et al.*, 2019). More often FA are derivatized and samples analysed using RP-HPLC or GC (Bielawska *et al.*, 2010).

1.3 Association of selected analytes with diseases

The above-mentioned analytes are involved in many important biological processes, regulation of metabolism included. Their concentrations are stable (regulated properly) under physiological conditions and long-term increase or decrease in this concentration may indicate the disease state. Thus, they can be used as non-specific biomarkers reflecting the current state of metabolism. The most common disease associated with changes in the concentration of all selected analytes is *diabetes mellitus*. As summarized in Song *et al.* (2018), the changes in the concentration of AA can be related to many other diseases, for example different types of cancer, kidney or liver diseases and others. Keto acids are important intermediates of metabolic pathways, and the product of AA catabolism. Hence, elevated levels of AA (especially branched-chain AA) associated with decreased catabolism in obesity and diabetes or other alterations in AA metabolism can be reflected also in the levels of KA (Zhang *et al.*, 2017; Zhou *et al.*, 2013). Also changes in the concentration of FA (elevation or reduction of some FA or changes in their ratio) reflect impaired metabolism and can be a biomarker for early detection of disease state (Sobczak *et al.*, 2019; Wang *et al.*, 2003).

2 Aim of the study

The aim of this study was to establish sufficiently sensitive separation techniques for the determination of selected analytes (amino acids, keto acids, fatty acids) from a small volume of a biological material in DBS sample, analysis of samples obtained from volunteers, set up of a preliminary reference range of selected analytes in whole blood, statistical evaluation of measured data and overall evaluation of the DBS sample use as an alternative to whole blood sample collection in metabolic screening.

3 Experimental part

3.1 Sample collection and preparation

A total of 60 whole blood-donors were included in this study (32 women and 28 men, average age in every group was 39 years). The written informed consent was obtained from all participants. The subjects were a common representative sample of the population, only information about age and gender were obtained.
Before blood collection, spots estimated for FA analysis were treated with an antioxidant solution (0.1% BHT in ethanol). About 15 μL of BHT solution was pipetted onto the filter paper and left to air dry at room temperature for at least 30 minutes and then stored at -20 °C (not more than 9 months). Other spots (analysis of AA and KA) were left untreated.
The middle finger of non-dominant hand was first disinfected and then pricked with a sterile lancet. The very first blood drop was wiped with cellulose paper. Other blood drops were collected on Specimen Collection Paper (#903; Whatman, Dassel, Germany) following the principles of proper sample preparation. DBS samples were left to air dry at room temperature for at least 2.5 hours and then stored at -20 °C with desiccant in a plastic sealed bag (not more than 9 months).
The whole blood used for the preparation of DBS standards was collected by trained medical personnel from a cubital vein of an individual into vacuum tubes with EDTA (Vacuette Detection Tube, No. 454246; Greiner Labortechnik, Kremsmünster, Austria) just before use. The haematocrit level was measured afterwards from capillary blood by microhematocrit method, and its value was always within the reference range (men 0.40-0.52, women 0.34-0.46).

3.2 Amino acid analysis

The disc with a diameter of 6 mm was punched out from the DBS sample and transferred to an Eppendorf test tube. Then 150 μL of 75% ACN containing DL-norvaline (internal standard, 10 μmol/L) was added. After 30 minutes of elution in an ultrasonic bath, the sample was centrifuged (5 min, 1970 × g at 4 °C) and 100 μL from the top of thus prepared sample was transferred to an insert of a chromatographic vial and analysed with HPLC-MS. The chromatographic separation of AA was carried out with an Ascentis® Express OH5 column (150 × 2.1 mm; 2.7 μm) fitted with an Ascentis® Express OH5 guard column (5 × 4.6 mm; 2.7 μm) at 37 °C. Mobile phase A was a mixture of ACN and 10 mmol/L ammonium formate (90:10, v/v), pH 4.5 ± 0.1. Mobile phase B was a mixture of ultra-pure water and 10 mmol/L ammonium formate (90:10, v/v), pH 4.5 ± 0.1. The gradient was applied as follows: from 0 to 8 min, 95–47 % B (linear gradient); from 8 to 15 min, 95 % B. The flow rate was kept constant at 0.25 mL/min. The injection volume was 2 μL. For the detection, a triple quadrupole mass spectrometer with electrospray ionization was used. The ion source parameters were set as follows: capillary voltage: 5 kV, heating gas flow: 10 L/min; drying gas flow: 10 L/min, nebulizing gas flow: 2 L/min; interface temperature: 300 °C, DL temperature: 250 °C and heat block temperature: 400 °C. Data were obtained in selective positive multiple reaction monitoring modes (MRM). The concentration of AA was

determined from a calibration curve, which was constructed using the corresponding peak area ratio of the analyte/IS.

3.3 Keto acid analysis

The disc with a diameter of 6 mm was punched out from the DBS sample, transferred into well-caped 1.5 mL polypropylene tubes, and soaked with 200 μL of 1 mmol/L hydrochloric acid containing α-ketocaproate (2 μmol/L) for 60 min. Then, 50 μL of 6.3 mol/L perchloric acid was added, the content was vortexed for 60 s, incubated (4 °C, 10 min), and centrifuged (4430 × g, 4 °C, 10 min). The supernatant (150 μL) was transferred into a 1.5 mL well-capped polypropylene tube. Further, 150 μL of derivatization reagent (150 μg of OPD in a mixture of 25 mmol/L sodium dithionite, 0.7 mol/L hydrochloric acid and 1.0 mol/L 2-mercaptoethanol) was added. The mixture was incubated at 55 °C for 30 min, after cooling filtered through a nylon filter (pore size 0.2 μm, 4 mm diameter, Supelco, Bellefonte, PA, USA) and transferred into 1.0 mL amber vials. The chromatographic analysis of highly fluorescent 2-quinoxalinol derivatives was accomplished using a gradient elution on a LichroCart 125 × 4 mm, Purospher Star RP-18e, 5 μm, analytical column fitted with a LichroCart 4 × 4 mm, Purospher Star RP-18e, 5 μm, guard column (Merck, Darmstadt, Germany) at 37 °C. Mobile phase A consisted of 25% ACN in water (v/v) and phase B was 100% ACN. The gradient was applied in the following sequences: from 0 to 3 min, 2 % B; from 3 to 13 min, 35-38 % B (linear gradient); from 13 to 18 min, 100 % B. The flow rate was kept constant at 0.5 mL/min. The optimum response of the fluorescent derivatives was observed when the excitation and emission wavelengths were set to 350 and 410 nm, respectively. The concentration of KA was determined from a calibration curve, which was constructed using the corresponding peak area ratio of the analyte/IS.

3.4 Fatty acid analysis

The disc with a diameter of 6 mm was punched out from the DBS sample and transferred to a well-capped glass test tube. Then 200 μL of methanol containing heneicosanoic acid (internal standard, 6.75 μg/mL) was added. After 1 hour elution, the disc was removed and 300 μL of derivatization reagent (methanol:acetyl chloride, 25:2, v/v) was added. The sample was derivatized for 1 hour at 100 °C. After cooling to room temperature, the mixture was neutralized by adding 400 μL of 6% KHCO3. The FAME were extracted to 500 μL of hexane twice (10 min shaking, 5 min centrifugation 1225 × g at 4 °C). Extracts of one sample were pooled and evaporated under the stream of nitrogen, then dissolved in 100 μL dichloromethane. The sample thus prepared was completely transferred to an insert of a chromatographic vial and analysed with GC-FID. The chromatographic separation of FAME was carried out with an HP88 capillary column (100 m × 0.25 mm id; Agilent Technologies, Santa Clara, USA) coated with 88% cyanopropyl and 12% aryl-polysiloxane (with a film thickness of 0.25 μm) as a stationary phase. Helium was used as the carrier gas with a constant flow rate of 3 mL/min. The injection volume was 1 μL at the temperature of 250 °C and with an inlet split ratio 10:1. The separation was performed under the following temperature programme: 80 °C held for 3 min, then increased to 180 °C at a rate of 10 °C/min, held at this temperature for 3 min, then increased to 220 °C at a rate of 1 °C/min, held at this

temperature for 1 min and finally increased to 240 °C at a rate of 5 °C/min and held at this temperature for 40 min. The total analysis time was 101 min; the detector temperature was set to 250 °C. FA were qualified by direct comparison of retention time to those of known standards and quantified as a percentage of total FAs measured in the sample. Quantity of FAs with dominant representation was expressed also as a concentration, using the calculation from relative response factor equation $c_x=(RF\times c_{IS}\times Area_x)/Area_{IS}$.

3.5 Statistical analysis

The data were analysed using GraphPad Prism 8.0.1 (GraphPad Software, Inc., La Jolla, CA, USA). All tests were performed with $\alpha = 0.05$. For evaluation of the difference between men and women, data were first subjected to the test of normality (four built-in analyses in Graph Pad programme – Anderson-Darling, D´Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov test). The data which passed the normality test were assumed to have Gaussian distribution and were further analysed using an unpaired t-test. In case data were assumed to have different variances (F-test), Welch correction was applied. Data that did not pass the normality test were further analysed using a non-parametric Mann-Whitney test.
In the case of Gaussian distribution (four normality tests mentioned above), ordinary one-way ANOVA followed by Tukey multiple comparison test was used to evaluate the influence of age on different analyte levels. In the case of significantly different SDs, Brown-Forsythe and Welch ANOVA tests were used instead. If data were not assumed to have Gaussian distribution, Kruskal-Wallis test followed by Dunn´s multiple comparison test were used for evaluation.
The relationship between individual AA, KA and FA values was evaluated as a correlation matrix using the Pearson correlation coefficient.
P value <0.05 was considered statistically significant.

4 Results

4.1 Optimization of methods

All methods used for the determination of selected FA, AA, and KA, are the result of optimization process. Several parameters were optimized to obtain the highest sensitivity and the best separation of the selected analytes in the shortest time. Briefly, different non-polar stationary phases as well as different organic and buffered mobile phases were used in HPLC methods together with different gradient elution conditions. Also, different derivatization reagents were tested – for the determination of AA it was OPA and NDA, for the determination of KA it was OPD, dansylhydrazine, DDB, and DMB. Time of derivatization and temperature were optimized as well. For GC method, two different stationary phases were tested and several different derivatization reagents (BF_3 in methanol/ethanol/butanol and mixture of methanol and acetyl chloride). Several temperature programs were tested for separation of esters formed. The best results were achieved using conditions described above. Standard solutions as well as real DBS samples were used for the study of optimal conditions. The method parameters for determination of selected FA are summarized in Table 1 and for determination of selected AA and KA in Table 2.

Table 1 Analytical parameters of GC-FID method for the determination of selected FA from DBS sample

		Amount		Concentration	
	RRF*	AVG (SD) (%)	CV (%)	AVG (SD) (μmol/L)	CV (%)
C14:0	1.069	1.08 (0.08)	7.46	6.4 (0.6)	9.7
C15:0	1.080	0.27 (0.02)	5.48	1.6 (0.1)	6.4
C16:0	1.032	24.82 (0.32)	1.28	126.7 (5.5)	4.4
***cis* C16:1 n10**	-	1.20 (0.02)	1.54	-	-
***cis* C16:1 n7**	-	0.06 (0.01)	12.88	-	-
C17:0	1.043	0.39 (0.02)	4.11	1.9 (0.1)	5.5
C18:0	1.044	19.27 (0.79)	4.10	89.7 (4.7)	5.2
***cis* C18:1 n9**	1.039	16.07 (0.32)	1.97	74.9 (2.1)	2.8
***cis* C18:1 n7**	-	1.53 (0.07)	4.80	-	-
all-*cis* C18:2 n6	1.069	17.74 (0.35)	1.95	85.8 (3.2)	3.7
all-*cis* C18:3 n6	1.057	0.15 (0.02)	13.49	0.7 (0.1)	11.5
all-*cis* C18:3 n3	1.046	0.21 (0.01)	6.23	1.0 (0.1)	8.7
C20:0	1.003	0.40 (0.04)	8.93	1.6 (0.2)	10.4
***cis* C20:1 n9**	1.021	0.19 (0.02)	10.54	0.7 (0.1)	12.7
all-*cis* C20:3 n6	1.086	1.82 (0.02)	1.35	8.2 (0.3)	3.9
all-*cis* C20:4 n6	1.007	8.10 (0.16)	1.99	34.0 (1.3)	3.7
all-*cis* C20:5 n3	1.172	0.53 (0.03)	4.95	2.6 (0.2)	6.9
C22:0	1.011	0.59 (0.02)	4.19	2.2 (0.1)	4.8
all-*cis* C22:4 n6	-	1.15 (0.04)	3.14	-	-
all-*cis* C22:5 n6	-	0.27 (0.04)	16.40	-	-

		Amount		Concentration	
	RRF*	AVG (SD) (%)	CV (%)	AVG (SD) (μmol/L)	CV (%)
all-*cis* C22:5 n3	-	0.82 (0.06)	6.85	-	-
all-*cis* C22:6 n3	1.116	2.16 (0.10)	4.72	9.3 (0.5)	5.4
C24:0	1.018	0.64 (0.07)	10.26	2.3 (0.3)	12.1
***cis* C24:1 n9**	1.005	0.55 (0.08)	14.07	1.9 (0.3)	14.3

*RRF = $(Area_x/c_x) / (Area_{IS}/c_{IS})$

Intra-assay precision of the GC-FID method for FA determination (n = 10) from DBS samples. Both ways of results expression are included (percentage content of all FA evaluated and concentration calculated from the relative response factor).

Table 2 Analytical parameters of HPLC-ESI-MS/MS and HPLC-FLD method for the determination of selected AA and KA from DBS sample GC-FID method for the determination of selected FA from DBS sample

	A – low conc. CV (%)	**A** – high conc. CV (%)	**B** – low conc. CV (%)	**B** – high conc. CV (%)	LOD (μmol/L)	LOQ (μmol/L)	Working range of CC (μmol/L)
2-AB	1.7	9.3	6.2	6.5	0.3	1.0	1.0-75.1
Ala	2.9	1.9	9.5	7.4	2.5	7.7	7.7-851.3
Arg	0.5	2.6	7.7	3.1	0.6	1.9	1.9-273.3
Asn	9.1	3.4	10.6	0.0	0.4	1.2	1.2-144.1
Cit	0.2	2.6	9.1	7.6	0.3	0.8	0.8-79.1
Gln	5.5	3.1	8.5	7.4	2.4	7.4	7.4-1369.8
Glu	3.3	5.5	5.1	8.7	0.6	1.8	1.8-186.0
Gly	1.2	3.0	4.0	2.7	5.9	17.8	17.8-809.9
His	3.1	1.8	6.9	5.5	0.4	1.3	1.3-195.9
Ile	2.7	4.7	4.8	7.6	0.3	1.0	1.0-195.8
Leu	3.8	5.2	2.5	1.1	1.1	3.4	3.4-312.9
Lys	4.0	1.7	6.1	7.4	1.4	4.4	4.4-295.0
Met	4.0	5.7	2.4	7.0	0.2	0.5	0.5-102.4
Phe	1.7	4.8	8.0	0.1	0.3	0.9	0.9-140.0
Pro	3.6	4.2	0.0	2.9	0.9	2.8	2.8-384.0
Ser	1.5	4.6	7.8	3.8	3.6	11.1	11.1-264.0
Thr	5.7	3.0	1.5	9.0	1.2	3.7	3.7-353.1
Trp	3.9	4.2	8.8	8.7	0.2	0.6	0.6-117.8
Tyr	2.5	5.2	4.4	6.1	0.4	1.3	1.3-155.4
Val	2.7	3.8	7.0	7.2	1.9	5.8	5.8-590.0
2-OG	6.9	5.4	9.2	8.3	0.1	0.1	0.1-25.7
PYR	4.7	4.4	5.1	5.2	3.3	9.9	9.9-243.6
KIV	3.8	3.4	6.1	5.9	0.2	0.6	0.6-27.5
KMV	3.9	3.1	6.2	5.9	0.5	1.6	1.6-50.5
KIC	4.5	4.2	7.4	7.0	0.6	1.8	1.8-54.2

Intra-assay (n=10) and inter-assay (n=6) precision, LOD, LOQ and average parameters of 10 calibration curves for HPLC-ESI-MS/MS method for AA and HPLC-FLD method for KA determination from DBS samples. LOD and LOQ were calculated using the following equations: LOD = 3.3 *Sa/b*; LOQ = 10 *Sa/b*, where *Sa* is standard deviation of the intercept and *b* is slope of the calibration curve; the x-intercept (in μmol/L) is the point at which the line crosses the x axis (where the y value equals 0). CC – calibration curve.

4.2 Statistical analysis

A total of 60 DBS samples were analysed with chromatographic methods and 20 AA, 5 KA, and 24 (respectively 18) FA were quantified in each whole blood sample. The results were statistically evaluated. Statistically significant differences ($\alpha = 0.05$) between men and women were observed for five AA, arginine, alanine, glutamine, proline, and valine, three KA, α-ketoisovaleric, α-ketoisocaproic, and α-ketomethylvaleric acid, and eight FA, myristic, palmitic, stearic, oleic, linoleic, α-linolenic, gondoic, and arachidonic acid (see Table 3). All above-mentioned analytes were higher in men.

For the evaluation of age-dependency of measured analytes three age groups were created as follows: < 30 years, 30 – 45 years, ≥45 years. Statistically significant differences ($\alpha = 0.05$) were observed for two AA (asparagine and serine), and two FA (α-linolenic and γ-linolenic acids). Both AA seem to gradually decrease with age. α-Linolenic acid has no age-depending behaviour according to our results whereas the level of γ-linolenic acid seems to increase with age. No statistically significant differences were observed for the selected KA. All statistically significant results are summarized in Table 4.

Analyses of correlation were performed by calculating Pearson correlation coefficient to see a relationship between selected AA, KA, and FA. The statistically significant correlations were observed between some AA, some KA, and some FA.

Table 3 The statistical evaluation of gender influence on tested analytes

Concentration (μmol/L)	Men	Women	p value
Arg	93.47	80.67	*
2-AB	21.73	20.93	-
Ala	446.60	396.10	*
Asn	89.84	80.74	-
Cit	50.16	46.58	-
Glu	181.00	175.80	-
Gln	514.40	442.00	***
Gly	369.30	330.80	-
His	107.50	103.60	-
Ile	139.60	123.80	-
Leu	188.20	163.80	-
Lys	263.60	263.30	-
Met	31.43	30.89	-
Phe	87.85	88.39	-

Concentration (μmol/L)	Men	Women	p value
Pro	246.60	200.50	**
Ser	251.40	221.00	-
Thr	127.80	114.80	-
Trp	61.01	60.57	-
Tyr	72.01	68.76	-
Val	266.20	235.10	*
2-OG	11.34	9.85	-
PYR	227.80	213.80	-
KIV	16.29	13.74	**
KIC	32.30	25.69	**
KMV	27.50	22.28	**
C14:0	7.4	5.8	*
C15:0	1.9	1.6	-
C16:0	187.4	155.7	**
C17:0	1.8	1.6	-
C18:0	88.5	75.9	*
cis C18:1 n9	115.1	83.6	****
all-*cis* C18:2 n6	110.6	94.6	*
all-*cis* C18:3 n6	0.9	0.7	*
all-*cis* C18:3 n3	1.8	1.8	-
C20:0	2.4	1.5	-
cis C20:1 n9	1.2	0.9	**
all-*cis* C20:3 n6	6.2	5.4	-
all-*cis* C20:4 n6	36.8	33.5	*
all-*cis* C20:5 n3	2.3	2.3	-
C22:0	3.6	3.8	-
all-*cis* C22:6 n3	7.8	7.9	-
C24:0	5.3	5.5	-
cis C24:1 n9	7.4	8.0	-

Data are presented as mean values (n=28 for men, n=32 for women). The data with Gaussian distribution were analysed using an unpaired t-test (when data were assumed to have different variances, Welch correction was applied). Data which did not pass the normality test were analysed using a non-parametric Mann-Whitney test. All tests were performed with $\alpha = 0.05$. Statistically significant results are described as follows: *$p<0.050$, ** $p<0.010$, *** $p<0.0005$, **** $p<0.0001$.

Table 4 The statistical evaluation of age influence on tested analytes

	A	B	C	p value	p value (A vs. B)	p value (A vs. C)	p value (B vs. C)
Concentration of AA (μmol/L)							
Asn	90.93	89.88	74.95	*	-	*	-
Ser	275.60	249.20	185.30	*	-	*	-

	A	B	C	p value	p value (A vs. B)	p value (A vs. C)	p value (B vs. C)
Concentration of FA (μmol/L)							
all-cis C18:3 n3	1.97	1.62	1.72	***	***	*	-
all-cis C18:3 n6	0.54	0.79	0.97	**	-	**	-

Data are expressed as mean values (n=19 for group A, n=20 for group B, n= 21 for group C). The ordinary one-way ANOVA followed by Tukey multiple comparison test was used for data with Gaussian distribution (in the case of significantly different SDs, Brown-Forsythe and Welch ANOVA tests were used instead). The Kruskal-Wallis test followed by Dunn´s multiple comparison test was used for data which did not pass a normality test. All tests were performed with $\alpha = 0.05$. Statistically significant results are described as follows: *$p<0.050$, ** $p<0.010$, *** $p<0.0005$. **A** refers to age group < 30 years, **B** refers to 30 - 45 years, **C** refers to ≥45 years.

4.3 Preliminary reference range

The composition of whole blood and plasma samples differs since erythrocytes, leukocytes as well as thrombocytes are separated by centrifugation from whole blood to obtain plasma. Thus, also the concentration of selected analytes in these two types of samples can be different. For this reason, it is not right to automatically use reference range set up for plasma also for DBS samples, but it is important to set up new reference range after method validation. When monitoring patient regularly, also individual reference range can be set and that has even higher informative value. Preliminary reference range of selected analytes from our study is shown in Table 5.

Table 5 Preliminary reference range of selected AA, KA, and FA in whole blood of adults

	Concentration (μmol/L)	SD (μmol/L)	MIN (μmol/L)	MAX (μmol/L)
2-AB	21.3	9.4	2.9	59.6
Ala	419.7	99.2	237.5	709.7
Arg	86.6	24.1	33.1	135.9
Asn	85.0	21.8	51.9	146.1
Cit	48.3	10.2	28.4	78.3
Glu	178.2	41.0	105.9	314.2
Gln	475.8	76.8	295.4	654.1
Gly	348.7	109.2	127.5	636.3
His	105.4	18.3	71.9	148.9
Ile	131.2	40.2	58.6	235.0
Leu	175.2	57.7	70.1	399.4
Lys	263.5	69.8	155.3	455.00
Met	31.1	9.7	14.9	68.1
Phe	88.1	17.9	51.6	129.3
Pro	222.1	65.8	81.5	363.6
Ser	235.2	109.5	83.9	497.8
Thr	120.9	27.9	67.2	198.5
Trp	60.8	10.4	43.0	88.9

	Concentration (µmol/L)	SD (µmol/L)	MIN (µmol/L)	MAX (µmol/L)
Tyr	70.3	16.1	27.4	107.4
Val	249.6	55.1	126.9	377.2
2-OG	10.6	4.9	1.5	21.7
PYR	220.4	68.6	126.3	435.7
KIV	14.9	4.2	7.8	30.5
KIC	28.8	8.8	7.0	48.5
KMV	24.7	8.2	6.4	48.6
C14:0	6.5	2.9	2.7	14.7
C15:0	1.7	0.7	0.7	3.5
C16:0	170.5	43.6	106.4	296.5
C17:0	1.7	0.4	1.1	3.1
C18:0	81.8	20.6	55.6	127.8
cis C18:1 n9	98.3	35.5	62.1	249.2
all-*cis* C18:2 n6	102.1	24.9	57.3	208.5
all-*cis* C18:3 n3	2.2	1.5	0.9	10.1
all-*cis* C18:3 n6	0.8	0.5	0.1	2.3
C20:0	1.6	0.3	1.0	2.5
cis C20:1 n9	1.0	0.4	0.5	2.4
all-*cis* C20:3 n6	5.8	1.5	3.4	9.6
all-*cis* C20:4 n6	35.1	6.2	19.1	50.6
all-*cis* C20:5 n3	2.3	0.4	1.5	3.8
C22:0	3.7	0.8	2.4	7.0
all-*cis* C22:6 n3	7.9	2.0	3.4	13.4
C24:0	5.4	1.9	3.3	16.7
cis C24:1 n9	7.7	4.2	1.8	37.0

5 Conclusions

Three different separation techniques were optimized in this work to enable determination of selected AA, KA, and FA from DBS sample. All presented methods are the results of selection based on performance-price ratio. All of them are sensitive enough for the determination of selected analytes from a few microliters of whole blood in DBS sample, and thus enabling metabolomic screening and eventual detection of disease state. Even though there are some analytical issues (haematocrit effect, sensitivity of analytical techniques) because of which the use of DBS sample instead of whole blood or plasma samples can be challenging, for screening purposes the DBS sample is more than appropriate. The presented methods were used for analysis of 60 samples obtained from blood-donors by finger prick. The concentration of 20 AA, 5 KA, and 18 FA was evaluated. The results were used for setting up of preliminary reference range of analytes in whole blood in adult population. Further, all results were statistically evaluated. Statistically significant higher concentrations in men were found for 16 analytes. Two AA and two KA showed statistically significant age-dependency. Several statistically significant correlations were found as well. Based on many benefits of DBS technique and regarding sensitive methods optimized in this work, we can assume that the DBS sample is a suitable alternative of sample collection technique and that it can be used in the screening of metabolism for possible detection of disease states that are associated with changes in selected analyte levels.

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