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Utilization of dried blood spot technique and HPLC-MS method in metabolomics

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Abstract

This paper describes several metabolites that can be used as biomarkers for the diagnosis of various diseases. For their determination, it is advisable to use liquid chromatography with mass spectrometry detection due to its sensitivity and selectivity. Furthermore, the dried blood spot collection technique is described, which is characterized by a non-invasive method of sample collection, simple transport, and storage. The experimental part is focused on the development of separation methods for the determination of amino acids, allantoin, and uric acid in blood samples obtained by the dried spot technique. Optimization and validation of methods including their application to real samples and discussion of obtained results are also included.

Abstrakt

V této práci je popsána řada metabolitů, které je možné využít jako biomarkery pro diagnostiku řady onemocnění. K jejich stanovení je vhodné využít kapalinovou chromatografii s hmotnostní detekcí kvůli její citlivosti a selektivitě. Dále je popsána technika odběru tzv. suché kapky krve, která se vyznačuje neinvazivním způsobem odběru vzorku, jednoduchým transportem a skladováním. Experimentální část je zaměřena na vývoj separačních metod pro stanovení aminokyselin, alantoinu a kyseliny močové ve vzorcích krve získaných technikou suché kapky. Součástí práce je také optimalizace a validace metod zahrnující aplikaci na reálné vzorky a diskuze získaných výsledků.

Keywords

Allantoin, amino acids, uric acid, metabolomics, dried blood spot, HPLC-MS

Klíčová slova

Alantoin, aminokyseliny, kyselina močová, metabolomika, suchá kapka krve, HPLC-MS

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List of abbreviations

AA	Amino acid
ALA	Allantoin
BEH	Ethylene bridge hybrid
DBS	Dried blood spot
GC	Gas chromatography
HCT	Hematocrit
HILIC	Hydrophilic interaction chromatography
HPLC	High-performance liquid chromatography
IS	Internal standard
ME	Matrix effect
MRM	Multiple reaction monitoring
MS	Mass spectrometry
RP	Reversed phase
UA	Uric acid

1 Introduction

Currently, there is a growing interest in technologies capable of profiling biological systems. One of these technologies is metabolomics, which deals with the analysis of relatively small molecules in biological systems. The main goal of metabolomics is the understanding of metabolic pathways with their subsequent use for disease prediction and the analysis of biomarkers, which can help with disease diagnosis or therapy monitoring. New methods are constantly being developed for metabolomic analysis, which can determine up to several hundreds of metabolites simultaneously. For these purposes, liquid chromatography (HPLC) coupled with mass spectrometry (MS) is used most commonly. The combination of HPLC and MS enables desirable selective, sensitive, and relatively fast analysis of metabolites.

The metabolome of any given biological system contains a wide range of low-molecular-weight metabolites. The detected amount of these metabolites during analysis can be influenced by the timing and method of sampling, as well as by storage and handling of the sample. In metabolomic research, it is necessary to take into account the pre-analytical processes and biobanking. Mishandling samples before the analysis can result in negative effects on the robustness and reproducibility of the obtained data. Additionally, the need of large sample sets for studies requires as least invasive sample collection as possible, their simple transport to the laboratory, and the high stability of the metabolites. Above mentioned requirements are met by the dried blood spot (DBS) sample collection technique, which is commonly used in newborn screening of metabolic disorders and has the potential to become an alternative to the conventional whole blood sampling method.

1.1 Dried blood spot

The DBS technique became popular in 1963 when Robert Guthrie used it for sample collection in newborn screening for phenylketonuria. The DBS technique is based on applying a few drops of capillary blood onto a specific filter paper. Currently, the DBS technique is used to determine the levels of drugs, environmental contaminants, metabolites, proteins, antibodies, DNA, and RNA. It is also the standard technique for collecting blood samples in newborn screening for various metabolic diseases worldwide [1–3]. The blood for the sample preparation can be collected from various parts of the body, while the heel and fingertips are most frequently used. Before sampling, the skin must be disinfected with 70% alcohol and then the skin is pierced with a specific sterile needle. The first drop of blood is aspirated with gauze and the other drops are applied onto the sampling filter paper in predetermined circles. The obtained DBS samples are left to dry in a horizontal position at laboratory temperature for 2-3 hours. Contact with other surfaces and exposure of the sample to direct sunlight should be avoided during the drying process [4].

The DBS technique has a lot of advantages. The major advantage is a less invasive way of sample collection. A small injection into the finger with a specific needle is almost painless and can be performed by patients themselves without any specific

training. The DBS samples also show the good stability of the analytes for up to several months. In addition, the dried sample matrix inactivates most of the pathogens, thereby reducing the biological risks associated with sample transport. Moreover, samples can be sent to the laboratory by post without the need to freeze them due to the high analytes' stability. However, humidity and high temperatures in mailboxes may cause analytes degradation [1,5,6].

The quantitative analysis of biomarkers in DBS samples relies on the assumption that the individual sections used for the analysis provide volumetric measurements comparable to liquid blood samples. However, several factors influence the quantitative results of the analysis of a section from the DBS sample, which was created by applying a non-volumetric blood sample. The factors affecting precision and accuracy include blood volume applied onto the collection card, hematocrit (HCT), distribution of the analytes in the spot, stability of the analytes, method of addition of internal standard, and extraction efficiency [7–9].

1.2 Determination of selected analytes

The analysis of the DBS samples is still challenging in some cases, due to low sample volumes. For the routine determination of amino acids (AA) from DBS samples, the pre-column derivatization in combination with HPLC-MS is commonly performed [10]. Several reagents are utilized for the derivatization. The most often used are *ortho*-phthalaldehyde, DANSYL-chloride, 1-brombuthane and 2,4-dinitrofluorobenzene [11–13]. For the determination of AA without derivatization, hydrophilic interaction liquid chromatography (HILIC) is preferably used in conjunction with MS detection. The retention behavior of AA is highly dependent on the choice of buffer and pH of the mobile phase. The most frequently applied buffer is ammonium formate in the concentration range of 5-30 mmol/L and in the pH range from 3 to 6. The separation is usually performed on columns with an amide or C18 hybrid stationary phase (BEH). The biggest advantage of using the HILIC method is the determination of AA in their native form [14–16].

The determination of uric acid (UA) is performed in clinical laboratories by the enzymatic method routinely. However, a serum is used for the analysis and many interfering compounds may influence results [17]. To obtain more accurate results, more sensitive and selective methods should be utilized. Gas chromatography (GC) with MS detection and isotope dilution is a definitive method for the determination of UA [18], however, HPLC is more preferred nowadays. Different types of detectors can be used for the detection, including electrochemical [19], UV [20], and MS [21,22] detector. The analysis of allantoin (ALA) is still considerably difficult and it is not commonly done. The levels of ALA can be determined by HPLC with UV detection after conversion to glyoxalate and its derivatization with dinitrophenylhydrazine [23]. Another option is the GC-MS method in combination with methylsilyl derivatization [24]. An alternative without derivatization is the HPLC-MS method. Separation can be performed on columns with the reversed stationary phase (RP) [25], however due to the high polarity of ALA, it is preferable to employ HILIC separation [26].

2 Aim of the study

The aim of this dissertation thesis was to develop and validate a sensitive method for the simultaneous determination of ALA and UA, a method for the determination of 20 AA without derivatization, and a method for the determination of phenylalanine and tyrosine. Furthermore, to apply the developed methods to the analysis of DBS samples collected from voluntary donors. The next objective was to compare the results obtained from DBS samples with identical whole blood samples, and finally to evaluate the usage of the dried blood spot technique as an alternative to the whole blood sample collection for metabolomic and clinical studies.

3 Experimental part

3.1 Sample collection and preparation

A total of 100 volunteer whole-blood donors (49 men and 51 women, age range from 21 to 85 years with average age of 50 years) participated in this study. Each donor was informed about the study design and signed an informed consent form before the blood collection. The study itself was approved by the ethics committee of the Hospital of Pardubice. Regarding the representativeness of the sample population, donors did not have to meet any specific criteria to be enrolled to the study. Only the age and gender of donors were known.

Blood samples were collected from the cubital vein into EDTA-coated tubes by qualified personnel. The collected blood samples were pipetted (approximately 50 µL) with an automatic pipette onto sampling collection cards (Whatman 903® protein saver cards) immediately after the collection. The blood was applied in the form of a drop ensuring the tip of the pipette did not touch the filter paper. In addition, exactly 20 µL of whole blood per spot was pipetted for phenylalanine and tyrosine analysis. The blood samples were then allowed to dry at laboratory temperature in a horizontal position for 3 hours. After that time samples were placed in a hermetically sealed polypropylene bag and stored at -80 °C for a maximum of 3 months.

Blood samples for methods optimization were obtained from 2 voluntary donors whose HCT value was within the range of our studied population (0.34-0.46 for women and 0.40-0.52 for men). Venous blood was also collected into EDTA-coated tubes by qualified personnel and then pipetted onto collection cards (50 µL). The HCT value was verified immediately after blood collection using the microhematocrit method.

3.2 Allantoin and uric acid analysis

A disc, 6 mm in diameter, was punched from DBS and transferred into a polypropylene Eppendorf tube. Then, 150 µL of 85% acetonitrile (ACN) in water containing IS (isotopically labelled uric acid-1,3-$^{15}N_2$, 10 µmol/L and allantoin-5-^{13}C,1-^{15}N, 0.1 µmol/L) was added. The tube was closed and a mixture with the disc was sonicated for 30 minutes in the ultrasonic bath. After extraction, the mixture was centrifuged (1970 *g*, 5 min) and the supernatant was transferred into a vial for analysis by HPLC-MS. For the liquid whole blood, 10 µL of blood was mixed with 150 µL of 85% ACN in water containing IS. After protein precipitation, the mixture was centrifuged (1970 *g*, 10 min) and the supernatant was transferred into a vial for analysis. The chromatographic analysis of UA and ALA was performed by HPLC using isocratic elution on a Discovery® HS F5 (150 × 2.1 mm; 3 µm) analytical column, fitted with a Discovery® HS F5 Superguard™ (20 × 2.1 mm; 3 µm) guard column (Supelco, Bellefonte, PA, USA) at 40°C. The mobile phase was a mixture of ACN and deionized water (10:90, *v/v*) containing 0.1% formic acid. The flow rate of the mobile phase was kept constant at 0.2 mL/min and the injection volume was 5 µL. The time of the analysis was 3 min.

3.3 Amino acid analysis

A disc, 6 mm in diameter, was punched from DBS and transferred into a polypropylene Eppendorf test tube. Then 150 µL of 75% ACN in water containing IS (isotopically labelled Phe-D5, 10 µmol/L) was added. The tube was closed and a mixture with the disc was sonicated for 30 minutes in the ultrasonic bath. After extraction, the mixture was centrifuged (1970 *g*, 5 min) and the supernatant was transferred into a vial for analysis by HPLC-MS. For the liquid whole blood, 10 µL of blood was mixed with 150 µL of 75% ACN in water containing IS. After protein precipitation, the mixture was centrifuged (1970 *g*, 10 min) and the supernatant was transferred into a vial for analysis. The chromatographic analysis of AA was performed by HILIC HPLC using gradient elution on 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 40 °C. Mobile phase A was a mixture of ultra-pure water and 10 mmol/L ammonium formate (90:10, *v/v*), pH 4.5 ± 0.1. Mobile phase B was a mixture of ACN and 10 mmol/L ammonium formate (90:10, *v/v*), pH 4.5 ± 0.1. The gradient was applied in the following sequence: from 0 to 8 min, 95-47% B (linear gradient); from 8 to 15 min, 95% B. The flow rate of the mobile phase was kept constant at 0.25 mL/min. The injection volume was 2 µL.

3.4 Phenylalanine and tyrosine analysis

Whole DBS (containing 20 µL of blood) or a disc (6 mm in diameter) was punched from DBS and transferred into a polypropylene Eppendorf test tube. Then 400 µL of 100% methanol (500 µL in case of whole DBS analysis) containing IS (isotopically labelled Phe-D5, 10 µmol/L) was added. The tube was closed and a mixture with a disc was vortexed for 20 minutes. After extraction, the mixture was centrifuged (1970 *g*, 5 min) and the supernatant was transferred into a vial for analysis by HPLC-MS. For the liquid whole blood, 20 µL of blood was mixed with 500 µL of 100% methanol containing IS. After protein precipitation, the mixture was centrifuged (1970 *g*, 10 min) and the supernatant was transferred into a vial for analysis. The chromatographic analysis of phenylalanine and tyrosine was performed by RP HPLC using isocratic elution on a LiChroCart Purospher Star RP-18e (125 × 4 mm; 5 µm) analytical column, fitted with a LiChroCart Purospher Star RP-18e (4 × 4 mm; 5 µm) guard column (Merck, Darmstadt, Germany) at 40°C. The mobile phase was a mixture of methanol and deionized water (1:1, *v/v*) containing 0.1% formic acid. The flow rate of the mobile phase was kept constant at 0.75 mL/min and the injection volume was 1 µL. The time of the analysis was 2.5 min.

3.5 MS detection

For the detection, the triple quadrupole mass spectrometer equipped with electrospray ionization was used. The ion source was set as follows: interface temperature: 300 °C, desolvation line temperature: 250 °C, heat block temperature: 400 °C, nebulizing gas flow: 2 L/min, drying gas flow: 10 L/min, heating gas flow: 10 L/min. Data were

acquired in selective negative multiple reaction monitoring mode (MRM) for UA and ALA, and in selective positive MRM for AA. The amount of analytes was quantified from the corresponding peak area ratio of the analyte/IS using chromatography software. The concentration of analytes in the samples was determined from a calibration curve.

3.6 Method validation

Methods validation was performed according to the Guidelines on bioanalytical method validation published by European Medicines Agency. The method was validated in terms of linearity, limit of detection and quantification, precision, accuracy, recovery, selectivity, matrix effects (ME), effect of blood volume, effect of HCT, and the stability of clinical patient dried blood samples.

4 Results

4.1 Optimization and validation of methods

Presented methods for the determination of UA, ALA, and selected AA are the results of the optimization process. To obtain the highest sensitivity, selectivity, and good separation in the shortest time, several analytical parameters were optimized. Briefly, three different analytical columns were tested: Discovery HS F5, Ascentis® Express OH5 column, and LiChroCart Purospher Star RP-18e. The composition of the mobile phase and the effects of the ionic strength of the mobile phase were tested to achieve the best retention, peak shape, and MS intensity. Different ratios of organic solvents (ACN and methanol) with various concentrations of ammonium formate (5, 10, and 15 mmol/L), formic acid (0.1%, 0.2%, 0.5%, and 1% *v/v*) and pH (pH values 3, 3.5, and 4) were examined. Changes in the mobile phase's ionic strength and pH values showed no significant effects on retention time and peak shape of the UA and ALA. On the other hand, the retention behavior of AA was highly influenced by pH values and by the ionic strength of the mobile phase. Moreover, the presence of ammonium formate in the mobile phase greatly decreased the MS signal of all analytes in comparison with formic acid, nevertheless the buffer was necessary for the reproducibility of AA's retention time. For the UA, ALA, phenylalanine and tyrosine determination, formic acid was used as an additive to enhance analytes MS ionization. Our tests showed that different concentrations of formic acid in the mobile phase had no significant effects on the signal intensity. Multiple gradients and isocratic elutions were tested to accomplish the best resolution in the shortest time. The mass spectrometer operated in a negative mode for UA and ALA and in a positive mode for AA determination, which provided a higher intensity and lower signal-to-noise ratio. The flow of the heating gas and drying gas were also optimized. The best results were achieved using the conditions described in the previous chapter. The quantitative analysis was done in multiple reaction monitoring (MRM) modes to ensure the highest possible selectivity.

ME were evaluated on six DBS samples from different donors. Due to the natural occurrence of the ALA and UA in DBS samples, ME were determined by comparing the area of the isotopically labelled ALA and UA standard peaks of the post-extraction spiked sample with that of the neat matrix. The following equation was used for the calculation: (peak area of the post-extraction spiked sample / peak area of the neat matrix) * 100. ME for AA were determined by comparing the area of the AA peaks of the post-extraction spiked sample with that of the neat matrix. ME were calculated according to the following equation: [(peak area of the post-extraction spiked sample – peak area of the sample without spiking) / peak area of the neat matrix] * 100. The overview of the ME is summarized in Table 1.

For the optimization of methods, standard solutions and real DBS samples were used. The analytical parameters of the method for the determination of selected AA are listed in Table 2 and for the determination of UA, ALA, and phenylalanine and tyrosine are in Table 3.

Table 1 Matrix effects for the analyzed analytes.

AA	ME (%)	AA	ME (%)	AA	ME (%)
Ala	84-96	**Leu**	69-83	**Gly**	82-91
2-AM	73-83	**Lys**	54-85	**His**	70-79
Arg	67-89	**Met**	57-70	**Ile**	77-99
Asn	73-88	**Phe**	97-101	**Trp**	121-131
Cit	58-72	**Pro**	84-96	**Tyr**	87-95
Glu	57-65	**Ser**	69-77	**Val**	88-102
Gln	76-86	**Thr**	77-84		

		Method using RP	
Analyte	**ME (%)**	**AA**	**ME (%)**
Allantoin	58-64	**Phe**	92-103
Uric acid	82-118	**Tyr**	91-101

ME, matrix effects; RP, reversed phase; AA, amino acid.

Table 2 Analytical parameters of HPLC-MS/MS method using the hydrophilic interaction chromatography mode for the determination of selected amino acids from dried blood spot samples.

	***A* – low conc. CV (%)**	***A* – high conc. CV (%)**	***B* – low conc. CV (%)**	***B* – high conc. CV (%)**	**LOD**	**LOQ**	**Working range of CC (µmol/L)**
					(µmol/L)		
Arg	0.5	2.6	7.7	3.1	0.6	1.9	1.9-273.3
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
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
Glu	3.3	5.5	5.1	8.7	0.6	1.8	1.8-186.0
Gln	5.5	3.1	8.5	7.4	2.4	7.4	7.4-1369.8
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

	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)
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

Intra-assay (**A**, n=10) and inter-assay (**B**, n=6) precision, LOD, LOQ and average parameters of 10 calibration curves for HPLC-MS/MS method for amino acids determination from dried blood spot samples. LOD and LOQ were calculated by the equations: LOD = 3.3 *Sa/b*; LOQ = 10 *Sa/b*, where *b* is slope of the calibration curve and *Sa* is standard deviation of the intercept. CC, calibration curve; conc., concentration; LOD, limit of detection; LOQ, limit od quantification.

Table 3 Analytical parameters of HPLC-MS/MS methods using the reversed phase mode for the determination of allantoin, uric acid, phenylalanine, and tyrosine from dried blood spot samples.

	A – L conc. CV (%)	*A* – M conc. CV (%)	*A* – H conc. CV (%)	*B* – L conc. CV (%)	*B* – M conc. CV (%)	*B* – H conc. CV (%)	LOD (µmol/L)	LOQ (µmol/L)
UA	1.9	1.6	4.2	1.7	2.2	2.6	0.16	0.48
ALA	2.2	1.9	1.7	0.8	1.9	4.0	3.26	9.89
Phe	3.0	2.8	3.1	1.9	1.8	4.9	0.18	0.56
Tyr	1.9	1.5	4.4	5.0	2.7	4.8	0.52	1.57

Intra-assay (**A**, n=10) and inter-assay (**B**, n=6) precision, LOD, LOQ and average parameters of 10 calibration curves for HPLC-MS/MS method for AA determination from DBS samples. LOD and LOQ were calculated by the equations: LOD = 3.3 *Sa/b*; LOQ = 10 *Sa/b*, where *b* is slope of the calibration curve and *Sa* is standard deviation of the intercept. CC, calibration curve; conc., concentration; L, low; M, medium; H, high; LOD, limit of detection; LOQ, limit od quantification.

During the optimization process, the effectiveness of various extraction solvents, times of extraction, and sonication or shaking were tested for the elution of analytes from

the DBS samples. Discs of DBS samples were soaked with different extraction solvents: ACN, methanol, a mixture of ACN and deionized water, a mixture of methanol and deionized water with the addition of formic acid, or without it. For the enhancement of the elution, the different times of sonication in an ultrasound bath and different times of shaking were tested. The best results were obtained using the extraction conditions described in the previous chapter.

The typical DBS sample contains approximately 50 μL of whole blood with an average diameter of 12 mm. The quality of the DBS and the volume of blood applied onto the collection filter paper can have a significant impact on the results. The impact of blood volume on the selected analytes' concentration was evaluated by applying an increasing volume of whole blood onto the collection filter paper. In the case of all analytes except arginine, DBS samples with smaller blood volumes produced lower concentrations, and with the increasing spotted blood volume also increased concentration. The DBS samples with blood volume ranging from 50 to 75 μL provided results with no considerable differences. In order to achieve accurate results, DBS samples and calibrators should contain the same blood volume, and discs for the analysis should be punched from the DBS sample in the same location.

HCT value is considered one of the main factors, which affect the characteristics of a DBS sample (drying time, homogeneity, and extraction of analytes), as HCT affects blood viscosity. Samples with a high value of HCT have a higher blood viscosity, which directly influences the distribution of erythrocytes and serum across the collection filter paper [7]. The effect of a HCT value on the concentration of analytes was tested at different HCT levels (20%, 30%, 40%, 50%, and 60%) without spiking the compounds and by spiking the compounds at different concentration levels. The results showed a low impact of HCT level on measured UA and ALA concentrations using DBS cards. ALA showed a difference of less than 10.5% and UA less than 10% from the concentration measured at the middle HCT level (40%) within the studied range. This implies, that the influence of the HCT value is irrelevant to the quantification of UA and ALA. On the other hand, the HCT value had a relatively significant effect on the concentration of all selected AA. An increasing trend was observed for all AA, in which the concentration of AA increased with the growing value of HCT except for arginine. For some AA, concentrations were up to 30% higher in samples with a HCT value of 60% compared to samples with a HCT value of 30%.

4.2 Determination of selected analytes in dried blood spot and liquid blood samples

Newly developed HPLC-MS/MS methods were used for the measurement of ALA, UA, and AA levels in 100 DBS samples and 100 identical liquid blood samples from voluntary donors. The samples were processed and analyzed according to the protocol described in the experimental section. Data were expressed as median with interquartile range (IQR) and are listed in Table 4. The measured concentrations were in the physiological range for all analytes. In the case of phenylalanine and tyrosine analysis, the levels of phenylalanine and tyrosine were additionally determined in a whole blood

spot with a blood volume of 20 μL along with the concentration in a disc punched from the DBS sample and liquid blood with a volume of 20 μL. Volumetric analysis of the whole DBS sample with a fixed volume of blood was tested as an alternative, which might be able to eliminate the effects of HCT level. Surprisingly, lower concentrations of phenylalanine and tyrosine were found in whole DBS samples compared to liquid blood samples with the same blood volume. In contrast, discs punched from DBS samples showed nearly identical median phenylalanine and tyrosine concentrations as liquid blood samples, see Table 5.

Table 4 The median concentration of amino acids, uric acid, and allantoin in dried blood spot and liquid blood samples.

	Concentration in DBS (IQR) (μmol/L)	**Concentration in liquid blood (IQR) (μmol/L)**
Ala	362.6 (134.6)	382.9 (300.0)
2-AB	19.0 (8.1)	21.2 (10.9)
Arg	76.1 (11.9)	81.6 (8.8)
Asn	66.9 (22.0)	64.3 (36.2)
Cit	32.8 (8.7)	30.4 (11.2)
Glu	125.8 (37.9)	86.5 (25.2)
Gln	455.1 (105.6)	489.2 (279.8)
Gly	282.5 (59.9)	281.8 (68.8)
His	78.1 (22.3)	71.9 (63.6)
Ile	88.5 (18.4)	93.2 (27.8)
Leu	110.2 (29.5)	112.9 (27.1)
Lys	148.6 (40.5)	141.4 (43.3)
Met	26.5 (9.0)	23.6 (23.5)
Phe	68.0 (14.0)	66.5 (15.6)
Pro	163.2 (65.7)	195.3 (168.3)
Ser	111.8 (26.7)	118.0 (27.3)
Thr	101.3 (37.9)	115.4 (80.5)
Trp	38.4 (7.6)	39.1 (16.4)
Tyr	80.7 (20.5)	75.3 (20.0)
Val	194.9 (48.2)	221.9 (122.9)

	Concentration in DBS (IQR) (µmol/L)	Concentration in liquid blood (IQR) (µmol/L)
Uric acid	239.3 (75.7)	220.1 (90.8)
Allantoin	5.6 (1.3)	3.6 (0.9)

DBS, dried blood spot; IQR, interquartile range

Table 5 The median concentration of phenylalanine and tyrosine in dried blood spot and liquid blood samples determined by the method using the reversed phase mode.

AA	Concentration in disc from DBS (IQR) (µmol/L)	Concentration in whole DBS (IQR) (µmol/L)	Concentration in liquid blood (IQR) (µmol/L)
Phe	65.1 (9.4)	57.5 (11.3)	65.6 (14.1)
Tyr	87.4 (17.6)	79.9 (18.6)	88.5 (17.5)

DBS, dried blood spot; IQR, interquartile range

4.3 Comparison of concentrations in dried blood spot and liquid blood samples

The comparison of analytes' concentrations obtained from DBS samples with their concentrations in whole liquid blood was made to verify the correctness of the results. The results measured in whole blood and DBS samples were plotted onto scatter plots to investigate the correlation between derived concentrations. For all selected analytes, the plots showed a linear relationship between concentrations in whole blood and DBS specimens. The regression equations and correlation coefficients are listed in Table 6 and Table 7. To verify the linear relationship, correlation analysis using Spearman Rank Order Correlation was performed. A statistically significant correlation between concentrations in DBS samples and concentrations in liquid blood samples was found for all analytes. The correlation coefficients were above 0.73 for all monitored AA except for arginine, which was the only one that showed a worse correlation with the coefficient value of 0.6811. Additionally, for phenylalanine and tyrosine, correlation analysis showed a better correlation of the concentration measured in the liquid blood samples with the concentration in the disc from DBS samples, than in the case of the concentration measured in the whole DBS samples with the concentration in the liquid blood samples (Table 7).

Table 6 Summary of the correlation coefficients for the dependence of the amino acids, allantoin, and uric acid concentrations in dried blood spot samples on their concentrations in liquid blood samples.

AA	Regression equation	Correlation coefficient	Spearman correlation coefficient
Trp	$y = 1.4683x - 15.7897$	0.8622	0.7304
Phe	$y = 0.9267x + 5.7299$	0.9085	0.8270
Ile	$y = 1.0474x + 0.5499$	0.9263	0.8779
Leu	$y = 0.9305x + 10.545$	0.9574	0.9165
Met	$y = 2.0286x - 25.1409$	0.9109	0.8353
Val	$y = 1.4789x - 58.5798$	0.8389	0.7683
Tyr	$y = 0.8854x + 5.0707$	0.9255	0.8785
Pro	$y = 1.5837x - 43.4844$	0.8958	0.8908
2-AB	$y = 1.1163x - 0.4766$	0.8801	0.8309
Ala	$y = 1.5999x - 163.1246$	0.8961	0.8943
Thr	$y = 1.6341x - 48.8639$	0.9127	0.8947
Gly	$y = 0.9286x + 15.9173$	0.8638	0.8277
Arg	$y = 0.8228x + 17.5632$	0.8444	0.6811
Glu	$y = 0.5438x + 16.4382$	0.8536	0.8176
Ser	$y = 0.9902x + 9.4328$	0.8926	0.8557
Gln	$y = 1.9467x - 381.7732$	0.8903	0.8426
Asn	$y = 1.5229x - 37.4201$	0.9260	0.9076
Cit	$y = 0.8718x + 1.4713$	0.8694	0.7814
His	$y = 2.4401x - 112.8474$	0.9068	0.8951
Lys	$y = 0.9927x - 2.3106$	0.8406	0.7476
Uric acid	$y = 1.1248x - 42.3962$	0.9300	0.9093
Allantoin	$y = 0.555x - 0.5268$	0.9276	0.9016

AA, amino acid

Table 7 Summary of the correlation coefficients for the dependence of the phenylalanine and tyrosine concentrations in dried blood spot samples on their concentrations in liquid blood samples measured by the method using the reversed phase mode.

AA	Dependence	Regression equation	Correlation coefficient	Spearman correlation coefficient
Phenylalanine	DBS disc vs LB	$y = 1.0376x - 1.5476$	0.9235	0.8947
	Whole DBS vs LB	$y = 1.0229x + 7.1515$	0.9223	0.8894
	DBS disc vs whole DBS	$y = 0.9309x - 2.9933$	0.9190	0.8928
Tyrosine	DBS disc vs LB	$y = 0.9833x + 2.9390$	0.9314	0.9069
	Whole DBS vs LB	$y = 0.8309x + 22.6503$	0.9191	0.8749
	DBS disc vs whole DBS	$y = 1.0501x - 11.9679$	0.8992	0.8624

LB, liquid blood; AA, amino acid; DBS, dried blood spot.

The results of the phenylalanine and tyrosine concentrations obtained by the method using RP mode were compared with the results obtained by the method using the stationary phase HILIC. The comparison was made to verify results and to determine whether the method for the determination of phenylalanine and tyrosine using the RP mode can serve as a faster alternative to the method using HILIC mode. The scatter plots showed a statistically significant linear correlation between the phenylalanine and tyrosine concentrations obtained by the method using HILIC mode and their concentrations obtained by the method using RP mode for DBS samples, as well as for liquid blood samples. To verify the linear relationship, correlation analysis using Spearman Rank Order Correlation was performed. An overview of the correlation coefficients is listed in Table 8.

Table 8 Summary of the correlation coefficients of the dependence of the concentration measured by the method using the stationary phase HILIC on the concentration measured by the method using the reversed phase mode.

	Phenylalanine		Tyrosine	
	DBS	Liquid Blood	DBS	Liquid blood
Correlation coefficient	0.9244	0.9161	0.9452	0.9096
Spearman correlation coefficient	0.8644	0.8725	0.9152	0.8738

DBS, dried blood spot

5 Conclusion

In the presented thesis, three HPLC-MS/MS methods were developed and validated for the determination of selected 20 AA, ALA, and UA from the DBS samples. One-step extraction by organic solvent was used to extract analytes from DBS samples. For increasing extraction efficiency, sonication or shaking of samples was performed. The presented methods are sufficiently sensitive and selective for determining the concentration of given analytes in DBS samples, which are characterized by a small volume and good analyte stability. The methods underwent an optimization process according to generally accepted standards and all analytical parameters were satisfactory. Results showed that the volume of blood applied onto the collection cards influence the concentration of all monitored analytes. In order to obtain correct results, the same volume of blood, approximately 50 μL, should be applied onto the collection cards if possible. Furthermore, it was found that the value of HCT has a relatively significant effect on AA levels in DBS samples. This problem can be eliminated by preparing standards in blood with a HCT value that is expected in the monitored population, or by using new collection devices with a fixed volume of blood applied onto the collection cards. On the other hand, the effect of a HCT value on the concentration of UA and ALA was negligible. The applicability of the methods was tested on 100 DBS samples from voluntary donors. The obtained concentrations were in the physiological range for all determined analytes. Additionally, a comparison of concentrations of analytes in DBS samples with their concentrations in identical liquid blood samples showed a linear relationship and a statistically significant correlation.

From the number of benefits of the DBS technique and the presented results, it follows that sample collection by DBS appears to be a suitable alternative to the classic collection of venous blood by a puncture. Finally, DBS samples also have the potential to be implemented in metabolomic studies with a large cohort of patients, as well as to have the potential to be used for screening metabolic disorders and treatment monitoring.

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7 List of published works

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