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ORIGINAL ARTICLE

Second-tier test for quantification of underivatized amino acids in dry blood spot for metabolic diseases in newborn screening

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Abstract The quantitative analysis of amino acids (AAs) in single dry blood spot (DBS) samples is an important issue for metabolic diseases as a second-tier test in newborn screening. An analytical method for quantifying underivatized AAs in DBS was developed by using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). The sample preparation in this method is simple and ion-pairing agent is not used in the mobile phase that could avoid ion suppression, which happens in mass spectrometry and avoids damage to the column. Through chromatographic separation, some isomeric compounds could be identified and quantified, which cannot be solved through only appropriate multiple reactions monitoring transitions by MS/MS. The concentrations of the different AAs were determined using non-deuterated internal standard. All calibration curves showed excellent linearity within test ranges. For most of the amino acids the accuracy of extraction recovery was between 85.3 and 115 %, and the precision of relative standard deviation was $<$7.0 %. The 35 AAs could be identified in DBS specimens by the developed LC–MS/MS method in 17–19 min, and eventually 24 AAs in DBS were quantified. The results of the present study prove that this method as a second-tier test in newborn screening for metabolic diseases could be performed by the quantification of free AAs in DBS using the LC–MS/MS method. The assay has advantages of high sensitive, specific, and inexpensive merits because non-deuterated internal standard and acetic acid instead of ion-pairing agent in mobile phase are used in this protocol.

Keywords Newborn screening · Dry blood spot · Underivatized amino acids · LC–MS/MS

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Introduction

Inborn errors of metabolism (IEM) or inherited metabolic diseases (IMD) are always autosomal recessive hereditary disorders. More than 500 disorders have been found that date back to early twentieth century since Garrod put forward the IEM concept (Garrod 1908). For a number of inborn disorders, the irreversible injury could be avoided by proper treatment and early diagnosis. Therefore, the faster and simple analytical method is very important in the newborn screening (NBS).

The used DBS was obtained from finger or heel pricks and spotted onto filter paper. The analysis of human blood dates back to 1963 for nearly 50 years when Dr. Robert Guthrie used the DBS specimens for the analysis of phenylalanine to identify newborns that suffered from phenylketonuria (Guthrie and Suzi 1963). This novel approach of blood collection brought about the population screening of newborns and other clinical testing (Parker and Cubit 1999; Wilcken and Wiley 2008). Direct analysis of DBS for NBS could significantly improve the efficiencies of screening laboratories, allowing for decreased

sample handling. In addition, it could let material costs down, and faster analysis. On the whole, the DBS offers more advantages than conventional whole blood, plasma or serum sample collection (Mei et al. 2001; Edelbroek et al. 2009)

Determination of AAs from single dry blood spot samples is important for treatable congenital disorders in newborn screening. The widely used analysis method of amino acids was with butyl-esterification followed by flow injection MS/MS analysis (Chace et al. 1993, 1995, 2003; Zytkovicz et al. 2001; Chace and Kalas 2005; Dietzen and Weindel 2010). Although this NBS method for amino acids in DBS has been proved to be acceptable for identification of several metabolism disorders, there have been reports about the lack of its ability to differentiate isobaric biomarkers since no chromatographic separation in this method and inability to differentially identify certain disorders (Chace et al. 2003). Moreover, the quantitative results of amino acids may be unstable because the artificial error of the samples derivative process for DBS. There are also a few reports of amino acids with underivatization followed by flow injection MS/MS analysis (De Jesus et al. 2010; Wang et al. 2011). However, the analytes are limited by the nonspecific ion transition. For example, Leu and Ile have the same ion transition $132 > 86$ and cannot differentiate by MS/MS method. Therefore, the use of chromatograph column is necessary to differentiate the isobaric biomarkers for NBS.

Since AAs lack large hydrophobic side chains, mobile phase modifiers were always used in order to improve the separation of AAs efficiently using reversed-phase LC. In addition, the modifier should be volatile to avoid a compromise of the ionization in the ion source of mass spectrometry. To overcome these shortcomings, the volatile ion-pairing reagents was usually used to enable the separation of free AAs with a reversed-phase HPLC and MS/MS to enable the detection of underivatized AAs (Qu et al. 2002; Piraud et al. 2003, 2005a, b; Zoppa et al. 2006; Waterval et al. 2009). However, the involved ion-pair reagent in the mobile phase allows omitting the derivatization step. However, the ion-pair reagent may eventually cause ion suppression in the mass spectrometry and may shorten the lifetime of the column by using it with the high proportion of aqueous phase in LC separation.

In our study, an analysis method of DBS was developed by using a C18 reverse chromatographic separation coupled with MS/MS that is capable of identifying 35 AAs in a 17–19 min total analysis time. This chromatographic separation eliminates the need for derivatization of specimens to separate these isomers.

Experimental

Materials

The amino acid standards A9906 were obtained from Sigma-Aldrich (St. Louis, MO, USA) which containing β-alanine (β-Ala), α-alanine (α-Ala), L-α-aminoadipic acid (Aad), L-α-amino-n-butryic acid (Abu), γ-amino-*n*-butyric acid (GABA), D,L-β-aminoisobutryic acid (β-AIB), L-arginine (Arg), L-aspartic acid (Asp), L-citrulline (Cit), creatinine (Cre), L-cystathionine (Hcy(Ala)), L-cystine ($(Cys)_2$), L-glutamic acid (Glu), glycine (Gly), L-histidine (His), 3-methyl-histidine (His-3Me), L-homocystine ($(Hcy)_2$), δ-hydroxylysine (Hyl), hydroxyl-L-proline (Hyp), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-ornithine (Orn), L-phenylalanine (Phe), L-proline (Pro), L-sarcosine (Sar), L-serine (Ser), taurine (Tau), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr) and L-valine (Val). The other standards of homo-cysteine (Hcy), L-cysteine (Cys), L-glutamine (Gln) and *N,N*-dimethylphenylalanine (*N,N*-Phe) were obtained from Sigma-Aldrich (St. Louis, MO, USA), too. HPLC grade formic acid and acetic acid were obtained from Tedia (USA). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ). Water was obtained from a Milli-Q Ultra pure Water Purification Systems (Millipore, Brussels, Belgium). Amino acids calibrations and spike standards were prepared from pure powder or commercially available standards. They were stored at −20 °C before use. Calibration curves were established through spiked with blood AAs (with matrix) for the assay of DBS samples.

Sample preparation procedure

Blood spot samples were collected from newborns tested by the local Newborn Screening Program. Samples from a group of healthy newborns 3 days old ($n = 10$) and a group of phenylketonuria (PKU) newborns 3 days old and 30 days old ($n = 5$) were collected on to S&S903 specimen collection paper and allowed to dry for at least 24 h before the measurements of AAs concentrations. Blood spots were immediately used or stored at 4 °C. Samples were prepared for analysis that have been previously reported (Wang et al. 2011). The different was the supernatant acquired from the last step not used to analysis directly. It was transferred to a new 0.5 mL Eppendorf tube and was evaporated by nitrogen, and then dissolved in 50 μL H_2O 0.12 % acetic acid before analysis. An explanation of the study was given to the parents of the patients and a standard informed consent, which was reviewed and approved by the Jilin department of health involving

human medical ethical committee, was obtained from all subjects.

Chromatograph and instrumentation

LC analysis was performed using Acquity-Ultra Performance LC system (Waters, USA) equipped with an autosampler. The column used for separation in LC–MS/MS analysis was a Venusil ASB C_{18} HPLC column (4.6 mm × 250 mm, 5 μm particle size, 150 and 300 Å pore size) from Agela Technologies.

Tandem mass spectrometry conditions

Mass spectrometry experiments and optimization of the method were performed using a Xevo TQ MS spectrometer with electrospray ion source (ESI) (Waters, Manchester, UK). The mass spectrometer was used in MRM under positive ion mode. To ensure the sensitivity of detection, we divided the analytes into three groups according to their retention times, and only one group of analyte was monitored simultaneously during each running period (Table 1 was for detail). The source voltage was 3 kV, the source temperature and desolvation temperature were 150 and 350 °C, respectively. The gas flow desolvation was 800 L/h, and the collision gas argon was kept at a pressure of 1.7×10^{-3} mbar.

Calibration curves

Amino acids standard solutions were added to a whole blood sample at levels 0.50 to 200.00 μmol/L. Then they were spotted on to the filter paper and were dried for about 24 h before analysis. Dried blood spots were prepared for analysis that have been previously reported (Wang et al. 2011). Moreover, each set of calibration curve for AAs in DBS was prepared in water/acetic acid (100:0.12, v/v) at the last step of sample preparation procedure before analysis. The concentration of *N*,*N*-Phe as internal standard for the calibration was 5 μmol/L. The calibration curves were prepared by plotting analyte/internal standard extracting ion current (XIC) peak area ratios versus concentrations of each analyte. Determination of AAs in DBS samples was performed using weighted least-squares regression analysis of the standard curves.

Method validation

The extract recoveries and accuracies of AAs from blood spots were determined by standard additions of AAs aqueous solutions to a whole blood sample at three levels (150, 750, and 1,500 μmol/L) for AAs. They were carefully pipetted onto filter paper and dried overnight. Then cut a diameter = 3.2 mm discs from DBS and ≈3.4 μL whole blood (Chace et al. 2003) for analysis. Precision of the assay was calculated by repeat analysis of the same blood sample and was estimated as the coefficient of variation (CV %) of the replicate measurements, both intra-day and inter-day.

Data processing

Data were acquired with MassLynx4.0 and processed for calibration and for quantification of the analytes with TargetLynx software (Micromass UK). Data statistics were used by the SIMCA-P 11.5 version software and SPSS 18.0 version software.

Results

MS/MS analysis optimization

Special conditions of MS/MS of each analyte are important for the development of a satisfactory quantification method by LC–MS/MS. Therefore, the intellistart function was firstly used in our study to find the most specific and sensitive detection parameters of each amino acid in MRM mode. Every amino acid solution (the concentrations of each analyte were 50 μmol/L) were directly injected into the mass spectrometer at a rate of 20 μL/min by a syringe pump. The tandem mass spectrometry conditions were as follows: lowest fragment ion mass was 40.00 Da, cone voltage ranges were 10–50 V, collision energy ranges were 0–40 eV, number of daughter transitions were 2, MS tune method was described as the "Tandem mass spectrometry conditions" section in Experimental. The most abundant product ion of each analyte has been chosen in order to obtain specific ion transitions for following the AAs in MRM under positive ion mode as described in Table 1. Among these specific ion transitions, most AAs lost neutral fragment of a formic acid and have a product at $[M+H-46]^+$ by a rearrangement. The transitions and corresponding optimal cone voltage and collision energies chosen for MRM of AAs are listed in Table 1.

Study of chromatographic condition

Even with special conditions of MS/MS in the study, the analytes separated by LC is evidently needed to diminish or eliminate the interferences from DBS sample matrix. For example, the interferences between Leu and Ile, α-Ala and Sar, etc. can be eliminated through their LC retention time. Therefore, the next step was to analyze the AAs in the LC–MS/MS method with the C_{18} column applying different mobile phase compositions to obtain the best condition

Table 1 Ion transitions and instrumental parameters for their LC–MS/MS quantification in MRM mode use acetonitrile as organic phase for the amino acids and internal standard and the retention times

AA + IS	MRM	CV	CE	Monitor period	RT	LOQ
Gly	76.00 > 30.00	20	18	0–8.5	5.82	36
β-Ala	90.00 > 72.00	15	10	0–8.5	5.07	21
α-Ala	90.00 > 44.00	18	18	0–8.5	5.91	3.5
Sar	90.00 > 44.00	18	18	0–8.5	6.97	4.2
GABA	104.02 > 86.05	15	10	0–8.5	5.07	1.2
	104.02 > 87.00	15	10	0–8.5		
β-AIB	104.02 > 86.05	15	10	0–8.5	5.71	3.0
	104.08 > 57.00	15	12	0–8.5		
Abu	104.08 > 58.09	15	18	0–8.5	6.31	2.1
	104.02 > 86.05	15	10	0–8.5		
Ser	106.16 > 60.06	18	16	0–8.5	5.87	1.8
	106.16 > 88.0	18	12	0–8.5		
Cre	114.09 > 86.01	20	15	0–8.5	5.53	3.0
	114.09 > 44.18	20	18	0–8.5		
Pro	116.18 > 70.02	20	15	0–8.5	7.02	1.5
Val	118.13 > 72.06	15	10	0–8.5	7.60	0.9
	118.13 > 55.00	15	15	0–8.5		
Thr	120.09 > 74.00	15	15	0–8.5	6.05	1.2
	120.09 > 120.00	15	11	0–8.5		
Cys	121.99 > 58.93	10	20	0–8.5	6.35	2.4
	121.99 > 104.98	10	10	0–8.5		
Tau	126.06 > 108.04	15	15	0–8.5	6.02	12
	126.06 > 44.00	15	25	0–8.5		
Hyp	132.00 > 67.94	15	14	0–8.5	6.24	1.5
	132.00 > 86.00	15	10	0–8.5		
Orn	133.02 > 70.08	20	15	0–8.5	4.56	3.9
	133.02 > 116.00	20	10	0–8.5		
Asp	134.09 > 88.06	30	20	0–8.5	5.79	1.2
	134.09 > 116.00	20	11	0–8.5		
Hcy	136.00 > 90.00	15	12	0–8.5	7.31	1.5
	136.00 > 118.00	15	9	0–8.5		
Lys	147.02 > 84.09	20	14	0–8.5	4.73	2.4
	147.02 > 129.01	20	10	0–8.5		
Gln	147.02 > 84.09	20	15	0–8.5	6.12	2.1
	147.02 > 130.01	20	10	0–8.5		
Glu	148.03 > 84.00	16	15	0–8.5	6.72	2.1
	148.03 > 130.02	16	10	0–8.5		
His	155.97 > 110.07	20	15	0–8.5	4.66	1.2
	155.97 > 93.00	20	22	0–8.5		
Aad	162.09 > 98.08	15	18	0–8.5	7.19	1.5
	162.09 > 116.0	15	15	0–8.5		
Hyl	163.01 > 128.08	15	14	0–8.5	4.55	2.4
	163.01 > 145.00	15	10	0–8.5		
His-3Me	170.03 > 124.07	22	15	0–8.5	4.96	1.2
	170.03 > 81.00	22	25	0–8.5		
Arg	175.00 > 70.02	18	20	0–8.5	4.90	0.9
	175.00 > 158.00	18	10	0–8.5		
Cit	176.10 > 159.03	15	13	0–8.5	6.44	1.8

Table 1 continued

AA + IS	MRM	CV	CE	Monitor period	RT	LOQ
	176.10 > 113.07	15	18	0–8.5		
Hcy (Ala)	223.16 > 134.01	20	15	0–8.5	5.80	2.4
	223.16 > 117.0	20	18	0–8.5		
$(Cys)_2$	241.03 > 73.99	20	20	0–8.5	5.83	2.7
	241.03 > 152.01	20	15	0–8.5		
$(Hcy)_2$	269.08 > 136.04	15	12	0–8.5	7.63	3.0
	269.08 > 90.01	15	20	0–8.5		
Ile	132.05 > 86.00	15	10	8.5–13.5	10.14	9.0
	132.05 > 69.02	15	12	8.5–13.5		
Leu	132.05 > 86.00	15	10	8.5–13.5	10.76	8.1
	132.05 > 44.01	15	20	8.5–13.5		
Met	150.23 > 104.08	16	15	8.5–13.5	9.37	1.2
	150.23 > 133.10	16	10	8.5–13.5		
Tyr	182.02 > 136.01	18	16	8.5–13.5	12.83	2.1
	182.02 > 165.02	18	12	8.5–13.5		
Phe	166.00 > 120.00	18	15	13.5–16.0	15.01	0.6
	166.00 > 103.02	18	18	13.5–16.0		
Trp	205.00 > 146.03	30	18	13.5–16.0	15.69	1.5
	205.00 > 188.01	30	15	13.5–16.0		
N,N-Phe	194.08 > 148.00	20	18	13.5–16.0	15.68	0.6
	194.08 > 133.00	20	22	13.5–16.0		

AA amino acids, *IS* internal standard, *CE* collision energy (eV), *CV* cone voltage (V), *MRM* specific mass transition, *RT* retention time (min), *LOD* limit of quantification (p mol on column)

both the separation of each analyte and the analysis time. We found that acetic acid as the modifier added to methanol–water gradient effluent was optimal for above purpose. The mobile phase consisted of two kinds of solvents: water containing 0.12 % acetic acid (solvent A, pH = 2.5) and 33 % methanol containing 0.9 % acetic acid aqueous (solvent B, pH = 3.0) at a flow rate 0.5 mL/min. The optimized elution program was as follows: from 0 to 2.5 min a constant 0 % B, from 2.5 to 5.0 min a linear gradient from 0 % B to 17 % B, from 5.0 to 8.0 min a linear gradient from 17 % B to 33 % B, from 8.0 to 8.5 min gradient from 33 % B to 100 %. The column was held at 100 % B from 8.5 to 19 min. Then returned to 0 % B from 19 to 20 min, and allowed to equilibrate for 15.0 min. Run-to-run time was 35 min. The injected volume was 3 μL.

In the present study, we also used the acetonitrile instead of methanol as an organic phase. The mobile phase consisted of two kinds of solvents: water containing 0.12 % acetic acid (solvent A, pH 2.5) and 33 % acetonitrile containing 0.9 % acetic acid aqueous (solvent B, pH 2.8) at a flow rate of 0.5 mL/min. The optimized elution program was as follows: from 0 to 2.5 min a constant 0 % B, from 2.5 to 5.0 min a linear gradient from 0% B to 17 % B, from 5.0 to 8.0 min a linear gradient from 17 % B to 33 % B, from 8.0 to 8.5 min gradient from 33 % B to 100 %. The column was held at 100 % B from 8.5 to 17 min. Then returned to 0 % B from 17 to 18 min, and allowed to equilibrate for 12.0 min. Run-to-run time was 30 min. The injected volume was 3 μL. The advantage of acetonitrile as solvent B of mobile phase was found that it could improve the peak shapes of some AAs and could short the total time of analysis. However, there was also the disadvantage for using acetonitrile that the Trp and *N,N*-Phe could not be separated in the chromatographic column. The details were shown in Fig. 1a, b). Comparing with methanol or acetonitrile as organic solvents in LC separations was considered. The advantage of use methanol as organic phase was that the Trp and internal standard *N,N*-Phe could be separated by liquid chromatography. Though the Trp and internal standard *N,N*-Phe could not be separated by use acetonitrile as organic phase in liquid chromatography, it could short the total analysis time for 5 min and could improve the peak shapes of AAs. Therefore, acetonitrile was selected as the organic mobile phase to analyze the concentration of AAs in DBS samples.

Because the column in our study could be applied to the solution condition of pH 1.0–7.0 and could be applied to the mobile phase of 100 % water. Therefore, the liquid

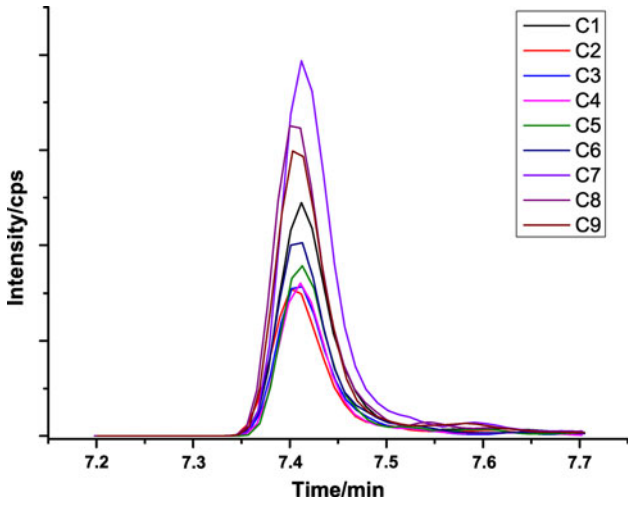

Fig. 1 Val retention time reproducibility. Overlay of Val extracted ion chromatograms (m/z 118.13 > 72.06) of DBS samples from nine different healthy newborns, showing sample-to-sample retention time reproducibility

chromatography condition was used with 0.12 % acetic acid in water to improve the separation. The favorable condition conduced the effective separation among the polar amino acids in reversed-phase chromatography column. The gradient conditions were optimized through separating degree of analytes and the total time of analysis. Under the optimized LC conditions, a satisfied LC–MS/MS chromatographic separation was achieved in the assay of the DBS sample extract (listed in Table 1). The time of equilibration was 12.0–15.0 min. The reproducibility performed perfectly for parallel sample analysis by the equipment of Acquity-Ultra Performance LC system. For example, the extracted ion chromatograms for Val in a DBS sample from nine different healthy newborns were compared in Fig. 1. The mass window for each sample was m/z 118.13 > 72.07 and the results showed good sample-to-sample retention time reproducibility.

The advantage of LC–MS/MS method was that the amino acid isomers could be separated effectively. For example, Leu and Ile, Gln and Lys, GABA and β-AIB, they had the same $[M + H]^+$ ions and the same ion transition. They could not be identity only by MS/MS without LC separated. Using LC–MS/MS method Hcy and $(Hcy)_2$ can also be separated to avoid the false positive result since the same ion transition 136 > 90 of $(Hcy)_2$ and Hcy. The diseases such as homocystinuria and hyperhomocysteinaemia can be identified through the qualitative and quantitative analysis of Hcy and $(Hcy)_2$ from DBS; the maple syrup urine disease can be identified through the qualitative and quantitative analysis of Val, Leu and Ile; the tyrosinemia type I disease can be identified through the qualitative and quantitative analysis of Tyr, Met and Phe; the phenylketonuria disease can be also identified through the qualitative and quantitative analysis of Tyr and Phe. The details are shown in Fig. 2a, b).

Calibration, linearity and limits of quantification

The linearity of the method was obtained by using six concentration levels of a standard mixture of AAs, supplemented with 5 μmol/L *N*,*N*-Phe as internal standard. The calibration curves were plots of AAs to internal standard peak area ratios versus their corresponding concentration. All AAs signals were recorded in XIC mode. The concentrations chosen for calibration plots were in the range 0.5–200 μmol/L for each AAs, whereas *N*,*N*-Phe concentrations were kept constant at 5 μmol/L. In both cases, regressions were found to be linear all over the concentration range studied, with correlation coefficients higher than 0.9900. The limits of quantification for each AAs at a signal to noise ratio (S/N) of 10 is shown in Table 1.

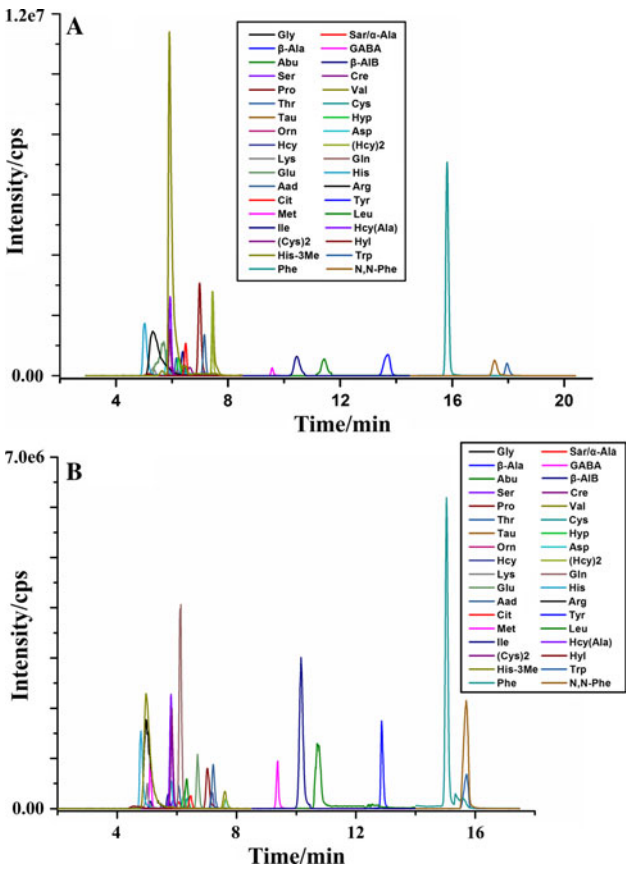

Fig. 2 The chromatograms of LC–MS/MS for AAs from DBS sample with added AAs Standard: 30 AAs were monitored simultaneously for the first 8 min, 4AAs for the following 6 min, and 2 AAs and the internal standard for the last 6 min. **a** The mobile phase was methanol. **b** The mobile phase was acetonitrile

Table 2 Precision, accuracy and recovery of the underivatized amino acids MS/MS assay in blood spots samples (μmol/L, mean ± SD)

AAs offset to whole blood	150 μmol/L (AA)			750 μmol/L (AA)			1,500 μmol/L (AA)		
	Mean ± SD (recovery %)	Intra-day SD (CV)	Inter-day SD (CV)	Mean ± SD (recovery %)	Intra-day SD (CV)	Inter-day SD (CV)	Mean ± SD (recovery %)	Intra-day SD (CV)	Inter-day SD (CV)
Gly	159.0 (106.0)	9.1 (4.7)	11.7 (5.3)	775.0 (103.3)	84.5 (6.9)	96.7 (2.5)	1514.8 (101.0)	65.3 (4.3)	29.3 (2.0)
β-ALA	150.3 (100.2)	15.3 (5.2)	20.0 (6.3)	733.8 (97.8)	11.1 (1.5)	51.1 (7.0)	1482.5 (98.8)	44.6 (3.0)	60.2 (4.1)
α-Ala	147.5 (98.3)	9.0 (6.1)	21.8 (6.8)	712.7 (95.0)	39.1 (5.5)	56.3 (5.9)	1469.3 (97.9)	28.7 (2.0)	66.8 (4.5)
Sar	107.5 (71.7)	7.5 (4.0)	10.6 (6.9)	617.0 (82.2)	59.5 (6.6)	34.8 (5.6)	1279.0 (85.3)	121.8 (4.5)	88.5 (3.9)
GABA	171.8 (114.5)	7.4 (4.3)	10.2 (5.9)	785.0 (104.7)	33.8 (4.3)	57.0 (5.3)	1487.3 (99.2)	32.1 (2.2)	46.4 (3.1)
β-AIB	150.3 (100.2)	4.8 (3.2)	9.4 (6.2)	766.3 (102.2)	81.1 (3.6)	52.8 (6.9)	1527.5 (101.8)	33.2 (2.2)	44.4 (2.9)
Abu	170.3 (113.5)	3.5 (2.0)	7.1 (4.2)	847.3 (112.9)	79.7 (4.4)	69.8 (6.2)	1543.3 (102.3)	29.3 (1.9)	60.8 (3.9)
Ser	169.8 (113.2)	9.5 (5.6)	16.9 (6.0)	805.0 (107.3)	49.7 (6.2)	45.9 (5.7)	1463.3 (97.6)	31.9 (2.2)	76.9 (5.3)
Cre	169.5 (113.0)	13.9 (4.2)	4.6 (2.7)	821.3 (109.5)	36.9 (4.5)	21.3 (2.6)	1455.3 (97.0)	14.6 (1.0)	41.3 (2.8)
Pro	171.3 (114.2)	4.6 (2.7)	15.2 (8.9)	786.0 (104.8)	83.6 (10.6)	13.1 (1.7)	1470.8 (98.1)	10.2 (0.7)	17.6 (1.2)
Val	166.0 (110.7)	2.6 (1.6)	18.6 (11.2)	797.5 (106.3)	80.2 (3.1)	36.1 (4.5)	1486.0 (99.1)	26.2 (1.8)	61.6 (4.1)
Thr	152.0 (101.3)	10.6 (7.0)	6.5 (4.3)	753.3 (100.4)	62.7 (2.3)	6.8 (0.9)	1497.5 (99.8)	17.9 (1.2)	61.8 (4.1)
Cys	151.8 (101.2)	8.3 (5.4)	17.7 (6.7)	716.3 (95.5)	37.7 (5.3)	70.5 (6.8)	1456.8 (97.1)	42.0 (2.9)	87.9 (6.0)
Tau	172.3 (114.8)	3.1 (1.8)	9.0 (5.2)	708.3 (94.4)	19.5 (2.8)	20.1 (2.8)	1483.8 (98.9)	43.3 (2.9)	78.9 (5.3)
Hyp	172.5 (115.0)	4.3 (2.5)	14.2 (6.2)	779.8 (104.0)	18.8 (2.4)	57.6 (5.4)	1451.0 (96.7)	31.7 (2.2)	70.7 (4.9)
Orn	108.0 (72)	11.5 (5.6)	14.2 (7.1)	535.0 (71.3)	15.0 (2.1)	39.4 (5.5)	1153.0 (76.9)	31.2 (2.1)	42.7 (2.9)
Asp	143.8 (95.6)	12.1 (5.4)	9.8 (6.8)	725.0 (96.7)	60.8 (2.4)	27.0 (3.7)	1462.8 (97.5)	56.6 (3.9)	68.3 (4.7)
Hcy	117.8 (78.5)	8.5 (4.2)	3.5 (2.9)	732.0 (97.6)	18.8 (2.6)	30.6 (4.2)	1447.3 (96.5)	52.2 (3.6)	71.6 (5.0)
Lys	168.3 (112.2)	7.7 (4.6)	18.6 (6.1)	839.3 (111.9)	31.8 (3.8)	92.2 (5.0)	1548.3 (103.2)	31.2 (2.0)	82.5 (5.3)
Gln	155.0 (103.3)	6.8 (4.4)	23.6 (15.2)	729.8 (97.3)	21.0 (2.9)	74.3 (4.2)	1487.0 (99.1)	62.4 (4.2)	95.3 (6.4)
Glu	170.3 (113.5)	6.5 (3.8)	11.3 (6.7)	852.0 (113.6)	47.7 (5.6)	29.5 (3.5)	1499.5 (99.9)	25.8 (1.3)	27.0 (1.8)
His	171.3 (114.2)	6.9 (4.0)	6.8 (3.9)	792.0 (105.6)	18.1 (2.3)	76.8 (4.7)	1452.3 (96.8)	19.5 (1.3)	51.6 (3.6)
Aad	125.5 (83.7)	10.6 (8.5)	15.0 (12.0)	726.0 (96.8)	37.2 (5.1)	23.1 (3.2)	1542.8 (102.9)	49.4 (3.2)	35.5 (2.3)
Hyl	153.8 (102.5)	9.0 (5.9)	15.8 (7.3)	724.0 (96.5)	6.5 (0.9)	15.0 (2.1)	1448.8 (96.6)	23.5 (1.6)	57.5 (4.0)
Arg	164.5 (109.7)	8.5 (5.2)	15.8 (6.6)	755.8 (100.8)	60.4 (2.0)	35.3 (4.7)	1499.2 (99.9)	56.6 (3.8)	53.3 (3.6)
Cit	166.3 (110.8)	4.3 (2.6)	10.5 (6.3)	783.8 (104.5)	23.6 (3.0)	89.7 (4.4)	1541.0 (102.7)	33.8 (2.2)	87.6 (5.7)
Hcy(Ala)	145.3 (96.7)	6.2 (4.3)	6.5 (4.5)	740.0 (98.7)	51.9 (2.0)	28.6 (3.9)	1492.3 (99.5)	43.3 (2.9)	96.6 (6.5)
$(Cys)_2$	171.8 (114.5)	4.6 (2.7)	11.4 (6.6)	785.8 (104.8)	21.3 (2.7)	22.6 (2.9)	1430.5 (95.4)	28.6 (2.0)	80.5 (5.6)
$(Hcy)_2$	163.8 (109.2)	11.9 (4.3)	15.9 (6.7)	805.5 (107.4)	55.1 (6.8)	44.2 (5.5)	1449.8 (96.7)	57.6 (4.0)	53.4 (3.7)
Ile	143.5 (95.7)	4.0 (2.8)	9.2 (6.4)	725.8 (96.8)	43.4 (4.7)	64.7 (5.9)	1462.5 (97.5)	48.8 (3.3)	61.3 (4.2)
Leu	170.3 (113.5)	5.3 (3.1)	9.5 (5.6)	720.0 (96.0)	67.0 (3.3)	61.1 (4.5)	1488.0 (99.2)	48.8 (3.3)	40.6 (2.7)
Met	155.0 (103.3)	7.7 (5.0)	12.8 (7.2)	776.5 (103.5)	11.3 (1.4)	30.2 (3.9)	1482.7 (98.9)	20.0 (1.3)	40.1 (2.7)
Tyr	142.3 (94.8)	3.1 (2.2)	5.7 (3.9)	750.0 (100.0)	37.5 (5.0)	15.9 (2.1)	1513.1 (100.9)	37.1 (2.4)	52.5 (3.5)
Phe	142.3 (94.8)	4.8 (3.4)	5.7 (4.0)	710.0 (94.7)	37.5 (5.3)	15.7 (2.2)	1570.3 (104.7)	74.7 (4.8)	84.0 (5.3)
Trp	144.0 (96.0)	3.8 (2.6)	6.2 (4.3)	698.3 (93.1)	16.6 (2.4)	31.0 (4.4)	1465.5 (97.7)	36.6 (2.5)	16.7 (1.1)

Intra-day, $n = 3$

Inter-day, $n = 3$

Method validation

The validation studies were determined including intra-day precision and accuracy, and inter-day precision and accuracy. The recoveries of AAs were also determined at three levels. Three separate DBS samples were prepared and analyzed on three different days. Results from the first day were used to calculate the intra-day accuracy and precision. Results from all the 3 days were used to calculate the inter-day precision, accuracy and recovery. The details are shown in Table 2.

In summary, most recoveries were 85.3–115.0 % and relative standard deviations (RSD) were <7.0 %. As a result, the 35 AAs were determined in DBS by this method.

Application to DBS samples

The new above-characterized procedure was applied to the analysis of AAs in DBS from healthy newborns ($n = 10$) and from classic PKU newborns ($n = 5$). The levels of each AAs in the healthy newborn group and PKU newborn group were shown in Figs. 3, 4.

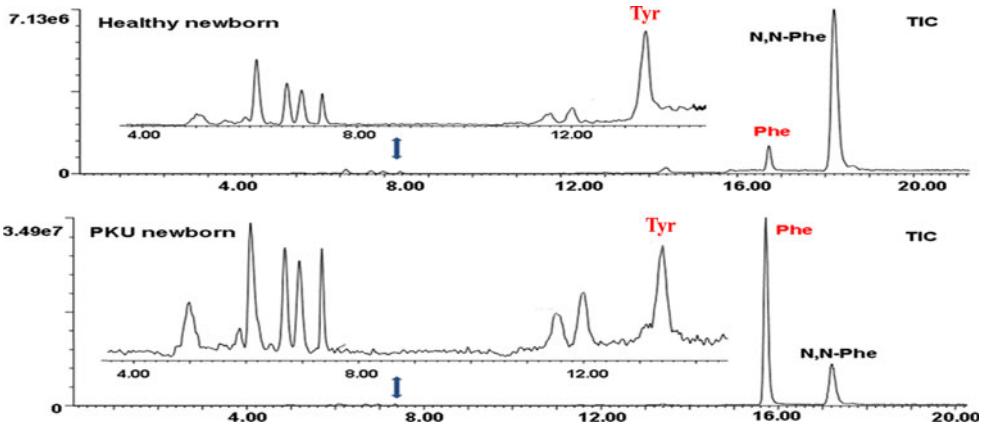

Fig. 3 The LC–MS/MS Chromatograms of AAs from healthy newborn and PKU newborn

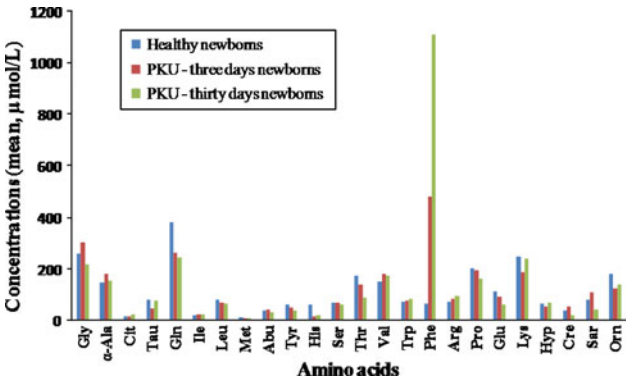

Fig. 4 Concentration of AAs in healthy controls and in PKU patients (3 days newborns and 30 days newborns) by the LC–MS/MS method

As shown from Fig. 3, the concentration of Phe was obviously increased in the DBS of PKU newborn patient. And the ratio of Phe/Tyr was increased accordingly when the concentration of Phe obviously increased and the concentration of Tyr remained constantly unchanged or slightly decreased.

In the present study, for the first time we reassessed PKU patients diagnosed through newborn screening and quantified Phe and other 23 AAs in their DBS with this method. The details are shown in Fig. 4. The other 11 amino acids (GABA, β-AIB, β-Ala, Hcy, Aad, Hyl, Hcy (Ala), $(Cys)_2$, $(Hcy)_2$, Asp and Cys) were not found in DBS samples. The significant differences were found in the concentration of a number of amino acids between classic PKU group and healthy control group. In the PKU 3 days newborns ($n = 5$), the level of Orn, Met, Tau, Gln and His decreased significantly ($P < 0.05$, $P < 0.01$ and $P < 0.001$), the level of Phe increased significantly ($P < 0.01$). And in the PKU-30 days without low diet phenylalanine therapy newborns ($n = 5$) the levels of Gln, His, Glu, Tyr, Cre, Thr and Sar decreased variously ($P < 0.05$, $P < 0.01$ and $P < 0.001$), the levels of Phe, Trp and Cit raised obviously ($P < 0.05$, $P < 0.01$ and $P < 0.001$). In conclusion, the increased level of Phe and reduced levels of Gln and His were appeared in the DBS from PKU newborns who were 3 days old and 30 days old compared with the DBS from healthy control newborns. The concentrations of Tau and Met were decreased in the DBS from PKU 3 days newborns compared with in the DBS from healthy newborns. However, they were normal when the PKU newborns were treated as healthy newborns for 30 days. However, the concentrations of Cit, Tyr, Ser, Thr, Glu, Trp and Cre were no significant differences in the DBS between PKU 3 days newborns and healthy newborns. In addition, except for Trp was increased, they were all decreased when the PKU newborns were treated as healthy newborns for 30 days.

Multivariate profile-wide predictive models were constructed using partial least-squares discriminant analysis (PLS-DA) by SIMAC-P 11.5 version software package. The results can be obtained by score plot and scatter loading plot of the PLS-DA. First, each dot stands for a specimen in the score plot. The dots will cluster obviously when the specimens have the similar concentrations of AAs. Secondly, each dot stands for an amino acid of each specimen in the scatter loading plot. The scatter loading plot can get the information about how the weight of these variables of the contribution in the classification. For example, the dots are far away from the origin of the coordinates, which can indicate that the amino acid plays an important role in classification between healthy newborns and PKU newborns. In our study, the acquired data about quantification of 24 AAs in DBS from healthy newborns and PKU newborns were processed by the PLS-DA statistical method. The R^2X was 0.728, R^2Y was 0.902 (goodness of fit) and Q^2 was 0.746 (goodness of prediction) by the two first principal components (PCs) in healthy newborns group and PKU newborns group. These data indicated that this model is credible with good goodness of fit and good goodness of prediction. The score plot demonstrated obvious differentiation between the PKU 3-days newborns and healthy newborns profiles is shown in Fig. 5a. The healthy newborns were clustered on the right

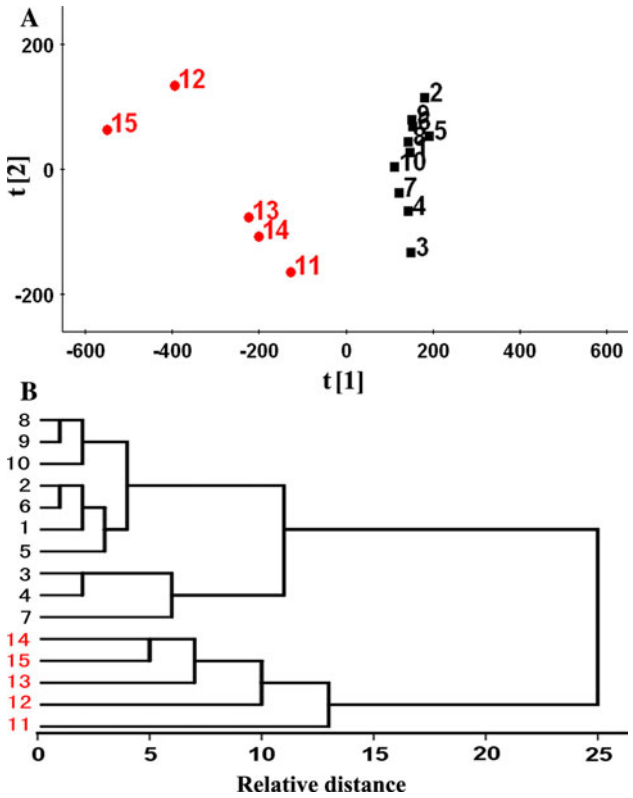

Fig. 5 PLS-DA scores of the two first PCs (t[1] and t[2]) (**a**) and average linkage, Pearson correlation coefficient dendrogram obtained by hierarchical cluster analysis (**b**). 1–10, healthy newborn samples. 11–15, PKU 3 days newborn samples

(No. 1–10) and the PKU 3 days newborns were clustered on the left (No. 11–15). The newborns could be divided into two groups by this method. The loading plot is shown in Fig. 6. There are three amino acids concentrations of Phe, Gln and Lys played remarkable role in the different classified groups because of their variable importance values were all above 1.0. And combined with the *t* test from the concentrations of analytes between classic PKU group and healthy control (Lys, $P > 0.05$; Phe, $P < 0.01$ and Gln, $P < 0.01$). We have, therefore, taken a holistic and data-driven to identify the metabolic signature increased amino acid Phe in PKU patients. Furthermore, we find that the decreased amino acid Gln in DBS can be considered as auxiliary diagnosis of classical PKU.

Hierarchical cluster analysis (HCA) is a statistical method for finding relatively homogeneous clusters of cases based on measured characteristics. This hierarchical clustering process can be represented as a tree, or dendrogram, where each step in the clustering process is illustrated by a join of the tree. HCA was applied use algorithm of average linkage and Pearson correlation coefficient through SPSS 18.0 version software package. The dendrogram generated is shown in Fig. 5b. It showed an adequate separation, forming two groups at a relative distance lower than 13.5. The healthy newborns (No. 1–10) and the PKU 3 days newborns (No. 11–15) were divided into two groups. This result was consistent with the score plot result of PLS-DA (Fig. 5a).

Discussion

The established method has merits as follows: (1) Non-deuterated *N*,*N*-Phe as internal standard can guarantee accurate quantitative results and can decrease the expense of analysis. The price of deuterated internal standard was much higher than the price of Non-deuterated internal standard *N*,*N*-Phe. For example, *N*,*N*-Phe (98 %) was about 50 $ for 5 g from Sigma-Aldrich (St. Louis, MO, USA) and L-Phe (D8, 98 %) was about 416 $ for 1 g from Cambridge Isotopes (Andover, MA, USA). Nearly 40 times difference was in the price of deuterated internal standard Non-deuterated internal standard; (2) The acetic acid instead of ion-pair agent in the mobile phase can improve the separation of AAs in liquid chromatography and can eliminate the damage to the column and the ion source of mass spectrometry caused by the use of ion-pair agent; (3) The analysis time is shorter than the method which use the same length column. In the study of Armstrong et al.

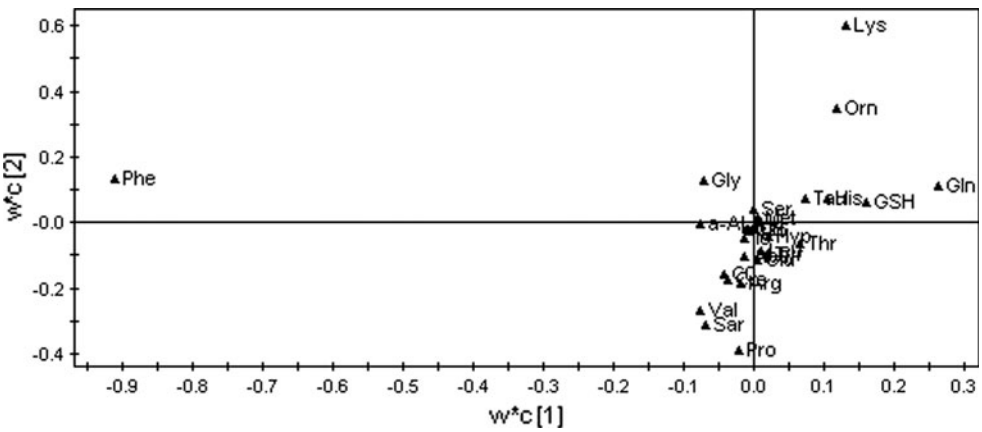

Fig. 6 Loading plot from a PLS-DA model of 24 AAs in the DBS between the PKU 3 days newborns and the healthy newborns

Table 3 Quantification of Phe and Tyr from DBS collected from 5 classical PKU newborns (3 days/30 days) by fluorescence and by the developed LC–MS/MS method (μmol/L)

Sample ID	Phe by fluorescence	Tyr by this method	Phe by this method	Phe/Tyr by this method
3 days				
1	170.0	67.5	297.0	4.4
2	578.2	24.0	576.0	24.0
3	336.4	73.5	393.0	5.3
4	286.7	45.0	360.0	8.0
5	656.9	34.5	765.0	22.2
30 days				
1	158.0	37.5	225.0	6.0
2	1353.0	28.5	1488.0	52.2
3	274.5	42.0	327.0	7.8
4	347.3	43.5	453.0	10.4
5	3083.0	45.0	2823.0	62.7

(2007) and Qu et al. (2002), the ion-pair agent was used in the mobile phase and the short columns were used in their analysis. The length of columns were 50 and 150 mm respectively. But the analysis time of their developed methods were 18 and 36 min. And the analysis time of our developed method was 17 and 19 min by LC–MS/MS with length of 250 mm column. Furthermore, our technique allows for the assessment of Maple Syrup Urine Disease (MSUD) and PKU, etc. by analysis of the dry blood spot from the newborn. This method shows the ability to analyze dry blood spot for the quantification of biomarkers related to metabolism disorders, and an advancement that can greatly improve the efficiencies and can decrease the cost of newborn screening. However, an important qualification was needed in our developed method. That was the column must be applied to the mobile phase of 100 % water and could be applied to the solution condition of pH 1.0–7.0.

In summary, the PLS-DA and HCA statistical analysis described here are preferable to differentiate the healthy newborn from the PKU newborn by the quantification of AAs in DBS samples. These methods also can be considered as auxiliary diagnosis of classical PKU.

Phe and His are essential amino acids and the Gln is conditionally essential amino acid in human being (National Research Council 1998). Neu had reported that enteral glutamine supplementation for very low birth weight infants could decrease the morbidity (Neu et al. 1997). So, we suggest that the therapy of low phenylalanine diet alone for classical PKU should be better replaced by the therapy of low phenylalanine and a certain high amount of His and Gln diet.

Samples numbered 1–5 were collected from newborns diagnosed as affected by PKU using commercial neonatal phenylalanine kits (PerkinElmer Life and Analytical Sciences, Wallac Oy, Mustionkatu 6, Turku, Finland) and retested with the new method of LC–MS/MS in our study. The results for Phe and Tyr concentrations and the molar ratios of Phe to Tyr in those samples are summarized in Table 3. The concentrations for Phe by the fluorescent method and the developed LC–MS/MS method are in substantial agreement. For the control group, all the Phe/Tyr ratios were <1.5 ($n = 10$). For all cases of classical PKU, all the Phe/Tyr ratios were more than 4.0 ($n = 5$). We found that both the concentrations of Phe and the molar ratios of Phe/Tyr were remarkably higher in PKU-positive groups than in healthy newborns. Furthermore, we found that the Phe/Tyr ratio from the DBS of PKU 3 days is higher than the DBS of PKU 30 days in the same numbered PKU newborn. Therefore, positives connected with only using Phe concentration as the marker for PKU diagnosis, which is the basis for the fluorescent method can also be insured using the ratio of Phe/Tyr with the proposed protocol.

Conclusion

We have presented a reliable, simple, and sensitive method for the simultaneous quantifying of 24 AAs in a single run in DBS samples. Through there are other methods that have been developed that can detect amino acids separately. However, the proposal we developed has lots of advantages as follows. Firstly, the injection was made right after a very simple extraction step free of interferences without the derivatization step. The simple extraction step greatly reduced the time-consuming sample preparation procedures required by most other methods. Hence, the errors introduced by the procedures of derivatization also could be eliminated. High selectivity was achieved by the combination of sufficient LC separation and specific MRM for characteristic transitions. Secondly, the use of *N*,*N*-Phe as internal standard obviously decreased the expenses of analysis. Thirdly, the use of acetic acid instead of ion-pair agent in mobile phase also improved the separation of analytes in liquid column. At last, we applied the established method to analyze AAs in DBS between healthy newborns and PKU newborns. And we found that not only the increased Phe more than 120 μmol/L was a diagnostic standard of PKU, but also the decreased Gln and the ratio of Phe/Tyr more than 2 could be as assisted diagnosis of PKU through this method. Therefore, a false positive for PKU diagnosis caused by solely using Phe concentration as the marker can be avoided with this method. The protocol is valuable for the routine quantification of AAs from biological samples, such as a second-tier test in the screening of metabolic diseases for which AAs are markers.

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