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Rapid and simultaneous determination of twenty amino acids in complex biological and food samples by solid-phase microextraction and gas chromatography–mass spectrometry with the aid of experimental design after ethyl chloroformate derivatization

Mohana Krishna Reddy Mudiam [a,*], Ratnasekhar Ch. [a], Rajeev Jain [a], Prem Narain Saxena [b], Abhishek Chauhan [a], R.C. Murthy [a]

[a] *Analytical Chemistry Section, CSIR-Indian Institute of Toxicology Research, PO Box 80, Mahatma Gandhi Marg, Lucknow 226 001, Uttar Pradesh, India*
[b] *Chemical and Pollutant Analysis Laboratory, CSIR-Indian Institute of Toxicology Research, PO Box 80, Mahatma Gandhi Marg, Lucknow 226 001, Uttar Pradesh, India*

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ABSTRACT

Amino acids play a vital role as intermediates in many important metabolic pathways such as the biosynthesis of nucleotides, vitamins and secondary metabolites. A sensitive and rapid analytical method has been proposed for the first time for the simultaneous determination of twenty amino acids using solid-phase microextraction (SPME). The protein samples were hydrolyzed by 6 M HCl under microwave radiation for 120 min. Then the amino acids were derivatized by ethyl chloroformate (ECF) and the ethoxy carbonyl ethyl esters of amino acids formed were extracted using SPME by direct immersion. Finally the extracted analytes on the SPME fiber were desorbed at 260 °C and analyzed by gas chromatography–mass spectrometer (GC–MS) in electron ionization mode. Factors which affect the SPME efficiency were screened by Plackett–Burmann design; most significant factors were optimized with response surface methodology. The optimum conditions for SPME are as follows: pH of 1.7, ionic strength of 733 mg, extraction time of 30 min and fiber of divinyl benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). The recovery of all the amino acids was found to be in the range of 89.17–100.98%. The limit of detection (LOD) of all derivatized amino acids in urine, hair and soybean was found to be in the range of 0.20–7.52 $\mu g L^{-1}$, 0.21–8.40 $\mu g L^{-1}$ and 0.18–5.62 $\mu g L^{-1}$, respectively. Finally, the proposed technique was successfully applied for the determination of amino acids in complex biological (hair, urine) and food samples (soybean). The method can find wide applications in the routine analysis of amino acids in any biological as well as food samples.

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1. Introduction

Amino acids are building blocks of proteins and play essential role in energy metabolism, neurotransmission and lipid transport. They are involved in many important metabolic processes that are vital to the health, growth, development and reproduction of organisms. Due to vital role of amino acids in many metabolomic pathways, they are one of the important targets for metabolomic profiling studies. Several metabolic disorders can be diagnosed by determining amino acid concentration levels e.g. urine phenylketonuria (PKU) can be diagnosed by measurement of ratio of L-phenyl alanine to L-tyrosine [1] and maple syrup urine disease (MSUD) can be diagnosed from the ratio of L-leucine plus L-isoleucine to L-phenyl alanine [2]. Diabetic risk assessment can be predicted by amino acid profiling [3]. The ability to separate and quantitate amino acids is essential for the characterization and structural elucidation of polypeptides and proteins.

Several analytical methods were reported for the determination of amino acids. Wide range of fluorescent labeling reagents such as ninhydrin, fluorescamine, dansyl chloride, 7-fluoro-4-nitrobenz-2,1,3-oxodiazole (NBD), o-phthalaldehyde (OPA), and naphthalene-2,3-dicarboxaldehyde (NDA) were used for the analysis of amino acids using liquid chromatography–fluorescence detector [4–10]. The limitations of these reagents include (i) sensitive to light and unstable nature of the reagent which enforces to prepare fresh reagent before analysis, (ii) difficulty in the derivatization of secondary amino acids, sulphur amino acids and (iii) unstability of the compounds formed after derivatization limit the application of these reagents for routine analysis of amino acids [11]. In addition to HPLC, gas chromatography, liquid

* Corresponding author. Tel.: +91 522 2627586; fax: +91 522 2611547.
E-mail address: mohanitrc@gmail.com (M.K.R. Mudiam).

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chromatography and capillary electrophoresis coupled to mass spectrometry are the other techniques used for the analysis of amino acids [12–18].

Gas chromatography–mass spectrometry (GC–MS) is a powerful analytical tool with reproducible quantitative capabilities for simultaneous identification and quantification of multiple compounds in different biological samples [19,20]. Further, shorter run time, better chromatographic resolution, low ion suppression, higher precision and complete automation of GC–MS make it amenable for amino acid analysis after derivatization with silylated reagents or alkyl chloroformates [21–25]. Solid-phase micro extraction (SPME) developed by Arthur and Pawliszyn is a solvent-free extraction technique that incorporates sample extraction, concentration and introduction into injector port of gas chromatography (GC) in a single step and applicable to the wide range of analytes [26–32]. This technique is fast, portable, easy to use. In recent years, coupling of SPME with GC–MS is widely used as solvent free analysis method and has several advantages with respect to sensitivity, selectivity and repeatability of the analysis.

Deng et al. have reported a method for amino acid analysis using SPME followed by GC–MS analysis [33]. However, the reported method has capability to analyze very few amino acids (V, L, I, F and Y) using SPME after isobutyl chloroformate derivatization in head space mode. This is due to the non/semi-volatile nature of amino acids even after derivatization limits the use of SPME for simultaneous analysis of amino acids. Further, no analytical method has been reported for simultaneous determination of twenty amino acids in food and biological samples using alkyl chloroformate derivatization followed by SPME and GC–MS analysis.

In the present communication, we aim to develop an analytical method for the rapid and simultaneous determination of twenty amino acids using SPME followed by GC–MS analysis in complex biological samples like hair and urine after ethyl chloroformate derivatization. Influence of important factors affecting the SPME was screened and optimized by experimental design. At first step, Plackett–Burmann design (PBD) was used to screen the significant factors that affect the SPME efficiency and in the second step central composite design (CCD) combined with desirability function (DF) was used to optimize the factors for better extraction efficiency.

2. Experimental

2.1. Reagents and materials

Reagents used in this study were of analytical grade unless otherwise stated. The amino acid standards (purity, 99.5%), pyridine, ECF and L-ascorbic acid were procured from Sigma (St. Louis, MO, USA). Hydrochloric acid (HCl) and ethanol were obtained from Merck Laboratories (Darmstead, Germany). Sodium chloride, sodium sulphate and magnesium sulphate were procured from Qualigens Laboratories (Mumbai, India). SPME fibers such as 100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane/divinyl benzene (PDMS/DVB), 85 μm polyacrylate (PA), 60 μm carbowax/polyethylene glycol (CW/PEG), 85 μm carboxen/PDMS, divinylbenzene/carboxen/polydimethyl siloxane (DVB/CAR/PDMS) and manual SPME holder were procured from Supelco (Bellfonte, USA). Prior to the experiments all the new fibers were conditioned as per the supplier's recommendation.

2.2. Preparation of standards

Standard stock solution containing 100 mg L^{-1} of each amino acid was prepared in 0.1 M HCl and stored at 4 °C. Working standards of amino acids were freshly prepared by diluting stock solution with 0.1 M HCl.

2.3. Collection and digestion of hair samples for amino acid analysis

Hair fibers distant from the proximal root end were collected from healthy Indian male volunteers with the prior consent. Hair samples were washed with hot water for 15 min to remove any external contamination and with a mixture of chloroform and methanol (1:1, v/v) in order to remove any external lipid. The dried samples were cut into small pieces. All samples after washing were hydrolyzed according to a reported procedure with a little modification [34]. Briefly, the dried hair sample (100 mg) was hydrolyzed with 10 mL of 6 M HCl in microwave for 120 min under nitrogen. Before the hydrolysis, the hair was immersed in the 6 M HCl for 5 min. The microwave radiation was initiated at 100 W for 5 min and then increased to 700 W at the rate of 20 W/s. The vials were then allowed to cool to room temperature and then the resultant mixture was subsequently diluted and stored at −20 °C till further analysis.

2.4. For urine samples

0.5 mL of urine was diluted with 1 mL of water and placed in the 4 mL micro reaction vial. The rest of the procedure was exactly same as the one used for the derivatization and SPME.

2.5. For soybean samples

Dried soybean seeds were grind into powder form with mortar and pestle. 200 mg of grinded soybean samples were hydrolyzed with 20 mL of 6 M HCl under microwave irradiation. 150 μL of hydrolyzed sample was used for the further analysis.

2.6. Derivatization and SPME

To an aliquot of hydrolyzed sample, 60 μL of ethanol, 50 μL of pyridine and 60 μL of ECF were added and vortexed for 30 s till the carbon dioxide gas leaves off. To this, an amount of 730 mg of Na_2SO_4 was added and pH was adjusted to 1.7 using 0.1 M HCl. Then the solution was made up to 4 mL with water. The SPMEs were performed by placing the DVB/CAR/PDMS (65 μm) fiber into this 4 mL solution (direct immersion) for 30 min. After each extraction, the fiber was rinsed with water to remove any excess of polar non-volatile compounds and dried it before placing in injector port of GC–MS. Desorption time and temperature were 5 min and 260 °C, respectively. All experiments were carried out in triplicate and the average values were reported.

2.7. GC–MS analysis

GC–MS analysis was performed by using Trace GC ultra gas chromatograph coupled to a Quantum XLS mass detector (Thermo Scientific, FL, USA). GC is equipped with TG-17MS capillary column (50% phenyl methylpolysiloxane, 30 m × 0.25 μm film thickness × 0.25 mm i.d.). The injection was carried out in splitless mode at an injector temperature of 260 °C. High purity helium gas (99.999%) was used as a carrier gas with a flow rate of 1.0 mL min^{-1}. The oven temperature programming was as follows: the initial oven temperature held at 80 °C for 2 min, then increased to 230 °C at a rate of 10 °C min^{-1} and held for 3 min and then increased to 280 °C at a rate of 20 °C min^{-1} and hold for 10 min. The ion source and interface temperature were set at 220 °C and 290 °C, respectively. All the samples were analyzed in selected ion monitoring (SIM) mode.

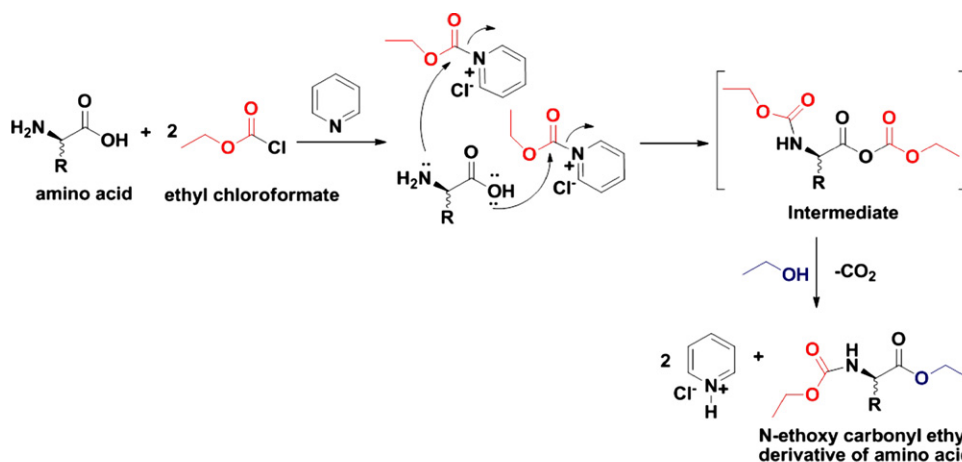

Fig. 1. Schematic representation of derivatization of amino acid with ethyl chloroformate in the presence of pyridine and ethanol.

2.8. Assay validation and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as per the IUPAC procedure (IUPAC Gold Book, http://goldbook.iupac.org/L03540.htmL) [35]. Five replicates were used to determine LOD and LOQ for each amino acid using the developed method. Recoveries were carried out by spiking the standard amino acids in urine, hair and soybean samples at a concentration range of 50–900 $\mu g\,L^{-1}$. Precision is the ability of the assay to consistently reproduce a result when sub-samples are taken from the same specimen. Intra and inter-day precisions were checked by carrying out five independent assays of sample in a day and for five successive days ($n = 5$, five replicates of samples) and the values were expressed as percent relative standard deviation (%RSD). The calibration graph was constructed by spiking amino acid solution in urine and hair at a concentration range of 50–10,000 $\mu g\,L^{-1}$.

2.9. Statistical data handling and processing

Design of experiments (PBD and CCD) was constructed and the results were evaluated using the statsoft statistical software package "statistica 10.0" (Tulsa, OK, USA). Calculations were based on the sum of the area of all the peaks obtained during GC–MS analysis.

3. Results and discussion

3.1. Optimization of derivatization conditions

Due to the presence of highly polar groups ($-NH_2$, $-OH$, $-COOH$) and their non volatile nature, amino acids need to be derivatized for the GC–MS analysis. In recent years, alkyl chloroformates were used as derivatizing agents with the advantages like in situ derivatization in aqueous medium at room temperature. The efficiency of alkyl chloroformates such as ethyl, isobutyl, benzyl, phenyl and trichloromethyl chloroformates for the derivatization of amino acids was evaluated in this study for the optimum derivatization conditions. Ethyl acetate was used as an extraction solvent for the optimization of derivatization conditions. The derivatization with isobutyl chloroformate was found to have more sensitivity than any other chloroformates. But it forms a large number of bi-products than the other chloroformate derivatization techniques. Ethyl chloroformate derivatization has shown the optimum response for amino acids next to isobutyl chloroformate with minimum number of bi-products. The reaction mechanism for the derivatization of amino acids using ethyl chloroformate in the presence of an aliphatic alcohol and pyridine is shown in Fig. 1. In order to find out the optimal conditions for derivatization, volume of ethyl chloroformate, pyridine and ethanol has been studied. Initially volume of derivatizing reagent, ECF was optimized in the range of 10–100 μL at a constant volume of 60 μL of pyridine and ethanol. Highest derivatizing efficiency of amino acids derivatization with ECF was found at a volume of 60 μL (Fig. 2). Further, the volume of ethanol and pyridine was optimized in the range of 10–100 μL at a constant volume of 60 μL of ECF. The highest derivatizing efficiency is obtained at 60 μL of ethanol and 50 μL of pyridine (Fig. 2). These conditions were selected for further experiments.

3.2. Optimization of conditions for SPME

To select the optimal conditions for the SPME, initially fiber type and ionic salt were screened for their optimum performance in the analysis of amino acids after ECF derivatization. After the selection of fiber type and ionic salt, the other factors which can affect the extraction efficiency such as ionic strength, pH, extraction time, stirring speed, dilution effect, desorption temperature, desorption time were screened and optimized with the aid of design of experiments (PBD and CCD).

3.2.1. Screening of SPME fiber

Selection of the SPME fiber is a crucial factor for the SPME because the efficiency of the extraction process is dependent on the coating on the fiber. At the first step six different fibers PDMS,

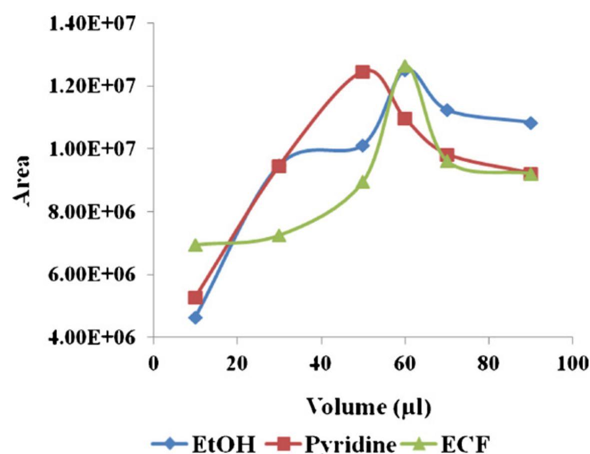

Fig. 2. Optimization of pyridine, ethyl chloroformate and ethanol.

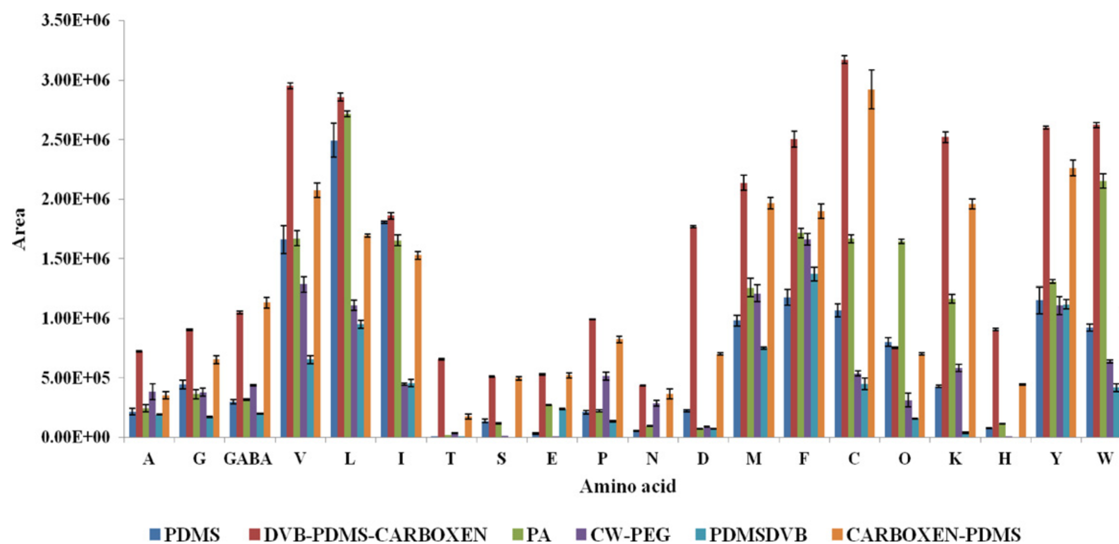

Fig. 3. Screening of SPME fiber.

DVB/CAR/PDMS, PA, CW/PEG, PDMS/DVB, CARBOXEN/PDMS were screened at pH 7 and sodium sulphate as ionic medium. Best extraction efficiencies were obtained with DVB/CAR/PDMS as shown in Fig. 3. After the selection of the fiber, experiments were performed to evaluate the type of extraction mode (either head space or direct immersion). In head space SPME, some of the amino acids such as T, S, N, E, O, K, H, Y, W could not be detected. This may be due to non-volatile nature of these amino acids even after derivatization as they contain unreactive polar functional groups such as —OH in case of T, S, $—CONH_2$ in case of N, imide group in case of H with ECF. But the direct immersion SPME could able to extract all the twenty amino acids chosen for this study. The comparison of the extraction efficiency of both the extraction modes is compared as depicted in Fig. 4.

3.2.2. *Screening of ionic salt*

Ionic strength had shown very significant effect for the SPME of amino acids. The ionic salts such as sodium sulphate and magnesium sulphate were evaluated for their optimal performance to increase the SPME efficiency. In the absence of salt, the extraction efficiency was found to be less, due to high solubility of ECF derivatives in water when ethanol used as a reaction medium. Solubility of the ECF derivatives of amino acids in water was drastically reduced by adding 400 mg of sodium sulphate to the 4 mL of the reaction media after derivatization. Sodium sulphate was found to give better response than magnesium sulphate.

3.2.3. *Screening and optimization of SPME factors*

The factors which can affect the extraction efficiency of SPME such as pH, ionic strength, desorption temperature, extraction time, desorption time, stirring speed, effect of dilution were screened using PBD and their values were further optimized by CCD.

3.2.3.1. *Plackett–Burmann design.*

Experimental PBD was used to evaluate the main factors which can affect the extraction efficiency. It involves k factors with $k+1$ runs. It is very useful in order to detect the most important factors that can affect significantly the SPME with minimum number of experiments. In this study, a 2^{7-4} PBD was applied in order to evaluate the main factors responsible for optimum SPME out of seven factors tested. PBD is a highly useful design to rapidly screen the significant factors from a multivariate system by eliminating the interactions so that the main effects are calculated with reduced number of experimental runs. The above mentioned variables were screened at two levels as

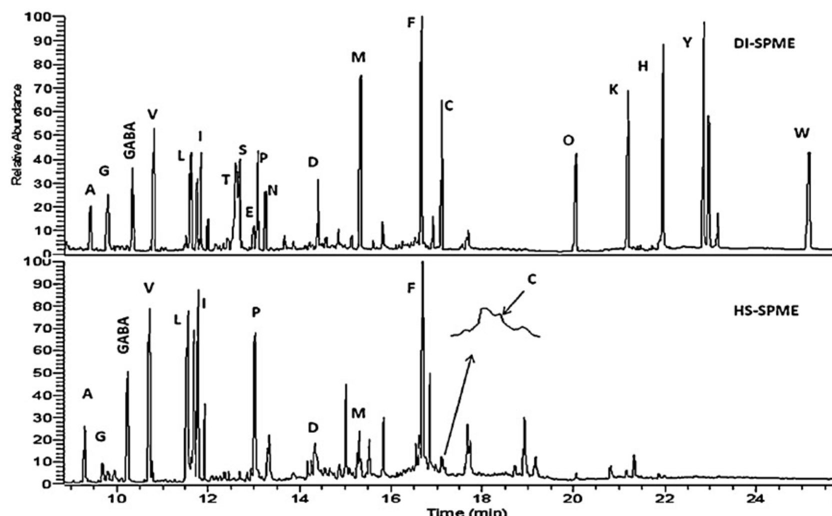

Fig. 4. GC–MS chromatogram for the comparison of SPME mode of derivatized amino acids.

Table 1
Factors and their levels for Plackett–Burmann design.

S.no.	Factors	Levels	
		Low (−1)	High (+1)
1.	Ionic strength (mg)	0	600
2	pH	3	9
3	Desorption temperature (°C)	220	260
4	Extraction time (min)	10	30
5.	Desorption TIME (min)	0.5	5
6	Stirring speed (rpm)	100	300
7	Dilution (mL)	1.5	4

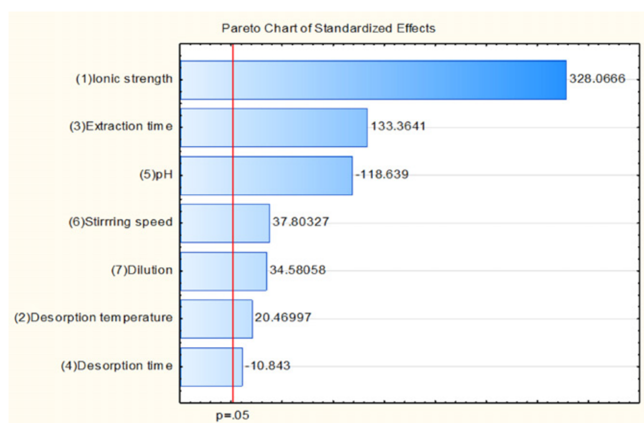

Fig. 5. Standardized main effect Pareto chart for the Plackett–Burmann design of screening experiment.

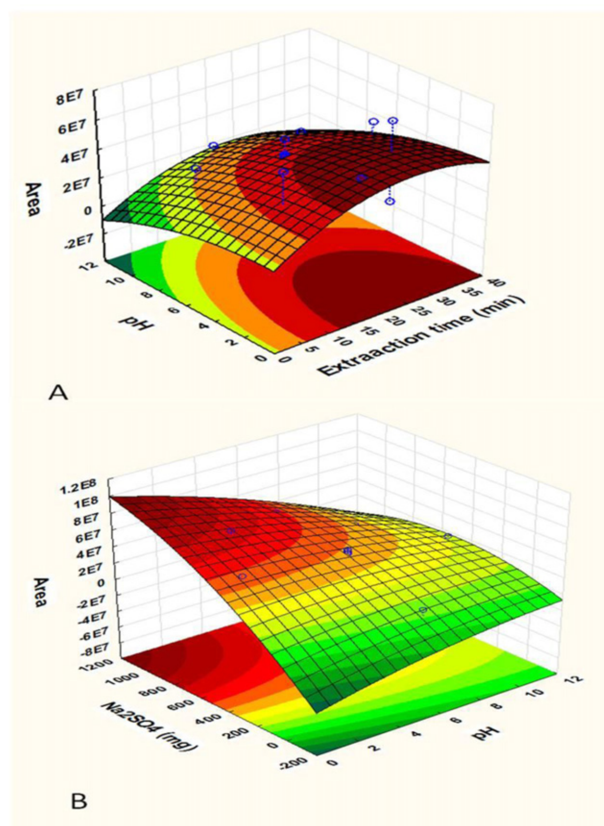

Fig. 6. Estimated response surface for derivatized amino acids using CCD by plotting. (A) Extraction time vs pH and (B) pH vs concentration of Na_2SO_4.

shown in Table 1. In total, the design consists of eight experiments with three replicates (i.e. 24 experiments). All these experiments were randomly carried out in order to nullify the bias which affects the change in experimental conditions. The sum of the peak areas

Table 2
Factors and their levels for central composite design.

Factors	Levels			Star point α = 1.68179	
	Low (−1)	Central (0)	High (+1)	−α	+α
(1) Ionic strength (Na_2SO_4 in mg)	200	500	800	0	1004
(2) Extraction time (min)	10	20	30	3	37
3) pH	3	6	9	1.45	11.5

Table 3
Mass fragmentations of N-ethoxycarbonyl amino acid ethyl esters generated by 70 eV electron impact GC–MS analysis [35].

Amino acid	Retention time (R_t) (min)	Molecular ion (M^+) (m/z)	Major fragment ions (m/z)	Quantifier ions (m/z)
A	9.26	189	116, 88, 72, 70	116
G	9.64	175	102, 74, 57, 175, 129,	102
GABA	10.21	203	130, 86, 58, 74	130
V	10.68	217	144, 101, 116, 72, 98, 55	116, 144
L	11.51	231	158, 102, 43, 58, 72	102, 158
I	11.74	231	158, 102, 130, 69, 74	102, 158
T	12.49	219	145, 129, 101, 74	129
S	12.55	205	132, 129, 101, 86, 74	132
E	12.91	275	157, 101, 84, 56	84
P	13.00	215	142, 70, 98, 114, 215	142
N	13.18	232	141, 174, 215, 113, 69, 102, 74	141
D	14.37	261	188, 142, 116, 74, 56	188, 142
M	15.30	249	61, 114, 129, 175, 74, 101	175
F	16.68	265	91, 176, 102, 192, 220, 265	176
C	17.13	221	74, 102, 220, 132, 174	220
O	20.06	304	142, 70, 212, 258, 98, 114	142
K	21.31	318	156, 56, 84, 226, 272 102, 128	156
H	22.34	255	238, 154, 166, 136, 81	238, 254
Y	23.02	353	107, 135, 192, 264, 74, 164	107
W	25.28	302	130, 117, 215, 101, 77	130

Table 4
LOD, LOQ, linearity, precision and recovery of urine, hair and soybean sample.

Amino acid	Linearity range (×10³ μg L⁻¹)	R^2			LOD (μg L⁻¹)			LOQ (μg L⁻¹)			Precision (%RSD)		%Recovery		
		Urine	Hair	Soybean	Urine	Hair	Soybean	Urine	Hair	Soybean	Intra-day	Inter-day	Urine	Hair	Soybean
A	0.10–9.0	0.9662	0.9891	0.9998	0.32	0.39	0.33	1.06	1.30	1.12	1.26–2.18	3.56–5.15	96.10	93.19	95.16
G	0.08–10.0	0.9648	0.9936	0.9930	0.29	0.43	0.37	0.96	1.45	1.26	1.96–4.91	2.16–6.32	92.62	96.52	95.38
GABA	0.08–10.0	0.9829	–	–	0.29	–	–	0.97	–	–	1.94	4.32	95.47	–	–
V	0.10–8.0	0.9777	0.9918	0.9986	0.20	0.24	0.26	0.65	0.81	0.88	0.94–3.48	4.18–5.60	94.49	95.92	94.20
L	0.10–8.0	0.9860	0.9920	0.9785	0.20	0.21	0.18	0.67	0.68	0.59	2.36–3.16	3.63–4.30	90.77	97.28	98.21
I	0.07–10.0	0.9970	0.9959	0.9948	0.29	0.32	0.26	0.97	1.10	0.87	1.16–1.86	1.31–2.39	96.15	93.65	95.18
T	0.10–10.0	0.9509	0.9938	0.9997	0.46	0.50	0.75	1.53	1.62	2.50	0.16–1.98	2.91–5.58	97.58	98.25	98.71
S	0.06–10.0	0.9820	0.9981	0.9917	1.09	1.28	0.87	3.61	4.28	2.91	2.14–2.31	2.86–3.84	90.56	99.59	96.21
E	0.05–10.0	0.9468	0.9863	0.9563	5.58	7.09	4.89	18.60	23.40	16.13	3.16–4.15	4.28–6.23	91.49	97.89	96.31
P	0.05–9.0	0.9893	0.9984	0.9944	0.30	0.37	0.47	0.95	1.26	1.61	1.16–2.28	2.16–3.89	90.54	91.68	93.13
N	0.20–10.0	0.9530	–	–	1.62	–	–	5.35	–	–	2.89	3.92	93.34	–	–
D	0.09–9.0	0.9868	0.9925	0.9992	0.71	0.76	0.60	2.41	2.52	1.92	1.10–1.59	2.60–4.15	91.79	96.25	96.21
M	0.10–8.0	0.9578	0.9946	0.9952	0.30	0.33	0.32	0.93	1.10	1.08	1.85–3.34	2.59–5.11	89.17	93.54	94.18
F	0.10–8.0	0.9864	0.9960	0.9939	0.29	0.28	0.23	0.98	0.76	4.81	0.95–3.53	4.16–6.26	92.91	94.26	95.27
C	0.20–8.0	0.9912	0.9956	0.9997	0.36	0.57	0.46	1.18	1.89	1.53	1.15–2.16	3.15–5.36	93.95	91.74	95.31
O	0.20–9.0	0.9790	–	–	0.64	–	–	2.21	–	–	3.18	3.91	95.70	–	–
K	0.20–10.0	0.9779	0.9935	0.9915	0.32	0.33	0.34	1.06	1.08	1.13	1.58–2.21	2.56–5.57	100.45	93.89	96.40
H	0.3–10.0	0.9554	0.9929	0.9985	7.52	8.40	5.62	24.78	27.80	17.20	1.16–2.30	3.16–4.82	93.20	90.91	92.36
Y	0.05–10.0	0.9798	0.9814	0.9955	0.31	0.31	0.38	1.13	1.04	1.27	2.14–3.16	3.21–4.57	100.98	95.61	93.46
W	0.06–10.0	0.9917	0.9926	0.9934	0.39	0.52	0.55	1.31	1.74	1.80	2.84–3.14	2.98–4.36	96.54	97.86	95.26

Table 5
Amino acids of hair and soybean samples.

	Hair sample ($n = 15$)		Soybean sample ($n = 9$)	
	Mean	SD	Mean	SD
A	3.10–3.86	0.53	1.51–1.78	2.16
G	4.90–5.28	0.19	1.62–1.93	1.18
V	5.11–5.58	1.26	1.21–1.77	1.26
L	5.16–6.13	2.85	2.41–2.68	3.41
I	1.80–2.66	1.63	1.51–1.78	1.26
T	4.39–4.81	0.21	1.21–1.48	2.01
S	4.61–5.44	3.46	2.31–2.76	1.48
E	3.66–4.16	5.28	4.51–4.96	3.56
P	5.81–6.26	3.25	1.71–1.96	4.18
D	3.04–3.60	2.28	4.21–4.86	2.77
M	1.10–1.92	3.41	0.20–0.36	1.93
F	3.31–3.68	2.19	2.01–2.35	2.60
C	4.91–5.73	4.41	0.30–0.51	3.92
K	3.80–4.57	2.39	2.10–2.36	4.78
H	0.43–1.18	3.36	1.10–1.31	4.36
Y	1.26–1.91	2.32	1.18–1.34	2.29

Results expressed as $(\times 10^3)$ μg L⁻¹ of samples.

of the twenty amino acids was taken for the design of experiments as a response. An analysis of variance (ANOVA) test was used to evaluate data and the factors which are statistically significant in SPME were determined using *t*-test with 95% probability. The effect of the factors and their significance in the screening experiments were expressed in the form of Pareto chart as shown in Fig. 5. Ionic strength is the most significant factor with positive effect on extraction efficiency. After ionic strength, extraction time and pH are the other two significant factors which can affect SPME. Desorption temperature, desorption time, stirring speed and dilution effects are very less significant than the above three factors. For further experiments, the less significant factors such as stirring speed of 300 rpm, dilution of 4 mL, desorption time of 5 min, desorption temperature of 250 °C were chosen in order to homogeneous mixing of analytes and complete evaporation of derivatives of amino acids for GC–MS analysis. The most significant factors such as ionic strength, pH and extraction time were considered for the next optimization step using CCD approach.

3.2.3.2. Central composite design. Central composite is considered as a response surface design for the optimization of the significant factors in order to obtain the best response. CCD consists of a 2^k factorial runs augmented with $(2k+1)$ star points. The three factors such as ionic strength, pH and extraction time at 3 levels low (−1), central (0), high (+1) were selected for optimization. The experimental range of the factors used to optimize SPME conditions are shown in Table 2. Extraction time, ionic strength and pH were selected in the range of 10–30 min, 200–800 mg and 3–9, respectively for CCD matrix. Central composite design of 2^3 with $2 \times 3 + 1$ star points placed at a distance of $+\alpha$ and $-\alpha$ from the central point. In order to establish the rotatability conditions, an α value was selected at 1.682, as the CCD generates information equally in all directions. The runs at the center of the experimental field were performed in three times. The levels of these factors, as well as location of their star points are given in Table 2. In total, CCD matrix design involves 18 runs and all the experiments were run in a random manner to minimize the effect of uncontrolled variables and the responses were expressed as the sum of the peak areas.

The data obtained was evaluated by ANOVA. The quantity of the fit of the polynomial model was expressed as coefficient of determination; R^2 and adjusted R^2 are equal to 0.95133 and 0.89657, respectively. The large adjusted R^2 value indicates a good relationship between the experimental data and the fitted model. Response surface model was developed by considering all the significant

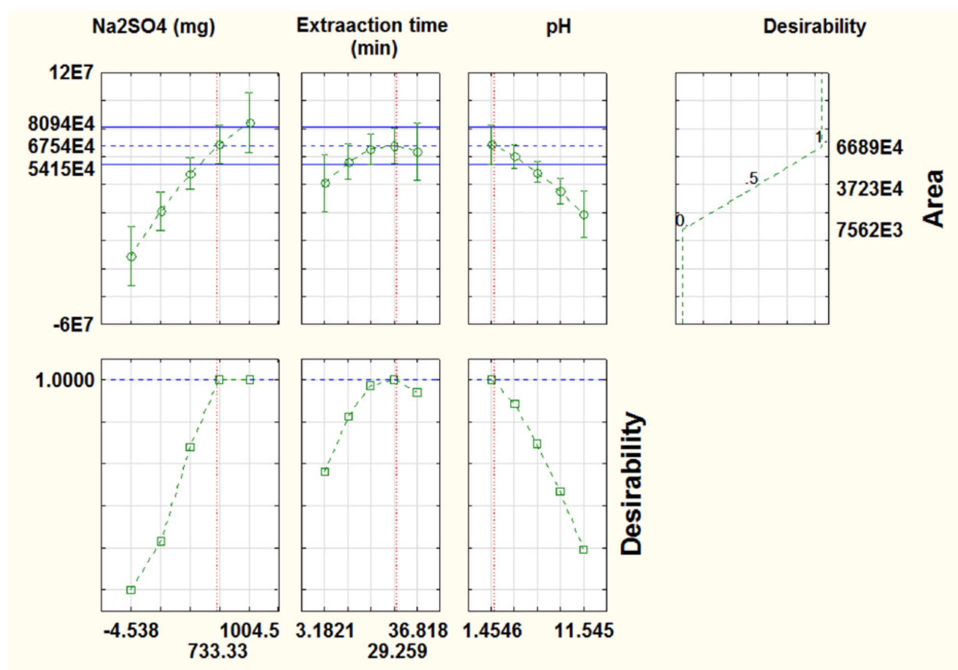

Fig. 7. Profiles for predicted values and desirability.

interactions in CCD. The response surface plots are shown in Fig. 6. Fig. 6A shows the response surface plot between the extraction time and pH with ionic strength of 500 mg. Optimum extraction time was found to be in the range of 25–40 min. This may be attributed to the fact that, in aqueous solution all the analytes must diffuse through the static layer of water to reach the fiber coating due to this it requires longer equilibrations times. Higher extraction efficiencies for pH were obtained in the range of 1–3. It may be due to neutral behavior of N-ethoxy carbonyl ethyl ester derivatives of amino acids under acidic conditions and produce maximum SPME efficiency. Fig. 6B shows the response surface plot between Na_2SO_4 and pH with an extraction time of 20 min. Maximum extraction efficiencies were obtained in the range of 600–800 mg. It may be due to salting out effect, with the decreased aqueous solubility distribution constant (K_{eS}) increases and consequently response is improved.

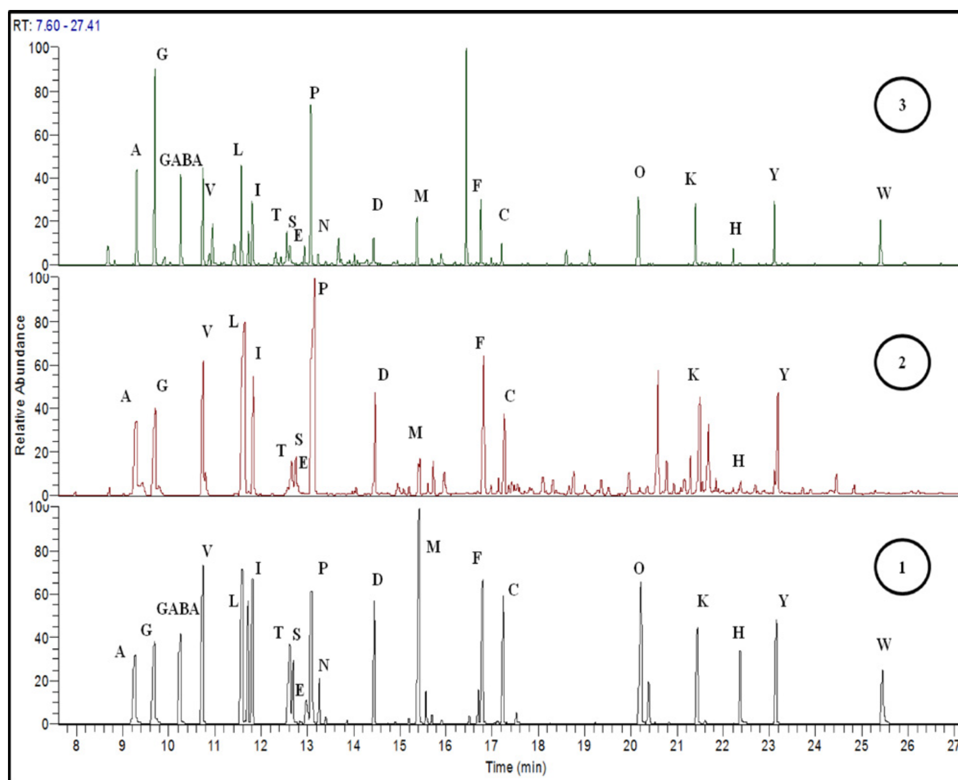

Fig. 8. GC–MS chromatogram for the amino acid analysis of (1) standard mixture (2) real hair sample and (3) spiked urine sample.

Optimum conditions were predicted by using desirability function. The prediction profile consists of a series of graphs, one for the each independent variable and inspecting the prediction profile graphs which can show the factor levels that can produce the most desirable response on the dependent variable. The values assigned for the predicted values ranging from 0.0 (undesirable) to 1.0 (desirable). The prediction profiles and desirability graphs are shown in Fig. 7. On the basis of these considerations, the optimum values obtained for the variables tested as follows: amount of Na_2SO_4 required is 733 mg, extraction time of 30 min and pH of 1.7.

3.3. Analytical validation

Retention times, molecular ions and major fragment ions of derivatized amino acids are shown in Table 3 [35]. The method developed for the analysis of amino acids using SPME followed by GC–MS analysis after ECF derivatization was validated w.r.t. linearity, intra and inter-day precision, recovery and then it was applied to real hair and soybean samples. Calibration curves were constructed by plotting the graph between peak area and concentration of ECF derivatives of amino acids in the concentration range of 50–10,000 $\mu g\,L^{-1}$. The linear correlation coefficients (R^2) for all amino acids were found to be in the range of 0.9468–0.9970.

The LOD and LOQ were calculated as per the IUPAC procedure [36]. The values obtained are depicted in Table 4 for urine samples, hair samples and soybean samples. The LOD and LOQ for urine samples were found to be in the range of 0.20–7.52 $\mu g\,L^{-1}$ and 0.65–24.78 $\mu g\,L^{-1}$, respectively. The LOD and LOQ for hair samples were found to be in the range of 0.21–8.40 $\mu g\,L^{-1}$ and 0.68–27.80 $\mu g\,L^{-1}$, respectively. The LOD and LOQ for soybean samples were found to be in the range of 0.18–5.62 $\mu g\,L^{-1}$ and 0.59–17.20 $\mu g\,L^{-1}$, respectively. Intra and inter-day precisions were expressed by percent relative standard deviation (%RSD) and were calculated by analyzing five replicates of amino acid standards spiked at 100 and 500 $\mu g\,L^{-1}$ in urine, hair and soybean samples, respectively. Intra and inter-day precisions for urine samples were found to be in the range of 0.16–4.91 and 1.31–6.32, respectively. Intra-day and inter-day precisions for hair samples were found to be in the range of 1.15–4.15 and 2.28–6.23, respectively. Intra and inter-day precisions for soybean samples were found to be in the range of 1.16–3.98 and 2.39–5.16, respectively The recovery of amino acids from five independent analyses was estimated by applying the analytical method to urine, hair and soybean samples that were spiked with amino acids at a concentration of 50 and 600 $\mu g\,L^{-1}$ for urine samples, 100 and 800 $\mu g\,L^{-1}$ for hair samples and 80 and 900 $\mu g\,L^{-1}$ for soybean samples, respectively. The results are depicted in Table 4. The recoveries were found to be in the range of 89.17–100.98% for urine samples, 90.91–99.59% for hair samples and 92.36–98.71% for soybean samples, respectively. The developed method was compared with previously reported methods with respective LOD, LOQ, linearity, recovery and found to be superior as compared to earlier reported methods depicted in Table 6.

3.4. Application of method to hair and soybean samples

To evaluate the efficiency of the present method, the method was applied to human hair samples (15 no.) collected from farmers and soybean seed samples (9 no.). Effect of antioxidant (L-ascorbic acid) on the digestion of samples was also studied and found to have a significant role in the digestion of both hair and soybean samples. It was observed that cysteine was not detected and the recovery of methionine was significantly reduced if the digestion of hair samples performed in the absence of ascorbic acid. This may be due to the oxidative degradation of the sulphur amino acids in the presence of HCl. Tryptophan was not found due to its

Table 6
Comparison of analytical parameters with previously reported methods.

Analytical parameter	Method	Amino acids																			
		A	G	GABA	V	L	I	T	S	E	P	N	D	M	F	C	O	K	H	Y	W
LOD ($\mu g\,L^{-1}$)	LLE [24]	2.67	2.25	–	21.06	7.8	15.7	21.42	315	132.3	31.05	39.6	39.9	134.1	49.5	–	39.6	43.8	465	54.3	6.12
	LLE [14]	80.1	138	–	9.36	40.6	10.5	220	220	271.9	92	244	93.1	7.45	6.6	18.15	–	21.9	139.5	14.48	30.6
	EME [37]	267	750	–	58.5	45.9	65.5	–	–	–	115	–	–	149	57.75	–	–	109.5	77.5	1810	71.4
	SPME[a]	0.32	0.29	0.29	0.20	0.20	0.29	0.46	1.09	5.58	0.30	1.62	0.71	0.30	0.29	0.36	0.64	0.32	7.52	0.31	0.39
R^2	LLE	0.999	0.999	–	0.999	0.999	0.999	0.998	0.997	0.999	0.996	0.998	0.999	0.995	0.999	–	0.997	0.996	0.998	0.998	0.998
	LLE	0.995	0.999	–	0.999	0.998	0.999	1.000	0.999	0.992	0.994	0.997	0.999	0.999	0.998	0.998	––	0.999	0.991	0.999	0.999
	EME	0.997	0.997	–	0.997	0.997	0.998	0.999	–	–	0.999	–	–	0.998	0.998	–	–	0.997	0.998	0.996	–
	SPME	0.999	0.993	0.983	0.991	0.992	0.996	0.994	0.998	0.986	0.998	0.953	0.993	0.995	0.996	0.996	0.979	0.994	0.993	0.981	0.993
Recovery	LLE	100.2	103.7	–	102.9	98.5	104.3	104.5	104.6	103.0	103.7	–	101.0	94.3	99.5	–	–	105.3	123.7	104.8	–
	LLE	102.1	100.7	–	103.2	94.6	101.5	90.2	97.8	99.8	101.3	91.5	96.2	93.1	90.1	104.8	–	92.6	107.4	99.7	90.6
	EME	2.1	1.4	95.47	5.7	9.6	10.0	0.5	–	–	–	–	–	5.5	10.1	–	–	4.7	5.9	1.2	–
	SPME	96.10	92.62		94.49	90.77	96.15	97.58	90.56	91.49	90.54	93.34	91.79	89.17	92.91	93.95	95.70	100.45	93.20	100.98	96.54
Precision	LLE	2.0	3.1	–	2.4	3.0	2.5	–	–	3.1	3.2	3.5	8.1	7.1	2.5	–	3.7	2.4	4.9	4.0	2.9
	LLE	2.8	2.1	–	2.5	3.5	3.2	8.3	6.6	3.2	3.8	4.5	3.6	6.1	2.3	9.3	–	2.7	3.2	2.2	2.6
	EME	7.8	4.0	–	6.1	3.4	8.1	2.1	–	–	2.8	–	–	8.1	9.7	–	–	5.4	2.9	5.6	6.7
	SPME	1.26	1.96	1.94	0.94	2.36	1.16	0.16	2.14	3.16	1.16	2.89	1.10	1.85	0.95	1.15	3.18	1.58	1.16	2.14	2.84

[a] Present method.

degradation during acidic hydrolysis. The results of sixteen amino acids from fifteen healthy hair samples and soybean seeds are presented in Table 5. The GC–MS chromatogram for the real hair samples is shown in Fig. 8.

4. Conclusion

Ethyl chloroformate derivatization followed by SPME–GC/MS analysis provides an effective platform for the analysis of amino acids in complex biological and food samples such as hair, urine and soybean seeds. The proposed method is rapid, sensitive and can easily be performed in aqueous media at room temperature. Further it does not require any pre-purification, cleanup and/or lyophilization steps before analysis. The method developed has wide applications for the routine analysis of amino acids in complex biological samples in any clinical and toxicological laboratories and food samples.

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