Food Chemistry 143 (2014) 97–105

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Rapid and sensitive ultrasonic-assisted derivatisation microextraction (UDME) technique for bitter taste-free amino acids (FAA) study by HPLC–FLD

Guang Chen [a,b,c,d], Jun Li [c,d], Zhiwei Sun [c,d], Shijuan Zhang [a,b], Guoliang Li [a,b], Cuihua Song [c,d], Yourui Suo [a], Jinmao You [a,c,d,*]

[a] Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China
[c] The Key Laboratory of Life-Organic Analysis, Qufu Normal University, Qufu, Shandong 273165, China
[d] Key Laboratory of Pharmaceutical Intermediates and Analysis of Natural Medicine, Qufu Normal University, Qufu, Shandong 273165, China

ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 8 July 2013
Accepted 19 July 2013
Available online 27 July 2013

Keywords:
Ultrasonic-assisted derivatisation microextraction (UDME)
Free amino acids (FAA)
Bitter foods
Bitter taste threshold (BTT)
Content of free amino acid detected at BTT amount level (CABT)

ABSTRACT

Amino acids, as the main contributors to taste, are usually found in relatively high levels in bitter foods. In this work, we focused on seeking a rapid, sensitive and simple method to determine FAA for large batches of micro-samples and to explore the relationship between FAA and bitterness. Overall condition optimisation indicated that the new UDME technique offered higher derivatisation yields and extraction efficiencies than traditional methods. Only 35 min was needed in the whole operation process. Very low LLOQ (Lower limit of quantification: 0.21–5.43 nmol/L) for FAA in twelve bitter foods was obtained, with which BTT (bitter taste thresholds) and CABT (content of FAA at BTT level) were newly determined. The ratio of CABT to BTT increased with decreasing of BTT. This work provided powerful potential for the high-throughput trace analysis of micro-sample and also a methodology to study the relationship between the chemical constituents and the taste.

1. Introduction

Amino acids (AA) are of great importance for various biological processes and the essentiality of relevant foods to human has long been recognised. Research showed that most of AA could respond to the sensor of taste receptor, implying the remarkable contribution of twenty AA to food taste (Nelson et al., 2002). It is noteworthy that AA in food extractives are the principal contributors to bitter taste and their contents in some bitter foods are usually higher than those in non-bitter foods (Kano & Goto, 2003; Kirimura, Shimizu, Kimizuka, Ninomiya, & Katsuya, 1969). Therefore, the relationship between AA and foods might play an important guiding role for choosing some foods to supplement amino acids in our daily life, or for medical heath care (Grover & Yadav, 2004; Mazer, Marchio, & Acosta, 2003).

However, the relationship between bitter taste and free amino acids (FAA) is still unknown and needs to be further investigated. In this work, a new concept CABT has been proposed, which can provide the more accurate information than average content of entire-food-body and can ensure that the research is conducted at low concentration levels. BTT values tested by volunteers have indicated that the minimum food sample amount causing the discernible bitter taste is low to *mg* level; and furthermore the corresponding analyte amounts are in the range of nmol/L before analysis. However, it is well known that most AA show neither natural UV absorption nor fluorescence, thus the sensitive analysis of

Abbreviations: AA, amino acids; AC, *Allium chinense*; AO, *Arundinaria oleosa*; Ala, alanine; Arg, arginine; Asp, aspartic acid; ACN, acetonitrile; AMB, *Allium macrostemon bunge*; ANN, artificial neural network; BA, *Bitter almond*; BBD, Box–Behnken design; BMC, *Black momordica charantia*; BTT, bitter taste threshold; Cys, cystein HCl; CHF, chloroform; CABT, content of free amino acid detected from the BTT amount level of food; DL, *Dendrocalamus latiflorus*; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; EASC, 10-ethyl-acridine-3-sulfonyl chloride; FE, *Fagopyrum esculentum*; FT, *Fagopyrum tataricum*; FAA, free amino acids; FLD, fluorescence detection; Glu, glutamic acid; GABA, C-aminobutyric acid; Gly, glycine; His, histidine; HPLC, high performance liquid chromatography; Ile, isoleucine; Leu, leucine; LT, *Lettuce*; Lys, lysine; LOD, limit of detection; LOQ, limit of quantification; LLOQ, lower limit of quantification; MC, *Momordica charantia*; Met, methionine; Orn, ornithine; Pro, proline; Phe, phenylalanine; RCB, ratio of CABT to BTT; RSM, response surface methodology; Ser, serine; SOL, *Sonchus oleraceus l*; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; UDME, ultrasonic-assisted derivatisation microextraction; Val, valine; XA, *Xinjiang Almond*.

* Corresponding author at: Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China. Tel.: +86 537 4456305; fax: +86 537 4456305.
E-mail address: jmyou6304@163.com (J. You).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.07.099

trace AA in some food samples has been traditionally difficult unless some sensitivity-enhancing techniques are used. For example, direct mass spectrometric analyses provided the limit of detection (LOD) of μmol/L (Nagy, Takáts, Pollreisz, Szabó, & Vékey, 2003; Thiele et al., 2008). In such a study, a feasible method must be established for the determination of trace AA in large batches of micro-samples. Therefore, chemical derivatisation technique must be introduced to improve sensitivity and selectivity. But, to the best of our knowledge, most derivatisation techniques for simultaneous detection of multiple AA provided unsatisfactory LOD or LOQ (limit of quantification) (Gatti, Gioia, Leoni, & Andreani, 2010; Głowacki, Bald, & Jakubowski, 2011; Jiménez-Martin, Ruiz, Pérez-Palacios, Silva, & Antequera, 2012; Jámbor & Molnár-Perl, 2009; Kelly, Blaise, & Larroque, 2010; Kvitvang, Andreassen, Adam, Villas-Bôas, & Bruheim, 2011; Shi et al., 2009; Tan, Tan, Zhao, & Li, 2011). Though several sensitive methods (Li et al., 2011; Visser et al., 2011) have been established for the determination of AA in various samples, the analytical procedures with a total run time of >60 min (single run) are very time-consuming. Ultra-high-performance liquid chromatography (UPLC) (Armenta et al., 2009; Boogers, Plugge, Stokkermans, & Duchateau, 2008) has been widely used in many research since it dramatically shorten the separation time in comparison with the conventional LC system, but it is difficult to popularise UPLC in most laboratories owing to its expensive price. Although the above methods have made it possible to isolate and detect AA in various samples, their pretreatment procedures are very complex, time-consuming, and solvent-wasting, and are not suitable for large batches of samples. Therefore, the principal purpose of this study was to develop a simple, rapid, highly sensitive and high-throughput method for the determination of FAA in micro-sample, thus, the taste-FAA in different bitter foods can be conveniently studied.

In our previous study (You et al., 2009), 10-ethyl-acridine-3-sulfonyl chloride (EASC) fluorescent reagent and its application for the determination of free amines were described. This reagent exhibited not only very high fluorescence sensitivity but also high water solubility, and was suitable for the rapid separation of target compounds. In this study, a novel UDME method coupled with EASC labelling technique for the determination of FAA in bitter food samples was developed. Two robust multivariate statistical methods namely artificial neural network (ANN) (Hanrahan, 2010) and response surface methodology (RSM) (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008) were used to perform the multi-objective optimisation of the micro-extraction and derivatisation. Determination of FAA in different micro-samples was performed using the UDME–HPLC–FLD method in a high-throughput manner. The relationships between BTT values and the bitterness of twelve foods were investigated. At the same time, BTT values were correlated with the two newly defined parameters CABT and RCB. To the best of our knowledge, the present work showed the lowest LLOQ for the detection of FAA with the shortest run time. The established methodology provided important reference for exploring the relationship between the chemical constituent and the taste.

2. Materials and methods

2.1. Materials and chemicals

Six fresh bitter foods and six control samples belonging to the same genus with weaker bitterness were purchased from Taobao mall (AMB and BMC) and Qufu east-gate market, respectively. They were as follows: *Sonchus oleraceus l* (SOL), *Fagopyrum tataricum* (FT), *Arundinaria oleosa* (AO), *Allium chinense* (AC), *Momordica charantia* (MC), *Bitter almond* (BA), *Lettuce* (LT), *Fagopyrum esculentum* (FE), *Dendrocalamus latiflorus* (DL), *Allium macrostemon bunge* (AMB), *Black momordica charantia* (BMC), and *Xinjiang Almond* (XA). Twenty amino acid standards were purchased from Sigma Chemical Co. (St. Louis, MO), which were cystein HCl (Cys), histidine (His), ornithine (Orn), arginine (Arg), lysine (Lys), serine (Ser), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), glycine (Gly), tryptophan (Trp), alanine (Ala), tyrosine (Tyr), c-aminobutyric acid (GABA), proline (Pro), methionine (Met), valine (Val), phenylalanine (Phe), isoleucine (Ile) and leucine (Leu). Derivatisation reagent 10-ethyl-acridine-3-sulfonyl chloride (EASC) was prepared as previously described in our laboratory (You et al., 2009). All other chemicals used were analytically pure and purchased from Tianjin Damao Chemical Reagent Co., Ltd. Water was prepared by Milli-Q water system.

2.2. Investigation on bitter taste threshold (BTT) of food

Ninety participants (50–50% male–female) were chosen from chemistry and chemical engineering department of Qufu Normal University by means of a sensitivity test which was carried out by comparing taste of food with that of pure water. Based on taste evaluation method (Stone, Bleibaum, & Thomas, 2012), the entire-food-body were divided into ten different parts, each one of which was equally divided into several pieces according to the BTT values from tentative tests. Then, each two of these pieces were used to investigate the BTT: one for taste test and the other for weigh and analysis. The average BBT values achieved from ten parts of food sample were compared, and the minimum sample amount that caused the discernible bitter taste was defined as the final BTT value.

2.3. Preparation of solutions

Standard solutions of AA (3.0×10^{-3} mol/L) were prepared by dissolving appropriate amounts of AA in 1.0 mL of 0.3 mol/L hydrochloric acid and diluted to 50 mL with sodium borate buffer solution (0.2 mol/L, pH 9.46). The final AA concentration was 1.0×10^{-4} mol/L. A series of standard solutions (0.2, 2.0, 20.0, 100, 200, 400, 600 and 900 nmol/L) were prepared by diluting stock solutions with ACN. Quality control (QC) solutions for method evaluation were prepared at three concentration levels (low: 0.5 nmol/L for Leu, Gly and Thr, 1 nmol/L for others; middle: 100 nmol/L; high: 1000 nmol/L). All QC and stock solutions were divided into small aliquots and stored at −20 °C in darkness until use. Derivatisation reagent solution (10^{-3} mol/mL) was prepared by dissolving 1.61 g of EASC with 5 mL of ACN and was then diluted with ACN to get the low concentration solutions.

2.4. Samples pretreatment procedure

To improve the pretreatment efficiency, the ultrasonic-assisted derivatisation microextraction (UDME) technique was developed: 3 mg of food sample (MC as representative sample) was dried by a stream of nitrogen gas, milled to particle sizes of 60 μm in an ice bath, and then transferred into the ampoule containing 3 μL of EASC (0.0007 mol/L), 10 μL of hydrochloric acid (0.03 mol/L), 167 μL of ACN and 100 μL of sodium borate buffer (0.2 mol/L, pH 9.46); The mixture was allowed to react in an ultrasonic water bath at 31 °C for 11.5 min in pH* (final pH) value of 6.51. The obtained mixture was filtered through a 0.2 μm membrane for analysis. Traditional pretreatment process with extraction followed by derivatisation was compared with UDME. In brief, to 200 μL of hydrochloric acid solution (pH* 6.12) in a 2-mL vial, the dried and milled sample (3.0 mg) was added. After being kept in ultrasonic water bath for 40 min at 66 °C, the mixture was filtered and the supernatant was transferred into another ampoule which was then dried by nitrogen blow at room temperature. To the dried

Table 1
Multivariate optimisation.

Run[a]	Variables[b]				Response[c]			Validation[g]		
	X_p	X_m	X_T	X_t	Exp.[d]	BBD[e]	BBD-ANN[f]		BBD	BBD-ANN
UDME										
1	8	5.5	70	10	52.68	57.21	59.76	*Multi-criteria*		
2	6	5.5	30	15	21.74	32.10	21.74	AME	10.3624	11.6828
3	8	8	50	20	64.34	68.39	64.34	CE	0.9967	0.9969
4	8	5.5	50	15	58.62	59.32	59.27	MAE	−0.0038	−0.0974
5	6	8	50	15	61.34	64.73	46.15	RMSE	4.5386	4.3382
6	10	5.5	50	20	60.79	61.45	60.79	MRE (%)	1.5046	−0.2586
7	8	5.5	70	20	46.35	42.55	46.35	R^2	0.9266	0.9375
8	6	3	50	15	11.27	10.55	13.82			
9	8	8	30	15	60.51	52.15	60.51	*Nonparametric tests*		
10	10	5.5	70	15	52.34	48.56	52.34	*p*-value	0.8572	0.8313
11	10	8	50	15	56.51	57.93	68.19			
12	8	5.5	50	15	60.06	59.32	59.27	Optima	BBD	BBD-ANN
13	6	5.5	70	15	40.98	41.52	40.24	X_p	7.82	6.51
14	10	5.5	30	15	37.15	43.19	37.61	X_m	7.49	3.58
15	6	5.5	50	10	51.15	43.18	51.15	X_T	42.91	30.96
16	10	3	50	15	38.14	35.46	38.14	X_t	20.00	11.41
17	8	3	70	15	20.16	21.22	20.13	Response	70.66	68.92
18	8	3	30	15	21.63	12.91	21.63	Exp.	66.25	65.94
19	8	5.5	50	15	59.01	59.32	59.27			
20	8	8	70	15	57.21	58.63	57.21			
21	8	5.5	30	10	21.44	25.94	13.41			
22	10	5.5	50	10	53.54	51.86	53.54			
23	6	5.5	50	20	57.64	52.02	57.64			
24	8	3	50	10	18.32	20.85	18.32			
25	8	3	50	20	26.34	34.85	26.34			
26	8	5.5	30	20	62.86	59.03	57.44			
27	8	8	50	10	65.89	63.96	70.99			
28	8	5.5	50	15	59.13	59.32	59.27			
29	8	5.5	50	15	59.82	59.32	59.27			
Extraction										
1	7.5		50	25	26.58	26.31	26.60	*Multi-criteria*		
2	6		50	10	50.31	47.98	50.28	AME	2.3324	1.3582
3	7.5		30	40	20.34	18.01	19.95	CE	0.9991	0.9995
4	7.5		70	40	29.33	28.65	29.79	MAE	0.0004	−0.0449
5	7.5		70	10	22.56	24.89	22.54	RMSE	1.3135	0.9167
6	6		50	40	56.72	57.40	56.61	MRE (%)	0.2801	0.0028
7	9		50	10	25.31	24.63	25.24	R^2	0.9893	0.9951
8	7.5		30	10	14.35	15.03	14.52			
9	7.5		50	25	25.99	26.31	26.60	*Nonparametric tests*		
10	7.5		50	25	26.10	26.31	26.60	*p*-value	0.8802	0.7063
11	9		30	25	15.84	15.84	14.92			
12	7.5		50	25	26.35	26.31	26.60	Optima	BBD	BBD-ANN
13	6		70	25	55.49	55.49	52.29	X_p	6.12	8.31
14	7.5		50	25	26.52	26.31	26.60	X_T	65.64	43.34
15	6		30	25	42.17	43.82	43.53	X_t	40.00	29.67
16	9		70	25	26.33	24.68	26.37	Response	59.76	57.92
17	9		50	40	19.62	21.95	20.10	Exp.	58.69	57.16
Derivatisation										
1	10	5.5	70	12.5	44.97	45.91	44.05	*Multi-criteria*		
2	7	5.5	30	12.5	27.95	28.10	26.66	AME	1.8416	2.1904
3	8.5	5.5	70	20	48.63	49.10	48.80	CE	0.9992	0.9980
4	8.5	3	70	12.5	41.52	41.68	40.81	MAE	0.0013	−0.5435
5	7	5.5	50	5	33.91	35.57	33.91	RMSE	0.9514	1.5013
6	8.5	8	30	12.5	31.97	32.40	32.28	MRE (%)	0.0474	−1.5408
7	10	5.5	30	12.5	34.65	36.49	34.48	R^2	0.9836	0.9705
8	8.5	3	50	20	42.32	43.26	42.16			
9	8.5	8	50	5	39.65	39.80	40.57	*Nonparametric tests*		
10	8.5	5.5	50	12.5	53.31	52.64	52.37	*p*-value	0.8571	0.8313
11	8.5	5.5	30	20	34.65	34.40	36.84			
12	10	3	50	12.5	41.65	40.20	40.56	Optima	BBD	BBD-ANN
13	10	5.5	50	5	39.65	41.06	39.34	X_p	8.77	7.63
14	8.5	5.5	50	12.5	52.14	52.64	52.37	X_m	5.92	3.97
15	8.5	5.5	50	12.5	52.67	52.64	52.37	X_T	57.44	39.02
16	8.5	3	50	5	33.81	34.18	32.82	X_t	14.95	9.58
17	8.5	5.5	50	12.5	52.62	52.64	52.37	Response	54.83	52.95
18	10	8	50	12.5	48.97	47.30	48.93	Exp.	53.94	51.67
19	8.5	5.5	50	12.5	52.47	52.64	52.37			
20	7	8	50	12.5	39.62	39.38	39.87			
21	8.5	5.5	70	5	41.66	40.23	41.17			
22	10	5.5	50	20	48.62	47.55	45.81			
23	8.5	8	70	12.5	45.92	46.53	46.74			

(*continued on next page*)

Table 1 (*continued*)

Run[a]	Variables[b]				Response[c]			Validation[g]	
	X_p	X_m	X_T	X_t	Exp.[d]	BBD[e]	BBD-ANN[f]	BBD	BBD-ANN
24	8.5	8	50	20	44.62	45.34	46.16		
25	8.5	3	30	12.5	29.56	29.54	24.25		
26	7	5.5	70	12.5	45.71	44.96	45.71		
27	8.5	5.5	30	5	30.81	28.65	28.36		
28	7	5.5	50	20	44.51	43.70	42.96		
29	7	3	50	12.5	38.79	38.78	36.48		

[a] The total runs of experiments were designed from BBD (UDME: 29 runs of experiments designed for ultrasonic assisted derivatisation microextraction; Extraction:17 runs for extraction experiments; Derivatisation: 29 runs for derivatisation experiments).
[b] Investigated variables including: pH value (X_p), molar ratio of fluorescent reagent to analytes (X_m), temperature (X_T) and time (X_t).
[c] Responses from FLD (peak area).
[d,e,f] Experimental and predicted responses from BBD and BBD-ANN.
[g] Validation including AME: absolute maximum error, CE: coefficient of efficiency, MAE: mean absolute error, RMSE: root mean squared error, MRE: mean relative error, R^2: correlation of determination) and nonparametric tests (p-value).

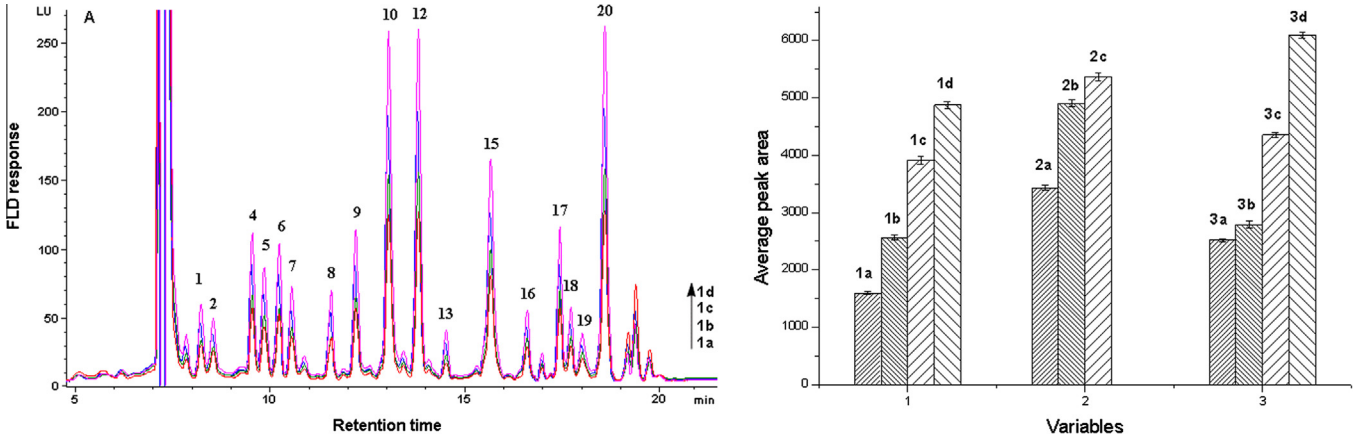

Fig. 1. Effect of solvent, cosolvent, and basic compounds on fluorescence response (A): the effect of four solvents (1a, 1b, 1c, 1d) on fluorescence responses (with *Momordica charantia* (MC) as representative), and the marked peaks were: 1, Cys; 2, His; 3, Orn; 4, Arg; 5, Lys; 6, Ser; 7, Asp; 8, Glu; 9, Thr; 10, Gly; 11, Trp; 12, Ala; 13, Tyr; 14, GABA; 15, Pro; 16, Met; 17, Val; 18, Phe; 19, Ile and 20, Leu. (B) 1 for solvents: 1a (DCM), 1b (DMF), 1c (CHF), 1d (ACN); 2 for cosolvent: 2a (ethanol–water (30:70 v/v)), 2b (HCl (0.03 M)–ethanol (70:30 v/v)), 2c (hydrochloric acid (0.03 M)) ; 3 for basic compounds: 3a (Na_2CO_3), 3b (K_2CO_3), 3c (NaOH), 3d (sodium borate buffer solution 0.2 M, pH 9.46), with the standard deviations included).

system, 5 μL EASC (0.0007 mol/L), 175 μL ACN and 100 μL sodium borate buffer (0.2 mol/L, pH 9.46) were added (pH* 8.77), and the mixture was then allowed to react in a water bath at 57 °C for 15 min.

2.5. Optimisation of conditions

The single-variable experiments were carried out to evaluate the effects of solvent composition on derivatisation yields. The selected solvents were acetonitrile (ACN), *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM) and chloroform (CHF). The co-solvents, which were used to improve the derivatisation yields were ethanol–water (30:70 v/v), hydrochloric acid (0.03 mol/L) and ethanol–HCl (0.03 mol/L) (70:30 v/v), respectively. Several types of basic catalysts including sodium borate buffer solutions (0.2 mol/L, pH 9.46), NaOH, K_2CO_3 and Na_2CO_3 were used to adjust pH value of the system to the desired value. In addition, two robust multivariate methods were applied. Multivariate combinations from Box–Behnken design (BBD) with Design-Expert 8.0.6 software were: pH value (X_p), molar ratio of fluorescent reagent to analytes (X_m), temperature (X_T) and time (X_t), data is shown in Table 1. Average peak area and optimal combination of four variables (X_p, X_m, X_T, X_t) were predicted through polynomial function fitting (Bezerra et al., 2008). With the programme Matlab R2010a, variable combinations from BBD were imported into a back-propagation ANN (i.e., BBD-ANN) combined with genetic algorithm (GA) (Cséfalvayová, Pelikan, Kralj Cigic, Kolar, & Strlic, 2010) programme to search the optimum. The two models were validated by the multi-criteria and nonparametric tests (Conover, 1980; Modarres, 2009) (see Table 1), through which the accuracy could be reflected, the best model could be indicated, and thereby the optimal variable combinations for UDME, individual extraction and derivatisation could be determined. In optimisation experiments, *Momordica charantia* contained a low analyte concentration and was therefore selected as a representative sample of bitter foods. Tyr was selected as the representative analyte as it caused lower responses in FLD than do others.

2.6. Instrumentation and conditions

Agilent HP 1100 series (Waldbron, Germany) equipped with quaternary pump (model G1311A), vacuum degasse (model G1322A), fluorescence detector (FLD) (model G1321A) and auto samplers (model G1329A, injection 10 μL) were applied. LC system was controlled by HP Chemstation software. Derivatives were separated on a reversed phase Akasil-C_{18} column (250 mm × 4.6 mm, 5 μm). A Paratherm U2 electronic water bath (Hitachi, Tokyo, Japan) was used to control temperature. Ultrasonic instrument (SB-5200DTD, 40 kHz, Xinzhi Biotech Co., Ningbo, China) was used for UDME. Mobile phases were A and B (A: CH_3CN/H_2O, *v*/*v* = 95:5; B: CH_3CN/H_2O, *v*/*v* = 5:95, containing 10 mmol/L formic acid/ammonia buffer, pH 3.7). The linear gradient conditions applied were as follows: 0–1 min, 100–85% B; 1–3 min, 85–75% B; 3–8 min, 75–62.5% B; 8–14 min, 62.5–50% B; 14–15 min, 50–40%

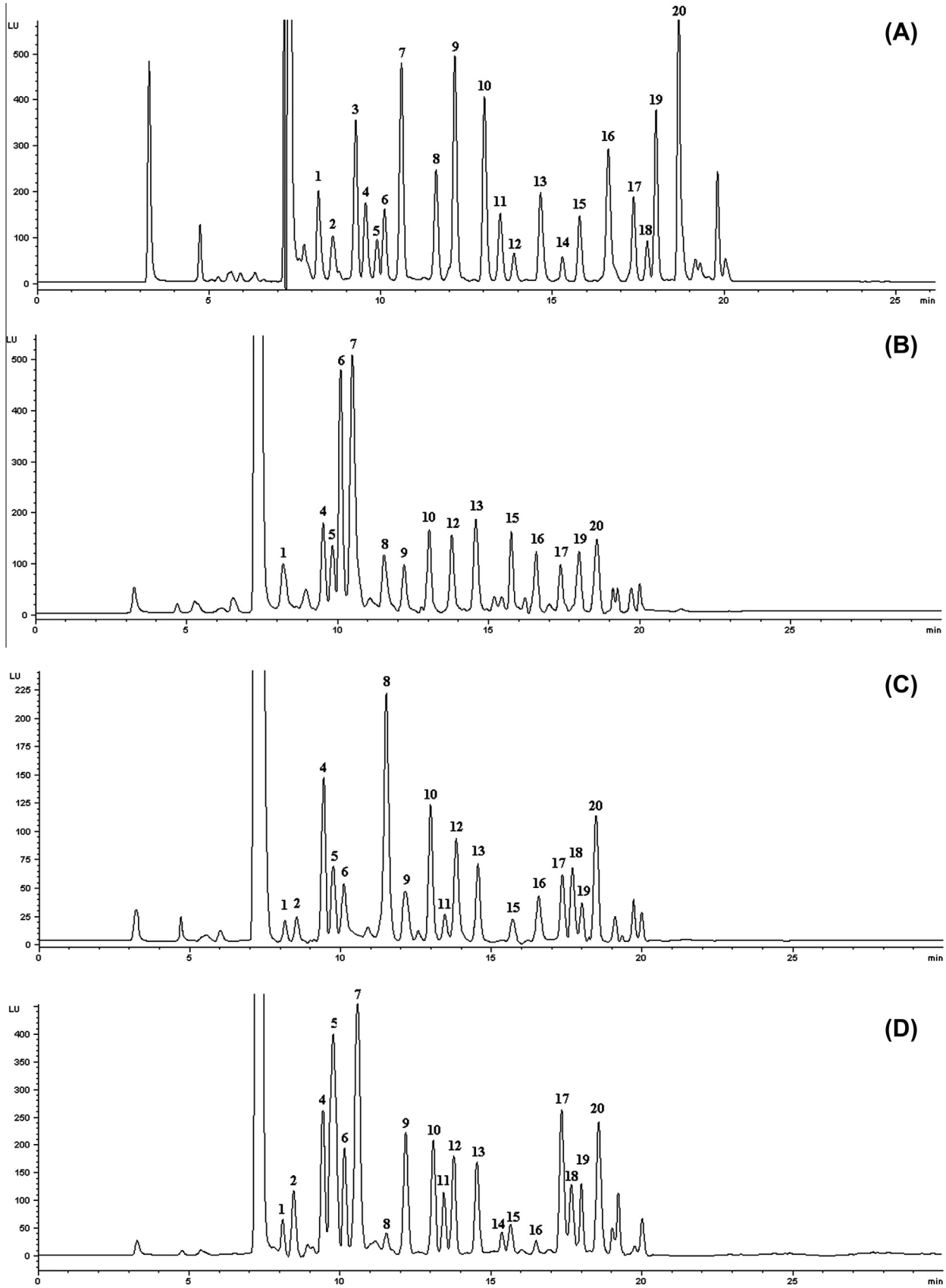

Fig. 2. Chromatograms for standard (A) amino acids (2.1 nmol/L for injection) and bitter foods (B) *Sonchus oleraceus l* (SOL), (C) *Fagopyrum tataricum* (FT), (D) *Arundinaria oleosa* (AO); Marked peaks were: 1, Cys; 2, His; 3, Orn; 4, Arg; 5, Lys; 6, Ser; 7, Asp; 8, Glu; 9, Thr; 10, Gly; 11, Trp; 12, Ala; 13, Tyr; 14, GABA; 15, Pro; 16, Met; 17, Val; 18, Phe; 19, Ile and 20, Leu.

B; 15–17 min, 40–0% B, 17–20 min, 100% A (post time 5 min). Flow rate was constant at 1.0 mL/min and column temperature was set at 30 °C. Fluorescence excitation and emission wavelengths were λ_{ex} 262 nm and λ_{em} 425 nm, respectively.

2.7. Method validation

2.7.1. Calibration curve, linearity correlation and sensitivity

Lower limit of quantification (LLOQ) defined as the lowest concentration on the standard curve was determined to indicate the method sensitivity. Based on LLOQ and ULOQ (FDA, 2001) (upper limit of quantification), triplicate calibration standards for each AA at seven concentration points were analysed. Calibration curves were obtained by linear regression analysis of the peak area (Y) versus the injected concentration (X). Linear equations were established to determine the concentration of analytes.

2.7.2. Accuracy, precision and extraction recovery

Six replicates analyses of the calibration standards were carried out to obtain the accuracy and precision which were evaluated by the relative error percentage (RE%) and relative standard deviations (RSD%), respectively. The intermediate precision was determined by performing the same operations over six days under different operating condition at LLOQ and ULOQ levels. Precision in samples matrices were determined by performing six replicated analyses of bitter food samples (here, the analyte concentrations in *Sonchus oleraceus l* (SOL: 3.1 mg), *Fagopyrum tataricum* (FT: 2.6 mg), *Arundinaria oleosa* (AO: 2.3 mg), *Allium chinense* (AC: 1.3 mg), *Momordica charantia* (MC: 3.0 mg) and *Bitter almond* (BA: 3.0 mg) were in the linear range). Three batches of samples, each one of which consisted of six replicates of spiked samples at three QC levels, were analysed on three consecutive validation days to obtain the recovery following the equation: recovery (%) = $S_b/S_a \times 100\%$, where S_b and S_a are the peak area values of each spiked QC concentration before and after the derivatisation extraction, respectively.

2.7.3. Application to bitter foods

BTT values of six bitter foods and their control samples were investigated by the statistical taste test (Section 2.2). Sample at its BTT amount level was pretreated and analysed with UDME–HPLC–FLD. For higher reliability, batch analyses of different parts from food sample were performed. The CABT and RCB values of twelve foods were obtained and their relationships to the bitterness of food were discussed.

3. Results and discussion

3.1. Optimisation of UDME: single variable optimisation

Six variables (solvent, cosolvent, temperature, time, molar ratio of reagent to analytes, pH value) were chosen for optimisation. Solvent experiments revealed that acetonitrile (ACN) was superior to other three in terms of fluorescence responses (Fig. 1A). The strongest fluorescence was observed in solvent ACN with hydrochloric acid (0.03 M) added (Fig. 1B-2c), which was presumably caused by the synergetic effect of the protonation by HCl and the miscibility of ACN with HCl. In addition, no increase of response was observed within 48 h, indicating that ACN–HCl system might prevent protein from being hydrolysed even in an acidic atmosphere. It should be noted that the acidic solvent was helpful to extract the FAA, whereas the weak basic atmosphere was beneficial to the derivatisation reaction of amino group with sulfonyl chloride functional group from EASC molecule. There was a significant decrease in the fluorescence responses with addition of an excess of Na_2CO_3, K_2CO_3 and NaOH (Fig. 1B-3a, 3b, 3c). This should be attributed to the hydrolysis of derivatives in an excess of alkaline atmosphere. In addition, a low alkaline medium will also lead to low yields in derivatisation. The results indicated that the highest yield could be obtained in ACN–HCl system with addition of 85–110 μL of sodium borate buffer solution (Fig. 1B-3d) and no obvious fluorescence differences were observed within 48 h.

3.2. Optimisation of UDME: multivariate optimisation

Interactions of variables in the UDME procedure are very complicated, thus they must be thoroughly investigated with multivariate methods (Bezerra et al., 2008). Among the above variables, X_p, X_m, X_T and X_t were delivered into multivariate models as the initial input data, since the four variables were more closely interrelated. As shown in Table 1, the experimental and predicted responses from BBD and BBD-ANN models were validated by multi-criteria

Table 2
Linear regression equations, correlation coefficients, linear range, LOD, LLOQ and repeatability for peak areas and retention time.

AA[a]	$Y = A \times X + B$ [b]	Coefficient	Linear range (nmol/L)	LOD[c] (nmol/L)	LLOQ[c] (nmol/L)	Retention time (RSD %) (min)	RSD of peak area (%)
Cys	$Y = 10731.84X + 50.13$	0.9990	1.83–633.04	0.54	1.83	8.17 (0.07)	1.61
His	$Y = 7940.97X + 13.66$	0.9993	2.46–576.28	0.82	2.46	8.59 (0.03)	1.29
Orn	$Y = 11042.36X + 81.75$	0.9995	0.57–704.48	0.15	0.57	9.26 (0.06)	0.38
Arg	$Y = 8132.39X - 40.22$	0.9994	0.82–759.13	0.25	0.82	9.55 (0.02)	0.97
Lys	$Y = 5681.66X - 116.39$	0.9993	3.64–624.38	1.05	3.64	9.88 (0.11)	1.06
Ser	$Y = 8995.99X - 30.68$	0.9995	1.16–683.69	0.40	1.16	10.10 (0.07)	0.51
Asp	$Y = 10980.24X - 204.30$	0.9987	0.50–429.59	0.15	0.50	10.59 (0.15)	1.89
Glu	$Y = 9448.3X - 9.82$	0.9991	0.87–478.57	0.26	0.87	11.60 (0.06)	0.34
Thr	$Y = 16306.15X + 93.98$	0.9998	0.31–661.19	0.09	0.31	12.14 (0.12)	0.22
Gly	$Y = 9469.87X - 7.33$	0.9996	0.29–896.78	0.07	0.29	13.00 (0.16)	1.69
Trp	$Y = 7597.84X + 50.13$	0.9992	0.65–770.42	0.38	1.27	13.47 (0.12)	0.65
Ala	$Y = 6852.57X + 65.41$	0.9994	3.28–842.79	0.94	3.28	13.87 (0.14)	0.73
Tyr	$Y = 9190.65X - 57.86$	0.9995	0.65–473.62	0.19	0.65	14.64 (0.05)	0.86
GABA	$Y = 8531.26X - 21.65$	0.9991	5.43–815.69	1.71	5.43	15.28 (0.09)	0.47
Pro	$Y = 7634.78X + 16.67$	0.9989	0.74–712.02	0.22	0.74	15.78 (0.13)	0.88
Met	$Y = 10838.99X + 6.52$	0.9997	0.39–488.45	0.11	0.39	16.61 (0.04)	0.11
Val	$Y = 6520.04X - 3.99$	0.9993	0.56–516.42	0.16	0.56	17.35 (0.04)	0.35
Phe	$Y = 3496.77X + 19.69$	0.9992	2.83–623.84	0.85	2.83	17.74 (0.08)	1.72
Ile	$Y = 5348.06X - 98.83$	0.9989	0.39–637.24	0.12	0.39	18.00 (0.17)	1.21
Leu	$Y = 6454.56X + 6.52$	0.9994	0.21–692.68	0.06	0.21	18.66 (0.03)	0.36

[a] AA: The twenty amino acids.
[b] X: Injected concentration (nmol/L); Y: Peak area.
[c] LOD were established based on a signal-to-noise ratio of 3 and LLOQ were the lowest concentration on the standard curve that can be quantitated within acceptable range.

and nonparametric tests (Conover, 1980; Modarres, 2009). Both models provided satisfactory results of CE (coefficient of efficiency, 0.9967 and 0.9969) and R^2 (correlation of determination, 0.9260 and 0.9375) for UDME. The absolute values of MRE (mean relative error) were <2%, indicating that the two models were fully operational and the predicted responses could be correlated well with the experimental responses(Cabrera & Prieto, 2010). The *p*-values were >0.05, demonstrating that the differences between experimental and predicted values were not statistically significant and both the two models simulated the statistic characteristic at the 95% confidence level(Modarres, 2009). The higher response (66.25) was obtained from BBD. Though higher temperature (42.91) was beneficial to extraction, it was not recommended owing to the potential hydrolysis of protein by enzymes in fresh food sample (Shen, Guo, Dai, & Zhang, 2012). In contrast, lower temperature (30.96) from BBD-ANN prevented the hydrolysis of protein to a great extent. Furthermore, the lower molar ratio (3.58) offered the low depletion and the shorter time (11.41) improved the efficiency of analysis. Therefore, it was more preferable to choose the results from BBD-ANN models as the experimental conditions.

To make a comparison, individual extraction and derivatisation procedures were optimised according to the same conditions as described above (Table 1). Obviously, both the two responses were lower than that obtained by UDME, implying that the individual procedures were less effective than UDME technique even in their respective optimum conditions. Moreover, the individual procedures were complex and time-consuming in the practical application. Consequently, UDME offered the advantages of high yields, low depletion, high efficiency and simple operation. The variable combination (X_p: 6.51, X_m: 3.58, X_T: 30.96, X_t: 11.41) was thought to be optimal and its approximation (X_p: 6.51, X_m: 3.58, X_T: 31, X_t: 11.5) was applied for later experiments.

Table 3
The bitter taste threshold (BTT), content of individual and total free amino acids (CABT) and ratio of CABT to BTT (RCB) in bitter foods and their control samples.

Samples[a]	SOL	LT	FT	FE	AO	DL
BTT[b]	0.307 ± 0.008	0.986 ± 0.035	0.263 ± 0.015	0.618 ± 0.031	0.235 ± 0.009	0.305 ± 0.021
Cys	0.168 ± 0.007	–	0.142 ± 0.002	0.063 ± 0.002	0.272 ± 0.006	0.517 ± 0.024
His	–	0.060 ± 0.002	0.284 ± 0.002	0.162 ± 0.009	0.356 ± 0.006	0.043 ± 0.002
Orn	–	–	–	–	–	–
Arg	0.322 ± 0.015	0.182 ± 0.005	1.052 ± 0.069	0.656 ± 0.024	0.995 ± 0.032	0.768 ± 0.038
Lys	0.256 ± 0.008	0.117 ± 0.005	0.484 ± 0.012	0.404 ± 0.015	1.237 ± 0.033	–
Ser	0.765 ± 0.023	0.133 ± 0.007	0.320 ± 0.007	0.481 ± 0.017	0.576 ± 0.016	0.317 ± 0.005
Asp	0.842 ± 0.023	0.681 ± 0.039	–	0.577 ± 0.017	1.354 ± 0.058	0.214 ± 0.014
Glu	0.216 ± 0.013	0.543 ± 0.016	1.474 ± 0.072	0.900 ± 0.025	0.081 ± 0.001	0.159 ± 0.006
Thr	0.137 ± 0.005	0.140 ± 0.004	0.299 ± 0.015	0.248 ± 0.005	0.599 ± 0.009	0.513 ± 0.014
Gly	0.273 ± 0.011	0.122 ± 0.004	0.495 ± 0.025	0.494 ± 0.019	0.555 ± 0.016	0.675 ± 0.014
Trp	–	–	0.136 ± 0.005	0.030 ± 0.001	0.185 ± 0.005	–
Ala	0.270 ± 0.003	0.149 ± 0.009	0.672 ± 0.018	0.575 ± 0.024	0.887 ± 0.026	0.743 ± 0.018
Tyr	0.385 ± 0.018	0.036 ± 0.003	0.404 ± 0.007	0.386 ± 0.009	0.833 ± 0.039	0.258 ± 0.008
GABA	–	–	–	–	0.236 ± 0.010	–
Pro	0.264 ± 0.011	0.183 ± 0.004	0.267 ± 0.011	0.284 ± 0.011	0.365 ± 0.009	0.168 ± 0.007
Met	0.223 ± 0.005	0.025 ± 0.001	0.374 ± 0.011	0.443 ± 0.028	0.202 ± 0.007	–
Val	0.188 ± 0.007	0.187 ± 0.007	0.619 ± 0.024	0.524 ± 0.013	0.917 ± 0.063	0.160 ± 0.003
Phe	–	0.162 ± 0.006	0.742 ± 0.021	0.155 ± 0.006	0.413 ± 0.018	0.274 ± 0.010
Ile	0.257 ± 0.009	0.169 ± 0.004	0.382 ± 0.007	0.337 ± 0.009	0.476 ± 0.020	0.183 ± 0.006
Leu	0.266 ± 0.011	0.209 ± 0.006	0.946 ± 0.047	0.363 ± 0.005	0.786 ± 0.029	0.067 ± 0.002
CABT (total)[c]	4.664 ± 0.065	3.098 ± 0.043	8.664 ± 0.199	7.084 ± 0.306	10.696 ± 0.232	5.059 ± 0.008
RCB[d]	15.172 ± 0.548	3.141 ± 0.052	32.930 ± 1.485	11.468 ± 0.189	45.439 ± 1.072	16.607 ± 0.718
	AC	AMB	MC	BMC	BA	XA
BTT[b]	0.026 ± 0.004	0.196 ± 0.008	0.004 ± 0.000	0.016 ± 0.002	0.003 ± 0.00	0.963 ± 0.010
Cys	–	0.027 ± 0.001	0.068 ± 0.002	0.021 ± 0.001	0.094 ± 0.003	0.118 ± 0.005
His	0.037 ± 0.001	0.096 ± 0.005	0.043 ± 0.001	0.002 ± 0.000	0.175 ± 0.008	0.140 ± 0.009
Orn	0.032 ± 0.002	–	–	–	–	–
Arg	0.049 ± 0.001	0.189 ± 0.004	0.186 ± 0.006	0.036 ± 0.002	0.534 ± 0.029	0.788 ± 0.031
Lys	0.148 ± 0.003	0.213 ± 0.011	0.124 ± 0.0056	0.022 ± 0.001	0.244 ± 0.007	0.122 ± 0.008
Ser	0.896 ± 0.038	0.146 ± 0.003	0.185 ± 0.008	–	0.424 ± 0.009	0.243 ± 0.008
Asp	0.037 ± 0.001	0.416 ± 0.008	0.094 ± 0.003	0.004 ± 0.000	0.589 ± 0.025	0.925 ± 0.057
Glu	0.029 ± 0.001	1.093 ± 0.053	0.121 ± 0.002	0.021 ± 0.001	0.888 ± 0.023	1.369 ± 0.048
Thr	0.579 ± 0.019	0.141 ± 0.005	0.187 ± 0.005	0.158 ± 0.004	0.288 ± 0.017	0.231 ± 0.007
Gly	0.013 ± 0.000	0.136 ± 0.005	0.360 ± 0.009	0.532 ± 0.021	0.355 ± 0.016	0.477 ± 0.012
Trp	0.036 ± 0.002	–	–	–	–	–
Ala	0.058 ± 0.002	0.036 ± 0.001	0.368 ± 0.011	0.017 ± 0.001	0.342 ± 0.015	0.425 ± 0.017
Tyr	0.023 ± 0.001	0.076 ± 0.003	0.054 ± 0.002	0.070 ± 0.002	0.138 ± 0.004	0.211 ± 0.008
GABA	–	–	–	–	–	–
Pro	0.079 ± 0.002	0.355 ± 0.013	0.267 ± 0.008	0.040 ± 0.002	0.282 ± 0.013	0.693 ± 0.018
Met	0.341 ± 0.016	0.072 ± 0.002	0.059 ± 0.002	0.971 ± 0.018	0.126 ± 0.002	0.028 ± 0.001
Val	1.279 ± 0.031	0.263 ± 0.008	0.174 ± 0.010	0.076 ± 0.003	0.421 ± 0.011	0.529 ± 0.017
Phe	0.332 ± 0.017	0.225 ± 0.009	0.092 ± 0.002	0.069 ± 0.001	0.114 ± 0.002	0.429 ± 0.012
Ile	0.759 ± 0.044	0.211 ± 0.005	0.085 ± 0.004	0.086 ± 0.003	0.178 ± 0.006	0.311 ± 0.011
Leu	0.067 ± 0.002	0.311 ± 0.011	0.389 ± 0.010	0.754 ± 0.029	0.406 ± 0.015	0.598 ± 0.028
CABT (total)[c]	4.724 ± 0.172	4.005 ± 0.087	2.744 ± 0.064	2.879 ± 0.099	5.327 ± 0.283	7.637 ± 0.041
RCB[d]	178.939 ± 4.318	20.393 ± 0.942	784.029 ± 35.360	175.531 ± 7.926	1775.689 ± 114.017	7.935 ± 0.129

[a] The studied bitter food and their control samples: *Sonchus oleraceus l* (SOL) and Lettuce (LT); *Fagopyrum tataricum* (FT) and *Fagopyrum esculentum* (FE); *Arundinaria oleosa* (AO) and *Dendrocalamus latiflorus* (DL); *Allium chinense* (AC) and *Allium macrostemon bunge* (AMB); *Momordica charantia* (MC) and *Black momordica charantia* (BMC); *Bitter almond* (BA) and *Xinjiang Almond* (XA).
[b] BTT (g), the bitter taste threshold of the fresh food.
[c] The CABT (mg/100 g) of total free amino acids obtained from the sum of above individual CABT value.
[d] RCB, the ratio of CABT (total) to the BTT; –, not detected.

3.3. Separation and detection method development

Generally, the complete separation of the amino acid mixture required tedious procedure with a relatively long run time (Gatti et al., 2010; Jiménez-Martin et al., 2012; Kelly et al., 2010; Li et al., 2011; Nagy et al., 2003; Thiele et al., 2008; Wang et al., 2010; Zhao et al., 2013). To improve separation efficiency, several conventional chromatographic columns, Spherisorb C_{18} (200 mm × 4.6 mm, 5 μm), Hypersil C_{18} (200 mm × 4.6 mm, 5 μm), Thermo hypersil Gold (200 mm × 4.6 mm, 5 μm) and Akasil-C_{18} column (250 mm × 4.6 mm, 5 μm) were evaluated, and labelling reagents (DBCEOC-Cl (Sun et al., 2011), DBCEC (Li et al., 2011) and EASC) were also studied comparatively. Though it is difficult to rapidly separate multiple AA with the conventional column, the complete HPLC separation and detection of twenty EASC-derivatised AA in only 20 min was achieved on Akasil-C_{18} column (Fig. 2A). As observed, EASC molecule and its derivatives can be easily eluted by organic phase, the elution gradient beginning with an rapid increase of organic phase in a short interval (0–1 min) was designed in order to rapidly sequence the derivatives on column. Accordingly, the excess fluorescent reagent was eluted prior to the AA derivatives, and other hydrophilic impurities would be eluted following derivatives. As expected, twenty FAA from food samples were all detected in 20 min (Fig. 2 and Supplementary Fig. 1), which was much more efficient than those reported elsewhere. In addition, another advantage is that no additional column cleaning is required in the process of continuous injection. Thus the twenty FAA could be simultaneously detected more efficiently and conveniently with the developed UDME-HPLC–FLD method.

3.4. Calibration curve, linearity correlation and sensitivity

As can be seen from Table 2, each analyte showed good linearity with correlation coefficient of >0.9987. The relative standard deviations of retention time and peak area were <0.17% and <1.89%, respectively. The LOD and LLOQ were 0.06–1.71 nmol/L and 0.21–5.43 nmol/L, respectively. These results demonstrated that the method presented more satisfactory analytical sensitivity for detection of FAA than those reported methods (Armenta et al., 2009; Boogers et al., 2008; Gatti et al., 2010; Jiménez-Martin et al., 2012; Kelly et al., 2010; Li et al., 2011; Liming, Jinhui, Xiaofeng, Yi, & Jing, 2009; Nagy et al., 2003; Tan et al., 2011; Thiele et al., 2008; Wang et al., 2010; Zhao et al., 2013). The excellent selectivity and sensitivity might be attributed to the low interferences and strong fluorescence responses of UDME technique. Meanwhile the lower LLOQ ensured trace analysis (<μg/g in sample) of micro-sample by pre-column derivatisation.

3.5. Accuracy, precision and extraction recovery

Accuracy, precision and extraction recovery were summarised in Supplementary Table 1. RE% for accuracy ranged from -3.87% to 3.99%. The RSD% for intra-day and inter-day precision were found to be in the range of 1.13–4.18% and 1.05–3.94%, respectively. Intermediate precision varied from less than 2% (at ULOQ concentration level) to 8.48% (at LLOQ level). Recoveries of the analytes at three examined concentrations levels were in the range from 93.7% to 108.4%. The precisions within different samples matrices were determined in Supplementary Table 2, and they were generally less than 6% and frequently no more than 4%. The acceptable validation might reveal that UDME could avoid the matrix effect in an effective way.

3.6. Method comparison

Comparisons between the proposed method and several reported methods were made in terms of simplicity, sensitivity and rapid analysis (Supplementary Table 3). Remarkably, this method provided the shortest time, the lowest LOQ and LOD for instrument. Although the tested samples were various and their pretreatment procedures were different, this method still showed the advantages in simplicity and efficiency of the experiments. Furthermore, the high efficiency with a total analytical run time of 35 min should be more convenient for works with large batches of samples to be analysed.

3.7. Application to bitter food

The average minimum amounts causing the discernible bitter taste tested by ninety participants were investigated as the BTT values (Table 3). Bitter samples showed lower BTT values than their control samples. The CABT values of individual and total FAA were obtained in Table 3. Overall, bitter foods had the higher CABT values than their control samples, whereas the CABT of MC and BA were lower than those of control samples (Fig. 3A and Supplementary Fig. 2). As can be seen from Fig. 3B, the bitter foods showed higher RCB values than their control samples. Moreover, with the decreasing of BTT, the RCB of bitter samples increased significantly. That is, for the studied bitter foods, the RCB value increased with increasing of bitterness (decrease in BTT).

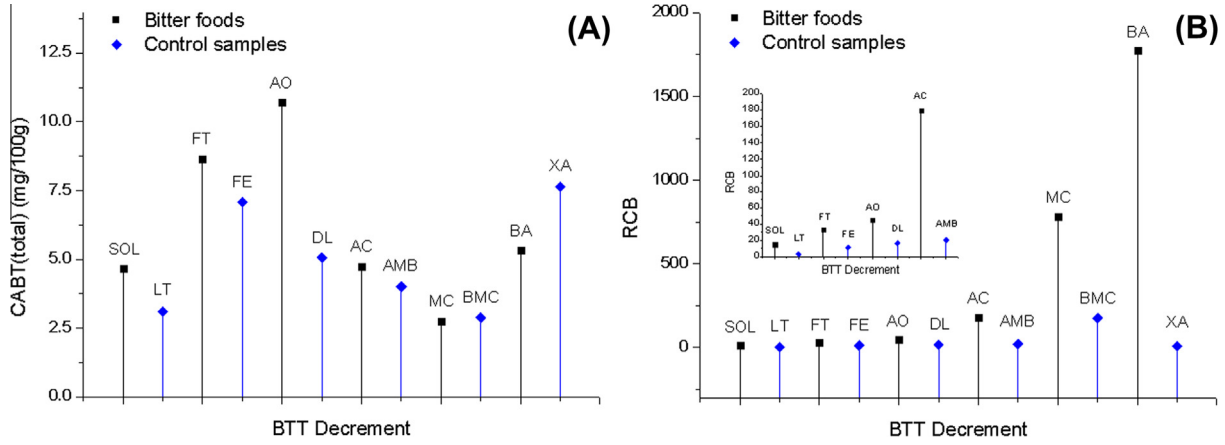

Fig. 3. The variation trends of the CABT (content of free amino acid at bitter taste threshold (BTT) amount level of food) and RCB (ratio of CABT to BTT) with the BTT decreasing.

4. Conclusions

A highly efficient and sensitive method has been proposed using a simple ultrasonic-assisted derivatisation microextraction (UDME) technique. The UDME has been proved to be more effective than the traditional pretreatment procedures and it significantly reduced the operational complexity. Derivatisation by fluorescent reagent EASC allowed the development of a sensitive and rapid method for the analysis of amino acids in combination with a conventional reversed-phase HPLC column within 20 min. The high sensitivity allows direct analysis of micro-sample generated from the trace test, such as the taste threshold test. Moreover, the method was validated to be suitable for analysis of free amino acids in a variety of food matrices. Six daily bitter foods and their control foods were investigated. The preliminary relationships (CABT versus BTT, RCB versus BTT) were achieved and the further research would be promising.

Acknowledgements

This work was supported by the 100 Talents Program of The Chinese Academy of Sciences (No. 328); National Natural Science Foundation of China (General Program) (21275089); Key Laboratory of Bioorganic Analysis Shandong Province; Key Laboratory of Pharmaceutical Intermediates and Analysis of Natural Medicine Shandong Province; the research project fund (XJ201102) of Qufu Normal University; the undergraduate research-training programs (2011A026).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.07.099.

References

Armenta, J. M., Cortes, D. F., Pisciotta, J. M., Shuman, J. L., Blakeslee, K., Rasoloson, D., et al. (2009). Sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ Tag ultra performance liquid chromatography–electrospray ionization-MS/MS with multiple reaction monitoring. *Analytical Chemistry, 82*(2), 548–558.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965–977.

Boogers, I., Plugge, W., Stokkermans, Y. Q., & Duchateau, A. L. L. (2008). Ultra-performance liquid chromatographic analysis of amino acids in protein hydrolysates using an automated pre-column derivatisation method. *Journal of Chromatography A, 1189*(1), 406–409.

Cabrera, A. C., & Prieto, J. M. (2010). Application of artificial neural networks to the prediction of the antioxidant activity of essential oils in two experimental *in vitro* models. *Food Chemistry, 118*(1), 141–146.

Conover, W. (1980). *Practical nonparametric statistics* (2nd ed.). New York.: John Wiley & Sons.

Cséfalvayová, L., Pelikan, M., Kralj Cigic, I., Kolar, J., & Strlic, M. (2010). Use of genetic algorithms with multivariate regression for determination of gelatine in historic papers based on FT-IR and NIR spectral data. *Talanta, 82*(5), 1784–1790.

FDA (2001). Guidance for industry: Bioanalytical method validation. In *US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research*. Rockville, MD: CDER.

Gatti, R., Gioia, M. G., Leoni, A., & Andreani, A. (2010). 2, 5-Dimethyl-1H-pyrrole-3, 4-dicarbaldehyde as a precolumn derivatization reagent for HPLC/UV detection of amino acids. *Journal of Pharmaceutical and Biomedical Analysis, 53*(2), 207–211.

Głowacki, R., Bald, E., & Jakubowski, H. (2011). An on-column derivatization method for the determination of homocysteine-thiolactone and protein N-linked homocysteine. *Amino Acids, 41*(1), 187–194.

Grover, J., & Yadav, S. (2004). Pharmacological actions and potential uses of *Momordica charantia*: A review. *Journal of Ethnopharmacology, 93*(1), 123–132.

Hanrahan, G. (2010). Computational neural networks driving complex analytical problem solving. *Analytical Chemistry, 82*(11), 4307–4313.

Jámbor, A., & Molnár-Perl, I. (2009). Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride: Literature overview and further study. *Journal of Chromatography A, 1216*(15), 3064–3077.

Jiménez-Martin, E., Ruiz, J., Pérez-Palacios, T., Silva, A., & Antequera, T. (2012). Gas chromatography–mass spectrometry method for the determination of free amino acids as their dimethyl-tert-butylsilyl (TBDMS) derivatives in animal source food. *Journal of Agricultural and Food Chemistry, 60*(10), 2456–2463.

Kano, Y., & Goto, H. (2003). Relationship between the occurrence of bitter fruit in cucumber (*Cucumis sativus* L.) and the contents of total nitrogen, amino acid nitrogen, protein and HMG-CoA reductase activity. *Scientia Horticulturae, 98*(1), 1–8.

Kelly, M. T., Blaise, A., & Larroque, M. (2010). Rapid automated high performance liquid chromatography method for simultaneous determination of amino acids and biogenic amines in wine, fruit and honey. *Journal of Chromatography A, 1217*(47), 7385–7392.

Kirimura, J., Shimizu, A., Kimizuka, A., Ninomiya, T., & Katsuya, N. (1969). Contribution of peptides and amino acids to the taste of foods. *Journal of Agricultural and Food Chemistry, 17*(4), 689–695.

Kvitvang, H. F. N., Andreassen, T., Adam, T., Villas-Bôas, S. G., & Bruheim, P. (2011). Highly sensitive GC/MS/MS method for quantitation of amino and nonamino organic acids. *Analytical Chemistry – Columbus, 83*(7), 2705–2711.

Li, G., Cui, Y., You, J., Zhao, X., Sun, Z., Xia, L., et al. (2011). Determination of trace amino acids in human serum by a selective and sensitive pre-column derivatization method using HPLC–FLD–MS/MS and derivatization optimization by response surface methodology. *Amino Acids, 40*, 1185–1193.

Liming, W., Jinhui, Z., Xiaofeng, X., Yi, L., & Jing, Z. (2009). Fast determination of 26 amino acids and their content changes in royal jelly during storage using ultra-performance liquid chromatography. *Journal of Food Composition and Analysis, 22*(3), 242–249.

Mazer, T. B., Marchio, A., & Acosta, P. (2003). *Medical food tablets containing free amino acids*. U.S. Patents Application.

Modarres, R. (2009). Multi-criteria validation of artificial neural network rainfall-runoff modeling. *Hydrology and Earth System Sciences, 13*(3), 411–421.

Nagy, K., Takáts, Z., Pollreisz, F., Szabó, T., & Vékey, K. (2003). Direct tandem mass spectrometric analysis of amino acids in dried blood spots without chemical derivatization for neonatal screening. *Rapid Communications in Mass Spectrometry, 17*(9), 983–990.

Nelson, G., Chandrashekar, J., Hoon, M. A., Feng, L., Zhao, G., Ryba, N. J. P., et al. (2002). An amino-acid taste receptor. *Nature, 416*(6877), 199–202.

Shen, Q., Guo, R., Dai, Z., & Zhang, Y. (2012). Investigation of enzymatic hydrolysis conditions on the properties of protein hydrolysate from fish muscle (*Collichthys niveatus*) and evaluation of its functional properties. *Journal of Agricultural and Food Chemistry, 60*(20), 5192–5198.

Shi, T., Tang, T., Qian, K., Wang, F., Li, J., & Cao, Y. (2009). High-performance liquid chromatographic method for determination of amino acids by precolumn derivatization with 4-chloro-3,5-dinitrobenzotrifluoride. *Analytica Chimica Acta, 654*(2), 154–161.

Stone, H., Bleibaum, R., & Thomas, H. A. (2012). *Sensory evaluation practices* (4th ed.). Elsevier Inc.

Sun, Z., You, J., Li, G., Zhao, X., Suo, Y., & Wang, X. (2011). Determination of amino acids in rat brain microdialysate with 1, 2, 5, 6-dibenzocarbazole-9-ethyl chloroformate as labeling reagent by high performance liquid chromatographic fluorescence detection and mass spectrometric identification. *Journal of Chromatography B, 879*, 1367–1374.

Tan, F., Tan, C., Zhao, A., & Li, M. (2011). Simultaneous determination of free amino acid content in tea infusions by using high-performance liquid chromatography with fluorescence detection coupled with alternating penalty trilinear decomposition algorithm. *Journal of Agricultural and Food Chemistry, 59*(20), 10839–10847.

Thiele, B., Füllner, K., Stein, N., Oldiges, M., Kuhn, A. J., & Hofmann, D. (2008). Analysis of amino acids without derivatization in barley extracts by LC–MS-MS. *Analytical and Bioanalytical Chemistry, 391*(7), 2663–2672.

Visser, W. F., Verhoeven-Duif, N. M., Ophoff, R., Bakker, S., Klomp, L. W., Berger, R., et al. (2011). A sensitive and simple ultra-high-performance-liquid chromatography–tandem mass spectrometry based method for the quantification of d-amino acids in body fluids. *Journal of Chromatography A, 1218*(40), 7130–7136.

Wang, L., Xu, R., Hu, B., Li, W., Sun, Y., Tu, Y., et al. (2010). Analysis of free amino acids in Chinese teas and flower of tea plant by high performance liquid chromatography combined with solid-phase extraction. *Food Chemistry, 123*(4), 1259–1266.

You, J., Zhao, H., Sun, Z., Xia, L., Yan, T., Suo, Y., et al. (2009). Application of 10-ethyl-acridine-3-sulfonyl chloride for HPLC determination of aliphatic amines in environmental water using fluorescence and APCI–MS. *Journal of Separation Science, 32*(9), 1351–1362.

Zhao, M., Ma, Y., Dai, L., Zhang, D., Li, J., Yuan, W., et al. (2013). A high-performance liquid chromatographic method for simultaneous determination of 21 free amino acids in tea. *Food Analytical Methods, 6*(1), 63–75.