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Highly selective and sensitive determination of free and total amino acids in *Apocynum venetum* L. (Luobuma tea) by a developed HPLC–FLD method coupled with pre-column fluorescent labelling

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Abstract

Amino acids (AA) are important chemical constituents of tea leaves remarkably influencing the quality of tea. In this study, free AA and total AA in *Apocynum venetum* L. (Luobuma tea) were estimated by HPLC equipped with fluorescent detector using 2-[2-(7H-dibenzo[a,g]carbazol-7-yl)-ethoxy] ethyl chloroformate (DBCEEC) as a fluorescent labelling reagent. Different parameters for derivatization and separation were optimized. AA were rapidly derivatized within 3 min at room temperature with DBCEEC. In conjunction with a gradient elution, a baseline resolution of 20 analytes was achieved on a reversed-phase Hypersil BDS C18 column. LC separation for the derivatized AA showed good reproducibility. Twenty AA were detected and showed significant linear responses with correlation coefficients (>0.9992). This developed method offered the low detection limit of 2.88–23.4 fmol.

Keywords: *amino acids analysis, HPLC–FLD analysis, pre-column derivatization,* Apocynum venetum *L*

Introduction

Apocynum venetum L. (Luobuma) is a perennial shrub, widely distributed throughout the Mediterranean area and northwestern China. Its leaves gained popularity among the Chinese people as a readily available source of tea for everyday use. Recently, different preparations made from Luobuma leaves have become a valuable health-promoting functional beverage in Japan and are marketed as anti-ageing nutritional supplements. Recent investigations confirmed that Luobuma leaf extract shows cholesterol-lowering, anti-hypertensive, anti-low-density lipoprotein oxidation, anti-lipid peroxidation effects and anxiolytic activities (Kim et al. 1998; Yokozawa et al. 1998; Yokozawa and Nakagawa 2004). Findings of current studies conclude that Luobuma tea possesses therapeutic benefits against cardiovascular disease, which is the most common cause of mortality and morbidity worldwide (Yokozawa and Nakagawa 2004).

Amino acids (AA) are important in human nutrition. Free AA (FAA) affect food palatability (Hermanussen et al. 2009) and are important chemical constituents with pronounced effect on the quality of tea (Ruan et al. 1999). In general, the taste of AA depends on their lateral chain. The majority of D-amino acids are sweet, L-amino acids are bitter and glutamic acid present umami taste (Belitz and Grosch 1997). In addition, the intensity of the gustative sensation depends on the hydrophobic character of the lateral chains (Belitz and Grosch 1997). γ-Amino-butyric acid (GABA) has received more attention

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because it can reduce psychological stress and boost immunity in humans (Yokogoshi 2009). Investigations revealed GABA plays an important role in sensation of taste (Obata et al. 1997), palatability of food and drink or chocolate (Hermanussen et al. 2009). Different bioactive ingredients have been detected in Luobuma leaf include rutin, D-catechin, quercetin and kaempferol (Cao et al. 2001; Grundmann et al. 2009). However, the FAA composition in Luobuma leaf remains poorly investigated. Therefore, quantitative determination of FAA is of immense importance to the farmers (picking, storage), processors (quality control), traders (trading price) and consumers (for further application of Luobuma leaf).

Most AA show neither natural UV absorption nor fluorescence (FL). Therefore, chemical derivatization is necessary to increase detection sensitivity and improve selectivity by means of high-performance liquid chromatography (HPLC) and electrophoretic microchips (Munro et al. 2000) or capillary electrophoretic (Prata et al. 2001; Lin et al. 2007) separation. These techniques have been widely used in food, chemical and biological sciences for AA analysis. Our recent investigation described the synthesis of a sensitive and selective labelling reagent 2-[2-(7H-dibenzo[a,g]carbazol-7-yl)-ethoxy] ethyl chloroformate (DBCEEC) for estimation of aliphatic amines (You et al. 2009) and the analytes labelled by DBCEEC showed excellent fluorescent sensitivity. In this study, a sensitive method was devised using DBCEEC as labelling reagent for determination of total AA (TAA) and FAA in Luobuma teas by HPLC equipped with fluorescence detector (FLD).

Experimental

Instrumentation

HPLC separation, mass spectrometry (MS) identification and analysis of samples were performed using Agilent 1100 Series HPLC/MS (HPLC–MS ion Trap SL, a complete LC–MS/MS). All the HPLC system devices were from the Agilent 1100 Series and consisted of an online vacuum degasser (Model G1322A), a quaternary pump (Model G1311A), an autosampler (Model G1329A), a thermostated column compartment (Model G1316A), a FLD (Model G1321A) and a diode array detector (Model G1315A). The MS 1100 Series LC–MSD Trap SL (ion trap) from Bruker Daltonics (Bremen, Germany) was equipped with an electrospray ionization (ESI) source. Ion source conditions: ESI in positive ion mode; nebulizer pressure, 241.3 kPa; dry gas temperature, 350°C; dry gas flow, 9.0 l/min and capillary voltage, 3500 V. Derivatives were separated on Hypersil BDS C18 column (200 mm × 4.6 mm, 5 μm; Yilite Co, Dalian, China). FL excitation and emission spectra were obtained on a 650-10S FL spectrophotometer (Hitachi, Japan). The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL, USA).

Chemicals and material

Twenty AA standards including L-alanine (Ala), L-arginine (Arg), L-aspartic acid (Asp), L-cystine (Cys), L-glutamic acid (Glu), L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), ornithine (Orn), cystine [$(Cys)_2$], glycine (Gly), L-methionine (Met), L-phenylalanine (Phe), L-proline (Pro), L-serine (Ser), L-threonine, (Thr), L-tyrosine (Tyr), L-valine (Val) and GABA were purchased from Sigma Corporation. HPLC-grade acetonitrile was obtained from Yuwang Company (China). Other reagents such as acetic acid were all analytically grade. Pure water was prepared by Milli-Q super pure water system. DBCEEC was synthesized according to our previous study (You et al. 2009). Luobuma leaves were obtained from Geermu (elevation 2800 m) and Delingha (elevation 2980 m), Qinghai province. Luobuma samples were thoroughly lyophilized and milled to a fine powder (20 mesh), kept for 7 days in a vacuum desiccator until a constant weight before submitted for chemical analysis.

Preparation of standard solutions

Individual stock solutions (1×10^{-2} M) of AA were prepared in water. The mixed standard solutions for HPLC analysis were prepared by diluting the stock solutions with 0.2 M sodium borate buffer (pH 9.0). The DBCEEC solution (0.01 mol/l) was prepared by dissolving 32.6 mg of DBCEEC in 10 ml of acetonitrile, and the corresponding low concentration of reagent (5.0 mmol/l) was diluted by acetonitrile.

Sample hydrolysis

Sample hydrolysis was done following a reported study with minor revision (Ayaz et al. 2011). A 50 mg sample (Luobuma leaves) was placed in a 50 × 6 mm test tube, 6 M hydrochloric acid (1000 μl) containing 0.1% (w/v) phenol was added and the test tube was sealed. After hydrolysis at 110°C for 24 h, the contents were evaporated to dryness under the stream of nitrogen. The precipitate was re-dissolved in 1.0 ml of borate buffer (pH 9.0) and filtered through a 0.2 μm nylon membrane filter. The final solution was made up to 2-ml with borate buffer (pH 9.0) and stored at 4°C until HPLC analysis.

FAA extraction from samples

The extraction of FAA was performed following the method developed by Ding et al. (2002) with slight modifications. An aliquot of 50 mg of Luobuma leaf was inserted into a test tube, 1 ml of hot water (80°C) was added and mixture was sonicated (high-frequency

setting) for 90 min in an ultrasonic bath. The solution was filtered through a 0.2-μm nylon membrane filter and made up to 2-ml with borate buffer (pH 9.0) and stored at 4°C until HPLC analysis.

Derivatization procedure

The derivatization proceeded in acetonitrile solution in a basic medium. About 80 μl of acetonitrile, 100 μl of 0.2 M sodium borate buffer (pH 9.0), 20 μl of AA and 200 μl DBCEEC were added successively into a 2.0-ml vial. The vial was sealed for 3 min at room temperature and 100 μl of 50% acetic acid was added into the solution until the final pH was 6.5. Finally, the derivatized sample solution was prepared and injected into the HPLC system for analysis. The derivatization process is shown in Figure 1.

High-performance liquid chromatography

Derivatives were separated on a reversed-phase Hypersil BDS C18 column (200 mm × 4.6 mm, 5 μm) in conjunction with a gradient elution. Eluent A was 30% acetonitrile (containing 30 mM, pH 3.60 ammonium/formic acid buffer); B was 50% acetonitrile (containing 30 mM, pH 3.60 ammonium/formic acid buffer); C was 95% acetonitrile. The flow rate was 1.0 ml min^{-1} and the column temperature was set at 35°C. The FL excitation and emission wavelengths were set at $\lambda_{ex} = 300$ nm and $\lambda_{em} = 400$ nm. The gradient elution programme was as follows: 0 min = 70% B and 0% C; 15 min = 100% B; 20 min = 95% B and 5% C; 40 min = 65% B and 35% C; 44 min = 35% B and 65% C; 55 min = 30% B and 70% C.

Results and discussion

FL spectra of DBCEEC–AA derivatives

Maximum excitation and emission wavelengths which were set in HPLC–FL detector were important for the detection sensitivity of AA derivatives. To determine the FL spectra of AA derivatives, every DBCEEC–AA sample was prepared by individual derivatization and purification on a solid-phase extraction column (ODS C18, Dalian Elite Analytical Instruments Co. Ltd, Dalian, China) and scanned on the 650-10S FL spectrophotometer. Results indicated that the maximum excitation and emission wavelengths for DBCEEC–AA derivatives were 300 and 400 nm, respectively.

Derivatization optimization

The derivatization is of great importance for the sufficient labelling of the analysed components. The main factors affecting derivatization yields are pH value of buffer, reaction time, temperature and concentration of DBCEEC. Several types of basic media were evaluated for the derivatization of AA, including phosphate buffers, carbonate buffers and borate buffers. The results indicated that borate buffers were the best one. The effect of the pH on derivatization yields was investigated in the pH range 7.7–10.5 using borate buffer solution (0.2 M). The maximum derivatization yields were achieved at pH 9.0. The higher pH values (>9.0) resulted in the lower derivatization yields because of the derivatives hydrolysis. Thus, pH 9.0 was chosen as the optimal derivatization pH.

The concentration of DBCEEC was critical for the labelling reaction and the FL intensity of DBCEEC derivatives increased with the increasing concentration of DBCEEC. The constant FL intensity was achieved upon the addition of a 2.5- to 5-fold molar reagent excess over the total molar amount of AA and increasing the reagent excess beyond this level exerted, the effect was not significant. With less than a 2.5-fold molar excess of derivatization reagent, the derivatization is incomplete and results in remarkably low detection responses. Therefore, we select fivefold molar reagent excess to total molar AA. Furthermore, the presence of water in reaction medium does not affect the derivatization yields. Derivatization of DBCEEC with AA could be achieved within 3 min at room temperature. The $(DBCEEC)_2$ was usually formed by the reaction of the hydrolysed DBCEECOH with the excess reagent DBCEEC. The presence of $(DBCEEC)_2$ and DBCEEC-OH did not interfere with the separation of AA derivatives by adjusting of mobile phase composition. According to our previous studies (You et al. 2003), 25% disubstituted His derivatives degraded to monosubstituted derivatives

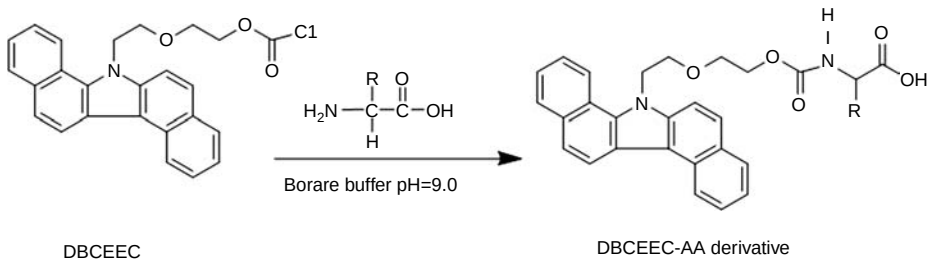

Figure 1. Scheme of derivatization reaction of DBCEEC with AA.

within 24 h. In this paper, we found that the degradation could be prevented by adding 100 µl of 50% acetic acid solution after derivatization.

HPLC separation optimization

The mobile phase pH could significantly affect the resolution. The separation for all AA derivatives with a good resolution could not be obtained by a constant pH. In previous studies (Brückner et al. 1994; Gatti et al. 2004; Schwarz et al. 2005), boric or phosphate buffer was often used to adjust the pH value of mobile phase in RP separation of AA. In this paper, formic acid/ammonia buffer was used to adjust pH value of mobile phases A and B, and could reduce the metal salt pollution in the mass ion chamber. To achieve a good resolution, pH value of mobile phase A was optimized on Hypersil BDS C18 column. The results showed that the difficult separation of Asp and Ser achieved baseline separation at acidic condition with pH 3.7. Furthermore, it was found that the pH value of mobile phase A at 3.7 could achieve a complete baseline resolution for all AA derivatives within the shortest time (Figure 2A).

MS identification by ESI/MS

The structure identification of AA derivatives was carried out by an online MS with ESI source in positive ion mode. The MS, MS/MS spectra and the cleavage mode of representative phenylalanine derivative are shown in Figure 3A, B. The molecular ions (MS, $[M + H]^+$ ion) and specific fragment ions (MS/MS) of 20 AA derivatives are showed in Table I. All derivatives exhibited an intense molecular ion peaks at $[M + H]^+$ ions. The collision-induced dissociation spectra (MS/MS) of molecular ions (MS, $[M + H]^+$ ion) produced intense and stable fragment ions at m/z 294.7, 338.7 and 356.6 (Figure 3). The selected reaction monitoring, which was based on the $[M + H]^+$ ion → m/z 294.7, 338.7 and 356.6 transition, was specific for DBCEEC-labelled AA derivatives (Figure 3). Although other endogenous amino compounds present in real samples were presumably co-extracted and derivatized by DBCEEC reagent, no interference was observed due to the highly intense molecular ions and the characteristic fragment ions.

Method validation

Reproducibility and recovery. A standard solution consisting of 25 pmol was analysed (used) to determine the reproducibility of the method. Relative standard deviations of retention time and peak area (RSD, %; $n = 6$) were less than 0.037% and 1.32%, respectively. The quantitative methods were established under the optimized conditions with FL detection. A known amount of AA standards was added to samples. The samples were treated according to the method described in the 'Experimental' section and derivatized with DBCEEC. The analyses were

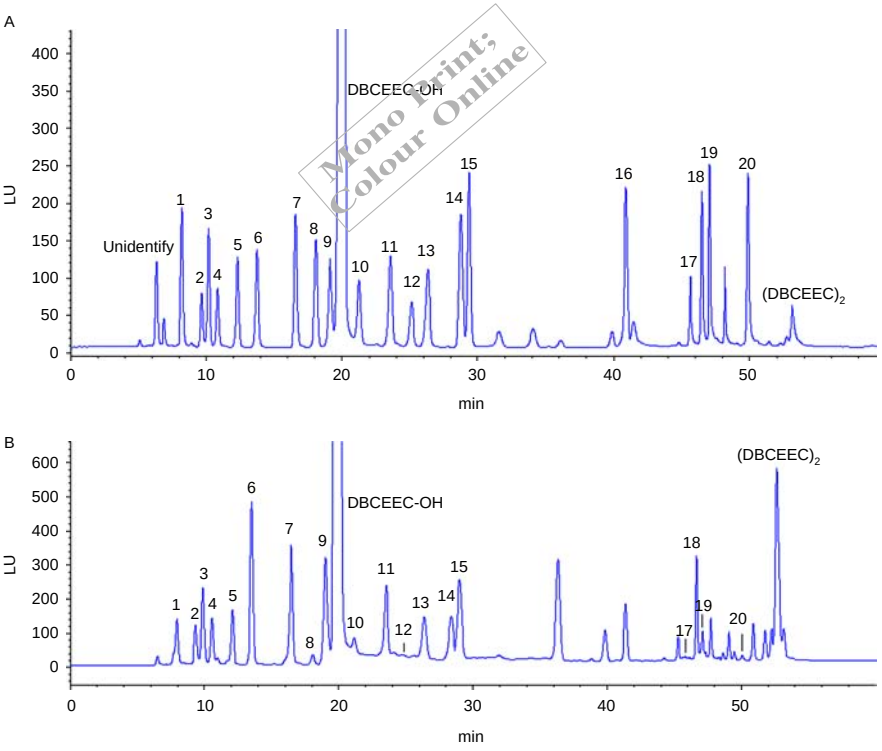

Figure 2. Typical chromatograms of AA standard derivatives (A), FAA derivatives in Luobuma from Geermu (B). Hypersil BDS C18 column: 4.6 mm × 200 mm × 5 µm; column temperature 35°C; flow rate 1.0 ml/min; excitation and emission: $\lambda_{ex}/\lambda_{em}$ = 300/400 nm. Peaks: 1 Arg; 2 Asp; 3 Ser; 4 Glu; 5 Thr; 6 Gly; 7 Ala; 8 GABA; 9 Pro; 10 Met; 11 Val; 12 Trp; 13 Phe; 14 Ile; 15 Leu; 16 $(Cys)_2$; 17 His; 18 Orn; 19 Lys; 20 Tyr.

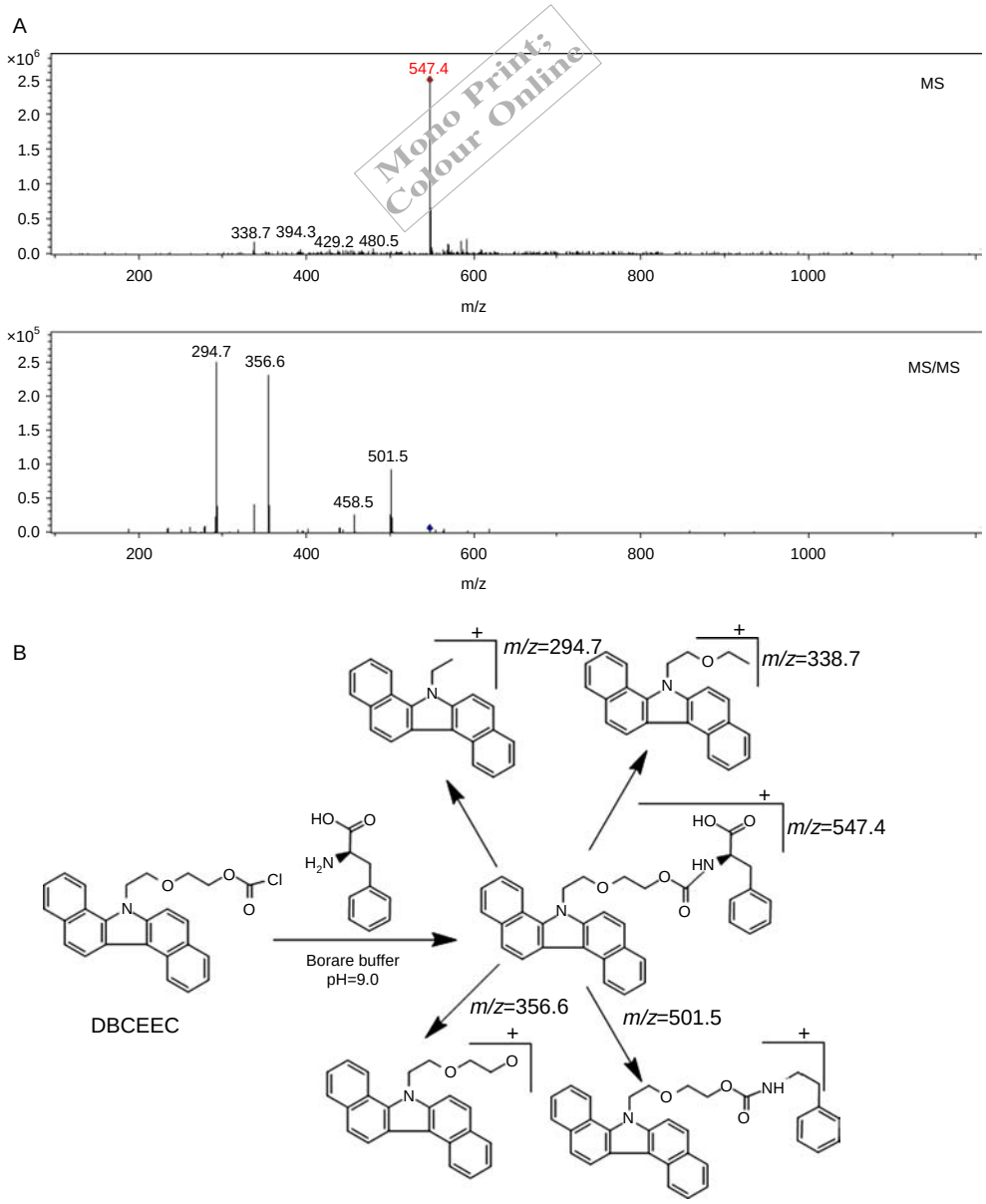

Figure 3. MS spectra of the representative phenylalanine derivative: (A) molecular ion MS and MS/MS and (B) the cleavage mode of protonated molecular ion.

carried out in duplicate and the experimental recoveries obtained were in the range 93–106%.

Correlation coefficient and limits of detection. Under the optimum experimental conditions, 20 standard AA derivatives solutions were analysed. The calibration graphs were established with peak area (Y) versus AA injection amounts (X, pmol). The linearity was established by the analysis of AA standard concentration range of 2500–25-fold, and the linear regression equations, correlation coefficients and detection limits for all AA derivatives were shown in Table I. All AA derivatives were found to give excellent linear responses with correlation coefficients (>0.9992). The detection limits (at a signal-to-noise ratio of 3:1) ranged from 2.88 to 23.4 fmol.

Analysis of AA composition in Luobuma leaf

TAA and FAA in Luobuma teas from Delingha and Geermu were determined under the optimal experimental conditions presented above. The peaks were identified by chromatographic retention time and online MS identification. The concentrations of TAA and FAA in samples are shown in Table II. The typical chromatogram of FAA derivatives in Geermu sample is shown in Figure 2B.

The results in Table II revealed that the TAA content of Delingha sample (83.25 mg/g) presented slightly higher than Geermu sample (81.76 mg/g). Essential AA in Geermu and Delingha samples accounted for 53.20% and 53.31%, respectively (Table II). Glutamic (9.82 and 10.01 mg/g) was the most abundant of AA in Luobuma leaf followed by

Table I. Linear regression equations [*Y*, peak area; *X*, injected amount (pmol)], correlation coefficient, detection limits, MS and MS/MS data of AA derivatives and reproducibility for retention time and peak area.

AA	Linear equation $Y = AX + B$	Correlation coefficient	Detection limits (fmol)	MS and MS/MS data	Retention time RSD (%)	Peak area RSD (%)
Arg	$Y = 78.16X - 64.81$	0.9995	8.22	556.8, 294.5, 338.5, 356.4	0.013	1.31
Asp	$Y = 38.22X - 42.33$	0.9993	7.06	515.6, 294.5, 338.5, 356.4	0.007	1.13
Ser	$Y = 57.92X - 10.63$	0.9999	9.81	487.6, 294.5, 338.5, 356.4	0.009	1.61
Glu	$Y = 40.25X - 42.56$	0.9994	2.65	529.6, 294.5, 338.5, 356.4	0.005	1.08
Thr	$Y = 59.74X + 1.48$	0.9998	6.97	501.7, 294.5, 338.5, 356.4	0.007	1.36
Gly	$Y = 58.36X + 7.27$	0.9998	23.4	457.6, 294.5, 338.5, 356.4	0.011	1.25
Ala	$Y = 67.12X + 7.16$	0.9993	5.28	471.6, 294.5, 338.5, 356.4	0.012	1.17
GABA	$Y = 80.03X + 24.54$	0.9998	3.22	485.5, 294.5, 338.5, 356.4	0.003	1.02
Pro	$Y = 64.41X + 8.13$	0.9998	3.48	497.5, 294.5, 338.5, 356.4	0.012	1.31
Met	$Y = 10.81X - 10.02$	0.9995	3.25	531,3, 294.5, 338.5, 356.4	0.014	1.24
Val	$Y = 76.18X + 14.32$	0.9997	4.74	499.6, 294.5, 338.5, 356.4	0.006	1.12
Try	$Y = 50.80X + 0.26$	0.9995	4.55	585.9, 294.5, 338.5, 356.4	0.040	1.04
Phe	$Y = 83.42X + 6.98$	0.9994	9.05	547.5, 294.5, 338.5, 356.4	0.012	1.17
ILe	$Y = 69.06X + 13.43$	0.9994	7.29	513.5, 294.5, 338.5, 356.4	0.009	1.22
Leu	$Y = 73.22X + 14.08$	0.9997	11.40	513.5, 294.5, 338.5, 356.4	0.018	1.15
$(Cys)_2$	$Y = 21.53X - 13.80$	0.9993	11.45	1004, 294.5, 338.5, 356.4	0.037	1.20
His	$Y = 42.31X - 21.70$	0.9992	15.86	918.7, 294.5, 338.5, 356.4	0.012	1.23
Orn	$Y = 64.60X - 18.19$	0.9996	3.24	894.8, 294.5, 338.5, 356.4	0.010	1.21
Lys	$Y = 84.21X - 7.72$	0.9998	4.15	908.2, 294.5, 338.5, 356.4	0.011	1.20
Tyr	$Y = 98.52X + 21.31$	0.9992	2.88	944.6, 294.5, 338.5, 356.4	0.007	1.17

leucine (8.80 and 8.89 mg/g). Other AA, such as threonine, lysine, aspartic, phenylalanine, valine, isoleucine, methionine, proline and alanine were present at concentrations ranging from 3.03 to 9.14 mg/g, and for the remaining AA, lower amounts were obtained (Table II).

FAA content from Geermu and Delingha samples were 3.85 and 4.04 mg/g, respectively, and essential

Table II. TAA and FAA contents in Luobuma teas from Geermu and Delingha (data are average values of three runs).

	AA	Luobuma leaf from Geermu		Luobuma leaf from Delingha	
		TAA	FAA	TAA	FAA
Acidic AA	Asp	4.33[a]	0.06[a]	4.41[a]	0.06[a]
	Glu	9.82[a]	0.29[a]	10.01[a]	0.31[a]
	Subtotal	14.15 (17.30)[b]	0.35 (9.09)[b]	14.43 (17.33)[b]	0.37 (9.16)[b]
Hydroxylated AA	Thr⋆	3.03[a]	0.14[a]	3.21[a]	0.14[a]
	Ser	2.34[a]	0.18[a]	2.36[a]	0.19[a]
	Subtotal	5.37 (6.57)[b]	0.32 (8.31)[b]	5.58 (6.70)[b]	0.33 (8.17)[b]
Aliphatic AA	Ala	8.79[a]	0.49[a]	9.14[a]	0.54[a]
	Pro	8.64[a]	0.06[a]	8.38[a]	0.06[a]
	Leu⋆	8.80[a]	0.03[a]	8.89[a]	0.02[a]
	Val⋆	5.37[a]	0.12[a]	5.48[a]	0.12[a]
	ILe⋆	5.98[a]	0.02[a]	6.10[a]	0.05[a]
	Gly	2.50[a]	0.04[a]	2.67[a]	0.06[a]
	Subtotal	40.08 (49.03)[b]	0.76 (20.00)[b]	40.66 (48.85)[b]	0.85 (21.04)[b]
Basic AA	Lys⋆	4.06[a]	0.19[a]	4.10[a]	0.21[a]
	His⋆	2.56[a]	0.09[a]	2.61[a]	0.07[a]
	Arg⋆	0.14[a]	0.05[a]	0.15[a]	0.05[a]
	Orn	0.10[a]	0.02[a]	0.10[a]	0.02[a]
	Subtotal	6.86 (8.39)[b]	0.35 (9.09)[b]	6.96 (8.36)[b]	0.35 (8.66)[b]
Aromatic AA	Tyr	0.35[a]	0.24[a]	0.37[a]	0.26[a]
	Phe⋆	4.90[a]	0.04[a]	5.10[a]	0.07[a]
	Trp⋆	ND	0.11[a]	ND	0.16[a]
	Subtotal	5.56 (6.79)[b]	0.39 (10.13)[b]	5.77 (6.94)[b]	0.49 (12.13)[b]
Sulphur-containing AA	Met⋆	8.35[a]	0.30[a]	8.43[a]	0.25[a]
	Subtotal	8.35 (11.24)[b]	0.30 (28.83)[b]	8.43 (11.14)[b]	0.25 (26.73)[b]
GABA		0.56[a]	0.56[a]	0.57[a]	0.57[a]
EAA		43.50 (53.20)[b]	1.10 (26.23)[b]	44.38 (53.31)[b]	1.14 (28.22)[b]
NEAA		38.26 (46.80)[b]	2.75 (73.77)[b]	38.87 (46.69)[b]	2.90 (71.78)[b]
TAAC		81.76[a]	3.85[a]	83.25[a]	4.04[a]

Note: GABA, γ-aminobutyric acid; EAA, essential amino acids; NEEA, no-essential amino acids; TAA, total amino acids; FAA, free amino acids; TAAC, total amino acids content; [a] absolute content [mg/g, mg (AA)/g (Luobuma leaf)]; [b] absolute content [mg/g, mg (AA)/g (Luobuma leaf)], mass percent (%, ratio of the absolute content with that of all AA content).

Table III. Comparison of the reported methods for amino acid determination.

Reagents	Derivatization conditions	Separation method	LOD (nM)	Refs
OPA	Borate buffer (pH 11.0), at room temperature for 10–15 min	HPLC–FLD, Ex: 340 nm, Em: 445 nm	1.5–11.5	Yoshitake et al. (2006)
Dns-Cl	Carbonate buffer (pH 9.5), at room temperature in darkness for 1 h	HPLC–UV 254 nm	151–155	Naval et al. (2006)
DTAF	Borate buffer (pH 9.2), at 38°C for 30 min	CE–LIFD	1600–7000	Xiao et al. (2007)
DNFB	0.2 mol/L sodium bicarbonate solution, 60°C, 40 min in the dark	Colorimetric determination	No mention	Chen et al. (2009)
AQC	Borate buffer (pH 8.8), at room temperature	HPLC–FLD, Ex: 250 nm, Em: 395 nm	3.3–7.6	Liu et al. (1998)
FMOC	0.5 M sodium hydrogen carbonate/sodium carbonate buffer with pH 10.2, at 40°C for 10 min	HPLC–FLD, Ex: 262 nm, Em: 615 nm	28–346	Lozanov et al. (2007)
CNBF	H_3BO_3-$Na_2B_4O_7$ buffer (pH 9.0), 60°C for 30 min	HPLC–UV	2700–6500	Shi et al. (2009)
DBCEEC	Borate buffer (pH 9.0), room temperature for 3 min	HPLC–FLD, Ex: 300 nm, Em: 400 nm	0.27–1.5	This work

Note: OPA, *o*-phthalaldehyde; DnsCl, dansyl chloride; DTAF, 5-(4,6-dichloro-striazin-2-ylamino) fluorescein; CNBF, 4-chloro-3,5-dinitrobenzotrifluoride; AQC, 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate; DNFB, 2,4-dinitrofluorobenzene; FMOC, 9-fluorenylmethyloxycarbonyl chloride.

AA accounted for 26.63% and 28.22%. In all FAA, GABA presented the highest content, followed by alanine, glutamine and tyrosine (Table II). As could be seen in Table II, GABA contents from Geermu and Delingha samples were 0.56 and 0.57 mg/g, respectively. Isoleucine showed the lowest content of 0.02 and 0.05 mg/g.

AA in Luobuma leaf were classified by their lateral chain (Table II). The aliphatic AA presented the highest content (40.08 and 40.66 mg/g), followed by acidic AA (14.15 and 14.43 mg/g), sulphur-containing AA (8.35 and 8.43 mg/g), basic AA (6.86 and 6.96 mg/g) and aromatic AA (5.56 and 5.77 mg/g). Meanwhile, hydroxylated AA presented the lowest contents of 5.37 and 5.58 mg/g. The content variation of acidic, hydroxylated, aliphatic, basic, aromatic and sulphur-containing AA between Geermu and Delingha samples was not significant ($p > 0.05$).

Comparison of the reported methods for AA determination

The overall comparison of the improved method with the reported methods is presented in Table III. In these methods, several reagents [e.g. *o*-phthalaldehyde (OPA), dansyl chloride (DnsCl), 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC), 2,4-dinitrofluorobenzene (DNFB) and 9-fluorenylmethyloxycarbonyl chloride (FMOC)] were used for AA determination. Compared with the reported methods, the developed method using DBCEEC as derivatization reagent showed many advantages. For example, it offered the detection limits of 0.27–1.5 nM, which was significantly lower than the methods mentioned in Table III. The full derivatization can be achieved within only 3 min at room temperature, which was more rapid and mild than the reagents in Table III such as Dns-Cl (1 h), 5-(4,6-dichloro-striazin-2-ylamino) fluorescein (30 min), DNFB (60°C, 40 min), 4-chloro-3,5-dinitrobenzotrifluoride (60°C, 30 min) and FMOC (40°C, 10 min).

Conclusion

In this study, a developed HPLC–FLD method using a sensitive labelling reagent (DBCEEC) for the simultaneous determination of 20 AA in Luobuma tea has been established. This method showed many advantages such as low detection limit, rapid derivatization, mild derivatization conditions and excellent applicability. The AA date in this study should be useful for the further understanding and development of Luobuma tea.

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