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Original Research Article

Determination of amino acids in black garlic using high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride

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Abstract

Purpose: *To quantify the amino acids obtained from black garlic (Allium sativum L.) using high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl).*

Methods: *Homogenized black garlic samples were derivatized with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) reagent in borate buffer at 30 °C for 60 min. The black garlic samples were hydrolyzed with 5.0 mL HCl (6 N) and neutralized to pH 5 – 6 by adding 30 mL NaOH (1 N). Amino acids were measured using high-performance liquid chromatography coupled with a fluorescence detector after hydrolysis and derivatization with FMOC-Cl reagent. Gradient elution mixture consisted of acetonitrile, methanol, and water at a flow rate of 1.0 mL/min, 0.2 % ammonium acetate, and 0.1 % formic acid.*

Results: *The ranges of the limits of detection and quantification were 30 and 100 g/mL, respectively. The overall intra- and inter-day variations (%RSD) for the precision findings were smaller than 3.43 and 4.63 %, respectively. The accuracy of the developed method was good, ranging from 90.9 to 106.8 %. The amino acids of six black garlic products from the market were determined using the suggested approach. The findings indicated that glutamic acid, the main amino acid, is present in the maximum concentration (564.64 – 1333.9 mg/100 g) in black garlic.*

Conclusion: *The validated method is for the determination of the amino acids in black garlic and would be a helpful tool for the quality control of products containing black garlic.*

Keywords: *Amino acid, Black garlic, Derivatization, High-performance liquid chromatography (HPLC), 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl)*

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INTRODUCTION

Garlic (*Allium sativum* L.) is widely used in food and medicine to treat various illnesses. Black garlic is described as fresh garlic that has undergone a controlled fermentation process at a high temperature (60 – 90 °C) and humidity (80 – 90 %) [1]. This technique darkens garlic cloves,

gives them a sweet flavor, and alters their chewiness. S-allyl cysteine (SAC), ascorbic acid, 1,2-dimercaptocyclopentane, diallyl disulfide, and ferulic acid are only a few of its active ingredients [2]. However, despite the fact that amino acids constitute one of the nutritious components of black garlic, very few studies have focused on their quantification [3-5].

There are two main methods for amino acid analysis: (1) pre- or post-column derivatization with fluorescence reagents and analysis by capillary electrophoresis (CE) chromatography, liquid chromatography (LC), gas chromatography (GC), and fluorescence detector (FLD) probes; and (2) direct quantification using mass spectrometry (MS) probes (LC-MS, GC-MS). The second method has high sensitivity, accuracy, and a very short analysis time (less than 5 min), but requires expensive laboratory equipment and highly trained technicians [3,6,7]. Till date, many studies have reported the application of 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl)-based derivatization reagents to analyze amino acids in sample matrices such as infusions of tea [8,9], *Arabidopsis thaliana* seedlings [10], and fruit juices [11,12]. This is because FMOC-Cl reagents are widely available, inexpensive, reactive, and specific for both primary and secondary amines [13]. The aim of this study was to develop a novel black garlic amino acid determination procedure using HPLC with FMOC-Cl, which can be applied to black garlic preparations sold commercially.

EXPERIMENTAL

Chemicals and solvents

Free amino acid reference standards including histidine (His), arginine (Arg), serine (Ser), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), glycine (Gly), alanine (Ala), methionine (Met), valine (Val), proline (Pro), phenylalanine (Phe), isoleucine (Iso), leucine (Leu), lysine (Lys) and tyrosine (Tyr) and FMOC derivatization reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, n-hexane, and HPLC-grade water were obtained from Honeywell (Charlotte, NC, USA). Ammonium acetate, formic acid, disodium tetraborate decahydrate, citric acid, and sodium citrate dihydrate were purchased from Merck (Darmstadt, Germany).

Black garlic

Black garlic samples were obtained from supermarkets, pharmacies, and functional food stores in Vietnam. The samples were homogenized by grinding and maintained at -4 °C in black glass containers before analysis.

Standard preparation

The amino acids were prepared as individual standard stock solutions (4000 g/mL) in HCl (1 N), and it was discovered that these solutions were stable for around 12 months. By diluting the stock standard solution with water to the necessary concentrations, an intermediate standard solution was created that contained 1000 nmol/mL of each standard chemical and was stable for around six months. The intermediate standard solution was diluted with acetonitrile and water at a ratio of 1:1 to give varied amounts for the working standard solutions, which were then neutralized to pH 5 – 6 using NaOH (1 N). These were protected from light and stored at 4 °C.

Hydrolysis

The protein hydrolysis was carried out by the procedure described by Crestfield *et al* [14]. Homogenized black garlic samples (100 – 200 mg) were accurately weighed in a glass test tube (10 x 150 mm) and 5.0 mL of HCl (6 M) was added to the tube. Subsequently, air was purged from the tube by vacuum, and the tube was sealed with a screw cap. Hydrolysis was conducted at 110 ± 1 °C for 17 h in an oven. After the tube had cooled to room temperature, the hydrolysate sample was transferred to a 50 mL volumetric flask, neutralized to pH 5 – 6 by adding approximately 30 mL NaOH (1 N), and diluted with water.

Optimization of derivatization conditions

After being diluted in acetonitrile, the hydrolysate sample or standard solution (75 µL) was pipetted into a centrifuge tube. After that, 150 µL of borate buffer (1 M, pH 9) was added and vortexed for 1 min. The vial was then held at 45 °C for 60 min while 150 µL of the FMOC-Cl reagent (dissolved in acetonitrile) was added. The surplus FMOC-Cl was removed by partitioning twice with 0.5 mL n-hexane after adding a neutralizing solution (0.3 % formic acid in a combination of acetonitrile and water, 50 %). The lower layer was filtered through a 0.22 µm nylon filter and transferred to an auto-sampler vial. The derivatization conditions were optimized with respect to gradient concentrations of FMOC-Cl (400, 800, 2000, 4000, and 8000 µg/mL) and reaction times (5, 10, 20, 30, 60, and 90 min). The effectiveness of derivatization was assessed using the sum of the amino acid peak areas.

Chromatographic conditions

Using an Agilent LC 1100 series (Agilent Technologies, Mississauga, ON, Canada) equipped with a G-1310A pump, G-1316A column thermostat, G-1313A auto-sampler, and G1315B fluorescence detector, method development, quantification, and validation tests were carried out.

An Agilent Zorbax Eclipse AAA column (150 mm × 4.6 mm x 3.5 mm) was used for the chromatographic separation. The mobile phases included 0.1 % formic acid in acetonitrile (A), 0.2 % ammonium acetate / 0.1 % formic acid in water (B), and 0.1 % formic acid in methanol (C). Table 1 lists the gradient elution procedures. The injection volume was 20 L, and a 1.0 mL/min flow rate was used for all chromatographic separations. The optimal wavelengths for excitation and emission were 260 and 320 nm, respectively. Agilent Technologies ChemStation software, which was also utilized to monitor and control all analytical parameters was used to reprocess the chromatographic results.

Table 1: Gradient elution program

Time (min)	Flow rate (mL/min)	Mobile phase ratio		
		A (%)	*B (%)*	*C (%)*
0.0	1.0	25	10	65
0.5	1.0	25	10	65
14.5	1.0	22	18	60
15.0	1.0	32	10	58
20.0	1.0	35	9	56
20.5	1.0	39	16	45
26.5	1.0	46.5	7.5	46
27.5	1.0	55	5	40
34.0	1.0	60	5	35
34.5	1.0	95	5	0
35.0	1.0	100	0	0
36.5	1.0	100	0	0
37.0	1.0	25	10	65
40.0	1.0	25	10	65

Method validation

In accordance with the recommendations of the Association of Official Analytical Chemists (AOAC) and the International Council for Harmonization (ICH), the proposed technique was validated for selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, and accuracy.

RESULTS

Optimized sample preparation

The effect of the concentration of FMOC-Cl (400 – 8000 µg/mL) on derivatization yield was compared and is shown in Figure 1. The FMOC-Cl molar concentrations related to the total volume of the reaction mixture varied from 0.15 to 3.0 mM. In the present study, the sum of the peak area of amino acids increased steadily with an increase in the concentration of FMOC-Cl from 400 to 2000 µg/mL and remained nearly unchanged from 2000 to 8000 µg/mL. In addition, tyrosine skyrocketed to a peak at 4000 µg/mL as shown in Figure 1 b. It is evident that the derivatization of tyrosine was incomplete at 400 – 2000 µg/mL FMOC-Cl.

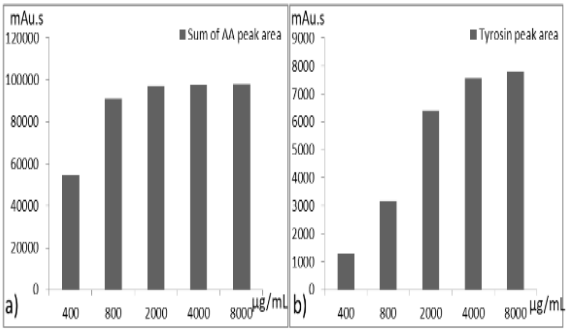

Figure 1: Derivatization yield (expressed as amino acid peak areas) as a function of FMOC-Cl concentrations, (a) the sum of peak areas, (b) Tyrosine peak areas

The reaction times were investigated including 5, 10, 20, 30, 60, and 90 min. The total peak areas of the amino acids in the sample were compared to those in the reference sample in order to assess the derivatization process. The derivatization yields rose before the reaction time reached 60 min, as shown in Figure 2, and then considerably dropped at 90 min. Therefore, it was decided and agreed that a 60-min reaction time was adequate.

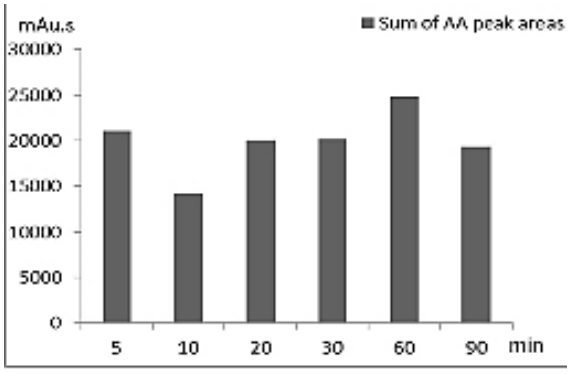

Figure 2: Results of reaction time investigation

Optimized chromatographic conditions

Optimal chromatographic conditions were determined by analyzing a mixed standard solution (100 nmol/mL) diluted from a working standard solution and the hydrolysate solution of

the black garlic sample. In this study, four different gradient elutions were investigated: methanol – 0.2 % ammonium acetate in water; acetonitrile – 0.2 % ammonium acetate in water; acetonitrile – methanol – 0.2 % ammonium acetate in water and 0.1 % formic acid in acetonitrile – methanol – 0.1 % formic acid; and 0.2 % ammonium acetate in water.

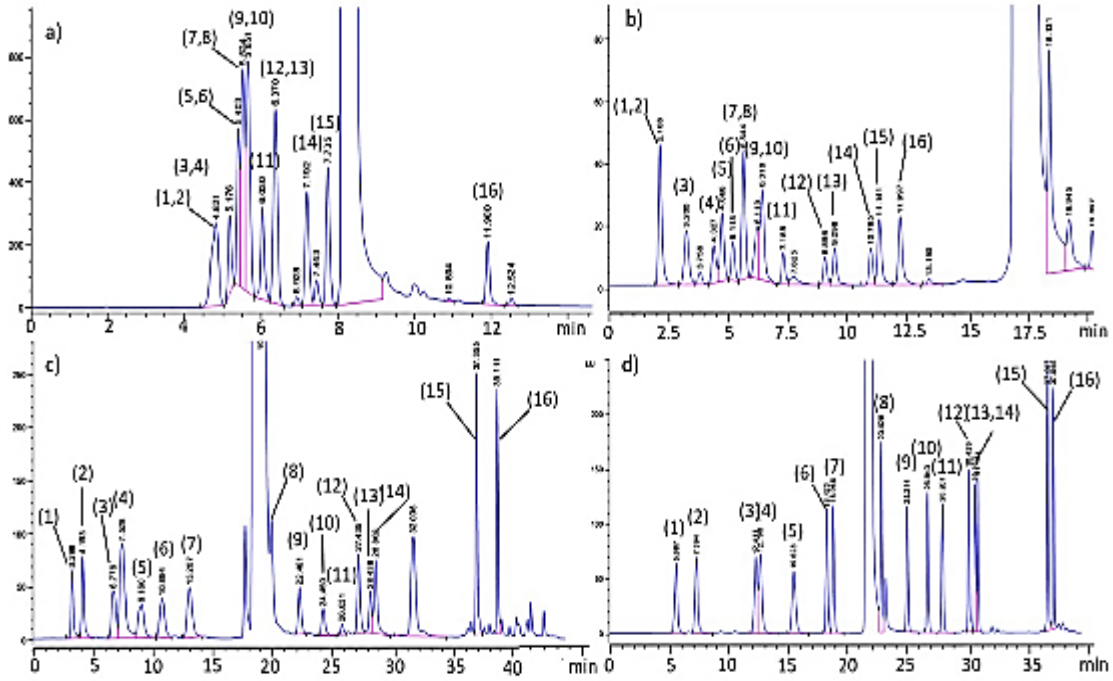

Figure 3: HPLC chromatogram of four different elution programs, (a) MeOH – 0.2 % ammonium acetate in water, (b) MeCN – 0.2 % ammonium acetate in water, (c) MeCN – MeOH – 0.2 % ammonium acetate in water, (d) 0.1 % formic acid in MeCN – MeOH – 0.1 % formic acid, 0.2 % ammonium acetate in water. (1)-Histidine, (2)-Arginine, (3)-Serine, (4)-Aspartic acid, (5)-Glutamic acid, (6)-Threonine, (7)-Glycine, (8)-Alanine, (9)-Proline, (10)-Methionine, (11)-Valine, (12)-Phenylalanine, (13)-Isoleucine, (14)-Leucine, (15)-Lysine, (16)-Tyrosine

Method validation results

System stability

The system stability was tested by carrying out six replicate injections of a mixed standard solution (20 nmol/mL) and determining the theoretical plate number (N), resolution (Rs). The %RSD values of the peak area and RT of all analytes were less than 2.0 %. Therefore, the proposed method met this requirement.

Specificity

Specificity was tested using HPLC to analyze the black garlic sample. This was assessed by comparing the retention time of each amino acid standard reference with those of the peaks obtained by analyzing a hydrolysate sample of black garlic. As shown in Figure 4, the HPLC method could distinguish amino acids from other constituents of the sample matrices. There was no interference with amino acid peaks in the analyzed samples.

Linearity, and limits of detection and quantification

Six different concentrations of 16 amino acids, ranging from 10.0 to 200 nmol/mL, were added to the working standard solution. The HPLC system received triplicate injections of each sample. Plotting the average of the peak area responses with concentration for each sample resulted in the calibration curves, which were used to test the linearity. Table 2 lists the outcomes of the regression equations and square correlation coefficients (r^2). The LOD and LOQ values of the analytes varied from 30 to 100 g/mL.

Precision

The precision of the method was verified by evaluating intra- and inter-day precision. Relative standard deviation (RSD) was used as a measure of accuracy. The intra-day precision was examined by analyzing six samples in a single day, whereas the inter-day precision was determined by analyzing six samples each day for three days. The precision results are shown in

Table 2 and indicated that the overall intra- and inter-day variations (%RSD) were less than 3.43 and 4.63 %, respectively.

Accuracy

Recovery studies were carried out to look into the accuracy of this method. Three different concentrations of the reference compounds were added to the blank samples: low (100 µg/mL), medium (200 µg/mL), and high (300 µg/mL). Following that, the hydrolyzed samples were measured using the aforementioned techniques. The outcomes showed that the approach was established to have excellent accuracy, with a total recovery of between 90.9 and 106.8 % (Table 2).

Applicability of results

The proposed method was used to determine the amino acid contents of six black garlic. Table 3 shows the results.

DISCUSSION

Amino acids in both hydrolysate and plasma samples have previously been determined using several derivatization reagents, including dabsyl, dansyl, FMOC, ortho-phthalaldehyde (OPA), and phenylisothiocyanate (PITC). Among these, reactions with OPA/thiol or FMOC are most commonly applied in the analysis of amino acids. Both reagents have the advantages of fast reaction times, availability as an aqueous solution, and high selectivity and sensitivity for derivatized amino acids in both UV and FL detectors. It is likely that FMOC-Cl is the best derivatization reagent for determining amino acids in black garlic because of the instability of the isoindole formed from amino acids and OPA, as well as the instability of the reagent itself.

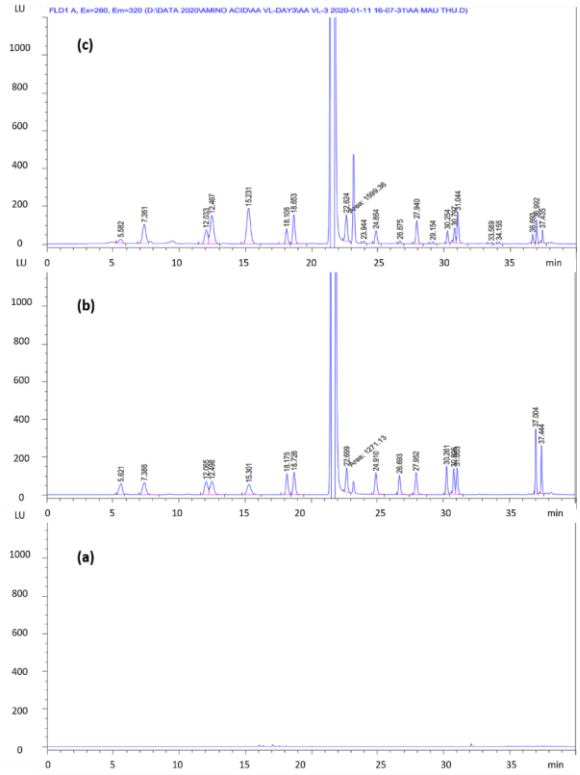

Figure 4: HPLC chromatogram of: (a) the extraction solvent, (b) mixed standards solution, (c) 16 analytes amino acids in black garlics. (1)-Histidine, (2)-Arginine, (3)-Serine, (4)-Aspartic acid, (5)-Glutamic acid, (6)-Threonine, (7)-Glycine, (8)-Alanine, (9)-Proline, (10)-Methionine, (11)-Valine, (12)-Phenylalanine, (13)-Isoleucine, (14)-Leucine, (15)-Lysine, (16)-Tyrosine

Table 2: Precision, accuracy and calibration parameters

Amino acid	Recovery (%)			Precision (n=6)		Calibration curve		
	Low-level	*Mid-level*	*High-level*	*Intra-day RSD (%)*	*Inter-day RSD (%)*	*Slope*	*y-intercept*	r^2
His	102.3	101.9	98.7	1.07	1.74	49.99	65.89	0.9998
Arg	97.8	100.7	94.9	0.84	4.16	59.47	-39.77	0.9999
Ser	96.4	106.8	103.6	1.55	3.45	58.06	122.8	0.9995
Asp	90.9	95.3	93.9	0.99	1.34	64.83	78.45	0.9998
Glu	99.0	104.7	100.7	0.69	1.59	54.70	100.7	0.9996
Thre	104.6	101.4	98.7	0.57	2.93	58.95	113.7	0.9997
Gly	105.9	101.6	97.4	2.92	2.92	64.48	30.09	0.9999
Ala	93.6	95.2	96.7	1.45	3.53	49.32	68.72	0.9998
Pro	96.0	97.4	96.6	2.17	4.41	62.59	53.06	0.9998
Met	102.7	97.3	92.4	1.18	4.63	49.32	68.72	0.9998
Val	95.5	98.8	97.8	0.48	2.48	58.04	136.5	0.9995
Phe	99.5	96.9	95.3	1,97	3.05	56.09	102.6	0.9996
Iso	94.7	101.1	99.7	3.43	2.16	51.59	83.85	0.9996
Leu	93.5	99.6	100.6	2.19	1.90	55.01	131.6	0.9995
Lys	96.7	96.2	95.4	2.38	4.37	95.05	181.5	0.9996
Tyr	102.8	104.2	99.1	1.86	2.92	77.39	-143.2	0.9994

Table 3: Content of amino acids in black garlic samples

Amino acid	Content of amino acids (mg/100g)					
	BG1	***BG2***	***BG3***	***BG4***	***BG5***	***BG6***
His	63.71±3.91	51.15±4.51	144.19±1.23	69.21±1.48	151.61±7.67	119.82±2.48
Arg	507.47±14.42	215.77±2.77	1284.34±9.95	417.36±1.30	787.88±6.45	431.96±2.54
Ser	323.84±6.43	190.92±1.16	481.75±5.03	280.69±11.49	345.28±10.66	285.35±5.85
Asp	620.65±2.05	354.47±1.06	1040.96±2.45	457.18±4.53	584.49±3.16	493.17±8.83
Glu	975.99±5.65	564.64±5.18	1333.90±6.01	743.94±9.99	873.31±23.96	883.70±10.56
Thre	184.26±9.51	105.64±1.22	271.24±8.41	149.29±2.32	204.79±5.79	168.49±2.86
Gly	453.45±10.96	226.84±2.75	598.04±10.27	303.78±7.37	467.69±10.28	353.08±3.40
Ala	341.79±20.31	183.51±1.32	419.45±14.49	293.75±2.61	359.96±3.37	309.50±9.58
Pro	132.45±2.74	17.48±0.71	179.75±2.51	83.82±1.19	153.87±3.65	110.33±1.10
Met	81.64±4.99	8.52±0.56	44.45±0.67	20.07±0.20	39.42±2.54	33.42±0.59
Val	258.59±6.10	154.87±9.01	368.03±3.21	209.71±5.71	286.75±8.76	241.88±3.46
Phe	121.84±4.21	63.79±0.79	185.27±5.33	103.91±1.43	136.73±3.74	134.95±2.99
Iso	146.61±0.73	85.19±0.83	205.67±12.90	106.31±2.19	169.58±5.54	145.38±6.93
Leu	327.62±0.61	203.26±3.61	468.93±8.58	252.88±2.76	366.65±10.83	307.25±3.11
Lys	83.88±3.71	23.80±2.18	204.27±5.15	58.76±0.72	102.81±6.91	69.59±0.65
Tyr	126.37±6.12	65.70±3.33	155.74±5.88	100.53±2.24	115.83±2.05	103.91±4.19
Total	***4717.87±199.62***	***2515.54±63.15***	***7385.97±193.36***	***3651.20±29.63***	***5146.66±5146.66***	***4191.78±65.80***

The ideal concentration of FMOC-Cl for derivatizing amino acids is 4000 µg/mL (approximately 1.5 mM). Compared to earlier report [13], this value for the optimum derivatization conditions of FMOC and amino acids was three times higher. This might be because the volume ratio of FMOC solutions in the two conditions was different, and the reaction temperature of the proposed method (45 °C) was greater than the prior condition (ambient temperature) reported by Jámbor and Molnár-Perl [13].

To evaluate FMOC-amino acid derivatives, acidic modifiers are often added to the mobile phase to reduce ionization of the carboxyl groups of the analytes and the silanol groups of the stationary phase. Furthermore, the addition lessens the possibility of peak tailing by reducing the interaction between the analytes and ionized silanol [15]. Acetonitrile offered a higher peak shape and separation of derivatives than methanol. The poor solubility of the borate buffer and interference from the black garlic samples in acetonitrile may cause obstruction at the frit of the HPLC column since His and Arg were poorly maintained even at low acetonitrile ratios. Therefore, a combination of acetonitrile, water, and methanol was used. The initial ratios of methanol, acetonitrile, and water was 10:25:65, which provided adequate retention of more hydrophilic analytes (such as derivatives of His, Arg, Ser, Asp, Glu, Thr, Gly, and Ala). The combination of water, methanol, and acetonitrile with increasing and decreasing methanol ratios throughout the run time provided satisfactory separation of all analytes. According to the chromatogram, the addition of formic acid to the mobile phase allowed for the generation of a narrower FMOC-Cl peak width, which improved the separation of Ala from FMOC-Cl. All amino acid derivatives and matrices were well-separated under these conditions after a run that lasted approximately 40 min.

The results showed that black garlic contains glutamic acid (the major amino acid in the highest quantity), aspartic acid, arginine, and glycine with quantities of 564.64 – 1333.9 mg/100 g, 354.47 – 1040.96 mg/100 g, 215.77 – 1284.34 mg/100 g, and 226.84 – 598.04 mg/100 g, respectively. Using pre-column derivatization with o-phthalaldehyde, Kang [16] was able to determine the presence of 14 amino acids in black garlic in Korea. Tryptophan was found in black garlic at the highest concentrations, followed by histidine, valine, glycine, arginine, and aspartic acid. The lowest concentrations of other amino acids, such as histidine, lysine, methionine, and proline, were 51.15, 23.80, 8.52, and 17.48 mg/100 g, respectively. The total amino acid content per 100 g ranged from 2515.54 to 7385.97 mg.

CONCLUSION

Amino acids have been hydrolyzed and derivatized using readily accessible and affordable reagent, FMOC-Cl. The chemicals are separated qualitatively using HPLC, which is effective, precise, and accurate. It can therefore be utilized to concurrently ascertain the amino acids in products containing black garlic.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. This study was conceived and designed by Ngoc-Van Thi Nguyen. The data were collected by Ngan Kim Huynh Nguyen, Lan Hoang Vuong and Nga Ngoc Thi Phan. The data analysis was done by Ngoc-Van Thi Nguyen and Ngan Kim Huynh Nguyen. The article was written by Ngan Kim Huynh Nguyen with reviews and corrections from Ngoc-Van Thi Nguyen.

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