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An LC-MS/MS-SRM Method for Simultaneous Quantification of Four Representative Organosulfur Compounds in Garlic Products

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Abstract The quantitative analysis of organosulfur compounds is important for the quality control of various garlic products along with studying their molecular functionality and nutraceutical properties. In this study, a liquid chromatography-tandem mass spectrometry-selected reaction monitoring (LC-MS/MS-SRM) method with electrospray ionization detection was developed and validated for the rapid, simultaneous quantification of four representative organosulfur compounds in garlic: alliin, S-allyl-L-cysteine, γ-glutamyl-S-allyl-L-cysteine, and allicin. Stable SRM transitions were achieved for these compounds under optimized conditions, and the linear range extended from 1 to 2000 ng/mL. The limits of detection and quantification ranged from 0.003 to 0.058 ng/mL and from 0.01 to 0.19 ng/mL, respectively. Excellent recovery and reproducibility at different spiking levels were achieved. The method was successfully applied to the simultaneous quantification of organosulfur compounds in fresh garlic samples. This highly selective and sensitive LC-MS/MS-SRM method is expected to be a useful tool for studying molecular functionality and the quality control of garlic products.

Keywords Garlic · LC-MS/MS · SRM · Organosulfur compounds · Quantification

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Introduction

Garlic (*Allium sativum* L.), a well-known vegetable, has been used as not only a flavor but also a medical herb and a source of nutritional supplements worldwide. Garlic has been reported to exhibit extensive biological activities (Butt et al. 2009; Santhosha et al. 2013; Suleria et al. 2015), including antioxidant (Capasso 2013), antifatigue (Morihara et al. 2007), anticancer (Nicastro et al. 2015), and antimicrobial (Lanzotti et al. 2014) activities. Garlic may also have a positive role in immunonutrition (Nantz et al. 2012) and help prevent cardiovascular diseases (Castro et al. 2010; Rahman 2007a). The main bioactive components in garlic are organosulfur compounds (Yun et al. 2014). Allicin, which is responsible for garlic's strong odor, is the most powerful medicinal compound derived from garlic. Allicin is converted from its precursor alliin by the enzyme alliinase when tissue damage occurs, and it readily breaks down into a series of sulfur-containing metabolites (Block 1985). S-allyl-L-cysteine (SAC) is another major bioactive compound in garlic, which is converted from γ-glutamyl-S-allyl-L-cysteine (GSAC) by γ-glutamyl transpeptidase (Ichikawa et al. 2006a). Due to its relative stability (Kodera et al. 2002), SAC has been used as a compliance marker in clinical studies involving garlic consumption (Amagase et al. 2001). Evidence has also suggested that SAC can be converted to alliin (Hughes et al. 2005; Jones et al. 2007). Therefore, alliin, SAC, GSAC, and allicin represent the organosulfur compounds of garlic and can be used to monitor the quality of garlic and its products along with their potential biological activities.

In addition to raw garlic, various garlic supplements are available in the market, including garlic powders, garlic oils, garlic oil macerates, aged garlic extracts, and black garlic. The content of organosulfur compounds in garlic and garlic products is usually low and varies with the methods used to process and store the product (Beato et al. 2012; Gorinstein et al. 2009; Ichikawa et al. 2006a; Liang et al. 2015). Moreover, in the era of molecular nutrition, quantitative analysis of active component is indispensable for the in-depth study of their functionalities. Therefore, the development of a highly sensitive and selective method for quantifying representative organosulfur compounds is important for the quality control and molecular functionality study of garlic products.

Currently, methods based on high-performance liquid chromatography (HPLC) are most commonly used for the quantification of organosulfur compounds in garlic (Arnault et al. 2003; Beato et al. 2012; Ichikawa et al. 2006a; Ichikawa et al. 2006b). However, HPLC often shows relatively low resolving power, and the quantification of organosulfur compounds using HPLC methods is complicated because of interference from other compounds, particularly for the analysis of garlic products with complex matrices. Although gas chromatography–mass spectrometry (GC-MS) has been employed to analyze garlic sulfur compounds directly or after derivatization (Kubec et al. 1999), the degradation of compounds such as allicin during GC limit this method's applicability (Mondy et al. 2001). Moreover, our early attempts to develop a quantification assay for these organosulfur compounds revealed that alliin, SAC, and GSAC cannot be volatilized under normal GC conditions without sample derivatization.

When considering the specificity, sensitivity, and stability of samples during analysis, liquid chromatography-tandem mass spectrometry-selected reaction monitoring (LC-MS/MS-SRM), which has been used to quantify trace-level analytes in complex matrices, shows promise for the analysis of organosulfur compounds in garlic (Diana Di Mavungu et al. 2009; Gianazza et al. 2014; Guo et al. 2012; Kitteringham et al. 2009). Among the organosulfur compounds of garlic, only SAC has been analyzed with LC-MS/MS-SRM to date (Lee et al. 2015; Lee et al. 2014); the LC-MS/MS-SRM analyses of alliin, GSAC, and allicin, and most importantly, the simultaneous analysis of all four of these garlic compounds have not yet been reported.

In this study, a rapid, sensitive, and specific LC-MS/MS-SRM method was developed for the simultaneous quantification of alliin, SAC, GSAC, and allicin in garlic products. The method was validated with respect to the specificity, linearity, limits of detection and quantification, and accuracy. As a proof of concept, two fresh garlic samples were analyzed using the new method. This method is expected to be used in the quality control of garlic products during their production as well as in studies on the metabolism and bioavailability of garlic. It will also be useful to study the molecular functionality of garlic products.

Material and Methods

Chemicals and Reagents

The reference standards of L-(+)-alliin (CAS No. 556-27-4) and allicin (CAS No. 539-86-6) were purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). SAC (CAS No. 21593-77-1) and GSAC (CAS No. 91216-95-4) were purchased from Sigma-Aldrich (Munich, Germany) and Pharmacopeial Convention, Inc. (Rockville, MD, USA), respectively. The purities of all compounds were greater than 98 %. LCMS-grade acetonitrile was obtained from Wako (Tokyo, Japan), and water was supplied by a Milli-Q Ultrapure water system (Millipore Corporation, Bedford, Mass., USA). A 0.01 M aqueous solution of hydrochloric acid (HCl) was used for sample preparation.

Sample Preparation

Mixtures of the four standards in serial concentrations were prepared in a 0.01 M HCl solution. Two fresh garlic samples were obtained from the local supermarket (Lumiere, Fukuoka, Japan): sample no. 1 was cultivated in Aomori Prefecture, Japan, and sample no. 2 was cultivated in Shandong Province, China. The fresh garlic cloves (10–15 g) were crushed using a Millser IFM-620DG (Iwatani, Osaka, Japan). The crushed garlic was then extracted with 30 mL of 0.01 M HCl solution at 40 °C for 25 min with the assistance of a sonicator (550 W, Elmasonic S 100H, New Jersey, USA). After centrifugation at 3500 rpm for 5 min, the supernatant (extract 1) was collected, and the pellet was again extracted with 15 mL of 0.01 M HCl for 5 min. The supernatant (extract 2) was collected and combined with extract 1. The supernatant was diluted to 50 mL with the HCl solution and centrifuged again at 3500 rpm for 5 min. The final supernatant was collected and used for the LC-MS/MS analysis. All the standards and samples were filtered through 0.2-μm membrane filters before analysis and kept at 4 °C before and during analysis.

LC-MS/MS Analysis

LC-MS/MS analyses were performed using an Agilent 1260 Infinity LC system coupled to an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The separation was conducted on a SUPELCO Discovery HS F5 column (3 μm, 3 × 150 mm; Sigma-Aldrich, Germany) using a binary mobile phase composed of A (water supplemented with 0.1 % formic acid) and B (acetonitrile). The mobile phase program was as follows: 0–15 min, B 0–100 %; 15–25 min, B 100 %. The flow rate was 0.4 mL/min, and the column oven was maintained at 30 °C. The injection volume was 5 μL.

The mass spectrometer was operated using an Agilent Jet Stream electrospray ionization (ESI) source in positive ion mode. The optimal MS parameters were as follows: capillary voltage, 3.5 kV; nebulizer gas pressure, 50 psi; dry gas temperature, 280 °C; dry gas flow rate, 10 L/min; sheath gas temperature, 350 °C; and sheath gas flow rate, 12 L/min. Quantitative analysis was performed in SRM mode.

Results and Discussion

Optimization of Analysis Conditions

Optimal separation is a critical factor in the simultaneous detection of multiple targets in complex matrices. Alliin, SAC, GSAC, and allicin have different polarities. The high polarity (XLogP3 = −3.5, PubChem) of alliin usually complicates the retention by common reverse-phase LC columns. To appropriately separate these four organosulfur compounds and obtain the relatively long retention time necessary to distinguish alliin from the void volume peak, several reverse-phase LC columns have been tested (data not shown). An octadecyl column (3.0 × 150 mm, 1.8 μm) and a pentafluorophenylpropyl (PFPP) column (3.0 × 150 mm, 3 μm) were found to be able to achieve the abovementioned two goals. The latter column exhibited a longer retention time for alliin (3.53 vs. 3.33 min) and was thus chosen for this study. PFPP modified silica columns usually have longer retention for polarity compounds compared to octadecyl columns, which might relate to the strong Lewis acid property of PFPP (Marin and Barbas 2006).

The SRM transitions for these compounds were optimized by single-MS full-scan mode followed by product ion scan mode using the standard compounds. ESI in positive ion mode was found to have the best performance for the mobile phase conditions investigated. Agilent Jet Stream thermal gradient focusing technology can enhance the sensitivity of ESI-MS by improving the desolvation and spatial focusing of ions (Mordehai and Fjeldsted 2009). Herein, an Agilent Jet Stream electrospray ionization (ESI) source was applied to ensure the sensitivity of detection for these four garlic compounds. The precursor ions were $[M+H]^+$. The predominant stable fragment from the precursor ion in MS/MS was selected as the product ion. The precursor–product ion pairs (quantification transitions) for the compounds were found to be *m/z* 178.1-88.1 (alliin), *m/z* 162.1-73.1 (SAC), *m/z* 291.1-145.0 (GSAC), and *m/z* 163.0-41.2 (allicin; Fig. 1). The corresponding fragmentation patterns for these four compounds are also illustrated in Fig. 1. The optimal collision energies and fragmentor voltages for the quantification transitions of alliin, SAC, GSAC, and allicin were 4 and 70 eV, 12 and 70 eV, 12 and 100 eV, and 16 and 50 eV, respectively. The transitions m/z 178.1 > m/z 42.0 (alliin), m/z 162.1 > m/z 41.3 (SAC), m/z 291.1 > m/z 73.0 (GSAC) and m/z 163.0 > m/z 73.0 (allicin) were used for qualification. The corresponding collision energies for the qualification transitions were 10, 20, 20, and 20 eV, respectively. The intensity ratios of the qualification

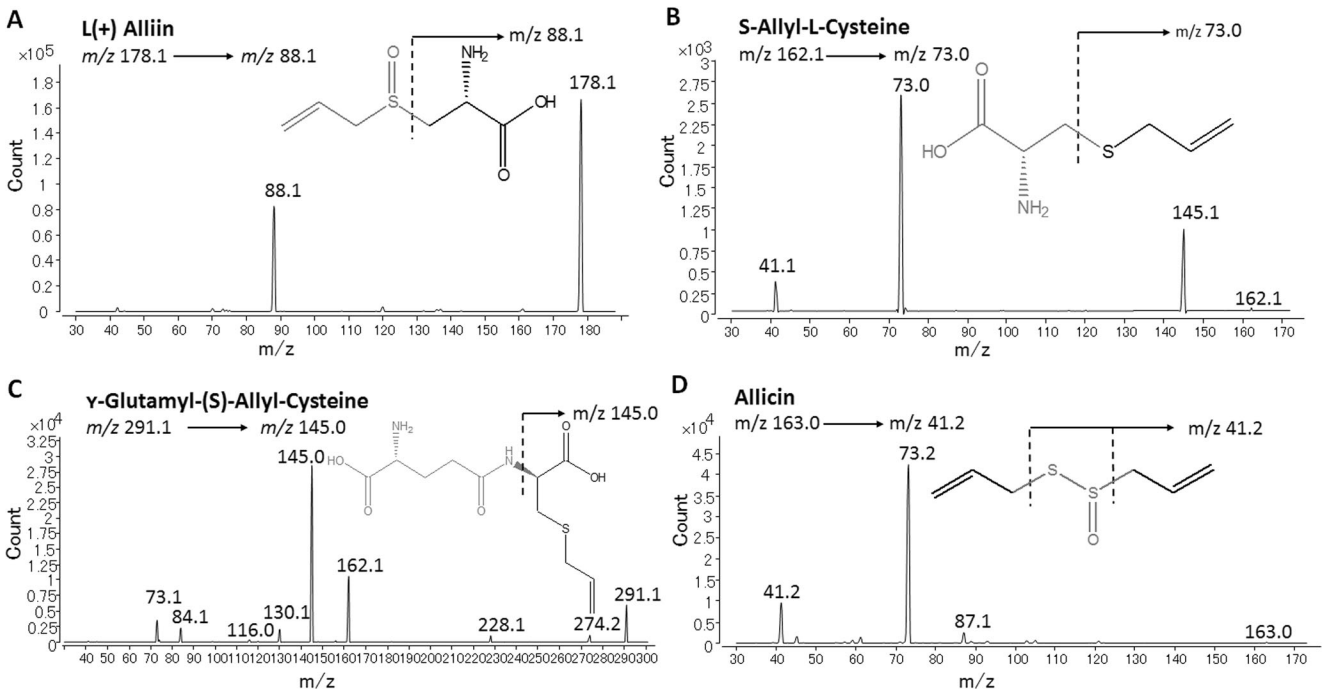

Fig. 1 Product ion spectra and the main fragmentation pathway of $[M+H]^+$ for L(+) alliin (**a**), S-allyl-L-cysteine (**b**), γ-glutamyl-(S)-allyl-cysteine (**c**), and allicin (**d**). The precursor–product ion pairs (transitions) and corresponding fragmentation pathways for these four compounds are also illustrated

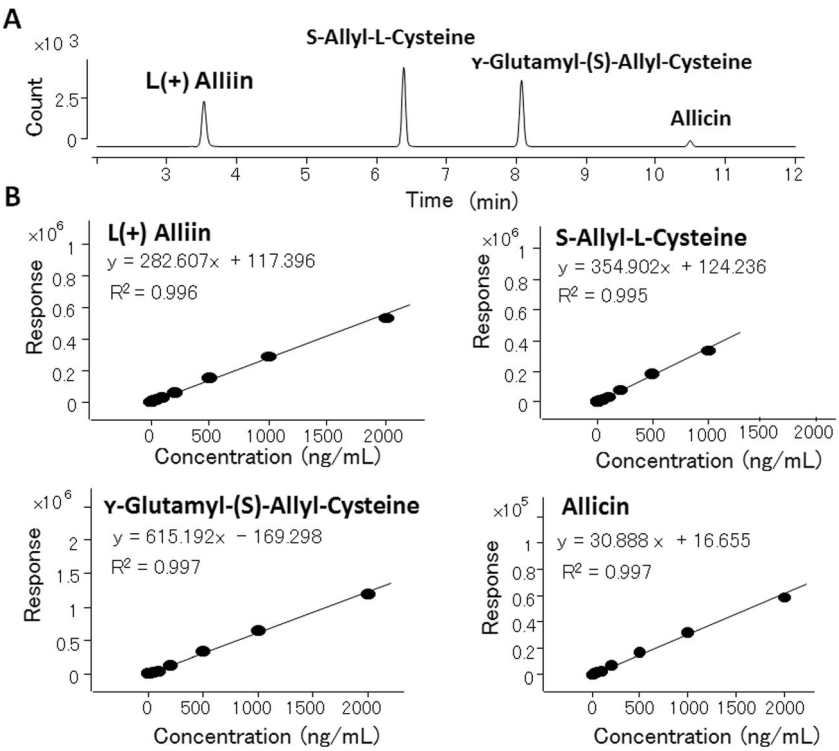

Fig. 2 SRM chromatogram for the simultaneous detection of four standard compounds (10 ng/mL each) (**a**) and their calibration curves (**b**)

ion to the quantification ion were 34.3% (alliin), 34.2% (SAC), 37.5% (GSAC), and 24.3% (allicin), which were also satisfactorily reproduced between the standards and testing samples (data not shown). Those optimized MS/MS parameters were automatically obtained by Agilent MassHunter Optimizer software.

The stability of garlic organosulfur compounds is always an important issue for their quantification. In particular, allicin is extremely unstable due to the presence of a thiol group (Fujisawa et al. 2008; Monosulphide and Monosulphide 2001; Prati et al. 2014), which is rapidly metabolized into diallyl sulfide, diallyl disulfide, ajoene, etc. (Rahman 2007b). Low pH has been found to inhibit the activity of alliinase and slow the decomposition of alliin and allicin (Lawson and Hughes 1992; Monosulphide and Monosulphide 2001). Here, we used HCl aqueous solution

Table 1 Validation parameters for the determination of alliin, SAC, GSAC, and allicin using LC-MS/MS-SRM

Analytes	Linearity range (ng/mL)	S/N (0.1 ng/mL)	LOD (S/N = 3, ng/mL)	LOQ (S/N = 10, ng/mL)	Retention time ($n = 5$)		Apparent recovery rate		
					Intra-day (min)	Inter-day RSD (%)	Added (ng/mL)	Recovery (%)	RSD (%) ($n = 5$)
L(+) Alliin	1–2000	35.0	0.009	0.03	3.53 ± 0.00	0.08	1	102.9 ± 1.9	1.9
							10	95.7 ± 6.6	6.9
							50	103.1 ± 0.1	0.14
							500	101.6 ± 1.3	1.3
SAC	1–1000	64.9	0.005	0.02	6.40 ± 0.00	0.11	1	101.3 ± 2.8	2.7
							10	104.5 ± 4.2	4.0
							50	108.7 ± 1.6	1.4
							500	101.6 ± 5.9	5.8
GSAC	1–2000	106.8	0.003	0.01	8.07 ± 0.00	0.12	1	109.1 ± 1.8	1.6
							10	93.7 ± 2.6	2.8
							50	98.8 ± 5.0	5.1
							500	103.7 ± 4.3	4.2
Allicin	1–2000	5.2	0.058	0.19	10.48 ± 0.00	1.62	1	97.6 ± 2.2	2.3
							10	107.3 ± 0.9	0.8
							50	110.8 ± 2.1	1.9
							500	103.5 ± 6.3	6.1

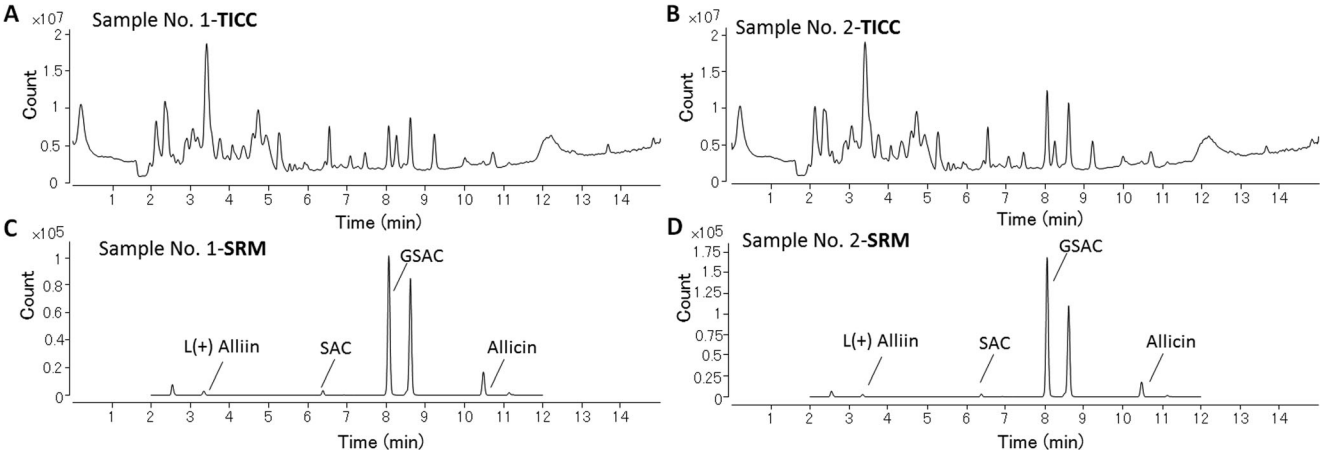

Fig. 3 Chromatograms of fresh garlic samples Nos.1 and 2. **a**, **b** Total ion current chromatogram (TICC) of 100-fold diluted extract of fresh garlic No.1 and No.2, respectively. **c**, **d** SRM chromatograms of 500-fold diluted extracts of fresh garlic No.1 and No.2, respectively

(0.01 M, pH 2.0) as the solvent to prepare the samples (Ichikawa et al. 2006b), and the samples were kept at 4 °C. All four studied compounds were stable under these conditions for at least 72 h (data not shown).

Method Validation

To evaluate the potential for the practical application of the proposed method, five critical parameters were studied: linearity, limit of detection (LOD), limit of quantification (LOQ), apparent recovery, and repeatability. The linearity was determined using 8–12 levels of mixed standard solution (1–5000 ng/mL). A good SRM chromatographic resolution was achieved (Fig. 2a). The calibration curves for all analytes were linear over a wide concentration range with correlation coefficients (r) greater than 0.99 (Fig. 2b and Table 1). The injected 0.1 ng/mL of alliin, SAC, GSAC, and allicin generated signal-to-noise ratios (S/N) of 35, 64.9, 106.8, and 5.2, respectively. The LOD and LOQ were determined as the concentrations with S/N = 3 and S/N = 10, respectively. The LODs for these compounds ranged from 0.003 to 0.058 ng/mL, while the LOQs ranged from 0.01 to 0.19 ng/mL (Table 1).

The apparent recovery and repeatability were evaluated at five spiked levels (Table 1). Different amounts of these four compounds were spiked with the sample extraction solution. The apparent recoveries were determined in duplicate to sextuplicate basing on the calibration curves. The apparent recoveries for alliin, SAC, GSAC, and allicin ranged from 95.7 to 103.1 %, 101.3 to 108.7 %, 93.7 to 109.1 %, and 97.6 to 110.8 %, respectively. The relative standard deviation (RSD) for these compounds at different spiked levels ranged from 0.14 to 6.9 %, which suggests that the repeatability of the method is satisfactory. Furthermore, the retention times were conservative in the intra- and inter-day tests (Table 1). These results indicate that the proposed method is satisfactory in terms of linearity, sensitivity, and precision.

Method Application

To demonstrate the applicability of the LC-MS-SRM method to real garlic samples, the method was applied to two fresh garlic samples, No.1 and No.2, which were cultivated in Japan and China, respectively. The TICC (total ion current chromatogram) and SRM chromatograms obtained from 100- to 500-fold diluted fresh garlic extracts are shown in Fig. 3. Although the garlic matrix is complex, clear SRM peaks were observed for alliin, SAC, GSAC, and allicin at the expected retention times (Fig. 3c, d).

The method was successfully applied to quantify the representative organosulfur compounds in the fresh garlic samples (Table 2). The extracts of the samples were diluted 10-fold and 500-fold for the quantitation of alliin and other three compounds, respectively. Alliin was detected at a very low concentration (0.14 and 0.19 μg/g fresh weigh for No.1 and No.2, respectively), whereas high concentrations of allicin were detected (2322.9 and 2601.5 μg/g fresh weigh in No.1 and No.2, respectively), suggesting that most of the alliin in the samples was enzymatically converted to allicin. The

Table 2 Contents of alliin, SAC, GSAC, and allicin in fresh garlic

Sample[a]	Content (μg/g FW)[b]			
	L (+) Alliin	SAC	GSAC	Allicin
No. 1	0.14 ± 0.01	36.1 ± 0.06	979.6 ± 4.0	2322.9 ± 2.2
No. 2	0.19 ± 0.01	40.38 ± 0.38	1883.9 ± 10.6	2601.5 ± 1.8

[a] Sample nos. 1 and 2 are fresh garlic samples cultivated in Japan and China, respectively

[b] μg/g FW: microgram per gram of fresh weigh (FW) garlic; each value is the mean ± standard deviation (n = 6)

concentrations of all four compounds were slightly higher in sample No.2 than in sample No.1. The variation in the concentrations of these compounds between the two garlic samples might be related to the differences among the cultivars, planting area, and maturity. The contents of alliin, GSAC, and allicin in fresh garlic were previously determined by HPLC to be in the range of 5400–14500 μg/g, 1900–8200 μg/g, and 2500–5000 μg/g, respectively (Iberl et al. 1990; Saito, 2008), while the content of SAC in raw garlic was reported as 22.73 μg/g (Bae et al. 2012). Herein, the contents of SAC, GSAC, and allicin were close to the reported ranges determined by HPLC, whereas the concentration of alliin was not in agreement with the HPLC result. In other two previous studies with HPLC quantification, the content of alliin was also found to be high in the dry garlic (Yoo et al. 2014; Montano et al. 2011). The relatively high alliin content previously reported may be because alliin was determined before being converted to allicin. Another possible explanation is that quantification was affected by the peak overlap caused by the solvent or another component. Our proposed LC-MS/MS-SRM method eliminates the effects of overlapping peaks.

When compared to usual HPLC quantification methods, our proposed LC-MS/MS-SRM method has significant advantages on specificity and sensitivity. Because in the LC-MS/MS-SRM method, the quantification was based on a more sensitive ion signal and the qualification was based on the retention time plus highly selective MS/MS ion pairs. When compared to the existing LC-MS/MS-SRM method for SAC (Lee et al. 2015; Lee et al. 2014), the advantages of our method is the optimized condition not only for quantifying SAC but also for quantifying other three representative garlic compounds simultaneously. These advantages give the method great potential to be used for the quality control of various garlic products that might have trace-level of organosulfur compounds or contain complex matrices.

Conclusion

An LC-MS/MS-SRM method for the rapid, simultaneous quantification of alliin, SAC, GSAC, and allicin in garlic products was developed. The method showed good linearity over a wide concentration range with very low LOD and LQD. Excellent recovery and reproducibility at different spiking levels were achieved. In a proof-of-concept study, this method was successfully applied to the simultaneous detection of four organosulfur compounds in fresh garlic. This highly selective and sensitive LC-MS/MS-SRM method for the simultaneous quantification of alliin, SAC, GSAC, and allicin will be a useful tool for the study of molecular functionality and for the reliable quality control of garlic products.

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Compliance with Ethical Standards

Conflict of Interest Qinchang Zhu declares that he has no conflict of interest. Kenichi Kakino declares that he has no conflict of interest. Chika Nogami declares that he has no conflict of interest. Koichiro Ohnuki declares that he has no conflict of interest. Kuniyoshi Shimizu declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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