Simple and Rapid Analytical Technique to Resolve the Two Isomers, α-alanine and β-alanine using GC-MS/MS

Pyone Pyone Yee* Dr. Warawan Eiamphungporn** Dr. Sakda Yainoy** Dr. Vichanan Yamkamon***
Dr.Thummaruk Suksrichavalit****

ABSTRACT

A simple and rapid analytical method was developed using gas chromatography-tandem mass spectrometry (GC-MS/MS) to resolve β-alanine which is popular nowadays as food supplement among trained athletics to enhance muscle endurance and athletic capacity from its isomer, α-alanine in foods and nutritional judgment. Both amino acids were derivatized with BSTFA + 1% TMCS and then, derivatives were efficiently determined by GC-MS/MS with multiple reaction monitoring (MRM), relative retention times at 6.23 and 7.24 mins. The limit of quantification (LOQ) of α-and β-alanine were 0.03 and 0.24 μg mL^{-1} with high linearity (R^2 = 0.9974 and 0.9846 respectively). Trimethylsilyl derivatives were >80% stable up to two hours at room temperature. Further method validation would be accomplished in recovery performance, precision and selectivity to certify application in nutritional and pharmaceutical perspectives.

Keywords: Gas chromatography-tandem mass spectrometry (GC-MS/MS), α-alanine, β-alanine

* *Student, Master of Science program in Medical Technology (International Program), Faculty of Medical Technology, Mahidol University*

** *Assistant Professor, Department of Clinical Microbiology and Applied Technology, Faculty of Medical Technology, Mahidol University*

*** *Lecturer, Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University*

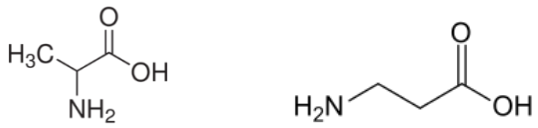
**** *Lecturer, Department of Data Mining and biomedical informatics, Faculty of Medical Technology, Mahidol University*

Introduction

Amino acids are basic elementary units for protein synthesis, the energy source in protein metabolism and neurotransmission. Some amino acids have been used as nutritional or dietary supplementation. Natural beta-amino acid (β-alanine) coupling with histidine converts to dipeptide carnosine, it has physiological role in contracting muscle. Accordingly, β-alanine supplementation has been exhibited the improvement of muscle endurance, and the building block of carnosine (β-analyl-L-histidine) which offers pH buffering activity and conserves exercise-induced lactic acid production (Tiedje *et al.,* 2010). Efficiency of short-term β-alanine supplementation in intensively-trained athletes was apparent in massive training values and lesser fatigue (Hoffman *et al.,* 2008). Food-addition of which overcame limited usage of β-alanine to convert carnosine, that was strongly correlated with energy output and worked out (Sale *et al.,* 2010). Although the accurate underlying mechanisms of memory strengthening of β-alanine have not known yet, six successive days supplementation of 10-20 mg kg^{-1} beta-alanine delineated learning and memory improvement in both young and aged-mice (Dhingra *et al.,* 2006).

In order to study the constituent of β-alanine in regular meal or pharmacological interests which was commonly monitored similar to other amino acids analysis (AAAs) using gas chromatography hyphenated with flame ionization detection (GC-FID), or high performance liquid chromatography in combination with either post-column ninhydrin derivation or pre-column derivatization using *o*-phthalaldehyde and liquid chromatography coupled with mass spectrometry without derivatization (LC-MS) (Krumpochova *et al*., 2015).

In addition, β-alanine possesses the same chemical structure and physical properties with its isobaric compound, alpha-alanine (α-alanine) (Fig. 1). The retention times of which from the liquid chromatography were approaching each other (8.704 and 8.907 mins) (Goldman, 2010). Therefore, this could be hinder the accurate determination of the two isomers. Furthermore, development of AAAs were mainly focused on 20 or more natural amino acids determination. Ordinarily, free β-alanine was analyzed together with creatinine in biological samples or with arginine and glutamine in nutritional products (Baxter *et al.,* 2012). Difference between this two isomers (alpha- and beta-alanine) had been theoretically studied (Abirami *et al.,* 2005). However, simple and fast GC-MS/MS analysis to separate and quantitate alpha and beta-alanine in nutritional and dietary aspects have never been studied.



α-alanine β-alanine

Figure 1 Schematic structures of α-alanine and β-alanine

Objectives of the study

Aim of this study is to develop a simple, rapid and accurate method for identification of β-alanine and α-alanine using gas chromatography-tandem mass spectrometry (GC-MS/MS) after silylation derivatization.

Materials and Methods (Methodology)

Study design

Studies involved optimization of derivatization reaction (silylation), GC-MS/MS conditions, and method validation, then stability and selectivity investigation of trimethylsilyl derivatives.

Experimental Protocols

Chemicals and reagents

Amino acid standards (α-alanine and β-alanine with 98% purity) were purchased from Sigma-Aldrich Corporation (St. Louis, MO). *N, O*-Bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane [BATFA + TMCS, 99:1 (Sylon BFT), derivatization grade for GC] was obtained from SUPELCO (Bellefonte, USA). More than 99.5% purity acetonitrile and water (HPLC grade) were procured from Chemex Industry Co.Ltd (Thailand). The certified reference amino acids mixture solution was obtained from Sigma-Aldrich Corporation (St. Louis, MO).

Preparation of standard stock solution

Standard stock solutions of α-alanine (1 mg mL^{-1}) and β-alanine (1 mg mL^{-1}) were prepared by diluting 10 mg of each amino acid in HPLC grade water (10 mL), and then stored in -20 °C until use.

Silylation Derivatization

The standard solutions of 100 µL α-alanine and β-alanine were put into GC analysed vials which were undergone overnight vacuum drying at 400 mmHg. After completely dry, further 100 µL of BSTFA + 1% TMCS was added. To optimize silylanization, derivatization was carried out at 100 °C for 30 min, 1 h, 1.5 h and 2 h. Derivative of each metabolite was mixed up with 100 µL of acetonitrile, then transferred into GC insert. 1 µL of the derivatives was injected into GC-MS/MS by automation.

Gas Chromatography

Bruker 456 Gas Chromatography (GC) coupled with Bruker Scion Triple Quadrupole Mass Spectrometer (Bruker Corporation) accompany with GERSTEL multipurpose sampler MPS was used for this entire study. The GC conditions were listed in table 1. Optimization the column oven temperature for both were performed at three temperature programs (table 2).

Table 1 Gas chromatography conditions

Parameter	Value
Analytical column	Rxi ®-5Sil MS (30 m × 0.25 mm × 0.25 µm, RESTEK, USA)
Carrier gas	Helium (> 99.995% purity)
Flow rate	1.0 mL min^{-1}
Injection type	Splitless
Injection volume	1 µL
Temperature of injection	260 °C

Table 2 Optimization of column oven temperature program

Condition	Initial Temp.	Hold Time	Ramp Temp.	Final Temp.	Hold Time
(a)	100 °C	2 min	20 °C min^{-1}	300 °C	3 min
(b)	80 °C	2 min	8 °C min^{-1}	280 °C	3 min
(c)	80 °C	2 min	15 °C min^{-1}	280 °C	3 min

Tandem Mass Spectrometry (MS/MS) condition

Overall tandem mass spectrometry conditions were: ion source temperature, 230 °C; positive ion mode, electron impact ionization at 70 eV; full scan mode, mass range 50-500 a.m.u; selected-ion monitoring (SIM) mode, specified ion fragments for silyl derivatives and retention times. At last optimized the collision energy (CE) for particular transition ions and multiple reaction monitoring (MRM) method was manipulated.

Method Validation

Linearity ranges were determined from standard calibration curves of alpha- and beta-alanine, the concentration range between 0.03 - 500 µg mL^{-1}. The limit of determination (LOD) and limit of quantification (LOQ) were calculated at signal to noise ratio reach to 3:1 and 10:1 respectively. All experiments were triplicated. Recovery performance will be analysed known concentrations of alpha- and beta-alanine (2, 5, 10 µg mL^{-1}) spiking in commercial dietary products. Intra-day and day-to-day precision (n=20) of 10 µg mL^{-1} spiked samples might also be carried out for evaluation of RSD%.

Stability of trimethylsilyl derivatives of amino acids were monitored at 30 min interval until 5 hours. Method selectivity will be investigated using certified reference materials.

Results

In derivatization, reaction temperature at 100 °C for 1.5 hrs furnished an optimum yield for α-alanine and β-alanine (Fig. 2A-B). An outstanding peak shape and clear chromatographic separation between α -and β-alanine were favoured by oven temperature program of initial was 80 °C, ramped up 15 °C min^{-1} to 280 °C and finally hold 3 mins at 280 °C (Fig. 3). Under optimal conditions, α-alanine was firstly eluted from the column at 6.23 mins, followed by β-alanine at 7.24 mins (Fig. 3). Thus, run time for both amino acid was completed at total 7.5 mins.

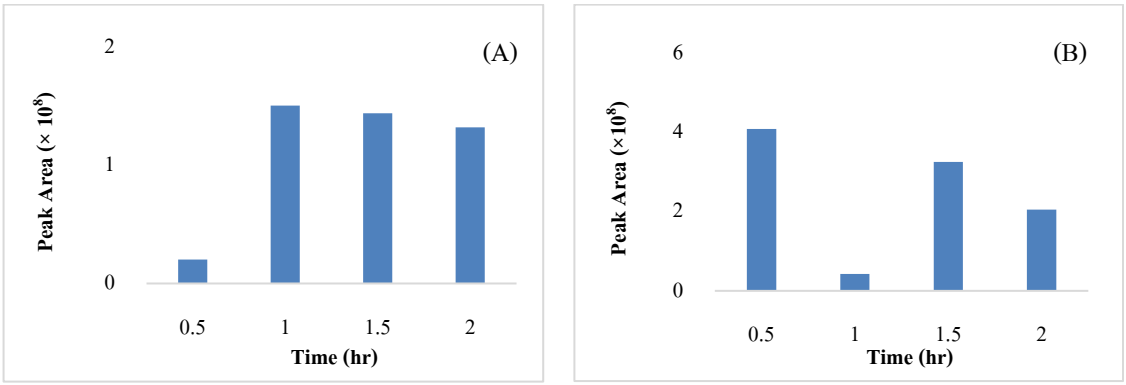


Figure 2 Derivatization (silylation) reaction optimization, reaction temperature was fixed at 100 °C, 1.5 hrs reaction time was suitable to set up an optimum for both (A) peak area vs reaction time of α-alanine (B) peak area vs reaction time of β-alanine.

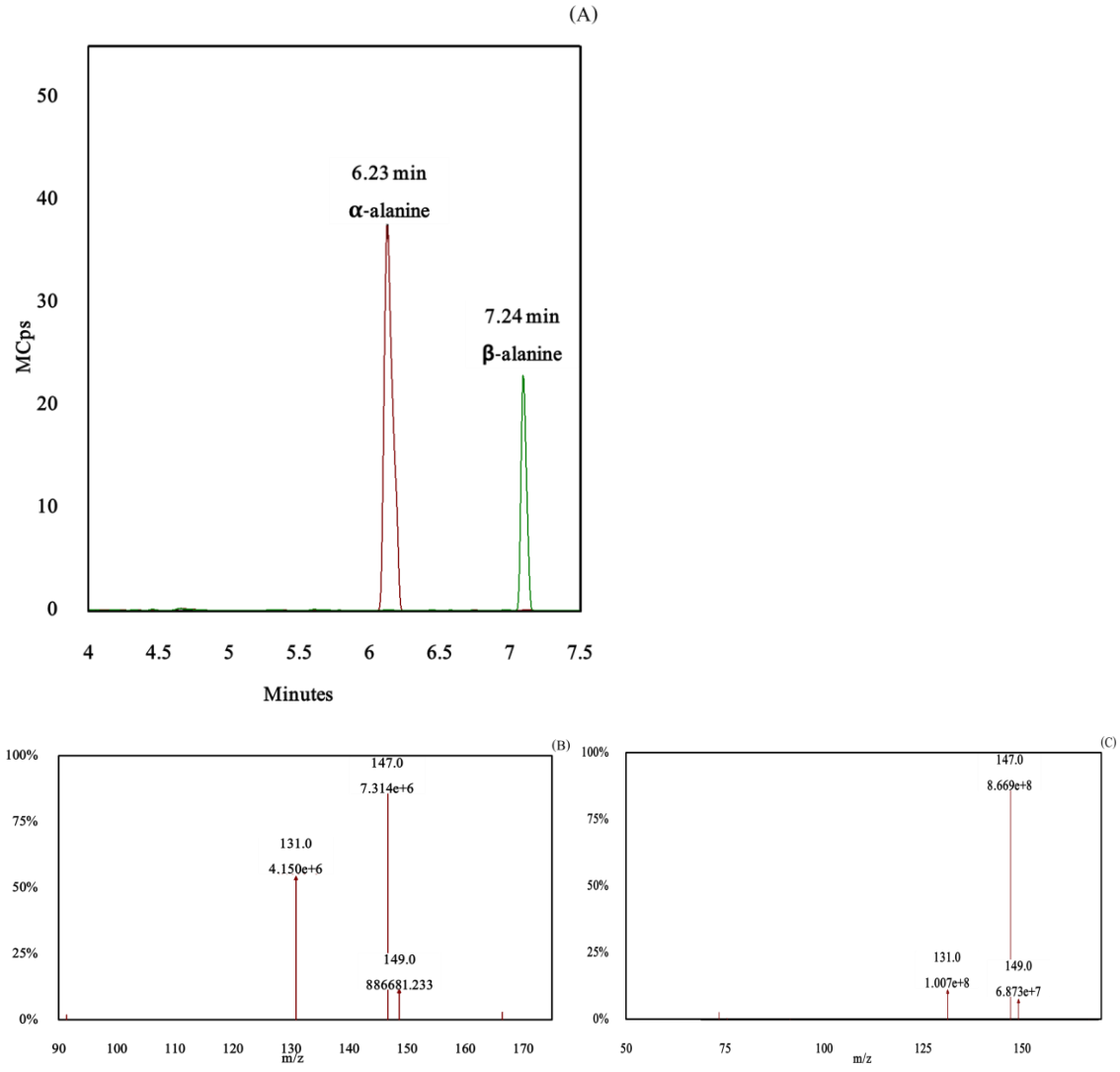


Figure 3 Total ion chromatogram (TIC) of α-alanine and β-alanine using optimal MRM mode (A), Mass spectrum of α-alanine (B), Mass spectrum of β-alanine (C).

Full scan mass spectra of α- and β-alanine were selected; the target ions as 73, 116, 147, 190 and 73, 102, 147, 176 respectively for further selected ion monitoring (SIM) (table 3). The specific transition ions were figured out to build up multiple reaction monitoring (MRM) which enhanced sensitivity and specificity for detection and quantification of isobaric compounds. An ultimate MRM condition for α- and β-alanine are summarized in table 4.

Table 3 Full-scan and Selected Ion Monitoring of alpha- and beta-alanine

Analyst	EI Mass Spectrum (*m/z*)	Retention Time (min)	Target Ions (SIM)
α-alanine	59, 73, 116, 147,190, 218	6.23	73, 116, 147, 190
β-alanine	59, 73, 102, 116, 147, 176, 218	7.24	73, 102, 147, 176

m/z mass by charge ratio

Table 4 MS/MS parameters for α- and β-alanine

Analyst	MW	Retention Time (min)	Q1	Q3	CE (eV)
α-alanine	89	6.23	116	91	5
			147	131	10
			190[a]	147[a]	15
			190	149	5
			190	167	5
β-alanine	89	7.24	102	91	5
			147	131	20
			147	91	5
			176[b]	147[b]	5
			176	149	5

MW molecular weight, Q1 quadrupole 1, Q3 quadrupole 3, CE collision energy

[a] Specific transition ions for quantification of α-alanine

[b] Specific transition ions for quantification of β-alanine

The optimal conditions were successfully performed. This simple and rapid analytical method for α- and β-alanine showed wide range of linearity (R^2 = 0.9974 and 0.985) (Fig. 4 & 5). LOD of α-alanine and β-alanine were achieved ~ 0.03 and 0.12 μg mL^{-1}, meanwhile LOQ were 0.03 and 0.24 μg mL^{-1} as demonstrated in table 5.

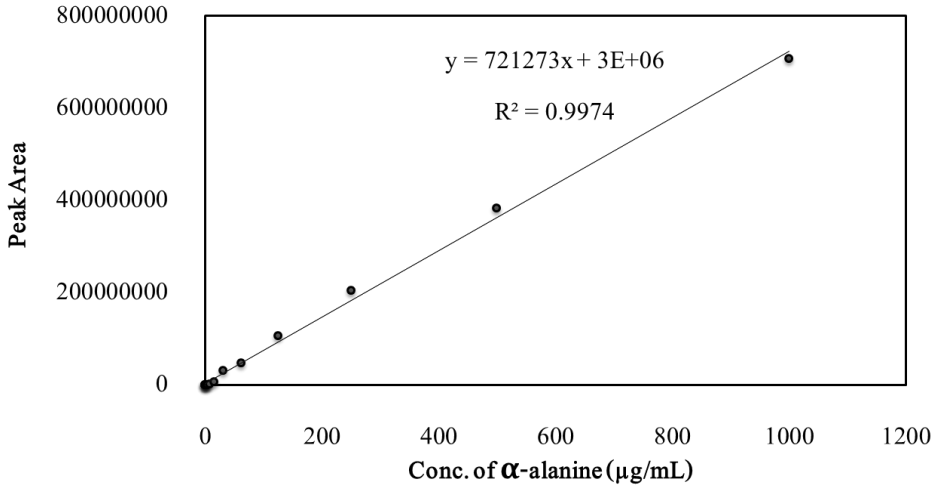


Figure 4 Correlation of the analytical results for α-alanine with newly developed method (concentration range: 0.03 – 500 µg mL^{-1} with two-fold serial dilutions) (n=3).

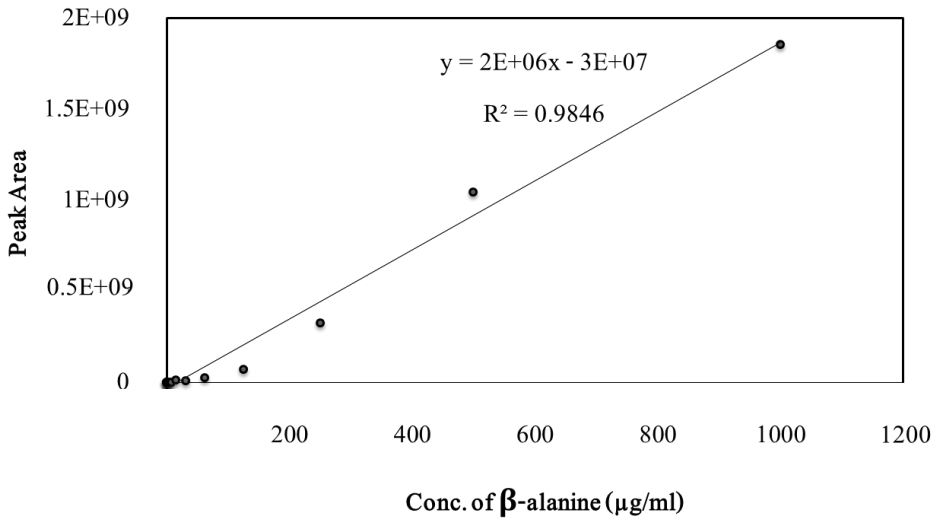


Figure 5 Correlation of the analytical results for β-alanine with newly developed method (concentration range: 0.03 – 250 µg mL^{-1} with two-fold serial dilutions) (n=3).

Table 5 Calibration and quantitative results of GC-MS/MS (All experiments were triplicated)

Analyst	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Linearity (µg mL^{-1})	Linearity equations	R^2
α-alanine	0.03	0.03	0.03 - 500	y = 721273x + 3E+06	0.9974
β-alanine	0.12	0.24	0.03 - 250	y = 2E+06x - 3E+07	0.9846

The stability study of these derivatives was also proved. The results showed that two derivatives were stable (> 80%) up to 2 hours at room temperature (Fig.6), then gradually declined until 47% for α-alanine and 24% for β-alanine after 5 hours.

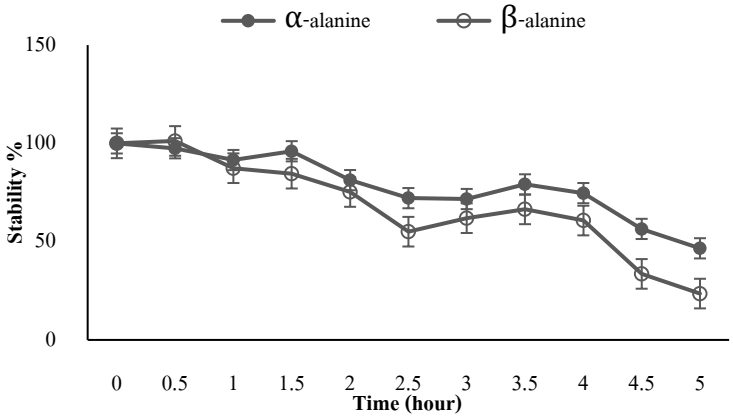


Figure 6 Stability studies of trimethylsilyl derivatives of α-alanine (close circle) and β-alanine (open circle). All experiments were triplicated.

Discussion and Conclusion

Chromatographic separation of α-alanine and β-alanine

Various derivatization procedures were applied to improve chromatographic separation, to reduce polarity of the target analysts and to enhance the sensitivity for low molecular weight compounds (Chen *et al.,* 2014, Zhu *et al.,* 2016). The most prevalence and simplest method, silylation was employed in this study (Wu *et al.,* 2011). Reaction time that is the major influencing factor upon derivatization efficiency was studied at fixed temperature (100 °C). Although the highest efficiency of α-alanine and β-alanine yielded in different reaction time (Fig. 1A & B), the optimal reaction for both was affordable at 100 °C for 1.5 hrs by second highest peak area.

After selection of suitable Rxi ®-5Sil MS column, upmost crucial in developing GC separation is designating column oven temperature for analysis. Therefore, several GC column oven temperatures were studied to achieve an acceptable resolution for α- and β-alanine. The researcher had been developed HPLC method to analyse 16 amino acids including alanine in commercial fruit juices to determine nutritional value (Fabiani *et al.,* 2002) but beta-alanine was excluded. In other previous study using GC-MS method, the times to elute from column were quite close (Goldman *et al.,* 2010). Thus, the one could probably be disappeared in the shoulder of another compound. Different gradient temperature programs had been developed and investigated (table 2) based on the protocol of Wu et al. (Wu *et al.,* 2011). Three temperature programs were investigated. The first condition was failed to exhibit sharp and symmetric chromatographic peak. The second condition explicated better TIC, however, it had peak broadening and tailing. Finally, the last condition provided outstanding peak shape and clear chromatographic separation as shown in Fig. 3.

Mass spectra study of amino acids derivatives

Since α-alanine and β-alanine possess the same mass spectra, target ions were selected for SIM mode with great caution (table 3). Highest abundance transition ions *m/z* 190 □ 147 (α-alanine) and *m/z* 176 □ 147 (β-alanine) were selected to analyse in MRM mode (table 4). This circumstance brought a satisfactory separation of between α-alanine and β-alanine with different response factor.

Analytical Performance

The result from method validation demonstrated that linearity of the calibration curve was studied with standard concentrations of α-and β-alanine which covered the range of 0.03-500 μg mL^{-1} and 0.03-250 μg mL^{-1} with correlation coefficient (R^2) of 0.9974 and 0.9846 correspondingly. These results were suitable enough to implement in nutritional study. Other analytical parameter; LOD and LOQ for α-alanine was about ~0.03 and 0.12 μg mL^{-1}, for β-alanine was 0.12 and 0.24 μg mL^{-1} which allowed adequate sensitivity as well. In future perspectives, recovery performance is hopefully to be within 80-120%, good precision and selectivity either. Moreover, stability of the derivatives are better to know to accomplish accurate determination. Silyl derivatives of amino acids were feasible by GC-MS, however, some derivatives became undetectable after particular time of derivatization (Gao *et al.,* 2013). In this study, we found that trimethylsilyl derivatives had 80% stability up to two hours at room temperature, then it was dramatically declined. Therefore, GC-MS/MS analysis for silyl derivatives of α and β-alanine should be carried out either immediately or within two hours after derivatization.

To be concluded, simple and rapid GC-MS/MS analysis for separation and quantification of the two isomers, α-and β-alanine had been developed, even though two further analytical assessments (recovery performance and precision) are still needed to be completed. Being the competence of GC-MS/MS minimized matrix interference and improved the sensitivity and specificity. Additionally linearity, LOD and LOQ of the developed method were satisfactory to apply in the study of nutritional value or dietary supplement.

Acknowledgements

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