

Analysis of γ -Aminobutyric Acid Content in Fermented Plant Products by HPLC/UV

Dong Gu Lee · Sunghun Cho · Jamin Lee · Seon Haeng Cho · Sanghyun Lee* 

Received: 12 June 2015 / Accepted: 21 August 2015 / Published Online: 31 December 2015
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Abstract γ -Aminobutyric acid (GABA) content in fermented plant products and their main plant materials (aerial part of *Acanthopanax sessiliflorus*, fruit of *Crataegus pinnatifida*, and whole plant of *Morus alba*) was determined by high-performance liquid chromatography. GABA was quantified using a reverse-phase column with a gradient elution program (water:acetonitrile =90:10 to 0:100 for 40 min). UV detection was conducted at 280 nm. GABA content was measured in fermented plant products (15.07 mg/g), aerial part of *A. sessiliflorus* (4.49 mg/g), fruit of *C. pinnatifida* (10.59 mg/g), and whole plant of *M. alba* (2.31 mg/g). The presence of GABA in fermented plant products, including *A. sessiliflorus*, *C. pinnatifida*, and *M. alba* is important in industrial application for health supplements.

Keywords *Acanthopanax sessiliflorus* · constituent · γ -Aminobutyric acid · industrial application

Introduction

Acanthopanax sessiliflorus belongs to the family Araliaceae, and it is used as a traditional medicine in Korea, China, Japan, and Manchuria (Lee, 1979). *A. sessiliflorus* is widely used as an herbal medicine because of its anti-tumor, anti-inflammatory, analgesic,

and immunostimulatory activities. It is used in the treatment of cardiovascular and cerebrovascular diseases (Lee, 1979; Zhou, 1985; Lee et al., 2003; Song et al., 2011). The root bark of *A. sessiliflorus* is widely used for treating rheumatism, reinforcing kidney function, strengthening bones, and promoting blood circulation (Song et al., 2012b). In addition, the roots have various analgesic, anti-inflammatory, anti-pyretic, and diuretic activities (Brekhman and Dardymov, 1969; Shohael et al., 2005). Several constituents such as lignin, asarinin, sesamin, helioxanthin, and savinin have been isolated from the roots (Zhang et al., 2003). Quercetin, a pharmacologically and therapeutically effective compound, was isolated from the fruits (Zhu et al., 2013). In Korea and some other Asian countries, many beverages and tablets contain *A. sessiliflorus*. Recently, consumption of *A. sessiliflorus* has been increasing dramatically (Zhao et al., 2001).

Crataegus pinnatifida belongs to the family Rosaceae (locally called hawthorn), and it is widely distributed in Europe, North America, and northeastern part of China (Edwards et al., 2012). *C. pinnatifida* has been widely used as a medicinal and food material in China and the European countries (Fong and Bauman, 2002). To date, more than 160 compounds have been isolated from *C. pinnatifida*, including flavonoids, terpenes, lignans, and organic acids (Ahn et al., 1998; Edwards et al., 2012; Song et al., 2012a; Huang et al., 2013; Liu et al., 2013; Sendker et al., 2013). The leaves of *C. pinnatifida* are widely used for heart problem, such as declining cardiac performance, deficiency in coronary blood supply, and mild forms of arrhythmia (Pöpping et al., 1995; Al Makeddi et al., 1996).

Morus alba is a deciduous tree that belongs to the family Moraceae, and it is widely cultivated in tropical, subtropical, and temperate regions (Agarwal and Kanwar, 2007). *M. alba* has been used in East Asia as an herbal medicine for healing various diseases, such as lung-heat, cough, hematemesis, dropsy, beriberi, dysuria, and it has anti-inflammatory, anti-viral, and anti-tumor activities (Zhou and Li, 1997; Zhishen et al., 1999; Nam et al., 2002; Shi et al., 2001; Du et al., 2003; Park et al., 2003).

γ -Aminobutyric acid (GABA) is a non-protein amino acid that is widely distributed in nature and acts as a major inhibitory

D. G. Lee · S. Cho · J. Lee · S. Lee
Department of Integrative Plant Science, Chung-Ang University, Anseong 17546, Republic of Korea

S. H. Cho
Gongju National University of Education, Gongju 32553, Republic of Korea

*Corresponding author (S. Lee: slee@cau.ac.kr)

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neurotransmitter in the central nervous system (Manyam et al., 1981; Abdou et al., 2006). GABA is known to have hypotensive, diuretic, and tranquilizing effects. GABA intake could also help treat various neurological disorders, such as seizures, Parkinson's disease, stiff-man syndrome, and schizophrenia (Shin and Lee, 2002; Aoki et al., 2003; Oh et al., 2003; Hayakaw et al., 2004; Oh and Oh, 2004). GABA has various biological activities, so it is an important compound. Many studies have analyzed triterpenoids, lignans, and phenylpropanoids in plants, including those belonging to *Acanthopanax* (Jakobs et al., 1993; Inoue et al., 2003; Wong et al., 2003; Jin et al., 2004; An et al., 2008; Kim et al., 2012), but few studies have analyzed GABA content in plant materials.

In this study, GABA content in fermented plant products, including aerial part of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant of *M. alba* was analyzed using high-performance liquid chromatography (HPLC).

Materials and Methods

Plant materials. Aerial parts (leaf, stem, and fruit) of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant (leaf, stem, and root) of *M. alba* were cultivated in and collected from Gongju, Korea, and they have identified by Prof. S. H. Cho, Gongju National University of Education, Korea.

Preparation of fermented plant products. Fermented plant products were prepared using traditional methods; these products were composed up of 12 types of plant materials (aerial part of *A. sessiliflorus*, 12%; fruit of *C. pinnatifida*, 8%; whole plant of *M. alba*, 8%; sprout of *Phyllostachys bambusoides*, 8%; fruit of *Benincasa hispida*, 8%; sprout of *Brassica oleracea*, 8%; sprout of *Rhaphanus sativus*, 8%; aerial part of *Venanthe japonica*, 7%; aerial part of *Sedum sarmentosum*, 7%; root of *Phragmites longivalvis*, 7%; fruit of *Quercus acutissima*, 2%; and sprout of *Pinus densiflora*, 2%), mineral water (8%), malt sugar (3%), and honey (5%).

Chemicals and equipment. HPLC-grade solvents such as acetonitrile (ACN), methanol (MeOH), and distilled water (H₂O) were purchased from J. T. Baker[®] (USA), and GABA (Fig. 1) was purchased from Sigma-Aldrich (USA). Evaporation was conducted using an evaporator system (Eyela rotary system, Japan) under reflux *in vacuo*. HPLC was performed using a Waters Breeze system (Waters Co., USA) equipped with a Waters 1525 binary HPLC pump and a 2489 system UV/VIS detector.

Sample preparation. To analyze GABA in fermented plant products and their main plant materials, 20 g of each sample was extracted with 100% MeOH (3×100 mL) under reflux and evaporated *in vacuo*. The residue was dissolved in 1 mL of H₂O and filtered with a 0.45-μm filter. The resulting solution was used for HPLC analysis.

HPLC conditions. HPLC of GABA for qualitative and quantitative analyses was performed using a reverse phase system. An INNO[®] C18 (4.6×250 mm, 5 μm) column was used with a mobile phase

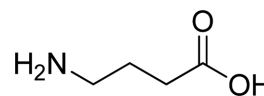


Fig. 1 Chemical structure of GABA.

that consisted of H₂O and ACN. A gradient solvent system of H₂O and ACN (90:10 to 0:100 for 40 min) was used for the elution program. UV detection was conducted at 280 nm. The injection volume was 10 μL, and the flow rate was 1 mL/min. All injections were performed in triplicate.

Limit of detection and limit of quantification. Validation of the HPLC method with GABA as the standard was performed using limit of detection (LOD) and limit of quantification (LOQ). The linearity of the method was established by triplicate injections in the range of 0.015–0.5 mg/mL. Standard calibration solutions were prepared at seven different concentration levels (0.5, 0.25, 0.15, 0.125, 0.0625, 0.0312, and 0.015 mg/mL) and injected in triplicate. Calibration curves were constructed by linear regression of the peak area-ratios (Y) of GABA versus the concentration (X) in mg/mL; relative standard deviation was used as a measure of repeatability. Percent recoveries were evaluated by calculating the ratio of the amount detected versus the amount added. The values of LOD and LOQ were determined separately at a signal to noise ratio (S/N) of 3 and 10, respectively.

Calibration curve. A stock solution (1 mg/mL) of GABA was prepared in water, and then successively diluted to 50% to create different concentrations. Analyte content was determined from the corresponding calibration curves. The calibration functions of GABA were calculated using the peak area (Y), concentration (X, μg/10 μL), and mean values (n=3)±standard deviation (SD).

High resolution LC/MS-MS. LC/MS-MS analysis was performed in a 5600 Q-TOF LC/MS/MS system (AB Sciex, USA) using a Ultimate 3000 RSLC HPLC system (Dionex, USA), including a degasser, an autosampler, diode array detector, and a binary pump. The LC separation was performed on a Hypersil GOLD column (2.1×50 mm, 1.9 μm, Thermo Scientific, USA) with a mobile phase A (0.1% formic acid in water) and a mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.25 mL/min. The linear gradient was as follows: 0–4 min, 99% A; 4.1–8.0 min, 50% A; and 8.1–13.0 min, 5% A. The autosampler was set at 5°C. The injection volume was 100 μL. Mass spectra were acquired under positive electrospray ionization with an ion spray voltage of -4500 V. The source temperature was 450°C. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 65, and 55 psi, respectively.

Table 1 Calibration curve for GABA

Name	t _R	Calibration equation ^a	Correlation factor, r ² ^b
GABA	35.76	Y=1226.5X-749.25	0.9909

^aY=Peak area, X=Concentration of standards (mg/mL).

^br²=Correlation coefficient for three data points from the calibration curve.

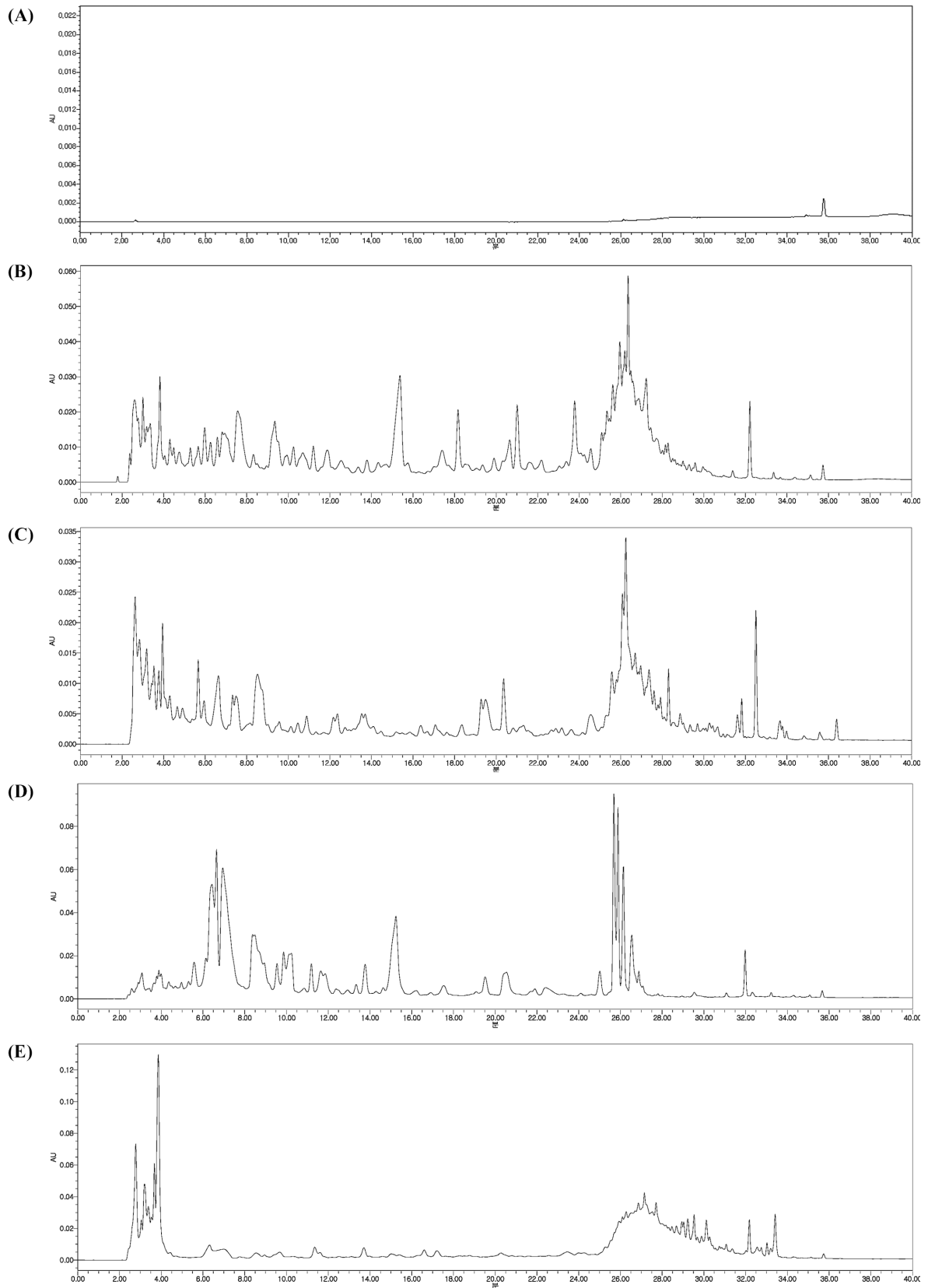


Fig. 2 HPLC chromatograms of GABA (A), fermented plant products (B), aerial part of *A. sessiliflorus* (C), fruit of *C. pinnatifida* (D), and whole plant of *M. alba* (E).

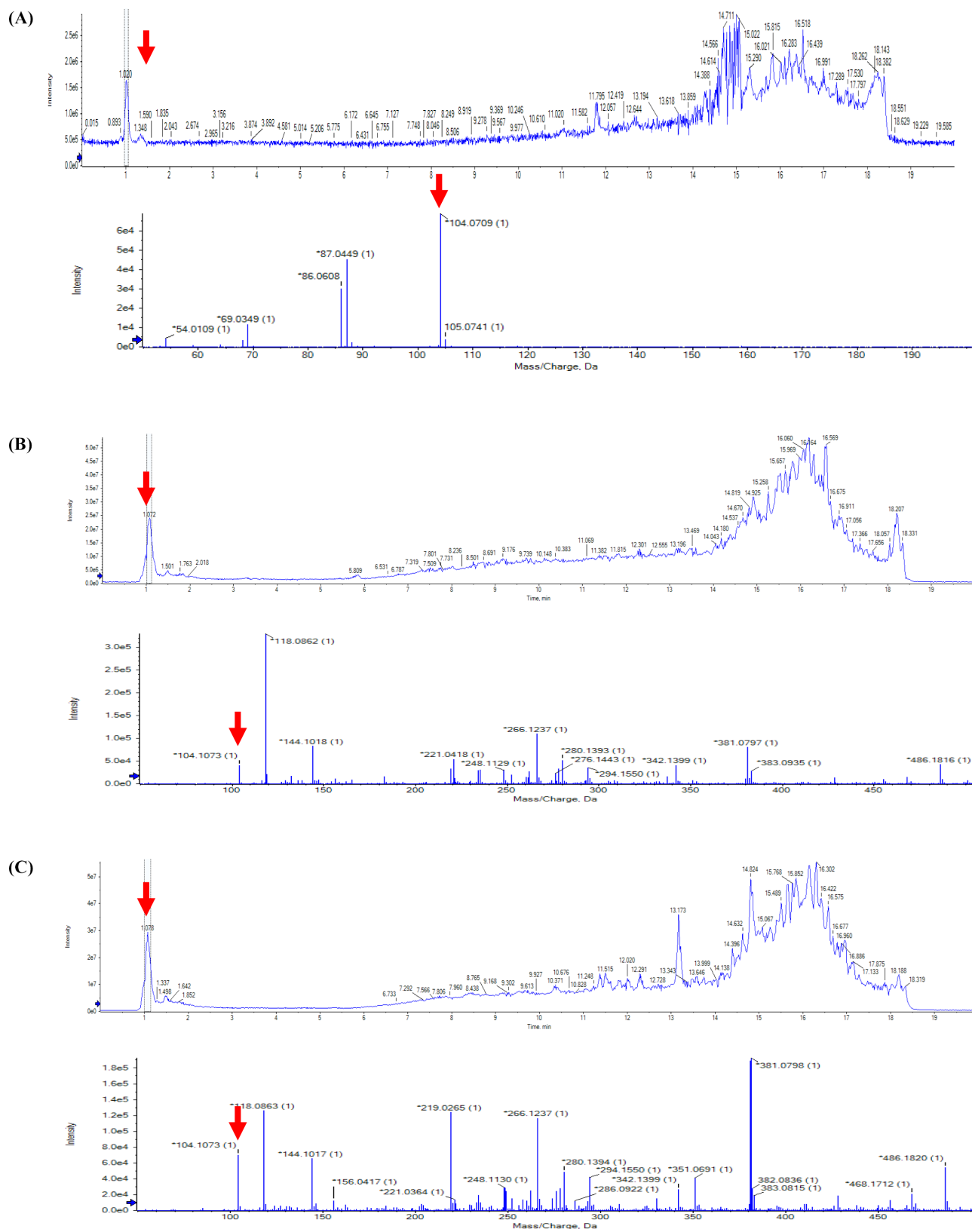


Fig. 3 LC/MS-MS chromatograms of GABA (A), fermented plant products (B), aerial part of *A. sessiliflorus* (C), fruit of *C. pimatifida* (D), and whole plant of *M. alba* (E).

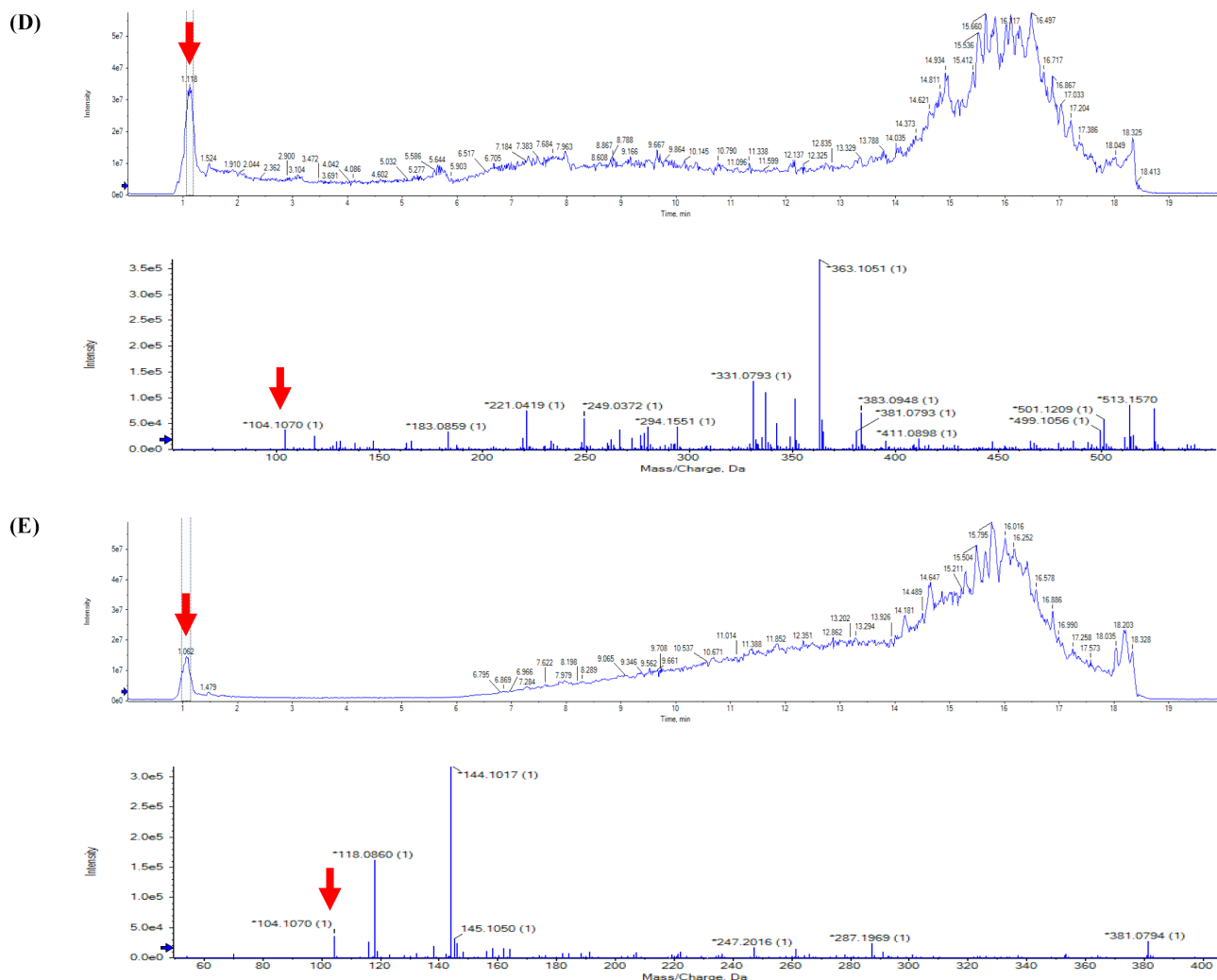


Fig. 3 Continued.

Results and Discussion

Qualitative and quantitative analyses of GABA content in fermented plant products and their main plant materials (aerial part of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant of *M. alba*) were performed using HPLC with a reverse phase system. The retention time for GABA was 35.76 min. The HPLC conditions used in the analysis showed good linearity for the five standard GABA solutions ($r^2=0.9909$) (Table 1). The calibration equation and retention time are shown in Table 1. GABA content in the fermented plant products, aerial part of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant of *M. alba* was determined on the basis of material specifications. The HPLC chromatograms of the fermented plant products, aerial part of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant of *M. alba* (100% MeOH extracts) are shown in Fig. 2. The optimized electrospray ionization condition should be sensitive enough to GABA with positive ion detection mode. The collision-induced dissociation of the precursor ion $[M]^+$ at m/z 104 resulted in the neutral loss of ammonia and the

formation of the product ions at m/z 87 (Fig. 3). Using an optimized analytical method, GABA content in the fermented plant products (15.07 mg/g), aerial part of *A. sessiliflorus* (4.49 mg/g), fruit of *C. pinnatifida* (10.59 mg/g), and whole plant of *M. alba* (2.31 mg/g) was successfully and simultaneously determined (Table 2). Our results showed that maximum GABA content was found in the fermented plant products (15.07 mg/g).

LOD and LOQ values were determined at signal to-noise ratios (S/N) of 3 and 10, respectively. LOD and LOQ values for ten

Table 2 GABA content in fermented plant products and their main plant materials

Sample	Content (mg/g)
Fermented plant products	15.07±2.03
Aerial part of <i>A. sessiliflorus</i>	4.498±0.630
Fruit of <i>C. pinnatifida</i>	10.59±0.95
Whole plant of <i>M. alba</i>	2.31±0.49

Data are the mean±SD (n=4) in mg/g of the dried samples.

Table 3 LOD and LOQ for GABA

Name	Regression equation ^a	r ² ^b	Linear range (mg/mL)	LOD (mg/mL)	LOQ (mg/mL)
GABA	Y=185.35X+10.972	0.9995	0.015–0.5	0.103	0.357

^aY=Peak area, X=Concentration of standards (mg/mL).

^br²=Correlation coefficient for three data points from the calibration curve.

marker compounds were determined by performing ten injections of each compound at concentrations that incrementally approached LOD and LOQ. The LOD and LOQ values of the ten analytes ranged from 0.103 to 0.357 mg/mL, respectively (Table 3). It is very significant that GABA, which is widely used as a health supplement, has been identified in the fermented plant products, aerial part of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant of *M. alba*.

GABA has several biological activities, such as hypertensive, diuretic, and tranquilizing effects (Shin and Lee, 2002; Aoki et al., 2003; Oh et al., 2003; Wong et al., 2003; Hayakaw et al., 2004; Oh and Oh, 2004). GABA is widely found in bacteria and higher plants, such as tea, rice, and soybean (Tsushida et al., 1987; Komassuzaki et al., 2005; Wang et al., 2010). Recently, GABA tea has become popular in Taiwan. GABA tea is a special tea enriched with GABA by anaerobic conditions of fresh tea-leaves (Tsushida et al., 1987). GABA content in the tea was found to be 1.734 mg/g (Lin et al., 2012). Maoyecha tea has shown that the amount of GABA could be increased by repeated treatment of bacteria sequence fermentation. After the treatment, GABA content was significantly increased (Tsushida et al., 1987; Huang et al., 2011).

In conclusion, the presence of GABA in the fermented plant products, including aerial part of *A. sessiliflorus*, fruit of *C. pinnatifida*, and whole plant of *M. alba* is important in agricultural crop production for increasing the amounts of clinically available medicine and health supplements. The inherent selectivity and sensitivity of the LC/MS-MS with electrospray ionization makes this analytical method potentially applicable to the detection and quantification of GABA in other food products (Zazzaroni et al., 2009). In addition, GABA content in the fermented plant products is higher than that in GABA tea and Maoyecha tea. Therefore, the fermented plant products can potentially be used as materials for manufacturing GABA products.

Acknowledgments This study was performed with the support of Gongju National University of Education (School Enterprise) Sillyok, Republic of Korea. The authors thank the staff and crew of the National Center for Inter-University Research Facilities (Seoul National University) for their assistance with the NMR and GC/MS experiments.

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