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Short communication

Improved quantification of γ-aminobutyric acid in rice using stable isotope dilution gas chromatography–mass spectrometry

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ABSTRACT

An accurate method for the analysis of γ-aminobutyric acid (GABA) in rice grain was developed using trimethylsilyl (TMS) derivatization and stable isotope dilution gas chromatography-mass spectrometry. When this method was used with GABA-d_6 as an internal standard (IS), the observed GABA concentration was maintained at 100% of the initial concentration with increasing storage time of the vial in the autosampler. In contrast, when using ribitol as an IS and multiple injections from one vial or single injections from different vials, the observed GABA concentration was 85 and 113% of the initial concentration upon increased storage time, respectively. The improved method recoveries at two different spike levels were between 93.3 and 97.8%, with relative standard deviations of less than 3.3%. The GABA content of resveratrol-enriched transgenic rice was compared with that of its non-transgenic counterpart from two field sites, and statistically non-significant differences were observed between the two grains.

1. Introduction

γ-Aminobutyric acid (GABA) and γ-aminobutanoic acid are amino acids and metabolic products of plants and microorganisms resulting from the decarboxylation of glutamate to produce glutamate decarboxylase (GDC) (Narayan & Nair, 1990). It has been shown that plants increase the concentrations of GABA to regulate environmental stresses (Narayan & Nair, 1990). In animals and humans, GABA acts as a neurotransmitter produced from its metabolic precursor glutamic acid (Petroff, 2002). Therefore, GABA has been shown to play a role in neurological function, such as in Alzheimer's disease (Seidl, Cairns, Singewald, Kaehler, & Lubec, 2001), epilepsy and anxiety disorders (Wong, Bottiglieri, & Snead, 2003), and intelligence degradation (Palmer, et al., 2012). It has been proposed that the intake of GABA-enriched foods could help treat such disorders; hence, it is important to develop accurate and precise quantification methods for GABA in food products (Diana, Quílez, & Rafecas, 2014; Hayakawa et al., 2004).

Recently, various methodologies using chromatography coupled to mass spectrometry (MS) for quantifying GABA have been reported, such as liquid chromatography (LC)-MS (Zazzeroni, Homan, & Thain, 2009), capillary electrophoresis (CE)-MS (Akamatsu & Mitsuhashi, 2013), and gas chromatography (GC)-MS (Struys, Guerand, Ten Brink, & Jakobs, 1999; Farthing, Farthing, Gress, & Sweet, 2017) because MS possesses high selectivity and sensitivity as a detector. Most of the chromatographic analyses of GABA require a derivatization in order to improve separation and introduce a chromophore to allow detection. The differences found in reported methods for GABA determination often lie in the methods used to derivatize GABA. For example, Marfey's reagent (2,4-dinitro-5-fluorophenyl-L-alaninamide) has been used to derivatize GABA for LC-MS detection (Vemula, Kitase, Ayon, Bonewald, & Gutheil, 2017). The CE-MS method requires a derivatization step using *o*-phthalaldehyde or 7-fluoro-4-nitrolenzoxadiazole (Su, Lin, Cheng, & Jen, 2010; Song, Shenwu, Dhossche, & Liu, 2005). For use with GC–MS, reagents such as methylchloroformate, propylchloroformate, bis-methylsilane trifluoroacetamide (BSTFA), and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) have been used to derivatize GABA. Of these, the most commonly used derivatization procedure is silylation using MSTFA (Kagan et al., 2008; Kaspar, Dettmer, Gronwald, & Oefner, 2009; Ding et al., 2016). However, stability problems are often observed during the derivatization process that converts non-volatile compounds into volatile derivatives [trimethylsilyl (TMS) derivatives]. Thus, this process must be undertaken with caution to ensure accuracy. Some studies have proposed methods to improve the poor stability of TMS derivatives. Noctor et al. (2007) reported that TMS-

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derivatives of amino acids were unstable and had to be analysed within 2.5 h to ensure stability. Quéro et al. (2014) reported TMS derivatives with low stability when ribitol was used as an internal standard (IS). This was overcome by controlling the storage temperature. However, these studies require additional procedures before injection. A recent study addressed the problem of low stability by using stable isotope dilution GC–MS in which arbutin—a phenol glycoside—was used (Lee et al., 2018). LC and LC-MS has been successfully used for the determination of GABA (Ding et al., 2018; Vemula, et al., 2017). However, to the best of our knowledge, no methods have yet been reported for measuring and improving the poor stability of GABA using a stable isotope dilution GC–MS method.

In this study, we evaluated the stability of GABA TMS derivatives as a function of sample storage time. In addition, we developed a new analytical method for GABA quantification to overcome the low stability of GABA derivatives and low accuracy of the previous method using ribitol as an IS. Baek et al. (2013) developed resveratrol-enriched transgenic rice (RR) by introducing a stilbene synthase gene derived from *Arachis hypogaea* into Dongjin (DJ), a commercial rice variety. The safety assessment of genetically modified (GM) food is based on the concept of substantial equivalence, which is founded on the basic idea of comparing a transgenic crop variety with consumer-accepted cultivars. GABA is found in rice germ. Thus, the GABA level in RR was analysed and compared with that in DJ for determination of the degree of substantial equivalence.

2. Materials and methods

2.1. Chemicals

γ-Aminobutyric acid (GABA; ≥99%), γ-aminobutyric acid-2,2,3,3,4,4-d_6 (GABA-d_6; 97 at.% Deuterium), and ribitol (adonitol; ≥99%) were purchased from Sigma-Aldrich (USA). Chloroform (HPLC grade) was purchased from Honeywell Burdick & Jackson (Korea), and methanol (HPLC grade) was purchased from Daejung Chemical & Metal Co., Ltd. (Gyeonggi-do, Korea). All water used for sample extraction was produced by a Millipore water purification system (Milli-Q Direct 8, Millipore, France). MSTFA (≥98.5%) was purchased from Sigma-Aldrich (USA) and 2% methoxamine hydrochloride in pyridine (MOX reagent) was purchased from Thermo Scientific (USA) for derivatization.

2.2. Rice sample preparation

Details of the rice sample cultivation and preparation conditions have been described in a previous study (Kim et al., 2016). Rice samples of RR and DJ were cultivated at Suwon (latitude, 37° 16′ 20.02″ N; longitude, 126° 59′ 2.85″ E) and Miryang (latitude, 35° 29′ 31.25″ N; longitude, 128° 44′ 30.99″ E) in 2013, sowed in April, and harvested in October (Table S1). The rice cultivation was a randomized complete block, and standard commercial agronomic practices were used to produce a commercially viable crop. The plot size was 10.5 m^2, including 10 rows ~7 m wide with 30 cm seed spacing. Essential elements provided by the applied fertilizer were N (9.0 kg/1000 m^2), P (4.5 kg/1000 m^2), and K (5.7 kg/1000 m^2). After harvesting, the whole grain samples were dried to a final moisture content of 10%. The samples were ground with a cyclone mixer mill (HMF-590, Korea) and a mortar and pestle and stored in a deep freezer at −80 °C.

2.3. Sample extraction

The extraction of GABA was performed with a slight modification to the polar metabolite extraction method used by Kim et al. (2014). Rice flour samples (10 mg) were extracted with 1 mL of methanol/water/chloroform = 2.5:1:1(v/v/v) supplemented with GABA-d_6 (1 μg) or ribitol (2 μg) as the IS. After the mixtures were vortex milled, they were incubated using a thermomixer (model 5355, Eppendorf AG, Germany) at 1200 rpm for 30 min at 37 °C. The mixtures were centrifuged for 3 min at 16,000*g* at 4 °C using a centrifuge (Tomy MX-307, Japan). Then, 800 μL of the supernatant was transferred to a new 2 mL tube, and 400 μL of water was added. After vortexing this tube, it was re-centrifuged for 3 min at 16,000*g* at 4 °C. Then 900 μL of the supernatant was transferred to a new 2 mL tube, and the solvent was evaporated using a centrifugal concentrator (CC-105, Tomy, Japan) for 4 h, followed by freeze-drying for 16 h. For the derivatization process, 80 μL of MOX reagent was added and mixed with a thermomixer for 90 min at 1200 rpm and 30 °C. Then 80 μL of MSTFA was added, and the solution was mixed again using the thermomixer for 30 min at 1200 rpm and 37 °C. The derivatized sample was analysed by GC-quadrupole MS (GC-qMS).

2.4. GC-qMS analysis

The analysis was performed using a GCMS-QP2010 Ultra System (Shimadzu, Japan) equipped with an autosampler (AOC-20i, Shimadzu, Japan). A DB-5 column (30 m length, 0.25 mm diameter, 1 μm thickness, Agilent Technologies, USA) was used. Samples with a volume of 1 μL were injected using a split mode with a 10:1 split ratio. The temperatures of injection, the ion source, and the interface were 280 °C, 200 °C, and 280 °C, respectively. Helium was used as a carrier gas (99.999%), and the column flow was 1.10 mL/min. The initial oven temperature was 100 °C, which was maintained for 4 min. Then, the oven temperature was increased to 320 °C at a rate of 10 °C/min and maintained at the final temperature for 11 min; the total GC program time was 37 min. The MS detector was used in EI mode, and the selected ion monitoring (SIM) acquisition mode was used at *m/z* 232 (GABA 2TMS), *m/z* 238 (GABA-d_6 2TMS), *m/z* 304 (GABA 3TMS), *m/z* 310 (GABA-d_6 3TMS), and *m/z* 319 (ribitol 5TMS). The ionization voltage was set at 70 eV. The retention times (RT) and mass spectra of all analytes were identified by comparison with an analyte standard.

2.5. Stability of TMS derivatization

The stability of derivatization was confirmed by observing changes in the GABA derivative peak areas (GABA 2TMS and GABA 3TMS) over 6 h. To eliminate systematic errors, we performed derivatization of 1 μg/mL aliquots of GABA at the same time. The prepared samples were analysed every 2 h for 6 h and replicated three times. After sample preparation, two different injection conditions were tested: multiple injection (several measurements from the same vial) and one-time injection (measurement from the same sample in different vials). For both injection conditions, samples were injected every 2 h.

Additionally, by observing the ratio of GABA 3TMS/ribitol 5TMS and GABA 3TMS/GABA-d_6 3TMS, quantitative changes were confirmed over 6 h. Derivatization was performed on aliquots of 1 mL of mixture at the same time. The mixture was composed of GABA 1 μg/mL, GABA-d_6 1 μg/mL, and ribitol 2 μg/mL. The analysis conditions were the same as mentioned above. Briefly, the samples were injected every 2 h for 6 h and maintained at room temperature until the end of the