Determination of γ-Aminobutyric Acid in Food Matrices by Isotope Dilution Hydrophilic Interaction Chromatography Coupled to Mass Spectrometry

Raniero Zazzeroni*, Andrew Homan, and **Emma Thain**
Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, MK44 1LQ, United Kingdom

Abstract

The estimation of the dietary intake of γ-aminobutyric acid (GABA) is dependent upon the knowledge of its concentration values in food matrices. To this end, an isotope dilution liquid chromatography–mass spectrometry method has been developed employing the hydrophilic interaction chromatography technique for analyte separation. This approach enabled accurate quantification of GABA in apple, potato, soybeans, and orange juice without the need of a pre- or post-column derivatization reaction. A selective and precise analytical measurement has been obtained with a triple quadrupole mass spectrometer operating in multiple reaction monitoring using the method of standard additions and GABA-d_6 as an internal standard. The concentrations of GABA found in the matrices tested are 7 µg/g of apple, 342 µg/g of potatoes, 211 µg/g of soybeans, and 344 µg/mL of orange juice.

Introduction

Chromatographic analysis of amino acids traditionally requires either pre- or post-column derivatization in order to improve separation and introduce a chromophore to allow detection by UV or fluorescence (1–5). The sensitivity of existing methodologies is widely acknowledged (6) and various combinations of derivatization chemistry and chromatographic separation are used for the determination of free amino acids in food and other complex matrices. Improvements in derivatization reactions for the determination of amino acids in food hydrolysate have been reported using pre-column derivatizations with butylisothiocyanate and benzylisothiocyanate (7).

A high-performance liquid chromatography (HPLC) separation with fluorescence detection has also been successfully used for the determination of γ-aminobutyric acid (GABA) in honey and bee-pollen following derivatization with *o*-phthaldialdehyde in the presence of 2-mercaptoethanol, yielding isoindolic derivatives detectable in the picomoles range (8). The classical analytical approach is hampered, however, by the requirement to optimize and carefully control the derivatization procedure in order to obtain good reproducibility of results. Furthermore, the unambiguous identification of amino acids cannot be achieved by UV or fluorescence detection due to the potential co-elution of matrix interferences (9). The analysis of GABA by conventional reversed-phase chromatography with octadecyl columns is limited by its high hydrophilicity, resulting in the analyte eluting with the column void volume of mobile phase. The lack of a chromophore is also a distinct feature of this amino acid (10,11). Hydrophilic interaction chromatography (HILIC) has been successfully implemented for the analysis of amino acids and other small polar organic molecules in complex matrices (12–15). In HILIC, polar organic molecules are retained by hydrophilic stationary phases eluted with more hydrophobic mobile phases (16). The high content of organic modifier in the mobile phase makes this chromatographic technique highly suitable for coupling to electrospray mass spectrometry and was selected as the technique of choice for the analysis of GABA in its native form in complex food matrices. A recent review of HILIC described in detail the mechanism underpinning the chromatographic separation and more generally provided a comprehensive review of the available separation materials and applications (17). A sulfoalkylbetaine zwitterionic stationary phase HPLC column was used for the chromatographic separation of GABA. The zwitterionic moiety is a potent osmolyte with a strong ability of binding water to surfaces. The mobile phase was a binary combination of methanol and ammonium bicarbonate solution at pH 7 with formic acid.

A hyphenated analytical method using positive electrospray ionization and a triple quadrupole mass spectrometer (MS) operating in multiple reaction monitoring (MRM) mode was

* Author to whom correspondence should be addressed.

developed, eliminating the requirement for a derivatization reaction step. The method allowed low level detection of GABA in its native form and unequivocal identification of the analyte.

Experimental

Reagents and solutions

Ammonium bicarbonate (> 99 %) was supplied by Sigma (Gillingham, UK). HPLC-quality methanol was supplied by Rathburn Chemicals Ltd. (Walkerburn, Scotland); formic acid Analar 98/100% was supplied by BDH (Poole, UK). One M sodium hydroxide was supplied by Aldrich (Milwaukee, WI) and 1M hydrochloric acid was supplied by Fisher Scientific (Loughborough, UK). The Ultrapure water used in the preparation of mobile phase and standard solutions was Milli-Q Plus (Millipore, Watford, UK). GABA and γ-aminobutyric acid-2,2,3,3,4,4-d_6 (GABA-d_6, 97 atom % D) were supplied by Sigma and stored at ambient temperature. Five hundred milligram C18 solid phase extraction (SPE) cartridges were supplied by International Sorbent Technologies (Glamorgan, UK).

Determination of GABA in apples

Apples (epicarp and mesocarp) were freeze-dried, macerated with a pestle and mortar to a fine powder, and mixed. A 0.4 g sample and a 0.3 mL volume of 80 µg/mL GABA-d_6 standard solution in water were extracted overnight with 40 mL of 40 mM ammonium bicarbonate pH 7–methanol (10:90). The samples were centrifuged at 4000 g for 10 min and the supernatants transferred quantitatively into 50-mL volumetric flasks diluting to volume with 40 mM ammonium bicarbonate pH 7–methanol (10:90). Each solution was further diluted 1 mL to 10 mL with 40 mM ammonium bicarbonate pH 7–methanol (10:90) and analyzed by LC–MS. Fortified samples were prepared by spiking 0.4 g of sample with 0.15, 0.4, and 0.6 mL of 80 µg/mL GABA and a 0.3 mL volume of 80 µg/mL GABA-d_6, and processed as detailed previously. The standard addition regression line for the analysis of GABA in apples comprised five replicates of the native sample and duplicate samples for each of the three fortification levels of 12, 32, and 48 µg of GABA equivalent to 24, 64, and 96 ng/mL in the final extract solutions.

Determination of GABA in orange juice

Approximately 20 mL of freshly squeezed orange juice purchased from a local food store was centrifuged at 4000 *g* for 10 min. A 1 mL volume of supernatant and a 2 mL volume of 200 µg/mL GABA-d_6 standard solution in water were diluted to 100 mL with 40 mM ammonium bicarbonate pH 7–methanol (90:10). Fortified samples were prepared by spiking 1 mL of supernatant with 0.5, 1.0, and 1.5 mL volumes of 500 µg/mL GABA and 2 mL volume of 200 µg/mL GABA-d_6. All samples were purified by SPE as follows: A 500 mg C18 SPE cartridge was conditioned with 3 mL of methanol followed by 3 mL of 40 mM ammonium bicarbonate buffer pH 7. A 2-mL volume of the sample solution was passed through the cartridge and discarded. A further 2 mL of sample solution was passed through the cartridge and collected. A 1 mL volume of this collected solution was diluted to 100 mL with 40 mM ammonium bicarbonate pH 7–methanol (10:90) and analyzed by LC–MS.

The standard addition regression line for the analysis of GABA in orange juice comprised five replicates of the native sample and duplicate samples for each of the three fortification levels of 250, 500, and 750 µg of GABA equivalent to 25, 50, and 75 ng/mL in the final extract solutions.

Determination of GABA in potatoes

Approximately 200 g of potatoes were sliced into thin strips (~ 2 mm thickness), freeze-dried, and then macerated with a pestle and mortar to a fine powder. A 0.2 g sample and 3 mL volume of 80 µg/mL GABA-d_6 standard solution in water were extracted overnight with 40 mL of 40 mM ammonium bicarbonate pH 7–methanol (10:90). The samples were centrifuged at 4000 *g* for 20 min. The supernatants were transferred quantitatively into 50-mL volumetric flasks, diluting to volume with 40 mM ammonium bicarbonate pH 7–methanol (10:90). Each solution was further diluted 1 mL to 100 mL with 40 mM ammonium bicarbonate pH 7–methanol (10:90) and analyzed by LC–MS. Fortified samples were prepared by spiking 0.2 g sample with 0.10, 0.25, and 0.40 mL volumes of 1000 µg/mL GABA, 3 mL volume of 80 µg/mL GABA-d_6, and processed as detailed previously.

The standard addition regression line for the analysis of GABA in potatoes comprised five replicates of the native sample and duplicate samples for each of the three fortification levels of 100, 250, and 400 µg of GABA equivalent to 20, 50, and 80 ng/mL in the final extract solutions.

Determination of GABA in dried soybeans

A domestic food mill was used to reduce the dried soybeans to a coarse powder. A 0.5 g powdered sample and 1-mL volume of 100 µg/mL GABA-d_6 standard solution in water were extracted overnight with 20 mL of ultrapure water–methanol (10:90) in 1 mM sodium hydroxide. The samples were centrifuged at 4000 *g* for 20 min. The supernatants were transferred quantitatively into 25-mL volumetric flasks, diluting to volume with ultrapure water–methanol (10:90) in 1 mM sodium hydroxide. A 5 mL volume of the sample solution was collected and diluted with 5 mL of ultrapure water–methanol (10:90) in 1 mM hydrochloric acid. Fortified samples were prepared by spiking 0.5 g soybeans with 0.6, 1.2, and 1.8 mL volumes of 100 µg/mL GABA and 1 mL volume of 100 µg/mL GABA-d_6 and processed as detailed previously.

All samples were purified by SPE as follows: a 500 mg C18 SPE cartridge was conditioned with 3 mL of methanol followed by 3 mL of 40 mM ammonium bicarbonate buffer pH 7. A 2 mL volume of the sample solution was passed through the cartridge and discarded. A further 2 mL of sample solution was passed through the cartridge and collected. A 1 mL volume of this collected solution was diluted to 50 mL with 40 mM ammonium bicarbonate pH 7–methanol (10:90) and analyzed by LC–MS.

The standard addition regression line for the analysis of GABA in dried soybeans comprised five replicates of the native

sample and duplicate samples for each of the three fortification levels of 60, 120, and 180 µg of GABA equivalent to 24, 48, and 72 ng/mL in the final extract solutions.

GABA and GABA-d_6 standard solutions

Internal standard calibration curve. Calibration standards containing 20, 40, 80, 120, and 200 ng/mL GABA were prepared in 40 mM ammonium bicarbonate (pH 7)–methanol (10:90). Each calibration standard contained GABA-d_6 at 40 ng/mL.

Fortification solutions. Fortification solutions of 80, 100, 500, and 1000 µg/mL of GABA and 80, 100, and 200 µg/mL of GABA-d_6 were prepared in ultrapure water.

LC–MS analysis. LC–MS–MS was carried out on a Quattro Micro triple quadrupole mass spectrometer (Waters, Manchester, UK) interfaced to an 1100 HPLC system (Agilent, Stockport, UK). A SeQuant (Umea, Sweden) zwitterionic stationary phase column (ZIC-HILIC, 2.1 × 150 mm, 3.5 µm particle size, 200 Å pore size) with a strong cation exchange and a strong anion exchange guard cartridges (Phenomenex, Macclesfield, UK) was utilized for the separation. The column temperature was set at 28°C. The mobile phase consisted of 40 mM ammonium bicarbonate (pH 7) with formic acid (A) and methanol (B). The initial condition was set at 90% B, maintained at initial condition for 7 min; then decreased to 10% B over 7 min, maintained at 10% B for 8 min; brought back to initial condition in 1 min and kept at 90% B for a further 22 min. The total run time was 55 min at a flow rate of 0.12 mL/min. The thermostatically-controlled autosampler was kept at 10°C, and a 2 µL aliquot was injected onto the column. The MS was equipped with an electrospray interface operating in positive ionization mode. A voltage of 3.0 KV for the capillary, 20 V for the cone, and temperatures of 120°C for the source, and 210ºC for the nitrogen desolvation gas flowing at 800 L/h were used for the analysis. The following MRM transitions with a dwell time of 0.5 s were selected: GABA (*m/z* 104 → *m/z* 87); GABA-d_6 (*m/z* 110 → *m/z* 93).

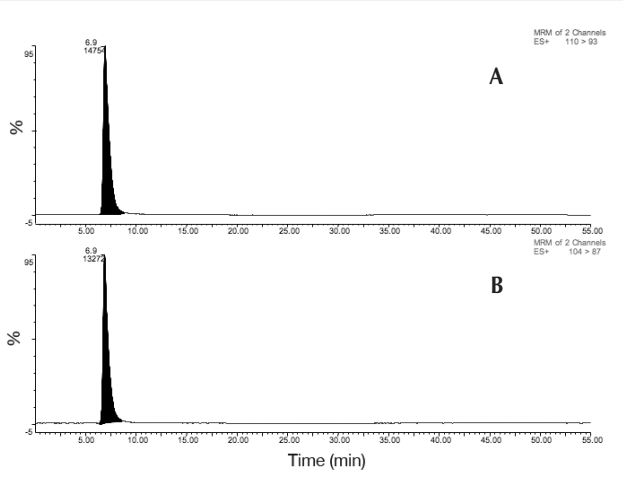

Figure 1. LC–MS trace of GABA-d_6 (A) and GABA (B) in apple extract solution.

Results and Discussion

For the development and optimization of the LC and MS conditions, different buffer solutions in the LC eluent were evaluated. Formic acid, ammonium acetate, and ammonium bicarbonate in combination with either acetonitrile or methanol were investigated. Ammonium bicarbonate 40 mM at pH 7 with formic acid gave optimum instrumental sensitivity. An ~ 15% improvement in peak symmetry, resulting from a reduction of peak tailing, was observed with higher concentrations of ammonium bicarbonate, but 40 mM was selected as a good compromise between the chromatography requirements, the potential accumulation of inorganic residue in the MS interface, and the salt solubility in the highly organic mobile phase. Acetonitrile and methanol were evaluated in a generic HILIC gradient, starting with a high percentage of organic modifier in the mobile phase, and ending with a mainly aqueous eluent after 14 min. A prolonged re-equilibration time of the HPLC column at the low flow rate used for the chromatographic separation was introduced to ensure reproducibility of retention time over multiple injections. The final choice of organic modifier was dictated by the sample matrix and the limit of detection (LOD) required for the analysis. The use of acetonitrile under identical instrument conditions resulted in a considerably longer retention time but lower instrument response. Conversely, methanol resulted in a shorter retention time (baseline resolution from the solvent void volume peak) but higher instrument response. Methanol was selected as the organic modifier of choice as the increased sensitivity enabled us to carry out a significant dilution of sample extracts that, combined with the inherent selectivity of detection by mass spectrometry in MRM mode, resulted in LC–MS chromatograms with no appreciable matrix effect and solely characterized by the peak of interest (Figure 1). Cationic and anionic guard cartridges were used to prevent potential irreversible binding of matrix components to the column zwitterionic stationary phase. The guard cartridges were replaced after each batch of ~ 15 samples to ensure column performance and maintain constant column backpressure.

A solution of GABA was infused into the MS for the optimization of the instrument parameters and the selection of the MRM transition. The collision-induced dissociation of the precursor ion $[M+H]^+$ at *m/z* 104 (GABA) and *m/z* 110 (GABA-d_6) resulted in the neutral loss of ammonia and the formation of the product ions at *m/z* 87 and *m/z* 93, respectively. These

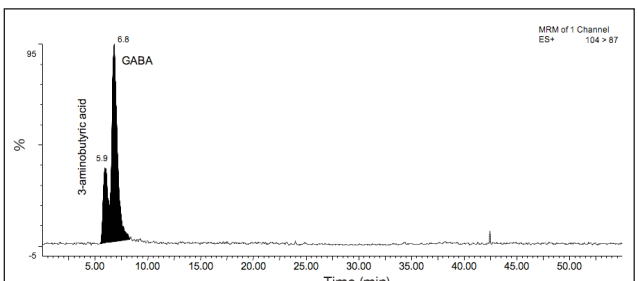

Figure 2. LC–MS trace of GABA (50 ng/mL) and 3-aminobutyric acid (5 µg/mL).

MRM transitions were used for the quantification of GABA in all matrices. The analytical methods proved to be selective towards isobaric interferences 2-aminobutyric acid and 3-aminobutyric acid. The isomer 2-aminobutyric acid was not detectable at the MRM used for the analysis of GABA, whereas 3-aminobutyric acid was detectable but was partially resolved from the analyte of interest and characterized by a response factor approximately 300-fold lower than GABA (Figure 2).

The natural occurrence of GABA in all the four food matrices investigated and the consequent unavailability of certified matrix blanks led to the selection of the standard additions technique in combination with an isotopically labeled internal standard as the preferred quantification method. The standard addition regression lines were obtained by plotting the fortified amount of GABA in the final sample dilutions (*X* axis) versus the response factor (RF) (*Y* axis) calculated as:

$$RF = \text{GABA peak area} \left[\frac{\text{GABA-d}_6 \text{ conc. (ng/mL)}}{\text{GABA-d}_6 \text{ peak area}} \right] \qquad \text{Eq. 1}$$

The accuracy of the analytical method was expressed as the mean percentage recovery of six fortified samples at three concentration levels, whereas the intra-batch precision was expressed as the percentage relative standard deviation of five replicate analyses of the native samples.

For apples, the equation describing the standard addition regression line for the analysis of GABA was:

Response = 1.04686 × GABA (ng/mL) + 53.82429, R^2 = 0.997 (Figure 3).

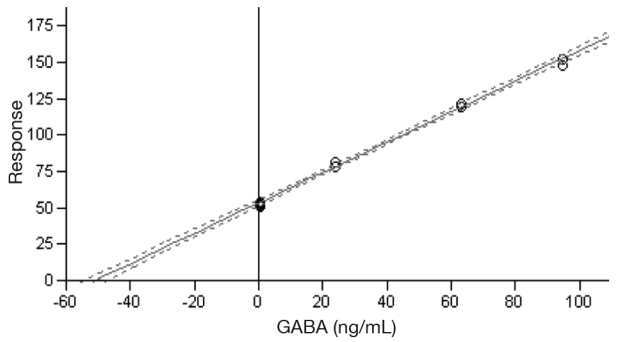

Figure 3. Apple. The sample was fortified with 12, 32, and 48 µg of GABA equivalent to 24, 64, and 96 ng/mL in the final extract solutions. The regression line is shown with the 95% confidence interval used for the calculation of the concentration range.

Table I. Calculated GABA Concentration*

Food matrix	GABA concentration	Concentration range
Apple	7.11 µg/g	6.60–7.67 µg/g
Orange juice	344.16 µg/mL	312.78–378.46 µg/mL
Potato	342.34 µg/g	314.66–373.47 µg/g
Soybeans	211.16 µg/g	187.64–237.89 µg/g

* The 95% confidence interval of the intercept (standard additions technique) was used for the calculation of the concentration range. Each of the four regression lines comprised five replicates of the native sample and six fortified samples (duplicate samples at three fortification levels).

The calculated accuracy and intra-batch precision of the analytical measurement were 104.5% and 2.7%, respectively.

For orange juice, the equation describing the standard addition regression line for the analysis of GABA was:

Response = 0.91023 × GABA (ng/mL) + 31.32661, R^2 = 0.996.

The calculated accuracy and intra-batch precision of the analytical measurement were 104.4% and 3.9%, respectively.

For potatoes, the equation describing the standard addition regression line for the analysis of GABA was:

Response = 1.10445 × GABA (ng/mL) + 86.78951, R^2 = 0.994.

The calculated accuracy and intra-batch precision of the analytical measurement were 107% and 2.9%, respectively.

For soybeans, the equation describing the standard addition regression line for the analysis of GABA was:

Response = 1.07579 × GABA (ng/mL) + 45.66127, R^2 = 0.992.

The calculated accuracy and intra-batch precision of the analytical measurement were 100.5% and 5.3%, respectively.

The concentration of GABA in the food matrices was calculated from the standard addition curves as follows:

$$\text{Intercept}_{X\text{ axis}} = \left| \text{Intercept}_{Y\text{-axis}} / \text{Slope} \right| = \text{GABA (ng/mL, final extract solution)} \qquad \text{Eq. 2}$$

$$\text{GABA concentration} = \text{Intercept}_{X\text{-axis}} \times \text{Dilution factor} \qquad \text{Eq. 3}$$

The GABA concentration range was calculated from Equation 3 using the upper and lower values of the $\text{Intercept}_{X\text{ axis}}$ obtained from the 95% confidence interval of the standard addition curve (JMP Statistical Analysis software v6, SAS Institute Inc., Cary, NC) (Figure 3).

The linearity of detector response was demonstrated over the GABA concentration range of 20–200 ng/mL (the LOD of 5 ng/mL was derived experimentally as the analyte peak area with a 3 to 1 signal-to-noise ratio), and all final dilutions of sample extracts were optimized to maintain the analyte concentration within this range.

The calculated values of GABA obtained with this new analytical method (Table I) compare well with the limited published data (18,19), despite the reported variation in concentration attributed to plant growing conditions and food processing methods (20).

Conclusions

A new HILIC–MS method was successfully developed for the analysis of GABA in four food matrices in an effort to address the lack and sometimes inconsistent availability of data compromising our ability to assess its dietary intake. The simple extraction procedures combined with no requirement

for analyte derivatization are distinct advantages over traditional analytical methods. Although existing methods are available for the analysis of small polar chemicals (amino acids and peptides) by HILIC–MS, our effort was focused solely on GABA. We have been able to optimize the analytical strategy and obtain a low LOD with good accuracy and precision of analytical measurement. To achieve this, an isotopically labeled internal standard (GABA-d_6) in combination with the standard additions technique was employed. The inherent selectivity and sensitivity of the triple quadrupole MS with electrospray ionization makes this analytical method potentially applicable to the detection and quantification of GABA in other food products and complex matrices.

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