A Validated Method for Gas Chromatographic Analysis of γ-Aminobutyric Acid in Tall Fescue Herbage

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γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter in animals that is also found in plants and has been associated with plant responses to stress. A simple and relatively rapid method of GABA separation and quantification was developed from a commercially available kit for serum amino acids (Phenomenex EZ:faast) and validated for tall fescue (*Festuca arundinacea*). Extraction in ethanol/ water (80:20, v/v) at ambient temperature yielded detectable amounts of GABA. Clean separation from other amino acids in 28 min was achieved by gas chromatography (GC) with flame ionization detection (FID), using a 30 m, 5% phenyl/95% dimethylpolysiloxane column. The identity of the putative GABA peak was confirmed by GC with mass spectrometric (MS) detection. The relatively small effects of the sample matrix on GABA measurement were verified by demonstrating slope parallelism of GABA curves prepared in the presence and absence of fescue extracts. Limits of quantification and detection were 2.00 and 1.00 nmol/100 μL, respectively. Method recoveries at two different spike levels were 96.4 and 94.2%, with coefficients of variation of 7.3 and 7.2%, respectively.

KEYWORDS: GABA; norvaline; *Festuca arundinacea*; method validation; GC−FID; GC−MS; dimethylpolysiloxane; propyl chloroformate; derivatization; kit

INTRODUCTION

γ-Aminobutyric acid (GABA, **Figure 1A**) is a nonprotein amino acid found in many plants, animals, and microorganisms (*1*). GABA was first identified as a component in some bacterial cultures (*2, 3*), and the presence of GABA in plant tissue was first reported in a study of potato tuber amino acids (*4*). GABA was found soon afterward in yeast cultures and mammalian tissues and later found to be an inhibitory neurotransmitter in animals, as reviewed by Roberts (*5*), and it has been shown to lower blood pressure in humans and other animals (*6*). In plants, GABA accumulation has been documented in response to stresses, such as anoxia (*7*), as well as decreasing cellular pH, temperature changes, and mechanical handling (*8*). GABA has also been associated with plant growth responses, because GABA concentrations change in embryos of developing wheat seeds (*9*), and in some plants, GABA is a breakdown product of polyamines, compounds that play a role in growth regulation (*10, 11*) as well as stress responses (*12*). Because GABA has been detected in tall fescue herbage (*13*) and can cross the blood-

brain barrier under some circumstances (*14*), GABA ingested by grazing animals could potentially modulate the "fescue toxicosis" syndrome, which, as reviewed by Strickland et al. (*15*), leads to reduced animal performance on tall fescue pastures. Because tall fescue is the most abundant forage grass in the transition zone between the temperate North and subtropical South of the eastern half of the U.S., any factor that could affect animal performance on tall fescue may affect the success of animal production. The method reported herein should aid in future studies to determine if any relationship exists between forage GABA content and tall fescue toxicosis.

Numerous methods of GABA analysis have been developed on the basis of the instrumentation available, the sensitivity required, and the organism/tissue being analyzed. Ackermann (*2*) and Abderhalden et al. (*3*) determined the presence of GABA in bacterial cultures by precipitating GABA as a platinum salt and comparing its melting point to that of synthetic GABA platinate. The first report of GABA in plant tissue was based on paper chromatography of potato extracts, using a ninhydrin spray to detect amino acids (*4*). Column chromatography on ion-exchange, starch, or cellulose media was employed by various researchers to isolate GABA from plants and yeast in quantities sufficient for crystallization (*16*). From the time of their commercialization, both high-performance liquid chromatography (HPLC) (*7, 11, 17–19*) and gas chromatography

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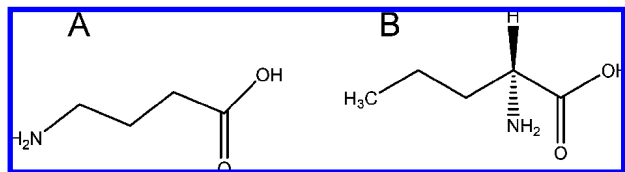§ University of Kentucky.

Figure 1. Structures of (A) GABA and (B) norvaline.

(GC) (*20–25*) have been used to detect GABA, sometimes sequentially (*11*). The differences in published HPLC and GC methods of GABA analysis often lie in the procedure used to derivatize GABA. While derivatization for HPLC usually involves adding a chromophore that allows GABA to be detected by fluorescence (*7, 11, 17–19*) spectroscopy, derivatization for GC usually involves forming a volatile compound (*7, 11, 20–25*). Structural confirmation of the derivatized GABA can then be achieved via chromatography coupled to mass spectrometry, whether GC−MS (*7, 11, 20–25*) or LC−MS (*26*).

The applicability of any of the published methods depends upon laboratory resources and the matrix from which GABA is to be extracted. Given those criteria, the objectives of this study were (a) to develop a rapid GC−FID method for analyzing GABA in grass herbage and (b) to find a derivatization method that could readily be optimized. A commercially available kit for GC−FID analysis of free amino acids, complete with derivatization reagents and a column, was chosen. The use of this kit was reported in studies of amino acids in garlic (*22*) and betalain-producing fruits and vegetables (*23*). A similar kit from the same company, optimized for GC−MS instead of GC−FID, was used in a study of GABA and other amino acids in transgenic wheat (*24*) and in a study of amino acids in honey (*25*). For the study described herein, the derivatized forage plant extracts were too complex for separation on the column provided with the kit, which had been intended for analysis of amino acids in serum. However, the extracts separated well on an alternative column. The derivatization method was modified slightly for forage tissue extracts, and the separation method was fully validated.

MATERIALS AND METHODS

Plant Tissue. Tall fescue [*Schedonorus arundinaceus* (Schreb.) Dumort = *Festuca arundinacea* (Schreb.), (*27*)] herbage was harvested from an established endophyte-free (*Neotyphodium coenophialum*) Kentucky-31 pasture at the Spindletop farm of the University of Kentucky Agricultural Experiment Station Farm in Fayette County during two seasons (November 2004 and June 2007). Herbage from 5 cm above the soil surface was collected, freeze-dried, ground to pass through a 100-mesh screen (Cyclotec 1093 sample mill, FOSS North America, Eden Prairie, MN), and stored in sealed containers at room temperature or −20 °C until use.

Reagents and Chemicals. GABA, norvaline, serine, and proline were purchased from Sigma-Aldrich (St. Louis, MO). Derivatization reagents and amino acid standards (unless otherwise specified) came with the Phenomenex (Torrance, CA) EZ:faast kit for GC−FID free (physiological) amino acid analysis. Ethanol (EtOH) was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY).

Extraction. Disposable 15 mL centrifuge tubes made of polypropylene (Becton-Dickinson, Franklin-Lakes, NJ) or polyethylene terephthalate (PET; Corning, Corning, NY) were used for extractions. For optimization of chromatographic parameters, 20 mg of freeze-dried, ground tall fescue was extracted with 2 mL of EtOH/water (80:20, v/v) by shaking (2 h, 25 °C, 45° angle, and 300 rpm) in an Innova 4300 incubator shaker (New Brunswick Scientific, Edison, NJ). Extraction was followed by centrifugation (20 min, 1850*g*, 22 °C; CR4 22 benchtop centrifuge, Jouan, Winchester, VA).

For recovery analyses and quantification of GABA in unknown samples, 200 mg of ground tissue was extracted at ambient temperature for 2 h with 5 mL of EtOH/water (80:20, v/v, spiked with 200 nmol of norvaline/mL as the internal standard and 0, 150, or 600 nmol of GABA/mL) in a horizontal position on a rocker (50−55 rpm; CR300, FinePCR, Seoul, South Korea) [Attempts were initially made to extract GABA from fescue using an accelerated solvent extractor (ASE 200, Dionex Corp., Sunnyvale, CA), which automates extraction of samples under elevated temperature and pressure. However, yields of GABA-spiked samples indicated considerable GABA loss (data not shown), whereas those losses were not observed when tissue was extracted by shaking at room temperature]. These extracts were centrifuged (5 min, 3830*g*; Sorvall RC-6, SH-3000 rotor, Thermo Fisher Scientific, Inc., Waltham, MA) and then filtered through a Whatman #4 filter.

Sample Cleanup and Derivatization. A 100 *μ*L aliquot of supernatant from each centrifuged and filtered extract was derivatized with an EZ:faast kit for free (physiological) amino acid analysis by GC−FID (Phenomenex). The derivatization process is described quite briefly in the kit manual, but more detail on the process is provided by Kugler et al. (*23*), and reagent information is in the Materials Safety Data Sheets (MSDSs) for the kit. The derivatization protocol was similar to that described by Kugler et al. (*23*), with the following differences: norvaline was added at the start of extraction, instead of at the start of derivatization; derivatization reactions were allowed to proceed for 90 instead of 70 s; and a centrifugation step (1 min, 5300*g*; Sorvall Legend Micro 21 centrifuge, Thermo Fisher Scientific) was added after the addition of 100 *μ*L of 1 N HCl. This additional step allowed for cleaner removal of the organic phase, which was then transferred to an autosampler vial for analysis by GC−FID.

Analysis. Derivatized samples were injected (1.5 *μ*L) into a GC−FID (3900, Varian, Inc., Walnut Creek, CA) using a 5% phenyl/95% dimethylpolysiloxane capillary column (VF-5ms, 30 m × 0.25 mm, 0.25 *μ*m film thickness, Varian, Inc.). The following parameters were used: injector temperature, 250 °C; helium flow, 1.0 mL/min; makeup gas flow, 23 mL/min; hydrogen flow, 30 mL/min; air flow, 300 mL/min; oven program, 80 °C with a 2 min hold, increased to 320 °C at 10 °C/min with a 2 min final hold. The total run time was 28 min.

GABA was quantified via external calibration curves based on 5 or 6 dilutions of the commercial standard (5−100 nmol per derivatized sample). Norvaline was used as an internal standard, and peaks were quantified by peak integration, using the Star Workstation (Varian). Standard curves were constructed using GABA/norvaline peak area ratios [(GABA response/norvaline response)/(nmol of GABA/nmol of norvaline)]. A new standard curve was run when quality control samples indicated a shift in detector response. Resolution between GABA and adjacent peaks was calculated as $R_s = 2\Delta t/(w_1 + w_2)$ (*28*), where w = peak width (in seconds) at baseline.

Peak Identity Confirmation. To confirm the identity of the putative GABA peak resolved by GC−FID, a 25 mg tall fescue sample was extracted as described for the optimization of chromatographic parameters and derivatized with and without a 10 nmol spike of GABA. The change in area of the putative GABA peak was calculated to determine if the increase corresponded to the amount of GABA added. For structural confirmation by GC−MS, a 40 mg sample of tall fescue was extracted in the same way as the 25 mg sample and derivatized using the same kit, except that the final reagent (1 N HCl) was substituted with the final reagent [isooctane/chloroform (80:20, v/v)] from the Phenomenex EZ:faast kit for free amino acid analysis by GC−MS. The resulting sample was injected onto a GC−MS (CP-3800 equipped with Saturn 2000 MS, Varian), using an ionization energy of 70 eV and the same column and parameters (i.e., column flow rates, injector temperatures, and oven program) described for GC−FID analysis. The mass range scanned was *m/z* 10−250. The GABA peak was verified using the proprietary EZ:faast mass spectral library (Phenomenex) supplied with the kit.

Sample Matrix Interference. Portions (200 mg) of ground tissue from one tall fescue accession were extracted in 5 mL of EtOH/water (80:20, v/v) spiked with synthetic GABA at 0, 150, or 600 nmol/mL and with norvaline (200 nmol/mL) as an internal standard. Tissue extractions for each GABA spike were performed in triplicate on each of 3 days. GABA/norvaline ratios in tissue extracts were used to graph a mass addition curve, whose slope was compared to the slope of the external calibration curve used to quantify the GABA in the extracts.

Matrix effects were determined by comparing the slopes, using a *t* test (*29*). The pooled standard error of slopes ($S_{b(p)}$) was calculated using eq 1.

$$S_{b(p)} = S_{y \cdot x(p)} \left(\left(1/\sum x^2 \right)_1 + \left(1/\sum x^2 \right)_2 \right)^{1/2} \quad (1)$$

$S_{y \cdot x(p)} = (((n_1 - 2)S^2_{y \cdot x(1)} + (n_2 - 2)S^2_{y \cdot x(2)})/((n_1 - 2) + (n_2 - 2)))^{1/2}$, where *n* is the number of data points. In this case, $n_1 = 6$ for the external calibration curve and $n_2 = 27$ for the mass addition curve (9 data points per spike level).

A *t* value was calculated as $t = |b_1 - b_2|/S_{b(p)}$, where b_1 and b_2 are the slopes of the two curves. The *t* value, with $(n_1 + n_2 - 4)$ degrees of freedom, was compared at the 95% confidence level to the *t* value for the corresponding number of degrees of freedom.

Recovery and Extraction Efficiency. Native GABA content was determined from the 0 nmol/mL spikes described in the previous section. The recovered amount of GABA added to the tissue extracts was calculated from an external calibration curve. Recovery was calculated with respect to the sum of the native GABA and the actual amount of the GABA spike. This determination was made 3 times for spikes at both the lower (150 nmol/mL) and upper (600 nmol/mL) ends of the linear range.

Linearity and Range. The linear range of this method was determined by plotting calibration curves for a series of standards prepared by serial dilution of a stock GABA standard solution [29.1 *μ*mol GABA/mL in EtOH/water (80:20, v/v)]. The lower limit of the linear dynamic range, also known as the limit of detection (LOD), was determined as the concentration at which a signal-to-noise ratio of 3:1 was routinely obtained (*30*). The limit of quantification (LOQ), defined as the concentration at which a signal-to-noise ratio of 10:1 was routinely obtained (*30*), was determined as well.

RESULTS AND DISCUSSION

Choice of Column and Separation Parameters. GABA extracts were originally separated on an amino acid analysis GC column (10 m × 0.25 mm, Zebron ZB-AAA, Phenomenex). Under the recommended separation parameters (32 °C/min from 110 to 320 °C, 1.5 mL/min flow, split 1:15 injection of 2.0 *μ*L at 250 °C, detector temperature of 320 °C), GABA coeluted with serine (Ser) in an amino acid standard mixture supplied with the EZ:faast kit. Various changes in starting temperatures and ramping rates failed to resolve GABA and Ser standards, and in tall fescue extracts, two compounds seemed to coelute with GABA (**Figure 2A**). Coelution of GABA and Ser on a ZB-AAA column was also reported by Kugler et al. (*23*). After a temperature increase from 40 to 320 °C at 4 °C/min failed to resolve GABA (data not shown), the ZB-AAA column was replaced with a 5% phenyl/95% dimethylpolysiloxane column (VF-5ms, 30 m × 0.25 mm, 0.25 *μ*m film thickness, Varian, Inc.), which resolved GABA and the previously coeluting compounds (**Figure 2B**). Temperature programming was subsequently modified to 80−320 °C at 10 °C/min for an optimal speed of analysis. The flow rate was decreased from 1.5 to 1 mL/min to provide a slight improvement in baseline resolution (**Figure 2C**). Injection of a derivatized mixture of synthetic Ser, GABA, and proline (Pro) confirmed that none of the three amino acids coeluted when using these optimized parameters and the VF-5ms column (chromatogram not shown). Peak resolution was 4.7 for GABA and Ser and 1.7 for GABA and Pro. Both values meet the requirement for baseline resolution ($R_s > 1.5$) (*28*). The improved resolution provided by the VF-5ms column may have resulted partly from the increased length. However, the VF-5ms column may have been better suited than the ZB-AAA column for GABA separation, because it is similar in length and composition to the 25 m × 0.2 mm dimethylpolysiloxane column used by Rastogi and Davies (*11*) to identify methylated dansyl derivatives of GABA and other polyamine

metabolites in tomato pericarp tissue. The structural differences between methylated dansyl GABA and a propyl chloroformate derivative of GABA demonstrate that the VF-5ms column is suitable for separating a wide range of compounds. However, because the ZB-AAA column composition is proprietary, it is difficult to speculate about the role that differences in stationary phases of the respective columns may play in the improved resolution.

Verification of GABA Peak Identity. When a tall fescue extract was injected with and without a 10 nmol spike of GABA, the putative GABA peak increased in area by an amount corresponding to 9.6 nmol, supporting the identification of that peak as GABA (data not shown). When a tall fescue extract was analyzed by GC−MS, GABA partially coeluted with proline, emerging as a shoulder on the proline peak (**Figure 3A**). No attempt was made to optimize the GC−MS separation because the goal was to identify the GABA peak, and the mass spectrum of the GABA peak in that sample (**Figure 3B**) closely matched the mass spectrum of derivatized GABA from the Phenomenex EZ:faast library (**Figure 3C**). Fragments at *m/z* 41, 56, 69, 86, 112, 130, and 231 were present in both the sample and the library spectra. Because the product of the EZ:faast derivatization, according to Phenomenex, is proprietary, no information on the structures of the observed GC−MS fragments is provided with the library. However, because an application note from LECO Corp. reported a *m/z* of 232 for GABA derivatized with the EZ:faast kit and analyzed by HPLC coupled to time-of-flight MS (HPLC−ESI−TOF) (*26*), it is likely that the small fragment at *m/z* 231 in the GC−MS spectra (parts **B** and **C** of **Figure 3**) represents the molecular ion of the GABA derivative. The positive electrospray ionization (ESI) method used in the application note of LECO (*26*) is a "soft" ionization that generates few, if any, protonated fragments (*31*). The *m/z* of 232 reported for the GABA derivative probably represents a singly protonated compound, with a molecular weight of 231.

Linearity and Range. The lower limit of the linear dynamic range was 1.00 nmol of GABA per 100 *μ*L of derivatized sample. The typical range of linearity for this assay proved to be 1.00−100 nmol of GABA per 100 *μ*L, with the LOQ being 2.00 nmol/100 *μ*L.

Sample Matrix Interference. The external calibration curve used in recovery calculations had a slope of 0.0128 ($R^2 = 0.988$). The mass addition curve generated from the recovery studies based on yields of tissue extracts spiked with 0, 150, and 600 nmol of GABA/mL had a slope of 0.0117 ($R^2 = 1.00$). The *t* value obtained as described in the Materials and Methods was 2.01, which did not exceed the *t*-table value of 2.04 (*32*) at the 95% confidence level for 29 degrees of freedom (see the Materials and Methods for determination of the number of degrees of freedom). The matrix effect was therefore considered slight and unlikely to interfere with quantifying GABA in forage grass tissue extracts.

Internal Standard Selection. The derivatization procedure specified by the kit included the addition of 20 nmol of an internal standard, norvaline, in the first step. The loss of GABA during derivatization was taken into account by quantifying GABA relative to norvaline, because these amino acids have fairly similar structures (**Figure 1B**) and, hence, could be expected to react similarly to the derivatization. However, the loss of GABA during extraction could not be accounted for by this method. Consequently, the time of addition of norvaline was changed to be at the start of extraction instead of the start
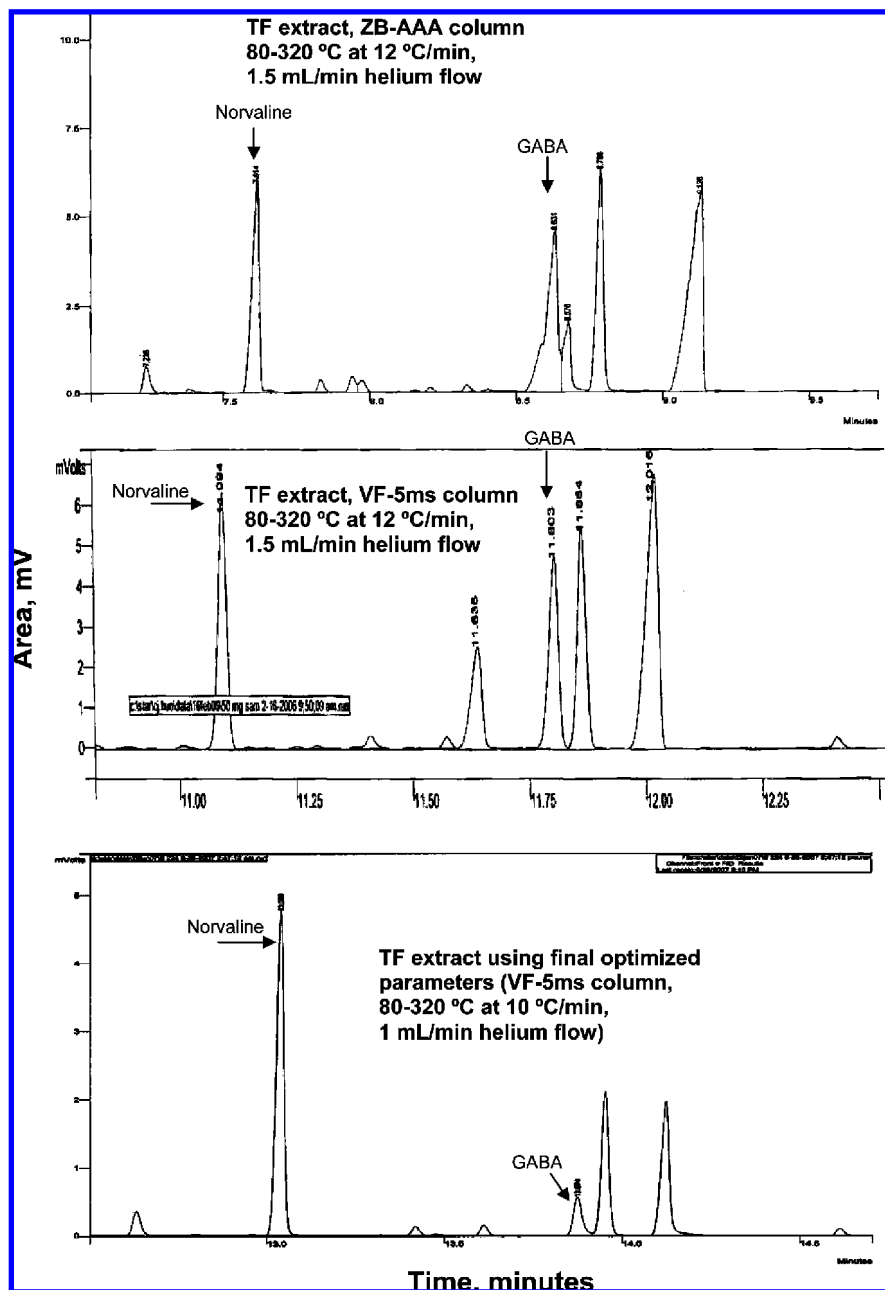
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Figure 2. GC−FID separation of GABA on (A) a 10 m Zebron ZB-AAA column, and (B) a 30 m 5% phenyl/95% dimethylpolysiloxane column, using the same separation parameters for both columns. (C) Optimized separation of GABA on the 30 m 5% phenyl/95% dimethylpolysiloxane column. Norvaline was used as the internal standard.

of derivatization. In this way, the amount of norvaline measured in the derivatized extract reflected losses from both extraction and derivatization.

Recovery and Extraction Efficiency. Mean extraction recoveries for GABA in tall fescue, using the final developed GC−FID method, were 96.4% at the lower end of the linear range (tall fescue extract spiked with 150 nmol of GABA/mL), while those for the upper end of the linear range (tall fescue extract spiked with 600 nmol of GABA/mL) were 94.2% (**Table 1**). Coefficients of variation (CVs) for the replicated spikes were similar (7.3 and 7.2% for the low and high spikes, respectively). Both CVs indicated an acceptable level of reproducibility over the linear dynamic range. This reproducibility is demonstrated in the replicated determinations of GABA on a dry matter basis shown in **Table 2**. Basal GABA content of tall fescue accession 224, which was used for all recovery determinations in **Table 1**, decreased about 50% in the yield studies of **Table 2**.

However, the difference can be explained by the fact that the samples used for the two studies were gathered a few months apart (June 2007 and November 2007). All GABA determinations had CVs below 5% for triplicate intraday analyses. The reproducibility of GABA determinations was partly due to the extraction volume, because extraction of the same amounts of tissue with 3 or 4 mL of solvent had given CVs ranging from 4 to 45%.

GABA yields for tall fescue in this study, expressed as mg/kg dry matter (DM), were 4−18-fold higher than those determined by Baker et al. (*24*) for wheat flour (30−80 mg/kg DM), using the EZ:faast kit for GC−MS analysis of free amino acids and 2−6-fold higher than those determined by Galleschi et al. (*9*) for wheat embryos (126−180 mg/kg DM) on the basis of spectrophotometric absorbance of ninhydrin-sprayed samples eluted from paper chromatograms. Those differences may reflect differences in endogenous GABA levels in wheat and fescue.
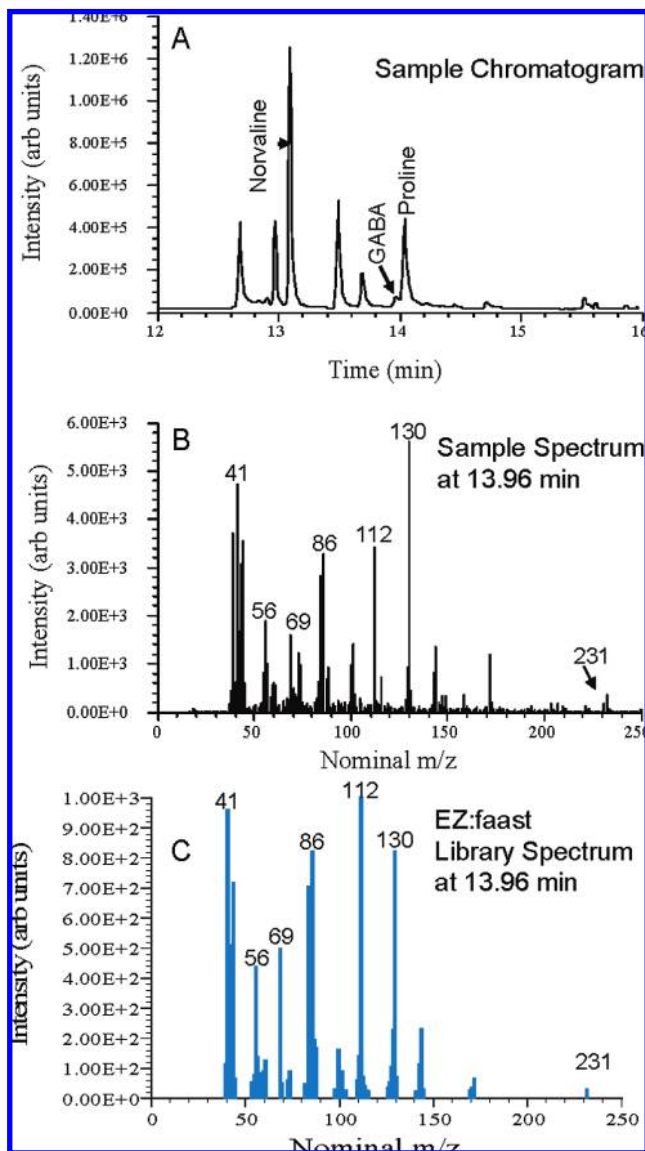
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Figure 3. Mass spectral confirmation of GABA. (A) GABA as shoulder on the proline peak under GC−MS analysis. (B) GABA peak mass spectrum from test sample. (C) GABA spectrum from EZ:faast library spectra.

Table 1. Method Recovery for GABA (200 mg of Tissue in 5 mL of Extraction Solvent Spiked with 0, 15, and 60 nmol/100 μL Extract, Equating to 0, 386.7, and 1546.8 mg/kg Dry Matter, Respectively) and Interday Variation of GABA Yield in Tall Fescue Extractions ($n = 9$)[a]

	spike level for GABA (nmol/100 μL)		
	0	15	60
GABA recovered (mg/kg DM)	775.0	1120.2	2187.5
standard deviation (mg/kg DM)	117.9	81.4	158.0
coefficient of variation (%)[b]	15.2	7.3	7.2
recovery (%)		96.4	94.2

[a] Nine separate extractions per spike level of a randomly chosen tall fescue accession [#224, also used for determination of intraday precision (**Table 2**) but collected on November 6, 2007]. Three extractions per day per spike level were performed over a 3 day period. [b] Interday coefficient of variation.

However, GABA yields from tall fescue in this study are about 5% of those determined for tall fescue by Bond et al. (*13*) (10−13 g/kg DM). Those differences may be partly due to the different detection methods, because Bond et al. (*13*) used HPLC and detected the ninhydrin-reacted eluate by absorbance (*33*).

Table 2. GABA Method Intraday Precision as Determined by Replicated Extractions of Five Randomly Chosen Tall Fescue Forage Accessions (200 mg of Tissue/Subsample)

		tall fescue accession number[a]				
		117	118	222A	224	316
GABA (mg/kg DM)	Rep 1	323.89	434.39	312.76	383.99	552.94
	Rep 2	312.09	418.20	315.33	400.96	552.12
	Rep 3	318.66	397.21	311.73	402.65	535.66
mean (mg/kg DM)		318.22	416.6	313.27	395.87	546.91
standard deviation		5.91	18.64	1.86	10.32	9.75
coefficient of variation (%)		1.86	4.47	0.59	2.61	1.78

[a] All samples were collected on June 20, 2007.

On the other hand, because GABA can accumulate in response to various stresses, the differences in our results and those of Bond et al. (*13*) may reflect differences in plant stress during growth and sample handling. Given the difference in GABA yields for tissue from the same plant on different collection dates (**Tables 1** and **2**), seasonal differences may play a role as well.

In summary, a commercial kit for analyzing serum amino acids on GC−FID was optimized for analysis of GABA in tall fescue herbage, following modifications of the derivatization procedure and a change in the column used. GABA is cleanly separated in less than 30 min, using a column commonly found in laboratories doing GC analysis. Although relatively expensive, the kit provides a derivatization procedure that generates little solvent waste and is easy to use. The method is currently being evaluated for analysis of GABA in rumen fluid as well as in additional forage grasses.

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