Optimal Extraction Procedure for High-Performance Liquid Chromatographic Determination of α,γ-Diaminobutyric Acid in Flatpea, *Lathyrus sylvestris* L.

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Interest in the regulation of α,γ-diaminobutyric acid (DABA) in flatpea (*Lathyrus sylvestris* L.) tissues and concern about the influence that this compound might have on the health of ruminant livestock that are fed flatpea forage prompted studies to develop a quantitative extraction procedure for this basic nonprotein amino acid. Comparison of alcohol, organic solvent, and acid based media for extraction of free amino acids from flatpea tissues showed that soaking tissue in hot (35 °C) 50% (v/v) aqueous ethanol or using Soxhlet extractors with 35% (v/v) ethanol gave maximum yields. *S*-β-(4-Pyridylethyl)-DL-penicillamine (Pep) was found to be an acceptable internal standard. Extracts were analyzed by high-performance liquid chromatography following precolumn derivatization of amino acid constituents with *o*-phthalaldehyde (OPA). Calculation of DABA concentrations with DABA/Pep peak area ratio calibration curves, derived from standard solutions subjected to the extraction and analytical procedures, provided a reproducible means to quantify levels of DABA in the tissue extracts. The optimal procedure is compatible with a requirement for rapid, reliable, quantitative analysis of large numbers of samples.

Flatpea, *Lathyrus sylvestris* L., is a long-lived, perennial legume that is a potentially valuable forage plant for regions where production of conventional agricultural legumes is limited by edaphic and climatic conditions. Despite its numerous desirable characteristics, flatpea has not been listed among recommended forage species because it contains L-α,γ-diaminobutyric acid (DABA), a nonprotein amino acid whose effect on ruminant livestock is uncertain (Foster, 1989a). Quantitative extraction of DABA from flatpea herbage is essential for studies aimed at defining tissue levels of this compound and correlating these levels with responses of animals that consume the herbage.

Much of the research concerning DABA in plant tissues has focused on identification of species that contain this compound (Bell, 1962a,b, 1964; Przybylska and Rymowicz, 1965; Ressler et al., 1961; Simola, 1966, 1968; VanEtten and Miller, 1963). Seeds have been investigated most frequently (Bell, 1962a,b; Ressler et al., 1961; VanEtten and Miller, 1963), but relative comparisons of DABA levels in different organs of several *Lathyrus* species have been published (Przybylska and Rymowicz, 1965; Simola, 1966, 1968). Concentration determinations have been limited to a few reports. Ressler et al. (1961) found that the DABA (monohydrochloride) content of seeds of *L. latifolius* ranged from 0.51% to 0.67%; markedly higher levels, 1.4%, were recorded for seeds of *L. sylvestris* Wagneri. VanEtten and Miller (1963) reported that seeds of *L. sylvestris* contained 2.7% DABA monohydrochloride, a value corresponding to 10.3 ± 0.09% of the nitrogen present in the seed. These levels far exceeded those in seeds of 30 other plant species that also contained DABA (VanEtten and Miller, 1963). Subsequently, Ressler (1964) reported DABA levels in flatpea seeds, seedlings (roots and green parts only), and hay (harvested at the early stage of senescence) to be 0.91%, 2.55%, and 0.02% of the dry weight, respectively. Fresh forage and hay prepared from "Lathco" flatpea (flowering/pod-filling stage) were reported to contain 0.46% and 0.28% DABA, respectively, on a dry weight basis (Pavelka, 1985).

These results suggest that the amount of DABA in flatpea tissues may be quite variable. However, there has been little consistency among the procedures used to extract DABA. Bell (1962a) prepared seed extracts by soaking finely ground seeds at room temperature in a solution composed of equal parts, by volume, of ethanol and 0.1 N hydrochloric acid. In Simola's (1968) study, extracts were obtained by combining fluid fractions derived from an initial soaking of fresh tissue in 94% ethanol and a subsequent homogenization and overnight soaking of the insoluble residue in 70% ethanol at room temperature. Przybylska and Rymowicz (1965) employed a multistep procedure using 80% ethanol. The aqueous extract obtained from a brief extraction of the residue in boiling water was adjusted to 80% ethanol and combined with the ethanol extracts. Ressler and co-workers (Ressler et al., 1961; Nigam and Ressler, 1964) used 30% ethanol to isolate DABA from hexane-extracted ground seeds, and presumably Ressler (1964) used the same procedure to extract DABA from the root and aerial tissues. Acid hydrolysis was used by VanEtten and Miller (1963) to obtain DABA from mature, ground, hexane-extracted seeds. In Pavelka's (1985) study, DABA was removed from tissue samples by extracting ground material first in methanol–chloroform–water–formic acid (12:5:2:1 (v/v)) and then in 2% formic acid in 20% methanol. For analysis, the aqueous phase from the initial extract was combined with the second extract.

None of the studies cited above addressed recovery of DABA. This research represents the first effort to optimize procedures for extraction of DABA from flatpea herbage. A rapid, reproducible, quantitative procedure was identified as illustrated by examples of its application.

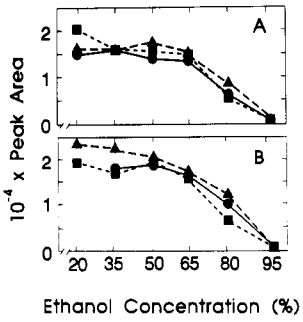

Figure 1. Yields of DABA from flatpea leaf tissue extracted with aqueous ethanol. Fresh, frozen tissue (panel A) (2 g dry weight equivalent) or lyophilized tissue (panel B) was placed either in cellulose thimbles (●, ▲) or directly in flasks (■) of Soxhlet extraction units. Prior to Soxhlet extraction some thimbles with their contents were soaked for 30 min in the extraction medium (35 °C) (▲).

MATERIALS AND METHODS

Materials. DABA, *S*-β-(4-pyridylethyl)penicillamine (Pep), norvaline, norleucine, α-aminoadipic acid, vinylglycine, and β-alanine standards were obtained from Sigma. Solvents and water were HPLC grade. All other chemicals used were of the highest purity available.

Tissue and Preextraction Treatment. For initial comparison of extraction procedures, flatpea (*Lathyrus sylvestris* L. var. "Lathco") leaves were collected from a single plant maintained in a greenhouse. Fully expanded leaves were harvested, quick-frozen in liquid nitrogen, and crushed with a mortar and pestle to a particle size of <2 mm. Subsamples of 2.0 ± 0.1 g of crushed, fresh, frozen tissue were weighed into 125-mL Florence flasks and into 25 mm × 80 mm cellulose extraction thimbles. Subsamples either were stored frozen (−20 °C) until extracted (1–5 days) or were lyophilized and stored desiccated at −20 °C prior to extraction. Lyophilized, field-grown flatpea tissue and pelleted flatpea hay harvested at the seed-ripening stage were ground to pass a 0.5-mm screen using a Udy cyclone mill and were used to obtain additional information about specific extraction procedures. [Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.]

Amino Acid Extraction: Alcohol-Based Procedures. Extraction media (90 mL) consisting of 20, 35, 50, 65, 80, and 100% (v/v) ethanol in water were placed in Florence flasks, thimbles were placed in 30-mm-i.d. Soxhlet tubes, and extraction was performed until the equivalent of approximately 2200 mL of medium had cycled through the Soxhlet tube (45 cycles, 50-mL Soxhlet chamber). Some subsamples of both dried and fresh, frozen tissue in thimbles were presoaked in hot (35 °C) extraction medium for 30 min prior to extraction with the same medium in a Soxhlet apparatus. A time course study was conducted to determine the number of Soxhlet cycles necessary to remove all of the free DABA from the tissue. In companion studies, tissue was extracted by soaking in 35% and 50% (v/v) ethanol for 1 or 1.5 h at 35 °C or for 48 h at −20 °C without subsequent Soxhlet extraction. Extraction media composed of various concentrations of methanol in water and ethanol–water–NH_4OH (50:48:2 (v/v)) were used with Soxhlet extractors to determine their influence on the recovery of DABA and the internal standard.

Amino Acid Extraction: Organic Solvent Based Procedures. Lyophilized and fresh, frozen tissues were extracted with methanol–chloroform–water (12:5:3 (v/v)) by using the approach of Bieleski and Turner (1966). As a second approach, 400 mg dry weight equivalent of tissue was placed in a 25 mm × 80 mm cellulose thimble (without internal standard) and extracted by soaking in 50 mL of chloroform for 1 h at room temperature. Tissue and thimbles were then air-dried at room temperature, an internal standard was added, and the sample was extracted with 50% (v/v) aqueous ethanol in a Soxhlet apparatus as described above. The Soxhlet extract was processed for analysis. The reverse sequence of steps was also investigated: a 90-mL Soxhlet extract, cooled to room temperature, was combined with 50 mL of chloroform, shaken vigorously in a separatory funnel for 10 s, and allowed to stand for 10 min. The aqueous ethanol layer was collected and analyzed. Alternatively, chloroform extraction was performed after the Soxhlet extract had been concentrated to dryness and resuspended in 10 mL of 50% (v/v) aqueous ethanol.

Table I. Influence of Duration of Extraction and Media Composition on DABA Yield from Lyophilized Flatpea Leaf Tissue Subjected to Soxhlet Extraction

no. of cycles	extraction medium	DABA yield,[a] mg/g dry wt	LSD[b]
5	50% ethanol	13.5	
10	50% ethanol	14.3	
20	50% ethanol	13.1	
30	50% ethanol	12.9	
45	50% ethanol	13.0 ± 0.80	A
45	50% methanol	14.3 ± 0.96	A

[a] Extracts were prepared from subsamples from a batch harvest of greenhouse-grown tissue. Data are reported for single observations or as means ± SD for $n = 4$. Yields were calculated with a DABA standard curve generated by HPLC analysis of a stock solution of pure DABA. [b] Least significant difference. Means followed by the same letter are not significantly different at the 0.05 level.

Amino Acid Extraction: Acid-Based Procedures. Perchloric acid (7% (v/v)) extraction of free amino acids was accomplished by using a modification of the procedure described by Saifer (1971). For comparison, subsamples from the same batch of tissue were extracted for 90 min with 35% and 50% ethanol, either in Soxhlet extractors or by incubating tissue in extraction medium in a 35 °C shaking water bath as described above.

Sample Preparation for Amino Acid Analysis. Tissue residues remaining in extracts prepared by boiling tissue directly in the extraction medium were removed by filtration through Whatman no. 2 filter paper. Filter paper and retentate were rinsed with the corresponding extraction medium, and the rinse solution was combined with the original extract. These extracts, and those obtained from the other procedures, were placed in a 35 °C water bath and concentrated to dryness under a stream of air. Residues obtained from alcohol-based extraction procedures were resuspended in 10 mL of the corresponding extraction medium. Residues from organic solvent based extraction procedures were resuspended in 10 mL of 50% aqueous ethanol or 10% (v/v) 2-propanol (Bieleski–Turner-type samples). A 2.5-mL aliquot of each extract was centrifuged at 2000*g* for 10 min. Pellets were washed 2 times with 2.5 mL of the corresponding resuspension medium, and all supernatants for a given sample were combined and adjusted to a volume of 7.5 mL with resuspension medium. Samples (0.25-mL aliquots), followed by successive washes of 0.5 mL of water and 1.0 mL of methanol, were passed through an activated C_{18} Sep-Pak cartridge (Waters). Combined eluates, adjusted to 2.0 mL with water, were ready for amino acid analysis. Subsequently, centrifugation steps were found to be unnecessary and 0.25-mL aliquots of the 10-mL resuspension were applied directly to Sep-Pak cartridges. Perchloric acid extracts and some ethanolic extracts were filtered through a 0.22-μm Millex-GV (Millipore) filter and assayed directly.

Analytical Procedures. DABA was analyzed by high-performance liquid chromatography following precolumn derivatization with *o*-phthalaldehyde as described previously (Foster, 1989b). Peak areas were determined with a Nelson Analytical Model 4416X chromatography data system. Standard solutions of DABA (45 mM) and Pep (5–15 mM) were assayed directly and subjected to selected sample-processing procedures prior to analysis to estimate recoveries and generate calibration curves.

Statistical Analyses. Analysis of variance on means and mean comparisons by least significant difference test at the 0.05 level were performed by using SAS (SAS Institute, 1985).

RESULTS

Figure 1 illustrates the influence of various components of the extraction procedure on yields of DABA from

Table II. Influence of Extraction Procedure and Tissue Treatment on Yield of DABA from Flatpea Leaves

procedure	medium	tissue[a]	yield,[b] mg/g dry wt	LSD[c]
Soxhlet[d]	50% ethanol	fresh, frozen	8.8 ± 0.93	BCD
Soxhlet[d]	50% ethanol	lyophilized	7.9 ± 1.21	CD
hot soak[e]	50% ethanol	fresh, frozen	11.5 ± 2.29	A
hot soak[e]	50% ethanol	lyophilized	10.0 ± 0.70	AB
cold soak[f]	50% ethanol	fresh, frozen	9.8 ± 1.06	ABC
cold soak[f]	50% ethanol	lyophilized	9.7 ± 0.69	ABC
Bieleski–Turner[g]	CMW (12:5:3 (v/v)); aqueous ethanol	fresh, frozen	8.5 ± 1.98	BCD
Bieleski–Turner[g]	CMW (12:5:3 (v/v)); aqueous ethanol	lyophilized	7.8 ± 0.35	BCD

[a] Extracts were prepared from subsamples from a batch harvest of mature, field-grown flatpea. [b] Yields were calculated with a DABA standard curve generated by HPLC analysis of a stock solution of pure DABA. Data are reported as means ± SD for $n = 4$. [c] Least significant difference. Means followed by the same letter are not significantly different at the 0.05 level. [d] Extraction was performed through 45 cycles. [e] Extraction was performed for 1 h at 35 °C. [f] Extraction was performed for 48 h at −20 °C. [g] Bieleski and Turner, 1966; CMW = chloroform–methanol–water.

Table III. Influence of Chloroform Extraction on Yields of DABA from Flatpea Leaf Tissue

extraction sequence	yield,[a] mg/g dry wt	LSD[b]
Soxhlet[c]	12.4 ± 0.20	A
chloroform, Soxhlet[c]	12.1 ± 0.47	A
Soxhlet,[c] chloroform	12.9 ± 0.47	A
Soxhlet,[c] resuspension,[d] chloroform	10.5 ± 0.68	B
Bieleski–Turner[e]	8.3 ± 0.35	C

[a] Extracts were prepared from subsamples of lyophilized tissue from a single batch harvest. Yields were calculated with a DABA standard curve generated by HPLC analysis of a stock solution of pure DABA. Data are reported as means ± SD for $n = 3$. [b] Least significant difference. Means followed by the same letter are not significantly different at the 0.05 level. [c] Extraction medium for the Soxhlet step was 50% (v/v) aqueous ethanol. [d] Soxhlet extract was concentrated to dryness and resuspended in 10 mL of 50% (v/v) aqueous ethanol. [e] Bieleski and Turner, 1966; chloroform–methanol–water (12:5:3), aqueous ethanol extraction sequence.

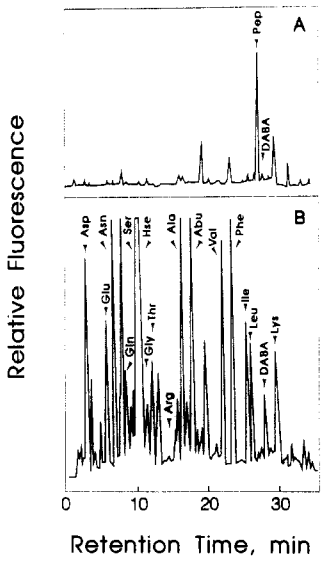

Figure 2. Chromatographic profile of flatpea leaf amino acids and *S*-β-(4-pyridylethyl)penicillamine (Pep). Panel A: area of Pep peak corresponding to a concentration of 9.55 μg/mL of the solution assayed. Panel B: 50% (v/v) aqueous ethanol Soxhlet extract of flatpea pellets prepared from hay harvested at the pod-filling/ripening stage.

flatpea leaf tissue. Quantities of DABA extracted from either the dry or the fresh, frozen tissue with a given extraction medium were similar, whether extraction was accomplished by boiling the tissue directly in the medium, by extracting the tissue with a Soxhlet apparatus, or by first soaking thimbles and their contents in hot medium and then extracting the tissue with the same medium in a Soxhlet apparatus. The soaking procedure tended to result in slightly higher yields of DABA but did not enhance recoveries of other major free amino acid constituents in the extracts (data not shown). Yields of DABA, especially, varied with the concentration of ethanol in the extraction medium. Virtually none of the DABA was extracted with 100% ethanol. Maximum yields of this lower homologue of lysine were not obtained unless the water content of the aqueous ethanol extraction solution was at least 50% (v/v). Other major amino acid constituents (Lys, Arg, Asn, Glu, Ser, homoserine (Hse), Asp, Ala, Val, Ile, Leu, Phe) were extracted effectively when the ethanol content of the medium was 80% (v/v) or less (data not shown). The seemingly higher yields of DABA from the dry tissue reflect lower quantities of dry matter processed with fresh, frozen samples because of accumulation of frost/condensate on tissue during the weighing process.

DABA is readily removed from flatpea leaf tissue. A time course evaluation in which lyophilized leaf tissue from greenhouse-grown plants was subjected to Soxhlet extraction with 50% aqueous ethanol revealed that recovery of DABA after only five cycles was comparable to those obtained for longer times with a mean yield of 13.4 ± 0.57 mg/g dry weight for the five time points (Table I).

Preliminary indications that soaking procedures enhanced yields of DABA from flatpea leaf tissue (Figure 1) were supported by data given in Table II. Although differences were small, yields of DABA extracted solely by soaking tissue in hot (35 °C) ethanol were higher than those obtained by Soxhlet extraction or by soaking tissue in cold medium. Lower yields, compared to data reported in Table II, are due to the use of more mature, field-grown plant material.

Results obtained by applying the multistep extraction sequence described by Bieleski and Turner (1966) were comparable to those obtained by the more direct Soxhlet procedure (Table II), suggesting that incorporation of an organic solvent in the extraction medium was not beneficial. This result was verified by data presented in Table III. Inclusion of a chloroform extraction step at various points in the 50% aqueous ethanol Soxhlet procedure did not enhance yields. All Soxhlet-based procedures gave better results than did the Bieleski and Turner (1966) protocol.

Quantification of peak areas to obtain yield data presented in Tables I–III was accomplished by use of external standards. Requirements for quantitative extraction of DABA from flatpea tissues prompted consideration of the internal standard approach to quantification. Selection of a suitable internal standard was complicated by the complex chromatographic profiles obtained for flatpea samples (Figure 2). Retention times for vinylglycine, β-alanine, α-aminoadipic acid, norvaline, and nor-

Table IV. DABA Recovery from Flatpea Pellets Prepared from Hay Harvested at the Pod-Filling/Ripening Stage of Growth

extraction procedure[a]	DABA peak area[b]	DABA,[b,c] mg/g dry wt	DABA/Pep peak area ratio[b,d]	DABA,[d,e] mg/g dry wt
Soxhlet, 35% ethanol				
with Sep-Pak clean up	47145 ± 2131 (4.5)	9.3 ± 0.4	0.6419 ± 0.0492 (7.7)	12.2 ± 0.9
without Sep-Pak clean up[f]	52867 ● 2367 (4.5)	10.4 ± 0.4	0.6059 ± 0.0366 (6.0)	11.6 ± 0.6
Soxhlet, 50% ethanol				
with Sep-Pak clean up	32318 ± 3361 (10.4)	6.5 ± 0.6	0.4238 ± 0.0396 (9.3)	8.2 ± 0.7[g]
without Sep-Pak clean up[f]	42758 ± 3308 (7.7)	8.5 ± 0.6	0.4523 ± 0.0338 (7.5)	8.7 ± 0.6
soak, 35% ethanol				
without Sep-Pak clean up[f]	47539 ± 2237 (4.7)	9.4 ± 0.4	0.5749 ± 0.0315 (5.5)	11.0 ± 0.6
soak, 50% ethanol				
without Sep-Pak clean up[f]	54171 ± 2162 (4.0)	10.7 ± 0.5	0.6572 ± 0.0264 (4.0)	12.5 ± 0.5
7% perchloric acid[f]	38868 ± 2179 (5.6)	7.8 ± 0.4	0.5088 ± 0.0230 (4.5)	9.8 ± 0.4

[a] Soxhlet extraction was performed for 90 min. Soaking procedures were conducted at 35 °C for 90 min. [b] Replicate subsamples from a single batch of crushed flatpea pellets were extracted. Data are reported as means ± SD, $n = 4$. Coefficients of variation (%) are reported in parentheses. [c] DABA was quantified by using a peak area calibration curve generated with standard solutions of DABA not subjected to any extraction procedure ($y = 846x - 2137$, $r = 0.998$; y = DABA peak area, x = DABA concentration in μg/mL). [d] Pep concentration in extracts was 15.9 μg/mL. [e] DABA was quantified by using a DABA/Pep peak area ratio calibration curve generated with standard solutions of DABA and Pep not subjected to any extraction procedure ($y = 0.00867x - 0.02176$, $r = 0.998$; y = DABA/Pep peak area ratio, x = DABA concentration in μg/mL). [f] Extracts were filtered through a 0.22-μm Millex-GV filter and assayed directly. [g] Yield of DABA = 9.0 ± 0.7 mg/g dry weight, based on calibration curve ($y = 0.00835x - 0.04390$; y = DABA/Pep peak area ratio, x = DABA concentration in μg/mL) derived from standard solutions subjected to the extraction procedure.

leucine, compounds typically used as internal standards for amino acid analyses, coincided with those for flatpea constituents (data not shown). Pep eluted in a clean region of the flatpea chromatograms (retention time = 27.2 min), close to DABA (retention time = 28.0 min) (Figure 2).

The influence of extract preparation procedures on recovery of DABA and Pep was investigated by analyzing a series of stock solutions, varying in known concentrations of the pure amino acids, before and after processing through the Soxhlet extraction (50% (v/v) aqueous ethanol) and Sep-Pak clean-up procedures. Correlation coefficients ($r \geq 0.997$) for regression equations relating chromatographic peak areas and the expected concentrations of the amino acids in assayed samples indicated a good correlation between the dependent and independent variables for amino acids in both nonextracted and extracted, Sep-Pak-treated standard solutions. Analysis of Pep and DABA yields in extracted samples, however, revealed differential recoveries of the two amino acids. Total loss of Pep consistently ranged between 14% and 16% for all concentrations of stock solutions (1–4 mg/mL) examined. Approximately 10% of this loss occurred during the Soxhlet extraction and extract resuspension steps. Losses of DABA were concentration dependent, varying from 14% to 41% for stock solutions ranging in concentration from 1 to 9 mg/mL. Recovery of DABA following the extraction and resuspension steps was essentially 100%; loss of DABA during the Sep-Pak procedure (15–40%) was equivalent to the total loss observed. Within-run and between-run coefficients of variation were <10%.

Peak area ratio calibration curves constructed from data derived by chromatographic analysis of Soxhlet-extracted, Sep-Pak-prepared standard solutions provide a means to estimate tissue levels of DABA. The regression equation, $y = mx + b$, for the best line through the data points, where y is the peak area ratio for DABA/Pep and x is the expected concentration of DABA (μg/mL) in the 50% (v/v) aqueous ethanol extracted, Sep-Pak-processed standards, does not intercept the origin ($y = 0.00835x - 0.04930$, $r = 0.998$). This characteristic was also exhibited by DABA/Pep peak area ratio calibration curves generated from data derived from analyses of the corresponding nonextracted standard solutions ($y = 0.00867x - 0.02176$, $r = 0.998$) and extracted standards prior to the Sep-Pak procedure ($y = 0.01014x - 0.03595$, $r = 0.998$). Good correlation existed in each case.

Extracts prepared from pelleted flatpea hay (0.5 g) by a 90-min Soxhlet extraction with 50% (v/v) aqueous ethanol and Sep-Pak clean up gave DABA/Pep ratios of 0.37499 ± 0.06395 ($n = 3$). Calculations based on the peak area ratio calibration curve for extracted standards ($y = 0.00835x - 0.04390$) estimated tissue levels of DABA to be 8.0 ± 1.2 mg/g dry weight. These values correspond to 5.9 ± 0.7 mg/g dry weight, obtained by use of DABA as an external standard ($y = 846x - 2137$, $r = 0.998$; y is the DABA peak area, x is the DABA concentration in μg/mL), and 7.6 ± 0.9 mg/g dry weight, calculated by using the regression equation relating DABA peak area to expected DABA concentration in extracted, Sep-Pak-processed standards ($y = 692x - 3780$, $r = 0.997$; y is the DABA peak area, x is the DABA concentration in μg/mL).

Results of further analyses of DABA extraction procedures are illustrated in Table IV. Whether recoveries were estimated by using calibration curves based on DABA peak area or the DABA/Pep peak area ratio, maximum yields of DABA on a dry weight basis were obtained by soaking tissue in 50% ethanol or by using 35% ethanol with Soxhlet extractors. Yields obtained by soaking tissue in 35% ethanol tended to be only sllightly lower. On the other hand, yields of DABA were markedly less in perchloric acid extracts and in Soxhlet extracts obtained with 50% ethanol. The coefficient of variation for each procedure was ≤10%.

Compared to values obtained by calibration with nonextracted standards (Table IV), estimates of the DABA concentration in tissue extracts (50% ethanol Soxhlet procedure) obtained with peak area ratio calibration curves for standard solutions subjected to the 50% ethanol Soxhlet extraction procedure ($y = 0.00835x - 0.04390$; y is the DABA/Pep peak area ratio, x is the DABA concentration in μg/mL) were slightly higher (9.0 ± 0.7 mg/g dry weight). That this estimate was lower than those obtained with the 35% ethanol Soxhlet procedure or the 50% ethanol soak procedure (Table IV) was consistent with incomplete removal of DABA from the tissue. Inclusion of NH_4OH in the ethanol extraction medium to discourage binding of basic amino acids to tissue residue resulted in poor resolution of the DABA peak, appeared not to enhance recovery of DABA, and decreased recoveries of Pep by 25%.

Recovery of DABA from Soxhlet extracts prepared with C_{18} Sep-Pak cartridges was 15% higher for 35% ethanol extracts, compared to the 50% ethanol extracts when the

DABA peak area alone was used for quantification (Table IV). Use of the DABA/Pep peak area ratio calibration curve compensated for this differential effect, and comparable yields were obtained for extracts prepared with or without the Sep-Pak procedure.

DISCUSSION

Saifer (1971) observed poor yields of basic amino acids from brain tissue extracted with ethanol, chloroform–methanol, acetone–HCl, and picric acid. Improved yields of basic amino acids with water, compared with 70% or 80% ethanol, as the extraction medium have been observed by other researchers (Lazarus, 1973; Oland, 1959). Evidence gathered in this study clearly indicates that 80% ethanol, typically used for extraction of free amino acids from plant tissues (Oland, 1959), is unacceptable for extraction of DABA from flatpea. Maximum yields of DABA obtained by extracting tissue with 35% ethanol in a Soxhlet system or by soaking tissue in 50% aqueous ethanol at 35 °C (Figure 1, Tables II and IV) suggest that a critical balance exists between water solubilization of DABA and ethanol disruption of cellular membranes during the extraction process. Complete recovery of DABA from standard solutions subjected to Soxhlet extraction confirmed that the gradient extraction had little adverse effect when the concentration of ethanol in the stock extraction medium was sufficiently low that extraction in the Soxhlet tube was accomplished in media containing enough water to solubilize the DABA (Figure 1). Ammonia-containing media, however, did not provide the improvement in DABA yield that was observed for arginine in other studies (Bieleski and Turner, 1966; Hackett et al., 1965). Further, the positive influence of perchloric acid on isolation of basic amino acids from brain tissue (Saifer, 1971) was not observed with DABA in flatpea.

Correction for losses based on analytical recovery of an internal standard, added to the tissue at the outset, has been strongly encouraged in amino acid analyses (Gardner, 1985). Friedman et al. (1973) found Pep to be superior to a number of other compounds as an internal standard for basic amino acid analysis by ion-exchange chromatography. Elution of the OPA derivative of Pep in a clean region of the reverse-phase chromatogram with a retention time similar to that for DABA makes Pep a reasonable choice as an internal standard for DABA analyses. The reproducibility of relative responses of DABA and Pep in extraction and Sep-Pak procedures is quite good; thus, DABA/Pep peak area ratios can provide better estimates of DABA in tissue than might be obtained with the DABA peak area alone. Errors, of course, are associated with area determinations for each of the two peaks.

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