Analysis of *Solanum* Alkaloids Using Internal Standardization and Capillary Gas Chromatography[†]

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A capillary gas chromatographic method has been developed for quantitation of the principal steroidal alkaloids of *Solanum chacoense* and *Solanum tuberosum*. The method uses tomatine as an internal standard and requires less than 100 mg of dry weight of plant tissue. Internal standard recoveries were approximately 80–95%. Glycoalkaloids were concurrently extracted and hydrolyzed using 1 N HCl in methanol. The underivatized aglycons, solanidine, leptinidine, tomatidine, and acetylleptinidine, the peak areas of which constituted between 70 and 80% of the total peak area of the chromatogram, were resolved in less than 20 min using a nonpolar (Rt_x-1) megabore fused silica column, with retention times of 10.34, 14.47, 15.44, and 15.96 min, respectively. Retention times varied less than 0.2%. The procedure was applied to the quantitation of steroidal alkaloids from *S. chacoense* and *S. tuberosum* leaves and tubers, for which relative standard errors were typically less than 2% of the mean.

INTRODUCTION

The need to quantitate glycoalkaloids in potato cultivars has been emphasized repeatedly (Gregory, 1984; Osman et al., 1978; Osman, 1980). While levels below 20 mg/100 g of fresh weight of tuber pose no threat to consumers, excessive levels of specific glycoalkaloids impart bitterness (Sinden and Deahl, 1976) and may be toxic or teratogenic (Jadhav et al., 1981; Kuć, 1975; Maga, 1980; Morris and Lee, 1984; Roddick, 1986). Therefore, quantitation of individual glycoalkaloids is critical, especially in breeding programs that introduce germplasm from wild potato species for the development of new potato cultivars. Wild species, such as *Solanum chacoense* Bitter, commonly accumulate high levels of glycoalkaloids, and one *Solanum tuberosum* cultivar, Lenape, which had *S. chacoense* in its ancestry (Akeley et al., 1968), was withdrawn from production because of its high glycoalkaloid content (Zitnak and Johnston, 1970). Nonetheless, certain accessions of *S. chacoense*, which have high levels of leptine glycoalkaloids (Sinden et al., 1986b), have demonstrated excellent resistance to the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Carter, 1987; Sinden et al., 1986a; Sturckow and Löw, 1961), and have become important genetic resources for potato breeding programs (Deahl and Sinden, 1987; Ross, 1966; Sinden et al., 1986a,b).

The importance of quantitating potato glycoalkaloids and its challenge to potato researchers are reflected in the number of methods that have been developed. These methods vary in their approach and include colorimetric assays (Bushway et al., 1980; Coxon et al., 1979; Fitzpatrick and Osman, 1974; Sanford and Sinden, 1972), thin-layer chromatography (TLC) (Coxon and Jones, 1981; Deahl and Sinden, 1987; Hunter et al., 1976; Jellema et al., 1980, 1981; Shih and Kuć, 1974), high-performance liquid chromatography (HPLC) (Crabbe and Fryer, 1980; Morris and Lee, 1981), and gas chromatography (GC) (King, 1980; van Gelder, 1985). However, these chromatographic methods lack an accurate measure of glycoalkaloid recovery during sample preparation. To estimate recoveries, King (1980) fortified selected samples with glycoalkaloids already present in the tissue, but this technique fails to accurately account for sample-to-sample differences in recovery.

Further, substantial and variable losses in recovery can be incurred if glycoalkaloids are precipitated from crude extracts by adding concentrated ammonia (ammonia precipitation; Gregory et al., 1981; Jellema et al., 1981; Morris and Lee, 1981; Sinden et al., 1986b). Despite being used routinely in preparing extracts of *S. tuberosum* for glycoalkaloid analysis, ammonia precipitation does not quantitatively precipitate glycoalkaloids of some wild *Solanum* species, including *S. chacoense*. Losses greater than 60% have been reported (Sinden et al., 1986b).

In this paper, we report the development of a procedure that combines the extraction and hydrolysis of glycoalkaloids into a single step and uses capillary GC to quantitate principal steroidal glycoalkaloid (SGA) aglycons from *S. tuberosum* and *S. chacoense* leaves and tubers (Figure 1). This procedure requires only a minimal amount of plant material (less than 100 mg of dry weight), avoids ammonia precipitation during sample preparation, and uses a readily available internal standard for consistent, accurate quantitation.

MATERIALS AND METHODS

Plant Material. Tubers of *S. tuberosum* cv. Atlantic were field-grown using standard cultivation practices at the OARDC, Wooster, OH. Tubers of *S. chacoense*, PI 458310-1, were harvested from plants grown in the greenhouse. Foliar samples of *S. tuberosum* and *S. chacoense* were taken from plants (30–50 cm tall) grown in the greenhouse in a sand/soil/peat moss (1:1:1) potting mixture. Foliar samples also were taken from *S. chacoense* plantlets cultured in vitro for 30 days (30 °C, 12-h photoperiod using fluorescent lighting) on Murashige and Skoog (1962) basal media containing 3% sucrose.

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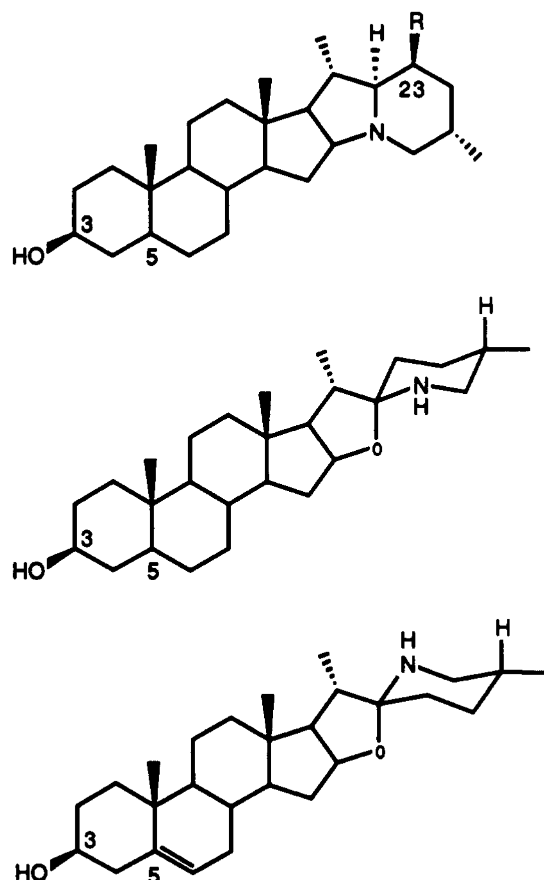

Aglycone	Aglycone Structure	Corresponding Glycoalkaloids
Demissidine	R=H	Demissine
Solanidine	Δ^5,R=H	Solanine, Chaconine
Leptinidine	Δ^5,R=OH	Leptinines (I-IV)
Acetylleptinidine	Δ^5,R=OC(O)CH_3	Leptines (I-IV)
Tomatidine		Tomatine
Solasodine		Solasonine, Solamargine

Figure 1. Structures of *Solanum* C_{27}-steroidal alkaloids.

Alkaloid Standards. Demissidine, solanidine, solasodine, tomatidine, and tomatine were purchased from Sigma Chemical Co. (St. Louis, MO).

Purification of Acetylleptinidine. Freeze-dried leaves (ca. 10 g of dry weight) of *S. chacoense*, PI 458310-1, were homogenized for 5 min in 350 mL of 2% acetic acid in methanol using a Waring blender. The homogenate was vacuum filtered through Whatman No. 1 filter paper, and the filtrate was evaporated to near dryness at 50 °C in vacuo. The residue was redissolved in 50 mL of 1 N HCl in methanol, capped under nitrogen, and hydrolyzed at 70 °C for 4 h in a shaking water bath. After cooling to room temperature, the pH of the hydrolysate was raised to 10 with concentrated ammonium hydroxide and centrifuged at 12000*g* for 6 min. The supernatant was partitioned twice (25 mL each) against chloroform, and the combined chloroform phases, which contained the aglycons, were evaporated to near dryness at 40 °C in vacuo.

The residue was redissolved in chloroform and subjected to two successive flash chromatography steps. The first step used a 1 × 4 cm silica gel (E. M. Merck, 40–60 μm) column, onto which approximately 100 mg (300 μL) of the preparation was applied. The column was eluted with 5 mL of hexane followed by 20 mL of ethyl acetate/hexane (2:8); 1-mL fractions were collected. Fractions containing acetylleptinidine according to TLC (see below) were combined, evaporated to dryness at 50 °C under a stream of nitrogen, and redissolved in chloroform. Twenty-five milligrams (50 μL) of the pooled acetylleptinidine-containing fractions were loaded onto a 0.8 × 25 cm silica gel (E. M. Merck, 40–60 μm) column, and acetylleptinidine was purified by first washing the column with 10 mL of ethyl acetate/hexane (1:9) and then eluting acetylleptinidine with ethyl acetate/hexane (2:8). Fractions of 1.2 mL were collected. The composition of each fraction was determined by TLC (see below). Fractions containing acetylleptinidine were combined, evaporated to dryness at 50 °C under a stream of nitrogen, and then recrystallized from methanol.

TLC was performed using silica gel 60 (E. M. Merck, 0.25 μm) plates and ethyl acetate/hexane (1:1) for development. Spots were visualized with iodine vapor or Dragendorff's reagent (Sigma).

Preparation of Leptinidine. Leptinidine was prepared by hydrolyzing (70 °C for 30 min) recrystallized acetylleptinidine in 0.83 N sodium hydroxide in methanol/water. After cooling, the hydrolysate was partitioned twice against benzene; the combined benzene phases contained leptinidine, which was verified by TLC and GC (see below). Leptinidine was recrystallized from methanol.

Sample Preparation. Methodology is shown in Figure 2. Freeze-dried foliar or tuber samples were ground to a fine powder using a mortar and pestle. Leaf (10–20 mg of dry weight) or tuber (60–80 mg of dry weight) tissue was placed into a 10-mL screw-cap vial, and then 200 μg of tomatine (internal standard; dissolved in methanol) and 3 mL of 1 N HCl in methanol were added. Samples were capped under nitrogen and concurrently extracted and hydrolyzed in a shaking water bath for 4 h at 70 °C. After cooling to room temperature, extracts were made alkaline (pH ≥10) with 2 mL of concentrated ammonium hydroxide and then centrifuged for 10 min at 1800*g* to remove insoluble materials. The supernatant was partitioned against 2 mL of benzene. Samples were centrifuged at 1800*g* for 5 min to hasten phase separation. One milliliter of the benzene phase was removed and evaporated to dryness at 50 °C under a stream of nitrogen. The residue was redissolved in 0.5 mL of chloroform and either subjected to GC directly or applied to a silica gel Sep-Pak cartridge (Waters Associates, Milford, MA). Aglycons were eluted from the Sep-Pak with 8 mL of pyridine/ethyl acetate/hexane (2:5:5 v/v/v), and the eluate was evaporated to dryness at 50 °C under a stream of nitrogen. The residue was redissolved in 0.5 mL of chloroform before GC analysis.

GC Analysis. One-microliter aliquots were injected onto a 15 m × 0.53 mm i.d. × 0.25 μm Rt_x-1 fused silica column (Restek Corp., Bellefonte, PA) fitted to an HP 5890A gas chromatograph

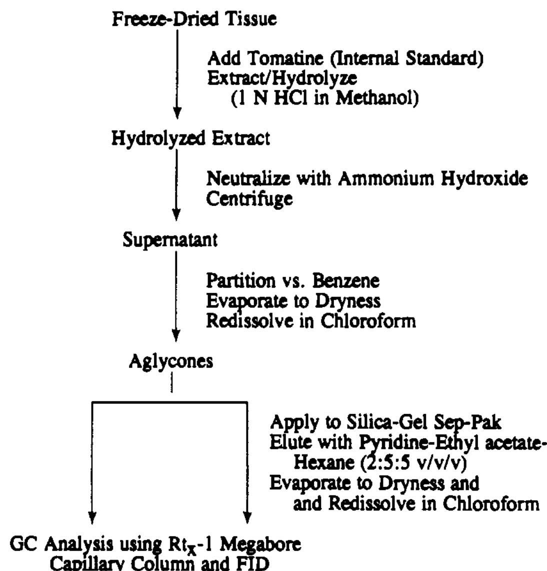

Figure 2. Flow diagram for extraction and quantitative analysis of *Solanum* alkaloids from *S. tuberosum* and *S. chacoense*. See Materials and Methods for details.

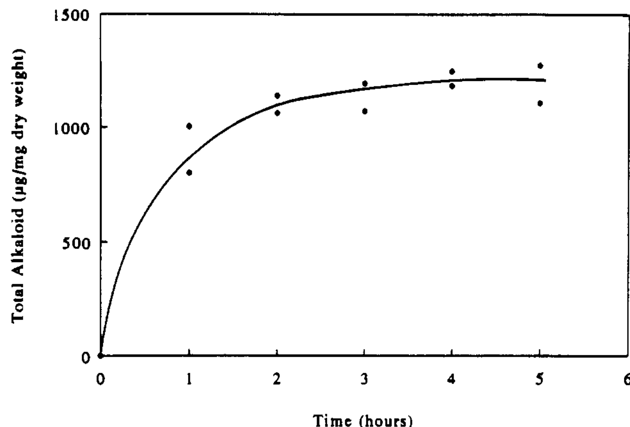

Figure 3. Time course for concurrent extraction and hydrolysis of *S. chacoense*, PI 458310-1, alkaloids from freeze-dried leaf tissue. Each point represents one replicate. See Materials and Methods for details.

equipped with a flame ionization detector (FID). The injector and detector temperatures were 270 and 280 °C, respectively. The column temperature was programmed from 210 to 260 °C at 2 °C/min. Helium was used as the carrier gas at a linear velocity of 45 cm/s.

RESULTS AND DISCUSSION

An overview of our procedure (Figure 2) reveals key elements that contributed to accurate SGA aglycon quantitation while minimizing technical input. These include (1) use of an internal standard (tomatine), (2) concurrent extraction and hydrolysis, and (3) megabore (530 μm i.d.) capillary GC of aglycons.

Tomatine as an Internal Standard. Coxon (1984) noted that there is no standard method for glycoalkaloid analysis. Ideally, any standard method should include an internal standard for reproducible, accurate quantitation. For *S. chacoense* and *S. tuberosum* cv. Atlantic, tomatine satisfied all of the criteria for an internal standard: (1) it was not a normal constituent, (2) it was chemically and physically similar to the indigenous alkaloids, (3) its aglycon was resolved from the other analytes in the samples, (4) it eluted near the peaks of interest, and (5) it was readily available. Tomatine will not be a suitable internal standard for (glyco)alkaloid analysis of all *Solanum* species, specifically those for which tomatidine cannot be resolved from indigenous components or tomatine is a normal constituent of the sample, but it can be used for (glyco)alkaloid analysis of several wild *Solanum* species and *S. tuberosum* cultivars. Tomatine was added to our samples prior to extraction and hydrolysis (Figure 2) to account for losses that may have occurred during preparation and analysis.

Even though several methods have been developed for *Solanum* (glyco)alkaloid quantitation using one or more of a number of colorimetric and chromatographic techniques, none of those routinely used accurately account for sample-to-sample variation due to sample workup. King (1980) obtained an average estimate of recovery by fortifying selected samples of *S. tuberosum* tubers and foliage with solanine, but the use of an internal standard is the technique of choice, since it minimizes errors due to sample preparation, apparatus, and technique.

Extraction and Hydrolysis. Our procedure combines extraction and hydrolysis into a single step and eliminates losses that can occur during grinding, filtration, and transfers. The parallel nature of sample preparation extends throughout the procedure, permitting several samples to be handled simultaneously, which reduces technical input, shortens sample preparation time, and permits "same-day" analysis. Any losses that were incurred during sample processing were accounted for by the internal standard, for which recoveries were (mean ± SEM, $n = 9$) 0.96 ± 0.06 and 0.77 ± 0.04 for leaves and tubers, respectively.

Traditional extraction and hydrolysis of glycoalkaloids in the absence of ammonia precipitation is usually a sequential process. Plant samples are extracted using a blender or homogenizer, filtered, and then concentrated before acid hydrolysis (Gregory et al., 1981; King, 1980). This three-step procedure requires constant technical input. In addition, samples may have to be processed serially in one or more of these steps, which increases sample preparation time.

In our procedure, tissues were concurrently extracted and hydrolyzed for 4 h at 70 °C, after which time total alkaloid recoveries were maximized (Figure 3). After 1 and 3 h, total alkaloid recoveries were only 75 and 90%, respectively, of those achieved at 4 h. Incubation for 5 h did not increase recoveries.

Accurate quantitation of aglycons can be affected by the method of hydrolysis. King (1980) and van Gelder (1984) have found that conventional aqueous acid hydrolysis of glycoalkaloids results in degradation of some aglycons, including solanidine and tomatidine. To avoid this degradation, King (1980) hydrolyzed potato glycoalkaloids in ethanolic HCl and consistently isolated unmodified aglycons. Van Gelder (1984, 1985) used a two-phase system to isolate unmodified aglycons, in which aglycons were trapped in carbon tetrachloride following hydrolysis in aqueous acid. We used methanolic HCl (Gregory et al., 1981; Sinden et al., 1986b) for simultaneous extraction and hydrolysis of glycoalkaloids. GC and TLC of glycoalkaloid standards hydrolyzed under our conditions revealed no evidence of aglycon degradation since no additional peaks (GC) or additional spots (TLC) were detected as compared to GC and TLC of aglycon standards.

Since our procedure does not depend on ammonia precipitation during preparation of crude extracts, it is suitable for the analysis of both wild and cultivated *Solanum* species. Ammonia precipitation has been used reliably for precipitating solanine and chaconine from crude extracts of many *S. tuberosum* cultivars (Gregory et al., 1981; Jellema et al., 1981; Morris and Lee, 1981;

Table I. Relative Response Factors (*F*), Retention Times (t_R), Resolution (*R*), and Separation Number (SN) of *Solanum* Glycoalkaloid Aglycons[a]

aglycon	*F*	t_R	*R*	SN
solanidine	1.52	10.339		
			15.2	12
leptinidine	1.20	14.473		
			2.9	1
tomatidine	1.00	15.444		
			1.4	0
acetylleptinidine	1.30	15.960		

[a] See Materials and Methods for chromatographic details.

Sinden et al., 1986b) but results in substantial and variable losses (more than 60%) in recovery when used in preparing extracts of some wild *Solanum* species (Gregory et al., 1981; Sinden et al., 1986b). Sinden et al. (1986b) reported for one *S. chacoense* clone that only 34% of the leptines, 37% of the leptinines, and 36% of solanine and chaconine were ammonia-precipitated. However, for another clone of *S. chacoense*, 78% of the leptines, 62% of the leptinines, and 91% of solanine and chaconine were recovered in the ammonia precipitate. Thus, ammonia precipitation not only fails to quantitatively precipitate glycoalkaloids unique to some wild species but, in these species, fails to quantitatively precipitate glycoalkaloids that are normally ammonia insoluble (e.g., solanine and chaconine).

For the data reported here, and for routine analysis, we extracted and hydrolyzed glycoalkaloids from 10–20 or 60–80 mg of dry weight of leaf or tuber tissue, respectively, in only 3 mL of solvent. However, we have also quantitated aglycons from as little as 0.3 mg of dry weight of leaf tissue (Lawson, unpublished results). These sample and solvent requirements are substantially lower than those reported previously. For example, glycoalkaloids have been extracted from 5 g of fresh weight or more of leaf tissue in approximately 20 mL of solvent (Sinden et al., 1986b) and 10–300 g of fresh weight of tuber tissue in 75–100 mL or more of solvent (Coxon et al., 1979; Herb et al., 1975; King, 1980; van Gelder, 1984). It may be thought that larger sample sizes are needed for representative sampling. We tested whether our sampling method was representative by measuring the alkaloid contents of three subsamples of a single plant sample and found that standard errors were approximately 1–2% of the mean. For instance, from a single leaf sample, alkaloid levels were (mean ± SEM, $n = 3$) 1762 ± 21 μg of solanidine/g of dry weight, 1986 ± 34 μg of leptinidine/g of dry weight, and 5413 ± 18 μg of acetylleptinidine/g of dry weight.

Chromatographic Performance. Quantitation by GC of glycoalkaloid aglycons has proved to be an accurate means of determining glycoalkaloid content (King, 1980; van Gelder, 1985). Response factors, retention times, resolution, and separation number were calculated for each alkaloid or alkaloid pair (Table I). Since tomatine was the internal standard, its aglycon, tomatidine, was assigned a response factor of $F = 1$. Chromatographic conditions were optimized on the basis of the resolution of tomatidine and acetylleptinidine, since these two alkaloids were the most difficult to separate. These conditions provided near-baseline resolution of tomatidine and acetylleptinidine ($R = 1.4$; Table I) with an analysis time of less than 20 min (Figure 4); an R value of 1.5 constitutes baseline separation (Poole and Schuette, 1984). Thus, these conditions provided a good compromise between resolution and analysis time. Higher initial temperatures (i.e., 220 and 230 °C) shortened analysis time but reduced resolution. A lower initial temperature (i.e., 200 °C) provided baseline separation of tomatidine and acetylleptinidine ($R = 1.5$) but increased analysis time by more than 6 min.

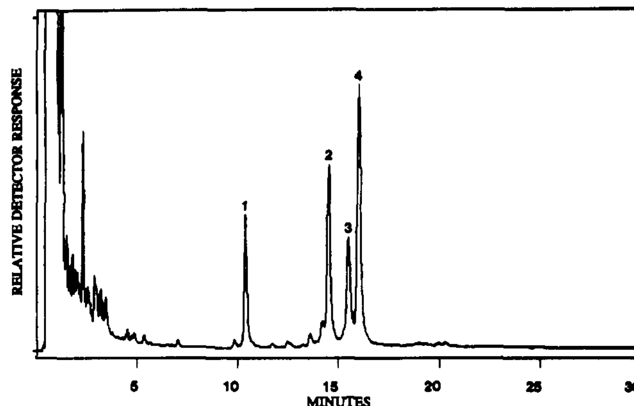

Figure 4. Chromatogram of *S. chacoense*, PI 458310-1, leaf extract. See Materials and Methods for chromatographic details. Peaks: 1, solanidine; 2, leptinidine; 3, tomatidine (internal standard); 4, acetylleptinidine.

Van Gelder (1985) reported that column temperatures below 240 °C caused significant fronting (up to 52% peak asymmetry) in leading peaks and peak broadening, which he attributed to condensation of the sample in the column. We observed none of these effects at an initial column temperature of 210 °C. On the other hand, van Gelder (1985) provided evidence of aglycon decomposition at high temperatures (≥280 °C). We avoided aglycon thermal decomposition by operating injector and column temperatures at or below 270 °C. GC of aglycon standards under our optimized conditions revealed no evidence of degradation since no additional peaks were detected.

We also tested our method for its effectiveness in resolving other steroidal alkaloids. Solasodine, an important starting material for the synthesis of pharmaceutical steroids (Roddick, 1986), was resolved from leptinidine ($R = 1.0$) and tomatidine ($R = 2.0$). Demissidine, which has been implicated in plant resistance to insect feeding (Tingey, 1984), was not resolved from solanidine using our operating conditions. When chromatographed individually, demissidine had a retention time 0.1 min longer than solanidine. Therefore, if the analyst required resolution of these two alkaloids, a 30-m column could be used to obtain $R = 0.6$. Solasodine differs structurally from tomatidine in that solasodine is both Δ^5 and 22*R*,25*R* rather than 22*S*,25*S* configuration, whereas demissidine differs structurally from solanidine only in that demissidine is not Δ^5 (Figure 1), which may account for the ability of our system to resolve solasodine from tomatidine but not demissidine from solanidine. Van Gelder (1985) obtained resolution of solanidine and demissidine ($R = 1.9$) using a 50 m × 0.22 mm i.d. × 0.12 μm CP-Sil 5 capillary column. King (1980) used 3-β-trifluoroacetate derivatives to distinguish between these two alkaloids.

For total glycoalkaloid measurements, colorimetric assays (Bushway et al., 1980; Coxon et al., 1979; Fitzpatrick and Osman, 1974; Sanford and Sinden, 1972) have proved to be very useful. However, modern liquid or gas chromatography is necessary for quantitation of individual (glyco)alkaloids. Each technique has its limitations. For glycoalkaloid analysis, GC is unattractive principally because of the high operating temperatures (>300 °C) necessary to achieve elution, even if samples are derivatized (Herb et al., 1975). TLC has been limited primarily to qualitative or semiquantitative analysis (Coxon and Jones, 1981; Deahl and Sinden, 1987; Hunter et al., 1976; Shih and Kuć, 1974), although a few exceptions exist (Jellema et al., 1980, 1981). HPLC provides resolution of glycoalkaloids, but due to the absence of a good UV-absorbing

Table II. Levels of Principal SGA Aglycons from *S. tuberosum* Cv. Atlantic and *S. chacoense*, PI 458310-1, Leaves and Tubers Grown in the Greenhouse (GH), Field (F), or Tissue Culture (TC)

	μg/g of dry wt[a]		
source	solanidine	leptinidine	acetylleptinidine
S. tuberosum			
tubers (F)	590 ± 38	ND[b]	ND
leaves (GH)	6288 ± 139	ND	ND
S. chacoense			
tubers (GH)	2877 ± 43	38 ± 0	ND
leaves (GH)	1731 ± 13	5990 ± 114	8909 ± 153
leaves (TC)	6807 ± 83	1419 ± 25	2102 ± 36

[a] Values are the mean ± SEM of three replications. [b] Not detected.

chromophore, detection limits are 0.1 μg at best (Morris and Lee, 1981). The detection limit of underivatized alkaloids was approximately 3 ng using our megabore capillary GC method.

Analysis of *S. tuberosum* and *S. chacoense* Alkaloids. The alkaloid contents of *S. tuberosum* and *S. chacoense* leaves and tubers were quantitated using our GC procedure (Figure 4; Table II). The purity of all alkaloid peaks was verified by GC–MS (Lawson et al., unpublished results). Leaves contained higher levels of alkaloids than tubers in both *S. chacoense* and *S. tuberosum*, which is consistent with previous studies [reviewed in Gregory (1984) and Maga (1980)]. Greenhouse-grown *S. chacoense* leaves contained higher (2.5×) total alkaloid levels than *S. tuberosum* leaves, and *S. chacoense* contained leptinidine and acetylleptinidine, which were not detected in *S. tuberosum*. Acetylleptinidine was the predominant alkaloid of *S. chacoense* leaves (54%) followed by leptinidine (36%) and solanidine (10%), which is similar to the proportions calculated from data reported by Sinden et al. (1986b). However, the trend was different in *S. chacoense* plantlets grown in vitro, the leaves of which contained predominantly solanidine (66%), followed by acetylleptinidine (20%) and leptinidine (14%). These data suggest that *S. chacoense* alkaloid levels are quantitatively influenced by the environment (Sinden et al., 1984).

Acetylleptinidine was not detected in *S. chacoense* tubers, suggesting that the biosynthesis of this alkaloid is under developmental regulation, since it was detected in *S. chacoense* leaves. The absence of acetylleptinidine in PI 458310-1 tubers is consistent with a previous paper (Sinden et al., 1986b).

We have reported the use of tomatine as an internal standard, which was added prior to extraction, for the quantitation of *Solanum* alkaloids. Underivatized alkaloids were quantitated using megabore capillary GC. We have demonstrated the applicability of this procedure for the analysis of *S. chacoense* and an *S. tuberosum* cultivar but believe that (1) the concurrent extraction and hydrolysis, (2) the parallel nature of sample preparation, and (3) the analytical capability of capillary GC have application for (glyco)alkaloid analyses of other wild and cultivated *Solanum* species. We are currently using this procedure to investigate the developmental regulation and the biochemistry of *Solanum* alkaloid biosynthesis in *S. chacoense*.

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