Comparison of Glycoalkaloid Content of Fresh and Freeze-Dried Potato Leaves Determined by HPLC and Colorimetry

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As part of a program to control the biosynthesis of *Solanum* glycoalkaloids in potatoes, we used an HPLC assay to measure the specific α-chaconine and α-solanine content of greenhouse-grown potato leaves at different stages of maturity. Comparative studies were done with the bromophenol blue titration assay for total glycoalkaloids. We found that foliar glycoalkaloids can be extracted with 5% aqueous acetic acid, that analysis of glycoalkaloids with freeze-dried leaf powders was more reproducible than with fresh leaves, and that the bromophenol colorimetric method gave higher values than HPLC. As little as one leaf from a growing plantlet can be analyzed with the HPLC assay. Large variations in both α-chaconine and α-solanine contents of fresh leaves suggest that potato foliar glycoalkaloids should be determined by HPLC from freeze-dried rather than fresh leaves.

Keywords: *Glycoalkaloids; α-chaconine; α-solanine; potato leaves; biosynthesis; high-performance liquid chromatography; colorimetry; transgenic plants; pathogen resistance*

INTRODUCTION

Glycoalkaloids are nitrogen-containing secondary plant metabolites found in the Solanaceae (Friedman and McDonald, 1996; Maga, 1994). As part of an effort to devise methods for keeping levels of glycoalkaloids in potatoes as low as possible while maintaining acceptable resistance to plant pathogens and insects, we previously described improved HPLC methods to measure glycoalkaloids in different parts of the potato plant, including tubers, leaves, and sprouts, and in commercial potato products such as chips, fries, and skins (Dao and Friedman, 1994; Friedman and Dao, 1992). In the course of these studies, a need developed to determine the glycoalkaloid content of potato leaves harvested at different stages of maturity in order to assess the effectiveness of gene manipulation in transgenic plants. Since this involved working with very young greenhouse-grown plantlets with only a few small leaves, a method was needed that would be sensitive and reliable using a very small amount of material. To accomplish our objective, the HPLC method was used to determine the α-chaconine and α-solanine content of both fresh and freeze-dried leaves. Experiments on the recovery of α-chaconine and α-solanine added to dried leaves and a comparative estimation of glycoalkaloid content with the bromophenol blue colorimetric method for total glycoalkaloids were also done.

Ideally, the most desirable potato plant would have high glycoalkaloid content in leaves to provide protection against insects and pathogens and low levels in tubers to reduce the potential health hazard to humans.

MATERIALS AND METHODS

Materials. Solvents were of HPLC spectroquality grade unless otherwise stated. α-Chaconine and α-solanine were purchased from Sigma Chemical Co., St. Louis, MO.

Potato Plants. Leaves of Lemhi Russet and No. 42015 varieties were obtained from plants grown in a greenhouse by D. Rockhold in connection with other experiments. D. L. Corsini and J. J. Pavek of the USDA-ARS Potato Breeding Program, Aberdeen, ID, provided the NDA 1725 potato plants that were planted in our greenhouse from sprouted tubers (greenhouse conditions: daytime temperature, 85−90 °F (29−32 °C); nighttime temperature, 50−55 °F (10−13 °C); relative humidity, 50−60%; light, natural). The leaves were harvested after each desired growing period (*e.g.*, after 3 weeks, plant A; after four weeks, plant B; etc.). Growing time was counted from the time when the plant first emerged from the soil surface. For consistency in sampling, the lowest 5−6 branches of each plant were harvested. About 35−50 leaflets, depending on the plant's age, were collected. The harvested leaves were analyzed for glycoalkaloids immediately (fresh samples). The rest were flash-frozen with liquid nitrogen and lyophilized at −40 °C and a vacuum of 100 μm in a Virtis lyophilizer (Model No. 10-145 MRBA, Gardiner, NY). The freeze-dried samples were then ground in a Wiley mill to pass a 35-mesh screen and stored in capped glass jars at 4 °C in a refrigerator.

Extraction Method. *Fresh Leaves.* A single large leaf or two small leaves (ca. 200−300 mg) were selected randomly and homogenized with 20 mL of 5% aqueous acetic acid using a Sorvall mixer set at high speed for 2 min. The slurry was transferred to a 50 mL Erlenmeyer flask and continuously stirred with a magnetic stirrer for 3 h. The sample was then vacuum filtered through Whatman No. 4 filter paper, and the residue was reextracted for an additional 3 h with another 15 mL of 5% acetic acid and filtered. The two filtrates were combined and transferred to a 125 mL separatory funnel. The pH was adjusted to 10−11 with ammonium hydroxide, and the alkaline extract was partitioned twice with 20 mL of water-saturated butanol. The combined butanol extracts were evaporated to dryness with the aid of an air vacuum rotovapor, and the residue was redissolved in either 1 mL of methanol−acetonitrile−water (10:55:35 (v/v/v)) for direct analysis by HPLC or in 5 mL of absolute methanol for colorimetric analysis.

Freeze-Dried Leaves. Freeze-dried leaf powder (10−20 mg) was stirred with 20 mL of 5% acetic acid by a magnetic stirrer for 3 h and vacuum filtered through Whatman No. 4 filter paper. The residue was reextracted for an additional 3 h with 15 mL of 5% acetic acid and filtered. The combined filtrates were treated as above.

HPLC Procedure. A Beckman 334 HPLC system with a 427 integrator and a 165 UV−vis variable wavelength detector was used. The column was a 3.9 × 300 mm Resolve 5μ Spherical C_{18} from Waters division, Millipore Corp., Milford, MA (Friedman and Levin, 1992). The mobile phase consisted

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S0021-8561(95)00282-2

Table 1. Glycoalkaloid Content (mg/100 g Fresh Weight) of Fresh and Freeze-Dried Potato Leaves (No. 42015) As Determined by HPLC

		α-chaconine		α-solanine		total	
treatment	N^a	mean	SE	mean	SE	mean	SE
dried	13	214.5	2.1	105.5	1.3	319.9	3.3
fresh	13	210.8	7.7	102.7	4.8	313.5	12.0
F		13.61		13.24		13.42	
prob > F		<0.0001		<0.0001		<0.0001	
t		0.46		0.57		0.52	
prob > $\|t\|$		0.65		0.58		0.61	

[a] Number of separate determinations.

Table 2. Recovery of α-Chaconine and α-Solanine Added to Freeze-Dried Lemhi Russet Potato Leaves As Determined by HPLC

	amount of spiking (μg)	% recovery[a]	
		α-chaconine	α-solanine
	50	94.0 ± 6.0	88.9 ± 4.8
	100	95.7 ± 5.2	99.6 ± 3.1
	150	92.5 ± 3.9	98.1 ± 2.4
av		94.1 ± 1.6	95.5 ± 5.7

[a] The original glycoalkaloid content of the leaves (in mg/100 g fresh weight) was as follows: α-chaconine, 26.54 ± 1.16; α-solanine, 46.82 ± 1.85. All values are the means of three separate determinations ± standard deviation.

Table 3. Glycoalkaloid Content (mg/100 g Fresh Weight) of Fresh NDA 1725 Potato Leaves at Different Stages of Maturity As Determined by HPLC

age		α-chaconine		α-solanine		total	
(weeks)	N^a	mean	95% CI[b]	mean	95% CI	mean	95% CI
3	6	20.2	14.9, 27.3	9.6	5.0, 15.6	29.8	23.0, 38.5
4	6	33.4	24.7, 45.2	16.9	10.6, 24.7	50.3	38.9, 65.1
6	6	31.0	22.9, 41.9	11.8	6.6, 18.4	42.3	32.7, 54.7
7	6	38.1	28.2, 51.6	19.9	13.0, 28.4	62.4	48.3, 80.7
9	4	111.4	77.0, 161.3	50.1	36.3, 66.1	161.2	117.7, 220.8

[a] Number of separate determinations. [b] 95% confidence intervals.

of 35% acetonitrile containing 100 mM of monobasic ammonium phosphate. The pH was adjusted to 3.5 with phosphoric acid. The solvent flow rate was 1 mL/min, and the UV absorbance detector was set at 200 nm. The size of the injection samples was 20 μL.

Colorimetric Procedure. For comparison, the total glycoalkaloids content of the extracts was determined by the bromophenol blue colorimetric procedure of Fitzpatrick and Osman (1974) as previously described (Dao and Friedman, 1994). The methanol solutions were titrated with 0.067/10% bromophenol/phenol in absolute methanol and converted to a solanine equivalent from an α-solanine standard curve.

Recovery Study. To help assess the validity of our methods, a series of preliminary spiking experiments were carried out to establish the extent of recovery of added glycoalkaloids from potato foliage. Specifically, various amounts of α-chaconine and α-solanine were added to 20 mg of freeze-dried Lemhi potato leaf powders available to us from an earlier study. The samples were thoroughly mixed, extracted as above, and analyzed by HPLC.

Statistics. The data in Tables 1 and 2 were analyzed using SAS PROC GLM and PROC TTEST (SAS, 1987). For Table 3, α-chaconine and total glycoalkaloid data were transformed by logs and solanine data by square roots in order to stabilize the variance among weeks. The transformed data were then subjected to analyses of variance between and within weeks. Means and 95% confidence intervals (95% CI) were constructed in the transformed scale and then retransformed back to the original scale for presentation. Transformations were not necessary for the data in Table 4. Analyses of variance were run on the original scales of measurement. For Table 5, the log transformation was used for the data on fresh leaves, resulting in the indicated retransformed means and 95% confidence intervals. Transformation was not necessary for the freeze-dried leaf data. The results of the statistical analyses are described below for each set of data.

Table 4. Glycoalkaloid Content (mg/100 g Fresh Weight) of Freeze-Dried NDA 1725 Potato Leaves at Different Stages of Maturity As Determined by HPLC

age		α-chaconine		α-solanine		total	
(weeks)	N^a	mean	95% CI[b]	mean	95% CI	mean	95% CI
3	3	21.5	18.1, 24.9	10.9	9.1, 12.7	32.4	27.4, 37.4
4	3	32.8	29.5, 36.2	12.4	10.6, 14.2	45.2	40.3, 50.2
6	3	27.4	24.0, 30.8	12.5	10.8, 14.3	40.0	35.0, 45.0
7	3	50.5	47.1, 53.9	22.0	20.2, 23.7	72.5	67.5, 77.4
9	3	93.3	89.9, 96.7	33.7	31.9, 35.5	127.0	122.0, 131.9

[a] Number of separate determinations. [b] 95% confidence intervals.

Table 5. Total Glycoalkaloid Content (mg/100 g Fresh Weight) of Fresh and Freeze-Dried NDA 1725 Potato Leaves As Determined by the Bromophenol Blue Method

	total glycoalkaloids					
	fresh leaves			freeze-dried leaves		
age (weeks)	N^a	mean	95% CI[b]	N	mean	95% CI
3	6	64.1	50.4, 81.5	*c*	*c*	*c*
4	*c*	*c*	*c*	3	63.7	49.4, 78.3
6	5	123.1	94.6, 160.1	*c*	*c*	*c*
7	6	130.6	102.7, 166.0	3	104.1	89.5, 118.7
9	2	181.7	119.9, 275.3	3	195.9	181.3, 210.5

[a] Number of separate determinations. [b] 95% confidence intervals. [c] The titration end point was difficult to detect. See text.

RESULTS AND DISCUSSION

Previous Studies. Methodologies for the analysis of potato glycoalkaloids and related compounds include gas chromatography (Herb et al., 1975; Lawson et al., 1992; van Gelder, 1985; van Gelder et al., 1989), HPLC (Bushway, 1982; Bushway et al., 1979, 1986; Carman et al., 1986; Crabbe and Fryer, 1980; Friedman and Levin, 1992; Jonker et al., 1992; Kobayashi et al., 1989; Kozukue et al., 1987; Morris and Lee, 1981; Saito et al., 1990), and immunoassays (Hellenäs, 1986; Plhak and Sporns, 1992; Stanker et al., 1994, 1996; Ward et al., 1988). No single method has gained widespread acceptance. Since the pioneering studies by Bushway and colleagues (Bushway, 1982; Bushway et al., 1979, 1986), Crabbe and Fryer (1980), and Morris and Lee (1981), HPLC has been continuously improved with respect to sample preparation and cleanup, column selection, and peak detection. Its use is increasing because it can distinguish individual glycoalkaloids and aglycons without derivatization. Most HPLC methods, however, require a relatively large amount of sample (1−50 g) and involve purification of the glycoalkaloids either by precipitation with strong base (Bushway et al., 1979; Friedman and Dao, 1992) or by passing through a Sep-Pak C_{18} cartridge (Carman et al., 1986; Kobayashi et al., 1989; Morris and Petermann, 1985; Saito et al., 1990). Small losses due to partial solubility even in strong base or to adsorption on the cartridge, though they may be insignificant in larger scale analyses, may make determination unreliable on very small samples, such as on very young potato plants.

The following selected examples based on our own studies illustrate continuing improvement in the analysis of glycoalkaloids: (a) a theoretical and practical foundation was developed to facilitate and optimize HPLC of closely related glycoalkaloids and aglycones based on acidic properties of column packing (Friedman and Levin, 1992, 1995); (b) the practical value of this

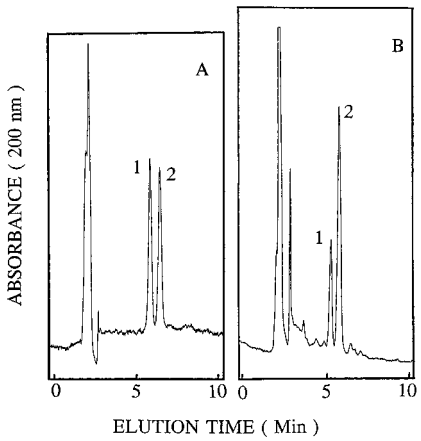

Figure 1. HPLC chromatograms (Resolve C_{18} column; flow rate, 1 mL/min of 100 mM ammonium phosphate; monobasic, in 35% acetonitrile adjusted to pH 3.5 with phosphoric acid). (A) Standard containing 50 μg/mL of α-solanine and α-chaconine. Peaks: 1, α-solanine; 2, α-chaconine. (B) Potato foliar extract. Peaks: 1, α-solanine; 2, α-chaconine.

approach was confirmed by the separation of eight hydrolysis products of α-chaconine and α-solanine (Friedman and McDonald, 1995a; Friedman et al., 1993); (c) a new HPLC method based on pulsed amperometric detection appears useful for glycoalkaloids such as α-tomatine and solasonine lacking chromophoric sites (Friedman et al., 1994); and (d) new monoclonal antibodies for enzyme-linked immunosorbent assays (ELISA) have the ability to differentiate between glycoalkaloids and aglycones (Stanker et al., 1994).

Previously, large numbers of fresh potato leaves and tubers were analyzed for glycoalkaloid content by various methods to relate genetic variations in composition (Deahl et al., 1993; Lampitt et al., 1943; Morris and Petermann, 1985; Sanford et al., 1994; Tingey et al., 1978; van Gelder et al., 1989). The reported studies and the results described below suggest the need to further optimize and standardize analytical methods for potato glycoalkaloids (Friedman and McDonald, 1995b, 1996).

Results. Figure 1 illustrates the separation of α-chaconine and α-solanine by HPLC both in pure form and in a potato foliage extract. Table 1 presents the preliminary analysis of glycoalkaloids on fresh and freeze-dried leaf samples using our extraction procedure. The samples were obtained from a 50-day-old potato plant. For Table 1, variance ratio, *F* tests were used to show that the variances among replicate samples are significantly higher for fresh than for dried samples, while the means are not significantly different via unequal variance tests. There was a much wider range of glycoalkaloid content in the fresh leaves than in the freeze-dried leaves even though the samples were harvested from the same plant at the same time. The range of α-chaconine for fresh leaves was 185.0−258.4 mg/100 g fresh weight but was only 199.6−229.5 mg/100 g for the dried leaves; α-solanine ranged from 70.6 to 126.6 mg/100 g for fresh leaves but was only 98.6 to 118.3 mg/100 g for the dried leaves. Similarly, results in Tables 3−5 show in most cases significantly larger statistical variations for the fresh leaves than for the dried leaves.

It should be noted that the observed values of glycoalkaloids in a single potato plant at different stages of maturity may not reflect the true trend in glycoalkaloid biosynthesis during growth because of biological variability among plants of the same cultivar. However, such variability is not expected to affect the comparisons of methods of analysis, the main objective of this study. It should also be emphasized that the purpose of this plant growth study was not to assess changes in glycoalkaloid formation during the growth of potato plants of any particular cultivar, although the tables do seem to indicate certain trends, but rather to provide a source of small and large leaves for comparative analysis by the two different methods.

Because of the smaller variation in freeze-dried samples, we conducted recovery experiments on freeze-dried samples only. Table 2 shows the percentage of recovery of α-chaconine and α-solanine added to freeze-dried potato foliage powder before extraction. These spiking experiments revealed that our modified extraction method recovered 89−99% of added α-chaconine and α-solanine. However, recovery experiments of this sort are only good for ensuring that there is little or no loss of glycoalkaloids in the cleanup and analysis after extraction. Because glycoalkaloids bound in a plant matrix may react differently or may be more difficult to solubilize than those added to the surface of plant material, these types of recovery experiments may not always be accurate in measuring extraction efficiency.

Bushway et al. (1985) determined that freeze-dried potatoes were more difficult to extract than fresh samples and developed a tetrahydrofuran−water−acetonitrile−acetic acid (500:300:200:10) solvent that would increase extraction efficiency. Comparing our acetic acid extraction method with that of Bushway and colleagues, we found that the results were comparable and that the acetic acid extract gave a better base line for our HPLC chromatograms.

Since the exploratory studies suggested that our methods could be applied to measure foliar glycoalkaloids, we carried out a series of detailed studies with greenhouse-grown NDA 1725 potatoes—a high-glycoalkaloid variety in which we hope to reduce glycoalkaloid production through genetic suppression of enzymes involved in glycoalkaloid biosynthesis (Stapleton et al., 1991, 1992, 1994; Moehs et al., 1996a,b).

Table 3 lists the levels of α-chaconine and α-solanine in fresh leaves at various ages of NDA 1725 potato plants. Generally, both α-chaconine and α-solanine increased with growing time. α-Chaconine increased from 20.2 (3 weeks) to 111.4 mg/100 g fresh weight (9 weeks) and α-solanine increased from 9.6 to 50.1 mg/100 g fresh weight over the same period. Table 3 also shows that the 6-week samples contained less α-chaconine and α-solanine than the 4-week samples. Although the reason for this difference is not known, the foliage of the 6-week plant appeared slightly yellowish instead of the normal dark green color of the other samples.

Additional studies on the glycoalkaloid levels of small and large leaves (results not shown) revealed that the size of the leaf was unrelated to glycoalkaloid content.

Table 4 illustrates the relationship of growing time and glycoalkaloids in freeze-dried NDA 1725 potato leaf samples. The average total glycoalkaloids (α-chaconine and α-solanine) increased from 32.4 for 3-week-old leaves to 127 mg/100 g fresh weight for 9-week-old samples. The 6-week samples also showed slightly lower glycoalkaloid content compared to the 4-week samples. Possible reasons for the decrease are not immediately apparent. The glycoalkaloids of the NDA 1725 potato foliage increased about 4−5-fold as the plant aged from 3 to 9 weeks. The ratio of α-chaconine to α-solanine ranged from 1.9 to 2.7. In general, both α-chaconine and α-solanine in potato leaves increased

with growing time. Similar results in Arran Signet potato leaves were reported by Lampitt et al. (1943).

For comparison, the bromophenol colorimetric method was also used to determine the total glycoalkaloids of NDA 1725 potato leaves. Table 5 lists the total glycoalkaloid values of fresh and freeze-dried NDA 1725 potato leaves. The values determined by this method are $1^1/_2-2$ times higher than those measured by HPLC (Table 4). The standard deviations were very high, especially for the fresh leaves. During titration the color changes from blue to blue-green, to yellow-green, and finally to the yellow-orange end point, the original color of the reagent. The green color of chlorophyll in the extract solution interfered with an accurate determination of the titration end point, causing a wide range of values and making it difficult to report accurate results for some of the samples (Table 5). We do not know the reason for the significantly higher values for glycoalkaloids, but it is possible that minor constituent glycoalkaloids or other nitrogen-containing compounds may also react with bromophenol blue, causing erroneously high results. This suggests that the bromophenol blue method may not always be a reliable technique for measuring glycoalkaloids of potato leaves, or foliage in general, with any extraction procedure that does not (a) result in nearly colorless solutions and (b) eliminate interfering compounds. Similar results were previously reported for potato tubers and commercial potato products (Friedman and Dao, 1992) and green potato peels (Dao and Friedman, 1994). The green color and other compounds, if present, do not seem to interfere with HPLC methods.

Conclusions. The described extraction−HPLC procedure can accurately measure α-chaconine and α-solanine of potato foliage. It requires only a very small amount of material (*e.g.*, a single leaf). The results show good recovery of both α-chaconine and α-solanine. Large variations in both α-chaconine and α-solanine contents of fresh leaves suggest that potato foliar glycoalkaloids should be determined by HPLC from freeze-dried rather than fresh leaves. Additional studies on leaves from different potato varieties and field-grown potatoes are needed to confirm the generality of this conclusion.

Freeze-drying offers numerous advantages: (a) it stops enzyme-catalyzed, wound-induced, and moisture-dependent compositional changes which may affect glycoalkaloid content; (b) it permits storage and transportation of samples for analysis at different time periods and by different investigators; (c) it allows analysis of other potato constituents including proteins, polyphenols, and protease inhibitors (Dao and Friedman, 1994; Friedman, 1992, 1996); and (d) it makes it possible to relate composition to nutrition and safety, *i.e.*, the same samples can be both used for analysis of composition and incorporated into diets for animal feeding studies (Friedman et al., 1996).

In summary, our objective was to demonstrate the validity of the analytical method using dry powders derived from single plants. This method would also be useful for a variety of applications, *e.g.*, to demonstrate trends in glycoalkaloid biosynthesis and degradation at different stages of plant growth. In all such studies, however, it would be important to use appropriate sampling techniques to take into account possible biological variability.

ACKNOWLEDGMENT

We thank Gary M. McDonald for excellent technical assistance, David Rockhold for greenhouse-grown potato plants, B. E. Mackey for statistical analysis, and S. C. Morris and the reviewers for helpful comments.

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Received for review May 10, 1995. Revised manuscript received February 22, 1996. Accepted May 8, 1996.[⊗]

JF9502820

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1996.