Determination of Potato Glycoalkaloids Using a Liposome Immunomigration, Liquid-Phase Competition Immunoassay

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Polyclonal anti-solanine antibodies were raised and used in the assembly of a liposome immunomigration, liquid-phase competition strip immunoassay. In this format, a similar cross-reactivity was observed between the glycoalkaloids α-solanine and α-chaconine. The strip assay was implemented to quantitate total glycoalkaloids (TGA) from potatoes. Recoveries in spiked potato samples using the strip assay and water:acetic acid:sodium bisulfite extracting solvent were in the range of 84−111%. The values of the TGA quantitations by the strip assay as compared with those obtained by HPLC, in nonspiked tubers coming from cloned potato samples donated by potato breeders, were equivalent and highly correlated ($r^2 = 0.91$). The strip assay proved advantageous over HPLC for extra-laboratory measurements such as the rapid identification of samples that should be rejected due to an elevated TGA content.

Keywords: *Glycoalkaloids; potato glycoalkaloids; liposomes; immunoassay; liposome immunoassay; α-solanine; α-chaconine*

INTRODUCTION

Liposome immunomigration, liquid-phase competition (LIM-LPC) is one of the most promising immunoassay formats recently reported (Durst et al., 1993). Compared with the enzyme-linked immunosorbent assay (ELISA), the LIM-LPC assay requires a shorter incubation time, and washing steps and substrate turnover reactions are not needed. Also, this methodology has the potential for extra-laboratory measurements where highly trained personnel and sophisticated equipment may not be available.

The LIM-LPC immunoassay is based on the competition between sample glycoalkaloid molecules and solanine-/biotin-tagged, dye-loaded liposomes for the antibody binding sites. In this format, anti-biotin antibodies are immobilized in a discrete zone on nitrocellulose strips that are then placed into test tubes with all the reagents in solution (anti-solanine antibodies, liposomes, and sample). After a short incubation time, the presence of analyte inhibits the antibody binding of liposomes, and the unbound liposomes migrate by capillary action up the nitrocellulose strip. They are captured at the anti-biotin zone, while the antibody-bound liposome aggregate on the strip at the meniscus of the reaction mixture (Figure 1). The strip is allowed to air-dry, and the color intensity in the capture zone is measured by densitometry using a desktop scanner and associated conversion software.

Immunoassays are promising methodologies for monitoring toxic glycoalkaloids (Morgan et al., 1985; Morris and Lee, 1984). In this regard, α-solanine and α-chaconine account for about 95% of the total glycoalkaloid (TGA) content in commercial potatoes (Paseshnichenko and Guseva, 1956; Morgan and Lee, 1990). They are quite stable under ordinary cooking conditions. Research indicates that the TGA content of potatoes is reduced 50% by peeling (Sizer et al., 1980), but if the

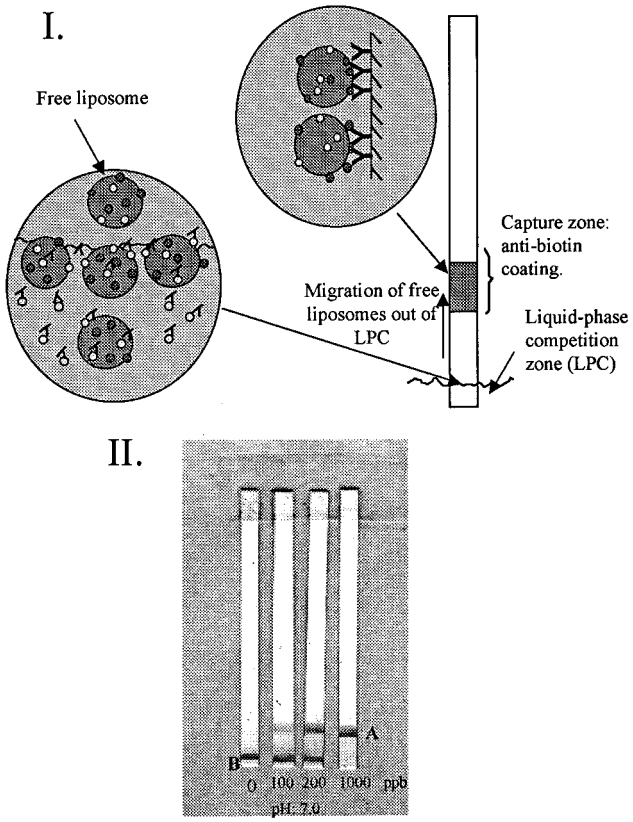

Figure 1. Mechanism of the LIM-LPC format (I) and quantitation of results (II). Y represents anti-biotin antibody; ***y*** represents anti-solanine antibody; ○ represents solanine tag on liposomes; ● represents biotin tag on liposomes; A represents capture zone (anti-biotin IgG zone); and B represents aggregation zone (red band that appears at the meniscus of reacting solution).

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content of TGA is high, it is also found in the deeper tuber flesh (Valkonen et al., 1996). However, other research indicates that, properly processed and monitored, potato peels are potentially useable as a source of dietary fiber for animals and humans (Arora and Camire, 1994).

There are two main deleterious effects associated with TGA. One mechanism of toxicity reported is membrane disruption, resulting in disturbances of the intestinal tract (Morris and Lee, 1984). Another mechanism is anticholinesterase activity (Slanina, 1990). These effects are associated with abnormally high levels of glycoalkaloids in potatoes (>20 mg/100 g). This condition occurs when unfavorable environmental conditions retard maturity (Sinden and Webb, 1972; Gosselin et al., 1988) or cause stress on the plant (Hlywka et al., 1994). It can also occur as a result of pest damage, tuber injury (Fitzpatrick et al., 1978; Mondy and Chandra, 1979; Wu and Salunkhe, 1976), or light exposure (Percival et al., 1993).

While ELISA immunoassays are generally used for monitoring TGA by plant breeders and harvesters, they are not suitable for the potato industry (Friedman and McDonald, 1997) where there is a need for an extra-laboratory immunoassay. Preliminary studies successfully evaluated the LIM-LPC strip assay for the quantitation of potato glycoalkaloids in pure buffered solutions (Glorio and Durst, 1996). This paper reports the performance of this methodology in its intended food matrix together with a comparison of the quantitation results between LIM-LPC and an HPLC technique (Hellenäs et al., 1995; Hellenäs and Branzell, 1997) in the determination of TGA in potato tubers.

MATERIALS AND METHODS

Reagents. The following reagents were obtained: dipalmitoylphosphatidylethanolamine (DPPE), sodium borohydride, sodium cyanoborohydride, poly(vinylpyrrolidone) (molecular wt 10 000) (PVP), cholesterol, α-solanine, α-chaconine, rabbit γ-globulin, and gelatin as reagent grade (Sigma Chemical Co., St. Louis, MO); DPPE-biotin (Molecular Probes, Eugene, OR); anti-biotin IgG, (Whatman, Hillsboro, OR); sulforhodamine B (Eastman Chemical Co., Rochester, NY); nitrocellulose membrane sheets (Sartorius, Goettingen, Germany); dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) (Avanti Polar Lipids, Alabaster, AL); keyhole limpet hemocyanin (KLH) (Boehringer Mannheim Biochemica, Indianapolis, IN); and HPLC-grade acetonitrile and common laboratory reagents (Fisher Scientific, Pittsburgh, PA and Sigma Chemical Co., St. Louis, MO).

Bioanalytical Reagents. Solanine−KLH, an immunogen for the production of polyclonal antibodies, and solanine−DPPE, used for tagging the analyte to the surface of liposomes, were prepared by adapting a procedure reported earlier (Morgan et al., 1983; Butler and Tse-Eng, 1982). In this procedure for preparing the immunogen (Glorio-Paulet, 1997), aldehyde groups generated in the solanine glycoside by periodate oxidation react with free amino groups of the KLH. When preparing the DPPE−solanine conjugate, after transferring the oxidized glycoside to an organic phase, the aldehyde groups react with the free amino group of the DPPE. In both cases, a Schiff base is formed that is stabilized by subsequent reduction. In the case of solanidine−DPPE, this was followed with purification by preparative TLC.

Polyclonal antibodies were obtained by purification of antiserum (McKinney and Parkinson, 1987; Temponi et al., 1989) obtained from Flemish giant/chinchilla cross rabbits at the Cornell University Veterinary College research service facility. The rabbits were immunized with solanine−KLH following Cornell standard procedures (Protocol 88-18-96). The antibody obtained was acid treated (Ishikawa et al., 1980) until pH reached 2.5 using a solution containing 0.05 M glycine and 0.1 M NaCl at pH 2.0. The antibody solution was maintained at this pH for 10 min and then adjusted to neutrality. Antibodies were stored at −20 °C until ready for use. The protein content of the antibody solution was determined using a protein test kit from Bio-Rad (Hercules, CA)

Production of Liposomes. The reverse-phase evaporation procedure (O'Connell et al., 1985; Siebert et al., 1993, 1995; Szoka et al., 1980) was used to prepare biotin- and solanine-tagged liposomes. In this procedure, a warm aqueous solution of sulforhodamine B (150 mM) was added to the lipids (47.2 mol % DPPC, 4.9 mol % DPPG, 47.8 mol % cholesterol, 0.1 mol % biotin−DPPE, and 1.0 mol % solanine−DPPE) dissolved in an organic solution [chloroform:isopropyl ether:methanol; 6:6:1 (v/v)] under a low flow of nitrogen and sonicated for 5 min at 42 °C. After removal of the organic solvent using rotary evaporation (Büchi 461 Rotovapor; Flawil, Switzerland), the liposomes were extruded once through a series of polycarbonate filters of 3, 0.4, and 0.2 μm arranged in tandem. Separation of liposomes from the free dye followed, using column gel chromatography (Sephadex G-50) and dialysis, while protected from light. The liposomes, stored in dark containers at 4 °C, had an average diameter of 180 nm and were determined to be 98% intact (Glorio-Paulet, 1997).

Preparation of the LIM-LPC Strips. Liposome immunommigration strips (Siebert et al., 1995) were prepared by applying a line of protein solution in PBS (0.7 mg of anti-biotin IgG/0.3 mg of γ-globulin) on 8 × 15.7 cm Sartorius (Goettingen, Germany) nitrocellulose membrane sheets (8 μm nominal pore size) using a microprocessor-controlled Linomat IV TLC sample applicator from Camag Scientific (Wrightsville Beach, NC). These membranes were then vacuum-dried at 15 psi (room temperature) for 1 h, followed by a 1 h blocking step using a blocking solution consisting of 0.002% PVP, 0.007% gelatin, and 0.001% Tween-20 in Tris-buffered saline, pH 7.0. The membranes were again dried in a vacuum oven for about 12 h. Strips 0.5 cm wide × 8 cm long were cut from the membrane sheets using a paper cutter.

Protocol for the LIM-LPC Strip Assay. In a small (10 × 75 mm) glass test tube, 30 μL of sample solution was mixed with 30 μL of liposome solution and 30 μL of anti-solanine antibody (70 μg/mL). Tris-buffered saline (TBS), pH 7.0, was used as a diluent for all reagents. The mixture was incubated for 15 min at room temperature. After being incubated, a test nitrocellulose strip (prepared as described above) was inserted, and the solution was allowed to migrate to the top, at which point the strip was removed and air-dried.

The color at the capture zone of the strip (Figure 1) was measured using a ScanJet IIc scanner from Hewlett-Packard (Camas, WA) and Scan Analysis Densitometer software from Biosoft (Ferguson, MO) using the background subtraction settings of the software. Results were reported as a gray-scale density (net density units; NDU). A calibration curve was constructed from these data.

Evaluation of Cross-Reactivity Using the Bioprobe. The reactivity of the antibodies with α-chaconine and α-solanine was investigated using the strip assay. B/B_o values were then calculated from the following equation: $B/B_o = (A - P)/(C - P)$ where B/B_o represents the ratio of sample-dependent unbound liposomes vs total unbound liposomes, A is the readings in net density units (NDU) at the capture zone when standard sample is present; P is NDU at the capture zone when no analyte is present in the sample, and C is NDU at the capture zone when the concentration of analyte is at the upper limit of quantitation and consequently is the signal at its maximum. The IC_{50} in ppb is calculated at the point in which B/B_o accounts for 0.5 (50%) of the signal.

HPLC System. The HPLC apparatus consisted of a Rainin (Emeryville, CA) Microsorb-MV HPLC reverse-phase C-18 column (spherical silica-based packing, 5 μm particle size, 300 Å pore size, 25 cm length); HPLC 757 absorbance detector (Applied Biosystems, Foster City, CA); Rheodyne model 7125 sample injector (20 μL loop); column temperature control device (Kratos Analytical Instruments, Ramsey, NJ); HPLC

pump (Varian, Walnut Creek, CA); and an Ommi Scribe recorder (Houston Instruments, Austin, TX).

The HPLC protocol was previously reported (Hellenäs et al., 1995). Samples and standards prepared from commercially available α-chaconine and α-solanine were manually injected via a 20-μL fixed-loop sample injector connected to the HPLC system described, using a 100-μL microsyringe. The isocratic mobile phase consisting of acetonitrile:0.022 M potassium phosphate buffer, pH 7.6 (55:45 v/v), was pumped at room temperature at a flow rate of 1.5 mL/min. The detector was set at a wavelength of 208 nm, and the sensitivity was set at 0.005 absorbance units, full-scale (AUFS), with a filter rise time of 1.0 s. The resolution (R) between the two glycoalkaloids was calculated (Bowers et al., 1994), and triplicate analyses were performed. Quantitation was achieved by means of a calibration curve constructed from glycoalkaloid standards that were prepared by weighing commercially obtained pure substances and dissolving them in the solvent used for the extraction of the glycoalkaloids

Potato Samples. Commercial Idaho baking potatoes were obtained locally and used for spiking studies. The TGA value for the nonspiked samples was found to be in the range of 85−120 mg/kg. For the nonspiked studies, different levels of TGA were expected from several potato clone samples that were kindly provided by the Department of Plant Breeding and Biometry at Cornell University. The samples provided were labeled as P118-14, N133-66, Q308-48, Superior, N133-93, L235-4, Snow-den, N142-72, N142-53 and N 133-66. The potatoes were exposed to some elevated temperatures during transportation and were stored in paper bags at 4 °C for 1 month prior to analysis by both methodologies.

Extraction of Glycoalkaloids from Tubers. Duplicate subsamples were randomly selected from the potatoes. A potato slice (5 g) was extracted in 4 min using a GLH tissue homogenizer (OMNI, Gainesville, VA) and 20 mL of a solvent mixture consisting of water:acetic acid:sodium bisulfite (95:5:0.5). In the spiking tests, the glycoalkaloid standards were added after the potato sample had been cut into small pieces and mixed with the extracting solvent but prior to homogenization. Once the samples were homogenized, a centrifugation followed at 1000 rpm for 30 min. The potato extracts were subjected to further sample cleanup and preparation procedures as schematized in Figure 2.

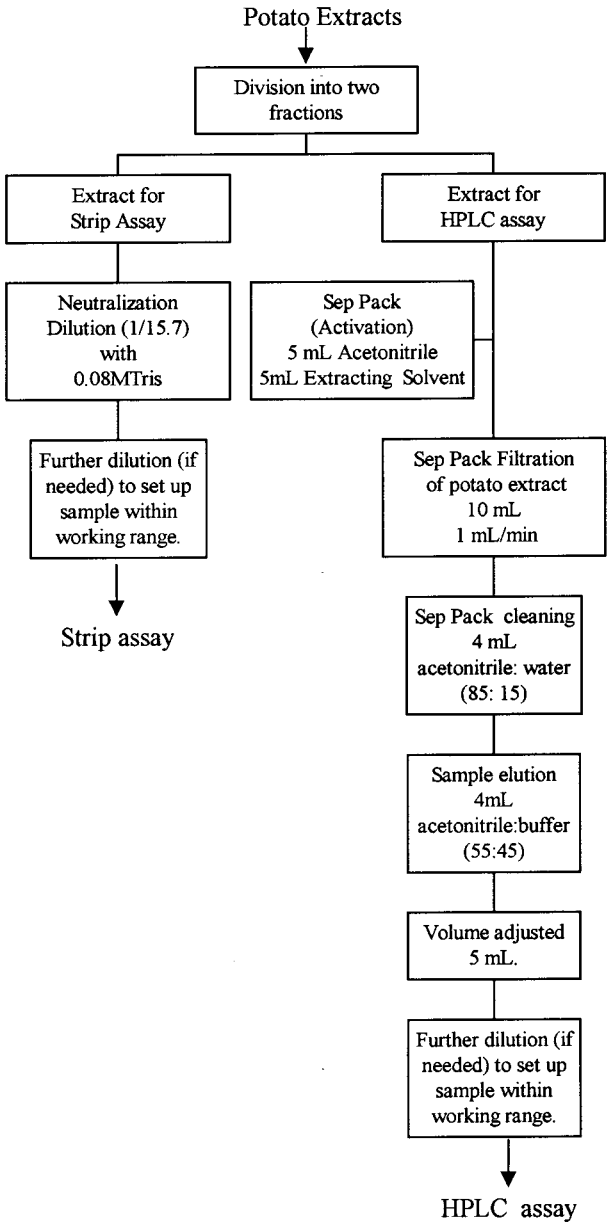

Figure 2. Sample cleanup and preparation procedure for TGA determinations of potato extracts for both LIM-LPC and HPLC methods.

RESULTS AND DISCUSSION

The integrity of the liposomes is a requirement for the implementation of this immunoassay since their signal amplification effectiveness depends on the amount of marker they are able to transport to the capture zone. During preliminary investigations, it was found that chloroform and other organic solvents such as dioxane lysed liposomes when used in concentrations higher than 10 vol %. In contrast, no lysis was observed when using neutralized potato extracts in the water:acetic acid:sodium bisulfite extraction solvent at any concentration. Furthermore, this aqueous acid solvent is relatively inexpensive and less toxic than the organic solvents. No attempts were made to study the liposome stability in the acidified solvent systems without first neutralizing them since physiological conditions are needed to optimize the antigen−antibody recognition process (Jourdan et al., 1996).

Results of the Cross-Reactivity. Studies with the polyclonal antibody used in this assay indicated a high cross-reactivity between α-solanine (100%), $IC_{50} = 305.3$ ppb, and α-chaconine (89%), $IC_{50} = 342.3$ ppb. The cross-reactivity with other alkaloid fractions for this type of antibody was reported earlier (Robins et al., 1985) indicating a rather high value for aglycones of potato glycoalkaloids such as demissidine, which are also steroidal molecules with structural similarities with solanidine, but in which there is a difference from the solanidine structure close to the site of conjugation in carbon 3 of the steroidal molecule. A smaller cross-reactivity, however, was observed for substances in which most of the difference is in the distal part from the point of conjugation in the molecule.

Recently, Glorio-Paulet working with another group (in collaboration with the International Potato Center in Lima, Peru) studied the cross-reactivity of this antibody in an indirect ELISA format. The results presented in the 1999 Annual Meeting of the American Chemical Society in New Orleans (Pérez et al., 1999) were in agreement with Robins et al. (1985). A high cross-reactivity for demissidine was observed (even though in the preliminary studies, when the method was under development, the results for demissidine appeared somewhat lower), and for α-tomatine the cross-reactivity was always low.

Consequently, this assay was designed to quantitate the total main fraction of glycoalkaloids of edible com-

mercial potatoes but does not identify any individual glycoalkaloid. Glycoalkaloid levels of concern in potatoes are reported to be >200 mg of TGA/kg of potato (Morris and Lee, 1984). Since both fractions have been shown to be toxic, the LIM-LPC strip assay will easily indicate if potatoes are above or below this level (Friedman and McDonald, 1997).

However, in a recent paper from Friedman et al. (1998), a novel and improved monoclonal anti-glycoalkaloid antibody was reported in which a lower cross-reactivity was achieved for demissidine and other important potato glycoalkaloid fractions for the ELISA format developed by Envirologix, Inc. Consequently, if needed, it should be possible to achieve better specificity in the LIM-LPC using this new monoclonal antibody.

Quantitation of TGA in Potatoes by HPLC vs LIM-LPC. From these research results, the LIM-LPC strip assay showed clear advantages in its extra-laboratory capabilities over HPLC, starting with the cleanup procedure (Figure 2). After the sample extraction, the potato extracts for the strips assay required only neutralization with 0.08 M Tris and further dilution with pH 7.0 Tris-buffered saline to bring the final concentration within the measurement range of the assay. In contrast, samples for HPLC analysis needed solid-phase extraction and purification prior to dilution, requiring more time, materials, and reagents. It was found that the range of dilution of the final purified sample for the strip assay (dependent on the initial sample analyte concentration) was between 1/20 to 1/680 with pH 7.0 TBS (included in this number is a previous 1/15.7 dilution with 0.08 M Tris base for solvent neutralization). For the HPLC assay, the dilution range was between 1/2 and 1/16 with acetonitrile: water (85:15 v/v).

Moreover, during the quantitation of TGA, the strip assay required about 15 min of incubation and an additional 8 min for capillary migration. During this 23 min, 20 or more samples can easily be run simultaneously by one analyst. That means it takes about 1 min per sample, a considerably shorter assay time as compared with other instrumental methods such as HPLC, where about 7 min (Hellenäs and Branzell, 1997), in the best of the cases, was required per injection of sample for the last peak of α-chaconine to appear. In our laboratory, 10 min was required per sample for the HPLC analysis.

For the strip assay, a typical dose−response curve was obtained for a direct competitive immunoassay. The working range was found to be ca. 0.1−0.7 ppm TGA. When a semilogarithmic fit is applied to the data in the working range, the calibration curve is linearized and amenable to a mathematical transformation of the type $y = m \log(x) + b$, which for these immunoassay data in the range between 0.1 and 0.7 ppm was highly correlated ($r^2 = 0.95$). When the Rodbards-Ciba approach (Brady, 1995; James and Joseph, 1995) was applied to the calibration data (in the logarithmic range), a limit of detection of 0.11 ppm TGA was obtained. The limit of quantitation, or the level above which quantitative results can be reported with a certain degree of confidence (theoretically calculated as 10 times the standard deviation plus the mean reading of the background), was found to be 0.14 ppm TGA. The percent coefficient of variation (% CV) was between 3.5 and 15%.

For the HPLC, calibration curves were constructed by relating the peak height of the eluting peak with the concentration of injected analyte, and a linear relationship was observed in the range between 10 and 50 ppm glycoalkaloid concentration in the standard solutions injected into the column. Retention times in our HPLC system were 8.2 min for α-solanine and 10 min for α-chaconine; the resolution of the peaks (R) was found to be 2.44.

Table 1. Recoveries of TGA in Spiked Potato Samples

assay type	concn spiked in potato (mg/kg)	deg of dilution	alkaloid recovered: mean (mg/kg)	n	%	% CV
Recoveries of α-Solanine in Spiked Idaho Baking Potato Samples						
strips	50	157	53	3	105	21
strips	200	393	187	3	94	13
strips	400	683	337	3	84	8
HPLC	50	4	44	3	87	4
HPLC	200	8	162	3	81	0
HPLC	400	16	348	3	87	1
Recoveries of Glycoalkaloid Mixtures[a] in Spiked Idaho Baking Potato Samples						
strips	50	157	55	3	109	2
strips	200	236	223	3	111	7
strips	400	420	327	3	80	11
HPLC[b]	50	4	56	2	112	8
HPLC[b]	200	8	213	2	106	42
HPLC[b]	400	16	374	2	93	27

[a] Glycoalkaloid mixture: α-chaconine/α-solanine (2/1). [b] Data obtained with UV detector at 202 nm.

Results of the spiking tests are shown in Table 1. For this immunoassay, no undesirable matrix effects were found for potato samples when spiked with different levels of the main fraction of potato glycoalkaloids. Recoveries ranged from 80 to 111% demonstrating the feasibility of using the strip methodology for monitoring typical levels of TGA in potatoes.

The recoveries at the approximate natural levels of TGA in potatoes, which normally contain an excess of α-chaconine (Bushway and Ponnampalam, 1981) were evaluated. In both methodologies, comparable recoveries were obtained. Some of the losses that account for recoveries lower than 100% in the HPLC analyses may be due to irreversible adsorption on the Sep-Pak cartridges. However, less variability in the results were observed for the HPLC system (% CV between 0.58 and 3.78) when the optimum wavelength (208 nm) for the detector was used.

Using both methodologies, the glycoalkaloid content of potato clones was studied, and the results are shown in Table 2. Using data from the strip assays, it was possible to identify the clones (N 133-93, L 235-4, N 142-72, and P 118-14) that had glycoalkaloid levels below the safe tolerance level of 200 mg/kg as well as to identify those that should be rejected due to their dangerous TGA content (Q 308-48, N 142-53, and N133-66). These results were confirmed by the HPLC determinations, and there was an excellent correlation of results of the values obtained by both assay methods (y = LIM-LPC results; x = HPLC results; $y = 1.041x - 38.685$; $r^2 = 0.91$). Both determinations showed a linear relationship with a slope very close to unity.

CONCLUSION

The results of this research demonstrate the feasibility of using the liposome immunomigration strip assay for the determination of the main fraction of potato glycoalkaloids (α-solanine + α-chaconine). Also shown

Table 2. Glycoalkaloid Content of Potato Clones

	glycoalkaloid content (mg/kg)					
	HPLC		LIM strips		% CV	
clone sample	*n*	mean	*n*	mean	HPLC	LIM strips
N 133-93	3	79	3	58	4.2	7.9
L 235-4	3	137	3	59	4.2	7.7
L 235-4	3	118	3	73	1.2	2.7
N 142-72	3	142	3	92	7.3	2.8
P 118-14	3	151	3	98	2.7	1.9
Q 308-48	3	457	3	604	1.7	2.4
Q 308-48	3	376	3	388	0.4	7.0
N 142-53	3	237	3	145	3.1	3.3
N 142-53	3	604	3	480	7.6	21.1
N 133-66	3	444	3	480	1.0	3.6
N 133-66	3	501	3	450	1.7	8.3
Superior[a]	3	46	3	49	5.1	1.5
Superior	3	5.2	3	2.4	3.3	14.6
Superior	3	52	3	24	3.3	14.6
Superior	3	84	3	32	10.0	9.0
Snow-den[b]	3	439	3	402	3.5	30.3

[a] Introduced as control of low glycoalkaloid level. [b] Introduced as control of high glycoalkaloid level.

was the potential of the assay for screening at potato breeding locations and at storage or retailer facilities. The LIM-LPC strip assay, due to the simplicity of its procedure, does not need highly trained personnel and can be performed without sophisticated equipment in any facility, such as in a raw materials receiving area of a food processing plant, at a retail store, or on a farm. In this regard, LIM-LPC showed a clear advantage in its extra-laboratory capabilities over HPLC. LIM-LPC also required less sample cleanup and shorter analysis time per sample. In addition, it uses less toxic solvents, and the colorimetric signal of the liposome marker can be easily quantitated

Further improvements in the strip assay in terms of its portability and "user friendliness" for field use are possible, such as the elimination of the test tube format by the incorporation of the strip into a disposable cassette (Durst et al., 1993) or the reading of the signal by means of a portable scanner such as a Colortron (Light Source Inc., Larkspur, CA) (Reeves and Durst, 1995). Also, with new approaches for obtaining antibodies, such as recombinant and monoclonal antibody technology (Stanker and Beier, 1996; Kamps-Holtzapple and Stanker, 1996; Friedman et al., 1998), the assay may also hold the potential for highly specific recognition of individual glycoalkaloids and their subsequent identification.

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