Immunoaffinity Sample Purification and MALDI-TOF MS Analysis of α-Solanine and α-Chaconine in Serum

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A sample purification technique was developed for the detection of potato glycoalkaloids (GAs) in blood serum by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). GAs were extracted from spiked serum (5 mL) using a C_{18} solid-phase extraction cartridge. The GAs were then selectively captured on antibody-coated agarose beads. The agarose beads were washed with water and the GAs eluted with 25 μL of methanol. MALDI-TOF MS was used to detect the GAs in the methanol eluent. Immunoaffinity sample purification of the GAs effectively reduced the signal suppression observed during the analysis of unpurified samples. α-Chaconine and α-solanine were detected in serum spiked with 1 ng/mL of each GA.

Keywords: *Potato; glycoalkaloid; solanidine; antibody; affinity column; solid-phase extraction*

INTRODUCTION

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has become a valuable tool for the analysis of biomolecules. Its ability to analyze complex mixtures often makes it more suitable than other MS techniques for biological extracts. Picomole sample quantities are usually adequate for analysis. MALDI-TOF MS has been used to characterize a wide range of compounds including proteins (*1*), oligosaccharides (*2*), phospholipids (*3*), alkaloids (*4*), and tannins (*5*). Although MALDI-TOF MS is tolerant of impurities compared to other MS techniques, maximum sensitivity is achieved from pure analyte in solution (*6*). Femtomole detection limits have been reported when the sample is free from impurities (*7*). Detection limits are severely compromised when biological extracts are analyzed (*8*).

Affinity purification is a widely used strategy for isolating biomolecules from biological fluids. The technique exploits the selective biochemical binding interactions between such molecules as antibody and antigen or between avidin and biotin. MALDI-TOF MS is well suited for detecting analytes isolated by affinity purification. MALDI-TOF MS has been coupled with immunoaffinity purification to analyze lysozyme (*9*), cytochrome *c* (*10*), and myotoxin (*11*); avidin−biotin affinity has been used to detect biotinylated bradykinin and insulin (*12*); and metal ion affinity has been used to isolate several metal-binding peptides (*10*). The binding molecules are typically immobilized on agarose beads (*10*, *12*). When the beads containing the binding molecule are exposed to the sample solution, the analyte is captured on the bead. After the analyte is captured on the beads, the beads can be washed to remove unwanted impurities and the analyte is then eluted for MALDI-TOF MS analysis. Alternatively, the binding molecule can be immobilized directly on the MALDI probe (*9*, *13*). The entire MALDI probe is immersed in the sample solution, and the analyte is retained directly on the probe. Immobilization of the binding molecule on a solid support was completely avoided by Nakanishi et al. (*14*), who used immunoprecipitation to purify transferrin for MALDI-TOF MS analysis.

Potato glycoalkaloids (GAs) are of interest because of their potential toxicity to humans (*15*, *16*). GAs can be determined in potatoes by a variety of methods (*17*). It is much more difficult to analyze GAs in animal tissue (e.g., serum) after potato consumption because concentrations are ∼1000 times less than in potatoes. Immunoassays are able to detect GAs in serum but are not able to distinguish individual GAs or metabolic products (*18*). Hellenäs et al. (*19*) developed a high-performance liquid chromatography (HPLC) procedure that was able to detect individual GAs at serum concentrations; however, extensive sample cleanup was required to remove interfering compounds.

In the present paper, we explore the feasibility of using MALDI-TOF MS to detect individual GAs in serum. MALDI-TOF MS has been used previously to detect and quantitate individual GAs in potatoes (*4*). To detect the lower concentrations found in serum, more rigorous sample preparation will be required. We describe the development of antibody-coated particles for the isolation of potato GAs from serum. Several sample purification strategies are explored. Substantial improvement in method detection limits can be achieved when chromatographic and immunoaffinity sample purification procedures are used.

MATERIALS AND METHODS

Preparation of Immunogen. All GA standards (>95% analytical purity) were purchased from Sigma Chemical Co., St. Louis, MO. Solanidine was conjugated to *Limulus polyphemus* hemocyanin (LPH) for rabbit immunization using a modification of the procedure used by Plhak and Sporns (*20*). The first step involved the formation of the succinylated

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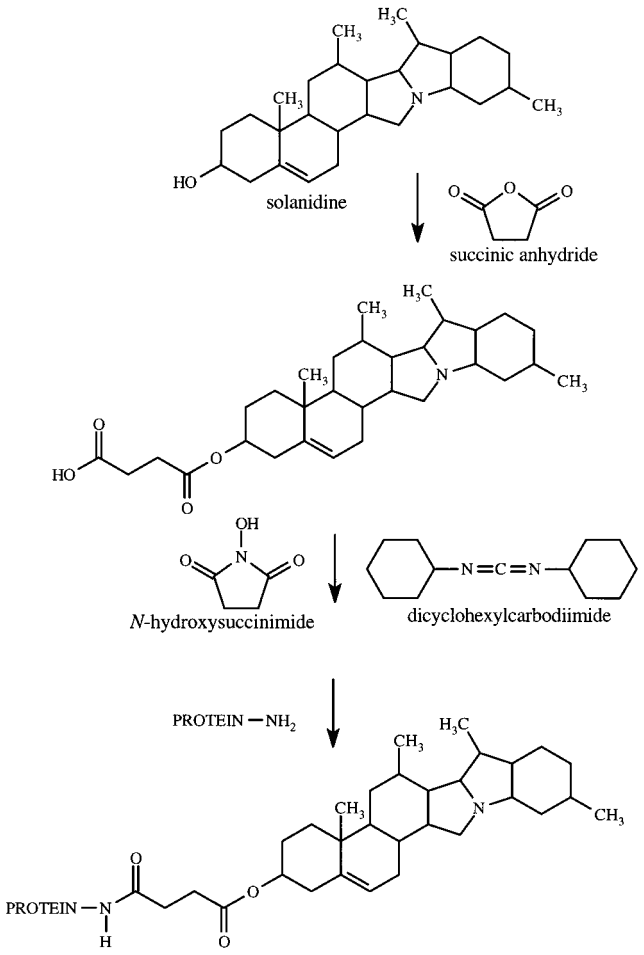

Figure 1. Succinylation of solanidine and conjugation of protein to succinylated solanidine via an active ester intermediary.

alkaloid (Figure 1). 4-(Dimethylamino)pyridine (218 μmol) and succinic anhydride (4290 μmol) were added to 10 mL of pyridine. Solanidine (39.7 μmol) was then added to the pyridine solution and heated under reflux at 55 °C for 74 h. The reaction was followed by TLC. Additional succinic anhydride was added after 24 h (3230 μmol) and 48 h (1890 μmol) when unreacted solanidine appeared to persist. The reaction was stopped with 20 mL of water and extracted with 3 × 25 mL of methylene chloride. The combined organic extract was washed with 25 mL of 1% acetic acid, followed by 3 × 25 mL of water. The organic extract was rotoevaporated to dryness (coevaporation with toluene was required) and dried overnight under vacuum in the presence of phosphorus pentoxide. A yield of 141% of theoretical yield indicated the product still contained impurities.

The succinylated solanidine was further purified with a silica solid-phase extraction (SPE) cartridge. The cartridge was washed with 5 mL of methanol before 28 mg of the impure succinylated solanidine dissolved in ~2 mL of methanol was loaded. Fractions (0.5 mL each) were eluted with methanol and monitored by TLC. Fractions 2−14 were combined, rotoevaporated, and dried overnight under vacuum in the presence of phosphorus pentoxide. Brown crystals were recovered giving a yield of 120%. No contaminants were revealed by thin-layer chromatography (TLC). MALDI-TOF MS produced a strong signal at 499 (succinylated solanidine, $M + H^+$) and no signal at 399 (solanidine, $M + H^+$).

Succinylated solanidine was then conjugated to LPH using an active ester method (Figure 1). The active ester was prepared by stirring succinylated solanidine (28.3 μmol), *N*,*N*′-dicyclohexylcarbodiimide (28.1 μmol), and *N*-hydroxysuccinimide (53.9 μmol) in 1 mL of dry dimethylformamide for 24 h at 4 °C. To improve the solubility of the active ester in aqueous solution, 2 mL of 1,4-dioxane was added to the dimethylformamide solution immediately prior to the introduction of the LPH. The dimethylformamide−dioxane solution was then added to 1 mL of 0.1 M sodium carbonate containing 16 mg of LPH and stirred for 24 h at 4 °C. The entire reaction mixture was dialyzed against 1 L of 8 M urea for 24 h, 4 L of 50 mM ammonium carbonate for 24 h, 4 L of 25 mM ammonium carbonate, and finally 4 L of water for 24 h. All dialyses were performed at room temperature. The contents of the dialysis tube were lyophilized and stored at 4 °C. Elemental analysis indicated 3 mol of solanidine/mol of bovine serum albumin (BSA) equivalent on a C basis. TLC showed that solanidine could not be removed from the LPH−solanidine conjugate by washing with methylene chloride. Boiling the conjugate in 2 N sodium hydroxide for 2 h did hydrolyze the solanidine−LPH ester linkage, indicating that solanidine was covalently bound to the LPH rather than simply adsorbed.

Immunization of Rabbits. Preimmunization blood samples were taken from two female Flemish Giant × Dutch Lop Ear rabbits. Each rabbit was injected with ~0.7 mg of the LPH−solandine conjugate in 1 mL of a phosphate-buffered saline (PBS)/Freund's complete adjuvant (1:1, v/v) emulsion. The rabbits received 0.5 mL gluteally and 2 × 0.25 mL subscapularly. Two monthly boosts were made in the same manner except with Freund's incomplete adjuvant. Blood samples were collected 2 weeks after each boost and centrifuged at 2150*g* for 15 min, and the serum was tested for antibody titer.

Preparation of Conjugates for Enzyme Immunoassay. Preparation of the BSA−solanidine conjugate was similar to that of the LPH−solanidine conjugate. Succinylated solanidine (22.5 μmol), *N*,*N*′-dicyclohexylcarbodiimide (24.7 μmol), and *N*-hydroxysuccinimide (55.6 μmol) were stirred in 1 mL of dry dimethylformamide for 24 h at 4 °C. The reaction mixture was then added to 1 mL of phosphate-buffered saline (PBS) containing 132 mg of BSA and stirred for 24 h at 4 °C. The entire reaction mixture was dialyzed and lyophilized the same as the LPH conjugates. Elemental analysis indicated 9 mol of solanidine/mol of BSA on a C basis. Basic hydrolysis of the conjugate released solanidine, indicating that solanidine was covalently bound to the BSA. Solubility of the conjugate was a problem when MALDI-TOF analysis was attempted. The addition of solanidine drastically reduced the solubility of BSA in aqueous solution. MALDI-TOF MS of the BSA−solanidine that was soluble in 0.1% trifluoroacetic acid indicated 2 mol of solanidine/mol of BSA. Presumably, the portion of the conjugate that did not dissolve was more heavily substituted as the elemental analysis suggested.

Evaluation of Sera. Antibody titer was determined using a checkerboard immunoassay. BSA−solanidine was dissolved in PBS with a concentration of 36 μg/mL, and 1:5 serial dilutions were made in PBS. Each conjugate dilution was applied to a microtiter plate row (200 μL/well) with 200 μL of PBS in the final row. The plates were stored for 18 h at 4 °C to allow the conjugate to coat the wells. The next day the conjugate solutions were removed, and each well was coated with 200 μL of 1% BSA in PBS for 1 h at room temperature. The plate was then washed with 3 × 200 μL of PBS with Tween (PBST) for 5 min each wash.

Rabbit serum was diluted 1:500 with 0.05% BSA in PBST, and 1:3 serial dilutions were made in 0.05% BSA in PBST. Each serum dilution was applied (200 μL/well) to a column of the coated microtiter plate with a blank of 0.05% BSA in PBST in the last column. The plate was incubated for 2 h at room temperate. The wells were then emptied and washed as before with 3 × 200 μL of PBST. Goat anti-rabbit antibody−peroxidase conjugate was diluted 1:3000, added to each well (200 μL/well), and allowed to incubate for 2 h at room temperature. The wells were then emptied and washed as before. The peroxidase substrate was prepared by dissolving 10 mg of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) in 100 mL of 0.05 M phosphate−citrate buffer, pH 5.0, and then adding 25 μL of 30% hydrogen peroxide (Sigma A-9941 product literature, 05/96). The peroxidase substrate was added to each

well (200 μL) and absorbance measured at 405 nm after 40 min at room temperature. The titer was determined as the minimum serum dilution required to give an absorbance reading 3 times greater than background levels (no serum).

Affinity Purification of Antibodies. GammaBind Plus Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gel was prepared in a 10 × 1.0 cm glass column according to the instructions of the manufacturer. GammaBind contains protein G that binds the Fc region of rabbit immunoglobulin G. Equal volumes of rabbit serum and binding buffer [0.01 M sodium phosphate, 0.15 M sodium chloride, and 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 7.0] were mixed and filtered through a 0.22 μm Millex-GS filter unit (Waters Corp., Milford, MA). The diluted serum (4 mL) was loaded onto the column at 1 mL/min. The column was washed with 80 mL of binding buffer at 1.5 mL/min. Antibody (immunoglobulin G) was eluted in 4 mL fractions with 0.5 M acetic acid, pH 3.0, at 1 mL/min. The absorbance of the eluent was monitored at 278 nm. Each fraction was immediately neutralized with 2.8 mL of 1 M Tris-HCl, pH 9.0. Fractions exhibiting absorbance at 278 nm were pooled and dialyzed against PBS (0.01 M phosphate, 0.9% sodium chloride, pH 7.3). The antibodies were finally concentrated to ~1 mg/mL in a 30K centrifugal concentrator (Pall Canada Ltd., Missisauga, ON) and stored at −20 °C.

Preparation of Antibody-Coated Silica Particles. An aliquot of the purified antibody solution was desalted on an Econo-Pac 10 DG desalting column (Bio-Rad Laboratories, Hercules, CA) and lyophilized. Antibody was covalently coupled to carboxylate-modified 0.30 μm diameter silica particles via a one-step procedure according to the manufacturer's suggested protocol (Tech note #13c, Bangs Laboratories, Fishers, IN). The lyophilized antibody was dissolved in 0.05 M morpholinoethanesulfonic acid, pH 5.5, at a concentration of 1 mg/mL. The antibody solution (1 mL) was mixed with 20 mg of the silica particles and 0.2 mL of a 1% solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in water in a siliconized microcentrifuge tube. The mixture was allowed to react for 2 h at room temperature with slow rotation. The coated particles were removed from the suspension by centrifugation at 650*g* for 1 min. Unreacted carboxylate groups were blocked by washing the particles twice with 1 mL of 0.2% gelatin in 0.05 M phosphate, 0.1% sodium chloride, pH 6.6. The silica particles were recovered by centrifugation as above. The particles were stored as a 2% suspension in the gelatin containing phosphate buffer.

Preparation of Antibody-Coated Agarose Beads. Lyophilized antibody was coupled to CNBr-activated Sepharose 4B (particle diameter = 45−165 μm) according to the manufacturer's instructions (Amersham Pharmacia Biotech AB, Uppsala, Sweden). CNBr-activated Sepharose 4B (0.15 g) was suspended in 1 mL of cold 1 mM HCl and washed first with 30 mL of 1 mM HCl on a sintered glass funnel, followed by two washings with 2 mL of carbonate buffer (0.1 M carbonate, 0.5 M sodium chloride, pH 8.3). The washed gel was scraped from the funnel and added to 1 mL of carbonate buffer containing 4 mg of the antibody. The mixture was allowed to react overnight at 4 °C with slow rotation. The antibody-coated agarose bead suspension was centrifuged at 1800*g* for 10 min and the supernatant removed. The beads were resuspended in 1 mL of 0.2 M glycine, pH 8.0, and centrifuged as above. The unreacted sites on the beads were blocked by again suspending the beads in 1 mL of 0.2 M glycine and slowly rotating for 2 h at room temperature. The blocked beads were applied to a 4.0 × 0.7 cm column and washed with five cycles of carbonate buffer followed by acetate buffer (0.1 M acetate, 0.5 M sodium chloride, pH 4.0). The flow rate was 1 mL/min, and 10 mL of buffer was used for each wash. The beads were finally washed with 25 mL of PBS. The column was drained until the total volume was 1 mL (beads plus PBS). The volume of the agarose gel was ~0.5 mL after settling.

SPE Sample Cleanup. The SPE procedure was based on that of Saito et al. (*21*). Classic C_{18} Sep-Pak (Waters, Milford, MA) cartridges were conditioned with 8 mL of methanol, followed by 8 mL of water. Porcine serum (5 mL) was loaded at 1 mL/min. The SPE cartridge was washed with 5 mL of 20% methanol. The GA was eluted with 2 mL of methanol, discarding the first 0.5 mL. An aliquot of the eluent was used directly for MALDI-TOF MS analysis.

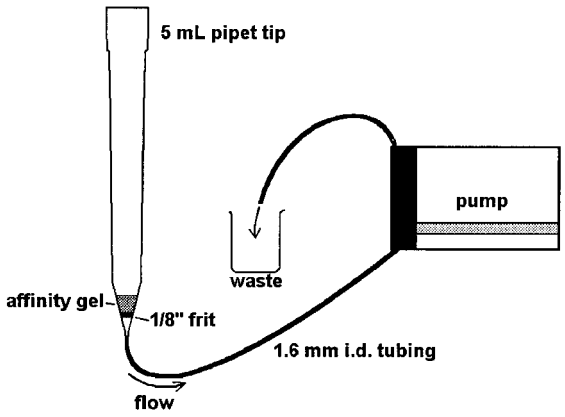

Figure 2. Immunoaffinity column prepared in a 5 mL pipet tip.

Extraction of GAs from Serum with Silica Immunoaffinity Particles. Antibody-coated silica particles (0.1 mL of a 2% solution) were incubated with 1 mL of porcine serum for 2 h at room temperature with slow rotation in a siliconized microcentrifuge tube. The particles were precipitated by centrifugation at 650*g* for 1 min, and the supernatant was removed. The particles were resuspended in 1 mL of water by drawing them repeatedly through a Pasteur pipet followed by centrifugation as above. The particles were washed twice in this manner. After removal of the second water wash, the GAs were eluted by suspending the washed particles in 20 μL of methanol and centrifuging at 650*g* for 5 min. The supernatant was used for MALDI-TOF MS analysis.

Extraction of GAs from Serum with Agarose Immunoaffinity Beads. The agarose beads prepared previously were vortexed in the column to produce a suspension of ~0.5 mL of agarose/mL of PBS. An aliquot (50 μL) was added to 5 mL of porcine serum and incubated for 2 h at room temperature with slow rotation. The suspension was transferred to a 5 mL pipet tip with a 2 mm × $^1/_8$ in. (3.2 mm) o.d. frit (Supelco, Bellefonte, PA) wedged in the tip (Figure 2).

The pipet tip column was attached to a peristaltic pump and the sample solution drawn through the frit at 1.5 mL/min. The retained agarose beads were washed with 5 mL of water. The GAs were eluted by adding 25 μL of methanol to the washed beads and allowing the beads to sit for 5 min. The methanol was then pushed through the agarose and frit with a 5 mL pipettor. The methanol eluent was collected for MALDI-TOF MS analysis.

MALDI-TOF MS. MALDI-TOF MS was performed using a Proflex linear mode instrument, Bruker Analytical Systems Inc. (Billerica, MA). The instrument was equipped with a 337 nm nitrogen laser. Positive ions were accelerated with a 20 kV potential and time-delayed extraction. GA analysis was performed using 2,4,6-trihydroxyacetophenone (THAP) as the matrix. A saturated solution of THAP was prepared in acetone and then diluted 1:1 (v/v) with acetone. This matrix solution was applied (0.3 μL) to the MALDI probe and allowed to air-dry. GA solutions (1 μL) were spotted directly on top of the matrix crystals and air-dried. The laser attenuation was typically set at 30, with less attenuation necessary when more contaminants were present in the GA solution. Sinapinic acid was used as the matrix for MALDI-TOF analysis of the BSA conjugates. A saturated solution of sinapinic acid in 0.1% trifluoroacetic acid (TFA)/acetonitrile (2:1, v/v) was mixed 1:1 (v/v) with the conjugate dissolved in TFA/acetonitrile (4:1, v/v). About 1.5 mL was spotted on the MALDI probe and allowed to air-dry. Each spectrum represents the sum of 200 laser pulses directed at several positions on the sample spot.

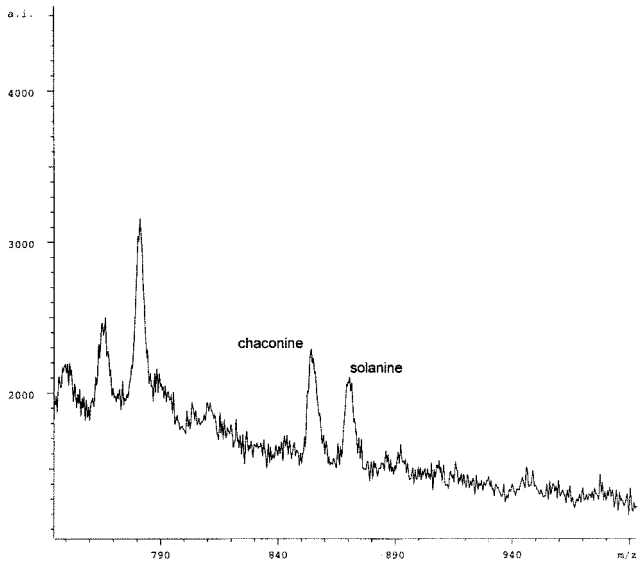

Figure 3. MALDI-TOF spectrum of ~25 fmol of α-chaconine and 25 fmol of α-solanine. Sample solution concentration was 25 ng/mL of each GA.

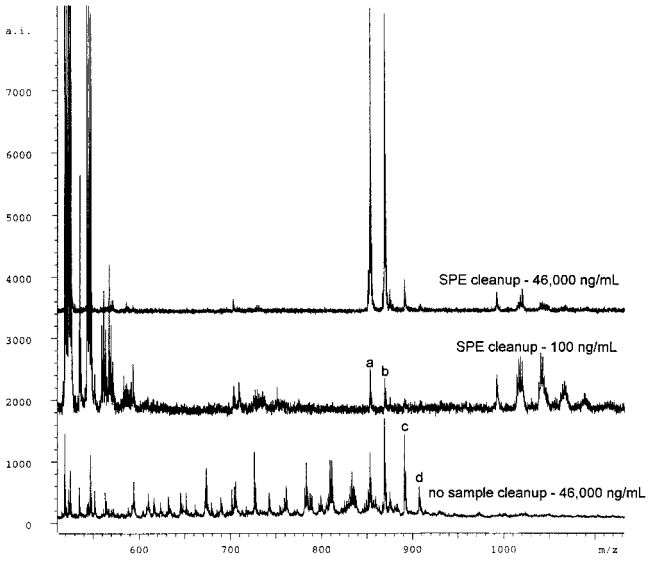

Figure 4. MALDI-TOF mass spectra of GAs in porcine serum (a, α-chaconine plus H^+; b, α-solanine plus H^+; c, α-chaconine plus K^+; d, α-solanine plus K^+).

RESULTS AND DISCUSSION

GA Antibody Production. The immunized rabbits exhibited a strong response to the LPH−solanidine immunogen. The checkerboard immunoassay of the first test bleed produced a titer of 1/500000 serum dilution with 1 μg/mL conjugate applied to the microtiter plate. The titer improved to 1/800000 serum dilution after the second boost when the blood was harvested. The titer of the antibody after purification on the GammaBind column was 6.0×10^{-6} mg of antibody/mL, indicating that antibody affinity for solanidine was preserved during the purification process.

MALDI-TOF MS of GAs. Figure 3 shows the spectrum generated from a 25 ng/mL solution of each GA (~25 fmol of each GA on the probe). The peaks are clearly identifiable with a signal-to-noise (S/N) ratio of 5. The response confirms that MALDI-TOF MS is a sensitive method of analysis. With liquid secondary ion mass spectrometry, 200 fmol is the lowest reported amount of GA detected (*22*).

Signal suppression is commonly encountered when mixtures are analyzed by MALDI-TOF MS. Harvey (*6*) showed that the peak intensity of a given oligosaccharide was greatest when the sample solution contained only the one oligosaccharide. The peak intensity of the original oligosaccharide consistently decreased with each new oligosaccharide added to the sample solution. Likewise, the signal suppression encountered in complex biological mixtures can be substantial (*13*). Analysis of porcine serum fortified with α-solanine and α-chaconine confirmed that this signal can be severely suppressed in a biological sample (Figure 4). The minimum GA concentration that could be reliably detected when serum was spotted directly on the MALDI probe was 46000 ng/mL, representing a 2000-fold decrease in sensitivity.

The same fortified serum sample was treated with the SPE sample cleanup procedure developed by Saito et al. (*21*) for potato extracts to be analyzed by HPLC. Salts and other hydrophilic components are removed from the serum by the SPE procedure. MALDI-TOF MS analysis of the cleaned up serum showed a considerable improvement in GA signal intensity (Figure 4). Subsequent fortification experiments showed that 100 ng of GA/mL was reliably detected in serum using the C_{18} SPE cleanup procedure.

Immunoaffinity Sample Purification. Initial attempts at immunoaffinity purification focused on using antibody-coated polystyrene particles as the GA capture device. Results were generally unsatisfactory. The polystyrene particles had a density of 1.06, only slightly more dense than water. They also tended to stick to the walls of microcentrifuge tubes, making centrifugation difficult. Paramagnetic polystyrene particles were easier to use. However, all polystyrene particles appeared to exhibit nonspecific affinity for GAs. Particles coated with nonspecific IgG, rather than GA antibody, were able to extract GAs from PBS when GA concentrations were >1 μg/mL. The nonspecific affinity for GAs was attributed to the hydrophobic nature of the polystyrene. The relatively hydrophobic GAs probably have more affinity for polystyrene than for the aqueous solution in which they are dissolved.

The next strategy was to isolate the GAs on an immunoaffinity column (*23*). Antibody was immobilized on hydrophilic agarose beads (CNBr-activated Sepharose 4B); hence, hydrophobic interactions would be minimized. A 1.0 × 0.3 cm column of the agarose gel was prepared, and sample was loaded at 0.5 mL/min. The column was washed with water, and the GAs were eluted with methanol. The column effectively isolated GA from PBS but was not able to isolate GA from serum. Possibly the viscosity of the serum inhibited diffusion of GA toward the immobilized antibody. The flow rate of 0.5 mL/min resulted in a short residence time of the sample in the gel, ~8 s.

To increase the time available for antibody−antigen binding, the antibody-coated agarose beads were mixed with GA-fortified serum for 2 h as described under Materials and Methods. The increased reaction time allowed the immobilized antibody to bind the GAs in the serum, and the bound GAs were detectable by MALDI-TOF MS. The lowest GA concentration detected in serum was 100 ng/mL, representing a 450-fold increase in sensitivity over unpurified serum (Figure 5). The same improvement in sensitivity was observed with the SPE sample cleanup procedure.

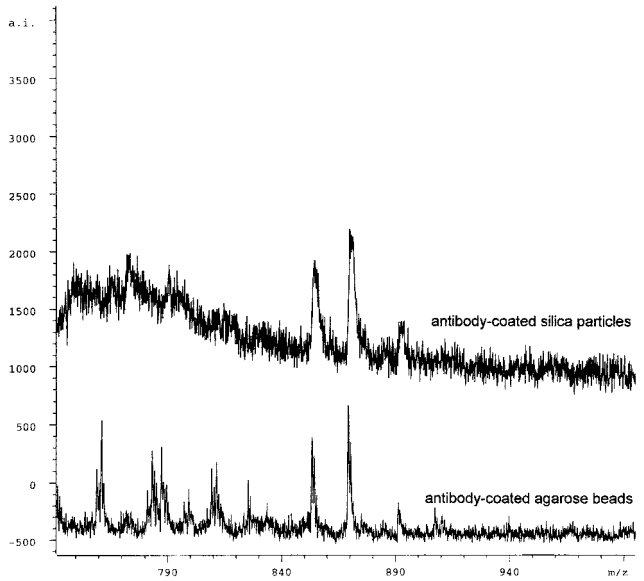

Figure 5. MALDI-TOF MS spectrum of serum fortified with 100 ng/mL α-chaconine and 100 ng/mL α-solanine using immunoaffinity purification.

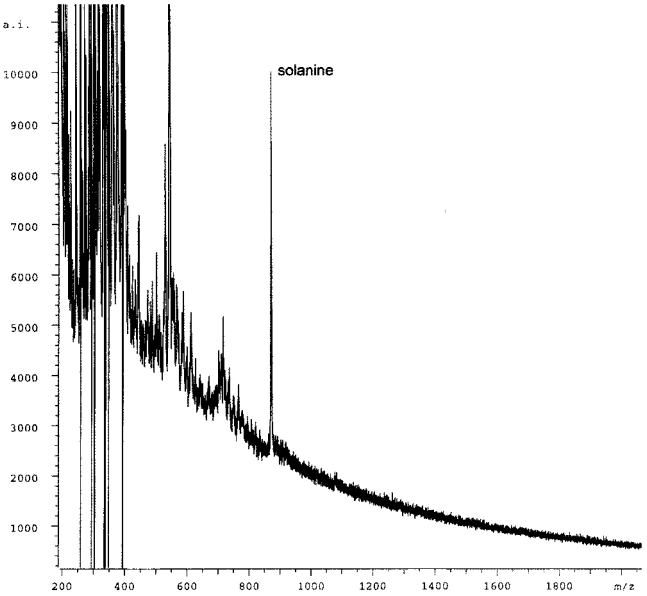

Figure 6. MALDI-TOF spectrum of 100 ng/mL α-solanine in PBS extracted with agarose affinity beads. Affinity beads were applied directly to the MALDI probe.

As an alternative method of eluting GA, application of the beads directly to the MALDI probe was investigated (*12*). Immunoaffinity beads were exposed to the sample and washed as previously. Rather than elute with methanol, the washed beads were suspended in 25 μL of matrix solution. An aliquot (2 μL) of this mixture was applied on top of THAP crystals previously applied to the probe. When the spot had dried, the agarose beads were removed with a blast of compressed air. Acceptable spectra were produced in this manner, although the baseline rose toward the low-mass region (Figure 6). As there was no significant reduction in analysis time, applying the beads directly to the probe was not used any further.

GAs could also be isolated on antibody-coated silica particles (Figure 5). Silica particles had two advantages over polystyrene particles. Silica is hydrophilic, eliminating the hydrophobicity problem of polystyrene particles. It was also easier to remove the silica particles from suspensions because they had a much higher density than polystyrene (1.96 versus 1.06). Silica particles needed to be centrifuged gently (650*g*) to prevent the pellet from becoming too compact. If more centrifugation was used, it was difficult to resuspend the silica as individual particles. In terms of overall convenience, the agarose beads were easier to use because collecting and washing the beads on the pipet tip column required less attention than centrifuging the silica particles each time they were washed.

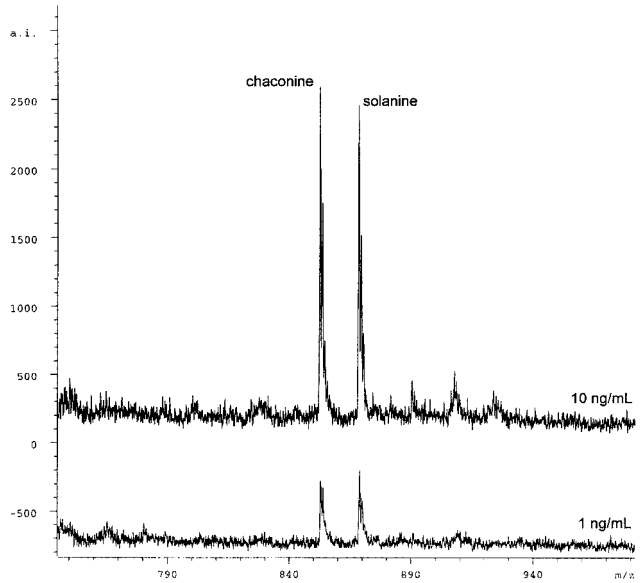

Figure 7. MALDI-TOF spectrum of serum GAs purified by SPE and immunoaffinity on agarose beads.

SPE Coupled with Immunoaffinity Purification. Assuming all of the GA in the serum sample was bound by the immobilized antibody and that all of the GA was subsequently eluted, agarose affinity purification of the 100 ng of GA/mL of serum sample should theoretically result in 200 pmol of GA on the probe. The detection limit of pure GA was previously shown to be at least 25 fmol (Figure 3). The peak intensities of the immunoaffinity-purified serum samples were still much less than expected. Given that immunoaffinity purification is highly selective, it is unlikely that contaminants suppressed the GA signal. It is more probable that the immobilized antibody did not bind all of the GA in the serum. As with the immunoaffinity column, diffusion of the GAs toward the antibodies may have been inhibited by the viscosity of the serum, or protein−protein interactions between the antibody and serum proteins may have made the antibody binding sites less available. When serum was not present, agarose affinity beads were very effective in isolating GAs from a 100 ng/mL solution (Figure 6).

On the basis of this information, a sample purification procedure was developed coupling SPE and agarose affinity beads. GA-fortified serum (5 mL) was cleaned up with the SPE procedure as before. The methanol eluent (1.5 mL) from the SPE cartridge was diluted to 10 mL with PBS and then extracted using the agarose affinity bead procedure. By combining SPE and immunoaffinity purification, 1 ng of GA/mL of serum could be detected with MALDI-TOF MS (Figure 7). There was a 200-fold concentration effect as the result of reducing the serum volume from 5 mL to 25 μL. The remaining increase in sensitivity (another 200-fold) can be ex-

Table 1. Effect of Sample Purification on MALDI-TOF Analysis of GAs in a 5 mL Serum Sample

purification	sample vol (mL)	detection limit (ng/mL)
none	5	46000 (Figure 4)
C_{18} SPE	2	100 (Figure 4)
immunoaffinity	0.025	100 (Figure 5)
C_{18} SPE + immunoaffinity	0.025	1 (Figure 7)

plained by the removal of signal-suppressing contaminants from the serum sample (Table 1).

Harvey et al. (*18*) used a radioimmunoassay to determine that the concentration of total GAs in the serum of healthy individuals ranged from 3.3 to 125 nM (~3−110 ng/mL). Hellenäs et al. (*19*) reported that after a single serving of potatoes, serum levels of α-chaconine peaked at 14.4 ng/mL and those of α-solanine at 7.7 ng/mL, on average. The present MALDI-TOF MS method was able to detect individual GAs at concentrations typically found in serum. Sample preparation was simpler than required for HPLC (*19*), and MALDI-TOF MS offers the further advantages of unambiguous analyte identification based on molecular mass and the possibility of identifying GA metabolites. GA analysis represents only one application for immunoaffinity sample purification coupled with MALDI-TOF MS. In principle, the same approach can be used to detect subparts per million drug and pesticide residue levels in a variety of food and agricultural products.

ABBREVIATIONS USED

BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GA, glycoalkaloid; LPH, *Limulus polyphemus* hemocyanin; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PBS, phosphate-buffered saline; PBST, PBS with Tween; S/N, signal-to-noise; SPE, solid-phase extraction; THAP, 2,4,6-trihydroxyacetophenone; TLC, thin-layer chromatography; TOF, time-of-flight.

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