A Capillary Electrophoresis Laser-Induced Fluorescence Method for Analysis of Potato Glycoalkaloids Based on a Solution-Phase Immunoassay. 1. Separation and Quantification of Immunoassay Products

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Solution-phase immunoassays are typically faster and more precise than ELISAs. This research developed a solution-phase for the immunoassay of potato glycoalkaloids (GAs) based on quantification by capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. Solanidine coupled to 4′-(aminomethyl)fluorescein and a polyclonal antibody solution were used as the immunoreagents. Unbound fluorescent solanidine was detected by CE-LIF (excitation 488 nm, emission 520 nm). Optimum resolution of immunoassay products was achieved with a buffer consisting of 50 mM phosphate, 10% (v/v) methanol, and 1.5 mM SDS, pH 7.5. A plot of signal vs log [GA] produced a sigmoidal curve typical of immunoassays. Analysis of extracts of sprouted Yukon Gold potato tubers and nonsprouted Yukon Gold tubers resulted in total [GA] of 98 μg/g (RSD 9%) and 55 μg/g (RSD 9%), respectively. The findings indicated that CE-LIF coupled with a solution-phase immunoassay can be used to quantify total GA in potatoes.

Keywords: *Amino(methylfluorescein); solanidine; chaconine; solanine; tuber*

INTRODUCTION

For more than 100 years, potato glycoalkaloids (GAs) have been chemically analyzed by a wide range of techniques (Friedman and McDonald, 1997). Currently, both high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) methodologies are used for this analysis. HPLC methods are more frequently used even though detection by UV absorbance is problematic because GAs lack a strong UV or visible chromophore. Extensive sample cleanup is also required to remove interfering compounds that would hinder GA detection at 200−208 nm. This, in turn, increases the analysis time and opportunity for sample loss and error. More recently, Friedman et al. (1994) reported using HPLC with pulsed amperometric detection for analysis of GAs in tomatoes, but to date, it has not been reported for GAs in potatoes.

A number of ELISA methodologies also have been developed for the measurement of total GAs in potatoes (Morgan et al., 1983; Plhak and Sporns, 1992; Stanker et al., 1994). Solid-phase ELISA immunoassays require the attachment of one of the reactants to the solid phase, most commonly a microtiter plate. This presumably slows the rate of antibody−antigen binding because the solid phase constrains the movement of one of the reactants. The necessary multiple washing steps also increase analysis time and decrease the method's precision. Pritchett et al. (1995) affirmed that solid-phase immunoassays were slower and had poorer reproducibility than solution-phase immunoassays. However, the detection of the reaction products in solution-phase immunoassays can be complicated and difficult. Thomson and Sporns (1995) described a solution-phase fluorescence polarization immunoassay for GAs in potatoes that relied on the increase in the polarization of fluorescence when the fluorescent antigen was bound to the antibody relative to the polarization of fluorescence of the free antigen in solution. Alternatively, the products of the antibody−antigen reaction can be analyzed by chromatographic or capillary electrophoretic techniques (Shahedo and Karnes, 1998). In the case of a competitive immunoassay, sample antigen and labeled antigen are allowed to react with a limited amount of antibody. If a high amount of sample antigen is present, antibody binding sites will be occupied by sample antigen, and labeled antigen will be left free in solution. Correspondingly, a low concentration of sample antigen decreases the amount of labeled antigen left free in solution. When the products of this immunoassay are analyzed electrophoretically or chromatographically, the area of the labeled antigen peak is proportional to the amount of sample antigen in the test material.

Capillary electrophoresis (CE) is well suited for immunoanalytical work. The high resolving power of CE can allow separations that would be difficult or impossible by HPLC. Short run times, automated sample injection, and multicapillary arrays permit high sample throughput (Pritchett et al., 1995). Analyte detection has been a problem with CE because of small injection volumes and short optical pathways. The development of laser-induced fluorescence (LIF) detectors has recently led to substantial improvements in sensitivity. Sub-attomole levels of analyte have been detected

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conventionally (Shea, 1997), whereas Chen et al. (1994) found a detection limit of 6 molecules (10 yoctomoles) for a rhodamine dye using CE with a sheath-flow LIF detector. Sensitive CE-LIF immunoassays using fluorescein-labeled antigens have been reported for insulin (Schulz et al., 1995), chloramphenicol (Blais et al., 1994), cortisol (Schmalzing et al.,1995), and morphine and phencyclidine (Chen and Evangelista, 1994). Therefore, the objective of the present work was to develop a CE-LIF method using a precolumn immunoassay technique based on the competition between fluorescein-labeled alkaloid and the native GAs extracted from fresh potato tubers. In this paper, we describe the influence of buffer composition and CE-LIF instrument parameters on the separation of GA immunoassay products.

EXPERIMENTAL SECTION

Materials and Reagents. Instrumentation. During method development, fluorescence excitation and emission spectra were determined with a Perkin-Elmer LS-50 luminescence spectrometer (Perkin-Elmer, Ltd., Beaconsfield, England). A Beckman P/ACE System 2100 CE instrument with a LIF detector was used for all separations (Beckman Coulter Inc., Fullerton, CA). The LIF detector was equipped with a 488 nm laser for excitation and a 520 nm emission filter. Separations were performed on an uncoated fused-silica capillary (Polymicro Technologies Inc., Phoenix, AZ), total length 27 cm, effective length 20 cm, i.d. 50 μm. Column conditioning prior to each injection was done by rinsing the capillary with 0.1 N NaOH for 1 min, followed by reconditioning with buffer for 2.5 min. Unless otherwise stated, the applied voltage was 10 kV, column temperature 25 °C, and sample injection by pressure for 2 s. Data were collected with Beckman System Gold software, version 8.10.

Materials. The fluorescent label, 4′-(aminomethyl)fluorescein (AMF) was purchased from Molecular Probes, Inc. (Eugene, OR), and the other compounds used were >95% analytical purity: α-chaconine, solanidine, sodium dodecyl sulfate (SDS), sodium cholate, 4-(dimethylamino)pyridine (Sigma Chemical Co., St. Louis, MO); α-solanine (Indofine Chemical Co., Inc., Somerville, NJ); pyridine (Fisher Scientific, Edmonton, AB); succinic anhydride, sodium borate (BDH Inc., Edmonton, AB); *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, *N*,*N*-dimethylformamide (Aldrich Chemical Co., Milwaukee, WI). Rabbit polyclonal antiserum was prepared previously using a solanidine−*Limulus polyphemus* hemocyanin immunogen (Plhak and Sporns, 1992). Phosphate-buffered saline (PBS) solution composition was 0.9% NaCl (w/v) and 50 mM phosphate, pH 7.5.

Procedures. Synthesis of Fluorescently Labeled Solanidine. Fluorescently labeled solanidine (AMF-SOL) was prepared according to the method of Thomson and Sporns (1995). AMF-SOL was isolated from the reaction mixture using normal-phase preparative thin-layer chromatography on silica gel Kieselgel $60F_{254}$ 20 × 20 cm, 1000 μm thick plates (E. Merck, Darmstadt, Germany) and a solvent system of ethyl acetate/methanol/aqueous ammonia (79:20:1, v/v/v). The band corresponding to AMF-SOL was scraped from the plate, extracted with methanol:methylene chloride (3:1, v/v) and subsequently filtered. The solvent was removed under reduced pressure and the residue was taken up in pure methylene chloride. This extract was filtered through glass wool and then evaporated under vacuum. The residue was then dried over phosphorus pentoxide. A stock solution (5040 nM) of AMF-SOL was prepared in methanol and stored in the dark at 4 °C. Standards for determining the linearity of CE-LIF response were prepared in PBS containing 10% (v/v) methanol and ranged from 0 to ca. 250 nM AMF-SOL.

Antibody Dilution Curve. Antibody solutions were made from the rabbit serum collected by Plhak and Sporns (1992). The serum was diluted (v/v) with PBS to cover ranges between 1:4 and 1:300. The serum dilutions were mixed 1:1 (v/v) with 400 nM AMF-SOL in PBS containing 20% (v/v) methanol in polypropylene microcentrifuge tubes and allowed to equilibrate for 30 min at room temperature. The dilution ranges in the final solutions were 1:8 to 1:600 and the AMF-SOL concentration in the final solution was 200 nM. After equilibration, the serum dilutions were analyzed by CE-LIF, and the AMF-SOL peak area was plotted against serum dilution.

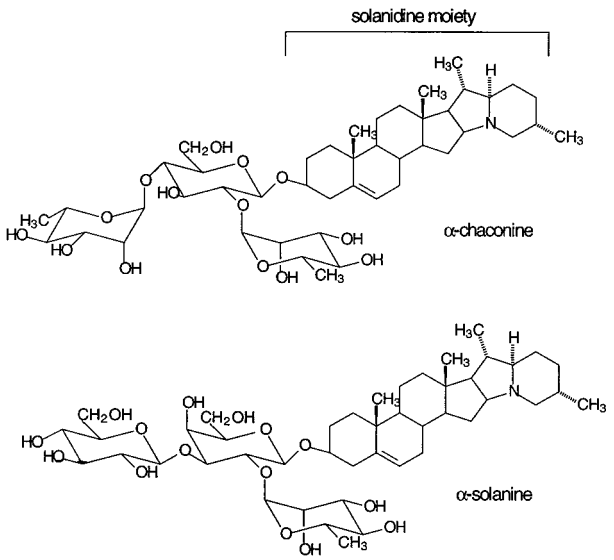

Figure 1. Structural diagrams of α-chaconine and α-solanine.

GA Extraction From Potatoes. GAs were extracted from freeze-dried potatoes using a modification of a procedure described by Plhak and Sporns (1992). Samples (1.00 g) were initially moistened with 5 mL of Milli-Q water. The sample was then extracted three times by homogenizing in 15 mL of HPLC-grade methanol for 1 min using a Polytron homogenizer (Kinematica AG, Littau, Switzerland). After each extraction, the sample was centrifuged using a Beckman J2-21 centrifuge (Beckman Coulter Inc., Fullerton, CA) at 1960*g* for 5 min. The supernatants from each of the three extractions were then combined and filtered through a Whatman No. 4 filter into a 50 mL volumetric flask. The extract was brought to volume with methanol and stored at 4 °C until it could be analyzed. Prior to analysis, 0.5 mL of the extract was diluted to 50 mL with PBS and methanol so that the final methanol content was 10% (v/v).

RESULTS AND DISCUSSION

Fluorescently Labeled Solanidine. The immunoassay component of the analytical procedure was based on the competition between fluorescently labeled alkaloid and native GAs from the potato extract. The antibody used in this experiment recognizes an epitope on the alkaloid portion of the GA molecule, and hence it was not imperative to preserve the carbohydrate moiety (Plhak and Sporns, 1992). The most common alkaloid moiety of potato GAs is solanidine; the two major potato GAs, solanine and chaconine, differ only in the carbohydrates attached to solanidine (Figure 1). GA antibody exhibited strong affinity (K_{aff} approximately 4×10^8) for the fluorescent solanidine molecule synthesized by Thomson and Sporns (1995), and consequently it was decided to synthesize the same fluorescent conjugate for the present work.

The synthesized AMF-SOL exhibited peak excitation at 497 nm and peak emission at 518 nm, compared to 492 and 512 nm, respectively, for the 4′-(aminomethyl)fluorescein starting material. CE-LIF detection of the AMF-SOL revealed the presence of several contaminants that fluoresced similarly (Figure 2). The identity

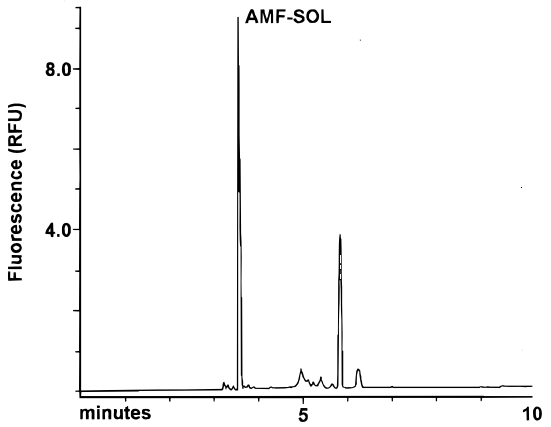

Figure 2. CE-LIF electropherogram of stock solution of AMF-SOL showing the presence of several contaminants.

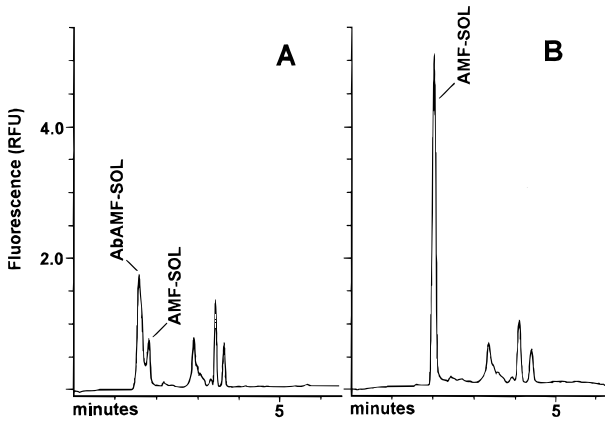

Figure 3. CE-LIF electropherograms showing the effect of anti-GA serum on AMF-SOL peak area (A) and the effect of anti-brochocin C serum on GA peak area (B).

of these contaminants was not determined. CE-LIF detection of the original 4′-(aminomethyl)fluorescein confirmed that the newly synthesized AMF-SOL was not contaminated with any starting material. Treatment of the AMF-SOL solution with anti-GA serum reduced the peak area of only the AMF-SOL peak, and this indicated that none of the contaminants had noticeable affinity for the GA antibody. To test for nonspecific binding, the AMF-SOL solution was treated with serum raised against a brochocin C (Kwok, 1997) that was not expected to show any affinity for GA. The AMF-SOL peak area was not affected by treatment with anti-brochocin serum (Figure 3), and thus further purification was deemed unnecessary because (1) the contaminants appeared well resolved from the main AMF-SOL peak, and (2) none of the contaminants showed affinity for the GA antibody.

Effect of pH on Fluorescence of AMF-SOL. The fluorescence intensity of AMF-SOL increased rapidly with an increase in pH and reached a plateau at ca. pH 7.5. The effect of pH on fluorescence intensity is consistent with data reported for similar fluorescein derivatives (Bieniarz et al., 1994; Babcock and Kramp, 1983) and reflects the more intensely chromophoric dianionic species. The pH curve implies a pK_a ca. 6.5 for the phenolic hydrogen of AMF. To optimize fluorescence intensity, a CE buffer with a pH > 7.5 would have been preferred, but antibody affinity was expected to be strongest near a more neutral pH value. GA solubility is also compromised at higher pH values. On the basis of these considerations, CE buffers with pH 7.5 were developed.

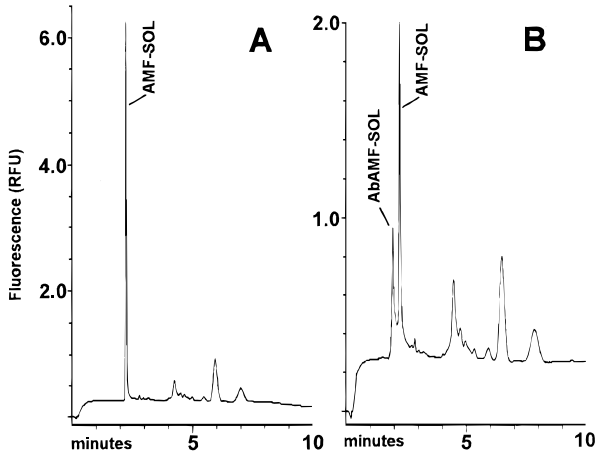

Figure 4. CE-LIF separation of unbound AMF-SOL from AMF-SOL-antibody complex using 50 mM borate, 10% (v/v) methanol, pH 7.5 as the running buffer: (A) 200 nM AMF-SOL; (B) 200 nM AMF-SOL in the presence of anti-GA serum.

CE-LIF Analysis of Immunoassay Products. Buffer composition is one of the principal tools affecting separation in CE. Coupling an immunoassay to CE-LIF introduces numerous limitations to buffer selection because harsh conditions will reverse antigen−antibody binding. It is noteworthy that either extremes in pH or high concentrations of organic solvent will induce the antibody to release the antigen (Harlow and Lane, 1988). The search for a suitable buffer was begun using 50 mM phosphate, pH 7.5, and under these conditions the AMF-SOL peak had a somewhat irregular shape and inconsistent reproducibility. A standard curve produced with AMF-SOL (no precolumn immunoassay) using this buffer resulted in a correlation coefficient of 0.982. The solubility of AMF-SOL in such an aqueous buffer was also a potential problem, and this necessitated evaluation of the effects of different concentrations of methanol in the buffer on retention time and AMF-SOL peak shape. The addition of up to 10% (v/v) methanol had little effect on peak shape, and the correlation coefficient for the standard curve improved slightly to 0.991. Nevertheless, increasing the methanol content to 15% (v/v) resulted in peak tailing. Plhak and Sporns (1992) had successfully performed GA ELISAs in up to 50% methanol, indicating that 10% methanol should readily be tolerated in our running buffer. However, when anti-GA serum was introduced to the AMF-SOL solution, free AMF-SOL could not be adequately resolved from bound AMF-SOL with the 50 mM phosphate, 10% (v/v) methanol running buffer.

The ionic strength of the phosphate buffer was increased from 50 mM to 75 mM, resulting in an increase of the migration time for the AMF-SOL by 0.3 min, but the resolution was not improved. Separation of free AMF-SOL and bound AMF-SOL was then evaluated using cholate (25 mM, pH 7.50) and borate (50 mM, pH 7.50) buffers, each containing 10% (v/v) methanol. The cholate buffer was not able to resolve the AMF-SOL from its impurities, thereby eliminating it from further consideration. However, the borate buffer did partially resolve the unbound AMF-SOL from the bound AMF-SOL (Figure 4). Borate buffers have often been used to separate sugars because borate forms an anionic complex with the hydroxyl groups on the sugar (El Rassi, 1996). In this instance, the AMF-SOL molecule

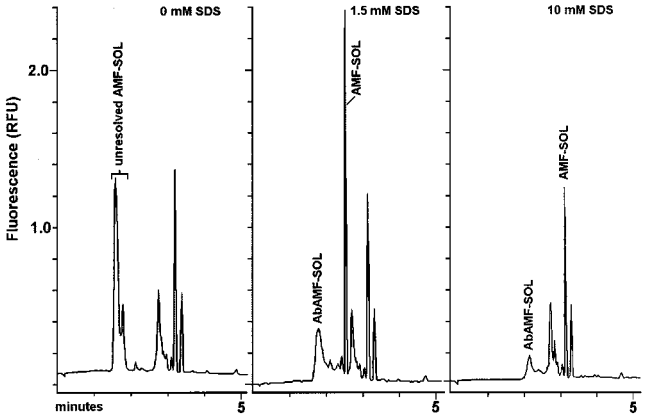

Figure 5. Effect of SDS concentration on the resolution of unbound AMF-SOL from AMF-SOL−antibody complex. SDS was added to 50 mM phosphate, 10% (v/v) methanol, pH 7.5.

did not contain a carbohydrate group and the borate probably acted simply as a buffer and not as an anionic complexing agent. Although the borate buffer showed some resolution potential, the peak areas were substantially less compared to those separated with the phosphate buffer. The reason for this may relate to poor solubility of AMF-SOL in the borate buffer.

Surfactants such as SDS and cetyltrimethylammonium bromide (CTAB) have been used at concentrations below their critical micelle concentration (CMC) to alter endoosmotic flow and improve solubility of hydrophobic solutes (Hu et al., 1995). When added at levels higher than the CMC, the mode of separation changes from capillary zone electrophoresis (CZE) to micellar electrokinetic capillary chromatography (MECC). The CMC for SDS is 8.1 mM (Beckman, 1994). SDS was added to the 50 mM phosphate, 10% (v/v) methanol buffer at 1.5 mM, 10 mM, and 100 mM. AMF-SOL migration time increased with SDS concentration in the buffer. Figure 5 shows that the addition of 1.5 mM SDS did resolve free AMF-SOL from bound AMF-SOL. Concentrations above the CMC further resolved the free AMF-SOL from bound AMF-SOL, but detector response decreased and resolution of free AMF-SOL from the AMF-SOL impurities decreased. Subsequently, all separations were performed at 25 °C, using 50 mM phosphate containing 1.5 mM SDS and 10% (v/v) methanol, pH 7.5.

The role of SDS in improving the resolution of free AMF-SOL and bound AMF-SOL is not clear. Hydrophobic interactions between SDS and the alkaloid may have resulted in a shift in migration time. Alternatively, SDS has a strong affinity for a protein moiety and may have shifted the antibody migration time away from that of AMF-SOL. Other researchers have reported that antibody−antigen binding remains unaffected by up to 75 mM SDS (Steinman et al., 1995) even though SDS has the potential to open the tertiary structure of a protein, which possibly could alter the affinity of the antibody for the antigen. Nevertheless, our results indicate that 1.5 mM SDS in the running buffer facilitates satisfactory separation of the compounds of interest. An Ohm's Law plot revealed that current increased linearly with voltage to about 10 kV. Reduction of voltage from 12 to 10 kV made a noticeable improvement in the quality of the results, although run time increased. An AMF-SOL standard curve generated at 10 kV produced a correlation coefficient of 0.998.

Effect of Injection Mode. Injection of samples for analysis using CE-LIF can be performed by pressurizing the sample vial for a set period of time or by applying a voltage to the sample vial so that the solute migrates into the capillary as a result of both electrophoretic migration and electroosmotic flow. A 2 s pressure injection was found to be adequate and any attempt at electrokinetic injection was unsuccessful. Multiple injections of 200 nM AMF-SOL indicated a relative standard deviation (RSD) of 5−6% for peak areas. This level of injection precision was constant throughout the course of the experiment.

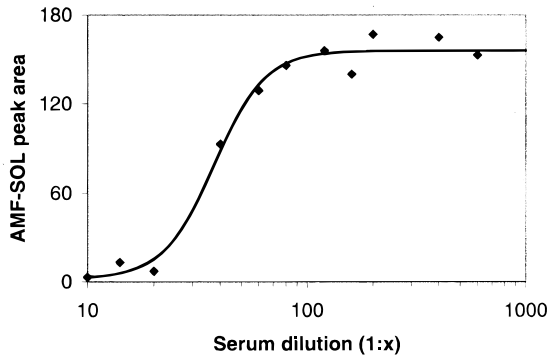

Figure 6. Serum dilution curve showing the effect of anti-GA serum dilution on AMF-SOL peak area.

Immunoassay Conditions. Trial-and-error assessments indicated that analysis of potato extracts was feasible when an AMF-SOL concentration of 200 nM was used in the immunoassay reaction mixture. To determine the optimal serum dilution for the immunoassay, dilution curves were prepared by allowing 200 nM AMF-SOL to react with various amounts of serum. The optimal serum dilution required for the competitive immunoassay was then determined. The curve prepared with 200 nM AMF-SOL showed that a serum dilution of 1:30 resulted in an approximately 80% reduction in the AMF-SOL peak area (Figure 6). Under these conditions, it was estimated that the addition of native GA from a potato extract should induce the greatest increase in the unbound AMF-SOL peak area. The high serum concentration appears to be a characteristic of fluorescent solution-phase immunoassays (Thomson and Sporns, 1995).

The finalized immunoassay protocol in the present study contained a 1:30 serum dilution and 200 nM AMF-SOL in the final solution. Specifically, 250 μL of the test solution (GA standard or diluted potato extract) was mixed with 250 μL of 600 nM AMF-SOL solution and 250 μL of the 1:10 serum dilution. The mixture was allowed to equilibrate for 30 min before analysis by CE-LIF. Peak areas were used to determine total GA concentration in the potato extracts.

Potential for Quantification of GAs in Potatoes. The AMF-SOL, by itself, exhibited a linear response ($r > 0.990$). When the products of the immunoassay were analyzed by CE-LIF, the area of the unbound AMF-SOL peak was found to be proportional to [GA] in the test solution. Competitive immunoassays are typically plotted logarithmically and have a relatively concise linear range. Figure 7 shows that a plot of the unbound AMF-SOL peak area versus the [GA] in the test solution produced a typical sigmoidal curve. Analysis of extracts of sprouted Yukon Gold potato tubers and nonsprouted Yukon Gold tubers resulted in total [GA] of 98 μg/g (RSD 9%) and 55 μg/g (RSD 9%), respectively. These values fall within the expected range of total [GA] for potato tubers (Friedman and McDonald, 1997). Matrix-assisted

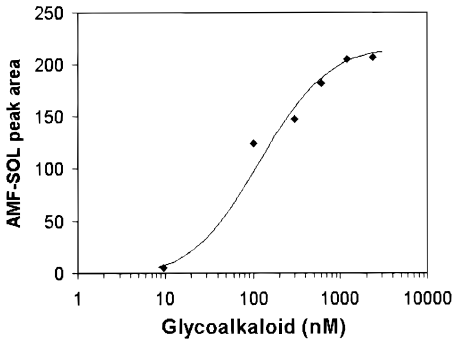

Figure 7. Sigmoidal calibration curve for total [GA] determined by a solution-phase immunoassay and CE-LIF.

laser desorption/ionization time-of-flight mass spectrometric analysis of the nonsprouted sample produced a value of 65 μg/g (Driedger and Sporns, 1999). Subsequent experiments are planned to determine the accuracy and precision of these initial findings.

Conclusions. It is possible to use CE-LIF to separate and quantify the products of a solution-phase GA immunoassay. Nevertheless, the separation of the antibody-bound AMF-SOL from unbound AMF-SOL proved surprisingly difficult given the large size difference of the molecules. The antibody−AMF-SOL complex has a molecular weight of ca. 150,000 compared to 841 Da for AMF-SOL. Apparently, the molecules have a similar mass-to-charge ratio despite their difference in size. The incorporation of sub-CMC levels of SDS into the running buffer was important for separation of the immunoassay products, and other surfactants may warrant evaluation in future studies. The fluorescence intensity of AMF-SOL decreased quickly when the pH was below 7.0. Hence, the useful range of the present buffer is between pH 7 to 8. Raising the buffer pH above 8 would be expected to affect antigen−antibody binding. Based on the present results, further experiments are planned to validate the precision and accuracy of coupling CE-LIF and a solution-phase immunoassay for analysis of GAs in potatoes.

ABBREVIATIONS USED

AMF, 4′-(aminomethyl)fluorescein; AMF-SOL, solanidine labeled with AMF; CE, capillary electrophoresis; CZE, capillary zone electrophoresis; CMC, critical micelle concentration; CTAB, cetyltrimethylammonium bromide; ELISA, enzyme-linked immunosorbent assay; GA, glycoalkaloid; HPLC, high-performance liquid chromatography; LIF, laser-induced fluorescence; MECC, micellar electrokinetic capillary chromatography; PBS, phosphate-buffered saline solution; RSD, relative standard deviation; SDS, sodium dodecyl sulfate.

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