# INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

University of Alberta

# Analysis of Potato Glycoalkaloids by Immunoassay Coupled to Capillary Electrophoresis or Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

by

**Darcy Renard Driedger** 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 2000

University of Alberta

Library Release Form

**Name of Author:** Darcy Renard Driedger

**Title of Thesis:** Analysis of Potato Glycoalkaloids by Immunoassay Coupled to Capillary Electrophoresis or Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry

**Degree:** Doctor of Philosophy

**Year this Degree is Granted:** 2000



Box 411
Duchess, AB T0J 0Z0

Date: 18 January 2000

University of Alberta

Faculty of Graduate Studies

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Analysis of Potato Glycoalkaloids by Immunoassay Coupled to Capillary Electrophoresis or Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry submitted by Darcy Renard Driedger in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science and Technology.

Dr. P. Sporns (Supervisor)

Dr. L. Li

Dr. T. Vasanthan

Dr. B. Ooraikul

Dr. R. Yada (External Examiner)

Date: 17 Jan 2000

## ABSTRACT

The use of separation or spectroscopic techniques to analyze the products of an immunoassay can result in faster analysis times, more accurate analyte identification, or increased sensitivity. The objective of this thesis was to develop methods for determining potato glycoalkaloids (GAs) by coupling immunoassays with capillary electrophoresis or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

A solution-phase immunoassay for the determination of GAs was developed based on quantitation by capillary electrophoresis with laser-induced fluorescence detection. Solanidine coupled to 4'-(aminomethyl)fluorescein and polyclonal antibody solution were used as the immunoreagents. Unbound fluorescent solanidine was detected by capillary electrophoresis. A calibration curve of signal vs log [GA] was linear from 50-400 nM. The relative standard deviation (RSD) for duplicate and day-to-day analyses averaged 5.7% and 12%, respectively. Spike recoveries ranged from 85-97% for spike levels ranging from 43-170 μg/g fresh potato.

MALDI-TOF MS was used to determine individual potato GA concentrations in tubers. Samples were extracted with 50% aqueous methanol, deposited on 2',4',6'-trihydroxyacetophenone crystals and analyzed by MALDI-TOF MS. Analyte ion intensities relative to an internal standard were used to determine α-chaconine and α-solanine concentrations. The RSD of triplicate measurements ranged from 1 to 16%, with an average of 9%. The day-to-day RSD for replicate determinations was 11%. Analyst prepared spike recoveries (50 μg/g) averaged 104% for α-chaconine (RSD 8%)

and 98% for α-solanine (RSD 4%). The method limit of detection was estimated to be 2 μg/g. The method was modified to determine GAs in potato pulp, potato protein concentrate, fruit water, and starch.

A sample purification technique was developed for detecting GAs in blood serum by 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 methanol. MALDI-TOF MS was used to detect the GAs in the methanol eluent. α-Chaconine and α-solanine were detected in serum spiked with 1 ng/mL of each GA. This represents the first known use of immunoaffinity purification with MALDI-TOF MS for food analysis.

**Acknowledgements**

Many individuals and organizations supported my Ph.D. work in a very tangible way, either by donating samples or instrument time or with financial support: Department of Agricultural, Food and Nutritional Science; American Chemical Society, Dr. W. Bergthaller, Federal Centre for Cereal, Potato and Lipid Research; Patricia Duplessis, Alberta Agriculture, Food and Rural Development; Faculty of Graduate Studies and Research; Dr. O. Hindsgaul, Department of Chemistry; Natural Sciences and Engineering Research Council; Potato Development Incorporated; Alan Stuart, Potato Growers of Alberta; Joan Turchinsky, AFNS; University of Alberta. Their support is gratefully acknowledged. I would also like to acknowledge Amanda Fraser for her assistance with some of the ELISA analyses.

I am thankful for the contributions made by the members of my supervisory and examining committees: Drs. Liang Li (Department of Chemistry); Mike McBurney; Eileen LeBlanc; Thava Vasanthan; Feral Temelli, Buncha Ooraikul, and Rickey Yada (University of Guelph). I am especially grateful for my supervisor, Dr. Peter Sporns, who taught me many things, provided excellent support, and inspired me.

Len Steele and Jian Wang provided many ideas, a lot of technical assistance, some thoughtful critique, and plenty of humor throughout my time at the University of Alberta.

## Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| AchE | acetylcholinesterase |
| CAN | acetonitrile |
| AMF | 4'-(aminomethyl)fluorescein |
| BSA | bovine serum albumin |
| BuChE | butyrylcholinesterase |
| $C_{18}$ | octadecyl silane |
| CE | capillary electrophoresis |
| Chol | cholesterol |
| CMC | critical micelle concentration |
| CZE | capillary zone electrophoresis |
| ELISA | enzyme-linked immunosorbent assay |
| EOF | electroosmotic floe |
| EtOH | ethanol |
| GA | glycoalkaloid |
| HOAc | acetic acid |
| HPLC | high performance liquid chromatography |
| LIF | laser-induced fluorescence |
| LPH | *Limulus polyphemus* hemocyanin |
| LSIMS | liquid secondary ion mass spectrometry |
| m/z | mass-to-charge ratio |
| MALDI | matrix-assisted laser deorption/ionization |
| MECC | micellar electrokinetic chromatography |
| MeOH | methanol |
| MLOD | method limit of detection |
| MS | mass spectrometry |
| PBS | phosphate-buffered saline |
| PBST | phosphate-buffered saline with Tween |
| PC | phosphatidyl choline |
| pI | isoelectric point |
| PPC | potato protein concentrate |
| RSD | relative standard deviation |
| SDS | sodium dodecyl sulfate |
| SPE | solid-phase extraction |
| TFA | trifluoroacetic acid |
| THAP | 2',4',6'-trihydroxyacetophenone |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| TOF | time-of-flight |
| UV | ultraviolet |

# Chapter 1

## Introduction

Members of the *Solanum* genus synthesize a variety of nitrogen-containing steroidal glycosides commonly termed glycoalkaloids (GAs). α-Chaconine and α-solanine account for at least 95% of the GAs in commercial potato varieties (*Solanum tuberosum*)(Friedman and McDonald, 1997). These two GAs share the same solanidine aglycone and differ only in the sugar moieties at position 3 of the aglycone (Table 1-1). Potato varieties that contain other GAs have usually been derived from crosses with wild varieties. For example, the Kennebec variety of potato reported to contain α- and β-solamarine was derived from wild *S. demissum* Lindl. (Shih and Kuć, 1974) and solasodine glycosides have been identified in *S. tuberosum* × *S. vernei* crosses (van Gelder and Scheffer, 1991). In an unusual case, Sinden and Sanford (1981) identified solamarine in White Rose, a potato cultivar with only *S. tuberosum* as known ancestors. Tomatine is a tomato (*Lycopersicon esculentum*) GA that very rarely occurs in the *Solanum* genus, despite having an aglycone structure similar to the solamarine aglycone (Figure 1-1).

GAs are distributed throughout the potato plant (Table 1-2). The highest concentrations appear to be associated with the parts of the plant with greatest metabolic activity, especially the sprouts and the flowers (Achterberg et al., 1979). Tuber GA levels are comparatively low but can become elevated when exposed to stress, such as

**Table 1-1 Selected *Solanaceae* glycoalkaloids**

| Glycoalkaloid | Glycoside | Aglycone |
|---|---|---|
| α-chaconine | chacotriose | solanidine |
| α-solanine | solatriose | solanidine |
| α-solamarine | chacotriose | tomatidenol |
| β-solamarine | solatriose | tomatidenol |
| tomatine | lycotetraose | tomatidine |

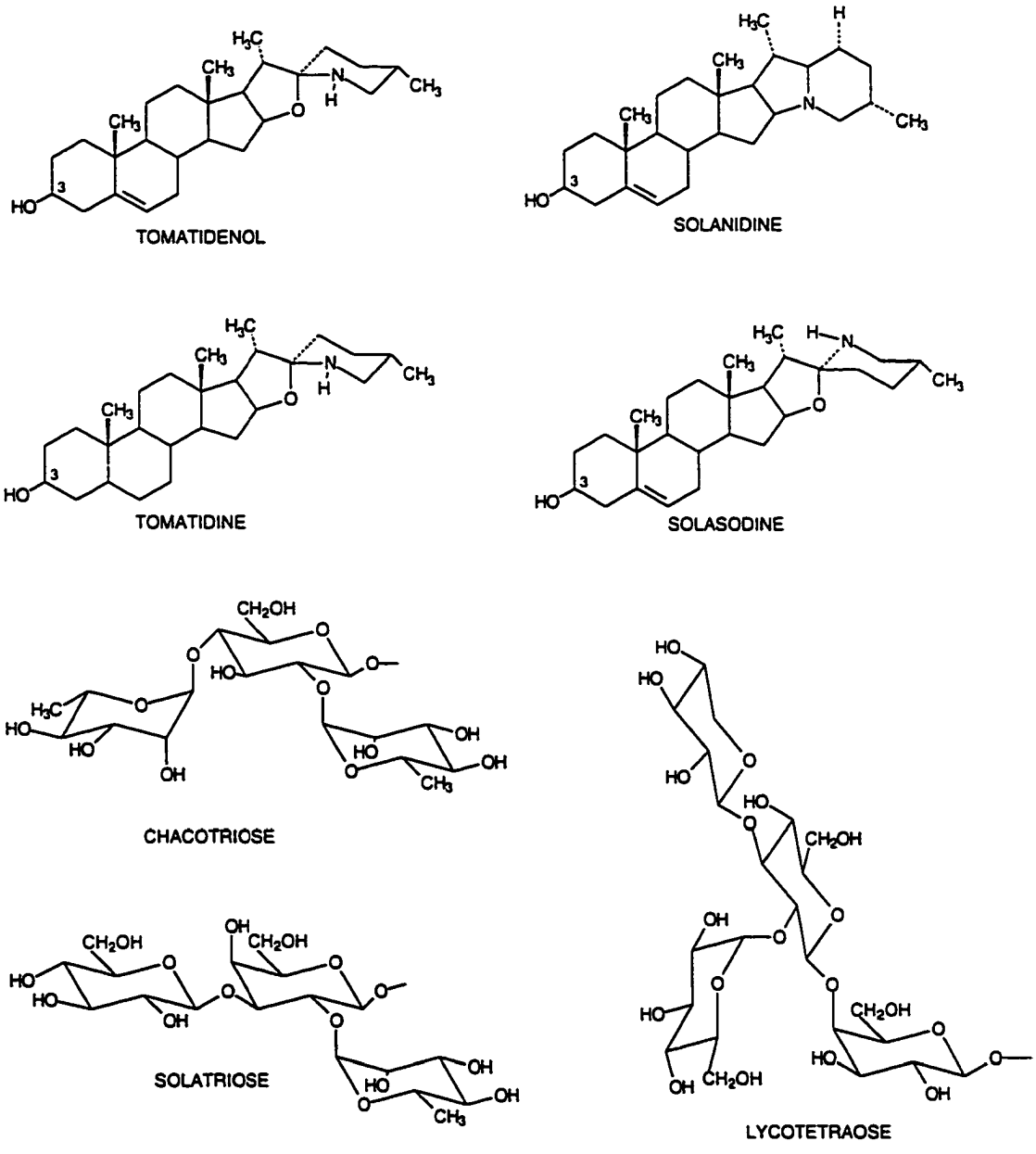


**Figure 1-1 Structures of selected *Solanaceae* aglycones and glycosides.**

**Table 1-2 Distribution of total glycoalkaloids in potato cultivar NDA 1725**

| | Total GA (mg/kg) |
|---|---|
| Roots | 860 |
| Main stems | 320 |
| Small stems | 450 |
| Leaves | 1,450 |
| Sprouts | 10,000 |
| Flesh | 150 |

Adapted from Friedman and Dao (1992)

injury or exposure to light or temperature extremes. The distribution in the plant and the response to stress have lead some researchers to postulate that GAs evolved to confer a degree of pest resistance to the plant. Correlations have been reported between foliar GA levels and resistance to the Colorado potato beetle (Sinden et al., 1980) and the potato leafhopper (Tingey et al., 1978). However, other studies have found no relationship between GA levels and resistance to peach aphids, potato aphids, fleabeetles, leafhoppers, or Colorado potato beetles (Tingey and Sinden, 1982; Flanders et al., 1992).

The potential for potatoes to be toxic has long been suspected (Harris and Cockburn, 1918). More recently, it has been confirmed that potato GAs can disrupt cell membranes in the digestive tract and other organs and can negatively affect the central nervous system. Because potatoes are so widely consumed, the consequences of elevated GA levels can be serious. Hall (1992) has claimed that GAs and cyanogenic glycosides are responsible for far more human illness and death than any other plant toxicants, however, data to support this claim do not exist and would be very difficult to obtain. Nevertheless, others have echoed these concerns, "Despite the fact that levels of GA are much lower in modern cultivars than in wild progenitors, if the potato were introduced today as a novel food, it is quite possible that its use would not be approved because of the presence of these potentially toxic compounds" (Smith et al., 1996). Morris and Lee (1984) have suggested that the widely used 200 mg/kg fresh weight upper limit for GAs in potato tubers only provides a 4-fold safety margin, while Hellenäs (1994) says the 200 mg/kg limit must be regarded as "unsatisfactory".

Despite the widespread consumption of potatoes and persistent concerns about the toxicity of potato GAs, remarkably little is known about how GAs are metabolized by the body. Toxicological research has been limited by the ability of common analytical techniques to detect GAs in blood and other organs. Various colorimetric, chromatographic, and immunoassay techniques can detect and quantify GAs in potato tubers where concentrations typically range from 10-100 mg/kg. However, GA concentrations in blood and other organs are approximately 1,000 times lower than in potato tubers. In order to directly measure GAs at this level, novel sample preparation and detection techniques will be required. Recently, immunopurification techniques have been used together with mass spectrometry to detect very low levels of snake venom toxin (Nelson et al., 1995), cytochrome c (Papac et al., 1994a), transferrin (Nakanishi et al., 1994), and lysozyme (Brockman and Orlando, 1995).

This review will address the significance of potato GAs in the human diet and current methods for GA analysis. It will also address the potential of using capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for GA analysis. Numerous other reviews of potato GAs have been written, with notable ones covering general properties of potato GAs (Jadhav et al., 1997), chemistry, analysis, safety, and plant physiology (Friedman and McDonald, 1997), metabolism and toxicity (Morris and Lee, 1984; Smith et al., 1996), and bioactivity (Plhak and Sporns, 1997). This paper will explore how recent advances in analytical chemistry might be applied to GA analysis, with special emphasis the problems associated with analyzing GAs in blood and other tissues.

## DIETARY AND TOXICOLOGICAL SIGNIFICANCE OF POTATO GLYOCALKALOIDS

### Glycoalkaloid Flavor

GAs impart a bitter taste to potatoes. It has been suggested that low GA concentrations are associated with typical mild or savory potato flavor (Ross et al., 1978).

However, as levels increase, the flavor imparted by GAs becomes decidedly bitter. Sinden et al. (1976) performed the most conclusive study on GA flavor to date. They reported that potatoes containing >140 mg GA/kg were consistently perceived as bitter. As concentrations approached 250 mg/kg, panelists reported a burning sensation that left a sore or scratchy throat that persisted for hours after tasting the sample. Kaaber (1993) reported that GA concentrations >170 mg/kg potatoes produced a burning sensation. Zitnak and Filadelfi (1985) confirmed the bitter and burning sensation of pure GA solutions.

The above studies would suggest that toxic levels of GA (>200 mg/kg) should be readily perceived by consumers, especially since GAs are generally unaffected by baking, microwaving, boiling, or frying (Bushway and Ponnampalam, 1981). However, when potatoes are consumed, they are often flavored with gravy, sauces, cheese, curry, or other spices, which would presumably mask the bitterness. The existence of GA poisoning cases is evidence that people will consume toxic amounts of GA despite the harsh flavor.

**Cell Membrane Lysis**

GAs appear to have two toxic effects on the body; they can disrupt cell membranes and they inhibit cholinesterase activity. Other mechanisms of toxicity, such as liver damage and teratogenicity, are still debated and have been thoroughly reviewed by Friedman and McDonald (1997). GAs have conclusively been shown to disrupt membranes in several types of cells, including erythrocytes (Roddick et al., 1988) and epithelial cells (Keukens et al. 1996). Although GAs contain a lipophilic alkaloid moiety and an hydrophilic sugar moiety, surfactant properties do not appear to play a major role in membrane disruption. GAs only affect membranes when sterols are present in the membrane (Keukens et al., 1992).

Roddick (1979) showed that potato GAs have the ability to associate or bind with various sterols. His later work found that cholesterol bound more readily with α-chaconine than with α-solanine (Roddick et al., 1988). α-Chaconine also caused more erythrocyte lysis than α-solanine. However, α-solanine and α-chaconine act

synergistically so that a 70% α-chaconine/30% α-solanine mixture is more hemolytic than pure α-chaconine. The membrane lytic effect of the aglycone solanidine appears to be intermediate to α-chaconine and α-solanine (Keukens et al., 1992). The type of sterol present in the membrane also affects the degree of lysis. In general terms, more lysis occurs when β-sitosterol or fucosterol are present than when cholesterol and ergosterol are present in the membrane layer (Keukens et al., 1992). Nevertheless, significant lysis does occur with cholesterol which is a major sterol in mammalian cell membranes.

The nature of the carbohydrate portion of the GA molecule appears to be very important in membrane disruption. α-Solanine and α-chaconine differ only in the composition of their trisaccharide substituents, yet α-chaconine is significantly more lytic. Removing any one sugar residue from α-chaconine also dramatically reduces its lytic ability (Keukens et al., 1995). On the other hand, α-solanine acts synergistically with α-chaconine to increase their lytic ability when they are present together. These data suggest that sugar-sugar interactions are important in GA action on membranes. The three-dimensional fit between sugar groups might be what determines the degree of membrane disruption. Keukens et al. (1995) have proposed a model of membrane disruption that involves the formation of membrane buds (Figure 1-2). Electron microscopy was used to show that tubular GA/sterol structures can be isolated from cholesterol containing vesicles that were exposed to GA. These tubular structures were taken as evidence of membrane buds. This model, however, does not explain how the aglycone can cause membrane disruption.

Several symptoms associated with GA poisoning also indicate that membrane disruption is an important mechanism of toxicity. Vomiting, diarrhea (sometimes including blood), and severe abdominal pain are typical of GA poisoning (Morris and Lee, 1984). These symptoms, as well as the burning sensation in the mouth, are all consistent with membrane disruption.

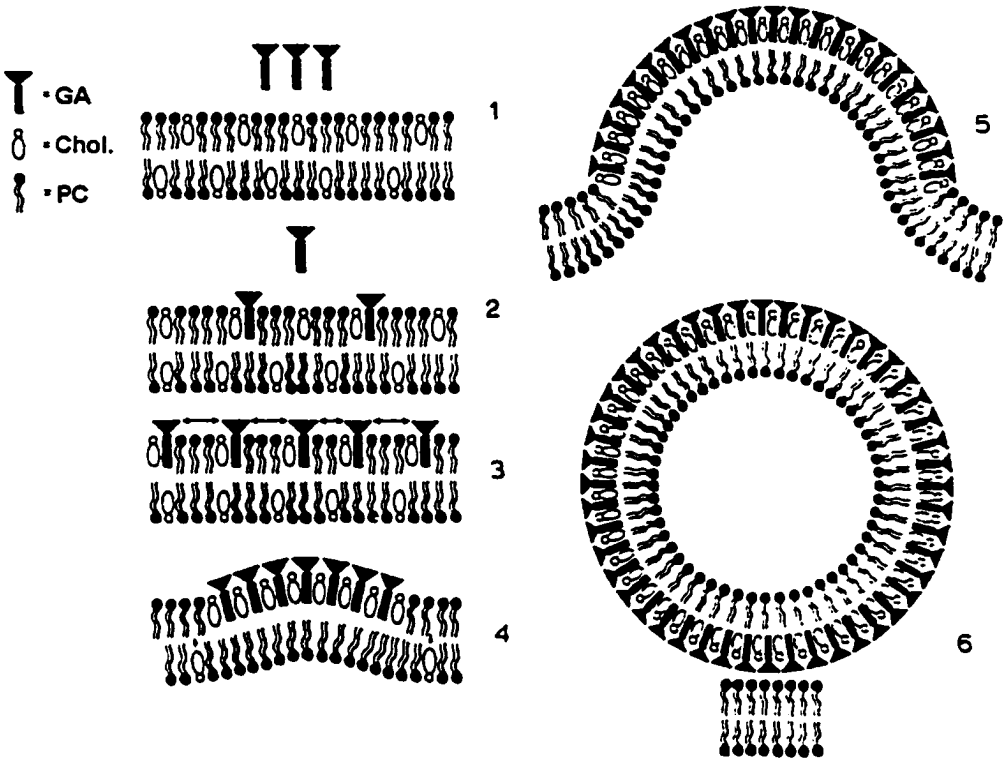


**Figure 1-2 Proposed Mechanism of Membrane Disruption by Glycoalkaloids.**

1. Insertion of the aglycone part in the membrane bilayer.
2. The glycoalkaloid binds on a 1:1 ratio with the sterol present.
3. When the density of GA in the membrane reached a certain level, sugar-sugar interactions between the sugar moieties of the GA cause the formation of a stable GA-sterol matrix.
4. Sterols from the inner membrane layer flip to replace the immobilized sterols in the matrix.
5. The matrix will cause the membrane to "bud" because of the relatively large sugar groups.
6. The bud will eventually close in on itself, creating a new membrane structure.

GA - glycoalkaloid
Chol - cholesterol
PC - phosphatidylcholine

**Cholinesterase Inhibition**

Cholinesterase enzymes are a key component of the nervous system. Acetylcholinesterase (AChE) is the main cholinesterase enzyme that hydrolyzes the neurotransmitter acetylcholine. The primary function of AChE is to clear acetylcholine from the receptor sites of neurons (nerve cells) and muscle cells. Nervous impulses are transmitted along a neuron until the impulse reaches the end of the cell and must be passed along to the next neuron. Acetylcholine is one of the transmitter chemicals that facilitates this transfer to the next cell. Acetylcholine is released from motor neurons in fairly well defined amounts of about 10,000 molecules in response to discrete nerve impulses. Acetylcholine diffuses across the synaptic cleft (or neuromuscular junction) and binds to receptor sites on the next neuron or effector cell. The binding of the transmitter causes an alteration in the membrane polarization and a new impulse may be generated in that cell.

Normally the time it takes for AChE to hydrolyze the acetylcholine is less than the time it takes for the membrane depolarization to decay. This fast response by AChE is required in order to clear the acetylcholine from the receptor sites before the next wave of acetylcholine arrives. If the acetylcholine is not hydrolyzed quickly, subsequent nerve impulses and muscle membrane depolarization will begin to overlap. The physiological effects of overlapping nerve impulses are muscle spasms, followed by complete loss of function due to continuous depolarization of the muscle cell membrane.

Butyrylcholinesterase (BuChE) is also able to hydrolyze acetylcholine and has several characteristics that differentiate it from AChE.

1. BuChE is the most prevalent soluble circulatory cholinesterase and is widely distributed in the body.
2. BuChE is less substrate specific than AChE and often interacts at higher rates.
3. Many mutations have been identified on the gene for BuChE, giving rise to numerous variants of BuChE (atypical BuChE) in the human population. In contrast, only one mutation has been identified for the AChE gene.

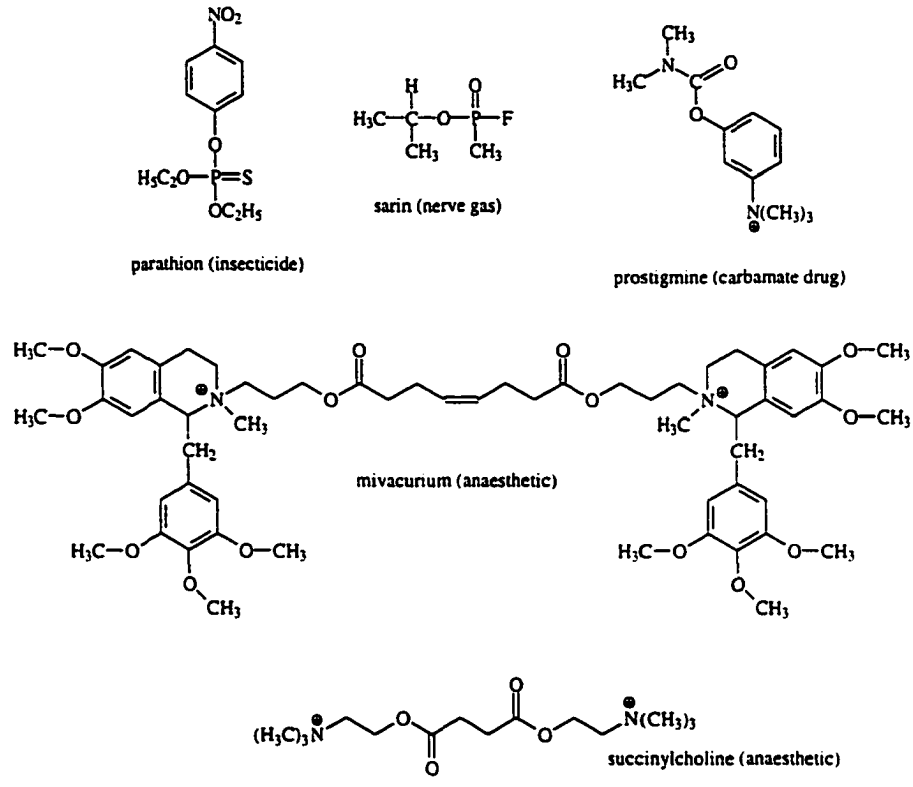


**Figure 1-3 Cholinesterase inhibitors.**

Because BuChE can interact with a wide range of substrates and is widely distributed in the body, it is thought that one of its major roles is to act as a scavenger of cholinesterase inhibitors so that AChE is protected (Loewenstein-Lichtenstein et al., 1995).

Several compounds are known to inhibit the cholinesterases (Figure 1-3). Organophosphates are effective pesticides and chemical warfare agents because of their ability to phosphorylate AChE and thereby render the enzyme ineffective (Marrs, 1993). Ester anaesthetics, such as succinylcholine (suxamethonium) and mivacurium, cause neuromuscular blockade by inhibiting cholinesterase (Davis et al., 1997). Carbamates also inhibit the enzyme. Schwarz et al. (1995) has reviewed the effects of drugs and poisons on cholinesterases. Potato GAs are some of the few natural cholinesterase inhibitors that can inhibit both AChE and BuChE (Krasowski et al., 1997) (Table 1-3).

**Table 1-3 Cholinesterase Inhibition by Potato Glycoalkaloids**

| Compound | Cholinesterase | Glycoalkaloid | Inhibition | Reference |
|---|---|---|---|---|
| solanidine | human BuChE | 100 μM | 65% normal<br>2-14% atypical | Neville et al., 1990 |
| α-solanine | eel ChE | 38 μM | 26% | Bushway et al., 1987 |
| solanidine | | 84 μM | 15% | |
| α-chaconine | | 39 μM | 27% | |
| α-chaconine | human BuChE | 2.9 μM | 70% | Nigg et al., 1996 |
| α-solanine | | 2.9 μM | 50% | |

The mechanism of inhibition is not well understood. Unlike succinylcholine and mivacurium, GAs do not have an ester group that can be acted upon by an esterase enzyme. Nigg et al. (1996) showed that the inhibition was reversible with dilution when human BuChE was treated with 2.88 μM α-chaconine and α-solanine. The aglycone had little effect on BuChE, indicating that the carbohydrate moiety of the GA is important in cholinesterase inhibition.

Apathy, weakness, and confusion are some of the reported symptoms of potato GA poisoning that are consistent with nervous system damage (Morris and Lee, 1984). In addition to concerns about the acute toxic effects of consuming high GA potatoes, Krasowski et al. (1997) suggest that the effect of GA on cholinesterase activity warrants further study for the following reasons:

1. Inhibition of BuChE has implications for the use anaesthetics that also affect BuChE.
2. GAs might affect other esterases. Several newer drugs (eg. remifentanil) rely on non-specific esterases to clear them from the body.
3. The reduced ability of "atypical" BuChE to bind GA may have given these individuals a survival advantage, that is, GAs may have been an evolutionary driving force.

Although membrane disruption and cholinesterase inhibition have usually been studied in isolation from each other, several authors have speculated on an interrelationship. Morris and Lee (1984) commented that it would not be uncommon for a person to consume almost 1 mg GA/kg body weight in a typical meal. A toxic dose might be less than 4 mg/kg body weight. They state that "at levels of α-chaconine and α-solanine approaching lethal doses in laboratory animals, absorption of GA in the body markedly increased; they began to accumulate in various tissues, especially in the liver, kidney and lungs. This GA intake across the intestinal wall and other cell walls may explain why the lethal dose and the 'normally' consumed non-lethal dose of GA can vary by only a factor of four or even less." It might be that cell membrane disruption facilitates greater absorption of GA in the gastrointestinal tract, allowing secondary effects to occur elsewhere in the body. Mitchell and Harrison (1985) postulate that GA might affect nerve cell membranes. If nerve membranes are depolarized, nerve impulses could be generated, producing symptoms that might be similar to cholinesterase inhibition.

**Fate of Ingested Potato Glycoalkaloids**

The fate of ingested GA is poorly understood. Because potatoes are widely consumed, GA are almost constantly present in the gut, with smaller amounts in the blood. Using a radioimmunoassay, Harvey et al. (1985b) reported that total serum GAs (including solanidine) ranged from 3.2 - 125 nM with a mean of 38.5 nM in normal healthy individuals (1 nM = ca 1 ng/mL). Solanidine alone ranged from 2.5 - 92.5 nM with a mean of 14.5 nM. Matthew et al. (1983), using an almost identical analytical technique, reported much lower plasma solanidine levels, 0.8 - 10.4 nM.

The level of GA in the blood appears to be dependent on the amount of potatoes consumed (Harvey et al. 1985a). In one of the few human studies on GA metabolism, subjects were given a single potato meal containing 0.4 mg α-solanine/kg body weight and 0.6 mg α-chaconine/kg body weight after fasting from potato products for the previous 48 hours (Hellenäs et al., 1992). Serum GA levels rose almost immediately, indicating that GAs are absorbed intact in the blood. Average serum α-solanine peaked

5.1 hours after ingestion at 8.8 nM and α-chaconine peaked 6.0 hours after ingestion at 16.9 nM. For both components, the peak serum level accounted for no more than about 0.2% of the amount ingested, an uptake level similar to that reported in rats (Nishie et al., 1971). Solanidine also was detected in the serum at levels ranging from 2.5 to 12 nM, peaking between 8 and 25 hours after ingestion. The presence of solanidine in the blood suggests that some of the potato GAs are metabolized into their aglycone, although the extent of this metabolism appears limited. The biological half-life of α-solanine in blood was calculated to be about 11 hours, α-chaconine about 19 hours.

Nishie et al. (1971) orally dosed rats with tritiated solanine and confirmed that 84% of the radioactivity was excreted in the feces. The urine accounted for 10% of the dose, indicating that at least 10% of the GAs were absorbed. The remaining 6% was assumed to have been deposited in various body tissues.

Claringbold et al. (1980) injected two humans with tritiated solasodine and monitored excretion of radioactivity. They reported that even when GAs were administered directly into the bloodstream, 20% of the dose was excreted in the feces. This suggests that fecal GA levels do not accurately represent unabsorbed GA; it appears some of the dose enters the bloodstream and is later shunted back to the gastrointestinal tract for excretion. Urinary excretion accounted for another 7% of the dose. Presumably, the remainder of the dose was deposited in body tissues. Animal studies indicate much of the absorbed GA is deposited in the liver and kidney (Clarinbold et al., 1980; Nishie et al., 1971).

Rapid uptake by the kidney and liver may explain the relatively low levels of GA in the blood. It may be that the GA resident time in blood is quite short. In reviewing the literature on GA metabolism, Nigg et al. (1996) estimated liver concentrations could rise as high as 267 μM solanidine, 293 μM α-solanine and 560 μM α-chaconine, concentrations a thousand times higher than in the blood. These estimates represent a worst case scenario but they are worrisome because Nigg et al. (1996) also showed that only 3 μM GA can cause significant inhibition of human BuChE.

Nishie et al. (1971) found that the $LD_{50}$ of α-solanine injected in rats intraperitoneally is ca 42 mg/kg body weight, whereas rats were unharmed by oral doses

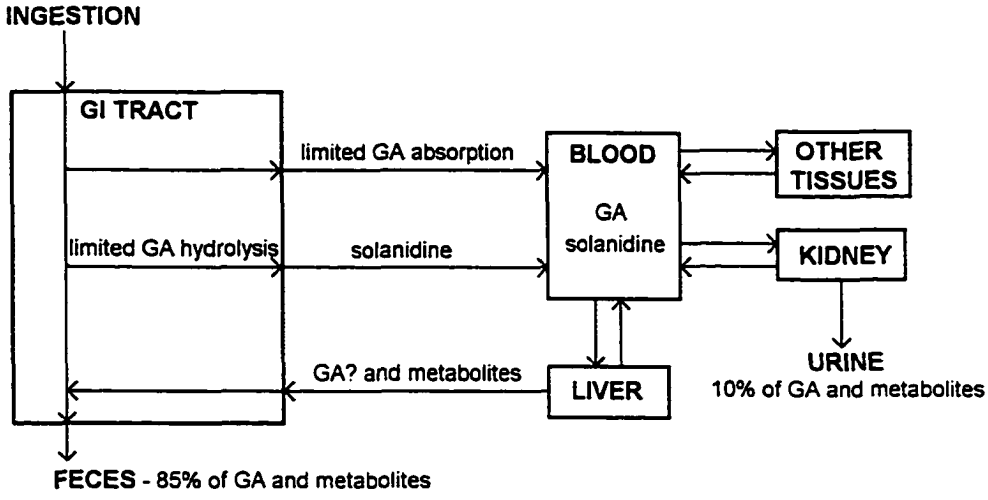


**Figure 1-4 Proposed fate of orally ingested glycoalkaloids.**

up to 1,000 mg/kg, again indicating that GAs are poorly absorbed in the gastrointestinal tract. Friedman and McDonald (1997) warn that rats appear much more tolerant of GAs than humans. Nevertheless, it appears that the body deals with dietary GAs in a number of ways (Figure 1-4). The lack of analytical techniques capable of identifying specific GAs and their metabolites at serum concentrations has severely limited our understanding of the fate of ingested GAs.

## Documented Poisoning

The number of documented GA poisoning cases is surprisingly low, given the widespread consumption of potatoes (Table 1-4). The case of the 78 children reported by McMillan and Thompson (1979) is often discussed because it is the only known case within the last 25 years. The poisoning occurred at a school in southeast London and 17 of the children were hospitalized. Symptoms included diarrhea, vomiting, malaise, delerium, and several students became comatose. The hospitalized children were subjected to numerous tests. Bacteriological tests were negative. Because of the neurological symptoms, plasma cholinesterase activity was measured revealing that 10 of

**Table 1-4 Documented cases of potato glycoalkaloid poisoning**

| Author | Description |
|---|---|
| Schroff (1865) in Swinyard and Chaube (1973) | adults |
| Pfuhl (1899) | 56 adults |
| Rothe (1918) | 41 persons |
| Harris and Cockburn (1918) | 61 persons, 1 child died |
| Bömer and Mattis (1924) | large number of persons |
| Griebel (1924) | large number of persons |
| Hansen (1925) | 7 persons, 2 died |
| Autenreith (1928) in McMillan and Thompson (1979) | 673 adults |
| Willimott (1933) | 61 persons, 1 died |
| Terbruggen (1936) | 1 child died |
| Rühl (1951) | adults |
| Reelah and Keem (1958) | 404 adults, 22 died |
| Wilson (1959) | 4 persons |
| McMillan and Thompson (1979) | 78 children, 3 critically ill |

Source: Morris and Lee (1984)

the 17 children had subnormal levels 6 days after eating the meal. All the children's plasma cholinesterase levels had increased five weeks later, with all but one considered normal. The source of the poisoning was identified as the first sitting of a midday meal at the school. The potatoes were the only item of food eaten in common by all the ill children. The remaining suspect potatoes had been discarded before it was realized the children were ill so it was not known how much GA was consumed. The potatoes were said by the school staff to be old and deteriorated. Assuming this case was truly GA poisoning, it shows the narrow margin between common gastrointestinal discomfort and serious neurological illness.

Many cases of GA poisoning almost certainly go unreported or misdiagnosed. The symptoms of mild GA poisoning resemble a mild case of food poisoning. Many medical practitioners may not even consider GA poisoning. O'Malley and McCurdy (1990) describe an incident where 30 migrant grape pickers became ill with gastrointestinal and neurological symptoms. The 16 workers who were tested showed moderate to severe cholinesterase depression. The field where they had been working had previously been treated with phosalone, a phosphorothioate insecticide. The authors concluded that the workers had suffered organophosphate poisoning from subacute exposure to the insecticide. However, unlike most organophosphates, phosphorothioates do not generally cause cholinesterase depression (Marrs, 1993). The grapes also showed residue levels well within California safety limits. The authors make no mention of GAs although the symptoms observed were consistent with GA poisoning.

## ANALYSIS OF POTATO GLYCOALKALOIDS

Many analytical methods have been developed for potato GAs. Earliest methods were gravimetric, while high-performance liquid chromatography (HPLC) and immunoassays have been used almost exclusively since 1985. No one method has become widely accepted and the AOAC Handbook does not list a method for GAs (AOAC International, 1999). The choice of method depends on the requirements of the

researcher and is often a choice between speed of analysis and quality of the results. GA analysis has been reviewed extensively by Friedman and McDonald (1997).

## Extraction

The chemical structure of GAs (Figure 1-1) provides an explanation why the quantitative extraction of GAs from plant material is difficult. The hydrophilic carbohydrate part and the hydrophobic alkaloid part combine to make a molecule that is at least slightly soluble in most solvents, but not very soluble in any. As a result, it is difficult to make a concentrated solution. The nitrogen in solanidine has a pKa of 6.7. Solubility of GAs in aqueous solutions is improved by low pH. Friedman and McDonald (1995) evaluated 11 solvent combinations that have been used for GA extraction:

2% acetic acid;

5% acetic acid;

3% acetic acid in ethanol;

methanol-acetic acid-water (94:1:6);

5% trichloroacetic acid in 50% methanol;

methanol-chloroform (2:1);

chloroform-acetic acid-methanol (10:1:9);

tetrahydrofuran-water-acetonitrile-acetic acid (5:3:2:0.1);

0.5% sodium bisulfite in 2% acetic acid;

0.02 M sodium 1-heptanesulfonate in 0.17 M acetic acid

1-heptanesulfonic acid in 1% acetic acid

They found that fresh (wet) samples were best extracted by methanol:chloroform (2:1) or by the tetrahydrofuran-water-acetonitrile-acetic acid (5:3:2:0.1) solvent first described by Bushway et al. (1985). Dry samples were best extracted by aqueous solvents, specifically 2% acetic acid.

## Early Methods

Earliest methods of GA analysis simply involved the precipitation of GAs with alkaline pH (Birner, 1969). In the 1950s and 1960s, more specific colorimetric methods were developed. Typically, GAs were reacted with an acidic solution of formaldehyde (Dabbs and Hilton, 1953; Clarke, 1958), with methyl orange (Birner, 1969), or with antimony trichloride in HCl (Bretzloff, 1971). A titrimetric method using bromothymol blue-phenol was developed by Fitzpatrick and Osman (1974). Other color reactions were also developed, but the paraformaldehyde-phosphoric acid method of Clarke (1958) probably was used most widely (Clement and Verbist, 1980). GAs were extracted by refluxing with 80% ethanol, the ethanol was evaporated and the residue taken up in 5% acetic acid. The GAs were then precipitated with ammonia. GAs remaining in solution were extracted with isoamyl alcohol and combined with the precipitated GAs. The residue was then treated with 1% paraformaldehyde in 90% phosphoric acid. The amount of GA in the extract was proportional to the development of a deep steel blue color. This particular method could not detect alkaloids with no double bond, such as tomatidine. All colorimetric methods could only measure total GAs and not provide information about specific compounds.

Thin layer chromatography (TLC) provided a way of identifying specific GAs (Jellema et al., 1980). Cadle et al. (1978) showed that individual GAs could be quantified using antimony trichloride and a densitometer for detection. There was reasonable agreement between the TLC results and total GA measured with the paraformaldehyde method. Jellema et al. (1981) also achieved good quantification using a fluorescent optical brightener to detect the GAs. Other chromatographic methods have now supplanted TLC for most quantitative work, although TLC is still commonly used for identifying GAs.

## Gas Chromatography

Gas chromatography offers excellent resolution, good sensitivity with flame ionization detection, and the advantage of automated analysis. The main limitation of gas

chromatography is that the analytes must be volatile and GAs are not. One strategy for analyzing total GAs involves hydrolyzing the samples in acid and then separating the aglycones on an OV-17 packed column (Osman and Sinden, 1977) or a poly(dimethylsiloxane) capillary (Van Gelder et al., 1989). Alternatively, the unhydrolyzed glycosides have been permethylated to increase their volatility (Herb et al., 1975; Osman et al., 1978). Permethylation allows the quantification of individual GAs. In either case, chemical modification of the GAs prior to separation on the column would be expected to reduce recoveries and add analysis time. Gas chromatographic analysis of potato GAs is not popular.

## High-Performance Liquid Chromatography

Bushway et al. (1979) developed the first published method for quantitative determination of potato GAs by HPLC. It is currently the most popular technique although no one protocol has become widely accepted (Table 1-5). Most methods involve the use of a solid-phase extraction cartridge for sample cleanup, separation on an amino or $C_{18}$ column, and detection by ultraviolet (UV) absorbance at ca 208 nm. Effective sample cleanup is important because many interfering compounds absorb UV light in this range. Unless automated, sample preparation adds a considerable amount to total analysis time. Both amino and $C_{18}$ columns are able to resolve α-chaconine, α-solanine, and solanidine. Some authors have also reported separation of GA hydrolysis products (Carman et al., 1986; Friedman and Levin, 1992).

The quality of the data produced by HPLC is generally good. Spike recoveries are typically >90% and relative standard deviations <10% (Table 1-5). A collaborative study carried out by the Nordic Committee on Food Analysis investigated inter-laboratory reproducibility (Hellenäs and Branzell, 1997). Twelve laboratories were given 6 homogenized tuber samples. The tubers were extracted with 5% acetic acid containing 0.5% $NaHSO_4$, cleaned up on a $C_{18}$ solid-phase extraction cartridge, and analyzed on a $C_{18}$ column. Relative standard deviations for α- chaconine and α-solanine were similar ranging from 8-13%, on samples containing 12-260 mg/kg.

## Table 1-5 HPLC methods for the determination of potato glycoalkaloids

| Reference | Sample matrix | Extraction and clean-up | HPLC conditions | Accuracy/precision |
|---|---|---|---|---|
| Bushway et al., 1979 | tubers, peels, sprouts | THF:$H_2O$:ACN (or MeOH:$CHCl_3$), ammonium precipitate | $NH_2$ column THF:$H_2O$:ACN (5:3:2) 208-225 nm | not evaluated |
| Bushway et al., 1980 | tubers | MeOH:$CHCL_3$ (2:1), ammonium precipitate | Waters CHO column, THF:ACN:$H_2O$ (53:17:30) 215 nm | s CV 1.6-4.6% c CV 1.9-6.3% 90-100% rec |
| Bushway and Ponnampalam, 1981 | potato products | MeOH:$CHCl_3$ (2:1), extract organic with HOAc, ammonium precipitate | $NH_2$ column THF:$H_2O$:ACN (5:3:2) 208-225 nm | s 93-98% rec c 98-101% rec CVs 2.2-11% |
| Morris and Lee, 1981 | tubers | HOAc, ammonium precipitate | $C_{18}$ or $C_8$ or silica ACN:$H_2O$:ethanol-amine (varied) 200 nm | not evaluated |
| Eldridge and Hockridge, 1983 | nightshade berries | MeOH reflux (2 h) | silica column ACN:$H_2O$ (78:22) 200 nm | s 105% rec c 101% rec |
| Bushway et al., 1983 | potato peels | THF:ACN: $H_2O$:HOAc extract organic with HOAc, ammonium precipitate | $NH_2$ column THF: $H_2O$:CAN (5:3:2) 208-225 nm | not evaluated |
| Bushway et al., 1986 | tubers, chips | THF:ACN: $H_2O$:HOAc $C_{18}$ Sep-Pak | $NH_2$ column THF: $H_2O$:CAN (5:3:2) 208-225 nm | not evaluated |
| Carman et al., 1986 | tubers, skins | THF:heptanesulfonic acid, $C_{18}$ Sep-Pak | $C_8$ column ACN:phos (1:1) 202 nm | s 90% rec c 96% rec |
| Hellenas, 1986 | tubers | water:HOAc:$NaHSO_3$, $C_{18}$ Sep-Pak | $C_{18}$ column ACN: $H_2O$:ethanol-amine (35:65:0.05) 202 nm | rec 86% |
| Kozukue et al., 1987 | tubers and plant parts | $CHCl_3$:MeOH (2:1), ammonium precipitate | $NH_2$ column THF:phos:ACN (2:1:1) 208 nm | s 97% rec c 98% rec |
| Kobayashi et al., 1989 | plant shoots | MeOH, silica gel column, $C_{18}$ Sep-Pak | $NH_2$ column EtOH:ACN:phos 205 nm | s 84.5-97.3% rec c 90.7-112.8% rec s CV 9% c CV 10% |
| Saito et al., 1990 | potato products and starch | MeOH extract, $C_{18}$ or $NH_2$ Sep-Pak | $NH_2$ column ACN:phos (75:25) 208 nm | s 82.4-92.6% rec c 86.5-97.4% rec |
| Friedman and Dao, 1992 | tubers and potato products | THF: $H_2O$:ACN, ammonium precipitate | $C_{18}$ column ACN:sulfate (1:1) 200 nm | s 90.1-95.4% rec c 88.8-91.6% rec |
| Hellenas et al., 1992 | blood serum | $C_2$ Sep-Pak, cyanopropyl HPLC | silica column ACN:phos (80:20) 200 nm | s 86.3-91.5% rec c 88.5-93.0% rec CV 1.2-7.5% |

**Table 1-5 HPLC methods for the determination of potato glycoalkaloids (continued)**

| Reference | Sample matrix | Extraction and clean-up | HPLC conditions | Accuracy/precision |
|---|---|---|---|---|
| Friedman and Levin, 1992 | tubers and hydrolyzed extracts | THF: $H_2O$:ACN:HOAc add heptanesulfonic acid, $C_{18}$ Sep-Pak | $C_{18}$ column ACN:phos (35:65) 200 nm | not evaluated |
| Jonker et al., 1992 | wild potato tubers | MeOH:water, CN Sep-Pak | $C_8$ or $C_{18}$ column ACN:tris (6:4) 208 nm | s 98% ± 9 rec c 96% ± 9 rec |
| Hlywka et al., 1994 | tubers | HOAC:MeOH, add heptanesulfonic acid, $C_{18}$ Sep-Pak | $C_{18}$ column ACN:phos 208 nm | not evaluated |
| Zhao et al., 1994 | extruded potato peels | THF:ACN: $H_2O$:HOAc ammonium precipitate | $C_8$ column $H_2O$:ACN:THF (30:35:1) 205 nm | not evaluated |
| Sanford et al., 1994 | potato foliage | EtOH:HOAc, add heptanesulfonic acid, $C_{18}$ Sep-Pak | $C_{18}$ column ACN:phos (1:1) 210 nm | not evaluated |
| Percival and Duncan, 1994 | tubers | $H_2O$:HOAc:$NaHSO_3$, $C_{18}$ Sep-Pak | $C_{18}$ column ACN: $H_2O$:ethanol-amine (65:35:0.05) 202 nm | ave 98% rec |
| Hellenas et al., 1995 | tubers | $H_2O$:HOAc:$NaHSO_3$, $C_{18}$ Sep-Pak | $C_{18}$ column ACN:water (55:45) 202 nm | ave > 96% rec CVs 4.2%, c 3.5% (among days) |
| Dao and Friedman, 1996 | leaves | HOAc, extract HOAc with butanol | $C_{18}$ column ACN:phos (35:65) 200 nm | s 92.5-95.7% rec c 88.9-99.6% rec |

ACN, acetonitrile; THF, tetrahydrofuran; HOAc, acetic acid; phos, phosphate buffer; MeOH, methanol; EtOH, ethanol

Hellenäs et al. (1992) developed the only HPLC method sensitive enough to detect individual GAs at concentrations found in blood. The GAs were extracted from serum with a $C_2$ solid-phase extraction cartridge and evaporated to dryness. The dry sample was taken up in 50% acetonitrile and separated on a HPLC cyanopropyl column. Two fractions were collected, one containing solanidine and one containing the glycosides. The fractions were concentrated and then analyzed on a silica column with ultraviolet detection at 200 nm. This procedure produced a sensitivity of 0.3 ng/mL.

Recoveries averaged 87% with the relative standard deviation of five replicates ranging from 1.2-7.5%. The authors claimed they were able to analyze 20 samples in four days.

**Immunoassays**

The first immunoassay developed for GAs was a radioimmunoassay technique developed by Vallejo and Ercegovich (1979). Anti-solanidine serum (which also cross reacted 100% with α-solanine and α-chaconine) and tritiated solanidine were added to a potato extract and allowed to incubate. Bound and unbound antibody fractions were separated using dextran-coated charcoal and the radioactivity of the bound fraction was measured. The amount of tritiated solanidine present in the bound fraction was inversely related to the total amount of solanidine, α-solanine and α-chaconine in the original sample. Recoveries ranged from 96-116%. Morgan et al. (1983) developed the first enzyme-linked immunosorbent assay (ELISA), greatly simplifying potato analysis. Since then, several ELISAs have been developed for potato GAs using both polyclonal and monoclonal antibodies (Table 1-6). EnviroLogix, Inc. (Portland, ME) produces a commercial kit for potato GA analysis based on a competitive ELISA where GA in the sample extract competes with enzyme-labeled solanidine for a limited number of antibody binding sites on the test wells. The relative standard deviations of duplicate extracts from tubers ranged from 5-17%, with kit results in good agreement with HPLC results (Friedman et al., 1998).

ELISAs have the advantage of being relatively easy to perform. However, incubation times and numerous washing steps typically require a whole day to complete. Thomson and Sporns (1995) developed a fluorescence-polarization immunoassay for potato GAs that eliminates the need for multiple incubations and washings. Equal volumes of a potato extract, fluorescently-labeled solanidine, and anti-GA serum were allowed to equilibrate for 30 min before measuring the polarization of fluorescence. The amount of polarization was inversely related to the amount of GA in the sample extract. More recently liposome-based immunoassays have been described, using both strip assays and flow injection analysis (Kim and Durst, 1999).

**Table 1-6 Potato glycoalkaloid ELISA methods**

| Authors | Type of antibody |
|---|---|
| Morgan et al., 1983 | polyclonal |
| Plhak and Sporns, 1992 | polyclonal |
| Plhak and Sporns, 1994 | monoclonal |
| Stanker et al., 1994 | monoclonal |
| Peréz et al., 1999 | polyclonal |

Immunoassays have also been used to analyze GAs in serum. Matthew et al. (1983) and Harvey et al. (1985a,b) used a radioimmunoassay similar to the one developed by Vallejo and Ercegovich (1979) to analyze total GAs and solanidine in human serum. To measure only solanidine, a chloroform extract of the serum was analyzed. The glycosides were not extracted by the chloroform. The assay was sensitive to 1 ng/mL total GA with relative standard deviations of ca 20%.

**Mass Spectrometry**

Mass spectrometry has typically been used to study the structure of GAs or to confirm their identity. Liquid secondary ion mass spectrometry (LSIMS) was used to confirm the structure of several potato GAs ( Price et al., 1985; Chen et al., 1994). Evans et al. (1993) were able to detect 200 fmol of GA using LSIMS. van Gelder et al. (1989) used gas chromatography-mass spectrometry to identify alkaloids present in several *Solanum* species.

Abell and Sporns (1996) developed a novel method that was able to very rapidly quantify individual GAs using MALDI-TOF MS. Freeze-dried potato tubers were extracted with 50% aqueous methanol. A drop of the extracted GAs was placed on top of a layer of 2,4,6-trihydroxyacetophenone crystals that had been applied to a MALDI probe. The sample was then analyzed by MALDI-TOF MS. No fragmentation of the GAs was reported. Quantification of the individual GAs, based on peak intensity, was possible when an internal standard (tomatine) was used. The MALDI-TOF MS results

showed good agreement with HPLC results. Relative standard deviations of triplicate measurements ranged from 5-32%.

## CAPILLARY ELECTROPHORESIS

### Principles of Capillary Electrophoresis

Electrophoresis performed in a gel is a very well established technique for analyzing proteins based on electrical charge and size. Gel electrophoresis, however, is not amenable to automation and its ability to resolve similar proteins is not particularly good. In 1983, Jorgenson and Lukacs showed electrophoretic separations were possible in a fused silica capillary. Not only did this prove that electrophoresis could be performed in a novel medium, but it also opened up the potential to develop the type of instrumentation typical of HPLC. Automated sample injection, various modes of sample detection, and computer-run data acquisition has made capillary electrophoresis (CE) an attractive technique for certain types of analyses.

The term "electrophoresis" is used to describe the migration of a charged molecule in an electrical field. Gel electrophoresis was first developed for the analysis of proteins and it is useful to use the protein molecule as a model to describe the mechanism of electrophoresis. At low pH, the amino groups on a protein molecule become protonated, and therefore the protein carries a net positive charge. When placed in a buffer that contains a two electrodes, the protein will migrate toward the negative electrode when an electric current is applied because opposite charges attract. This migration based on a charged molecule in an electrical field is referred to as "electrophoretic mobility".

One of the ways CE is distinguished from traditional gel electrophoresis is by the use of instrumentation more commonly associated with HPLC. Electroosmotic flow (EOF) is the other major distinction between CE and traditional electrophoresis. EOF explains why the running buffer itself will flow through the capillary, normally from the

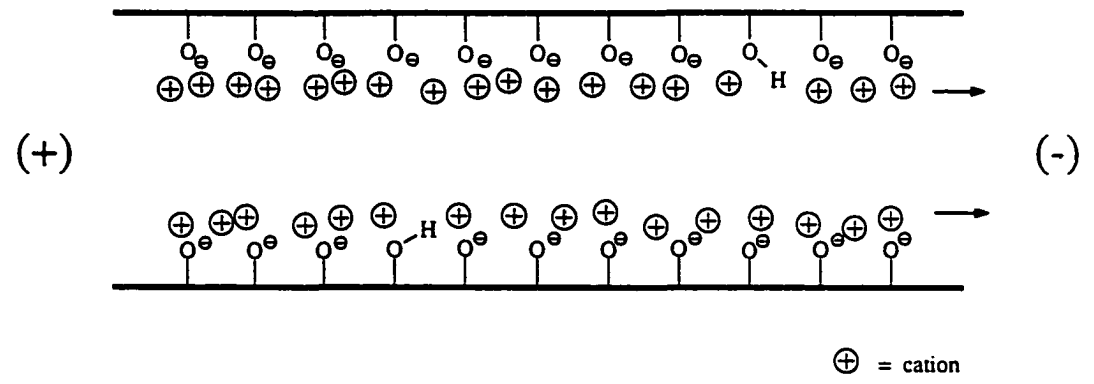


**Figure 1-5 Electroosmotic flow in a silica at low pH.**

anode side to the cathode side. The silanol groups of the silica columns used for CE have a isoelectric point (pI) of approximately 1.5 (Beckman, 1991). When the buffer in the capillary is about pH 4 or greater, most of the silanol groups are deprotonated ($SiO^-$). Cations from the running buffer are attracted to the negatively charged silanol groups on the capillary wall and form a type of electrical double-layer (Figure 1-5). When voltage is applied to the system, the cations associated with double-layer migrate toward the negative electrode. Their movement, in effect, drags the rest of the buffer along in the capillary, creating EOF.

This flow is unique to capillary electrophoresis because of the relatively large surface area of the capillary wall that can be negatively charged. EOF does not occur in traditional slab or tube gel electrophoresis. A useful analogy is to think of the samples in the gel migrating toward the cathode while the gel itself also moves toward the cathode. In the same way, samples in a capillary move toward the cathode due to their electrophoretic mobility, as well as being swept along by EOF. This has been called the "superpositioning" of flow onto solute mobility (Heiger, 1992).

As in traditional gel electrophoresis, it is possible to carry out CE in various modes. These modes differ in the buffer systems used, additives and type of capillary.

Capillary zone electrophoresis and micellar electrokinetic capillary chromatogrpahy are the most popular modes.

**Capillary zone electrophoresis** (CZE) was the first mode of CE developed and is its simplest form. In most of the previous discussion, CZE was the assumed mode. Migration of the solutes in CZE is based solely on the charge-to-mass ratios. Neutral molecules can not be separated. The term "zone" refers to idea that ideally each solute migrates in a distinct band, or "zone", separate from other solutes in the capillary. Fused silica capillaries are almost always used for CZE. Therefore, buffer selection is the key to optimizing CZE. Many types of buffers are compatible with CZE. The general rule of thumb is to use a concentration of 50 to 100 mM and adjust the pH to one or two pH units below or above the pI of the solute of primary interest. The pH of the buffer must be at least one pH unit away from the pI of the solute to ensure that the solute is charged. A difference of two pH units is better.

In many cases, a simple phosphate buffer adjusted to the correct pH will produce very good separations, especially of proteins. Nevertheless, various additives and unique buffer systems have been developed that expand the scope of CZE. Chiral separations can be achieved with the addition of cyclodextrins to the buffer (Kuhn et al., 1992). Borate buffers will form a cyclic anionic complex with carbohydrates, allowing the separation of saccharides which are primarily neutral in character (El Rassi, 1996). Surfactants such as sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) can be added in small concentrations to alter EOF and improve the solubility of hydrophobic solutes (Hu et al., 1995).

**Micellar electrokinetic capillary chromatography** (MECC), also called micellar electrokinetic chromatography, was developed for the separation of anionic, cationic and neutral solutes all in one run. Terabe et al. (1983) reported micellar separations even before the fused silica capillary became widely accepted as the best medium for CE. MECC is similar to CZE in many respects. Solutes migrate in zones. It uses a homogeneous buffer system. Buffer pH strongly affects migration rates of the solutes and the strength of EOF. The main difference between the two modes is the addition of micelles to the running buffer of MECC systems.

Micelles are aggregates of surfactant molecules (Heiger, 1992). When placed in an aqueous solution at a high enough concentration, certain surfactants will aggregate together and re-orient themselves in a rough sphere with their hydrophobic tails pointing toward the inside of the sphere, and with the hydrophilic heads sticking outward towards the aqueous medium. SDS and CTAB are two of the most common surfactants used in MECC. Micelles will form when surfactant concentrations exceed a value known as the critical micelle concentration (CMC). The CMC for SDS is 8.1 mM; for CTAB it is 0.92 mM (Beckman, 1991). As discussed earlier, these two surfactants can be used with CZE where they are used to alter EOF and improve the solubility of more hydrophobic solutes. However, the concentration of surfactants used in CZE is too low to allow the formation of micelles, so the basic mechanism of separation is not affected in CZE.

The presence of micelles in the running buffer changes the mode of separation. Under CZE conditions, the neutral solutes would all migrate together with the EOF. When SDS micelles are added to the running buffer, the micelles will be carried by the EOF toward the cathode, but being anionic, they will migrate slower than the EOF. Because many neutral molecules have some hydrophobic character, they are attracted to the inner hydrophobic region of the SDS micelle. Therefore, the rate of migration of neutral molecules depends on how they partition between the micelle and the running buffer. While the neutral solute is "captured" by the micelle, its migration rate is slowed to that of the micelle. When the micelle "releases" the neutral solute back into the aqueous buffer, the neutral solute again migrates with EOF at a faster rate. The more hydrophobic the neutral solute is, the more time the solute will remain "captured" by the micelle, giving it a slower migration time. Neutral molecules with no hydrophobic character will have little affinity for the SDS micelle and will migrate at more or less the rate of the EOF. In this way neutral molecules are separated on the basis of their hydrophobicity.

**Capillary gel electrophoresis** is characterized by the use of a polymer filled capillary as opposed to an empty capillary. The polymer is usually polyacrylamide, which makes capillary gel electrophoresis directly comparable to slab gel electrophoresis.

**Capillary isoelectric focusing** is an example of a focusing separation as opposed to a moving zone separation. The key to performing an isoelectric separation is to suppress the EOF so that a pH gradient develops in the capillary. Solutes in the capillary migrate to the pH region which matches their pI. In contrast to other CE separations, a steady state is achieved, ie. the solutes become tightly focused at their pI and do not elute from the capillary. After a steady state has been achieved, sodium chloride is often added to one of the electrode reservoirs which mobilizes the proteins and elutes them from the capillary in the direction of the salt.

**Capillary isotachophoresis** is a unique mode of electrophoresis where solutes are stacked between a leading electrolyte and a trailing electrolyte. The mechanism of separation is the same as that which occurs in the stacking gel of a polyacrylamide gel electrophoretic system. The solute bands are very efficiently compressed and separated.

## Instrumentation

A basic CE system consists of a capillary, two buffer reservoirs, a detector, and a power supply (Figure 1-6). Sample is introduced by dipping one end of the capillary into the sample solution and applying pressure or an electrical field. The capillary is then moved to the buffer reservoir and an electrical field is applied. The analytes are separated as they migrate through the capillary. Detection occurs as the analytes pass an optical window near the end of the capillary.

Sample injection is very important for good quantification and for obtaining reproducible resolution. Sample volumes are very small, often less than 1 nL. It is important not to overload the sample as this will result in a long sample plug in the capillary. For best resolution, the sample plug should be no longer than 2% of the total capillary length. Hydrodynamic and electrokinetic injection techniques are the most common methods for introducing the sample to the capillary. Hydrodynamic sample injection uses pressure to get the sample into the capillary. This is done by pressurizing the sample vial to push the sample into the capillary. Electrokinetic injection relies on

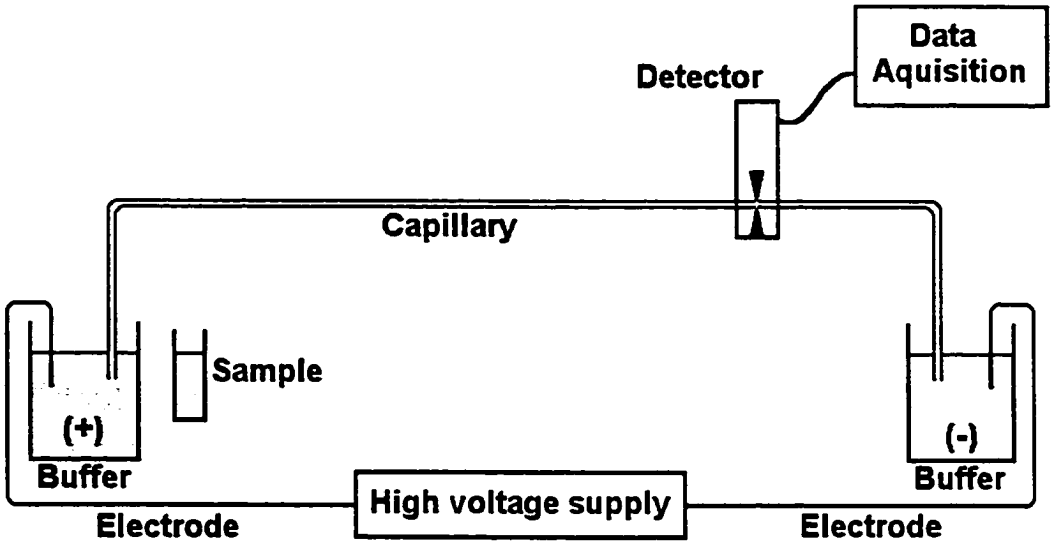


**Figure 1-6 Schematic of a capillary electrophoresis system.**

electrical field instead of pressure to get the sample into the capillary. The injection-end of the capillary is placed into the sample vial and a voltage is applied. The solutes migrate into the capillary as a result of electrophoretic migration and electroosmotic flow, just as they would move through a capillary during analysis.

Separation is performed on a simple fused silica capillary with a polyimide external coating. This is the same type of flexible capillary that is used in gas chromatographs except that CE capillaries usually have no functional groups attached to the silica. A typical length is 50 to 75 cm. Longer capillaries allow more resolution but shortness is desirable in terms of analysis time and reducing voltage and heat problems. Typical inside diameters range from 25 μm to 75 μm with smaller diameters giving better resolution and larger diameters allowing greater sample loading.

UV/visible absorption and fluorescence are the most common methods of detection. Detection occurs through a window near the end of the capillary. As a result,

the optical path is very short (the diameter of the capillary). The short optical path and the small injection volume mean that the concentration limit of detection for CE is often poorer than for HPLC. Refractive index, conductivity, radioactivity, chemiluminescence and amperometric detection have been reported in the literature but are not available commercially (Weinberger, 1993). CE is also well suited for coupling with electrospray ionization mass spectrometry (Severs and Smith, 1997).

Beckman Coulter (Fullerton, CA), BioMolecular Instruments (Santa Fe, NM), Bio-Rad Laboratories (Hercules, CA), Hewlett-Packard (Palo Alto, CA), and Waters Corporation (Milford, MA) are some of the current commercial suppliers of CE instruments. Several reviews of CE and food analysis have been published (Cancalon, 1995; Lindeberg, 1996).

## MALDI-TOF MASS SPECTROMETRY

### Principles of Mass Spectrometry

Mass spectrometry is a very useful technique for determining the structure of a molecule. A mass spectrometer is used to ionize a molecule and then measures the mass of that ion. The most popular way to ionize a molecule historically has been by electron impact ionization. A heated filament generates a beam of electrons that is directed to a sample ionization chamber (ion source) (Figure 1-7). When a volatile molecule is introduced from a capillary column or a syringe and encounters the electron beam, an electron is extracted from the molecule. Because electrons have nearly no mass, the weight of the analyte does not change significantly. The loss of an electron, however, does convert the analyte to a positively charged ion. Ions can then be accelerated into a mass analyzer by applying an electrical field to the ion source. The electrical field is generated by applying a positive voltage to a repeller plate. The positively charged ions are accelerated in the direction opposite of the repeller plate because like charges repel.

Chemical ionization is similar to electron impact except that a reagent gas, such as ammonia or methane, is introduced to the ionization chamber. Both electron impact and

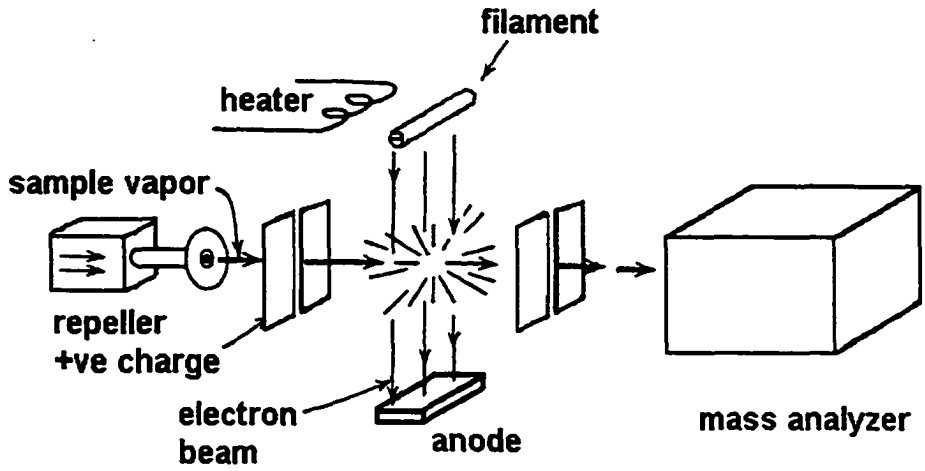


**Figure 1.7 Electron impact ionization.**

chemical ionization require that the analyte be volatile. This effectively limits mass spectrometry to small molecules. Both ionization techniques are also relatively energetic. The intact molecular ion typically fragments immediately after ionization. The resulting spectrum will then reveal the fragments of the molecule. The intact parent ion may not even be observed. The fragmentation makes it very difficult to interpret the spectra of mixtures of analytes. It also requires that the sample be relatively pure when introduced to the mass spectrometer.

Once the analyte has been ionized, the mass must be measured in a mass analyzer. Several options are available for determining the mass of ions. Quadrupole mass analyzers are based on the filtering effect achieved by varying the electrical potential of four cylindrical electrodes. They are commonly coupled to electron impact ion sources. Time-of-flight mass analysis is based on the time it takes for an ion to travel a specified distance through a vacuum. Time-of-flight is almost always used with MALDI because with this ionization technique, the time of ion formation can be very accurately determined. Other mass analyzers include sector analyzers, ion traps, and fourier transform ion cyclotron resonance analyzers.

## Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry

The problems associated with electron impact and chemical ionization have been largely addressed with the development of softer ionization techniques such as secondary-ion mass spectrometry, electrospray, and MALDI. MALDI is of particular interest to food scientists because it is relatively tolerant of impurities. Food extracts tend to be complicated mixtures of many different molecules. The MALDI technique was first demonstrated by Karas and Hillenkamp (1988) using a crystalline matrix. At about the same time, Tanaka et al. (1988) reported MALDI using a liquid matrix. Most subsequent work has been done with crystalline matrices and Franz Hillenkamp and Michael Karas are generally credited with developing the technique. Beavis (1992) provides an interesting account of the early development of MALDI.

Sample preparation for MALDI requires that the sample be mixed with an excess of an UV-absorbing matrix. Commonly used matrices include 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, sinapinic acid, and 2',4',6'-trihydroxyacetophenone. A drop (1-2 μL) of the mixture is placed on a stainless steel or gold surface and the solvent allowed to evaporate. This leaves a layer of crystals on the MALDI probe. When the crystals are irradiated with a laser, the energy is absorbed by the matrix, which becomes partially vaporized and carries the sample along into the vapor phase (Figure 1-8). During the desorption, the sample molecules become ionized. The mechanism of ionization is still unclear (Chan et al., 1992). The ions are accelerated from the ion source into the TOF mass analyzer by applying a voltage potential. The time required for the ions to travel the length of the drift tube is proportional to their mass-to-charge ratio and a mass spectrum can be generated.

Resolution was one of the major weaknesses of early MALDI-TOF instruments. Irradiating the matrix-analyte mixture in the ion source created a plume in which the analyte ions were distributed. The broad nature of the peak generated when the ions hit the detector reflected the broad nature of this plume. The expansion of the plume in the ion source was countered with a technique variously known as delayed extraction, time-

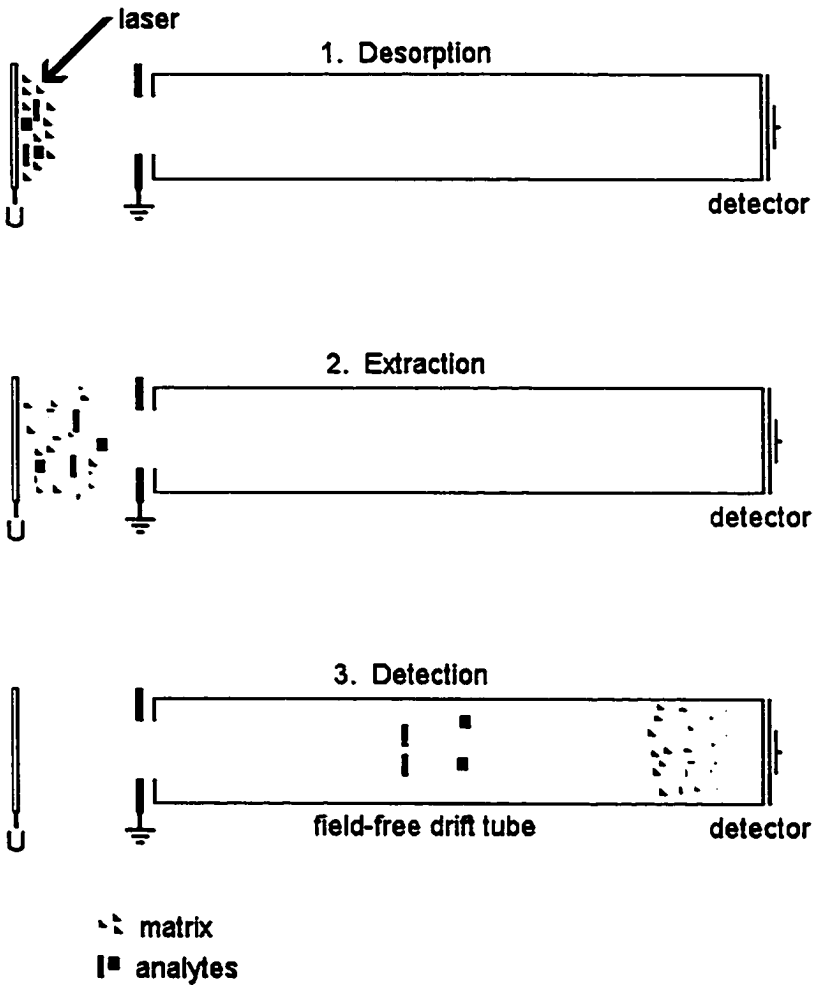


**Figure 1-8 Schematic of MALDI-TOF MS.**

lag focusing, pulsed extraction, or dynamic extraction. Rather than applying the extraction voltage simultaneously with the laser pulse, the extraction was delayed by several hundred nanoseconds after the laser pulse. Ions near the repeller plate received more energy and were able to "catch up" with the ions farther away from the repeller plate. By adjusting the delay carefully, the analyte ions could be made to arrive at the detector in a tight band, rather than a diffuse cloud (Brown and Lennon, 1995).

MALDI-TOF MS has become especially popular in the biological sciences. The ionization process is very gentle, allowing the intact ionization of oligosaccharides (Wang and Sporns, 1999) and large proteins (Hillenkamp, 1991). Perhaps most importantly, sample purification is not as critical for MALDI-TOF as for other MS techniques. Because few or no fragments are produced during ionization, spectra of sample mixtures are easily interpreted. The matrix itself also seems to act as a sample purifier. For example, 2',4',6'-trihydroxyacetophenone crystals produce good carbohydrate spectra but seem to suppress peptides and proteins that might interfere. Sinapinic acid appears to do the opposite. Low salt concentrations are tolerated, although high concentrations (>mM) will also suppress analyte signal (Siuzdak, 1994).

MALDI-TOF MS has been reviewed by Hillenkamp et al. (1991) and Karas (1997). Sporns and Wang (1999) have reviewed food analysis by MALDI-TOF MS. BioMolecular Instruments, formerly Finnigan MAT, (Santa Fe, NM), Bruker Daltonics (Billerica , MA), Comstock, Inc. (Oak Ridge, TN), Kratos Analytical (Chestnut Ridge, NY), and PerSeptive Biosystems (Foster City, CA) are some of the current suppliers of commercial instruments.

## Quantitative MALDI-TOF MS

MALDI-TOF MS is primarily used to identify and characterize large molecules. However, there is a growing body of literature showing that MALDI-TOF MS can also be used as a quantitative technique. For example, Abell and Sporns (1996) showed that MALDI-TOF MS could quantitate GAs in potato tubers significantly faster than the alternative HPLC method. The time saving was mainly the result of less sample purification. Detection based on molecular mass also provides additional confidence in identifying the analyte. Numerous other compounds relevant to food and agricultural chemistry have been quantitatively determined by MALDI-TOF MS (Table 1-7). Quantitative MALDI-TOF MS was more generally reviewed by Muddiman et al. (1995).

**Table 1-7 Literature on quantitative MALDI-TOF MS of food-related compounds**

| Analyte | Internal Standard | Reference |
|---|---|---|
| aminotriazole (pesticide) | none | Benazouz et al., 1998 |
| amperozide (drug) | [$^{13}C_4$]-amperozide | Jespersen et al., 1995 |
| anthocyanins | cyanidin-3-rutinoside | Wang and Sporns, 1999 |
| chaconine, solanine | tomatine | Abell and Sporns 1996 |
| cyclodextrins | maltohexaose | Bartsch et al., 1996 |
| dinoterb (pesticide) | dinitrophenol | Benazouz et al., 1998 |
| DOPA | [$^{13}C_6$]-DOPA | Duncan et al., 1993 |
| gluten gliadins | none | Camafeita et al., 1997 |
| gluten avenins | none | Camafeita and Mendez, 1998 |
| lactoferrin | serum albumin | Nelson et al., 1994 |
| lysozyme, myoglobin | cytochrome c | Tang et al., 1993 |
| oligosaccharides | other oligosaccharides | Whittal et al., 1995; Pitt and Gorman, 1997 |
| oligosaccharides | none | Harvey, 1993 Wang et al., 1999 |
| oligosaccharides | N-acetylgalactosamine | Kazmaier et al., 1998 |
| rennin | melittin | Jespersen et al., 1995 |
| sulfonamide antibiotics | acetaminophen | Ling et al., 1998 |
| triacylglycerides | matrix peak | Asbury et al., 1999 |

There are several problems associated with quantitative MALDI-TOF MS. Any quantitative method of analysis requires that measurements be highly repeatable. With MALDI-TOF MS, signal intensity and resolution vary greatly from laser shot to laser shot. The distribution of the analyte in the dried matrix is rarely homogenous so that in one area of the sample spot, no signal response may be detected, while other areas of the same sample spot show strong signals. High concentrations of one compound can suppress the signal of less concentrated analytes when a mixture is being analyzed (Gusev et al., 1996). Evidence of this can often been seen when comparing the signals of a pure analyte and the same analyte in a more complex mixture of other compounds (Harvey, 1993). The analyte signal is less intense in the mixture than in the pure system, presumably because the signal is suppressed by other components in the mixture. Matrix and cationized adducts can cause poor resolution at higher mass ranges by causing peak broadening. At lower mass ranges where adducts are often resolved from each other, this

is less of a problem, but adducts do make interpretation of spectra more difficult. Numerous strategies have been described to counteract these problems.

**Internal Standards**: There are some reports of quantitative MALDI-TOF MS that use external standards for calibration (Harvey, 1993; Preston et al., 1993; Camfeita et al., 1997). However, most quantitative MALDI-TOF MS methods use an internal standard to counter shot-to-shot variability. It is assumed that the analyte and internal standard will be similarly distributed in the matrix crystal, thereby minimizing the effects of non-homogeneous analyte distribution in the matrix. The selection of an appropriate internal standard can pose some problems. Gusev et al. (1996) lists four characteristics of an ideal internal standard.

1. The internal standard must be completely resolved from the analyte.
2. The internal standard must be stable during analysis.
3. The internal standard must be chemically similar to the analyte, but not react with the analyte. There is some evidence this requirement may be relaxed if the analytical system is well controlled. Good accuracy has been demonstrated using cytochrome c as an internal standard for bovine insulin although chemically the compounds are not similar (Gusev et al., 1993; Gusev et al., 1996).
4. The internal standard must be close to the analyte in mass and concentration. Signal supression can occur if concentrations are very different. It has been proposed that the internal standard concentration be near the concentration of the highest expected analyte concentration. This appears to be especially critical if the interal standard is not chemically similar to the analyte (Gusev et al., 1996).

An isotope analogue would be the best internal standard. Isotopes have been used for the quantification of DOPA and acetylcholine (Duncan et al., 1993) and amperozide (Jespersen et al., 1995). Because the mass resolution of MALDI-TOF MS is limited, isotope analogues are useful only for low molecular weight analytes. Isotopes may also not be available.

**Standard Addition**: Calibration curves have been prepared by standard additions to the sample material (Nelson et al., 1994; Abell and Sporns, 1996). Sample extracts will contain many compounds of no interest that likely have some suppressing effect on

the analyte signal. Generating a calibration curve by means of standard addition to the sample material is one way of ensuring that both standards and samples are equally suppressed.

**Spectrum Collection:** To clarify terminology, the circle of crystals formed when a drop of matrix-sample solution evaporates will be called a *spot*, and the area within the spot that is irradiated by a laser shot will be called a *point*. There is a great deal of variation in the methodology used to collect MALDI mass spectra. The simplest procedures simply fire the laser in a set pattern a set number of times and collect an averaged spectrum (Tang et al., 1996; Abell and Sporns, 1996; Muddiman et al., 1994). No point on the sample spot is irradiated more than once. Usually around 100 shots are averaged in one spectrum. The next step in complexity entails firing the laser multiple times (usually less than 20) at a single point and repeating this at several points on the same sample spot (Harvey, 1993; Bartsch et al., 1996). Approximately 100 shots are averaged in one spectrum. In both cases, several spectra are collected for quantification.

Gusev et al. (1995) used the most complicated spectrum collection technique but they have also reported the most systematic evaluation of signal reproducibility in MALDI-TOF MS. An area in the sample spot that produced a rich signal was identified and the laser was fired repeatedly at that point. The first 10 - 20 shots were ignored, but the next 30-50 shots were collected. Another signal-rich area on the spot was then located and the cycle repeated. A total of 100-200 shots were averaged for one spectrum. Four to six spectra were collected for quantification. Shot-to-shot variability was improved by omitting the first 10 - 20 shots taken at any one point. The higher signal variability of the first shots was explained by surface contamination.

**Signal Intensity vs Peak Area:** There is no consensus in the literature whether to use peak intensity (peak height) or peak area for quantification. Signal intensity is normally used although Tang et al. (1993) suggested peak area could also be used. Peak area would be less affected by poor resolution than signal intensity.

**Matrix Crystal Formation:** The matrix selected for MALDI-TOF MS clearly affects the analyte signal quality. There are many matrices available and some experimentation is usually required to identify a matrix that is suitable for a given

analyte. Optimal signal reproducibility is obtained from matrices that generate homogenous crystals. Homogeneity can sometimes be improved by the addition of a co-matrix. Fucose/ferulic acid and gentisic acid/5-methoxysalicylic acid combinations have been used successfully (Gusev et al., 1995). Accelerated solvent drying using a stream of high-purity nitrogen can also promote homogenous crystalization (Gusev et al., 1995). Other researchers have used fast-evaporating solvents to improve crystal homogeneity (Vorm et al., 1994). Harvey (1993) obtained improved crystal formation by manually scratching the sample with a syringe during drying.

**Laser Fluence**: Selection of the optimal laser power can affect signal reproducibity. While laser power just above the threshold of ionization results in maximum mass resolution, matrix crystal heterogeneity requires that power be set somewhat higher to ensure that signal can be obtained from multiple points on the same spot. Gusev et al. (1995) described a protocol where laser power was adjusted to 50% above the threshold of ionization. Threshold of ionization was defined as the minimum laser power to produce a molecular ion peak with a signal-to-noise ratio of 3 - 4 after 20 shots.

## Immunoaffinity-Based Sample Preparation and MALDI-TOF MS

MALDI-TOF MS can provide accurate molecular weight information from <20 fmol of analyte (Vorm and Mann, 1994). However, the small volumes applied to the MALDI probe mean that solution concentration detection limits are typically in the nM range. When crude extracts from biological samples are analyzed, μM or mM detection limits may be all that is possible. Various affinity-based sample purification techniques have been used to isolate analytes for analysis by MALDI-TOF MS. Avidin-biotin interactions have been used to isolate DNA (Jurinke et al., 1996), bradykinin and insulin (Schriemer and Li, 1996); single stranded DNA was used to isolate lactoferrin (Hutchens and Yip, 1993); metal-affinity chromatography to isolate metal-binding proteins (Papac et al., 1994a); and immunoaffinity purification techniques that exploit antibody-antigen

**Table 1.8 Immunoaffinity sample purification techniques coupled with MALDI-TOF MS.**

| Reference | Analyte | Concentration in original sample | Immunoaffinity purification technique |
|---|---|---|---|
| Nakanishi et al., 1994 | transferrin | unknown | analyzed immuno-precipitates |
| Papac et al., 1994a | cytochrome c | 50,000 ng/mL in phosphate buffer | antibody-coated agarose beads |
| Papac et al., 1994b | gastrin-releasing peptide | undetermined concentration in PBS | antibody-coated agarose beads |
| Nelson et al., 1995 | myotoxin a | 2 ng/mL in blood | antibody-coated agarose beads |
| Brockman and Orlando, 1995 | lysozyme | 3 ng/mL in tears | antibody-coated MALDI probe |
| Brockman and Orlando, 1996 | γ-INF | 10 ng/mL in PBS(?) | antibody-coated MALDI probe |
| Wang et al., 1996 | amyloid β-protein | 0.4 ng/mL in cultured cell media | immuno-precipitation on protein G/A beads |
| Liang et., 1998 | SNX-111 (peptide) | 3 ng/mL in plasma | antibody-coated MALDI probe |

interactions to extract biological molecules from biological fluids have been used to isolate numerous analytes (Table 1-8).

Wang et al. (1996) achieved the lowest detection limit with immunoaffinity purification at 0.4 ng amyloid β-protein/mL cultured cell media. The purification procedure entailed incubating the cell media for at least 5 h with the amyloid β-protein antibody. The antibody-antigen complex was then isolated on protein G/A-agarose beads (3 h) and finally eluted from the beads with the MALDI matrix solution. In a much

simpler procedure, Liang et al. (1998) achieved similar detection limits by immobilizing antibody directly on a MALDI probe. The probe was immersed in a blood plasma sample for 20 minutes to allow antibody-antigen binding. The probe was then rinsed with water and 3 μL of matrix solution was applied over top of the bound analyte. Immobilizing the antibody directly on the MALDI probe was very effective for isolating the SNX-111 peptide, however, only one sample can be analyzed per probe and the volume of sample solution must be large enough to immerse the probe. Nelson et al. (1995) detected 2 ng myotoxin a/mL of blood by allowing 50 μL of blood to equilibrate with a suspension of antibody-coated agarose beads for 45 min. The beads were separated and eluted with 4 μL of MALDI matrix solution. The eluent was spotted on the MALDI probe. In addition to the purification achieved, the myotoxin a was concentrated 12.5-fold.

In general, immunoaffinity purification coupled with MALDI-TOF MS appears capable of detecting analytes in biological fluids in the 1-10 ng/mL range. Serum GA levels were previously shown to range from ca 3-100 ng/mL. Based on this information, it may be possible to detect GAs in serum using MALDI-TOF MS.

## THESIS OBJECTIVES

The relative recent availability of commercial CE (ca 1988) and MALDI-TOF MS (ca 1991) instruments has made these techniques much more accessible to analytical food laboratories. Not many food applications have been developed to date, although CE is gaining in popularity because of its lower cost. The overall objective of the thesis is to explore the potential of using CE and MALDI-TOF MS for GA analysis. Specific objectives can be summarized as follows:

1. To develop and validate a CE method for determining GAs in potatoes.
2. To validate a MALDI-TOF MS method for quantifying α-chaconine and α-solanine in tubers with respect to accuracy, reproducibility, range and limits of detection.

3. To validate the MALDI-TOF MS method for quantifying α-chaconine and α-solanine in non-tuber samples.
4. To determine the distribution of endogenous toxic GAs during processing of potatoes into starch, potato protein concentrate, and potato pulp.
5. To explore the potential of using solid-phase extraction and immunopurification techniques with MALDI-TOF MS to detect GAs at concentrations typically found in blood after consuming potatoes.

## REFERENCES

Abell, D.A. and Sporns, P. 1996. Rapid quantitation of potato glycoalkaloids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Agric. Food Chem. 44: 2292-2296.

Achterberg, C.L., Clauson, D.M., Blease, J.A. and Barden, E.S. 1979. A new procedure for the mass extraction and collection of potato glycoalkaloids. Am. Potato J. 56: 145-148.

AOAC International, 1999. *Official Methods of Analysis of AOAC International*, 16th ed., 4th revision. AOAC International, Gaithersburg, MD.

Asbury, G.R., Al-Saad, K., Siems, W.F., Hannan, R.M. and Hill, H.H. 1999. Analysis of triacylglycerols and whole oils by matrix-assisted laser desorption/ionization time of flight mass spectrometry. J. Am. Soc. Mass Spectrom 10: 983-991.

Bartsch, H., Konig, W.A., Strassner, M. and Hintze, U. 1996. Quantitative determination of native and methylated cyclodextrins by matrix assisted laser desorption/ionization time of flight mass spectrometry. Carb. Res. 286: 41-53.

Beckman. 1991. *Introduction to Capillary Electrophoresis*. Beckman Instruments, Inc., Fullerton, CA.

Beavis, D.B. 1992. Matrix-assisted ultraviolet laser desorption: evolution and principles. Org. Mass Spectrom. 27: 653-659.

Benazuoz, M., Hakim, B. and Debrun, J.L. 1998. Study of laser desorption/ionization time-of-flight mass spectrometry for the analysis of pesticides. Rapid Commun. Mass Spectrom. 12: 1018-1020.

Birner, J. 1969. Determination of total steroid bases in *Solanum* species. J. Pharm. Sci. 58: 258-259.

Bömer A. and Mattis, H. 1924. The solanine content of potatoes. Z. Unters. Nahr-. Genussm. Gebrauchsgegenstände. 47: 97-127. [German] Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Bretzloff, C.W. 1971. A method for the rapid estimation of glycoalkaloids in potato tubers. Am. Potato J. 48: 158-162.

Brockman, A.H. and Orlando, R. 1995. Probe-immobilized affinity chromatography/ mass spectrometry. Anal. Chem. 67: 4581-4585.

Brockman, A.H. and Orlando, R. 1996. New immobilization chemistry for probe affinity mass spectrometry. Rapid Commun. Mass Spectrom. 10: 1688-1692.

Brown, R.S. and Lennon, J.J. 1995. Mass resolution by incorporation of pulsed ion extraction in a matrix-assisted laser desorption/ionization linear time-of-flight mass spectrometer. Anal. Chem. 67: 1998-2003.

Bushway, R.J. and Ponnampalam, R. 1981. α-Chaconine and α-solanine content of potato products and their stability during several modes of cooking. J. Agric. Food Chem. 29: 814-817.

Bushway, R.J.; Barden, E.S.; Bushway, A.W.; Bushway, A.A. 1979. High-performance liquid chromatographic separation of potato glycoalkaloids. J. Chromatogr. 178: 533-544.

Bushway, R.J.; Barden, E.S.; Wilson, A.M.; Bushway, A.A. 1980. Analysis of potato glycoalkaloids by high-performance liquid chromatography. J. Food Sci. 45: 1088-1089.

Bushway, R.J.; Bureau, J.L.; McGann, D.F. 1983. Alpha-chaconine and alpha-solanine content of potato peels and potato peel products. J. Food Sci. 48: 84-86.

Bushway, R.J., Bureau, J.L. and Stickney, M.R. 1985. A new efficient method for extracting glycoalkaloids from dehydrated potatoes. J. Agric. Food Chem. 33: 45-46.

Bushway, R.J.; Bureau, J.L.; King, J. 1986. Modification of the rapid high-performance liquid chromatographic method for the determination of potato glycoalkaloids. J. Agric. Food Chem. 34: 277-279.

Bushway, R.J., Savage, S.A. and Ferguson, B.S. 1987. Inhibition of acetylcholinesterase by solanaceous glycoalkaloids and alkaloids. Am. Potato J. 64: 409-413.

Cadle, L.S., Stelzig, D.A., Harper, K.L. and Young, R.J. 1978. Thin-layer chromatographic system for identification and quantification of potato tuber glycoalkaloids. J. Agric. Food Chem. 26: 1453-1454.

Camafeita, E. and Méndez, E. 1998. Screening of gluten avenins in foods by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Mass Spectrom. 33: 1023-1028.

Camafeita, E., Alfonso, P., Mothes, T. and Méndez, E. 1997. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric micro-analysis: the first non-immunological alternative attempt to quantify gluten gliadins in food samples. J. Mass Spectrom. 32: 940-947.

Cancalon, P.F. 1995. Capillary electrophoresis: a new tool in food analysis. J. AOAC Int. 78:12-15.

Carman, A.S.; Kuan, S.S.; Ware, G.M.; Francis, O.J.; Kirschenheuter, G.P. 1986. Rapid high-performance liquid chromatographic determination of the potato glycoalkaloids α-solanine and α-chaconine. J. Agric. Food Chem. 34, 279-282.

Chan, T.-W.D., Colburn, A.W., Derrick, P.J., Gardiner, D.J. and Bowden, M. 1992. Suppression of matrix ions in ultraviolet laser desorption: scanning electron microscopy and raman spectroscopy of the slid sample. Org. Mass Spectrom. 27: 188-194.

Chen, S., Derrick, P.J., Mellon, F.A. and Price, K.R. 1994. Analysis of glycoalkaloids from potato shoots and tomatoes by four-sector tandem mass spectrometry with scanning-array detection: comparison of positive ion and negative ion methods. Anal. Biochem. 218: 157-169.

Claringbold, W.D.B., Few, J.D., Brace, C.J. and Renwick, J.H. 1980. The disposition and metabolism of [$^3$H]-solasodine in man and Syrian hamster. J. Steroid Biochem. 13: 889-895.

Clarke, E.G.C. 1958. Identification of solanine. Nature (London) 181: 1152-1153.

Clement, E. and Verbist, J.F. 1980. Determination of solanine in *Solanum tuberosum* L. tubers: comparative study of 9 colorimetric methods. Lebensm.-Wissen. u. Technol. 13: 202-206. [French]

Dabbs, D.H. and Hilton, R.J. 1953. Methods for analysis for solanine in tubers of *Solanum tuberosum*. Can. J. Technol. 31: 213-220.

Dao, L. and Friedman, M. 1996. Comparison of glycoalkaloid content of fresh and freeze-dried potato leaves determined by HPLC and colorimetry. J. Agric. Food Chem. 44: 2287-2294.

Davis, L., Britten, J.J. and Morgan, M. 1997. Cholinesterase: its significance in anaesthetic practice. Anaesthesia 52: 244-260.

Duncan, M.W., Matanovic, G. and Cerpa-Poljak, A. 1993. Quantitative analysis of low molecular weight compounds of biological interest by matrix-assisted laser desorption ionization. Rapid Comm. Mass Spectrom. 7: 1090-1094.

Eldridge, A.C. and Hockridge, M.E. 1983. High-performance liquid chromatographic separation of eastern black nightshade (*Solanum ptychanthum*) glycoalkaloids. J. Agric. Food Chem. 31: 1218-1220.

El Rassi, Z. 1996. *High Performance Capillary Electrophoresis of Carbohydrates.* Beckman Instruments, Inc., Fullerton, CA.

Evans, S., Buchanan, R., Hoffman, A., Mellon, F.A., Price, K.R., Hall, S., Walls, F.C., Burlingame, A.L., Chen, S. and Derrick, P.J. 1993. Structural characterization of a glycoalkaloid at the femtomole level by means of four-sector tandem mass spectrometry and scanning-array detection. Org. Mass Spectrom. 28: 289-290.

Fitzpatrick, T.J. and Osman, S.F. 1974. A comprehensive method for determination of total potato glycoalkaloids. Am. Potato J. 51: 318-323.

Flanders, K.L., Hawkes, J.G., Radcliffe, E.B. and Lauer, F.L. 1992. Insect resistance in potatoes: sources, evolutionary relationships, morphology and chemical defenses, and ecogeographical associations. Euphytica 61: 83-111.

Friedman, M. and Dao, L. 1992. Distribution of glycoalkaloids in potato plants and commercial potato products. J. Agric. Food Chem. 40: 419-423.

Friedman, M. and Levin, C.E. 1992. Reversed-phase high-performance liquid chromatographic separation of potato glycoalkaloids and hydrolysis products on acidic columns. J. Agric. Food Chem. 40: 2157-2163.

Friedman, M. and McDonald, G.M. 1995. Extraction efficiency of various solvents for glycoalkaloid determination in potatoes and potato products. Am. Potato J. 72: 66A (abstr.).

Friedman, M. and McDonald, G.M. 1997. Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Crit. Rev. Plant Sci. 16: 55-132.

Friedman, M., Bautista, F.F., Stanker, L.H. and Larkin, K.A. 1998. Analysis of potato glycoalkaloids by a new ELISA kit. J. Agric. Food Chem. 46: 5097-5102.

Griebel, C. 1924. Solanine content of the 1922 potato harvest. Z. Unters. Nahr-. Genussm. Gebrauchsgegenstände 47: 436-438. [German] Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Gusev, A.I., Wilkinson, W.R., Proctor, A. and Hercules, D.M. 1993. Quantitative analysis of peptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Appl. Spectrosc. 47: 1091-1092.

Gusev, A.I., Wilkinson, W.R., Proctor, A. and Hercules, D.M. 1995. Improvement of signal reproducibility and matrix/comatrix effects in MALDI analysis. Anal. Chem. 67: 1034-1041.

Gusev, A.I., Wilkinson, W.R., Proctor, A. and Hercules, D.M. 1996. Direct quantitative analysis of peptides using matrix assisted laser desorption ionization. Fres. J. Anal. Chem. 354: 455-463.

Hall, R.L. 1992. Toxicological burdens and the shifting burden of toxicology. Food Technol. 46(3): 109-112.

Hansen, A.A. 1925. Two fatal cases of potato poisoning. Science 61: 340-341.

Harris, F.W. and Cockburn, T. 1918. Alledged poisoning by potatoes. Analyst (London) 43: 133-137.

Harvey, D.J. 1993. Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides. Rapid Commun. Mass Spectrom. 7: 614-619.

Harvey, M.H., McMillan, M., Morgan, M.R.A. and Chan, H.W.S. 1985a. Solanidine is present in serum of healthy individuals and in amounts dependent on their dietary potato consumption. Hum. Toxicol. 4: 187-194.

Harvey, M.H., Morris, B.A., McMillan, M. and Marks, V. 1985b. Measurement of potato steroidal alkaloids in serum and saliva by radioimmunoassay. Human Toxicol. 4: 503-512.

Heiger, D.N. 1992. *High Performance Capillary Electrophoresis - An Introduction.* 2nd ed. Hewlett-Packard Co., Waldbronn, Germany.

Hellenäs, K.-E. 1986. A simplified procedure for quantification of potato glycoalkaloids in tuber extracts by h.p.l.c.; comparison with ELISA and a colorimetric method. J. Sci. Food Agric. 37: 776-782.

Hellenäs, K.-E. 1994. Glycoalkaloids in Potato Tubers - Aspects of Analysis, Occurrence and Toxicology. Ph.D dissertation. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Hellenäs, K.-E. and Branzell, C. 1997. Liquid chromatographic determination of the glycoalkaloids α-solanine and α-chaconine in potato tubers: NMKL interlaboratory study. JAOAC. Int. 80: 549-554.

Hellenäs, K.-E., Nyman, A., Slanina, P., Lööf, L. and Gabrielsson, J. 1992. Determination of potato glycoalkaloids and their aglycone in blood serum by high-performance liquid chromatography: application to pharmacokinetic studies in humans. J. Chromatogr. 573: 69-78.

Hellenäs, K.-E.; Branzell, C.; Johnsson, H.; Slanina, P. 1995. Glycoalkaloid content of early potato varieties. J. Sci. Food Agric. 67: 125-128.

Herb, S.F., Fitzpatrick, T.J. and Osman, S.F. 1975. Separation of potato glycoalkaloids by gas chromatography. J. Agric. Food Chem. 23: 520-523.

Hillenkamp, F., Karas, M., Beavis, R.C. and Chait, B.T. 1991. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. Anal. Chem. 63: 1193-1202.

Hlywka, J.J.; Stephenson, G.R.; Sears, M.K.; Yada, R.Y. 1994 Effects of insect damage on glycoalkaloid content in potaotes (*Solanum tuberosum*). J. Agric. Food Chem. 42: 2545-2550.

Hu, A.Z., Cruzado, I.D., Hill, J.W., McNeal, C.J. and Macfarlane, R.D. 1995. Characterization of lipoprotein a by capillary zone electrohoresis. J. Chromatogr. A 717: 33-39.

Hutchens, T.W. and Yip, T.T. 1993. New desorption strategies for the mass spectrometric analysis of macromolecules. Rapid Commun. Mass Spectrom. 7: 576-580.

Jadhav, S.J., Lutz, S.E., Mazza, G. and Salunkhe, D.K. 1997. Potato glycoalkaoids: chemical, analytical, and biochemical perspectives. Ch. 6 in *Antinutrients and Phytochemicals in Food*, F. Shahidi (Ed.), p. 94-114, ACS Symposium Series 662, American Chemical Society, Washington, D.C.

Jellema, R., Elema, E.T. and Malingre, T.M. 1980. Optical brighteners ans thin-layer chromatography detection agents for glycoalkaloids and steroid alkaloids in solanum species. II. Blankophor BA 267%, BBU neu and KU, and Tinopal CBS-X and 5 BMS-X. J. Chromatogr. 189: 406-409

Jellema, R., Elema, E.T. and Malingre, T.M. 1981. Fluorodensitometric determination of potato glycoalkaloids on thin-layer chromatograms. J. Chromatogr. 210: 121-129.

Jespersen, S., Niessen, W.M.A., Tjaden, U.R. and van der Greef, J. 1995. Quantitative bioanalysis using matrix-assisted laser desorption/ioniation mass spectrometry. J. Mass Spectrom. 30: 357-364.

Jonker, H.H., Koops, A.J. and Hoogendoorn, J.C. 1992. A rapid method for the quantification of steroidal glycoalkaloids by reversed phase HPLC. Potato Res. 35: 451-455.

Jorgenson, J.W. and Lukacs, K.D. 1983. Capillary zone electrophoresis. Science 222: 266-272.

Jurinke, C., Zöllner, B., Feucht, H.-H., Jacobs, A., Kirchhübel, J., Lüchow, A., van den Boom, D., Laufs, R. and Köster, H. 1996. Detection of hepatitis B virus DNA in serum samples via nested PCR and MALDI-TOF mass spectrometry. Genet. Anal. Biomol. Eng. 13: 67-71.

Kaaber, L. 1993. Glycoalkaloids, green discoloration and taste development during storage of some potato varieties (*Solanum tuberosum* L.). Norw. J. Agric. Sci. 7: 221-229.

Karas, M. 1997. Matrix-assisted laser desorption ionization MS: a progress report. Biochem. Mass Spectrom. 24: 897-900.

Karas, M. and Hillenkamp, F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal. Chem. 60: 2299-2301.

Kazmaier, T., Roth, S., Zapp, J., Harding, M. and Kuhn, R. 1998. Quantitative analysis of malto-oligosaccharides by MALDI-TOF mass spectrometry, capillary electrophoresis and anion exchange chromatography. Fres. J. Anal. Chem. 361: 473-478.

Keukens, E.A.J., de Vrije, T., Fabrie, C.H.J.P., Demel, R.A., Jongen, W.M.F and de Kruijff, B. 1992. Dual specificity of sterol-mediated glycoalkaloid induced membrane disruption. Biochim. Biophys. Acta 1110: 127-136.

Keukens, E.A.J., de Vrije, T., van den Boom, C., de Waard, P., Plasman, H.H., Thiel, F., Chupin, V., Jongen, W.M.F. and de Kruijff, B. 1995. Molecular basis of glcoalkaloid induced membrane disruption. Biochim. Biophys. Acta 1240: 216-228.

Keukens, E.A.J., de Vrije, T., Jansen, L.A.M., de Boer, H., Janssen, M., de Kroon, A.I.P.M., Jongen, W.M.F. and de Kruijff, B. 1996. Glycoalkaloids selectively permeabilize cholesterol containing biomembranes. Biochim. Biophys. Acta 1279: 243-250.

Kim, M. and Durst, R.A. 1999. Development of two sensitive liposome-based immunoassays for the determination of potato glycoalkaloids - strip assay and flow injection analysis. Presented at Ann. Mtg., Inst. Food Technologists, Chicago, IL, July 24-28.

Kobayashi, K.; Powell, A.D.; Toyoda, M.; Saito, Y. 1989. High-performance liquid chromatographic method for simultaneous analysis of α-solanine and α-chaconine in potato plants cultured *in vitro*. J. Chromatogr. 462: 357-364.

Kozukue, N.; Kozukue, E.; Mizuno, S. 1987. Glycoalkaloids in potato plants and tubers. HortScience 22: 294-296.

Krasowski, M.D., McGehee, D.S. and Moss, J. 1997. Natural inhibitors of cholinesterases: implications for adverse drug reactions. Can. J. Anaesth. 44: 525-534.

Kuhn, R., Stoeklin, F. and Erni, F. 1992. Chiral separations by host-guest complexation with cyclodextrin and crown ether by capillary zone electrophoresis. Chromatographia 33: 32-40.

Liang, X., Lubman, D.M., Rossi, D.T., Nordblom, G.D. and Barksdale, C.M. 1998. On-probe immunoaffinity extraction by matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 70: 498-503.

Lindeberg, J. 1996. Capillary electrophoresis in food analysis. Food Chem. 55: 73-94.

Ling, Y.-C., Lin, L. and Chen, Y.-T. 1998. Quantitative analysis of antibiotics by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 12: 317-327.

Loewenstein-Lichtenstein, Y., Schwarz, M., Glick, D., Nørgaard-Pedersen, B., Zakut, H. and Soreq, H. 1995. Genetic predisposition to adverse consequences of anti-cholinesterases in 'atypical' BCHE carriers. Nature Med. 1: 1082-1085.

Marrs, T.C. 1993. Organophosphate poisoning. Pharmacol. Ther. 58: 51-66.

Matthew, J.A., Morgan, M.R.A., McNerner, R., Chan, H.W. and Coxon, D.T. 1983. Determination of solanidine in human plasma by RIA. Food Chem. Toxicol. 21: 637-641.

McMillan, M. and Thompson, J.C. 1979. An outbreak of suspected solanine poisoning in schoolboys. Quarterly Journal of Medicine, News Service 48, No. 190, p. 227-243.

Mitchell, B.K. and Harrison, G.D. 1985. Effects of Solanum glycoalkaloids on chemosensilla in the colorado potato beetle: A mechanism of feeding deterrence. J. Chem. Ecol. 11: 73-83.

Morgan, M.R.A., McNerney, R., Matthew, J.A., Coxon, D.T. and Chan, W.-S. 1983. An enzyme-linked immunosorbent assay for total glycoalkaloids in potatoes. J. Sci. Food Agric. 34: 593-598.

Morris, S.C. and Lee, T.H. 1981. Analysis of potato glycoalkaoids with radially compressed high-performance liquid chromatographic cartridges and ethanolamine in the mobile phase. J. Chromatogr. 219: 403-410.

Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Muddiman, D.C., Gusev, A.I., Proctor, A., Hercules, D.M., Venkataraman, R. and Diven, W. 1994. Quantitative measurement of cyclosporin A in blood by time-of-flight mass spectrometry. Anal. Chem. 66: 2362-2368.

Muddiman, D.C., Gusev, A.I. and Hercules, D.M. 1995. Application of secondary ion and matrix-assisted laser desorption-ionization time-of-flight mass spectromtry for the quantitative analysis of biological materials. Mass Spectrom. Rev. 14: 383-429.

Nakanishi, T., Okamoto, N., Tanaka, K. and Shimizu, A. 1994. Laser desorption time-of-flight mass spectrometric analysis of transferrin precipitated with antiserum: a unique simple method to identify molecular weight variants. Biol. Mass Spectrom. 23: 230-232.

Nelson, R.W., McLean, M.A. and Hutchens, T.W. 1994. Quantitative determination of proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 66: 1408-1415.

Nelson, R.W., Krone, J.R., Bieber, A.L. and Williams, P. 1995. Mass spectrometric immunoassay. Anal. Chem. 67: 1153-1158.

Neville, L.F., Gnatt, A., Loewenstein, Y. and Soreq, H. 1990. Aspartate-70 to glycine substitution confers resistance to naturally occurring and synthetic anion-site ligands on in-ovo produced human butyrylcholinesterase. J. Neurosci. Res. 27: 452-460.

Nigg, H.N., Ramos, L.E., Graham, E.M., Sterling, J., Brown, S. and Cornell, J.A.. 1996. Inhibition of human plasma and serum butyrylcholinesterase (EC 3.1.1.8) by alpha-chaconine and alpha-solanine. Fundam. Appl. Toxicol. 33: 272-281.

Nishie, K., Gumbmann, M.R. and Keyl, A.C. 1971. Pharmacology of solanine. Toxicol. Appl. Pharmacol. 19: 81-92.

O'Malley, M.A. and McCurdy, S.A. 1990. Subacute poisoning with phosalone, an organophosphate insecticide. West. J. Med. 153: 619-624.

Osman, S.F. and Sinden, S.L. 1977. Analysis of mixtures of solanidine and demissidine glycoalkaloids containing identical carbohydrate units. J. Agric. Food Chem. 25: 955-957.

Osman, S.F., Herb, S.F., Fitzpatrick, T.H. and Schmiediche, P. 1978. Glycoalkaloid composition of wild and cultivated tuber-bearing *Solanum* species of potential value in potato breeding programs. J. Agric. Food Chem. 26: 1246-1248.

Papac, D.I., Hoyes, J. and Tomer, K.B. 1994a. Direct analysis of affinity-bound analytes by MALDI/TOF MS. Anal. Chem. 66: 2609-2613.

Papac, D.I., Hoyes, J. and Tomer, K.B. 1994b. Epitope mapping of the gastrin-releasing peptide/anti-bombesin monoclonal antibody complex by proteolysis followed by matrix-assisted laser desorption ionization mass spectrometry. Protein Sci. 3: 1485-1492.

Percival, G., Dixon, G. and Sword, A. 1994. Glycoalkaloid concentration in potato tubers following continuous illumination. J. Sci. Food Agric. 66: 139-144.

Peréz, J., Glorio-Paulet, P., Trognitz, B., Delgado, C. and Espanola, N. 1999. Development an evaluation of an enzyme immunoassay for the quality control of total glycoalkaloids in improved potato tubers. Presented at 218$^{th}$ National Mtg. of Amer. Chem. Soc., New Orleans, LA, Aug. 22-26.

Pfuhl, E. 1899. Mass sickness caused by poisoning from potatoes with high solanine content. Deutsch. Med. Wochenschr. 25: 753-755. [German] Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Pitt, J.J. and Gorman, J.J. 1997. Oligosaccharide characterization and quantitation using 1-phenyl-3-methyl-5-pyrazolone derivatization and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Biochem. 248: 63-75

Plhak, L.C. and Sporns, P. 1992. Enzyme immunoassay for potato glycoalkaloids. J. Agric. Food Chem. 40: 2533-2540.

Plhak, L.C. and Sporns, P. 1994. Development and production of monoclonal antibodies for the measurement of solanidine potato glycoalkaloids. Am. Potato J. 71: 297-313.

Plhak, L.C. and Sporns, P. 1997. Biological activities of potato glycoalkaloids. Ch. 7 in *Antinutrients and Phytochemicals in Food*, F. Shahidi, (Ed.), p. 115-126, ACS Symposium Series 662, American Chemical Society, Washington, D.C.

Preston, L.M., Murray, K.K. and Russell, D.H. 1993. Reproducibility and quantitation of matrix-assisted laser desorption ionization mass spectrometry: Effects if nitrocellulose on peptide ion yields. Biol. Mass Spectrom. 22: 544-550.

Price, K.R., Mellon, F.A., Self, R., Fenwick, G.R. and Osman, S.F. 1985. Fast bombardment mass spectrometry of *Solanum* glycoalkaloids and its potential for mixture analysis. Biomed. Mass Spectrom. 12: 79-85.

Reelah, L.A. and Keem, A. 1958. A case of mass poisoning by solanine. Sov. Med. 22: 129-131. [Russian]

Roddick, J.G. 1979. Complex formation between Solanaceous steroidal glycoalkaloids and free sterols in vitro. Phytochemistry 18: 1467-1470.

Roddick, J.G., Rijnenberg, A.L. and Osman, S.F. 1988. Synergistic interaction between potato glycoalkaloids alpha-solanine and alpha-chaconine in relation to destabilization of cell membranes: ecological implications. J. Chem. Ecol. 14: 889-902.

Ross, H., Pasemann, P. and Nitzsche, W. 1978. The dependence of the glycoalkaloid content of potatoes on location and year of production and its relation to taste. Z. Pflanzenzüchtg. 80: 64-79. [German]

Rothe, J.C. 1918. Illness following the eating of potatoes containing solanine. Z. Hyg. 88: 1-12. [German] Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Rühl, R. 1951. Contribution to pathology and toxicology of solanine. Arch. Pharm. Weinheim Ger. 284: 67-74. [German] Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Saito, K.; Horie, M.; Hoshino, Y.; Nose, N. 1990. High performance liquid chromatographic determination of glycoalkaloids in potatoes. J. Chromatogr. 508: 141-147.

Sanford, L.L.; Deahl, K.L.; Sinden, S.L. 1994. Glycoalkaloid content in foliage of hybrid and backcross populations from a *Solanum tuberosum* × *S. chacoense* cross. Am. Pot. J. 71: 225-235.

Schriemer, D.C. and Li, L. 1996. Combining avidin-biotin chemistry with matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 68: 3382-3387.

Schwarz, M., Glick, D., Loewenstein, Y. and Soreq, H. 1995. Engineering of cholinesterases explains and predicts diverse consequences of administration of various drugs and poisons. Pharmac. Ther. 67: 283-322

Severs, J.C. and Smith, R.D. 1997. Capillary electrophoresis - electrospray ionization mass spectrometry. Ch. 10 in *Electrospray Ionization Mass Spectrometry*, R.B. Cole (Ed.), p. 343-382, Academic Press, John Wiley and Sons, Inc.

Shih, M.J. and Kuć, J. 1974. α- and β-Solamarine in Kennebec (*Solanum tuberosum*) leaves and aged tuber slices. Phytochemistry 13: 997-1000.

Sinden, S.L. and Sanford, L.L. 1981. Origin and inheritance of solamarine glycoalkaloids in commercial potato cultivars. Am. Potato J. 58: 305-325.

Sinden, S.L., Deahl, K.L. and Aulenbach, B.B. 1976. Effect of glycoalkaloids and phenolics on potato flavor. J. Food Sci. 41: 520-523.

Sinden, S.L., Sanford, L.L. and Osman, S.F. 1980. Glycoalkaloids and resistance to the colorado potato beetle in *Solanum chacoense* Bitter. Am. Potato J. 57 :331-343.

Siuzdak, G. 1994. The emergence of mass spectrometry in biochemical research. Proc. Natl. Acad. Sci. USA 91: 11290-11297.

Smith, D.B., Roddick, J.G. and Jones, J.L. 1996. Potato glycoalkaloids: some unanswered questions. Trends Food Sci. Technol. 7: 126-131.

Sporns, P. and Wang, J. 1999. Exploring new frontiers in food analysis using MALDI-MS. Food Res. Int. 31: 181-189.

Stanker, L.H., Kamps-Holtzapple, C. and Friedman, M. 1994. Development and characterization of monoclonal antibodies that differentiate between potato and tomato glycoalkaloids and aglycons. J. Agric. Food Chem. 42: 2360-2366.

Swinyard, C.A. and Chaube, S. 1973. Are potatoes teratogenic for experimental animals? Teratology 8: 349-358.

Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y. and Yoshida, T. 1988. Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2: 151-153.

Tang, K., Allman, S.L., Jones, R.B. and Chen, C.H. 1993. Quantitative analysis of biopolymers by matrix-assisted laser desorption. Anal. Chem. 65: 2164-2166.

Tang X.D., Sadeghi, M.E., Olumee, Z., Vertes, A., Braatz, J.A., McIlwain, L.K. and Dreifuss, P.A. 1996. Detection and quantitation of β-2-microglobulin glycosylated end products in human serum by matrix assisted laser desorption/ionization mass spectrometry. Anal. Chem. 68: 3740-3745.

Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A. and Ando, T. 1983. Electrokinetic separations with micellar solutions in open-tube capillaries. Anal. Chem. 56: 111-113.

Terbruggen, A. 1936. Fatal solanine poisoning. Beit. Pathol. 97: 391-395. [German] Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Thomson, C.A. and Sporns, P. 1995. Fluorescence polarization immunoassays for potato glycoalkaloids. J. Agric. Food Chem. 43: 254-260.

Tingey, W.M. and Sinden, S.L. 1982. Glandular pubescence, glycoalkaloid composition, and resistance to the green peach aphid, potato leafhopper, and the potato fleabeetle in *Solanum berthaultii*. Am. Potato J. 59: 95-106.

Tingey, W.M., MacKenzie, J.D. and Gregory, P. 1978. Total foliar glycoalkaloids and resistance of wild potato species to *Empoasca fabae* (Harris). Am. Potato J. 55: 577-585.

Vallejo, R.P. and Ercegovich, C.D. 1979. Analysis of potato for glycoalkaloid content by radioimmunoassay (RIA). In *Methods and Standards for Environmental Measurements*, p. 333-340. Publication 519. National Bureau of Standards, Washington, DC.

van Gelder, W.M.J. and Scheffer, J.J.C. 1991. Transmission of steroidal glycoalkaloids from *Solanum vernei* to the cultivated potato. Phytochemistry 30: 165-168.

van Gelder, W.M.J., Tuinistra, L.G.M.Th., van der Greef, J. and Scheffer, J.J.C. 1989. characterization of novel steroidal alkaloids from tubers of *Solanum* species by combined gas chromatography-mass spectrometry. Implications for potato breeding. J. Chromatogr. 482: 13-22.

Vorm, O. and Mann, M. 1994. Improved mass accuracy in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of peptides. J. Am. Soc. Mass Spectrom. 5: 955-958.

Vorm, O., Roepstroff, P. and Mann, M. 1994. Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. Anal. Chem. 66: 3281-3287.

Wang, J. and Sporns, P. 1999. Analysis of anthocyanins in red wine and fruit juice using MALDI-MS. J. Agric. Food Chem. 47: 2009-2015.

Wang, J., Sporns, P. and Low, N.H.. 1999. Analysis of food oligosaccharides using MALDI-MS: quantification of food fructooligosaccharides. J. Agric. Food Chem. 47: 1549-1557.

Wang, R., Sweeney, D., Gandy, S.E. and Sisodia, S.S. 1996. The profile of soluble amyloid β protein in cultured cell media. J. Biol. Chem. 271: 31894-31902.

Weinberger, R. 1993. *Practical Capillary Electrophoresis*, Academic Press, Inc., San Francisco, CA.

Whittal, R.M., Palcic, M.M., Hindsgaul, O. and Li, L. 1995. Direct analysis of enzymatic reactions of oligosaccharides in human serum using matrix-assisted laser desorption ionization mass spectrometry. Anal. Chem. 67: 3509-3514.

Willimott, S.G. 1933. An investigation of solanine poisoning. Analyst 58: 431-439.

Wilson, G.S. 1959. A small outbreak of solanine poisoning. Month. Bull. Ministrry Health Public Health Lab. Serv. 18: 207-210. Cited in Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Zhao, J.; Camire, M.E.; Bushway, R.J.; Bushway, A.A. 1994. Glycoalkaloid content and *in vitro* glycoalkaloid solubility of extruded potato peels. J. Agric. Food Chem. 42: 2570-2573.

Zitnak, A. and Filadelfi, M.A. 1985. Estimation of taste thresholds of three potato glycoalkaloids. J. Can. Inst. Food Sci. Technol. 18: 337-339.

# Chapter 2

## Capillary Electrophoresis Laser-Induced Fluorescence Method for Analysis of Potato Glycoalkaloids based on a Solution-Phase Immunoassay.[1]

## INTRODUCTION

For more than one hundred 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 above 210 nm. Extensive sample clean-up is 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 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 ELISAs require the attachment of one of the reactants to the solid phase, most commonly a microtiter plate. This slows the rate of antibody-antigen binding because one of the reactants cannot move freely. Multiple washing steps also increase analysis time and decrease the method's precision. Pritchett et al. (1995) reported that solution-phase immunoassays were faster and more reproducible than solid-phase immunoassays. However, the detection of the reaction products in solution-phase

[1] A version of this chapter has been accepted for publication. Driedger, D.R, LeBlanc, R.J., LeBlanc, E.L. and Sporns, P. 2000. J. Agric. Food Chem.

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 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 (Figure 2-1).

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). The development of laser-induced fluorescence (LIF) detectors has led to substantial improvements in sensitivity. Sub-attomole levels of analyte have been detected conventionally (Shea, 1997), whereas Chen et al. (1994) reported 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.,1995a), morphine and phencyclidine (Chen and Evangelista, 1994). The objective of the present work was to develop a CE-LIF method using a pre-column 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 and then evaluate the specificity, range, precision, and accuracy of the method.

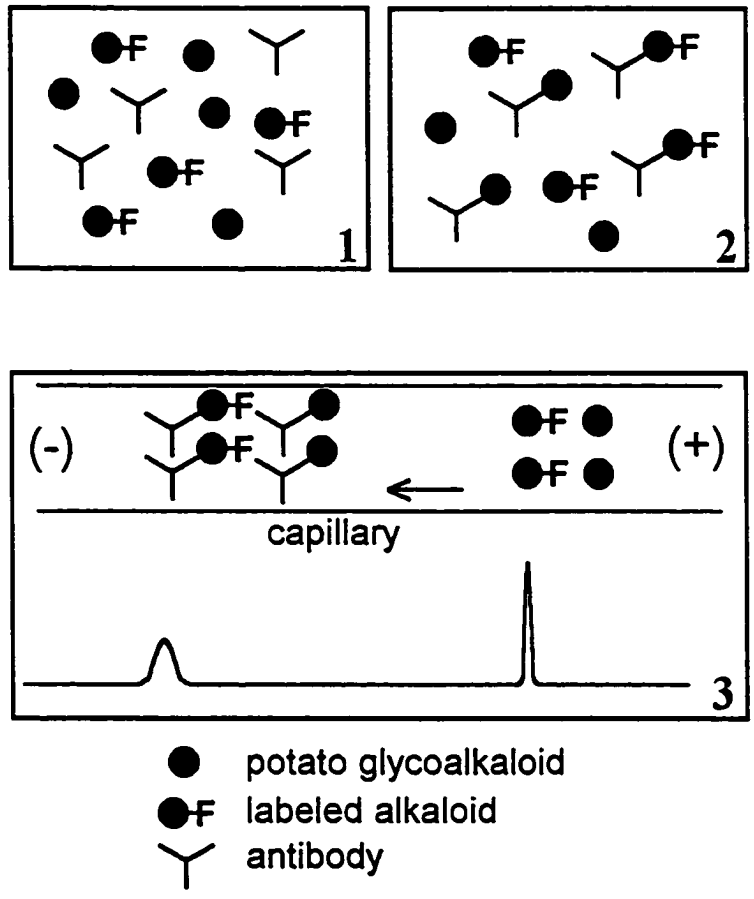


**Figure 2-1 Competitive immunoassay coupled to CE-LIF. (1) Potato GA, labeled-alkaloid, and antibody are mixed, (2) potato GA and labeled-alkaloid compete for antibody binding sites, (3) reaction products are separated by CE-LIF.**

## MATERIALS AND METHODS

**Instrumentation.** 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 sec. Data was 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-dimethylaminopyridine (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). Polyclonal antiserum was prepared previously (Plhak and Sporns, 1992). Phosphate buffered saline (PBS) solution composition was 0.9% NaCl (w/v) and 50 mM phosphate, pH 7.5.

Potato (*Solanum tuberosum*) samples A-F were originally described by Abell and Sporns (1996). Samples A and B were cultivar Russet Burbank stored 8 and 12 months, respectively. Samples C and D were cultivars Shepody and Yukon Gold, respectively, both stored 8 months. Samples E and F were obtained commercially. Sample E was peeled and Sample F was unpeeled. A second Yukon Gold potato sample was obtained from Alberta Agriculture, Food and Rural Development for repeatability studies and had been stored in a commercial seed potato storage facility for approximately seven months after the 1997 harvest.

**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, evaporated under vacuum, and the residue 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 prepared 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. After equilibration, the serum dilutions were analyzed by CE-LIF and the AMF-SOL peak area was plotted against serum dilution.

**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 Milli-Q water. The sample was then extracted three times by homogenizing in 15 mL 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 1,960 × *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).

**GA Determination in Potatoes.** A stock solution of 240 μM total GA was prepared in methanol using ca equal amounts of α-chaconine and α-solanine. Calibration standards were prepared with ca 0, 50, 100, 200, 300 and 400 nM total GA in PBS with

10% methanol (v/v). Standards were analyzed in duplicate. The test solution (250 μL of a standard or a 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 at room temperature before analysis by CE-LIF. Extracts were assayed in duplicate and each assay injected in duplicate. Peak areas were used to determine total GA concentration in the extracts. GA concentration in fresh potatoes was calculated assuming 80% moisture in the tuber (Abell and Sporns, 1996).

## 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, α-solanine and α-chaconine, differ only in the carbohydrates attached to solanidine. GA antibody exhibited strong affinity ($K_{aff}$ approximately $4 \times 10^8$) 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 nm and 512 nm respectively for the 4'-aminomethylfluorescein starting material. CE-LIF detection of the AMF-SOL revealed the presence of several contaminants that fluoresced similarly (Figure 2-2). The identity of these contaminants was not determined. CE-LIF detection of the original 4'-methylaminofluorescein 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 non-specific binding, the AMF-SOL solution was treated with serum raised against a brochocin C

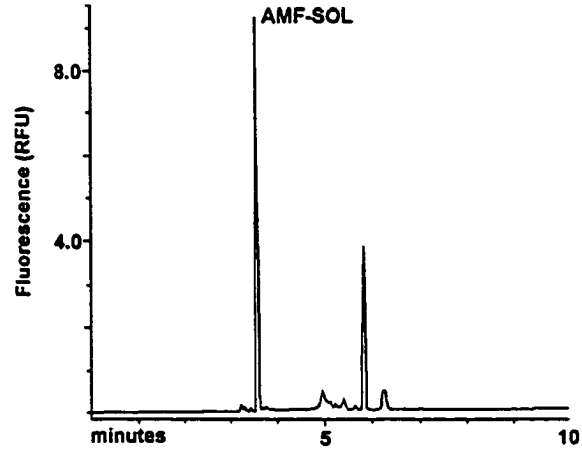


**Figure 2-2 CE-LIF electrophrogram of stock solution of AMF-SOL showing the presence of several contaminants.**

peptide (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 2-3) 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 with pH and reached a plateau at ca pH 7.5 (Figure 2-4). 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 pKa 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. Based on these considerations, CE buffers with pH 7.5 were developed.

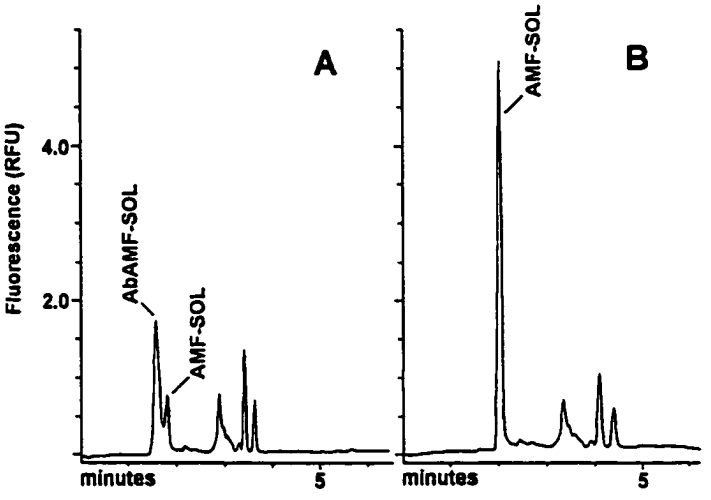


**Figure 2-3 CE-LIF electropherogram showing the effect of anti-GA serum on AMF-SOL peak area (A) and the effect of anti-brochocin C serum on GA peak area (B).**

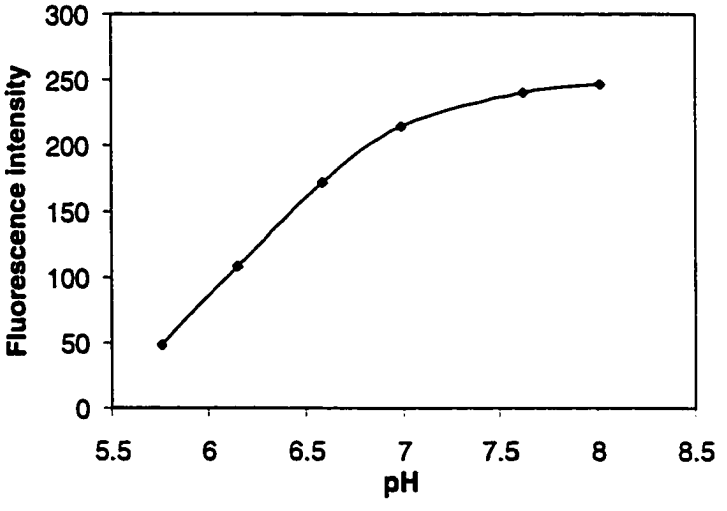


**Figure 2-4 Effect of pH on the fluorescence intensity of AMF-SOL in phosphate buffer at an excitation of 488 nm and an emission of 520 nm.**

**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. 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 peak areas were inconsistent. A standard curve produced with AMF-SOL (no pre-column 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. 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. The borate buffer, however, partially resolved the unbound AMF-SOL from the bound AMF-SOL (Figure 2-5). 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 did not contain a carbohydrate group and the borate probably acted simply as a buffer and not as an anionic complexing agent. Although the

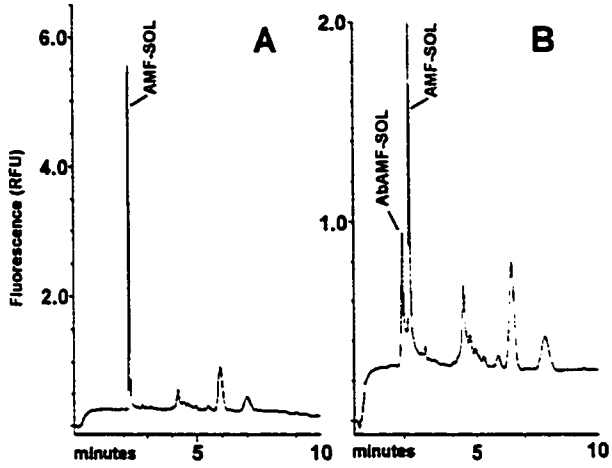


**Figure 2-5 CE-LIF separation of unbound AMF-SOL from the AMF-SOL-antibody complex using 50 mM borate, 10% (v/v) methanol, pH 7.5 as the running buffer. (A) 200 nM AMF-SOL; (B) 200 nM AMF-SOL in the presence of anti-GA serum**

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. Figure 2-6 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-

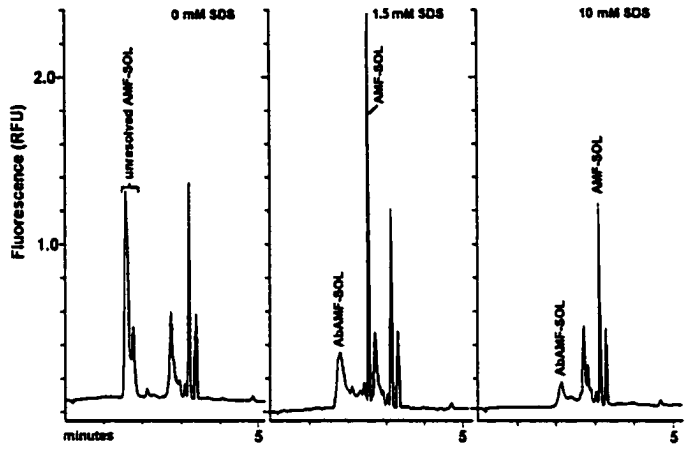


**Figure 2-6 Effect of SDS concentration on the resolution of unbound AMF-SOL from AMF-SOL-antibody complex. SDS was added to 50 mM phosphate, 10% (v/v) methanol, pH 7.5.**

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. Figure 2-7 shows that

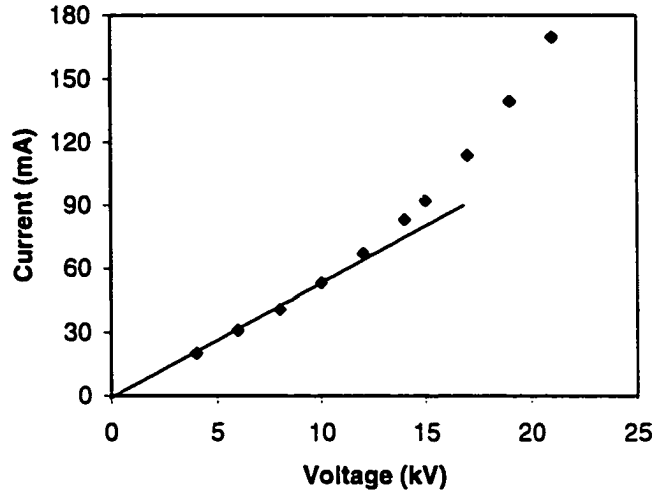


**Figure 2-7 Ohm's Law plot for the 50 mM phosphate, 1.5 mM SDS, 10% (v/v) methanol, pH 7.5 buffer.**

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 sec 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.

**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 antibody concentration for the immunoassay, antibody dilution curves were prepared by allowing 200 nM AMF-SOL to react with various amounts of antibody. The optimal concentration of antibody required for the competitive immunoassay was then determined. The antibody dilution 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 2-8). 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.

**Calibration and Range for Quantitative Determination of GAs in Potatoes.** The area of the unbound AMF-SOL peak was proportional to the GA concentration in the test solution (Figure 2-9). When plotted on a semilogarithmic scale, a straight line calibration curve was generated with standards encompassing 50 – 400 nM GA (Figure 2-10). Extension of the range beyond these values produced a sigmoidal curve typical of competitive immunoassays (Schmalzing et al., 1995a). For quantification, the range was limited to the straight-line portion of the curve. The latter allowed for quantification of GA in tubers from 43 µg/g to 340 µg/g on a fresh weight basis under the specified protocol for sample preparation. Samples E and F contained less than 40 µg/g GA and were reported as having trace amounts of GA. It is expected that levels less than 40 µg/g could be quantified by CE-LIF by using more concentrated sample extracts but since the

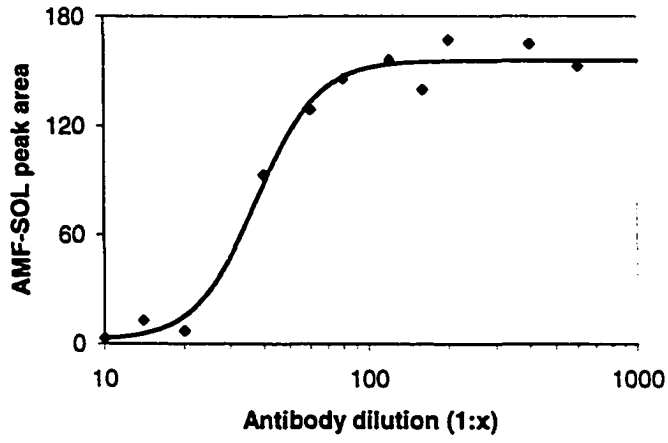


**Figure 2-8 Antibody dilution curve showing the effect of anti-GA serum dilution on AMF-SOL peak area.**

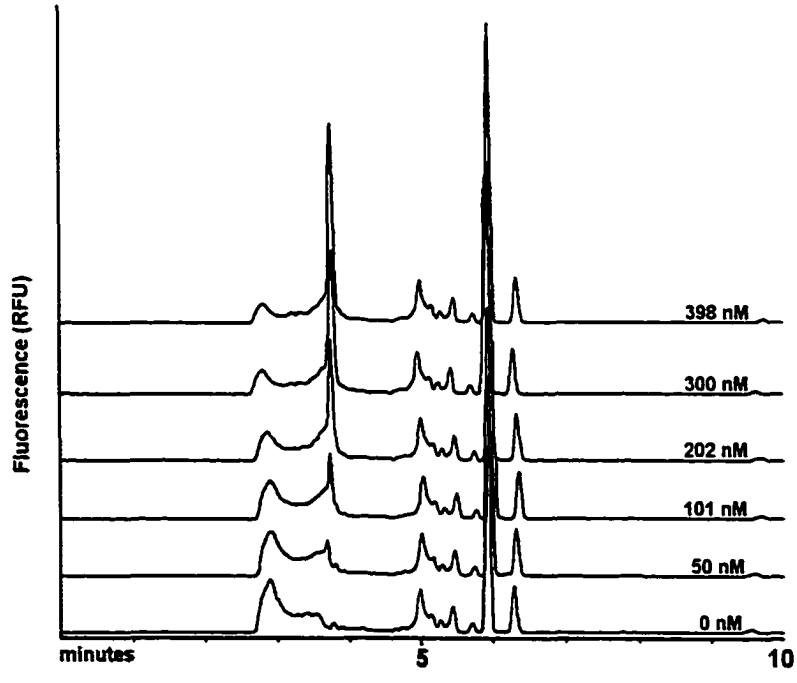


**Figure 2-9 Effect of GA concentration on unbound AMF-SOL peak area.**

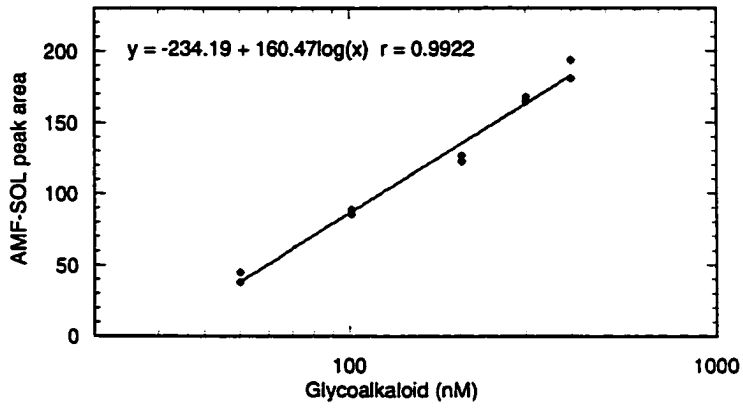


**Figure 2-10 Calibration curve for the determination of GAs by CE-LIF-based immunoassay.**

generally recognized acceptable concentration of GA in tubers is 200 μg/g, no further effort was made to accurately quantify the samples of < 40 μg/g GA.

**Accuracy.** Without standard reference material, it was impossible to conclusively determine the accuracy of the method. Nevertheless, the accuracy was estimated with spike recoveries and by comparing results with different analytical methods. Spike recoveries ranged from 63% to 97% with the lowest recovery coming from the lowest spike level (Table 2-1). Similar recoveries were reported by Schmalzing (1995a,b). Data from the same potato samples analyzed by HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are presented in Table 2-2. While each method successfully identified high and low GA samples, there were differences in actual values. HPLC values tended to be somewhat higher than CE-LIF and MALDI-TOF MS values. However, if CE-LIF values are adjusted for spike recoveries (typically 90%), agreement between CE-LIF and HPLC data improves. Different extraction procedures no doubt account for some of the discrepancies; CE-LIF

**Table 2-1 Accuracy of potato GA determination by CE-LIF**

| GA added (μg/g) | GA found (μg/g) | Recovery (%) |
|---|---|---|
| 22 | 14 | 63 |
| 43 | 41 | 97 |
| 86 | 73 | 85 |
| 170 | 160 | 93 |

Initial concentration in potato sample 43 μg/g.

**Table 2-2 Comparison of GA analysis by CE-LIF, HPLC and MALDI-TOF MS**

| | Total glycoalkaoids (μg/g, fresh weight basis) | | |
|---|---|---|---|
| Sample | CE-LIF | HPLC | MALDI-TOF MS |
| A | 210 | 252 | 217 |
| B | 220 | 340 | 364 |
| C | 97 | 131 | 92 |
| D | 98 | 107 | 76 |
| E | trace | 12 | 16 |
| F | trace | 22 | 29 |

HPLC and MALDI-MS data published by Abell and Sporns (1996).

and HPLC samples were extracted with methanol, while MALDI-TOF MS samples were extracted with methanol:water 1:1 (v/v) (Abell and Sporns, 1996). Nevertheless, it should be noted that previous comparisons of GA analytical methods exhibited similar agreement between immunoassay and HPLC data as was observed in our experiment (Plhak and Sporns, 1992; Hellenäs, 1984). Recently, somewhat better agreement between ELISA and HPLC was reported by Friedman et al. (1998).

**Repeatability.** The immunoassay and separation by CE-LIF were performed on duplicate aliquots from the same extract. The RSD for the duplicates ranged from 0.9% to 9.7% with an average of 5.7% (n=14). To determine the variability due to the instrument, a 200 nM AMF-SOL in PBS solution was analyzed as an instrument check sample three or four times with each sample queue. On a given day, the RSD for peak

areas for the instrument check sample averaged 5%, indicating that the instrument accounted for a large proportion of the intra-assay variability. This suggests that the use of an internal standard may be helpful in clarifying some of the variability due to the instrument. The day-to-day repeatability of the method was estimated by analyzing a Yukon Gold potato extract on four separate days. A mean value of 53 μg/g was obtained with a RSD of 12%. The repeatability of the present CE-LIF method is comparable to other solution-based immunoassays (Schultz et al., 1995; Schmalzing et al., 1995b).

**Specificity.** The specificity of an immunoassay depends upon the cross-reactivity of the antibody. In the present immuno-CE-LIF method, the area of the unbound AMF-SOL peak will be affected only by compounds that exhibit an affinity for the anti-GA serum. The serum used in this experiment was well characterized by Plhak and Sporns (1992). They showed that the serum had a strong affinity for solanidine alkaloids (α-chaconine and α-solanine), slightly lower affinity for the structurally similar demissidine alkaloids (demissine and commersonine), and very little affinity for the spiral alkaloids (tomatidine and solasodine). Given that the antibody had little affinity for spiral potato alkaloids, the likelihood that other less-related compounds could interfere with the method is remote. The strong affinity for solanidine alkaloids makes the antibody suitable for most potato analyses since α-chaconine and α-solanine make up at least 95% of the GAs found in domestic potato varieties (Friedman and McDonald, 1997). However, this procedure may underestimate total GA levels in non-domesticated potato varieties, which can contain a variety of non-solanidine alkaloids (Maga, 1980).

**Conclusions.** These results indicate that a solution-phase immunoassay coupled to CE-LIF allows for rapid quantification of total GAs in potatoes. Incubation of the sample extract with the antibody solution required 30 min and was followed by a 10 min CE-LIF separation (Table 2-3). Since CE-LIF injection is automated, the analyst simply has to mix the reagents in a microcentrifuge tube and allow them to incubate before transferring the contents to a CE vial. By adjusting volumes, it is likely the whole immunoassay could be performed in the CE vial itself. The short analysis time and potential for fully automated operation make this technique attractive for routine analysis

**Table 2-3 Estimated time required for analyzing 10 samples by various GA methods**

| | Time required (min) | | | |
|---|---|---|---|---|
| | CE-LIF | HPLC[1] | MALDI-TOF MS[2] | ELISA[3] |
| Sample preparation or immunoassay | 60 | 600 | 100 | 400 |
| Separation and/or detection | 140 | 300 | 100 | 5 |
| Total time | 200 | 900 | 200 | 405 |

[1] Saito et al., 1990
[2] Abell and Sporns, 1996
[3] Plhak and Sporns, 1992

of large numbers of samples. Conversely, the simplicity of the method makes it convenient for the analysis of small numbers of potato GA samples as well.

## REFERNCES

Abell, D.A. and Sporns, P. 1996. Rapid quantitation of potato glycoalkaloids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Agric. Food Chem. 44: 2292-2296.

Babcock, D.F. and Kramp, D.C. 1983. Spectral properties of fluorescein and carboxyfluorescein. J. Biol. Chem. 258: 6389.

Beckman Instruments, Inc. 1994. *Introduction to Capillary Electrophoresis*, Document No. 360643; p 29.

Bieniarz, C., Young, D.F. and Cornwell, M.J. 1994. Thiolate and phosphorothioate functionalized fluoresceins and their use as fluorescent labels. Bioconj. Chem. 5: 31-39.

Blais, W.B., Cunningham, A. and Yamazaki, H. 1994. A novel immunofluorescence capillary electrophoresis assay system for the determination of chloramphenicol in milk. Food Agric. Immunol. 6: 409-417.

Chen, F.-T.A. and Evangelista, R.A. 1994. Feasibility studies for simultaneous immunochemical multianalyte drug assay by capillary electrophoresis with laser-induced fluorescence. Clin. Chem. 40: 1819-1822.

Chen, D.Y., Adelheim, K., Cheng, X.L. and Dovichi, N.J. 1994. A simple laser-induced fluorescence detector for sulforhodamine 101 in a capillary electrophoresis system: Detection limit of 10 yoctomoles or six molecules. Analyst 119: 349-352.

El Rassi, Z. 1996. *High Performance Capillary Electrophoresis of Carbohydrates*, Beckman Instruments, Inc., Fullerton, CA.

Friedman, M. and McDonald, G.M. 1997. Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Crit. Rev. Plant Sci. 16: 55-132.

Friedman, M., Bautista, F.F., Stanker, L.H. and Larkin, K.A. 1998. Analysis of potato glycoalkaloids by a new ELISA kit. J. Agric. Food Chem. 46: 5097-5102.

Friedman, M., Levin, C.E. and McDonald, G.M. 1994. α-Tomatine determination in tomatoes by HPLC using pulsed amperometric detection. J. Agric. Food Chem. 42: 1959-1964.

Harlow, E.; Lane, D. 1988. *Antibodies. A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.

Hellenäs, K.-E. 1994. Glycoalkaloids in Potato Tubers - Aspects of Analysis, Occurrence and Toxicology. Ph.D disertation. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Hu, A.Z., Cruzado, I.D., Hill, J.W., McNeal, C.J. and Macfarlane, R.D. 1995. Characterization of lipoprotein *a* by capillary zone electrophoresis. J. Chromatogr. A 717: 33-39.

Kwok, S.K.A. 1997. Enzyme-linked immunosorbent assay development for advanced glycated end products and brochocin-c. M.Sc. dissertation, University of Alberta, Edmonton, Canada.

Maga, J. 1980. Potato glycoalkaloids. Crit. Rev. Food Sci. Nutr. 12: 371-405.

Morgan, M.R.A., McNerney, R., Matthew, J.A., Coxon, D. and Chan, H.W.-S. 1983. An enzyme-linked immunosorbent assay for total glycoalkaloids in potato tubers. J. Sci. Food Agric. 34: 593-598.

Plhak, L.C. and Sporns, P. 1992. Enzyme immunoassay for potato glycoalkaloids. J. Agric. Food Chem. 40: 2533-2540.

Pritchett, T., Evangelista, R.A. and Chen, F.-T.A. 1995. Capillary electrophoresis-based immunoassays. Bio/Technology 13: 1449-1450.

Saito, K.; Horie, M.; Hoshino, Y.; Nose, N. 1990. High performance liquid chromatographic determination of glycoalkaloids in potatoes. J. Chromatogr. 508: 141-147.

Schmalzing, D., Nashabeh, W. and Fuchs, M. 1995a. Solution-phase immunoassay for determination of cortisol in serum by capillary electrophoresis. Clin. Chem. 41: 1403-1406.

Schmalzing, D.; Nashabeh, W.; Yao, X.-W.; Mhatre, R.; Regnier, F.E.; Afeyan, N.B.; Fuchs, M. 1995b. Capillary electrophoresis-based immunoassay for cortisol in serum. Anal. Chem. 67: 606-612.

Schultz, N.M., Huang, L. and Kennedy, R.T. 1995. Capillary electrophoresis-based immunoassay to determine insulin content and insulin secretion from single islets of Langerhans. Anal. Chem. 67: 924-929.

Shahdeo, K. and Karnes, H.T. 1998. Combining immunoassays with chromatographic and electrophoretic separation techniques – a review. Mikrochim. Acta 129: 19-27.

Shea, D. 1997. Analysis of brevetoxins by micellar electrokinetic capillary chromatography and laser-induced fluorescence detection. Electrophoresis 18: 277-283.

Stanker, L.H., Kamps-Holtzapple, C. and Friedman, M. 1994. Development and characterization of monoclonal antibodies that differentiate between potato and tomato glycoalkaloids and aglycons. J. Agric. Food Chem. 42: 2360-2366.

Steinman, L., Caslavska, J. and Thormann, W. 1995. Feasibility study of a drug immunoassay based on micellar electrokinetic capillary chromatography with laser induced fluorescence detection: Determination of theophylline in serum. Electrophoresis 16: 1912-1916.

Thomson, C.A. and Sporns, P. 1995. Fluorescence polarization immunoassays for potato glycoalkaloids. J. Agric. Food Chem. 43: 254-260.

# Chapter 3

## Glycoalkaloid Concentration in Alberta Potatoes Determined by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry[2]

### INTRODUCTION

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful technique for quick and accurate determination of the molecular mass of large molecules. A pulse from a laser is used to ionize an analyte embedded in an UV-absorbing matrix, allowing the ionization of molecules up to 1.5 million Da with minimal molecular fragmentation (Schriemer and Li, 1996). While MALDI-TOF MS is used primarily as a qualitative technique, several laboratories have also reported using MALDI-TOF MS quantitatively (Gusev et al., 1996; Jespersen et al., 1995; Whittal et al., 1995; Bartsch et al., 1996). The most serious limitation to using MALDI-TOF MS quantitatively is that signal intensity can vary tremendously from one laser shot to the next. Signal reproducibility is primarily affected by sample preparation techniques, but also by signal suppression from other ions, fluctuations in laser fluence, and adduct formation. Despite these problems, quantitative MALDI-TOF MS remains attractive because sample preparation and analysis time can be very fast and because analyte identification on the basis of molecular mass is so selective.

Sample preparation for MALDI-TOF MS requires that the analyte be embedded in crystals of an UV-absorbing matrix, usually accomplished by preparing a solution of the analyte and matrix and allowing the solvent to evaporate after applying a drop of the mixture to a sample probe. The distribution of analyte in the resulting crystals is rarely homogenous so that in one area of the sample spot, no signal response may be detected, while other areas of the same spot show strong signals. Practically all reports of

[2] A version of this chapter has been published. Driedger, D.R. and Sporns, P. 1999. J. AOAC Int. 82: 908-914.

quantitative MALDI-TOF MS employ an internal standard to counter this shot-to-shot variability. The ideal internal standard must be similar to the analyte in terms of chemical structure, mass and concentration (Gusev et al., 1996). Isotope analogues would be ideal but frequently can not be resolved and are difficult to obtain. A second approach to reducing shot-to-shot variability is to promote the formation of small uniform matrix crystals, thereby improving the distribution of analyte in the matrix. This has been done using co-matrices (Gusev et al., 1995) and by accelerating the solvent evaporation (Vorm et al., 1994; Nicola et al., 1995).

Glycoalkaloids (GAs) are present in practically all potatoes and are considered a health concern. They have been shown to be toxic at doses only four times higher than might be consumed in a 500 g serving of potatoes (Morris and Lee, 1984). α-Chaconine and α-solanine are the only two GAs found in significant concentrations in commercial potato varieties, although numerous other GAs have been identified in wild varieties (Friedman and McDonald, 1997). GA determination has traditionally been done by liquid chromatography requiring sample clean-up on a solid-phase extraction cartridge and concentration before injection with subsequent UV detection near 208 nm (Saito et al., 1990; Hellenäs, 1986; Kobayashi et al., 1989; Bushway et al., 1986). Immunoassay procedures have also been developed to measure total GAs and are available commercially.

Abell and Sporns (1996) were the first to report quantification of potato GAs using MALDI-TOF MS. Their procedure involved simply spotting the crude sample extract on a MALDI probe and collecting the spectra. Results were comparable to HPLC results. The following method is an adaptation of that procedure and is used to determine the GA concentration of Alberta potatoes stored through the winter. Calibration considerations, repeatability, accuracy, selectivity, and detection limits of the MALDI-TOF MS GA method are documented.

## MATERIALS AND METHODS

**Potato Tuber Preparation and Extraction.** Potato tubers were washed and then shredded through a kitchen food processor. The shredded potatoes were freeze-dried, ground with a coffee grinder to pass through a 20-mesh screen, and stored at 4°C until needed. Freeze-dried potato (400 mg ± 4 mg) was suspended in 10 mL of extraction solvent and placed in an ultrasound water bath for 1 min. The extraction solvent was methanol:water (1:1, v/v) containing ca 10 μg/mL tomatine as an internal standard. The sample was shaken for 1 h at 200 rpm on an orbital shaker (Junior Orbital Shaker, Lab-Line Instruments, Inc., Melrose Park, NJ). An aliquot (ca 1 mL) was transferred to a microcentrifuge tube and centrifuged at 650 × *g* for 10 min. The supernatant was used for MALDI-TOF MS analysis. Each sample was extracted in triplicate.

**Preparation of Calibration Curve.** Standard GA solutions contained both α-chaconine and α-solanine in approximately equal concentrations of ca 0, 4, 8, 12, 16 and 20 μg/mL. Standards were prepared in methanol:water (1:1 v/v) containing ca 10 μg/mL tomatine as an internal standard. Note that commercial tomatine has been shown to contain significant levels of dehydrotomatine (Friedman et al., 1994), however, this should not preclude its use as an internal standard.

Calibration curves were prepared using a series of standard additions to a potato sample shown to have very low or undetectable levels of α-chaconine and α-solanine. Peeled, freshly harvested samples are potentially good sample blanks. Six blank samples were extracted in the same way normal samples were extracted, except standard solutions were used as the extraction solvent. The intensity of the GA peaks as a percentage of the internal standard was plotted against concentration of the standard. Second order polynomial regression analysis was applied to the data.

**MALDI-TOF MS.** A saturated solution of 2',4',6'-trihydroxyacetophenone in acetone was prepared as the MALDI matrix solution. The matrix was applied to the MALDI probe by spotting ca 0.3 μL of the saturated solution on the probe and allowing the acetone solvent to evaporate (< 3 sec). The sample extract (0.5 μL) was applied on top of the 2',4',6'-trihydroxyacetophenone crystals and allowed to dry for 10 min. The

probe was then rinsed with water for about 3 sec and allowed to dry for 1 h under a household fan. Each extract was spotted in triplicate.

Mass spectra were generated on a Kompact MALDI I (Kratos Analytical, Ramsey, NJ) using a 337 nm laser with a maximum output 6 mW on a point ca 100 μm in diameter. Positive ions were accelerated with a 20 kV potential. Spectra were analyzed with Kompact 4.0 software. Optimal laser power was determined each day spectra were generated by selecting the maximum power setting that still allowed baseline resolution between α-chaconine and α-solanine. The window between no response and poor resolution could be less than 5% of total laser output. Spectra were collected by firing the laser step-wise across the entire spot. A single spectrum was generated by averaging 100 shots. The average peak intensities relative to the tomatine internal standard were calculated for each extract. Potassium adduct peaks were observed as peaks 39.1 mass units higher than the analyte molecular mass. If potassium adducts were present, the intensity of the potassium adduct peak was added to the intensity of the protonated peak for calculating the GA concentration.

The concentration of each GA in the extract was determined by comparison with a calibration curve prepared the same day. The concentration of GA in the freeze-dried potato was calculated as follows:

$$\text{Glycoalkaloid } (\mu g/g) = \frac{C \times V}{W}$$

where C is the GA concentration in the extract (μg/mL), V is the volume of solvent used to extract the sample (mL), and W is the weight of freeze-dried sample (g). GA concentration in fresh potatoes was calculated assuming 80% moisture in the tuber (Abell and Sporns, 1996).

## RESULTS AND DISCUSSION

**Instrument Response.** Several parameters were found to be helpful in reducing shot-to-shot variability. Preparing the matrix in acetone, rather than in a methanol:water

solution as previously reported (Abell and Sporns, 1996), resulted in visually much smaller crystals. The finer crystals led to more consistent signal response, presumably because the analytes were more homogeneously distributed in the matrix crystals. Rinsing the probe with water after spotting the sample effectively reduced the presence of potassium adducts and produced cleaner spectra because interfering salts were removed (Figure 3-1). Day-to-day repeatability was also improved by daily determining the optimal laser power. However, the laser power was kept constant during any one day because the relative response of the individual analytes differed when laser power was adjusted.

**Calibration.** The relationship between peak intensity and GA concentration was best described by a second-order polynomial curve (Figure 3-2). The deviation from linearity is thought to be caused by suppression of the α-chaconine and α-solanine signals at higher concentrations. This might be the result of a competition between matrix and analytes for cations during ionization (Schriemer and Li, 1997). It is postulated that α-chaconine and α-solanine do not compete for protons as effectively as the internal standard tomatine, resulting in a non-linear calibration curve. In spite of this, signal response as a percentage of the internal standard response did correlate well with analyte concentration, with coefficient of correlation values consistently above 0.97 (Tables 3-1 and 3-2).

**Repeatability.** The same potato sample was analyzed in triplicate (3 extractions) on seven separate days over a four month period. The relative standard deviation (RSD) of triplicate analysis on a single day ranged from 1 – 15% for α-chaconine, and from 3 – 16% for α-solanine (Tables 3-1 and 3-2). This compares favorably with reported ELISA repeatability results (Sporns et al., 1996) and is comparable to liquid chromatographic results (Kobayashi et al., 1989). Day-to-day RSD values were 10% for α-chaconine and 12% for α-solanine.

**Accuracy.** The accuracy of the method was estimated using spike recoveries. Potato samples were spiked with ca 50 μg/g of each GA. Mean recovery for α-chaconine was 104% with a day-to-day RSD of 8% (Table 3-1). Mean recovery for α-solanine was 98% with a day-to-day RSD of 4% (Table 3-2). Total GA concentration in

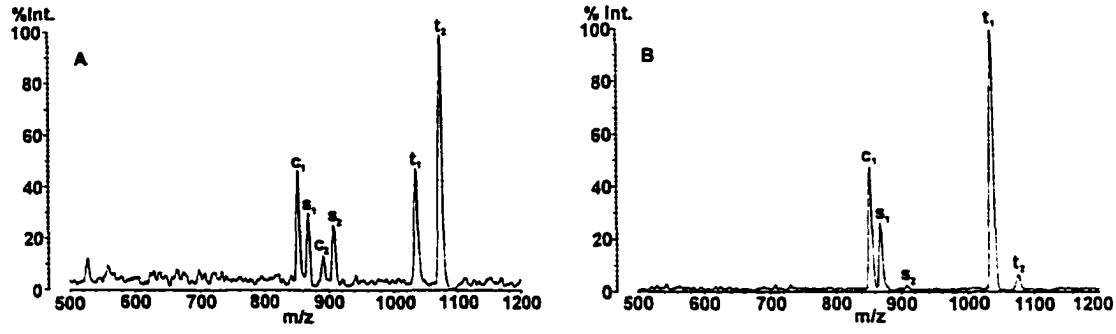


**Figure 3-1 MALDI-TOF mass spectra of Yukon Gold potato containing 49 μg/g α-chaconine and 27 μg/g α-solanine with tomatine as an internal standard. $c_1$, α-chaconine + $H^+$ (MW 853); $s_1$, α-solanine + $H^+$ (MW 869); $c_2$, α-chaconine + $K^+$ (MW 891); $s_2$, α-solanine + $K^+$ (MW 907); $t_1$, tomatine + $H^+$ (MW 1035); $t_2$, tomatine + $K^+$ (MW 1073). A: Probe not rinsed. B: Probe rinsed with water after spotting the samples.**

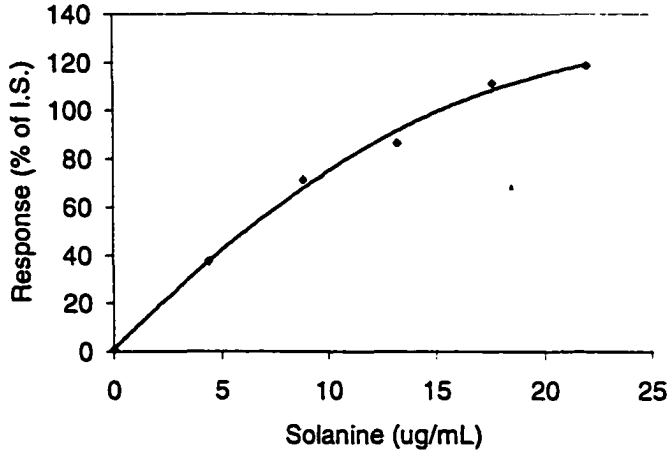


**Figure 3-2 Second-order polynomial calibration curve for α–solanine using MALDI-TOF MS.**

**Table 3-1 α-Chaconine in potatoes determined by MALDI-TOF MS. Repeatability of reference sample results, spike recoveries and coefficients of correlation**

| Day | Reference sample (μg/g) | Reference sample RSD (%) | Spike recovery, (%) | Coefficient of correlation |
|---|---|---|---|---|
| 1 | 41 | 15 | 102 | 0.999 |
| 2 | 35 | 8 | 103 | 0.997 |
| 3 | 49 | 11 | 118 | 0.993 |
| 4 | 45 | 1 | 91 | 0.987 |
| 5 | 42 | 6 | 110 | 0.992 |
| 6 | 41 | 12 | 100 | 0.998 |
| 7 | 46 | 4 | 103 | 0.994 |
| Mean | 43 | 8 | 104 | 0.994 |
| RSD, % | 10 | - | 8.4 | 0.4 |

**Table 3-2 α-Solanine in potatoes determined by MALDI-TOF MS. Repeatability of reference sample results, spike recoveries and coefficients of correlation**

| Day | Reference sample (μg/g) | Reference sample RSD (%) | Spike recovery, (%) | Coefficient of correlation |
|---|---|---|---|---|
| 1 | 23 | 16 | 94 | 0.993 |
| 2 | 20 | 10 | 97 | 0.997 |
| 3 | 27 | 3 | 101 | 0.999 |
| 4 | 20 | 6 | 93 | 0.978 |
| 5 | 23 | 9 | 103 | 0.999 |
| 6 | 21 | 16 | 98 | 0.999 |
| 7 | 23 | 5 | 98 | 0.999 |
| Mean | 22 | 9 | 98 | 0.995 |
| RSD, % | 12 | - | 3.6 | 0.8 |

potato tubers determined by MALDI-TOF MS has previously been shown to correlate well with liquid chromatographic results (Abell and Sporns, 1996).

These data indicate that MALDI-TOF MS can accurately measure potato tuber GA levels, however, it should be noted that all calibration curves were prepared by standard additions to tuber material. This was done because it was observed that the presence of tuber material in the solution spotted on the probe dramatically decreased the signal response for all GAs. For example, 5 nM α-solanine in a tuber extract produced a peak 5 – 10 times less intense than a 5 nM α-solanine standard made up in pure solvent. This illustrates how the presence of other compounds can greatly affect the desorption and ionization of an analyte. To compensate for this effect, the calibration curve should be prepared in a sample matrix as similar as possible to the samples being analyzed.

**Method Limit of Detection.** The method limit of detection (MLOD) was defined as the concentration of GA in potato tubers required to generate a signal equal to 3 times the baseline noise (peak-to-peak). The MLOD was estimated from fresh potatoes with low GA levels. Under the conditions specified in the method, the MLOD for both α-chaconine and α-solanine was approximately 2 μg/g in potato tissue (Figure 3-3). In principle, the limit of detection could be improved by increasing the amount of sample used in the extraction procedure.

**Selectivity.** The selectivity of the method is primarily limited by the ability of the instrument to resolve the analyte from other compounds of similar molecular mass. For low mass analytes (MW < 400), one can expect to see interfering compounds simply because so many different molecules exist in this mass range. Matrix and matrix cluster ions also tend to occupy the spectrum below *m/z* 500. However, as the molecular mass of the analyte increases, the probability of there being interfering compounds of similar mass becomes less, so that interferences are rare for analytes larger than MW 500. The resolving power of MALDI-TOF mass spectrometers has also improved dramatically over the last few years with the introduction of delayed extraction (also called time-lag-focussing or pulsed ion extraction). Resolution values of 6,000 fwhm (full width at half maximum) are now possible, meaning that the instrument is roughly capable of resolving *m/z* 3,000 from *m/z* 3,001 (product literature from Bruker Daltonics, Billerica, MA).

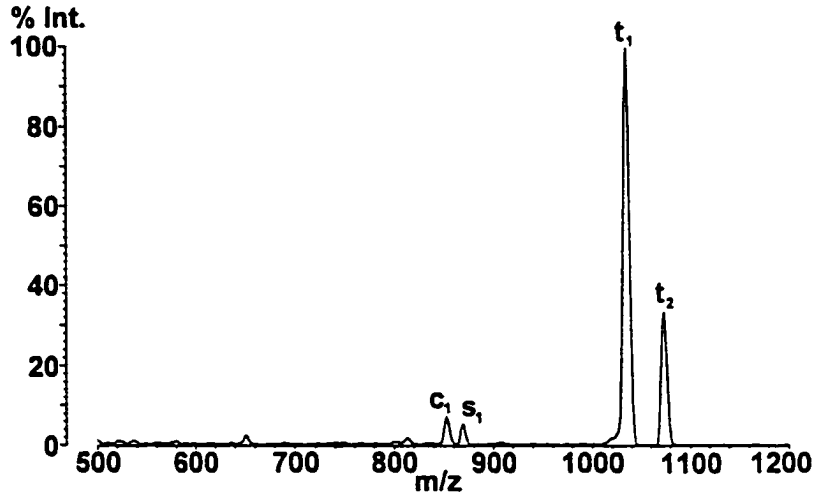


**Figure 3-3 MALDI-TOF mass spectrum of a peeled potato sample containing ca 2 μg/g α-chaconine and 2 μg/g α-solanine. $c_1$, α-chaconine + $H^+$ (MW 853); $s_1$, α-solanine + $H^+$(MW 869); $t_1$, tomatine + $H^+$ (MW 1035); $t_2$, tomatine + $K^+$ (MW 1073).**

Spectra of potato extracts showed no observable interferences. One of the limitations of chromatographic determination of GAs is the difficulty in resolving hydrolysis products from α-chaconine and α-solanine (Friedman and Levin, 1992). Since the loss of one sugar unit changes the mass of a GA ca 180 mass units, resolution of α-GAs from β-GAs (loss of one sugar unit) and γ-GAs (loss of two sugar units) is easily achieved with even the earliest MALDI-TOF mass spectrometers (Figure 3-4).

**Glycoalkalkaloid Levels in Stored Alberta Potatoes.** Most of the samples collected for this experiment were seed potatoes that had been stored in commercial bins for ca seven months. The seed market prefers small tubers, less than 10 ounces (280 g), because large tubers may yield significant numbers of blind seed pieces, without eyes, when they are cut prior to planting (Secor and Gudmestad, 1993). Average tuber size was <280 g for all samples used in this study. Both extended storage time and small tuber size are associated with elevated levels of GA (Friedman and McDonald, 1997), therefore, it is expected that these potatoes would represent a higher than normal

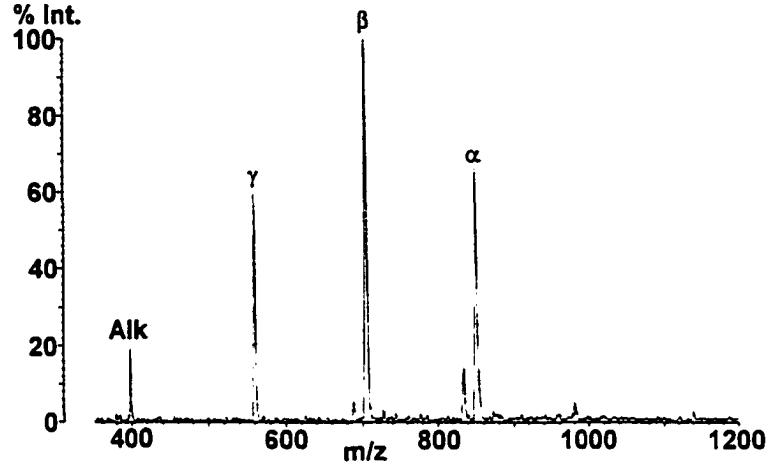


**Figure 3-4 MALDI-TOF mass spectrum of hydrolysis products of α-chaconine. α, α-chaconine + $H^+$ (MW 853); β, β-chaconine + $H^+$ (MW 707); γ, γ-chaconine + $H^+$ (MW 561); Alk, solanidine + $H^+$ (MW 399).**

exposure to GAs for typical Alberta consumers. Most samples visually appeared to be in good condition with the exception of Shepody-1 and Amisk samples. About 20% of Shepody-1 had rotted. The rotted tubers were culled before analysis. The Amisk sample had sprouted about 1 cm. The sprouts were also removed prior to analysis, yet Amisk still contained the highest GA levels of all the samples analyzed. While some of the potato samples did exhibit slightly elevated GA levels, all were well within the 200 μg/g limit generally regard as acceptable (Friedman and McDonald, 1997) (Table 3-3).

**Conclusions.** These results indicate that MALDI-TOF MS is able to quantify α-chaconine and α-solanine in potato tubers with minimal sample requirements and minimal sample preparation. There can be a high degree of confidence in the identity of the analyte because detection is based on molecular mass. The potential for using MALDI-TOF MS as a quantitative technique is vast. It should be remembered that this is a new technique; the first reports of MALDI-TOF MS were published only 11 years ago

**Table 3-3 Glycoalkaloids (μg/g) in Alberta potatoes determined by MALDI-TOF MS.**

| Sample | α-chaconine | α-solanine | total GA |
|---|---|---|---|
| Red Norland | 24.1 | 8.7 | 32.8 |
| Norkota | 17.0 | 10.0 | 37.0 |
| Shepody-2 | 25.4 | 18.7 | 44.1 |
| Norkota Russet | 21.7 | 23.8 | 45.5 |
| Chipeta | 30.1 | 24.3 | 54.4 |
| Red LaSoda | 43.2 | 13.8 | 57.0 |
| Ranger Russet | 39.6 | 22.7 | 62.3 |
| Atlantic | 36.7 | 27.6 | 64.3 |
| Niska | 38.8 | 31.6 | 70.4 |
| Shepody-1 | 37.5 | 34.8 | 72.3 |
| Yukon Gold-1 | 34.3 | 39.8 | 74.1 |
| Yukon Gold-2 | 48.7 | 27.2 | 75.9 |
| Russet Burbank | 48.6 | 35.9 | 84.5 |
| Amisk | 73.0 | 28.3 | 101.3 |

(Karas and Hillenkamp, 1988; Tanaka et al., 1988). Consequently, instruments are still expensive and undergoing rapid changes. Unlike chromatography and capillary electrophoresis, MALDI-TOF instrumentation has so far not been designed explicitly for quantitative analysis. For example, most sample probes can accommodate no more than 20 spots. This is more a problem of design than a limitation of the technology. As the technology continues to mature and more applications are developed, it is expected that MALDI-TOF MS will become an increasingly important tool for the analytical chemist.

## REFERENCES

Abell, D.A. and Sporns, P. 1996. Rapid quantitation of potato glycoalkaloids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Agric. Food Chem. 44: 2292-2296.

Bartsch, H., Konig, W.A., Strassner, M. and Hintze, U. 1996. Quantitative determination of native and methylated cyclodextrins by matrix assisted laser desorption/ionization time of flight mass spectrometry. Carb. Res. 286: 41-53.

Bushway, R.J., Bureau, J.L. and King, J. 1986. Modification of the rapid high-performance liquid chromatographic method for the determination of potato glycoalkaloids. J. Agric. Food Chem. 34: 277-279.

Friedman, M. and Levin, C.E. 1992. Reversed-phase high-performance liquid chromatographic separation of potato glycoalkaloids and hydrolysis products on acidic columns. J. Agric. Food Chem. 40: 2157-2163.

Friedman, M. and McDonald, G.M. 1997. Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Crit. Rev. Plant Sci. 16: 55-132.

Friedman, M., Levin, C.E. and McDonald, G.M. 1994 α-Tomatine determination in tomatoes by HPLC using pulsed amperometric detection. J. Agric. Food Chem. 42: 1959-1964.

Gusev, A.I., Wilkinson, W.R., Proctor, A. and Hercules, D.M. 1995. Improvement of signal reproducibility and matrix/comatrix effects in MALDI analysis. Anal. Chem. 67: 1034-1041.

Gusev, A.I., Wilkinson, W.R., Proctor, A. and Hercules, D.M. 1996. Direct quantitative analysis of peptides using matrix assisted laser desorption ionization. Fres. J. Anal. Chem. 354: 455-463.

Hellenäs, K.-E. 1986. A simplified procedure for quantification of potato glycoalkaloids in tuber extracts by h.p.l.c.; comparison with ELISA and a colorimetric method. J. Sci. Food Agric. 37: 776-782.

Jespersen, S., Niessen, W.M.A., Tjaden, U.R. and van der Greef, J. 1995. Quantitative bioanalysis using matrix-assisted laser desorption/ioniation mass spectrometry. J. Mass Spectrom. 30: 357-364.

Karas, M. and Hillenkamp, F. 1988. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal. Chem. 60: 2299-2301.

Kobayashi, K., Powell, A.D., Toyoda, M. and Saito, Y. 1989. High-performance liquid chromatographic method for simultaneous analysis of α-solanine and α-chaconine in potato plants cultured *in vitro*. J. Chromatogr. 462: 357-364.

Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Nicola, A.J., Gusev, A.I., Proctor, A., Jackson, E.K. and Hercules, D.M. 1995. Application of the fast-evaporation sample preparation method for improving quantification of angiotensin II by matrix-assisted laser desorption/ionization. Rapid Commun. Mass Spectrom. 9: 1164-1171.

Saito, K., Horie, M., Hoshino, Y. and Nose, N. 1990 High performance liquid chromatographic determination of glycoalkaloids in potatoes. J. Chromatogr. 508: 141-147.

Schriemer, D.C. & Li, L. 1996. Detection of high molecular weight narrow polydisperse polymers up to 1.5 million daltons by MALDI mass spectrometry. Anal Chem. 68: 2721-2725.

Schriemer, D.C. & Li, L. 1997. Mass discrimination in the analysis of polydisperse polymers by MALDI time-of-flight mass spectrometry. 1. Sample preparation and desorption/ionization issues. Anal. Chem. 69: 4169-4175.

Secor, D.A. & Gudmestad, N.C. 1993. Handling and planting seed tubers. Ch. 4 in *Potato Health Management*, R.C. Rowe (Ed.), p. 27-34. APS Press, St. Paul, MN.

Sporns, P., Abell, D.C., Kwok, A.S.K., Plhak, L.C. & Thomson, C.A. 1996. Immunoassays for toxic potato glycoalkaloids. Ch. 19 in *Immunoassays for Residue Analysis*, ACS Symposium Series 621, R.C. Beier & L.H. Stanker (Ed.), p. 256-272. American Chemical Society, Washington, DC.

Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y. and Yoshida, T. 1988. Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2: 151-153.

Vorm, O., Roepstroff, P. and Mann, M. 1994. Improved resolution and very high sensitivity in MALDI TOF of matrix surfaces made by fast evaporation. Anal. Chem. 66: 3281-3287.

Whittal, R.M., Palcic, M.M., Hindsgaul, O. and Li, L. 1995. Direct analysis of enzymatic reactions of oligosaccharides in human serum using matrix-assisted laser desorption ionization mass spectrometry. Anal. Chem. 67: 3509-3514.

# Chapter 4

## Glycoalkaloid Concentration in By-Products of Potato Starch Extraction as Measured by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry[3]

## INTRODUCTION

Potato starch is a valuable commodity in certain sectors of the food, paper, and textile industries because of its high viscosity and large number of phosphate groups. While approximately 100 million pounds of potato starch are recovered annually as a by-product from chip and french fry plants, this is not enough to supply the North American market. Significant quantities of potato starch are imported from Europe, where several companies process potatoes principally for starch recovery (Alexander, 1995). Processing plants dedicated to starch extraction are handicapped by the seasonal availability of potatoes and the need for specialized storage of the tubers. Compared to maize and wheat starch, there is a relative lack of valuable by-products (Hasse and Platte, 1996). Nevertheless, the unique properties of potato starch allow it to command a higher price than most other starches.

Potato protein is one of the commonly exploited by-products of starch extraction. In a typical process, "fruit water" is removed from potatoes by grinding the tubers in a rasper and decanting the liquid (Westfalia Separator AG, 1982). The fruit water is acidified, heated, and the resulting precipitate is recovered as potato protein concentrate (PPC). PPC is a high quality protein that is widely used as an animal feed supplement. Starch is extracted from the potato mash left after the removal of the fruit water. The pulp remaining after starch removal is also used as an animal feed, and its potential for

[3] A version of this chapter has been published. Driedger, D.R. and Sporns, P. 1999. J. Food Process. Preserv. 23:377-390.

yeast, vitamin, and alcohol production has been explored (Slominska and Starogardzka, 1987; Klingspohn et al., 1993).

Knorr (1977) cites toxic factors as one of the potential limitations of PPC. Potato tubers can commonly contain glycoalkaloid (GA) levels that some researchers claim may be only four-fold less than that required to induce deleterious effects in humans (Morris and Lee, 1984). Potato GAs disrupt cell membranes and are potent neurotoxins (Friedman and McDonald, 1997). There is concern that these GAs may be concentrated in the by-products of starch extraction, thereby limiting the value of PPC and the pulp.

GAs are not easy to analyze due to their heterogeneity and lack of a useful chromophore. Several HPLC procedures have been reported, but because detection must be by UV absorbance at 200-208 nm, extensive sample clean-up is required to remove interfering compounds (Saito et al., 1990; Hellenäs et al., 1995). Immunoassays represent a rapid, simple method for quantifying total GA, but can not distinguish individual GAs (Morgan et al., 1983; Plhak and Sporns, 1992). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a relatively new technique that has shown potential for quantitative GA analysis in tubers (Abell and Sporns, 1996). The great advantage of MALDI-TOF MS is simplified sample preparation; for GAs, a drop of crude potato extract is placed on an instrument probe and allowed to dry before instrumental analysis. HPLC analysis, in comparison, typically requires purification through a solid-phase extraction cartridge and a concentration step before chromatographic analysis.

This investigation reports the protein and GA concentrations in the various fractions of a simulated potato starch extraction process. The effect of tuber protein on the yield and protein concentration of PPC will be determined. GA analysis by MALDI-TOF MS will indicate the potential toxicological risks of using PPC or potato pulp as a food or feed ingredient.

## MATERIALS AND METHODS

**Potato Fractionation.** Potato samples were supplied by Alberta Agriculture, Food and Rural Development and the Potato Growers of Alberta. Samples had been

kept in commercial storage facilities for approximately seven months. The potato fractionation procedure presented in Figure 4-1 was modeled after industrial starch extraction (Westfalia Separator AG, 1982). The food processor was a Braun kitchen appliance. Centrifugation was performed with a relative centrifugal field (RCF) of 2,740 × *g* for 10 min. A Waring blender was used to blend the de-watered potato. The crude starch was washed with water by making a slurry with approximately equal volumes of starch and water, followed by centrifugation at 2,740 × *g* for 10 min. The starch was washed with ethanol and the ethanol removed with vacuum filtration. Both pulp and starch were dried at room temperature. PPC was prepared by precipitating protein from the fruit water in an autoclave. After cooling, the fruit water was centrifuged at 2,740 × *g* for 10 min to separate the precipitated PPC. The PPC was freeze-dried to remove residual moisture.

**Analytical procedures.** Specific gravity was determined by weighing the tubers in air and in water (Smith, 1987). Moisture was determined using AOAC method 930.15 (AOAC, 1990). Protein (N x 6.25) was determined by the Kjeldahl procedure (Bradstreet, 1965).

GAs were extracted using 50% methanol as the extraction solvent. Fruit water was an exception; the fruit water was simply diluted six-fold with 60% methanol so that the final methanol content was 50%. Tomatine was added to the extraction solvent at a concentration of approximately 10 µg/mL to serve as an internal standard for later MALDI-TOF MS analysis. Potato tubers were freeze-dried before extraction. The appropriate amount of sample was suspended in extraction solvent (200 mg sample/5 mL solvent for all samples except PPC; 100 mg PPC/5 mL solvent) and placed in an ultrasonic water bath for 1 min. Samples were then shaken on an orbital shaker for 1 h at 200 rpm. An aliquot was transferred to a microcentrifuge tube and centrifuged at 3,000 rpm for 10 min. The supernatant was immediately analyzed for α-chaconine and α-solanine by MALDI-TOF MS. Each sample was extracted in triplicate.

A saturated solution of 2',4',6'-trihydroxyacetophenone in acetone was used for preparing MALDI matrix crystals. Approximately 0.3 µL of the saturated 2',4',6'-trihydroxyacetophenone was deposited on the MALDI probe. Very fine, uniform crystals

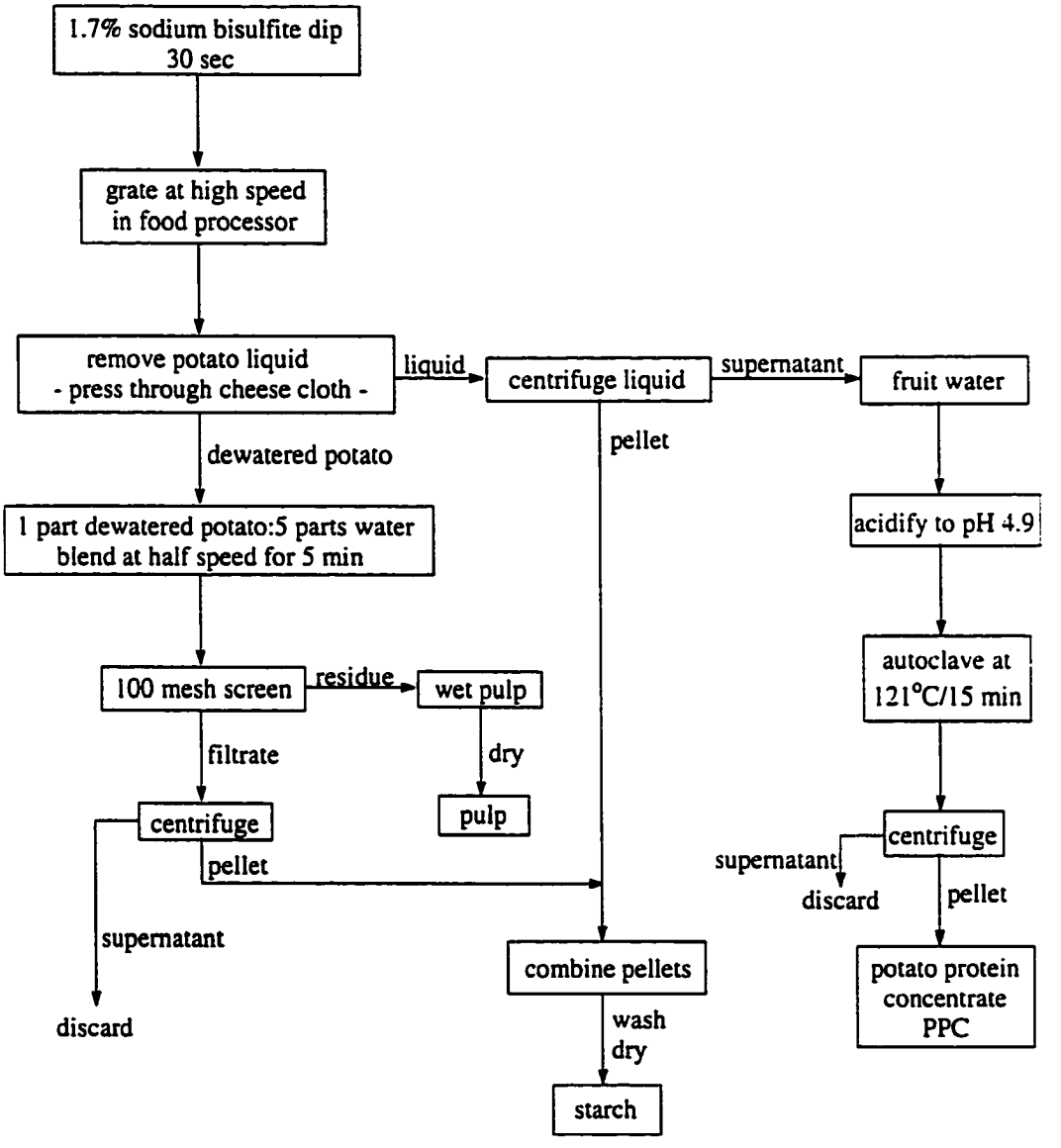


**Figure 4-1 Fractionation process for the extraction of starch from potato tubers.**

formed when the acetone was allowed to evaporate. Sample extract (0.5 μL) was deposited on top of the matrix crystals and allowed to dry for 10 min. Each extract was spotted in triplicate. After the solvents had evaporated, a squirt bottle was used to rinse the crystals on the probe with water for about 3 sec. The probe was allowed to dry for an additional 60 min before analysis.

Optimal laser power was considered to be the maximum power that still allowed baseline resolution between α-chaconine and α-solanine. The optimal power setting had to be experimentally determined each day that analyses were performed. The window between no response and poor resolution could be less than 5% of total laser output. The positive ion mode was selected. Spectra were collected by firing the laser step-wise across the entire spot. The average intensity of 100 shots from each spot was recorded. The tomatine internal standard was well resolved from α-chaconine and α-solanine (Figure 4-2). Response factors differed slightly among all three GAs, necessitating the preparation of separate calibration curves for both α-chaconine and α-solanine. The average peak intensities relative to the tomatine internal standard of each extract were compared to a calibration curve prepared the same day. Potassium adduct peaks may be observed as peaks 39.1 mass units higher than the analyte molecular mass. If potassium adducts were present, the intensity of the potassium adduct peak was added to the intensity of the protonated peak. Calibration curves were prepared using a series of standard additions to potato samples known to have very low or undetectable levels of α-chaconine and α-solanine. Second order polynomial regression analysis was used to calculate the calibration curves (Abell and Sporns, 1996).

## RESULTS AND DISCUSSION

**Potato Fractionation.** Potato processing plants have historically had problems with waste disposal because of high BOD in the effluent water (Strolle et al., 1973). In starch extraction plants, one approach to this problem has been to recover as much protein as possible from the effluent. Typically this is done by rasping the tubers, decanting the fruit water and then recovering the protein from the fruit water before

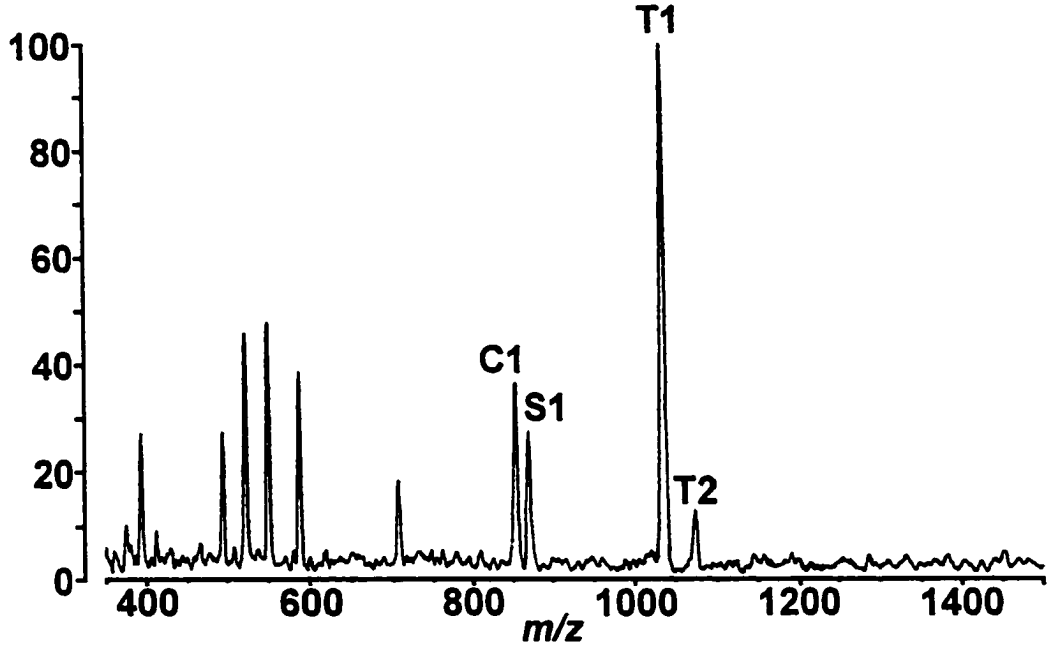


**Figure 4-2 MALDI-TOF mass spectrum of glycoalkaloids present in a potato protein concentrate extract. C1, α-chaconine + $H^+$ (MW 853); S1, α-solanine + $H^+$ (MW 869); T1, tomatine + $H^+$ (MW 1035); T2, tomatine + $K^+$ (MW 1073).**

disposing of the liquid. The recovered protein also has economic value as a feed ingredient.

The potato fractionation protocol used in this experiment was modeled on an industrial starch extraction process (Westfalia Separator AG, 1982). The yield of fruit water averaged 33.5%, representing about 40% of the moisture available in the tubers (Table 4-1). Industrial decanters are somewhat more efficient, removing about 60% of the moisture (Strolle et al., 1973). As expected, the yield of fruit water generally decreased with increasing specific gravity of the tuber sample ($r = 0.876$). The protein

concentration in the fruit water ranged from 2.0% to 3.0%, accounting for approximately half the protein available in the tuber.

Industrially, protein is often precipitated by slight acidification of the fruit water, followed by steam injection. In this experiment, an autoclave was used to mimic steam injection. Yield of PPC ranged from 0.521% to 0.837% of the initial tuber weight (Table 4-1). The yield of PPC did not correlate well with the yield of fruit water ($r = 0.182$) nor did PPC yield correlate with tuber protein ($r = 0.257$). Protein levels in the PPC ranged from 53.4% to 69.9%, representing an average of 23% of the tuber protein. Previous investigations using pilot-scale equipment report 37% protein recovery (Knorr et al., 1977). The lower recovery in the present experiment can most likely be attributed to the poor efficiency of fruit water removal from the rasped potatoes. Nevertheless, the protein concentration in the PPC is similar to previous reports (Knorr et al., 1977)

**Glycoalkaloid Determination by MALDI-TOF MS.** Our lab has used MALDI-TOF MS extensively for GA analysis in whole potato tubers, however, initial attempts to quantify GAs in tuber fractions were disappointing. Day-to-day variations in reference sample results were unacceptably high and spike recoveries were poor. The problem was eventually attributed to the way calibration was performed. Calibration curves were originally prepared by a series of standard additions to low-GA freeze-dried tubers. However, repeatability and spike recoveries improved markedly when calibration curves were prepared using standard additions into a sample matrix similar to the samples being tested. For example, standard additions to a PPC sample were used to generate a calibration curve for the analysis of PPC samples. Table 4-2 presents repeatability data and Table 4-3 spike recovery data for tuber, fruit water, PPC and pulp analysis. No GAs were detected in the starch. Figure 4-2 shows a typical MALDI-TOF mass spectrum of a PPC extract.

The process of analyte desorption and ionization during MALDI appears to be complex and is poorly understood. It is clear that there are interactions between ions. For example, 10 nM α-solanine solution in pure methanol exhibits a signal intensity approximately 5 times greater than 10 nM α-solanine in a tuber extract. This inhibition of analyte signal by other compounds in the extract has been termed "signal suppression"

**Table 4-1 Yield and protein content of fruit water and PPC normalized to 100 g tubers**

| | | Fruit Water | | PPC | |
|---|---|---|---|---|---|
| Sample | Tuber Protein (g) | Yield (g) | Protein (g) | Yield (g) | Protein (g) |
| Shepody 1 | 1.54 (0.01) | 31.0 | 0.760 (0.002) | 0.679 | 0.416 (0.008) |
| Norkota | 1.66 (0.01) | 30.8 | 0.770 (0.021) | 0.542 | 0.310 (0.002) |
| Chipeta | 1.68 (0.02) | 29.9 | 0.807 (0.004) | 0.652 | 0.453 (0.011) |
| Ranger Russet | 1.69 (0.07) | 29.5 | 0.813 (0.000) | 0.620 | 0.408 (0.006) |
| Amisk | 1.70 (0.00) | 32.9 | 0.781 (0.001) | 0.553 | 0.373 (0.003) |
| Russet Burbank | 1.72 (0.01) | 32.8 | 0.903 (0.003) | 0.600 | 0.388 (0.008) |
| Atlantic | 1.79 (0.02) | 32.1 | 0.980 (0.007) | 0.748 | 0.513 (0.009) |
| Dark Red Norland | 1.87 (0.03) | 35.7 | 0.804 (0.002) | 0.521 | 0.312 (0.005) |
| Yukon Gold 1 | 1.89 (0.01) | 33.2 | 1.01 (0.00) | 0.837 | 0.496 (0.048) |
| Yukon Gold 2 | 1.98 (0.11) | 33.1 | 0.902 (0.006) | 0.784 | 0.450 (0.002) |
| Niska | 2.03 (0.06) | 35.5 | 0.954 (0.039) | 0.675 | 0.472 (0.033) |
| Shepody 2 | 2.08 (0.02) | 36.4 | 0.971 (0.004) | 0.739 | 0.467 (0.008) |
| Norkota Russet | 2.10 (0.01) | 32.2 | 0.905 (0.004) | 0.535 | 0.361 (0.001) |
| Red LaSoda | 2.18 (0.01) | 44.5 | 0.932 (0.003) | 0.699 | 0.374 (0.001) |

Protein measured in duplicate, standard deviation in parenthesis.

**Table 4-2 MALDI-TOF MS glycoalkaloid analysis repeatability data for Yukon Gold potato fractions (wet weight basis)**

| Sample | Chaconine (μg/g) | Solanine (μg/g) | Total (μg/g) |
|---|---|---|---|
| Tuber (80% moisture) | | | |
| Day 1 | 45 (1) | 20 (1) | 65 |
| Day 2 | 42 (2) | 23 (2) | 65 |
| Day 3 | 46 (2) | 23 (1) | 69 |
| Mean | 44 | 22 | 66 (c:s 2.0) |
| Fruit Water (98% moisture) | | | |
| Day 1 | 29 (3) | 40 (2) | 69 |
| Day 2 | 22 (3) | 35 (1) | 57 |
| Day 3 | 22 (1) | 38 (2) | 60 |
| Mean | 24 | 38 | 62 (c:s 0.6) |
| PPC (15% moisture) | | | |
| Day 1 | 330 (26) | 210 (11) | 540 |
| Day 2 | 390 (22) | 260 (46) | 650 |
| Day 3 | 390 (77) | 230 (56) | 620 |
| Mean | 370 | 230 | 600 (c:s 1.6) |
| Pulp (15% moisture) | | | |
| Day 1 | 270 (34) | 230 (14) | 500 |
| Day 2 | 300 (26) | 190 (21) | 490 |
| Day 3 | 280 (82) | 240 (85) | 520 |
| Mean | 280 | 220 | 500 (c:s 1.3) |

Glycoalkaloids measured in triplicate, standard deviations in parentheses.
c:s, α-chaconine to α-solanine ratio

**Table 4-3 MALDI-TOF MS glycoalkaloid spike recovery data**

| | Chaconine (μg/g) | | | Solanine (μg/g) | | |
|---|---|---|---|---|---|---|
| Sample | Added | Recovered | % | Added | Recovered | % |
| Tubers | 12 | 12 | 102 | 13 | 13 | 98 |
| Fruit Water | 26 | 28 | 107 | 32 | 32 | 101 |
| Protein Concentrate | 450 | 480 | 107 | 430 | 360 | 84 |
| Pulp | 460 | 440 | 95 | 250 | 230 | 93 |

(Gusev et al., 1996; Harvey, 1993) and may be caused, in part, by competition for cations during the ionization process (Chan et al., 1992). In practical terms this means that sample matrix is an important consideration when performing quantitative analysis by MALDI-TOF MS. All sample extracts, including standards, must have similar composition so that signal suppression is "standardized" for all samples. If this type of sample homogeneity is maintained, good quantification can be achieved.

The reproducibility and spike recovery data indicate that MALDI-TOF MS can be used to reliably quantify GAs in a variety of potato fractions. Sample preparation is simple; 0.5 μL of potato extract is applied to a stainless steel probe and allowed to evaporate before instrumental analysis. The instrumental analysis can be performed in approximately one minute (100 laser shots). The speed of analysis exceeds even that of an ELISA, and unlike ELISA methods, MALDI-TOF MS can also quantify individual GAs. HPLC analysis of potato GAs is similar to MALDI-TOF MS in that it is able to quantify α-chaconine and α-solanine individually, but HPLC does require significantly more time-consuming sample preparation. MALDI-TOF MS also offers a higher degree of confidence in the identity of the analyte because molecular mass is determined. The precision of MALDI-TOF MS GA analysis compares favorably to ELISA precision and is similar to HPLC precision (refer to Chapter 3). As the technology matures, it is expected that MALDI-TOF MS will become an increasingly important tool for the food analyst.

**Fate of Glycoalkaloids During Starch Extraction.** The Yukon Gold potato sample used to study the fate of GAs during starch extraction contained 66 μg total GA/g

(fwb), well within the recommended maximum limit of 200 μg/g (Friedman and McDonald, 1997). The PPC derived from these potatoes contained a GA concentration (600 μg/g) almost 10 times higher than the fresh tubers. The higher value can be partially explained by simply taking into account moisture loss; the tuber GA concentration would be 380 μg/g on an equivalent moisture basis. Nevertheless, it is evident that the GAs also partition into the PPC to some extent, suggesting that GAs are associated with some component of the PPC, either with the protein itself or some other cellular component.

It is worth noting that the ratio of α-chaconine to α-solanine in the tubers was 2.0, which falls within the expected range for potatoes (Friedman and McDonald, 1997). The ratio of α-chaconine to α-solanine in the fruit water decreased to 0.6, but the ratio then reverted back to 1.6 in the PPC. The disproportionate amount of α-solanine in the fruit water suggests that α-chaconine is more strongly associated with some insoluble cellular material than is α-solanine. The α-solanine appears to be more freely dispersed in the fruit water. This is somewhat surprising since the molecular structures of the two GAs are so similar (Figure 4-3). On the other hand, significant differences in cell membrane lytic activity (Roddick et al., 1988) hint at important differences between α-chaconine and α-solanine, possibly having to do with the spatial orientation of the carbohydrate moieties.

The total GA concentration in the pulp remaining after starch extraction was 500 μg/g, despite extensive washing of the pulp with water (Table 4-2). The α-chaconine to α-solanine ratio was 1.3, just slightly lower than in the PPC. Since the pulp washing equipment used industrially is more efficient than our lab-scale procedure, several pulp samples produced from pilot plant equipment were also tested (Table 4-4). The pilot plant produced pulp did contain lower total GA levels than the lab pulp, probably because the pulp was washed more efficiently. It is also possible that initial GA levels in the pilot plant tubers were lower than in the lab tubers (the pilot plant tubers were not available for testing). Despite the more efficient washing, GA levels were still higher than 200 μg/g in 3 of the 5 pilot plant produced pulp samples.

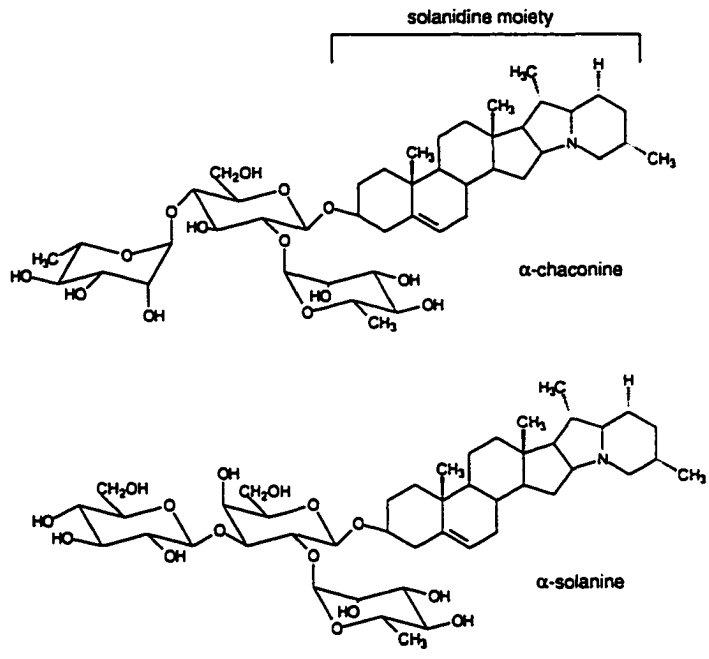


**Figure 4-3 Chemical structure of potato glycoalkaloids α-chaconine and α-solanine.**

**Table 4-4 Glycoalkaloid concentration in pilot plant produced potato pulp (wet weight basis)**

| | Glycoalkaloid (μg/g) | | |
|---|---|---|---|
| Potato Variety | Chaconine | Solanine | Total |
| Yukon Gold | 190 (8) | 80 (6) | 270 |
| Atlantic | 150 (9) | 100 (14) | 250 |
| Russet Burbank | 120 (6) | 70 (13) | 190 |
| Shepody | 130 (13) | 50 (6) | 180 |
| Snowdon | 120 (13) | 100 (18) | 220 |

Glycoalkaloids measured in triplicate, standard deviation in parentheses.

There were no GAs detected in the starch. When the starch was extracted from the potatoes, it was washed with ethanol to facilitate drying and this might have removed some GA. Nevertheless, previous studies have shown potato starch to contain < 4 μg/g GA (Saito et al., 1990).

**Conclusion.** Differences in tuber protein levels were not large enough to produce observable differences in PPC yield when using acidification and heat to recover fruit water protein. On average, 23% of tuber protein can be recovered when 40% of the available fruit water is removed from rasped potatoes. MALDI-TOF MS was shown to have the ability to rapidly and reliably quantify GAs in a variety of potato fractions. The GA concentration of the dried PPC was approximately 10 times that of the fresh tubers. The GA concentration in dry potato pulp was similar to PPC. The high concentrations of GA in both the PPC and potato pulp raises concerns about toxicity and suggests that GA levels should be monitored in these products.

## REFERENCES

Abell, D.C. and Sporns, P. 1996. Rapid quantitation of potato glycoalkaloids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Agric. Food Chem. 44: 2292-2296.

Alexander, R.J. 1995. Potato starch: new prospects for an old product. Cereal Foods World 40: 763-764.

AOAC. 1990. *Official Methods of Analysis*, 15th ed. Association of Official Analytical Chemists, Washington, DC.

Bradstreet, R.B. 1965. *The Kjeldahl Method for Organic Nitrogen.* Academic Press, New York and London.

Chan, T.-W.D., Colburn, A.W., Derrick, P.J., Gardiner, D.J. and Bowden, M. 1992. Suppression of matrix ions in ultraviolet laser desorption: Scanning electron microscopy and raman spectroscopy of the solid samples. Org. Mass Spectrom. 27: 188-194.

Friedman, M. and McDonald, G.M. 1997. Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Crit. Rev. Plant Sci. 16: 55-132.

Gusev, A.I., Wilkinson, W.R., Proctor, A. and Hercules, D.M. 1996. Direct quantitative analysis of peptides using matrix assisted laser desorption ionization. Fresenius J. Anal. Chem. 354: 455-463.

Harvey, D.J. 1993. Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides. Rapid. Commun. Mass Spectrom. 7: 614-619.

Hasse, N.U. and Platte, J. 1996. Properties of potato starch in relation to varieties and environmental factors. Starch 48: 167-171.

Hellenäs, K.-E., Branzell, C., Johnsson, H. and Slanina, P. 1995. Glycoalkaloid content of early potato varieties. J. Sci. Food Agric. 67: 125-128.

Klingspohn, U., Bader, J., Kruse, B., Vijai Kishore, P., Schugerl, K., Kracke-Helm, H.-A. and Likidis, Z. 1993. Utilization of potato pulp from starch processing. Process Biochem. 28: 91-98.

Knorr, D. 1977. Protein recovery from waste effluents of potato processing plants. J. Food Technol. 12: 563-580.

Knorr, D., Kohler, G.O. and Betschart, A.A. 1977. Potato protein concentrates: the influence of various methods of recovery upon yield, compositional and functional characteristics. J. Food Process. Preserv. 1: 235-247.

Morgan, M.R.A., McNerney, R., Matthew, J.A., Coxon, D. and Chan, H.W.-S. 1983. An enzyme-linked immunosorbent assay for total glycoalkaloids in potato tubers. J. Sci. Food Agric. 34: 593-598.

Morris, S.C. and Lee, T.H. 1984. The toxicity and teratogenicity of *Solanaceae* glycoalkaloids, particularly those of the potato (*Solanum tuberosum*): a review. Food Technol. Aust. 36: 118-124.

Plhak, L.C. and Sporns, P. 1992. Enzyme immunoassay for potato glycoalkaloids. J. Agric. Food Chem. 40: 2533-2540.

Roddick, J.G., Rijnenberg, A.L. and Osman, S.F. 1988. Synergistic interaction between potato glycoalkaloids α-solanine and α-chaconine in relation to destabilization of cell membranes: ecological implications. J. Chem. Ecol. 14: 889-902.

Saito, K., Horie, M., Hoshino, Y. and Nose, N. 1990. High performance liquid chromatographic determination of glycoalkaloids in potatoes. J. Chromatogr. 508: 141-147.

Slominska, L. and Starogardzka, G. 1987. Application of a multi-enzyme complex in the utilization of potato pulp. Starch 39: 121-125.

Smith, O. 1987. Potato Chips. Ch. 10 in *Potato Processing*, 4th ed., W.F. Talburt and O. Smith (Ed.), p. 371-489, Van Nostrand Reinhold Company, New York.

Strolle, E.O., Cording, J. and Aceto, N.C. 1973. Recovering potato proteins coagulated by steam injection. J. Agric. Food Chem. 21: 974-977.

Westfalia Separator AG. 1982. Information on Starch Production Magazin International. No. 4., p. 3-10. Print number 9997-6922-000/1182. Westfalia Separator AG, Oelde, Germany.

# Chapter 5

## Development of Antibodies Against the Steroidal Sapogenin Diosgenin

### INTRODUCTION

Tomatine is the major glycoalkaloid (GA) of the tomato plant (*Lycopersicon esculentum*) and is potentially toxic (Friedman et al., 1994). Tomatine has not been reported in *Solanum tuberosum* potato varieties. The low level of tomatine found in ripe tomatoes is of little concern, however, unripe green tomatoes may contain up to 300 μg tomatine/g fresh weight (Eltayeb and Roddick, 1984). Green tomatoes are occasionally used in foods (eg. chutneys).

The literature describes the development of numerous antibodies with affinity for solanidane GAs, such as α-chaconine and α-solanine (Morgan et al., 1983; Plhak and Sporns, 1992; Stanker et al., 1994). There has been less success in developing antibodies against spirosolane alkaloids, such as tomatidine or solasodine (Figure 5-1). Ishiyama et al. (1996) reported an antibody for solamargine (solasodine with a chacotriose substituent) using a human serum albumin-solasonine immunogen. These antibodies were used to develop an ELISA, and later, an immunoaffinity column for extracting GA from *Solanum khasianum* fruit (Putalun et al., 1999). Stanker et al. (1994) identified a monoclonal line of antibodies that showed affinity for α-chaconine and α-solanine, as well as for tomatine. The development of an assay for tomatine based on this unusual antibody has still not been reported. Abell (1997) speculated that tomatidine is a poor immunogen because the N-containing ring tautomerizes to an open form (Figure 5-2). In fact, rotation around the bond between $C_{20}$ and $C_{22}$ is possible, giving rise to a new structure where the ring-nitrogen changes position and the methyl group at $C_{25}$ shifts from equatorial to axial (Boll and von Philipsborn, 1965). It may be that this tautomerism prevents the development of a strong immunogenic response; in effect, the immune system is presented with a moving target.

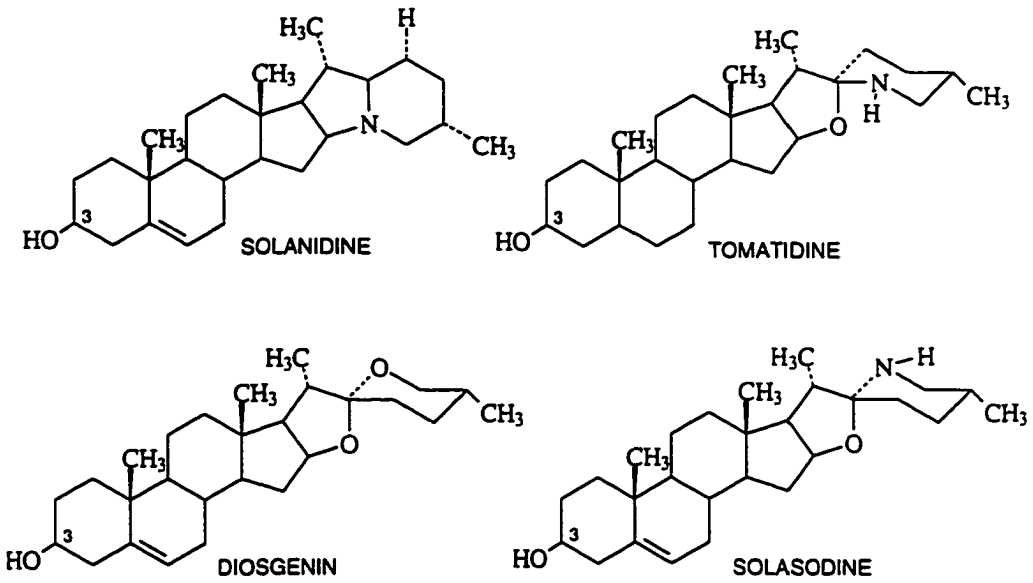


**Figure 5-1 Aglycone structures of α-chaconine and α-solanine (solanidine), tomatine (tomatidine), diosgenin, and solasodine.**

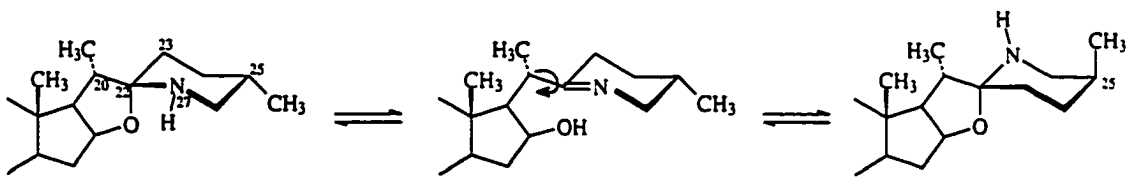

**Figure 5-2 Proposed ring flipping mechanism in tomatidine.**

We propose that tomatine-specific antibodies might be produced if an animal were presented with an immunogen that is similar in structure to tomatine, but one that is not capable of tautomerism. Diosgenin is a steroidal sapogenin (aglycone of a saponin) found in wild yams and fenugreek that has a structure similar to tomatine (Figure 5-1). Because diosgenin contains no nitrogen, its bond topology is expected to be very stable. The objective of this study is to develop an antibody with strong affinity for tomatine using a diosgenin conjugate as the immunogen.

## MATERIALS AND METHODS

**Preparation of Immunogen.** Disogenin hemisuccinate was conjugated to *Limulus polyphemus* hemocyanin (LPH) for rabbit immunization using a modification of the procedure used by Plhak and Sporns (1992). 4-(Dimethylamino)pyridine (229 μmol) and succinic anhydride (4,240 μmol) were added to 10 mL pyridine. Diosgenin (103 μmol) was then added to the pyridine solution and heated under reflux at 55°C. The reaction was followed by TLC. Additional succinic anhydride (2,000 μmol) was added after 24 h when unreacted diosgenin appeared to persist. After 72 h, the reaction was stopped with 20 mL water and extracted with 3 × 25 mL methylene chloride. The combined organic extract was washed with 25 mL 1% acetic acid, followed by 3 × 25 mL water. The organic extract was rotoevaporated to dryness (co-evaporation with toluene was required), and dried overnight under vacuum in the presence of phosphorus pentoxide.

The succinylated diosgenin was further purified with a silica solid-phase extraction cartridge. The cartridge was washed with 5 mL methanol before loading 55 mg of the impure succinylated diosgenin dissolved in ca 6 mL methanol. Fractions (0.5 mL each) were eluted with methanol and monitored by TLC. Fractions 3-15 were combined, rotoevaporated, and dried overnight under vacuum in the presence of phosphorus pentoxide. Light tan crystals were recovered giving a yield of 60%. TLC revealed a very small amount of unreacted diosgenin. MALDI-TOF-MS, however, was unable to confirm the presence of starting material. Strong signals were observed at 516, 538, and 554 (succinylated diosgenin, $M + H^+$, $M + Na^+$, $M + K^+$, respectively), indicating that succinylated diosgenin had been formed.

Succinylated diosgenin was then conjugated to LPH using an active ester method. The active ester of diosgenin was prepared by stirring succinylated diosgenin (36.1 μmol), N,N'-dicyclohexylcarbodiimide (35.9 μmol), and N-hydroxysunnimide (79.9 μmol) in 1 mL 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 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 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 45 mol diosgenin/mol BSA equivalent on a C basis. TLC showed that not all the diosgenin was covalently bound to the LPH because some of the diosgenin could be removed from the conjugate by washing with methylene chloride. Nevertheless, hydrolysis of the washed conjugate in 2 N sodium hydroxide produced additional diosgenin, indicating that a substantial amount of diosgenin was also covalently bound to the LPH.

**Immunization of Rabbits.** Preimmunization blood samples were taken from 2 male Flemish Giant × Dutch Lop Ear rabbits. Each rabbit was injected with ca 0.7 mg of the LPH-diosgenin conjugate in 1 mL of a PBST/Freund's complete adjuvant (1:1, v/v) emulsion. The rabbits received 0.5 mL gluteal and two 2 × 0.25 mL sub-scapular. Three monthly boosts were made in the same manner except with Freund's incomplete adjuvant. Blood samples were collected 2 weeks after each boost, centrifuged at 2,150 × *g* for 15 min, and the serum tested for antibody titer.

**Preparation of Conjugates for Enzyme Immunoassay.** Preparation of the BSA-diosgenin conjugate was similar to that of the LPH-diosgenin conjugate. Succinylated diosgenin (19.4 μmol), N,N'-dicyclohexylcarbodiimide (19.4 μmol), and N-hydroxysuccinimide (63.4 μmol) were stirred in 1 mL dry dimethylformamide for 24 h at 4°C. This reaction mixture was added to 140 mg BSA in 1 mL PBS. The entire reaction mixture was dialyzed and lyophilized the same as the LPH conjugates. Elemental analysis was ambiguous but TLC of the hydrolyzed conjugate again confirmed that covalent binding took place. MALDI-TOF MS of the conjugate soluble in 0.1% trifluoroacetic acid indicated 3 mol diosgenin/mol BSA, however, only a small proportion of the conjugate was soluble in trifluoroacetic acid.

**Enzyme Immunoassay.** Antibody titer was determined using a checkerboard immunoassay. BSA-diosgenin was dissolved in PBS with a concentration of 35 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 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 coated with 200 μL of 1% BSA in PBS for 1 h at room temperature. The plate was then washed with 3 × 200 μL PBST for 5 min each wash.

Rabbit serum was diluted 1/30 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 PBST. Goat anti-rabbit antibody-peroxidase conjugate was diluted 1/3000, added to each well (200 μL/well), and allowed to incubate 2 h at room temperature. The wells were then emptied and washed as before. The peroxidase substrate was prepared by dissolving 10 mg 2,2'-azino-bis(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) 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).

For analysis of diosgenin, solasodine, tomatine and α-solanine samples, an indirect competitive ELISA was performed. Sample solutions were prepared in methanol with concentrations ranging from $10^{-5}$ - $10^{3}$ μM. Higher concentrations were not prepared because of solubility concerns. Microtiter plates were coated with 200 μL of 2 μg/mL BSA-diosgenin, blocked,and washed as above. Sample solutions (100 μL/well) were added to the plate, followed immediately by 100 μL of serum diluted 1/5,000. The plates were incubated and otherwise treated as above.

## RESULTS AND DISCUSSION

The titer of the anti-diosgenin serum was calculated as 1/50,000 serum dilution with 1 μg/mL diosgenin conjuate used to coat the microtiter plate. Indirect competitive ELISA of diosgenin standards resulted in the expected sigmoidal calibration curve (Figure 5-3). The $I_{50}$ for diosgenin was calculated to be 0.8 μM (the concentration that reduced absorbance 50%). The $I_{50}$ values for solasodine and tomatine could not be accurately calculated because it was not possible to generate a complete sigma curve, however, the $I_{50}$ for solasodine and tomatine were estimated to be about 30 and 100 μM, respectively. The serum had almost no affinity for α-solanine, a solanidane steroid. Not surprisingly, the degree of affinity was related to the degree of similarity between the molecular structures of the hapten and the analyte. The low but discernible affinity the serum exhibited for tomatine indicates that structurally similar haptens, rather than identical haptens, may be used to develop antibodies. As expected, affinity values are lower using this strategy.

It is not known if the affinity of the serum for tomatine was strong enough to facilitate the development of an ELISA for tomatine. Tomato fruit would not be expected to contain significant quantities of compounds, such as diosgenin, that would interfere with the ELISA. The serum may be adequate for tomatine analysis in green tomatoes, but this would have to be verified experimentally. The development of a reliable ELISA would greatly simplify tomatine analysis (Friedman et al., 1994).

The development of a diosgenin-specific antibody does create other research opportunities. Since the 1940s, diosgenin has been extracted from wild yams and used as a starting material for the synthesis of progesterone and other steroids (Rouhi, 1995). Fenugreek and wild yam, two sources of diosgenin, have been used medicinally in several cultures (Foster and Duke, 1990; Takechi et al., 1991). Wild yam, in particular, is widely sold as a natural health supplement. Specific health claims usually include a hypocholesterolemic effect and in improvement in hormone balance based on the structural similarity between diosgenin and progesterone. There is very little reference in

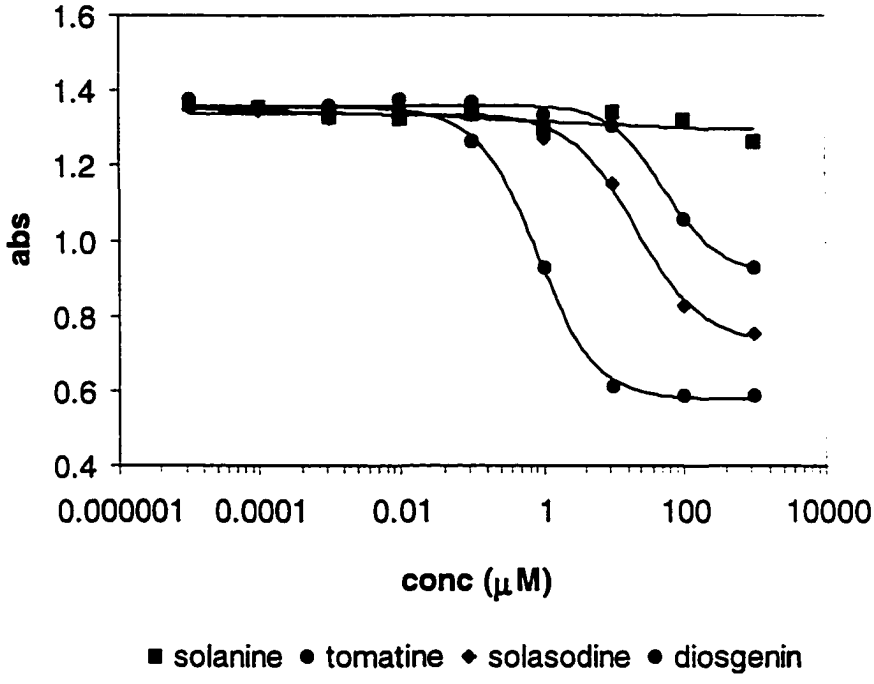


**Figure 5-3 Enzyme immunoassay of diosgeinin, solasodine, tomatine, and α-solanine.**

the scientific literature to the health benefit of diosgenin. Takechi et al. (1991) showed that diosgenin was anti-fungal and that the glycoside of diosgenin, dioscin, was hemolytic. A saponin-rich extract of fenugreek was shown to be hypocholesterolemic in dogs (Sauvaire et al., 1991). As fenugreek production is being promoted for western Canada (Taylor et al., 1997), clarifying the health effect of diosgenin-rich nutraceuticals and functional foods may be of some benefit. Diosgenin antibodies might be used to develop an ELISA, immunoaffinity columns, or other analytical tools.

## REFERENCES

Abell, D.C. 1997. Analysis of potato glycoalkaloids by ELISA and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Ph.D. thesis dissertation, University of Alberta, Edmonton, Canada.

Boll, P.M. and von Philipsborn, W. 1965. NMR studies and the absolute configuration of *Solanum* alkaloids (Spiroaminoketal alkaloids). Acta Chem. Scand. 19: 1365-1370.

Eltayeb, A.E. and Roddick, J.G. 1984. Changes in the alkaloid content of developing fruits of tomato (*Lycopersicon esculentum* Mill.). 1. Analyses of cultivars and mutants with different ripening charcteristics. J. Exp. Bot. 35: 252-260.

Foster, S. and Duke, J.A. 1990. *A Field Guide to Medicinal Plants: Eastern/Central Medicinal Plants*, p. 204. Houghton Mifflin Co., Boston, MA.

Friedman, M., Levin, C.E. and McDonald, G.M. 1994 α-Tomatine determination in tomatoes by HPLC using pulsed amperometric detection. J. Agric. Food Chem. 42: 1959-1964.

Harvey, M.H., Morris, B.A., McMillan, M. and Marks, V. 1985b. Measurement of potato steroidal alkaloids in serum and saliva by radioimmunoassay. Human Toxicol. 4:503-512.

Ishiyami, M., Shoyama, Y., Murakami, H. and Shinohara, H. 1996. Production of monoclonal antibodies and development of an ELISA for solamargine. Cytotechnology 18: 153-158.

Morgan, M.R.A., McNerney, R., Matthew, J.A., Coxon, D.T. and Chan, W.-S. 1983. An enzyme-linked immunosorbent assay for total glycoalkaloids in potatoes. J. Sci. Food Agric. 34: 593-598.

Plhak, L.C. and Sporns, P. 1992. Enzyme immunoassay for potato glycoalkaloids. J. Agric. Food Chem. 40: 2533-2540.

Putalun, W., Tanaka, H. and Shoyama, Y. 1999. Rapid separation of solasodine glycosides by an immunoaffinity column using anti-solamargine monoclonal antibody. Cytotechnology 31: 151-156.

Rouhi, A.M. 1995. Researchers unlocking potential of diverse, widely distributed saponins. Chem. Eng. News, September 11, 1995, p. 28-35.

Sauvaire, Y., Ribes, G., Baccou, J.-C., Loubatieres-Mariami, M.-M. 1991. Implication of steroid saponins and sapogenins in the hypocholesterolemic effect of fenugreek. Lipids 26: 191-197.

Stanker, L.H., Kamps-Holtzapple, C. and Friedman, M. 1994. Development and characterization of monoclonal antibodies that differentiate between potato and tomato glycoalkaloids and aglycons. J. Agric. Food Chem. 42:2360-2366.

Takechi, M., Shimada, S. and Tanaka, T. 1991. Structure-activity relationships of the saponins dioscin and dioscinin. Phytochemistry 30: 3943-3944.

Taylor, W.G., Zaman, M.S., Mir, Z., Mir, P.S., Acharya, S.N., Mears, G.J. and Elder, J.L. 1997. Analysis of steroidal sapogenins from amber fenugreek (*Trigonella foenum-graecum*) by capillary gas chromatography and combined gas chromatography/mass spectrometry. J. Agric. Food Chem. 45: 753-759.

# Chapter 6

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

## 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 (Nelson et al., 1994), oligosaccharides (Wang et al., 1999), phospholipids (Harvey, 1995), alkaloids (Abell and Sporns, 1996), and tannins (Ohnishi-Kameyama, 1997). Although MALDI-TOF MS is tolerant of impurities compared to other MS techniques, maximum sensitivity is achieved from pure analyte in solution (Harvey, 1993). Femtomole detection limits are possible when the sample is free from impurities (Vorm and Mann, 1994). Detection limits are severely compromised when biological extracts are analyzed (Hutchens and Yip, 1993).

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 immuno-affinity purification to analyze lysozyme (Brockman and Orlando, 1995), cytochrome c (Papac et al., 1994), and mytoxin (Nelson et al., 1995); avidin-biotin affinity has been used to detect biotinylated bradykinin and insulin (Schriemer and Li, 1996); and metal ion affinity has been used to isolate several metal-binding peptides (Papac et al., 1994). The binding molecules are typically immobilized on agarose beads (Papac et al., 1994; Schriemer and Li, 1996). 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 then eluted for MALDI-TOF MS analysis. Alternatively, the binding molecule can be immobilized directly on the MALDI probe (Brockman and Orlando, 1995; Liang et al., 1998). The entire MALDI probe is immersed in the sample solution and the anayte is retained directly on the probe. Immobilization of the binding molecule on a solid support was completely avoided by Nakanishi et al. (1994) who used immuno-precipitation to purify transferrin for MALDI-TOF MS analysis.

Potato glycoalkaloids (GAs) are of interest because of their potential toxicity to humans (McMillan and Thompson, 1979; Phlak and Sporns, 1997). GAs can be determined in potatoes by variety of methods (Friedman and MacDonald, 1997). It is much more difficult to analyze GAs in animal tissue (eg. serum) after potato consumption because concentrations are ca 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 (Harvey et al., 1985). Hellenäs et al. (1992) developed an 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 report, 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 (Abell and Sporns, 1996). In order 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 using chromatographic and immunoaffinity sample purification procedures.

## MATERIALS AND METHODS

**Preparation of Immunogen.** Solanidine was conjugated to *Limulus polyphemus* hemocyanin (LPH) for rabbit immunization using a modification of the procedure used

by Plhak and Sporns (1992). The first step involved the formation of the succinylated alkaloid (Figure 6-1). 4-(Dimethylamino)pyridine (218 µmol) and succinic anhydride (4,290 µmol) were added to 10 mL 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 24 h (3,230 µmol) and 48 h (1,890 µmol) when unreacted solanidine appeared to persist. The reaction was stopped with 20 mL water and extracted with 3 × 25 mL methylene chloride. The combined organic extract was washed with 25 mL 1% acetic acid, followed by 3 × 25 mL water. The organic extract was rotoevaporated to dryness (co-evaporation 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 cartridge. The cartridge was washed with 5 mL methanol before loading 28 mg of the impure succinylated solanidine dissolved in ca 2 mL methanol. 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 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 6-2). 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 dry dimethylformamide 24 h at 4°C. To improve the solubility of the active ester in aqueous solution, 2 mL of 1,4-dioxane was added 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 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.

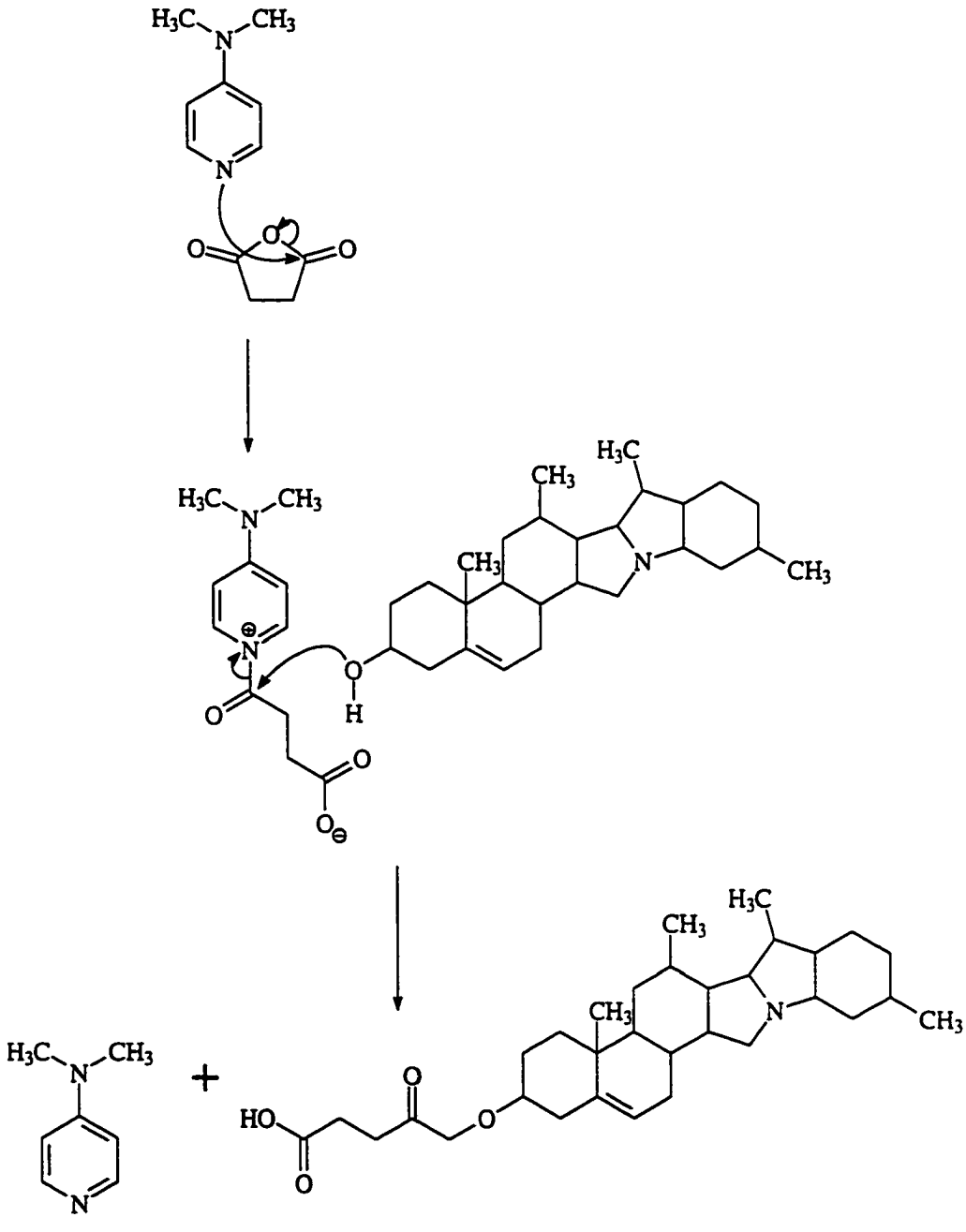

**Figure 6-1 Succinylation of solanidine.**

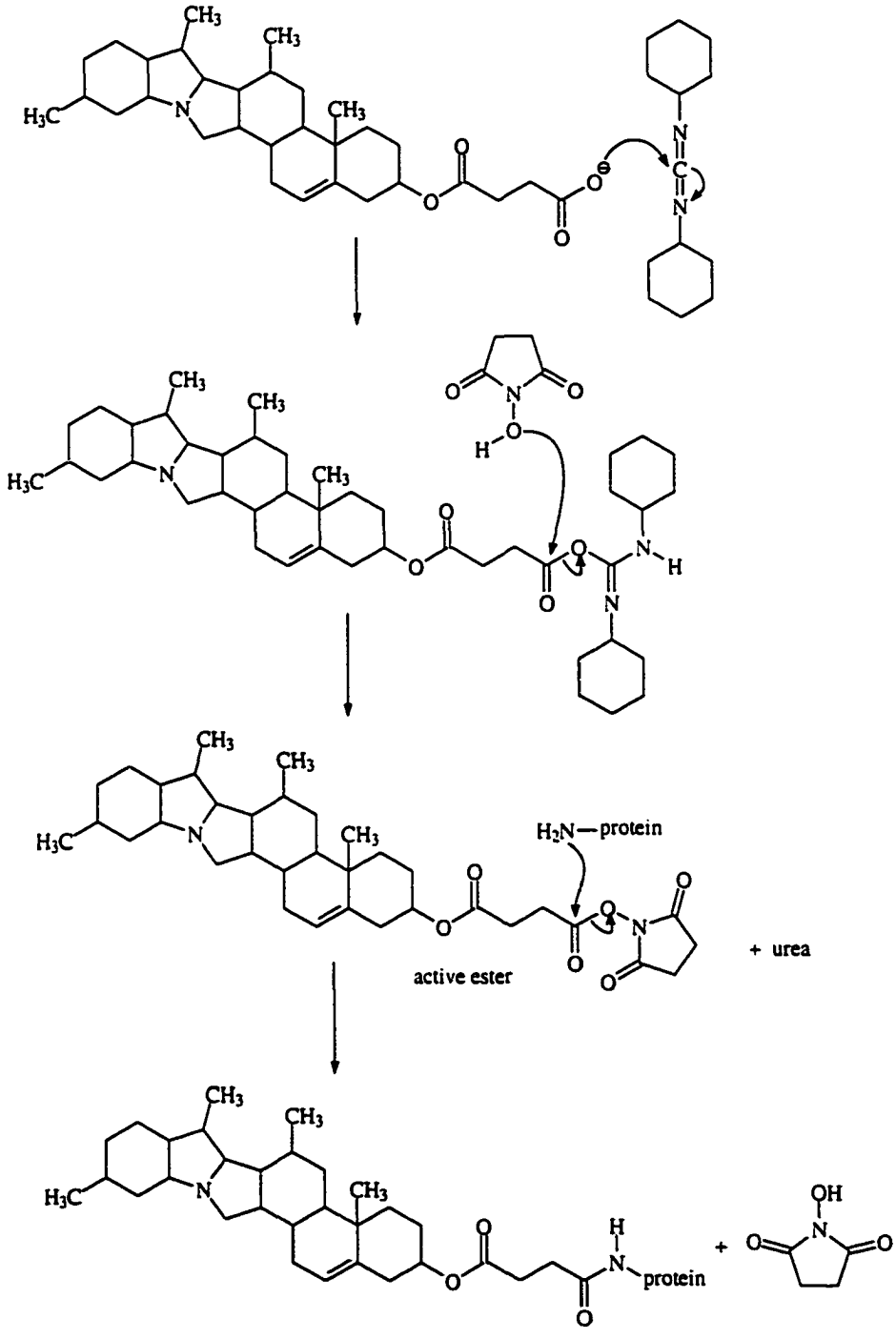


**Figure 6-2 Conjugation of protein to succinylated solanidine via an active ester intermediary.**

Elemental analysis indicated 3 mol solanidine/mol 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 2 female Flemish Giant × Dutch Lop Ear rabbits. Each rabbit was injected with ca 0.7 mg of the LPH-solandine conjugate in 1 mL of a PBST/Freund's complete adjuvant (1:1, v/v) emulsion. The rabbits received 0.5 mL gluteal and two 2 × 0.25 mL sub-scapular. Two monthly boosts were made in the same manner except with Freund's incomplete adjuvant. Blood samples were collected 2 weeks after each boost, centrifuged at 2,150 × *g* for 15 min, and the serum 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 dry dimethylformamide 24 h at 4°C. The reaction mixture was then added to 1 mL of PBS containing 132 mg 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 solanidine/mol 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 of the BSA-solanidine that was soluble in 0.1% trifluoroacetic acid indicated 2 mol solanidine/mol 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 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 coated with 200 μL of 1% BSA in PBS for 1 h at room temperature. The plate was then washed with 2 × 200 μL 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 PBST. Goat anti-rabbit antibody-peroxidase conjugate was diluted 1/3000, added to each well (200 μL/well), and allowed to incubate 2 h at room temperature. The wells were then emptied and washed as before. The peroxidase substrate was prepared by dissolving 10 mg 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 100 mL of 0.05M 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) 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, 0.01 M 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 on to 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 fraction 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 phosphate-buffered saline (PBS,

0.01 M phosphate, 0.9% sodium chloride, pH 7.3). The antibodies were finally concentrated to ca 1 mg/mL in a 30 K 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 am Econo-Pac 10 DG desalting column (Bio-Rad Laboratories, Hercules, CA) and freeze-dried. Antibody was covalently coupled to carboxylate-modified 0.30 μm diameter silica particles via a an one-step procedure according to the manufacturer's suggested protocol (Tech note #13c, Bangs Laboratories, Fishers, IN). The freeze-dried 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 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.** Freeze-dried 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 1 mM HCl on a sintered glass funnel, followed by 2 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 1,800 × *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 5 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 PBS. The column was drained until the total volume was 1 mL (beads + PBS). The volume of the agarose gel was ca 0.5 mL after settling.

**Solid-Phase Extraction Sample Cleanup**. The solid-phase extraction (SPE) procedure was based on Saito et al. (1990). 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 methanol, discarding the first 0.5 mL. An aliquot of the eluent was used directly for MALDI-TOF analysis.

**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 water by drawing them repeatedly through a pasteur pipet followed by centrifugation as above. The particles were washed twice in this manner. After removing the second water wash, the GAs were eluted by suspending the particles in 20 µL methanol and centrifuging at 650 × *g* for 5 min. The supernatant was used for MALDI-TOF MS analyzed.

**Extraction of GAs from Serum with Agarose Immunoaffinity Beads.** The agarose beads prepared previously were vortexed in the column to produce a suspension of ca 0.5 mL agarose/mL 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 × $\frac{1}{8}$" o.d. frit (Supelco, Bellefonte, PA) wedged in the tip (Figure 6-3). 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 water. The GAs were eluted by adding 25 µL methanol to the washed beads and allowing the beads to sit for 5 min. The methanol was then pushed

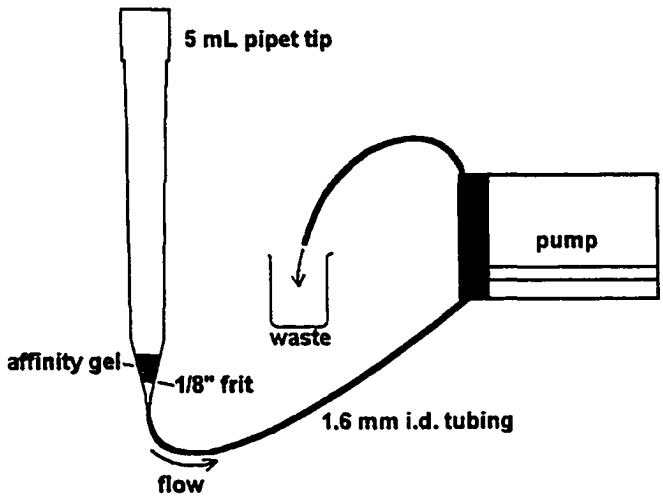


**Figure 6-3 Immunoaffinity column prepared in a 5 mL pipet tip.**

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 mass spectrometry 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). Ca 1.5 mL was spotted on the MALDI probe an allowed to air dry. Each spectrum represents the sum of 200 laser pulses directed at several positions on the sample spot.

## 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 1/500,000 serum dilution with 1 μg/mL conjugate applied to the microtiter plate. The titer improved to 1/800,000 serum dilution after the second boost when the blood was harvested. The titer of the antibody after purification on the GammaBind column was $6.0 \times 10^{-6}$ mg antibody/mL, indicating that antibody affinity for solanidine was preserved during the purification process.

**MALDI-TOF MS of GAs.** Figure 6-4 shows the spectrum generated from a 25 ng/mL solution of each GA (ca 25 fmol of each GA on the probe). The peaks are clearly identifiable with a 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 (Evans et al., 1994).

Signal suppression is commonly encountered when mixtures are analyzed by MALDI-TOF MS. Harvey (1993) 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 (Liang et al., 1998). Analysis of porcine serum fortified with α-solanine and α-chaconine confirmed that signal can be severely suppressed in a biological sample (Figure 6-5). The minimum GA concentration that could be reliably detected when serum was spotted directly on the MALDI probe was 45,000 ng/mL, representing a 2,000-fold decrease in sensitivity.

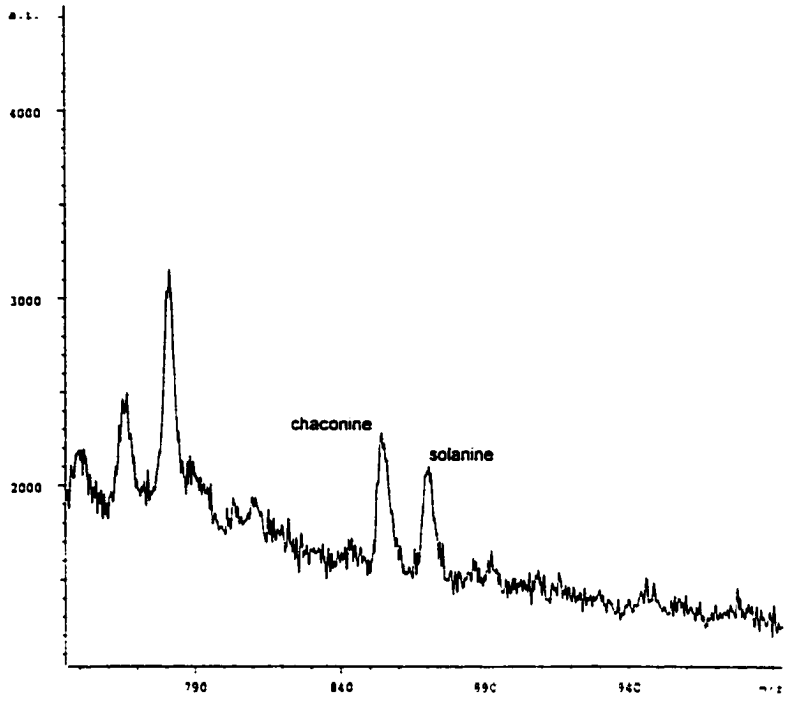


**Figure 6-4 MALDI-TOF spectrum of ca 25 fmol α-chaconine and 25 fmol α-solanine. Sample solution concentration was 25 ng/mL of each glycoalkaloid.**

The same fortified serum sample was treated with the SPE sample cleanup procedure developed by Saito et al. (1990) 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 shows a large improvement in GA signal intensity (Figure 6-5). Subsequent fortification experiments showed that 100 ng 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 non-

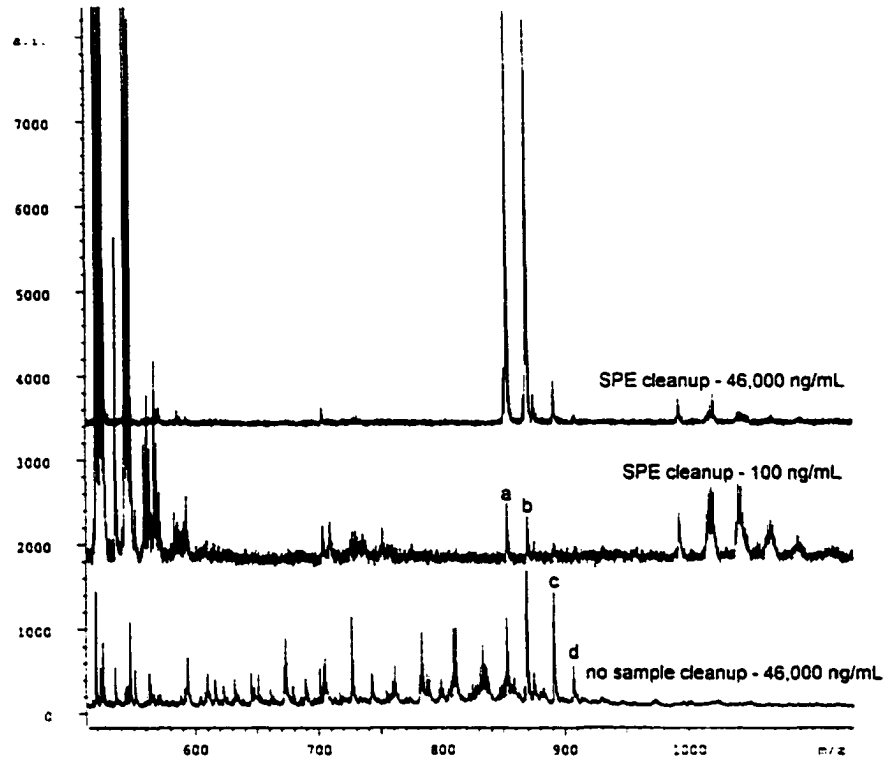


**Figure 6-5 MALDI-TOF mass spectra of glycoalkaoids in porcine serum (a, α-chaconine + $H^+$; b, α-solanine + $H^+$; c, α-chaconine + $K^+$; d, α-solanine + $K^+$).**

specific affinity for GAs. Particles coated with non-specific IgG, rather than GA antibody, were able to extract GAs from PBS when GA concentrations were >1 μg/mL. The non-specific affinity for GAs was attributed to the hydrophobic nature of the polystyrene. The relatively hydrophobic GAs probably have more affinity for polystyrene than they have for the aqueous solution in which they are dissolved.

The next strategy was to isolate the GAs on an immunoaffinity column (Yu et al., (1998). 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 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 resident time of the sample in the gel, ca 8 sec.

To increase the time available for antibody-antigen binding to occur, the antibody-coated agarose beads were mixed with GA-fortified serum for 2 h as described in the Methods and Materials section. 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 6-6). The same improvement in sensitivity was observed with the SPE sample cleanup procedure.

As an alternative method of eluting GA, application of the beads directly to the MALDI probe was investigated (Schriemer and Li, 1996). 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 6-7). As there was no appreciable 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 6-6). 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 vs 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.

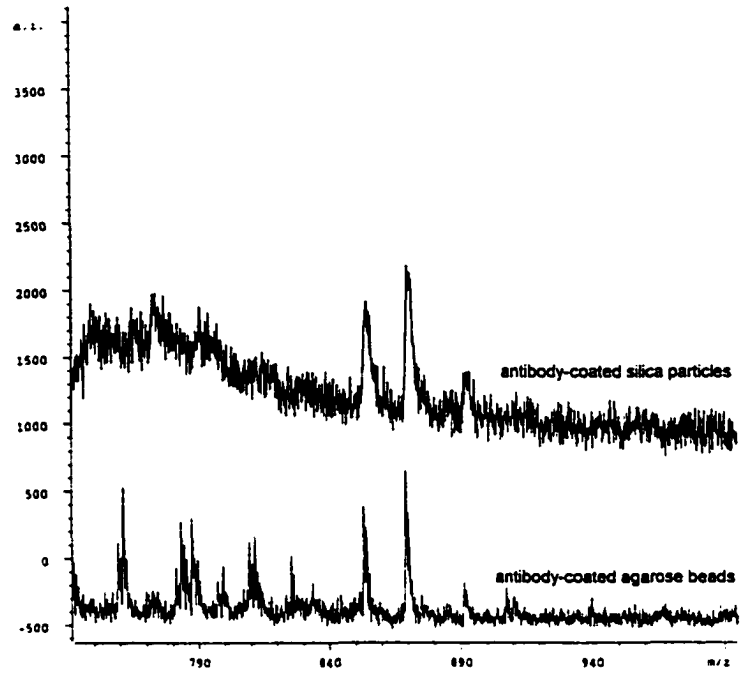


**Figure 6-6 MALDI-TOF MS spectrum of serum fortified with 100 ng/mL α-chaconine and 100 ng/mL α-solanine using immunoaffinity purification.**

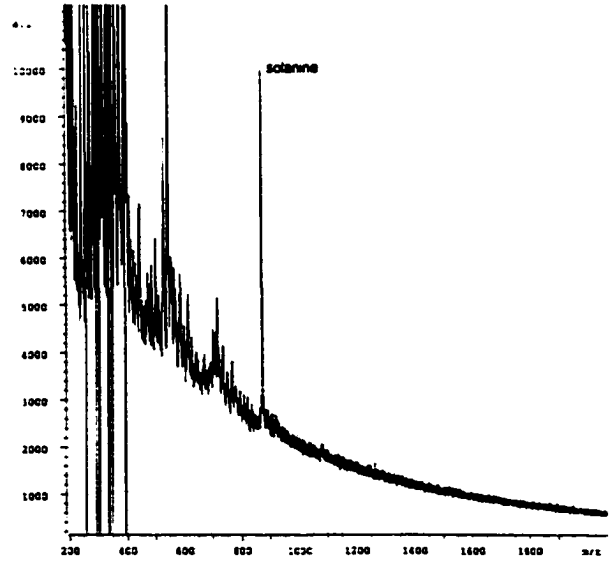


**Figure 6-7 MALDI-TOF spectrum of 100 ng/mL α-solanine in PBS extracted with agarose affinity beads. Affinity beads were applied directly to the MALDI probe.**

**SPE Coupled with Immunoaffinity Purification.** Assuming all the GA in the serum sample was bound by the immobilized antibody, and that all the GA was subsequently eluted, agarose affinity purification of the 100 ng GA/mL 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 6-4). 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 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 6-7).

Based on 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 GA/mL serum could be detected with MALDI-TOF MS (Figure 6-8). 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 explained by the removal of signal suppressing contaminants from the serum sample (Table 6-1).

Harvey et al. (1985) used a radioimmunoassay to determine that the concentration of total GAs in the serum of healthy individuals ranged from 3.3-125 nM (ca 3-110 ng/mL). Hellenäs et al. (1992) reported that after a single serving of potatoes, serum levels of α-chaconine peaked at 14.4 ng/mL and 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 less time-consuming than required for HPLC (Hellenäs et al., 1992). MALDI-TOF MS also offers the advantages of unambiguous analyte identification based on molecular mass and the possible identification of GA metabolites.

**Table 6-1 Effect of sample purification on MALDI-TOF analysis of glycoalkaloids in a 5 mL serum sample**

| Purification | Sample volume (mL) | Detection limit (ng/mL) |
|---|---|---|
| No purification | 5 | 45,000 (Figure 6-5) |
| $C_{18}$ SPE | 2 | 100 (Figure 6-5) |
| Immunoaffinity purification | 0.025 | 100 (Figure 6-6) |
| $C_{18}$ SPE + immunoaffinity purification | 0.025 | 1 (Figure 6-8) |

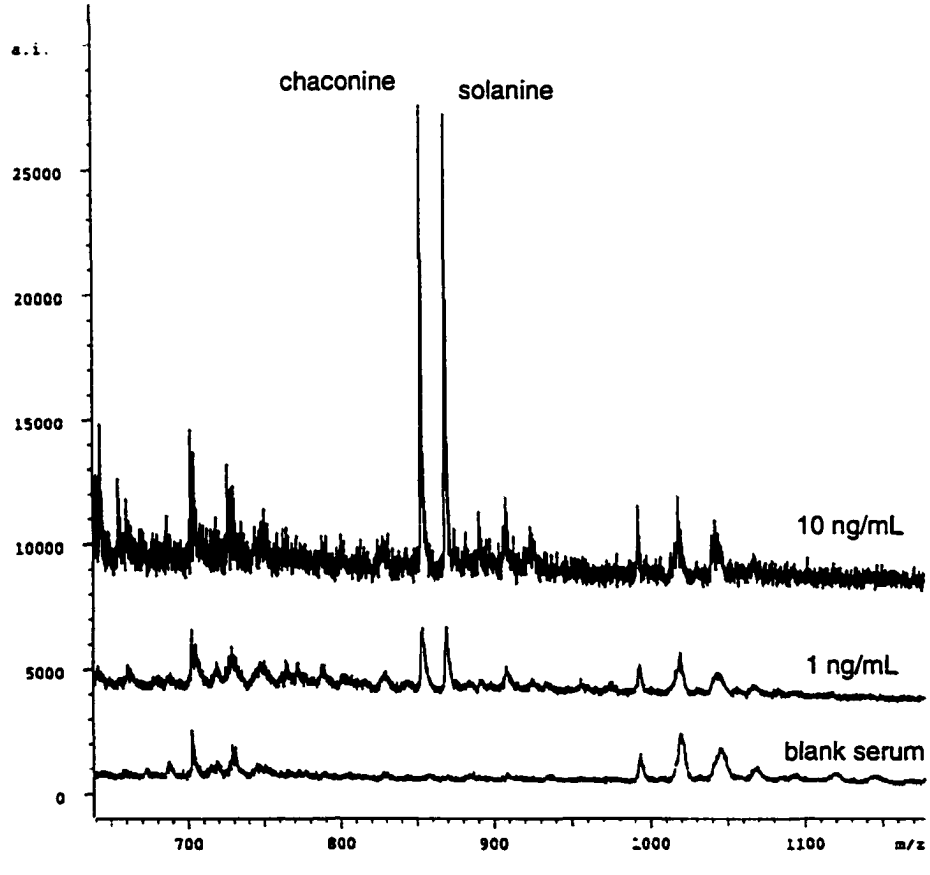


**Figure 6-8 MALDI-TOF spectrum of serum glycoalkaloids purified by SPE and immunoaffinity on agarose beads.**

## REFERENCES

Abell, D.A. and Sporns, P. 1996. Rapid quantitation of potato glycoalkaloids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J. Agric. Food Chem. 44: 2292-2296.

Brockman, A.H. and Orlando, R. 1995. Probe-immobilized affinity chromatography/ mass spectrometry. Anal. Chem. 67: 4581-4585.

Friedman, M. and McDonald, G.M. 1997. Potato glycoalkaloids: chemistry, analysis, safety, and plant physiology. Crit. Rev. Plant Sci. 16: 55-132.

Harvey, D.J. 1995. Matrix-assisted laser desorption/ionization mass spectrometry of phospholipids. J. Mass Spectrom. 30: 1333-1346. LOOK UP

Harvey, D.J. 1993. Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides. Rapid Commun. Mass Spectrom. 7: 614-619.

Harvey, M.H., Morris, B.A., McMillan, M. and Marks, V. 1985. Measurement of potato steroidal alkaloids in serum and saliva by radioimmunoassay. Human Toxicol. 4: 503-512.

Hellenäs, K.-E., Nyman, A., Slanina, P., Lööf, L. and Gabrielsson, J. 1992. Determination of potato glycoalkaloids and their aglycone in blood serum by high-performance liquid chromatography: application to pharmacokinetic studies in humans. J. Chromatogr. 573: 69-78.

Hutchens, T.W. and Yip, T.T. 1993. New desorption strategies for the mass spectrometric analysis of macromolecules. Rapid Commun. Mass Spectrom. 7: 576-580.

Liang, X., Lubman, D.M., Rossi, D.T., Nordblom, G.D. and Barksdale, C.M. 1998. On-probe immunoaffinity extraction by matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 70: 498-503.

McMillan, M. and Thompson, J.C. 1979. An outbreak of suspected solanine poisoning in schoolboys. Quarterly Journal of Medicine, News Service 48(190): 227-243.

Nakanishi, T., Okamoto, N., Tanaka, K. and Shimizu, A. 1994. Laser desorption time-of-flight mass spectrometric analysis of transferrin precipitated with antiserum: a unique simple method to identify molecular weight variants. Biol. Mass Spectrom. 23:230-232.

Nelson, R.W., Krone, J.R., Bieber, A.L. and Williams, P. 1995. Mass spectrometric immunoassay. Anal. Chem. 67: 1153-1158.

Nelson, R.W., McLean, M.A. and Hutchens, T.W. 1994. Quantitative determination of proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal. Chem. 66: 1408-1415.

Ohnishi-Kameyama, M., Yanagida, A., Kanda, T. and Nagat, T. 1997. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. Rapid Commun. Mass Spectrom. 11: 31-36. LOOK UP

Papac, D.I., Hoyes, J. and Tomer, K.B. 1994. Direct analysis of affinity-bound analytes by MALDI/TOF MS. Anal. Chem. 66: 2609-2613.

Plhak, L.C. and Sporns, P. 1997. Biological activities of potato glycoalkaloids. Ch. 7 in *Antinutrients and Phytochemicals in Food*, F. Shahidi (Ed.), ACS Symposium Series 662, p. 115-126. American Chemical Society, Washington, D.C.,

Plhak, L.C. and Sporns, P. 1992. Enzyme immunoassay for potato glycoalkaloids. J. Agric. Food Chem. 40: 2533-2540.

Saito, K.; Horie, M.; Hoshino, Y.; Nose, N. 1990 High performance liquid chromatographic determination of glycoalkaloids in potatoes. J. Chromatogr. 508: 141-147.

Schriemer, D.C. and Li, L. 1996. Combining avidin-biotin chemistry with matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 68: 3382-3387.

Vorm, O. and Mann, M. 1994. Improved mass accuracy in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of peptides. J. Am. Soc. Mass Spectrom. 5: 955-958.

Wang, J., Sporns, P. and Low, N.H. 1999. Analysis of food oligosaccharides using MALDI-MS: quantification of fructooligosaccharides. J. Agric. Food Chem. 47: 1549-1557.

Yu, W., Dorner, J.W. and Chu, F.S. 1998. Immunoaffinity column as cleanup tool for a direct competitive enzyme-linked immunoassay of cyclopiazonic acid in corn, peanuts, and mixed feed. J. AOAC Int. 81: 1169-1175.

# Chapter 7

## Conclusions and Future Research Possibilities

**Capillary Electrophoretic Determination of Glycoalkaloids in Tubers.** The concentration of total GA in potato tubers was rapidly determined using CE-LIF to analyze the products of a competitive solution-phase immunoassay. The procedure was easy to perform and results were shown to be accurate and reproducible. The method was well suited for analyzing small numbers of samples, but could also be adapted for screening large numbers of samples as might be generated in a potato breeding program. Sample throughput would be enhanced if CE autosamplers were adapted to accept 96-well microtiter plates rather than in individual vials, and if an array of capillaries were used rather than a single capillary. However, sample extraction is still the most time-consuming part of the method and there would be little point in further increasing the speed of the analytical part.

While the method performed satisfactorily, several modifications have been identified that might improve the quality of the results:

1. Improve the resolution between the unbound fluorescent solanidine (AMF-SOL) and the AMF-SOL-antibody complex. This may be possible using acetonitrile or ethanol as a modifier in the mobile phase.
2. Use an internal standard to improve precision. A substantial amount of the method variability was attributable imprecise injection volumes.
3. Improve the purity of the AMF-SOL. The quality of the results would not likely be improved by better purifying the AMF-SOL, however, the electropherograms would have neater appearance.
4. Use monoclonal antibodies instead of polyclonal antibodies. The polyclonal serum used in the experiment introduced all the components of blood serum to the sample mixture. Cleaner separations might be possible with monoclonal antibodies because the rest of the serum is eliminated and because monoclonal antibodies are more homogenous than polyclonal antibodies.

It is likely that immunoassay-CE-LIF could be used for many of the food analyses where ELISAs are currently used, eg. mycotoxins, drug residues, pesticide residues, and allergens. Analysis time would be shortened considerably because solution-phase immunoassays are inherently faster than solid-phase immunoassays, plate washing steps are eliminated, and capillary electrophoretic instrumentation is already highly automated.

**Quantitative MALDI-TOF MS of Glycoalkaloids in Tubers.** Accuracy, reproducibility, range, and detection limits were documented for the determination of α-chaconine and α-solanine in potato tubers by MALDI-TOF MS. In general, validation data for the MALDI-TOF MS method were comparable to published HPLC values. MALDI-TOF MS, however, required less sample preparation and provided more precise analyte identification than HPLC.

Shot-to-shot variation in analyte intensity remains the limiting factor in performing quantitative analysis by MALDI-TOF MS. Internal standards and more homogenous matrix crystals reduce, but do not eliminate, the variability in analytical results. To some extent, increasing the number of laser shots used to generate a spectrum can also be used to reduce variability. However, more research is required to reduce shot-to-shot variability. Improving the homogeneity of the analyte-matrix crystal layer would be beneficial. Some of the original MALDI work was done with glycerol as the matrix (Tanaka et al., 1988). Further investigating the use of liquid matrices may represent a new approach for obtaining more reproducible spectra as it may be easier to produce a homogenous solution than a homogenous analyte-crystal mixture.

Commercial MALDI-TOF instruments have been designed explicitly for qualitative rather than quantitative analysis. Several design changes would strengthen the ability to perform quantitative analysis. Current data handing software is well-suited for accurately determining mass-to-charge ratios, but it is generally difficult to get accurate peak height or peak area information. While sample analysis is very rapid, analysis of the peak intensity data is very time-consuming. Quantitative analysis would be much easier if data handling software had the same peak integration capability that chromatography software has. With respect to sample ionization, some MALDI-TOF

instruments (eg. Kratos Kompact I) can be programmed to systematically sample a spot when firing the laser. Other instruments (eg. Bruker Proflex), require the operator to aim the laser manually. Manually aiming the laser is useful for obtaining high quality spectra, however, representative sampling of the spot is preferable for quantitative analysis because analyst bias is minimized. Neither the software capability nor the laser firing process represents a fundamental design limitation of the instrument. Both problems could be addressed with existing technology.

Despite the problems associated with quantitative analysis, more than 50 papers have been published since 1997 where MALDI-TOF MS has been used quantitatively. Many of these papers simply report that a standard curve was generated. Our research shows that results generated by MALDI-TOF MS are accurate and reproducible. Before commercial and regulatory labs seriously consider MALDI-TOF MS for quantitative analysis, it will be necessary to show that results are reproducible among different labs with different instruments and analysts. This will require an interlaboratory study similar to the kind sponsored by AOAC International (AOAC International, 1999).

**Determination of Glycoalkaloids in Non-Tuber Potato Samples by MALDI-TOF MS.** α-Chaconine and α-solanine were successfully determined in potato pulp, protein, starch, and fruit water by MALDI-TOF MS, however, the quality of the results was very dependent on how the calibration curve was generated. It was essential that the calibration curve be generated by a series of standard additions into a sample similar to the type of sample to be analyzed. For example, it was necessary to use standard additions to potato pulp to generate a calibration curve for analyzing potato pulp. The signal suppression characteristics of each sample type appeared to be unique. This is an important factor to consider when performing quantitative MALDI-TOF MS.

**Fate of Glycoalkaloids During Extraction of Potato Starch.** The starch extraction study revealed how GAs are concentrated in the pulp and protein by-products of industrial starch extraction. The GA concentration in the potato protein concentrate was 10-times the concentration in the original tubers. No GA could be detected in the potato starch. The toxicity and potential flavor problems of the protein concentrate will likely limit its use as a food supplement. The protein concentrate may find some use as a

supplement for livestock feed, especially if the animals can be shown to be more tolerant of dietary GAs than humans.

**Analysis of Glycoalkaloids in Blood Serum by MALDI-TOF MS.** A method capable of detecting 1 ng GA/mL serum was developed using solid-phase extraction and immunoaffinity purification of the sample prior to analysis by MALDI-TOF MS. This level of sensitivity is adequate for monitoring potato GAs in human serum (Harvey et al., 1985; Hellenäs et al., 1992). Immunoaffinity purification was carried out on antibody-coated agarose beads or silica particles. Antibody-coated polystyrene particles did not produce good results and were difficult to use because of their low density (they were difficult to centrifuge).

Because the immunoaffinity purification procedure is highly selective and also concentrates the sample (from 5 mL to 25 μL), it may be possible to use HPLC with UV detection to analyze the purified GAs. After extensive sample clean up, Hellenäs et al. (1992) used HPLC to detect GAs in a 2 mL serum sample containing 0.3 ng GA/mL. Nevertheless, MALDI-TOF MS provides faster results and more precise analyte identification than HPLC.

At present, our method is only capable of detecting GAs in serum. Nelson et al. (1995) described the quantification of a myotoxin purified on antibody-coated agarose beads using an internal standard and MALDI-TOF MS. In order to quantify serum GAs, a suitable internal standard will have to be developed. It would be preferable to develop an internal standard that is recognized by the GA antibody so that it could be incorporated early in the extraction procedure. Future research might investigate the reaction of a water-soluble acid chloride with solanidine to create an internal standard that would have appropriate solubility characteristics and would be recognized by the GA antibody. 2-Acetoxypropionyl chloride is one possible candidate for this reaction (Figure 7-1). Another approach would be to incorporate both tomatidine and solanidine antibodies on the immunoaffinity bead. This would allow the use of tomatine as the internal standard as is currently done with potato tuber analysis (Chapter 3). However, tomatidine antibodies have been difficult to produce (Chapter 5).

The immunoaffinity purification of serum GAs was carried out with purifed polyclonal antibodies. Future research should investigate the use of monoclonal

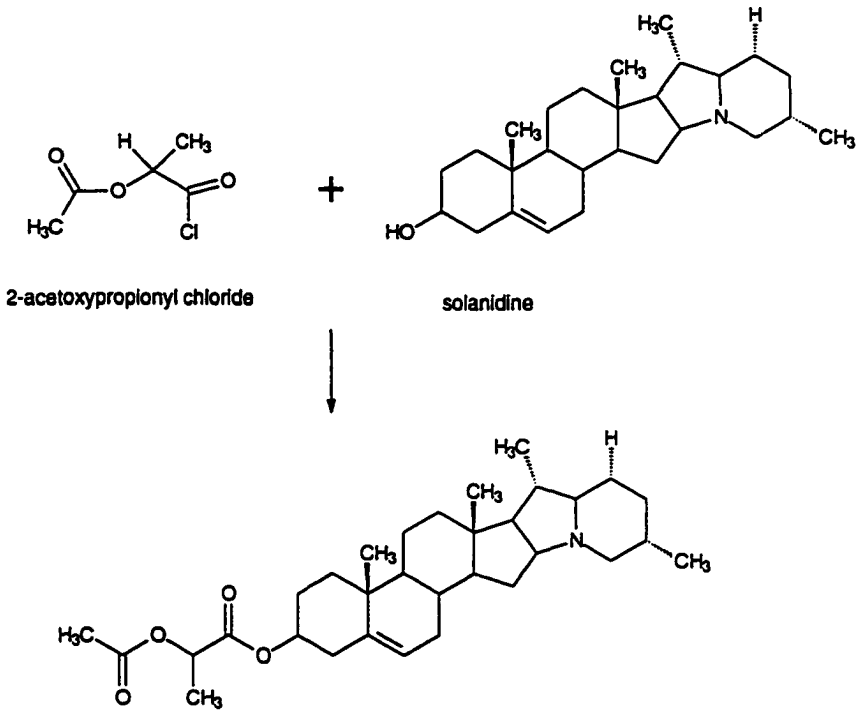


**Figure 7-1 Proposed internal standard for MALDI-TOF MS analysis of potato glycoalkaloids in serum.**

antibodies. Monoclonal antibodies have more uniform affinity for antigen than polyclonal antibodies, allowing gentler and more effective elution of the antigen from the beads (Harlowe and Lane, 1988). It is likely that only a portion of the GAs were eluted from the polyclonal antibody-coated agarose beads used in this study.

The ability to detect ng/mL concentrations of GA in biological tissues provides an opportunity to further investigate the fate of ingested GAs. Current knowledge is based on radioisotope data from animals that were dosed with tritiated GAs (Nishie et al., 1971; Claringbold et al., 1980). All that can be concluded from these studies is that the tritium was eventually excreted from the animals; it is not known if or how the tritiated GAs were metabolized. MALDI-TOF MS could be used to determine if intact GAs are

excreted in the feces and urine. Subsequent experiments might focus on GA concentrations in different tissues of the animals. Data from these experiments would almost certainly shed light on the mechanism of toxicity of potato GAs. GA analysis only represents one application for immunoaffinity sample purification/MALDI-TOF MS. The ability to detect part per million concentrations in biological samples opens up many opportunities for drug and pesticide residue analysis in food and agricultural commodities.

## REFERENCES

AOAC International, 1999. *Official Methods of Analysis of AOAC International*, 16th ed., 4th revision. AOAC International, Gaithersburg, MD.

Claringbold, W.D.B., Few, J.D., Brace, C.J. and Renwick, J.H. 1980. The disposition and metabolism of [$^3$H]-solasodine in man and Syrian hamster. J. Steroid Biochem. 13: 889-895.

Harlowe, E. and Lane, D. 1988. *Antibodies: a Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 511-552.

Harvey, M.H., Morris, B.A., McMillan, M. and Marks, V. 1985. Measurement of potato steroidal alkaloids in serum and saliva by radioimmunoassay. Human Toxicol. 4: 503-512.

Hellenäs, K.-E., Nyman, A., Slanina, P., Lööf, L. and Gabrielsson, J. 1992. Determination of potato glycoalkaloids and their aglycone in blood serum by high-performance liquid chromatography: application to pharmacokinetic studies in humans. J. Chromatogr. 573: 69-78.

Nelson, R.W., Krone, J.R., Bieber, A.L. and Williams, P. 1995. Mass spectrometric immunoassay. Anal. Chem. 67: 1153-1158.

Nishie, K., Gumbmann, M.R. and Keyl, A.C. 1971. Pharmacology of solanine. Toxicol. Appl. Pharmacol. 19: 81-92.

Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y. and Yoshida, T. 1988. Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. Rapid Commun. Mass Spectrom. 2: 151-153.