UNIVERSITY OF ALBERTA

Analysis of Potato Glycoalkaloids by ELISA and Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy.

BY

Darcy Cameron Abell ©

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

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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 ELISA and Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectroscopy submitted by Darcy Cameron Abell in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Chemistry.

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Abstract

While potatoes are one of the worlds most important food crops, they also contain toxic glycoalkaloids (GA). Although GA levels in commercial varieties are low, there is concern over the introduction of higher GA levels and different GA through breeding programs. Although there are a number of methods for GA analysis, most are not suitable for rapid screening of a large number of samples. ELISAs have been developed for the GA in commercial varieties, but are not available for less common GA such as tomatine.

Methods were developed to synthesize tomatine-protein conjugates for development of an ELISA for tomatine. The limited solubility of tomatine required modifications to an active-ester method for linking the glycoalkaloid to the protein. By using N-hydroxy sulfosuccinimide to form the active ester rather than N-hydroxysuccinamide, the solubility of the intermediate in aqueous solvents was increased allowing for a high number of tomatine groups to be added to BSA (7.4 groups/BSA molecule).

Both polyclonal antibodies (PAb) and monoclonal antibodies (MAb) were produced against tomatine-protein conjugates. While both antibodies displayed good recognition of tomatine-protein conjugates, the competition with tomatine was low and there was no recognition of tomatidine up to 100 μM levels. The PAb competed better then the MAb with tomatine and tomatine conjugates. In both cases there was greater recognition of atine when bound to the protein than free tomatine. The results of antibody testing indicate the antibody binding is to the carbohydrate portion of the molecule, including the linking arm and a portion of the carrier protein. The lack of recognition of the alkaloid portion (tomatidine) is likely due to the spiroaminoketal moiety present and the tautomerism between a ring form and open form.

Using the relatively new mass spectrometry method, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a rapid and simple method for the analysis of α-solanine and α-chaconine was developed. Initial studies into the quantitation of GA using MALDI-TOF MS demonstrated a method suitable for

routine GA analysis. Using tomatine as an internal standard, this method produced quantitative results similar to traditional HPLC analysis with a large decrease in assay time. This represents the first example of MALDI-TOF MS for the quantitation of a food constituent.

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List of Abbreviations

Ab . . . . . antibody

Abs . . . . . absorbance

Ag . . . . . antigen

Ag-Ab . . . . . antigen-antibody complex

BSA . . . . . bovine serum albumin

DCC . . . . . dicyclohexyl carbodiimide

DMAP . . . . . dimethylaminopyridine

DMF . . . . . dimethylformamide

DMSO . . . . dimethylsulfoxide

EDC . . . . . 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

ELISA . . . . . enzyme linked immunosorbant assay

FPIA . . . . . fluorescence polarization immunoassay

GA . . . . . glycoalkaloid

HAT . . . . . hypoxanthine/aminopterin/thymine media

HPLC . . . . . high pressure liquid chromatography

HSFM . . . . . hybridoma serum free media

HT . . . . . hypoxanthine/thymidine media

LPH . . . . . *Limulus polyphenus* haemocyanin

m/z . . . . . mass to charge ratio

MAb . . . . . monoclonal antibody

MALDI . . . . matrix assisted laser desorption/ionization

MS . . . . . mass spectrometry

PAb . . . . . polyclonal antibody

PBS . . . . . phosphate buffered saline

PBST . . . . . phosphate buffered saline with Tween 20

PFHM . . . . . protein free hybridoma media

TLC . . . . . thin layer chromatography

TOF . . . . . time-of-flight

1 Introduction

1.1 Glycoalkaloids

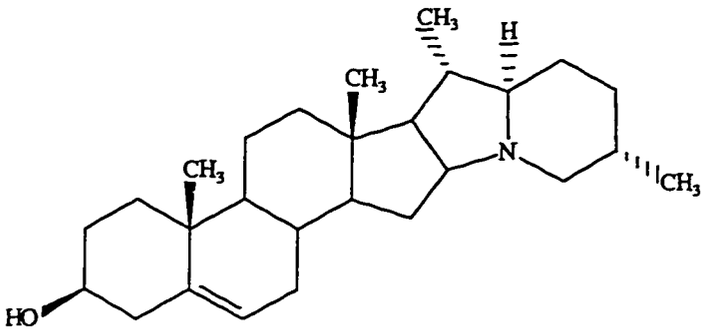
The potato tuber (*Solanum tuberosum*) is a very common and valuable food source due to its high yield per acre and high nutrient levels (Maga, 1980). Although the potato contains many important nutritional factors, the plant and tubers also contain toxic glycoalkaloids (GAs) which pose a threat to human health.

Glycoalkaloids are nitrogen containing steroidal glycosides and over 90 different steroidal alkaloids have been identified from *Solanum* species (Friedman and McDonald, 1997). The majority of GA occurring in potato species can be divided into two major classes, the solanidanes and spirasolanes, based on the alkaloid portion of the molecule (Figure 1.1 and 1.2). There are several common carbohydrate moieties found in the GA (Figure 1.3) and these may be bound to various alkaloids to form the GA (Table 1.1). α-Chaconine and α-solanine are the only GA found in commercial potato cultivars.

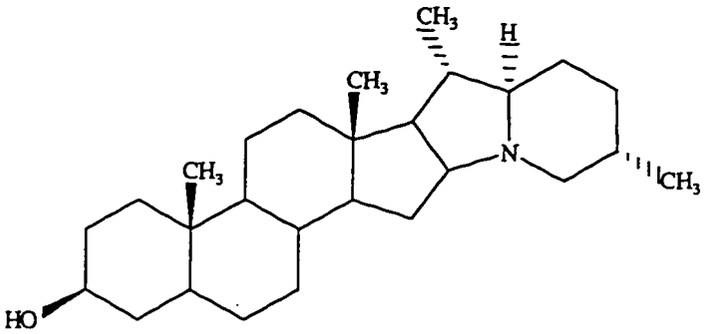
Table 1.1 Composition of Common Glycoalkaloids

Glycoalkaloid	**Alkaloid**	**Glycoside**
α-chaconine	solanidine	β-chacotriose
α-solanine	solanidine	β-solatriose
demissine	demissidine	β-lycotetraose
α-solamargine	solasodine	β-chacotriose
α-solasonine	solasodine	β-solatriose
α-tomatine	tomatidine	β-lycotetraose
α-solamarine	tomatidenol	β-solatriose

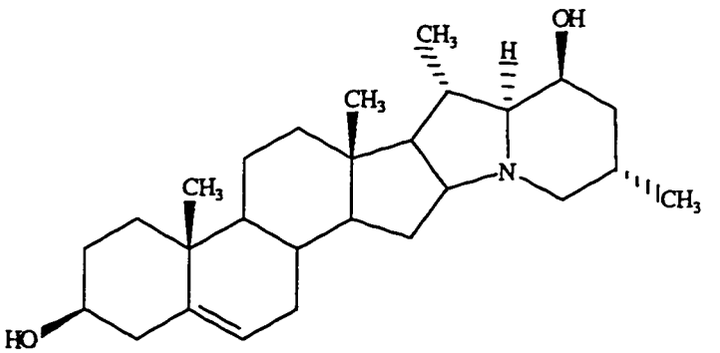
Glycoalkaloids are found in all tissues of the potato plant, but are concentrated in the actively growing tissues such as berries and shoots (Table 1.2). The concentration of GA in the tuber is dependent on many pre- and post-harvest factors, including light exposure, temperature stress, tuber damage, and genetic factors (Sharma and Salunke, 1989). The



solanidine

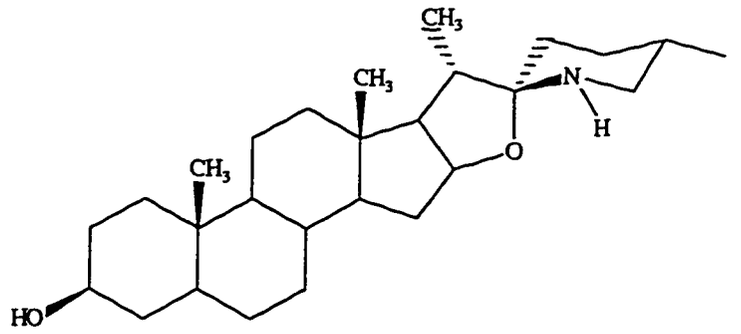


demissidine

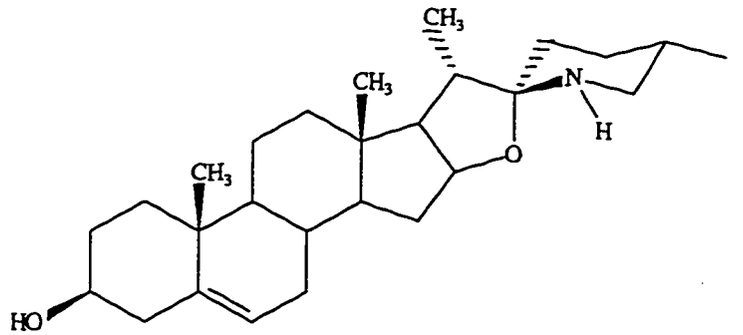


leptinidine

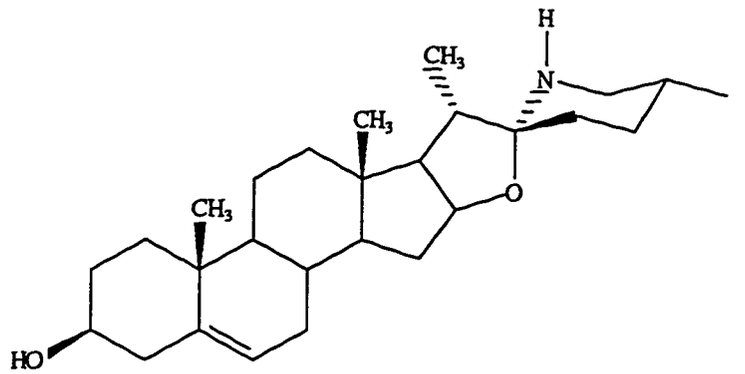
Figure 1.1 Solanidane Alkaloids



tomatidine

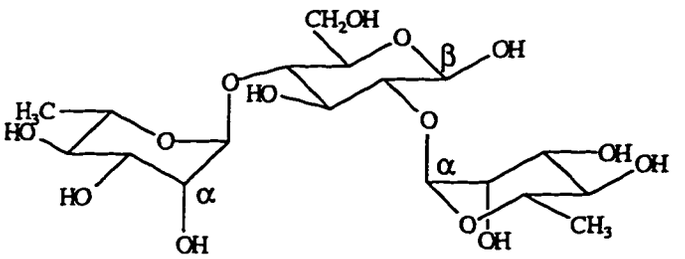


tomatidenol

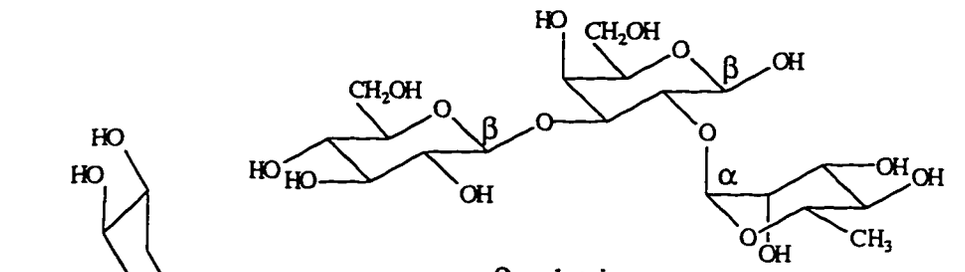


solasodine

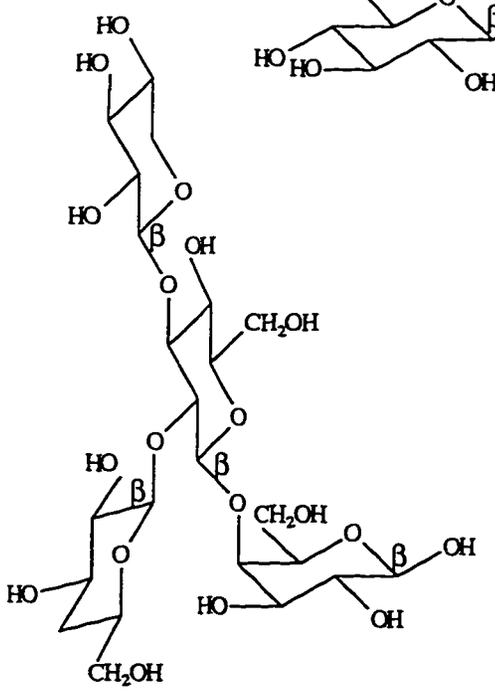
Figure 1.2 Solasodine Alkaloids



β-chacotriose



β-solatriose



β-lycotetraose

Figure 1.3 Common Glycosides

tuber levels will vary with growing conditions, but are known to be increased when the plant or tuber is subjected to stress. The increases in GA levels in response to stress may be related to their role as a defence mechanism for the plant. GA are known to confer resistance to fungi and insects and protect the potato plant from herbivores (Fewell and Roddick, 1993)

Table 1.2 Distribution of Glycoalkaloids in Potato Tissue.

Tissue	Total GA (mg/100 g) Commercial Cultivar	Total GA (mg/100 g) High GA Cultivars
Stems	3	32-45
Leaves	40-100	145
Flowers	300-500	----
Sprouts	200-400	275-1000
Tuber Periderm	30-60	----
Tuber Peel	15-30	850
Tuber Flesh	1-5	110

Adapted from Friedman and McDonald (1997)

GA toxicity is well documented, and a number of cases of poisoning to varying degrees, including death, have been reported and include both humans and animals (Morris and Lee, 1984; Harvey et al., 1985). GA toxicity is manifested through both gastrointestinal disturbances as well as neurological effects (Nishe, 1971). GA were at one time implicated as a teratogen, but studies have not shown teratogenic affects and the GA are not generally regarded as teratogenic (Slanina, 1990; Friedman et al., 1992; Crawford and Myhr, 1995; Swinyard and Chaube, 1973). Studies have actually found the solasodine glycosides to be an effective topical treatment for skin cancer (Cham et al., 1991).

The gastrointestinal disturbances from GA are caused by disruption of sterol containing membranes (Nishie et al., 1971). While the effect may vary between the different GA, synergistic effects have been found (Keukens et al., 1992). The extent of the toxicity is dependent on both the sugar moiety and the alkaloid portion and can differ greatly, even with

only slight changes in the carbohydrates (Keukens et al., 1992; Roddick et al., 1992).

Neurological effects such as shallow breathing, rapid pulse and coma are due to the inhibition of acetylcholinesterase by GA. As well as concerns regarding toxicity, there are new concerns with GA levels and the elimination of drugs from the body as many new drugs are eliminated from the body through the cholesterinase system (Sitar, 1996; Schwarz, 1995; Parnetti, L).

An upper safety level of 20 mg total GA/100 g potato tissue (Groen et al., 1993) has been recognized, which represents only a 4- to 5-fold safety factor between the average GA level and a potentially toxic dose; therefore, GA are considered by some to be the most serious toxic components of the human diet (Hall, 1992). There are several instances of commercial cultivars, Lenape (Zitnak and Johnston, 1970) and Magnum Bonum (Hellenäs et al., 1995) being restricted from the market due to high GA levels. As well, GA are not appreciably destroyed by cooking, baking or frying (Jadhav et al., 1981).

From a human health standpoint, it is desirable for breeders to eliminate GA in potatoes; however, this is not the case for several reasons. At low levels, GA are a component of potato flavour (Ross et al., 1978), and confer disease and pest resistance to the potato plant (Morgan et al., 1983; Fewell et al., 1993). GA production is also reliant on a number of different genes (Sandford and Sinden, 1972) which causes difficulties in elimination through breeding. Another reason for the lack of attention to GA levels is the difficulty and expense required for routine analysis of the thousands of crosses performed annually. As breeders continue to use wild varieties of potatoes in breeding programs, there is also a concern with the introduction of glycoalkaloids other than α-solanine and α-chaconine into the diet (Table 1.3).

Table 1.3 Glycoalkaloids found in various *Solanum* species.

Species	GA Present	Total Tuber GA (mg/100g)	Reference
S. jamesii	α-tomatine	128	Johns and Alonso (1990)
S. fendlori	α-solanine, α-chaconine	64	Johns and Alonso (1990)
S. canasense	dehydrocommersonine, demissine	62	Johns and Alonso (1990)
S. vernei	solasodine GA	109	van Gelder et al. (1988)
S. nigrum	α-solamargine, α-solasonine	67-648	Ridout et al. (1989)
S. acaule	α-tomatine	----	Schriber (1968)
S. tuberosum	α-solanine, α-chaconine	2-15	Slanina (1990)

A number of different techniques exist for the analysis of GA in potato material (Coxon, 1984; Friedman and McDonald, 1997; van Gelder, 1991). The simplest methods include gravimetric, colorimetric and titrimetric techniques, but all three methods lack specificity and sensitivity. Gravimetric methods can suffer from incomplete precipitation due to differences in GA solubilities. While several colorimetric methods exist using either dye binding (Coxon et al., 1979) or reaction with reagents such as antimony trichloride (Smittle, 1971) or paraformaldehyde (Wang et al., 1972) these are time consuming and rely on dangerous reagents. The results also vary as the reactivity and resulting coloured products varies among the GA. Methods based on the titration of the aglycone portion of GA after hydrolysis suffer from high losses during extraction and hydrolysis (Jadhav et al., 1981). Thin layer chromatography can be used for the qualitative analysis of GA and their aglycones and is applicable to most GA. Although TLC is not used for quantitation, it is suitable for screening large numbers of samples (Svendsen and Verpoorte, 1983).

The most common methods for the analysis of GA rely on chromatographic techniques

such as gas chromatography (GC) or high performance liquid chromatography (HPLC). Both are expensive and time-consuming.

Although GC is a sensitive technique, the low volatility of the GA complicates the analysis. GA require derivatization and high column temperatures which can reduce the life of the GC column (Morgan et al., 1985). GA can also be hydrolysed to their corresponding aglycones and analyzed using capillary GC methods (Lawson et al., 1992), but this does not allow for the quantitation of the individual GA present. Using this method α-solanine and α-chaconine would both be analysed as solanidine, the alkaloid.

Several HPLC methods exist for the analysis of GA (Saito et al., 1990; Bushway et al., 1988; Friedman and Dao, 1992). Due to the lack of a strong chromophore on the GA, UV detection must be in the 200-208 nm region. Due to the wide range of compounds which absorb in this region of the spectrum, extensive sample cleanup is required. As well, GA which lack a double bond for UV absorption, such as α-tomatine, are poorly detected. The use of pulsed amperometric detection of the sugar moiety of GA has recently been used to improve the detection of GA after separation with HPLC (Friedman et al., 1994).

Immunoassays are a newer and promising method of analysis, and a number of analyses for GA have been reported (Morgan et al., 1983; Barbour et al., 1991; Phlak and Sporns, 1992; 1994; Stanker et al., 1994). Immunoassays rely on the specificity of antibodies to eliminate the problems of extensive purification and extraction of samples. Immunoassays are also rapid and inexpensive to perform and are well suited to large sample throughput. The first enzyme immunoassay developed for GA correlated well with established methods (Morgan et al., 1983), although background absorbances were high. An ELISA developed by Phlak and Sporns (1994) overcame this problem by immunizing using a conjugate with higher hapten-protein ratio which resulted in higher antibody titers and decreased background. A fluorescence polarization immunoassay was developed by Thomson and Sporns (1995) to improve on the variability inherently associated with solid phase immunoassays.

1.2 Immunoassays

Immunoassays are based on the interaction between an antibody (Ab) and an antigen (Ag) which form a non-covalent complex. Due to the specificity of Ab toward a given antigen, and the strength of the Ag-Ab interaction, antibodies can be used to develop sensitive and specific assays.

Before proceeding with assay development an Ab directed towards the compound of interest is needed. Specific Ab can be developed by injecting an animal with the Ag in order to invoke an immune response and stimulate the production of antibodies. The compound of interest is usually injected along with an adjuvant, in order to increase the immune response. Common adjuvants consist of bacterial cell wall components and oil. The bacterial components are highly immunogenic and promote a strong immune response. The oil is used to create a water in oil emulsion to allow for slower dissolution and release of the Ag.

In order to invoke an immune response, the Ag must be foreign to the host animal and must be large enough (>10 000 Da) to invoke an immune response (Erlanger, 1980). Low molecular weight analytes are referred to as haptens, and are too small to produce an immune response. In order to allow for Ab production, haptens must first be coupled to a carrier protein. By raising Abs against a hapten-protein conjugate, Abs can be produced which will also recognize the free hapten (Coleman et al., 1989).

Common carrier proteins are bovine serum albumin (BSA), ovalbumin, keyhole limpet haemocyanin (KLH) and *Limulus polyphemus* haemocyanin (LPH). While there is no one protein carrier which is preferred, proteins with high solubility are usually preferred to simplify conjugation and handing. The number of haptens bound to the protein must be high enough to promote a good immune response. Research has suggested that one hapten for every fifty amino acid residues in the protein used will result in a good antibody response (Harlow and Lane, 1988). The majority of antibodies produced using protein conjugates will be toward the portion of the hapten which is the farthest away from the linking region (Tijssen, 1985). Because of this, the method of linking and the region where the hapten is

linked will have a large effect on the antibodies produced.

The choice of linking method used is determined primarily by the functional groups present on the hapten. Common reactive functional groups are carboxyl, hydroxyl and amino groups. When hydroxyl groups are present, they are derivatized to allow for coupling with the protein. The most common methods of linking rely on reaction of an activated hapten with the amino groups on lysine residues of the protein (Erlanger, 1980). The use of various carbodiimides as activating agents is common as both aqueous and organic reagents are available. A two step coupling procedure is preferred in which an activated intermediate is produced followed by coupling to the carrier protein. This can eliminate cross linking of the carrier protein. For haptens containing vicinal diols, sodium periodate can be used to produce dialdehydes, which can then be reacted with amine groups on the protein. The conjugate is then reduced with sodium borohydride to produce a stable conjugate.

The size of an Ab binding site corresponds to 3-7 glucose molecules (Nisonoff, 1982) and, therefore, can incorporate not only the hapten, but also portions of the protein and linking agent. Often haptens are not linked to proteins directly, but rather through a linking arm such as succinamide. The length of this linking arm can affect the specificity and the affinity of the antibodies against haptens (Szurdoki et al., 1995). Typical serum after immunization will contain antibodies directed not only against the hapten, but also against the linking arm, the carrier protein and combinations of all three.

The Ab in serum from an immunized animal are referred to as polyclonal antibodies (PAb) due to the large number of potential Ab specificities present. While PAb are simple and inexpensive to produce, the lack of a defined specificity and reproducible production is a disadvantage. Monoclonal antibodies (MAb), first developed by Köhler and Milstein (1975), overcome this disadvantage as they allow the production of antibodies with a defined specificity. The first step in producing PAb or MAb against haptens is to immunize a host animal with the hapten-protein conjugate. Typically, rabbits or goats are for PAb production, while mice are used for MAb production. If PAb are desired, the blood is collected, either

through bleeding or sacrifice. After allowing for clotting of the blood, the serum is collected and frozen. This serum often can be used for the required assays without any purification.

To produce MAb, rather than collecting the blood, the spleen of the animal is removed to recover the antibody producing B cells. These cells will only survive for a few days in tissue culture media, therefore, they are fused with an immortal myeloma tumour cell line. Myeloma cells lines are used which do not secrete Ab and which lack hypoxanthine-guanine phosphoribosyltransferase (HGPRT), an enzyme required for an alternative biosynthetic pathway for the production of purine bases. By culturing the hybridomas cells in a media containing hypoxanthine, aminopterin and thymine (HAT media), the hybridomas cells can be selected. Aminopterin blocks the *de novo* biosynthetic pathway for purine and pyrimidine synthesis and as a result, cells which lack HGPRT cannot use the alternative pathway and will not survive. After growth selection for hybridomas, the cells can be screened for production of the desired Ab. Once isolated, the desired cells are subjected to a series of cloning steps to ensure that only one species of cell, or clone, is present. Once the appropriate monoclonal cell line is selected, the MAb can be collected from culture media. As cell lines can be frozen in liquid nitrogen for later culturing, the amount of MAb that can be produced is unlimited.

While MAb are a well defined species, they are much more expensive to produce than PAb, and MAb will generally have a lower affinity than PAb. As PAb and MAb have advantages and disadvantages (Table 1.4), the best choice will depend on the intended use.

Table 1.4. Comparison Between Polyclonal and Monoclonal Antibodies.

Antibody Type	Polyclonal Antibodies	Monoclonal Antibodies
Determinant	Several	Single
Specificity	Variable between animals Partial cross-reactivity Seldom too specific	Standard Unexpected cross reactivity may occur May be too specific
Affinity	Variable with bleed	May be selected during cloning
Yield of antibody	Up to 1 mg/mL	Up to 100 μg/mL
Contaminating IgG	Up to 100 %	None
Minimum cost	Usually below $250	Greater than $25 000

Adapted from Campbell (1984).

Once a suitable Ab is available, an assay can be developed. While many assays can be developed using Ab, only enzyme-linked immunosorbent assays (ELISAs) will be discussed.

ELISAs are based on the binding of either Ab or Ag to a solid support surface, typically polystyrene microtitre plates, followed by the formation of the Ag-Ab complex using one of a variety of assay formats. The specificity of the Ab for the Ag allows for complex mixtures to be tested, and minimizes interference from other compounds. The amount of bound Ab is quantified using an enzyme label such as horseradish peroxidase or alkaline phosphatase. After allowing for antibody binding, a colourless substrate solution is added which is converted to a coloured product by the enzyme. The amount of colour produced is measured by spectrometry. There are several common assay formats, but the most common are the sandwich ELISAs and competitive ELISAs.

Sandwich ELISAs are commonly used with large antigens such as proteins (Figure 1.4). In order to develop a sandwich ELISA, two Ab's which each recognize a different epitope, or binding site, on the Ag are required. The microtitre plate is first coated with a primary antibody, followed by the addition of a test solution. The Ab on the plate will bind any Ag present in the solution. After removal of the test solution and washing of the microtitre plate,

a secondary Ab is added which also binds to the analyte. If the secondary Ab is labelled, the bound Ag can be quantified directly. A more common method is an indirect assay in which an unlabelled secondary Ab is used and a tertiary labelled Ab is used which is directed against the secondary Ab. This third Ab is usually anti-species specific and may be labelled with a variety of enzymes. The indirect method is usually preferred due to the commercial availability of labelled, species specific Ab and the amplification effect of the tertiary Ab due to multiple binding (Porstman et al., 1982).

While sandwich ELISAs are the most sensitive form of ELISAs, they are not suitable for low molecular weight compounds as there is insufficient area to allow for two Ab to bind. A more common ELISA setup is a competitive ELISA (Figure 1.5). While the sensitivity of a competitive ELISA is lower than the sandwich ELISA, it is suitable for both low and

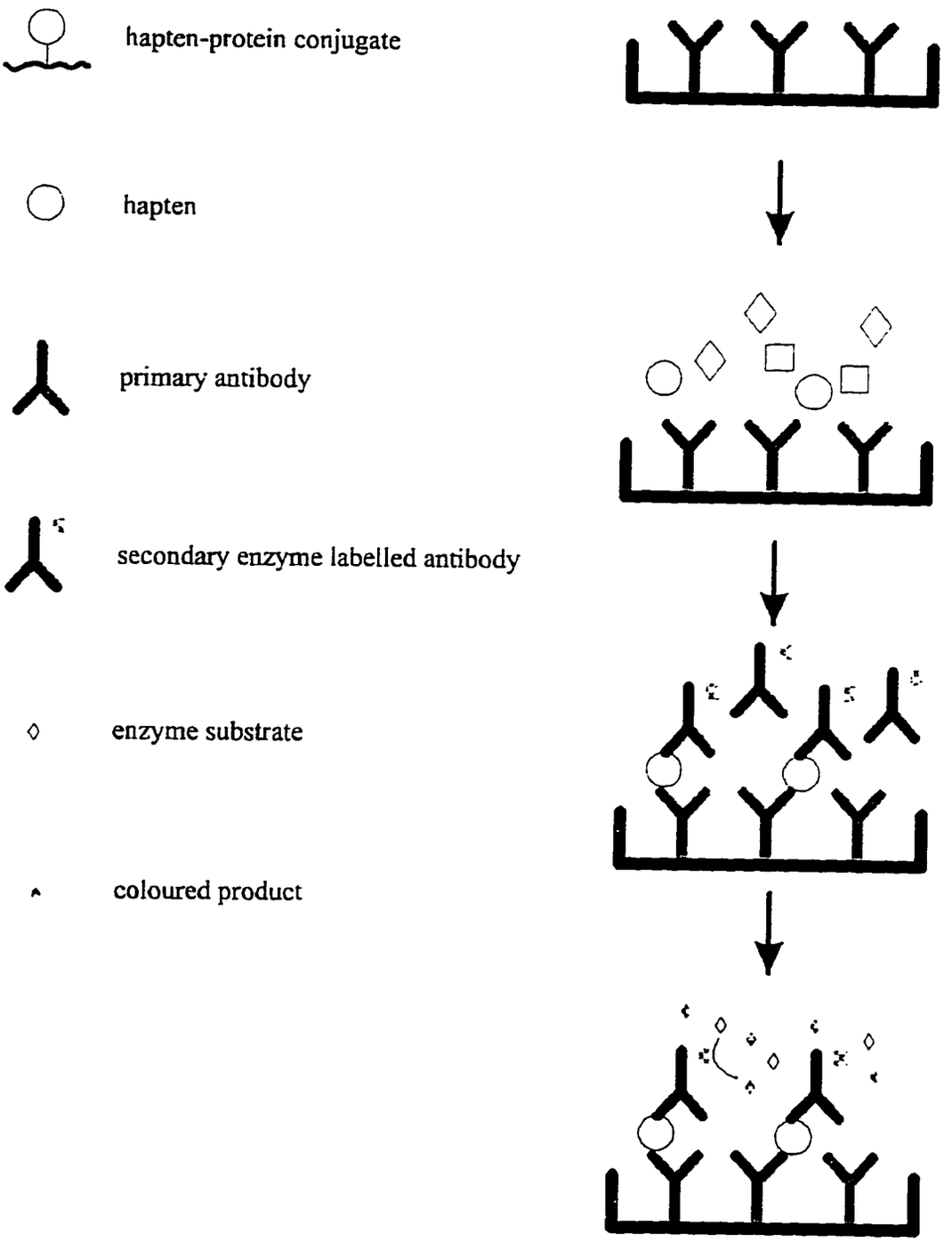


Figure 1.4 Sandwich ELISA Format

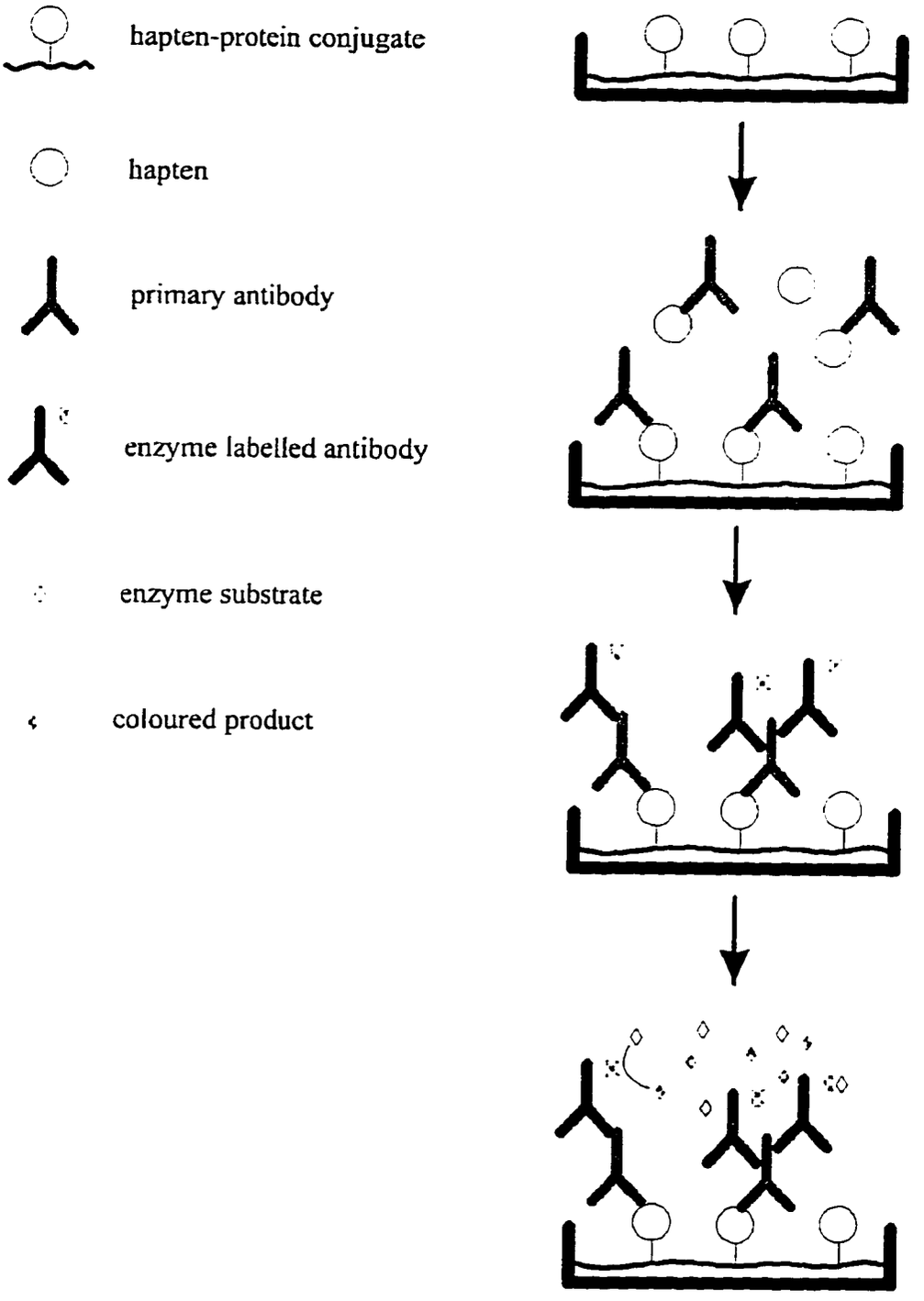


Figure 1.5 Indirect Competitive ELISA Format

high molecular weight analytes. The competitive ELISA only requires one Ab specific to the analyte. To perform a competitive ELISA the microtitre plate is first coated with a known amount of the analyte, or in the case of haptens, a hapten-protein conjugate. The next step of the assay is to add the test sample as well as the Ab specific to the compound of interest. The concentration of Ab used must be limiting, such that there is a competition between the bound and free antigen for the limited number of binding sites on the Ab. Upon removal of the solution from the microtitre plate, Ab which is bound to the free Ag in solution is lost, while some Ab will remain bound to the Ag on the plate. Similar to the sandwich ELISA, the Ab can be labelled for direct quantitation, or a secondary, labelled Ab can be used. The use of a secondary Ab is preferred due to the commercial availability and increased sensitivity.

The data generated through either a sandwich ELISA or competitive ELISA can be modelled using a sigmoidal curve fit. The sigmoidal curve is calculated using the equation:

$$y = \frac{(a-d)}{1+\left(\frac{x}{c}\right)^b} + d$$

where x = concentration of sample; y = absorbance; a = upper asymptote; b = slope of curve; c = inflection point of curve (I_{50}); d = lower asymptote

When using a sandwich ELISA the upper asymptote will be reached when sufficient Ag is present to bind all the available Ab sites. Conversely, in a competitive ELISA, the maximum absorbance is obtained when there is no free analyte present. The lower asymptote represents the background absorbance in the assay. The inflection point of the curve is commonly referred to as the I_{50} value as it is the concentration required to reduce the maximum absorbance by 50 % in a competitive ELISA. The I_{50} value can be used to compare assay sensitivity toward different analytes, although this does not indicate the limit of quantitation or detection limit.

1.3 MALDI-TOF MS

Mass spectrometry (MS) is widely used as a tool for chemical analysis. All MS techniques rely on the ionization of a sample molecule in the gas phase followed by separation based on its mass-to-charge ratio (*m/z*). Early MS techniques used electron impact ionization to generate gas phase ions. Electron impact ionization uses a beam of electrons to ionize the molecule of interest which also leads to fragmentation of the molecule. While fragmentation patterns are useful in determining structural features, they make identification of the parent molecule difficult. Early methods were also limited to low molecular weight compounds.

The development of other ionization methods such as chemical ionization, fast atom bombardment, and plasma desorption, increased the useful mass range of MS into the low kilodaltons while decreasing the amount of fragmentation of the parent molecule. In 1988, Karas and Hillenkamp described a new ionization technique, matrix-assisted laser desorption/ionization (MALDI). MALDI allows for the ionization of large molecules of over one million Daltons (Schreiemer and Li, 1996) and results in very little or no fragmentation of the molecule of interest (Harvey, 1994). MALDI is usually coupled with a time-of-flight (TOF) detection system as TOF has no upper *m/z* limit and is suitable for a pulsed ionization source such as MALDI.

The main areas of research involving MALDI-TOF MS are related to mass determinations of large biomolecules such as proteins, DNA, oligonucleotides and protein digests (Gusev et al., 1995; Siuzdak, 1994; Fenselau, 1995). MALDI-TOF MS can also be used for analysing proteins directly from membranes such as nitrocellulose (Vesling and Fenselau, 1995). Patterson et al. (1995) described the applicability of MALDI-TOF MS to C-terminal sequencing of proteins and peptides.

While much of the research on MALDI-TOF MS applications is focused on high molecular weight compounds, there are a number of applications for small molecules as well. A number of compounds, including hormones, amino acids, antibiotics and opiates have been successfully tested (Lidgard and Duncan, 1995). A method to quantify cyclosporin A and its

metabolites has been reported by Muddiman et al. (1995). The coupling of antibody capture techniques with MALDI-TOF MS has been described with a variety of compounds (Brockman and Orlando, 1995).

MALDI uses low molecular weight UV-absorbing compounds to act as a matrix vehicle for desorption and ionization of molecules of interest. The sample of interest is co-crystallized with 500-50 000 fold excess of a UV absorbent matrix, placed in a vacuum and pulsed with a UV laser to desorb and ionize the matrix. The absorption of the energy by the matrix and its rapid heating results in a sublimation plume, carrying the analyte into the gas phase as well. Heat which is generated in the process is dissipated quickly in sublimation, preventing cleavage of the analyte. This soft ionization method provides intact molecules in the gas phase, principally as cations (Harvey, 1994). The ionized molecules are usually protonated or charged through an interaction with a sodium or potassium ion. The MALDI-generated ions are then accelerated by passing though an electrical potential before passing though a drift tube and onto a detector. As the initial velocities of the ions are independent of mass, their velocity after acceleration is dependent only on their mass. Therefore the higher the molecular weight of the ion, the greater the time required to reach the detector.

MALDI-TOF MS analysis can be performed with picomole quantities of sample with reported sensitivity in the femtomole range (Juhasz and Costello, 1992; Gusev et al., 1995). However, by employing ion traps and multiple re-measurement of an ion packet, attomole quantities of samples have been analysed (Solouki et al., 1995). Although the amount of sample required is low, the effective concentration is high (μM) due to the small sample volumes. MALDI-TOF MS is tolerant of high salt concentrations and can be used with crude sample mixtures and sample clean up is often not required. If high levels of salts are present, adducts of the analyte with sodium and potassium will often be present in the spectrum. Additionally, since the solvent is removed before analysis, MALDI-TOF MS is compatible with most solvent systems. MALDI-TOF MS is applicable to most compounds, although some, are difficult to analyse. Compounds such as polylysine can be analysed, but due to the degree of protonation, the spectra produced are not useful. As well, samples which readily

absorb UV light, such as rhodamine, may fragment resulting in minimal parent ion peaks.

The mass resolution of MALDI-TOF MS is much lower than traditional MS methods. Calculated on the basis of full width at half maximum (whereby the resolution is the mass of the peak divided by the width of the peak at 50 % of its height) simple linear instruments have a mass resolution of up to 500. The addition of ion reflector systems to the linear TOF instruments allow for mass resolution of up to 6000, but suffer from a loss of particles due to fragmentation. Using complex instruments which use Fourier transform ion cyclotron resonance it is possible to achieve resolution as high as 100 000, but this limits the *m/z* range of the instrument (Pasa-Tolic et al., 1995).

A newer method of increasing resolution is to incorporate delayed extraction in the instrument (Vestal et al., 1995). Delayed extraction can be used to minimize the effect of the initial ion velocity distribution on the ion flight time. As a result, ions are extracted after a brief delay of several hundred nanoseconds rather than immediately after formation. Ions which have an lower initial velocity are closer to the repelling potential and therefore are accelerated to a slightly higher velocity than those with a higher initial velocity. By properly adjusting the potential and the delay, ions can be more accurately focused at the detector. Using delayed extraction, mass resolution of 4000 - 6600 can be achieved in a linear instrument, with greater resolution achieved with a longer flight tube. When combined with an ion reflector system, mass resolution of up to 8600 can be achieved (Vestal et al., 1995).

While the use of MALDI-TOF MS with high molecular weight compounds is of great value, it also has potential for quantification of both high and low molecular weight compounds (Gusev et al., 1995). The signal strength observed with MALDI is subject to a large amount of variation, therefore, an internal standard must be used for quantitation. While internal standards are commonly used to allow for accurate mass determinations (Wu et al., 1995), quantitation does not require a high degree of accuracy in calculated mass. A good internal standard for quantitation should be chemically similar to the analyte but must differ in mass. While isotopically enriched molecules are the ideal choice, they are expensive and

difficult to obtain (Jespersen et al., 1995). Furthermore, due to the low mass resolution of MALDI-TOF MS instruments, isotopically enriched standards are only suitable for low mass analytes. Chemical modification of a purified analyte is a relatively simple method of preparing internal standards. This modification must change the mass sufficiently to allow for resolution of the internal standard and analyte and avoid any overlap of adduct or other peaks. Due to a varying instrument response for different compounds, standard curves need to be generated for each analyte to be measured, even when using a single standard to measure several different compounds.

The choice of matrix will have a large effect on the spectrum observed when analysing compounds by MALDI-TOF MS. While a number of compounds can be used for matrixes in MALDI-TOF MS, certain matrixes have been found to produce better results within a class of compounds (Table 1.5). Further modifications such as the addition of 50 % formic acid can also be used to improve sample spectra.

Table 1.5. Matrixes for MALDI-TOF MS.

Common Name	Chemical Name	Uses	Comments	Reference
thymine	2,4-dihydroxy-5-methylpyrimidine	proteins, peptides	broad proteins, selective	Beavis and Chait (1989)
gentisic acid	2,5-dihydroxybenzoic acid	proteins, peptides	good with mixtures	Strupat et al. (1991)
nicotinic acid	3-pyridinecarboxylic acid	proteins, peptides, RNA	severe adduct formation	Beavis and Chait (1989)
sinapic acid	2,5-dihydroxybenzoic acid	peptides, proteins, oligosaccharides	non-selective, good for mixtures	Beavis and Chait (1989)
THA	2,4,6-trihydroxyaceto phenone	glycoalkaloids, acidic oligosacchardies, glycopeptides	strong signal, low detection limit	Abell and Sporns (1996), Papac et al. (1996)

Given the speed associated with MALDI-TOF MS analysis, coupled with its ability to produce intact molecules, this study was undertaken to investigate the potential of applying MALDI-TOF MS to GA analysis.

2 Materials and Methods

2.1 Reagents

2.1.1 Chemicals

Tetramethylbenzidine (TMB), α-tomatine, tomatidine, α-solanine, solanidine, α-chaconine, solasodine, bovine serum albumin (BSA), *Limulus polyphenus* haemocyanin (LPH), hypoxanthine and thymidine were obtained from Sigma-Aldrich Canada Ltd., Mississauga, Ontario. RPMI media, protein free hybridoma media (PFHM), hybridoma serum free media (HSFM), calf serum, fetal calf serum, penicillin/streptomycin, Freund's complete adjuvant, Freund's incomplete adjuvant and aminopterin were obtained from Gibco-BRL (Life Technologies), Burlington, Ontario. Goat anti-rabbit IgG-horseradish peroxidase conjugate, goat anti-mouse IgG-horseradish peroxidase conjugate and urea peroxide were obtained from Calbiochem, San Diego, California, U.S.A. AG1-X2 acetate anion exchange resin was obtained from BioRad, Mississauga, Ontario. Lissamine (lissamine rhodamine B ethanediamine) was obtained from Molecular Probes, Eugene, Oregon, USA. RIBI adjuvant system for mice was obtained from RIBI Immunochemical Research, Inc., Hamilton, Montana, U.S.A. 50 % PEG in HEPES buffer was obtained from Boehringer-Manheim, Laval, Quebec. 2,4,6-Trihydroxyacetophenone was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U.S.A.

All other reagents were reagent grade or better. All water used was purified using a Milli-Q system (Millipore Corp., Bedford, MA).

2.1.2 Standard Solutions

2.1.2.1 Phosphate Buffered Saline 10X (PBS 10X)

Sodium chloride (90.0 g), sodium phosphate (11.08 g) and potassium dihydrogen phosphate (3.0 g) were dissolved in ~ 950 L water and the pH adjusted to 7.3 with 6 N sodium hydroxide. The total volume was made up to 1 L with water. Aliquots were frozen at -20 °C until needed.

2.1.2.2 Phosphate Buffered Saline

PBS 10X (100 mL) was diluted to ~ 950 mL with water and the pH adjusted to 7.3. The volume was then made up to 1 L.

2.1.2.3 Phosphate Buffered Saline with Tween (PBST)

Tween 20 (0.5 g) and 100 mL of PBS 10X was diluted to ~ 950 mL with water, the pH adjusted to 7.3, and the volume made up to 1L.

2.1.2.4 Citrate Buffer

A 0.1 M citric acid solution was prepared by dissolving 21.01 g citric acid monohydrate in ~950 mL water and making the volume up to 1 L. A 0.1 M sodium citrate solution was prepared by dissolving 29.41 g sodium citrate in ~950 mL water and making the volume up to 1 L. Appropriate volumes of citric acid monohydrate (0.1 M) and sodium citrate (0.1 M) solution were mixed to give a final pH of 4.0.

2.1.2.5 3,3',5,5'-Tetramethylbenzidine (TMB) Solution

TMB-dihydrochloride (0.01 g) was dissolved in 1.0 mL of dimethyl sulphoxide. An aliquot of this solution was added to an appropriate volume of 0.1 M citrate buffer, pH 4.0, containing 1 mg/mL urea peroxide to give 0.1 mg/mL TMB. The solution was prepared immediately before use.

2.1.2.6 Thin Layer Chromatography (TLC) Systems

The organic layer of chloroform: methanol: 1% ammonia (2:2:1) was used for TLC of the glycoalkaloids and their derivatives. Ethyl acetate: methanol: 1% ammonia (80:20:1) was used for TLC of the alkaloids and their derivatives. Glycoalkaloids were visualized by spraying with a saturated solution of antimony trichloride in chloroform and heating. For visualization of all spots a 5% solution of sulfuric acid in ethanol was sprayed on the plate and the sample charred on a hot plate.

2.1.2.7 Tissue Culture Media

Complete RPMI media consisted of RPMI (450 mL), calf serum (20 mL), 200 μM glutamine (6 mL), 100 μM pyruvate (6 mL), 50 mM oxaloacetate (6 mL), and penicillin/streptomycin (6 mL). Serum free RPMI was prepared as above with the omission of the calf serum. HAT media consisted of RPMI (75 mL), fetal calf serum (20 mL), 200 μM glutamine (1 mL), 100 μM pyruvate (1 mL), 50 mM oxaloacetate (1 mL), penicillin/streptomycin (1 mL) and 100 μM hypoxanthine/ 16 μM thymidine/ 0.4 μM aminopterin (1 mL). HT media consisted of RPMI (75 mL), fetal calf serum (20 mL), 200 uM glutamine (1 mL), 100 μM pyruvate (1 mL), 50 mM oxaloacetate (1 mL), penicillin/streptomycin (1 mL) and 100 μM hypoxanthine/ 16 μM thymidine (1 mL).

2.2 Food Samples

Potato samples A-D were donated by Dr N. R. Knowles, Department of Agricultural, Food and Nutritional Science, University of Alberta. Samples A and B were *Solanum tuberosum* cv. Russet Burbank stored for 8 and 20 months, respectively. Samples C and D were *S. tuberosum* cv. Shepody and Yukon Gold, respectively, both stored for 8 months. All samples were stored in the dark at 4 °C and 95% relative humidity. Potato samples E and F were commercially obtained *S. tuberosum* tubers. Sample E was peeled while sample F was unpeeled. Two to three whole tubers were cut into 1 cm^3 pieces, freeze-dried, and ground sufficiently to pass through a 20-mesh screen. Samples were stored at 4 °C until needed. Samples E and F were previously prepared and analysed by Phlak and Sporns (1994).

2.3 Equipment

Potato samples were freeze dried using a Virtis Pilot Scale Freeze Drier (Virtis Co. Inc., Gardiner, NY, U.S.A). Freeze dried samples were ground using a Braun Model KSM2 coffee grinder (Braun Canada Ltd., Mississauga, ON). Standard curve fitting was done with Microsoft Excel 5.0 Solver (Microsoft, Redmond, WA, U.S.A). MALDI-TOF MS was performed on a Kompact MALDI I (Kratos Analytical, Ramsey, NJ, U.S.A) and a HP G2030A MALDI (Hewlett Packard, Mississauga, ON). Enzyme immunoassays were performed using Immulon 2 polystyrene microtiter plates (Dynatech Laboratories, Inc.,

Chantilly, VA, U.S.A) and read on a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA, U.S.A). Plates were sealed with Linbro Adhesive Plate sealers (ICN Biomedicals, Costa Mesa, CA, U.S.A). Cell cultures were grown in 24 and 96 well Linbro tissue culture plates (Flow Laboratories, Inc., Mclean, VA, U.S.A) as well as 25 cm^2 and 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY, U.S.A). Cell counts were performed using an Neubauer Improved counting chamber (Fisher Scientific, Edmonton, AB). Solvent removal was performed with a Büchi Rotavapor RE 121 with Büchi 461 water bath (Büchi, Switzerland) or Savant SC100 Speedvac (Savant, Farmingdale, NY, U.S.A). An Accumet 925 pH/ion meter (Fisher Scientific, Edmonton, AB) was used for pH measurements. Dialysis was performed using Spectra/Por Membrane 2, MWCO 12,000 - 14,000 (Spectrum Medical Industries, Inc., Los Angeles, CA, U.S.A). Freeze drying of conjugates and intermediates was performed using a Virtis Benchtop Freeze Dryer (Virtis Co. Inc., Gardiner, NY, U.S.A). Absorbance measurements were obtained using 10 mm path length quartz cuvettes (Hellma, Concord, ON) in a HP 8452A diode array spectrometer (Hewlett Packard, Mississauga, ON). Thin layer chromatography was performed on Whatman AL SIL G/UV plates for analytical work and Whatman K 5F Silica Gel plates for preparative work (Millipore, Milford, MA, U.S.A). Sep-Pak C18 cartidges (Millipore, Milford, MA, U.S.A) were used for sample clean up in HPLC analysis.

2.4 Animals

All animals were obtained through and maintained by Animal Services, Biological Sciences Department, University of Alberta. Rabbits were female Flemish Giant/Lop Ear crosses, 4-6 weeks old. Mice were 3-4 week old female Balb/C mice.

2.5 Methodology

2.5.1 Synthesis of Tomatine and Tomatidine Intermediate Conjugates for ELISA

2.5.1.1 Succinylation of Tomatidine

Dimethylaminopyridine (DMAP) (4.16 mg, 34 µmol) and succinic anhydride (480 mg, 4.78 mmol) were dissolved in 10 mL dry pyridine. Tomatidine (100.38 mg, 222 µmol) was added and allowed to react for 8 h at 57 °C. The mixture was acidified to pH 4.5 with 12 N

HCl and extracted with methylene chloride (3 x 10 mL). The combined extractions were dried over sodium sulphate and the solvent removed under vacuum.

2.5.1.2 Succinylation of Tomatidine in Toluenesulfonic acid

Tomatidine (20.3 mg, 44 µmol) was dissolved in 12 mL dimethylsulfoxide (DMSO) and the solution heated to 55 °C. Toluenesulfonic acid (47.3 mg, 249 µmol) was added followed by succinic anhydride (28.2 mg, 282 µmol). After 3 h an additional 27.2 mg (272 µmol) of succinic anhydride was added and the reaction monitored by TLC. No reaction was observed after 24 hours.

2.5.1.3 Synthesis of Multiply Succinylated Tomatine

Tomatine (51.1 mg, 45 µmol) was dissolved in 3 mL dry pyridine at room temperature. Succinic anhydride (9.89 mg, 99 µmol) was added and the solution stirred for 24 h with an attached drying tube. Water (20 mL) was added and the reaction mixture stirred for 15 minutes. The mixture was then evaporated to dryness and stored under desiccation.

Confirmation of the succinylation was obtained by IR spectroscopy. To confirm that the succinylation of the nitrogen had not occurred, a sample was subjected to a mixed acid hydrolysis (van Gelder, 1984) and the reaction mixture tested by TLC for the presence of the alkaloid tomatidine. Succinylated tomatine was added to 2 mL of 2 N HCl and 4 mL of carbon tetrachloride. The solution was then refluxed at 85 °C for 4 hours and monitored by TLC.

2.5.1.4 Synthesis and Purification of Mono and Di-Succinylated Tomatine

Tomatine (201.4 mg, 0.195 mmol) was dissolved in 5 ml dry pyridine and the solution cooled to 4°C. Succinic anhydride (78.3 mg, 0.782 mmol) was added and the solution stirred at 4°C with an attached drying tube. The reaction was monitored by TLC. After 72 hours, 10 ml of water was added and the solution stirred for 10 minutes at room temperature. The mixture was evaporated to near dryness, 10 ml of water was added, and then evaporated to dryness.

The succinylated tomatine products were separated by anion exchange chromatography. An AG1-X2 acetate resin anion exchange column 37 cm x 1.5 cm was prepared. Ammonium acetate (1 M, 150 mL) followed by 100 mL water was passed through the column at a flow rate of 1 ml/min, giving a linear flow rate of 0.57 cm/min. The reaction products from the succinylation were dissolved in 2 mL of water and added to the column. The flask was rinsed with 2 mL of water which was also added to the column. After the sample application, the column was eluted with 100 mL of water followed by 1.6 L of a linear ammonium acetate gradient (0.0 M to 0.5 M) and finally 300 mL of 1.0 M ammonium acetate. Ten minute fractions were collected during elution of the compounds. Fractions were spotted (3 μL) on an aluminum backed TLC plate and visualized by 5% sulfuric acid in ethanol and charring. Positive fractions were combined and the samples freeze dried to remove solvent and ammonium acetate.

Composition of fractions and relative percentages were identified using fast atom bombardment (FAB) mass spectroscopy or MALDI-TOF MS.

2.5.2 Synthesis of Protein Conjugates

2.5.2.1 Synthesis of LPH Conjugate

Unpurified tomatine hemisuccinate (39.4 mg, 32 μmol), dicyclohexyl carbodiimide (DCC) (20.8 mg, 101 μmol) and N-hydroxysuccinimide (17.8 mg, 155 μmol) were dissolved in 1 mL dimethylformamide (DMF) and stirred at 4 °C for 15.5 h. The DMF solution was filtered through glass wool into 3 mL PBS containing 40.0 mg (0.611 μmol) LPH and the solution stirred for 8.5 h at 4 °C. The reaction mixture was transferred to dialysis tubing and dialysed against 8 M urea (1 L, 24 h), 50 mM ammonium bicarbonate (4 L, 24 h) and 25 mM ammonium bicarbonate (4 L, 24 h). The contents of the dialysis tubing were then lyophilized.

2.5.2.2 Synthesis of BSA Conjugate with Active Ester Method

Unpurified tomatine hemisuccinate (6.58 mg, 5.3 μmol), DCC (1.63 mg, 7.9 μmol) and N-hydroxysuccinimide (0.86 mg, 7.5 μmol) were dissolved in 1 mL DMF and stirred at 4 °C for 24 h. The DMF solution was filtered through glass wool into 3 mL PBS containing 49.83

mg (0.76 μmol) BSA and the solution stirred for 24 h at 4 °C. The reaction mixture was transferred to dialysis tubing and dialysed against 8 M urea (1 L, 24 h), 50 mM ammonium bicarbonate (4 L, 24 h) and 25 mM ammonium bicarbonate (4 L, 24 h). The contents of the dialysis tubing were then lyophilized.

2.5.2.3 Synthesis of BSA Conjugate with Water-Soluble Carbodiimide

Tomatine hemisuccinate (75 % disuccinate, 25 % trisuccinate, 8.81 mg, 7.3 μmol) was dissolved in 3 mL water, 38.7 mg (202 μmol) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added and the solution stirred for 1 h. Half of the solution was then transferred to a second flask and BSA (23.0 mg, 0.35 μmol) in 1.5 mL of water was added slowly. After stirring for 2.5 h, an additional 15.3 mg (80 μmol) of EDC was added and the solution stirred for 0.5 h. Sodium acetate (1 M, 660 μL) was added to stop the reaction and the solution stirred for 15 min. The reaction mixture was then transferred to dialysis tubing and dialysed against 50 mM ammonium bicarbonate (1 L) for 15.5 h. This was followed by two rounds of dialysis against 25 mM ammonium bicarbonate (1 L) for 3 h each. The conjugate was then lyophilized. After lyophilization, 21.6 mg of conjugate was recovered.

2.5.2.4 Synthesis of BSA Conjugate using N-hydroxy sulfosuccinimide

Tomatine hemisuccinate (75 % disuccinate, 25 % trisuccinate, 36.4 mg, 27.8 μmol) was dissolved in 8 mL DMF and the solution cooled to 4 °C. N-hydroxy sulfosuccinimide (74.9 mg, 345 μmol) and DCC (148.0 mg, 717 μmol) was added while stirring and the solution stirred for 20 h. The solution was evaporated to reduce the volume to approximately 0.5 mL and then 3 mL DMF added. BSA (203.4 mg, 3.11 μmol) was dissolved in 2 mL PBS and 1 mL added to the flask along with 1 mL methanol, followed by 3 mL of 50 % methanol in PBS, followed by an additional 2 mL methanol. The mixture was allowed to react for 19 h at room temperature. The reaction mixture was evaporated to dryness and 15 mL of 8 M urea added. The resulting solution was filtered through Whatman #1 filter paper, and the filter rinsed with 5 mL 8 M urea followed by 2 x 5 mL water. The filtrate was dialysed against 2 L of 50 mM ammonium bicarbonate (6 h), 4 L 25 mM ammonium bicarbonate (50 h), and 4 L water (24 h). The conjugated protein was then lyophilized. After lyophilization

18.3 mg of conjugate was recovered and designated as TOM-BSA-H. Formation of the conjugate was confirmed by analysis using MALDI-TOF MS.

2.5.3 Synthesis of MALDI-TOF Internal Standards

2.5.3.1 Periodate Oxidation and Borohydride Reduction of Chaconine

Chaconine (10.0 mg) was dissolved in 2 mL of methanol and 1 mL of sodium periodate solution (500 mM) added. After stirring for 30 min at room temperature, the reaction mixture was placed in an ice bath and 0.5 mL methanol followed by 0.5 mL of sodium borohydride solution (1.0 g/mL) was added. During the reaction, the pH was maintained between 8.5 and 10.5 by adding 1 N HCl as necessary. After stirring for 17 hours, 2 mL of methanol was added followed by 2 mL of acetone to destroy any unreacted borohydride.

The reaction mixture was centrifuged at 2000 rpm for 15 min and the supernatant collected. The solvent was removed by rotary evaporator and 1 mL methanol added to the precipitate. Water (10 mL) was added to the precipitate remaining in the centrifuge tubes. Both the water and the methanol solutions were tested by MALDI-TOF MS.

2.5.3.2 Butylation of Chaconine

Chaconine (10.01 mg, 1.7 μmol) was dissolved in 1 mL of dry pyridine and 10 μL (61.3 μmol) of butyric anhydride added while stirring. After 4 h, an additional 10 μL (61.3 μmol) of butyric anhydride was added. After stirring for 14 h the reaction was stopped by the addition of 0.5 mL water. A sample of the reaction mixture was purified by TLC using a 4.5 cm wide TLC strip. After separation, 0.5 cm was removed from each side of the TLC and developed. Using the developed sides as a guide, four bands were scraped from the TLC plate. The scrapings were transferred to a 0.5 mL microcentrifuge tube and 400 μL methanol added. A 100 μL sample of this solution was added to 100 μL water for testing by MALDI-TOF MS.

2.5.4 Rhodamine-Tomatine Conjugates

2.5.4.1 Synthesis of Conjugates

Two conjugates were synthesized, one using a primarily monosuccinylated tomatine, and a second using primarily disuccinylated tomatine. Both conjugates were synthesized using the same method. Tomatine hemisuccinate (6.57 mg - monosuccinyl or 6.09 mg - disuccinyl) was dissolved in 1 mL DMF. N-hydroxysuccinimide (3.41 mg - monosuccinyl or 5.43 mg - dissucinyl) and DCC (5.79 mg - monosuccinyl or 10.96 mg - disuccinyl) were added and the solution stirred for 4 h at room temperature. Lissamine (6.58 mg - monosuccinyl or 11.77 mg - disuccinyl) was dissolved in 250 µL DMF and added dropwise while stirring. After stirring for 18.5 h, 20 µL of triethylamine was added and the solution stirred an additional 3 h. The solvent was removed by under reduced pressure, 400 µL methanol added, solvent removed, and the remaining material dissolved in 400 µL of methanol. The species in each conjugate were determined by MALDI-TOF MS using THA as a matrix with a 1:1000 dilution of the methanol solution from the reaction. The monorhodamine conjugate was designated as FL1 and the dirhodamine conjugate as FL2.

2.5.4.2 Purification of Conjugates

Both FL1 and FL2 were purified by preparative TLC using a methanol:ethyl acetate:1% ammonia (25:75:1) solvent system using Whatman K5F Silica Gel 20 x 20 cm plates, 250 µm thickness. After loading the plates (3 plates/conjugate) they were developed twice, dried and the bands scraped from the plate; 9 bands for FL1 and 10 bands for FL2. The conjugates were eluted from the silica gel using 5 x 2 mL methanol. The methanol was removed by using a speedvac concentrator and the individual fractions purified a second time, using methanol:ethyl acetate:1% ammonia (30:70:2) solvent system and a single development. The most dominant band from each fraction was collected. The products were then extracted from the silica gel using a narrow sintered glass column with methanol (4-6 mL) and the solvent removed using a speedvac concentrator. The samples were transferred with 3 x 200 mL methanol to a 1.5 mL microcentrifuge tube and the solvent removed by speedvac. The final yield of conjugates FL1 (monorhodamine) and FL2 (dirhodamine) was 2 mg of each.

2.5.5 Enzyme Immunoassays

2.5.5.1 Standard Indirect Competitive Enzyme Immunoassay Protocol

An appropriate dilution (typically between 1 and 5 ppm) of the protein-hapten conjugate (200 µL/well) was added to the microtitre plate, the plate sealed with an acetate sealer, and the plate incubated overnight at 4 °C. The next morning the coating conjugate was shaken from the plate and the plate blotted on paper towels. A 1% solution of BSA in PBS (200 µL/well) was added as a blocking solution and the plate sealed as before. The plate was incubated for 1 h at room temperature. All other incubation steps were also at room temperature. The plate was shaken and blotted, then washed using a standard washing procedure consisting of 3 washes with PBST (200 µL/well). The competitor compound was serially diluted with either 0.05% BSA in PBST or methanol depending on solubility. Serum diluted in 0.05% BSA in PBST (100 µL/well) was added followed by 0.05% BSA in PBST (50 µL/well) and methanol (50 µL/well) with the competitor compound in the appropriate solution. The plate was incubated for 2 h in a sealed plastic bag with a wet paper towel to maintain humidity. After washing, goat anti-rabbit IgG, horseradish peroxidase conjugated, diluted 1:3000 in PBST was added (200 µL/well). When using monoclonal antibodies, goat anti-mouse IgG, horseradish peroxidase conjugated, diluted 1:6000 was used. The plate was incubated 2 h and then washed. The standard TMB solution in citrate buffer was added (200 uL/well) and the colour allowed to develop for 15 min at which time 50 µL of 2 M sulfuric acid was added. The difference between the absorbance at 450 nm and the absorbance at 650 nm was read using a microtitre plate reader.

2.5.5.2 Checkerboard Enzyme Immunoassay

A standard checkerboard assay was used for determining the levels of coating conjugate and the appropriate sera dilution. The procedure was similar to the competitive ELISA with the following modifications. For the coating of the plate, the 1:10 serial dilutions of the coating conjugate in PBS, beginning with 100 ppm were used. In the competition step, 100 µL of serial dilutions of the antibody sera and 100 µL of 0.05 % BSA in PBST were added to the wells in place of a competitor compound. The rest of the procedure was as described in section 2.5.5.1.

2.5.5.3 Data Analysis

Standard curves for ELISA were generated using Microsoft Excel Solver using the following sigmoidal curve fit:

$$y = \frac{(a-d)}{(1+(c/x)^b)} + d$$

where a = lower asymptote

b = slope

c = concentration decreasing the maximum absorbance by 50 %

d = upper asymptote

x = concentration

y = calculated absorbance

The best fit line was calculated by minimizing the sum of squares between the log of the experimental absorbance and the log of the absorbance as calculated from the four curve parameters. The correlation coefficients represent the degree of correlation between the experimental and calculated y values of the curve.

2.5.6 Polyclonal Antibody Production

2.5.6.1 Injection Protocol

Flemish Giant x Lop Ear cross rabbits at 4 weeks old were used for production of polyclonal antibodies using the tomatine-BSA conjugate BSA-TOM-H described under section 2.5.2.4. A 1 mg/mL solution of the conjugate in sterile PBS was mixed 1:1 with Freund's complete adjuvant and 1.5 mL/rabbit injected (4 x 0.25 mL subcutaneous, 0.5 mL intramuscular). Booster injections were performed on days 22, 56 and 70 using Freund's incomplete adjuvant with the same concentration of antigen.

Test bleeds were performed on days 29 and 77 and were tested using a checkerboard ELISA to determine the titer. On day 82 the rabbit was sacrificed by cardiac puncture and the blood collected. The blood was allowed to clot for 1 h at room temperature and then centrifuged at 2000 rpm for 15 minutes. The sera was removed, aliquoted into 1.5 mL vials

and stored at -20 °C.

2.5.6.2 Testing of Titre

For the checkerboard ELISA the plate was coated with 50, 10, 2 and 0.4 ppm of BSA-TOM-H and the sera was tested in 1:10 serial dilutions from 10^2 - 10^7.

2.5.7 Monoclonal Antibody Production

2.5.7.1 Immunization of Mice

Mice were immunized using the RIBI adjuvant system. Conjugate LPH-TOM (see section 2.5.2.1) in sterile PBS (0.5 mg/mL) was combined with the RIBI reagent and 0.4 mL/mouse injected intraperitoneal. Injections were repeated on days 44, 80 and 122. A test bleed was taken after the third injection (tail, 200 uL). The blood was allowed to clot for 45 min at room temperatures in a serum separator tube, and then centrifuged at 14,000 rpm for 2 min and the serum collected. The titer of the sera was then tested using a checkerboard ELISA.

Plates were coated using BSA-TOM-L (1 ppm)(see section 2.5.2.2). Sera was diluted in 1:100 with 0.05% BSA in PBST and 1:10 serial dilutions to 10^7 tested. Rows G and H of the microtiter plate were used as blanks and no coating conjugate was added. Sera diluted 1:10,000 was added to the uncoated wells.

2.5.7.2 Myeloma Growth

Myeloma cells (strain NS-1) were grown in complete RPMI media at 37 °C and 7% CO_2 in 75 cm^2 culture flasks. Cells were subcultured every 2-5 days to maintain a cell concentration of 10^5 to 10^6 cells/mL. Viable cell counts were performed with a 1:1 mixture of cell media and 0.25% (w/v) trypan blue in PBS using a Neubauer counting chamber.

The viability of the cells was tested two days before the fusion and subcultured 1:30 to allow for sufficient cells for the fusion. On the day of the fusion the cells were centrifuged at 1250 rpm for 10 min. The supernatant was pooled, filter sterilized, and saved as

conditioned media for use in cell cloning. The pellets were combined and suspended in 50 mL serum-free RPMI, then centrifuged as above. This was repeated, then the cells suspended in 10 mL serum-free RPMI and a viable cell count done. The cells were then placed in the incubator for a short period of time, until needed.

2.5.7.3 Harvesting of Spleen Cells

Three days after the final boost, one mouse was sacrificed by CO_2 asphyxiation and its spleen removed under sterile conditions. The spleen was washed in a petri dish containing 10 mL serum-free RPMI and then transferred to a second petri dish containing 10 mL serum-free RPMI. The spleen was macerated between two ground glass slides to release the cells and the cells in media transferred to a 50 mL centrifuge tube. After centrifugation at 1200 rpm for 5 min the cells were resuspended in 4 mL 0.85% ammonium chloride and incubated for 3 min. Serum-free RPMI (6 mL) was added and the cells centrifuged as above. The pellet was washed twice with 10 mL serum-free RPMI and then suspended in 10 mL serum-free RPMI and counted.

2.5.7.4 Fusion

The splenocytes (1.5×10^8 cells) and the myeloma cells (2.0×10^7 cells) were combined (8:1 ratio), centrifuged at 1800 rpm for 5 min, and the supernatant removed completely. One mL of 50% PEG in HEPES buffer was added with stirring over 1 min, followed by gentle stirring for 1 min. Serum-free RPMI (1 mL) was added over 1 min, followed by another 4 mL over 3 min and 7 mL over 1.5 min. The cells were centrifuged at 1200 rpm for 5 min and then resuspended in 100 mL HAT. The cells were plated out in 6 tissue culture microtitre plates (160 µL/well)

2.5.7.5 Growth and Screening of Hybridomas

Three days after the fusion an additional 100 µL/well of HAT medium was added and the number of hybridomas cell clusters counted. On day 6, 50 µL of supernatant was removed from the wells for testing and 50 µL of fresh HAT medium added back. The supernatant was added to microtitre plates which had been coated with BSA-TOM-L (1

ppm), then blocked and washed as described earlier. Methanol (100 μL) and 0.05% BSA in PBST (50 μL) was added along with the supernatant. The standard procedure was then followed, with the exception that the colour development was stopped after 10 min rather than 15 min.

On day 7, 48 of the wells with the highest absorbances from the screening were tested for competition with tomatine in an ELISA. Tomatine was tested at 20, 0.2, 0.002 and 0 μM concentration. The coating was as above, and the competitive ELISA followed the standard procedure with the following change in the competition step. For the competition, 20 uL of supernatant, 100 μL of the tomatine solution in methanol, and 80 μL of 0.05 % BSA in PBST was used. The supernatant which was removed was replaced with 80 μL of fresh HAT medium.

On day 10, 100 μL of the media from the top 24 competitors was transferred to a 24 well tissue culture plate and 400 μL HT medium added. The cloning of these cells is described in the next section. For the remaining wells, 100 μL media was removed and 100 μL HT medium added. On day 12 this was repeated with 150 μL HT media. On day 14 the cells from all the 96 well plates were poured into 50 mL centrifuged, concentrated and combined. The cells were then frozen for later use or selection if needed. The supernatant was filter sterilized using a 0.22 μm filter and saved as conditioned media for use in cloning.

2.5.7.6 Growth in 24 Well Plate

On day 12, 300 μL/well was removed and 300 μL/well HT medium added. On day 13, 240 μL was removed for ELISA testing and 300 μL HT medium added. The supernatant was tested for competition with tomatine (100 μM, 10 μM, 0.1 μM), solasodine (100 μM, 10 μM) and solanidine (100 μM). The standard competitive ELISA was used with BSA-TOM-L (1 ppm) as a coating conjugate and for the competition step, 30 μL supernatant, 50 μL 0.05% BSA in PBST, and 100 μL competitor solution (Table 2.1).

Table 2.1. Competitve ELISA with Hybridoma Supernatant.

Hybridoma / Competitor Compound	A1	B1	C2	C3	A4	B4	C5	D6
Tomatine, 100 μM	0.35	0.76	0.77	0.45	0.60	0.65	0.81	0.89
Tomatine, 10 μM	0.31	0.91	0.83	0.58	0.68	0.87	0.89	1.00
Tomatine, 0.1 μM	0.34	1.13	0.98	0.88	0.86	0.89	0.92	1.07
Methanol	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Solasodine, 100 μM	0.32	0.79	0.68	0.78	0.98	0.96	0.92	1.00
Solasodine, 10 μM	0.33	0.89	1.05	0.65	1.11	1.10	0.90	0.94
Solanine, 100 μM	0.33	0.80	1.02	0.93	1.05	0.96	1.02	0.96

All plates were coated with 1 ppm BSA-TOM-L and competition was tested using 100 μL of the test compound, 30 μL of supernatent and 50 μL 0.05 % BSA in PBST. Results are given as A/A_0 where A_0 is the maximum absorbance (methanol). Values are the results of a single determination.

2.5.7.7 Cloning

For cloning, 8 wells of hybridomas from the 24 well plate which showed positive results in competitive ELISA were selected. Each cell line was cloned using the following procedure of limiting dilution in soft agar.

Conditioned media (150 mL) from previous cell production was fortified with fetal calf serum (30 mL) and 200 mM glutamine (1.5 mL). A series of six 1:2 serial dilutions of the cell line in fortified conditioned media were performed. A soft agar solution was prepared by adding 2.5 mL of melted agar (2.4% agar in 0.15 M NaCl) to 10 mL of fortified conditioned media. This mixture was dispensed in 2 mL aliquots into 6 sterile tubes along with 100 μL of the serial dilutions of the cells. After mixing 0.5 mL of each dilution in agar was dispensed into a 24 well plate in duplicate. The plate was then placed in the incubator at 37 °C and 7% CO_2.

After 1 week of growth, individual clumps of cells were visible in the wells. Using an inverse phase microscope in a laminar flow hood, 8 - 11 clones of each cell line were selected

and transferred to a 96 well plate by mouth pipet. A sterile 25 µL borosilicate glass capillary tube connected to ~ 2 ft of latex tubing with two 0.2 µm filter inline and mouth pipetting was used to select clumps of clones from those wells which had the least amount of growth. The cells were deposited into 100 µL of fortified conditioned media in a 96 well plate.

After 2 days of growth, 100 µL of complete RPMI was added to all the wells, and the four clones of each cell line which had the greatest growth were transferred to a 24 well plate. After 2 additional days of growth, 240 µL of media was removed for testing by competitive ELISA and replaced with 200 µL of complete RPMI media. Cell lines were tested for competition against tomatine, solasodine and solanidine using the standard competitive assay procedure (Table 2.2).

Table 2.2. Competitive ELISA with Hybridomas After Single Cloning.

Clone / Competitor Compound	A4-1	C3-1	A4-3	D6-2	C3-3	D6-3
Tomatine, 100 µM	0.49	0.54	0.29	0.41	0.49	0.37
Tomatine, 10 µM	0.79	0.79	0.62	0.76	0.73	0.66
Tomatine, 1 µM	0.90	0.83	0.77	0.64	0.79	----*
Methanol	1.00	1.00	1.00	1.00	1.00	1.00
Solasodine, 100 µM	0.85	0.86	0.86	0.96	0.85	0.84
Solasodine, 1 µM	0.94	1.04	0.89	0.97	1.01	----*
Solanine, 100 µM	0.71	0.88	0.53	0.72	0.96	0.51

* no experimental value

All plates were coated with 1 ppm BSA-TOM-L and competition was tested using 50 µL of the test compound, 30 µL supernatent and 120 µL 0.05 % BSA in PBST. Results are given as A/A_0 where A_0 is the maximum absorbance (methanol). Values are the results of a single determination.

Cell lines with the best competition (6 lines) were cloned a second time using the cloning procedure described earlier.

2.5.7.8 Final Screening and Transfer to Flasks

After 4 days of growth in a 96 well plate, 4 clones from each cell line were transferred to a 24 well plate with 300 µL of complete RPMI media. After an additional 3 days of growth, 90 µL was removed for testing, and 200 µL removed for subculturing into a second 24 well plate containing 700 µL complete RPMI. Complete RPMI (200 µL) was added back to all the wells.

After testing for competition against tomatine using the competitive ELISA procedure, 6 cell lines with the greatest amount of competition and growth were selected for freezing in liquid nitrogen (Table 2.3). The cell lines were subcultured into 25 cm^2 flasks by taking 0.5 mL and adding this to 10 mL complete RPMI. After 4 days of growth the cells lines were subcultured 1:10 into two 75 cm^2 flask for a total of 60 mL of media per cell line. The cell lines were frozen as described in section 2.4.4.2. After counting the cells and centrifuging each clone, the supernatant was saved and frozen at -20°C for testing.

Table 2.3. Competitive ELISA with Hybridomas after Second Cloning.

Clone / Competitor Compound	A4-1-1	C3-1-1	C3-1-3	C3-1-5	A4-3-1	A4-3-8
Tomatine, 1000 µM	0.40	0.32	0.29	0.35	0.36	0.40
Tomatine, 10 µM	0.75	0.85	0.83	0.85	0.93	0.72
Methanol	1.00	1.00	1.00	1.00	1.00	1.00

All plates were coated with 1 ppm BSA-TOM-L and competition was tested using 50 µL of the test compound, 30 µL supernatent and 120 µL 0.05 % BSA in PBST. Results are given as A/A_0 where A_0 is the maximum absorbance (methanol). Values are the results of a single determination.

2.5.7.9 Freezing and Thawing of Cell Lines

Before freezing the cells were centrifuged at 1200 rpm for 5 min and resuspended in 9% DMSO in fetal calf serum at 4 °C. The cells were suspended in a sufficient volume to give approximately 1 x 10^7 cells/mL. The cells were transferred to cryovials (0.5 mL/vial) and placed in a styrofoam box at -70 °C to allow a cooling rate of approximately 1 °C/min. The next day the vials were transferred to a liquid nitrogen storage tank.

Cells were thawed quickly after being removed from liquid nitrogen, transferred to 10 mL complete RPMI, and centrifuged at 1200 rpm for 5 min. The supernatant was removed and the pellet suspended in 10 mL complete RPMI and transferred to a tissue culture flask.

2.5.7.10 Growth Rates and Production

Cell line A4-1-1 was used for the growth rate and production studies. A vial of the cells was removed from liquid nitrogen and thawed as described in 2.5.7.9. The cells were subcultured 1:2 on days 4 and 8 with complete RPMI. On day 13, the cell lines were subcultured 1:10 into 4 different growth media, complete RPMI, RPMI with 5% fetal calf serum (5% RPMI), protein free hybridoma media (PFHM), and hybridoma serum free media (HSFM). Two flasks were used for each media. The number of viable cells was counted at day 0, 2 and 5. After the final count, the cells were centrifuged and the supernatant frozen at -20 °C for testing by ELISA. The pellet from the PFHM was resuspended in 100 mL PHFM in a 75 cm^2 flask.

The antibody production from each flask was tested by ELISA. The competitive ELISA procedure was used, replacing the competitor solution with methanol. Serial dilutions of each supernatant from 1/10 to $1/10^5$ were tested using this procedure.

2.5.7.11 Monoclonal Antibody Purification

Cell line A4-1-1 was grown in both PFHM and complete RPMI until cell viability began to decrease. The antibodies were purified from both media using the following procedure. The media was poured into 50 mL centrifuge tubes and centrifuged at 300 x g for 10 minutes. The supernatant was poured off and kept at 4 °C overnight. The next morning the supernatant was centrifuged at 3000 x g for 30 minutes. The supernatant was collected, sufficient ammonium sulphate added to give a 50 % saturated solution, and the solution stored overnight at 4 °C. After centrifugation at 3000 x g for 30 min, the supernatant was raised to 60 % saturation with ammonium sulphate. After 2 hours, the solution was again

centrifuged at 3000 x g for 30 min and the supernatant collected. For the PFHM media, and additional step of 80 % saturated ammonium sulphate was performed.

The pellets from each of the precipitations were dissolved in PBS (4 tubes/step, 2 mL PBS/tube). The purified antibodies in PBS were combined to give 8 mL of PBS solution. This solution was dialysed in 12-14,000 MWCO dialysis tubing against PBS for 19 h, the PBS changed, and the solutions dialysed an additional 24 h. The contents of each dialysis tube was diluted to 25 mL with PBS and the protein content determined by measuring the absorbance at 280 nm.

2.5.8 Analysis of Tomatine Intermediates and Protein Conjugates by MALDI-TOF MS

The GA, alkaloids and their reaction products were analysed using both the Kompact MALDI I and the HP G2030A MALDI. The matrix used was a saturated solution of 2,4,6-trihydroxyacetophenone in 50:50 methanol:water. Samples in 50:50 methanol:water were analysed by spotting 0.5 - 1.0 μL of sample and 1.0 μL of the matrix solution and drying. For the Kratos MALDI the laser energy was set to 80, while the HP G2030A was set with a laser energy of 2-3 μJ.

Proteins and protein conjugates were analysed by dissolving the sample in water at a concentration of approximately 10 μM. Samples were tested using the HP G2030A MALDI. The matrix solution used was a saturated solution of sinapinic acid. The matrix solution (1 μL) was added on the probe and dried, followed by 1 μL of the sample. For proteins or conjugates which were difficult to analyse, an additional 0.5 μL of matrix solution along with 0.5 μL of 50 % formic acid was added. The laser was set to an energy output of approximately 2.8 μJ for the analysis.

2.5.9 Quantitation of Glycoalkaloids by MALDI-TOF MS

2.5.9.1 Equilibrium Extraction

A 100 μM solution of tomatine in water:methanol (50:50 v/v) was used for all extractions. Freeze dried potato (400 mg) was shaken vigorously with 10 mL of extraction solution for 1 minute in a 20 mL vial. Samples containing very high levels of GAs were treated similarly, except only 200 mg of tissue was used. The sample was then placed on a Junior Orbit Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) for 1 hr at 200 rpm. Approximately 5 mL of the solution was poured into a 14 x 100 mm test tube and centrifuged at 1500 rpm for 5 min. Supernatant (1 mL) was transferred to a microcentrifuge tube and stored at -20 °C until analysed.

2.5.9.2 MALDI-TOF MS

MALDI testing was performed using a Kompact MALDI I using a 337 nm laser with a maximum output of 6 mW. The equipment was operated using a Sun SPARCstation with Kompact 4.0.0 software with SunOS (Release 5.30). Samples were tested using a 20 sample stainless steel probe. Sample (0.5 μL) was placed on the probe and allowed to air dry. A saturated solution (1 μL) of THA in methanol:water (50:50 v/v) was added on top of the sample and allowed to air dry. The samples were scanned with a power setting of 80, positive high detection, and averaged over 100 shots. Peak heights relative to the internal standard tomatine were compared to a standard curve prepared with spiked potato tissue samples in 10 mL of extraction solvent up to a concentration of 55 μg/ml of α-solanine or α-chaconine.

2.5.10 Quantitation of Glycoalkaloids by HPLC

2.5.10.1 Extraction of GAs for HPLC

A modified method of Saito et al. (1990) was used for extraction. A 1 g sample of freeze dried potato was shaken vigorously with 1 mL of water for 1 min; this was followed by the addition of 20 mL of methanol and shaking for 2 min. The mixture was vacuum filtered through Whatman No. 1 filter paper. The vial and filter paper were washed with 2 x 10 mL methanol. The filtrate was diluted to 50 mL with methanol. A 5 mL aliquot was then mixed with 8 mL of water and added to a Sep-Pak cartridge which had been conditioned with 10

mL methanol and 10 mL water. The cartridge was washed with 5 mL 40% methanol followed by elution of the glycoalkaloids with 15 mL methanol. The sample was concentrated and taken up in 1 mL methanol.

2.5.10.2 HPLC

The extracted sample was injected through a 20 µL loop onto a 300 x 3.9 mm µBondapak NH_2 (Bio-Rad Laboratories Ltd., Mississauga, ON) column at 25 °C. The mobile phase was acetonitrile:20 mM potassium dihydrogen phosphate (75:25 v/v) delivered at a flow rate of 1.0 mL/min using a Beckman Model 110A/332 pump (Beckman Instruments Inc., Fullerton, CA). A Bio-Rad UV monitor, Model 1305 (Bio-Rad Laboratories Ltd.) set at 208 nm was used for detection. Output was monitored using a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA). Peak heights were compared against a linear standard curve using similarly prepared standards at concentrations ranging from 0 to 100 mM. All samples were extracted in triplicate and each extraction analysed in triplicate.

3 Results and Discussion

3.1 Immunoassay Conjugates

3.1.1 Synthesis of Succinylated α-Tomatine

α-Tomatine and tomatidine do not contain reactive groups suitable for a direct link to a protein, therefore it was necessary to activate either the glycoalkaloid or alkaloid before conjugation. Previous work with the glycoalkaloids used a hemisuccinate intermediate to link the alkaloid solanidine to proteins (Phlak and Sporns, 1992) as shown in Figure 3.1. Although this is a simple and high yield procedure, the proper intermediate could not be made with tomatidine due to the reactivity of the ring nitrogen.

The tomatidine molecule differs from the solanidine alkaloid in the structure of the terminal ring and the nature of the ring nitrogen. In solanidine, the ring nitrogen is tertiary and unreactive, while tomatidine contains a secondary ring nitrogen in a spiroaminoketal structure. This spiroaminoketal structure allows for tautomerism with an open ring form (Schreiber, 1968; Quyen et al., 1991), greatly increasing the reactivity of the nitrogen. When α-tomatine and succinic anhydride are allowed to react under the conditions specified by Phlak and Sporns (1992) a disuccinylated compound is produced resulting from succinylation of both the hydroxyl and amino group (Fig 3.2). Acetic anhydride is known to preferentially react with the hydroxy group of the spirosolane at low temperatures (Bite and Tuzson, 1958; Kuhn, 1952), but the succinic anhydride was not sufficiently reactive under the same conditions. Further modifications of the reaction conditions, including omission of a catalyst (dimethylaminopyridine) and reaction under acidic conditions were attempted, but were not successful. Attempts to block the nitrogen using an acid-labile *t*-butyl carbonyl group were not successful, probably due to the steric hindrance of the secondary nitrogen.

Another common method of linking carbohydrate containing compounds to proteins is to first oxidize the vicinal diols on the sugars using sodium periodate, then incubate the resulting compound with the protein. The conjugate is then reduced using sodium borohydride to form a stable conjugate. While this method is successful with the solanidane

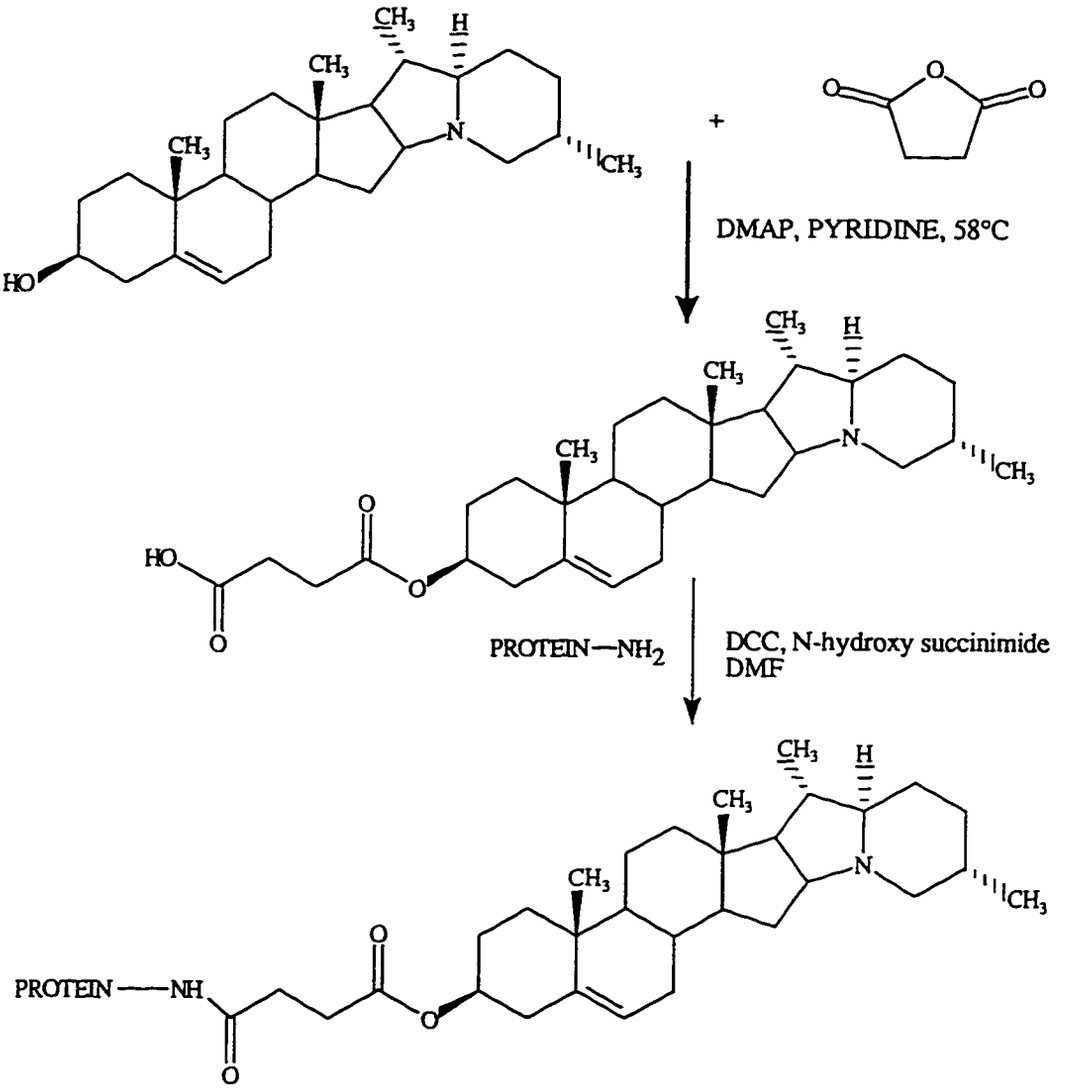


Figure 3.1 Succinylation of α-Solanine

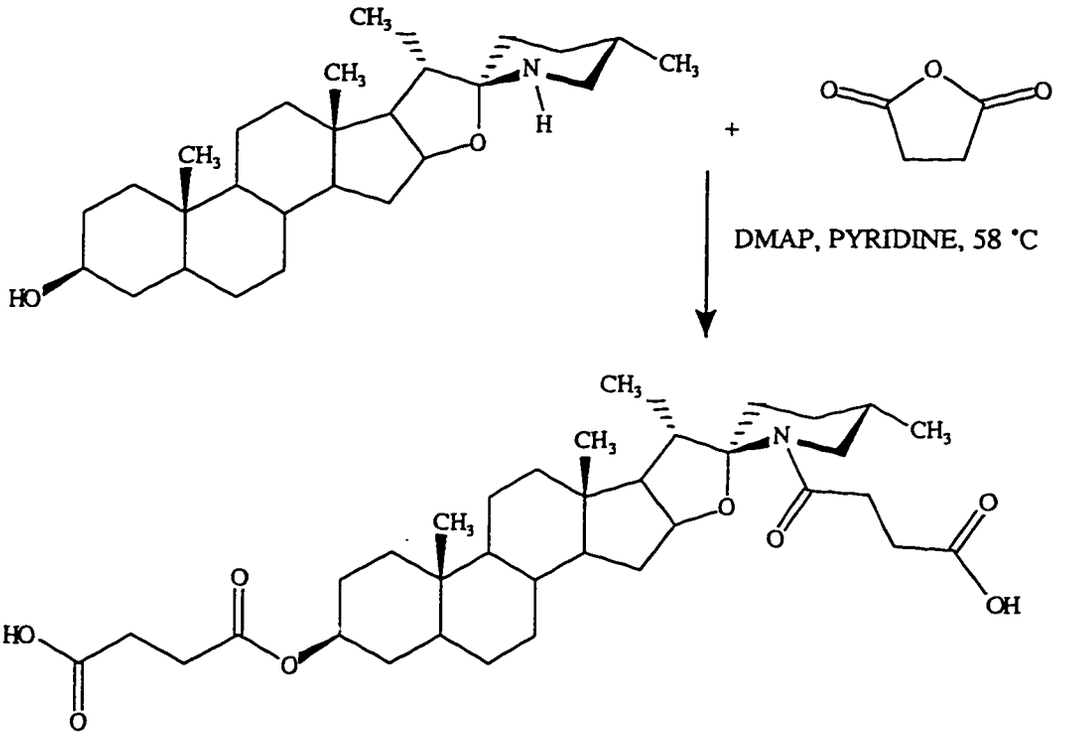


Figure 3.2 Succinylation of Tomatidine

glyoalkaloids (Phlak and Sporns, 1992) it cannot be used with the spirasolane glycoalkaloids such as α-tomatine. The open chain form of the alkaloid is reactive towards sodium borohydride and is reduced (Fig 3.3). Barbour et al. (1991) reported an ELISA for α-tomatine using conjugates prepared in this method, the titre of the serum produced were very low and the assay did not work when used with extracts from tomato foliage.

Since the succinylation of the alkaloid was not successful, the glycoalkaloid was used for the synthesis. As the glycoalkaloid contains three primary hydroxy groups, it was hoped that the greater reactivity of primary hydroxy groups would allow for a selective succinylation while maintaining an unreacted ring nitrogen. Work with α-tomatine under varying reaction conditions had shown that succinic anhydride did not react with the ring nitrogen without the presence of dimethylaminopyridine (DMAP) and temperatures above 40 °C. By reacting the α-tomatine at low temperatures without DMAP, the sugars could be selectively succinylated without any reaction with the ring nitrogen (Fig 3.4). The reaction was initially performed at -20 °C and 4 °C, and although the reaction was slower at -20 °C, the end products (as monitored by TLC) were similar. Further reactions were carried out at 4 °C.

Confirmation of the oxygen succinylation and the absence of succinylation of the nitrogen was confirmed by IR spectroscopy (Fig 3.5) and a mixed acid hydrolysis. The IR spectra of succinylated α-tomatine showed no evidence of the amide bond which was present after the succinylation of tomatidine. To further confirm that the nitrogen was not affected, an acid hydrolysis of the carbohydrate groups was performed. The acid hydrolysis of the succinylated α-tomatine produced two spots on TLC, one corresponding to tomatidine, and the other the tomatidiene. Tomatidiene is the unsaturated form of tomatidine and is a known product of α-tomatine hydrolysis (Schrieber, 1968; van Gelder, 1984).

Reaction of α-tomatine with succinic anhydride produced multiple products, ranging from one to nine succinyl groups per α-tomatine molecule depending on the length of time the reaction was allowed to proceed. The multiple products were distinguished by TLC and

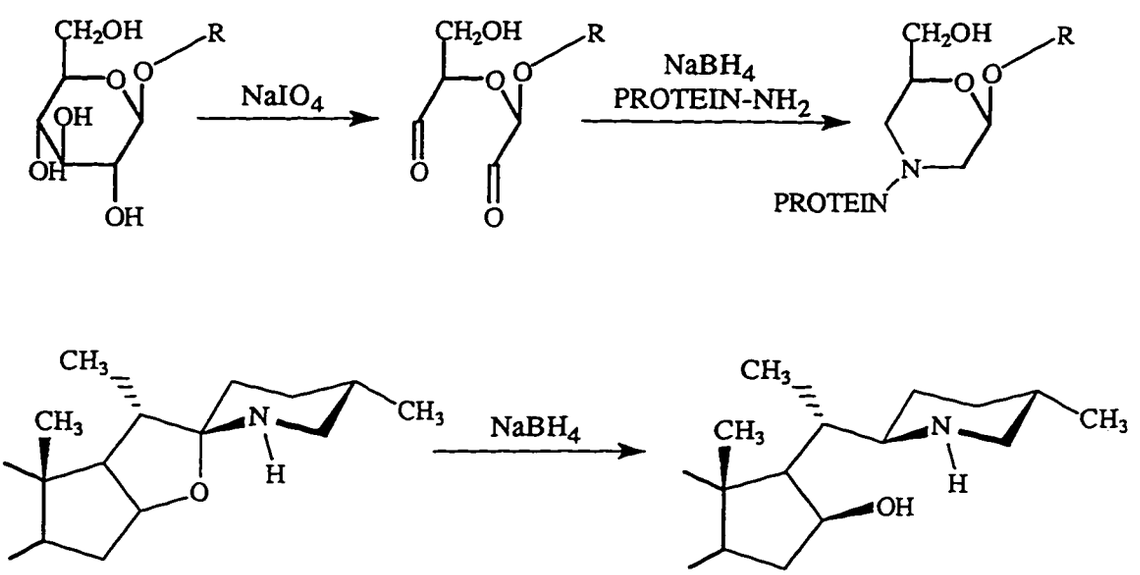


Figure 3.3 Sodium Periodate Oxidation/Sodium Borohydride Reduction of α-Tomatine

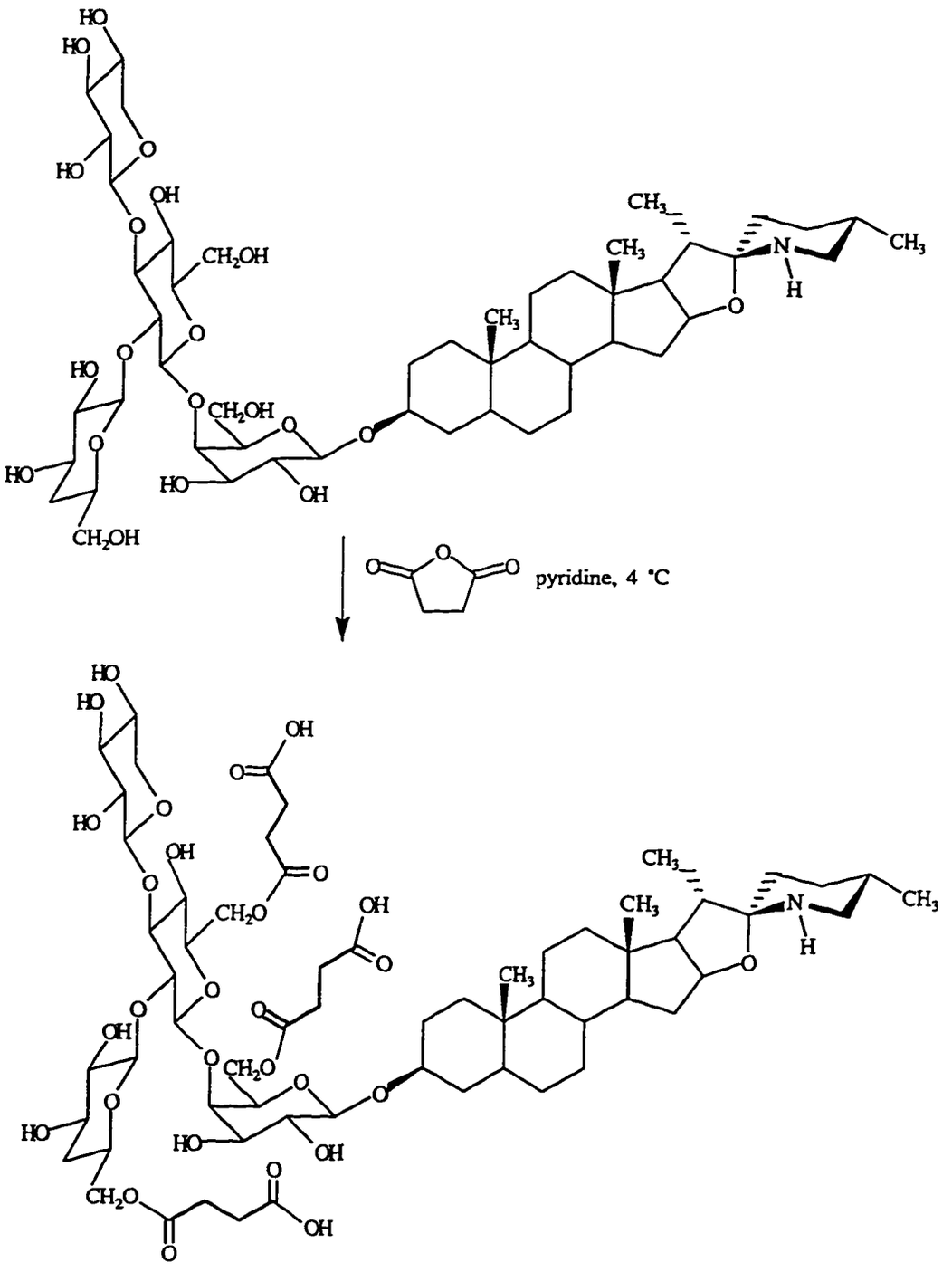


Figure 3.4 Selective Succinylation of α-Tomatine

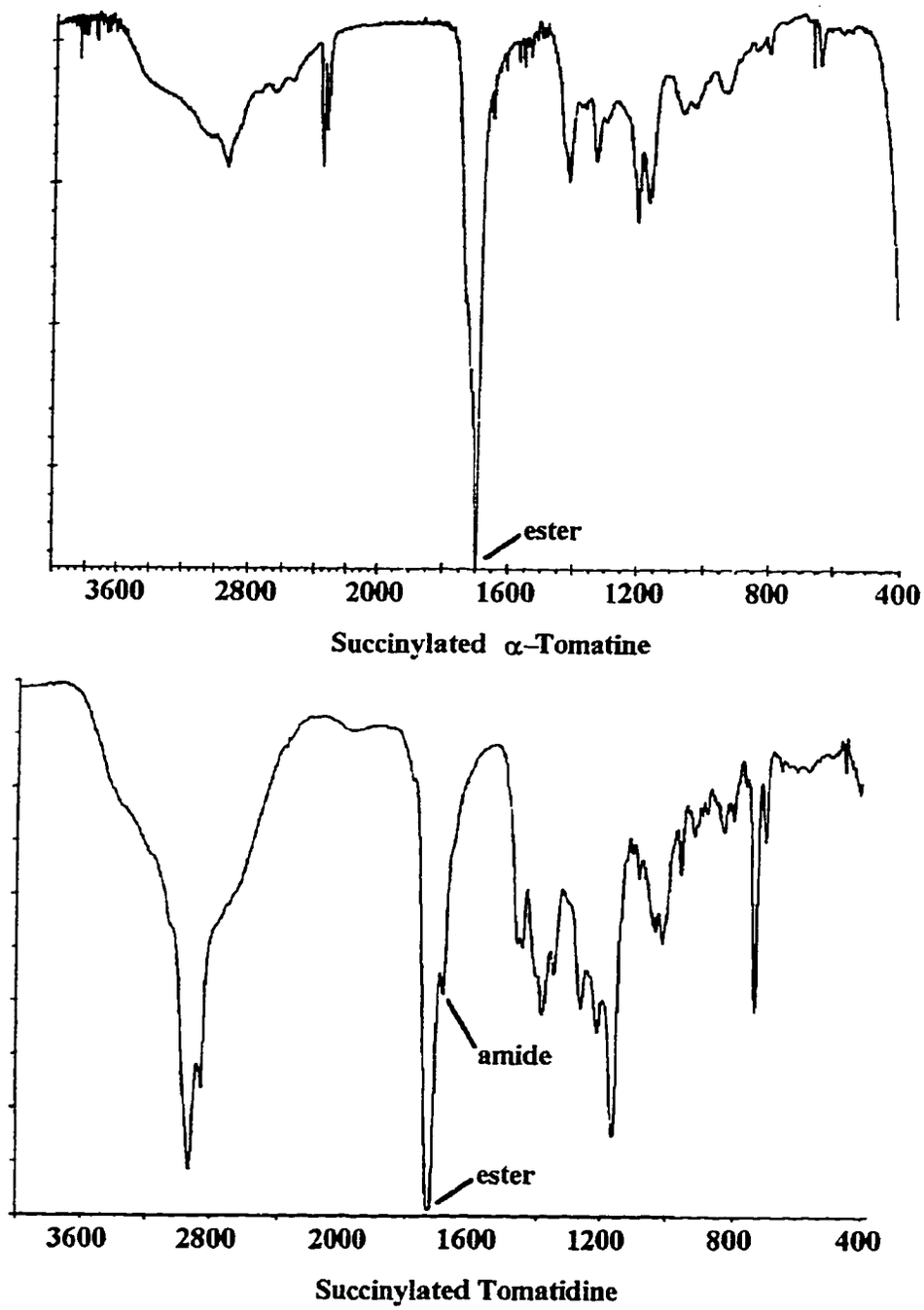


Figure 3.5 IR Spectra of Succinylated α-Tomatine and Disuccinylated Tomatidine

by MALDI-TOF MS (Fig 3.6). This likely represents the succinylation of the primary hydroxyl groups as well as the secondary hydroxyl groups on the C-2 sugar positions. Although the crude mixture of multiply succinylated α-tomatine can be used for further conjugation, it was preferable to purify the individual species which was performed using an acetate anion exchange column and eluting with ammonium acetate. After collecting the fractions they were tested by spotting on a silica TLC plate and charring. There were four series of positive fractions which were all separated by a number of fractions containing no visible product, and these were combined to give four fractions. These four fractions were tested by either fast atom bombardment MS or MALDI-TOF MS to determine the degree of succinylation. The fractions were found to have up to four succinyl groups attached (Table 3.1). Although the reaction mixture initially contained tomatine with greater than four succinyl groups, compounds with greater than four groups were not eluted from the column under the conditions used. Tomatine and monosuccinylated tomatine were eluted from the column with water, as the addition of the charged hemisuccinate gave the glycoalkaloid an overall neutral charge under the buffer conditions used (pH 7.0). The disuccinylated tomatine appeared in fraction 2, 3, and 4 although when the fractions were initially tested by spotting and charring, there was clearly separation between the combined fractions. The presence of disuccinyl tomatine in several pooled fractions is not the results of a very broad band eluting from the column, but rather several species of disuccinylated tomatine present. The disuccinylated species have sufficiently different conformations resulting from succinylation of the various available reactive sites to allow for separation on the anion column. When the purified fractions were tested by TLC, several spots were also observed.

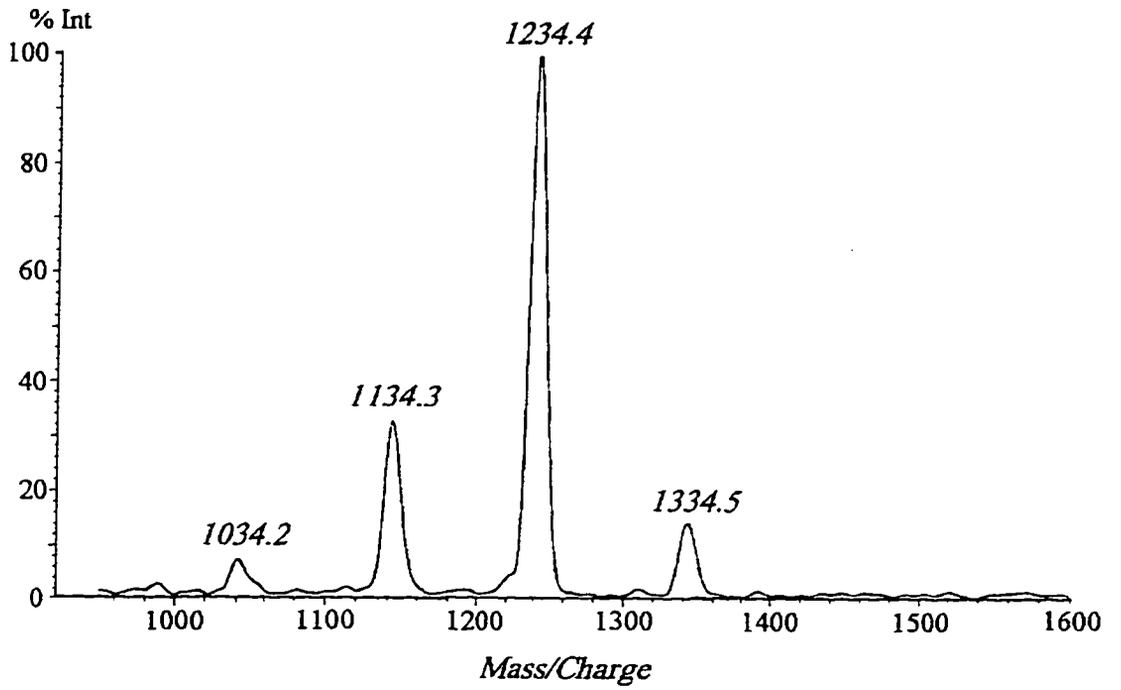


Figure 3.6 MALDI-TOF MS of Partially Purified Succinylated α-Tomatine

One μL of a saturated matrix solution of THA in methanol:water (50:50) was added to probe and allowed to dry followed by 1 μL of sample. Sample is the summation of 100 shots using power setting of 80 on Kratos MALDI I. Starting material (tomatine, 1034.2 *m/z*) was present as well as mono-, di-, and tri-succinylated tomatine.

Table 3.1. Purified Succinylated Tomatine.

Fraction	Compounds Present	Recovery (mg)	Yield (%)
TOM1-F1	unreacted (30 %), mono (70 %)	13.3	12.5
TOM1-F2	mono(100 %)	9.8	8.8
TOM1-F3	di (100 %)	13.8	11.4
TOM1-F4	di (90%), tri (10 %)	112.2	----*
TOM2-F1	unreacted (30 %), mono (70 %)	47.7	22.3
TOM2-F2	mono (5 %), di (95 %)	7.1	3.0
TOM2-F3	di (85 %), tri (15 %)	48.7	20.1
TOM2-F4	di (30 %), tri (45 %), tetra (25 %)	666.0	----*

* fractions still contained ammonium acetate

TOM1 and TOM2 represent two separate synthesis, while F1-F4 represent the combined fractions from TLC. Fractions were purified by anion exchance chromatography using AG X-1 acetate resin using a linear ammonium acetate gradient. Consituent percentages determined from FAB MS or MALDI-TOF MS.

3.1.2 Synthesis of Protein Conjugates

When using protein-hapten conjugates for immunoassays it is desirable to use two different protein conjugates, one for immunization and a second for the assay. Also, the use of two different carrier proteins eliminates antibodies developed against the immunizing carrier protein.

An LPH conjugate was synthesized with unpurified α-tomatine hemisuccinate using an active ester method. Due to limited solubility of the tomatine-LPH conjugate as well as the activated tomatine hemisuccinate, only 3-4 tomatine molecules per LPH molecule could be added. A similar situation occurred when BSA was used as the carrier protein. The results were similar when a water-soluble carbodiimide method was used in place of the active ester method. While these conjugates may be suitable for use in an assay, a higher degree of conjugation is desirable for conjugates used for immunization (Erlanger, 1980).

In order to link with a higher ratio of tomatine to protein, the conjugation was performed

using N-hydroxy sulfosuccinimide as the activating agent rather than N-hydroxy succinimide. The sulfo group increases the solubility of the active ester intermediate. While the solubility of the active ester was increased, it was still not soluble in water, but was soluble in methanol. Therefore the reaction was performed in 50:50 methanol:water to allow for solubility of both the active ester and the BSA. By performing the reaction under these conditions, an average of 7.4 tomatine molecules per BSA molecule was achieved as determined by MALDI-TOF MS. The distribution of mass in the MALDI-TOF MS spectra was narrow, ranging from 73000 to 88000 Da with an average molecular weight of 79113 Da.

Table 3.2. Summary of Protein-Tomatine Conjugates Prepared

Conjugate	Carrier Protein	Tomatine Form Used	Conjugation Method	# of Groups
BSA-TOM-VL	BSA	unpurified tomatine hemisuccinate	NHS, DCC	<0.01
BSA-TOM-L	BSA	unpurified tomatine hemisuccinate	NHS, DCC	0.8
BSA-TOM-M	BSA	85 % disuccinyl, 15 % trisuccinyl	NHS, EDC	1.3
BSA-TOM-H	BSA	65 % disuccinyl, 35 % trisuccinyl	S-NHS	7.4
LPH-TOM	LPH	unpurified tomatine hemisuccinate	NHS, DCC	3.4

Number or groups determined by MALDI-TOF MS.
NHS - n-hydroxy succinimide, S-NHS - n-hydroxy sulfosuccinimide
EDC - 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
DCC - dicyclohexylcarbodiimide

The LPH conjugate was used for the immunization of mice for the production of monoclonal antibodies. Although the number of tomatine groups were low, it was expected that there would still be a response by the animal. The heavily loaded BSA conjugate (BSA-TOM-H) was used for the immunization of rabbits for the polyclonal antibody production. The BSA conjugates were all soluble and were tested for suitability as a solid phase conjugate in ELISA. The LPH conjugate was insoluble in all solvents and therefore was not evaluated in the ELISA.

3.2 Monoclonal Antibodies

3.2.1 Immunization

After the initial injection of LPH-TOM followed by a booster injection, a test bleed and checkerboard ELISA confirmed the presence of antibodies toward the BSA-tomatine conjugate. Two additional boosts were performed in order to increase the specificity of the antibodies and ensure that the differentiation from IgM to IgG isotype had occurred.

3.2.2 Fusion and Cloning of Cell Lines

Three days after fusion and plating of the hybridomas, cell growth was observed in 38 % of the wells (total of 219) with the majority containing one visible colony. The hybridomas were screened using a competitive ELISA and then cloned twice. This produced 6 clones which were frozen for storage in liquid nitrogen. Clone A4-1-1 was used for further studies based on the results of a competitve ELISA against tomatine, tomatidine, solasodine and solanine.

3.2.3 Growth Rates and Antibody Production

Growth rates in a variety of different culture media and the antibody production was tested using clone A4-1-1. The clone was thawed from storage in liquid nitrogen and subcultured several times in complete RPMI before testing different culture media. The media tested were as follows: RPMI + 20 % (complete RPMI), RPMI + 5 % fetal calf serum (RPMI 5%), protein-free hybridoma media (PFHM) and hybridoma serum-free media (HSFM). The clone was subcultured 1:10 from complete RPMI media into duplicate flasks of each test media. The viable cell counts were determined at day 0, 2 and 5. At day 5 the cells were spun down and the supernatant of each flask tested for antibody titer using a standard ELISA format with no competition.

The cell growth was the greatest in the complete RPMI media (Figure 3.7), although PFHM was also good. The cells grew moderately well in the HSFM, but were clumping and did not appear healthy. The growth in the RPMI 5% was very poor and a large degree of clumping cells and unhealthy cells were observed. The antibody production in each medium

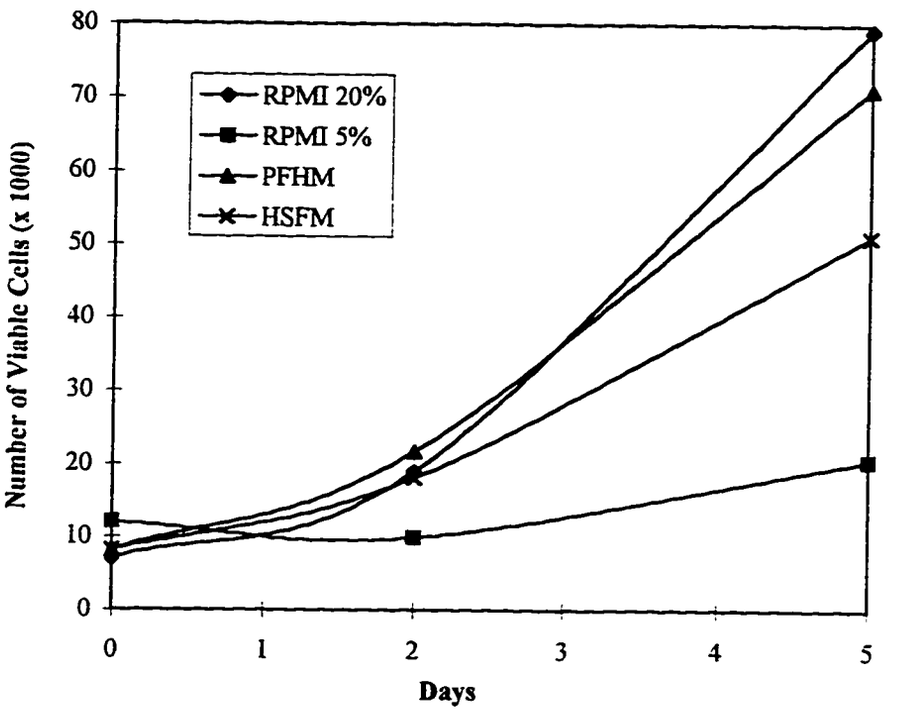


Figure 3.7 Cell Growth in Tissue Culture Media

Cells were inoculated into duplicate flasks of each tissue culture media. Counts were made at day 0, 2 and 5. Each point is an average of 8 cell counts, four on each flask.

was tested by ELISA and was closely related to the cell growth.

Since the cells grew well and produced high titers of antibody in PFHM media, it was the preferred media for production and purification of the monoclonal antibodies. Purification of the antibodies using a protein free medium allows for better purity and eliminates the potential for contamination by serum proteins.

3.2.4 Purification of Monoclonal Antibodies

Purification was performed with supernatant from 100 mL of both RPMI and PFHM media. The precipitate at 50 % and 60 % saturated ammonium sulfate solutions were collected for each media, and an additional 80 % fraction collected for PFHM. After resolubilization and dialysis of the precipitates the fractions were diluted to 25 mL in PBS. The protein content of the purified antibodies was determined by measuring the absorbance at 280 nm and the titer determined by ELISA 2 (Table 3.3). The absorbance at 280 nm was then converted to the concentration of IgG antibodies using a conversion factor of 1.35 (Harlow and Lane, 1988) as follows:

$$\text{Concentration of Antibody (mg/mL)} = \text{Abs}_{280} \div 1.35$$

The purified antibodies from the RPMI media had very high absorbances, most likely due to the presence of contaminating proteins as the titre for the supernatant was not significantly higher than the supernatent from the PFHM. Using a conversion factor of 1.35 for antibodies, the antibody concentration of the PFHM fractions was calculated to be 0.175 mg/mL, 0.078 mg/mL and 0.113 mg/mL for the 0-50 %, 50-60 % and 60-80 % fractions, respectively.

It is possible that the 0 - 50 % fraction of PFHM contains some cellular proteins other than the antibodies as the cells were lysed before collecting the supernatant. Any contamination in the last two fractions from 50 - 60 % and 60 - 80 % would be expected to be very low. While antibodies typically will precipitate from 40 - 55 % ammonium sulfate (Harlow and Lane, 1988), the 60 - 80 % fraction contained a substantial amount of protein.

Table 3.3. Antibody Yield from PFHM by Ammonium Sulfate Precipitation.

Sample	PFHM 0 - 50 % Saturated NH_4SO_4	PFHM 50 - 60 % Saturated NH_4SO_4	PFHM 60 - 80 % Saturated NH_4SO_4
Absorbance (280 nm)	0.130	0.058	0.083
Concentration of Antibody (mg/mL)	0.175	0.078	0.113
Total Antibody (mg)	4.38	1.95	2.83
Yield from Culture Media (μg/mL)	43.8	19.5	28.3

Absorbance readings are single replicates, using PBST as a blank. Total antibody yield based on 25 mL of solution. Yield from culture media based on 100 mL of media as starting material.

The titre of the purified antibody solutions from RPMI and PFHM media was determined to allow for comparisons between the protein levels and the antibody content. Each MAb solution was tested at a dilution of 1:50 using the standard competitive ELISA format with the omission of any competitor compound (Table 3.4).

Table 3.4. Titre and Activity of Purified Monoclonal Antibodies.

MAb Source	Average ELISA Abs (Ave Abs - Ave Blank)	Protein Concentration (mg/mL)	Immuno-Activity of Protein (Abs/mg/mL)
PFHM 0-50 %	0.980	0.175	5.60
PFHM 50-60 %	1.701	0.078	21.78
PFHM 60-80 %	0.484	0.113	4.30
RPMI 0-50 %	2.461	10.435	0.24
RPMI 50-60 %	1.411	0.997	1.41

MAb solutions were diluted 1:50 and 100 μL used in the standard competitive ELISA format. Average ELISA Abs value is an average of 6 replicates corrected for background Abs.

From the data in Table 3.4 it is clear that there were protein impurites present in the

purified MAb. For both the PFHM and the RPMI purifications, the greatest specific activity was in the fraction collected between 50 - 60 % saturated ammonium sulfate. The total yield of MAb from PFHM media of 9.16 mg from 100 mL of culture media (91.6 μg/mL) is not a true representation as the MAb is not pure. Typical yields are 50 μg/mL for monoclonal antibodies (Harlow and Lane, 1988), but may be as high as 100 μg/mL (Campbell, 1984).

3.3 Polyclonal Antibody Production

Polyclonal antibodies were produced using a tomatine-BSA conjugate containing 7.4 alkaloid molecules per BSA molecule (BSA-TOM-H). Although using a BSA conjugate for injection as well as coating can result in interference due to anti-BSA antibodies in the sera, this effect can be eliminated by including BSA in the dilution buffers. The relatively high concentration of BSA (0.05 %) in the solution eliminates the effect of anti-BSA antibodies by binding any anti-BSA antibodies which may be present.

A test bleed was performed after the second injection of the rabbits and the antibody titre tested. The two sera tested had titers of 1:100 000 which gave a maximum absorbance of 0.757 and 1.634, respectively. Dilutions of the sera of 1:1 000 000 still gave absorbance readings of greater than 3 times the background for both sera. After two additional boosts, tests bleeds were again tested for titer (Table 3.5). From the data below, there was clearly an increase in titer with the additional boosts. While some literature suggests sacrificing after a single boost, the additional boosts results in increased titers and improved specificity of the antibodies (Harlow and Lane, 1988).

Table 3.5. Results of Titre Testing of Polyclonal Sera.

Dilution	1:10 000	1:100 000	1:1 000 000
Sera SG7 bleed #1	2.823	0.629	0.214
Sera SG7 bleed #2	3.298	1.144	0.263
Sera SG8 bleed #1	3.522	1.541	0.232
Sera SG8 bleed #2	3.819	1.673	0.271

All plates were coated with 1 ppm BSA-TOM-H. Sera was diluted using 0.1 % BSA in PBST and 100 µL added to the wells for testing along with 100 µL of 0.1 % BSA in PBST. Values are the results of a single determination.

3.4 Polyclonal and Monoclonal Enzyme Immunoassays

Initial testing of the polyclonal and monoclonal antibodies in competitive assays did not show encouraging results against tomatine. Although there was evidence of competition against tomatine through a decreased absorbance, the competition only occurred at high concentrations of tomatine (100 μM). In order to evaluate the antibody binding thoroughly, a competitive ELISA was performed using four different BSA-tomatine conjugates for coating. These four conjugates all contained differing amounts of tomatine on the protein as shown in Table 3.6.

Table 3.6. Summary of Coating Conjugates Used in ELISA.

Conjugate	# of groups	Tomatine used	linking method
BSA-TOM-H	7.4	65 % disuccinyl, 35 % trisuccinyl	active ester with N-hydroxy sulfosuccinimide
BSA-TOM-L	0.8	unpurified tomatine hemisuccinate	active ester with N-hydroxysuccinimide
BSA-TOM-VL	< 0.01	unpurified tomatine hemisuccinate	active ester with N-hydroxysuccinimide
BSA-TOM-M	1.3	85 % disuccinyl, 15 % trisuccinyl	water soluble carbodiimide

The number of groups was determined by MALDI-TOF MS.

Both the MAb and PAb were tested for competition against tomatine, BSA-TOM-H, succinylated tomatine (disuccinyl) and BSA. The tomatine conjugates were tested at approximately 100 μM concentration of tomatine and all coating conjugates were used at 1 ppm. There were a large differences among the four different coating conjugates used (Table 3.7 - 3.8).

Table 3.7. Comparision Between Coating Conjugates Using Monoclonal Antibodies.

Competitor / Coating Conjugate	Tomatine	BSA-TOM-H	T-S-P2-10-F3	0.05% BSA	Blank
BSA-TOM-H	0.109	0.073	0.095	0.184	0.215
BSA-TOM-L	0.118	0.060	0.088	0.270	0.274
BSA-TOM-VL	0.130	0.038	0.047	0.079	0.125
BSA-TOM-M	0.042	0.033	0.041	0.036	0.043

All plates were coated with 1 ppm of the coating conjugate. Purified MAb from PFHM (0-50 % fraction) were diluted to 3.5 μg/mL using 0.05 % BSA in PBST and 100 μL added to the wells for testing along with 50 μL of the competitor compound dissolved in either methanol or 0.1 % BSA in PBST. Methanol or 0.05 % BSA in PBST (50 μL) was added as appropriate to give a final concentration of 25 % methanol in the well. The values are the mean absorbance values of a minimum of six replicates.

Table 3.8. Comparison Between Coating Conjugates Using Polyclonal Antibodies.

Competitor / Coating Conjugate	Tomatine	BSA-TOM-H	T-S-P2-10-F3	0.05% BSA	Blank
BSA-TOM-H	0.813	0.185	0.722	0.827	0.882
BSA-TOM-L	0.113	0.073	0.092	0.274	0.303
BSA-TOM-VL	0.207	0.029	0.096	0.157	0.314
BSA-TOM-M	0.283	0.033	0.062	0.239	0.406

All plates were coated with 1 ppm of the coating conjugate. Sera was diluted 1:250 000 using 0.05 % BSA in PBST and 100 μL added to the wells for testing along with 50 μL of the competitor compound dissolved in either methanol or 0.1 % BSA in PBST. Methanol or 0.05 % BSA in PBST (50 μL) was added as appropriate to give a final concentration of 25 % methanol in the well. The values are the mean absorbance values of a minimum of six replicates.

The highly substituted BSA conjugate (BSA-TOM-H) resulted in the highest antibody binding with the PAb and was also high with the MAb. This effect may simply be due to the higher number of tomatine molecules present. For the PAb the higher affinity could be expected as BSA-TOM-H was the conjugate used for immunization. While the amine link to the protein is consistent from conjugate to conjugate, the degree of succinylation of

tomatine varies between conjugates which may account for differences in the antibody binding. The PAb were also tested using a BSA-succinyl-sulfamerazine conjugate containing 6.9 sulfamerazine groups per BSA molecule (Garden and Sporns, 1994) and did not bind this conjugate.

The evaluation of the different competitor compounds provided the greatest amount of information regarding the best coating conjugate to use. Although BSA-TOM-H had the greatest degree of antibody binding, neither 100 μM tomatine or 100 μM succinylated tomatine could compete for antibody binding with this conjugate.

The superior choice for a coating conjugate was BSA-TOM-L which competed well with tomatine, succinylated tomatine and the tomatine-BSA conjugate. This coating conjugate did, however, have maximum absorbance readings 2.5 times lower than when BSA-TOM-H was used (Table 3.8). From the results of initial conjugate testing, BSA-TOM-L was selected for further competition testing. The concentration of BSA-TOM-L used for coating in the assay was increased from 1 ppm to 5 ppm to increase the maximum absorbance.

The MAb diluted to a concentration of 7 μg/mL was tested for competition against tomatine, solanine, succinylated tomatine, BSA-TOM-L and BSA-TOM-H. The standard competitive ELISA format was used with BSA-TOM-L at a concentration of 5 ppm for the coating conjugate. Each competitor compound was tested at concentrations of 100 μM, 10 μM, 5 μM, 1 μM, 500 nM, 100 nM, 50 nM, 10 nM, 5 nM, 1 nM and 100 pM. The results for the MAb are shown in Table 3.9 and Figure 3.9. The PAb was diluted 1:250 000 and tested similarily against tomatine, tomatidine, solasodine, solanine, succinylated tomatine, BSA-TOM-L and BSA-TOM-H (Table 3.10 and Figure 3.10).

Table 3.9. Competitive ELISA with Monoclonal Antibodies.

Competitor Compound	I_{50} Concentration (μM)*
Tomatine	> 100
Solanine	> 100
BSA-TOM-L	0.02
BSA-TOM-H	1.68

* I_{50} based on the calculated *c* value of the standard sigmoidal curve fit.
All plates were coated with 5 ppm of the BSA-TOM-L. Purified monoclonal antibodies from PFHM (0-50 % fraction) were diluted to 3.5 μg/mL using 0.05 % BSA in PBST and 100 μL added to the wells for testing along with 50 μL of the competitor compound dissolved in either methanol or 0.1 % BSA in PBST. Methanol or 0.05 % BSA in PBST (50 μL) was added as appropriate to give a final concentration of 25 % methanol in the well. Competition was tested from 100 μM to 100 pM in triplicate.

Table 3.10. Competitive ELISA with Polyclonal Antibodies.

Competitor Compound	I_{50} Concentration (μM)
Tomatine	6.71
Tomatidine	> 100
Solanine	> 100
Solasodine	> 100
T-S-P2-10-F3	1.84
BSA-TOM-L	0.35
BSA-TOM-H	5.8×10^{-3}
FL1-B	> 100

* I_{50} based on the calculated *c* value of the standard sigmoidal curve fit.
All plates were coated with 5 ppm of the BSA-TOM-L. Sera (100 μL) diluted 1:250 000 with0.05 % BSA in PBST was added to the wells for testing along with 50 μL of the competitor compound dissolved in either methanol or 0.1 % BSA in PBST. Methanol or 0.05 % BSA in PBST (50 μL) was added as appropriate to give a final concentration of 25 % methanol in the well. Competition was tested from 100 μM to 100 pM in triplicate.

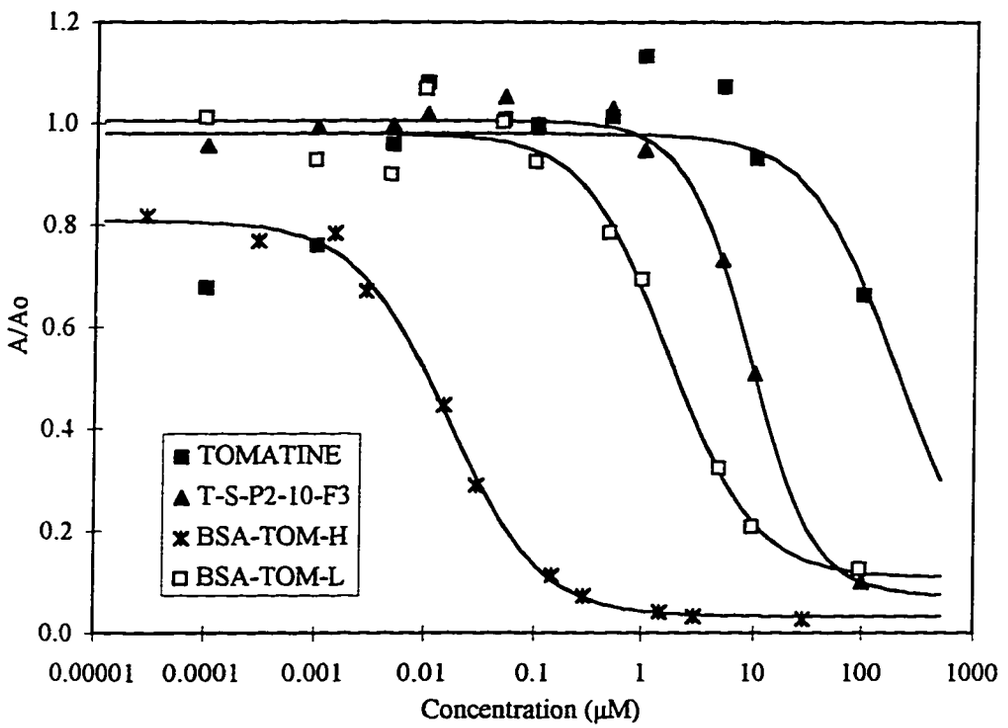


Figure 3.9 Monoclonal Competition Curves for Tomatine and Tomatine Conjugates

Points represent the average of six replicates. Plates were coated with 5 ppm BSA-TOM-L. Competition performed with 50 μL of competition solution in either methanol or 0.05 % BSA in PBST and 100 μL of serum diluted 1:250 000 with 0.05 % BSA in PBST. To give 25 % methanol, 50 μL of either methanol or 0.05 % BSA in PBST was added as required. The experimental absorbance was divided by the maximum absorbance to give A/Ao.

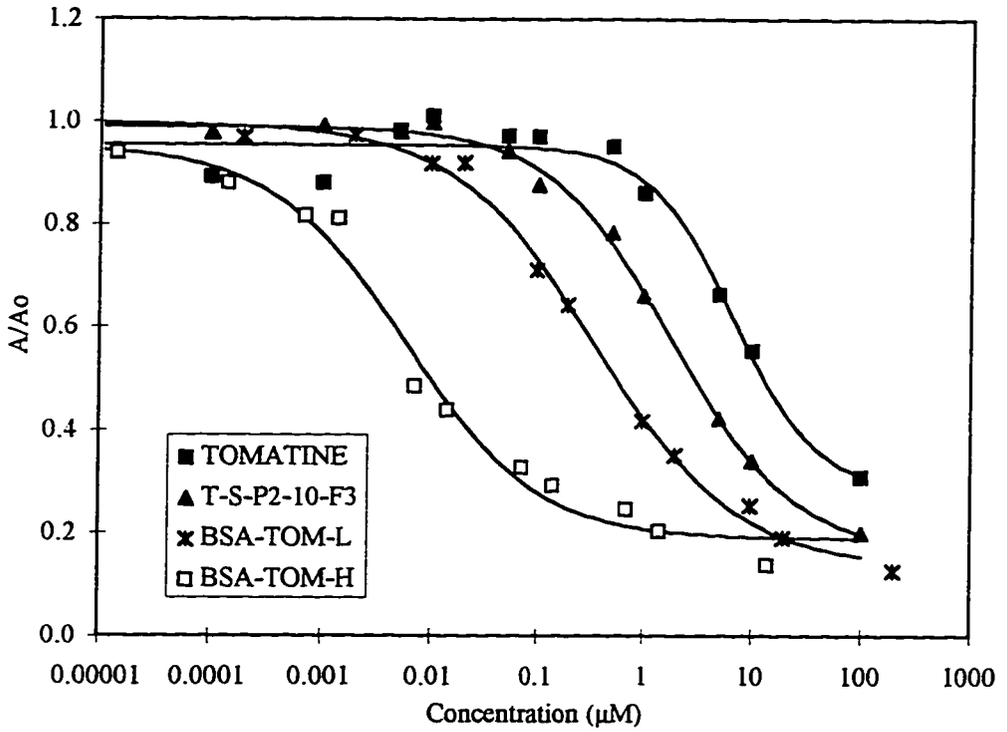


Figure 3.10 Polyclonal Competition Curves for Tomatine and Tomatine Conjugates

Points represent the average of six replicates. Plates were coated with 5 ppm BSA-TOM-L. Competition performed with 50 μL of competition solution in either methanol or 0.05 % BSA in PBST and 100 μL of serum diluted 1:250 000 with 0.05 % BSA in PBST. To give 25 % methanol, 50 μL of either methanol or 0.05 % BSA in PBST was added as required. The experimental absorbance was divided by the maximum absorbance to give A/Ao.

From the results of the monoclonal and polyclonal competitive assays, it is clear that the antibodies recognize the carbohydrate portion of tomatine over the alkaloid region. Both solasodine and tomatidine produced similar results in competitive assays supporting the theory of the antibody recognition towards the carbohydrate region of the molecule. If the antibodies were directed against the alkaloid portion containing the ring nitrogen, tomatine and tomatidine would be expected to compete similarly.

While the antibodies did recognize tomatine preferentially over the other alkaloids and glycoalkaloids tested, a stronger recognition was observed for succinylated tomatine and the protein-tomatine conjugates. When the results of competition are corrected for the amount of tomatine present on the protein conjugates, both produced very similar results (Table 3.11 and Figure 3.11).

Table 3.11. Corrected I_{50} Values for Protein Conjugate Using Polyclonal Antibodies..

Competitor Compound	I_{50} Concentration (μM)	I_{50} (μM) corrected for tomatine concentration
BSA-TOM-L	0.35	5.3×10^{-2}
BSA-TOM-H	5.8×10^{-3}	3.9×10^{-2}

Corrected tomatine concentrations based on calculated number of tomatine groups present of the protein and the average molecular weight of the tomatine intermediate used in synthesis of the conjugate.

Two rhodamine-tomatine conjugates were prepared for development of a fluorescence polarization immunoassay (FPIA). Rhodamine conjugates containing either one or two rhodamine groups attached to succinylated tomatine were synthesized. It was hoped that multiple fluorophores would decrease the antibody requirements for the assay and that this could be combined with the FPIA for solanine and chaconine developed by Thomson and Sporns (1994) to demonstrate a multi-component analysis. When the fluorescent conjugate was tested with the PAb, no antibody recognition was observed. This indicates that the antibody recognition is directed not only toward the carbohydrate portion of the GA and succinyl linking arm, but also a portion of the protein carrier. The large rhodamine group

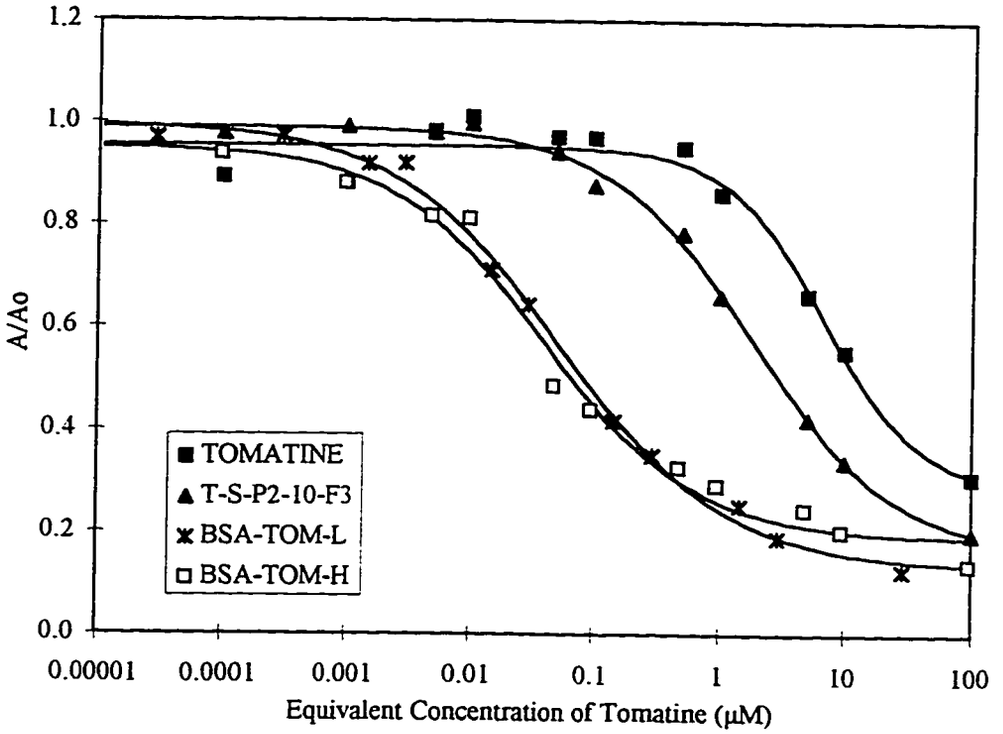


Figure 3.11 Competitive ELISA for Tomatine and Tomatine Conjugates Using Polyclonal Antibodies and Corrected for Tomatine Concentration

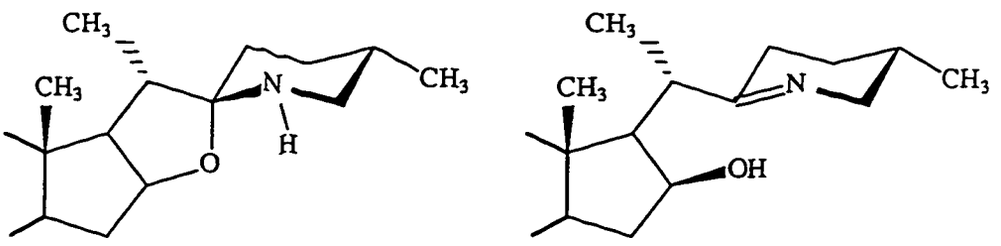
Points represent the average of six replicates. Plates were coated with 5 ppm BSA-TOM-L. Competition performed with 50 μL of competition solution in either methanol or 0.05 % BSA in PBST and 100 μL of serum diluted 1:250 000 with 0.05 % BSA in PBST. To give 25 % methanol, 50 μL of either methanol or 0.05 % BSA in PBST was added as required. The experimental absorbance was divided by the maximum absorbance to give A/Ao.

Concentration are based on the concentration of tomatine or conjugated tomatine present in solution.

blocked all antibody recognition of the compound.

It is proposed that the active tautomerism of the spiroaminoketal moiety of the tomatine molecule prevents antibodies from forming against this variable portion of the molecule (Figure 3.12). The presence of open and closed ring forms would hinder formation of a strong antibody complex and antibodies will preferentially be directed against the rigid succinylated carbohydrate moiety and the protein link.

It appears that the spiroaminoketal moiety and terminal ring of tomatidine is not sufficiently immunogenic to initiate significant antibody formation. This is in stark contrast to the solanidine derivatives, where the antibodies are directed primarily towards the alkaloid portion of the molecule in the region of the ring nitrogen. The lack of immunogenicity is most likely due to the lack of a rigid conformation in the alkaloid.



tomatidine - ring form

tomatidine - open form

Figure 3.12 Open and Closed Forms of Tomatidine

3.5 MALDI-TOF MS

3.5.1 Quantitation of Glycoalkaloids by MALDI-TOF Analysis

GAs are well suited to analysis by MALDI-TOF MS as they have very similar chemical properties, but differ enough in molecular weight to be resolved, and are difficult to analyse by more conventional methods. The mass resolution of present commercial MALDI-TOF instruments ranges from 500-8000, allowing for resolution of glycoalkaloids differing by as little as two Daltons. Furthermore, GA molecular weights are sufficiently high (840-1200 D)such that matrix peaks do not interfere with the analysis.

Prior to using MALDI-TOF MS as an analytical technique, a matrix in which the sample of interest desorbs and ionizes well must be found. Compounds of similar structure will typically produce similar results with a given matrix. A number of common matrices were tested including: sinapic acid, dihydroxybenzoic acid, α-cyano hydroxycinnamic acid, 4-hydrazinobenzoic acid, and trihydroxyacetophenone (THA). THA was found to produce the largest peaks with the greatest degree of baseline separation. Suggested sample amounts for MALDI-TOF MS are 1-10 pmol on the probe (Gusev et al., 1995; Rideout et al., 1993). GAs were tested at 1, 10, and 100 ng per spot (approximately 1, 10, and 100 pmol on the probe) and performed well at all three concentrations. Consequently, subsequent samples and standards were analysed with less than 10 ng/spot.

A high degree of variability is associated with MALDI-TOF MS due to variances in spotting, both from run to run and from spot to spot. Therefore, tomatine was used as an internal standard in the analysis. Tomatine is not found in commercial potato varieties, although it occurs in some wild varieties (Gregory et al., 1981). When pure chaconine, solanine, and tomatine samples are analysed using MALDI-TOF MS with only the extraction solution, each produces a single peak. Upon analysis of a potato sample, a potassium peak appears for each GA (Figure 3.13). This peak is 39 Daltons, the molecular weight of a potassium ion, higher than the actual molecular weight of the glycoalkaloid. Calculations were always carried out on the sum of the two peak heights. In the middle of the study it was learned that the tomatine was only about 80% pure, the other 20% containing a double bond

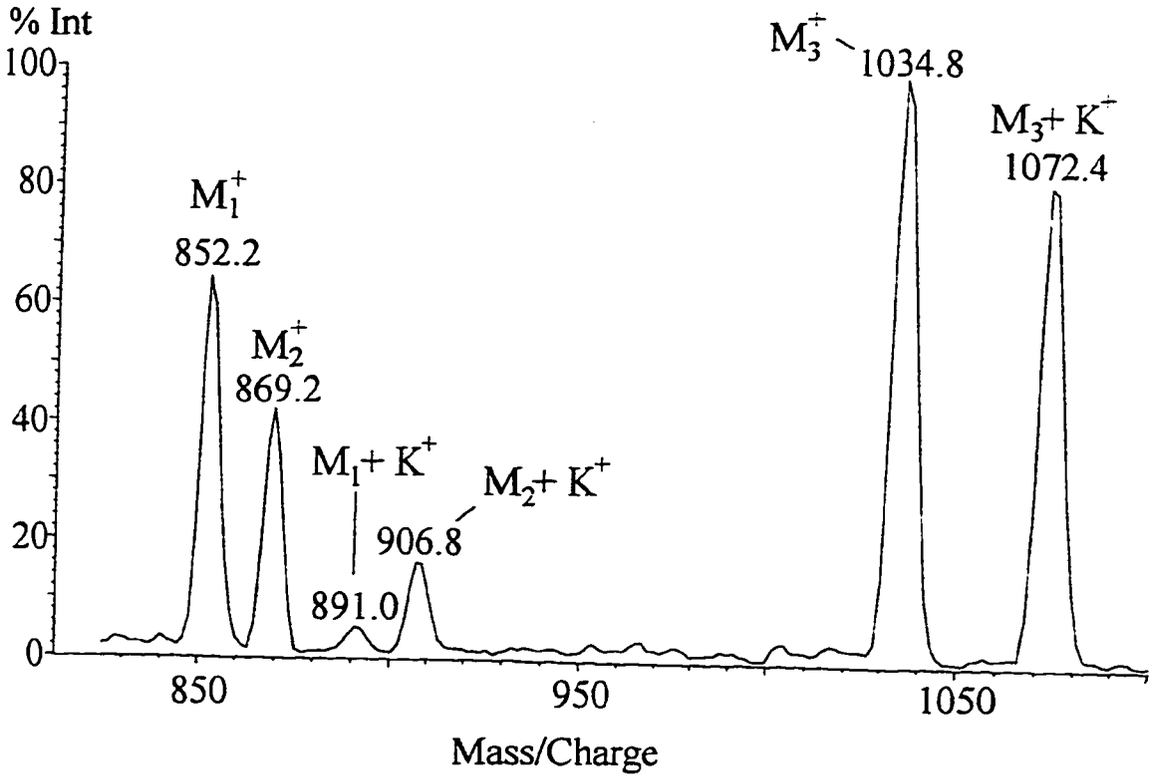


Figure 3.13 MALDI-TOF MS of α-Solanine, α-Chaconine and α-Tomatine

Sample (1 μL) was added to probe and allowed to dry followed by 1 μL of saturated matrix solution of THA in methanol:water (50:50). Sample is the summation of 100 shots using power setting of 80 on Kratos MALDI I. M_1 - alpha-chaconine, M_2 - alpha-solanine, M_3 - alpha-tomatine

(Bushway et al., 1994). While this presents a problem for HPLC analysis, the slight difference in mass (2 mass units) of the major impurity merely leads to some peak broadening for samples and did not preclude the use of the commercially available tomatine without purification.

A standard curve was produced in a range that would encompass the range of GA concentration in potato tissue up to 25 mg/100g. Potato tissue (200 mg) was added to the standards, because in the absence of potato tissue relative peak heights were much larger than when potato tissue was present. Consequently, a useful standard curve could not be generated in the absence of potato tissue. Although the potato tissue did contain a small amount of glycoalkaloids (<1.5 mg/100 g fresh weight), this did not have a large effect on the standard curve generated. Measurements were made on two separate days with triplicate spots and analysis in triplicate on each spot to eliminate spotting and equipment variation. A second-order polynomial curve through zero was then fit to the data (Figures 3.14 and 3.15). The second-order curve fit, rather than a linear model, can be explained by the fact that the presence of solanine or chaconine resulted in greater ionization of the tomatine as well. It is speculated that this ionization pattern may be due to the presence of the double bonds in the chaconine and solanine, allowing for better absorption of energy and a resulting increased energy transfer to tomatine.

The extraction method used when analysing potatoes for GAs is an important consideration. Since many extraction solvents used for GAs contain acid (Coxon, 1984), initial extraction solvents containing 5% acetic acid were evaluated; however, concentration of the acid during drying on the probe resulted in hydrolysis of the sugars from the GAs. While 100% methanol is a commonly used extraction solvent, a water:methanol mixture is preferred as it provides a medium which is easily applied to the MALDI probe. Testing of the extraction over time indicated that the majority of the GAs appeared after only a few minutes of extraction, but 60 minutes were required for complete equilibrium.

Results of MALDI-TOF MS and HPLC analyses are given in Table 3.12. Both analyses

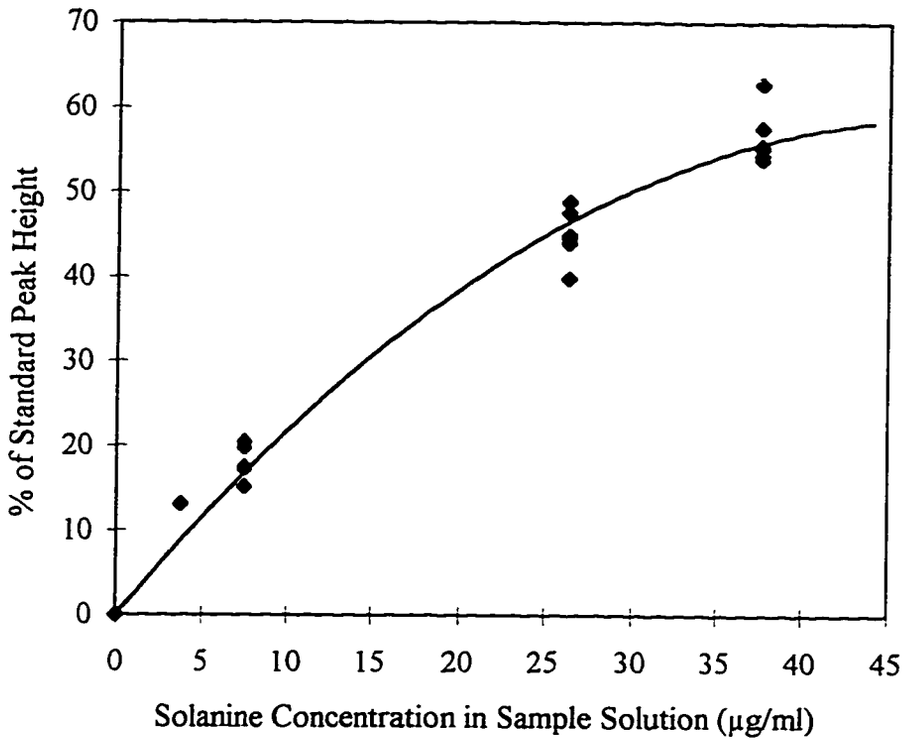


Figure 3.14 Standard Curve For MALDI-TOF MS Analysis of alpha-Solanine

Samples were spiked with varying levels of alpha-solanine using alpha-tomatine as the internal standard. The peak height of the solanine peak and the solanine/potassium peak were summed and are reported as a percentage of the height of the internal peak. Each point is the average of three determinations on each of three separate spots.

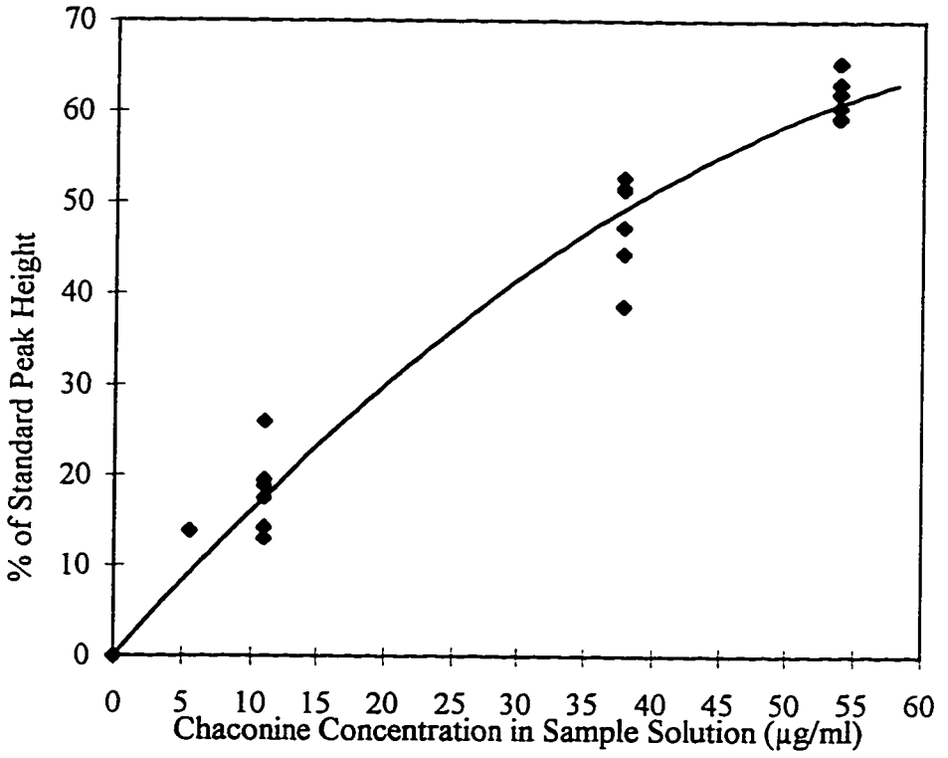


Figure 3.15 Standard Curve For MALDI-TOF MS Analysis of alpha-Chaconine

Samples were spiked with varying levels of alpha-chaconine using alpha-tomatine as the internal standard. The peak height of the solanine peak and the solanine/potassium peak were summed and are reported as a percentage of the height of the internal peak. Each point is the average of three determinations on each of three separate spots.

produced very similar results (R^2 = 0.98) and similar standard deviations. The standard deviations obtained compare well with HPLC results of Saito et al. (1990). It is interesting to note that samples A, C, and D, which were commercial cultivars stored for only 8 months, had relatively high GA levels (21.72, 9.18, and 7.62 mg/100 g, respectively). Sample A had levels above the recommended allowance for GAs (> 20 mg/100g). None of these samples showed excessive greening or sprouting before analysis, which is often used in industry as an indicator for high GA levels. This demonstrates the need for testing of potatoes, rather than reliance on secondary indicators. Another benefit of MALDI is the ability to detect other glycoalkaloids which may be present such as β-chaconine. Other glycoalkaloids could be identified by their molecular weight, and once a suitable standard curve is established, could also be quantified.

Table 3.12. Comparison of GA Analysis by MALDI-TOF MS and HPLC.

	MALDI			HPLC		
sample	α-chaconine	α-solanine	Total GA	α-chaconine	α-solanine	Total GA
A	13.20 (2.76)	8.52 (0.58)	21.72 (3.32)	13.30 (0.67)	11.87 (0.45)	25.17 (1.12)
B	23.44 (1.43)	12.92 (1.95)	36.36 (3.17)	19.36 (0.16)	14.66 (0.06)	34.02 (0.22)
C	5.98 (0.31)	3.19 (0.20)	9.18 (0.50)	8.08 (1.53)	5.00 (0.98)	13.08 (2.51)
D	4.63 (0.79)	2.99 (0.24)	7.62 (0.93)	6.59 (1.34)	4.12 (0.90)	10.71 (2.24)
E	0.85 (0.06)	0.76 (0.24)	1.62 (0.29)	0.85 (0.25)	0.35 (0.34)	1.20 (0.59)
F	1.96 (0.1)	0.98 (0.13)	2.94 (0.20)	1.75 (0.11)	0.43 (0.12)	2.18 (0.23)

All values are mg/100 g fresh weight basis assuming 80% moisture; values in parentheses are standard deviations between triplicate extractions of a sample.

The greatest advantage of MALDI-TOF MS is its speed of analysis. Even when triplicate extractions and triplicate analyses/extraction was considered, MALDI-TOF MS analysis was still much faster than HPLC. Each HPLC test required 10-12 min, or nearly 2 h/sample for triplicate analysis on triplicate extractions.. MALDI-TOF MS permitted triplicate analysis on an extraction in under 6 min, for a total of 20 min/sample, assuming triplicate extraction. Moreover, HPLC requires extensive cleanup of sample prior to analysis,

which is very labour intensive and represents an additional 30-40 min per sample (with triplicate extractions). This contrasts sharply to the MALDI-TOF MS method in which 10-15 samples could be prepared in the same time period. The major disadvantage to MALDI-TOF MS is the associated capital cost (> $75 000). However, MALDI-TOF MS is a very recently developed technology, and with increased application, it is anticipated that mass production of instruments will reduce their cost (Siuzdak, 1994).

3.5.2 Synthesis of Alternative Internal Standards for MALDI-TOF MS

While tomatine works well as an internal standard, it would be useful to have an internal standard which allows for the analysis of all the glycoalkaloids. The internal standard should be closely related in chemical structure to the GA and, while having a similar molecular weight, must not overlap with the analyte peaks. This can be achieved through chemical modification of a glycoalkaloid to produce a compound of either lower or higher molecular weight. Several methods were investigated for the production of internal standards.

To make a lower molecular weight standard, sodium periodate oxidation followed by sodium borohydride reduction can be used to remove a carboxyl group from the sugars. Sodium periodate oxidation will only react with the vicinal alcohols of the carbohydrate moiety and will result in the loss of a formaldehyde group. Chaconine, which has two reactive sites was used for the reaction, but some hydrolysis of the sugars occurred.

While the periodate oxidation/borohydride reduction reaction is commonly used for the linking of carbohydrate containing compounds to proteins, difficulties arose when using this reaction sequence with chaconine in solution. Excess periodate is usually destroyed using ethylene glycol, but this results in the formation of formaldehyde, and upon concentration of the reaction mixture a paraformadehyde precipitate is formed. Sucrose can also be used to remove unreacted periodate, but interferes with TLC analysis. Due to these problems, the periodate was not destroyed before adding the sodium borohydride. After reaction with sodium borohydride, acetone was added to destroy any remaining borohydride. Throughout the reaction, a precipitate was present, corresponding to iodine salts which are not soluble in

methanol.

The main concern with the reaction was the variety of end-products observed. Although the borohydride reduction results in two carbonyl groups, in the presence of water they will condense into a six membered ring (Figure 3.16). This method is not suitable for the synthesis of internal standards due to the presence of the multiple forms and the difficulty of purifying the end products.

Another method of creating a lower molecular weight internal standard is to partially hydrolyze the carbohydrate. If a single sugar were cleaved from chaconine, this would produce a good internal standard for the assay. This was investigated, but was not pursued due to the low yield of the desired derivative.

Due to the difficulties in creating lower molecular weight internal standards higher molecular weight standards were synthesized by adding substituents to α-chaconine. The addition of succinic acid using succinc anhydride is a simple procedure which could be used, but has several disadvantages. The reactivity of the succinic anhydride would likely result in multiple products as was observed with tomatine. As well the addition of succinic acid would result in a differently charged species which may not respond in the same manner as chaconine. For these reasons, a butyl group was chosen for the substituent group.

For the addition of sufficient mass to chaconine, pivaloyl chloride was first examined. It was hoped that the steric hinderance would prevent any reaction with the secondary alcohols, leaving only two reactive primary alcohols. When the reaction was tested, a large excess of pivaloyl chloride was required to initiate any reaction, and had to be performed at low temperatures (- 40 °C) in order to limit the reaction. As the reaction proceeded, a series of faster running spots was observed with TLC. The addition was confirmed by MALDI-TOF MS. Although this reaction was successful, purification was difficult and the reaction conditions were difficult to control.

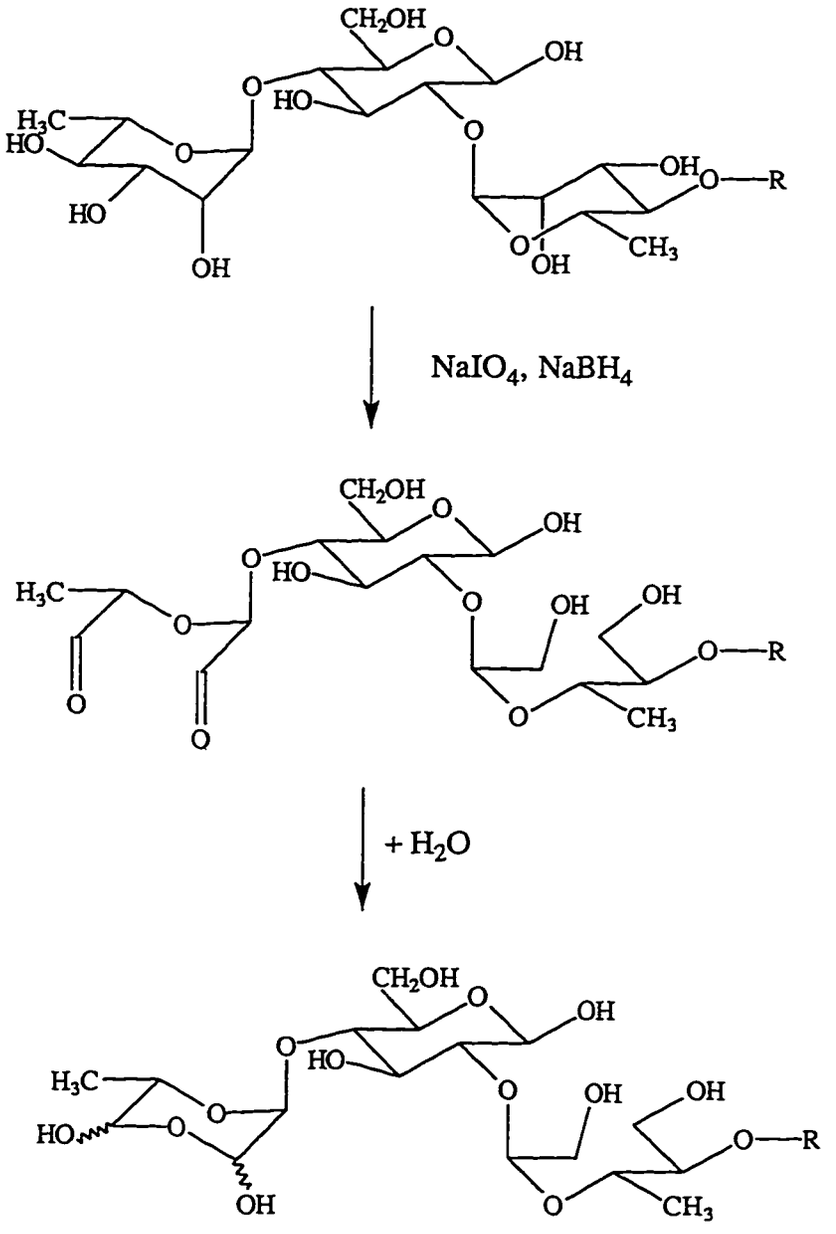


Figure 3.16 Condensation of a-Chaconine Oxidation/Reduction End Product with Water

In order to better control the reaction conditions, the less reactive butyric anhydride was used rather than pivaloyl chloride. This allowed the reaction to be performed at room temperature (20-25 °C). After stopping the reaction with the addition of water, analysis by MALDI-TOF MS showed starting material, mono-, di-, tri- and tetra-butylated chaconine were present (Figure 3.17). The reaction mixture was then analysed by TLC/MALDI-TOF MS (Figure 3.18). Of the four visible spots on TLC, the first was starting material and the next two were monobutylated chaconine. The fourth spot corresponded to the dibutylated chaconine. A sample of each of the four compounds was purified by separating the reaction mixture by TLC and scraping the positive zones from the TLC plate.

After collecting the samples from the TLC plate, methanol was added to fraction 2 and the solution tested by MALDI-TOF MS. A peak corresponding to the addition of a single group was found. Less than 1 % of the disubstituted chaconine was present in the sample, and it is expected that this could be eliminated through further purification. The monosubstituted chaconine is well suited for an internal standard although standard curves need to be produced to further test the applicability of this compound as an internal standard for GA quantitation.

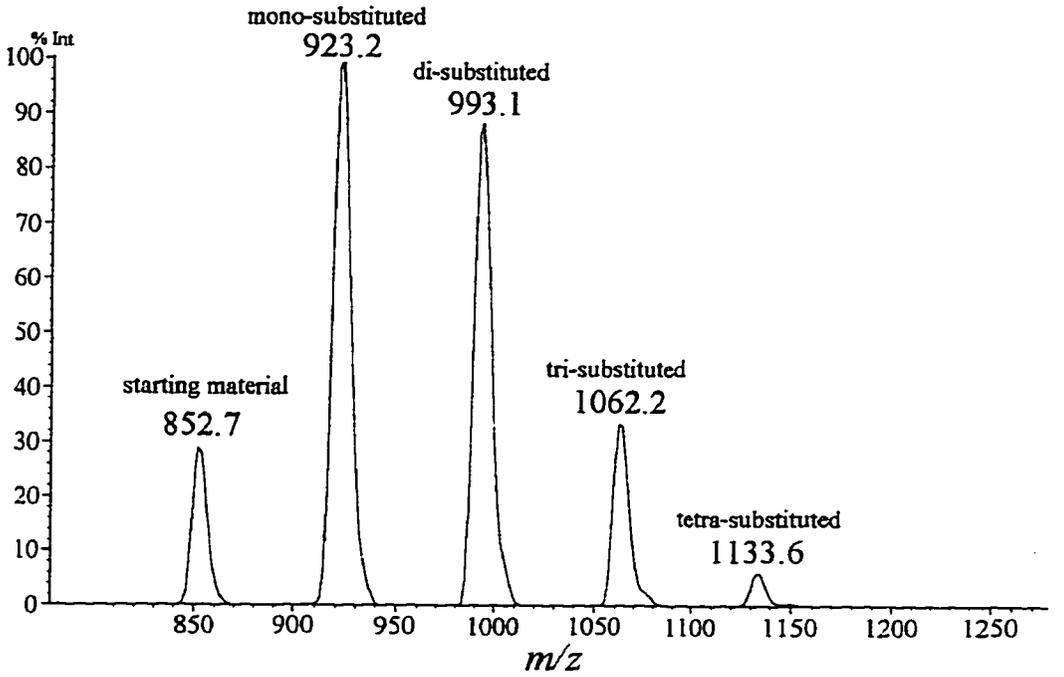


Figure 3.17 MALDI-TOF MS of Butylated α-Chaconine

One μL of a saturated matrix solution of THA in methanol:water (50:50) was added to probe and allowed to dry followed by 1 μL of sample. Sample is the summation of 100 shots using power setting of 80 on Kratos MALDI I. Starting material (chaconine, 852.7 *m/z*) was present as well as chaconine with up to four substituent groups added.

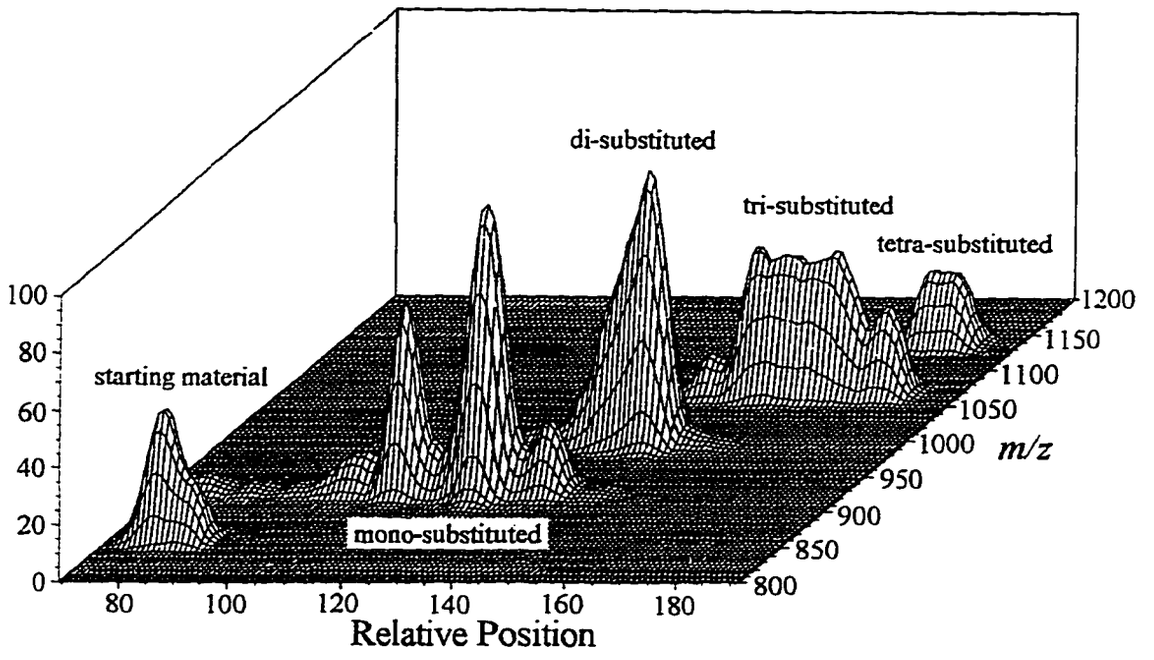


Figure 3.18 TLC/MALDI-TOF MS of Butylated Chaconine Mixture

After separation by TLC the TLC strip was affixed to the MALDI probe. A saturated solution of THA in methanol:water (50:50) was added to the entire strip (2 x 50 μL) and allowed to dry. The sample was obtained using a power setting of 80 on the KRATOS MALDI I. Each position along the TLC plate is the result of a single laser shot. The data was smoothed using a setting of 2.

4 Conclusions

Methods were developed to synthesize tomatine-protein conjugates with varying levels of substitution. While tomatine reacts readily with succinic anhydride, limiting the reaction and purifying the intermediates is more difficult. By performing the reaction at low temperatures without a catalyst, the number of succinyl groups added could be limited. The reaction mixture was then purified using an anion exchange column to produce mono-, di- and tri-succinylated tomatine. The limited solubility of succinylated tomatine required modifications to an active-ester method for linking the glycoalkaloid to the protein. By using N-hydroxy sulfosuccinimide to form the active ester rather than N-hydroxysuccinamide, the solubility of the intermediate in aqueous solvents was increased allowing for a greater number of tomatine groups to be added to BSA (7.4 groups/BSA molecule).

Both PAb and MAb were produced against tomatine protein conjugates. While both antibodies had high titres, the competition with tomatine was low and there was no recognition of tomatidine at the levels tested. The PAb competed better then the MAb with tomatine and tomatine conjugates. In both cases the antibody was found to recognize succinylated tomatine bound to the protein much more than free tomatine. Succinylated tomatine competed better in the ELISA than tomatine, but not as well as when bound to the protein. For the PAb the competition of the protein was found to be the same for two different conjugates when evaluated on the basis of the equivalent tomatine concentration present. The results of the antibody testing suggested the formation of antibodies to the carbohydrate portion of the molecule, including the succinyl linking arm and a portion of the carrier protein. The lack of recognition of the terminal ring of the alkaloid is likely due to the spiroaminoketal moiety present and the tautomerism between a ring form and open form. These two forms would decrease the affinity of antibody binding and inhibit the affinity maturation process of the immune response.

Two conjugates were prepared for use in a FPIA containing either one or two rhodamine groups attached to succinylated tomatine, although no antibody binding was observed with these compounds. It was hoped that multiple fluorophores would decrease the antibody

requirements for the FPIA. For further work in this area, it is possible to synthesize a multply substituted fluorophore of α-chaconine or α-solanine using the methods described earlier for tomatine which would allow for the investigation of the effects of multiple fluorophores on the antibody requirements and sensitivity of an FPIA.

Initial studies into the quantitation of GA using MALDI-TOF MS demonstrated a method suitable for routine GA analysis. Using tomatine as an internal standard, this method produced quantitative results similar to traditional HPLC analysis. The main advantage of MALDI-TOF MS was the speed of the analysis which required only 6 - 8 minutes per sample rather than the 40-50 minutes required for HPLC. As well the extensive sample extraction and cleanup procedure required for HPLC is not necessary for MALDI-TOF MS.

An internal standard was synthesized through the butylation of chaconine to allow the analysis of a greater range of GA including tomatine. Initial testing of butylated chaconine showed its applicability to GA analysis. Further testing of the new standard is required to determine standard response curves for various GA and to examine the internal standard when used with potato tissue.

It would be useful to test a wider range of GA using MALDI-TOF MS including some uncommon GA to determine which, if any, are not suitable for MALDI-TOF MS analysis.

As more drugs are designed which require the cholesterinase system for elimination from the body, the methods capable of analysing GA in tissue and blood samples will be required. Although the MALDI-TOF MS does not have a low enough sensitivity for these analyses, antibodies can be used to capture and concentrate samples. By combining the selectivity of antibody binding with the speed of MALDI-TOF MS, it may be possible to develop rapid and sensitive techniques for analysing GA in serum samples.

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Appendix

The appendix contains the raw and processed data for the following::

Sigmoidal Curve Calculations for Monoclonal Antibody Competition Curves Based on Concentration of Competitor Compound

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Concentration of Competitor Compound

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Equivalent Tomatine Concentration

Standard Curves Calculations for MALDI-TOF MS of GA

MALDI-TOF MS Analysis of Glycoalkaloids

Sigmoidal Curve Calculations for Monoclonal Antibody Competition Curves Based on Concentration of Competitor Compound

TOMATINE

a	0.966
b	14.490
c	11.496
d	0.662
R^2	0.487

SOLANINE

a	1.450
b	0.000
c	36.957
d	0.635
R^2	0.001

BSA-TOM-H

a	0.809
b	1.028
c	0.016
d	0.032
R^2	0.969

Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$
100	0.665	0.662	0.000	100	0.875	1.043	0.028	29.27	0.026	0.033	0.000
100	0.643	0.662	0.000	100	0.843	1.043	0.040	29.27	0.027	0.033	0.000
100	0.679	0.662	0.000	100	0.767	1.043	0.076	29.27	0.027	0.033	0.000
10	0.892	0.931	0.002	10	1.119	1.043	0.006	2.927	0.030	0.036	0.000
10	0.907	0.931	0.001	10	1.135	1.043	0.009	2.927	0.035	0.036	0.000
10	0.994	0.931	0.004	10	0.961	1.043	0.007	2.927	0.035	0.036	0.000
5	1.088	0.966	0.015	5	1.140	1.043	0.010	1.4635	0.038	0.040	0.000
5	1.032	0.966	0.004	5	1.069	1.043	0.001	1.4635	0.043	0.040	0.000
5	1.093	0.966	0.016	5	1.007	1.043	0.001	1.4635	0.042	0.040	0.000
1	1.102	0.966	0.018	1	1.155	1.042	0.013	0.2927	0.063	0.069	0.000
1	1.101	0.966	0.018	1	1.214	1.042	0.029	0.2927	0.074	0.069	0.000
1	1.193	0.966	0.052	1	1.083	1.042	0.002	0.2927	0.081	0.069	0.000
0.5	0.890	0.966	0.006	0.5	1.138	1.042	0.009	0.14635	0.112	0.104	0.000
0.5	1.016	0.966	0.002	0.5	1.015	1.042	0.001	0.14635	0.114	0.104	0.000
0.5	1.133	0.966	0.028	0.5	1.102	1.042	0.004	0.14635	0.115	0.104	0.000
0.1	0.832	0.966	0.018	0.1	1.172	1.042	0.017	0.02927	0.249	0.302	0.003
0.1	1.007	0.966	0.002	0.1	1.078	1.042	0.001	0.02927	0.310	0.302	0.000
0.1	1.149	0.966	0.033	0.1	1.072	1.042	0.001	0.02927	0.310	0.302	0.000
0.05	0.921	0.966	0.002	0.05	1.037	1.042	0.000	0.01464	0.386	0.437	0.003
0.05	1.023	0.966	0.003	0.05	1.030	1.042	0.000	0.01464	0.480	0.437	0.002
0.05	1.081	0.966	0.013	0.05	1.075	1.042	0.001	0.01464	0.473	0.437	0.001
0.01	0.917	0.966	0.002	0.01	1.144	1.042	0.010	0.00293	0.620	0.693	0.005
0.01	1.093	0.966	0.016	0.01	1.176	1.042	0.018	0.00293	0.662	0.693	0.001
0.01	1.230	0.966	0.070	0.01	1.155	1.042	0.013	0.00293	0.731	0.693	0.002
0.005	0.814	0.966	0.023	0.005	1.100	1.042	0.003	0.00146	0.758	0.747	0.000
0.005	0.960	0.966	0.000	0.005	1.084	1.042	0.002	0.00146	0.772	0.747	0.001
0.005	1.101	0.966	0.018	0.005	1.011	1.042	0.001	0.00146	0.822	0.747	0.006
0.001	0.614	0.966	0.124	0.001	1.012	1.042	0.001	0.00029	0.708	0.796	0.008
0.001	0.765	0.966	0.041	0.001	0.980	1.042	0.004	0.00029	0.758	0.796	0.001
0.001	0.902	0.966	0.004	0.001	0.928	1.042	0.013	0.00029	0.841	0.796	0.002
0.0001	0.600	0.966	0.134	0.0001	0.898	1.042	0.021	2.9E-05	0.798	0.808	0.000
0.0001	0.649	0.966	0.100	0.0001	0.932	1.042	0.012	2.9E-05	0.818	0.808	0.000
0.0001	0.784	0.966	0.033	0.0001	0.892	1.042	0.023	2.9E-05	0.837	0.808	0.001
	sum of squares		0.804		sum of squares		0.374		sum of squares		0.036

Sigmoidal Curve Calculations for Monoclonal Antibody Competition Curves Based on Concentration of Competitor Compound

BSA-TOM-L

a	0.981
b	1.107
c	1.679
d	0.110
R^2	0.984

T-S-P2-10-F3

a	1.005
b	1.448
c	9.112
d	0.073
R^2	0.979

Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$	Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$
95.76	0.104	0.120	0.000	100	0.093	0.101	0.000
95.76	0.120	0.120	0.000	100	0.104	0.101	0.000
95.76	0.154	0.120	0.001	100	0.106	0.101	0.000
9.576	0.200	0.221	0.000	10	0.521	0.508	0.000
9.576	0.208	0.221	0.000	10	0.497	0.508	0.000
9.576	0.216	0.221	0.000	10	0.506	0.508	0.000
4.788	0.319	0.318	0.000	5	0.686	0.730	0.002
4.788	0.337	0.318	0.000	5	0.677	0.730	0.003
4.788	0.313	0.318	0.000	5	0.830	0.730	0.010
0.9576	0.712	0.677	0.001	1	0.999	0.969	0.001
0.9576	0.671	0.677	0.000	1	0.924	0.969	0.002
0.9576	0.697	0.677	0.000	1	0.918	0.969	0.003
0.4788	0.794	0.807	0.000	0.5	0.986	0.992	0.000
0.4788	0.741	0.807	0.004	0.5	1.051	0.992	0.003
0.4788	0.822	0.807	0.000	0.5	1.051	0.992	0.003
0.09576	0.929	0.946	0.000	0.1	1.010	1.004	0.000
0.09576	0.900	0.946	0.002	0.1	0.956	1.004	0.002
0.09576	0.944	0.946	0.000	0.1	1.030	1.004	0.001
0.04788	0.999	0.964	0.001	0.05	1.074	1.005	0.005
0.04788	0.981	0.964	0.000	0.05	0.999	1.005	0.000
0.04788	1.027	0.964	0.004	0.05	1.084	1.005	0.006
0.00958	1.037	0.978	0.004	0.01	1.107	1.005	0.010
0.00958	1.042	0.978	0.004	0.01	1.108	1.005	0.011
0.00958	1.124	0.978	0.021	0.01	0.839	1.005	0.028
0.00479	1.008	0.979	0.001	0.005	0.973	1.005	0.001
0.00479	0.947	0.979	0.001	0.005	1.046	1.005	0.002
0.00479	0.745	0.979	0.055	0.005	0.970	1.005	0.001
0.00096	0.961	0.980	0.000	0.001	1.068	1.005	0.004
0.00096	0.910	0.980	0.005	0.001	0.998	1.005	0.000
0.00096	0.914	0.980	0.004	0.001	0.912	1.005	0.009
9.6E-05	1.008	0.981	0.001	0.0001	0.948	1.005	0.003
9.6E-05	1.015	0.981	0.001	0.0001	0.968	1.005	0.001
9.6E-05	1.014	0.981	0.001	0.0001	0.953	1.005	0.003
	sum of squares		0.115		sum of squares		0.115

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Concentration of Competitor Compound

TOMATINE

a	0.954
b	1.123
c	6.715
d	0.280
R^2	0.969

TOMATIDINE

a	1.410
b	0.027
c	36.120
d	0.385
R^2	0.518

BSA-TOM-H

a	0.954
b	0.706
c	0.006
d	0.188
R^2	0.984

Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$
100	0.336	0.311	0.001	100	0.834	0.890	0.003	14.08	0.148	0.191	0.002
100	0.302	0.311	0.000	100	0.898	0.890	0.000	14.08	0.130	0.191	0.004
100	0.287	0.311	0.001	100	0.794	0.890	0.009	14.08	0.136	0.191	0.003
10	0.614	0.543	0.005	10	0.931	0.906	0.001	1.408	0.208	0.204	0.000
10	0.531	0.543	0.000	10	0.873	0.906	0.001	1.408	0.208	0.204	0.000
10	0.516	0.543	0.001	10	0.834	0.906	0.005	1.408	0.190	0.204	0.000
5	0.730	0.672	0.003	5	0.944	0.911	0.001	0.704	0.289	0.213	0.006
5	0.620	0.672	0.003	5	0.901	0.911	0.000	0.704	0.223	0.213	0.000
5	0.641	0.672	0.001	5	0.971	0.911	0.004	0.704	0.223	0.213	0.000
1	0.937	0.883	0.003	1	0.944	0.923	0.000	0.1408	0.307	0.261	0.002
1	0.806	0.883	0.006	1	0.962	0.923	0.002	0.1408	0.280	0.261	0.000
1	0.840	0.883	0.002	1	0.882	0.923	0.002	0.1408	0.286	0.261	0.001
0.5	1.005	0.920	0.007	0.5	0.965	0.927	0.001	0.0704	0.358	0.300	0.003
0.5	0.904	0.920	0.000	0.5	0.928	0.927	0.000	0.0704	0.313	0.300	0.000
0.5	0.944	0.920	0.001	0.5	0.919	0.927	0.000	0.0704	0.310	0.300	0.000
0.1	0.998	0.948	0.003	0.1	0.983	0.939	0.002	0.01408	0.455	0.455	0.000
0.1	0.919	0.948	0.001	0.1	0.934	0.939	0.000	0.01408	0.425	0.455	0.001
0.1	0.992	0.948	0.002	0.1	0.989	0.939	0.003	0.01408	0.437	0.455	0.000
0.05	0.986	0.952	0.001	0.05	0.959	0.943	0.000	0.00704	0.488	0.545	0.003
0.05	0.913	0.952	0.001	0.05	0.962	0.943	0.000	0.00704	0.476	0.545	0.005
0.05	1.014	0.952	0.004	0.05	1.008	0.943	0.004	0.00704	0.491	0.545	0.003
0.01	1.026	0.954	0.005	0.01	0.983	0.955	0.001	0.00141	0.765	0.747	0.000
0.01	1.002	0.954	0.002	0.01	0.983	0.955	0.001	0.00141	0.946	0.747	0.039
0.01	1.005	0.954	0.003	0.01	0.995	0.955	0.002	0.00141	0.723	0.747	0.001
0.005	0.971	0.954	0.000	0.005	0.892	0.959	0.005	0.0007	0.789	0.813	0.001
0.005	0.968	0.954	0.000	0.005	1.017	0.959	0.003	0.0007	0.819	0.813	0.000
0.005	1.011	0.954	0.003	0.005	0.980	0.959	0.000	0.0007	0.840	0.813	0.001
0.001	0.889	0.954	0.004	0.001	1.038	0.970	0.005	0.00014	0.834	0.902	0.005
0.001	0.910	0.954	0.002	0.001	0.931	0.970	0.002	0.00014	0.928	0.902	0.001
0.001	0.846	0.954	0.012	0.001	0.956	0.970	0.000	0.00014	0.880	0.902	0.000
0.0001	0.861	0.954	0.009	0.0001	0.895	0.986	0.008	1.4E-05	0.898	0.943	0.002
0.0001	0.873	0.954	0.007	0.0001	0.913	0.986	0.005	1.4E-05	0.946	0.943	0.000
0.0001	0.944	0.954	0.000	0.0001	0.959	0.986	0.001	1.4E-05	0.973	0.943	0.001
		sum of squares	0.092			sum of squares	0.071			sum of squares	0.084

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Concentration of Competitor Compound

BSA-TOM-L

a	0.998
b	0.643
c	0.351
d	0.131
R^2	0.995

Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$
195	0.123	0.145	0.000
195	0.130	0.145	0.000
195	0.123	0.145	0.000
19.5	0.196	0.192	0.000
19.5	0.190	0.192	0.000
19.5	0.184	0.192	0.000
9.75	0.256	0.222	0.001
9.75	0.235	0.222	0.000
9.75	0.268	0.222	0.002
1.95	0.352	0.347	0.000
1.95	0.358	0.347	0.000
1.95	0.340	0.347	0.000
0.975	0.431	0.427	0.000
0.975	0.413	0.427	0.000
0.975	0.407	0.427	0.000
0.195	0.623	0.645	0.000
0.195	0.657	0.645	0.000
0.195	0.651	0.645	0.000
0.0975	0.714	0.734	0.000
0.0975	0.705	0.734	0.001
0.0975	0.714	0.734	0.000
0.0195	0.931	0.881	0.002
0.0195	0.901	0.881	0.000
0.0195	0.928	0.881	0.002
0.00975	0.877	0.919	0.002
0.00975	0.985	0.919	0.004
0.00975	0.895	0.919	0.001
0.00195	1.036	0.968	0.005
0.00195	0.991	0.968	0.001
0.00195	0.895	0.968	0.005
0.0002	0.946	0.991	0.002
0.0002	0.997	0.991	0.000
0.0002	0.967	0.991	0.001
	sum of squares		0.033

SOLANINE

a	1.028
b	7.972
c	7.538
d	0.798
R^2	0.939

Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$
100	0.797	0.798	0.000
100	0.782	0.798	0.000
100	0.815	0.798	0.000
10	0.809	0.820	0.000
10	0.806	0.820	0.000
10	0.845	0.820	0.001
5	1.045	1.020	0.001
5	1.009	1.020	0.000
5	1.006	1.020	0.000
1	1.100	1.028	0.005
1	1.006	1.028	0.001
1	1.052	1.028	0.001
0.5	1.073	1.028	0.002
0.5	1.073	1.028	0.002
0.5	1.036	1.028	0.000
0.1	1.045	1.028	0.000
0.1	0.970	1.028	0.003
0.1	0.982	1.028	0.002
0.05	1.009	1.028	0.000
0.05	0.994	1.028	0.001
0.05	1.018	1.028	0.000
0.01	0.997	1.028	0.001
0.01	1.000	1.028	0.001
0.01	1.079	1.028	0.003
0.005	1.067	1.028	0.001
0.005	0.967	1.028	0.004
0.005	1.021	1.028	0.000
0.001	1.018	1.028	0.000
0.001	1.039	1.028	0.000
0.001	1.067	1.028	0.001
0.0001	1.027	1.028	0.000
0.0001	1.027	1.028	0.000
0.0001	1.015	1.028	0.000
	sum of squares		0.032

SOLASODINE

a	1.545
b	0.027
c	30.629
d	0.286
R^2	0.556

Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$
100	0.852	0.905	0.003
100	0.836	0.905	0.005
100	0.873	0.905	0.001
10	0.855	0.925	0.005
10	0.885	0.925	0.002
10	0.845	0.925	0.006
5	0.967	0.931	0.001
5	0.964	0.931	0.001
5	0.945	0.931	0.000
1	0.979	0.945	0.001
1	1.042	0.945	0.010
1	0.988	0.945	0.002
0.5	0.970	0.951	0.000
0.5	0.961	0.951	0.000
0.5	1.061	0.951	0.012
0.1	0.955	0.964	0.000
0.1	1.003	0.964	0.001
0.1	0.997	0.964	0.001
0.05	0.979	0.970	0.000
0.05	0.967	0.970	0.000
0.05	1.006	0.970	0.001
0.01	0.958	0.984	0.001
0.01	1.085	0.984	0.010
0.01	1.009	0.984	0.001
0.005	0.952	0.990	0.001
0.005	1.003	0.990	0.000
0.005	0.939	0.990	0.003
0.001	0.961	1.003	0.002
0.001	1.048	1.003	0.002
0.001	0.945	1.003	0.003
0.0001	0.952	1.023	0.005
0.0001	0.973	1.023	0.002
0.0001	1.024	1.023	0.000
	sum of squares		0.084

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Concentration of Competitor Compound

T-S-P2-10-F3

a	0.990
b	0.753
c	1.838
d	0.158
R^2	0.988

FL1

a	0.991
b	4.747
c	3.136
d	0.831
R^2	0.788

Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$
100	0.190	0.197	0.000	46	0.843	0.831	0.000
100	0.192	0.197	0.000	46	0.819	0.831	0.000
100	0.213	0.197	0.000	46			
10	0.294	0.340	0.002	4.6	0.861	0.853	0.000
10	0.414	0.340	0.006	4.6	0.846	0.853	0.000
10	0.309	0.340	0.001	4.6			
5	0.449	0.424	0.001	2.3	0.957	0.961	0.000
5	0.414	0.424	0.000	2.3	0.966	0.961	0.000
5	0.405	0.424	0.000	2.3			
1	0.732	0.668	0.004	0.46	1.034	0.991	0.002
1	0.633	0.668	0.001	0.46	0.993	0.991	0.000
1	0.618	0.668	0.002	0.46			
0.5	0.825	0.763	0.004	0.23	1.064	0.991	0.005
0.5	0.735	0.763	0.001	0.23	0.975	0.991	0.000
0.5	0.793	0.763	0.001	0.23			
0.1	0.878	0.907	0.001	0.046	1.001	0.991	0.000
0.1	0.892	0.907	0.000	0.046	0.924	0.991	0.005
0.1	0.863	0.907	0.002	0.046			
0.05	0.988	0.939	0.002	0.023	1.007	0.991	0.000
0.05	0.933	0.939	0.000	0.023	1.004	0.991	0.000
0.05	0.907	0.939	0.001	0.023			
0.01	1.061	0.974	0.008	0.0046	1.016	0.991	0.001
0.01	0.945	0.974	0.001	0.0046	0.969	0.991	0.001
0.01	0.985	0.974	0.000	0.0046			
0.005	1.000	0.981	0.000	0.0023	0.984	0.991	0.000
0.005	0.930	0.981	0.003	0.0023	0.945	0.991	0.002
0.005	1.009	0.981	0.001	0.0023			
0.001	1.029	0.988	0.002	0.00046	1.061	0.991	0.005
0.001	0.950	0.988	0.001	0.00046	0.927	0.991	0.004
0.001	0.994	0.988	0.000	0.00046			
0.0001	1.079	0.990	0.008	4.6E-05	1.070	0.991	0.006
0.0001	0.918	0.990	0.005	4.6E-05	0.882	0.991	0.012
0.0001	0.936	0.990	0.003	4.6E-05			
	sum of squares		0.061		sum of squares		0.044

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Equivalent Tomatine Concentration

TOMATINE

a	0.954
b	1.123
c	6.715
d	0.280
R^2	0.969

TOMATIDINE

a	1.410
b	0.027
c	36.120
d	0.385
R^2	0.518

BSA-TOM-H

a	0.954
b	0.706
c	0.039
d	0.188
R^2	0.984

Conc uM	Exp Abs yi	Calc [ ] yc	$(yi-yc)^2$	Conc uM	Exp Abs yi	Calc [ ] yc	$(yi-yc)^2$	Conc uM	Exp Abs yi	Calc [ ] yc	$(yi-yc)^2$
100	0.336	0.311	0.001	100	0.834	0.890	0.003	95.76	0.148	0.191	0.002
100	0.302	0.311	0.000	100	0.898	0.890	0.000	95.76	0.130	0.191	0.004
100	0.287	0.311	0.001	100	0.794	0.890	0.009	95.76	0.136	0.191	0.003
10	0.614	0.543	0.005	10	0.931	0.906	0.001	9.576	0.208	0.204	0.000
10	0.531	0.543	0.000	10	0.873	0.906	0.001	9.576	0.208	0.204	0.000
10	0.516	0.543	0.001	10	0.834	0.906	0.005	9.576	0.190	0.204	0.000
5	0.730	0.672	0.003	5	0.944	0.911	0.001	4.788	0.289	0.213	0.006
5	0.620	0.672	0.003	5	0.901	0.911	0.000	4.788	0.223	0.213	0.000
5	0.641	0.672	0.001	5	0.971	0.911	0.004	4.788	0.223	0.213	0.000
1	0.937	0.883	0.003	1	0.944	0.923	0.000	0.9576	0.307	0.261	0.002
1	0.806	0.883	0.006	1	0.962	0.923	0.002	0.9576	0.280	0.261	0.000
1	0.840	0.883	0.002	1	0.882	0.923	0.002	0.9576	0.286	0.261	0.001
0.5	1.005	0.920	0.007	0.5	0.965	0.927	0.001	0.4788	0.358	0.300	0.003
0.5	0.904	0.920	0.000	0.5	0.928	0.927	0.000	0.4788	0.313	0.300	0.000
0.5	0.944	0.920	0.001	0.5	0.919	0.927	0.000	0.4788	0.310	0.300	0.000
0.1	0.998	0.948	0.003	0.1	0.983	0.939	0.002	0.09576	0.455	0.455	0.000
0.1	0.919	0.948	0.001	0.1	0.934	0.939	0.000	0.09576	0.425	0.455	0.001
0.1	0.992	0.948	0.002	0.1	0.989	0.939	0.003	0.09576	0.437	0.455	0.000
0.05	0.986	0.952	0.001	0.05	0.959	0.943	0.000	0.04788	0.488	0.545	0.003
0.05	0.913	0.952	0.001	0.05	0.962	0.943	0.000	0.04788	0.476	0.545	0.005
0.05	1.014	0.952	0.004	0.05	1.008	0.943	0.004	0.04788	0.491	0.545	0.003
0.01	1.026	0.954	0.005	0.01	0.983	0.955	0.001	0.00958	0.765	0.747	0.000
0.01	1.002	0.954	0.002	0.01	0.983	0.955	0.001	0.00958	0.946	0.747	0.039
0.01	1.005	0.954	0.003	0.01	0.995	0.955	0.002	0.00958	0.723	0.747	0.001
0.005	0.971	0.954	0.000	0.005	0.892	0.959	0.005	0.00479	0.789	0.813	0.001
0.005	0.968	0.954	0.000	0.005	1.017	0.959	0.003	0.00479	0.819	0.813	0.000
0.005	1.011	0.954	0.003	0.005	0.980	0.959	0.000	0.00479	0.840	0.813	0.001
0.001	0.889	0.954	0.004	0.001	1.038	0.970	0.005	0.00096	0.834	0.902	0.005
0.001	0.910	0.954	0.002	0.001	0.931	0.970	0.002	0.00096	0.928	0.902	0.001
0.001	0.846	0.954	0.012	0.001	0.956	0.970	0.000	0.00096	0.880	0.902	0.000
0.0001	0.861	0.954	0.009	0.0001	0.895	0.986	0.008	9.6E-05	0.898	0.943	0.002
0.0001	0.873	0.954	0.007	0.0001	0.913	0.986	0.005	9.6E-05	0.946	0.943	0.000
0.0001	0.944	0.954	0.000	0.0001	0.959	0.986	0.001	9.6E-05	0.973	0.943	0.001
		sum of squares	0.092			sum of squares	0.071			sum of squares	0.084

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Equivalent Tomatine Concentration

BSA-TOM-L

a	0.998
b	0.643
c	0.053
d	0.131
R^2	0.995

SOLANINE

a	1.028
b	7.987
c	7.557
d	0.798
R^2	0.939

SOLASODINE

a	1.545
b	0.027
c	30.629
d	0.286
R^2	0.556

Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$	Conc uM	Exp Abs y_t	Calc [ ] y_c	$(y_t-y_c)^2$
29.27	0.123	0.145	0.000	100	0.797	0.798	0.000	100	0.852	0.905	0.003
29.27	0.130	0.145	0.000	100	0.782	0.798	0.000	100	0.836	0.905	0.005
29.27	0.123	0.145	0.000	100	0.815	0.798	0.000	100	0.873	0.905	0.001
2.927	0.196	0.192	0.000	10	0.809	0.820	0.000	10	0.855	0.925	0.005
2.927	0.190	0.192	0.000	10	0.806	0.820	0.000	10	0.885	0.925	0.002
2.927	0.184	0.192	0.000	10	0.845	0.820	0.001	10	0.845	0.925	0.006
1.4635	0.256	0.222	0.001	5	1.045	1.020	0.001	5	0.967	0.931	0.001
1.4635	0.235	0.222	0.000	5	1.009	1.020	0.000	5	0.964	0.931	0.001
1.4635	0.268	0.222	0.002	5	1.006	1.020	0.000	5	0.945	0.931	0.000
0.2927	0.352	0.347	0.000	1	1.100	1.028	0.005	1	0.979	0.945	0.001
0.2927	0.358	0.347	0.000	1	1.006	1.028	0.000	1	1.042	0.945	0.010
0.2927	0.340	0.347	0.000	1	1.052	1.028	0.001	1	0.988	0.945	0.002
0.14635	0.431	0.427	0.000	0.5	1.073	1.028	0.002	0.5	0.970	0.951	0.000
0.14635	0.413	0.427	0.000	0.5	1.073	1.028	0.002	0.5	0.961	0.951	0.000
0.14635	0.407	0.427	0.000	0.5	1.036	1.028	0.000	0.5	1.061	0.951	0.012
0.02927	0.623	0.645	0.000	0.1	1.045	1.028	0.000	0.1	0.955	0.964	0.000
0.02927	0.657	0.645	0.000	0.1	0.970	1.028	0.003	0.1	1.003	0.964	0.001
0.02927	0.651	0.645	0.000	0.1	0.982	1.028	0.002	0.1	0.997	0.964	0.001
0.01464	0.714	0.734	0.000	0.05	1.009	1.028	0.000	0.05	0.979	0.970	0.000
0.01464	0.705	0.734	0.001	0.05	0.994	1.028	0.001	0.05	0.967	0.970	0.000
0.01464	0.714	0.734	0.000	0.05	1.018	1.028	0.000	0.05	1.006	0.970	0.001
0.00293	0.931	0.881	0.002	0.01	0.997	1.028	0.001	0.01	0.958	0.984	0.001
0.00293	0.901	0.881	0.000	0.01	1.000	1.028	0.001	0.01	1.085	0.984	0.010
0.00293	0.928	0.881	0.002	0.01	1.079	1.028	0.003	0.01	1.009	0.984	0.001
0.00146	0.877	0.919	0.002	0.005	1.067	1.028	0.001	0.005	0.952	0.990	0.001
0.00146	0.985	0.919	0.004	0.005	0.967	1.028	0.004	0.005	1.003	0.990	0.000
0.00146	0.895	0.919	0.001	0.005	1.021	1.028	0.000	0.005	0.939	0.990	0.003
0.00029	1.036	0.968	0.005	0.001	1.018	1.028	0.000	0.001	0.961	1.003	0.002
0.00029	0.991	0.968	0.001	0.001	1.039	1.028	0.000	0.001	1.048	1.003	0.002
0.00029	0.895	0.968	0.005	0.001	1.067	1.028	0.001	0.001	0.945	1.003	0.003
2.9E-05	0.946	0.991	0.002	0.0001	1.027	1.028	0.000	0.0001	0.952	1.023	0.005
2.9E-05	0.997	0.991	0.000	0.0001	1.027	1.028	0.000	0.0001	0.973	1.023	0.002
2.9E-05	0.967	0.991	0.001	0.0001	1.015	1.028	0.000	0.0001	1.024	1.023	0.000
	sum of squares		0.033		sum of squares		0.032		sum of squares		0.084

Sigmoidal Curve Calculations for Polyclonal Antibody Competition Curves Based on Equivalent Tomatine Concentration

T-S-P2-10-F3

a	0.990
b	0.753
c	1.838
d	0.158
R^2	0.988

FL1

a	0.991
b	4.747
c	3.136
d	0.831
R^2	0.788

Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$	Conc uM	Exp Abs y_i	Calc [ ] y_c	$(y_i-y_c)^2$
100	0.190	0.197	0.000	46	0.843	0.831	0.000
100	0.192	0.197	0.000	46	0.819	0.831	0.000
100	0.213	0.197	0.000	46			
10	0.294	0.340	0.002	4.6	0.861	0.853	0.000
10	0.414	0.340	0.006	4.6	0.846	0.853	0.000
10	0.309	0.340	0.001	4.6			
5	0.449	0.424	0.001	2.3	0.957	0.961	0.000
5	0.414	0.424	0.000	2.3	0.966	0.961	0.000
5	0.405	0.424	0.000	2.3			
1	0.732	0.668	0.004	0.46	1.034	0.991	0.002
1	0.633	0.668	0.001	0.46	0.993	0.991	0.000
1	0.618	0.668	0.002	0.46			
0.5	0.825	0.763	0.004	0.23	1.064	0.991	0.005
0.5	0.735	0.763	0.001	0.23	0.975	0.991	0.000
0.5	0.793	0.763	0.001	0.23			
0.1	0.878	0.907	0.001	0.046	1.001	0.991	0.000
0.1	0.892	0.907	0.000	0.046	0.924	0.991	0.005
0.1	0.863	0.907	0.002	0.046			
0.05	0.988	0.939	0.002	0.023	1.007	0.991	0.000
0.05	0.933	0.939	0.000	0.023	1.004	0.991	0.000
0.05	0.907	0.939	0.001	0.023			
0.01	1.061	0.974	0.008	0.0046	1.016	0.991	0.001
0.01	0.945	0.974	0.001	0.0046	0.969	0.991	0.001
0.01	0.985	0.974	0.000	0.0046			
0.005	1.000	0.981	0.000	0.0023	0.984	0.991	0.000
0.005	0.930	0.981	0.003	0.0023	0.945	0.991	0.002
0.005	1.009	0.981	0.001	0.0023			
0.001	1.029	0.988	0.002	0.00046	1.061	0.991	0.005
0.001	0.950	0.988	0.001	0.00046	0.927	0.991	0.004
0.001	0.994	0.988	0.000	0.00046			
0.0001	1.079	0.990	0.008	4.6E-05	1.070	0.991	0.006
0.0001	0.918	0.990	0.005	4.6E-05	0.882	0.991	0.012
0.0001	0.936	0.990	0.003	4.6E-05			
	sum of squares		0.061		sum of squares		0.044

Standard Curve Calculations for MALDI-TOF MS of Chaconine

Curve Parameters	
a	0.0000
b	1.7366
c	-0.0112

R^2
0.9742

Standard	Conc (uM)	Ave Exp Signal	Calc Signal	$(y_e-y_c)^2$
5	5.56	13.83	9.31	20.42
5	5.56			
5	5.56			
10	10.92	17.49	17.63	0.02
10	10.92	12.96	17.63	21.73
10	10.92	25.94	17.63	69.15
10	10.92	14.15	17.63	12.12
10	10.92	19.49	17.63	3.47
10	10.92	18.85	17.63	1.49
35	37.72	51.64	49.56	4.36
35	37.72	51.40	49.56	3.41
35	37.72	52.82	49.56	10.65
35	37.72	44.45	49.56	26.06
35	37.72	38.69	49.56	117.99
35	37.72	47.32	49.56	4.98
50	53.80	62.10	60.98	1.25
50	53.80	60.55	60.98	0.19
50	53.80	63.17	60.98	4.78
50	53.80	65.48	60.98	20.17
50	53.80	59.35	60.98	2.68
50	53.80	59.47	60.98	2.28

Sum of Squares	327.2

Standard Curve Calculations for MALDI-TOF MS of Solanine

Curve Parameters	
a	0.0000
b	2.4609
c	-0.0259

R^2
0.9841

Standard	Conc (uM)	Ave Exp Signal	Calc Signal	$(y_e-y_c)^2$
5	3.76	13.10	8.89	17.71
5	3.76			
5	3.76			
10	7.52	15.19	17.04	3.41
10	7.52	17.39	17.04	0.12
10	7.52	19.83	17.04	7.78
10	7.52	17.31	17.04	0.08
10	7.52	17.49	17.04	0.20
10	7.52	20.47	17.04	11.74
35	26.32	47.58	46.80	0.62
35	26.32	44.01	46.80	7.76
35	26.32	39.83	46.80	48.47
35	26.32	44.62	46.80	4.75
35	26.32	48.80	46.80	4.03
35	26.32	44.89	46.80	3.65
50	37.60	54.42	55.85	2.03
50	37.60	53.91	55.85	3.76
50	37.60	55.51	55.85	0.11
50	37.60	62.85	55.85	49.03
50	37.60	55.29	55.85	0.31
50	37.60	57.61	55.85	3.11

Sum of Squares	168.7

MALDI-TOF MS Analysis of Glycoalkaloids

Raw Data of Peak Heights

Blank cells were either zero values or not obtained

Sample wt (g)	0.3957	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Russet Burbank	Chac	10.45	16.79	16.70	16.27	15.90	17.59	77.82	67.65	77.88
8 month #1	Sol	6.59	14.28	13.30	11.21	13.14	14.15	59.68	51.26	58.67
	Chac + K							2.39	1.41	2.08
	Sol + K	4.63		4.01	7.63	8.15	3.19	16.70	19.18	13.33
	Tom	19.09	39.46	34.07	26.26	31.10	37.68	131.71	116.88	129.23
	Tom + K	18.57	29.90	20.53	27.82	26.96	22.98	75.34	67.56	62.07
	% Ht chac	27.75	24.21	30.59	30.09	27.39	29.00	38.74	37.44	41.80
	% Ht sol	29.79	20.59	31.70	34.84	36.67	28.59	36.89	38.19	37.64

Sample wt (g)	0.4070	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Russet Burbank	Chac	58.58	60.02	57.48	41.27	49.33	53.06	44.18	40.41	42.59
8 month #2	Sol	40.41	44.70	47.49	28.31	33.82	45.68	29.72	22.61	28.68
	Chac + K	4.32			3.43			1.81	1.47	3.00
	Sol + K	15.53	15.87	6.62	14.77	11.12	11.76	13.51	9.13	10.78
	Tom	88.14	111.40	133.43	75.98	87.22	122.09	70.01	60.39	69.27
	Tom + K	63.66	61.55	58.03	50.52	56.95	61.27	50.74	32.20	38.14
	% Ht chac	41.44	34.70	30.02	35.34	34.22	28.94	38.09	45.23	42.44
	% Ht sol	36.85	35.02	28.26	34.06	31.17	31.33	35.80	34.28	36.74

Sample wt (g)	0.3976	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Russet Burbank	Chac	28.09	34.41	28.22	8.73	5.76	11.95	74.63	61.18	98.47
8 month #3	Sol	16.15	23.84	13.17	7.54	8.03	8.27	42.22	39.31	46.81
	Chac + K	2.27	1.96				1.07		1.29	4.56
	Sol + K	8.03	10.02	4.75			3.19		8.39	20.37
	Tom	50.43	55.61	50.25	25.52	10.36	15.41	84.13	90.84	125.12
	Tom + K	37.25	48.16	24.36	12.70	4.44	8.33	39.86	33.39	73.90
	% Ht chac	34.63	35.05	37.82	22.84	38.92	54.84	60.19	50.29	51.77
	% Ht sol	27.58	32.63	24.02	19.73	54.26	48.27	34.05	38.40	33.76

MALDI-TOF MS Analysis of Glycoalkaloids

Raw Data of Peak Heights

Blank cells were either zero values or not obtained

Sample wt (g)	0.3977	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Shepedy	Chac	30.33	7.78	5.36	12.65	14.95	18.29	18.38	19.30	
#1	Sol	25.06	3.89	2.02	10.48	10.57	6.40	8.92	9.65	
	Chac + K		2.94			2.36	1.96			
	Sol + K	11.31	2.67	2.21	5.09	3.09	4.63	6.25	3.19	
	Tom	83.95	13.20	11.37	48.84	53.86	47.21	69.76	75.55	
	Tom + K	62.75	12.07	15.99	37.41	41.57	31.00	45.62	40.50	
	% Ht chac	20.67	42.42	19.59	14.67	18.14	25.89	15.93	16.63	
	% Ht sol	24.79	25.96	15.46	18.05	14.31	14.10	13.15	11.06	

Sample wt (g)	0.4062	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Shepedy	Chac	23.38	18.63	16.42	24.11	21.11	27.57	17.65	14.92	10.63
#2	Sol	9.80	14.06	7.69	10.42	12.81	19.36	6.22	8.43	6.50
	Chac + K	3.22	1.90		2.67			2.51	2.30	1.90
	Sol + K	5.73	8.52	4.53	5.64	6.13	9.47	3.65	5.55	4.78
	Tom	60.45	63.30	50.52	56.37	65.47	82.75	44.33	34.93	47.95
	Tom + K	51.87	41.36	34.56	48.38	62.44	90.90	26.23	38.82	46.51
	% Ht chac	23.68	19.62	19.30	25.57	16.50	15.88	28.57	23.35	13.26
	% Ht sol	13.83	21.57	14.36	15.33	14.81	16.60	13.99	18.96	11.94

Sample wt (g)	0.3972	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Shepedy	Chac	7.97	3.46	5.36	15.99	15.41	11.18	22.00	21.08	30.58
#3	Sol	4.90	2.91	3.80	9.10	6.10	7.38	7.17	11.46	18.66
	Chac + K	1.16				2.54		2.51	1.29	2.14
	Sol + K	2.85	2.14	2.57	4.81	2.11	3.77	2.18	4.81	5.48
	Tom	40.99	17.56	22.95	69.36	51.75	43.90	63.97	56.68	68.23
	Tom + K	24.72	13.45	20.40	40.78	45.83	43.69	29.56	45.99	40.81
	% Ht chac	13.89	11.16	12.36	14.52	18.40	12.76	26.21	21.79	30.01
	% Ht sol	11.79	16.29	14.69	12.63	8.41	12.73	10.00	15.85	22.14

MALDI-TOF MS Analysis of Glycoalkaloids

Raw Data of Peak Heights

Blank cells were either zero values or not obtained

Sample wt (g)	0.3979	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Yukon Gold #1	Chac	13.79	10.05	5.48	6.34	3.00	7.38	18.35	17.74	18.84
	Sol	8.18	8.36	5.85	3.86	1.78	3.22	13.02	8.88	11.43
	Chac + K	2.76		1.99	2.79	1.01		2.42		2.14
	Sol + K	3.52	3.68	3.55	2.30	1.53	2.42	4.26	4.96	2.14
	Tom	63.45	40.99	43.26	23.77	6.25	16.97	101.19	74.45	66.61
	Tom + K	37.10	31.56	35.08	16.45	9.80	15.90	46.81	46.69	38.02
	% Ht chac	16.46	13.85	9.54	22.70	24.98	22.45	14.03	14.64	20.05
	% Ht sol	11.64	16.60	12.00	15.32	20.62	17.16	11.68	11.42	12.97

Sample wt (g)	0.4002	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Yukon Gold #2	Chac	9.38	10.20	5.73	14.03	9.31	6.92	12.62	23.96	21.20
	Sol	8.39	5.73	7.63	8.79	5.70	4.11	10.05	17.37	13.60
	Chac + K		2.24	1.41	2.67	1.59	1.75	2.48	2.73	1.78
	Sol + K	3.68	2.85	2.88	4.96	3.52	2.33	4.93	5.12	3.62
	Tom	66.39	44.85	44.52	45.96	22.67	35.97	74.79	98.68	84.22
	Tom + K	37.47	37.93	31.53	45.93	36.64	32.63	50.09	70.13	57.35
	% Ht chac	9.03	15.03	9.39	18.17	18.38	12.64	12.09	15.81	16.23
	% Ht sol	11.62	10.36	13.82	14.96	15.55	9.39	12.00	13.32	12.16

Sample wt (g)	0.4077	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Yukon Gold #3	Chac	12.07	11.83	8.76	11.86	9.19	2.76	6.77	2.51	4.75
	Sol	6.65	9.83	7.84	9.71	5.06	3.12	3.89		4.60
	Chac + K									
	Sol + K	4.93	4.44	6.10	3.52	3.55	1.41	2.14	1.19	1.41
	Tom	51.04	48.13	47.86	60.02	26.72	18.08	25.25	6.80	14.74
	Tom + K	38.08	40.13	36.09	34.41	31.89	21.66	21.72	8.55	11.03
	% Ht chac	13.54	13.40	10.43	12.56	15.68	6.95	14.41	16.35	18.43
	% Ht sol	12.99	16.17	16.61	14.01	14.69	11.40	12.84	7.75	23.32

MALDI-TOF MS Analysis of Glycoalkaloids

Raw Data of Peak Heights

Blank cells were either zero values or not obtained

Sample wt (g)	0.2020	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Russet Burbank	Chac	11.52	11.52	13.54	18.32	11.83	29.23	30.09	21.20	10.66
20 month #1	Sol	8.18	8.18	9.59	10.97	9.38	23.07	16.12	9.77	4.38
	Chac + K		1.29		2.05	1.41				
	Sol + K	2.45	2.45	2.48	2.91	1.62	1.65	4.38	3.19	0.98
	Tom	22.12	22.12	15.13	39.74	26.72	55.24	54.50	47.55	30.97
	Tom + K	18.17	18.17	9.80	17.31	9.65	12.47	26.01	30.21	8.73
	% Ht chac	28.59	31.79	54.31	35.71	36.40	43.17	37.37	27.26	26.85
	% Ht sol	26.38	26.38	48.42	24.33	30.24	36.51	25.46	16.67	13.50

Sample wt (g)	0.1997	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Russet Burbank	Chac	19.24	16.36	27.48	26.65	26.75	34.13	28.74	42.52	37.65
20 month #2	Sol	12.99	6.16	19.21	16.08	15.81	21.20	13.02	25.83	25.83
	Chac + K	2.79		1.16					1.56	
	Sol + K	5.15	2.54	3.58	4.20	5.42	4.04	3.74	3.12	4.26
	Tom	55.24	37.87	45.96	60.72	59.77	64.86	61.12	86.55	103.55
	Tom + K	29.93	19.24	25.35	24.26	29.41	28.77	24.72	27.79	40.04
	% Ht chac	25.87	28.65	41.32	31.36	30.00	36.45	33.48	38.55	26.22
	% Ht sol	21.30	15.23	32.88	23.86	23.81	26.96	19.52	25.32	20.96

Sample wt (g)	0.1996	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Russet Burbank	Chac	8.92	7.94	12.75	10.32	25.52	11.00	38.14	55.24	49.85
20 month #3	Sol	9.25	7.20	6.89	11.86	20.50	7.32	17.92	27.76	29.63
	Chac + K						1.10	1.44	1.07	
	Sol + K	2.18		3.19	1.84	7.02	2.88	3.71	2.14	6.10
	Tom	17.19	18.78	17.56	39.71	47.21	30.58	93.93	92.52	119.00
	Tom + K	6.25	9.59	7.84	9.56	27.94	19.39	25.03	27.70	41.48
	% Ht chac	38.05	27.99	50.20	20.95	33.96	24.21	33.27	46.84	31.06
	% Ht sol	48.76	25.38	39.69	27.81	36.62	20.41	18.18	24.87	22.26

MALDI-TOF MS Analysis of Glycoalkaloids

Raw Data of Peak Heights

Blank cells were either zero values or not obtained

Sample wt (g)	0.4185	Spot 1			Spot 2			Spot 3		
Sample	**Peak**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**
Comm Peeled	Chac		2.45	0.98	2.79	2.27	2.82	1.75	1.81	2.70
#1	Sol	3.34		1.44	2.63	2.91	2.30	3.06	1.44	2.88
	Chac + K		1.16		2.48			2.67		1.78
	Sol + K	2.79		1.07	1.96	2.48	1.72	2.85	1.35	
	Tom	25.34	36.52	28.34	64.92	103.40	99.08	39.31	58.49	47.00
	Tom + K	15.93	26.72	23.77	47.37	75.03	74.14	29.23	38.39	28.34
	% Ht chac	0.00	5.71	1.88	4.69	1.27	1.63	6.45	1.87	5.95
	% Ht sol	14.85	0.00	4.82	4.09	3.02	2.32	8.62	2.88	3.82

Sample wt (g)	0.3994	Spot 1			Spot 2			Spot 3		
Sample	**Peak**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**
Comm Peeled	Chac	2.48	1.90	1.72	2.51	2.73		2.79	2.79	2.97
#2	Sol	2.85		3.03	2.48	2.09		2.82	2.82	3.77
	Chac + K	2.57	1.59		3.37	1.23		2.08	2.08	2.97
	Sol + K		1.90	3.22	2.54	1.65		2.08	2.08	
	Tom	82.78	83.85	105.48	61.76	80.18	33.33	91.45	91.45	146.60
	Tom + K	49.57	55.55	61.80	44.06	44.33	19.78	66.79	66.79	92.59
	% Ht chac	3.82	2.50	1.03	5.56	3.18		3.08	3.08	2.48
	% Ht sol	2.15	1.36	3.74	4.74	3.00		3.10	3.10	1.58

Sample wt (g)	0.4014	Spot 1			Spot 2			Spot 3		
Sample	**Peak**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**
Comm Peeled	Chac	3.22	3.22	3.19	2.85	1.93	1.35	1.72	1.56	2.24
#3	Sol	2.79	2.79	2.48	1.78	3.09	2.11	2.39	2.02	2.30
	Chac + K	2.30	2.30	2.24		1.41	1.69		1.26	
	Sol + K	2.21	2.21	1.81	2.67	2.11	2.05	1.62	2.18	1.96
	Tom	86.52	86.52	98.93	59.10	51.13	63.08	80.51	63.48	70.50
	Tom + K	55.61	55.61	68.96	34.96	51.75	45.34	48.93	48.62	45.53
	% Ht chac	3.88	3.88	3.23	3.03	3.25	2.80	1.33	2.52	1.93
	% Ht sol	3.52	3.52	2.56	4.73	5.05	3.84	3.10	3.75	3.67

MALDI-TOF MS Analysis of Glycoalkaloids

Raw Data of Peak Heights

Blank cells were either zero values or not obtained

Sample wt (g)	0.3977	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Comm Unpeeled #1	Chac	4.75	2.63	1.93	6.56	2.60	2.73	14.43	10.32	6.50
	Sol	2.88	2.63		3.37	2.30		4.56	4.38	3.55
	Chac + K	2.11			2.45		1.07	2.21		1.96
	Sol + K	1.93		1.04	2.48		1.35	3.12	2.08	2.63
	Tom	38.63	20.83	18.50	51.10	40.62	30.51	136.40	123.96	84.38
	Tom + K	28.95	26.38	21.05	34.38	30.12	22.03	80.61	79.63	63.11
	% Ht chac	10.15	5.57	4.88	10.54	3.68	7.23	7.67	5.07	5.74
	% Ht sol	7.12	5.57	2.63	6.84	3.25	2.57	3.54	3.17	4.19

Sample wt (g)	0.4062	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Comm Unpeeled #2	Chac	5.12	3.65	3.62	5.33	5.39	8.33	8.39	4.72	5.76
	Sol	3.58	3.19	2.30	1.72	1.69	2.27	3.95	4.38	2.67
	Chac + K	2.94	1.23	1.81	1.75		2.67			1.69
	Sol + K	1.41	2.24	2.11	1.19	2.11	2.85	1.99	1.87	1.41
	Tom	84.10	40.35	40.56	57.87	62.93	65.13	94.15	79.81	85.91
	Tom + K	42.56	38.63	35.66	30.27	56.22	45.37	50.83	49.14	50.49
	% Ht chac	6.36	6.18	7.12	8.03	4.52	9.95	5.79	3.66	5.46
	% Ht sol	3.94	6.88	5.79	3.30	3.19	4.63	4.10	4.85	2.99

Sample wt (g)	0.3972	Spot 1			Spot 2			Spot 3		
Sample	Peak	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
Comm Unpeeled #3	Chac	7.20	7.11	8.61	3.03	3.68	6.04	7.87	2.33	3.62
	Sol	2.60	5.73	3.77	3.74	2.94	1.62	3.98	1.50	3.55
	Chac + K		2.30	1.47	2.67	2.21			1.07	
	Sol + K	2.48	1.78	1.75	2.33	2.30	1.62	2.48	1.41	
	Tom	99.94	98.93	71.08	49.33	42.34	44.85	57.90	20.99	40.47
	Tom + K	69.24	81.99	57.23	38.57	27.27	31.53	32.48	24.23	37.59
	% Ht chac	4.26	5.20	7.86	6.48	8.46	7.91	8.71	7.52	4.64
	% Ht sol	3.00	4.15	4.30	6.91	7.53	4.24	7.15	6.44	4.55

MALDI-TOF MS Analysis of Glycoalkaloids

Calculations

Blank cells were either zero values or not obtained

	Curve Parameters						
	a	b	c		a	b	c
Chaconine	0.000	1.737	-0.011	Solanine	0.000	2.461	-0.026

Sample	Wt (g)
Russet Burbank-8 #1	0.3957

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	27.75	24.21	30.59	30.09	27.39	29.00	38.74	37.44	41.80	
% Ht sol	29.79	20.59	31.70	34.84	36.67	28.59	36.89	38.19	37.64	Ave
mg chaconine/100g	9.14	7.83	10.24	10.04	9.01	9.62	13.66	13.08	15.06	10.85
mg solanine/100 g	7.20	4.69	7.77	8.75	9.36	6.85	9.43	9.88	9.69	8.18

Sample	Wt (g)
Russet Burbank-8 #2	0.4070

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	41.44	34.70	30.02	35.34	34.22	28.94	38.09	45.23	42.44	
% Ht sol	36.85	35.02	28.26	34.06	31.17	31.33	35.80	34.28	36.74	Ave
mg chaconine/100g	14.48	11.58	9.74	11.84	11.38	9.33	13.00	16.28	14.94	12.51
mg solanine/100 g	9.16	8.57	6.57	8.27	7.40	7.44	8.82	8.34	9.12	8.19

Sample	Wt (g)
Russet Burbank-8 #3	0.3976

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	34.63	35.05	37.82	22.84	38.92	54.84	60.19	50.29	51.77	
% Ht sol	27.58	32.63	24.02	19.73	54.26	48.27	34.05	38.40	33.76	Ave
mg chaconine/100g	11.82	12.00	13.19	7.30	13.67	22.22	26.33	19.39	20.26	16.24
mg solanine/100 g	6.53	8.02	5.56	4.45	17.54	13.94	8.46	9.90	8.37	9.20

Overall	Chac	Sol	Total
Average	13.20	8.52	21.72
Std Dev	2.76	0.58	3.32

MALDI-TOF MS Analysis of Glycoalkaloids

Calculations

Blank cells were either zero values or not obtained

Sample	Wt (g)
Shepedy-8 #1	0.3977

	Spot 1			Spot 2			Spot 3			
	Run 1	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	
% Ht chac	20.67	42.42	19.59	14.67	18.14	25.89	15.93	16.63		
% Ht sol	24.79	25.96	15.46	18.05	14.31	14.10	13.15	11.06		**Ave**
mg chaconine/100 g	6.54	15.28	6.16	4.51	5.66	8.40	4.92	5.16	0.00	6.29
mg solanine/100 g	5.76	6.08	3.40	4.03	3.13	3.08	2.86	2.38	0.00	3.41

Sample	Wt (g)
Shepedy-8 #2	0.4062

	Spot 1			Spot 2			Spot 3			
	Run 1	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	
% Ht chac	23.68	19.62	19.30	25.57	16.50	15.88	28.57	23.35		
% Ht sol	13.83	21.57	14.36	15.33	14.81	16.60	13.99	18.96		**Ave**
mg chaconine/100 g	7.44	6.04	5.93	8.11	5.01	4.80	9.21	7.32	0.00	5.99
mg solanine/100 g	2.95	4.81	3.08	3.30	3.18	3.60	2.99	4.16	0.00	3.12

Sample	Wt (g)
Shepedy-8 #3	0.3972

	Spot 1			Spot 2			Spot 3			
	Run 1	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	
% Ht chac	13.89	11.16	12.36	14.52	18.40	12.76	26.21	21.79	30.01	
% Ht sol	11.79	16.29	14.69	12.63	8.41	12.73	10.00	15.85	22.14	**Ave**
mg chaconine/100 g	4.26	3.38	3.77	4.47	5.76	3.90	8.53	6.93	9.98	5.66
mg solanine/100 g	2.55	3.60	3.22	2.74	1.79	2.76	2.14	3.50	5.07	3.04

Overall	**Chac**	**Sol**	**Total**
Average	5.98	3.19	9.17
Std Dev	0.31	0.20	0.50

MALDI-TOF MS Analysis of Glycoalkaloids

Calculations

Blank cells were either zero values or not obtained

Sample	Wt (g)
Yukon Gold-8 #1	0.3979

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	16.46	13.85	9.54	22.70	24.98	22.45	14.03	14.64	20.05	
% Ht sol	11.64	16.60	12.00	15.32	20.62	17.16	11.68	11.42	12.97	Ave
mg chaconine/100 g	5.10	4.24	2.87	7.24	8.07	7.16	4.30	4.50	6.32	5.53
mg solanine/100 g	2.51	3.67	2.59	3.37	4.67	3.81	2.52	2.46	2.82	3.16

Sample	Wt (g)
Yukon Gold-8 #2	0.4002

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	9.03	15.03	9.39	18.17	18.38	12.64	12.09	15.81	16.23	
% Ht sol	11.62	10.36	13.82	14.96	15.55	9.39	12.00	13.32	12.16	Ave
mg chaconine/100 g	2.69	4.60	2.80	5.64	5.71	3.83	3.65	4.85	4.99	4.31
mg solanine/100 g	2.49	2.21	3.00	3.26	3.40	1.99	2.58	2.88	2.61	2.71

Sample	Wt (g)
Yukon Gold-8 #3	0.4077

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	13.54	13.40	10.43	12.56	15.68	6.95	14.41	16.35	18.43	
% Ht sol	12.99	16.17	16.61	14.01	14.69	11.40	12.84	7.75	23.32	Ave
mg chaconine/100 g	4.04	4.00	3.07	3.73	4.72	2.02	4.32	4.94	5.62	4.05
mg solanine/100 g	2.75	3.48	3.59	2.98	3.14	2.40	2.72	1.60	5.24	3.10

Overall	Chac	Sol	Total
Average	4.63	2.99	7.62
Std Dev	0.79	0.24	0.93

MALDI-TOF MS Analysis of Glycoalkaloids

Calculations

Blank cells were either zero values or not obtained

Sample	Wt (g)
Russet Burbank-20 #1	0.2020

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	28.59	31.79	54.31	35.71	36.40	43.17	37.37	27.26	26.85	
% Ht sol	26.38	26.38	48.42	24.33	30.24	36.51	25.46	16.67	13.50	Ave
mg chaconine/100 g	18.54	21.00	43.04	24.16	24.75	30.79	25.57	17.55	17.25	24.74
mg solanine/100 g	12.20	12.20	27.58	11.10	14.37	18.23	11.70	7.27	5.79	13.38

Sample	Wt (g)
Russet Burbank-20 #2	0.1997

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	25.87	28.65	41.32	31.36	30.00	36.45	33.48	38.55	26.22	
% Ht sol	21.30	15.23	32.88	23.86	23.81	26.96	19.52	25.32	20.96	Ave
mg chaconine/100 g	16.72	18.80	29.40	20.90	19.83	25.07	22.60	26.89	16.98	21.91
mg solanine/100 g	9.65	6.67	16.12	10.98	10.95	12.66	8.75	11.76	9.47	10.78

Sample	Wt (g)
Russet Burbank-20 #3	0.1996

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	38.05	27.99	50.20	20.95	33.96	24.21	33.27	46.84	31.06	
% Ht sol	48.76	25.38	39.69	27.81	36.62	20.41	18.18	24.87	22.26	Ave
mg chaconine/100 g	26.47	18.31	38.52	13.21	23.00	15.52	22.44	34.85	20.68	23.67
mg solanine/100 g	28.26	11.80	20.64	13.14	18.52	9.20	8.09	11.52	10.15	14.59

Overall	Chac	Sol	Total
Average	23.44	12.92	36.36
Std Dev	1.43	1.95	3.18

MALDI-TOF MS Analysis of Glycoalkaloids

Calculations

Blank cells were either zero values or not obtained

Sample	Wt (g)
Comm Peeled #1	0.4185

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	0.00	5.71	1.88	4.69	1.27	1.63	6.45	1.87	5.95	
% Ht sol	14.85	0.00	4.82	4.09	3.02	2.32	8.62	2.88	3.82	Ave
mg chaconine/100 g	0.00	1.61	0.52	1.31	0.35	0.45	1.82	0.52	1.67	0.92
mg solanine/100 g	3.10	0.00	0.96	0.81	0.59	0.46	1.74	0.57	0.75	1.00

Sample	Wt (g)
Comm Peeled #2	0.3994

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	3.82	2.50	1.03	5.56	3.18	0.00	3.08	3.08	2.48	
% Ht sol	2.15	1.36	3.74	4.74	3.00	0.00	3.10	3.10	1.58	Ave
mg chaconine/100 g	1.12	0.73	0.30	1.64	0.93	0.00	0.90	0.90	0.72	0.80
mg solanine/100 g	0.44	0.28	0.77	0.99	0.62	0.00	0.64	0.64	0.32	0.52

Sample	Wt (g)
Comm Peeled #3	0.4014

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	3.88	3.88	3.23	3.03	3.25	2.80	1.33	2.52	1.93	
% Ht sol	3.52	3.52	2.56	4.73	5.05	3.84	3.10	3.75	3.67	Ave
mg chaconine/100 g	1.13	1.13	0.94	0.88	0.94	0.81	0.38	0.73	0.56	0.83
mg solanine/100 g	0.72	0.72	0.52	0.98	1.05	0.79	0.64	0.77	0.76	0.77

Overall	Chac	Sol	Total
Average	0.85	0.76	1.62
Std Dev	0.06	0.24	0.29

MALDI-TOF MS Analysis of Glycoalkaloids

Calculations

Blank cells were either zero values or not obtained

Sample	Wt (g)
Comm Unpeeled #1	0.3977

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	10.15	5.57	4.88	10.54	3.68	7.23	7.67	5.07	5.74	
% Ht sol	7.12	5.57	2.63	6.84	3.25	2.57	3.54	3.17	4.19	Ave
mg chaconine/100 g	3.06	1.65	1.44	3.18	1.08	2.15	2.29	1.50	1.70	2.01
mg solanine/100 g	1.50	1.17	0.54	1.44	0.67	0.53	0.73	0.66	0.87	0.90

Sample	Wt (g)
Comm Unpeeled #2	0.4062

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	6.36	6.18	7.12	8.03	4.52	9.95	5.79	3.66	5.46	
% Ht sol	3.94	6.88	5.79	3.30	3.19	4.63	4.10	4.85	2.99	Ave
mg chaconine/100 g	1.85	1.79	2.08	2.35	1.30	2.94	1.68	1.05	1.58	1.85
mg solanine/100 g	0.80	1.42	1.19	0.67	0.65	0.95	0.83	0.99	0.61	0.90

Sample	Wt (g)
Comm Unpeeled #3	0.3972

	Spot 1			Spot 2			Spot 3			
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3	
% Ht chac	4.26	5.20	7.86	6.48	8.46	7.91	8.71	7.52	4.64	
% Ht sol	3.00	4.15	4.30	6.91	7.53	4.24	7.15	6.44	4.55	Ave
mg chaconine/100 g	1.25	1.54	2.35	1.93	2.54	2.36	2.61	2.24	1.37	2.02
mg solanine/100 g	0.62	0.87	0.90	1.46	1.59	0.88	1.51	1.36	0.95	1.13

Overall	Chac	Sol	Total
Average	1.96	0.98	2.93
Std Dev	0.10	0.13	0.20