CHROMATOGRAPHIC DETERMINATION OF GLYCOALKALOIDS IN EGGPLANT

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ABSTRACT

CHROMATOGRAPHIC DETERMINATION OF GLYCOALKALOIDS IN EGGPLANT

Novel modifications were applied to high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) for the separation and quantitation of the steroidal glycoalkaloids (SGAs) solanine, chaconine, solamargine, and solasonine as well as the steroidal glycoalkaloid aglycones (SGAAs) solasodine and solanidine. Because attempts to develop a gradient elution HPLC method were only marginally successfully and non-robust, it was deemed more practical to develop separate HPLC methods for either the SGAs or SGAAs of interest. Furthermore, a novel approach using methanol as a mobile phase modifier was still required to successfully separate solamargine and chaconine. Comparing potential mobile phase buffers, ammonium dihydrogen phosphate was chosen as the most efficient, stable, and economical. Separations were best realized isocratically at a column temperature of 50 °C for the SGAs and either 26 °C or 50 °C for the SGAAs. Progesterone was applied as an internal standard. Effects of pH were also tested. Figures of merit such as limit of detection (LOD), limit of quantitation (LOQ), and linear dynamic range are described herein.

Lastly, solid-phase microextraction (SPME) using on-fiber derivatization coupled with GC-MS was investigated for extraction and analysis of these SGAAs. A carbowax divinylbenzene (CW-DVB) coated SPME fiber was the most suitable. Solanidine could be extracted and identified directly using our SPME/GC-MS method while solasodine required a derivatization step involving trimethylsilylimidazole (TMSI). Although initial attempts were qualitatively reproducible, eventual degradation to fibers precluded complete study. Cholesterol as an internal standard was investigated.

ÖZET

PATLICANDA GLİKOALKALOİDLERİN KROMATOGRAFİK TAYİNİ

Bu çalışmada, α-solanine, α-chaconine, α-solasonine ve α-solamargine steroid glikoalkaloidleri (SGAs) ile solanidine, solasodine steroid aglikonlarının (SGAAs) ayırım ve tayininde kullanılan, mevcut yüksek preformanslı sıvı kromatografisi (HPLC) ve gaz kromatografisi-kütle spektrometrisi (GC-MS) yöntemlerinde değişiklikler yapılmıştır. Gradient elüsyon metodu ile istenilen ayırım elde edilememiş ve incelenen glikoalkaloidler ya da aglikonlar için farklı HPLC metotları geliştirilmesinin daha pratik olduğu görülmüştür. Solamargine ve chaconine maddelerinin tayini için mobil faza literatürde bulunan metotlardan farklı olarak metanol eklenmiştir. Diğer tampon çözelti türlerine kıyasla amonyum dihidrojen fosfat tampon çözeltisi daha verimli ve kullanımı ekonomiktir. Glikoalkaloidler için en iyi ayırım 50 °C kolon scıcaklığında, isokratik elüsyonla elde edilmiştir. Aglikonların ayırımı ise 50 °C ya da 26 °C kolon sıcaklıklarında, isokratik elüsyonla sağlanmıştır. Progesterone internal standard olarak kullanılmıştır.

Son olarak, aglikon ekstraksiyonu ve tayini için fiber üzerinde türevleme yapılarak katı faz mikroekstraksiyonu (SPME) ve GC-MS yöntemleri incelenmiştir. Ekstraksiyon için carbowax-divinylbenzene (CW-DVB) kaplı fiber uygun bulunmuştur. Solanidine doğrudan SPME/GC-MS metodu ile tayin edilebilmesine rağmen solasodine türevleme uygunlandıktan sonra gözlenebilmiştir. Yapılan ilk nitel analizler tekrarlanabilir olmasına rağmen fiberin bozunması tam bir çalışma yapılmasına engel olmuştur. Ayrıca kolesterolün internal standard olarak kullanımı araştırılmıştır.

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CHAPTER 1

INTRODUCTION

1.1. Eggplant

Agriculture has always been a basic element of the Turkish economy. Although the role of agriculture in the overall economy is gradually reducing, it maintains its importance due to its contribution to national income and employment, provision of raw material and capital to industry, direct or indirect contribution to export, and its ability to meet the food demand. Due to a rich diversity of climate and soil properties approximately 1500 commercial vegetable types are grown in Turkey. Eggplant is one of Turkey's most popular and important agricultural crops. Major world producers of eggplant are China, India, Egypt, and Turkey (Table 1.1) "(WEB_1 2006)". Between 1993-2002 the major world exporters of eggplant were Spain (21,8%), Mexico (21,7%), China (5,5%), Italy (3,4%), and Turkey (1,5%) "(WEB_2 2006)".

Solanum melongena L. (2n=24), commonly known as eggplant, aubergine, guinea squash or brinjal, is an economically important vegetable crop of tropical and temperate parts of the world. Eggplant fruits are quite high in nutritive value and can justifiably be compared with tomato as a good source of vitamins, dietary fiber and minerals (particularly iron) (Table 1.2) "(Salunkhe and Kadam 1998)". It has been used in traditional medicines. For example, tissue extracts have been used for treatment of asthma, bronchitis, cholera, and dysuria; fruits and leaves are beneficial in lowering blood cholesterol "(Kashyap et al. 2003)". Its production is severely affected by biotic and abiotic stresses, as the levels of resistance to those factors are insufficient. There are many wild species of eggplant showing resistance to important pests that influence commercial eggplant production. Among the wild relatives, which can be exploited to increase genetic variability, *S. torvum* has been identified to carry traits of resistance to most of the serious diseases affecting eggplant, particularly bacterial and fungal wilts, and nematodes. Interspecific hybrids between wild and cultivated species have been successful in only a few cases "(Gousset et al. 2005)".

Table 1.1. Eggplant Production

Source: (WEB_1 2006)

Country/Years	*2003 (MT)*	*2004 (MT)*	*2005(MT)*
China	16,029,029	16,530,287	17,030,300
India	7,830,000	8,200,000	8,200,000
Egypt	1,026,353	1,046,742	1,000,000
Turkey	935,000	900,000	880,000
Japan	395,800	390,700	395,000
Italy	368,991	362,296	373,635
Spain	175,629	46,671	60,000
World	29,338,998	30,142,663	30,523,867

Table 1.2. Chemical Composition of Eggplant (per 100 g edible portion)

(Source: Salunkhe and Kadam 1998)

Constituent	**Content**
Moisture	92.7%
Carbohydrates	4.0%
Protein	1.4%
Fat	0.3%
Fiber	1.3%
Ca	18 mg
Mg	16 mg
P	47 mg
Na	3.0 mg
K	2.0 mg
S	44 mg
Thiamine	0.4 mg
Riboflavin	0.11 mg
Vitamin C	12 mg
Energy	24 kcal

1.2. Plant Secondary Metabolites

Plants produce a large, diverse array of organic compounds known as secondary metabolites. These substances have no direct function in growth and development but more recently many secondary metabolites have been suggested to have important ecological functions in plants such as providing protection against herbivores and pathogens as well as aiding in pollen and seed dispersal.

Secondary metabolites (SM) are typically produced in a specific organ, tissue, or cell type at specific stages of development (e.g., during flower, fruit, seed or seedling development). They can be present in the plant in an active state or as a prodrug that becomes activated upon wounding, infection or in the body of a herbivore. Their concentration in a given plant often varies during a 24-hour period "(Raver et al. 1999)". In agriculturally important species, the composition of secondary metabolites in plant tissue may affect the quality of food or foodstuff produced for humans and animals.

Most secondary metabolites can be classified according to structural similarities, biosynthetic pathways, or the kinds of plants that make them. There are three major classes of secondary plant compounds based on a biosynthetic classification system (Table 1.3).

Terponoid compounds (1) are the largest class of secondary metabolites, with over 22,000 in number "(Raver et al. 1999)". The carbon skeletons of terpenoids are products of the condensation of five-carbon isoprenoid units (C_5H_8). Sterols, a subclass of triterpenes, are chemically similar to the steroidal hormones of animals and may be combined with nitrogen to form alkaloids, as in tomatine, or with sugars in steroidal glycosides like digitalin. Saponins, glycosidic terpenoids, consist of an aglycone or non-sugar molecule with a triterpenoid (C_{30}) or steroidal backbone link to one or more sugars. Terpenoids play various roles in plants. Some are photosynthetic pigments (carotenoids) or hormones regulating plant growth and development (gibberellins**,** abscisic acid), while others serve as essential components of cell membranes (sterols) or defensive compounds. They include the pyrethroids, essential oils, limonoids, phytoecdysones, cardenolides, and saponins) "(Taiz 2002)".

Table 1.3. Classes, Examples of Plant Secondary Metabolites
(Source: Craik et al. 2002)

Class/Subclass	Types of Compound	Example
Terpenoids		
Monoterpenoids	Monoterpene lactone	Nepetelactone
Sesquiterpenoids	Sesquiterpene lactone	Artemisinin
Diterpenoids	Gibberellins	Gibberillic acid
Triterpenoids	Saponins	Diosgenin
	Sterols	Sitosterol
Tetraterpenoids	Carotenoids	Lycopene
Terpenoid esters	Pyrethroids	Pyrethrin
Phenolics		
Phenols		Hydroquinone
Phenylpropanoids	Hydroxycinnamic acids	Caffeic acid
	Hydroxycoumarins	Umbelliferone
	Phenylpropenes	Eugenol
	Lignans	Pinoresinol
Flavonoids	Anthocyanins	Cyanidin
	Flavonols	Kaempferol
	Flavones	Luteolin
	Glycoflavones	Orientin
Nitrogeneous	Benzylisoquinoline	Morphine
Alkaloids	Bisindole	Vincristine
	Diterpenoid	Aconitine
	Indole	Serpentine
	Indolizidine	Swainsonine
	Pyridine	Nicotine
	Pyrrolyzidine	Senecionine
	Steroidal	Solanine
	Tropane	Atropine
	Quinoline	Quinine
	Quinolizidine	Anagyrin
		Canavanine
Toxic amino acids		Prunasin
Cyanogenic glycosides		Glucocapparin
Glucosynolates		Pipericide
Amides		Mescaline
Aromatic amide		

Phenolic compounds (Group 2) are a chemically heterogeneous group of nearly 10,000 individual compounds. They have the common structural feature of an aromatic ring with one or more hydroxyl substituents “(Craik et al. 2002)”. Many phenolic compounds serve in defense roles against herbivores and pathogens. Included in this group are lignin, tannins, furanocoumarins, salicyclic acid. Some function in mechanical support (lignin), in attracting pollinators and fruit dispersers (flavonoids), in absorbing harmful ultraviolet radiation (flavonoids), or in reducing the growth of nearby competing plants (phenylpropanoids and benzoic acid derivatives) “(Taiz 2002)”.

Nitrogen-containing compounds (Group 3) are the largest class of plant toxins. The alkaloids are a family of more than 15,000 nitrogen-containing secondary metabolites found in 20% of the species of vascular plants, bacteria, fungi, and even in animals “(Raver et al. 1999)”. As their name would suggest, most of them are alkaline. The nitrogen atom in these substances is usually part of a heterocyclic ring, a ring that contains both nitrogen and carbon atoms. The skeleton of most alkaloids is derived from aminoacids although moieties from other pathways, such as terpenoids are often combined. In addition, in a number of alkaloids (e.g., steroid alkaloids) the nitrogen (derived from glutamine or other NH_2 sources) is added in the final steps of a biosynthetic pathway, i.e. the alkaloid skeleton does not stem from amino acids “(Dey and Harborne 1997)”. The primary role of alkaloids in plants is for chemical defense: as phytotoxins, antimicrobials, insecticides, fungicides, and as feeding deterrents to insects, herbivorous mammals and mollusks “(Craik et al. 2002)”. At lower doses, however, many are useful pharmacologically (morphine, codeine).

1.3. Steroidal Glycoalkaloids

Glycoalkaloids, a class of nitrogen-containing steroidal glycosides are naturally occuring secondary metabolites commonly found in the Solanaceae family which includes many significant agricultural plants, such as tomato, potato, eggplant, pepper, nightshade, thorn apple, and capiscum. For example, solasodine, has been found in about 200 Solanum species “(Dinan et al. 2001)”. Glycoalkaloids are generally found in all plant organs, with the highest concentrations occuring in flowers, sprouts, unripe berries, young leaves or shoots (metabolically active parts). They are regarded as defensive allelochemicals against a number of pathogens and predators including fungi,

viruses, bacteria, insects, and worms “(Friedman 2005)”. Due to defensive character, development of new cultivars of tomato and potato with high foliar steroidal glycoalkaloid levels is underway. The types of steroidal glycoalkaloids produced by solanaceous plants differ from species to species. The differences can be manifested as a presence or absence of a C-C double bond, variety of functional groups (e.g., hydroxyl, acetyl) and sugar groups, as well as in the sterochemistry of these functional groups. “(Chen and Miller 2001)”. The most common *Solanum* glycoalkaloids are given in Table 1.4.

Table 1.4. The most common glycoalkaloids found in Solanum species ([a]Aglycone, [b]R=aglycone; Gal= β-D-galactose; Rham: α-L-rhamnose; Glu: β-D-glucpse; Xyl=β-D-xylose,[c]Minor SGAs may be artefacts or metabolites) (Source: Laurila 2004)

SGA[a]	Sugar Moiety	Glycoside Structure[b]
Solanidine glycosides		
α-Solanine	Solatriose	A:R-Gal – Glu Rham
β-Solanine[c]	Solabiose	B:R-Gal-Glu
γ-Solanine[c]	Galactose	C:R-Gal
α-Chaconine	Chacotriose	D:R:Glu – Rham Rham
β_1-Chaconine[c]	Chacobiose	E:R-Glu-Rham
β_2-Chaconine[c]	Chacobiose	F:R-Glu-Rham
ϒ-Chaconine[c]	Glucose	G:Glu
Dehydrocommersonine	Commertetratose	H:R:-Gal-Glu – Glu Glu
Demissidine glycosides		
Demissine	Lycotetraose	I:R:Gal-Glu – Glu Xyl
Commersonine	Commertetratose	same as H

(cont.on next page)

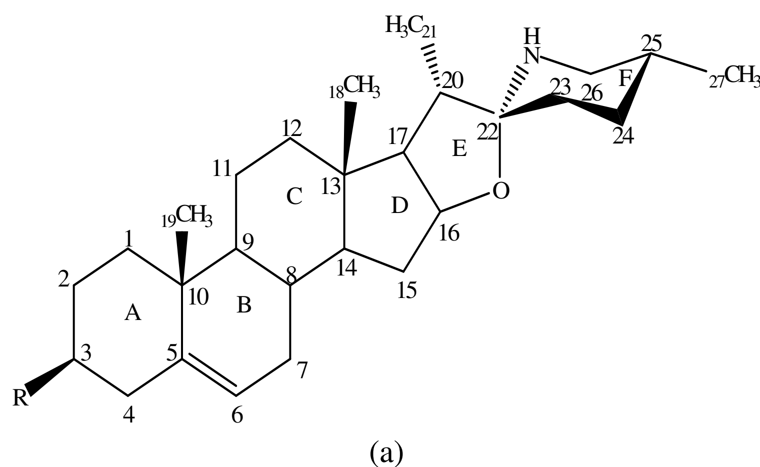
Table 1.4. (cont.)

SGA[a]	Sugar Moiety	Glycoside Structure[b]
Acetylleptinide glycosides		
Leptine I	Chacotirose	same as D
Leptine II	Solatriose	same as A
Tomatidenol glycosides		
α-Solamarine	Solatriose	same as A
β-Solamarine	Chacotriose	same as D
Solasodine glycosides		
Solasonine	Solatriose	same as A
Solamargine	Chacotriose	same as D
Tomatidine glycosides		
α-Tomatine	Lycotetraose	same as I
Sisunine(neotomatine)	Commertetratose	same as H

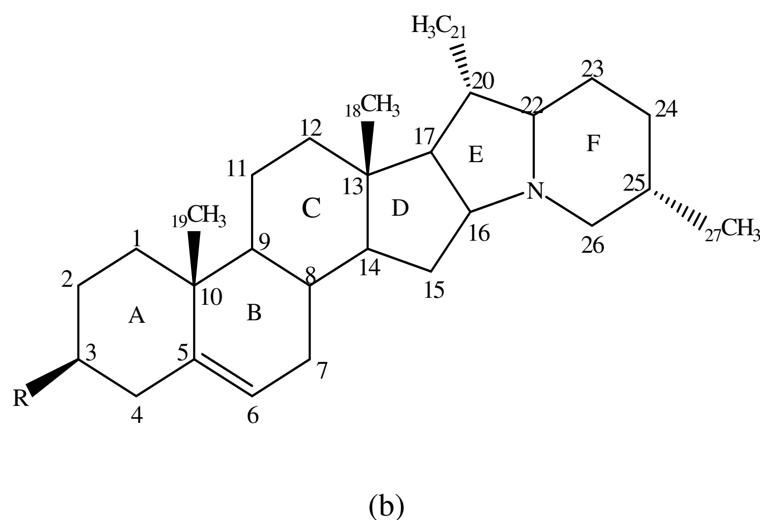
1.3.1. Chemical Structure of Glycoalkaloids

Steroidal alkaloids are characterised by the presence of an intact or modified steroid skeleton with nitrogen. Since nitrogen is inserted into a non-aminoacid residue these compounds belong to a subgroup of pseudoalkaloids (or isoprenoid alkaloids) "(Laurila 2004)". Structural variation in the family of plant steroidal glycoalkaloids is limited to two main groups, based on the skeletal type of the aglycone, examples of which are represented in Figure 1.1. One is the spirosolan type, similar to spirostan, but with nitrogen in place of the oxygen in ring F (forming a tetrahydrofuran and piperidine spiro-linked bicyclic system) (as in solasodine. Figure 1.1). Second is the solanidane type, where N connects spirostan rings E and F rings (as in solanidine Figure 1.1). All types can contain double bonds and hydroxyls in various positions. At least 90 structurally different steroidal alkaloids have been found in over 350 *Solanum* species "(Laurila 2004)". Nitrogen can be attached as a primary NH_2 group in position 3 or 20 (free or methylated), forming simple steroidal bases (e.g., conessine), ring-closed to skeletal or side-chain carbon (as a secondary NH) or annelated in two rings as a tertiary N (e.g., solanidine). This often influences the chemical character of the compound "(Dinan et al. 2001)".

Plants often contain alkaloids in glycosidic form as glycoalkaloids. Thus, steroidal glycoalkaloids contain three portions: a non-polar steroid unit and a basic portion with either a so called indolizidine or oxa-azaspirodecane structure which together form the aglycone part; a polar, water-soluble sugar moiety with three or four monosaccharides attached to the 3-OH group of the first ring of the aglycone. The common glycoalkaloid aglycones in eggplant and potato tubers are presented in Figure 1.1.



(a)



(b)

Figure 1.1. (a) solasodine, (b) solanidine

(Source: Chen and Miller 2001)

Solasonine and solamargine are the two major steroid alkaloid glycosides (SAGs) found in eggplants (*Solanum melongena*) and in at least 100 other Solanum species "(Blankemeyer et al. 1998)". Structurally, these two glycoalkaloids have the same steroidal part of the molecule (aglycone), solasodine, but differ in the nature of the carbohydrate side chain. The trisaccharide chain attached to the 3-hydroxy group of solasonine has a solatriose (branched α-L-rhamnopyranosyl-β-D-glucopyranosyl-β-galactopyranose) structure (Figure 1.2). The corresponding trisaccharide of solamargine has the structure of chacotriose (branched bis-α-L-rhamnopyranosyl-β-glucopyranose) (Figure 1.2). Alpha-solanine and α-chaconine are the most prevalent glycoalkaloids found in cultivated potato (*Solanum tuberosum*) with a solatriose and chacotriose sugar moiety respectively attached to the aglycone solanidine. The eggplant glycoalkaloids differ from those found in potatoes only in the structure of the steroidal part of the molecules. It has been reported that while solamargine and solasonine are in fruits of eggplant, solanine and chaconine are found in the leaves of these plants "(Chen and Miller 2001)".

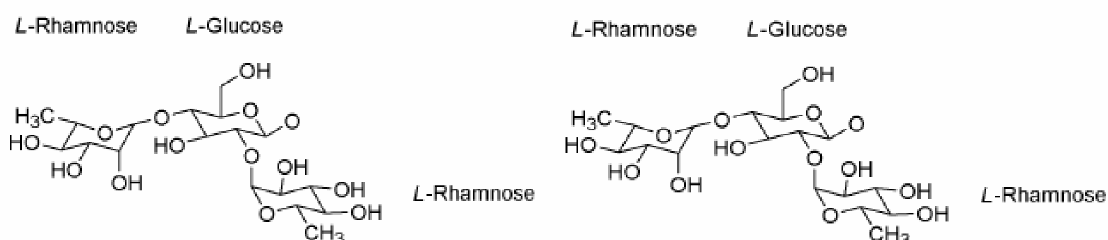


Figure 1.2. (a) solatriose group, (b) chacotriose group
(Source: Alt et al. 2005)

1.3.2. Toxicity of Glycoalkaloids

Glycoalkaloids are potentially toxic compounds. Typically potato tubers contain about 20-60 mg of total glycoalkaloid (TGA) per 100 g of freeze-dried matter, equivalent to 4-12 mg of TGA per 100 g fresh weight (fwt). At these concentrations glycoalkaloids enhance potato flavor. However, at concentrations greater than 20 mg

per 100 g fwt they impart a bitter taste and can cause gastroenteritic symptoms, coma, and even death. The toxic dose is considered to be approximately 2-5 mg kg^{-1} body weight (bw) whereas the lethal dose is probably 3-6 mg kg^{-1} "(Edwards and Cobb 1996)". Due to human toxicity, 200 mg TGA/kg fwt potatoes is accepted as the upper safety limit. The joint FAO/WHO Expert Committee on Food Additives (JECFA) considers a TGA content of less than 100 mg/kg potatoes FW of no concern. These are potato-based recommendations. Presently available epidemilogical and experimental data from human and laboratory animal studies are not sufficient to determine a realistic safe level of intake "(Mensinga et al. 2004)".

Several poisoning cases have been documented by the consumption of potatoes containing high levels of α-solanine, α-chaconine "(Chen and Miller 2001)".There are two main mechanisms of glycoalkaloid toxicity. First, GAs disrupt the cell membrane by causing the formation of destabilising complexes of the lipophilic moiety of the GAs with cholesterol "(Mensinga et al. 2004)". Recent cell culture and experimental animal studies have demonstrated that GAs may adversely influence intestinal permeability "(Mensinga et al. 2004)". Cell lysis in the gastrointestinal tract can lead to abdominal cramps, diarrhea, and eventually internal hemorrhanging "(Driedger et al. 2000)". With regard to membrane-disruptive activity, chacotriose-based glycoalkaloids are highly active compared to solatriose-based compounds "(Roddick et al. 2001)". Second, glycoalkaloids inhibit acetylcholinesterase (AchE, an enzyme in nerve impulse transmission) and butyrylcholinesterase (an enzyme that is possibly protective against specific toxins) activities. The physiological effects of cholinesterase inhibition include sweating, vomiting, diarrhoea, and muscle spasms. Severe poisoning may cause serious adverse events, such as paralysis, respiratory insufficiency, cardiac failure, and coma. Cases of lethal poisoning have been reported at estimated doses greater than 3 mg TGA/kg bw.

Most toxicological studies of glycoalkaloids of members of the Solanaceae have been focused on rats, mice, hamster, and rabbits. The LD_{50} for α-solanine, α-chaconine and tomatine in mice were 27, 30, and 34 mg/kg bw intraperitonally, respectively, and for most animals, the i.p. LD_{50} of the various glycoalkaloids were around 30 to 60 mg/kg bw. Toxicological studies revealed that the solanidanes seem to be more toxic than their corresponding spirosolanes-α-solamargine, solasonine and solasodine "(Chami et al. 2003)".

The toxicity of several GAs was tested using frog embryo teratogenesis assay-Xenopus (FETAX) "(Friedman et al. 1992a, Rayburn et al. 1994, Blankemeyer et al. 1992, 1998)". It was shown that glycoalkaloids alter the membrane potential of embryos and the active transport of sodium by frog skin. The type, order of attachment of the carbohydrate residues and the nature of the aglycone part appear to influence the developmental toxiciy and embryotoxicity of the steroidal aglycone. Following removal of the carbohydrates from the triglycosides, the developmental toxicity of SGAs in FETAX generally declined. The relative potency of α-solanine (which contains glucose, galactose, and rhamnose side chains) is lower than that of α-chaconine (having the sugars rhamnose, glucose, and galactose) although attached to the same aglycone. Although solasonine has the same carbohydrate residue as α -solanine, its activity in the frog embryo assay is between that of solanine and chaconine. Furthermore, mixtures of α-chaconine and α-solanine caused synergistic mortality or malformation in FETAX. However, toxicities observed for individual glycoalkaloids may not coincide with predicted toxicities of mixture. The results indicate that although potato glycoalkaloids show a synergistic effect in the distruption of membranes, they do not do so during inhibition of AchE "(Smith et al. 2001)". A recent human volunteer study demonstrated that daily consumption of potato products may cause accumulation of glycoalkaloids (α-solanine and α-chaconine). Single doses of up to 90.2 mg TGA (1.25 mg TGA/kg bw) did not create acute systemic effects. However, it was indicated that in one case at the exposure level of 1.25 mg TGA/kg bw, possibly due to local glycoalkaloid toxicity, some local gastrointestinal effects were observed "(Mensinga et al. 2004)".

Even though biological and toxicological properties of potato glycoalkaloids have been studied extensively, this does not appear to be the case for the two eggplant glycoalkaloids, solasonine and solamargine. Slight AchE inhibition by solamargine was reported "(Chami et al. 2003)". Alpha-solamargine isolated from fruits of *Solanum americanum* Miller was studied for its toxcity "(Chami et al. 2003)". Lethality studies in rats showed a dose-mortality relationship with an LD_{50} of 42 mg/kg bw intraperitoneally "(Chami et al. 2003)". No appreciable toxic effects were seen at doses below 35 mg/kg bw. In a survey of the effects of two eggplant glycoalkaloids on membrane potentials, survival, and organ malformations of the South Africa clawed frog, *Xenopus laevis*, the results showed that solamargine is more potent in distrupting cell membranes than is solasonine by a factor of between 2 and 3 "(Blankemeyer et al. 1998)". It was stated that since the two GAs share the same steroidal aglycone (solasodine), this difference is

presumably due to the different structures of the carbohydrate side chains. Therefore, the carbohydrate moiety has a significant role in influencing cell membrane disruptions and embryotoxic/teratogenic effects of glycoalkaloids "(Rayburn et al. 1994, Blankemeyer et.al 1998)".

1.3.3. Beneficial Effects of Glycoalkaloids

Although glycoalkaloids are toxic compounds at certain levels, they have some proposed beneficial effects. In recent years, medicinal uses of glycoalkaloids has been a focus of scientific and pharmacological attention. For example, solamargine and solasodine exhibit potent cytotoxicity to human hepatoma cells (Hep3B) by apoptosis which is the major process responsible for cell death in various physiological events "(Cheng et al. 1998)". Solasodine, solamargine, and solasonine from *Solanum incanum* L. showed liver protective effects against CCl_4-induced liver damage "(Lin et al. 1990)". Furthermore, α-chaconine, α-solanine, α–solamargine, α-solasonine, α-tomatine (being the most effective), and some of their hydrolysis products inhibit the growth of human colon (HT29) and liver carcinoma (HepG2) cells "(Lee et al. 2004)". Plasma low-density lipoprotein cholesterol and triglycerides in hamsters is lowered by α-tomatine. The immune response is enhanced by α-tomatine inducing cytokines in immunized animals "(Friedman 2002)". Solanine and chaconine either individually or as mixtures reduced the numbers of the cervical (HeLa), liver (HepG2), lymphoma (U937), stomach (AGS and KATO III) cancer cells "(Friedman et al. 2005)". Solamargine displayed a superior cytotoxicity in human lung, prostate (LNCaP and PC-3), and breast (T47D and MDA-MB-231) cancer cells "(Liu et al. 2004)". Moreover, solamargine and solasonine isolated from *Solanum sodomaeum* have been utilized to treat malignant human skin tumors including basal and squamous cell carcinomas "(Lee et al. 2004)".

Very recently a mixture of solamargine and solasonine has been developed to treat various cancer types such as glioblastoma multiform, colon rectal, bladder, liver, basal cell and squamous, metastasised melanoma to the lungs and other respiratory cancers, cell cancers. It was claimed that promising results obtained e.g., reduction in tumor size and growth rates and, extension of life from treatments of more than 40 patients "(WEB_3 2006)".

Furthermore, solasodine present in Solanaceae plants has gained significant importance globally. It can be converted to 16-dehydropregnenolone, a key intermediate in the synthesis of steroid drugs. "(Eltayeb et al. 1997)".

The leptines found in *Solanum chacoense Bitt*. are natural antifeedants to the Colorado potato beetle, *Leptinotarsa decemlineata*, with the Leptine I displaying deterrent activity on adult feeding and inhibiting larval development "(Sinden et al. 1986)". Moderate resistance to the Colorado potato beetle in the hybrids between *S.chacoense* and *S.tuberosum* has been reported "(Laurila 2004)". Solamargine, solasonine and tomatine inhibited larval growth of the red flour beetle, *Tribolum castaneum*. Tomatine also showed inhibitory activity on tobacco hornworm, *Manduca sexta* Johan "(Weissenberg 1997)". An extract of a mixture containing solamargine and solasonine mixture from the fruit of *S. mammosum* was shown to display a strong molluscicidal property on *Lymnaea cubensis* snails. Tomatine was reported to have anti-bacterial effects on gram positive bacteria that infect humans "(Jadhav et al. 1981)".

1.3.4. Factors Influencing Glycoalkaloid Levels

Several factors during growth, harvesting and post-harvest treatment as well as tuber size, maturity can affect glycoalkaloid accumulation. Genotype has a major effect and glycoalkaloid levels can vary between different cultivars (cvs). It was suggested that any environmental factor that causes a stress in a plant of the *Solanum* family can alter glycoalkalaoid content "(Laurila 2004)". For most cultivated potato varieties, the amounts of glycoalkaloids do not exceed 20mg/100 g fwt, remaining in the range of 2-13 mg/100g fwt with the ratio of α-chaconine to α-solanine maintained between 2:1 and 7:1. Commercial potatoes in the market place usually contain total glycoalkaloid around 100 mg/kg.

It was indicated that cvs with genetically determined high levels of glycoalkaloids would be more responsive to unfavourable environmental conditions and have greater tendency to accumulate excessive levels in their tubers than do cvs with low levels. Small size and immaturity of tubers have often been associated with higher glycoalkaloid levels. As tuber size increases the TGA content of individual tubers generally decreases and then levels off. However, it was demonstrated that glycoalkaloid accumulation generally continues as tuber size increases "(Papathanasiou

et al. 1999a)". Due to variability of several factors (cv. type, tuber size, maturity, e.g.) evaluation of the effects of differing climatic conditions has been complex. Unusually cold and wet conditions during tuber development and growth have been assumed to cause high glycoalkaloid levels. Cold, overcast days and high rainfall near harvest time were correlated with the very high glycoalkaloid levels found in the commercial potato cultivar Magnum Bonum in 1986 and as a result it was withdrawn from the market "(Hellenas et al. 1995)". Similarly, hot and dry conditions during plant growth have also been suggested to be responsible for elevated glycoalkaloid concentrations. Nevertheless, cvs may respond differently to temperature. Experiments showed that if early maturing cvs were kept at low temperature 12/9 ^{o}C day/night, TGA concentration did not increase. In contrast, it was reported that starch potatoes growing at the same temperatures (12/9 ^{o}C day/night) accumulated higher TGA levels. Combined stress treatments showed that waterlogging during the later stages of development and drought stress increased glycoalkaloid concentration in the cv. British Queen "(Papathanasiou et al. 1999b)".

Post-harvest conditions including light, storage time, and heat also affect glycoalkaloid formation. Synthesis of glycoalkaloids can be rapidly stimulated when tubers are exposed to illumination. It has been shown that exposure of tubers to sodium and fluorescent light results in a substantial increase in glycoalkaloid content. However, in some cases, the accumulation rates of glycoalkaloids in two commercial cvs after exposure to fluorescent and mercury light were reduced significantly. Continuous illumination decreased the ratio of α-chaconine/α-solanine which may influence toxicity since α-chaconine possesses higher toxicological potency "(Percival 1999)". Genotypes having similar initial concentrations can differ in their rates of accumulation during light exposure. Furthermore, glycoalkaloid levels increase and the increase has been found to be higher in green potatoes during storage. It was determined that storage under light for three to six months caused a greater increase than did storage in the dark "(Şengül et al. 2004)". The effect of storage time and temperature are difficult to separate. Higher storage temperatures resulted in greater glycoalkaloid concentrations "(Laurila 2004)". Studies conducted using the heat-susceptible potato cultivar known as Atlantic showed that TGA concentration increased by 74% after storage for four hours at 35 ^{o}C as compared with the same cultivar after storage for four hours at 22 ^{o}C. Conversely, a heat-resistant potato cultivar, LT7, showed a 50% reduction in TGA content after the same treatments "(Coria et al. 1998)".

Pest-related stress following foliar damage by Colorado potato beetles substantially increased the glycoalkaloid concentrations in tubers, whereas leafhoppers caused no change “(Hlywka et al. 1994)”. Concerning alterations in glycoalkaloids, plants show different responses to pathogens. As a result of inoculations with *Clavibacter michiganesis ssp. sepedonicus* (*Cms*), TGA levels in *S.acaule Bitt*., a wild potato species, were lowered “(Rokka et al. 2005)”. However, as a consequence of infection TGA levels in *S.tuberosum* were either higher or unchanged. Similarly, higher foilage tomatine accumulation was observed when a tomato cv. was infected with *C.michiganensis ssp. michiganensis* “(Rokka et al. 2005)”. Glycoalkaloid content is a genetically controlled trait “(Laurila 2004)”. Wild Solanum species that usually contain high levels of glycoalkaloids are widely used in breeding studies to introduce desirable charecteristics such as unique or improved resistance into cultivated species. This may result in high levels of glycoalkaloids and or new types of glycoalkaloids may be introduced. For instance, somatic hybrids between *S. tuberosum* and the wild species *S.circaeifolium* were found to be resistant to several pathogens, each containing high levels of glycoalkaloids and different glycoalkaloids of demissidine from both parents were detected in the tubers of somatic hybrids “(Kozukue et al. 1999)”.

1.3.5. Methods of Analysis for Glycoalkaloids

Glycoalkaloid analysis methodology (particularly for the potato) has been studied at length. The overall GA assay can be divided into three steps: extraction, clean-up and analysis. There is a wide range of extraction solvents employed in published methods. Most are based on a weak solution of acetic acid or combinations of acetic acid with different solvents such as methanol/chloroform, tetrahydrofuran-acetonitrile-methanol. Some methods use only methanol for the extraction. Usually samples are freeze-dried prior to extraction since freeze-drying offers several advantages: (1) it stops enzyme-catalyzed, wound induced, and moisture dependent compositional changes (2) once thoroughly dried, samples can be stored before analysis for long periods of time and (3) for plants mostly containing water SGAs are effectively concentrated by freeze-drying “(Dao and Friedman 1996)”. After extraction various clean-up methods for GA purification can be used. Precipitation with ammonia is one of them “(Lawson et al. 1992, Friedman et al. 2003)”. However, it is not reliable as

substantial and variable losses (more than 60%) in recovery was observed when used in preparing extracts of some wild *Solanum* species "(Gregory et al. 1981)". It failed to quantitatively precipitate GAs specific to some wild species and also GAs that are normally ammonia insoluble (e.g., solanine, chaconine) "(Gregory et al. 1981)". For liquid-liquid extraction (LLE) of SGAs from aqueous media, butanol has been used "(Sotelo and Serrano 2000)". The most commonly used clean-up method is solid-phase extraction (SPE). It is much simpler and gives better results than ammonia precipitation for some cases. Mostly silica based octadecyl (C_{18}) "(Carman et al. 1986, Edwards and Cobb 1996)", or amino (NH_2) "(Saito et al. 1990)" sorbents have been utilized for potato tuber extracts. The use of cyano (CN), phenyl (Ph), and octyl (C_8), cation-exchange phases has been reported as well "(Vaananen et al. 2000)". However, significant loss during SPE, variable results between batches for reproducibility of recoveries were recorded "(Friedman and Levin 1992b, Edwards and Cobb 1996)". Moreover, removal of organic solvent before any step is required as organic solvents prevent adsorption of GAs when C_{18} sorbents are used "(Edwards and Cobb 1996)". Nonetheless, SPE can be a powerful method to concentrate and purify the analytes of the complex matrices. Heptanesulfonic acid has been applied as an ion-pair reagent to help improve complete adsorption of SGAs for some methods utilizing solid-phase extraction "(Carmen et al. 1986)".

The complex nature of GA-dietary relationships necessitates accurate methods to measure the content of individual GAs and their metabolites. Rapid, simple, highly selective and reproducible assay systems are required for a large number of plants, and a limited small amount of samples and standards. There are many methods for the analysis of GAs reported in literature. Each method has relative advantages and disadvantages. The earlier methods including colorimetric, gravimetric, and titrimetric techniques lack the desired specificity and suffer from contamination by other compounds "(Gregory et al. 1981)". Thin-layer chromatography (TLC) is a simple and inexpensive method that has been used primarily for qualitative or semiquantitative analysis "(Carman et al. 1986, Kozukue et al. 1999)". High-performance liquid chromatography (HPLC) is the most commonly applied method for the analysis of entire glycosides and aglycones "(Edwards and Cobb 1996, Kittipongpatana et al. 1999, Sotelo and Serrano 2000, Friedman et al. 2003)". Gas chromatography (GC) is well-suited for the determination of glycoalkaloid aglycones with "(Herb et al. 1975)", or without "(Sinden et al. 1986, Lawson et al. 1992, Holstege et al. 1995)" derivatization

in potato materials. Even though GC is a destructive method, it can detect as little as 3 ng of glycoalkaloids. HPLC with UV detection can detect glycoalkaloids above 100 ng levels which is less sensitive than GC because glycoalkaloids lack an efficient UV-absorbing chromophore "(Lawson et al. 1992)". A newer method of analysis involves the use of immunoassays. Enzyme linked immunosorbent assay (ELISA) "(Sporns and Phalk 1992)", fluorescence polarisation immunoassay "(Thomson and Sporns1995)", solution phase immunoassay with capillary electrophoresis (CE) and laser induced fluorescence detection "(Driedger et al. 2000)" are examples of recently investigated methods for glycoalkaloid analysis. Immunoassay relies on the specificity of antibodies and offers the possibility of a sensitive, simple, rapid, and relatively cheap detection although the assays are unable to differentiate between α-chaconine and α-solanine "(Stanker et al. 1994)". Mass spectrometric methods, such as GC/MS, "(Laurila et al. 1999, Van Gelder et al. 1989)", and LC/MS "(Stobiecki et al. 2003, Zywicki et al. 2005)" have been applied to the determination of steroidal glycoalkaloids and their aglycones. Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was investigated for quantitative analysis of α-solanine and α-chaconine "(Abell and Sporns 1996)". These authors reported that similar results were obtained as for HPLC (with UV detection) for potato tubers. However, in spite of being a relatively fast technique, MALDI-TOF-MS is an expensive alternative. Non-aqueous capillary electrophoresis-UV (NACE-UV) has been described for the quantitation of solasodine and solasonine in eggplant samples "(Kreft et al. 2000)". Due to the lack of chromophore groups these compounds have low absorbance, and the detection limits are high. Coupling of NACE with ion-trap MS and MS/MS detection allowed separation and detection of α-chaconine, α-solanine, α-tomatine, solanidine and tomatidine in potato cvs. NACE-MS offers the advantage of being a rapid and sensitive assay of small volumes of samples, which in turn can reduce organic solvent usage "(Bianco et al. 2002)". Application of HPLC with chemiluminescence (CL) detection method for α-solanine and α-chaconine determination in potato tubers have been reported "(Kodamatani et al. 2005)". Detection limits of α-solanine and α-chaconine were 1.2 and 1.3 ng/ml, respectively. CL detection permitted the HPLC determination of GAs in small quantities of samples without any clean-up or concentration steps. NMR was applied for characterization of glycoalkaloids in potato "(Lawson et al. 1997, Weissenberg 2001)" and eggplant species "(Ripperger 1996, Usubillaga et al. 1996, Arthan et al. 2002)".

CHAPTER 2

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) ANALYSIS OF STEROIDAL GLYCOALKALOIDS IN EGGPLANT

2.1. Introduction

High-performance liquid chromatography (HPLC), together with its derivative techniques, is today the primary analytical separation tool in many industries. The reasons for the widespread use of the method is its sensitivity, its suitability for separating nonvolatile species or thermally fragile ones, and its adaptibility to accurate quantitative determinations.

Chromatography in general includes all separation techniques in which analytes partition between different phases that move relative to each other or where the analytes have different migration velocities. In liquid chromatography (LC), the mobile phase is liquid, while the stationary phase can be a solid or a liquid immobilized on a solid. HPLC consists of all liquid chromatographic techniques that require the use of elevated pressures to force the liquid through a packed bed of the stationary phase. Between 70 and 80% of all HPLC applications utilize reversed-phase chromatography (RPC) in which a nonpolar stationary phase is used in conjunction with polar, mainly aqueous mobile phases "(Neue 1997)". Its popularity is based largely on its ease of use and detection (especially with UV detectors), high efficiency, reproducibility, and widely accepted versatility.

Most stationary phases are silica-based bonded phases (typically, a silica support modified with a C_8 or C_{18} bonded phase), but polymeric phases based on inorganic substrates other than silica, and graphitized carbon are used as well. The detailed nature of reversed-phase retention is not understood completely, but it appears that retention can be approximated by a partition process. Sample molecules partition between the polar mobile phase and the nonpolar C_8 or C_{18} stationary phase, and the more hydrophobic (nonpolar) the substance, the stronger it is retained. For a given mobile phase composition, the result is a differential retention of compounds according to their

hydrophobicity. Hydrophilic compounds are less strongly held and elute from the column first. The RPC retention of a compound is determined by its polarity and experimental conditions such as type and strength of mobile phase, column type and temperature.

Relative retention of analytes is compared by considering their retention (capacity) factors. An analyte's retention factor is defined as the ratio of its concentration between the stationary phase (sp) and mobile phase (mp):

$$k = [\text{analyte}]_{sp} / [\text{analyte}]_{mp} \tag{2.1}$$

When two analytes are separated, the ratio of their retention factors will not be equal to one. A separate term, selectivity factor, α, is used to define this ratio (Skoog et al.1998).

$$\alpha = [k_{\text{analyte1}}] / [k_{\text{analyte2}}] \tag{2.2}$$

Both k and α can be adjusted by changing mobile phase composition or solvent strength. In RPC, retention of a compound is less for stronger, less polar solvents. Literature data suggest that RPC solvent strength varies as water (weakest) < methanol < acetonitrile (ACN) < ethanol < tetrahydrofuran < methylene chloride (strongest) "(Snyder et al. 1997)". Therefore, solvent strength decreases as solvent polarity increases. ACN-water mixtures can be used with UV detection at low wavelengths (185-210 nm). They also have much lower viscosities, resulting in somewhat higher plate numbers and lower column pressure "(Snyder et al. 1997)". Three properties of the column affect sample retention: type, concentration and surface area of the bonded phase. Retention generally increases as the chain length or hydrophobicity of the bonded-phase group increases. RPC retention of nonpolar, non-ionic compounds generally follows the pattern: unbounded silica (weak) << cyano < C_1 (TMS) < C_3 < C_4 < phenyl < C_8=C_{18} (strong) (Polystyrene and porous graphitic carbon columns are even more retentive than a C_{18} column, other factors being equal "(Snyder et al. 1997)". Alteration of temperature is seldom used to control sample retention since variation of solvent strength is more effective. An increase in temperature by 1 ^{o}C usually decreases k by 1 to 2% "(Vaananen et al. 1999)". Three basic variables can be used in RPC to change selectivity (α) for neutral samples: mobile phase composition (the most effective and suitable one), column type, and temperature. Change in temperature generally has

little effect; however, small changes in selectivity factor are enough for separating many analytes in a sample.

For ionic samples, controlling band spacing often requires changes in pH (often the most effective way to vary separation), percent composition of mobile phase (%B), solvent type, temperature, column type, and buffer concentration. Although temperature generally has a minor effect on band spacing for the RPC separation of neutral samples, this is not the case for ionic samples since several different retention related processes can be included in the separation, each responding differently to a change in temperature (e.g., changing ionization of sample components, silanol interactions involving the ionic species, hydrophobic retention of ionized vs. neutral molecules of the same compound, and variation of pH and pKa with temperature) "(Snyder et al. 1997)". It can be assumed that maximum changes in selectivity with temperature will occur for pH values that result in the partial ionization of analytes. For the combination of basic samples and silica based columns whose silanols are significantly ionized, the effect of buffer concentration on the RPC retention of ionic samples is expected to be important. These ionized silanol groups can strongly retain protonated bases or other cations by means of ion-exchange processes. This can result in increased retention, band tailing, and problems in column-to-column reproducibility. Silanol interaction can be reduced by selecting basic columns or by using a high buffer concentration (>10 mM) to increase competition from buffer cations and choosing buffer cations that are strongly held by the silanols ($Na^+ < K^+ < NH_4^+ <$ triethylammonium$^+ <$ dimethyloctylammonium$^+$) "(Snyder et al. 1997)".

HPLC-UV is the most widely used method for glycoalkaloid detection, because it is rapid, accurate, relatively easy-to-use, and reproducible. Furthermore, individual glycoalkaloids and aglycones can be determined without any derivatization and there is wide availability of such HPLC equipment. However, glycoalkaloids do not have a suitable UV chromophore, and thus, absorbance is measured at around 200 nm, where many compounds absorb light "(Kodamatani et al. 2005)". This limits the sensitivity of detection, which may be improved by the use of large sample sizes and a sample clean-up to overcome background noise.

Most of the reported methods apply reverse phase C_{18} or NH_2 columns with a mobile phase system containing usually ACN and a biological buffer (e.g., ammoniumphosphate(monobasic), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), potassiumdihydrogen phosphate (KH_2PO_4), triethylammonium phosphate

(TEAP)) for the determination of glycoalkaloids in potato samples. Mostly aglycones and glycoalkaloids were detected in separate runs under isocratic conditions at pH values less than 7. Using an NH_2 column and an ACN/KH_2PO_4 mobile phase system, Friedman investigated the effect of buffer concentration, mobile phase ratio, column temperature, and pH of the mobile phase on retention times of α-solanine and α-chaconine. It was concluded that all of the variables except pH significantly influenced the retention times "(Friedman et al. 2003)". Vaananen applied several buffers such as Tris-HCl, TEAP, and triethlyammonium acetate (TEAA) using C_{18} columns. At low pH separations (pH:3), retention times were found to decrease as compared to medium pH conditions. According to Vaananen, ACN/TEAP mobile phase system was found to be the best, eluting both SGAs and SGAAs under both isocratic and gradient elution conditions "(Vaananen et al. 1999)". They were able to obtain a reasonable separation of SGAs (α-solanine, α-chaconine, tomatine, solasodine and dehydrotomatine) and SGAAs (solanidine, dehydrotomatidine, and tomatidine) during a single run.

The aim of this work was to find suitable chromatographic conditions for the separation of glycoalkaloids α-solanine, α-chaconine, solamargine, solasonine and their aglycones, solanidine and solasodine. For this reason the effects of experimental conditions (type and strength of organic solvent, column temperature, type, concentration and pH of buffer) on the separation of these ionizable compounds were investigated. The resulting HPLC method is then planned to be used for current and future studies of SGAs and SGAAs in eggplant.

2.2. Experimental

All standards were obtained commercially, except for solamargine which was provided by Prof. Dr. Adelia Emila de Almeida (Faculdade de Ciencias Farmaceuticas-UNESP, Brazil). Alpha-solanine, α-chaconine, and progesterone were obtained from Sigma Aldrich (Germany). Solasodine and solanidine were obtained from both MP Biochemicals (OH, USA) and Research Plus (NJ, USA). Solasonine was obtained from Research Plus (NJ, USA). HPLC-grade acetonitrile (Sigma Aldrich, Germany) and methanol (Riedel-de Haen) were used for HPLC analysis. 1.0 mM triethylammonium phosphate (TEAP), 1.0 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), and ammonium dihydrogen phosphate (>99%) were purchased from Fluka

(Switzerland) and Merck (Germany). The buffers were filtered through a 0.45-μm polyamide filter (Sartorius, Germany). Standards were prepared in acetonitrile-water (1/1 v/v) acidifed with orthophosphoric acid and stored at 4 ^{o}C. Both NH_2 Nucleosil and C_{18} Nucleosil columns were purchased from HiChrom (USA).

2.3. General HPLC Conditions

A Shimadzu Class-VP (Kyoto, Japan) single piston high pressure liquid chromatograph with photodiode array detection was used. Due to the lack of a suitable chromaphore on the glycoalkaloids, UV detection at 205 and 208 nm was chosen. Flow rates were typically 1 mL/min unless otherwise indicated. Column temperature could also be adjusted up to 60 degrees with this instrument. Mobile phases were prepared fresh, sonicated and filtered through a 0.45 μm polyamide filter. The injector loop was 20 μL. Either isocratic or gradient elution could be employed.

2.4. Results and Discussion

The glycoalkaloids are particularly difficult to separate due to their similarity in structure. Solamargine and chaconine have identical sugar constituents, but different aglycones, i.e. solasodine and solanidine, respectively. Similarly, solasonine and solanine contain the same sugar moieties, but have the solasodine and solanidine aglycone backbones, respectively (Figure 1.1 and Figure 1.2). Therefore, conditions that can affect selectivity appreciably were varied. For this study, solanine, chaconine, solamargine, and solasonine are the steroidal glycoalkaloids (SGAs) of interest to our studies. Likewise, the steroidal glycoalkaloid aglycones of interest to this work will be referrred to as SGAAs. Only two SGAAs were important to our work, namely, solanidine and solasodine. For all work, a binary mobile phase system was set up where one delivery bottle contained the oganic solvent B (ACN for all work) and was designated "B''. The second delivery bottle contained the buffer (Tris-HCl, TEAP, ammonium dihydrogen phosphate).

In an attempt to separate glycoalkaloids and aglycones all in one chromatographic run with isocratic elution, an ACN/TEAP buffer (pH:3.14) solvent system was evaluated initially, since according to Vaananen this was found to be the

best mobile phase system, eluting reproducibily both the SGAs and the SGAAs they had chosen for their studies (α-solanine, α-chaconine, tomatine, solasodine, dehydrotomatine, solanidine, dehydrotomatidine, tomatidine) "(Vaananen et al. 1999)". A low pH mobile phase was preferred since column silanols are protonated, reducing their chromatographic activity. For basic samples, silanol interactions can lead to poor band shape. In addition a Nucleosil type column is proposed for separating basic compounds in this work. Under these conditions it was possible to separate the SGAAs of interest to our work while no isocratic conditions resulted in adequate separation of SGAs. In general the aglycones (SGAAs) eluted much faster (within 10 minutes) when the percentage of ACN (B%) in the mobile phase was increased to 70%. Unfortunately under such conditions, SGAs eluted too quickly to be detected, requiring that the ACN pecentage be decreased to 35%. However, decreasing the percentage of ACN to 35% resulting in longer run times (30 minutes) and broadening of the solasodine band. Increasing the molarity of TEAP to 35 mM and further to 50 mM decreased the retention times of SGAAs but did not improve the resolution of SGAs. Therefore, gradient elution was initially employed to enhance the separation of SGAs. ACN was the organic portion of the mobile phase for all the gradient study unless otherwise stated. With an ACN/Tris-HCl (25 mM using gradient method in Appendix E) buffer mobile phase (pH:6.0 acidified with 85% orthophosphoric acid) applied as a gradient, SGAs and SGAAs were not separated at all. In addition the column back pressure became too high. During development of a gradient method, water (pH:2.5 acidified with 85% orthophosphoric acid) was employed as the weak solvent of the mobile phase (A). The aglycones eluted as broad and tailing peaks when no buffer was used as compared to the case when 25 mM or 50 mM TEAP buffer (pH:3.14) was used (data not shown). The use of buffer help reduce tailing. Although retention times of SGAAs decreased with increasing buffer concentration and higher buffer concentrations provide increased buffer capacity, 25 mM TEAP was a good compromise since there was no difference in the separation of SGAs and it was more economical. Furthermore, whenever ionizable samples are separated, it is strongly advisable to control mobile phase pH by adding a buffer to obtain reproducible separations and prevent peak distortion "(Snyder et al. 1997)".

In the case of ionic samples, pH and temperature are important variables for controlling selectivity. Figure 2.1, Figure 2.2, Figure 2.3 and Figure 2.4 represent the effect of four column temperatures on the separation of SGAs and SGAAs with a

gradient from 27 to 100% ACN (details of this gradient method were given in Appendix E) over the course of eighteen minutes.

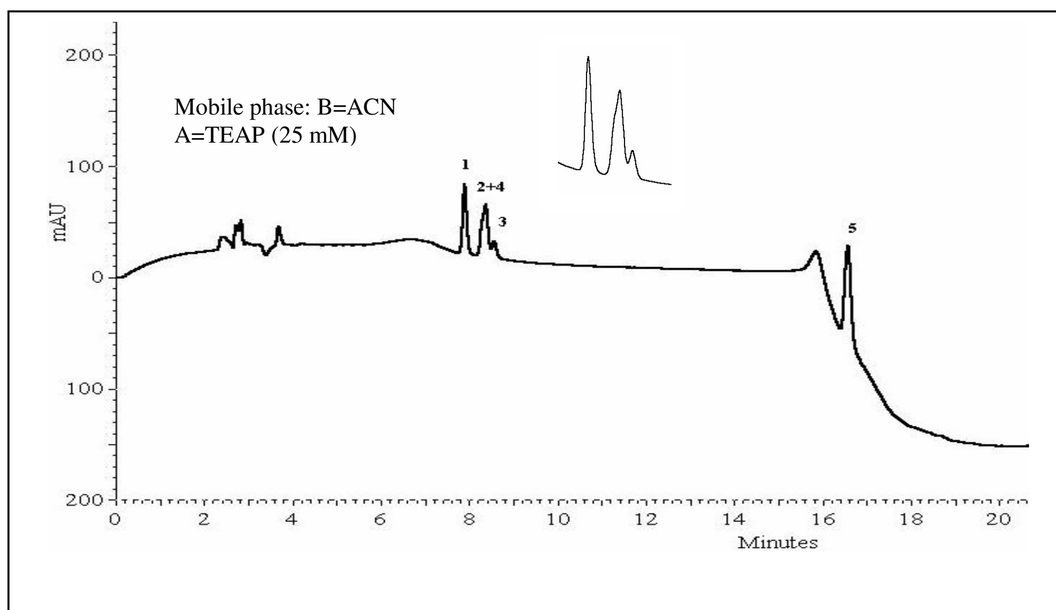


Figure 2.1. Gradient elution T: 35 °C pH: 3.02 F: 1.0 mL/min. 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine 5: solanidine

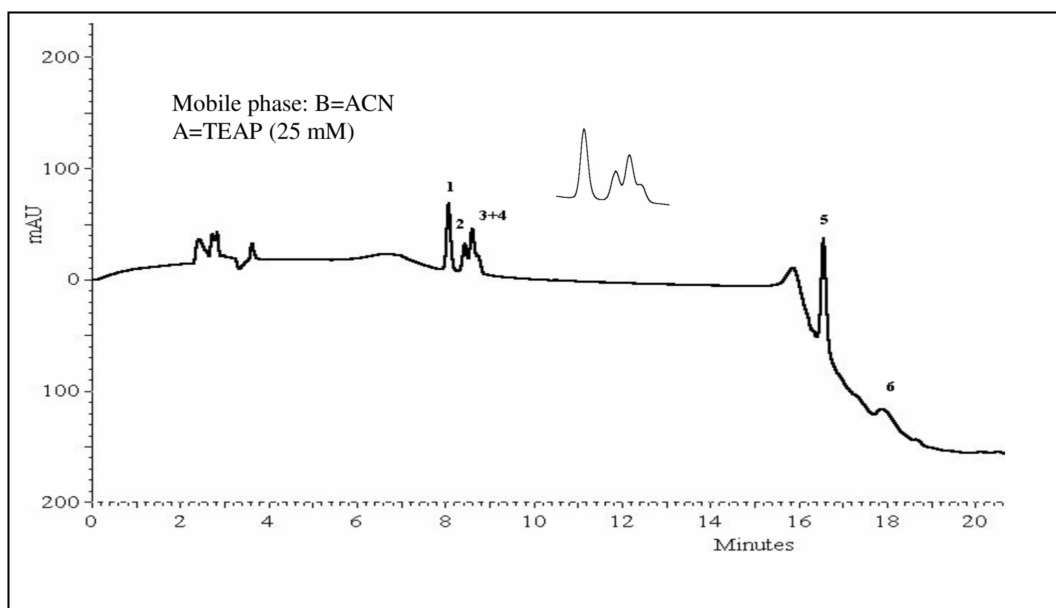


Figure 2.2. Gradient elution T: 40 °C pH: 3.02 F: 1.0 mL/min. 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine 5: solanidine 6: solasodine

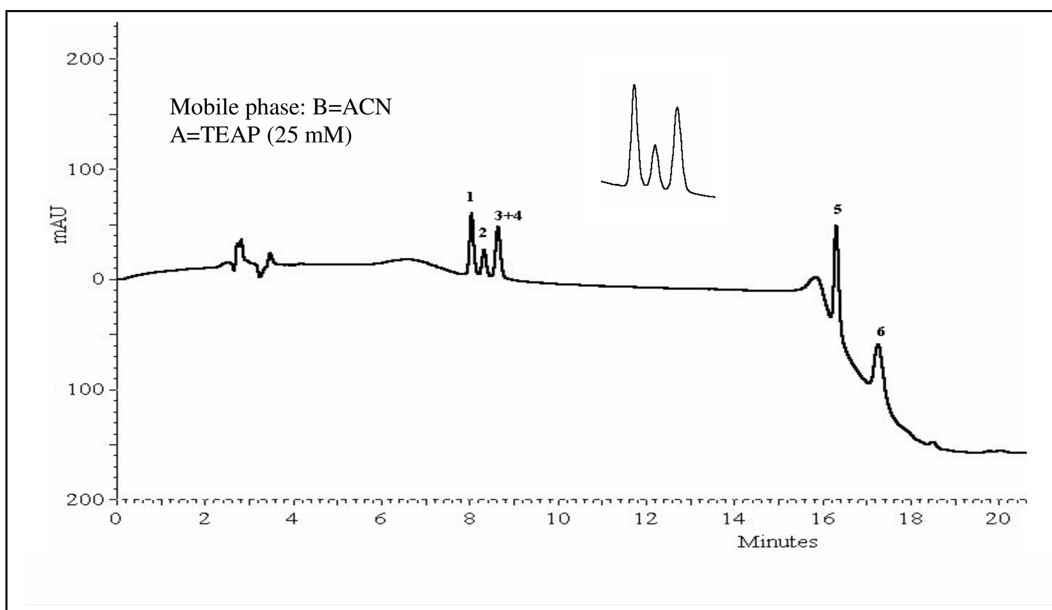


Figure 2.3. Gradient elution T: 50 °C pH: 3.02 F: 1.0 mL/min. 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine 5: solanidine 6: solasodine

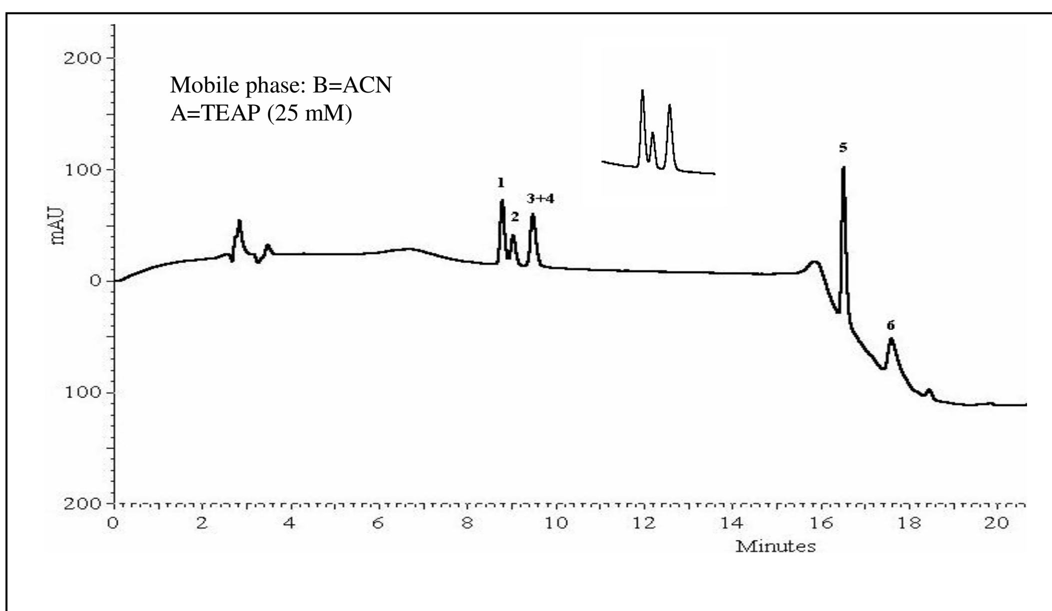


Figure 2.4. Gradient elution T: 55 °C pH: 3.02 F: 1.0 mL/min. 1: solasonine 2: α solanine 3: α chaconine 4: solamargine 5: solanidine 6: solasodine

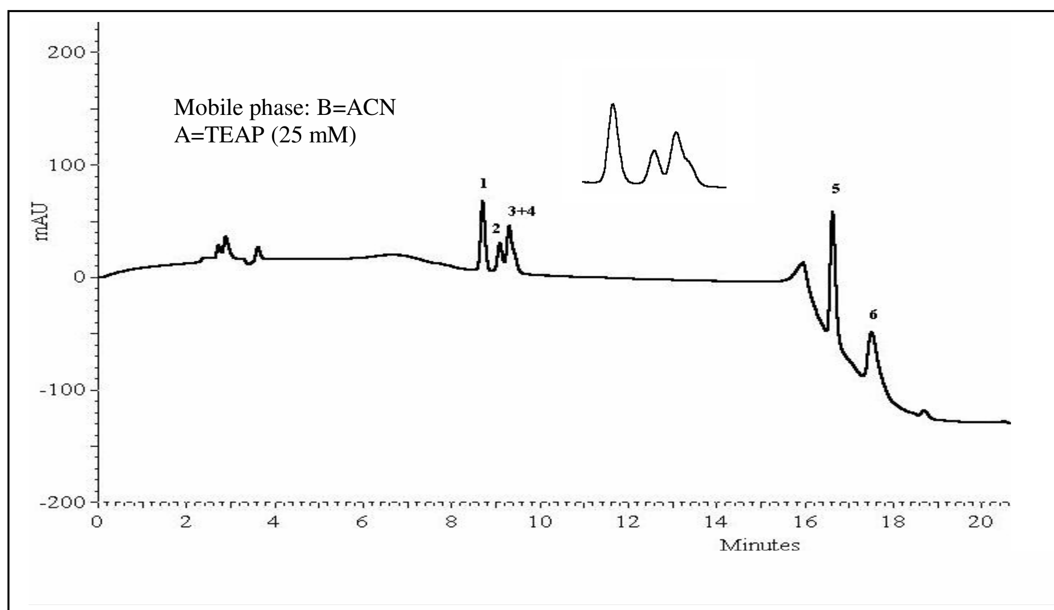


Figure 2.5. Gradient elution T: 40 ^{0}C pH: 2.3 F: 1.0 mL/min. 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine 5: solanidine 6: solasodine

At 35 ^{o}C (Figure 2.1) and 40 ^{o}C (Figure 2.2) the separation of band 1 was acceptable but bands 2, 3, and 4 overlapped. Band 6 was not detected during the run at 35 ^{o}C and was marginally detectable at 40 ^{o}C. Separation of band 2 was improved at 50 ^{o}C but bands 3/4 totally overlapped. Band 2 moved toward band 1 as the temperature was raised to 55 ^{o}C (Figure 2.4). Overall, the aglycone peaks became sharper as the temperature increased. Furthermore, the effect of pH on separation of glycoalkaloids was investigated at 40 ^{o}C. At lower pH (2.3) (Figure 2.5) the peak shapes of aglycones improved but for this pH, however, the separation of glycoalkaloids was still marginal.

Moreover, the effect of concentration of buffer (10 mM and 35 mM) was investigated with the same gradient at 40 ^{o}C (Appendix A Figure. A.1). Apart from band 1 the resolution of bands 2, 3 and 4 was not sufficient. With 10 mM TEAP buffer, solasodine eluted at 25 minutes (data not shown) and there was still no improvement in the separation of SGAs with 35 mM buffer compared to the 25 mM case. Therefore, 25 mM TEAP produced bettter results and it was more economical. Also a change in buffer concentration as a means of changing selectivity is usually not advisable, however, because silanol ionization is generally not reproducible from one batch of columns to the next, leading to variable retention and separation "(Snyder et al.1997)". To improve separation of SGAs, mixtures of MeOH-ACN and MeOH-TEAP were employed as the organic solvent (Appendix A Figure A.2). However, no significant improvement in the

separation of SGAs was observed with these mixtures as mobile phases. Unfortunately, the separation obtained at 40 ^{o}C using 25 mM TEAP and ACN could not be reproduced after several months of injections which presumably is due to column aging. As a result, gradient elution was found to be both ineffective and not robust for the separation of solamargine and α-chaconine. From this point on the gradient method of elution was abandoned in favour of several isocratic methods. The gradient method was more complex and did not provide acceptable separation of our SGAs. Also it proved to be impractically slow since for subsequent injections the column should be equilibrated at the starting concentration of the gradient.To ensure that the column was properly equilibrated, at least three sequential analytical gradient runs were obtained. It was found that replicate injections more than one hour apart would often display shifts in retention times. This was due to the rate of change of supplied mobile phase during the gradient run as well as the limited reproducibility of the single-piston type HPLC used. Lastly, because of the dramatic change in column pressure during the gradient method, a drastic shift in the chromatographic baseline occured approximately 15 minutes after injection. Although such a gradient was required to elute both SGAs and SGAAs in a single chromatographic run, such a shift in baseline is not desirable for quantitative analysis. In the end, it was decided that two or three separate isocratic methods would consume nearly the same amount of time and mobile phase; therefore, further attempts to improve or use a gradient method were abandoned. Unless otherwisw noted, all subsequent work described hereafter was done in one of three different isocratic modes as will be explained.

2.4.1. Effect of Buffer Type

For the weak solvent of the mobile phase, A, two different buffers ammonium dihydrogen phosphate (denoted here as AH_2P) (100 mM) and TEAP (25 mM) acidified to pH 2.5 with 85% orthophosphoric acid were employed at 26 ^{o}C and 50 ^{o}C. To improve precision in both measurement of retention times and signal intensities, progesterone was used as an internal standard (IS) for the determination of SGAAs. Moreover, it had structural similarity to the aglycones and was economical to obtain. The isocratic solvent conditions were 30% ACN and 70% buffer (either TEAP or AH_2P) for the separation of SGAs, but 60% ACN and 40% buffer was necessary for the

elution of SGAAs. Further increases in percent ACN (e.g. to 75%) caused formation of precipitates with ammonium dihydrogen phosphate (100 mM) as phosphate buffers are marginally soluble in solutions that contain high concentrations of organic solvent "(Snyder et al. 1997)". Ammonium dihydrogen phosphate buffer produced better separations of the SGAs at both 26 ^{o}C (Figure B.1) and 50 ^{o}C (Figure 2.6). However, bands 3 (chaconine) and 4 (solamargine) still overlapped. The SGAAs (solanidine and solasodine) were separated adequately with both TEAP and AH_2P buffers but the peaks were narrower with the ACN/ammonium dihydrogen phosphate buffer (Figure 2.7). Inexplicably the IS peak showed distortions akin to interferences; however such observations of interferences was intermittent and eventually nonexist as seen in subsequent studies in this work.

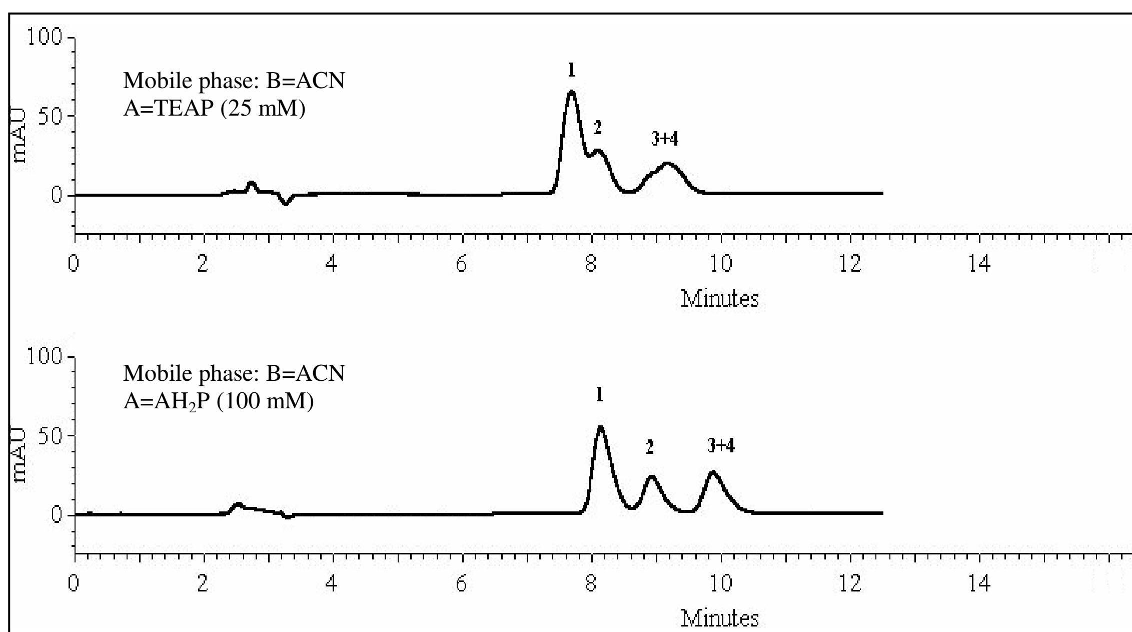


Figure 2.6. Effect of buffer type on separation of SGAs T: 50 ^{o}C pH: 2.5 F: 1.0 mL 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine

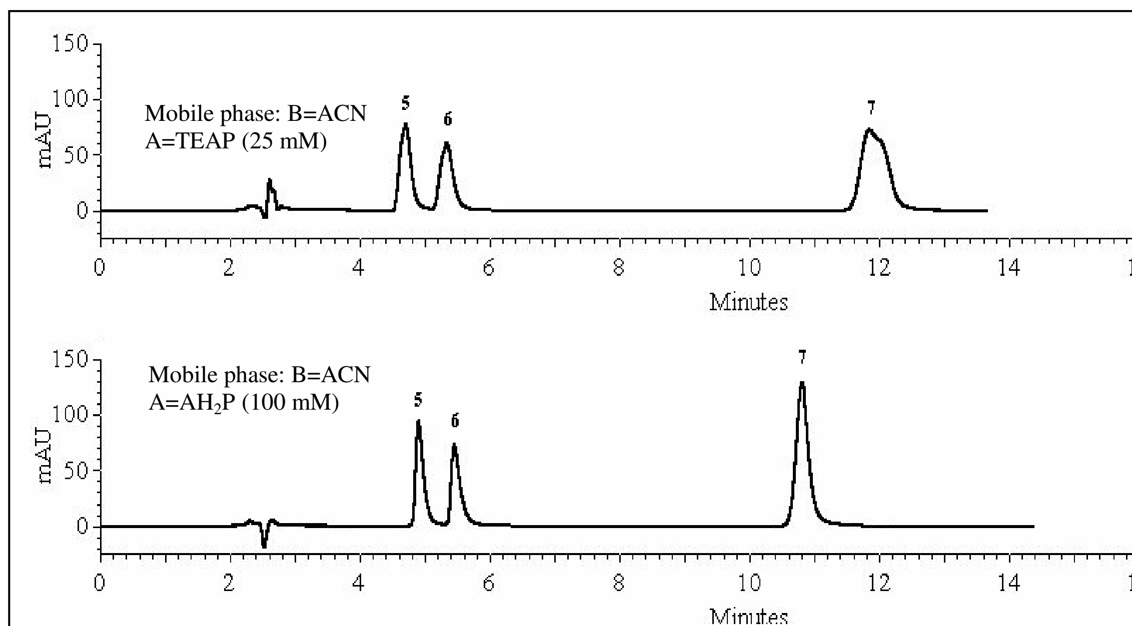


Figure 2.7. Effect of buffer type on the separation of SGAAs T: 50 ^{0}C pH: 2.5 F: 1.0 mL/min 5: solanidine 6: solasodine 7: progesterone as internal standard

In an attempt to improve the separation of the overlapping bands (chaconine and solamargine) TEAP and ammonium dihydrogen phosphate buffer were mixed. The resulting chromatogram is shown in Appendix B Figure B.2. When 20 mM TEAP and 5 mM ammonium dihydrogen phosphate buffers were mixed, similar separation was observed as when 100 mM ammonium dihydrogen phosphate buffer was employed. However, the separation was not reproducible when the same buffer mixture was applied another day (Figure B.3). As a result ammonium dihydrogen phosphate buffer (100 mM) were chosen as it produced better separations of the SGAs.

2.4.2. Effect of Temperature

The separation temperature can be selected to achieve different goals. Many LC methods specify ambient temperature, which means that the column temperature need not to be controlled. However, a primary requirement is that the column temperature not change to avoid possible shifts in retention and selectivity as room temperature varies. A temperature of 35 or 40 ^{o}C is usually a good starting point but ambient temperature is required if the method will be used in laboratories with HPLC instrumentation that lack column temperature control. Figure 2.8 shows the chromatogram obtained with

ACN/ammonium dihydrogen phosphate buffer (100 mM) (30/70) at two different temperatures. It can be seen that band pair 2+4 was the critical pair at 26 ^{0}C. Bands 1 and 2 were well separated. Band 4 moved toward band 3 and overlapped as the temperature was raised to 50 ^{o}C. Bands 1 and 2 were separated reasonably. The chromatogram indicates that variation in temperature results in selectivity changes for the separation of glycoalkaloids. As the temperature increased, SGAAs eluted earlier and peaks were sharper as compared with those at lower temperature (Figure 2.9). When TEAP buffer was used, temperature also influenced the separation of SGAs (Appendix C Figure C.1). Band 1 was separated reasonably at 26 ^{o}C while at 50 ^{o}C bands 1 and 2 were partially separated. The retention times of SGAAs decreased slightly at higher temperatures (Appendix C Figure C.2).

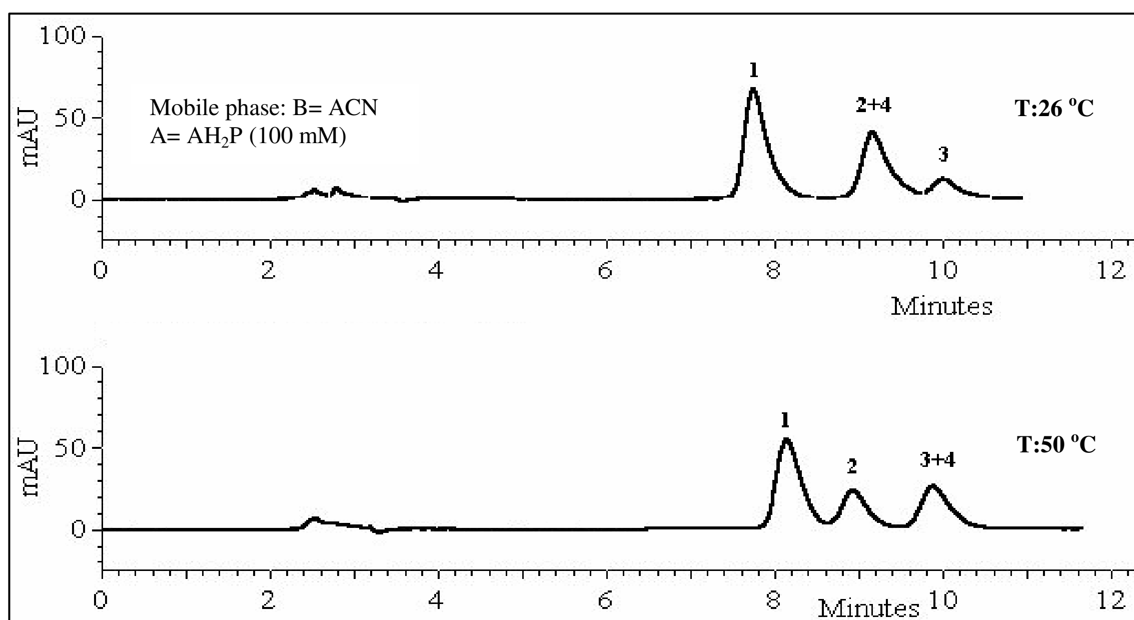


Figure 2.8. Effect of temperature on the separation of SGAs pH: 2.5 F: 1.0 mL/min 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine

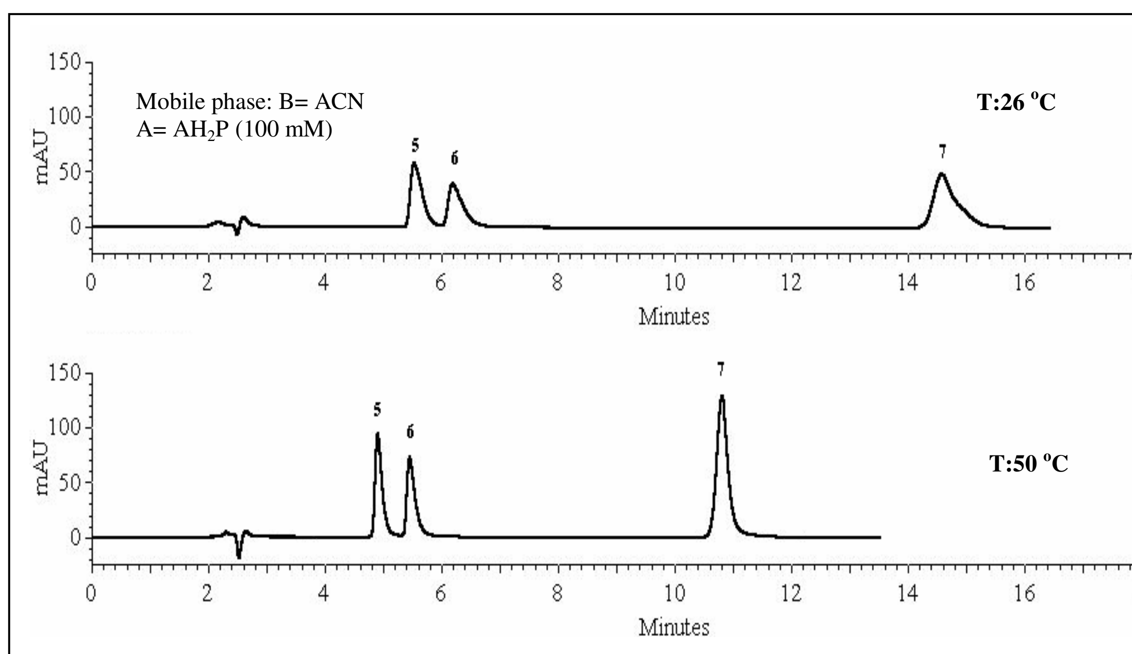


Figure 2.9. Effect of temperature on the separation of SGAAs pH: 2.5 F: 1.0 mL/min 5: solanidine 6: solasonine 7: progesterone as internal standard

2.4.3. Effect of Solvent Type

Another powerful approach to improve resolution is the use of organic solvent mixtures "(Snyder et al. 1997)". Thus methanol (MeOH), a weaker reverse phase solvent, was added to ACN. At 26 °C and with the addition of MeOH (10%), peak pair 3+4 (chaconine+solamargine) overlapped and band 2 (solanine) moved toward band 1 (solasonine) (Figure 2.10). At 50 °C when the organic modifier was ACN the critical overlapping band pair was 3+4 but remarkably, addition of 10% MeOH to ACN resulted in baseline separation of bands 3 and 4 (Figure 2.11). However, band 1 and 2 overlapped. At both temperatures, not only did the separation of solanidine and solasodine (SGAAs) improve, but also the overall retention times of both of these analytes were extended by manipulating the amount of methanol in ACN (mobile phase solvent B) as shown in Figure 2.12 and Appendix D Figure D.4. To the best of our knowledge this is the first time that the use of methanol in this manner has been shown to improve the separation of solamargine and chaconine.

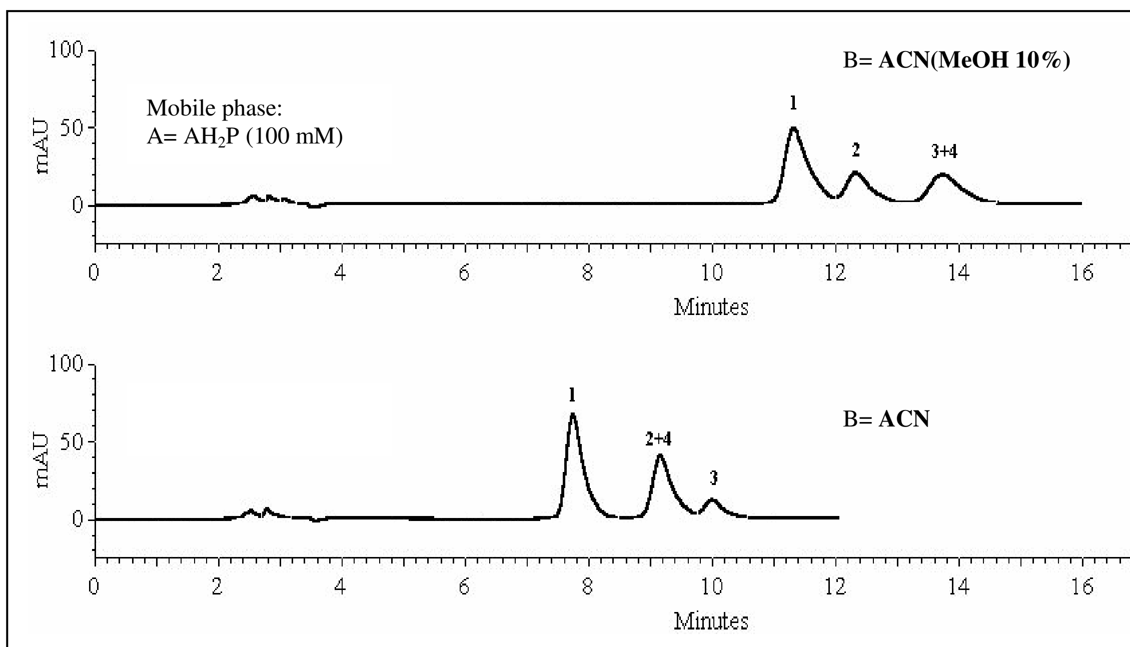


Figure 2.10. Effect of addition of methanol on the separation of SGAs T: 26 °C pH: 2.5 F: 1.0mL/min 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine

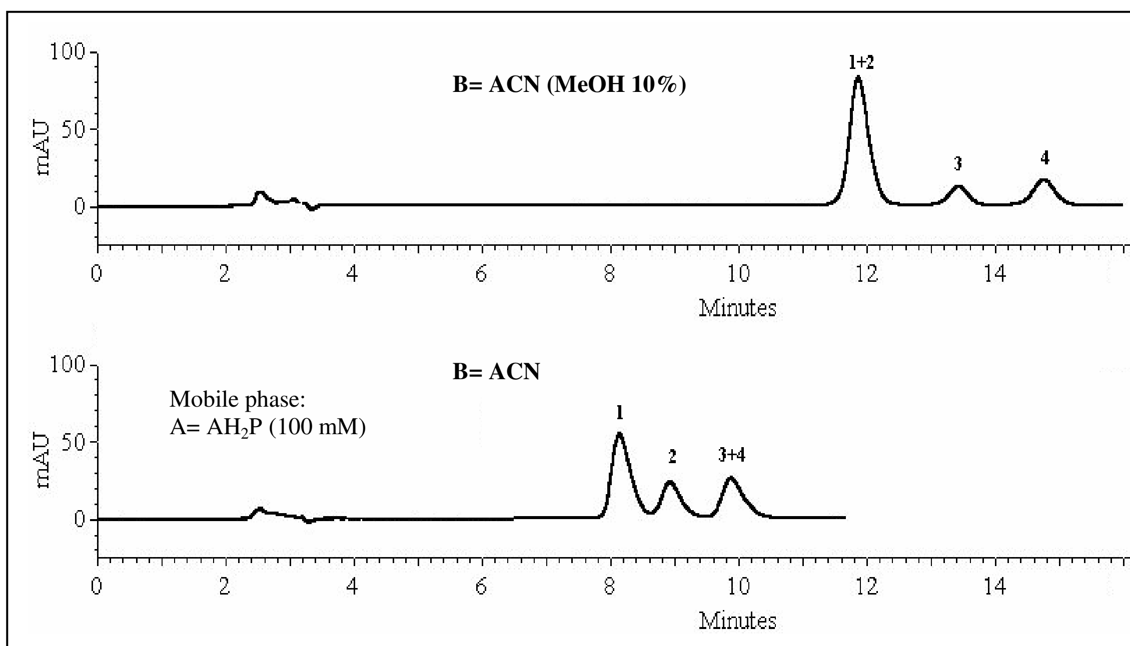


Figure 2.11. Effect of addition of methanol on the separation of SGAs T: 50 °C pH: 2.5 F: 1.0 mL/min 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine

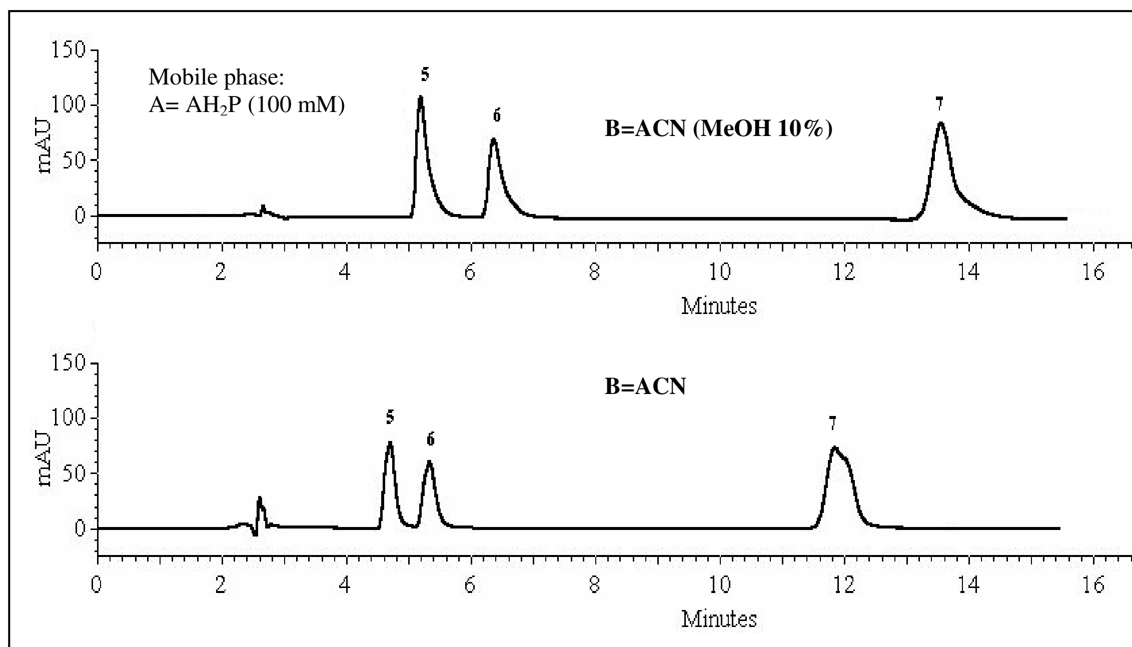


Figure 2.12. Effect of addition of methanol on the separation of SGAAs T: 50 ^{0}C pH: 2.5 F: 1.0 mL/min 5: solanidine 6: solasodine 7: progesterone as internal standard

When TEAP buffer (A) was used in conjunction with the addition of methanol to ACN (B), separation of chaconine and solamargine improved but in this case band 2 (solanine) overlapped band 1 (solasonine) at 26 ^{o}C (Appendix D Figure D1). At 50 ^{o}C separation of chaconine and solamargine improved while chaconine moved towards band pair 1+2 (Figure D2).

2.4.4. Effect of pH

A change in pH is an effective way to vary separation selectivity for ionic samples. At this point in this study, it became clear that AH_2P was the buffer of choice. Figure 2.13 therefore illustrates separations at different pH values when using ammonium dihydrogen phosphate buffer. Changing pH did not improve the separation of chaconine and solamargine. At pH 3.5 and 4.5, however, showed improvement in the separation of solanine and chaconine band spacing between 2 and 3 and hence their separation increased. At pH 4.5 separation of solasoine and solanine became worse. Overall lowering the pH of the mobile phase decreased the retention of SGAs.

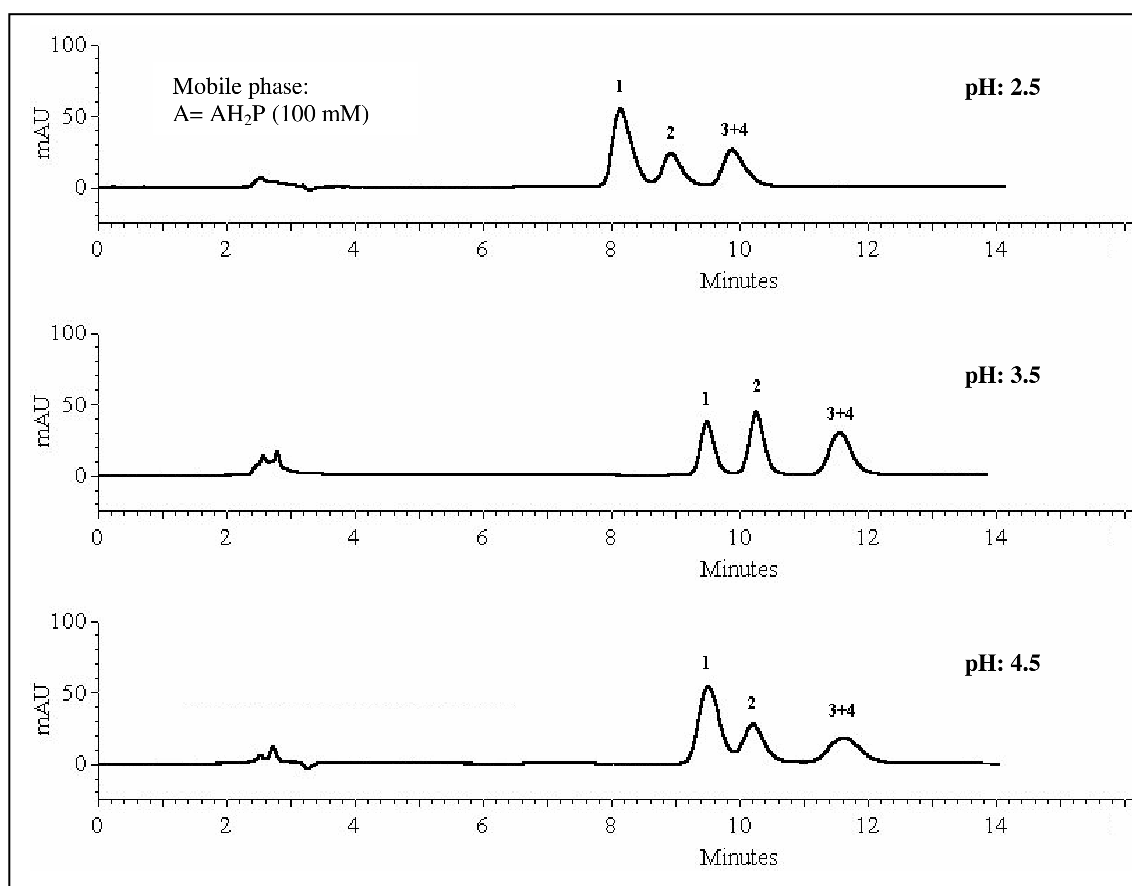


Figure 2.13. Effect of pH on the separation of SGA T:50 ^{0}C F:1.0 mL/min 1: solasonine 2: α-solanine 3: α-chaconine 4: solamargine

In conclusion, for the separation of the studied glycoalkaloids and their aglycones three methods were chosen. The best set of conditions for the separation of solamargine and chaconine were decided as follows: ACN (10%MeOH)/ammonium phosphate buffer (30/70), pH: 2.5, and temperature 50 ^{o}C (Method 1). Solanine and solasonine can be separated under two sets of conditions, namely ACN (10%MeOH)/ammonium phosphate buffer (30/70), pH: 2.5, and temperature 26 ^{o}C and ACN/ammonium phosphate buffer (30/70), pH:2.5, and temperature 50 ^{o}C. However, the presence of methanol and lower temperature increased the retention times of glycoalkaloids and peaks showed more tailing. The column back pressure was higher at lower temperatures with or without methanol due to higher solvent viscosities. Therefore, various combinations can be made as a compromise for the separation of solanine and solasonine from the following conditions: ACN/ammonium phosphate buffer (30/70), pH: 2.5, and temperature 50 ^{o}C (Method 2). The aglycones were

separated under all conditions discussed above. The chosen set of conditions for the separation of the aglycones (solasodine and solanidine) was as follows: ACN/ammonium phosphate buffer (60/40), pH:2.5, and temperature 26 ^{o}C (Method 3). If the HPLC system being used has a column heater, the same conditions but at 50 ^{o}C will give sharper peaks for the separation of solasodine and solanidine.

2.4.5. Calibration Results

Calibration was performed by injecting standard mixtures of solasodine and solanidine at levels ranging from 4 to 200 mg/L. The calibration plots for solanidine and solasodine are shown in Figure 2.14 to 2.17. Good linearity of response was found for solanidine and solasodine this concentration range belonging to cited interval, with correlation coeffiecients greater than 0.9950.

Based on the reative areas obtained in the chromatograms, solanidine absorbs UV radiation of 205 nm more efficiently than solasodine at the same concentration. Likewise, the same relative ratio of solanidine to solasodine was also seen at 208 nm as well; however, greater signal intensities were seen for both analytes at 205 nm. The average standard deviation of the background in general was 0.457 mg/L, which when multiplied by 10 yielded a practical estimated limit of quantitation of 4.6 mg/L for both solasodine and solanidine analyzed under these mobile phase conditions. The limit of detection (LOD) was 9 mg/L for solanidine and 1.6 mg/L for solasodine. Note this value was determined in a solution of ACN/water (1:1 v/v) acidified with orthophosphoric acid to pH: 3. Detection limits were not determined in an eggplant matrix.

For quantitative analysis, progesterone was used as an internal standard. Specifically, it was important to know if using an internal standard would improve measurement precision for our HPLC methods without adversely affecting accuracy. For analysis of solasodine, a value of 17.4 ± 0.2 ppm was determined for a prepared 18 ppm solasodine sample (error assumed to be less than 0.2 ppm) when using an internal standard. Without the internal standard, the calculated solasodine concentration was 16.8 ± 1.3 ppm. These results reflect an increase in precision, but a decrease in accuracy. Apparently for our HPLC work, precision is already very good without use of an internal standard. However, an internal standard or surrogate added to the extraction

sample before extraction may show a different result because the extraction may be more difficult to reproduce.

Progesterone was a good choice for internal standard because it should not present in the eggplant sample. Others have used cholesterol and even caffeine as internal standards for analysis of SGAs and SGAAs in potato "(Laurila et al. 1998)"; however, these compounds, especially caffeine, can be found in eggplant. Progesterone is not an ideal internal standard and when the potential precision has reached a limit, any improvement in precision may be offset by a decrease in accuracy. This is normal. Many assume that using an internal standard will automatically improve the results. It can improve precision but not accuracy.

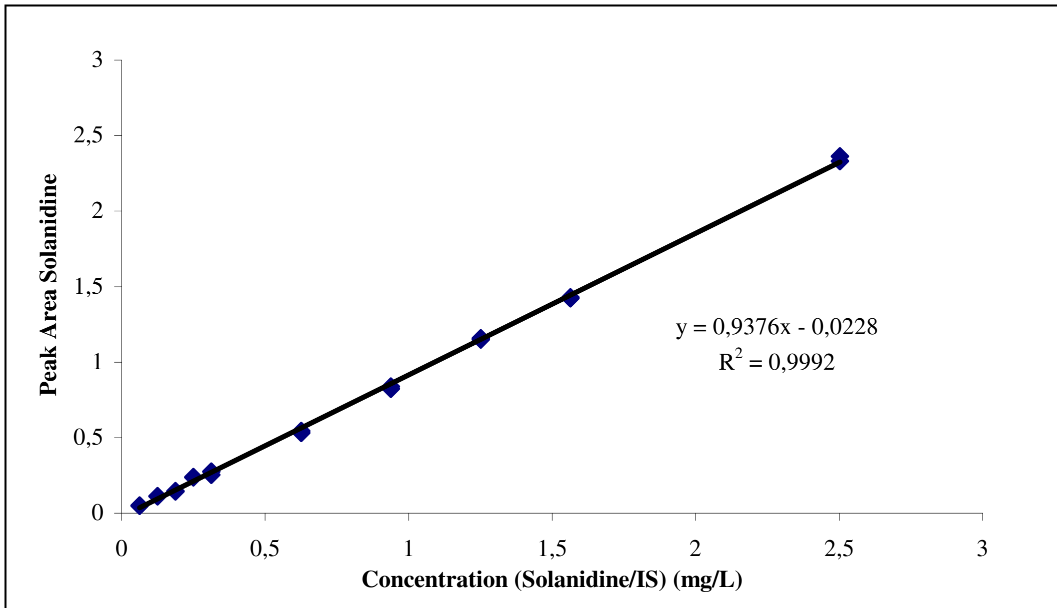


Figure 2.14. Calibration plot for solanidine in the concentration range of 4-200 mg/L

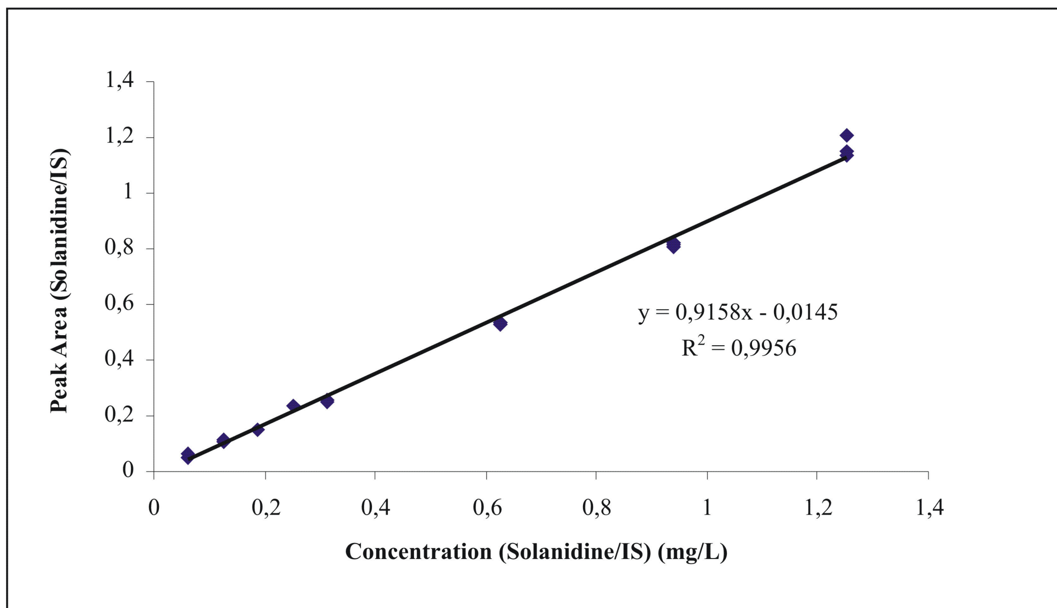


Figure 2.15. Calibration plot for solanidine in the concentration range of 4-100 mg/L

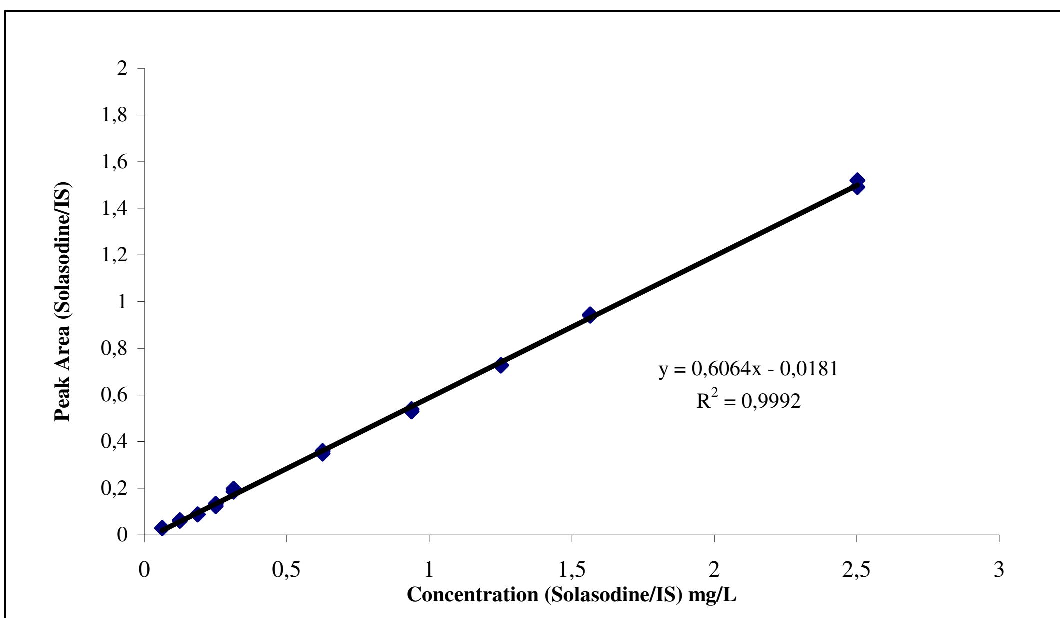


Figure 2.16. Calibration plot for solasodine in the concentration range of 4-200 mg/L

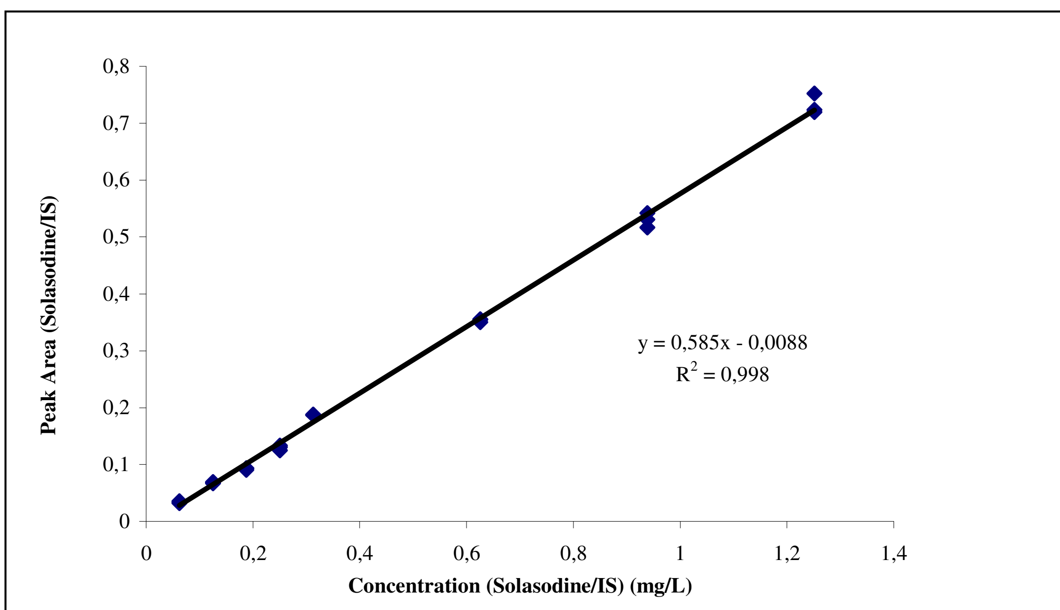


Figure 2.17. Calibration plot for solasodine in the concentration range of 4-100 mg/L

In Figures through 2.18 through 2.21 the calibration curves for the determination of solanidine and solasodine without internal standardization at 26 ^{o}C and 50 ^{o}C are shown.

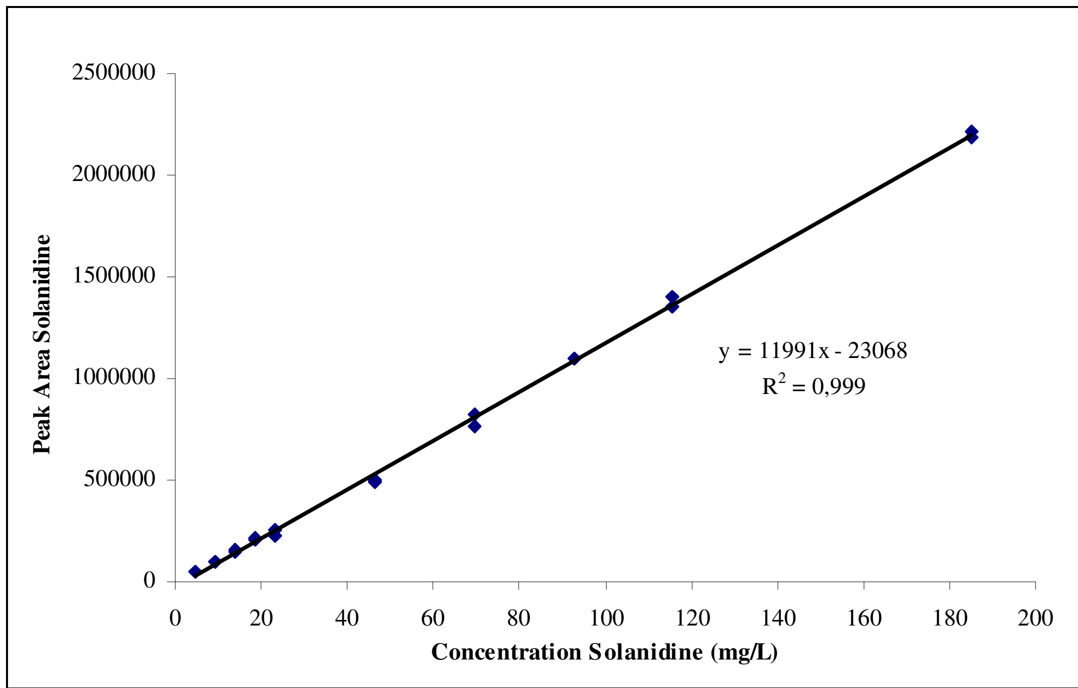


Figure 2.18. Calibration plot for solanidine in the concentration range of 4-200 mg/L without IS

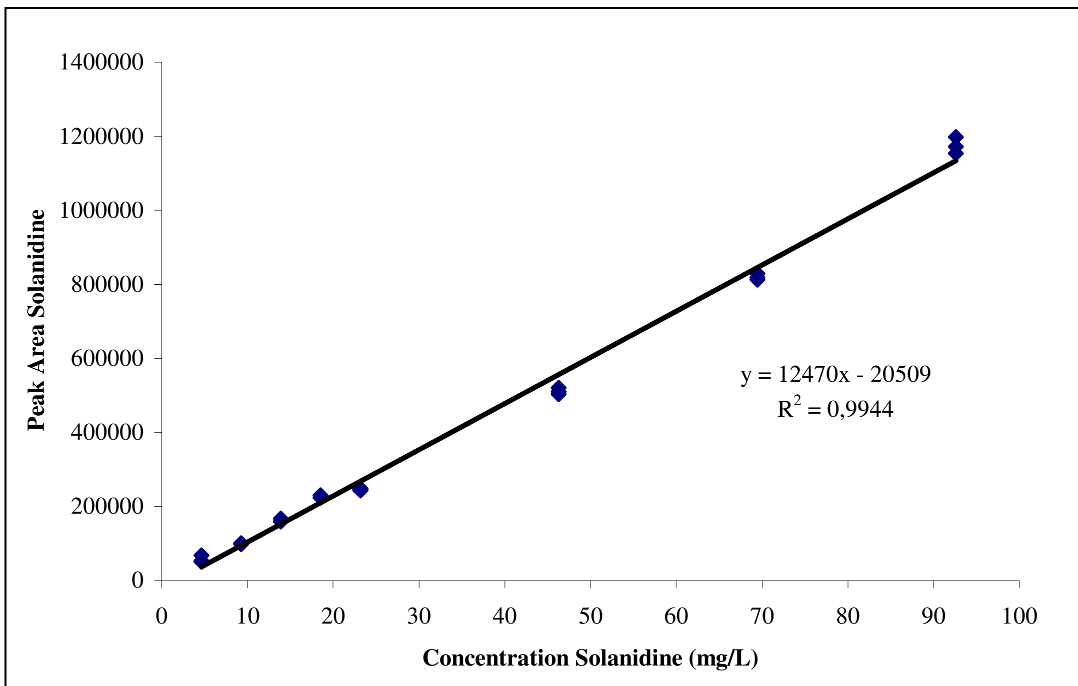


Figure 2.19. Calibration plot for solanidine in the concentration range of 4-100 mg/L without IS

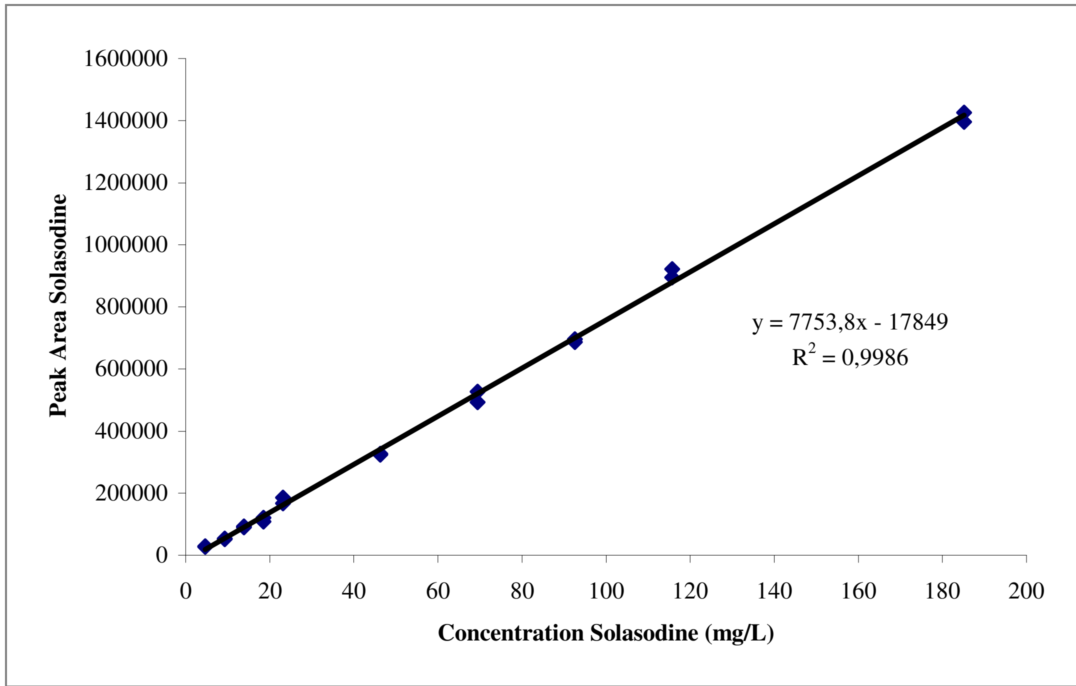


Figure 2.20. Calibration plot for solasodine in the concentration range of 4-200 mg/L without IS

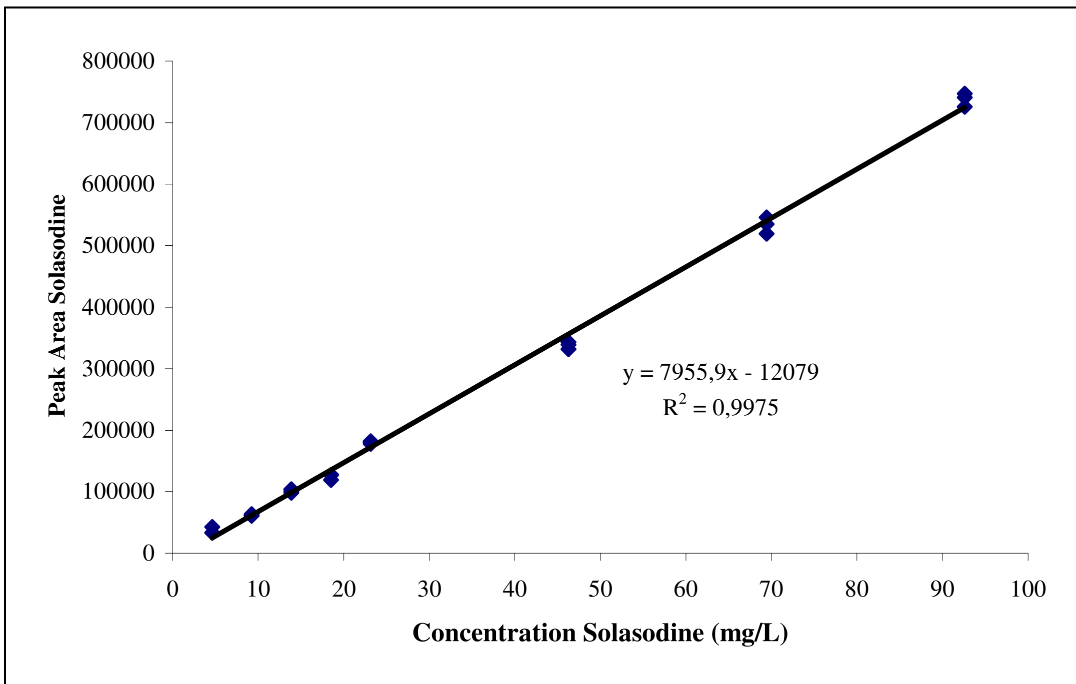


Figure 2.21. Calibration plot for solasodine in the concentration range of 4-100 mg/L without IS

CHAPTER 3

GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS) SOLID - PHASE MICROEXTRACTION (SPME)

3.1. Introduction

SPME is a sample-preparation technique, introduced in 1989 by Pawliszyn for volatile organic compounds analysis in an attempt to redress limitations inherent in SPE and LLE. It integrates sampling, extraction, concentration, and sample introduction into a single solvent-free step. SPME has been used in combination with GC and GC/MS and successfully applied to a wide variety of compounds, especially for the extraction of volatile and semi-volatile organic compounds from environmental, biological and food samples. Direct coupling of SPME with HPLC and HPLC-MS in order to analyse weakly volatile or thermally labile compounds not amenable to GC or GC-MS is also possible "(Vas and Vekey 2004)".

The commercially available apparatus shown in Figure 3.1 consists of a short-length (1-2 cm) narrow diameter fused-silica fibre coated with a stationary phase attached to a stainless steel guide rod. This is housed in a hollow septum-piercing needle into which the fibre can be withdrawn for protection when not in use. The whole needle/fibre assembly is contained in a holder, adjustable to allow for variable depth of fibre exposure either during sampling or desorption. A modified unit has become available for field sampling "(Mills and Walker 2000)". The fibre itself is a thin fused-silica optical fibre, coated with a thin (7-100 μm) polymer film (e.g., poly(dimethylsiloxane)-PDMS, poly(acrylate)-PA, poly(ethyleneglycol)-Carbowax) or a mixture of polymers blended with a porous carbon-based solid material (e.g. PDMS-Carboxen) "(Mullet and Pawliszyn 2003)".

SPME sampling can be performed in three basic modes: direct extraction, headspace extraction, and extraction with membrane protection. In the direct extraction mode, the coated fibre is inserted into the sample and the analytes are transported directly from the bulk of the sample to the extracting phase. In headspace mode, the analytes are extracted from the gas phase equilibrated with the sample. The main

purpose for this modification is to protect the fibre from adverse effects caused by non-volatile, high molecular weight substances present in the sample matrix (e.g. proteins, humic acid).

When the fibre is in the headspace, the analytes are removed from the headspace first, followed by indirect extraction from the matrix. Thus, volatile analytes are extracted faster than semivolatiles. In membrane-protected SPME, the fibre is separated from the sample by a selective membrane, which lets the analytes through while blocking the interferences. The primary reason for the use of membrane is to protect the fibre against matrix when dirty samples are analysed. While extraction from headspace serves the same purpose, membrane protection allows the analysis of less volatile compounds. The fiber is then transferred from the sample to the desorption chamber, typically a hot GC injection port, where the extracted analytes are desorbed and transported to the analytical column for separation and detection.

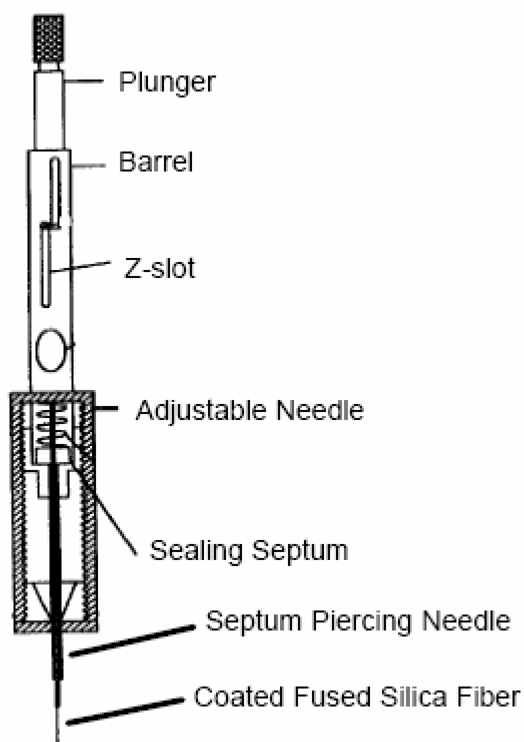


Figure 3.1. Schematic diagram of SPME assembly
(Source: Vas and Vekey 2004.)

SPME is not an exhaustive extraction. Extraction is based on gas-liquid or liquid-liquid partitioning. The analytes are adsorbed or absorbed by the fiber phase (depending on the nature of the coating) until an equilibrium is reached in the system. Equilibrium methods are more selective because they take full advantage of the differences in extracting-phase/matrix distribution constants to separate target analytes from interferences. Exhaustive extraction can be accomplished in SPME when the distribution constants are large enough. The amount of an analyte extracted by the coating at equilibrium is determined by the magnitude of the partition coefficient (distribution ratio) of the analyte between the sample matrix and the coating material. Once equilibrium is reached, the extracted amount is constant and is independent of further increases in extraction time. When equilibration times are long, shorter extraction times can be used. However, in such cases the extraction time and mass transfer conditions have to be strictly controlled to ensure good precision "(Pawliszyn 1999)". The partition equilibrium for a liquid polymeric extraction phase (absorbent) can be described mathematically by Eq.3.1 "(Wang et al. 2005)". In general, there is a linear relationship between the amount of the extracted analyte, n, and its initial concentration in the sample matrix, C_o, as described in equation 3.1 (for direct SPME) where K_{fs} is the fibre/sample distribution coefficient, V_f is the volume of the fibre coating and V_s is the volume of the sample.

$$n = \left(\frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s}\right) C_o \qquad (3.1)$$

For headspace analysis this equation should be expressed as shown in equation 3.2

$$n = \left(\frac{K_{fs} V_f V_s}{K_{fs} V_f + K_{hs} V_h + V_s}\right) C_o \qquad (3.2)$$

With this proportionality, the extracted quantity correctly reflects the concentration of the analyte in the sample matrix. For a sufficiently large sample volume (V_s) equations 3.1 and 3.2 can be reduced to:

$$n_f = K_{fs} V_f n_o \quad (3.3)$$

Eq. 3.3 indicates that the efficiency, and hence sensitivity of the extraction process is directly dependent on the distribution constant, K_{fs}. Therefore, imparting analyte selectivity to SPME is possible by modifiying any experimental parameter that will result in a higher distribution constant for that analyte relative to interferences in the sample matrix. This may be done by changing the sample pH, derivatization of analytes, or more fundamentally, altering the extraction coating on the SPME fiber.

Table 3.1 lists the most common commercially available coatings. PDMS is a high-viscosity rubbery liquid. PA is a solid crystalline coating that becomes liquid at desorption temperatures. Both PA and PDMS extract analytes via absorption. Carbowax/DVB, Carbowax/TR and Carboxen, are mixed coatings, in which the primary extracting phase is a porous solid extracting analytes via adsorption. The difference between these processes is that, in absorption, analytes dissolve in the coating and diffuse into the bulk of it during the extraction process, while in adsorption they stay on the surface of the solid "(Pawliszyn 1999)".

Table 3.1 Summary of commercially available SPME fibers
(Source: Mills and Walker 2000)

Fiber coating	**Film thickness (µm)**	**Polarity**	**Coating method**	**Maximum operating temperature (°C)**	**Technique**	**Compounds to be analysed**
Polydimethylsiloxane (PDMS)	100	Non-polar	Non-bonded	280	GC/HPLC	Volatiles
PDMS	30	Non-polar	Non-bonded	280	GC/HPLC	Nonpolar semi-voaltiles
PDMS	7	Non-polar	Bonded	340	GC/HPLC	Medium to nonpolar semi-volatiles
PDMS-divinylbenzene (DVB)	65	Bipolar	Cross-linked	270	GC	Polar volatiles

(cont.on next page)

Table 3.1 (cont)

Fiber coating	**Film thickness (µm)**	**Polarity**	**Coating method**	**Maximum operating temperature (°C)**	**Technique**	**Compounds to be analysed**
PDMS-DVB	60	Bipolar	Cross-linked	270	HPLC	General purpose
PDMS-DVB[a]	65	Bipolar	Cross-linked	270	GC	Polar volatiles
Polyacrylate (PA)	85	Polar	Cross-linked	320	GC/HPLC	Polar semivolatiles (phenols)
Carboxen-PDMS	75	Bipolar	Cross-linked	320	GC	Gases and volatiles
Carboxen-PDMS[a]	85	Bipolar	Cross-linked	**320**	GC	Gases and volatiles
Carbowax-DVB	65	Polar	Cross-linked	265	GC	Polar analytes (alcohols)
Carbowax-DVB[a]	70	Polar	Cross-linked	265	GC	Polar analytes (alcohols)
Carbowax-templated resin (TPR)	50	Polar	Cross-linked	240	HPLC	Surfactants
DVB-PDMS-Carboxen[a]	50/30	Bipolar	Cross-linked	270	GC	Odours and flavours

[a]Stableflex type is on a 2 cm length fibre

3.1.1. Derivatization for Gas Chromatography (GC)

Volatility and thermal stability of the compounds are desirable in gas chromatographic analysis. Luckily, by modifiying the functionality of a molecule (e.g., -OH, COOH, =NH, $-NH_2$, -SH, and other functional groups) with derivatizing reagents, it is possible to analyze compounds that otherwise are not readily monitored using GC.

The reduction in polarity of the compounds may also improve the gas chromatographic properties of the compounds by minimizing undesirable and non-specific column adsorption which can distort peak shapes. The resolution of closely related compounds not separated in the underivatized form can also be enhanced by using a suitable derivative. In some cases the mass spectrum of the underivatized molecule exhibits poor diagnostic ions. After derivatization, the fragmentation pattern may change so that structural elucidation is more clear. Mass spectra with ions of higher m/z ratios and higher abundance can be obtained. High-mass-ions have greater diagnostic value, since they are more specific than low-mass-ions, which can be easily influenced by interference from the fragment ions of contaminants such as those due to

column bleeding. An increase in the abundance of the molecular ion or a related ion can also be used for determination of the molecular mass. The production of more than one derivative can give helpful additional information for determining molecular mass. In addition derivatization can be used to favour the formation of highly stable, characteristic fragment ions for the GC-MS analysis of a structurally related group of compounds “(Segura et al. 1998)”.

A large number of reagents are used to prepare derivatives for gas chromatography, but most of the derivatization reactions belong to one of three categories: acylation, alkylation, or silylation. Of these, silylation is the most widely used for GC-MS. Silyl derivatives are formed when active proton displacement (in –OH, -SH or –NH groups) by an alkylsilylgroup occurs. Trimethysilylation is the most common silylation procedure. Trimethylsilyl derivatives are easy to prepare compared to acylation. During acylation a halogen acid is produced and a basic acceptor is normally required for neutralization. The removal of the excess acylating reagent is preferred as its presence can lead to problems during GC. A variety of trimethylsilylating reagents with different properties (e.g., volatility, reactivity, selectivity, by-product formation) have been developed including the trimethylhalosilanes, TMS-amines, TMS-esters and the TMS-amides (BSTFA, MSTFA).

Trimethylsilylimidazole (TMSI) has a strong silylation power for hydroxyl and carboxyl groups but does not react with amino groups. All silylation reagents and derivatives are sensitive to moisture; for this reason, reactions must be performed under anhydrous conditions “(WEB_4 2006)”.

Gas chromatography has been applied for the determination of the aglycones of steroidal glycoalkaloids in potato materials. Using GC with a nitrogen-specific detector “(Holstege 1995)” or FID detector “(Herb et al. 1975, Lawson et al. 1992)”, several aglycones can be separated and quantified in a single run. The aglycones can be analyzed without derivatization “(Van Gelder et al.1989)”, but it has been shown that high temperatures (>280 ^{o}C) can lead to aglycone decomposition and shorten the column life “(Lawson et al. 1992)”. Permethylation “(Herb et al. 1975)”, trimethysilylation “(Laurila et al. 1996)”, and acylation “(Laurila et al. 1999)” have also been used to convert aglycones into more volatile and thermally stable derivatives. Using both trimethylsilylation (MSTFA) and pentafluoropropionylation (pentafluoropropionic acid-PFAA), more specific and abundant fragmentation for

aglycones with a tomatidine type structure for GC-MS analysis can be produced "(Laurila et al. 1999)".

3.1.2. Derivatization and SPME

Most of the published SPME applications that include derivatization have been mainly focused on the treatment of polar compounds to convert them into more easily extractable, thermally stable, more volatile analytes with better chromatographic behaviour. There are three different SPME derivatization modes:

(1) derivatization in the sample matrix prior to SPME

(2) derivatization on the SPME fiber after sampling (generally, for analyte molecules that are less volatile than those of derivatizing reagent.)

(3) simultaneous sampling and on-fiber derivatization (for analyte molecules that are more volatile than those of the derivatizing reagent.)

In this initial study the possibility of combining SPME, followed by on-fiber derivatization, and gas chromatography mass spectrometry for the qualitative determination of steroidal glycoalkaloid aglycones using standard mixtures has been evaluated. Until now no other researcher has applied SPME to the analysis of aglycones. Here analytes were first extracted onto an SPME fiber and then derivatized using TMSI.

Before performing SPME experiments, glycoalkaloids were analyzed by GC-MS without derivatization. For this, different temperature programs were tested. However, when GC was used the aglycone solasodine needed to be derivatized prior to injection into the chromatographic system. Therefore, silyl derivatives of aglycones and cholesterol (internal standard) were first formed and detected by GC-MS.

3.2. Experimental

3.2.1. Chemicals and Materials

Standards of solanidine and solasodine were obtained from MP Biomedicals LLC (Ohio, USA) and Research Plus, Inc. (Manasquan, N.J., USA), respectively. The internal standard (I.S.) cholesterol (95% purity) was purchased from Alfa Aesar

(Karlsruhe, Germany). Each of the aglycone stock solutions (1.0 mg/mL) was prepared in methanol-acetic acid (95:5 v/v) unless otherwise stated, and stored at 4 ^{o}C. The derivatization reagent TMSI was purchased from Sigma-Aldrich (St.Louis, USA) in 1 mL ampoules. A manual SPME holder and fibers with different coatings: PDMS-DVB (65 µm film thickness), CAR-PDMS (75 µm film thickness), and CW-DVB (65µm and 70 µm film thickness-Stable Flex) were obtained from Supelco (Bellefonte, PA, USA). Before their first use, each fibre was conditioned as described in the supplier specifications followed by blank analysis to determine the quality of conditioning.

3.2.2. Derivatization for GC-MS without SPME

Separate aglycone standard solutions each containing 20 microliters were placed in a 4-mL vial and evaporated to dryness for 30 min using a rotary evaporator. After the vials were dried further at 105 ^{o}C for 5 min in an oven, they were stored in a dessicator until they were cooled to room temperature with loose cap. Since TMSI is extremely sensitive to moisture, it was transferred to a vial under an inert argon atmosphere in a glove bag (I^2R Glove Bag model X-27-27, USA) for later use. Twenty microliters TMSI and 50 µL dry acetonitrile were added via glass syringe to each vial in a glove bag to exclude moisture and the mixtures were placed in an oven at 60 ^{o}C for 15 min. After this, they were cooled to room temperature and 2 µL of each solution was injected into the chromatographic system.

3.2.3. Direct SPME with on-fiber Derivatization

Twenty microliters individual standard solutions of the analytes containing the internal standard were placed in a 4-cm length and 1 mm i.d. glass capillary fixed in a vial. The analysis methodology is illustrated in Figure 3.2. The needle of the fiber holder was inserted into the capillary and the fiber was immersed into the solution. The microextraction of solanidine was carried out at room temperature for 30 min. Solasodine was extracted for an hour. After finishing the extraction step, the SPME fiber was exposed to the vapours of TMSI in a 4-mL amber vial at 70 ^{o}C for an hour.

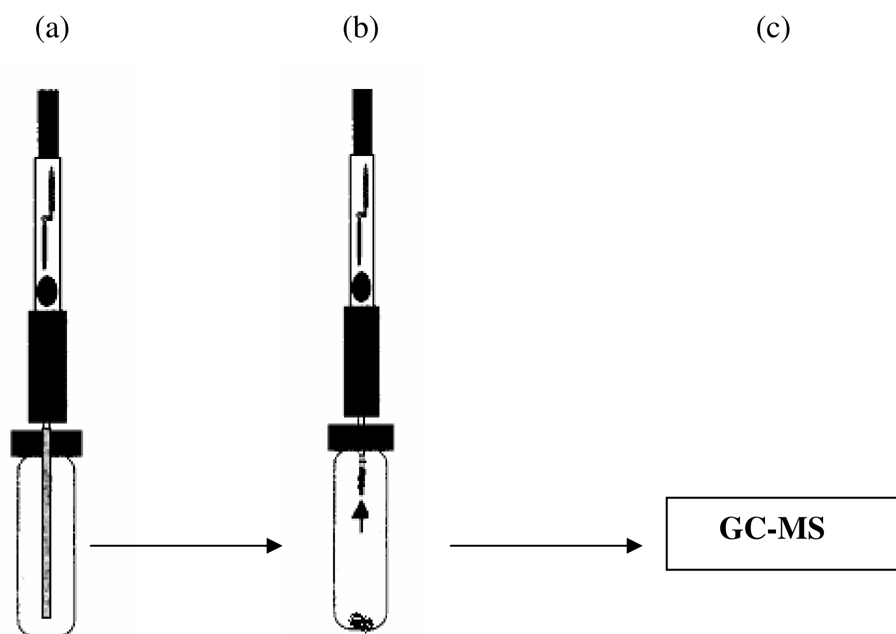


Figure 3.2. Schematic of the methodology for analysis of SGAs: (a) direct immersion extraction, (b) headspace derivatization, (c) GC-MS analysis

3.2.4. GC-MS Analysis

A Varian 2000 Ion Trap Mass Spectrometer as interfaced with a Varian Star 3400 C*x* Gas-Chromatograph (GC) (Walnut Creek, Calfornia USA) equipped with a split/splitless programmable temperature injector (SPI/1078, a 3.14 mm i.d. glass liner), and an SAC-5 type capillary column (30 mX0.25 mm i.d., df: 0.25 µm) was employed for these studies. Helium (99.999 %) was used as a carrier gas at a flow rate of 1.0 mL/min. For analysis without SPME, the GC oven and injector temperatures were kept constant at 275 ^{o}C and 300 ^{o}C, respectively. For analyis by SPME the GC oven was programmed as follows: 3 min at 160 ^{o}C, 20 ^{o}C/min to 280 ^{o}C (hold for 36 min). SPME fibers were desorbed during 5 min in the splitless mode, using the following temperatures: 240 ^{o}C for PDMS-DVB, 270 ^{o}C for CAR-PDMS, and 250 ^{o}C for CW-DVB. The GC-MS interface and ion-trap temperature were set at 280 ^{o}C and 200 ^{o}C, respectively. The ion-trap mass spectrometer was operated in normal EI mode (70 eV) and in full scan mode from m/z 40-650 for qualitative analysis.

3.3. Results and Discussion

Initial experiments were performed using a polar CW-DVB fiber since aglycones are basic, and therefore, polar compounds. Since solasodine is less volatile than solanidine, analysis by GC required derivatization. The fiber was dipped directly into a glass capillary containing a 20 μL standard mixture of solanidine and cholesterol in methanol containing 5% acetic acid (300 mg/L for each one). A capillary was preferred instead of a vial due to the high price and limited quantities of commercially available standards. That is to say that less standard was necessary for analysis when capillary tubes were used. With the capillary set up only a few microliters of sample was used for extraction. In a recent study employing adsorption type SPME sampling in a capillary, it was concluded that the shortened diffusion path favoured the achievement of equilibrium and small volume of sample reduced the possible interference from matrix. Furthermore, the large phase ratio (coating/sample) aid the complete extraction of the analytes having lower distribution constants “(Zhu et al. 2003)”.

Thirty minutes sampling time was chosen initially. After completing the extraction step the fibre was placed in the headspace of a 4-mL vial in which derivatization reagent was present at the bottom of the vial. The amount of TMSI affected the yield of derivatization. Under these conditions, two peaks were obtained for each compound, corresponding to the derivatized and non-derivatized forms (Figure 3.3).

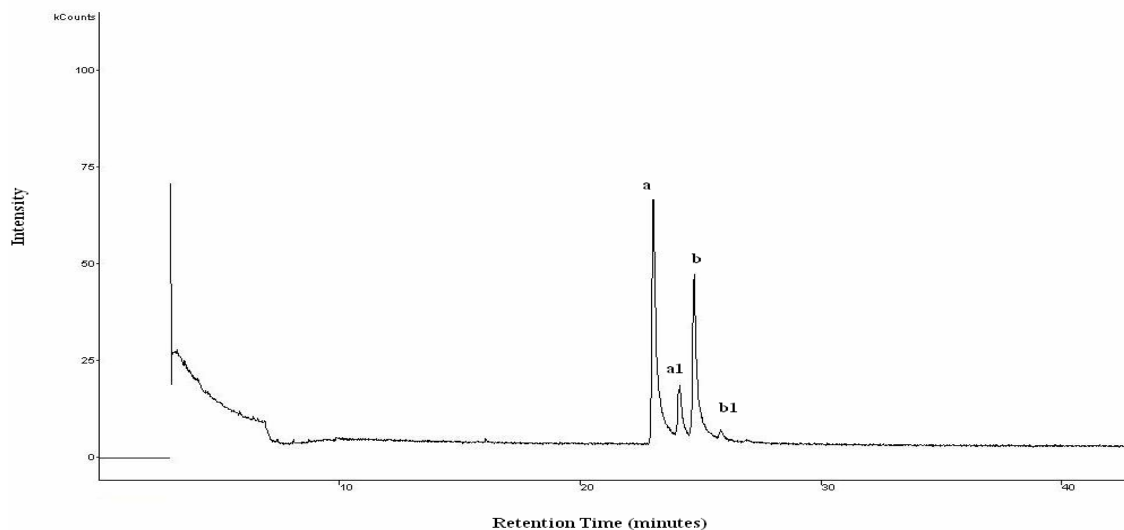


Figure 3.3. Total ion GC-MS chromatogram of derivatized standard solanidine solution (300 mg/L); a = underivatized cholesterol (IS); a_1 = derivatized cholesterol b = underivatized solanidine; b_1 = derivatized solanidine

Increasing the amount of TMSI to 40 µL and decreasing the concentration of solanidine and cholesterol (30 mg/L for each one) produced fully derivatized compounds. Total ion GC-MS chromatograms and mass spectra of each compound were obtained (Figure 3.4). Both solanidine and cholesterol produced mono-TMS derivatives with molecular ion peaks at m/z 469 and m/z 458, respectively. Under the same conditions derivatized solasodine could not be seen in GC-MS. For this reason, extraction and derivatization times were increased to 60 min. To avoid memory effects the CW-DVB fibers were additionally heated at 250 ^{o}C for 5 minutes after completing the chromatographic injection. Molecular masses of the aglycones are given in Table 3.2.

Table 3.2. Formulas and Molecular Masses of the Aglycones

Compound/Formula	**Molecular Mass**
Solanidine $C_{27}H_{43}NO$	397.60
Solasodine $C_{27}H_{43}NO_2$	413.60

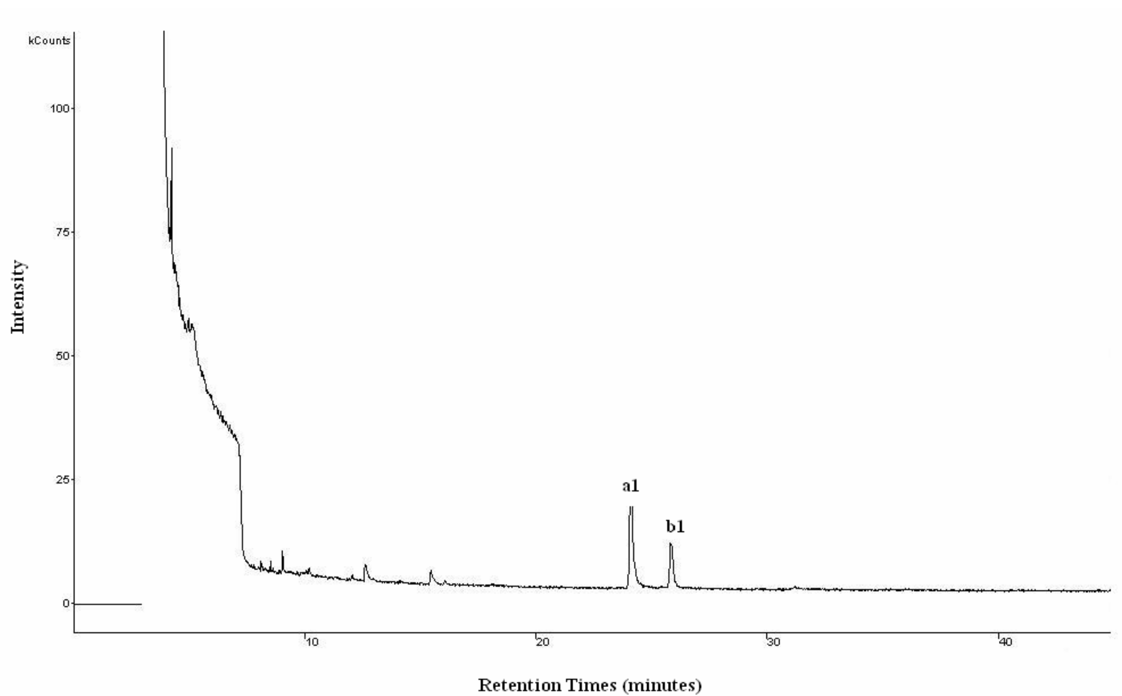


Figure 3.4. Total ion GC-MS chromatogram of derivatized standard solanidine solution (30 mg/L); a1 = cholesterol ; b1 = solanidine

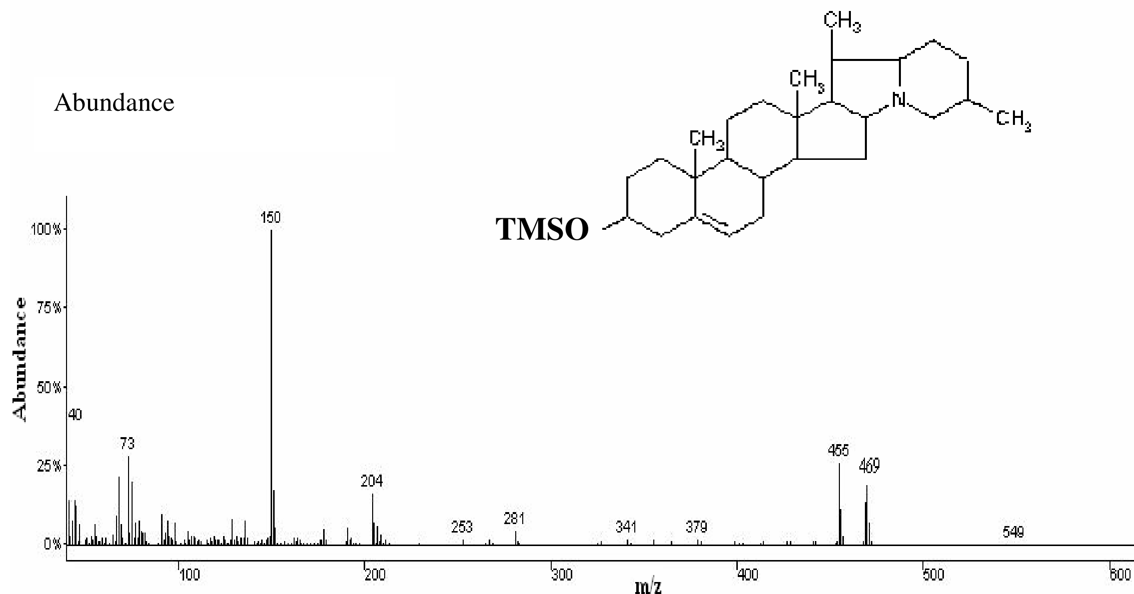


Figure 3.5. Mass spectrum of solanidine

After the first extraction, derivatization, and desorption cycle, the same GC-MS analyzed fiber was exposed to the vapours of 40 µL fresh aliquot of TMSI without any

added extraction step for 30 min. In its GC-MS spectrum (Figure 3.6), solasodine showed the di-TMS derivative after silylation with base peak at m/z 125 and at m/z 559 [M^++$2H^+$]. According to the literature the tetrahydrofuran ring opens, after which the formed hydroxyl group has been attached to the TMS group. Moreover, it has been stated that such a phenomenon can be related to the presence of the nitrogen ring, for example the silylation of diosgenin containing oxygen instead of nitrogen gave a mono-TMS derivative only "(Laurila et al.1999)". The peaks with retention times less than 20 minutes in Figure 3.6 and Figure 3.10 were most likely due to the fiber itself.

In order to investigate whether the solvent reacts with TMSI preventing derivatization of solasodine, the fiber was desorbed at 100 ^{o}C in the injector port for 5 min after the extraction step. Next, derivatization was applied using 40 μL TMSI for an hour. However, solasodine could not be seen via GC-MS even after solvent removal step. Then, without an extraction, a second derivatization was performed on the same sample as above. The total ion chromatogram is shown in Figure 3.10.

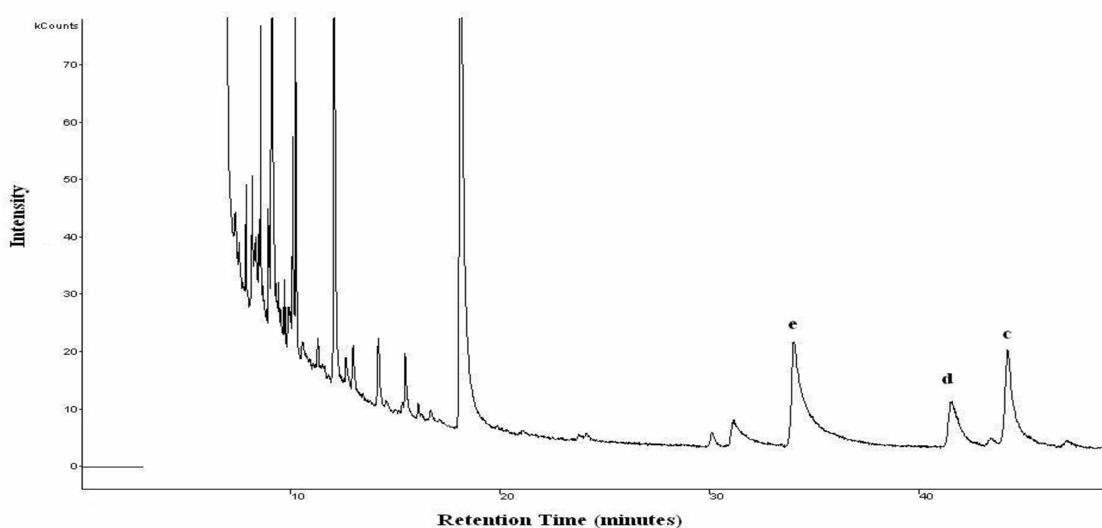


Figure 3.6. Total ion GC-MS chromatogram of derivatized standard solasodine solution (300 mg/L); c = solasodine

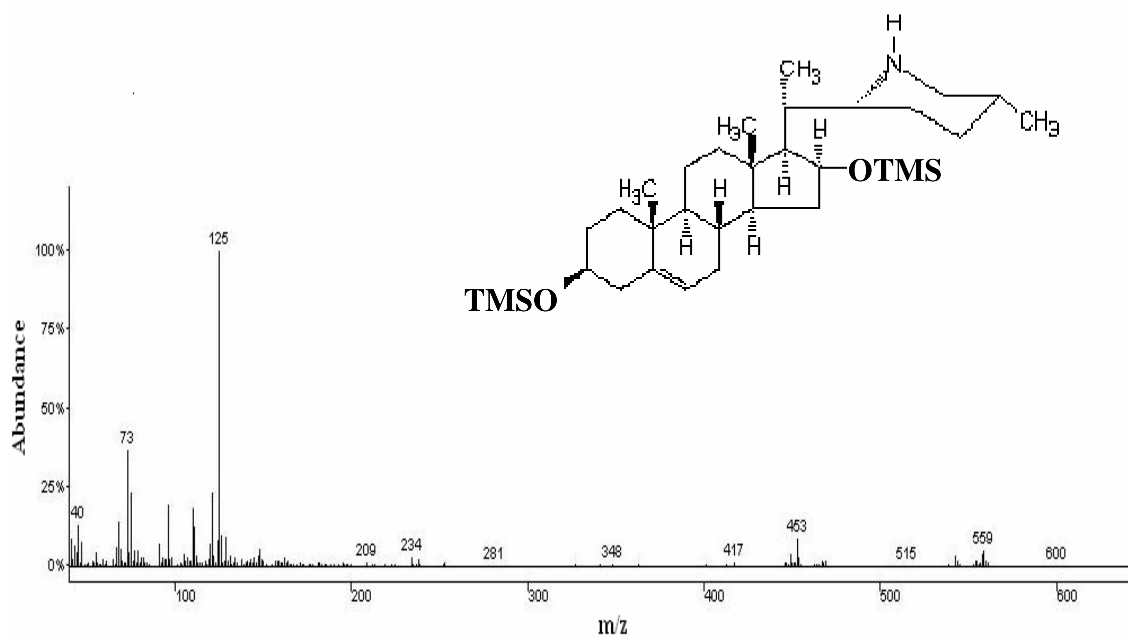


Figure 3.7. Mass spectrum of solasodine

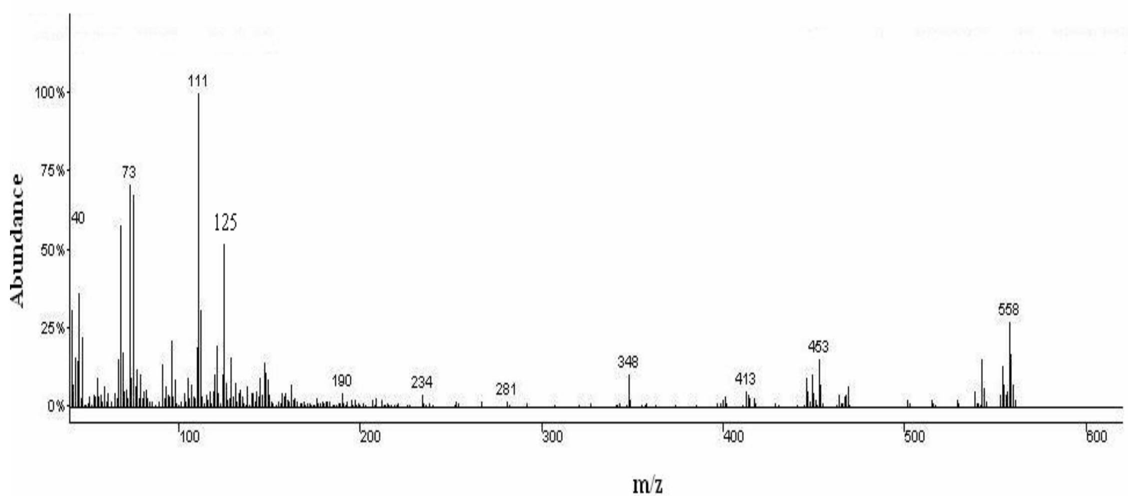


Figure 3.8 Mass spectrum of d in figure 3.6 (Refer to figure 3.6)

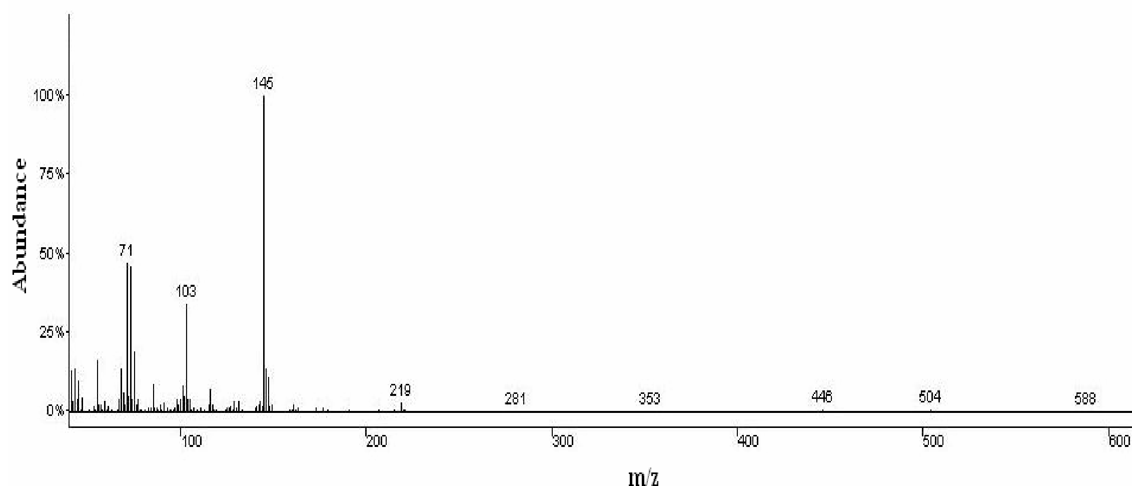


Figure 3.9 Mass spectrum of e in figure 3.6 (Refer to Figure 3.6)

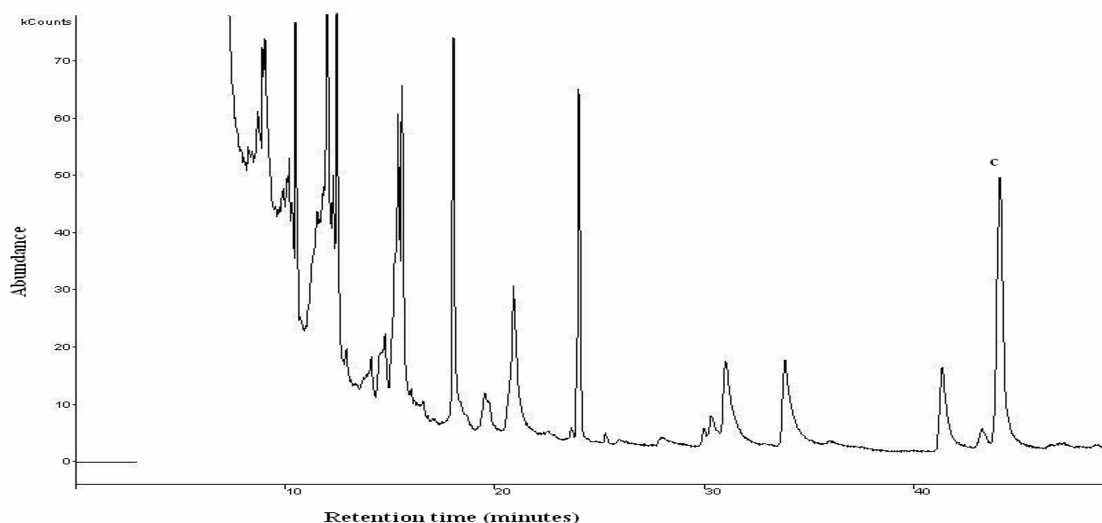


Figure 3.10. Total ion GC-MS chromatogram of standard solasodine solution (300 mg/L); c = solasodine (replicate analysis)

The unknown peak d has similar fragments as solasodine with m/z 125 and m/z 558 [M+H]. After several extraction and derivatization steps, the same CW-DVB fiber degraded to an unusable state. Unfortunately, TMSI vapour or methanol can slightly dissolve the polar coating. In further experiments, comprison trials were performed with PDMS-DVB and CAR-DVB phases. The fibers were immersed into the solution of solanidine and cholesterol (IS) overnight and then desorbed. PDMS-DVB extracted only cholesterol. However, neither solanidine nor cholesterol were observed in the GC-MS spectra after extraction using the CAR-PDMS fiber. This fiber is suited for SPME

analysis of molecules in the C_2-C_{12} range. Molecules larger than C_{12} are strongly held on the surface of the particle, and are difficult to desorb. This may require a high desorption temperature, 300-320 ^{o}C "(Pawliszyn 1999)". According to the manufacturer the maximum operating temperature of the CAR-PDMS is 320 ^{o}C thus in our experiment 270 ^{o}C was chosen as a desorption temperature to extend the life of the fiber. We also assumed that methanol might have damaged the CW-DVB fiber. It is better to prevent the exposure of the polar coating to the polar solvent. Therefore, the extraction was also carried out in a non-polar solvent. For these studies the capillary was filled with 20 µL solanidine standard solution (1000 mg/L) in methanol-acetic acid (95:5 v/v) and 20 µL hexane was added to the top of the solution (upper phase). The fiber was immersed for an hour in the hexane phase thereby preventing its contact with the lower polar phase and then desorbed in the GC injector. Under these conditions the solanidine peak was successfully obtained (Figure 3.11). Mass spectrum of solanidine was shown in Figure 3.12. However, unfortunately during the replicate extraction before derivatization the fiber coating was completely removed from the fiber, rendering it useless.

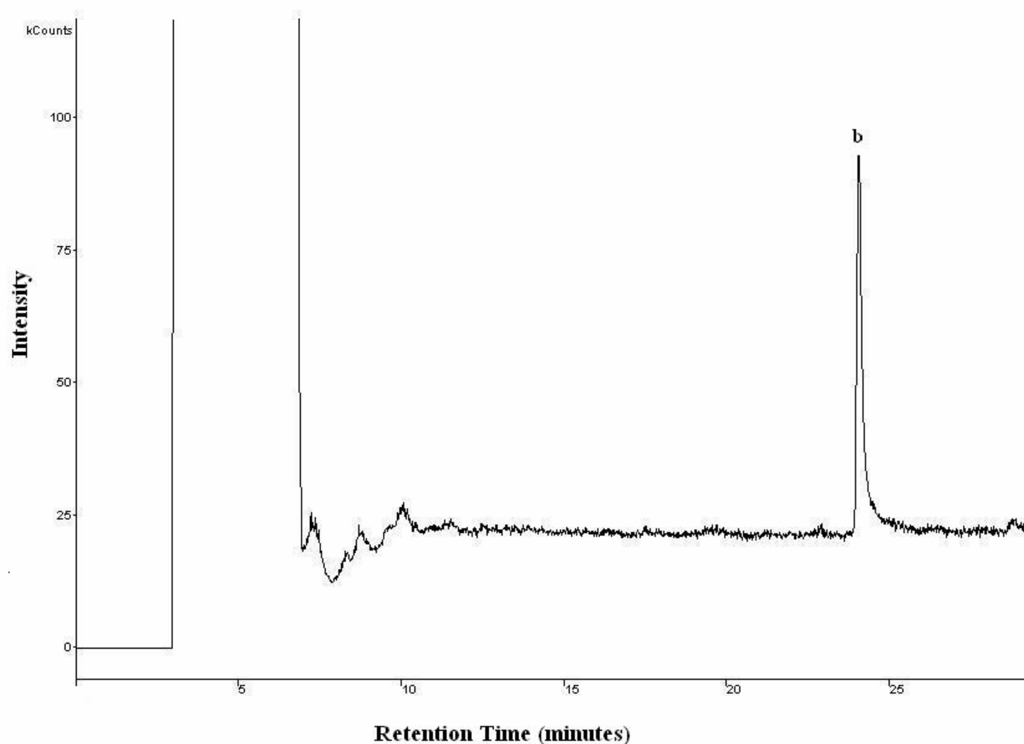


Figure 3.11. GC-MS chromatogram of solanidine solution (1000mg/ L);a = solanidine

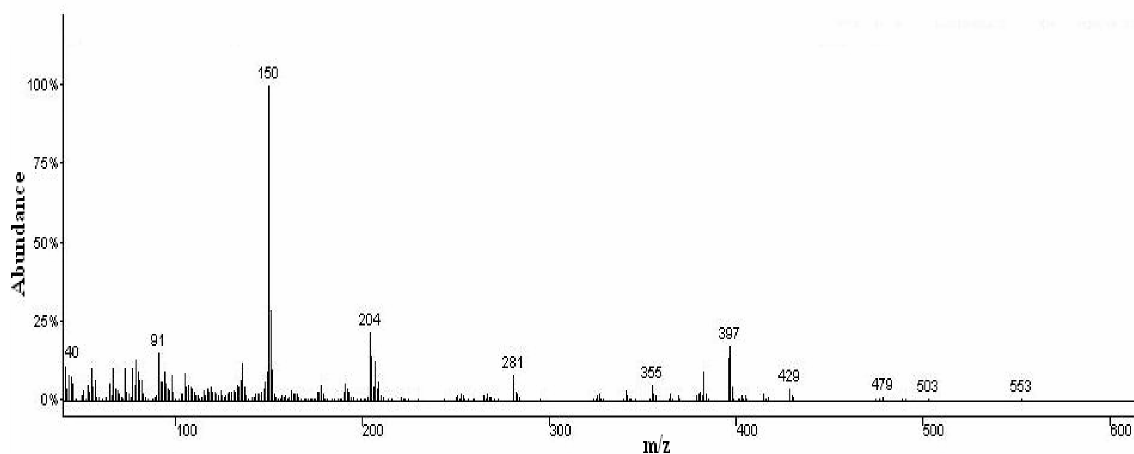


Figure 3.12. Mass spectrum of solanidine in hexane

The results of this initial testing of the applicability of the SPME to the analysis of steroidal glycoalkaloid aglycones are encouraging. The fact that the SPME does not require large amounts of expensive (and in some cases harmful) pure solvents and that the extraction and determination procedure is relatively simple make this technique particularly interesting. SPME on-fiber derivatization is an alternative sample preparation technique for the determination of less volatile compounds. The fiber serves as a solid, dry support allowing the use of water-sensitive derivatization reagents. Due to the poor volatility of solasodine, derivatization was needed prior to GC-MS to produce more volatile product. The polar CW-DVB phase was suitable for the extraction of aglycones containing polar functional groups. A methanol-acetic acid mixture was used to prepare the standards since it is one of the most widely used solvent system for the extraction of glycoalkaloids in potato samples. However, the CW-DVB fiber was unstable in methanol solution and/or damaged by the vapours of TMSI.

CHAPTER 4

CONCLUSION

The findings of this work showed that using a acetonitrile-triethylammonium phosphate (TEAP) buffer system gradient elution system was ineffective for the separation of α-solanine, α-chaconine, solasonine, and solamargine and their aglycones solanidine, solasodine all in one chromatographic run. Moreover, it is not robust, more complex and can be affected by the performance of the instrument (e.g. variation in pumping rate or stabilitiy). Ammonium dihydrogen phosphate (AH_2P)buffer produced better separation of glycoalkaloids and improved band shapes for the aglycones when compared with the triethylammonium phosphate (TEAP) buffer under the same conditions. The former is also cheaper and triethylammonium phosphate buffer (TEAP) can show instability during long-term storage.

Temperature has an important effect on the separation of glycoalkaloids for both isocratic and gradient elution. Changes in temperature can lead to variation in elution order of the glycoalkaloids under isocratic conditions. The most important parameter for the separation of glycoalkaloids was strength and type of the organic solvent in the mobile phase. Addition of methanol resulted in separation of solamargine and chaconine at 50 ^{o}C. These two compounds could not be separated with gradient elution using an acetonitrile-triethylammonium phosphate buffer. Moreover during isocratic elution at 26 ^{o}C solanine had the same retention time as solamargine. Therefore, optimum conditions for the separation of solamargine and chaconine were chosen as follows: ACN (10%MeOH)/ammonium phosphate buffer (30/70), pH: 2.5 and temperature 50 ^{o}C (Method 1). The separation of solasonine and solanine was not as difficult as for the other two glycoalkaloids (solamargine and chaconine). Various combinations can be made as a compromise for the separation of solanine and solasonine from the following conditions: ACN/ammonium phosphate buffer (30/70), pH: 2.5, and temperature 50 ^{o}C (Method 2). The aglycones were separated under all conditions discussed above. The best set of conditions for the separation of the aglycones (solasodine and solanidine) was chosen as follows: ACN/ammonium phosphate buffer (60/40), pH:2.5, and temperature 26 ^{o}C (Method 3). The validity of Method 1 was tested with aglycone standards. The detector peak area was linear over

the range of 4-230 mg/L for solanidine and solasodine. The limit of detection (LOD) was 0.9 mg/L for solanidine and 1.6 mg/L for solasodine. The limit of quantitation was estimated to 4.6 mg/L.

With a slightly polar phase capillary column, GC-MS was used for the determination of solanidine without its first being derivatized. Solasodine, however, required derivatization due to its lower vapour pressure. Using a normal derivatization procedure with GC-MS, neither the molecular ion nor fragments for the glycoalkaloids were seen in GC-MS. Promising results were obtained in the initial application of solid-phase microextraction (SPME) using on fiber derivatization and gas chromatography ion-trap mass spectrometry for the qualitative determination of glycoalkaloid aglycones. The extraction was simple and required neither large amounts of solvents nor valuable standards of aglycones. Furthermore, the SPME method simplified the use of water-sensitive derivatizing reagents for analysis of these analytes. The polar CW-DVB phase was found to be suitable for the extraction of glycoalkaloid aglycones since it also contains polar functional groups. However, the fiber was ultimately not stable in the extraction solvent (methanol-acetic acid) or vapours of the derivatizing reagent applied (TMSI).

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APPENDIX A

EFFECT OF BUFFER CONCENTRATION AND ADDITION OF METHANOL TO THE MOBILE PHASE

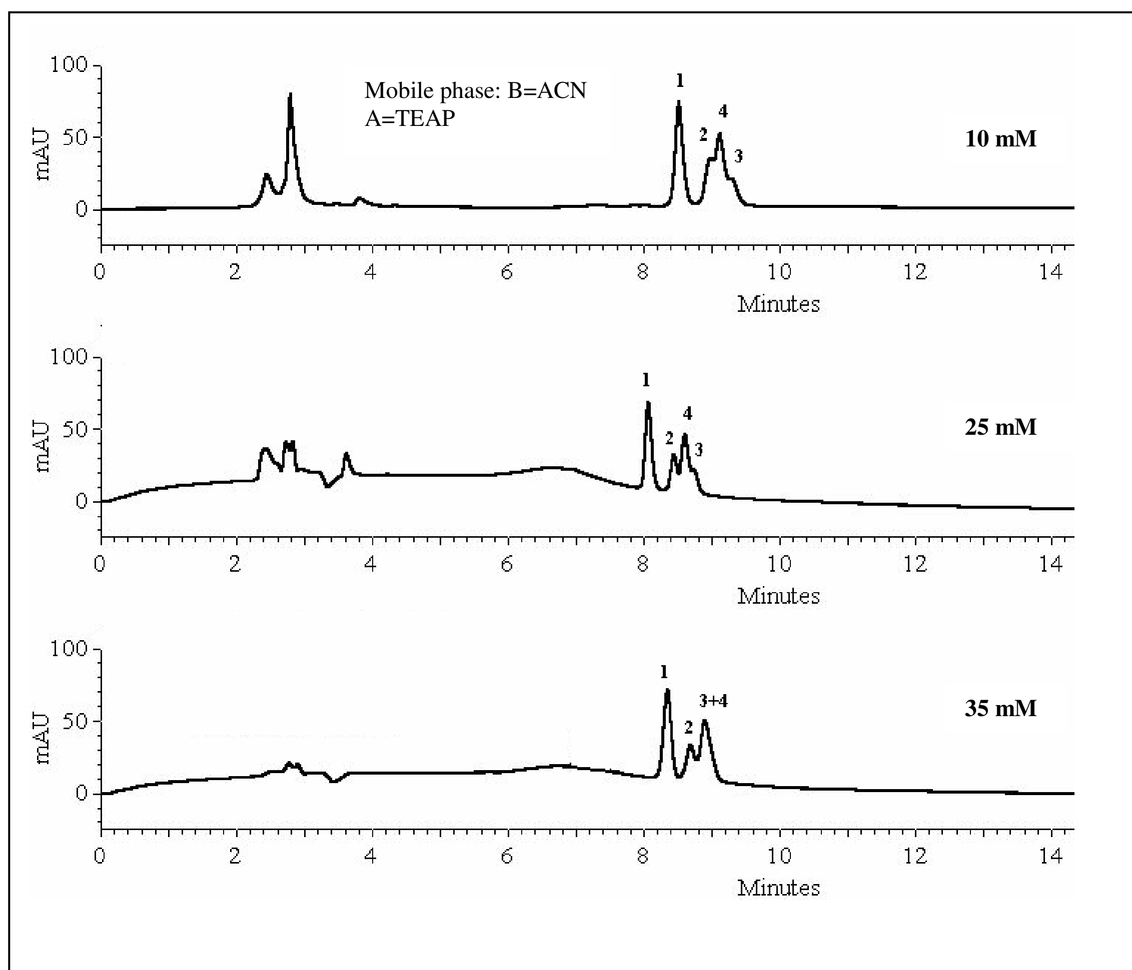

Figure A.1. Effect of buffer concentration durig gradient elution 27-100% ACN in 18 minutes T:40 °C pH:3.02 F:1.0 mL/min 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

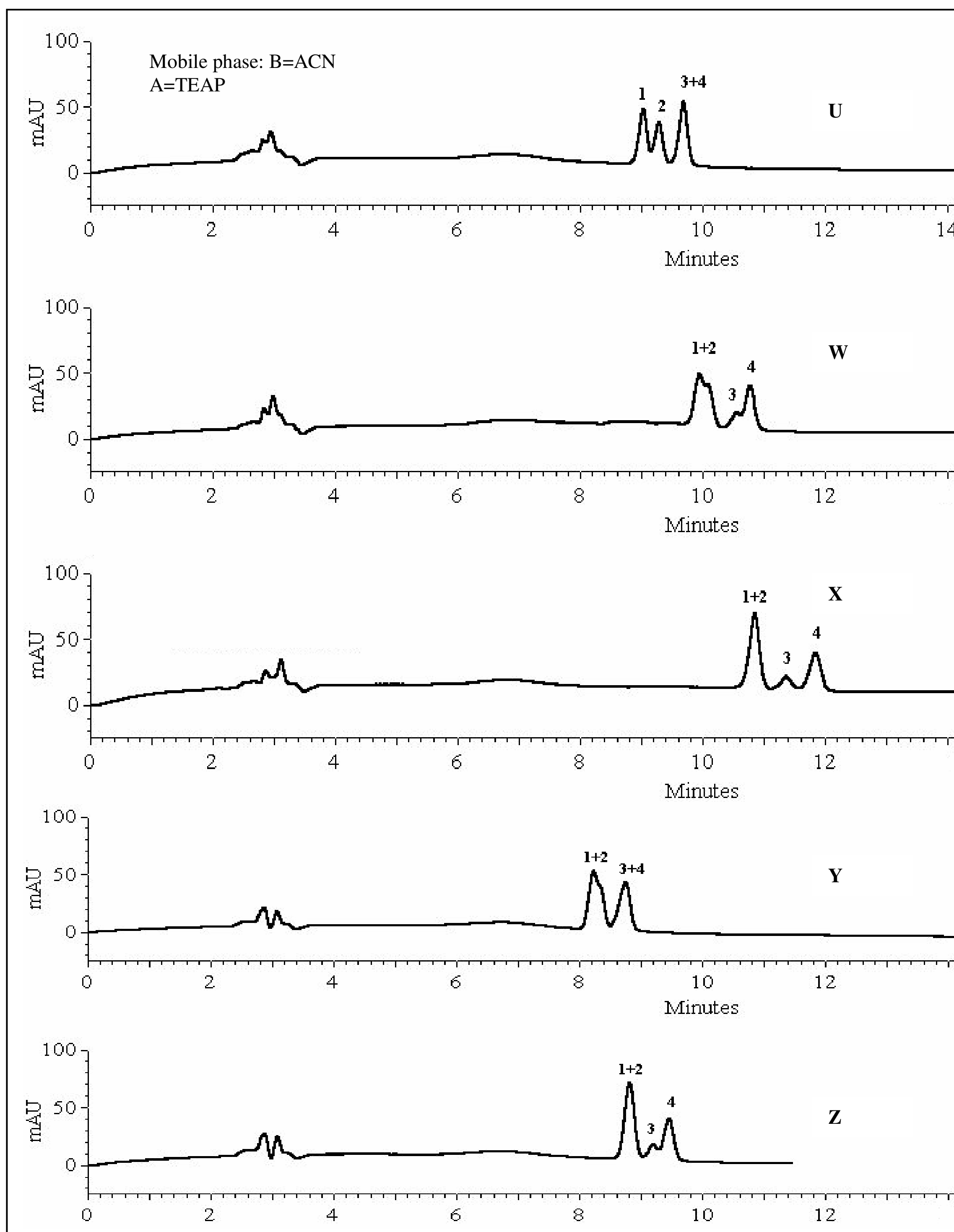

Figure A.2. Effect of methanol gradient elution 27-100% ACN in 18 minutes T:40°C pH:3.02 F:1.0 mL/min U:ACN (5%MeOH) W:ACN (10%MeOH) X:ACN (15%MeOH) Y:A=TEAP (5%MeOH) Z=B:ACN (5%MeOH) A=TEAP(5%MeOH) 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

APPENDIX B

OVERALL EFFECT OF BUFFER TYPE ON SEPARATION OF GLYCOALKALOIDS

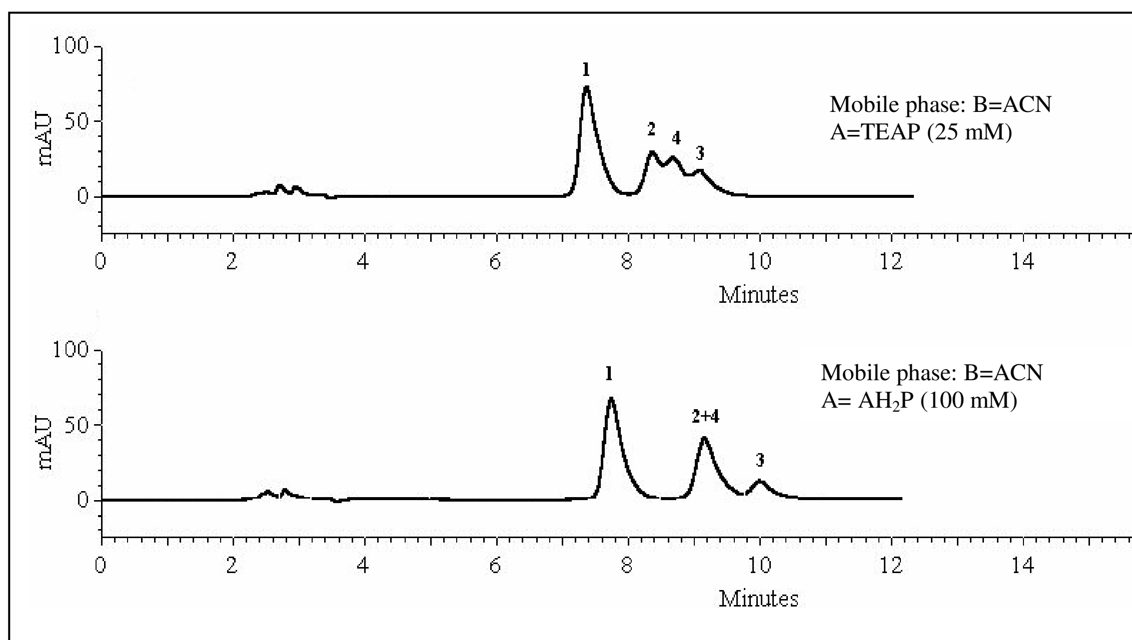

Figure B.1. Effect of buffer type during isocratic elution T:26 ^{0}C F:1.0 mL/min pH:2.5 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

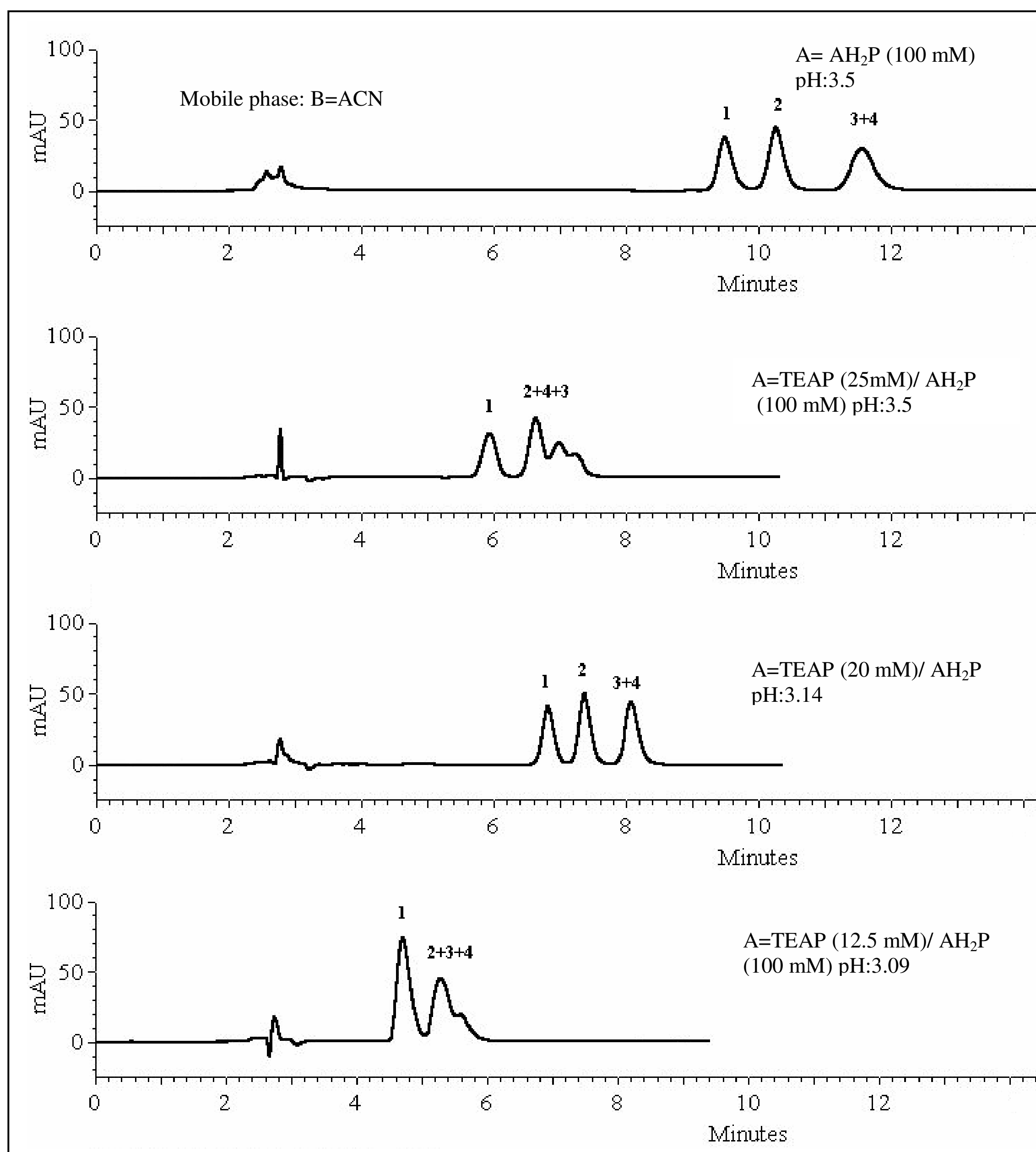

Figure B.2. Effect of additon of TEAP to ammonium dihydrogen phosphate during isocratic elution (SGAs) T:50 ^{o}C F:1.0 mL/min 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

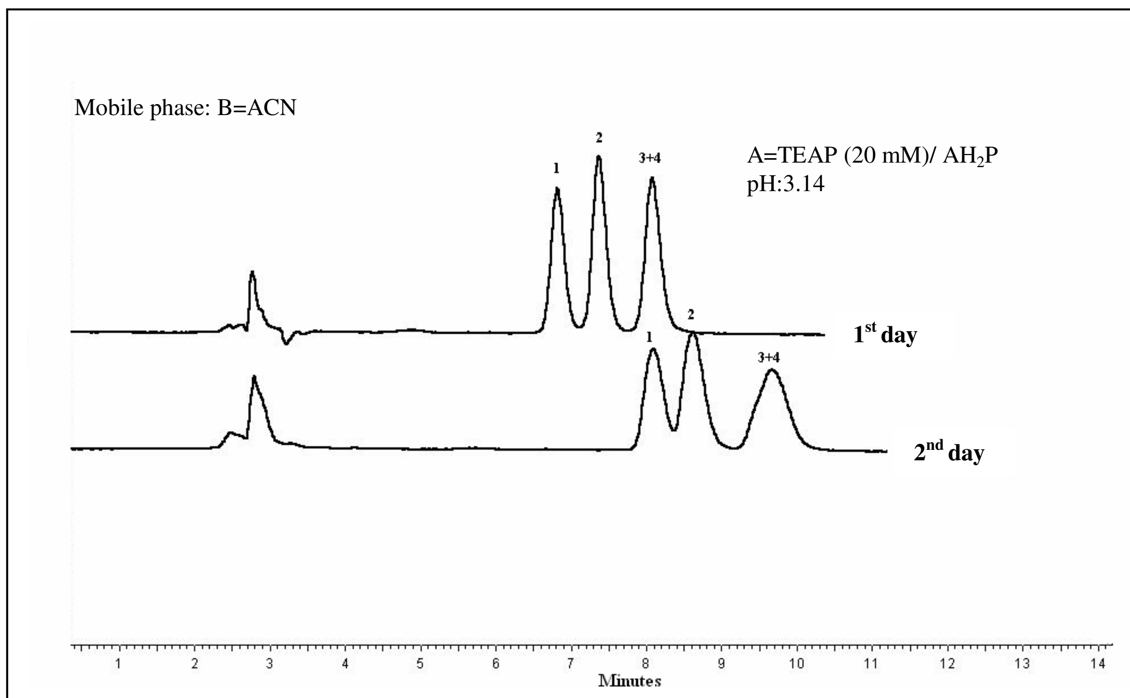

Figure B.3. Change of separation during isocratic elution (SGAs) 50 °C F:1.0 mL/min 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

APPENDIX C

EFFECT OF TEMPERATURE

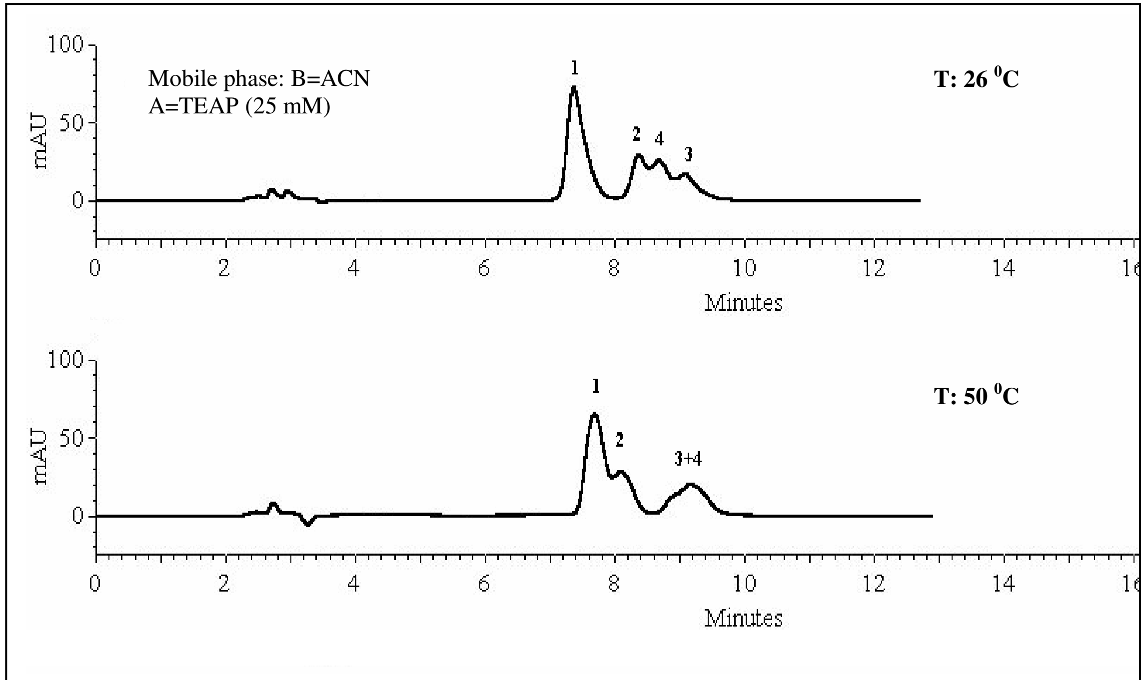

Figure C.1. Effect of temperature during isocratic elution (SGAs) pH:2.5 F:1.0 mL/min 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

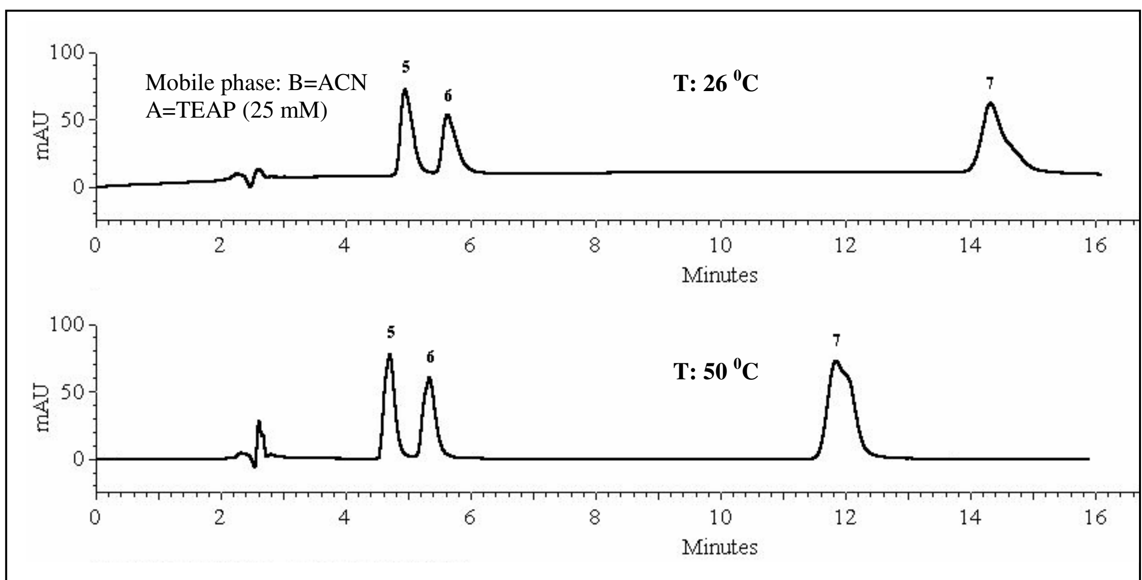

Figure C.2. Effect of temperature during isocratic elution (SGAAs) pH:2.5 F:1.0 mL/min 5:solanidine 6:solasodine 7:progesterone (I.S.)

APPENDIX D

EFFECT OF TYPE OF MOBILE PHASE

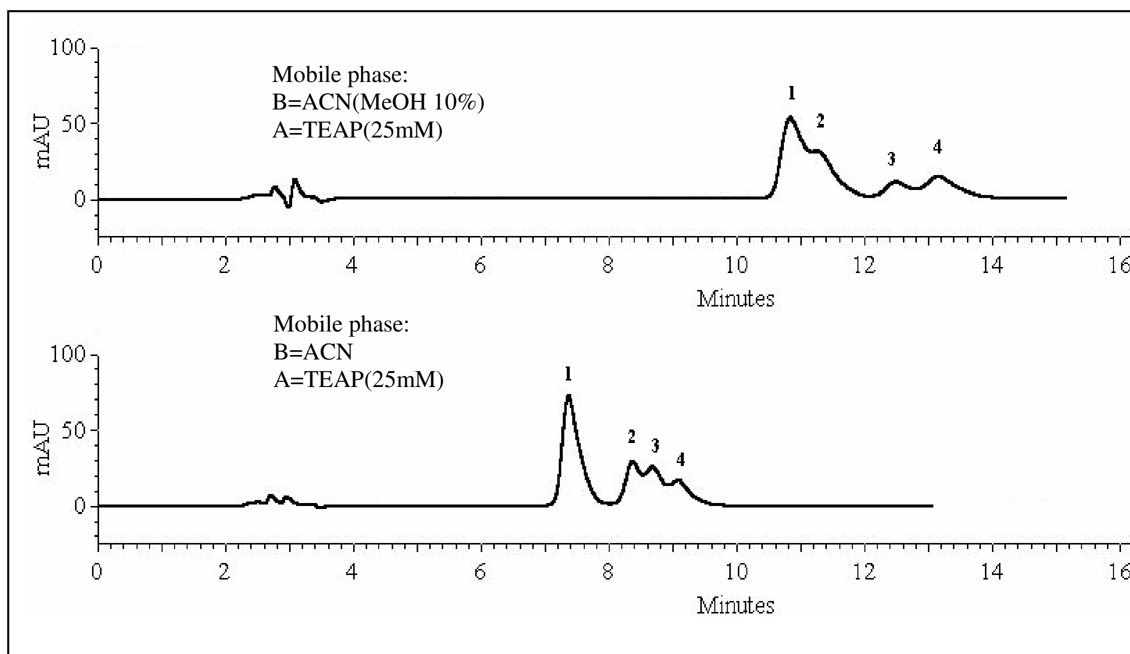

Figure D.1. Effect of addition of MeOH during isocratic elution T:26 ^{0}C F:1.0 mL/min pH:2.5 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

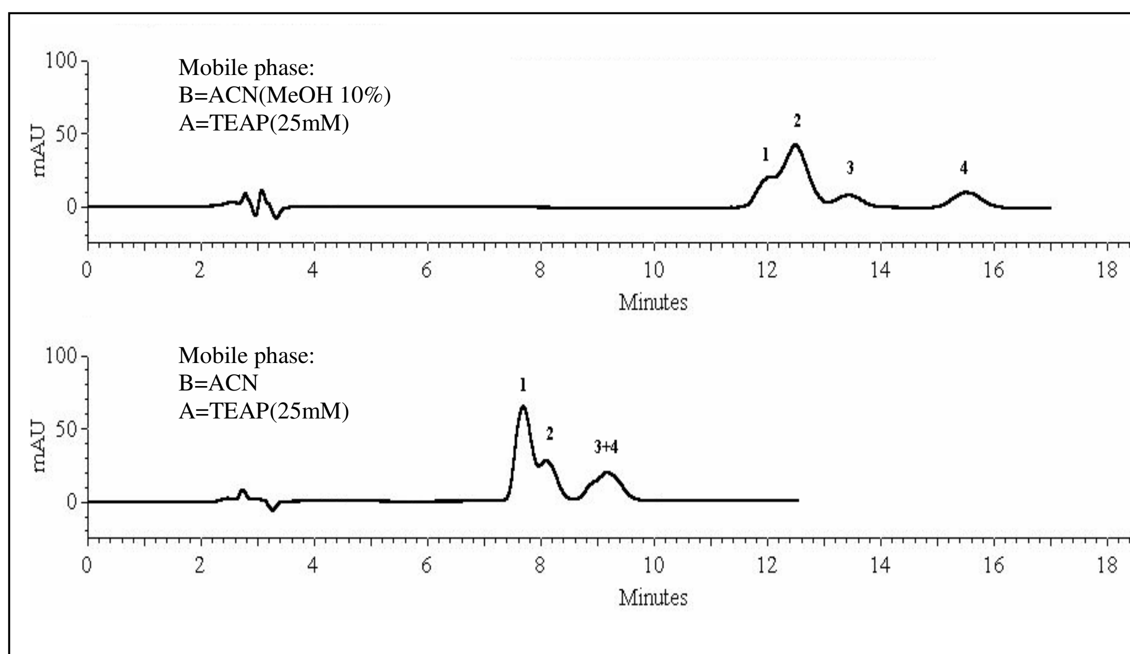

Figure D.2. Effect of addition of MeOH during isocratic elution T:50 ^{0}C F:1.0 mL/min pH:2.5 1:solasonine 2:α-solanine 3:α-chaconine 4:solamargine

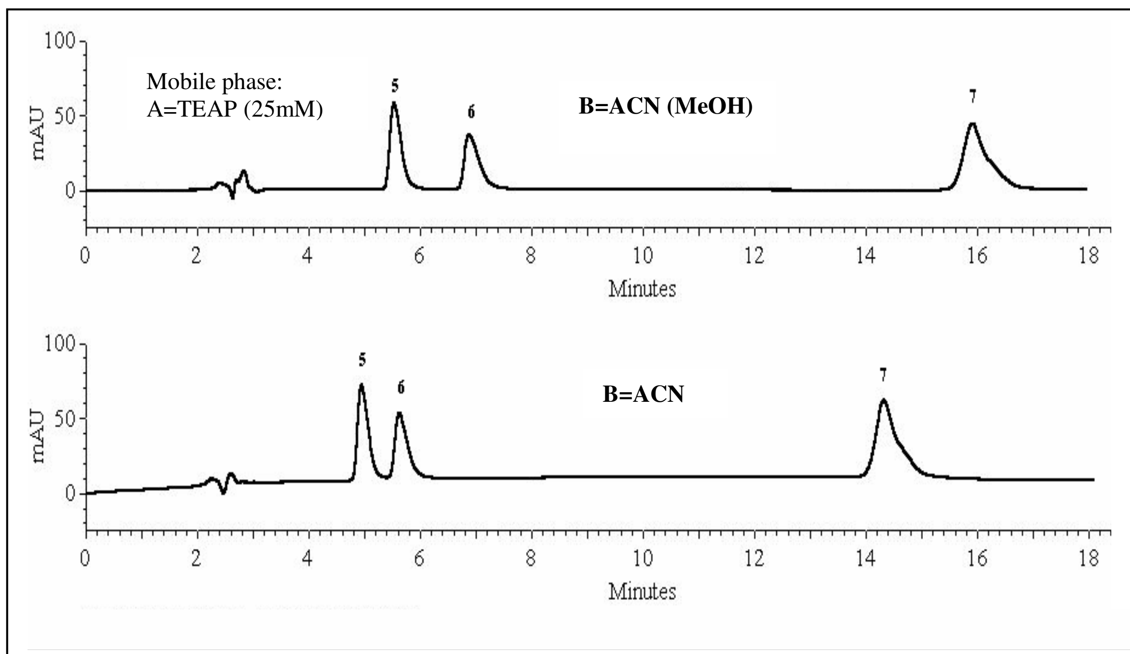

Figure D.3. Effect of addition of MeOH on separation of SGAAs T:26 ^{0}C F:1.0 mL/min pH:2.5 5:solanidine 6:solasodine 7:progesterone (IS)

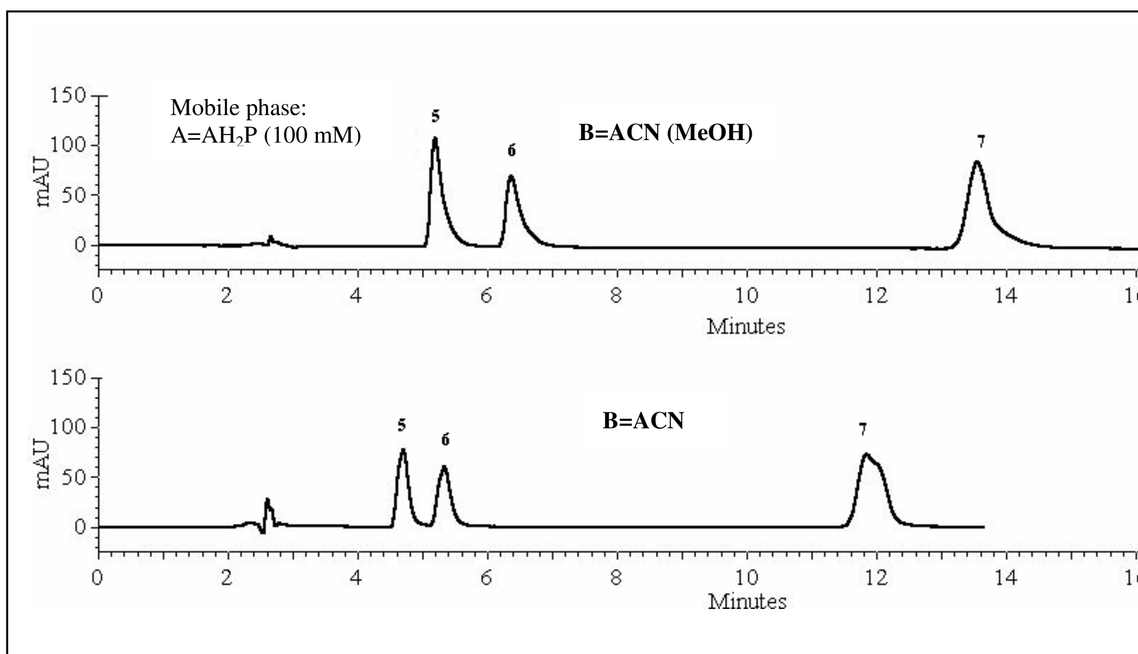

Figure D.4. Effect of addition of MeOH on separation of SGAAs T:26 ^{0}C F:1.0 mL/min pH:2.5 5:solanidine 6:solasodine 7:progesterone (internal standard)

APPENDIX E

GRADIENT METHOD

Table E.1. Gradient Method

Time (minute)	**ACN %**
0.01	27
0.1	28
0.2	29
0.3	30
0.4	31
0.5	32
1	33
2	34
3	35
4	35.5
5	36
6	36.5
7	37
8	38
9	40
10	50
11	100
12	100
13	100
24	100
15	100
16	100
17	100
18	100
19	27
20	27
21	27
22	27
23	27
24	27
25	27
26	27