GENETIC MAPPING AND CHARACTERIZATION OF EGGPLANT FOR GLYCOALKALOID CONTENT

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by

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İZMİR

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ABSTRACT

GENETIC MAPPING AND CHARACTERIZATION OF EGGPLANT FOR GLYCOALKALOID CONTENT

Most plants including eggplant, produce toxins against insects, pathogens and animals. The biggest class of these toxins is alkaloids. Generally plants produce alkaloids in a glycosidic form which are called glycoalkaloids. Glycoalkaloids have toxic effects on human health. For example, as a result of disrupting the cell membrane, the gastrointestinal track and other organs are damaged. Glycoalkaloids are not only toxic to human health but also they have beneficial effects. For example, they decrease cholesterol level, and have anticancer activity. And also they are used as a precursor for steroidal drugs. Therefore, isolation and determination of glycoalkaloids content is important.

For this aim, column chromatography was performed and solamargine and solasonine were obtained. Totally 122,8 mg solamargine, solasonine and solamargine and solasonine mix from *Solanum linnaeanum* were obtained. Their identities were confirmed via ^{1}H NMR and mass spectroscopy. For determination of glycoalkaloid content HPLC method was developed via changing different parameters such as mobile phase, pH, temperature, flow rate and column type. It was found that separation of glycoalkaloids by using RP column without phosphate buffer was not sufficient. On the other hand, separation of glycoalkaloids by using carbohydrate column was better but this type of column is not stable.

Furthermore, linkage mapping was performed by using CAPs and SSR methods; and 385 COSII markers and 221 SSR markers were tested. Of the tested markers, 38 of the COS II markers and 28 of the SSR markers were found to be polymorphic. A total of 52 of these markers were mapped.

ÖZET

PATLICANDA GLİKOALKALOİD İÇERİĞİNİN KARAKTERİZASYONU VE GENETİK HARİTALAMA

Patlıcan dahil çoğu bitki böceklere, patojenlere ve hayvanlara karşı toksinleri üretir. Bu toxinlerin en geniş sınıfı alkaloidlerdir. Genellikle bitkiler alkaloidleri glikoalkaloid olarak adlandırılan glikosidik formda üretirler. Glikoalkaloidlerin insan sağlığı üzerinde toksik etkileri vardır. Örneğin; hücre membranını parçaladıklarından dolayı gastrointestinal bölge ve diğer organlarda hasara yol açar. Glikoalkaloidler sadece toksik değildirler, yararlı etkileri de vardır. Örneğin kolesterol seviyesini düşürürler ve antikanser aktiviteleri vardır. Ayrıca steroidal ilaçların öncüsü olarak kullanılırlar. Bu nedenle glikoalkaloid izolasyonu ve glikoalkaloid içeriğinin belirlenmesi önem taşır.

Bu amaçla kolon kromatografisi gerçekleştirilmiş ve solamargine ve solasonine elde edilmiştir. *Solanum Lineanum türünden* toplamda 122.8 mg solamargine, solasonine, solamargine ve solasonine karışımı elde edilmiştir. ^{1}H NMR ve kütle spektroskopisi ile doğruluğu kontrol edilmiştir. Glikoalkaloid içeriğinin belirlenmesi için mobil faz, pH, sıcaklık, akış hızı ve kolon tipi gibi çeşitli parametreler değiştirilerek HPLC metodu geliştirilmiştir. Fosfat tamponu kullanmadan RP kolon kullanılarak glikoalkaloidlerin ayrımının yeterli olmadığı bulunmuştur. Buna karşılık karbohidrat kolonda glikoalkaloidlerin ayrımı daha iyidir, fakat bu tip kolonlar stabil değildir.

Ayrıca CAPs metodu ve COSII işaretleyicileri kullanılarak bağlantı haritası oluşturulmuştur.385 COS II işaretleyicisi ve 221 SSR işaretleyicisi kontrol edilmiş ve 38 tane COS II, 28 tane SSR işaretleyicisi polimorfik bulunmuş; bunlardan 52 tanesi haritalanmıştır.

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LIST OF SYMBOLS AND/OR ABBREVIATIONS

ACN: Acetonitril

C_5D_5N: Deuterated pyridine

CTAB: Cetyl trimethylammonium bromide

DAD: Diode array detection

dH_2O: Distilled water

dNTP: Deoxyribonucleotide triphosphate

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

ELSD: Evaporative light-scattering detector

GC: Gas Chromatography

H_3PO_4: Phosphoric acid

HCOOH: Acetic acid

HPLC: High performance Liquid Chromatography

LC/MS: Liquid Chromatography/ Mass Spectroscopy

MALDI-TOF/MS**:** Matrix-assisted laser desorption/ionization- Time of Flight/ MS

MeOH: Methanol

$MgCl_2$: Magnesium chloride

MS: Mass Spectroscopy

NaOH: Sodium hydroxide

$NH_4H_2PO_4$: Monoammonium dihydrogen phosphate

NMR: Nuclear Magnetic Resonance

PCR: Polymerase Chain Reaction

Rf: Retention factor

RP: Reverse phase

TAE: Tris base, acetic acid and EDTA

TE:Tris-EDTA

TFH: Triflouro acetic acid

TLC: Thin Layer Chromatography

VLC column: Vacuum liquid chromatographic column

CHAPTER 1

GLYCOALKALOID PURIFICATION

1.1. Introduction

The *Solanaceae* family (also known as *Nightshade* family) is a family that includes important agricultural plants such as tomato, potato, sweet pepper and eggplant (Simonovska and Vovk 2000). Eggplant belongs to genus *Solanum* L. and species *Solanum melongena* L. (USDA, 2008). Some wild relatives of *Solanum melongena* such as *S. linnaeanum, S. macrocarpon, S. aethiopicum,* and *S. incanum* contain resistance to biotic and abiotic stresses (Behera and Singh 2002). *S. incanum* is one important wild relative of *Solananum melongena*. It is known as 'sodom apple' and important for traditional medicine like other Solanum species. For example in East Africa it is used for chest pain, toothache, fever, stomachache and indigestion (Fukuhara and Kubo 1991).

Eggplant is an important crop in worldwide production (Figure 1.1). It is an important vegetable in terms of economy; for human nutrition; as a source of vitamins, minerals and dietary fiber; and in traditional medicine (Collonnier et al. 2001; Kashyap et al. 2003). Worldwide distribution of eggplant is very wide. China and India are the biggest producers of eggplant. Egypt, Turkey, United Kingdom, Sudan, Italy, Japan, Iraq and Indonesia are also important producers.

Turkey is an agrarian state where nearly 1500 commercial vegetables are produced as a result of appropriate climate conditions and soil diversity (Tek, 2006). Eggplant production is also important in our country. Eggplant is grown in Samsun, Bursa, Aydın, Antalya, İçel, Adana, Hatay, and Şanlıurfa which have moist and temperate climate conditions (Gezginler, 2008). Turkey ranks in third place in worldwide eggplant production (Figure 1.2).

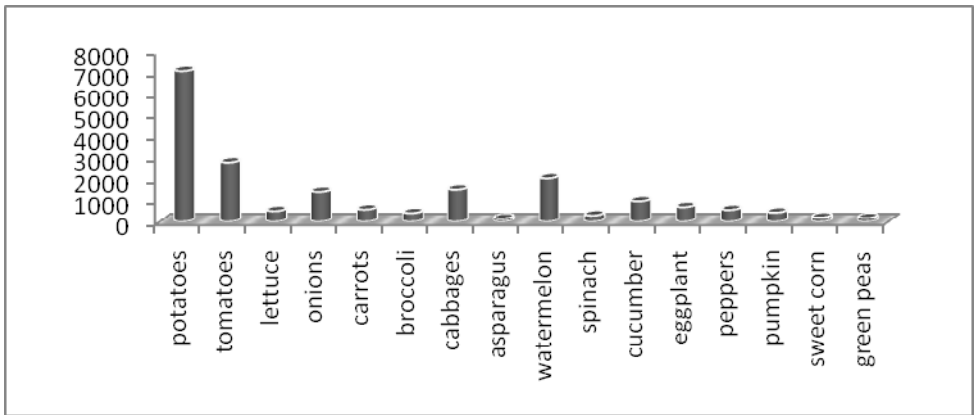


Figure 1.1. Worldwide production of vegetables and place of eggplant
(Source: Adapted from USDA, 2008).

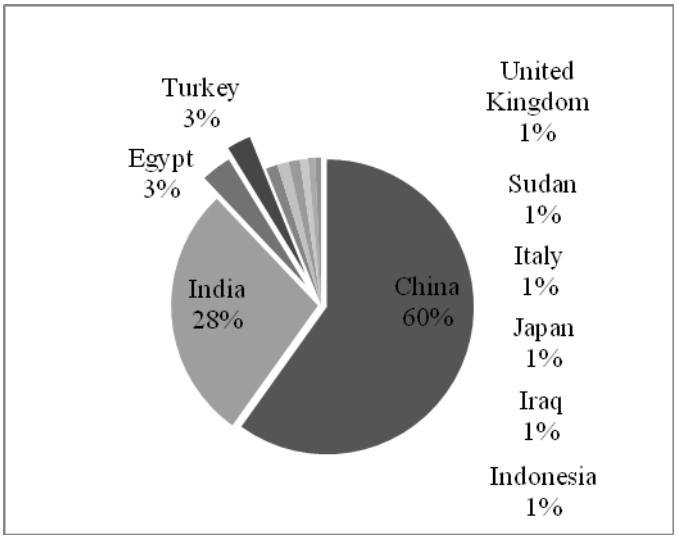


Figure 1.2. Worldwide production of eggplant
(Source: Adapted from Gezginler, 2008).

Eggplant is a very nutritive vegetable. It contains calcium, magnesium, phosphorus, potassium at high levels and other compounds that are important for human health (Table 1.1).

Table 1.1. Nutritional content of eggplant
(Adapted from USDA, 2008).

MACRONUTRIENTS	
Water	75.80 g
Protein	0.83g
Carbohydrates	4.67 g
Fiber	2.80 g
Sugars	1.93 g
Total fat	0.160 g
Saturated fat	0.029 g
Monosaturated fat	0.013 g
Polysaturated fat	0.062 g
Cholesterol	0µg

PHYTONUTRIENTS	
Phytosterols	6 mg
β-Carotene	13 mcg
β-Cryptoxanthin	0 mg
Lycopene	0 mcg
Lutein	0 mcg
Zeaxanthin	0 mcg

MICRONUTRIENTS	
Calcium	7 mg
Iron	0.20 mg
Magnesium	11 mg
Phosphorus	21 mg
Potassium	21 mg
Sodium	2 mg
Zinc	2 mg
Vit C	1.8 mg
Thiamin	0.032 g
Riboflavin	0.03 mg
Pantothenic acid	0.23 mg
Vit B6	0.069 mg
Vit B12	0 mcg
Folate	18 mcg
Vit A	22 IU
Vit E	0.25 mg
Vit K	2.9 mcg

1.2. Plant Metabolites

Plants produce primary and secondary metabolites. Although primary metabolites directly play essential roles in plant growth, development and reproduction, secondary metabolites are not directly involved in these processes. Primary metabolites include proteins, carbohydrates and fats and oils while secondary metabolites include flavanoids and similar phenolic and polyphenolic compounds, nitrogen-containing alkaloids, sulphur-containing compounds, and terpenoids (Zulak et al. 2007).

Secondary metabolites represent diversity in terms of chemical structure among plant species (Zulak et al. 2007). Secondary metabolites play important roles in many physiological processes, especially in response to environmental effects (Stobiecki et al.

2003). They are important naturally-occurring compounds because they help defend the plant against herbivores, insects, viruses and diseases but also help for pollination by attracting pollinators. Secondary metabolites are also used in industry as dyes, fibers, glues, oils, waxes, flavouring agents, drugs and perfumes (Crozier 2007).

In recent years, secondary metabolites have drawn attention for human nutrition. It is reported that they have beneficial effects on cancer, cardiovascular disease and Type II diabetes (Crozier 2007).

1.2.1. Classification of Secondary Metabolites

1.2.1.1. Phenolic Compounds

Phenolic compounds consist of at least one aromatic ring and one or more hydroxyl groups attached to an aromatic ring. Based on the number and arrangement of carbon atoms they can be classified as flavanoids and non-flavanoids (Crozier et al. 2007).

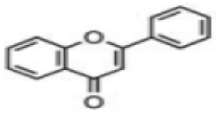
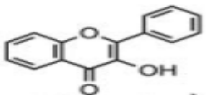
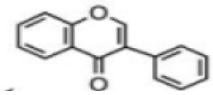
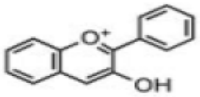
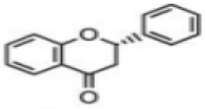
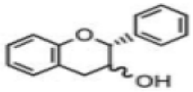
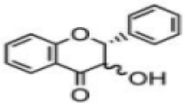
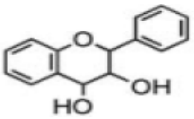
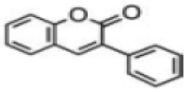
1.2.1.1.1. Flavanoids

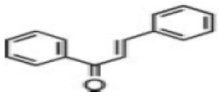
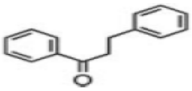
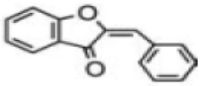
Flavanoids are polyphenolic carbons which are formed of fifteen carbons and two aromatic rings. They are the most abundant phenolic compounds that are found in plants. They play roles in UV protection, pigmentation, stimulation of nitrogen-fixing nodules and resistance against disease.

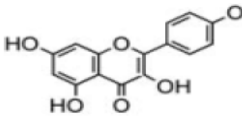
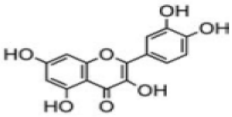
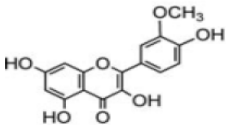
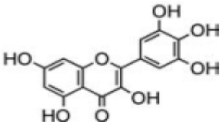
Flavanoids can be classifed as: major flavanoids such as flavon, flavanol, isoflavon, anthocyanidine, flavanone, flavan-3-ol and dihydroflavanol; and minor flavanoids such as dihydroflavanol, flavan-3,4-diol, coumarin, chalcone, dihydrochalcone and aurone (Table 1.2). Various substituents such as sugars, hydroxyl groups, methyl groups and isopentyl units can be attached to the basic flavanoid skeleton (Crozier et al. 2007).

Table 1.2. Classification of flavanoids and aglycones of flavanoids

(Source: Crozier et al. 2007).

Subgroups of flavonoids	Chemical structure
Flavone	
Flavanol	
Isoflavone	
Anthocyanidine	
Flavanone	
Flavan-3-ol	
Dihydroflavanol	
Flavan-3,4-diol	
Coumarin	

Subgroups of flavonoids	Chemical structure
Chalcone	
Dihydrochalcone	
Aurone	

Aglycones of flavonoids	Chemical structure
Kaempferol	
Quercetin	
Isorhammetin	
Myricetin	

1.2.1.1.2. Non-Flavanoids

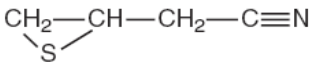
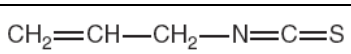
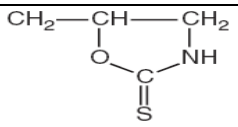
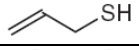
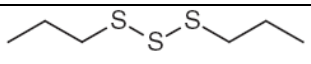
The major non-flavanoid compounds are C_6-C_1 phenolics acids, C_6-C_3 hydroxycinammates and their conjugated derivatives, and the polyphenolic C_6- C_2 -C_2 stilbenes. They have diverse functions. For example tannins, which belong to class one, bind to dietary proteins in the gut and inactivate herbivore digestive enzymes directly. The other example is stilbenes. They protect plants from fungal, bacterial, and viral pathogen attacks (Crozier et al. 2007).

1.2.1.2. Sulphur-Containing Compounds

Two plant species are the major sources of sulphur-containing compounds (Table 1.3). These compounds are synthesized via the glucosinolate-myrosinase system in cruciferous crops, such as cabbages, broccoli and watercress, and via the alliin-alliinase system in Allium crops, such as garlic, onions and leeks. Sulphur-containing compounds have beneficial effects on human health. Reduction of cancer risk, protection against atherolosclerosis and other inflammatory diseases can be considered among the benefits of these compounds (Mithen 2007; Humphrey and Beale 2007).

Table 1.3. Examples of sulphur-containing compounds

(Source: Mithen 2007)

Name of compound	Chemical structure
1-cyano-2,3-epithiopropane	
2-propenyl isothiocyanate	
5-vinyloxazolidine-2-thione	
Allyl mercaptane	
Dipropyl trisulphide	

1.2.1.3. Terpenes

Terpenes are also known as isoprenoids, because they are derived from isoprene which consists of five carbon atoms. However, the final chemical structures of terpenes are diverse. If terpenes are chemically modified, then the end products are called terpenoids. Although the major source of terpenes are plants, some insects can also produce them (Humphrey and Beale 2007).

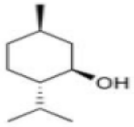
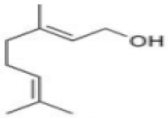
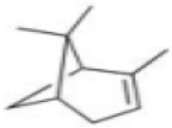
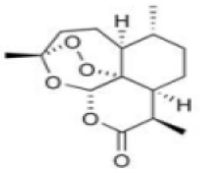
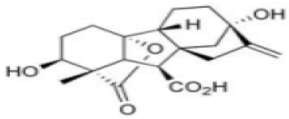
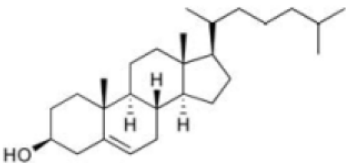
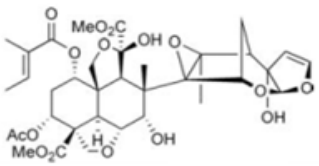
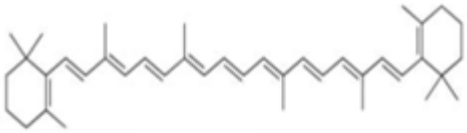
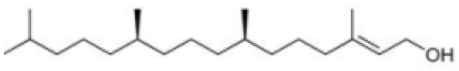
Terpenes are one of the most diverse groups of secondary metabolites with diverse functions such as contributing flavour and aroma, being precursor of antibiotics and anticancer drugs, plant and animal hormones and lipids, attracting insects and being mediators of the electron transport chain (Humphrey and Beale 2007). Terpens are classified based on number of isoprene units involved in the molecule (Table 1.4). For example, abscisic acid contains 4 isoprene units and carotenoids contain 8 isoprene units, therefore abscisic acid belongs to the diterpenes, and carotenoids belong to the tetraterpenes.

Table 1.4. Classification of terpens
(Source: Humphrey and Beale 2007).

Class	**Number of isoprene units**	**Example**
Hemiterpenes	*1*	*Tiglic acid, isoamyl alcohol*
Monoterpenes	*2*	*Menthol, myrcene*
Sequiterpenes	*3*	*Farnesol, Artemissin*
Diterpenes	*4*	*Abscisic acid, phytol*
Triterpenes	*6*	*Azadirachtin, oleanic acid*
Tetraterpenes	*8*	*Carotenoids,*

Table 1.5. Examples of terpens

(Source: Humphrey and Beale 2007).

Example of terpenes	Chemical structure
(-)-Menthol	
Geraniol	
(+)-α-Pinene	
Artemisinin	
Gibberellin A_1	
Cholesterol	
Azadirachrin	
β-Carotene	
Phytol	

1.3. Alkaloids

Most plants produce toxins against insects, pathogens and animals. The largest class of these toxins is alkaloids. Three hundred different plant families produce more than 10.000 different alkaloids. Approximately 2600 species of the *Solanaceae* family also produce alkaloids (Jensen et al. 2007). Eggplant (*Solanum melongena L.*), chili pepper (*Capsicum sp. L.*), tomato (*Lycopersicon esculentum Mill.*) and potato (*Solanum tuberosum L.*) all belong to the *Solanaceae* family (Zulak et al. 2007). All of the members of the *Solanaceae* family contain different alkaloids. Alkaloids can be classified as benzylisoquinoline alkaloids, tropane alkaloids, terpenoid indole alkaloids, purine alkaloids, pyrrolizidine alkaloids, quinolizidine alkaloids, and steroidal alkaloids (Ziegler and Facchini 2008).

Steroid alkaloids are characterized as "intact" or "modified" based on nitrogen localization. Nitrogen can integrate into the ring or can exist as a side chain. There are several subgroups based on nitrogen and arrangement of the ring. Nitrogen can be added to the 3^{rd} or 20^{th} carbon atom alone or methylated as a "primary NH_2" and so form "simple steroidal bases". Nitrogen can be added as a "secondary NH" and can cause closing of the carbon skeleton to form a ring structure. Finally, nitrogen can be added as "tertiary N" and this result in combining the two carbon skeleton rings. All these features affect the chemical characteristics of the compound (Dinan et al. 2001).

1.3.1. Glycoalkaloids and Their Aglycones

Generally plants produce alkaloids in glycosidic forms which are called glycoalkaloids. Glycoalkaloids are nitrogen-containing toxins and are found in the Apocynaceae, Buxaceae, Solanaceae, and Liliaceae families (Kreft et al. 2000; Dinan et al. 2001). Nitrogen binds the carbon skeleton on glycine, arginine or L-arginine (Kuronen et al. 1999).

Glycoalkaloids consist of three main portions: (i) a polar, water-soluble sugar residue which is composed of three or four monosaccharides, (ii) a nonpolar steroid portion and (iii) a basic portion which can be either indolizidine or oxa-azaspirodecane structure (Kuronen et al. 1999; Zulak et al. 2007) .

Steroidal glycoalkaloids are divided into two main groups according to the structure of the aglycone skeleton: “spirosolon type” and “solanidane type”. Both types can contain a double bond or hydroxyl group at different positions. And also both types can contain a sugar moiety (Dinan et al. 2001).

Steroidal glycoalkaloids can be found in all of the parts of the plant but especially they accumulate in metabolically active parts such as flowers, immature berries and young leaves or shoots. It is known that they are toxic but during fruit maturation, they are converted into nitrogen-free, non-toxic compounds (Dinan et al. 2001).

Aglycones are found in plants as hydrolyzed forms of glycoalkaloids. Specific glycosyltransferases remove the carbohydrate side chains from glycoalkaloids to form aglycones by hydrolysis. For example, in potato, solanidine is a hydrolysis product of α-chaconine and α-solanine. In tomato, tomatidine is a hydrolysis product of α-tomatine and in eggplant, solasodine is a hydrolysis product of solamargine and solasonine. In humans and animals, glycoalkaloids are converted to aglycones via enzymatic and non-enzymatic reactions. When enzymatic reactions are carried out by bacterial glycosidases in the gastrointestinal region, non-enzymatic reactions are carried out in the stomach by acid hydrolysis (Friedman et al. 2003).

Based on their structure aglycones are divided into 5 groups. These groups are solanidane, spirosolane, 22,26-epimonocholestones type, α-epiminocyclohemiketals, and 3-aminospirostones. Solanidane contains an indolizidine ring structure. Spirosolane contains an oxa-azaspirodecane alkaloid portion. Steroidal glycoalkaloids in the Solanaceae family belong to the first and second groups: solanidane and spirosolane (Jadhav et al.1997).

Saccharides bind to the 3-hydroxy position of aglycones to form glycoalkaloids. These saccharides can be D-glucose, D-galactose, D-xylose, D-rhamnose and they can bind in different combinations as tetra- or tri-saccharides (Vaananen 2007). If two L-rhamnose and D-glucose moieties bind to 3-hydroxy position of solanidine, chaconine is formed. If L-rhamnose, D-galactose and one D-glucose moieties bind to 3-hydroxy position of solanidine, solanine is formed. If two L-rhamnose and one D-glucose moieties bind to 3-hydroxy position of solasodine, solamargine is formed. If L-rhamnose, D-galactose and one D-glucose moieties bind to 3-hydroxy position of solasodine, solasonine is formed (Figure 1.3.) (Friedman 2006).

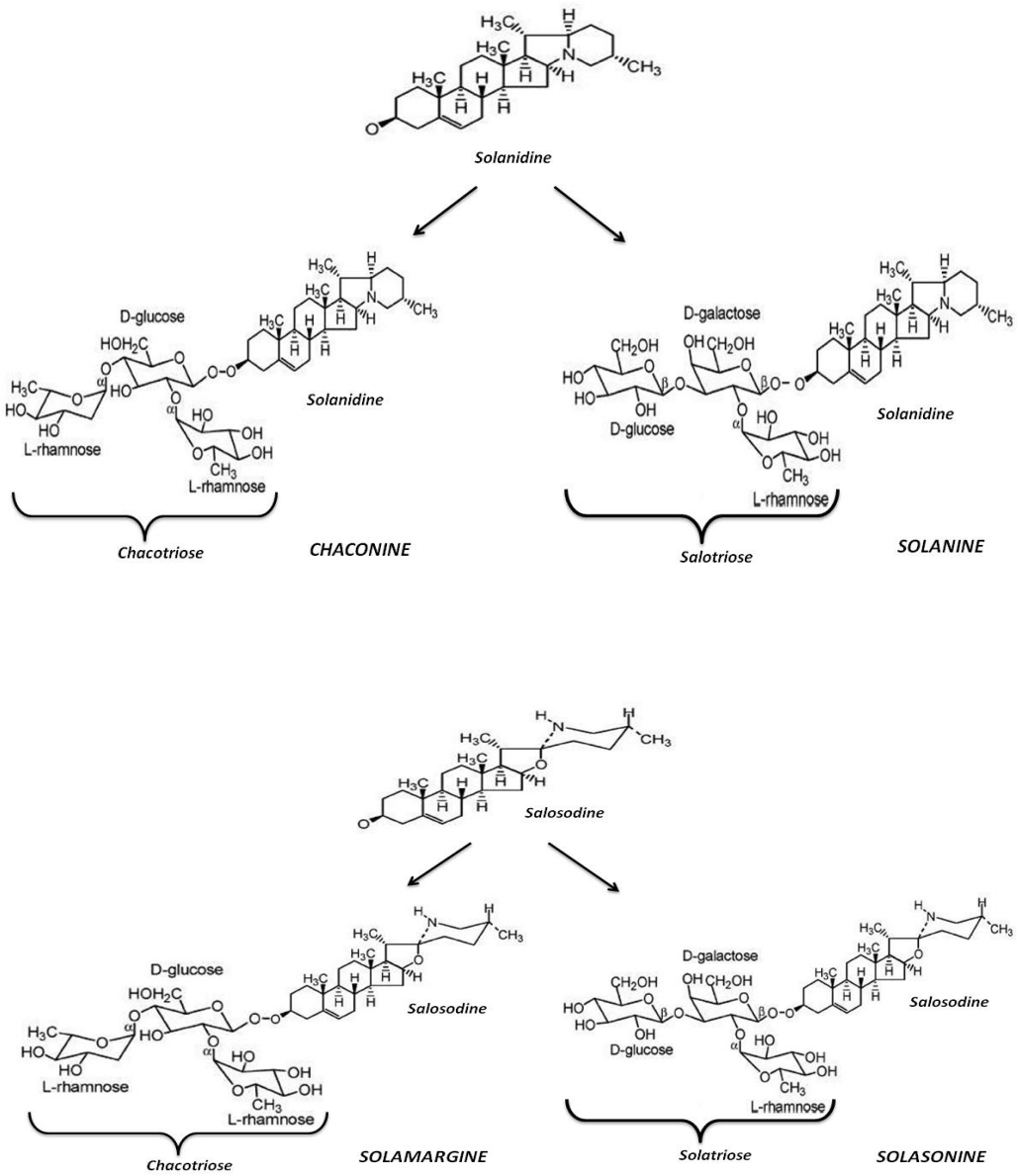


Figure 1.3 Chemical structure of eggplant glycoalkaloids

(Source: Friedman 2006).

1.3.2. Toxicity of Glycoalkaloids

Glycoalkaloids cause bitter flavor in foods. More importantly, they cause gastroenteric symptoms, coma and even death. It is thought that they are toxic to human health as a result of their effects on the nervous system and destruction of cell membranes. Steroidal glycoalkaloids bind to the cell membrane and form complexes with 3β-hydroxysterols. Thus, active transport of ions among the membrane is changed and this results in metabolic disorders (Vaananen 2007).

The adverse effects of glycoalkaloids can cause toxicity. For example, as a result of disrupting the cell membrane, the gastrointestinal track and other organs are damaged. Glycoalkaloids have anticholinesterase activity on the central nervous system. This results in accumulation of acetylcholine in the nervous system. Accumulation of acetylcholine can result in gastrointestinal defects, neurological disorders, coma and even death (Alt et al. 2005). Intoxication and death have been seen because of high glycoalkaloid content in potato (Stobiecki et al. 2003). Symptoms of steroidal glycoalkaloid toxicity are headache, rapid pulse, fever, hot skin, vomiting, diarrhea, gastroenteritis, burning sensation about the lips and mouth and colic pain in the abdomen and stomach (Friedman and McDonald 1996).

In potato, α-chaconine is the most toxic glycoalkaloid but also its effect can increase with other glycoalkaloids synergistically. Food processing like grilling, baking, or boiling does not affect the concentration of glycoalkaloids (Stobiecki et al. 2003). The toxic dose of glycoalkaloids is 2-5 mg/kg body mass and the lethal dose is 3-6 mg/kg body mass (Nema et al. 2008; Langkilde et al. 2009; Alt et al. 2005).

1.3.3. Beneficial Effects of Glycoalkaloids

Glycoalkaloids are not only toxic to human health but also they have beneficial effects (Friedman et al. 2003). They decrease cholesterol level, protect against *Salmonella typhimurium* infection, have anticancer activity and, increase the effects of malaria vaccine and anaesthetics which are cholinesterase inhibitors (Friedman 2006). In humans they inactivate *Herpes simplex*, *Herpes zoster*, and *Herpes genitalis* viruses (Sauerbrei and Wutzler 2007).

Solasodine is used against skin cancer and tomatidine is used in cancer chemotherapy (Plhak and Sporns 1997). In humans glycoalkaloids dampen the multidrug resistance of cancer cells (Friedman 2006). In many countries solasodine is used as a precursor of steroidal drugs, for example, an alternative to diosgenin which is a precursor of steroidal drugs (Kittipongpatana et al. 1999). According to one study which examined frog embryo, folic acid and glucose-6-phosphate and $NADP^+$ protect against α-chaconine toxicity (Friedman et al. 2003).

1.3.4. Glycoalkaloid Biosynthesis

Steroidal glycoalkaloids are synthesized from cholesterol which is one of the major end product sterols that is produced from a branch of the isoprenoid pathway in plants (Ziegler and Facchini 2008) (Figures 1.4, 1.5).

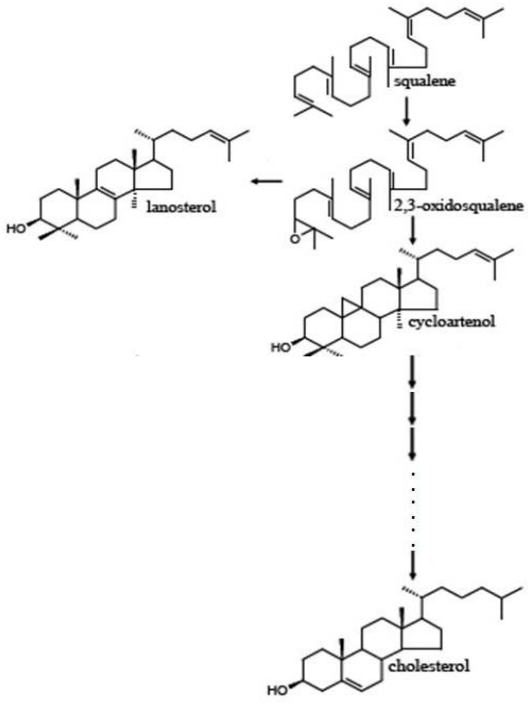


Figure 1.4. Cholesterol biosynthesis
(Source: Arnqvist 2007).

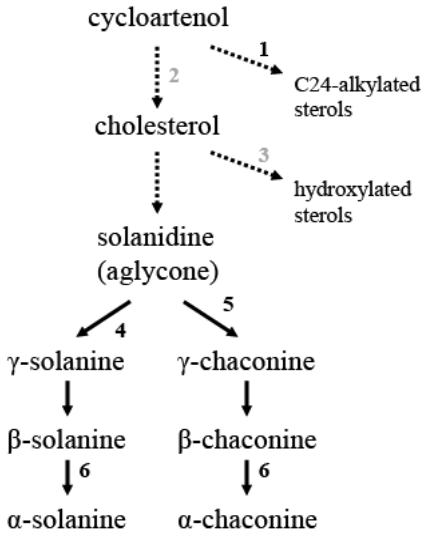


Figure 1.5. An example of glycoalkaloid synthesis in potato

(Source: Arnqvist 2007).

1.3.5. Parameters That Affect Glycoalkaloid Biosynthesis

Many environmental factors affect glycoalkaloid synthesis. For example, when potato tubers are exposed to daylight, their glycoalkaloid content increases (Stobiecki et al. 2003). It was shown that in some situations such as high temperature or low temperature glycoalkaloid synthesis increases. But this phenomenon is controversial. However, we definitely know that glycoalkaloid synthesis increases under stress conditions (Zrust 1997; Stobiecki et al. 2003; Wang et al. 2002; McCue et al. 2007).

Glycoalkaloid content increases as the result of physical wounding, poor growth conditions, climate and storage conditions (Kodamatani et al. 2005). For example, in potato tubers glycoalkaloid content increases when mechanical wounding occurs (Zrust 1997; McCue et al. 2007). Also a plant's genetic background affects glycoalkaloid synthesis (Turakainen et al. 2004). Glycoalkaloid levels are variable among species (Jensen et al. 2007). It was reported that the SMT1 gene in Arabidopsis as well as in most other plant species plays a role in cholesterol biosynthesis, hence this gene controls total glycoalkaloid synthesis. Furthermore Sgt1, Sgt2, and Sgt3 genes have roles in glycoalkaloid synthesis (Arnqvist 2007).

CHAPTER 2

HPLC METHOD DEVELOPMENT

2.1. Methods for Glycoalkaloid Determination

Glycoalkaloids can be detected by different methods such as colorimetry, TLC, GC, MS, GC/MS, HPLC, LC/MS, MALDI-TOF/MS, ELISA, and immunoassay. Needless to say, all methods have advantages and disadvantages (Kuronen et al. 1999). MALDI-TOF/MS cannot be used in routine analysis and α- chaconine and α-solanine cannot be separated from each other by using ELISA (Matsuda et al. 2004). In LC/MS detection we do not need derivatization (Kuronen et al. 1999). In GC-MS analysis, glycoalkaloid hydrolysis or derivatization is necessary (Matsuda et al. 2004). Glycoalkaloids are not volatile and they are too large to analyze by GC/MS.

The most commonly used conventional technique for determination of glycoalkaloid concentration is HPLC-UV (Matsuda et al. 2004). HPLC-UV is a widely used, rapid, accurate and reproducible technique. Generally C18 and NH_2 columns and as mobile phases like acetonitrile and biological buffers (commonly phosphate buffer) are used (Edwards and Cobb 1996)

However, glycoalkaloids do not have a suitable UV chromophore. Thus, they are measured at about 200nm by UV detection. This is a disadvantage, because many compounds absorb light at these wavelengths. In consequence, large sample size and extensive sample clean-up are needed to decrease background noise (Kodamatani et al. 2005). Although HPLC is a widely used technique to determine glycoalkaloid level, sometimes it can not separate glycoalkaloids well. The most important parameters in this technique are mobile phase composition, pH, column capacity and temperature (Friedman et al. 2003).

Currently, HPLC-ELSD has been explored for this aim because of the problems in UV detection. Evaporative light scattering detector is “universal” and it is suitable for non-chromophoric compounds such as glycoalkaloids (Dinkins et al. 2008; Paul et al. 2008; Sanchez-Mata et al. 2010).

In industry we need a rapid, accurate and cheap method for total glycoalkaloid determination. To this end, there are many methods but each of them has advantages and disadvantages. For example, derivatization, expensive chemicals, and/or extensive sample clean-up may be required or maybe the method lacks sensitivity. HPLC-UV is still used in industry to determine glycoalkaloid content, because it is rapid and accurate (Alt et al. 2005). For industrial usage, immunoassay is also preferred.

Immunoassay is a sensitive, rapid and cheap method compared to other methods. On the other hand, as a disadvantage, polyclonal antibodies are not specific and monoclonal antibodies are only specific to solanidine-based compounds. Thus, glycoalkaloids cannot be individually determined.

CHAPTER 3

GENOME MAPPING AND MOLECULAR MARKER SYSTEMS

3.1. Genome Mapping

Genetic tools are used to identify loci that affect phenotypic traits and also identify recombination distance between loci. Alternate allelic forms are present in different organisms of the same species. Based on recombination between alleles of different loci, linkage values among genetic factors can be calculated. By using these linkage relationships among all chromosomes, a genetic map of an organism can be constructed (Schneider 2005).

Thus, genetic maps are constructed based on recombination among polymorphic loci. Recombination occurs by chiasma formation between two sisters chromatids of each parent in the meiotic prophase (Figure 3.1). As a result of this process, 50% recombinant gametes and 50% non-recombinant gametes are produced. This event can cause a problem in genetic mapping if genetic markers located on the region of strand exchange undergo gene conversion and these results in nonreciprocal recombination (Schneider 2005).

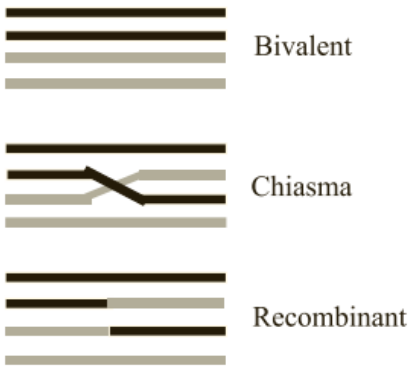


Figure 3.1. Recombination event by chiasma formation
(Source: Schneider 2005).

The possibility of recombination between two points of a chromosome depends on their physical distance. The nearer they are located to each other, the lower the probability of recombination and vice versa. The more distant they are from each other, the higher the probability of recombination (Schneider 2005).

Genetic maps provide us with an understanding of how genes are arranged in chromosomes. Genetic maps help marker assisted selection and marker assisted selection helps in breeding to verify and select resistance cultivars or other important traits such as yield, seed quality, etc. Also genetic maps can give information about species chromosome and gene evolution. (Grant et al. 2001)

The linear arrangement of genes on a chromosome is depicted by genetic maps which give the chromosomal order of genes and the distance of separation expressed as the percent of recombination between them. A genetic map cannot be used to determine physical location of genes or how far apart they are in base pairs. This is because the distances on such maps are not equivalent to physical distances. The unit of measurement is centiMorgan (cM) and equals 1% recombination between any two genetic loci. When nothing about the gene is known, linkage maps allow identification of the position of a gene. Therefore, generally this type of map is used as the first step toward isolating a gene responsible for a specific trait (Schneider 2005).

3.2. Quantitative Trait Locus (QTL) Mapping

Quantitative traits are controlled by multiple genes, each gene has a small effect and segregates according to Mendel's laws. QTLs are also affected by the environment at variable degrees. QTLs can be mapped and identified using appropriate mapping populations and their corresponding molecular genetic maps. (Lynch and Walsh, 1998).

3.3. Comparative Mapping

Similarities and differences in gene content and gene order between genera belonging to different taxa can be compared by using comparative genetic mapping studies (Seres et al. 2006). Comparative genome mapping provides an understanding of conservation of gene order of genes over large regions of chromosomes in different species. Also such maps give information about genome evolution and the location of a

gene in one species may help to determine its location in the genome of another species (Dear et al. 2005)

3.4. Mapping Populations

Individuals of one species or crosses among related species, which differ in the traits that will be studied constitute mapping populations. When choosing a mapping population two parameters are important: (i) polymorphism between the parental lines for the trait to be studied and (ii) reproductive mode (self-compatible or self-compatible) of the plant. It is a problem to obtain pure homozygous line in self-incompatible plants, because they show high genetic heterozygosity while self-compatible lines allow development of pure homozygous lines (Meksem and Kahl 2005).

F_2 plants, recombinant inbred lines (RIL), backcross (BC) populations, introgression lines assembled in exotic libraries, and doubled haploid lines (DH) can be used as mapping populations (Meksem and Kahl 2005).

3.4.1. F_2 Population

F_2 plants are the simplest form of a population. The degree of polymorphism can be observed at the phenotypic level and also identified with molecular markers at the nucleic acid level. For F_2 population development, a F_1 hybrid is first obtained by crossing two homozygous parents. F_1 progeny will be heterozygous and all individuals will have similar phenotype. To obtain a F_2 population that segregates for the traits between the parents, the F_1 plant is selfed. For a codominant marker, the expected segregation ratio is 1:2:1 (homozygous like P1:heterozygous:homozygous like P2) (Figure 3.2). F_2 populations cannot be easily preserved and F_3 plants which are generated from F_2 plants are not genetically identical. These are the disadvantages of using F_2 plants in genetic mapping (Meksem and Kahl 2005).

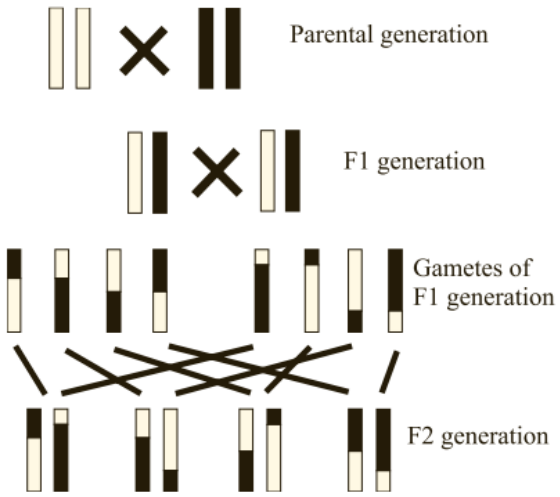


Figure 3.2. Generation of F_2 population
(Source: Schneider, 2005).

3.4.2. Recombinant Inbred Lines (RILs)

Recombinant inbred lines are generated by selfing individual plants of the F_2 generation (Figure 3.3). To generate RILs, plants should be self-compatible. RILs are also called single-seed descent lines because one seed from each line is the source for the next generation. Using RILs as a mapping population has advantages. Lines are permanent, because recombination can no longer change the genetic constitution. The other advantage is, the degree of recombination is higher compared to F2 populations (Schneider 2005).

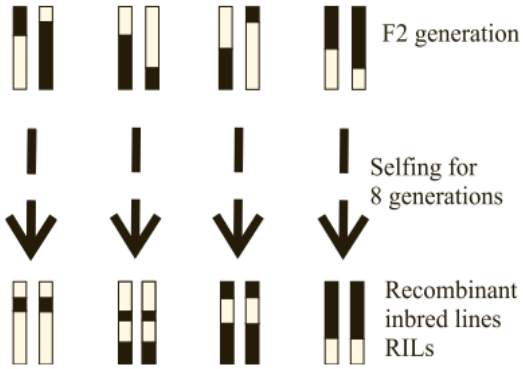


Figure 3.3. Generation of RILs
(Source: Schneider 2005).

3.4.3. Backcross Populations (BC)

To analyze specific DNA fragments derived from parent A (donor), an F1 plant is backcrossed to parent B (recipient). Unlinked donor fragments from parent A are separated by the segregation process and also linked fragments are reduced by recombination with the recurrent parent. To minimize number and size of donor fragments, backcrossing with the recurrent parent is repeated (Figure 3.4). Using backcross lines in breeding is an important tool, if a single trait, such as resistance, has to be introduced into a cultivar that already contains other desirable traits (Schneider 2005).

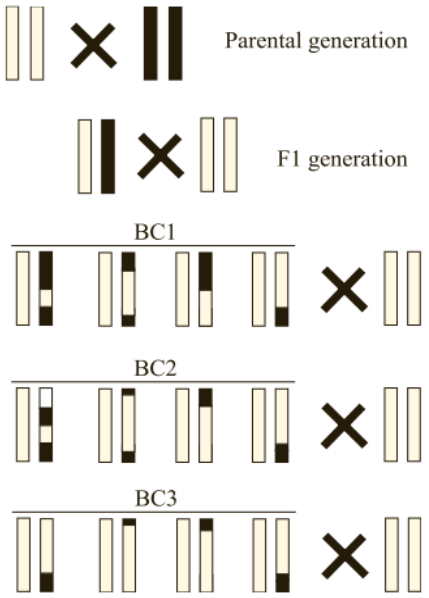


Figure 3.4. Generation of backcross lines
(Source: Schneider 2005).

3.4.4. Introgression Lines: Exotic Libraries

An exotic library comprises of a set of lines, each of which carries a single, defined chromosomal region that is derived from a donor species. Combining positive alleles for desirable traits on a cultivar is possible. An exotic library is generated by advanced backcrossing. Exotic libraries provide permanent resources for mapping (Zamir 2001).

3.4.5. Doubled Haploid Lines

Doubled haploids are homozygous because they contain two identical sets of chromosomes. Doubled haploids lines can be obtained from haploid lines. Because of homozygosity, doubled haploid lines constitute a permanent resource for mapping (Schneider 2005).

3.5. Molecular Marker Systems

Traditionally diversity and genetic maps were determined based on morphological characters. In terms of morphology, many characters are distinguished by the use of electron microscopy, biochemical and phytochemical analysis. Morphology is not sufficient to determine diversity. Because of advantages of molecular-based methods, they have replaced morphologic analysis in many cases (Buckler and Thornsberry 2002).

Molecular marker system can be classified as:

1. First generation markers based on restriction fragments
   a. *RFLP (Restriction Fragment Length Polymorphism)*

2. Second generation markers based on PCR
   a. *CAP (Cleaved Amplified Polymorphic Sequences)*
   b. *RAPD (Randomly Amplified Polymorphism)*
   c. *AFLP (Amplified Fragment Length Polymorphism)*
   d. *S-SAP (Sequence specific amplification polymorphism)*
   e. *SSR (Microsatellites or simple sequence repeat)*
   f. *ISSR (Inter-simple sequence repeat)*
   g. *VNTR (Variable Number of Tandem Repeats)*
   h. *STS (Sequence tagged sites)*
   i. *SCAR (Sequence characterized amplification region)*
   j. *SAMPL (Sequence amplification of microsatellite polymorphic loci)*

3. Third generation markers based on DNA sequencing.
   a. *SNP (Single nucleotide polymorphism)*

4. Genome scanning for expressed genes
    a. *EST (Expressed sequence tag)*
    b. *SRAP (Sequence related amplified polymorphism)*
    c. *TRAP (Target recognition amplification protocol)*
5. Markers using array technology
    a. *Microarrays*
    b. *DarT (Diversity array technology)*
6. Other marker systems
    a. *SSCP (Single-strand conformational polymorphism)*
    b. *DGGE (Denaturating gradient gel electrophoresis)*
    c. *TGGE (Temperature gradient gel electrophoresis)*
    d. *Methylation-sensitive PCR*

Mostly used methods were explainedbelow and table 3.1 shows comparision of broadly used marker systems.

3.5.1. RFLP

The RFLP method is based on digestion of DNA into fragments of different size by using restriction enzymes. RFLP method is reproducible and markers are codominant; so heterozygous and homozygous individuals are distinguishable. This method also has disadvantages. Probe selection is important. Probes must be heterologous. If not, developing cDNA or genomic DNA probes are necessary. Blotting and hybridization steps are difficult to automate and time-consuming. Moreover, good quality DNA is required (Buckler and Thornsberry 2002).

3.5.2. RAPD

In this technique products are amplified with randon primers, stained with ethidium bromide and after running on agarose gel, visualized under UV. The other arbitrary priming techniques are AP-PCR (Arbitrary Primed PCR) and DAF (DNA Amplification Fingerprinting) and differ from RAPD in terms of primer length, primer to template ratio, the gel matrix used, and visualization procedure. In these arbitrary priming techniques are rapid, easy to perform and automate because of absence of

blotting and hybridization steps. Another advantage is it does not require a large quantity of DNA. On the other hand, they are not reproducible and it is hard to stabilize PCR conditions (Buckler and Thornsberry 2002).

3.5.3. AFLP

In this technique, after restriction digestion, genomic DNA fragments are amplified via PCR. Amplified products are labeled radioactively or fluorescently and separated on sequencing gels. This method is not as reproducible as RFLPs and more demanding than RAPDs. Also more DNA is required than RAPDs. On the other hand, AFLP covers whole genome. Therefore, they are useful for mapping, fingerprinting and calculating distances between genotypes (Gupta and Rustgi 2004; Rajeev et al. 2007).

3.5.4. VNTR

VNTR is another powerful technique which is used in diversity studies. Hypervariable regions of the genome which are varying in number and length are used in this method. VNTRs can occur at many sites in the genome. VNTRs are highly distinctive, so it reveals the differences between species and also even individuals (Buckler and Thornsberry 2002).

3.5.5. SSR

SSRs are tandem repeats of short DNA sequences about 1-6 nucleotides. The advantages of using SSR markers are: (i) they have multi-allelism, (ii) they are codominant, therefore homozygous and heterozygous individuals in mapping population can be determined, (iii) they cover whole genome and (iv) they are applicable to high-throughput PCR-based methods (Stagel et al. 2008).

3.5.6. CAPS

In this technique, first DNA is amplified with selected primer by using PCR. Amplified DNA regions are digested with restriction enzymes. Fragments which are digested with restriction enzymes and undigested fragments differ in terms of length, so polymorphism can visualized on an agarose gel. CAPs markers are codominant, therefore homozygous and heterozygous individuals in mapping population can discriminated. Also this method does not require large amount of DNA and it is simple and rapid (Buckler and Thornsberry 2002).

Table 3.1. Comparision of broadly used marker systems (Meksem and Kahl 2005).

Marker/ technique	*PCR-based*	*Polymorphism (abundance)*	*Dominance*	*Repro-ducibility*	*Auto-mation*	*Running cost*
RFLP	No	Low/medium	Codominant	High	Low	High
RAPD	Yes	Medium/high	Dominant	Low	Medium	Low
SCARS/CAPS	Yes	High	Codominant	High	Medium	Medium
AFLP	Yes	High	Dominant	High	Medium/ high	Medium
SSR	Yes	High	Codominant	High	Medium/ high	Low
ISSR	Yes	High	Dominant	High	Medium/ high	Low
STS	Yes	High	Codominant/ dominant	High	Medium/ high	Low
SRAP/EST	Yes	Medium	Codominant	High	Medium	Low
IRAP/REMAP	Yes	High	Codominant	High	Medium/ high	Low
SNP	Yes	Extremely high	Codominant/ dominant	High	High	Low

3.6. Goals of the Study

Our main purpose was to isolate eggplant glycoalkaloids which are not commercially available or hard to find. Isolated glycoalkaloids can be used as standards for subsequent studies. Chromatographic determination of glycoalkaloids is an enormous problem because of their chemical structure similarities. To solve this problem we tried to improve an HPLC method to separate eggplant glycoalkaloids.

In addition, construction of a linkage map of eggplant will allow construction of QTL maps to identify genomic regions of agronomically important characters in eggplant such as glycoalkaloids. After determination of genetic markers that are tightly linked with QTLs, alleles related to nutritional and agronomic traits can be used for improvement of eggplant hybrids by using marker assisted selection. Thus, the second goal of this study was to identify polymorphic markers for future development of a linkage map of eggplant.

CHAPTER 4

MATERIALS AND METHODS

4.1. HPLC Method Development

There are several examples of methods which employ high performance liquid chromatography (HPLC) for the analytical determination of glycoalkaloids (Eanes et al. 2008; Eanes and Tek 2008; Friedman and Levin 1992; Dao and Friedman 1996; Friedman et al. 2003) The most popular HPLC methods make use of a hydrogen phosphate (salt) type buffer. However, this buffer has a tendency to precipitate in the tubing, pump, and column which makes its use undesirable. Likewise, UV detection is very commonly used for these analyses even though the glycoalkaloids exhibit poor UV-vis absorption. For this reason, it is desirable to consider other HPLC detectors such as mass spectrometric (MS) or evaporative light scattering (ELS) detectors. However, not only is the phosphate buffer problematic because it can precipitate in the chromatographic system, but also it can precipitate in a mass spectrometric system and significantly increase the background noise in an ELS system (due to small particles of the precipitate in the mobile phase). Any chromatographic method based on the use of this buffer makes it impossible to adapt the chromatographic method to modern detection methods such as MS or ELS. Therefore a method which would not make use of this buffer was sought. Both a Nuceosil C18-type column (Hi-Chrom) (Eanes et al. 2008) and a C18 XTerra column based on the work of Jensen et al. (Jensen et al. 2008; Jensen et al. 2007) were used with ammonium acetate buffer. Different mobile phase ratios (ACN/ammonium acetate-water or MeOH), pH (2.5, 3.5), flow rates (0.1, 0.2, 0.3, 0.5, 1), and column temperatures (26, 37,50),and drift tube temperatures for ELS detector (50^{O}C, 60 ^{O}C, 80 ^{O}C, 90 ^{O}C, 100 ^{O}C) were tested. Glycoalkaloid standards at different concentrations (50 ppm, 100 ppm, 200 ppm, 500ppm) were used. They were prepared in different solutions at 1000 ppm concentration as stock solutions, then diluted to the desired concentrations and also filtered through a syringe filter (0.45µm pore size). Mobile phases were prepared fresh, sonicated in an ultrasonic bath and filtered through a 0.45µm polyamide filter. Recent and ongoing investigations of the

use of a carbohydrate-analysis type column are underway (Eanes et al. 2008). Although not generally seen in the literature of the last two decades, carbohydrate-analysis type columns were among the first types to be employed for the separation of glycoalkaloids (Verpoorte et al. 1984). It is expected that this type of column may potentially give better separations; however, at present its short and long-term stability is still problematic, making it unsuitable at present for use with a large number of samples over an extended study period (Eanes et al. 2008).

4.2. Glycoalkaloid Purification

4.2.1. Glycoalkaloid Extraction

A total of 1 kg eggplant fruit was lyophilized until all water was removed and then freeze-dried fuits were ground by using an automatic mortar. The final weight of the freeze-dried fruits was 81.67 g and they were stored at -80 ^{o}C.

For extraction, 250mL n-hexane was added to the sample and shaken for 1 hour at 150 rpm on a shaker to remove lipids from the sample. Then the sample was filtered by using a Buchner funnel to separate the powder and n-hexane. Filter paper with a pore size of 0.45µm was used with the Buchner funnel. Additionally, 50 mL n-hexane was used to rinse the Erlenmeyer flask. N-hexane solution was checked for the presence of glycoalkaloids by using TLC. The TLC solvent was chloroform:methanol:water (61:32:7)(v/v/v). Then 30% sulfuric acid was used as a visualizing agent by heating to 100 ^{o}C. No glycoalkaloid presence was detected in the n-hexane solution.

After n-hexane treatment, 250 mL dichloromethane was added to the sample and shaken for 1 hour at 150 rpm on a shaker to remove pigments from the sample. Then the sample was filtered using a Buchner funnel to separate the powder and dichloromethane. The filter paper's pore size was 0.45µm. Additionally 50 mL dichloromethane was used to rinse the Erlenmeyer flask. The dichloromethane solution was checked for the presence of glycoalkaloids via TLC as described above. No glycoalkaloid presence was detected in the dichloromethane solution.

Powder was taken and reflux was done for 12 hours with 250 mL methanol (reflux 1). Then the sample was filtered by using a Buchner funnel to separate the powder and methanol. The filter paper's pore size was 0.45µm. Again powder was

taken and reflux was done for 3.5 hours with 250 mL methanol (reflux 2). Methanol solutions were checked for the presence of glycoalkaloids by using TLC as previously described. In both methanol solutions glycoalkaloid presence was detected.

Methanol was evaporated by using a rotary evaporator. Temperature was 50 ^{o}C and rotary speed was 90 rpm. Material remained like caramel on the inner surface of the round bottom flask.

4.2.2. Glycoalkaloid Isolation

Glycoalkaloid purification for this work was based on a combined and modified method from the literature (Abouzied et al. 2008; Dinan et al. 2001; Nakamura et al. 2008; Wanyonyi et al. 2002; Veissenberg 2001 and Usubillage et al. 1996).

Because of the presence of so much sugar in eggplant, a simple wash with water and methanol was employed. First, the extract was washed with 250 mL water to remove sugars. The water phase was filtered through a 0.45 µm polyamide filter in a Buchner funnel which contained 20 g silica gel. Then extract was washed with 1.250 mL methanol to obtain glycoalkaloids. The methanol phase was filtered in the same manner as the water phase. TLC was performed as previously described.

A total of 2 g silica gel was put in the flask which contained methanol phase which had been concentrated for dry implementation prior to application to a VLC column to separate impurities. The VLC column had a diameter of 4.5 cm and a height of 45 cm. To construct VLC column, 123.7 g silica gel was placed in the column with the starting solvent after sonication. Then 300 mL chloroform and 600 mL methanol were passed through the column, respectively, aided by a vacuum pump. Fractions of 150 mL were collected. The first and second fractions from methanol were combined. Finally the column was washed with 400 mL methanol.

For the water phase, three liquid-liquid partitions were performed with butanol at a ratio of water: butanol (4: 1, v/v). TLC was performed for both the water and butanol phases. The TLC solvent was chloroform:methanol:water (61:32:7)(v/v/v). Sulfuric acid (30%) was used as a visualizing agent. First, the butanol phase was evaporated and dry material was washed with 250 mL methanol after washing with 60 mL water. The water phase was filtered through a 0.45µm polyamide filter via a Buchner funnel which contained 20 g silica gel. The methanol phase was filtered in the

same manner as the water phase. The same procedure was used for the methanol phase likewise used for the VLC column that used for the butanol phase. According to the TLC results, the first and second methanol fractions from the butanol phase from the first liquid-liquid partition were combined with the methanol phase from first VLC column eluate.

To clean up the sample, an RP-column (4.5 cm diameter and 45 cm height) was constructed. For this, 106.6 g RP-silica gel was used. Gradient elution (Table 4.1) was achieved using a vacuum pump. Fractions were collected according to the volumes of gradient elution solvent used.

Table 4.1. Gradient elution method for RP column.

Volume (mL)	*Gradient (MeOH)*
380	50%
100	60%
100	70%
100	80%
100	90%
100	100%
200	100%
100	100%
100	100%
100	100%
100	100%
200	100%

For all fractions, TLC was performed and the TLC solvent was employed the same as the elution solvent for each fraction except for the 100% methanol fractions. For these fractions chloroform:methanol:water (61:32:7)(v/v/v) was used and 30% sulfuric acid was used as a visualizing agent. According to the TLC results, the 80%, 90% and first, second, and third 100% fractions were combined (Table 4.1).

Another column having a diameter of 3 cm and a height of 60 cmwas constructed. Methanol was evaporated with 1 g silica gel for dry implementation. A total of 100 g silica gel was filled into the column and isocratic elution was performed with chloroform:methanol:water (61:32:7)(v/v/v) solvent system. In total 137 fractions were collected and the volume of each fraction was 5ml. TLC was performed for all

fractions. According to the TLC results, fractions 9 through 32 were combined. These were the solamargine and solasonine containing fractions.

To separate fractions 9-32, a column (2.5 cm diameter and 30 cm height) was constructed with 50 g silica gel. Elution was done isocraticly with chloroform:methanol:water (61:32:7)(v/v/v) and 147 fractions were collected and volume of each fraction was 2 ml. TLC was performed for all fractions. The TLC solvent was chloroform:methanol:water (61:32:7)(v/v/v). Sulfuric acid (30%) was used as a visualizing agent. According to the TLC results fractions 37 through 63 contained solamargine, fractions 64 through 92 contained solamargine and solasonine mix and fractions 93 through 119 contained solasonine. The solamargine, solamargine and solasonine, and solasonine containing fractions were combined and evaporated separately.

To increase the amount of the extracted compounds, the solasonine and solamargine mix was separated again on a silica column (2,5 cm diameter and 60 cm height). Silica gel (30 g) was used. Sample was applied to the column after redissolved in 1ml of elution solvent. Isocratic elution was done using a chloroform:methanol:water (61:32:7) (v/v/v) solvent system. Fractions were collected (10 mleach) and checked for solamargine and solasonine presence with TLC. TLC method was the same as that mentioned earlier. According to the TLC results, fractions 18 through 22 contained solamargine, fractions 23 through 34 contained solamargine and solasonine mix and fractions 35 through 38 contained solasonine.

Fractions 23 through 34 were combined and evaporated for further separation. One more silica column (3 cm diameter and 60 cm height) was constructed with 150 g silica gel. The sample was dissolved in chloroform:methanol:water (61:32:7) (v/v/v). Isocratic elution was performed using the same as solvent system as the sample and 4 ml fractions were collected. Solamargine containing fractions 171 through 199 , mixed solamargine and solasonine containing fractions 200 through 210, and solasonine containing fractions 211 through 232 were combined and evaporated separately after checking with TLC by using same method above.

Finally, a Sephadex column (2 cm diameter and 45 cm height). was constructed to clean up solamargine containing fractions. Isocratic elution was done with methanol and the sample was redissolved in 1 ml of methanol. A total of 139 fractions were collected and the volume of each fraction was 1ml. Solamargine containing fractions 32 through 44 were combined and likewise solamargine containing fractions 45 through

123 were combined. As seen in figure 5.11a the former set was of a lower purity than the latter set.

The solvent of solamargine, solasonine, and solamargine and solvent mix were evaporated separately and lyophilized for two days afterwards resolving in 1 ml of tertiary butanol (Figure 4.1.a and 4.1.b summarizes glycoalkaloid purification process).

In the end, 5.9 mg of solamargine, 3.9 mg of solasonine, and3,1 mg of solamargine/solasonine mix were obtained. To calculate the efficiency and yield of the purification process 122,8 mg of solamargine and solasonine were mixed into 80,569 eggplant powder which did not contain glycoalkaloid. Thus, 12,9 mg of total glycoalkaloids were obtained. So, yield of the process was calculated to be 10,50%.

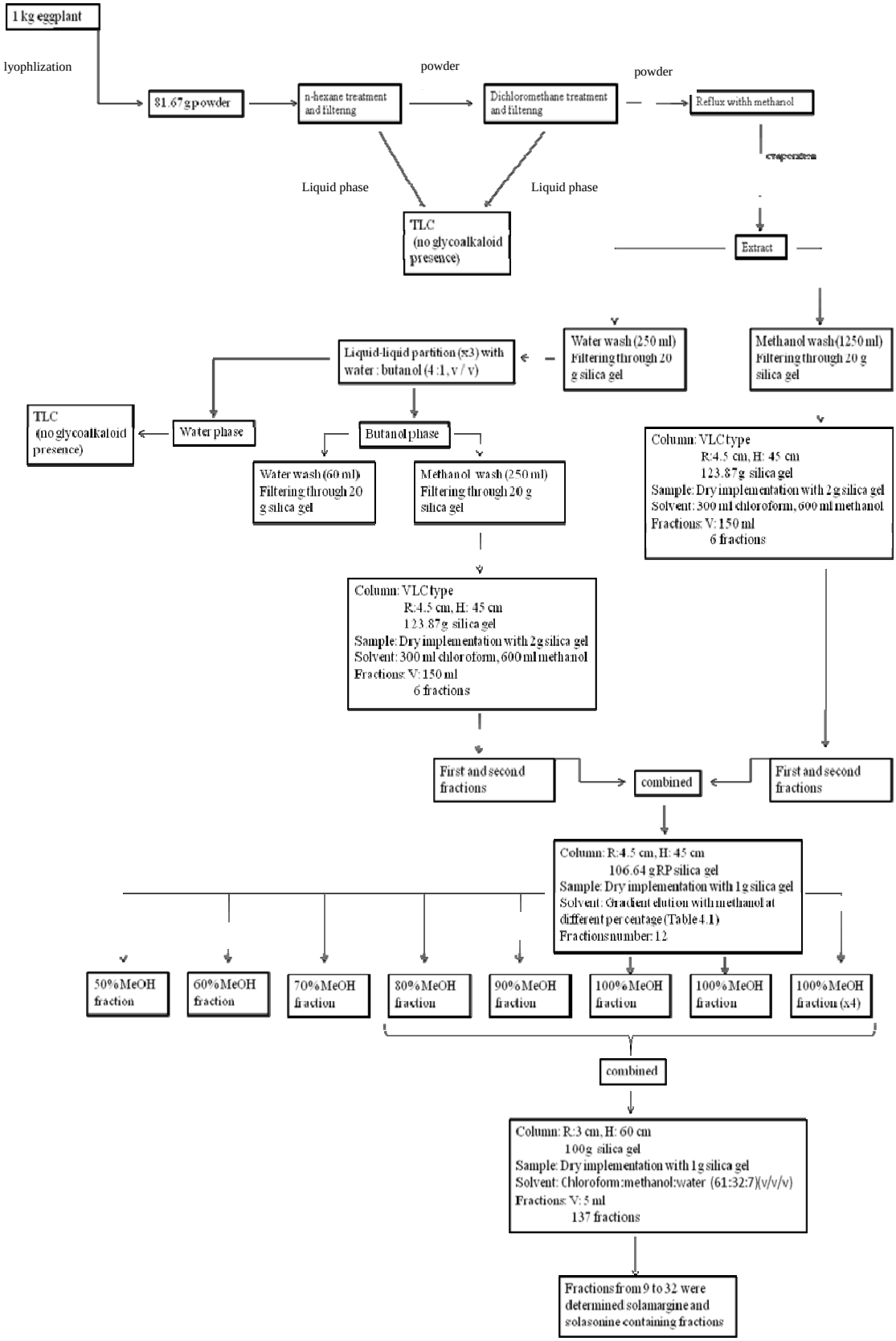


Figure 4.1. Glycoalkaloid purifiation process

(Cont. on next page)

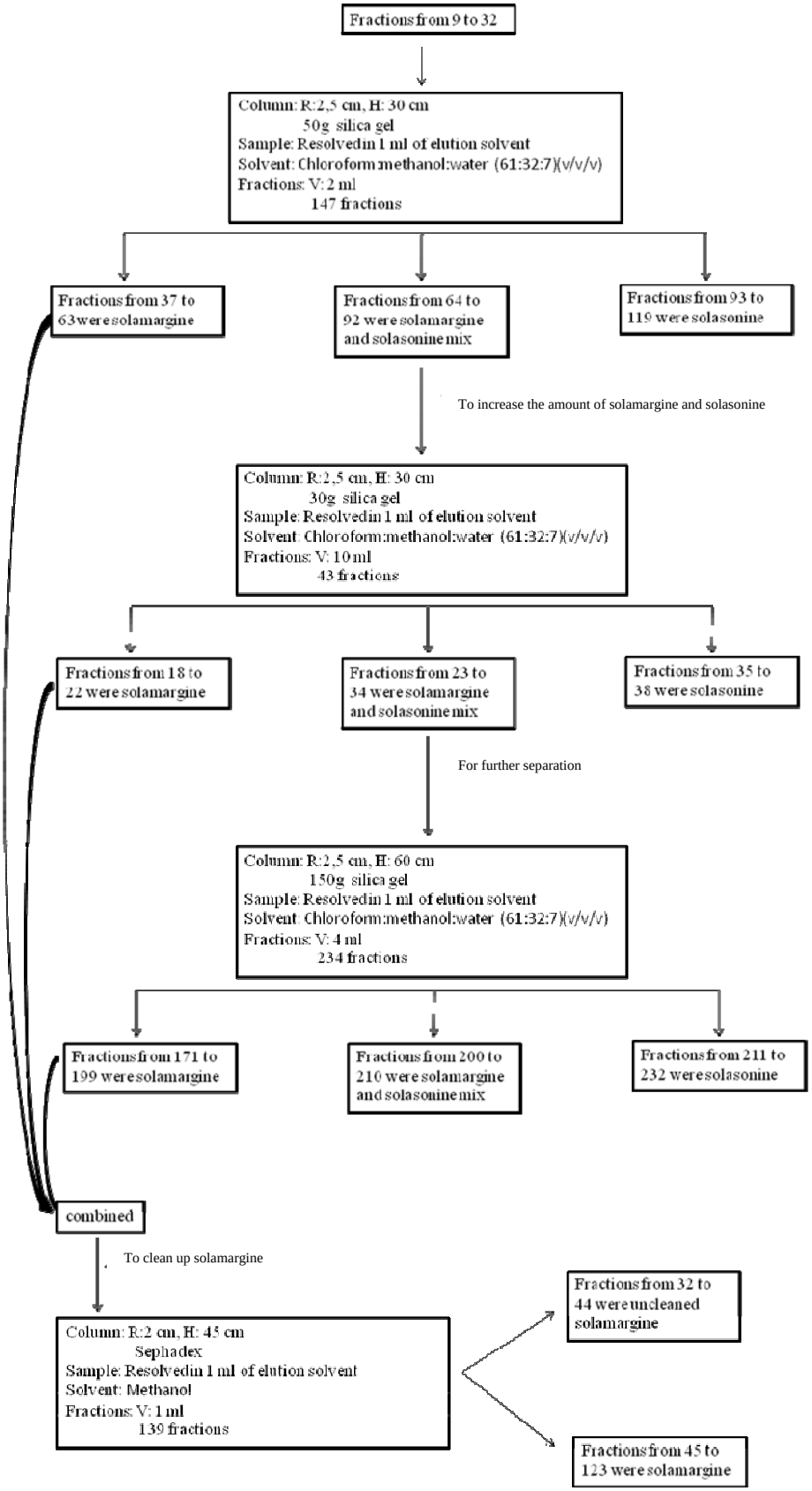


Figure 4.1. (Cont.)

To verify the accuracy of purified compounds ^{1}H NMR and mass spectroscopy were performed. For mass spectroscopy; 2,5-dihydrobenzoic acid (DHB) was used as a matrix. The 2 mg of matrix was dissolved in 100 µl of 20 % acetonitrile and 80 % 0.1 TFA in water. One layer sample preparation technique was used, whereby 10 mg/L standard solution of each glycoalkoid was mixed with a DHB matrix (1:1 ratio). The samples were dissolved in HPLC grade methanol and they were also mixed in the same matrix with the same ratio. The standards and samples were spotted on a stainless MALDI target and allowed to dry under ambient conditions.

The spectra were acquired with an autoflex III MALDI TOF/TOF MS system (Bruker Daltonics, Bremen, Germany) in positive reflectron mode. The ions were generated using a 337-nm laser beam from a nitrogen laser. Each spectrum was averaged over 1000 laser shots at a laser frequency of 50 %. The acquisition range was set to *m/z* 200 – 1000. Spectra acquired were reprocessed with the Igor Pro software package.

NMR experiments were performed on a Varian AS-400 spectrometer in C_5D_5N (99.95%, SigmaAldrich).

4.3. Linkage Mapping

4.3.1. Plant Material

The BC_2F_1 mapping population used in this project was developed by Sami Doğanlar by crossing *Solanum melongena* with *Solanum incanum*. F_1 hybrids were backcrossed to *Solanum melongena* to obtain a BC_1F_1 population, then BC_1F_1 individuals were backcrossed one more time with *Solanum melongena* to produce a BC_2F_1 population. A total of 148 individuals from 37 lines of the BC_2F_1 population were planted in the field in Antalya by MULTİ Tarım Seed Company.

4.3.2. DNA Extraction

For DNA extraction from individuals of the mapping population, ‘miniprep DNA isolation’ method was used (Bernatzky and Tanksley 1986). A handful of young

leaves of eggplant (*Solanum melongena Dusky X Solanum incanum* BC2 F1) was collected. Sodium bisulfate was added to cold CTAB extraction buffer to a final concentrationn of 4-8 g/L. Leaves and 90 mL extraction buffer were homogenized for 30 seconds in a blender. Extract was filtered through cheesecloth into 50 mL falcon tubes. Then sample was centrifuged 10 minutes at 4 ^{o}C, 3.000rpm. After removing supernatant, 1.75 mL extraction buffer was added to pellet. Pellets were mixed well by pipeting. After this, 1.75 mL nuclei lysis buffer and 0.6 mL 5% sarkosyl were added to samples. Samples were incubated 30-60 minutes at 65 ^{o}C. Then, 5 mL of chloroform:isoamyl alcohol (24:1, v:v) mix was added and samples were shaken well. Samples were centrifuged 10 minutes at 4 ^{o}C, 3.000rpm and supernatant was transfered into 15 mL falcon tube. An equal volume of cold isopropanol was added to supernatant and the falcon tube was shaken gently until DNA appeared. For washing, DNA was transferred into 70% ethanol by using Pasteur pipet. Ethanol was dried at room temperature and 250-300 mL TE (tris-EDTA) buffer was added to DNA. The amount and purity of DNA was checked by nanodrop. Final concentration of DNA was adjust to 50-80 ng/µL.

For *Solanum melongena* DNA "microprep DNA isolation" method was used (Fulton et al. 1995). In this method young leaves were collected into a 1.5 mL eppendorf tube. After adding 250 µL microprep buffer, sample was homogenized via drill. Again 500 µL microprep buffer was added and mixed by vortexing. Sample was incubated 30-60 minutes at 65 ^{o}C. After incubation 750 µL of chloroform:isoamyl alcohol (24:1, v/v) mix was added and sample was shaken well. Sample was centrifuged 5 minutes at 10.000rpm. Supernatant was transfered into new 1.5 mL eppendorf tube and 750 µL cold isopropanol was added. Sample again was centrifuged 5 minutes at 10000rpm. Isopropanol was decanted and 50 µL 70% ethanol was added to sample for washing. After removing ethanol 30-50 µL TE buffer was added. DNA was dissolved in TE at 65 ^{o}C in water bath. Sample was mixied by vortexing and centrifuged 5 minutes at 10.000 rpm. DNA. The amount and purity of DNA was checked by nanodrop. Final concentration of DNA was adjusted to 90-100 ng/µL.

For *Solanum incanum, DNA* was extracted by using Promega kit according to its protocol. The amount and purity of DNA was checked by nanodrop. Final concentration of DNA was adjusted to 90-100 ng/µL.

Buffers Used in DNA Extraction:

Extraction buffer (for 20L):

1275 g sorbitol (0.35M)

242 g Tris-base (0.1M)

37.2 EDTA-Na_2

Final pH was adjusted to 8.25 with HCl.

Nuclei Lysis Buffer (10L):

200g CTAB (Hexadecyltrimethyl ammonium bromide (%2))

2L Tris-HCl pH=8 (200 mM)

186.1g 0,25M EDTA (50mM)

1168.8g NaCl (2M)

%5 Sarkosyl Solution:

50g/L N-lauroylsarcosine sodium salt

Microprep Buffer:

For 5-6 samples:

2.5 mL extraction buffer

2.5 mL nuclei lysis buffer

1 mL 5% sarkosyl

0.01g sodium bisulphate (it was solved in extraction buffer.)

4.3.3. Survey and Mapping

4.3.3.1. Survey for mapping

First of all survey was done in parents DNA to detect markers with polymorphism (Figure 4.2). CAPs (Cleaved Amplified Polymorphic Sequence) and COS II (Conserved orthologous set II) markers were used to determine polymorphism. In this method, PCR was done and then amplified product was digested by restriction enzymes. Finally, restricted DNA was visualized by 3% agarose gel electrophoresis.

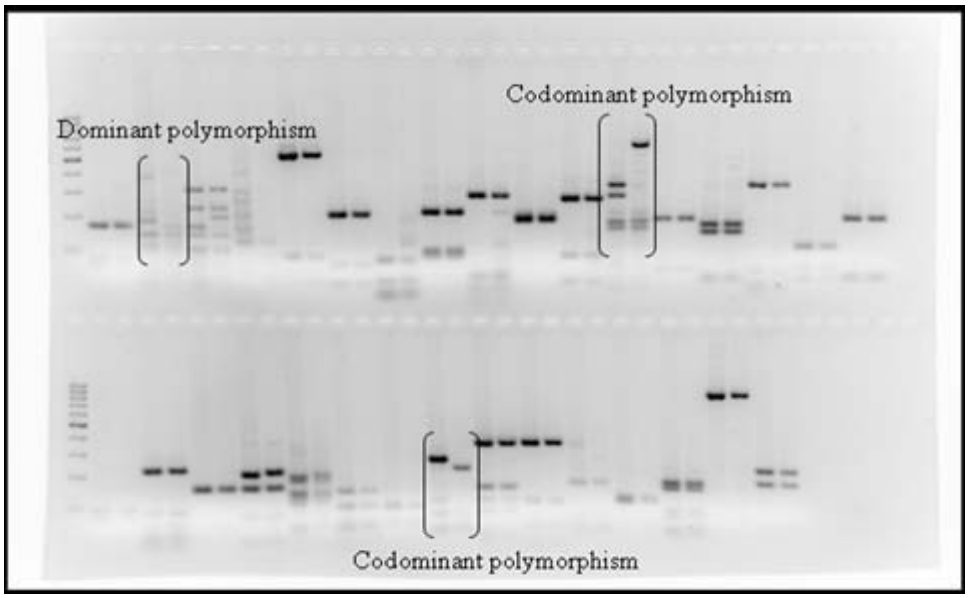


Figure 4.1. An example of survey which was done with COS II markers and restriction enzymes.

PCR method (25 µL reaction):

17.25 µL dH_2O

2.5 µL 10x PCR Buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH: 8.3)

1.5 µL $MgCl_2$ (1.5 mM)

0.5 µL dNTP (0.2 mM)

0.25 µL Taq polymerase (0.25 U)

2 µL DNA (90-100ng/µL)

0.5 µL F and R primer (10 pmol)

The PCR reaction was performed as shown in the figure 4.3:

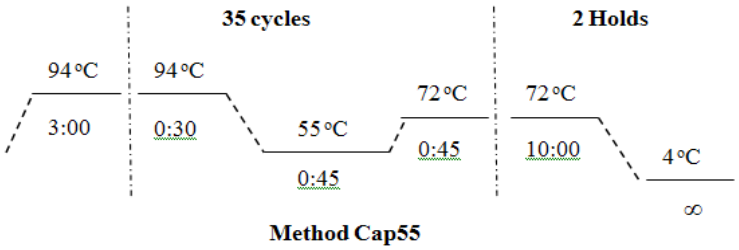


Figure 4.2. PCR conditions.

Enzyme Digestion for 10 µL PCR Product:

Enzyme mix was prepared. 7.5 µL dH_2O, 2µL 10x buffer and 0.5 µL enzyme were mixed. 10 µL enzyme mix was added to 10 µL PCR product and incubated at least 2 hours at enzyme's working temperature.

To visualize polymorphism, restricted DNA was run on 3% agarose gels. Agarose gels were prepared with 1xTAE buffer and visualized with UV light after ethidium bromide staining.

4.3.3.2. SSR Method

For survey of polymorphism, eggplant, pepper, tomato and *hirsutum* SSR primers were checked. First of all, PCR was done for each primer at 50 ^{O}C and 55 ^{O}C annealing temperature. Then polymorphism was detected on agarose gel using the same procedure aswith CAPs method. Polymorphic markers were applied on population by using same method with SSR survey.

PCR method (25 µL reaction):

18.75 µL	dH_2O
2.5 µL	10x PCR Buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, pH: 8.3)
0.5 µL	dNTP (0.2 mM)
0.25 µL	Taq polymerase (0.25 U)
2 µL	DNA (90-100ng/µL)
0.5 µL	F and R primer (10 pmol)

The PCR reaction was performed as shown in the figure :

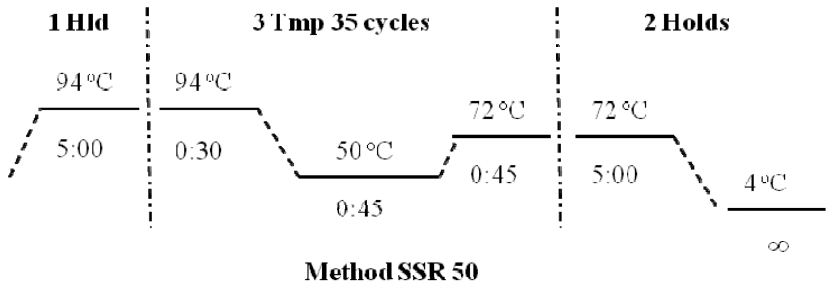


Figure 4.3 PCR conditions for SSR 50 method.

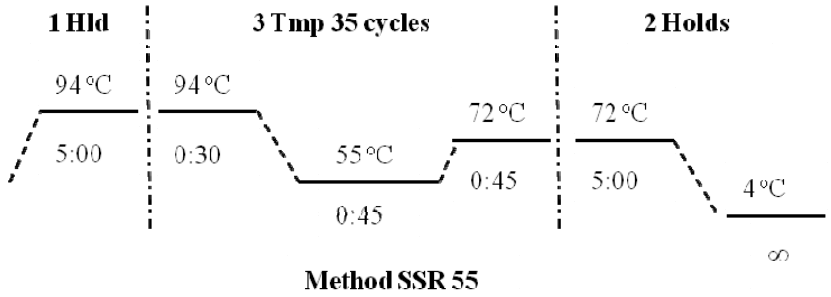


Figure 4.4. PCR conditions for SSR 55 method.

4.3.3.3. SRAP Method

In this method combinations of EM and ME primers were used for PCR. Following PCR, polymorphism was visualized on agarose gels which were the same as those used with previous methods.

PCR method (20 µL reaction):

9.5 µL dH2O

2.0 µL ۲ Buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3)

2.0 µL MgCl2 (1.5 mM)

0.7 µL dNTP (0.2 mM)

0.3 µL Taq polymerase (0.25 U)

1.5µL DNA (90-100ng/µL)

2 µL EM and ME primer (10 pmol)

The PCR reaction was performed as shown in the figure :

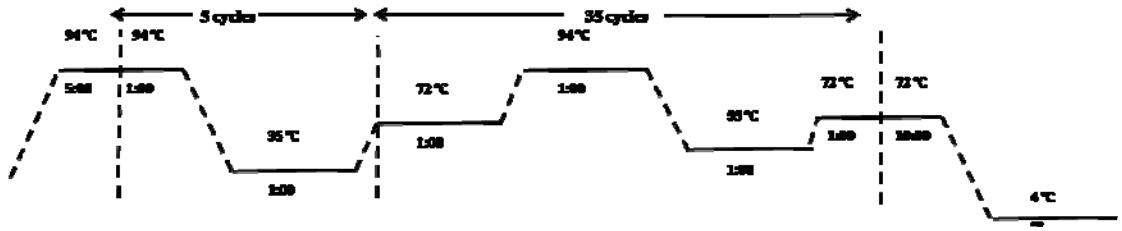


Figure 4.5. PCR conditions for SRAP method.

4.3.3.4. Construction of Map

Selected primers and restriction enzymes which provided polymorphism were applied to mapping population by using same PCR, enzyme digestion and visualizing method. After obtaining gel images of population individuals, scoring was done from 0 to 5. In the presence of codominant marker, 1 represents the similarity to recurrent parent, 2 represents the similarity to both parent (heterozygosity), 3 represents the similarity to donor parent. In the presence of dominant marker, 4 represents the similarity to donor parent, 5 represents the similarity to recurrent parent. For both dominancy, 0 represents missing data. Figure 4.4 shows scoring the mapping population in the presence of codominant marker.

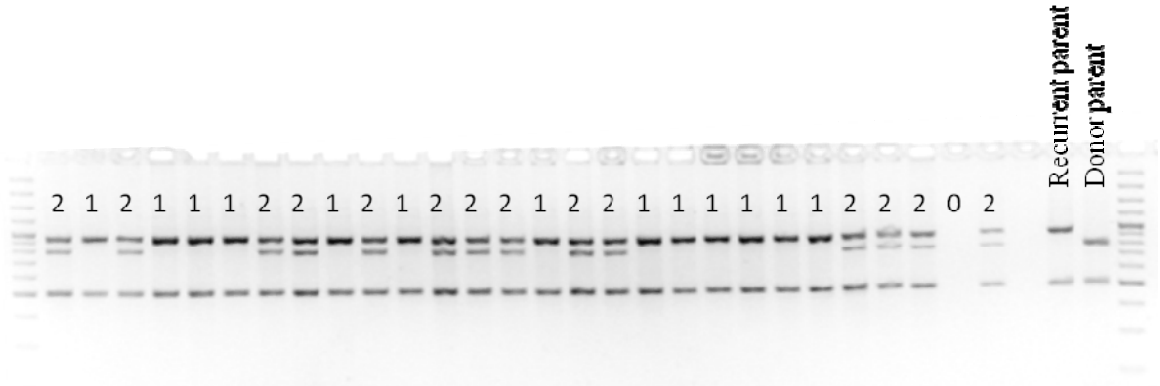


Figure 4.6. An example for applying to selected primer and enzyme to population.In the figure 5g 07910 primer and BsuRI enzyme were used.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. HPLC Method Development

In general, experimental results here have shown that better separation of the glycoalkaloids is obtained when using phosphate buffers, especiaally when employing a Nucleosil (reverse phase, C18) type column. Although separations are not necessarily of high resolution when using other buffers, this may not be such a problem for certain detection methods such as mass spectrometry that employs molecular ion peak isolation followed by MS/MS, thus making good chromatographic separations less critical. However, if a detector employing ELS is used, then most definitely phosphate buffers cannot be utilized as they will swamp the detector signal with excesssive background noise. Potentially the ELS would be an attractive detector for glycoalkaloid and Dinkins et al. (2008) have used this detector for their analyses of potato glycoalkaloids, making use of an amide type column for their work. Although it is known that amino types of columns exhibit "notorious" instability (Kuronen et al. 1999), and even though such instability has been experienced by others in this lab (Eanes et al. 2008) it may still be feasible for this lab to revisit this type of column again in the future. Another possible alternative that is underway in this lab is the use of a carbohydrate-analysis column (Verpoorte and Niessen 1994). This type of column was once employed in the early days of glycoalkaloid chromatographic separations. Initial experiments in our lab have shown that separations can be obtained but unfortunately, this column also exhibits instability and reproducibility problems with time (Eanes et al. 2008). In fact, Eanes expects that if the instability issue can be overcome, it may be possible to obtain in one chromatographic run a complete separation of all the glycoalkaloids of interest to our studies. (Eanes and Tek 2008) Future work may focus on the carbohydrate analysis column as well as the method employing a Nucleosil C18 column with phosphate buffer and UV detection (e.g. Eanes *et al.,* 2008), or either the Nucleosil C18 or XTerra C18 with ammonium acetate buffer and MS detection (where high quality separations are not essential).

5.2. Glykoalkaloid Isolation

After collecting extract, content of the each extract was checked and compared to standards (Figure 5.1). Both reflux 1 and reflux 2 contain solamargine and solasonine. Also sugars are present in reflux 1 and reflux 2 at high concentrations.

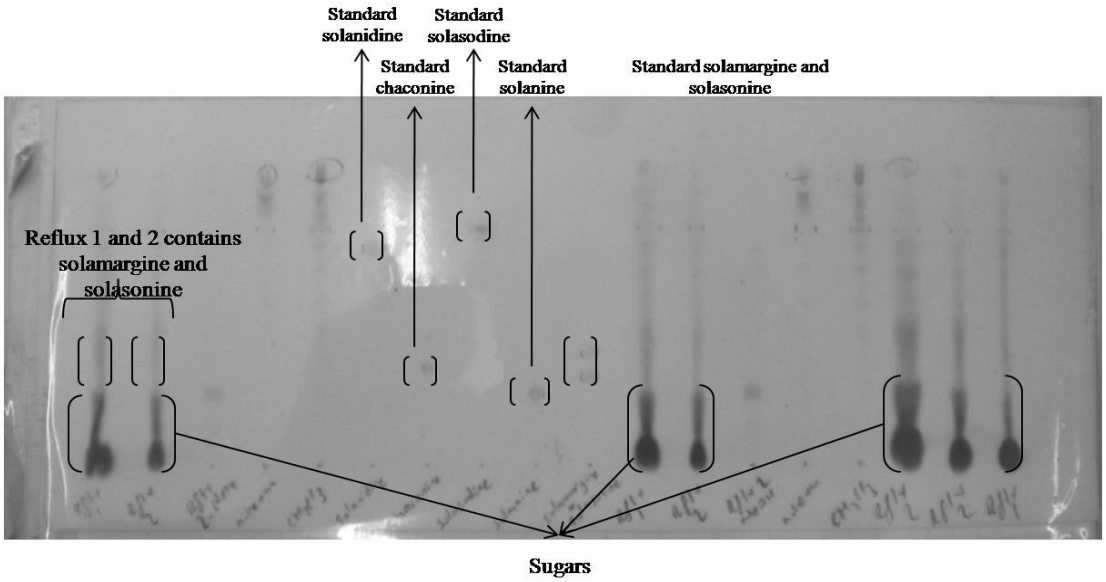


Figure 5.1. Comparison of refluxes, n-hexane and $CHCl_3$ treatment and glycoalkloid standards.

The methanol and water wash were checked for glycoalkaloid presence by TLC (Figure 5.2). The methanol wash contained glycoalkaloids and the water phase contained many impurities.

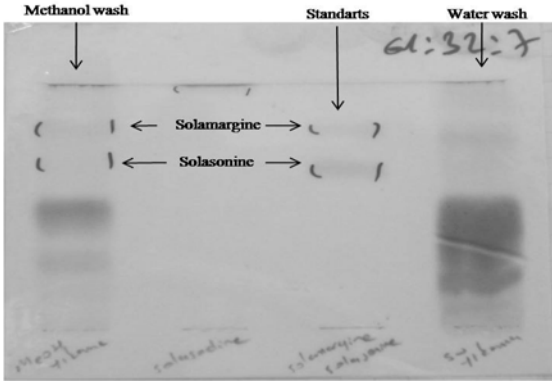


Figure 5.2 TLC of water and methanol wash.

To eliminate impurities, a VLC column was used for the fractions from the methanol wash. Butanol-water liquid-liquid extraction was done three times and all fractions were checked by using TLC (Figure 5.3).

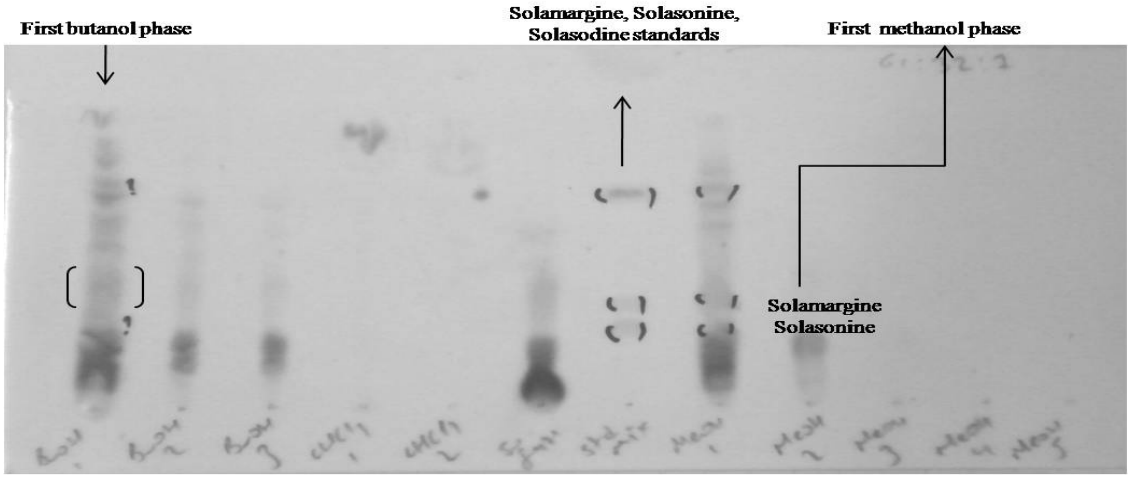


Figure 5.3. TLC of VLC column and liquid-liquid extraction.

The first fraction of butanol from the liquid-liquid extraction was taken and washed with methanol and water. For the methanol wash fraction, VLC was done. According to the TLC results, the first and second methanol fractions from butanol phase from the first liquid-liquid extraction were combined with the methanol phase from first VLC column (Figure 5.4).

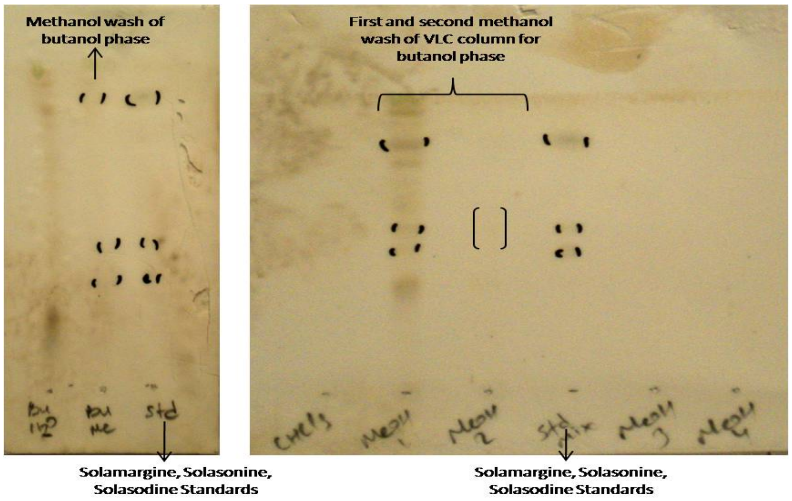


Figure 5.4. TLC of water and methanol wash and VLC column of butanol phase.

For the RP column, a gradient elution was choosen according to the result of TLC at different methanol concentration (Figure 5.5). According to TLC, methanol gradient was chosen as first 50% methanol, followed by 60% methanol, 70% methanol, 80% methanol, 90% methanol, and finally 100% methanol.

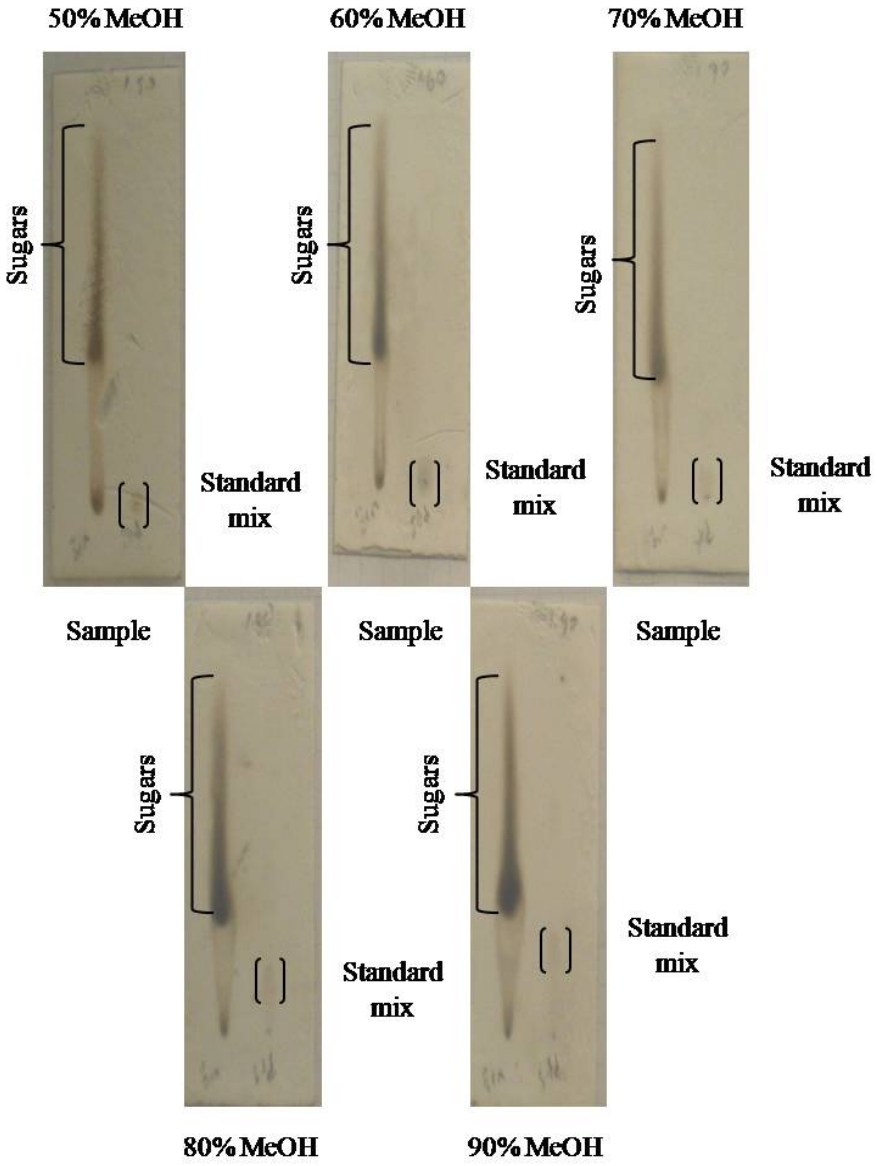


Figure 5.5. TLC for determine RP column solvent system.

Glycoalkaloids were retained on the RP column and most of the sugars were eluted when 50% methanol concentration was used. Glycoalkaloids eluted more easily at higher methanol concentrations (Figure 5.6)

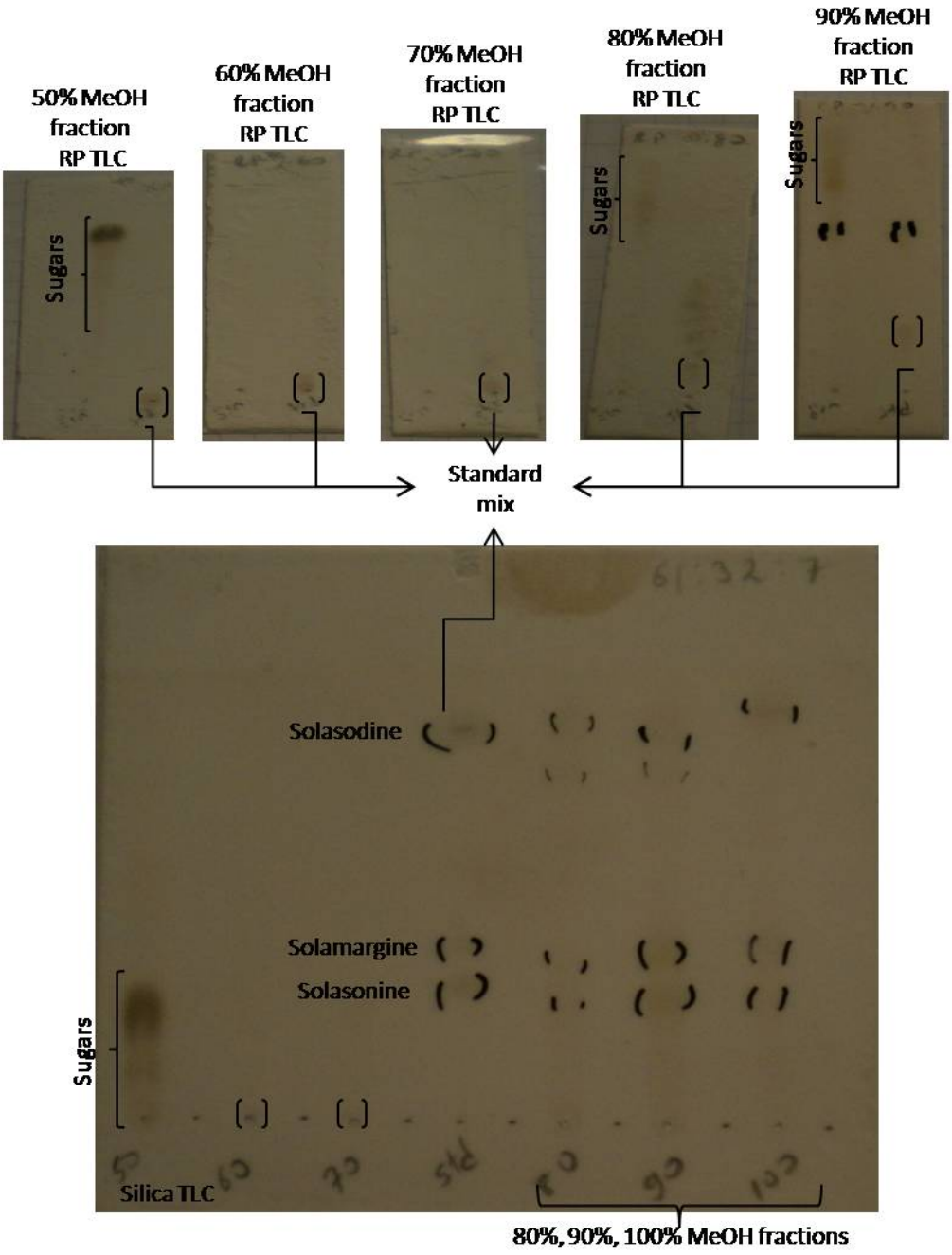


Figure 5.6. TLC of RP column fractions at RP and silica TLC.

According to the TLC results, the 80%, 90% and first, second, third 100% fractions were combined. The sample was cleaned up on a silica column. Solamargine and solasonine were obtained as a mix fractions 9 through 32 (Figure 5.7).

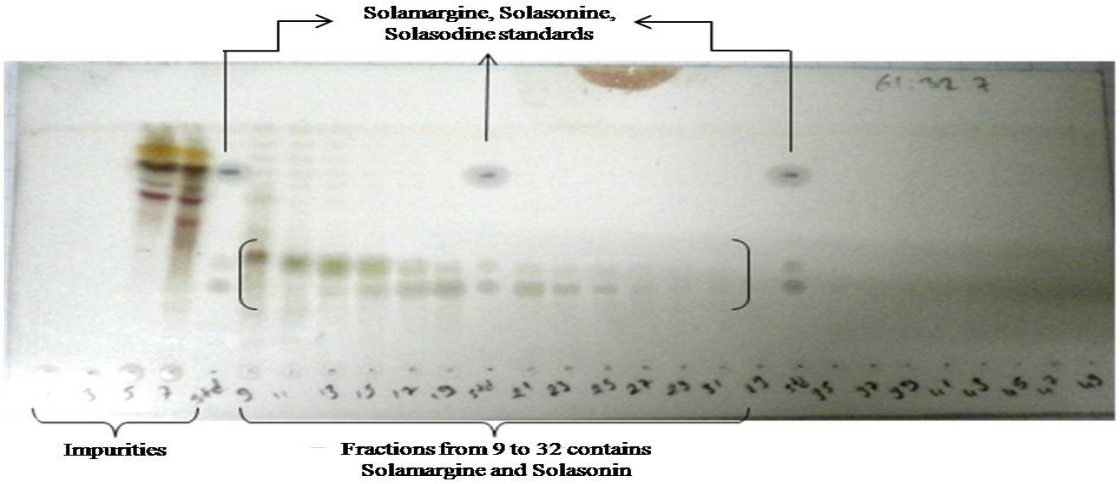


Figure 5.7. TLC of silica column fractions.

The solamargine and solasonine mix was separated on a silica column. Fractions 37 through 63 were determined to contain solamargine, 64 through 92 were determined to contain a solamargine and solasonine mix and 93 through 119 were determined to contain solasonine (Figure.5.8).

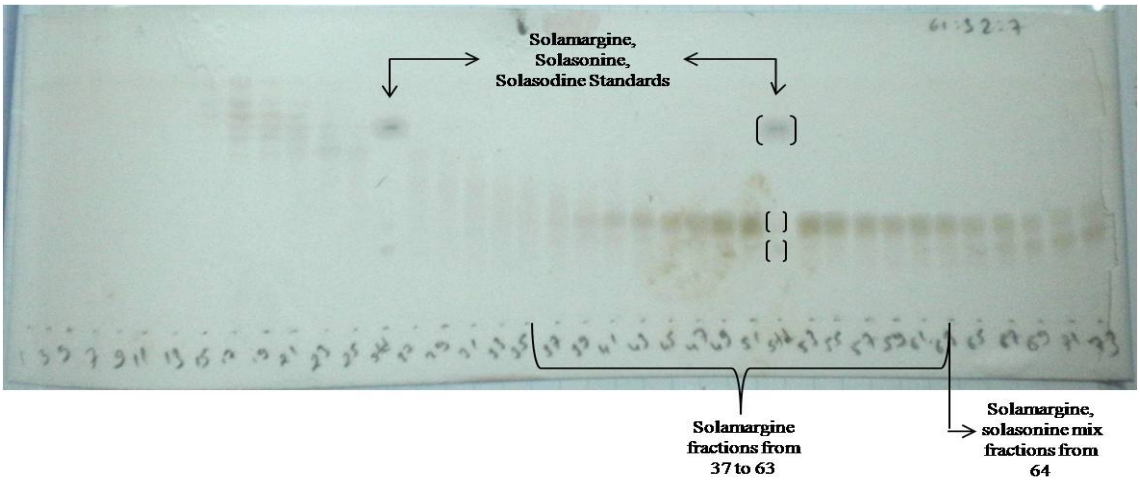


Figure 5.8. TLC of silica column fractions which shows solamargine and solasonine fractions.

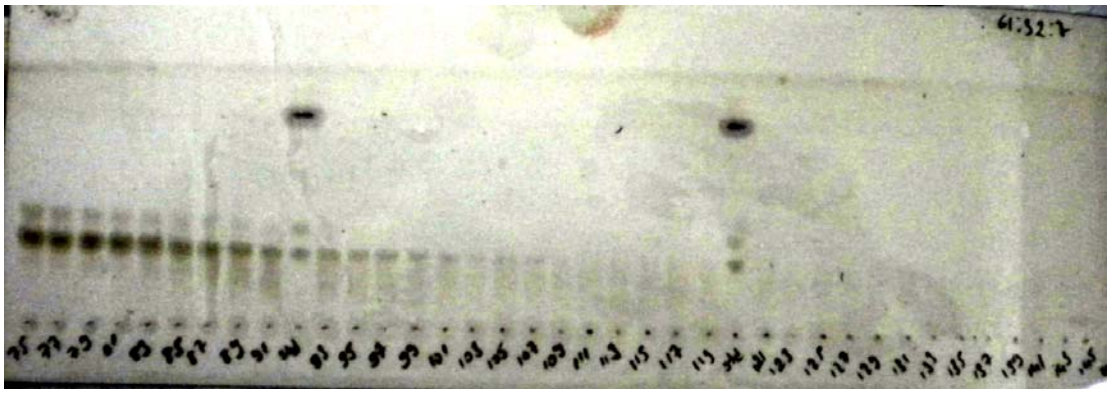


Figure 5.9. TLC of silica column fractions which shows solamargine and solasonine fractions.

To increase the amount of solamargine and solasonine, additionally two silica columns were done, consecutively (Figure 5.10a,b,c,d).

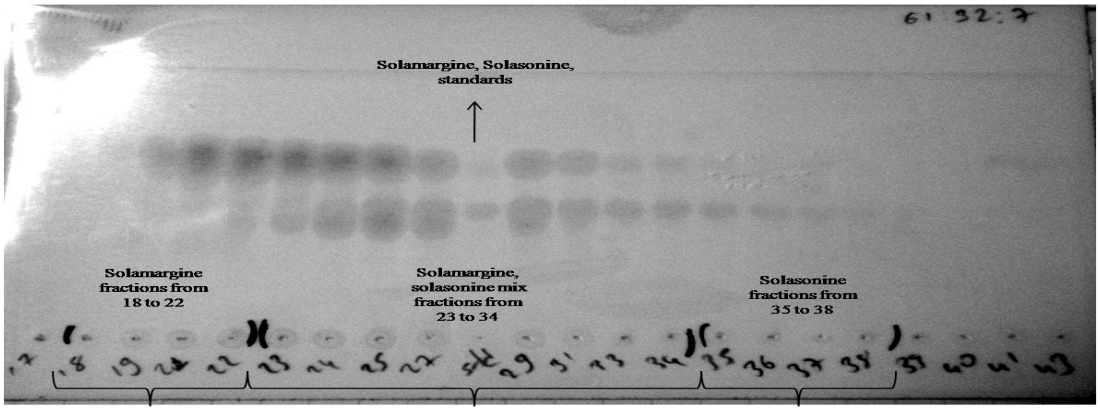


Figure 5.10.a. Solamargine and solasonine containing fractions.

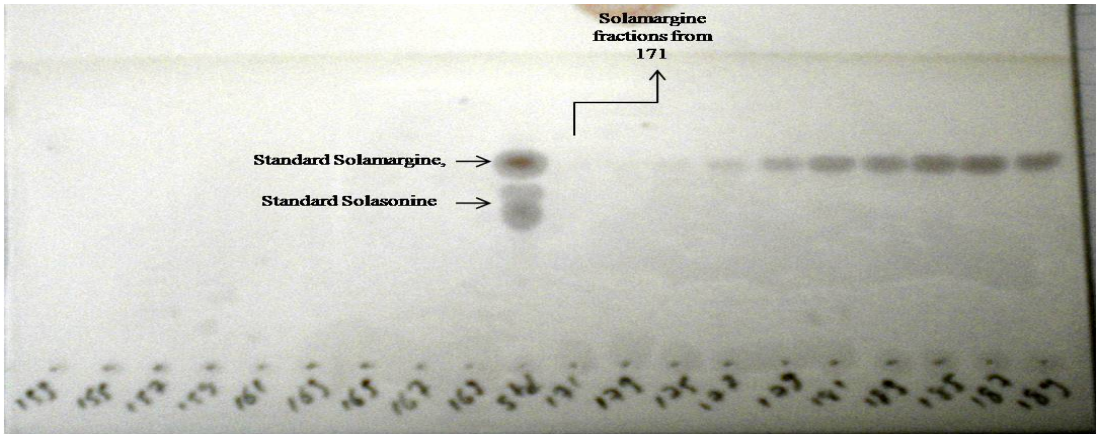


Figure 5. 10.b. Solamargine and solasonine containing fractions.

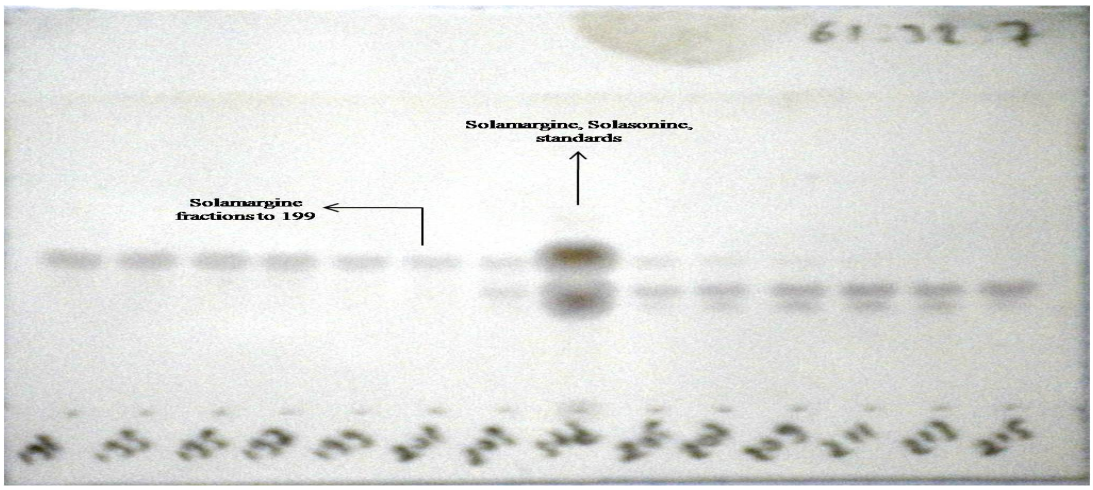


Figure 5.10.c. Solamargine and solasonine containing fractions.

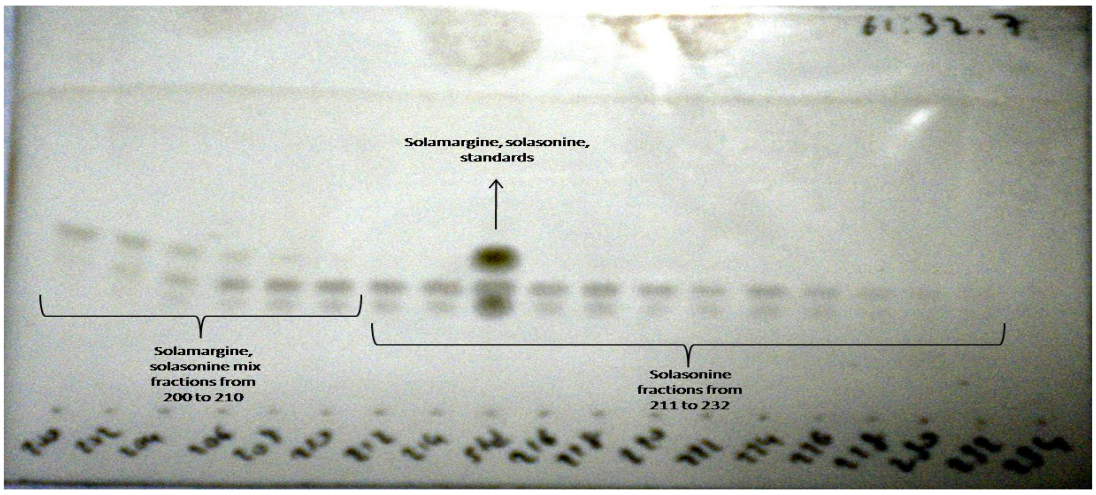


Figure 5.10.d. Solamargine and solasonine containing fractions.

Finally, solamargine was purified via Sephadex column (Figure 5.10 a,b).

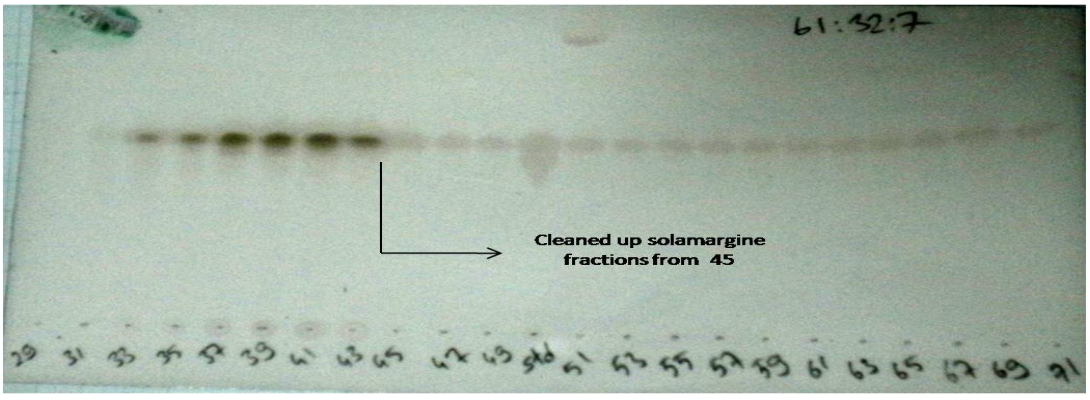


Figure 5.11.a. TLC of solamargine from Sephadex column.

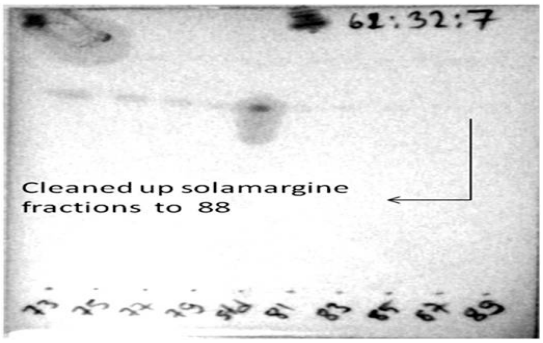


Figure 5.11.b. TLC of solamargine from sephadex column.

Isolated glycoalkaloids (solamargine and solasonine) were confirmed via TLC (Figure 5.11.), proton NMR and MS spectra were compared to literature values (Abouzied et al., 2008; Dinan et al., 2001; Nakamura et al., 2008; Wanyonyi et al., 2002; Veissenberg 2001 and Usubillage et al., 1996).

R_f values for solamargine:

Solamargine standard: R_f: 4.0/7.4= 0,540 cm

Purified solamargine: R_f: 4,1/7,5 = 0,546 cm

R_f values for solasonine:

Solasonine standard: R_f: 3,2 / 7.4= 0,432 cm

Purified solasonine: R_f: 3,1/7,3 = 0,426 cm

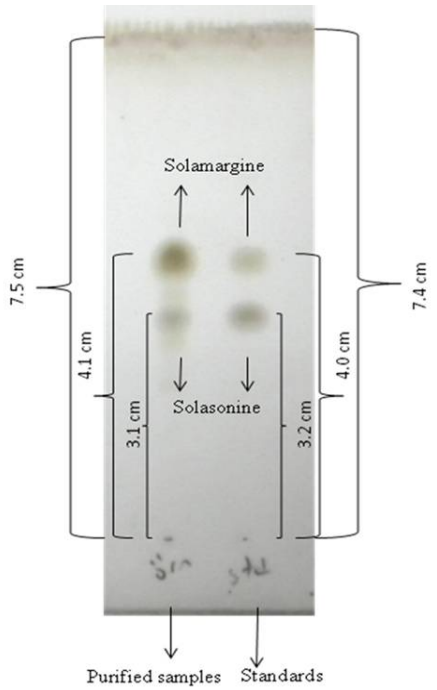


Figure 5.12. R_f calculation of standard and purified solamargine and solasonine.

Proton data of two compounds are given in Table 5.1. Also Figure 5.11 represents the solamargine NMR spectrum and Figure 5.12 represents solamargine NMR spectrum.

Solasonine (1) and solamargine (2) have been regarded as the common glycoalkaloidal constituents of Solanum species. These glycoalkaloids are both similar to theirmaglycone, solasodine. Compounds 1 and 2 were observed as major components in methanol extraction of *Solanum lineanum*. Both compounds contain a trisaccharidic sugar moiety: Compound 1 has α-L-rahmnose, β-D-glucose, β-D-galactose and compound 2 has α-L-rahmnose, β-D- rahmnose, β-D-glucose.

The ^{1}H NMR spectrum of 1 and 2 showed four tertiary methyl groups. Additionally, the resonances for three anomeric protons were observed at d 4.71 d, 6.56 d and 5.63 d for solamargine and 4.71 d, 6.68 d, and 6.24 d for solasonine. Thus, both compounds were considered to be a 22αN-spirosal-5-ene monoglycoside. Because of glycosylation, the H-3 signal was (for solasonine 3.90 ppm, 2.76 ppm, for solamargine 3.90 ppm, 2.44 ppm) obtained. In both molecules, H-16 is neighbor to oxygen , therefore its signal was obtained at the lower field. Also H-26 is neighbour to nitrogen, (for solamargine; 1.37 ppm, 1.39 ppm and for solasonine; 1.43 ppm, 1.56 ppm), therefore, its signal was obtained at the lower field.

Consequently, the structure of compound 1 has been established as (25 R)-3β-{O-α-L-rahmnopyranosyl-(1-2)-[O-β-D-glucopyranosyl-(1-3)]-β-D-galactopyranosyloxy}-22αN-spirosal-5-ene and compound 2 was established as (25 R)-3β-{O-α-L-rahmnopyranosyl-(1-2)-[O-α-L-rahmnopyranosyl-(1-4)]-β-D-glucopyranosyloxy}-22αN-spirosal-5-ene (Jensen et al. 2008).

Table 5.1.[1]H-NMR data of solomargine and solasonine

H	***Solasonine***	***Solomargine***
1	1.69, 1.10 ddd	1.93, 1.17
2	2.07, 1.94	2.06, 1.93
3	3.90 dddd, 2.76	3.90, 2.44
4	2.77, nd	2.79
5	-	-
6	4.97 brd	4.98
7	1.86, 1.42	1.83, 1.42
8	1.42	1.43
9	0.66	1.11
10	-	-
11	1.40, 1.42	1.40, 1.39
12	1.75, 1.10	1.75, 1.12
13	-	-
14	1.79, 2.29	1.80, 2.27
15	1.86- nd	1.83-nd
16	4.51	4.50
17	1.92	1.97
18	0.60	nd
19	0.56	1.17
20	2.07	2.26
21	nd	2.37
22	-	-
23	1.94, 2.12	1.98, 2.16
24	2.07, 1.95	2.06, nd
25	2.15	2.08
26	1.43, 1.56	1.37, 1,39
27	0.64	nd
1’	4.71	4.71
2’	4.62	4.68
3’	4.60	4.67
4’	4.34	4.31
5’	3.90	3.90
6’	3.94	4.22
1’’	6.68	6.56
2’’	4.73	4.74
3’’	4.81	4.76
4’’	4.85	4.88
5’’	4.97	4.91
6’’	2.45	2.44
1’’’	6.14	5.63
2’’’	4.84	4.76
3’’’	4.82	4.74
4’’’	4.74	4.71
5’’’	4.73	4.68
6’’’	4.60	4.6

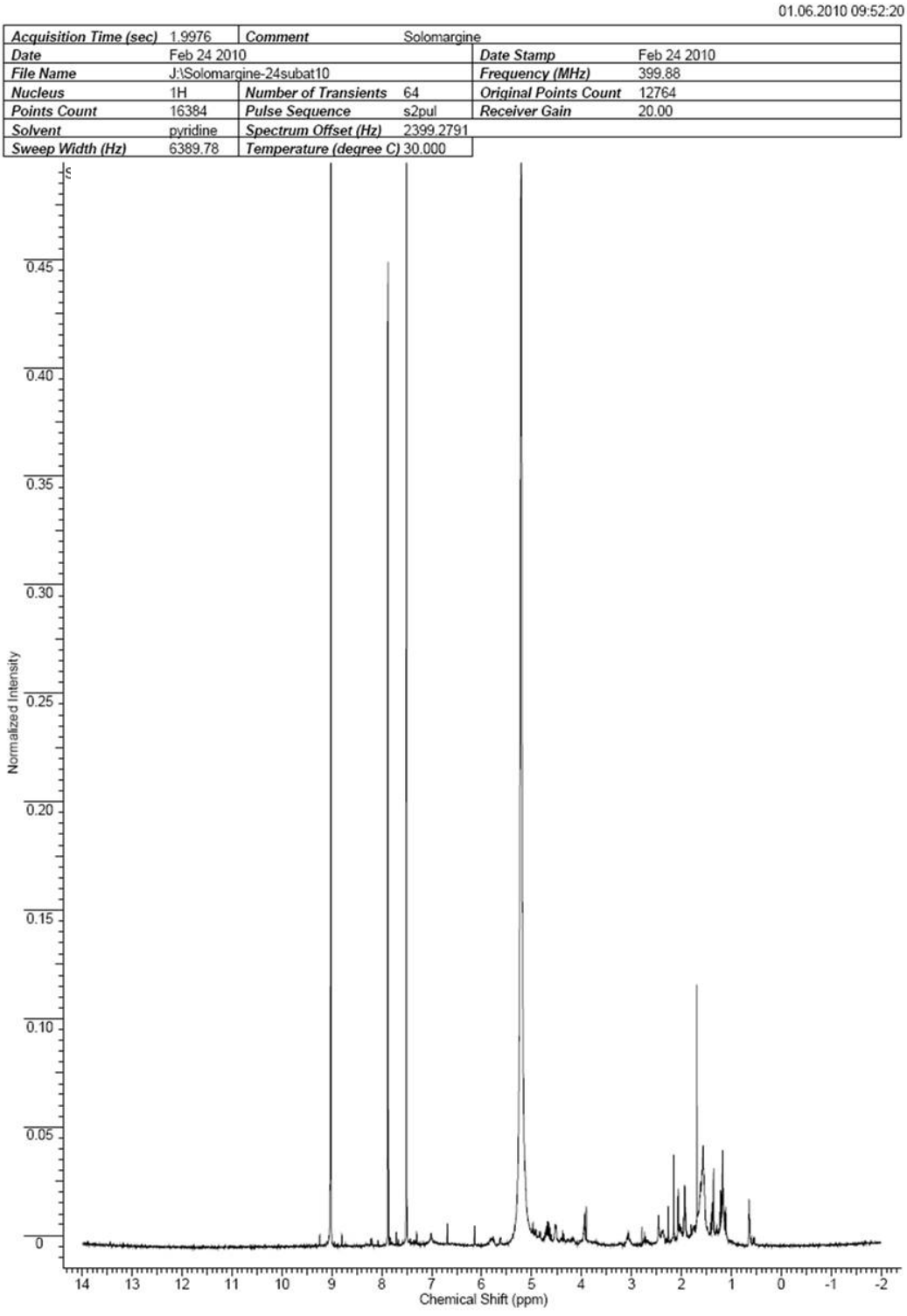


Figure 5.13. ^{1}H NMR spectrum of solamargine.

01.06.2010 09:53:16

Acquisition Time (sec)	1.9976	*Comment*	Solosonine		
Date	Feb 24 2010			*Date Stamp*	Feb 24 2010
File Name	J:\Solosonine-24subat10			*Frequency (MHz)*	399.88
Nucleus	1H	*Number of Transients*	16	*Original Points Count*	12764
Points Count	16384	*Pulse Sequence*	s2pul	*Receiver Gain*	20.00
Solvent	pyridine	*Spectrum Offset (Hz)*	2399.2791		
Sweep Width (Hz)	6389.78	*Temperature (degree C)*	30.000		

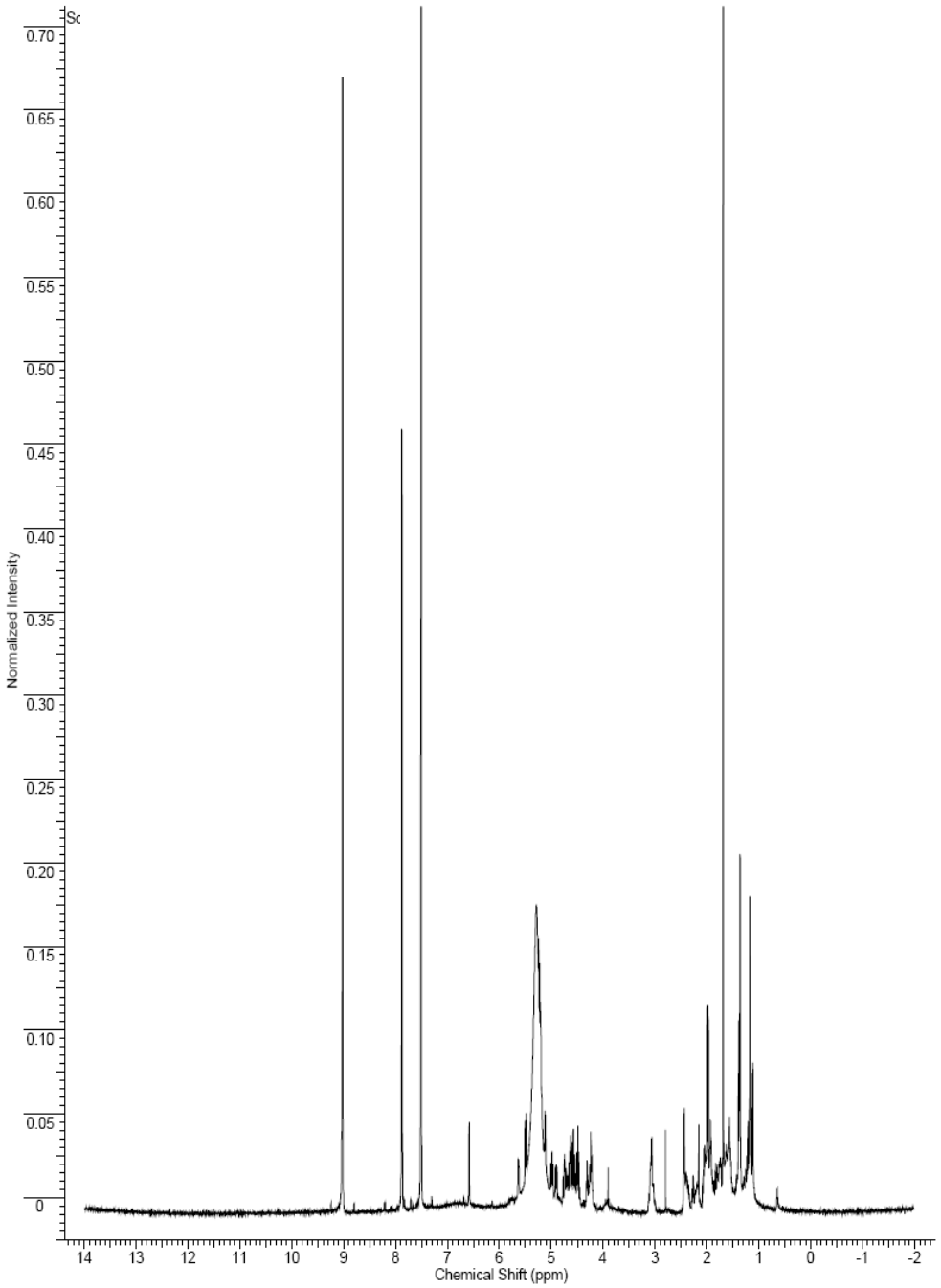


Figure 5.14.[1]H NMR spectrum of solasonine

The mass spectra of purified solamargine (Figure 5.15 and 5.16.) and solasonine (Figure 5.18) were compared to the mass spectra of solamargine (Figure 5.14) and solasonine (Figure 5.17) standards.

In the mass spectrum of purified solamargine $[M + H]^+$ ions at *m/z* 870 and 892. *m/z* 892 indicates binding of the sodium ion to the molecule ($[M + Na]^+$) from the MALDI matrix. When compared to the standard ($[M + H]^+$ ions at *m/z* 868), two additional attached protons were observed.In the mass spectra of both purified solasonine and solasonine standard at $[M + H]^+$ ions at *m/z* 884, this indicates that the purified compound was solasonine.

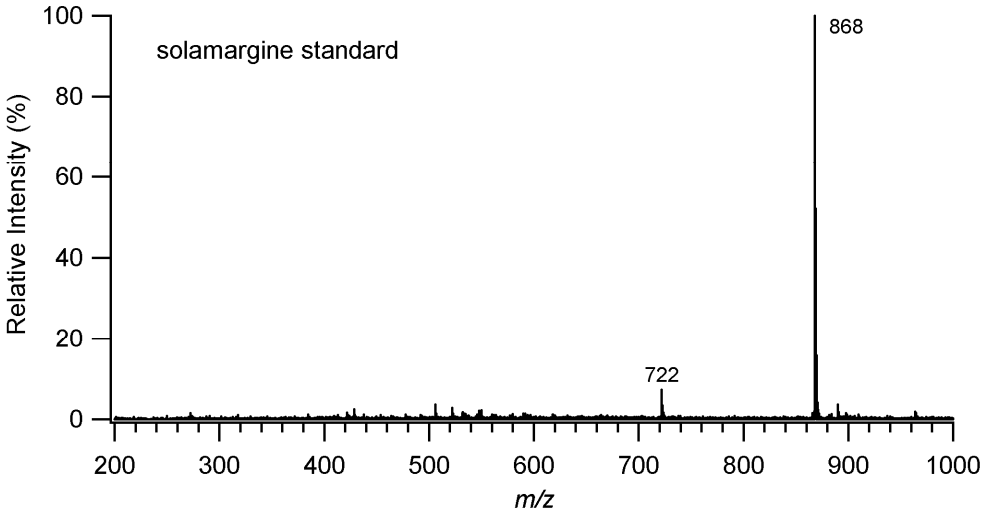


Figure 5.15. Mass spectrum of solamargine standard.

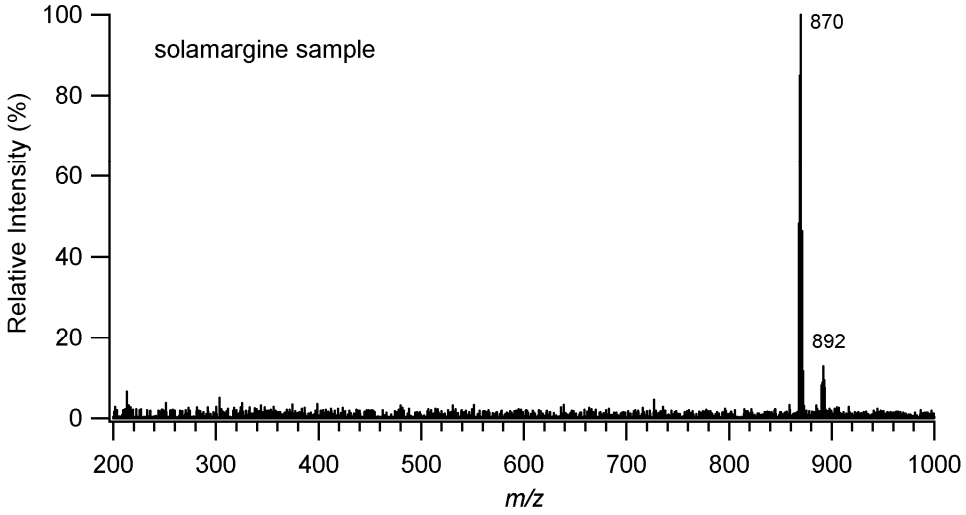


Figure 5.16. Mass spectrum of purified solamargine

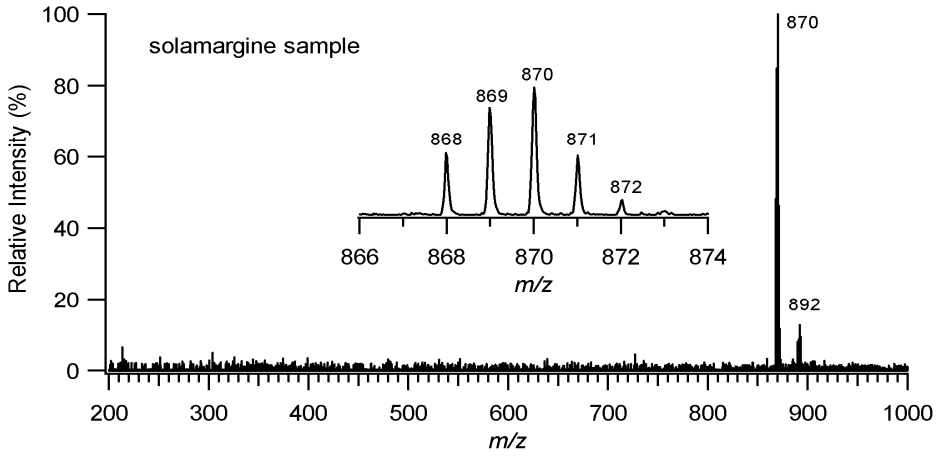


Figure 5.17. Mass spectrum of purified solamargine

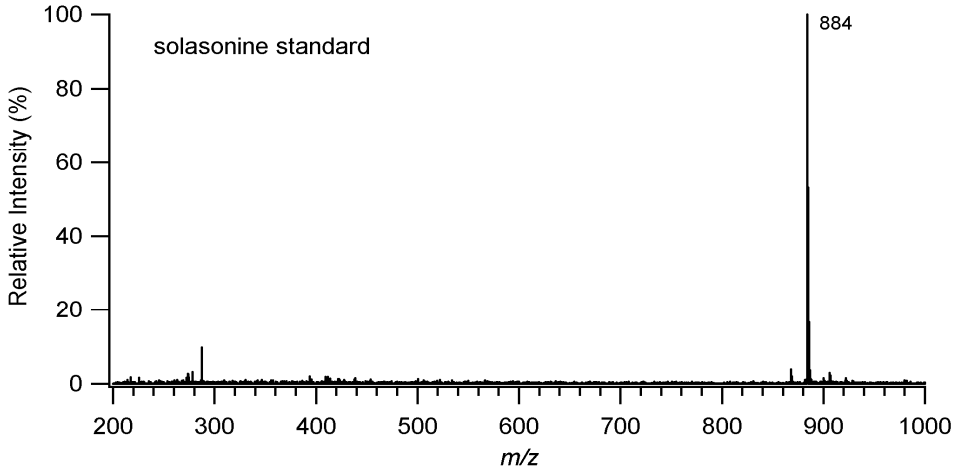


Figure 5.18. Mass spectrum of solasonine standard.

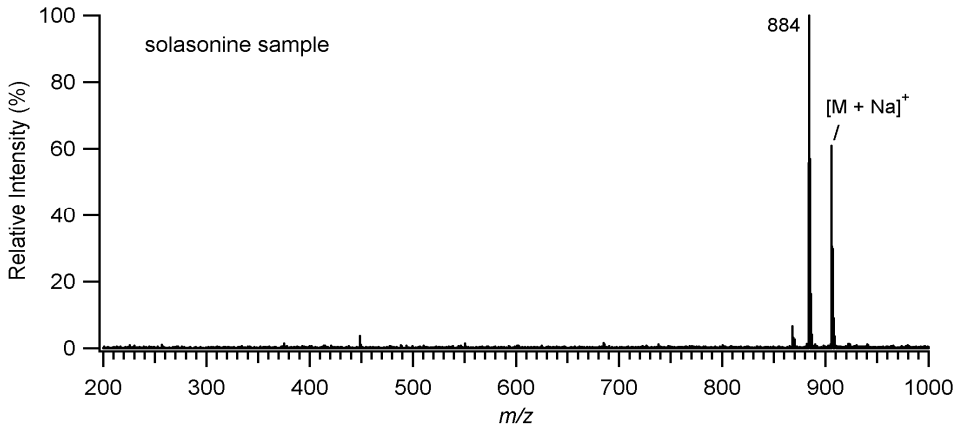


Figure 5.19. Mass spectrum of purified solasonine.

Based on the ^{1}H NMR and mass spectra data the chemical structure of purified solamargine and solasonine were drawn with ChemBioDraw ultra 11 software program (Figure 5.20).

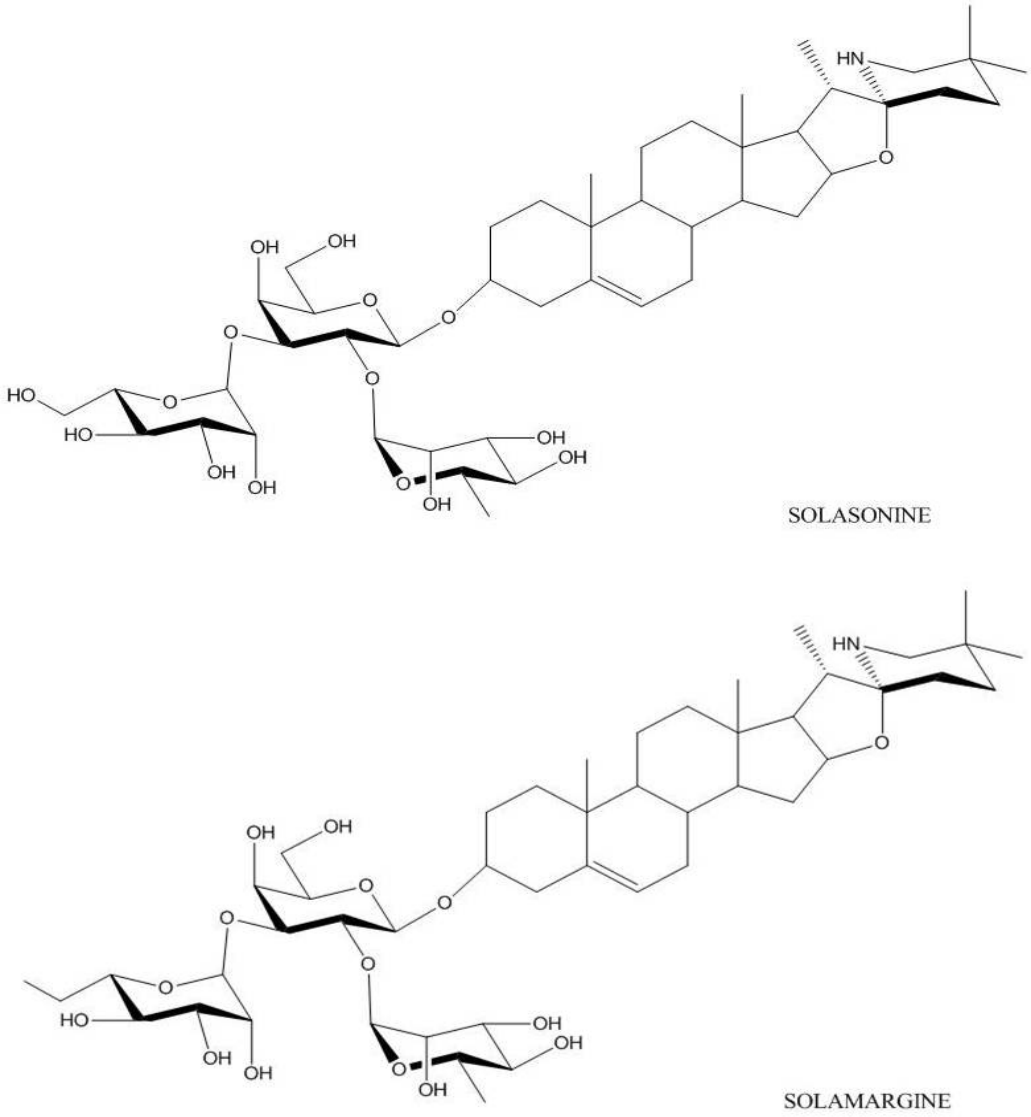


Figure 5.20. Chemical structure of solasonine and solamargine.

Weissenberg and his coworkers purified solamargine and solasonine from dried and ground berries of *S. khasianum* (150 g) via alumina column chromatography by using water and butanol as elution solvent followed by hot ethanol extraction. They obtained 7.8 g solamargine, 2.8 g solasonine, and also their mixture.

Usubillaga and his coworkers purified solamargine and solasonine from fresh berries of *S. sycophanta* via methanol extraction followed by acid extraction. They used also alumina column, and ethylacetate:methanol elution solvent system as a gradient. They obtained 50 mg of each compound.

Abouzid and his coworkers used ethanol for extraction of solanidine and chaconine, the other steroidal alkaloids found in the Solanaceae family, from dried berries of *S. distichum* (3,6 kg). They used silica column, and performed gradient

elution by using chloroform:methanol:ammonia, chloroform:ammonia, and methanol:water as elution solvent. At the end, they obtained 42 mg solanidine and 3 mg chaconine.

Wanyonyi and his coworkers purified solamargine and solasonine via HPLC following by column chromatography (for both, methanol:water was used gradiently as the elution solvent) after extraction with methanol from dried fruits of *S. lycocarpum* (5 kg). They obtained 133,6 mg solamargine, and 2,034 g solamargine and 11,62 mg solasonine.

In our work, we purified solamargine and solasonine via methanol extraction from dried fruits of *S. linnaeanum* (81,67 g) by using silica column chromatography, and chloroform:methano:water as elution solvent. Totally, we obtained 122,8 mg solamargine and solasonine.

In conclusion, Solanum species contain steroidal alkaloids at high levels. Glycoalkaloid extraction can be done by using ethanol or methanol, and they are well separated on both alumina and silica column. The species mentioned above except *S. sycophanta* contain higher levels of solamargine than *S. linnaeanum*. But, *S. linnaeanum* contains higher levels of solasonine than *S. lycocarpum* but not *S. khasianum*. On the other hand, the amount of the sample, maturation of the sample, and extraction and isolation procedures all affect the final amount of obtained purified compound .

5.3 Linkage Mapping

First, 385 COSII primers with different enzyme combinations were tested in parents to visualize polymorphism. Then 38 polymorphic primer and enzyme combinations were applied to mapping population. Table 5.2 represents the selected combinations which were then applied to the mapping population.

Table 5.2. Polymorphic primer and enzyme combinations.

Primers	*Enzymes*	*Primers*	*Enzymes*
At 1g 07080	*PCR polymorphic*	*At 3g 61140*	*BsuR l*
At 1g 14810	*BseG l*	*At 4g 00090*	*BsuR l*
At 1g 20575	*PCR polymorphic*	*At 4g 15530*	*BamH l*
At 1g 30110	*Pvul l*	*At 4g 31130*	*Tas ı*
At 1g 47830	*PCR polymorphic*	*At 4g 38630*	*Dra l*
At 1g 48300	*EcoR l*	*At 5 g 41480*	*Tas ı*
At 1g 53670	*PCR polymorphic*	*At 5g 07910*	*BsuR l*
At 2g 03120	*PCR polymorphic*	*At 5g 11480*	*BamH l*
At 2g 15890	*Csp6l*	*At 5g 11490*	*Dra l*
At 2g 238020	*Bme 1390 l*	*At 5g 12370*	*Dra l*
At 2g 24270	*PCR polymorphic*	*At 5g 13700*	*Csp6l*
At 2g 40760	*Pvul l*	*At 5g 23880*	*Rsa l*
At 3g 12300	*Pst l*	*At 5g 41040*	*Ban l*
At 3g 23590	*Hinf l*	*At 5g 44250*	*Rsa l*
At 3g 25120	*Hinf l*	*At 5g 54080*	*Pst l*
At 3g 44880	*Bme 1390 l*	*At 5g 61410*	*Pst l*
At 3g 47930	*Hinf l*	*At 5g 62390*	*Hinf l*
At 3g 57270	*BsuR l*	*At 5g 66090*	*Rsa l*
At 3g 52220	*BsuR l*	*At 5g 64730*	*PCR polymorphic*
At 1g 07080	*PCR polymorphic*	*At 3g 61140*	*BsuR l*
At 1g 14810	*BseG l*	*At 4g 00090*	*BsuR l*
At 1g 20575	*PCR polymorphic*	*At 4g 15530*	*BamH l*
At 1g 30110	*Pvul l*	*At 4g 31130*	*Tas ı*
At 1g 47830	*PCR polymorphic*	*At 4g 38630*	*Dra l*
At 1g 48300	*EcoR l*	*At 5 g 41480*	*Tas ı*
At 1g 53670	*PCR polymorphic*	*At 5g 07910*	*BsuR l*
At 2g 03120	*PCR polymorphic*	*At 5g 11480*	*BamH l*
At 2g 15890	*Csp6l*	*At 5g 11490*	*Dra l*
At 2g 238020	*Bme 1390 l*	*At 5g 12370*	*Dra l*
At 2g 24270	*PCR polymorphic*	*At 5g 13700*	*Csp6l*
At 2g 40760	*Pvul l*	*At 5g 23880*	*Rsa l*
At 3g 12300	*Pst l*	*At 5g 41040*	*Ban l*
At 3g 23590	*Hinf l*	*At 5g 44250*	*Rsa l*
At 3g 25120	*Hinf l*	*At 5g 54080*	*Pst l*
At 3g 44880	*Bme 1390 l*	*At 5g 61410*	*Pst l*
At 3g 47930	*Hinf l*	*At 5g 62390*	*Hinf l*
At 3g 57270	*BsuR l*	*At 5g 66090*	*Rsa l*
At 3g 52220	*BsuR l*	*At 5g 64730*	*PCR polymorphic*

Table 5.3. Presents enzyme's restriction sites which were used in CAPs method

(Source: Genscript 2010)

Enzyme	***Restriction Site***	***Enzyme***	***Restriction Site***
Alu l	AG^CT	*Hha I*	GCG^C
Alw21 I	GWGCW^C	*Hin6 l*	G^CGC
Apa l	GGGCC^C	*Hinc II*	GTY^RAC
BamH l	G^GATCC	*Hind III*	A^AGCTT
Ban I	G^GYRCC	*Hinf I*	G^ANTC
Bcl I	T^GATCA	*Kpn I*	GGTAC^C
Bgl ll	GCCNNNN^NGGC	*Mph 1103 l*	ATGCA^T
Bme 1390 l	CC^NGG	*Msp I*	C^CGG
Box l	GACNN^NNGTC	*Mval*	CC^WGG
BseD l	C^CNNGG	*NmuC l*	^GTSAC
BseG l	GGATGNN^	*Pst l*	CTGCA^G
BspT l	C^TTAAG	*Pvu l*	CGAT^CG
BsuR l	GG^CC	*Rsa l*	GT^AC
Cfo I	GCG^C	*Ssp l*	AAT^ATT
Csp6 I	G^TAC	*Tas l*	^AATT
Dpn I	GA^TC	*Taq I*	T^CGA
Dpn ll	^GATC	*Tru1 l*	T^TAA
Dra l	TTT^AAA	*Xap l*	R^AATTY
EcoR I	G^AATTC	*Xba I*	T^CTAGA
EcoR V	GAT^ATC	*Xmil*	GT^MKAC
Alu l	AG^CT	*Hha I*	GCG^C
Alw21 I	GWGCW^C	*Hin6 l*	G^CGC
Apa l	GGGCC^C	*Hinc II*	GTY^RAC
BamH l	G^GATCC	*Hind III*	A^AGCTT
Ban I	G^GYRCC	*Hinf I*	G^ANTC
Bcl I	T^GATCA	*Kpn I*	GGTAC^C
Bgl ll	GCCNNNN^NGGC	*Mph 1103 l*	ATGCA^T
Bme 1390 l	CC^NGG	*Msp I*	C^CGG
Box l	GACNN^NNGTC	*Mval*	CC^WGG
BseD l	C^CNNGG	*NmuC l*	^GTSAC
BseG l	GGATGNN^	*Pst l*	CTGCA^G
BspT l	C^TTAAG	*Pvu l*	CGAT^CG
BsuR l	GG^CC	*Rsa l*	GT^AC
Cfo I	GCG^C	*Ssp l*	AAT^ATT
Csp6 I	G^TAC	*Tas l*	^AATT
Dpn I	GA^TC	*Taq I*	T^CGA
Dpn ll	^GATC	*Tru1 l*	T^TAA
Dra l	TTT^AAA	*Xap l*	R^AATTY
EcoR I	G^AATTC	*Xba I*	T^CTAGA
EcoR V	GAT^ATC	*Xmil*	GT^MKAC

For survey by using SSR method, totally 221 SSR markers including 49 eggplant SSR markers (Table 5.4), 23 pepper SSR markers (Table 5.5), 88 tomato SSR markers (Table 5.6)and 61 *L. hirsutum* SSR markers (Table 5.7) were checked at both 50 ^{O}C and 55 ^{O}C annealing temperature. From these 221 SSR markers 28 of them showed polymorphism (Table 5.8).

For eggplant SSR markers; about 94% of them amplified products but none of them showed polymorphism. For pepper SSR markers; about 87% of them amplified and about 43% of them showed polymorphism. For tomato SSR markers, about 30% of them amplified products and about 16% of them were found polymorphic. Finally, 22% of *L. hirsutum* SSR markers amplified and about 11% of them were found polymorphic.

Table 5.4. List of tested eggplant SSR markers**.**

Primer	***Annealing Temperature***	***Polymorphism***
SM SSR 1	50^{o}C	Not Polymorphic
SM SSR 2	50^{o}C	Not Polymorphic
SM SSR 3	50^{o}C	Not Polymorphic
SM SSR 4	50^{o}C	Not Polymorphic
SM SSR 5	50^{o}C	Not Polymorphic
SM SSR 6	50^{o}C	Not Polymorphic
SM SSR 7	Not Amplified	
SM SSR 8	Not Amplified	
SM SSR 9	50^{o}C	Not Polymorphic
SM SSR 10	50^{o}C	Not Polymorphic
SM SSR 11	50^{o}C	Not Polymorphic
SM SSR 12	50^{o}C	Not Polymorphic
SM SSR 13	55^{o}C	Not Polymorphic
SM SSR 14	50^{o}C	Not Polymorphic
SM SSR 15	50^{o}C	Not Polymorphic
SM SSR 16	50^{o}C	Not Polymorphic
SM SSR 17	50^{o}C	Not Polymorphic
SM SSR 18	50^{o}C	Not Polymorphic
SM SSR 19	50^{o}C	Not Polymorphic
SM SSR 20	50^{o}C	Not Polymorphic
SM SSR 21	50^{o}C	Not Polymorphic

(Cont. on next page)

Table 5.4. (Cont.)

Primer	***Annealing Temperature***	***Polymorphism***
SM SSR 22	50°C	Not Polymorphic
SM SSR 23	50°C	Not Polymorphic
SM SSR 24	50°C	Not Polymorphic
SM SSR 25	50°C	Not Polymorphic
SM SSR 26	50°C	Not Polymorphic
SM SSR 27	50°C	Not Polymorphic
SM SSR 29	50°C	Not Polymorphic
SM SSR 30	50°C	Not Polymorphic
SM SSR 31	50°C	Not Polymorphic
SM SSR 32	Not Amplified	
SM SSR 33	50°C	Not Polymorphic
SM SSR 34	50°C	Not Polymorphic
SM SSR 35	50°C	Not Polymorphic
SM SSR 36	50°C	Not Polymorphic
SM SSR 37	50°C	Not Polymorphic
SM SSR 38	50°C	Not Polymorphic
SM SSR 39	50°C	Not Polymorphic
SM SSR 40	50°C	Not Polymorphic
SM SSR 41	50°C	Not Polymorphic
SM SSR 42	50°C	Not Polymorphic
SM SSR 43	55°C	Not Polymorphic
SM SSR 44	50°C	Not Polymorphic
SM SSR 45	50°C	Not Polymorphic
SM SSR 46	50°C	Not Polymorphic
SM SSR 47	50°C	Not Polymorphic
SM SSR 48	50°C	Not Polymorphic
SM SSR 49	50°C	Not Polymorphic
SM SSR 50	50°C	Not Polymorphic

Table 5.5. List of tested pepper SSR markers.

Primer	***Annealing Temperature***	***Polymorphism***
EM 104	Not Amplified	
EM 107	50°C	Not Polymorphic
EM 114	50°C	Polymorphic
EM 116	Not Amplified	
EM 117	55°C	Not Polymorphic
EM 119	50°C	Not Polymorphic
EM 120	50°C	Polymorphic
EM 126	50°C	Not Polymorphic
EM 127	50°C	Not Polymorphic
EM 128	50°C	Not Polymorphic
EM 131	50°C	Polymorphic
EM 133	50°C	Polymorphic
EM 134	50°C	Not Polymorphic
EM 135	50°C	Polymorphic
EM 139	55°C	Not Polymorphic
EM 140	55°C	Polymorphic
EM 141	55°C	Polymorphic
EM 145	50°C	Polymorphic
EM 146	Not Amplified	
EM 151	50°C	Polymorphic
EM 155	50°C	Polymorphic
EM 157	50°C	Not Polymorphic
EM 162	50°C	Not Polymorphic

Table 5.6. List of tested tomato SSR markers.

Primer	***Annealing Temperature***	***Polymorphism***
SSR 4	Not Amplified	
SSR 5	Not Amplified	
SSR 11	50°C	Polymorphic
SSR 14	55°C	Not Polymorphic
SSR 19	Not Amplified	
SSR 20	50°C	Polymorphic
SSR 22	55°C	Not Polymorphic
SSR 26	55°C	Not Polymorphic
SSR 27	Not Amplified	
SSR 32	55°C	Not Polymorphic
SSR 34	Not Amplified	
SSR 38	Not Amplified	
SSR 40	Not Amplified	
SSR 43	Not Amplified	
SSR 44	Not Amplified	
SSR 45	55°C	Not Polymorphic
SSR 46	55°C	Polymorphic
SSR 47	Not Amplified	
SSR 51	Not Amplified	
SSR 52	Not Amplified	
SSR 63	50°C	Not Polymorphic
SSR 65	50°C	Not Polymorphic
SSR 66	55°C	Not Polymorphic
SSR 67	Not Amplified	
SSR 69	Not Amplified	
SSR 70	55°C	Polymorphic
SSR 74	55°C	Not Polymorphic
SSR 76	Not Amplified	
SSR 80	50°C	Polymorphic
SSR 85	Not Amplified	
SSR 96	Not Amplified	
SSR 105	Not Amplified	
SSR 110	Not Amplified	
SSR 111	55°C	polymorphic
SSR 115	Not Amplified	
SSR 117	50°C	Not Polymorphic
SSR 124	Not Amplified	
SSR 128	Not Amplified	

(Cont. on next page)

Table 5.6. (Cont.).

Primer	*Annealing Temperature*	*Polymorphism*
SSR 134	Not Amplified	
SSR 135	Not Amplified	
SSR 136	Not Amplified	
SSR 146	Not Amplified	
SSR 150	Not Amplified	
SSR 152	Not Amplified	
SSR 155	Not Amplified	
SSR 156	Not Amplified	
SSR 162	Not Amplified	
SSR 188	Not Amplified	
SSR 192	Not Amplified	
SSR 218	Not Amplified	
SSR 222	Not Amplified	
SSR 223	55^{o}C	Polymorphic
SSR 231	Not Amplified	
SSR 237	Not Amplified	
SSR 241	Not Amplified	
SSR 244	55^{o}C	Polymorphic
SSR 248	50^{o}C	Not Polymorphic
SSR 270	55^{o}C	Not Polymorphic
SSR 276	Not Amplified	
SSR 285	Not Amplified	
SSR 286	Not Amplified	
SSR 300	Not Amplified	
SSR 301	Not Amplified	
SSR 306	55^{o}C	Polymorphic
SSR 308	Not Amplified	
SSR 310	55^{o}C	Not Polymorphic
SSR 311	Not Amplified	
SSR 320	Not Amplified	
SSR 327	Not Amplified	
SSR 333	Not Amplified	
SSR 335	Not Amplified	
SSR 344	Not Amplified	
SSR 349	55^{o}C	Polymorphic
SSR 350	50^{o}C	Not Polymorphic
SSR 356	Not Amplified	

Table 5.6. (Cont.).

Primer	***Annealing Temperature***	***Polymorphism***
SSR 383	Not Amplified	
SSR 450	55^{o}C	Polymorphic
SSR 478	Not Amplified	
SSR 479	Not Amplified	
SSR 557	Not Amplified	
SSR 578	55^{o}C	Not Polymorphic
SSR 586	55^{o}C	Not Polymorphic
SSR 590	Not Amplified	
SSR 593	Not Amplified	
SSR 594	Not Amplified	
SSR 603	Not Amplified	
SSR 605	Not Amplified	
SSR 638	Not Amplified	

Table 5.7. List of tested *Hirsutum* SSR marker**.**

Primer	***Annealing Temperature***	***Polymorphism***
shSSR 7	Not Amplified	
shSSR 21	55^{o}C	Polymorphic
shSSR 23	Not Amplified	
shSSR 33	Not Amplified	
SH 033	55^{o}C	Not Polymorphic
shSSR 36	Not Amplified	
shSSR 37	Not Amplified	
shSSR 42	55^{o}C	Polymorphic
shSSR 46	Not Amplified	
shSSR 50	Not Amplified	
shSSR 55	Not Amplified	
SH 056	50^{o}C	Not Polymorphic
shSSR 61	50^{o}C	Not Polymorphic
SH 62	Not Amplified	
SH 064	Not Amplified	
shSSR 67	Not Amplified	
shSSR 71	Not Amplified	
shSSR 74	50^{o}C	Polymorphic
shSSR 79	Not Amplified	
shSSR 84	Not Amplified	

(Cont. on next page)

Table 5.7. (Cont.).

Primer	***Annealing Temperature***	***Polymorphism***
shSSR 88	Not Amplified	
shSSR 90	Not Amplified	
shSSR 92	Not Amplified	
shSSR 94	Not Amplified	
shSSR 101	Not Amplified	
shSSR 138	50°C	Not Polymorphic
shSSR 286	Not Amplified	
shSSR 290	Not Amplified	
shSSR 291	Not Amplified	
shSSR 299	Not Amplified	
shSSR 317	Not Amplified	
shSSR 353	Not Amplified	
shSSR 397	Not Amplified	
shSSR 401	Not Amplified	
shSSR 460	Not Amplified	
shSSR 472	Not Amplified	
shSSR 480	Not Amplified	
shSSR 482	Not Amplified	
shSSR 488	Not Amplified	
SSR 17	Not Amplified	
SSR 39	50°C	Polymorphic
SSR 44	Not Amplified	
SSR 56	Not Amplified	
SSR 68	Not Amplified	
SSR 95	Not Amplified	
SSR 100	55°C	Polymorphic
SSR 258	50°C	Polymorphic
SSR 265	Not Amplified	
SSR 273	55°C	Not Polymorphic
SSR 283	Not Amplified	
SSR 288	55°C	Not Polymorphic
SSR 387	55°C	Not Polymorphic
SRR 438	Not Amplified	
SSR 439	Not Amplified	
SSR 440	Not Amplified	
SSR 454	Not Amplified	
SSR 455	55°C	Polymorphic
SSR 463	Not Amplified	
SSR 468	Not Amplified	
SSR 471	Not Amplified	
SSR 478	Not Amplified	

Table 5.8. List of polymorphic SSR markers.

Primer	***Annealing Temperature***
EM 114	50 ^{0}C
EM 120	50 ^{0}C
EM 131	50 ^{0}C
EM 133	50 ^{0}C
EM 135	50 ^{0}C
EM 140	55 ^{0}C
EM 141	55 ^{0}C
EM 145	50 ^{0}C
EM 151	50 ^{0}C
EM 155	50 ^{0}C
SSR 11	50 ^{0}C
SSR 20	50 ^{0}C
SSR 46	55 ^{0}C
SSR 70	55 ^{0}C
SSR 80	50 ^{0}C
SSR 111	55 ^{0}C
SSR 223	55 ^{0}C
SSR 244	55 ^{0}C
SSR 306	55 ^{0}C
SSR 349	55 ^{0}C
SSR 450	55 ^{0}C
shSSR 21	55 ^{0}C
shSSR 42	55 ^{0}C
shSSR 74	50 ^{0}C
SSR 39	50 ^{0}C
SSR 100	55 ^{0}C
SSR 258	50 ^{0}C
SSR 455	55 ^{0}C

For SRAP analysis, 182 combinations of EM and ME primers were checked for polymorphism; 42% of these combinations amplified products in both parents and 24% of these combinations showed dominant or codominant polymorphism (Table 5.9). In conclusion, pepper SSR markers showed the highest polymorphism while eggplant SSR markers did not show any polymorphism although they had the highest ratio of successful amplification.

Table 5.9. List of tested SRAP markers for polymorphism on parents.

	ME 01	ME 02	ME 03	ME 04	ME 05	ME 06	ME 07	ME 08	ME 09	ME 11	ME 12	ME 13	ME 14
EM 01	—	RP —	P	P	—	P	—	P—	P—	DP —	P—	P—	—
EM 02	RP —	—	DP —	P	RP —	—	—	P	P—	P	P	P	P—
EM 04	P	P	P	P	RP —	P	P	P	—	—	P—	RP —	DP —
EM 05	—	—	RP —	P—	—	—	P—	P—	DP —	—	—	—	—
EM 06	RP —	RP —	RP —	RP —	RP —	P	—	—	—	DP —	—	RP —	P
EM 09	—	P	P	P—	RP —	P	—	RP —	P	DP —	—	P—	P
EM 10	DP—	P	P	RP —	—	—	RP —	—	—	—	P—	P—	—
EM 11	—	—	—	—	—	RP —	—	—	DP—	—	—	RP —	P—
EM 12	—	—	P—	—	P—	P—	—	P	P	P	P—	P—	P
EM 13	P—	P—	—	DP —	—	RP —	—	RP —	RP —	P—	P—	P	—
EM 14	—	P	P—	P	P	P	DP —	—	P—	RP —	P—	P	P
EM 15	P	—	P—	P—	P	P—	—	RP —	—	P—	P	—	RP —
EM 16	P	—	RP —	RP —	P	P—	RP —	DP —	P	P	P	P	P—
EM 17	RP —	RP —	RP —	—	P—	P—	P—	—	—	RP —	—	DP —	DP —

—: Not amplified on both parents.

RP — : Not amplified on recurrent parent.

DP — : Not amplified on donor parent.

P— : Amplified on both parents but not polymorphic.

P : Amplified on both parents and shows polymorphism.

In conclusion 385 COS ll markers and 221 SSR markers were checked for polymorphism. Totally, 110 markers including 38 COS ll markers, 28 SSR markers and 44 SRAP markers combinations showed dominant and codominant polymorphism.Of these polymorphic markers, 52 COS II markers and SSR markers were mapped (Table 5.10).

To construct the map, MAPMAKER computer programme was used with linkage groups at min LOD 3.0, max Distance 50.0 and 3 linkage groups were found including group one consisting of 34 markers (Table 5.11), group two consisting of 9 markers (Table 5.12), group 3 consisting of 2 markers which were 4g 3863 and EM 120.Markers At 1g 4783 and SSR 455 were found to be unlinked to the other markers. To find chromosome numbers markers were compared with the other eggplant maps.

Table 5.10. List of mapped markers.

Primers	***Primers***
At 1g 0708	At 5g 1149
At 1g 1481	At 5g 1237
At 1g 2057	At 5g 1370
At 1g 3011	At 5g 2388
At 1g 4783	At 5g 4104
At 1g 4830	At 5g 4148
At 1g 5367	At 5g 4425
At 2g 0312	At 5g 5408
At 2g 1589	At 5g 6141
At 2g 2427	At 5g 6239
At 2g 3802	At 5g 6473
At 2g 4076	At 5g 6609
At 3g 1230	EM 114
At 3g 2359	EM 115
At 3g 2512	EM 120
At 3g 4488	EM 131
At 3g 4793	EM 133
At 3g 5222	EM 135
At 3g 5727	EM 140
At 3g 6114	EM 145
At 4g 0009	EM 151
At 4g 1553	shSSR 74
At 4g 3113	SSR11
At 4g 3863	SSR 20
At 5g 0791	SSR 100
At 5g 1148	SSR 223

Table 5. 11. Markers in linkage group 1.

Group I		
Markers	***Distance***	***Chromosome number***
37 At 5g 6473	11.6 cM	11
18 At 3g 5222	39.1 cM	Not Mapped
39 EM 133	5.8 cM	Not Mapped
48 shSSR 74	0.0 cM	Not Mapped
41 EM 114	0.0 cM	Not Mapped
43 EM 151	0.0 cM	Not Mapped
27 At 5g 1149	0.0 cM	Not Mapped
6 At 1g 4830	0.0 cM	5
7 At 1g 5367	0.0 cM	7
4 At 1g 3011	4.2 cM	10
26 At 5g 1148	12.1 cM	Not Mapped
52 SSR 20	6.0 cM	Not Mapped
12 At 2g 4076	32.2 cM	Not Mapped
11 At 2g 3802	20.0 cM	Not Mapped
13 At 3g 1230	19.6 cM	Not Mapped
19 At 3g 5727	2.9 cM	10
17 At 3g 4793	43.1 cM	Not Mapped
29 At 5g 1370	34.1 cM	3
28 At 5g 1237	1.5 cM	Not Mapped
35 At 5g 6141	0.0 cM	Not Mapped
38 At 5g 6609	0.0 cM	Not Mapped
44 EM 135	0.0 cM	Not Mapped
47 SSR 100	0.0 cM	Not Mapped
49 SSR 223	0.0 cM	Not Mapped
33 At 5g 4425	0.0 cM	Not Mapped
23 At 4g 3113	45.0 cM	Not Mapped
2 At 1g 1481	49.9 cM	1
14 At 3g 2359	47.2 cM	1
20 At 3g 6114	37.7 cM	Not Mapped
9 At 2g 1589	42.7 cM	Not Mapped
1 At 1g 0708	19.6 cM	Not Mapped
15 At 3g 2512	49.6 cM	Not Mapped
45 EM 131	34.5 cM	Not Mapped
10 At 2g 2427	----------	Not Mapped
558.9 cM 34 markers log-likelihood= -1220.46		

Table 5.12. Markers in linkage group 2.

Group 2		
Markers	***Distance***	***Chromosome number***
36 At5g6239	0.0 cM	3
22 At4g1553	10.4 cM	Not Mapped
53 ssr11	34.9 cM	Not Mapped
51 em140	0.0 cM	Not Mapped
21 At4g0009	0.8 cM	Not Mapped
8 At2g0312	0.5 cM	Not Mapped
3 At1g2057	0.6 cM	Not Mapped
34 At5g5408	1.5 cM	Not Mapped
31 At5g4104	----------	Not Mapped
48.8 cM 9 markers log-likelihood= -129.96		

CHAPTER 6

CONCLUSION

Glycoalkaloids are highly important secondary metabolites found in the *Apocynaceae, Buxaceae*, *Solanaceae*, and *Liliaceae* families, because of both their benefits and detriments. The goals of this study were to isolate glycoalkaloids which are not commercially avaliable, and develop an HPLC method to determine glycoalkaloid content, and to construct a linkage map for further works. Two of the eggplant glycoalkaloids, solamargine and solasonine, were purified by using column chromatography tecniques and verified by comparing to literature- based, TLC, ^{1}H NMR, and mass spectroscopy data. To develop an HPLC method different mobile phases, temperatures, pH, flow rates and columns were tested. Both RP column and carbohydrate column have advantages and disadvantages. RP columns are more stable than carbohydrate columns. Eggplant glycoalkaloids can be separated using a carbohydrate column but it is not reproducible because of the stability problem. On the other hand, a good separation cannot be obtained by using an RP column without using phosphate buffer. Phosphate buffer is a problem when it is used with ELSD detector or in LC/MS. ELSD detector provides better results. Further work on methods these method is warranted

For linkage mapping in eggplant, 38 polymorphic COSII primers and 28 polymorphic SSR primers were found and applied to the mapping population. When the map was constructed, only 3 linkage group was found. This does not represent a good map. Therefore another marker technique should be tested and the map should be reconstructed. In future work, QTLs for glycoalkaloid can be identified based on this linkage mapping.

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APPENDICES

Apendix A. Methods which were tested for HPLC method development

Method 1	
Mobile Phase	H_2O - 69,5 % ACN - 30 % HCOOH - 0,5 %
pH (for mobil phase)	not adjusted
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50ppm solasodine
Solvent for standards	ACN:H2O (1:1) (v:v) + 1drop of 85 % H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 2	
Mobile Phase	H_2O - 800 mL ACN - 200 mL HCOOH - 1,42 mL NaOH - 3,298 mL
pH (for mobil phase)	3,72
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100ppm and 200ppm solasodine
Solvent for standards	69,5% H_2O +30% ACN+0,5 % HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 3	
Mobile Phase	H_2O - 800 mL ACN - 200 mL HCOOH - 1,42 mL NaOH - 3,298 mL
pH (for mobil phase)	3,72
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100ppm and 200ppm solasodine
Solvent for standards	Same as mobil phase + 1 Drop H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 4	
Mobile Phase	H_2O - 800 mL ACN - 200 mL HCOOH - 1,42 mL NaOH - 3,298 mL
pH (for mobil phase)	3,72
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100ppm and 200ppm solasodine
Solvent for standards	MeOH + 5% HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 5	
Mobile Phase	H_2O - 800 mL ACN - 200 mL HCOOH - 1,42 mL NaOH - 3,298 mL
pH (for mobil phase)	3,72
Flow rate(mL/min)	0,1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm, 100ppm and 200ppm solasodine
Solvent for standards	MeOH + 5% HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 6	
Mobile Phase	H_2O - 800 mL ACN - 200 mL HCOOH - 1,42 mL NaOH - 3,298 mL
pH (for mobil phase)	3,72
Flow rate(mL/min)	0,5
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100ppm solasodine
Solvent for standards	MeOH + 5% HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 7	
Mobile Phase	H_2O - 450 mL ACN - 50 mL HCOOH – 0,71 mL NaOH – 1,649 mL
pH (for mobil phase)	3,72
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	200ppm solasodine
Solvent for standards	MeOH + 5% HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 8	
Mobile Phase	Pump A: 0.01M $NH_4H_2PO_4$ pH: 2,5 (0,1M 85% H_3PO_4) Pump B: 100% ACN 40 % from pump A, 60% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm, 100ppm and 200ppm solasodine
Solvent for standards	MeOH + 5% HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 9	
Mobile Phase	Pump A: 0.01M $NH_4H_2PO_4$ pH: 2,5 (0,1M 85% H_3PO_4) Pump B: 100% ACN 40 % from pump A, 60% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 20 ppm, 30 ppm, 50 ppm, 100ppm and 200ppm solasodine
Solvent for standards	ACN:H2O (1:1) (v:v) + 1drop of 85 % H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 10	
Mobile Phase	Pump A: 0.01M $NH_4H_2PO_4$ pH: 2,5 (0,1M 85% H_3PO_4) Pump B: 100% ACN
	70 % from pump A, 30% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 20ppm and 30ppm solasodine
Solvent for standards	ACN:H2O (1:1) (v:v) + 1drop of 85 % H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 11	
Mobile Phase	Pump A: 0.01M $NH_4H_2PO_4$ pH: 2,5 (0,1M 85% H_3PO_4) Pump B: 100% ACN
	75 % from pump A, 25% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 30ppm and 1000ppm solasodine
Solvent for standards	880 µl H2O and 220 µl H_3PO_4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 12	
Mobile Phase	Pump A: 0.01M NH4H2PO4 pH: 2,5 (0,1M 85% H3PO4) Pump B: 100% ACN
	75 % from pump A, 25% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 30ppm and 1000ppm solasodine
Solvent for standards	500 µl H2O + 500 µl MeOH + 2 drops of 85 % H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 13	
Mobile Phase	Pump A: 0.01M $NH_4H_2PO_4$ pH: 2,5 (0,1M 85% H_3PO_4) Pump B: 100% ACN 75 % from pump A, 25% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 30ppm and 1000ppm solasodine
Solvent for standards	500 µl H_2O + 500 µl MeOH + 2 drops of 85 % H_3PO_4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 14	
Mobile Phase	Pump A: 650 ml 0,1M $NH_4H_2PO_4$ 350 ml ACN pH: 3,5 (0,1M 65% H_3PO_4) Pump B: Same with pump A 50 % from pump A, 50% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	30ppm solasodine
Solvent for standards	500 µl H_2O + 500 µl MeOH + 2 drops of 85 % H_3PO_4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 15	
Mobile Phase	Pump A: 650 ml 0,1M $NH_4H_2PO_4$ 350 ml ACN pH: 3,5 (0,1M 65% H_3PO_4) Pump B: Same with pump A 50 % from pump A, 50% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	30
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50ppm solasodine
Solvent for standards	ACN:H_2O (1:1) (v:v) + 188 µl 0,1 M H_3PO_4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 16	
Mobile Phase	Pump A: 650 ml 0,1M NH4H2PO4 350 ml ACN pH: 3,5 (0,1M 65% H3PO4) Pump B: Same with pump A 50 % from pump A, 50% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10ppm, 20 ppm, 30 ppm and 100 ppm solasodine
Solvent for standards	ACN:H2O (1:1) (v:v) + 188 µl 0,1 M H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 17	
Mobile Phase	Pump A: 650 ml 0,1M NH4H2PO4 350 ml ACN pH: 3,5 (0,1M 65% H3PO4) Pump B: Same with pump A 50 % from pump A, 50% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	20 ppm, 30 ppm and 50 ppm solasodine
Solvent for standards	MeOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 18	
Mobile Phase	Pump A: 650 ml H2O 350 ml ACN 11,50 g NH4H2PO4 pH: 3,5 (0,1M 65% H3PO4) Pump B: Same with pump A 50 % from pump A, 50% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 30 ppm and 50 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 19	
Mobile Phase	Pump A: 100 mM NH4H2PO4 pH: 3,5 Pump B: 100% ACN 70 % from pump A, 30% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 30 ppm and 50 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 20	
Mobile Phase	Pump A: 100 mM NH4H2PO4 pH: 3,5 Pump B: 100% ACN 70 % from pump A, 30% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	50
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 21	
Mobile Phase	Pump A: 0,1 M NH4H2PO4 pH: 2,5 Pump B: 100% ACN 70 % from pump A, 30% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	50
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 22	
Mobile Phase	0,1M NH4H2PO4 pH: 2,5 (65;% H3PO4) 100% ACN 40% from pump A and 60% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	50
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm solasodine
Solvent for standards	MeOH
Detector	DAD
Column	RP column
Elution	Isocritic
Method 23	
Mobile Phase	0,1M NH4H2PO4 pH: 2,5 (65;% H3PO4) 100% ACN 40% from pump A and 60% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 20 ppm, 50 ppm, 100 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 24	
Mobile Phase	40 % 0,1M NH4H2PO4 pH: 2,5 (65;% H3PO4) 60% ACN 40% from pump A and 60% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	10 ppm, 20 ppm, 50 ppm, 100 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 25	
Mobile Phase	A- 0,1M NH4H2PO4 pH: 2,5 (65;% H3PO4) B- ACN 50% from pump A and 50% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm and 200 ppm solasodine, 100ppm and 200 ppm solanidine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 26	
Mobile Phase	A- 0,1M NH4H2PO4 pH: 2,5 (65;% H3PO4) B- ACN 70% from pump A and 30% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm and 200 ppm solasodine, 100ppm and 200 ppm solanidine combined at different ratios
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 27	
Mobile Phase	A- 0,1M NH4H2PO4; B- ACN (10 % MeOH) pH: 2,5 (65;% H3PO4) 40% from pump A and 60% from pump B
Flow rate(mL/min)	1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm and 200 ppm solasodine, 100ppm and 200 ppm solanidine combined at different ratios
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 28	
Mobile Phase	MeOH
Flow rate(mL/min)	0,1
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm and 1000 ppm solasodine
Solvent for standards	MeOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 29	
Mobile Phase	MeOH
Flow rate(mL/min)	0,2
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm and 1000 ppm solasodine
Solvent for standards	MeOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 30	
Mobile Phase	MeOH
Flow rate(mL/min)	0,3
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm, 200 ppm and 1000 ppm solasodine; 10 ppm, 20 ppm and 100 ppm progesterone
Solvent for standards	MeOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 31	
Mobile Phase	MeOH
Flow rate(mL/min)	0,5
Column temperature (◦C)	26
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm, 100 ppm and 200 ppm solasodine
Solvent for standards	MeOH- 5% HCOOH
Detector	DAD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 32	
Mobile Phase	MeOH
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	60
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	100 ppm and 200 ppm solasodine
Solvent for standards	MeOH- 5% HCOOH
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 33	
Mobile Phase	A=ACN B= H2O-0.1% TFH t=0 A=10% B=90% t=36 A=34% B=66%
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	40/ 80/100
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm and 100 ppm solasodine
Solvent for standards	25 µl H2O + 25 µl ACN + 1 drop of 65% H3PO4
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Gradient

Method 34	
Mobile Phase	A= 50 % MeOH B= 50% H2O
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	40/60/80
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm, 100 ppm, 200ppm and 1000ppm solasodine
Solvent for standards	80% µl H2O + 20% µl ACN + 0.1% H3PO4
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 35	
Mobile Phase	A= 50 % MeOH B= 50% H2O
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	98
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	200ppm and 1000ppm solasodine
Solvent for standards	80% µl H2O + 20% µl ACN + 0.1% H3PO4
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 36	
Mobile Phase	A= 70 % ACN B= 30% H2O
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	40/ 60/ 80/ 98
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm, 200ppm and 1000ppm solasodine
Solvent for standards	80% µl H2O + 20% µl ACN + 0.1% H3PO4
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Isocritic

Method 37	
Mobile Phase	MeOH
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	60
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm, 200ppm and 1000ppm solasodine
Solvent for standards	MeOH-5% HCOOH
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Isocritic
Method 38	
Mobile Phase	A= H2O-0.1% formic acid B= MeOH t=0 A=70% B=30% t=8 A=57% B=43% t=20 A=40% B=60% t=24 A=40% B=60%
Flow rate(mL/min)	0.8/ 1
Column temperature (◦C)	26
Drift tube temperature	40/ 60/ 80
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm and 100 ppm solasodine
Solvent for standards	80% µl H2O + 20% µl ACN + 0.1% H3PO4
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Gradient
Method 39	
Mobile Phase	A= H2O-0.1% formic acid-0.04% ammonia (25%) B= ACN t=0 A=95% B=5% t=6 A=80% B=20% t=40 A=65% B=35% t=45 A=0% B=100%
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	40
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	200 ppm and 1000 ppm solasodine
Solvent for standards	MeOH
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Gradient

Method 40	
Mobile Phase	A= H2O-0.1% formic acid-0.04% ammonia (25%) B= ACN t=0 A=95% B=40% t=20 A=80% B=90% t=40 A=65% B=90%
Flow rate(mL/min)	1
Column temperature (◦C)	26
Drift tube temperature	40
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	200 ppm and 1000 ppm solasodine
Solvent for standards	MeOH
Detector	DAD/ ELSD
Column	RP column (Hi-chrom)
Elution	Gradient
Method 41	
Mobile Phase	A= H2O-0.1% formic acid B= MeOH t=0 A=100% B=0% t=6.5 A=76% B=24% t=11,5 A=20% B=80% t=21.5 A=20% B=80% t=24,5 A=76% B=24%
Flow rate(mL/min)	0.2
Column temperature (◦C)	30
Drift tube temperature	40/ 60/ 80
Wavelength (nm)	200, 202, 205, 208,210, 254
Standards (Sample)	50 ppm and 100 ppm solasodine
Solvent for standards	80% µl H2O + 20% µl ACN + 0.1% H3PO4
Detector	DAD/ ELSD
Column	RP column (Xterra)
Elution	Gradient

Apendix B. Tested primers and enzyme combinations.

Primers	Enzymes	Polymorphic Enzymes
At 1g 01730	Not Amplified	-----
At 1g 02140	Kpn I, Apa I, Csp6I, BamH I,Box, NmuCI	Not Polymorphic
At 1g 02150	Kpn I, Apa I, Csp6I, BamH I,Box, Nmuc, Ssp I	Not Polymorphic
At 1g 02560	Kpn I, Apa I, Csp6I, BamHI,Box, NmuCI	Not Polymorphic
At 1g 02910	Not Amplified	-----
At 1g 03250	Not Amplified	Not Polymorphic
At 1g 03310	Kpn I, Apa I, Pst I, Tas I, Bspt I, Xmil	Not Polymorphic
At 1g 04530	Kpn I, Apa I	Not Polymorphic
At 1g 05055	Not Amplified	-----
At 1g 05350	Bme 1390 I, Apa I, Csp6I, BamHI, Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 05385	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 05970	Not Amplified	-----
At 1g 07080	Tru1 I, Apa I	Tru I, PCR Polymorphic, Apa I
At 1g 07960	Not Amplified	-----
At 1g 08940	KpnI, Apa I, Csp6,I BamHI, Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 09150	Not Amplified	-----
At 1g 09620	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 10020	KpnI, Apa I, Csp6I, BamHI,Box, NmuCI	Not Polymorphic
At 1g 10240	Not Amplified	-----
At 1g 10500	HinfI,KpnI, Apa I, Csp6,I BamHI, Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 11860	KpnI, Apa I, Csp6,I BamHI, Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 13380	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 14000	Not Amplified	-----
At 1g 14300	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 14310	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 14790	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 14810	BseG I, Apa I	BseG l
At 1g 16180	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 16870	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 17410	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 18270	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 18660	KpnI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 1g 19140	Not Amplified	-----
At 1g 19530	Not Amplified	-----
At 1g 20050	KpnI, Apa I	Not Polymorphic
At 1g 20575	SspI	PCR Polymorphic, Sspl
At 1g 21690	Not Amplified	-----
At 1g 22850	BsuRI, Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 23890	XapI, Apa I, SspI	Not Polymorphic
At 1g 24360	Not Amplified	-----
At 1g 26520	KpnI, Apa I, SspI	Not Polymorphic
At 1g 27385	KpnI, Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 29320	KpnI ,Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 29900	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 30110	BgI II, Pvul I, Mval	BgI II, Pvul l
At 1g 30360	KpnI ,Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 30580	Hhal, Apa I, Csp6I, BamHI,Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 30825	Not Amplified	-----
At 1g 31410	Not Amplified	-----
At 1g 31970	Not Amplified	-----
At 1g 32070	Not Amplified	-----
At 1g 32900	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 33970	KpnI ,Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 34370	MspI, Apa I	Not Polymorphic
At 1g 35720	KpnI ,Apa I, Csp6I, BamHI,Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 42990	KpnI, Apa I, Csp6I, BamHI,Box, NmuCI	Not Polymorphic
At 1g 43700	KpnI, Apa I, Csp6I, BamHI,Box, NmuCI, SspI	PCR polimorfik
At 1g 44446	KpnI, Apa I	Not Polymorphic
At 1g 44575	TaqI, Apa I	Not Polymorphic
At 1g 44760	KpnI, Apa I, Csp6I, BamHI,Box, NmuCI, SspI	Not Polymorphic
At 1g 44790	Not Amplified	-----
At 1g 46480	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 47830		PCR polimorfik
At 1g 48300	KpnI ,Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 50020	Hin6I, PvulI, Mval, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 51160	Apa I, Csp6I, BamHI,Box, NmuCI, PstI, Tas I, BspT I, Xmil	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 1g 52200	KpnI ,Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 52980	EcoR I, Apa I, Csp6I, BamHI,Box, NmuCI, SspI, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 53000	Not Amplified	-----
At 1g 53670	SspI	PCR Polymorphic, Ssp
At 1g 55170	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 55870	MspI, PvulI, Mval, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 55880	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 56345	PvulI, Mval, PstI, Tas I, BspT I, Xmil	Not Polymorphic
At 1g 60200	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 60440	Hind III, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 60640	EcoR I, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 61620	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 63610	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 63770	BsuR, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 63980	Csp6I,PvulI, Mval	Not Polymorphic
At 1g 65720	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 67325	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 67730	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 67740	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 69420	BseDI, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 1g 71810		Not Polymorphic
At 1g 71950	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 74520	Hind III, PvulI, Mval	Not Polymorphic
At 1g 74970	Not Amplified	-----
At 1g 75350	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 75670	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 76080	Not Amplified	-----
At 1g 76150	Alu I, PvuIl, Mval	Not Polymorphic
At 1g 77470	RsaI, Apa I, Csp6I BamHI, Box, NmuCI	Not Polymorphic
At 1g 78230	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 78620	KpnI, Apa I	Not Polymorphic
At 1g 78690	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 79790	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 80170	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 80360	Taq I, Pvull, Mval	Not Polymorphic
At 1g 80360	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 1g 80460	Alu I, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 2g 01110	EcoR V, Pvull, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 2g 01490	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 01720	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 03120		PCR polimorfik
At 2g 03510	Taql, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 04690	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 04700	Taql, Apa I	Not Polymorphic
At 2g 06005	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 06010	Not Amplified	-----
At 2g 13540	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 14260	Not Amplified	-----
At 2g 15890	BseG l, Apa I, Csp6I, BamHI, Box, NmuCI	Csp6l, NmuCl, BamHl
At 2g 16060	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 16920	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 18710	Hind III, Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 2g 18710	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 20360	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 20820	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 20860	EcoR V, Apa I	Not Polymorphic
At 2g 21620	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 22570	Not Amplified	-----
At 2g 23820	Bme1390 l	Bme1390 l
At 2g 24090	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 24270		PCR polimorfik
At 2g 24390	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 25110	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 25570	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 25950	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 26270	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 26590	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 26830	Hinf l,Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 2g 27290	Not Amplified	-----
At 2g 27450	Not Amplified	-----
At 2g 27730	Xapl,Apa I, Csp6I, BamHI, Box, NmuCI	Not Polymorphic
At 2g 28250	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 28490	Dpn I, Pvul l, Mval	Not Polymorphic
At 2g 28490	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 28880	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 29210	Ban I FD, Xbal, Csp6l	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 2g 30100	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 30200	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 30970	Ban I FD, Xbal, Csp6l	Not Polymorphic
At 2g 32090	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 32415	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 32950	Bme1390 l,Apa I, Csp6I, BamHI, Box, NmuC	Not Polymorphic
At 2g 32970	Mspl, Pvul l, Mval	Not Polymorphic
At 2g 33990	Not working	-----
At 2g 34560	Taq l, Pvul l, Mval	Not Polymorphic
At 2g 34560	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 34620	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 34860	Xap l, Pvul l, Mval	Not Polymorphic
At 2g 35610	KpnI, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 35920	EcoR I, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 35920	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 36230	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 37240	Taql, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 37330	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 37500	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 37510	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 38020	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 38025	Hind III, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 38730	Bme1390 I, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 39100	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 40490	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 40760	BseG I, Pvul l, Mval	Pvul l
At 2g 42750	EcoR I, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 2g 42810	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 43360	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 44310	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 46340	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 46820	Rsa l, Pvul l, Mval	Not Polymorphic
At 2g 46820	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 2g 47580	EcoR I, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 01480	Apa I, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 3g 02300	BsuRl, Pvul l, Mval	Not Polymorphic
At 3g 03440	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 04780	Taql, Pvul l, Mval	Not Polymorphic
At 3g 04870	Tru I, Apa I, Csp6 l, BamHl,Box, NmuCl	Not Polymorphic
At 3g 06050	Hind III,Apa I, Csp6 l, BamHl,Box, NmuCl	Not Polymorphic
At 3g 06730	Xapl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 07100	Taql, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 07565	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 08030	Pst l, Pvul l, Mval, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 08760	Dpn ll, Pst l, Pvul l, Mval, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 09090	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 09740	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 09920	BcI I, Pvul l, Mval	Not Polymorphic
At 3g 09925	Dra l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 10920	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 11210	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 11830	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 12290	Mph1103l, Pvul l, Mval, Pst l, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 12300	Dpn ll, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Pstl, Tas I, BspT I, Xmil
At 3g 13180	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 13235	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 13700	Csp6l, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 13940	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 14910	EcoR l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 15190	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 15290	Taql, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 15430	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 16150	BamHl, Pvul l, Mval, Pst l, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 16150	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 17000	EcoR l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 17040	Taql, Apa I, Pstl, Tas I, Bsp I, Xmil	Not Polymorphic
At 3g 17590	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 18860	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 19900	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 20020	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 3g 20390	Taq, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 20390	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 23400	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 23590	Hinf l	Hinf l
At 3g 24010	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 24050	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 24490	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 25120	Ban I FD, Bme1390 l, Hinfl	Hinf l
At 3g 25920	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 27200	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 44880	Ban I FD, Bme1390 l, Hinfl	Bme 1390 l
At 3g 44890	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 47640	Ban I FD, Bme1390 l, Hinfl	Not Polymorphic
At 3g 47930	Ban I FD, Bme1390 l, Hinfl	hinf l
At 3g 47990		Not Polymorphic
At 3g 51010	Mval, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 51840	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 52220	Ban I FD, BsuRl, Dra I	BsuR l
At 3g 52730	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 52860	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 53180	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 54360	Mspl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 54470	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 54770	BamHl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 55360	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 55800	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 56040	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 56130	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 57270	BsuRl, Pvul l, Mval	BsuRl, Pvul l, Mval
At 3g 57280	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 58470	Dpn II, Pvul l, Mval	Not Polymorphic
At 3g 58470	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 60830	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 3g 61140	Ban I FD, BsuRl, Dra I	BsuR l
At 3g 62010	Hhal, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 3g 62940	Dpn II, Pvul l, Mval	Not Polymorphic
At 3g 63190	Ban I FD, BsuRl, Dra I	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 4g 00090	Csp6l, BamHl, Box, NmuCl	Csp6l
At 4g 00560	Not Amplified	-----
At 4g 01880	EcoR I, Apa I, Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 4g 03280	EcoR V, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 04930	Kpnl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 09010	Csp6l, BamHl, Box, NmuCl, Dpn II, Pvul l, Mval	Not Polymorphic
At 4g 09010	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 10030	Csp6l, BamHl, Box, NmuCl, Taql, Pvul l, Mval, Pst l, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 10030	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 10050	Not Amplified	-----
At 4g 11120	Alw21l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 12230	Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 12590	BseD l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 13780	Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 4g 15420	Not Amplified	-----
At 4g 15520	Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 4g 15520	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 15530	BseG l, Apa I, Csp6l, BamHl, Box, NmuCl	BamHl
At 4g 16580	Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 4g 16580	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 16710	Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 4g 16710	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 17380	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 18593	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 18810	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 20410	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 21520	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 21710	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 22260	Taql, Pvul l, Mval	Not Polymorphic
At 4g 23100	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 24690	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 24830	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 26180	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 26680	Mspl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 26750	Ban I FD, BsuRl, Dra I	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 4g 27700	Taql, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 28530	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 29490	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 30220	EcoR I, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 30580	Hinfl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 31130	Rsal, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Tas I
At 4g 32770	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 33250	Not Amplified	-----
At 4g 33360	Not Amplified	-----
At 4g 33985	Hhal, Pvul l, Mval	Not Polymorphic
At 4g 34700	Hinc II, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 35250	BsuRl, Apa I, Csp, BamH,Box, Nmuc	Not Polymorphic
At 4g 35560	Bme1390l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 4g 37280	Not Amplified	-----
At 4g 38240	Taql, Pvul l, Mval	Not Polymorphic
At 4g 38630	Taql, Pvul l, Mval	PCR Polymorphic, Taql, Pvul l
At 4g 38810	Kpn l, Pvul l, Mval	Not Polymorphic
At 4g 38810	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 4g 39660	EcoR V, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 01350	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 5g 01990	Cfol, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 04590	Dpn I, Pvul l, Mval	Not Polymorphic
At 5g 04740	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 5g 04910	EcoR V, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 06130	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 5g 06370	BsuR l, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 06430	Not Amplified	-----
At 5g 07910	Ban I FD, BsuRl, Dra I	BsuR l
At 5g 07960	Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 08420	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 5g 09880	Rsal, Pvul l, Mval	Not Polymorphic
At 5g 09880	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 5g 11480	BamHl	PCR Polymorphic, BamHl
At 5g 11490	Ban I FD, BsuRl, Dra I	Dra l
At 5g 12200	Dpnl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 5g 12370	Ban I FD, BsuRl, Dra I	Dra l
At 5g 13030	Ban I FD, BsuRl, Dra I	Not Polymorphic
At 5g 13240	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 13450	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 13640	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 13700	Apa I, Csp6l, BamHl,Box, NmuCl	Csp6l
At 5g 14520	EcoR I, Apa I	Not Polymorphic
At 5g 16620	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 16710	Hinfl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 19690	Cfol, Apa I, Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 5g 20350	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 20890	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 23060	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 23120	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 23880	Dpn II, Pvul l, Mval	Rsal
At 5g 25630	Hinfl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 25630	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 25760	Taql, Pvul l, Mval	Not Polymorphic
At 5g 26030	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 26710	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 27390	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 27620	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 35360	Sspl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 37260	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 37360	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 39040	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 40530	Hinfl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 41040	Ban I FD, Pstl, Rsal	Ban l
At 5g 41350	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 41480	Hinfl, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Pstl, Tas I, BspT I, Xmil
At 5g 42740	Taql, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 44250	Ban I FD, Pstl, Rsal	Rsa I
At 5g 45410	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 47040	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 48300	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 48330	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 49830	Taql, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic

Primers	Enzymes	Polymorphic Enzymes
At 5g 49970	BamHl, Apa I, Csp6l, BamHl, Box, NmuCl	Not Polymorphic
At 5g 50720	Taql, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 50720	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 51160	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 51840	Dra l, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 51970	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 52820	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 54080	Ban I FD, Pstl, Rsal	Pst l
At 5g 54310	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 57655	Hinc II, Apa I, Csp6l, BamHl, Box, NmuCl, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 57970	Ban II, Pvul l, Mval	Not Polymorphic
At 5g 57970	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 58200	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 58410	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 59960	BseDl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 60160	Hinfl, Pvul l, Mval, Pstl, Tas I, BspT I, Xmil	Not Polymorphic
At 5g 60540	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 60540	Not working	PCR polimorfik
At 5g 60990	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 60990	Not working	Not Polymorphic
At 5g 61410	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 61410	Pst l	PCR Polymorphic
At 5g 62390	Hinfl, Pvul l, Mval	PCR Polymorphic, Hinfl, Pvul l, Mval
At 5g 63220	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 64350	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 64730	Taql, Pvul l, Mval	PCR Polymorphic, Taql, Pvul l, Mval
At 5g 64970	Ban I FD, Pstl, Rsal	Not Polymorphic
At 5g 66090	Ban I FD, Pstl, Rsal	Rsa l
At 5g 51110	Ban I FD, Pstl, Rsal	Not Polymorphic