SEPARATION AND CHARACTERIZATION OF SAPONINS FROM THE BARK EXTRACT OF THE SOUTH AMERICAN SOAP BARK TREE; QUILLAJA SAPONARIA MOLINA: (POTENTIAL IMMUNO-ADJUVANT ACTIVE COMPOUNDS)

MOTSHEGWANA OLENKIE TEBOGO

NOTE TO USERS

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(POTENTIAL IMMUNO-ADJUVANT ACTIVE COMPOUNDS)

by

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ABSTRACT

In recent years, researchers have demonstrated that the saponins derived from the bark extract of the South American tree, *Quillaja saponaria* Molina, possess an exceptionally potent immune stimulating capability. This has kindled much interest in their potential development as immuno- adjuvants. These saponins have also been shown to play a very important role in the formation of immune stimulating complexes (ISCOMs), which are a specific type of antigen/adjuvant delivery system consisting of a cage-like structure made up of a *Quillaja* saponin, cholesterol and phospholipids in a 1:1:1 ratio. The purified fraction of the bark extract, known as Quil-A, has been shown to contain more than 60 saponin compounds. The structures of several of these have been reported, while some have only been partially characterized. While the use of Quil-A as an adjuvant is well established in veterinary medicine, its use in humans has not been achieved due to its toxicological effects and the fact that its composition can not be standardized from batch to batch. Adjuvant research has become very important for the successful development of modern subunit vaccines as these vaccines do not elicit an immune response sufficient to grant an adequate protection to the subjects when administered alone.

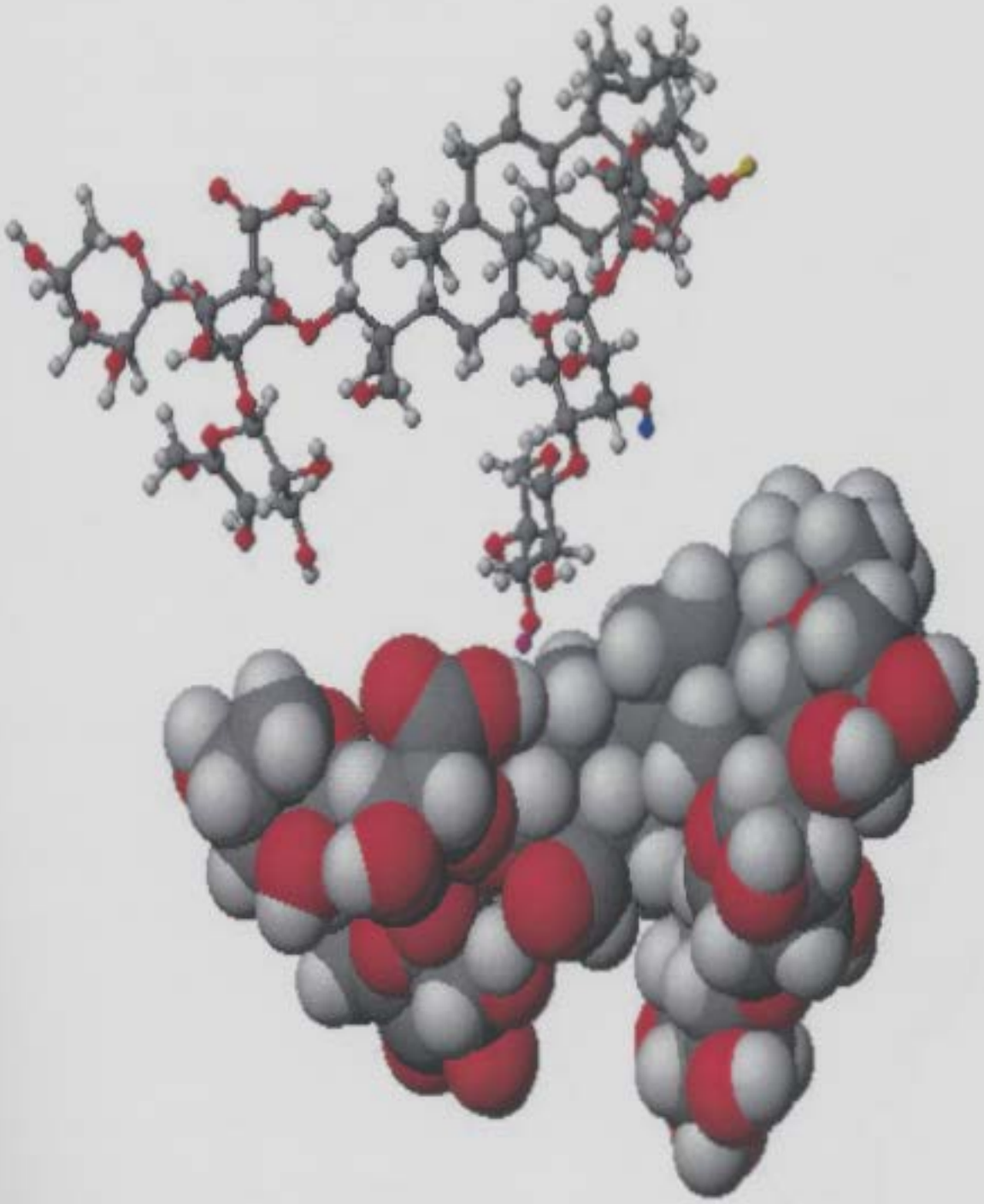
In this research saponin compounds from Quil-A, a commercially available bark extract of *Quillaja saponaria* Molina, were separated, purified and structurally characterized. The biological activity of the individual compounds or fractions

isolated to near purity have also been investigated. Compounds characterized that had novel structures were screened for possible adjuvant properties and compared with other compounds of known structures.

Preliminary High Performance Liquid Chromatography (HPLC) separation of the extract Quil-A by gradient elution using an ammonium acetate (10 mM)/acetonitrile/water solvent system on C5 reverse phase high performance liquid chromatography (RP- HPLC) column has resulted in the purification of a saponin compound having a molecular weight of 1560 m/z. Mass spectrometric analyses, which included utilization of techniques such as collision induced dissociation and tandem mass spectrometry, afforded a proposal of the molecular structure and fragmentation pattern of this compound designated QF-23. Large scale separation of the material Quil-A over fifty HPLC runs has enabled the acquisition of five more fractions in pure or near pure form, including two that are documented to have been previously isolated by other researchers, as well as a sufficient amount of pure compound QF-23 to facilitate the performance of nuclear magnetic resonance experiments (NMR) to confirm the structure of this compound. Liquid Chromatography/ Electrospray Ionization - Mass Spectrometry (LC/ESI-MS) has been employed to investigate further and more specifically the elution characteristics and composition of the crude material Quil-A. The results of this experiment has shown Quil-A to be a highly complex mixture of saponins and non-saponin components which have a tendency to co-elute. Over 80 HPLC peaks were

detectable by means of a photodiode array detector. Solid phase extraction technique was capable of eliminating some materials from Quil-A.

Biological investigation of some saponin fractions revealed a fraction that had considerably lower *in vitro* toxicity to mouse monocytes than the other fractions tested, while the toxicity of QF-23 was found to be moderate and comparable to that of the well-established QS-21. *In vitro* studies have shown the compound QF-23 to be capable of stimulating mouse monocytic cells to produce the cytokine IL-1 alpha, thus suggesting that it may have immuno-adjuvant properties.



Structure of *Quillaja* saponins

ACKNOWLEDGMENTS

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I would also like to thank any other person who has contributed in whatever way to the success of this project who I have not mentioned by name.

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GLOSSARY OF ABBREVIATIONS AND SYMBOLS

Ara	arabinose
APC	antigen presenting cells
Api	apiose
ASC	antibody secreting cells
ATCC	American Tissue Culture Collection
CD4	cluster of differentiation antigen 4
CD8	cluster of differentiation antigen 8
CD28	cluster of differentiation antigen 28
CD80	cluster of differentiation antigen 80
CD86	cluster of differentiation antigen 86
CI	chemical ionization
CID- MS/MS	collision induced dissociation-mass spectrometry/mass spectrometry
^{13}C-NMR	carbon thirteen nuclear magnetic spectrometry
COLOC	COrrelation LOng -range Coupling
CTL	cytotoxic T-lymphocytes
CVID-MS	cone voltage induced dissociation mass spectrometry
DAD	diode array detector
DCCC	droplet counter current chromatography.
DMSO	dimethylsulfoxide

EI	electron impact
EI-CI	electron impact-chemical ionization
ELISA	enzyme linked immuno-sorbent assay
ESI-MS	electrospray ionization-mass spectrometry
FAB	fast atom bombardment
FAB-MS	fast atom bombardment-mass spectrometry
FCA	Freund's complete adjuvant
FCS (FBS)	fetal calf serum (fetal bovine serum)
FDA	Food and Drug Administration
FIA	Freund's incomplete adjuvant
Fuc	fucose
Gal	galactose
GC/MS	gas chromatography/mass spectrometry
GLC	gas liquid chromatography
GLC-MSD	gas liquid chromatography-mass spectrometric detector
Glc UA	glucuronic acid
GRAS	generally recognized as safe
g	grams
HI_{50}	hemolytic index measured as concentration that gives 50% hemolysis
HIV-1	human immunodeficiency virus-type 1

HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
^{1}H-NMR	proton acquisition nuclear magnetic resonance spectroscopy
HPLC	high performance liquid chromatography
hr(s)	hour(s)
Hz	Hertz
IC_{50}	concentration of the sample causing 50% inhibition of cell growth
IgG1	immunoglobulin G1
IgG2a	immunoglobulin G2a
IgA	immunoglobulin A
IgE	immunoglobulin E
IL-1α	interleukin 1 alpha
IL-2	interleukin 2
IL-3	interleukin 3
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-10	interleukin 10
IFN-γ	interferon gamma

IR	infrared spectroscopy
ISCOM	immune stimulating complex
LC	liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
LC/EI-MS	liquid chromatography/electron impact-mass spectrometry
LC/ESI-MS	liquid chromatography/electrospray ionization-mass spectrometry
LPS	lipopolysaccharide
MEC (mec)	minimum effective concentration
mIL-1α	murine interleukin one alpha
mg	milligrams
MHC	major histocompatibility complex
MIC (mic)	minimum inhibitory concentration
min(s)	minute(s)
mL	milliliters
mm	millimeters
mM	milliMolar
mmoles	millimoles
MS	mass spectrometry
MSD	mass spectrometric detector
MTC (mtc)	minimum toxic concentration

MTT	3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide
Mwt	molecular weight
m/z	mass/charge
µL	micro liters
n	number of tests carried out in an experiment for biological testing.
NIST	National Institute of Standards and Technology
NK (cells)	natural killer cells
NMR	nuclear magnetic resonance spectroscopy
OD	optical density
O.D.	outer diameter
P	probability value
PBS	phosphate buffered solution
PMAA	partially methylated alditol acetate
ppm	parts per million
Qa	quillaic acid
QF	*Quillaja* fraction
QF-1	*Quillaja* fraction 1
QF-2	*Quillaja* fraction 2
QF- 9	Quillaja Fraction 9
QF-10	*Quillaja* fraction 10

QF-11	*Quillaja* fraction 11
QF-12	*Quillaja* fraction 12
QF-15	*Quillaja* fraction 15
QF-19	*Quillaja* fraction 19
QF-21	*Quillaja* fraction 21
QF-22	*Quillaja* fraction 22
QF-23	*Quillaja* fraction 23
QF-35	*Quillaja* fraction 35
QS	*Quillaja* saponin
QS-7	*Quillaja* saponin 7
QS-17	*Quillaja* saponin 17
QS-18	*Quillaja* saponin 18
QS-21	*Quillaja* saponin 21
RBC	red blood cells
Rha	rhamnose
rpm	revolutions per minute
RP-HPLC	reverse phase-high performance liquid chromatography
SD	standard deviation
SPE	solid phase extraction
t	time
Temp.	Temperature

TC_{50}	toxic concentration at 50% kill
TET	tetradine
TF	Thomsen Friedenreich antigen
TFA	trifluoroacetic acid
Th-1	T-helper cells (subset 1)
Th-2	T-helper cells (subset 2)
TI	therapeutic index
TIC	total ion chromatogram
TLC	thin layer chromatography
TNF-β	tumor necrosis factor beta
UV	ultra violet
vs	versus
Xyl	xylose

LIST OF PUBLICATIONS

Tebogo, Motshegwana O., Richardson, Vernon J., Banoub, Joseph, Purification and characterization of a novel saponin from the bark extract of *Quillaja saponaria* Molina: Constituents of this extract have potential immuno-adjuvant capabilities (manuscript under preparation for Rapid Communications in Mass Spectrometry).

Tebogo, Motshegwana O., Richardson, Vernon J., Banoub, Joseph, Jennings, Roy, *Quillaja*, The South American Soap Bark Tree and its immunoadjuvant saponin constituents: A review article (manuscript under preparation for Chemical Reviews).

Tebogo, M. O.; Richardson, V. J.; and Banoub, J., 2001, Separation and Characterization of Caponins from *Quillaja saponaria* Molina: Immuno-adjuvant Compounds (poster presentation), The Association of Faculties of Pharmacy of Canada and The Canadian Colleges of Clinical Pharmacy Conference 2001, Ottawa, Ontario. Canada

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*"**God** grant me the serenity to accept the things I cannot change, courage to change the things I can, and the wisdom to know the difference."*

For

"All things work together for good to them that love God, and if God be for us who can be against us."

Anonymous (Reinhold Neibuhr)

Romans 8:28 and 31

DEDICATION

To my daughter Gonyenya Shanti, with whom I share everything and whose smile has never failed to brighten even the darkest of my moments. My love for you is inexplicable. Equally I rejoice in the knowledge of your love for me. You truly are the greatest gift God has ever given me.

Chapter 1: Introduction

Quillaja is a genus of plants belonging to the family Rosaceae. It is native to the South American countries of Bolivia, Peru and Chile. Plants of this genus contain saponins, that have the characteristics of soap, and have been used by the native peoples to wash clothes. Hence, the name *Quillaja*, derived from the native word for "to wash", *quellean*, was given to the genus. The saponins derived from these plants have found important uses in a considerable number of industries: pharmaceutical, veterinary (vaccines), food, cosmetics, agriculture and so on (George, 1965; Birk 1969; Bomford, 1980; 1988; Price *et al.*, 1987; Chavali and Campbell, 1987; Chavali *et al.*, 1987; 1988; Hostettmann and Marston, 1995; Anaguade , 1999). Recently, these saponins have attracted a great deal of interest from researchers because of their immuno modulatory activity and potential use as vaccine adjuvants in humans (Chavali and Campbell, 1987; Chavali *et al.*, 1987; 1988; Bomford, 1980; 1988; 1992; Ahmeida, 1992; Audibert and Lice, 1993; Behboudi *et al.*, 1995;1997;1999). They have been shown to have potent immuno stimulatory activity inducing both the humoral and cell mediated responses (Chavali and Campbell 1987; Chavali *et al.*, 1987; 1988; Bomford, 1980; 1988; 1992; Ahmeida, 1992; Audibert and Lice, 1993; Behboudi *et al.*, 1995; 1997; 1999). They are also used as major components of immune stimulating complexes (ISCOMs) which are highly efficient delivery systems for antigen and adjuvant in vaccine

administration (Morein *et al.*, 1984; 1998; Rönnberg *et al.*, 1995). The saponins are most abundant in the *Quillaja saponaria* Molina species (Wallis , 1967; McClintock, 1984; Price et.al., 1987; Chavali *et al.*, 1987).

The need for the use of co-immune stimulators (adjuvants or co-adjutants) in the administration of certain vaccines, particularly those produced through attenuation and other methods (for example, those that result in the so called 'killed vaccines') has long been recognised as a result of the weak immunogenecity of the resultant antigen (Dalsgaard, 1974). In modern days, this problem has been accentuated even further by the new methods of vaccine development which result in highly purified antigens that are even weaker immunogens. These subunit vaccines are favoured over the conventional whole organism vaccines because they are capable of inducing highly specific and required immune responses (Vogel and Powell, 1995; Gizurason, 1996). A number of adjuvants are available for use in veterinary vaccines, but the choice is limited to one when it comes to adjuvants approved for human use (Audibert and Lice, 1993). For many years *Quillaja* saponins have found extensive use in veterinary vaccines. Human applications have greatly been hampered by their toxicity. A commercially available *Quillaja* saponin extract has been demonstrated to be a mixture of over 60 compounds that are difficult to separate (van Setten and van Werken, 1999). Fractionation and structural elucidation of these components, as well as structural/function studies, may facilitate the discovery of a nontoxic, adjuvant active component that may be suitable for human use.

1.1. Botanical information

1.1.1 The Genus *Quillaja*

As previously mentioned, the genus *Quillaja*, also natively called *Quillaia* or *Quillaiae,* belongs to the family Rosaceae (suborder: - Rosineae, order: - Rosales, grade: - Archichymadeaceae, subphylum: - Dicotyledons, phylum: - Angiospermae; flowering plants). The family consists of about 100 genera and over 2000 species. Members of this family usually contain saponins and cyanogenetic glycosides, which yield hydrocyanic acid under enzymatic influence. The plants normally have simple or compound leaves. Trees of this genus are well known for their saponin constituents. The genus *Quillaja* consists of only four species, namely; *Q. saponaria* Molina, *Q. poeppigii*, *Q. smegmadermos* de Candolle, and *Quillaja selloniana* also known as *Fontenellea braziliensis* (Wallis, 1967; Grieve, 1995). The species of this genus that has been of enormous interest is the *Q. saponaria* Molina species, as it is the major source of the saponins found to have a profound immunological activity.

1.1.2. *Quillaja saponaria* Molina

The *Quillaja Saponaria* Molina species was first described botanically and named in 1782 by a Chilean Jesuit named Juan Ignatius Molina. As mentioned earlier, he adapted the name of the genus *'Quillaja'* from the local names *'quillai'*

or *'cullay'*, themselves derived from the Chilean word *'quillean'* meaning 'to wash'. He selected *'saponaria'* as the species name because of the soap-like property of the bark (McLintock, 1984)[1].

The tree of *Quillaja saponaria* Molina, also known as Panama Wood, Soapbark Tree, *Quillaia*, *Quillay* or *Cullay*, (Wallis, 1967; Trease and Evans, 1996; Grieve, 1995) is a large tree (about 18 m high (Figure 1)), indigenous to Chile, Bolivia and Peru. Its bark has been long used by the native peoples for washing silk and wool. It was known to the Europeans in the early part of the 18th century but was not regularly imported until 1857 when it was sent to France under the name Bois De Panama indicative of the route by which it was sent. Since 1878, the tree has been introduced into India and California for medicinal and industrial purposes; the constituents were used in the cosmetic, pharmaceutical, and food industries as well as for agricultural and veterinary purposes. This soapbark tree is oak-like and evergreen. Its leaves are alternate, simple, oval, shallowly and remotely toothed, and one to two inches long. The leaves have short petioles while the flowers are greenish yellow when open and somewhat less than an inch across. They have five spreading sepals and petals, and ten stamens arching upwards. The fruits split at maturity into five spreading seed pods, each about half an inch long, opening along

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[1] Molina was born in Chile in 1737 and lived there until 1768 when he was expelled together with other Jesuits by the Spanish Monarch, Carlos III. He then settled in the city of Bologna, Italy until his death in 1829. In 1782, Molina published a classic work on the geography and the natural and civil history of Chile. In this work he described for the first time many Chilean plants among which was *Quillaja saponaria*. This work was first published in Italian, but it has since been translated into other European languages (McLintock, 1984).

one side with numerous winged seeds (see Figure 1).

The tree flowers and fruits from September to December (McLintock, 1984).The inner bark foams like soap when crushed and mixed with water. It was imported into Europe during the last century as an emulsifier, and for the less important use of producing froth on stale beer (McLintock, 1984). The bark constitutes about 38 - 44% of total dry mass of the tree (Plato *et al.*, 1986).

1.1.3. Pharmacognosy of the bark drug

The commercial bark consists almost entirely of phloem and is 100 X 20 X 0.6 cm in dimensions. It is brownish or yellowish in colour and has reddish-brown or blackish-brown longitudinal striations. The inner surface is yellowish brown and fairly smooth. The bark has a splintery fracture that separates into laminas. Minute glittering points of calcium oxalate can be seen sometimes with the naked eye, but are obvious on the smooth inner surface of the bark and on the fractured laminated surface when viewed with a lens. The bark is almost odourless but its powder is very sternutory (extremely irritating to the nostrils and fauces, causing prolonged fits of sneezing), and has an unpleasantly acrid and astringent taste. The powder froths abundantly when shaken with water and it yields a pale straw-coloured tincture (Wallis, 1967; Trease and Evans, 1972; 1996). Microscopical examination of a transversed section of the bark reveals a chequered appearance of medullary rays, cris-crossed by alternating bands of lignified and non-lignified phloem. The

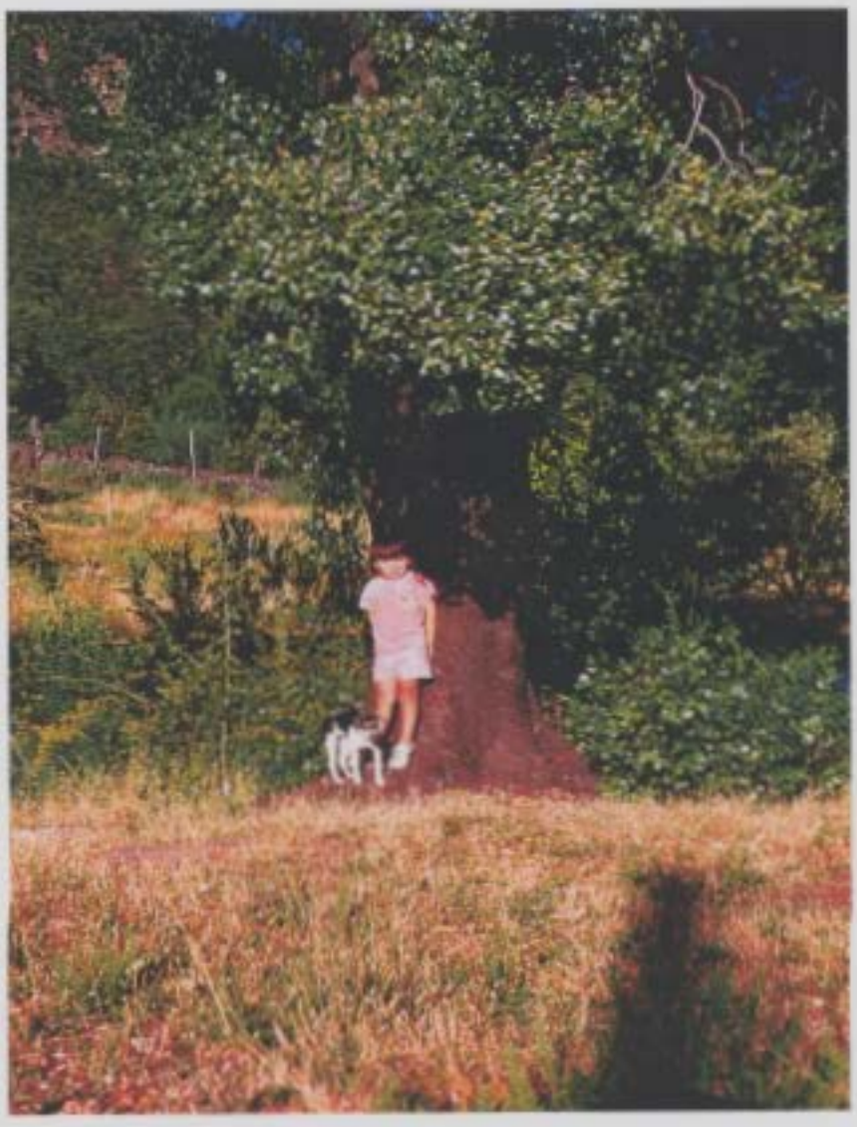


Figure 1: ***A picture of the Quillaja saponaria Molina tree.*** (Courtesy of Dr. Martin Gardner of the Royal Botanic Garden, Edinburgh, UK.)

phloem is tortuous and often accompanied by small groups of rectangular sclereids (Wallis, 1967; Trease and Evans, 1972; 1996). The histological characteristics of the bark are well described in detail in Wallis (1967).

1.2. Constituents of *Quillaja saponaria* Molina

The chief constituents of the soap bark tree are saponins found mainly in the inner bark. Upon hydrolysis, the saponins yield the principal sapogenin, quillaic acid (hydroxygypsogenin) and gypsogenin. The total concentration of saponins in the tree is 9- 10%. The bark also contains sugars, starch, calcium oxalate, and uronic acid.

Presently, there is not much information available about the composition of *Quillaja* saponins. A number of these saponins have been structurally characterised, such as QS-III, QS-21, QS-7, QS-18 (see Figures 2 and 3 on page 10 and Table 1 on page 11). But there are still some 50 compounds whose structural identities are not yet known (van Setten *et al.*, 1995). The difficulty in separation is postulated to be attributable to the formation of mixed micelles comprised of the different saponin species in aqueous solution, which prevents effective separation (Kensil, 1996).

The saponins and other constituents, as extractable material in water (ethanol or water /ethanol mixture), constitute 20 - 25% of the weight of the bark.

The saponins themselves are estimated to constitute 5% of the bark weight and 20% of the total extractable material. The rest of the extractables are tannins and phenolic compounds separable from the saponin extract (Qui-A) via dialysis, gel filtration and dialfiltration (Kensil, 1996). A typical analysis of the commercially available *Quillaja* extract powder (Quil-A) reveals the following content: moisture 5.0%, protein 0.5%, fat 0.4%, fibre 0.3%, ash 2.5%, carbohydrates (including saponins) 91.3% (Anagaude, 1999).

1.2.1. Saponin constituents of *Quillaja saponaria* Molina

Definition

Saponins are high molecular weight compounds of natural origin found mainly in higher plants and belong to a group of compounds known as glycosides that consist of a sugar portion and a non-sugar portion in their molecular structures. The non-sugar portion of glycosides is called the aglycone, and in most saponins it has soap-like characteristics from which the term saponin was derived. The aglycone of saponins may have a triterpene or a steroidal skeleton and is synthesised from squalene by the plant.

Many saponins have a hemolytic activity as well as the following characteristic properties that are also used for their identification and characterisation: bitter taste, forms stable foams in aqueous solutions, high toxicity

to fish and amphibians, and may form molecular complexes with cholesterol and other hydroxy steroids. However, a great deal of variability exists in these properties between saponins from various sources, and not all the saponins necessarily possess these properties (Birk, 1969). For these reasons, saponins are now defined on the basis of their molecular structure, such as triterpene glycosides or steroidal glycosides (Hostettman and Marston, 1995). The aglycone of the saponin molecule is also called the genin or sapogenin, and the terms monodesmosidic, bidesmosidic and tridesmosidic are used to describe the number of sugar chains attached to the aglycone, being one, two, and three, respectively. Oligosides refer to glycosides containing more than three to four monosaccharides (Hostettmann and Marston, 1995). *Quillaja* saponin is a mixture of acylated triterpenoid oligoglycosides (acylated saponins) (Higuchi *et al.*, 1987).

Saponins are widely distributed throughout the plant kingdom and have been identified in at least 400 species belonging to more than 80 different families. Some saponin containing plants are found as components of the human diet while others are found in animal feed. Saponins have been found in various parts of the plants, e.g., leaves, stems, roots, bulb blossom, bark, and fruits. They have been found to be localized in organelles which have high metabolic turnover rate such as the mitochondria, the chloroplasts, the hypocotyl and the cotyledon (Anisimov and Chirva, 1980, Taniyama *et al.*, 1988). This gave the impression that they may be important constituents with some physiologically significant role, perhaps the

regulation of the metabolism and the overall development of the organism (Anisimov and Chirva, 1980). A long standing theory that helps to explain their presence in plants, which can be as high as 30 % in some species, is that they protect the plant against fungal attack, suggested from the observation that there is often an increase in saponin content of the part of the plant undergoing microbial attack (Défago, 1977; Hostettman and Marston, 1995). Saponin concentrations in plant structures have been found to depend also on the environmental temperatures and vegetative period of the plant: e.g., flowering, fruit bearing etc. (Birk, 1969). *Quillaja* saponins occur in the inner bark of the *Quillaja saponaria* Molina tree and other species of the genus *Quillaja* (Korbert, 1887; Wallis, 1967; McLingtock, 1984; Trease and Evans, 1996).

History

The saponins contained in the bark of *Quillaja saponaria* Molina have been studied for a long time, in fact since as far back as 1903 (Labriola and Deulefeu, 1969). The isolation of *Quillaja* saponin was reported for the first time in 1887 and to this day *Quillaja* saponin has proven to be a complex and poorly separable mixture. Some of the very early scientists that studied the *Quillaja* saponin were Windaus *et al.*, who were the first to isolate the aglycone by acid hydrolysis in 1926 (Windaus *et al.*, 1926 in Labriola and Deulefeu, 1969). The aglycone was later named quillaic acid by Elliott *et al.*, 1939 (Labriola and Deulefeu, 1969), who also

determined its correct formula as $C_{30}H_{46}O_5$. In the early works with *Quillaja* saponin, D-galactose was the only other product identified by hydrolysis, besides the aglycone, (Labriola and Deulefeu, 1969). In 1969, Libroala and Deulefeu partially described the structure of the prosapogenin.

1.2.2. The Chemistry of the Quillaja saponins

Methods of extraction, isolation, purification and quantification of the saponins

Dalsgaard in 1970, employed dialysis, ion exchange and gel filtration chromatography (precoated silica gel layers developed with n-butanol, 96% ethanol and ammonia solution in the ratio 3:6:5 as the mobile phase) to the *Quillaja* extract to produce the product he named Quil-A. The extract was obtained from 5g of the *quilleae* cortex extracted with 50ml of distilled water stirred vigorously for three hours at room temperature, clarified by means of a Buchner funnel filtration followed by centrifugation at 800rpm for 15 min (Dalsgaard, 1970). Higuchi *et al.* (1987 and 1988) extracted saponins from the *Quillaja* bark using methanol and achieved purification through droplet counter current chromatography and reverse phase column chromatography. For the production of a good quality extract, Steinbeck, *et al.* (1995) recommended the use of the bark from young trees, as it was found to be less heterogenous. Kensil *et al.* (1991) isolated QS-21, -18, -17 and -7 (see

Figures 2 and 3 and Table 1 for structures) by silica chromatography from a water extract of *Quillaja* bark. The resulting products consisted of one peak each when analysed by reverse phase HPLC (White *et al.*, 1991). Using these chromatographic techniques, which utilised organic solvents, they separated saponin mixtures into 28 fractions. Four of these were tested and were found to have adjuvant activity (Kensil *et al.,* 1991). The reverse phase HPLC method of separation and purification (purification is achieved by repetitive injection of separated samples until a single peak is obtained, showing the presence of only one ion by mass spectrometry) was first applied by Kersten *et al.* in 1988. This is the method now favoured by most researchers, presumably because of its simplicity and relative effectiveness. In 1998, Thompson found that a C5 reverse phase column gave better separation than a C8 column, for a commercial *Quillaja* saponin extract called Spikoside.

The structure of the *Quillaja saponaria* Molina saponins

Quillaja saponins are didesmosidic triterpenoid oligoglycosides, which means that they are glycosides having multiple sugar chains attached at two different positions to an aglycone with a triterpenoidal skeleton. The aglycone is quillaic acid (3β-16 α -dihydroxy-23-oxolean-12-en-28-oic acid) and the sugars are attached at its C3 and C28 positions (see Figure 2, A typical molecular structure of *Quillaja*

saponins). The sugar at C3 is ether bound while the one at C28 is ester bound (Figure 2, A typical molecular structure of *Quillaja* saponins). Two structural features distinguish *Quillaja saponaria* saponins from saponins of other plant species. One is that they have an aldehyde group attached to the C4 position of the triterpene structure (see Figure 2) and the other is that some contain a fatty acid moiety at R_3 (Figures 2 and 3) (Kensil *et al.*, 1995; van Setten and van de Werken, 1996).

The common molecular structure of the *Quillaja saponaria* Molina saponins is described in Figure 2 and Table 1 presents a compilation of some of the structures reported thus far.

Structures of other compounds containing a rhamnose joined to the glucoronic acid rather than a xylose in forming the trisaccharide of the C-3 carbon have recently been reported (van Setten and van de Werken, 1996; Nord and Kenne, 1999). Lately, QS-21 has been demonstrated to isomerize into a regioisomer, which differs from the parent molecule at the position of attachment of the fatty acyl moiety to β-*D*-fucose sugar, as it can either be attached to the 3rd or 4th hydroxyl group (see Figure 2, page 14). The major isomer QS-21A has the fatty acyl moiety attached to the fucose at the 4th hydroxyl group (Jacobson *et al.*, 1996). The ratio of these isomers at equilibrium is 1:20 (Jacobson *et al.*, 1996).

Table 1: Structures of some characterized *Quillaja* saponins.

1. DS-1 and DS-2 were described by Highuchi *et al.* in 1987
2. QS-III was described by Highuchi *et al.* in 1988.
3. The compounds QS-7, QS-17, QS-18 and QS-21 were described by Kensil *et al.* in 1991.
4. HPLC fractions 1- 4 were characterized in 1995 (together with some forty or so others) by van Setten *et al.*
5. The structure of compound S2 was suggested by Nord and Kenne in 1999.

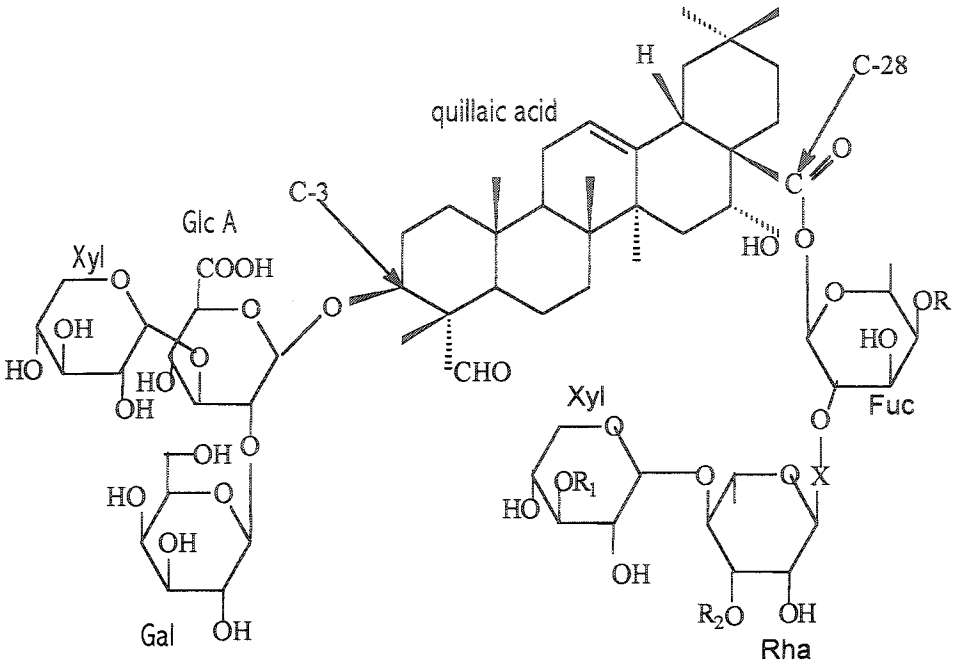


Figure 2
A typical molecular structure of Quillaja saponins
(R_1, R_2, R_3 and X are as stated in Table 1)

For some compounds R_3 =

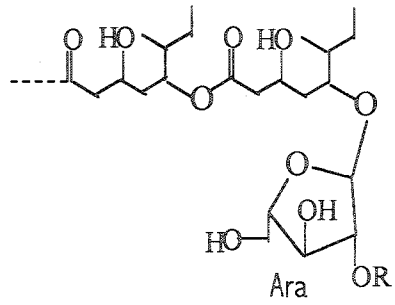


Figure 3
Structure of a fatty acyl moiety found in some Quillaja saponins
(R_4 is as stated in Table 1)

Table 1: Structures of some characterized *Quillaja* saponins.

(Refer to structures in Figures 2 and 3)

Saponin	**R_1**	**R_2**	**R_3**	**R_4**	**X**
DS-1	apiose	-H	-H	absent	absent
DS-2	apiose	glucose	-H	absent	absent
QSIII	apiose	glucose	Figure 3	rhamnose	absent
QS-7 (proposed structure)	apiose/xylose	glucose	CH_3CO-	absent	absent
QS-17	apiose	glucose	Figure 3	rhamnose	absent
QS-18	apiose	glucose	Figure 3	-H	absent
QS-21	apiose	-H	Figure 3	-H	absent
HPLC fraction 1	-H	hexose	-H	absent	$C_8H_{12}O_5$
HPLC fraction2	-H	-H	-H	absent	$C_8H_{12}O_5$
HPLC fraction 3	pentose	hexose	-H	absent	$C_8H_{12}O_5$
HPLC fraction 4	pentose	-H	-H	absent	$C_8H_{12}O_5$
Compound S2	-H	-H	Figure 3	-H	absent

Methods employed in the structural elucidation of saponins from *Quillaja saponaria* Molina

A typical mass spectrometric analysis would use tandem mass spectrometry as applied by Schopke *et al.* (1996) on triterpenoid saponins of *Bellis annua*. In the structural elucidation of saponins, issues that might be addressed can be broken down as follows: (1) the determination of the structure of the aglycone; (2) the composition and frequency of the monosaccharides in the carbohydrate moiety; (3) the nature of the linkages of the monosaccharides to one another; (4) the anomeric configuration of each glycosidically linked monosac-charide; and, (5) the location of the carbohydrate moiety on the aglycone.

A schematic representation of the molecular structural elucidation of saponins as recommended by Hostettetmann and Marston (1995) is depicted in Diagram 1.

The specific structural features that need to be determined are shown in Table 2, adapted from the review of van Setten and van de Weaken (1996) on the molecular structures of saponins from the *Quillaja saponaria*. The monomer mapping method has recently been developed by van Setten and van de Werken who applied it to 50 Quillaja Saponins (van Setten & van de Werken, 1994; van Setten *et al.*, 1995).The diazomethane degradation is thought to proceed through an epoxide intermediate, as studied by Higuchi *et al.* (1988). Several cleavage reactions need to be performed to obtain partially overlapping products. The data from these products, in comparison with the NMR data of the intact product, may lead to establishment of the ways in which the reaction products are linked in the original compound.

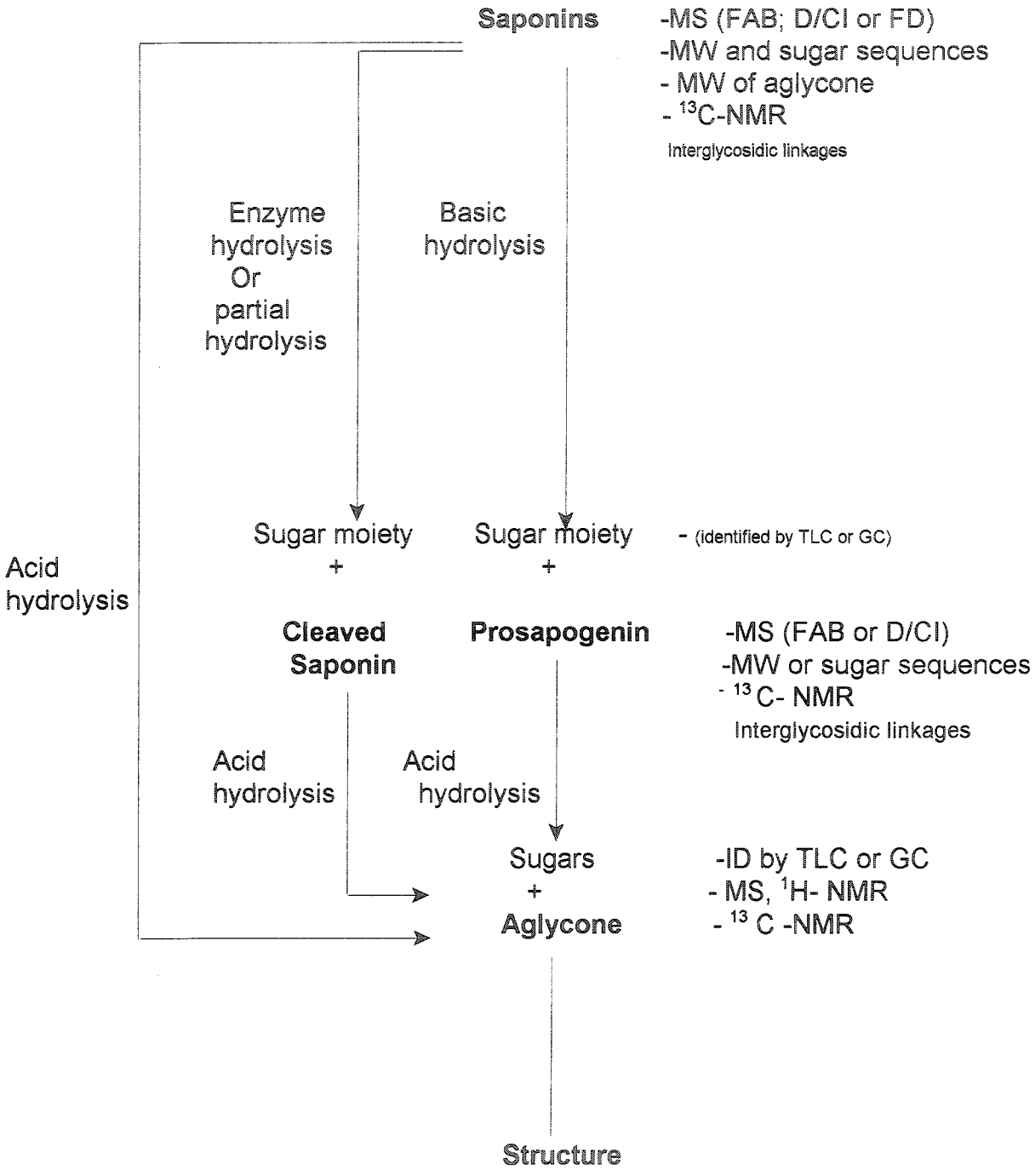


Diagram 1. Structure elucidation of saponins (Hostettmann& Marston, 1995)

Table 2: Specific structural features to be determined in structural elucidation of *Quillaja* saponins.

Structural feature	Technique
Molecular weight	- FAB-MS, EI-MS
Monomer composition	- Acid hydrolysis & TLC or GC/MS - monomer mapping
Primary sequence of 28-0 bound glycocyl moiety	- FAB-MS/MS -Partial acid catalyzed cleavage & identification of saccharide products by methylation analysis and/or TLC
Branching Pattern of 28-0 bound glycocyl moiety	-FAB-MS/MS -Partial acid catalyzed cleavage & identification of saccharide products by methylation analysis and/or TLC
Anomeric Configurations	^{1}H and ^{13}C NMR
Absolute Configuration	GC/MS after derivatization with a chiral agent
Acyl Moiety (non carbohydrate moiety)	^{1}H and ^{13}C NMR, FAB-MS, EI-MS, MS/MS
Aglycone	EI MS, IR, `H and ^{13}C NMR, TLC
Binding position to aglycone	^{1}H and ^{13}C NMR

One major problem with this approach is the risk of side reactions which may lead to production of molecules difficult to correlate to the original structure.The procedure for the alkaline hydrolysis is best described by Higuchi *et al.* (1987). Of the two methods employed, the one recommended by van Setten and van de Werken is the concentrated ammonia/methanol mixture 1:3 v:v reaction, as the reagents can be simply removed by evaporation (van Setten & van de Werken, 1996). Through this reaction, the sugars located in the R_4 portion of the structure, and the fatty acid present in the molecule can be identified and the decacylsaponin can be characterized. Basic hydrolysis cleaves the more sterically hindered ester formation at C-28 to yield the 3-0 glycoside, generally referred to as the prosapogenin.

Acid catalyzed cleavage reactions are also described in detail by Higuchi *et al.* (1987). These reactions yield the aglycone. Presently up to four different aglycones have been isolated from *Quillaja saponaria* Molina, three of which have already been characterized as quillaic acid, gypsogenin and gypsogenic acid (van Setten & van de Werken, 1996; Varshners *et al.*, 1985). There are two other important reactions which have been described and used by Higuchi *et al.* (1987) to describe the sugar types and linkages. These are partial acid catalyzed cleavage and methylation analysis. These methods are also described by Hostettemann and Marston (1996). Kensil *et al.* (1992) also applied the method of methylation analysis to *Quillaja* saponin, however, van Setten and van de Werken (1996) recommended the use of the method as described by Biermann in the Analysis of Carbohydrates by GLC (Biermann, 1988) as it results in only one partially methylated alditol

acetate (PMAA) product per monosaccharide (van Setten & van de Werken, 1996). Monomer mapping has recently been applied to 50 saponin components of Quil-A which were found to consist of pairs. Intra pair differences were shown to occur within the 3-0 bound glycosyl moiety, interchanged pentose and rhamnose, while inter- pair differences existed in the 28-0 bound glycosyl moiety (van Setten *et al.*, 1995).

Stability of *Quillaja* saponins and the extract of *Quillaja saponaria* Molina

The saponins of *Quillaja* saponaria Molina have been shown to isomerize at the fucose site. This isomerization involves the hydroxyl groups attched at positions C3' and C4' (Figure 2, page 14 and Table 1, page 15). The group attached at this position, C4' (which is in many saponins the fatty acyl domain, while in others it can only be a methyl group, or even a hydrogen atom as is the case in the compounds DS-1 and DS-2) migrates between the two hydroxyl groups in aqueous solution (Kensil *et al.*, 1995b; Kensil *et al.*, 1996; van Setten and van de Werken, 1996). Base catalyzed deacylation was found to be the major degradation reaction of the *Quillaja* saponin QS-21, and solutions of this compound are reported to be most stable at pHs between five and seven. Kensil *et al.* (1995a) and Cleland *et al.* (1996) have extensively studied the stability of QS-21 in vaccine formulation with the MN rgp 120 HIV-1 subunit vaccine. They found that the storage of QS-21 in aqueous solutions resulted in the interconversion of the QS-21 molecules into its two isomers designated QS-21A and QS-21B, both of which however are immunologically active. The compound was found to be most stable in its micellar

form, presumably due to the fact that the labile ester linkage bond becomes buried in the hydrophobic micellar environment. The maximum stability was found at a pH of 5.5, which is unusual as esters are usually less stable at lower pHs. The formulation that gave a shelf-life of over two years was 500mg/mL QS-21 in 20 mM sodium succinate and 150mM sodium chloride, buffered at pH 5.5.

Ester hydrolysis results in the loss of adjuvant properties (Kensil *et al.*, 1992) making it essential that before administration of the vaccine the QS-21 molecule must maintain its intact structure (Cleland *et al.*, 1996). The storage requirements for the commercially available preparations such as Quil-A, ISCOPREP-7.0.3™ and Stimulon are outlined in Vogel and Powel, 1995.

1.3. Pharmaceutical applications of *Quillaja saponaria* Molina

According to the literature, the use of *Quillaja* bark and its saponin can be traced as far back as the eighteenth century, when the native people took advantage its soap like properties and used it to wash their bodies and clothes. The saponin had an advantage over the true soaps because it was harmless to the fabrics and their colours, rendering it the best choice for silks, wools and sensitive skin. This property was later exploited by the American manufacturers who produced saponin containing soaps for use on sensitive skin. *Quillaja* saponins also have a very healthy stimulating action on the skin, and for this reason saponin soaps have been widely available in America for washing, shaving, shampooing and baths, for many years (Korert, 1911). Even to date *Quillaja* saponins are included in shampoos, liquid detergents, toothpastes and other cosmetic preparations (Tanaka *et al.*, 1996).

Another early use of *Quillaja* saponins was in the food industry where they have been added to soft drinks to slow the loss of effervescence after opening, and to stale beer to make it lively and drinkable again. (Korbert, 1911; Birk, 1969; McClintock, 1984; and Price *et al.*, 1987). In the United States, the United Kingdom and some other countries, the addition of the extract of the *Quillaja saponaria* Molina to foodstuff has been at times illegal (Steinbeck *et al.*, 1995; Grieve, 1995). Control was imperative because of the potential hazardous and toxic effects of these extracts. At present, the extract has been categorized as 'generally recognized as safe' (GRAS) by the U.S. Food and Drug Administration (title 21 CFR 172-510/FEMA no. 2973), and has now found applications in other food stuff such as milkshakes, cream sodas, mousses, cocktail mixes, and as a tasteless and odourless preservative, because of its antioxidant property. (Makkar, 1998; Anagaude, 1999; Garuda, 1999).

The historical pharmaceutical application of the *Quillaja* bark extract was as an emulsifying agent for tars and volatile oils (Wallis, 1967). Presently, it is being used, to some extent, as an antioxidant (Anagaude, 1999). Therapeutically, the soap bark has a long tradition of use as a treatment for chest problems. It has been employed for the treatment of bronchitis, especially in the early stages of the illness and works through increasing the amount of the respiratory tract fluid which in turn results in the clearing of the phlegm through coughing (expectorant action). It is not recommended for a dry cough, but gargles of warm salt solution with 10-20 drops of *Quillaja* extract have been employed in dry throat catarrh as a liquifying expectorant (Korbert, 1911). Other therapeutic uses of the *Quillaja* extract and its

saponins include, stimulant, mucosal irritant, nauseant, antihemorrhoidal, (in ointments), treatment of dandruff (as a constituent in dandruff shampoo), anti-inflammatory, antimicrobial, antihypercholesterolemic agent, astringent, anthelmintic (the tape worm is forced to lose its hold when it comes into contact with the saponin and it is then removed by the purgative action of the saponin), treatment for dermatosis, promotion of hair growth, prevention of tooth decay and halitosis (by inclusion in mouth washes), and so forth. It is presently being developed for use in humans as an immune stimulant. Its use in this regard is now well established in veterinary medicine, while the use of the extract in human vaccines does not yet comply with the requirements for medical products because the composition of the extract cannot be standardized from batch to batch. It has also been reported to cause toxicological effects in humans, such as; liver damage, respiratory failure and gastro-intestinal irritation (Kobert, 1911; Wallis, 1967; Dalsgaard, 1974; 1978; Oakenful, 1981; Maharaj *et al.*, 1986; Kersten, 1988; Dalsgaard *et al.*, 1995; van Setten *et al.*, 1995; van Setten and van de Werken 1996; Anagauda, 1999).

The *Quillaja* bark extract has lately received a considerable amount of attention from several groups of researchers for various novel applications. The extract has been proposed to decrease the ammonia level in the atmosphere; to suppress or stimulate microbial growth; to increase the binding of ammonia during ammoniation of straw and soil; to reduce odours from cattle manure in dairy barns; as livestock feed additives to reduce rumen motility and improve nutrient absorption that could be beneficial in fattening livestock and cholesterol reduction in the

animals; in waste purification and treatment applications, where the saponins would increase microbial reactions in both aerobic and anaerobic systems, enhance the biodegradation of oils and fats and enhance oxygen transfer (Price *et al.*, 1987; Crober *et al.*, 1991; Wallace *et al.*, 1994; Rouchi, *et al.*, 1995; Makar 1996; Sen *et al.*, 1998; Anagaude, 1999). *Quillaja* saponins have also found use in photographic materials, in photosensitized film as a surfactant, and in the manufacture of high temperature oils (Prado *et al.*, 1986; Tanaka *et al.*, 1996).

Physicochemical properties of the saponin constituents

Quillaja saponin is an off-white powder that provokes sneezing. Upon being tasted, it first has a sweet taste and then becomes bitter. It is water soluble and produces a stable foam at high aqueous dilutions. *Quillaja* saponins are highly soluble in aqueous solutions of pH 5 and above and less soluble in solutions of lower pH due to their acidic moiety (glucuronic acid) (van Setten,1996; Kensil, 1996). The commercial saponin is obtained from the *Quillaja* bark and is usually a mixture of quillaic acid and *Quillaja* sapotoxin though frequently it also includes a non-toxic modified quillaic acid, produced during the preparation. Quillaic acid, $C_{19}H_{30}O_{10}$, has been obtained as a colorless, amorphous powder that is highly sternutatory (sneeze provoking). Its aqueous solution is acidic and has an acrid taste. When boiled in mineral acids it yields *Quillaja* sapotoxin, galactose and another sugar that is non-fermentable and is dextrorotatory (believed to be a mixture of glucuronic acid and xylose). *Quillaja* sapotoxin, $C_{17}H_{26}O_{10}$, is also colorless, amorphous, sternutatory and acrid (Wallis, 1967).

Saponin mixtures in suspension have stabilizing properties (van Setten,

1996) and membrane permeation enhancing properties that make them ideal for components in devising drug delivery systems. This has been demonstrated with delivery of insulin and amino glucoside antibiotics by nasal and ocular routes, both of which can be presently only administered by intravenous and intramuscular routes (Recchia *et al.*, 1995). The semi-synthetic *Quillaja* saponin DS-1 (having the fatty acyl portion removed) has been found to be more effective in drug delivery than the crude *saponaria* mixture (Pillion *et al.*, 1995; 1996). DS-1 has also been studied in intestinal drug transportation and been found to be a good candidate as an absorption enhancing agent that can be co-formulated with orally delivered biopharmaceuticals, especially those presently presenting absorption difficulties (Chao *et al.*, 1998).

1.4. Immunological properties of *Quillaja saponaria* Molina

Saponins from *Quillaja saponaria* Molina have been identified as potent adjuvants and have been studied most thoroughly in the form of Quil-A. This saponin mixture has been shown to augment antibody responses to both T-dependent and T-independent antigens and to produce antigen specific helper T-lymphocyte activity and memory (Frebbe and Mullen, 1986; Wu, 1982). Since their discovery, saponins were extensively studied to determine their general characteristics and properties. In addition to these, numerous other *in vitro* and *in vivo* studies were carried out to determine the biological effects of the different saponins. It was found that most of the biological activity of the saponins arose from their surface activity and their ability to form complexes with steroids and proteins (Burkey, 1996). It was during early studies that the adjuvanticity of saponins was

discovered in France in 1930 (Galea, 1932; Rielriu *et al.*, 1936; in Bomford *et al.*, 1992). The adjuvanticity was believed to be at least in part due to the combination of the surface and cholesterol binding activity of the saponins (van Setten and van de Werken, 1996), which results in an initial effect on the mucosal immune system of secretion of mediators (cytokines) into the circulation. The mediators then initiate a multiple of cellular events that results in an enhanced immune response (Chavali *et al.*, 1987; 1988).

Co-adjutants (Adjuvants)

A number of viral antigens, such as the foot-and-mouth virus, were found to have very low antigenicity when used alone. This could be attributed to several factors. For example, in the case of the foot-and-mouth virus, the low antigenicity, which is an apparent contradiction given its high molecular weight if compared to other strong antigens, such as diptheria toxin (which are smaller), was attributed to the homologous nature of this virus. In addition, the foot-and-mouth virus acquires host antigens during infection which renders the virus less virulent. As a result of these factors, the foot-and-mouth virus has low antigenicity, necessitating the use of co-adjutants such as aluminum hydroxide. Some high molecular weight carbohydrates possess very high antigenicity. Saponins, which are high molecular weight compounds that include carbohydrate moieties, possess high antigenicity because of their heterogeneity, in addition to their high molecular weights (Espinet, 1951). Espinet, in 1951, reported the results of some studies involving saponins (including *Quillaja* saponins) as immune stimulants for the foot- and- mouth vaccine

(Espinet, 1951). However, saponins had been in use as co-adjutants in veterinary vaccines, such as those for pleuro-pneumonia, rinderpest, sheep pox virus, etc., long before Espinet's foot-and-mouth experiment (Espinet, 1951).

In 1970, Dalsgaard demonstrated the superiority of *Quillalja* saponins as adjuvants over other saponins available commercially (Dalsgaard, 1970) and in 1974 he purified a substance from the extract of *Quillaja saponaria* Molina that had improved adjuvant activity in the foot-and-mouth disease vaccination with fewer side effects in the animals immunised. He named it Quil-A (Dalsgaard, 1974).

The structure of the sapogenin (aglycone) of *Quillaja* saponins have previously been partially described by Labriola and Deulefeu in 1969 (also refer sections **1.2.1.** pages 9 and 10 and **1.2.2.** page 19) .

In 1984, Morein and his co-workers reported the use of Quil-A (then commercially available as Spikoside and Iscotec AB) in immune stimulating complexes, abbreviated ISCOMs, which they demonstrated to possess a potent immune stimulating capability with fewer side effects when administered to large animals (Morein, 1984). However, these ISCOMs were found to be highly toxic to mice (Kensil *et al.*, 1991, Simms *et al.*, 1997; 1998).

In one study the antigenic protein concentration in the ISCOMs was found to influence the immunological activity of the ISCOMs in an inverse relationship (Kersten *et al.*, 1988b). Chavali and Campbell (1987) specifically demonstrated the capability of *Quillaja* saponins to evoke a very strong immunocompetence in animals when administered with antigens, rendering protection of up to 100% (in mice with rabies vaccine administered orally). They observed an induction of cytotoxic T-lymphocyte activity and a potent memory response, in addition to an

enhanced humoral response (measured by IgG antibody titre), when the antigen was administered in conjunction with *Quillaja* saponins. Interestingly, they also reported an induction of an increased and prolonged activity of natural killer cells (non-specific immunity) in mice fed saponin alone. Also, they reported an induction of substances they termed soluble factors that are known to be cytokines, and that, these soluble factors had regulatory activities on the activation, proliferation and differentiation of the lymphocytes (Chavali and Campbell, 1987a; 1987b; Chavali *et al.*,1987; 1988).

The need for adjuvants

It has generally been observed that the ingestion of inactivated or killed infectious agents does not always result in an immune response, and if an immune response is elicited, most of the time it will be weak and mainly humoral rather than cellular (Ogra *et al.*,1980; Genco *et al.*, 1983). Still, some antigens are naturally very poor immunogens when administered on their own, and need to be administered in conjunction with certain substances that can boost their immune response. In the early years few substances were known to potentiate the immune response of antigens. Known adjuvants included aluminium hydroxide, oil emulsion adjuvants such as, Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) (Freund, 1956; Hebert, 1967; Bomford, 1980), formol, a lipoid amine designated CP 20.96, muramyl dipeptide, and liposomes (Espinet, 1951; Anderson *et al.*, 1983; Genco *et al.*, 1983; Leclerc *et al.*, 1985; Tauban *et al.*, 1983). Even then, the use of these substances was not without limitations. They had variable responses ranging from moderately effective to ineffective, depending on the

antigen used and the mode of delivery. Some of them, e.g. FCA and FIA, also demonstrated such undesirable reatogenic effects that their use even in animals is presently questionable (Gupter *et al* 1993). FIA in particular has been noted to cause carcinogenicity in mice (Potter and Boyce, 1962; McCardle, 1964; Muray *et al.*, 1972). FCA have also been shown to generate auto-immune responses in some cases because of its capability to stimulate systemic immune responses (Gupta and Siber, 1995).

Aluminium compounds were the first adjuvants described in 1926. These adjuvants have the shortcomings of not being active with many antigens and stimulating mainly humoral immune responses (Audibert and Lice ,1993). They however remain the safest and only licenced adjuvants for human use (Powell and Newman, 1995). Recently, the development of highly purified subunit vaccines has made the research on adjuvants an imperative part of vaccine development, because of the weak immunogenicity of these vaccines (Pietrobon and Kanda, 1992). To date, more than 80 substances with adjuvant properties are either available for use or being investigated. A compendium of them has been compiled by Vogel and Powell (Vogel and Powell, 1995). Among the most recently introduced adjuvants are cytokine adjuvants (Gu *et al.*, 1991; Brewster *et al.*, 1991; Hora *et al.*, 1992;), muramyl dipeptide adjuvants (Allison and Byars, 1992), and saponin adjuvants (Clark *et al.*, 1991; Gupta *et al.*, 1993; Bussiere *et al.*, 1995).

Saponin adjuvants

Since the discovery of their adjuvanticity in 1930, saponins found extensive use as adjuvants in parenterally administered veterinary vaccines. Some of the

veterinary vaccines in which saponin adjuvants have been employed include foot-and-mouth desease vaccine, rabies vaccines, antimalarial vaccines, and vaccines for babesiasis and triponomiasis (Espinet, 1951; Schneider *et al.*1971; Dalsgaard, 1970; 1974; 1978; Wictor *et al.*, l973; Strobbe *et al.*, 1976; Black, 1977; Solyom *et al.*, 1980; McColm *et al.*, 1982; Soulebout *et al.*, 1985; Bomford *et al.*, 1988). The superiority of saponins as adjuvants over other adjuvants such as aluminium hydroxide, formol, etc. has been demonstrated by Espinet (1951), Rivenson (1958), Schneider and Bengelsdorf (1963), Strobbe *et al.* (1964), Black (1977) and other researchers. Dalsgaard (1970) has shown that *Quillaja* saponins are better adjuvants than other saponins. Campbell *et al.* (1985) and Maharaj *et al.* (1986) investigated the expansion of the use of saponin adjuvants (including *Quillaja* saponins) in oral vaccines. They used an oral rabies vaccine on mice. The immune response induced by *Quillaja* saponins has been reported to be very strong. QS-21, one of the purified *Quillaja* saponins, has been found to induce the production and to enhance the immunological response up to 11 fold in some mice infected with a polysaccharide antigen, E. Coli 055:B5 Polysaccharide (White *et al*, 1991). Maharaj *et al.* (1986) observed a much higher antibody titre that lasted for at least six months with an oral rabies vaccine administered in conjunction with *Quillaja* saponin, even longer than when the vaccine was administered intraperitoneally. The antibody titre was found to be even higher when saponin was given hours before the antigen. As adjuvants, saponins, including *Quillaja* saponins, have been found to stimulate the production of complement fixating antibodies (Strobbe, 1973). Frebbe and Mullen (1986) have extensively studied the responding cell population to the immune stimulation induced when Quil-A is used as an adjuvant. In 1992,

Newman demonstrated that QS-21 elicits both heightened antibody and MHC class I restricted cytotoxic T-lymphocyte responses (Soltysik *et al.*, 1993; Kensil *et al.*, 1991; Newman *et al.*, 1992). QS-21 has also been demonstrated to increase antibody titre to cancer antigens, rendering it of considerable potential as a cancer vaccine adjuvant (Soltysik *et al.*, 1993). It has been shown to induce the production of a required immune response. QS-21 induced the production of major histocompatibility complex (MHC) class I restricted cytotoxic T-lymphocytes (CTL) (also known as cluster of differentiation antigen 8 ($CD8^+$) T-lymphocytes) where initially only MHC class II restricted ($CD4^+$) T-lymphocytes were induced. A typical immune response experienced with soluble proteins such as the HIV-1 subunit vaccine was used to demonstrate this effect. The induction of CD8+ cytotoxic T-cell immune response provides longer lasting protection than when only CD4+ T-helper cells are induced (Wu *et al.*, 1992; Akashi *et al.*, 1990). In addition, the enhanced proliferative response produced when formulations containing QS-21 were used was found to be virus group specific (a very important issue especially in the development of HIV-1 vaccines because of its genetic variability). The introduction of virus group specific responses was found only when QS-21 was the adjuvant (Wu *et al.*, 1992).

To date, protective immunity has been attained for a variety of antigens including viruses, bacteria, mycoplasma and parasites. Table 3 gives a list of examples of antigens that have been experimented with in the studies of the adjuvanticity of *Quillaja* saponins.

Table 3: Various antigens whose immune responses have so far been potentiated by the use of the saponins of *Quillaja* Saponaria.

Class of antigens	Type of antigen	Disease it causes	Animal model used	Results obtained and references
Veterinary antigens *(Almost all of the vaccines listed here are used practically)*	Anthrax vaccine	anthrax	cattle, sheep, goats	enhanced immunity (Espinet, 1951)
	pleuro-pneumonia	pleuro-pneumonia	cattle, sheep, goats,	protective immunity attained (Espinet, 1951)
	Rinderpest vaccine	rinderpest acrid	wild animals	protective immunity attained (Espinet, 1951)
	Foot-and-mouth vaccine	foot-and-mouth	cattle	protective immunity attained (Espinet, 1951)
	Rabies vaccine (parenteral)	rabies	red-fox, cats, dogs, mice	protective immunity attained (Espinet, 1951)
	Oral rabies vaccine ***(used only experimentally)***	rabies	mice	enhanced immunity with 100% protection (Maharaj et al.,1986)

Human antigens *(All human vaccines are still only experimental)*	*E coli* 018 polysaccharide (has very low antigenicity and no response in children)	sepsis, traveler's diarrhoea, urinary tract infections	Mice	greatly enhanced immune response (11 fold) production of additional isotypes. (White et al., 1991)
	Neonatal meningitis a subunit vaccine	respiratory infections		enhanced immune response (Couhglin et al., 1995)
	Influenza virus strain PR-8	influenza	mice	enhanced immune response even so when QS-21 is in iscom form. ⇓toxic effects ⇓local irritation (Rönberg et al., 1995)
	HIV-1 (a subunit antigens gp120 and p24)	HIV/AIDS, viremia	mice	enhanced response group specific response (Wu, 1992)
	Hemagglutinin, measles virus	encephalitis	mice	(Morein, et al., 1998)
	Immuno-affinity purified protein *Trypanosoma cruzi*	lethal infection	mice	(Morein, et al., 1998)
	Herpes simplex Virus -Type1 (detergent-solubilized subunit antigen)	herpes simplex	mice and guinea pigs	enhanced immunogenecity and excellent protection against systemic or local challenge (Erturk et al., 1991)
	Fusion protein, measles virus	encephalitis	mice	(Morein, et al., 1998)

ISCOMs (Immune stimulating complexes)

ISCOMs are a specific type of antigen/adjuvant containing structures that were first described by Morein *et al.* (1984). They may be regarded as a delivery system of antigen and adjuvant, created to make antigens optimally immunogenic by presenting the adjuvant and the antigen in the same particle, thus reducing the amount of antigen and adjuvant required for efficiently enhancing the immune response. The antigen and the adjuvant are presented in the most physically immunogenic form (several copies of the antigen/adjuvant are presented in a sub-microscopic particle that resembles particles of an infectious agent). Because of the uniformity of the particles, the antigen is targeted to lymphatic organs and cells (Morein *et al.*, 1998).

The ISCOM structure is composed of a *Quillaja* saponin (or some other saponin) matrix, cholesterol and phospholipids in a molar ratio of 1:1:1. The *Quillaja* saponin forms micelles at its critical micelle concentration of 0.03%. When in micelle form the saponin avails hydrophobic regions for interaction with membrane proteins of the antigens and forms complexes with them. The final resultant structure is cage-like, has a diameter of about 35 to 40 nm and is made up of ringlike subunits 12 nm in diameter. The structure is physically stable and can be frozen, freeze-dried and suspended in various buffers.

ISCOMs were found to be at least ten times more potent as adjuvats than lipid micelles formed by membrane proteins alone. The antigen in the ISCOM must

have a hydrophobic domain; it can be an envelope protein of a virus, a cellular membrane protein, a peptide domain or many other antigens. A variety of viral antigens have been used in ISCOM formation and they include influenza virus rabies virus, herpes simplex virus, HIV-1 virus, and so forth. Different ratios of antigen to saponin have been shown to have different effects on the immune response and on the size of the ISCOM.

Other species containing immunologically active saponins

It is worth noting that the adjuvant activity is not entirely confined to *Quillaja* saponins alone. It has also been reported in saponins of other genera, among which are *Gypsophila* and *Saponaria* genera. These saponins are structurally related to *Quillaja* saponins (Price *et al.*, 1987), including the presence of branched sugar chains at positions three and 28, which are hypothesised to be very important for adjuvant activity (Bomford *et al.*, 1992). Two of the adjuvant active saponins from *Gypsohila* have been separated, purified, and partly structurally characterized. Glycyrrhizic acid, a glycoside from *Glycyrrhiza glabra* L. (Leguminosae) has been demonstrated to possess a more profound *in vitro* immunomodulatory activity than *Quillaja* saponins (Chavali *et al.*, 1987). Recently, separation of some adjuvant active saponins from the root of *Polygala senega* L. found in the prairies of the Canadian province of Saskatchewan has been reported. The authors reported the toxicity of these saponins, measured by lethality in mice, to be less than that of

Quil-A at the same dose (Estrada *et al.*, 2000). However, they still have not proved the usefulness of this saponin in ISCOM technology.

Pharmacological properties and toxicity of the *Quillaja* saponins

Saponins in general are powerful irritants, local anaesthetics and muscular poisons. Due to their local irritant properties when injected hypodermically they cause intense pain and when applied to the nose they cause sneezing. They cause vomiting, diarrhoea, and gastroenteritis when taken orally in large doses. Local application can be harmful to the motor and sensory nerves and may cause a condition of the muscles known as rigour mortis, in which the muscles become brittle and structureless. Poisoning which can lead to death, has symptoms like headache, vertigo, vomiting, hot skin, rapid and feeble pulse, progressive muscular weakness, and finally coma (Grieve, 1999). Saponins combined with lipids (cholesterins and the lecithins) in equimolar concentrations loose their toxicity thereby rendering them more useful. However in this state they still exert their hemolytic activity on erythrocytes. The lipid membrane of the red blood cells develops holes and leak out hemoglobin. The amount of hemolysis, however, is only a physical measurement and is not indicative of the systemic toxicity of the saponin, as other factors such as phagocytosis, absorbability, gastric decomposition, and plasma cholesterol neutralization play a role in the overall toxic effect of a saponin (Kobert, 1911). This statement holds true for *Quillaja* saponins as well (Kensil, 1996).

Kensil *et al.* (1991), have studied the toxic and hemolytic activity of the *Quillaja* saponins. They measured toxicity in terms of lethality to mice. They found that Quil-A was lethal to mice at concentrations of 100-125 micrograms/kg body weight. The purified saponin QS-18 was the most lethal (at concentrations of 25 micrograms/kg body weight). It is also the most abundant in Quil-A. QS-21 and QS-7 had a large minimum toxic concentration(mtc)/minimum inhibitory concentration(mic) or minimum effective concentration(mec) ratio (mtc/mic (mec) ratio also known as therapeutic index (TI)). QS-21 was found to be the most hemolytic, while QS-7 was the least hemolytic. No correlation was found between hemolytic activity, lethality, and adjuvant activity. QS-21 which has higher hemolytic activity than QS-18 is comparatively less lethal to mice. In a study of toxicity and side effects of adjuvant formulations conducted by Simms *et al.* (1997; 1998), in their research of immune responses induced by different adjuvants administered with the herpes virus type-1 subunit vaccine, out of about five different adjuvants lethality was observed only in a group of mice receiving ISCOMs. In other words, ISCOMs were found to be the most toxic adjuvants in this experiment utilizing this particular animal model. However this was not the case when guinea pigs were used (Simms *et al.*, 2000; 2002). It is therefore quite apparent that the toxicity of the saponin adjuvants is an issue of great concern in the development of saponins as commercial veterinary and human vaccine adjuvants. However, other studies have been conducted which did not show any significant toxic effects in short-term feeding studies in rats (Gaunt *et al.*, 1974) and long term toxicity studies in mice

(Phillips *et al.*, 1979). The toxicity has been found to be less when the saponins are administered orally rather than parenterally.

Saponin safety by oral route has been evaluated on food grade *Quillaja* extracts. No carcinogenic effects or change in mortality rate were observed in these studies. Only a decrease in weight gain and antihypercholesterolemic effects were observed. The decrease in weight gain was attributable to some extent to the unpalatability of the saponin extract treated food. However, it is believed that the cumulative amount of the saponin to be used in immunization would be much lower than the concentration used in toxicity studies and that the two issues mentioned earlier (decreased weight gain and antihypercholesterolemic effects) would not be a problem at all (Kensil, 1996).

Local reactogenicity in parenterally administered vaccines with *Quillaja* saponins has been observed but disappears after about two weeks postimmunization. Incorporation into ISCOMs considerably reduces systemic toxicity and local irritation. Still a tremendous reduction in toxicity has been observed with Iscoprep 703, a fractionated *Quillaja* saponin (Sjölander *et al.*, 1997; Coulter *et al* 1998) . Safety studies in these saponins are still being carried out, and the latest reports on this subject are showing promising results (Sjölander *et al.*, 1997; Coulter *et al* 1998; Deliyannis *et al.*, 1998; Verschoor *et al.*, 1999; Harrison *et al.*, 1999).

Non-immunological studies involving *Quillaja* saponins

Several other studies involving *Quillaja* saponins have been carried out. Another saponin study demonstrated that *Quillaja* saponins from various commercial sources differed in their biological activity, with respect to their modulation of microbial growth in natural and artificial fermentation; this was measured by their effects on the growth of *E. coli* (Sen *et al.*, 1998).

One such study reported an observed drug interaction between *Quillaja* saponins and a compound abbreviated TET (tetradine), which is a calcium ion antagonist of Chinese herbal origin (Leung *et al.*, 1997). TET has been demonstrated to have antihypertensive and antisilicotic effects in experimental models and clinical trials (Gao *et al.*, 1965; Qian *et al.*, 1983; Leung *et al.*, 1988). TET was found to enhance membrane permiabilization effects of the *Quillaja* saponins.

Other studies involved the exploration of the micellar properties of *Quillaja* saponins in order to provide information for utilization as emulsifiers and to investigate the potential role of *Quillaja* saponins in the development of micellar based extraction process for the removal of cholesterol from milk (Mitra and Dungan, 1997) and for lowering plasma cholesterol levels in humans and animals.

Studies on rumen fermentation and *Quillaja* saponins are geared towards investigating the ammonia-binding effects of the saponins, which can be beneficial in alleviating the problem of ammonia emissions from excreta (Markar and Becker,

1997). Scientists point out that addition of saponins to animal feed will alter rumen fermentation, promote higher animal production and reduce environmental pollution by lowering the emission of polluting gases like carbon dioxide, methane and ammonia (Markar *et al.*, 1998). The potential applications of *Quillaja* saponins and other saponins for this purpose is currently being investigated.

Mechanism of action

Surface activity and cholesterol binding activity are in part responsible for the adjuvanticity (van Setten and van de Werken, 1996; Hostettman and Marston, 1995). The enhancement of the immune responses by *Quillaja* saponins in oral vaccines (oral rabies vaccine as the model) was postulated by Maharaj *et al.* (1986) to be a result of: protection of the antigen from degradation by digestive enzymes; complexing with the enzyme to present it in a more immunologically active form; and, stimulation of the interaction of the antigen with the lymphocytes by increasing the permeability of the intestinal membranes.

A detailed overview of the studies on the mechanism of action of *Quillaja* saponins has been carried out by Kensil (1996). A summary of this overview is given below. *Quillaja* saponins do not cause the formation of a depot of antigen, as is proposed as one of the mechanisms of action of other adjuvants like FIA. Instead, studies have shown that the *Quillaja* saponins act by increasing the following: the lymphocyte homing to injection site (free saponin); attraction of polymorphonuclear

cells to the injection site (ISCOMs); interferon gamma and interleukin-2 production; and, MHC class II expression on macrophages and antigen processing and presentation by macrophages. Researchers including Strobbe *et al.* (1973), Wu *et al.* (1992), Soltysik *et al.* (1995), and van Setten and van de Werken (1996), have in the course of their studies of the *Quillaja* saponins suggested mechanisms of action of these compounds that corresponded to the ones mentioned in the summary above. The recent studies of Hoshi and his co-workers (1999), on this subject and the review of Morein (1998), emphasize the increased cytokine and chemokine production to be responsible for the enhanced immune responses induced by the *Quillaja* saponins. These findings have been confirmed by other researchers (Spickler and Roth, 2003; Francis *et al.,* 2002 and McCluskie and Weeratna, 2001; Rao and Gurfinkel, 2000; Oda *et al.*, 2000 and Ragupathi *et al.*, 2000).

Cytokine induction by *Quillaja* saponins

The development of an effective immune response involves the lymphoid cells, the inflammatory cells and the hematopoetic cells. The complex interactions among these cells are mediated by a group of low molecular weight regulatory proteins or glycoproteins, collectively designated as cytokines. These substances are secreted by white blood cells and various other cells in response to a number of stimuli. These cytokines assist in regulating the development of the immune

effector cells, while others possess direct effector functions of their own. In general, cytokines serve as messengers of the immune system (Goldsby *et al.*, 2000). The cytokine interleukin-1 is the first cytokine released by activated macrophages in the response to antigens.

An effective immune response requires mainly two major groups of cells: lymphocytes and antigen presenting cells (APCs). B-lymphocytes and T-lymphocytes are the two major populations of the lymphocytes. While the B-lymphocytes can recognize antigen on its own through their B-cell receptors, the T-cell receptors can recognize only antigen that is bound to cell membrane proteins called Major Histocompatibility Complex (MHC). MHC molecules that function in this antigen presentation event are polymorphic glycoproteins found on cell membranes and are divided into two major classes: class I MHC molecules, expressed by nearly all nucleated cells, and class II MHC molecules, expressed only by antigen presenting cells. Antigen presenting cells include macrophages and monocytes: (macrophage precursors), B-cell lymphocytes, dendritic cells and various other cells.

The effects of adjuvants, including *Quillaja* saponins, on the two major populations of the lymphocytes (the B-lymphocytes (B-cells) and the T- lymphocytes (T-cells)) have been studied to a great extent by different researchers. The recent studies are those as mentioned in: Spickler and Roth, 2003; Ghosh *et al.*, 2002; Qiao *et al.*, 2003; Marchand *et al.*, 2003; Francis *et al.*, 2002; Marcian *et al.*, 2002;

Lunden *et al.*, 2002; Liu *et al.*, 2002; Huber *et al.*, 2002; Paykari *et al.*, 2002; Slingluff *et al.*, 2001; Berndart *et al.*, 2001; Diwan *et al.*, 2000; Hu *et al.*, 2001; Evans *et al.*, 2001; Shu *et al.*, 2001; Rao *and* Gurfinkel, 2001; Boyaka *et al.*, 2001; Hancock *et al.*, 2000., da Fonseca *et al.*, 2000; Shu *et al.*, 2000; Sabbatini *et al.*, 2000; Behboudi *et al.*, 1999; Sjölander and Cox, 1998; Cox *et al.*, 1998; Moore *et al.*, 1999; Sjölander *et al.*, 1998; and many others.

Two well defined sub-populations of T-cells are well recognized: T-helper (Th) cells that generally display CD4 molecules on their membrane, and T-cytotoxic (Tc) cells that generally display CD8 molecules on their membranes. A third type of T-cells, T-suppressor (Ts) cells, have been previously postulated, however, recent evidence suggests that it may not be distinct from the Th and Tc sub-populations (Goldsby *et al.,* 2000). A Th-cell recognizes only antigen bound to the MHC class II molecule and upon activation it secretes various cytokines which then play an important role in the activation of B-cells, T-cells, macrophages and various other cells that take part in the immune response including non-specific effector cells called natural killer (NK) cells. Tc-cells on the other hand are activated by antigen bound to MHC class I molecules and upon activation they proliferate and differentiate into cytotoxic T-lymphocytes (CTL) whose vital function is in monitoring the cells of the body and directly eliminating any virus infected cells, tumor cells, cells of a foreign tissue graft, or any other cells that display antigen.

Differences in the cytokines produced by Th- cells result in different types of immune responses. A Th-1 response produces a cytokine profile (IL-2, IFN-γ,

TNF-β) that supports mainly the following biological activities: inflamation; Th and Tc- cell proliferation, NK and CTL activity, inhibition of viral replication; activation of macrophages, increased expression of class I and class II MHC molecules; induction of class switch to IgG2a antibodies by proliferating B-cells and blockade of IL-4 induced class switch to IgE and IgG1 antibodies; increased phagocytosis and cytotoxicity and reduced Th-2 activity. A Th-2 response on the other hand produces a cytokine profile of IL-4, IL-5, IL-6, and IL-10, which in turn supports the following biological functions; B-cell proliferation and differentiation; class switch to IgG1 and IgE antibodies; IgA production; eosinophil growth and differentiation; plasma (a B-lymphocyte) cell production; antibody secretion; suppression of cytokine production by macrophages and therefore indirect reduction of cytokine production by Th-1 cells, and down regulates class II MHC expression by antigen presenting cells.

The cytokine IL-1 is mainly secreted by monocytes, macrophages, B-cells dendritic cells, endothelial cells and other cell types, and its activity is on the Th-cells; (co-stimulation of activation), B-cells (promotion of maturation and clonal expansion), NK cells (enhanced activity), macrophages and neutrophils (increases chemotactic attraction), and so forth. Hence, the measurement of the IL-1 induction activity of a substance is a very good indicator of its immunological activity. Studies on the cytokine induction properties of the *Quillaja* saponins have been carried out by various researchers, and the general belief is that they are able to induce both Th-1 like and Th-2 like responses, sometimes even equally (Sjölander *et al.,* 1998; Sasaki *et al.,* 1998; Simoes *et al.*, 1999; Smith *et al.*, 1999; McNeal *et al.*, 1999; Gosh *et al.*, 2002).

1.5. Other studies performed on the saponins of *Quillaja saponaria* Molina

It appears that since 1996, much of the work done in relation to *Quillaja* saponins was that concerning their immunostimulatory activity and ISCOM formation. Only a few structure elucidation or structure function relationship studies have been encountered (Steinbeck *et al.*, 1995; Rönberg *et al.*, 1995; Kensil *et al.*, 1996; So *et al.*, 1997; Nord and Guo *et al.*, 1998; Kensil *et al.*, 1998; Kenne,1999; Nyberg *et al.*, 1999).

Structure/function relationship studies

The early studies in structure activity relationship performed on QS-21 revealed that the aldehyde group at the C4 position in the triterpene was important for adjuvant activity and that it may actually be important for the adjuvanticity. Modifications at the carbonyl group of the glucunonic acid did not abolish the antibody stimulating activity. However, the required dose was raised substantially. The induction of cellular immunity was lost in most of the derivatives of the glucononic acidl (Soltysik *et al.*, 1995).

Studies have shown that DS-1, a deacylated saponin from *Quillaja saponaria,* differing from QS-21 only by not having the fatty acyl moiety, was inactive as an adjuvant (Kensil *et al.*, 1992), suggesting the possibility of the adjuvant activity of QS-21 residing within the fatty acid domain. However, Kensil *et al.* (1996) isolated this moiety and evaluated it for adjuvanticity. The fatty acyl moiety was found to be inactive as an adjuvant, leading to the conclusion that the

acyl moiety was essential for the adjuvant activity but was not sufficient on its own. The positioning of this acyl moiety was shown not to be crucial by Cleland *et al.*. (1996) who demonstrated that the domain in QS-21 underwent reversible migration from the 4-hydroxyl group of the fucose to the 3-hydroxyl group and that both isomers were biologically active.

Immunological studies

There has been a considerable number of studies on the immune stimulating activity of the *Quillaja* saponins. Most of the studies conducted used the saponins in ISCOM form as it was the form reported to elicit larger immune responses at minute concentrations of the saponins and, as a consequence, with minimal or no toxic effects in the model animals (Morein *et al.*, 1984). In most studies ISCOMs were found to have a much higher immune stimulating activity than other adjuvants such as Freund's Complete Adjuvant (FCA), Freund's Incomplete Adjuvant (FIA), allum adjuvant and so forth (Leenaars *et al.*,1994; Steineker *et al.*, 1995; Gupta and Siber, 1995; Rubbuelo *et al.*, 1995; Sjolander *et al.*, 1996; Hoshi *et al.*, 1998; Jiang *et al.*, 1999; Simms *et al.*, 1997). Interestingly, the non-ionic surfactant vesicle (NISV) delivery system, an adjuvant system described by Brewer *et al.* (1992), was found to induce greater lymphoproliferative responses than ISCOMs. However, ISCOMs have been found by other researchers to be superior in eliciting protective immunity (Deliyannis *et al.*, 1998; Cox *et al.*, 1998; da Fonseca *et al.*, 2000; Stittelaar *et al.*, 2000).

In almost all the antigens tested with ISCOMs, the isotypes mostly produced

were IgG1 and IgG2a, indicating both Th-1 and Th-2 responses (Kenney *et al.*, 1989; Ahmeida *et al.*, 1991; ten Hagen *et al.*, 1993; Hassan *et al.*, 1996; Sjölander *et al.*, 1996; 1997a; 1997b; 1999; Francis *et al.*, 2002; Connolly and Hill, 2002; Qiao *et al.*, 2003). However, some IgG2b and IgG3 antibodies are produced with some antigens, like influenza virus but not in large quantities, indicating the presence of some degree of Th-2 response for these antigens (Lövgren, 1988; Villacres-Erikson, 1992; Sjölander, 1996). IgG2a antibodies are very important in viral and parasitic infections as they activate complement, induce antibody dependant cellular toxicity and have been reported to have the greatest antigen neutralising capacity (Spiegelberg *et al.*, 1974; Kipps *et al.*, 1984; Coultier *et al.*, 1987; Hassan *et al.*, 1996; Sjölander *et al.*, 1996). ISCOMs are very efficient in inducing the proliferation and differentiation of B-cells that secrete antigen specific IgG antibodies (Antibody Secreting Cells). These ASCs were found to be localised in several organs including the draining lymph nodes, spleen and the bone marrow and were also found to persist for a long time. In addition, the B-cell memory induced by ISCOMs was found to be very potent (Höglund *et al.*, 1989; Classen and Osterhause, 1992; Sjölander *et al.*, 1996).

In addition to induction of humoral immune responses the administration of antigens in ISCOMs has been found to affect the resulting immune response in other aspects including activation of regulatory T-helper cells as demonstrated by the production of IL-2 and IFN-γ (Th-1 response) (Villacres-Erikson *et al.*,1992; Valensi *et al.*, 1994; Fossum *et al.*, 1994; Sjolander *et al.*, 1997a; 1997b), induction

of cytotoxic $CD8^+$ T- lymphocytes (Takashi *et al.*, 1990; van Binnendijk *et al.*, 1992; Lipford *et al.*, 1993), and induction of the activity of natural killer (NK) cells which is observed when *Quillaja* saponins are administered alone (Chavali and Campbell, 1987). ISCOMs have also been demonstrated to enhance the surface expression of MHC class II molecules on Antigen Presenting Cells (APCs) (Takashi et a., 1990; van Binnendijk *et al.*,1992). Studies have shown that the T-cell memory response induced by ISCOMs was also very potent (Sjölander *et al.*, 1997a). The saponins up-regulated the Th-1 and Th-2- like responses equally (joint responses) (Jiang *et al.*, 1999). In one study, Dotsika *et al.*, (1997), demonstrated that responses generated by ISCOMs can be manipulated by altering the composition of the triterpenoid saponin in the ISCOM, and that the levels of the saponin in the ISCOM can determine the type of T- cell response produced, whether Th-1-like or Th-2-like (that is humoral or cell mediated responses).

Latest developments in the research on the saponins of *Quillaja saponaria* Molina

It has recently been reported that an ISCOM formulation containing a mixture of fractions A and C (QH-A and QH-C), previously fractionated through reverse phase HPLC by Rönberg *et al.*, (1995), has highly promising properties of an ideal adjuvant. The formulation designated QH-703 (ISCOPREP 7.0.3™), consists of 70% QH-A and 30% QH-C, and lacks QH-B, the fraction found to be the most toxic of the three. The formulation was found to have strongly similar adjuvant properties compared to the semi-purified Quil-A but without the toxic effects of the

Quil-A. Interestingly, the major factor of this formula (QH-A) was initially thought to be immunologically inert, but it turned out to be a potent inducer of T-cell proliferation and production of the type I cytokines. It was found that the QH-A / QH-C ISCOMs induced higher levels of IgG2a antigen specific antibodies than those induced by QH-C ISCOMs on their own. Fraction QH-C has been demonstrated to strongly induce the production of IgG2a antibodies, which is indicative of a Th-1 response, and the production of the cytokines IFN-γ, TNF- β, 1L-2, and IL-3 (Johansson *et al.*, 1999). ISCOPREP 7.0.3™ has been developed for human use and is presently undergoing clinical evaluation (Villacres-Eriksson *et al.*, 1997; Behboudi *et al.*, 1996; Sjölander *et al.*, 1997; Behboudi *et al.*, 1997; Johansson *et al.*, 1999).

More structural characterization of *Quillaja* saponins is being carried out. The latest encountered is that of Nord and Kenne *et al.* (1999), who separated and characterized six fractions using mass spectrometry (MS), NMR and chemical methods. The latest number of saponins reported to be detected by MS from the *Quillaja* saponin bark is 60 (van Setten *et al.*, 1998). Behboudi *et al.* (1999) have demonstrated that the *Quillaja* saponins stimulate B and T-cell responses differently and to various degrees (i.e., T-h1 and Th-2 responses). The fraction QH-A is a potent stimulator of T-cell responses while fraction QH-C primarily stimulated B-cell responses. As such, it would be possible to develop vaccine formulations with defined characteristics according to a particular need. QH-A, QH-B, and QH-C are *Quillaja* saponin fractions which have been clinically and functionally defined. Their

use in ISCOMs has been extensively studied and a combination of QH-A and QH-C in influenza vaccine is currently under Phase 1 and Phase 2 clinical studies for humans (Romberg *et al.*,1995; Romberg *et al.*, 1997). QH-B and QH-C have strong adjuvant activity but they are also highly toxic, QH-B being the most toxic. Modification of the carbohydrate chain of QH-B was performed in an attempt to investigate the possibilities of the reduction in toxicity of this compound. The outcome was that the toxicity was reduced by this modification (carried out by oxidation with periodate) but so also was the adjuvanticity (Romberg *et al.*, 1997). The modified products, although they retained the cholesterol binding capacity, formed saponin-lipid complexes uncharacteristic of ISCOMs.

Furrier *et al.* (2002) have investigated (with positive outcomes) the possibility of intestinal activity of Quil-A ISCOMs in modulating local innate immune responses and antigen uptake. They used the protein antigens, ovalbumin, delivered orally. While Iosef *et al.* (2002) evaluated the systemic and intestinal antibody secreting cell responses and protection of a recombinant RFVP2/WaVP6 rotavirus-like-particle (2/6VLP) oral vaccine using ISCOMs in a gnotobiotic (Gn) pig model of human rotavirus (HRV) disease. The 2/6VLPs adhered to the ISCOM-matrix (2/6VLP-ISCOM) and were antigenic, but they failed to induce protection. However, when combined with attenuated (Att) HRV for oral priming, the 2/6VLP-ISCOM vaccine was effective as a booster and induced partial protection against virulent Wa HRV.

Bungener *et al.* (2002) and Chen *et al.* (2002) have reported studies in which other means of antigen presentation for the purposes of vaccine development were investigated and in which ISCOMs were included in the evaluative studies of these methods against others. Both groups of researchers reported the Quillaja saponins to perform well as adjuvants, though not the best in either of these studies. Reconstituted viral envelopes (virosomes), specifically fusion-active influenza virosomes, were found to perform as a better antigen delivery system than ISCOMs for the protein antigen ovalbumin (Bungener *et al.*, 2002). The activities were reported as 25, 5, and 0.7 (proportions) for liposomes, ISCOMs and virosomes respectively. Chen *et al.* (2002) on the other hand have reported that Semliki Forest virus particles (self-abortive viral particle that express respiratory syncytial virus (RSV) F and G proteins from an RNA replicon) produce a slightly better immune protection when administered alone than when these viral proteins were administered in ISCOM formulation (Hu *et al.*, 1998 and Chen *et al.*, 2002). The works of Robson *et al.* (2003) and Beacock-Sharp *et al.* (2003) suggest that dendritic cells may be the principal APCs responsible for the priming of CD8+ T-cells by ISCOMs *in vivo*. They further suggest that targeting the ISCOMs to the activated dendritic cells may further enhance their activity. Also among more recent studies involving ISCOMs are different methods of ISCOM construction and incorporation of various antigens (Konnings *et al.*, 2002; Guan *et al.*, 2002) and attempts at the development of synthetic and semi-synthetic compounds resembling

Quillaja saponins but without their toxicicity and stability problems (Marciani *et al.*, 2000; Kim and Gin, 2001). The semi-synthetic *Quillaja* saponin analog, GPI-100, has been reported to stimulate an antibody profile that corresponds to a Th-1 type immune response as well as to induce CTL production against exogenous antigens (Marciani *et al.*, 2000).

Clinical and veterinary applications of *Quillaja saponaria* Molina

Preclinical studies have undoubtedly proven that *Quillaja* saponins are useful as adjuvants in higher species for stimulating humoral and cell mediated immune responses.

Vaccine applications

The application of *Quillaja* saponins as adjuvants has highly promising uses in a large number of vaccines, including viral vaccines, bacterial vaccines, parasitic vaccines (such as against *P. yoelii*), and cancer immunotherapy (for example, in Thomsen Friedenreich (TF) antigen found in epithelial cancer cells). Some anticancer vaccines with *Quillaja* saponin adjuvants have proceeded to clinical trials. QS-21 in a Phase I clinical study of a melanoma immunotherapeutic vaccine was found to enhance antigen-specific IgM and IgG responses (Livingston *et al.*, 1994; Kensil *et al.*, 1996). In veterinary vaccines, the *Quillaja* saponins have

already found widespread use. ISCOMs have been used extensively in viral vaccines. ISCOMs consisting of feline leukemia virus gp 70, HIV-1 virus, influenza virus, bovine leukemia virus, HSV-1 virus, to mention a few, have been tested with promising results in animal models. The purified QS-21 has also been tested in viral vaccines in its conventional form and the results were highly promising.

In addition, bacteria vaccines such as *S. pneumoniae*, *E. coli*, *N. meningitidis* have been tested with Quil-A. The induction of CTL responses by *Quillaja* saponins has important consequences in the protection against intracellular bacteria such as *Mycobacterium avium*, *Listeria monocytogenes* and others. A number of routes of administration for the saponins in vaccine potentiation have been tried in preclinical research including nasal and rectal routes (Kensil *et al.*, 1996).

Clinical developments and trials

In 1994, Livingston *et al.* reported a clinical trial being carried out on the adjuvant QS-21 with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma. The results reported from this trial were favourable (Livingston *et al.*, 1994) and these authors were the first to report a Phase I clinical trial of a purified *Quillaja* saponin in humans (van Setten and van de Werken, 1996; Cleland et al., 1996). Another melanoma vaccine Phase I clinical trial has been carried out to evaluate the adjuvant activity of QS-21 in a vaccine containing the antigen MELIMMUNE-1 (a high molecular weight melanoma

proteoglycan chondriotin sulfate that mimics a murine anti-idiotype antibody). An influenza ISCOM vaccine prepared from a mixture of two of the fraction QH-A and QH-C, previously isolated and characterized by Rönberg *et al.* (1995), is presently in human clinical trials (Barr and Mitchell, 1996; Sjölander *et al.*, 1997a). The results of this trial are not yet available, however, reports of non-human primate studies conducted by an Australian company known as CSL Limited in collaboration with Chiron Corp of USA have shown promising results (Sjölander *et al.*, 1997a; 1997b; Coulter *et al.,* 1998; Deliyannis *et al.*, 1998; Verschoor *et al.*, 1999). The product being tested is called ISCOPREP 7.0.3™.

Quil-A itself has not been tested in clinical trials of human vaccines. The first purified saponin to be tested in clinical trials was QS-21 after being first evaluated in preclinical vaccine testing in rabbits, dogs, monkeys and baboons and in a vaccine for cats. QS-21 has now been tested in Phase I and II clinical trials for melanoma vaccines and HIV-1 subunit vaccines (Kensil, 1996). The early Phase I studies suggest that saponin adjuvants may be effective adjuvants in cancer immunotherapy and other vaccines. Other clinical trials of QS-21 and of other purified saponins such as QS-7, QH-A/QH-C (ISCOPREP 7.0.3™) with other types of antigens such as HIV-1, influenza, herpes simplex virus, and so forth, are underway (Kensil, 1996; Sjölander *et al.*, 1997a; 1997b; Coulter *et al* 1998; Deliyannis *et al.*, 1998; Verschoor *et al.*, 1999).

Most of the recent literature only indicate that clinical trials with ISCOPREP 7.0.3™ are in progress (e.g., Singh and O'Hagan, 2002 and Kersten and

Crommelin, 2003; Iconova, 2003; Vogel *et al.,* 2003), however, no results are available so far. There could be many reasons for this absence of results. One reason could be that some clinical trials may take a long time to conduct and complete. Another possibilty is that unfavorable results are being encountered. This speculation is definitely not unfounded as evidenced by the report of Potter and Jennings (2003), whose study attempted to find an intelligible explanation for the fact that, although animal studies have shown ISCOMs to be among the most potent adjuvants (Ben-Ahmeida *et al.*, 1993; Ghazi *et al.*, 1995; Stieneker *et al.*, 1995; Sjölander *et al.*, 1996; Coulter *et al.*, 1998; and Deliyannis *et al.*, 1998), the results obtained from human volunteers were disappointing (Martin, 1996). Potter and Jennings argued that animal and volunteer models are not comparable and postulate the underlying cause of the differences in the results obtained from animal and human studies to be due to the fact that ISCOMs possibly would only work better in un-primed subjects, at least with regard to antibody production while using influenza virus as the antigen (Potter and Jennings, 2003). Studies of superlative adjuvant activity of ISCOMs by previous researchers were carried out chiefly on un-primed mice, while volunteers are mostly primed by a previous infection and or previous immunization. Of course, the extrapolation of the findings of Martin (1996), and Potter and Jennings (2003) to ISCOPREP 7.0.3™ (and or other antigens) may not be correct, (Barr *et al.*, 1998; Kersten and Cromelin, 2003;Vogel *et al.*, 2003) as this product has a different saponin composition to that of ISCOM™ (Cox *et al.*,

1997; Isconova,2003) used by Potter and Jennings.

Still, the poor results obtained with *Quillaja* saponins in human studies could be attributed to the degradation of these products, *in vivo* or during vaccine storage to deacylated products which have been shown to possess less or no immunogenic properties (Kensil *et al.*,1992; Marciani *et al.*, 2002a).

Interestingly, this notion is being challenged by Liu *et al.*, (2002) who have found DS-1 (a deacylated *Quillaja* saponin) to induce a better Th-2-like response (induced higher IgG1 antibody production) than QS-21. However DS-1 was inactive in inducing IgG2a or CTL responses (Th-1 response). Marciani *et al.* (2002b) are, however, questioning the authenticity and nature of the QS-21 used by Liu *et al.* as (according to them) it appeared to have been produced under sub-optimal conditions.

In addition, other results from studies of the adjuvanticity of QS-21 and ISCOMs (in comparison to other saponins and other substances) have that these substances did not perform as well as previously reported (Ciarlet *et al.*,1998; Estrada *et al.*, 2000; Liu *et al.*, 2002; Iosef *et al.*, 2002; Bungener *et al.*, 2002a; 2002b; Chen *et al.*, 2002). In the study of Ciarlet *et al.* (1998) the activity of QS-21 was found to be inferior to that of Freud's adjuvant for virus-like particles (VLPs) administered parenteraly to rabbits in conjunction with these agents. VLPs are currently being investigated as a rotavirus vaccine (Ciarlet *et al* 1998). However, such results are few when compared to the number of studies that show *Quillaja*

saponins (including QS-21 and ISCOMs) to be adjuvants with phenomenal activity.

In spite of the few drawbacks mentioned earlier, *Quillaja* saponins are still being reported to perform superlatively as adjuvants in conjunction with various antigens. Reports of numerous studies: experimental animal studies, preclinical animal studies and clinical trials at different stages appear to be constantly being published, whereby, QS-21, Quil-A, and various purified fractions of Quil-A in their conventional (solution) and ISCOM formulations are being tested with different viral and bacterial vaccines. Many show favorable results, to mention a few: Marchand *et al.*, 2003; Qiao *et al* ., 2003; Furrie *et al.*, 2003; Heath *et al* 2003; Chen *et al.,* 2002; Choi *et al.*, 2002; Paykari *et al.,* 2002; Huber *et al.*, 2002; Slingluff *et al.*, 2001; Boyaka *et al.*, 2001; Sjölander *et al.*, 2001; Evans *et al.*, 2001; Hu *et al.*, 2001; Hancock *et al.,* 2000 Shu *et al.,* 2000; da Fonseca *et al*, 2000; Sabbatini *et al* 2000; Stittelaar *et al.*, 2000; Ragupathi *et al.*, 2000; Adluri *et al.*, 1999; Ennis *et al* 1999; McNeal *et al.*, 1999; Rimmelzwaan *et al.*, 1999; Livingston *et al.*,1998; Sjölander *et al* 1997b; Sasaki *et al.*,1998; Clelland *et al.*, 1998; Dotsika *et al.*, 1997; Rimmelzwaan *et al.*, 1997; Burns *et al.*, 1997; Donnelly, 1997. There have been over 300 publications on the subject since 1997.

Chapter 2: Research Plan

2.1 Scope

The major challenges in the research of *Quillaja* saponins as adjuvants have been:

Firstly, the difficulty in the separation of the crude extract into individual components, due to the amphiphillic nature of the components and their tendencies to form mixed micelles.

Secondly, the obtainable amount of each individual component during a separation is very small. This means that, the conventional rigorous, structural elucidation strategy involving NMR and MS is time consuming and difficult to implement. Our yields ranged from 0.05% to only 3% of the original crude extract when separated on a preparative column.

Use of organic solvents in adsorption and reverse phase HPLC however, has permitted the purification of several adjuvant active saponins from the extract to near homogeneity. Compounds such as DS-1, DS-2 QS-III, QS-21, QS-7, QS-17, QS-18, QS-L1, and fractions such as QH-A, QH-B, QH-C have been successfully separated and characterized (albeit some only partially).

Several elution protocols have been documented in the literature. In our lab, a method was developed from the modification of one of the published protocols (Higuchi *et al.*,1987; 1988) and the best results for analytical separation was obtained through gradient elution with a buffered acetonitrile and water mixture from a C5 reverse phase HPLC column. This method has been modified for large scale separation.

Depending on the method of separation and its efficiency, the number of components resolvable chromatographically has differed from one research group to another resulting in a very wide range of 23-60 as reported in the literature. Our separation has resolved the material into 80 components by adsorption reverse phase liquid chromatography/mass spectrometry (LC/MS) and an average of 70 peaks by reverse phase preparative HPLC.

To date only about eleven of these saponins out of a total of over fifty saponin peaks separable by HPLC, have been fully structurally characterized. Extensive immunological studies have been conducted on both Quil-A and QS- 21 (a purified *Quillaja* saponin having a molecular weight of 2012 (1989; without sodium) and a defined structure elucidated by Kensil *et al.* in 1991). While the use of Quil-A as an adjuvant is well established in veterinary medicine, its use in humans has not been established due to its toxicological effects and the fact that its composition cannot be standardized from batch to batch.

Some of the *Quillaja* saponins with documented chemical structures and, for some, their biological activity are included in Table 4.

Clearly, *Quillaja* saponins with fully characterized chemical structures and biological activities could be invaluable tools in vaccine research, especially so in the research and development of modern subunit vaccines which, despite their notable advantages over whole organism counterparts, possess the disadvantage of being weak immunogens and necessitates that adjuvant research be an integral part of vaccine research.

Table 4: Some *Quillaja* saponins with documented chemical structures

Compound	Molecular weight	Reference	Comments
DS-1	1512	Higuchi *et al.*, 1987	desacylsaponin
DS-2	1696	Higuchi *et al.*, 1987	desacylsaponin
QS-III	2296	Higuchi *et al.*, 1988	same as QS-17
QS-7	1886	Kensil *et al.*, 1991	
QS-17	2321	Kensil *et al.*, 1991	same as QS-III
QS-18	2150	Kensil *et al.*, 1991	
QS-21	2012 (+Na) 1989	Kensil *et al.*, 1991	most immunologically active
QS-L1	956	So *et al.*, 1997	

2.2 Study Objectives

The aim of this study has been to separate to purity (or near purity) some of the saponins of *Quillaja saponaria* Molina compounds, and to determine their structural identity and biological activity. Thus, this study has been comprised of two components: to isolate and determine the structure of the purified components, and to examine the toxicity, hemolytic activity and the adjuvant capability of the individual constituents.

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Chemical Analysis

- Quil-A was obtained from Superfos Biosector a/s (Denmark) as an off-white, crystalline solid.
- Ammonium acetate, A.C.S grade or better was obtained from Fisher Scientific Company (U.S.A.).
- Acetonitrile, HPLC grade or better, from Anachemica Ltd.
- Water, was distilled, deionized, and filtered using Millipore type HA filters of 0.22 μm pore size.
- Methanol, dimethylsolfoxide (DMSO), chloroform, isopropanol, hexane, benzene, trifluoroacetic acid (TFA), acetic anhydride, acetic acid, pyridine, and methyl iodide were obtained as HPLC grade or better from Fisher Scientific Company (U.S.A).
- Sodium bicarbonate, sodium borohydride, and sodium metal were obtained in A.C.S. grade or better from Sigma-Aldrich, Inc. (ON, Canada).
- Dowex 50W-X8, a strongly acidic cation exchange resin with 8 % divinylbenzene cross-linking (X 8) was obtained from The Dow Chemical Company (MD, U.S.A).

- Deuterated methanol (tetradeuteromethanol) of high purity (99.95 atom %) was obtained from MSD Isotope Laboratories (Montreal, QC. Canada).

3.1.2 Biological Testing

- Rat red blood cells (RBC) for the *in vitro* analysis of the hemolytic activity of the saponins were obtained from blood collected from Sprague-dawley rats from the laboratory of Dr. B. Van Vliet (Memorial University, St. John's, NL. Canada).
- Cells used in biological testing were of the J774 murine cell line, obtained from American Tissue Culture Collection (ATCC) (Rockville,MD. USA). They were grown to desired confluency at 37 ^{0}C in a 5 % CO_2 atmosphere and RPMI 1640 medium supplemented with fetal bovine (calf) serum (FCS) (10 % v/v), glutamine (2 mM), a penicillin/ streptomycin suspension(100 ug/ml and 100 µg/ml respectively) and sodium bicarbonate (0.15 % w/v). All cell culture materials mentioned above were purchased from Gibco Life Technologies (Gaitheisburg, MD, USA). Cells were grown in 75 cm^2 flasks manufactured by Falcon (distributed by Becton, Dickinson, ON, Canada).
- Enzyme linked immuno-sorbent assay (ELISA) kit for IL-1α cytokine determination was purchased from PeproTech Laboratories Inc. (Rocky Hill, NJ, USA).
- Other reagents for cell culture and other biological experiments including the

ELISA assay; Tween-20, Bovine serum albumin (BSA), Avidin peroxidase conjugate, ABTS (2,2'- azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) liquid substrate solution, phosphate buffered solution (PBS), lipopolysaccharide (LPS) from *E. Coli*,026:B6, Cat#L2654), and Nonidet^{R} P40 (NP40), were obtained from Sigma-Aldrich, Inc.(Ontario, Canada).

3.2 Apparatus and equipment

3.2.1 Chemical Analysis

- Millipore types HV and HA syringe filters of pore size 0.45 µm and 0.22 µm, respectively, were used for filtering solutions for HPLC, LC/MS and cell culture experiments.
- Solid Phase Extraction Kits were C8 silica packed columns of brand Strata^{TM} and size 1g packing material and 6ml volume obtained from Phenomenex. (CA, USA).
- HPLC analysis was carried out using analytical HPLC Phenomenex Jupiter C5 reverse phase HPLC column equipped with a guard column or cartridge (Specifications; 5 micrometre particle size, 300 Å pore size, and 4.6 mm x 250 mm length).
- Semi-preparative HPLC Phenomenex Jupiter C5 reverse phase HPLC column equipped with a guard column (Specifications; 15 micrometre particle size, 300 Å pore size, and 22.1 mm x 250 mm length).

- Hewlett Packard Series 1050 HPLC equipped with a Hewlett Packard 3396 integrator was used for preliminary HPLC analytical analyses.
- Varian Aerograph HPLC , model 502000-00 was used for semi-preparative HPLC analyses.
- Beckman system gold HPLC equipped with a 126 solvent delivery system, a 167 UV detector and a 406 interface analogue was also used for analytical HPLC analyses.
- HP 1100 series LC with diode array detector was employed for LC/MS experiments.
- HP 1100 series mass spectrometer with an elestrospray ionization source was employed for MS and LC/MS experiments.
- Automatic time-base fraction collector, Frac 100 Pharmacia Fine Chemicals was used to collect fractions separated by means of the semi-preparative HPLC equipment.
- GLC packed column of 1.5 % silar 7CP on Gas Chrom Q (specifications: 100-120 mesh; 183cm x 2mm id) obtained from Chrompack was used for GC-MS and gas liquid chromatography (GLC) analyses.
- Fused silica capillary GLC column of dimensions 0.32 mm id x25 m containing 3% OV-17 (Chompack) was used in GLC experiments.
- Sephadex LH-20 lipophilic gel permeation column, 40 cm x 19 mm id (Pharmacia) was used to separate mixtures produced in the permethylation reaction.

- Perkin- Elmer model gas chromatograph with a hydrogen flame detector was used for GLC experiments.
- Hewlett Packard gas liquid chromatography-mass spectrometric detector (GLC-MSD) equipment of model 5985 B GC/MS/DS equipped with a dual electron impact-chemical ionization (EI-CI) source was used for GC-MS experiments.
- Macromass VG Quattro II MS/MS (Quadrupole-Hexapole-Quadrupole) instrument fitted with electrospray ionization (ESI) source and coupled to HPLC (HP Series 1050) was used for MS and tandem mass spectrometric (MSMS) analyses.
- Bruker DRX-500 MHz NMR spectrometer used for NMR spectroscopy.

3.2.2 Biological Testing

- Bio-RAD Microplate reader Model 3550 (Bio-Rad, CA, USA) was used for all the calorimetric determinations.
- Sorvall RT6000 Refrigerated Centrifuge, Dupont Canada Inc. Mississauga, (Ontario, Canada) was used for large volume centrifuging.
- Automatic CO_2 Incubator- Model GCA Precision LE-8744 was used for cell culture.
- Brinkmann Eppendorf Centrifuge 5415 C was used for micro volume centrifuging.

- Beckman GS-6R centrifuge was used for centifuging of microtitre well plates.

3.3 Methods

3.3.1 Chemical Analysis

3.3.1.1 HPLC Separation, Isolation and Purification of the Saponin Fractions

i) Preliminary Analytical HPLC Separation

- Twenty microlitres of a 300 mg/ mL solution of Quil-A in 10 mM ammonium acetate was injected into the analytical column kept at a temperature of 35^{0}C.
- Peaks detected by UV absorbance at 214 nm were eluted from the analytical column by means of a linear gradient using the following water/ acetonitrile/10 mM ammonium acetate gradient with a flow rate of 1.5 mL/min; t = 0 - 75 %/15 %/10 %, t = 25 - 50 %/40 %/10 %.
- The column was washed with a 100mL of 100% acetonitrile after each run.
- 1.5 ml fractions were either lyophilized directly after collection or stored frozen at -70 ^{0}C to be defrosted and freeze dried the following day. Dried samples were stored at -70 ^{0}C until needed for further separation or mass spectral analysis.

ii) Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (LC/ESI-MS)

- Ten millilitres of a 300 mg/mL buffered solution of Quil-A in 10 mM ammonium acetate were eluted from the analytical column with a linear gradient of acetonitrile/water/10 mM ammonium acetate solution as follows; from 15: 75:10% to 40:60:10% over 40 min and from 40:60:10 % to 90:0:10% in 20 min then kept at this concentration for 15 minutes. The flow rate was kept at 1mL/min, ten percent of which was diverted into the ES source of the mass spectrometer.
- The LC/ESI-MS was carried out under the following conditions; the capillary voltage was set at *-29 eV,* the capillary temperature to 200^{0}C, and the spray voltage was set to *25 kV.* Nitrogen was used as sheath gas.
- The LC /UV traces were recorded online with a photodiode-array detector (HP 1100 serries).

iii) Semi-preparative HPLC Separation

- Two hundred and fifty milligrams of a buffered aqueous solution of Quil-A in 10 mM ammonium acetate solution was injected in to the column (kept at rom temperature) and eluted with the following water/acetonitrile/10 mM ammonium acetate linear gradient with a flow rate of 10 mL/min; t = 0 min - 75 %/15 %/10 %, t = 25 min - 50 %/40 %/10 %, t = 75 min - 0 %/90 %/10 %.

- Better purity was achieved when the peaks were collected individually by hand rather than by use of an automatic fraction collector as previously employed. By using manual collection, the peaks could be observed as they became eluted and could be collected in a timely manner from the beginning to the end of the peak.

3.3.1.2 Solid Phase Extraction (SPE) of Crude Material

- SPE cartridges containing 1g sorbent material were conditioned with one volume (volume of the cartridge = 6 mL) acetonitrile and rinsed with one volume of a buffer solution (10 mM ammonium acetate solution). The sample solution (50 mg in 500 µL buffer solution) was applied and aspirated through the cartridge at a flow rate of 1-3 mL/min by means of an SPE vacuum manifold and pump apparatus. The cartridge was then washed with 1mLof wash solution (15 % acetonitrile in 10 mM ammonium acetate solution).
- The cartridge was dried under full vacuum for 2 - 5 minutes before being eluted with 90 % acetonitrile in 10mM ammonium acetate solution. The elute was collected, dried, weighed and used in HPLC analysis.

3.3.1.3 Gas Chromatography- Mass Spectrometry (GC-MS)

i) Sugar Analysis

To determine the sugar composition of the saponin QF-23 the method outlined by Biermann and McGinnis, (1989), was adapted with some modifications. The method employed is as follows:

- Acid hydrolysis; one mg of the dried sample was dissolved in 2 mL of 2 M trifluoroacetic acid (TFA) and kept at 100 °C for 12 hours. The mixture was evaporated to dryness on a rotary evaporator followed by addition of a 2mL of a saturated aqueous solution of sodium borohydride ($NaBH_4$). One to two drops of glacial acetic acid was added, followed by addition of 2.0 mL of acetic anhydride in pyridine at a 1:1 ratio.
- The mixture was then kept at a 100 °C for a further eight hours, then evaporated on a rotary evaporator to dryness and dissolved in a sufficient amount of hexane (up to 1 mL) for GLC and GC-MS analyses.

Gas Liquid Chromatography (GLC) and Gas Chromatography- Mass Spectrometry (GC-MS) of Alditol Acetates

- GLC of the alditol acetates was performed on packed columns with 1.5 % silar 7CP on Gas Chrom using helium as the carrier gas (Banoub *et al.*, 1987).

- GLC-MS was performed on the Hewlett Packard GC-MS instrument also by electron impact (EI) and by chemical ionization (CI) using methane as the reagent and carrier gas for CI (Banoub *et al.*, 1987) and the GLC columns specified in the apparatus and equipment section (**3.2.1.**).
- The obtained mass spectra were identified by the NIST (National Institute of Standards and Technology) MS data library.

ii) Methylation Analysis

The absolute configuration of the sugars present in the saponin fraction QF-23 was determined essentially as described by Leontein *et al.* (1978) and Gerwig *et al.* (1978), which utilizes the trimethysilated (-)-2 butyl glycoside derivatives of the isolated mononsaccharides (Gerwig *et al.*, 1979; Banoub *et al.*, 1987) and by the method of circular dichroism measurement devised by Banoub and Shaw (1981). Methylation of the fraction was performed according to the method of Hakomori (1964), which is better understood as outlined by Björndal *et al.*, (1970), and by York *et al.*, (1985). In brief the method is as follows:

- one mg the dried saponin was dissolved in 1 mL of DMSO in a 6 mL hypo via and 1 mL of dimsyl sodium anion (methylsulfinyl carbanion; generated as outlined later) was added.
- The vial was flushed with nitrogen for one minute and stirred for an hour at room temperature with a magnetic stirrer, and with the vial capped with a

teflon lined stopper, vented with a 21 G hypodermic syringe.

- After one hour, 1 mL of methyl iodide was added to the mixture, and the mixture was stirred for a further four hours without venting.
- The methylation mixture was then separated on a gel permeation column (LH-20 lipophilic Sephadex) and eluted with chloroform. One mL fractions were collected from the Sephadex column and spotted on a silica gel TLC plate before being charred with a 50:50 mixture of sulphuric acid in ethanol.
- The fractions that showed charring were pooled and evaporated to dryness and used for GLC and GC-MS analyses as outlined earlier for sugar analysis using chloroform instead of hexane and the results were compared to those found in the literature (Björndal *et al.*, 1967; Jansson *et al.*, 1976)
- The configuration of the sugars were verified by the method of Banoub and Shaw (1981), (see also Banoub *et al.*, 1983).

Generation of the Dimsyl Sodium Anion.

- Fifty milligrams of sodium hydride in oil was washed three times in benzene to remove the oil and the benzene was dried off by adding sodium metal to the mixture in a hypo vial. Excess benzene was blown off under a stream of dry nitrogen.
- The sodium hydride was then dissolved in 1 mL of DMSO and after the addition of a magnetic stirring bar, the vial was flushed with dry nitrogen for

one minute and capped with a teflon lined stopper.

- The mixture was then stirred at 60 °C for 90 minutes to 2 hours. A 21 G hypodermic syringe pushed through the stopper was used to vent off the generated hydrogen. A clear pale greenish solution was produced.
- The anion mixture was stored at -70 °C and used when needed.

3.3.1.4 Mass Spectrometry (MS)

- Ten microlitres of the samples (buffered (with 10 mM ammonium acetate solution to basic pH) acetonitrile:water solutions) suspected to be pure, were injected into the Quatro II ESI MS/MS mass spectrometer and the solvent flow rate was kept at 0.4 µL/min.
- Ionization temperature of 200 ^{0}C was used. Scans were done over a range of 300 m/z to 3000 m/z. Spectra were taken at fragmentation voltage of 25 kV.
- The samples of interest were then dried under a gentle stream of nitrogen and stored at +4^{0} for future analyses.
- Low-energy collision induced dissociation tandem mass spectrometry (CID-MSMS) experiments were performed using argon as the collision gas in the collision cell. Cone voltage was set at 25kV and the collision energy at 180 eV.

3.3.1.5 Alkaline Hydrolytic Cleavage of Fraction QF-23.

- Basic hydrolysis was carried out on 1.5 mg of the saponin fraction QF-23 by boiling in 3 mL of 6 % $NaHCO_3$ in 50 % methanol for one hour.
- The reaction mixture was then neutralized with a few drops of Dowex 50W-X 8 and filtered through a sintered glass funnel.
- Finally the filtrate was evaporated to dryness and redissolved in 100 µL of acetonitrile/water mixture (50:50) for mass spectrometric analyses.

3.3.1.6 Nuclear Magnetic Resonance (NMR) Spectroscopy

- NMR spectra were recorded for 4.72 mg and 1.85 mg of QF-22 and QF-23 saponins, respectively, dissolved in deuterated methanol (CD_3OD), to approximately 0.4 % w/v concentration, at room temperature.
- The samples were analysed with a 5mm triple resonance inverse probe in 5 mm OD capillaries.
- Chemical shifts were reported in ppm referenced against the solvent peaks.

3.3.2 Biological Testing

3.3.2.1 Evaluation of the Hemolytic Activity of the Fractions

- Freshly collected and heparinized blood from Sprague-dawley rats was washed by centrifuging twice at 2000 rpm at 22 ^{0}C for 15 minutes with PBS (Oxoid, New Hampshire. UK), pH 7.4. Volume of PBS used for washing was

equal to volume of blood collected. The RBC pellet was then collected and further diluted with PBS in the following manner; 800 µL of RBC were diluted to 4 mL to end up with a one in five dilution.

- A 100 µL of 1 mg/mL solutions of the saponins in PBS were added in triplicate to microtitre well plates and serially diluted two fold to make ten different dilutions.
- Twenty-five µL of the diluted RBC was added to each plate well, and after 15 minutes the plate was centrifuged at 2000 rpm for 5 minutes at 22 ^{0}C.
- Fifty µL of the resultant supernatant was then transferred to new microtitre plates before reading the absorbance at 540 nm using a Bio-RAD Microplate Manager.
- Three wells per sample were used, into which was added 50 µL of a 0.1% w/v solution of the detergent NP40 instead of sample solution for 100 % lysis while three other wells were used containing only RBC and PBS to serve as blank and lysis was expressed as sample reading - blank reading/100 % lysis reading.
- The hemolytic activity of the compounds was quantitatively estimated graphically in terms of the hemolytic index at 50 % haemolysis (HI_{50}), which is the concentration of the saponin that gave 50 % haemolysis.

3.3.2.2 Toxicity Studies

- In the assay for intracellular dehydrogenase activity, cells of the J774 monocytic cell line grown to 90 % confluence were seeded in 96-well microtitre plates and incubated for 24 hrs (100 µL of a 10^4 viable cell/mL suspension per well). The medium was aspirated and serially diluted test samples were added to 100 µL of fresh medium and incubated for 24 hours.
- The tests were carried out with eight control wells (having no samples added to them), while another eight cells had no cells in them representing 100 % killing. Four wells were used for each sample dilution.
- The samples were aspirated off the cells and a 100 µL of fresh medium was added to the cells which were then allowed to recover for 24 hrs before aspirating the medium and adding to the wells a 100 µL of medium containing a 1 in 10 dilution of a 5 mg/mL stock solution (in PBS) of 3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) and incubating for a further 4 hrs at 37^0C.
- After incubation, the medium was aspirated, and 100 µL 0.04 *N* HCl in 10 % DMSO in isopropanol was added to the wells with vigorous agitation. The plates were then incubated for 20 minutes at room temperature before reading the absorbance at 595 nm with a Bio-RAD Microplate Manager plate reader.
- The concentration of the saponin sample causing 50 % cell death TC_{50} (toxic concentration at 50 %), was determined for each fraction.

3.3.2.3 *In vitro* Cytokine Production Determination as a Measure of Possible Adjuvant Aactivity

i) Study of the Kinetics of Cytokine Production

- Cells of the J774 murine monocytic cell line grown to 90 % confluency were coated on to 84 wells of a 96 well microtitire plates at 10^5 cells per well and allowed to equilibrate over a 24 hours period before being inoculated (after aspiration) with a solution of LPS in fresh medium at 0.5 µg/mL. A 100 µL of the LPS solution was added in 21 wells. Another 21 wells containing only cells and medium without LPS and 12 wells with no cells in them were subjected to the same conditions as for the ones containing the sample solution to act as controls.
- Plates were then incubated at 37 ^{0}C in a 5 % CO_2 atmosphere before the supernatant solutions were harvested from the cells at various intervals ranging from 12 to 84 hours in 12 hours increments, for IL-1α determinations.
- Readings obtained from the wells in which no cells were added were subtracted from the readings as background readings while those from the wells containing the cells and medium alone were subtracted from the wells in which samples were added for constitutive IL-1 production by the cells.

ii) Cytokine Induction by QF-23

- Cells of the J774 murine monocytic cell line grown to 90 % confluence were

coated on to 96 well microtitire well plates and allowed to attach and grow over a 24 hours period before being inoculated with the different concentrations of saponin fraction dissolved in fresh medium.

- The highest concentrations of saponin used was 10 % of the TC_{50} as determined in the toxicity studies. LPS was included as positive control. Untreated cells were incubated with medium alone to serve as constitutive control. While wells with medium alone were incubated concurrently with the test samples to serve as blank.
- The harvested supernatant medium solutions were assayed for the content of the cytokine interleukin-1 alpha (IL-1α) by sandwich enzyme linked immuno-sorbent assay (ELISA).

3.3.2.4. Enzyme Linked Immuno-Sorbent Assay (ELISA)

Initially the following ELISA protocol obtained from PetproTech laboratories Inc. (Rock Hill, NJ, USA) was followed for the determination of IL-1α production by the cells;

i) Plate Preparation

- The capture antibody provided with the murine IL-1α ELISA Development kit obtained from PeproTech Inc.(Cat # 900-K82, Lot # 10082), which consisted of 50 µg of affinity purified anti-mIL-1α, was reconstituted in 0.5 mL sterile water to obtain a concentration of 100 µg/mL. A sufficient amount of this

solution was further diluted to a concentration of 0.5 µg/mL and a 100 µL of this solution was immediately added to each ELISA plate well and the plate was sealed and incubated overnight at room temperature. The remaining capture antibody solution was aliquoted and stored frozen at -70 °C, where it can be kept for more than 6 months.

- After the incubation period, the wells were aspirated to remove the liquid and the plate was washed four times with 400 µL/well of wash buffer (0.05 % Tween -20 in PBS prepared from 1xPBS in sterile water from 10x PBS). After the last wash, the plate was inverted to remove residual buffer and blotted on paper towel before adding 300 µL of blocking buffer (1 % BSA in 1 x PBS) and incubating for at least an hour at room temperature. The wells were again aspirated and the plate was washed again with 400 µL/well of wash buffer.

ii) Preparation of Standard Solutions and Samples

- The murine IL-1α standard provided with the ELISA kit consisted of a 128 ng of recombinant mIL-1α which was reconstituted in 1 mL sterile water to make a 128 ng/mL solution which was aliquoted and stored frozen at -70 °C. A sufficient quantity of this solution was further diluted to a 3 ng/mL concentration with diluent (0.05 % Tween-20 and 0.1 % BSA in 1 x PBS). This solution was then serially diluted as required and a blank of diluent was also used.
- A 100 µL of standard or sample was added to each well in triplicate and incubated at room temperature for at least two hours.

iii) Detection of the IL-1α

- The provided 50 µg of the detection antibody (biotinylated antigen-affinity purified goat anti-mIL-1α) was reconstituted in 0.5 mL sterile water to a final concentration of 100 µg/mL. A sufficient volume of this solution was further diluted to a concentration of 0.5 µg/mL in diluent (the rest was aliquoted and stored at -70 ^{0}C).
- After the incubation period, the plate incubated with the standards and sample solutions was aspirated and washed four times with wash buffer and a 100 µL per well of the 0.5 µg/mL solution of the detection antibody was added immediately after preparation and the plate was incubated at room temperature for a further two hours.
- The plate was then aspirated and washed four times before a 100 µL of a 1:2000 dilute solution of avidin peroxidise (in diluent) was added and the plate incubated for 30 minutes at room temperature.
- The plate was again aspirated and washed ten times with wash buffer before adding a 100 µl of the substrate solution to each well. The plate was then incubated at room temperature for colour development which was monitored with an ELISA plate reader at 405 nm to obtain OD readings of not more than 0.2 absorbance units for zero standard concentration and 1.2 absorbance units for the highest standard concentration.

Although the murine interleukin one alpha (mIL-1α) ELISA development kit used in this experiment was purported to be adequate for the detection and the quantitative measurement of natural and/or recombinant mIL-1α in a sandwich ELISA format within the range of 32-2500 pg/mL problems were encountered with the above protocol which necessitated several trials that involved modification of the above procedure at various stages from adjustment of concentration of the kit components used (i.e. capture antibody, highest standard concentration, detection antibody), to trying out different incubation periods and the number of washings after each incubation, to determining the optimum avidin peroxidase dilution, and finally, the optimum wavelength for the determination of the optical density in monitoring colour development after the addition of the substrate was also investigated.

Subsequently the above protocol was modified as follows: Concentrations of the capture antibody, highest standard concentration and detection antibody used in the protocol were, 0.25 µg/mL, 1.5 ng/mL and 0.25 µg/mL, respectively, instead of 0.5 µg/mL, 3 ng/mL and 0.5 µg/mL; 150 µL (intead of 300 µL) of the block buffer was used; plates were washed five times instead of four times after each incubation period and fifteen times instead of ten times after incubation with a 1:4000 (optimum) dilution of avidin peroxidase. Best results were obtained when colour development was monitored every five minutes at 405nm and readings that gave the best standard curve were usually obtained between 15 and 40 minutes (see results section, pages 162-165).

3.3.2.5 Statistical Analysis

Data was analysed statistically using Prism GraphPad InStat Analysis software using non-parametric, unpaired, two tailed t-test. Tests having probability (P) values of less than 0.05 were considered significant.

Chapter 4: Results and Discussions

The following topics will be covered in results and discussions

1) Preliminary analysis
2) LC/MS elution profile of Quil-A
3) Large scale separation of components by means of semi-preparative HPLC
4) Solid phase extraction
5) Mass spectrometry
6) One pure compound having molecular weight 1560 (QF-23) structurally characterized
7) Analysis of the sugar content and linkages of QF-23
8) NMR spectroscopy performed for two compounds, QS- 21 molecular weight 1989 and for the compound QF-23 having molecular weight of 1560
9) Five more compounds of near homogeneity separated by semi-preparative RP-HPLC including two of the ones documented in the literature. The compounds having molecular weights 1217, 1257, 1353, 1389, 1856, 1989, and 955, the latter two have been identified before by other researchers.
10) Hemolytic activity studies
11) Toxicity evaluation of fractions QF-15,QF-21, QF-22 (QS-21), and QF-23

12) Studies of the kinetics of IL-1α production by a murine monocytic cell line

13) Evaluation of the immune stimulating activity of fraction QF -23

4.1. Chemical analyses

4.1.1. Separation, isolation and purification of saponin components

The purification of the *Quillaja* saponins has been carried out mainly using reverse phase high performance liquid chromatography (RP-HPLC). This required some initial investigations to determine the optimum conditions for separation. Initially the separation of these components in our laboratory was carried out by means of a preparative thin layer chromatography (TLC). Attempts using RP- HPLC in various gradients of acetonitrile: water on C18 and C8 stationary phases proved impractical and afforded poor separation of the components (Sharpe, 1997). Excellent separation was later achieved using C5 reverse phase HPLC column which was found to provide a superior resolution than when a C8 column was used (Thompson, 1998).

i) Preliminary Analysis

Preliminary investigations involved the separation of the crude extract Quil-A by means of an analytical column to investigate the elution characteristics of the material and to carry out method development and optimization. The results of this analysis set the foundation for large scale separation according to the guidelines

on HPLC method development found in the literature (Snyder *et al.*, 1988; 1997; Meyer, V. R., 1999).

Preliminary analytical separation of Quil-A using a C5 column gave rise to an elution profile (Figure 4) which resembled that found in the literature (van Setten *et al.*, 1995; So *et al.*, 1997). This profile shows clearly defined and well resolved peaks. On average, 50 peaks were apparent. A compound having a molecular weight of 1560 was resolved at this stage. This compound was easily obtainable in a highly pure form. The compound has since been isolated in the semi-preparative HPLC separation and called QF-23. Structure elucidation by means of mass spectrometric techniques, that included electrospray ionization - mass spectrometry (ESI-MS) in the negative mode (Figure 29), CID-MS/MS (Figure 28), CVID-MS (Figure 27) and some cleavage reactions, resulted in the proposal of the following structure for QF-23 as shown in Figure 5.

ii) Liquid Chromatography-Mass Spectrometry (LC-MS)

This procedure gave a tremendous amount of information about elution characteristics of the material separated and the individual components. The technique employed was the liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS).

The photodiode-array and the mass detector signals obtained during the LC/ESI-MS experiment, as shown in Figure 6 and Table 5, give a summary of the report obtained.

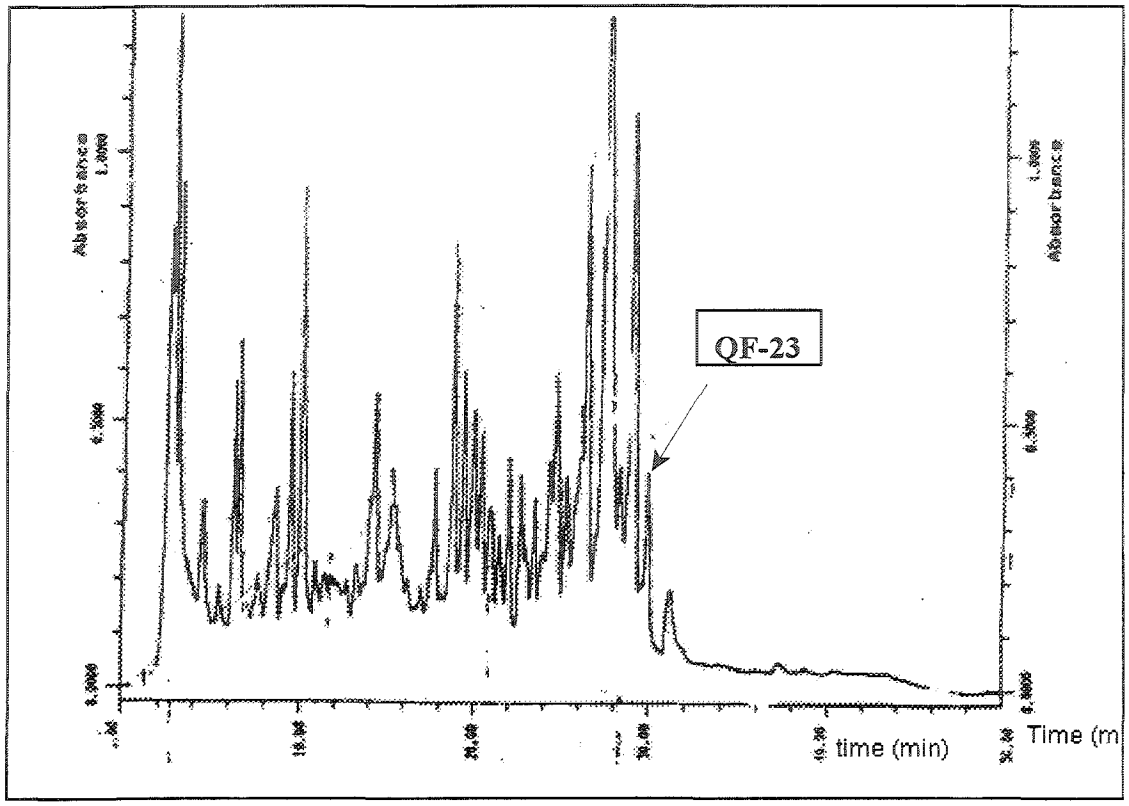


Figure 4. Elution profile of Quil-A as separated by means of RP-HPLC using a C5 analytical column. Absorbance was measured at 214nm. The time intervals on the x-axis are 0, 10, 20, 30, 40, and 50 minutes.

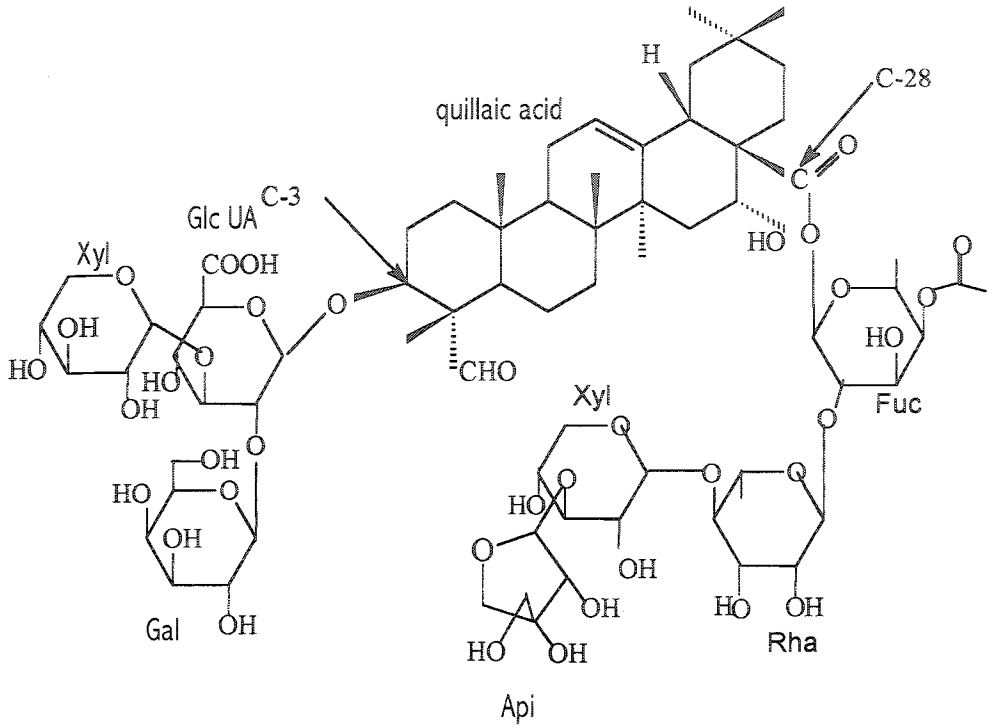


Figure 5: Proposed Structure of Compound QF-23

Glc UA = glucuronic acid, Xyl = xylose, Gal = galactose,

Fuc = fucose, Rha = rhamnose, and Api = apiose

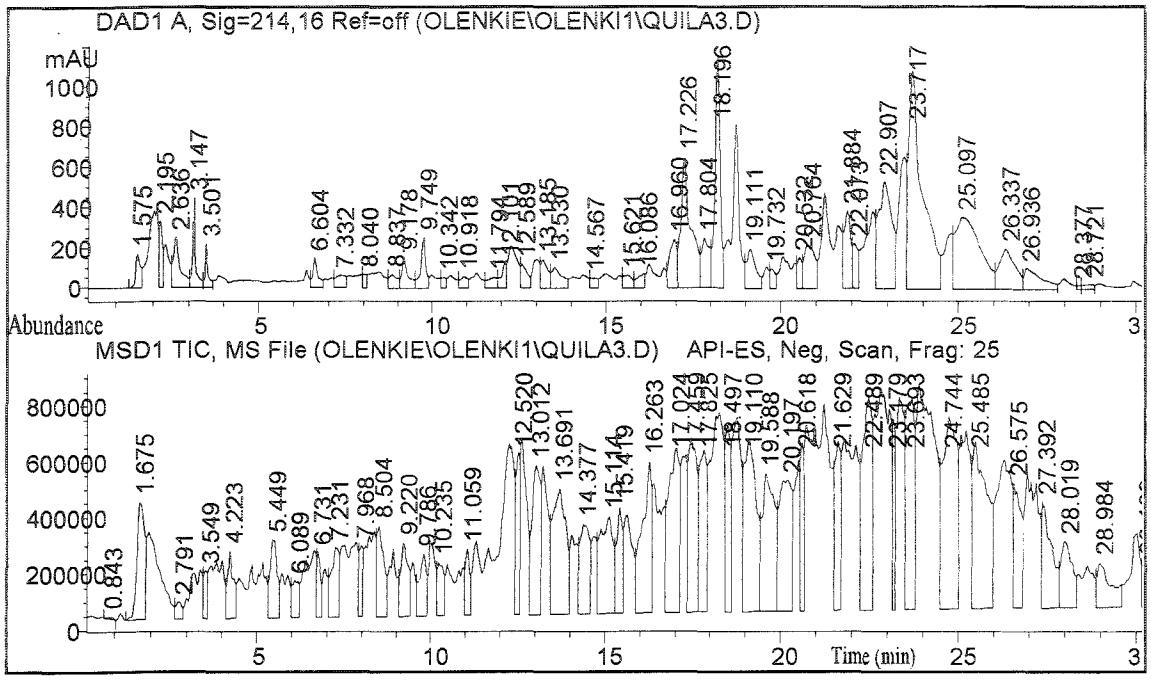


Figure 6: Diode array detector (DAD) signal (top) and mass spectral detector (MSD) signal (bottom) as obtained from the LC/ESI-MS analysis of Quil-A. The MSD signal is lagging behind the DAD signal by about one minute. Absorbance was measured at 214 nm in milli absorbance units (mAU). Absorbance in the MSD signal is given in counts.

Table 5 (Legend) **Summary of the full report obtained from the LC/MS experiment carried out on Quil-A.**

10 mL of a 300 mg/ml buffered solution of Quil-A in 10 mM ammonium acetate were eluted with a gradient of acetonitrile/water/10 mM ammonium acetate solution of; from 15: 75: 10% to 40:60:10% over 40 min and from 40:60:10% to 90:0:10% in 20 minutes then kept at this concentration for 15 minutes. The flow rate was kept at 1.5 mL/min, ten percent of which was diverted into the ES source of the MS instrument.The LC/ESI-MS was carried out on the analytical column and under the following conditions: the capillary voltage was set at -29 eV, the capillary temperature to 200°C, and the spray voltage was set to 25 *kV*. Nitrogen was used as sheath gas. The LC UV traces were recorded online with a photodiode-array detector.

The results given in this table are for one of three experiments that were carried out and found to give similar results.

*1 **Entry #;** Serial number of the entry into the table. In most cases the MSD signal has been matched up with a corresponding DAD signal, with the MSD signal lagging behind by anywhere from 0.02 to 0.238 of a minute (1.2-12 seconds) with most peaks differing in 0.15 minutes (9 seconds). Some MSD signals were found to not have corresponding DAD signals. This was attributed to the fact that the compounds whose ions were detected by the MSD at these times do not absorb very well in the specified UV range. Similarly some DAD signals could not be matched up with any MSD peaks, this could be due to the fact that the compounds that were detected by the DAD at those times do not ionize in the negative

mode. Still both cases could happen simply because the integration was not carried out at those times.

*2 **Peak #;** Corresponds to the peaks integrated in this report. The numbers in brackets correspond to the peak numbers included in the appendix.

*3 **Elution time;** MSD retention time

*4 **Most Abundant ions;** Ions having 100% abundance or closer in the TIC at this given retention time.

*5 **Total Abundance for the peaks;** Obtained manually from the MSD signal. The compound having molecular ion mass of 2297 m/z known as QS-III (Higuchi *et al.*, 1988) and QS-17 (Kensil *et al.*,1991) was found in the highest MSD peak at retention times 22.823 minutes.

*6 **Components;** reported after integration

*7 **Total number of ions;** Do not correspond with the number of components detected as some ions are seen as coming from a single component

*8 **Other ions detected;** Ions that are seen as coming from one component are entered in an identical fashion, differentiated by italics, bold, bold italics, and thereafter by (‘), (“) (‘”), etc. Peaks for which this data is not entered are included in the appendix and the corresponding peaks in the appendix are given in brackets.

*9 **Purity Assessment;** as obtained after integration of the MSD signal. All the peaks were seen as being impure, having more than one components. Some peaks did have as few as two components only.

*10 **DAD Purity assessment;** gives clearer quantitative peak purity calculations

*11 **# of spectra;** Components # as reported by the DAD were seen to be different from those reported by the MSD for corresponding peaks. Threshold limit of 999.000 is a default purity threshold limit.

*12 **UV absorption;** Provides a quantitative estimate of the components. Compound with molecular ion mass of 1353m/z (QF-15) was found in the highest DAD peak at retention time 18.197 minutes.

*13 **Peak elution time;** In the DAD signal is ahead of the MSD signal

*14 **Peak # DAD signal;** Some are included in the appendix. The numbers in brackets are definitely in the appendix.

*15 **Compounds' UV absorption characteristics;** Between 190 and 400 nm as recorded by the DAD. Has been classified as saponin like and non-saponin like for the purposes of this report (see Figure 7).

*16 **Time difference between the peaks;** Gives a good indication of whether the MSD and the DAD signals being matched could be considered as corresponding.

Table 5 **Summary of the full report obtained from the LC/MS experiment carried out on Quil-A**

MSD signal									DAD signal						
Entry # (*1)	Peak # (MSD signal) (*2)	Elution time (min) (*3)	Most Abundant ion (*4)	Total Abundance for this peak out of 800 000 (*5)	Total # of components detected (*6)	Total # of ions detected (*7)	Other ions detected (*8)	Purity Assessment (*9)	Purity assessment factor out of 1000 (*10)	# of spectra within purity threshold limit of 999.000 (*11)	UV absorption out of 1000 mAU (*12)	Peak elution time (min) (*13)	Pk # (DAD signal) (*14)	Compds UV absptn Characteristics (saponin or non-saponin) (*15)	Time difference between the peaks (MSD - DAD signal times) (min) (*16)
1	3	1.675	1110	450 000	-	-	530, 734, 755, 881,929,986, 1110,1131,1157, 1168,1506,1597, 1796,2121,2184, 2698,	im-pure	999.495	3/3	180	1.575	3	saponin	0.100
2	4	1.919	1061	340 000	-	-	1061,2039,2950	im-pure	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	999.142	5/5	400	2.020	6	saponin	
4	-	-	-	-	-	-	-	-	999.984	3/3	400	2.092	7	Saponin	-
5	-	-	-	-	-	-	-	-	999.979	3/3	400	2.195	8	saponin	-
6	-	-	-	-	-	-	-	-	999.982	3/3	100	2.341	9	saonin	-
7	5	2.791	1543	100 000	-	-	662,1560,2017,	im-pure	963.915	2/5	250	2.636	10	saponin + non-saponin?	.0155
8	6	3.213	1270	100 100	10	18	543,1261,1270, 1316,1362,1369, 1426,1845,1855, 2015,2034,2487, 2571,2660,2773	im-pure	-	-	-	-	-	-	-

9	7	3.391	603,1450, 2203	250 000	5	8	537,*603*,797,**850**, 1450,1020,*2045* **2204**	im-pure	995.211	7/7	400	3.147	12	saponin	0.244
10	8	3.547	2727	200 000	4	12	537,**547**,**785**,850 1020,***1197***,1450, **1602**,*2106*,*2677*, ***2727***,***2805***	im-pure	99.698	3/3	100	3.501	15	saponin	0.048
11	9	3.682	2006,2613	220 000	5	7	1018,*1448*,2007, *2360*,2614,2727, 2805	im-pure	-	-	-	-	-	-	-
12	10	3.828	1951	260 000	5	10	925,972.1476, *1633*,1795,**1951**, 2006, *2606*, 2727, **2944**	im-pure	999.914	3/3	50	3.741	18	saponin	0.087
13	11	3.994	926,2771	240 000	-	7	926,979,985, 1130,1723,2771, 2819	im-pure	989.363.	1/5	50	3.846	20	saponin	0.148
14	12	4.223	2558	280 000	6	9	*939*,1076,*1227*, 1344,**2450**,2497 **2553**, 2820,**2950**	im-pure	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	999.414	3/3	50	4.302	23	saponin	-
16	13	4.493	2382 2793	180 000	5	7	879,***1024***,**1946**, 2106,*2107*,***2382***,	im-pure	999.902	3/3	50	4.462	24	saponin	0.031
17	14	4.844	510	220 000	3	5	*510*,*1139*,1696, *1809*,2954	im-pure	999.950	3/3	50	4.698	26	saponin	0.146
18	15	5.160	2447	220 000	-	6	817,2182,2387, 2545,2760,2935	im-pure	99.980	3/3	50	5.047	28	saponin	0.113
19	16	5.449	700,1318, 1747,2203	230 000	7	9	**700**,***776***,*1307*, ***1318***,**1600**,1748, 1767,2036,2203, *2638*	im-pure	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	999.024	5/5	50	5.462	31	saponin	-
21	17	5.722	1996	180 000	2	4	*840*,**1487**,*1996*, **2501**	im-pure	99.834	3/3	50	5.695	32	saponin	0.027

22	18	5.884	655,1476	180 000	3	5	655,*1477*,**1614**, **1951**,*2445*	im-pure	-	-	-	-	-	-	-
23	19	6.089	1387,2471 ,2713	180 000	7	16	*624*, **871**, ***920***, 1083',1388,**1436**, **1594**,***1725***,1887', 1932,***1969***,2217'', ***2471***,2705',2713'' ,2746'',2948	im-pure	999.834	3/3	50	5.945	35	saponin	0.144
24	20	6.383	1057	250 00	6	12	*528*,798,**933**, ***1057***,***1059***,1151' ***1330***,***1407***,1433, **1555**,1854',2801, **2886**	im-pure	999.264	3/3	50	6.216	37	saponin	0.167
25	-	-	-	-	-	-	-	-	997.575	3/3	100	6.364	38	non-saponin	-
26	21	6.624	925,2317	250 00	5	8	**776**,**925**,**926**,*927*, ***1262***,2317,2679, 2862	im-pure	999.302	3/3	150	6.604	40	non-saponin	0.020
27	22	6.731	1541,1798	260 000	-	6	551,769,915,*927*, 1039,1127,*1262*, 1541,1747,1799,	im-pure	-	-	-	-	-	-	-
28	23	6.933	871,1027	240 000	5	6	***871***,***1028***,1214, 1688,1694,2138,	im-pure	999.539	3/3	50	6.901	43	saponin	0.032
29	24	7.231	2453,2568	260 000	6	8	525,*574*,620, *2351*,2452,**2453**, **2568**,	im-pure	999.570	3/3	50	7.083	45	saponin	0.148
30	25	7.480	955	260 000	-	5	797,955,969, 1065,2912	im-pure	999.140	2/7	50	7.332	47	saponin	0.152
31	26	7.817	955	260 000	8	19	543,773,911,955 962,1123,1259,1 330,***1398***,***2785***	im-pure	999.592	3/3	50	7.587	49	saponin	0.230
32	27	7.968	955	280 000	3	9	***511***,***797***,**798**,***962***, **969**,**1738**,*2109*, ***2162***,***2912***	im-pure	999.689	5/5	50	7.923	51	saponin	0.045
33	28	8.090	955	300 000	2	2	721,955	im-pure	999.927	3/3	50	8.039	53	saponin	0.051

34	29 (20)	8.223 (8.219)	955	320 000	7	14	*675*,721,*954*,955, ***963,969*,1257**, *1558*,1653',2089, 2228,2267,2462', 2700'	im-pure	-	-	-	-	-	-	-
35	30 (21)	8.364	955	340 000	3(2)	9(6)	***955,956*,970, 1572,2157,*2298, 2455,2873***	im-pure	99.989	3/3	100	8.258	56 (41)	saponin	0.106
36	31 (22)	8.504	624	360 000	-	8	624,797,955,970, 1689,2624,2711, 2862	im-pure	986.975	2/5	100	8.449	58 (43)	saponin	0.055
37	32 (23)	8.918	675,2120	280 000	-	7	629,675,1856, 2120,,2330,2430	im-pure	912.651	4/5	50	8.837	60 (45)	non-saponin?	0.081
38	33 (24)	9.220	1349	280 000	9	13	645, 674, *741*,763,***925***,944, 1123,***1311,1349***, ***1350***,1608,*1816*, 2580	im-pure	551.069	3/5	150	9.178	62 (47)	saponin + non-saponin	0.042
39	34 (25)	9.486	2143	220 000	4	5	2064,2143,*2211, 2289*, 2515	im-pure	-	-	-	-	-	-	-
40	35 (26)	9.786	1217	260 000	-	8	608,1217,1218, 1219,1220,1331, 1768,2207	im-pure	999.504	3/3	250	9.749	65 (50)	non-saponin	0.037
41	36 (27)	9.996	761, 1812	300 000	8	8		im-pure	983.882	1/3	100	9.976	67 (52)	saponin	0.020
42	41	11.059	1371,1866 2520	260 000	-	8	1371,1867, 2520	im-pure	994.498	3/3	50	10.918	-	saponin	0.141
43	44	12.244	1673	600 000	-	9	525,746,836,154 1,1689,1674,167 3,2149,2816	im-pure	973.384	2/5	150	12.099	82	saponin	0.145
44	45 -	12.520	1866	660 000	5	10	741,**837**, 843, **1661**,1675,1688, *1751,2062,2290*, 2842	im-pure	999.921	3/3	200	12.264	84 (65)	saponin	0.256

45	46	12.610	2842	680 000	9	16	741,782,636, 1312,1565,1674 1675,1687,1689, 2061,2753,2843, 2845.	im-pure	99.265	3/3	50	12.589	86 (67)	saponin	0.021
46	47 (36)	13.012	1565, 1566	580 000	7	14	633,782,**789**, ***1189*,1379**,1511, ***1565*,1566,*1567*, *1568'**,1579'',1580 '', 1692,***1895***,2398''	im-pure	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	im-pure	999.602	5/5	75	- (13.025	(68)	saponin	-
48	48 (37)	13.221	1511	560 000	5	12	***510*,*755***,789, **1431**,**1511**,***1512***, 1519',**1525**,1526', 1581,2604,	im-pure	999.920	3/3	150	13.184	90 (69)	saponin	0.037
49	- (39)	14.051	1505	260 000	2	3	731,1505,2254	im-pure	-	-	-	-	-	-	-
50	-	-	-	-	-	-	-	-	999.881	5/5	50	14.052	- (73)	saponin	-
51	59	17.024	1383	660 000	-	13	691,865,1353, 1354,1383,1384, 1497,1583,1715, 1730,1870,1882, 2733	im-pure	999.162	5/5	200	16.961	112 (85)	saponin	.063
52	-	-	-	-	-	-	-	-	999.742	7/7	600	17.226	114	saponin	
53	60 -	17.230	1383	660 000	4	8	***676*,1349,1353**, **1354**,1355,***1567***, ***1581*,*2199***	im-pure	-	-	-	-	-	-	-
54	61 -	17.459	1862,1876	660 000	7	18	***525*,649,*831***,937', ***1045***,1207''',**1339** ,**1340**,1861'', 1485''',1862'', 1863',1869'',1875 ',***1876***,1927,***2017***, *2187*	im-pure	-	-	-	-	-	-	-

55	62	17.825	1515,1699 2547	660 000	-	11	757,849,1515, 1516,1517,1699, 1701,1713,1875, 2434,2547	im-pure	999.953	5/5	150	17.804	117	saponin	0.021
56	63	18.184	1353	740 000	6	7	676,713,*1353*, *1354,1355*,1467, 1468	im-pure	"	"	"	"	"	"	"
57	- (47)	18.272	1353	740 000	6	7	**676**,713,*1353*, *1354,1355*,1467', 1468''	im-pure	999.990	3/3	**1150**	18.197	120 (89)	saponin	0.075
58	64 (48)	18.497	1485	680 000	8	18		im-pure	999.995	3/3	200	18.480	121 (91)	saponin	0.017
59	65 (49)	18.770	1221	680 000	-	9	1221,1222,1223	im-pure	999.994	3/3	780	18.718	123	saponin	0.052
60	67 (51)	19.588	1353	540 000	7	12		im-pure	999.820	3/3	50	19.577	127 (95)	saponin	0.011
61	68 (52)	20.197	1191	520 00	3	5	1191,1192,1193, 1305	im-pure	999.945	6/6	50	20.062	130	saponin	0.135
62	70	20.618	1433	700 000	3	6	716,1177,1433, 1434	im-pure	999.875	3/3	100	20.532	134	saponin	0.086
63	71 (54)	20.743	1433	640 000	7	14		im-pure	"	"	"	"	"	"	"
64	72 (55)	20.968	1475	680 000	4	8	737,**767**,*768*, 1405',**1475**,1476'' ,**1477**,1478'', 2214''	im-pure	999.865	5/5	150	20.763	137 (101)	saponin	0.205
65	- (58)	21.794	1059	640 000	8	15		im-pure	999.998	3/3	350	21.884	144 (106)	saponin	0.031
66	76	21.915	1059	560 000	2	5	*1059,1060,1061*, **1592,568**	im-pure	"	"	"	"	"	"	"
67	- (60)	22.489	1475	720 000	5	7	767,**831**,1475, 1476,*1477*,*1632*, 1799'	im-pure	"	"	"	"	"	"	"
68	79 (61)	22.661	1758	760 000	4	16		im-pure	999.867	7/7	350	22.602	149 (110)	saponin	0.059

69	80 (62)	22.823	2297	840 000	9	20		im-pure	-	-	-	-	-	-	-
70	81	23.079	2310	780 000	4	9	*1148*,1166,2298', *2303*,*2304*,2309, 2310,2311',*2577*	im-pure	999.915	8/8	500	22.907	152	saponin	0.190
71	82	23.179	2310	780 000	2	5	*1147*,*1148*,2295, *2304*,*2310*,	im-pure	-	-	-	-	-	-	-
72	83 (63)	23.359	2018,2150 2310	720 000	-	9	1155,2012,2018 2032,2134,2150, 2163,2296,2310	im-pure	-	-	-	-	-	-	-
73	84 (64)	23.693	2150	720 000	9	20		im-pure	999.520	5/5	600	23.448	156 (115)	saponin	0.245
74	-	-	-	-	-	-	-	-	976.979	2/5	1050	23.717	-	saponin	-
75	85	23.908	2149, 2166	850 000	-	-	1475,2018,2134, 2150,2163,2166	im-pure	999.977	5/5	1050	23.717	158	saponin	0.190
76	86 (66)	24.744	1856, 1989	700 000	-	-	1768,1856,1857, 1858,1987,2002, 2003,2004,2586	im-pure	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	999.876	3/3	200	24.791	- (119)	saponin	-
78	87 (67)	25.220	1989	640 000	-	-	1856,1989,1990, 2003	im-pure	998.797	7/7	200	25.097	162	saponin	0.123
79	88 (68)	25.485	1989	620 000	-	-	675,1855,1858,1 989,2002,2003,2 004	im-pure	-	-	-	-	-	-	-
80	89 (69)	26.299	2030, 2044	580 000	-	-	1014,1517,1856, 2031,2043	im-pure	-	-	-	-	-	-	-
81	90	26.575	2030, 2043	340 000	8	21	*853*,845,875,*882*, *1014*,1016,1022', 1033'',1691'',*170 5*,*1706*,1825'', 1869,1875'',*1897*, 1898',1899',2038, 2045,2046,2118,,	im-pure	999.471	7/7	150	26.337	166	saponin	0.238

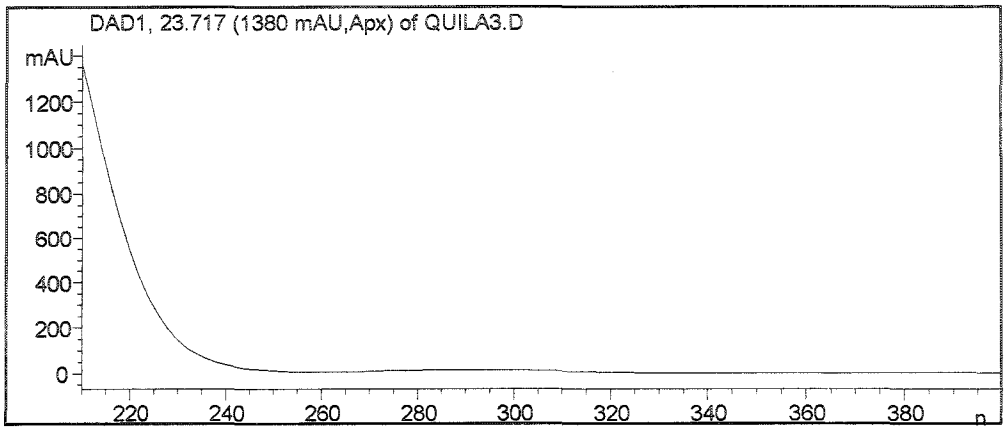
82	91	26.956	1560	480 000	4	8	809,1560,	im-pure	-	-	-	-	-	-	0.020
83	92 (72)	27.051	1560	540 000	5	8		im-pure	983.421	1/7	50	26.936	168 (126)	saponin	0.115
84	93	27.196	1560	460 000	5	6	810,1560,1561, 2024,2334,2683	im-pure	-	-	-	-	-	-	-
85	94 (74)	27.392	1560	380 000	4	6	810,1560,1561, 2024,2334,2683	im-pure	-	-	-	-	-	-	-
86	95	28.018	1856	220 000	-	-	756,985,1723, 1856,18581870, 1973,1974,1988	im-pure	980.865	2/3	25	27.981	173	saponin	0.037
87	99 (77)	30.008	1856, 1972	300 000	-	-	779,809,852, 2340	im-pure	997.569	3/3	25	29.936	182 (138)	saponin	0.072
88	122	39.418	2135	50 000	2	2	1518,2135,	im-pure	-	-	-	-	-	-	-

A group of compounds were noticed to have similar UV absorption characteristics (Figure 7) typical for saponin compounds (Ahmad and Basha, 2000). These were concentrated more in the portion of the chromatogram from ten to thirty minutes retention time. In this area the highest UV absorption was on average obtained at about 214 nm. Very few peaks along the spectra or chromatogram showed high purity (Figure 8), emphasizing the tendency of the components of this material to co-elute.

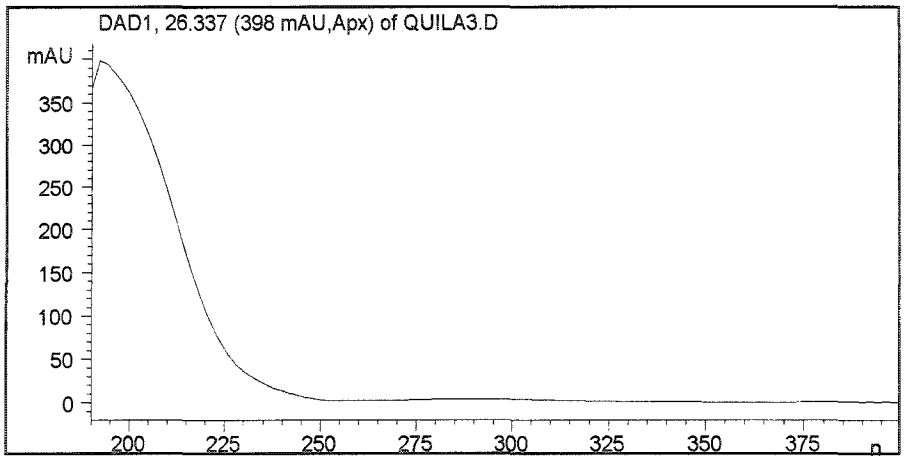
Some high molecular weight ions were detected at the beginning of the run, as evidenced from the inspection of the total ion chromatogram (TIC) of the experiment shown in Figure 9. Some of these peaks did not, however, show the "typical" saponin UV absorption characteristics shown before, but were nevertheless intriguing. Figures 7 and 9 show the UV absorption shown by some of these compounds. These compounds were found to be eluted from the column as early as the first five minutes. Inspection of the mass spectra revealed the presence of ions with very high molecular weights, many of which do not appear to have been identified anywhere in the literature (Table 6). The actual stability of these ions is however not known. They could also be due to the formation of dimers between the molecules of the stable components. Preliminary inspection however did not point towards this speculation. The ions belonging to sixteen molecules of the compounds previously detected by other researchers were identified in the mass spectra of this experiment (Table 7).

(Cont. on p.109)

a)



b)



c)

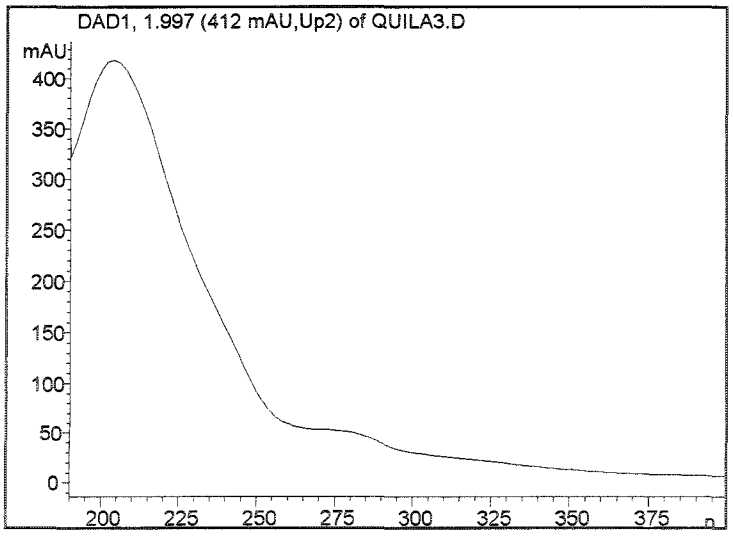


Figure 7 Characteristic Saponin UV absorptions *(continued on page 102)*

d)

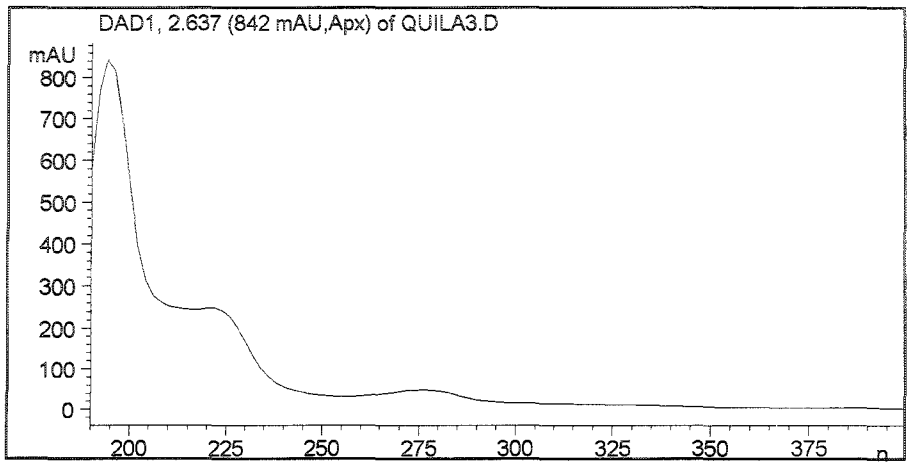


Figure 7 Characteristic saponin UV absorption as obtained from the diode array signal of the LC/MS experiment carried out on Quil-A. **a), b), c)** and **d)** represent the absorption characteristics of the compounds eluting at retention times 23.717, 26. 337, 1.997 and 2.637 minutes respectively.

Figure 8: An example of purity assessment of the peak at retention time 23.909 minutes in the MSD signal (Figure 8B). That same peak is detected at 23.717 minutes in the DAD signal (Figure 8A). Figure 8C shows the ions that are detectable in that peak.

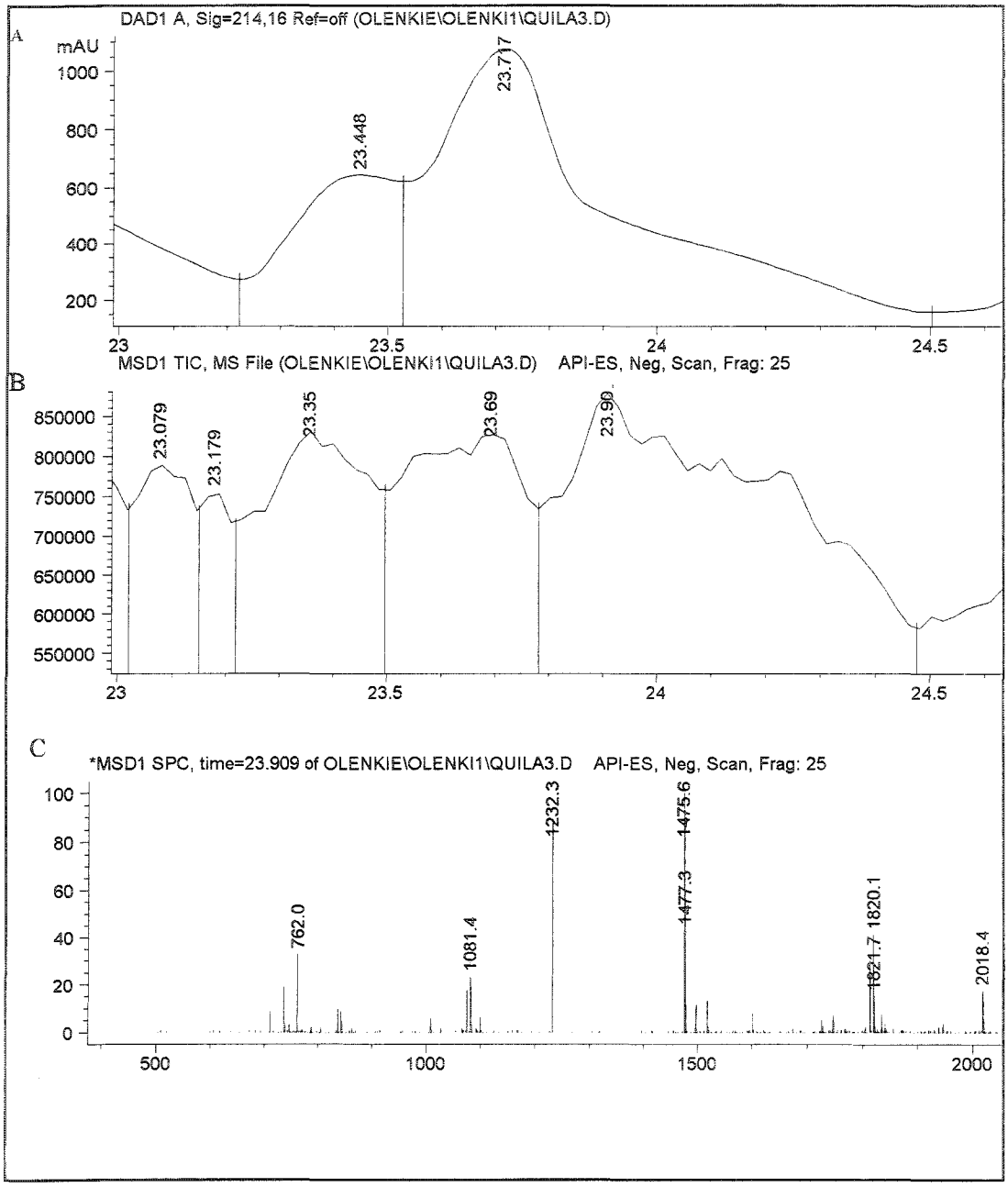
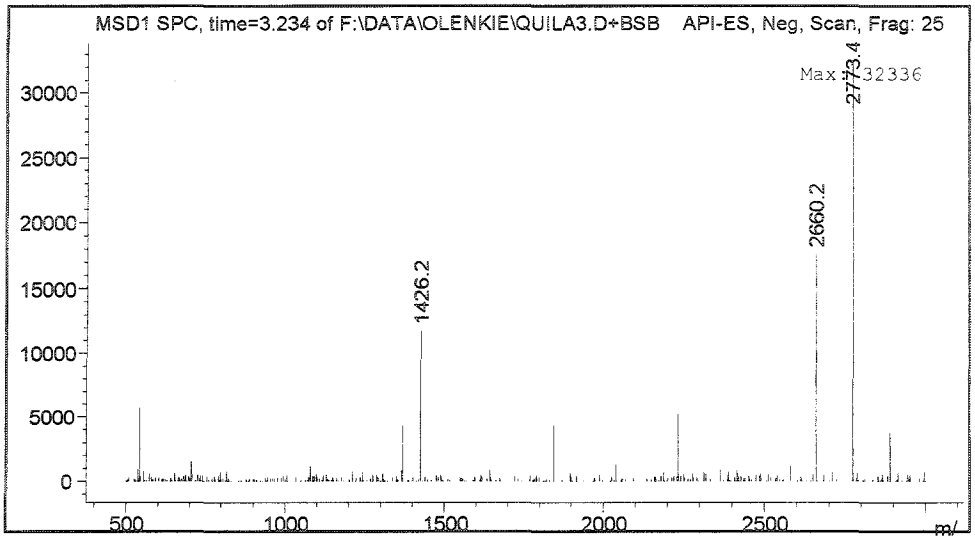


Figure 8: An example of purity assessment of a peak

a)



b)

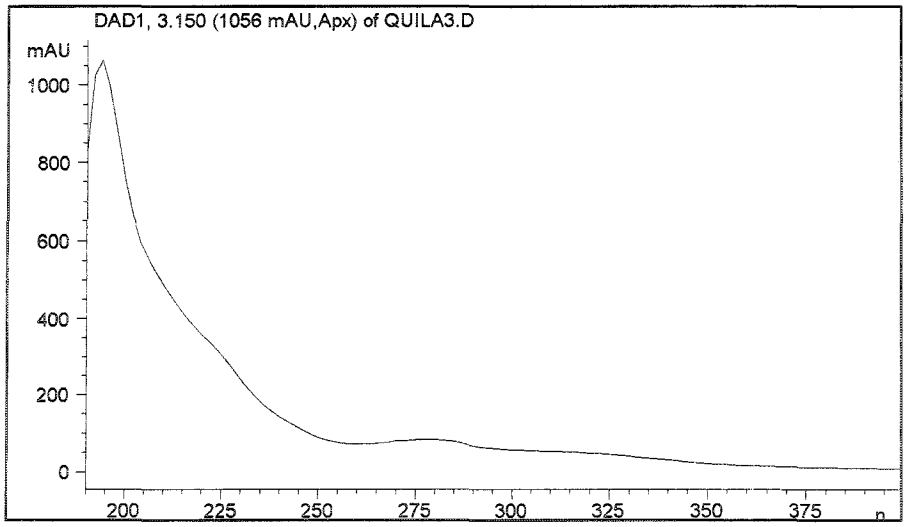


Figure 9: a) TIC showing high molecular weight compounds

c)

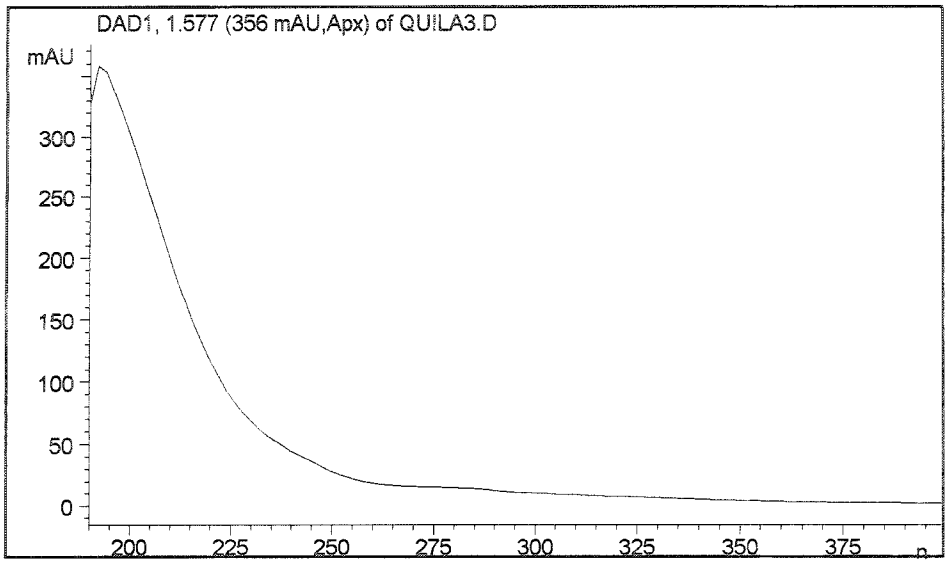


Figure 9: **a)** Total ion chromatogram (TIC) showing high molecular weight compounds with saponin like UV absorption characteristics being eluted early in the LC/MS separation of Quil-A. Ions having molecular weights of m/z 1426, 2660 and 2773 are clearly detectable.

b) Shows the absorption characteristics observed for these compounds.

c) Shows the absorption characteristics of compounds eluted as early as 1.577 minutes as being saponin like.

Table 6: Some of the high molecular weight compounds detected in the mass spectra of the LC/MS experiments that were not previously identified by other researchers

Molecular ion mass (m/z)	Elution Times (min)		UV absorption Caharacteri-stics
	MSD	DAD	
2438	17.825	17.804	saponin like
2447	5.160	5.047	saponin like
2450	4.223	-	-
2453	7.231	7.083	saponin like
2487	3.213	-	-
2509	5.722	5.695	saponin like
2515	9.485	-	-
2520	11.059	10.918	saponin like
2547	17.825	17.804	saponin like
2558	4.223	-	-
2567	7.231	7.083	saponin like
2571	3.213	-	-
2580	9.220	9.178	?
2613	3.682 15.419	15.395	saponin like
2624	8.504	8.449	saponin like
2638	5.449	-	-
2660	3.213	-	-
2677	3.549	3.501	saponin like
2679	6.624	6.604	non-saponin like
2698	1.675	1.575	saponin like
2705	6.089	5.945	saponin like

2713	6.089	5.945	saponin like
2727	3.549	3.501	saponin like
2746	6.089 14.684	5.945 14.560	saponin like
2771	3.994	3.846	saponin like
2773	3.213	-	-
2785	7.817	7.587	saponin like
2793	4.493	4.462	saponin like
2794	9.996	9.976	saponin like
2801	6.383	6.216	saponin like
2820	4.223	-	-
2848	12.610	12.589	saponin like
2862	6.624	6.604	
2872	8.364	8.258	saponin like
2912	7.968 16.378	7.923	saponin like
2935	5.160	5.047	saponin like
2950	4.223	-	-
2954	4.844 9.220 9.498	4.698 9.178	saponin like

Table 7: Ions belonging to molecules of the compounds previously detected by other researchers that were identified in the mass spectra of the LC/ESI-MS experiment.

Molecular ion mass (m/z)	Detected by Researchers	Compound Name (where known)	Elution Times (min)	
			MSD	DAD
956	So *et al.*, 1997	QS-L1	7.817- 8.504	7.587-8.20
1512	Higuchi *et al.*, 1987	DS-1	13.012 13.221 19.110	 13.184 19.110
1626	van Setten *et al.*, 1995		10.485 14.684	14.684 14.560
1640	van Setten *et al.*, 1995		19.110	19.110
1696	Higuchi *et al.*, 1987	DS-2	4.844 17.825 20.743 30.199	4.698 17.804
1788	van Setten *et al.*, 1995		18497 19.110 21.233 22.124	18.480 19.110 21.229 22.073
1862	van Setten *et al.*,1995		17.459 17.825	17.226 17.804
1869	van Setten *et al.*, 1995		17.459 26.575	17.226 26.337
1876	van Setten *et al.*, 1995		17.459 17.825	17.226 17.804
1886	Kensil *et al.*, 1991	QS-7	18.770 19.585	18.718 19.577
1989	Kensil *et al.*, 1991	QS-21	24.744 -5.485	23.971-25.097
2018	Kensil *et al.*, 1991		10.818 17.459 22.661	10.520 17.226 22.602
2150	Kensil *et al.*, 1991 van Setten *et al.*, 1995	QS-18	23.693 23.908	23.448 23.717
2296	Higuchi *et al.*, 1988	QS-III	8.364 22.124 22.823 23.179	8.258 22.073
2321	Kensil *et al.*, 1991	QS-17	6.624	6.604

Table 7: Ions of molecules previously detected by other researchers

Other very useful information that was obtainable from the LC-MS of Quil-A was the extracted ion chromatogram (EIC) (Figure 10). With this information it was possible to pinpoint the exact peak of a given molecular ion.

iii) Semi-preparative HPLC

The aim of this undertaking was to obtain large amounts of individual components or fractions in pure or near pure form. Sufficient quantities of the purified saponin fractions were necessary to facilitate further chemical analyses and biological investigations. The separation by means of a semi-preparative HPLC column resulted in an elution profile that surprisingly showed better separation than the one obtained by analytical separation (Figure 11). Batch to batch differences in the elution profiles were noticed. The UV detector used was limited to a maximum column loading of 250 mg of crude material. This amount resulted in very low yields, necessitating multiple runs before an amount of material sufficient for further analyses could be obtained. This exercise was found to be inconsistent, cumbersome and resulted in material loss, as the fractions collected were distributed in several containers. The ideal situation would have been to separate as much as 2.5 g of Quil-A all in one run. This would have required the purchase of a flow cell suitable for semi-preparative separations to be used with the analytical HPLC systems available in our laboratory or alternatively to obtain access to an HPLC system designed specifically for semi-preparative HPLC separations. *(to p. 113)*

Figure 10 A) Extracted Ion Chromatogram (EIC) for QF-22 (QS-21) Mwt 1989 (iii) and QF-23 Mwt 1560 (iv). The spectrum shows the ion detected at 1989 m/z to be concentrated more on the peak eluted at 25.485 minutes (MSD signal,(ii)) which corresponds to the peak eluted at 25 .097 minutes in the DAD signal (i). While the ion detected at 1560 m/z is concentrated at 26.387 minutes (MSD signal) which corresponds to the peak seen at 26.337 minutes in the DAD signal.

Also see legend to Figure 10 B (page 105) for more explanation.

(Y-axis of the rest of the figures except for the DAD signal give abundance of the ions in counts. Y-axis in the DAD signals gives UV absorbances in milli absorbance units (mAU). X- axis give retention times in minutes.)

A

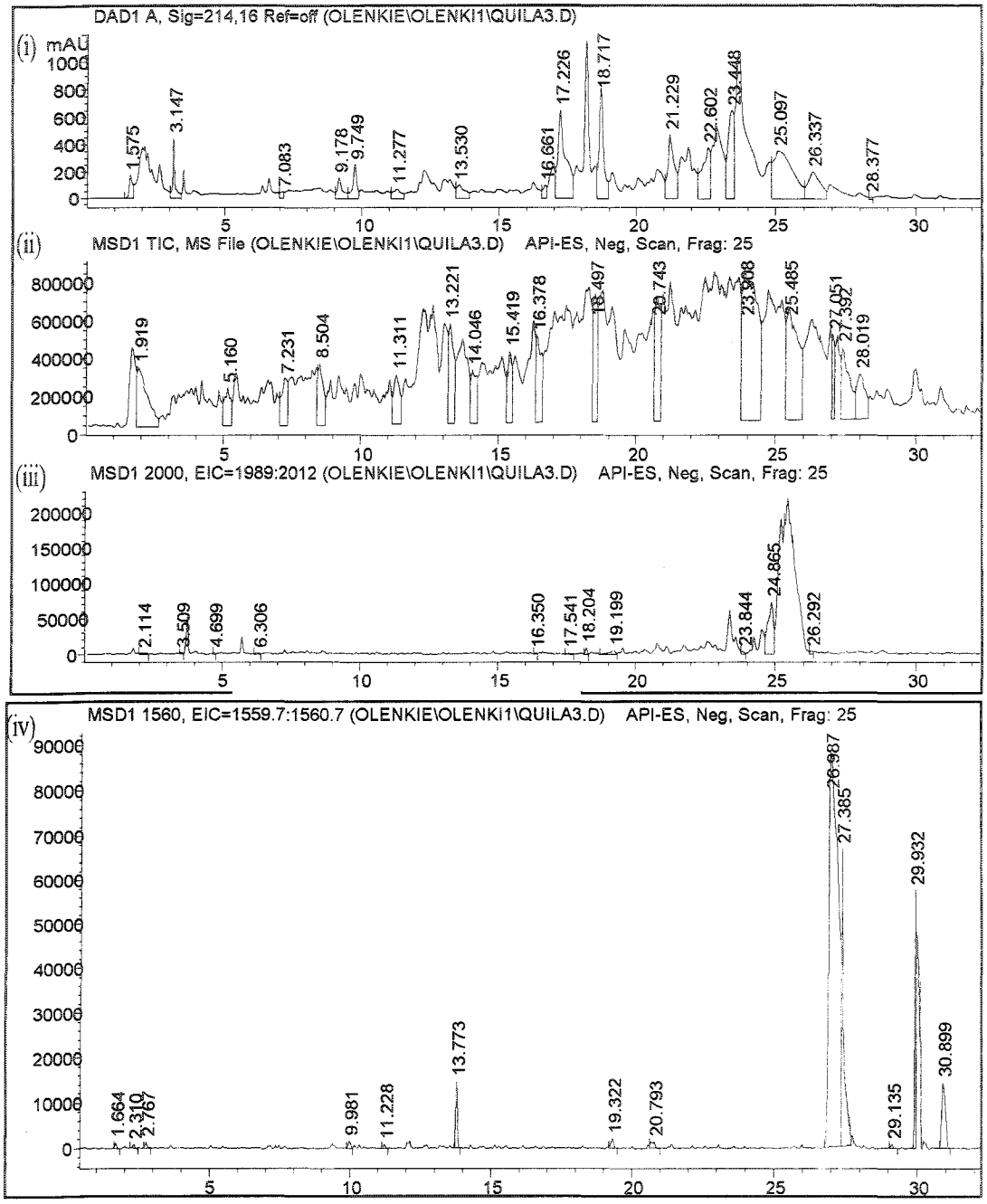


Figure 10 A) Extracted Ion Chromatograms (EIC)

Figure 10: B) EIC of ions, detected at m/z, 1257, QF-11 (iii), 1389, QF-15 (iv),1560 QF-23 (V), 1856, QF-21(vi), 955, QF-9 (QS-L1) (vii) and QF-22 (QS-21) (viii) m/z 1989. (i) and (ii) are the MSD and DAD signals.

(Y-axis of the rest of the figures except for the DAD signal give abundance of the ions in counts. Y-axis in the DAD signals gives UV absorbances in milli absorbance units (mAU). X- axis give retention times in minutes.)

The ions of the same molecular mass can be seen eluting at different points on the profile. Several probable explanations for this occurrence could be brought forward: **1**. These could be ions of the same compound that are for some reason showing different affinity properties to column material (e.g different isomers of the same molecule of the compound). Isomerization of the compounds of *Quillaja sapaonaria* Molina have been reported by other researchers before (van Setten *et al.*, 1995, Cleland *et al.*, 1996). In fact both the identified isomers of QS-21 were found to be immunologically active (Cleland *et al.*, 1996). **2.** These could be ions of compounds with different molecular structures but same molecular weight. The compounds could exist as such in the *Quillaja* extract, they could also have been formed as a result of the breakdown of other compounds while in the column, **3**. These ions could also be multiply charged ions of compounds of higher molecular weight. **4**. The ions could be daughter ions formed during fragmentation of ions of higher molecular weight compounds.

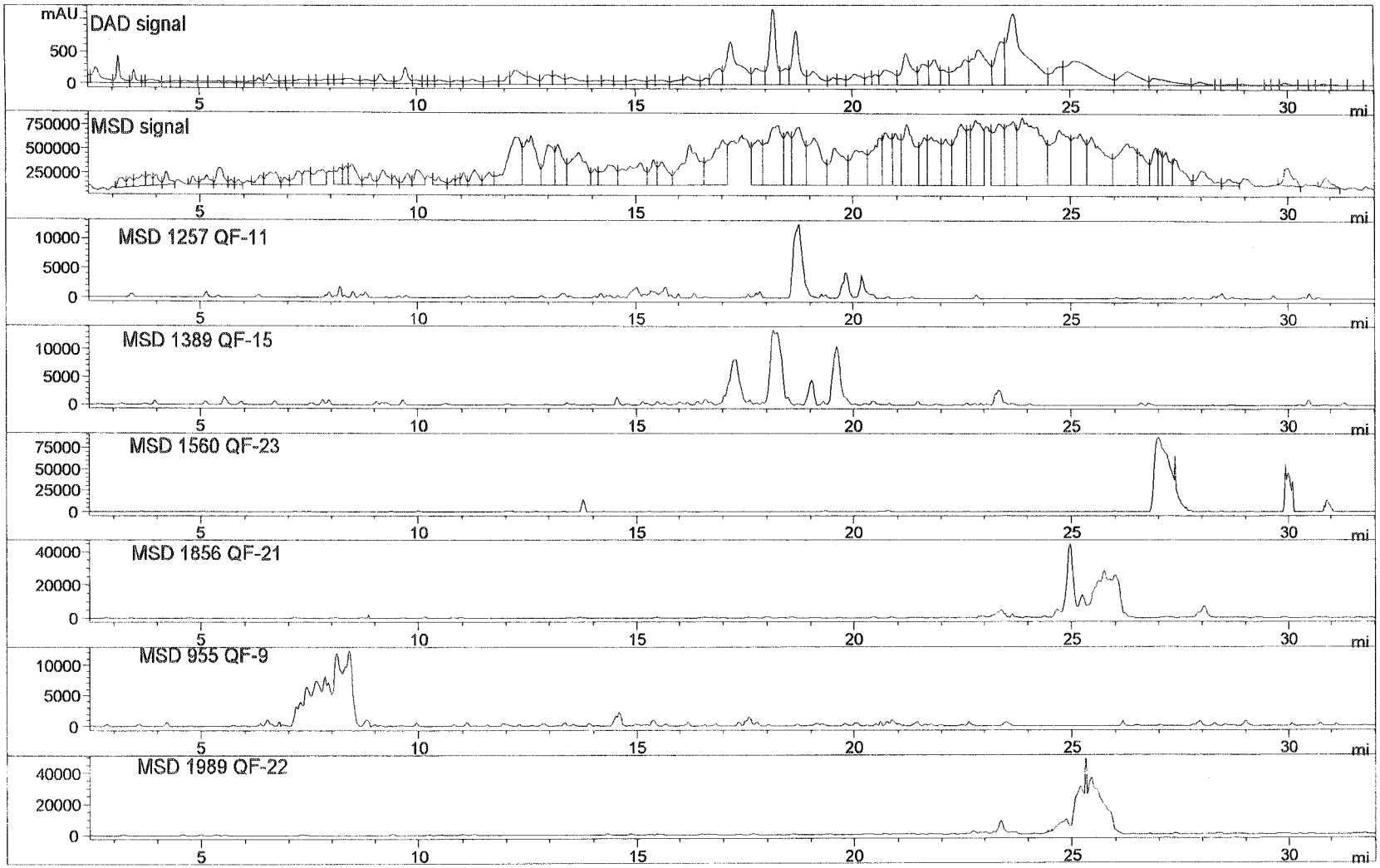


Figure 10: B) EIC of ions

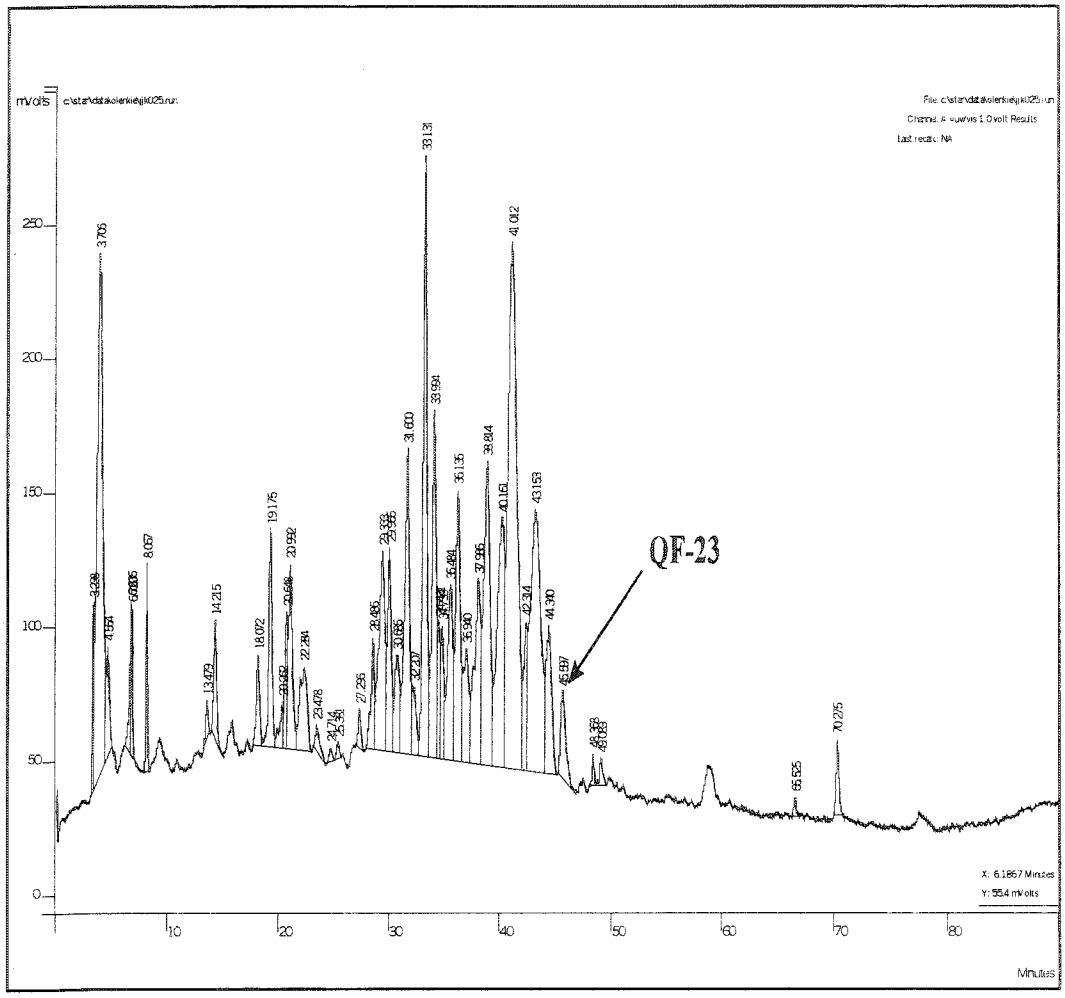


Figure 11: Elution profile of Quil-A from a semi-preparative column; C5 RP-HPLC with a guard column; 15 µm, 300 Å, 22.1 mm x 250 mm. Numbers on peaks refer to retention time in minutes.

Neither of these were possible. As a result of repetitive runs fraction QF-23 was obtained in pure form (Figure 12). An average of 1.0 mg was obtained from 250 mg of Quil-A. About fifty HPLC runs, with an average of 250 mg crude Quil-A were carried out. A total of 35 fractions were collected and recovery of each fraction varied from 0.2 mg to 13.4 mg dry weight. Fractions QF-30 to QF-35 were obtained in the lowest amounts while fractions QF-17, QF-20, QF-21 and QF-22 were obtained in larger amounts.

Name change

The name of the compound P5 (*Quillaja* saponin with molecular weight 1560), separated during preliminary analytical HPLC was changed to QF-23 (*Quillaja* Fraction number 23) after semi-preparative separation. The basis for the name change was due to the fact that this fraction was collected in the twenty-third fraction while using the automatic fraction collector set to collect fractions at two minutes intervals. In addition, the name QF-23 is in conformity with the naming system that has been commonly used by other researchers on this subject of *Quillaja* saponins; such as QS-21, QH-A, QS-L1, and DS -1.

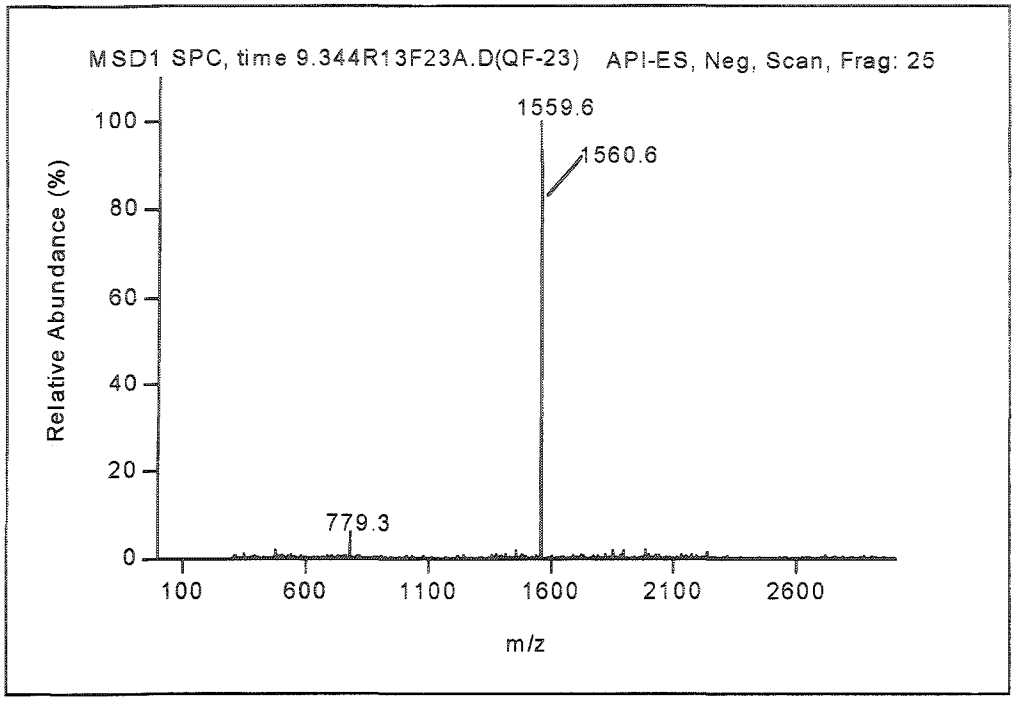


Figure 12: Mass spectrum of compound QF-23, molecular mass 1560 m/z, as obtained after purification by means of semi-preparative RP-HPLC. The singly charged ion of this molecule is detected at m/z 1559.9, while the ion detected at m/z 779.3 represents the doubly charged ion, $[M-2H]^{2-}$, of the molecule of QF-23

4.1.2 Drying of the fractions

Removal of the solvents employed in the purification of the Quil-A constituents was fundamental to the process and imperative for the successful analysis of the saponins. This proved to be a challenge. The fractions had to be obtained free of solvents, which were mainly organic (acetonitrile) and a buffered aqueous solution (water containing ammonium acetate mostly in 1mM concentration), because they interfered with subsequent analyses (both chemical and biological). For example, in biological testing fractions are diluted either in PBS or in cell culture media both of which are aqueous solutions and the presence of organic solvents in these instances would have detrimental consequences. Likewise, the presence of water in nuclear magnetic resonance experiments was undesirable as it would cause interference. The preservation and drying method employed should preserve the integrity of the constituents of the fractions and not lead to the hydrolysis or deterioration of the components. The feasibility and suitability of several drying methods were investigated, including freeze-drying, cryo-crystallization, air drying and drying under a gentle stream of the inert gas nitrogen. It was found that drying by blowing away the solvent under a gentle stream of nitrogen was a suitable method of drying as shown by the before and after spectra of the tested fractions in Figures13 and14, respectively, as being identical, showing no deterioration of the sample.

(cont. on p. 118)

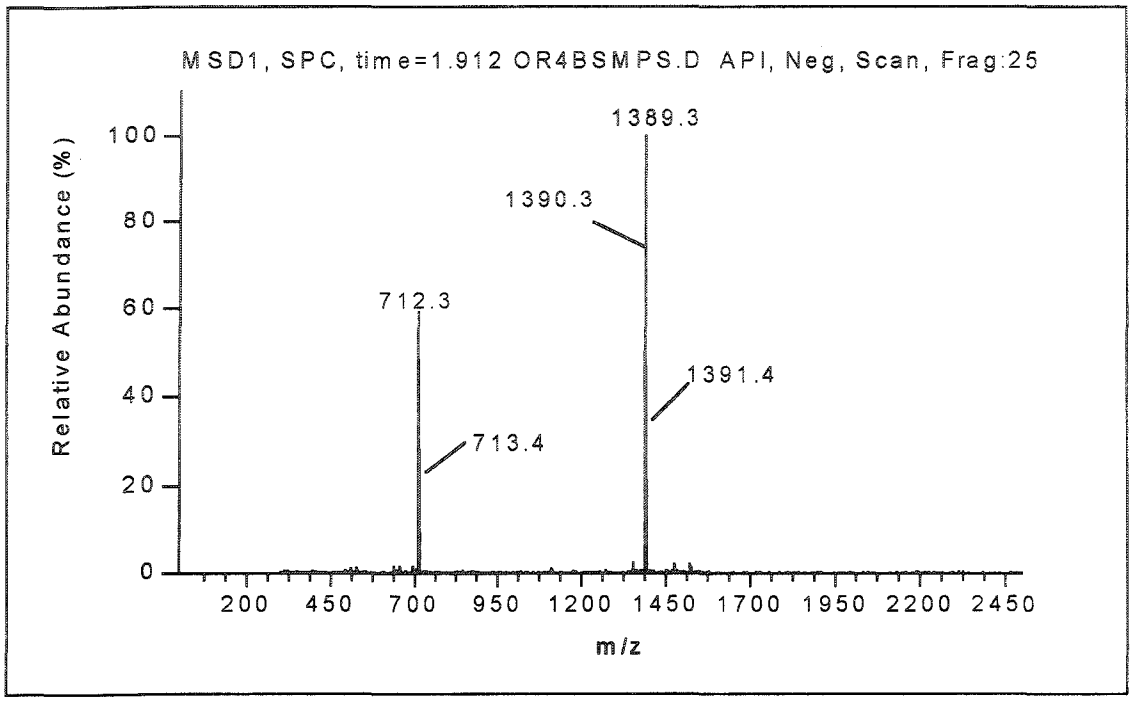


Figure 13 : ESI-MS mass spectrum of pure novel compound, QF-15, detectable at m/z 1389.3. The ions detectable at m/z 712.3 and 713.4 represent the potassium salt of the doubly charged ion of the original compound having molecular weight 1354 Dalton.

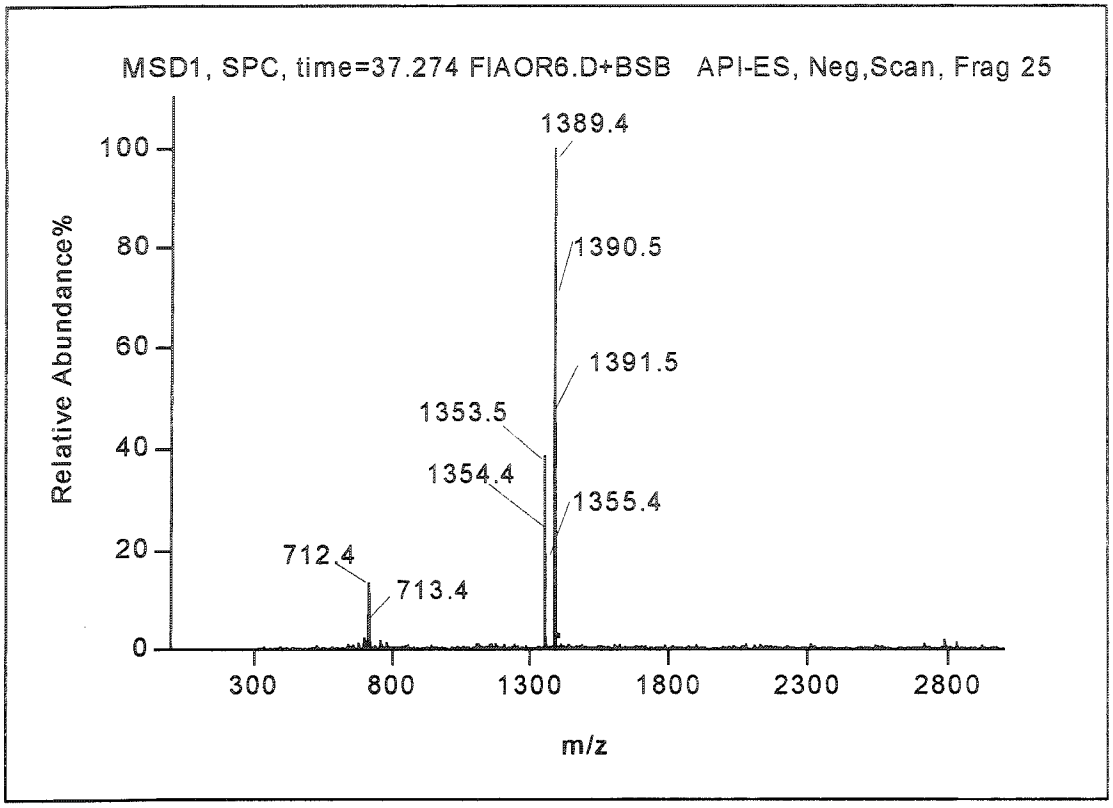


Figure 14: ESI-MS spectrum of pure novel compound QF-15 after drying and storage. The ion of this compound is detectable at 1353.5 m/z and as a potassium salt at 1389 m/z. The potassium salt of the doubly charged ion is observable at 712.4 and 713.4. All the ions detected in this spectrum represent one compound, having molecular weight 1354 Dalton. The other ions are probably due to isotope differences.

The peak observed at m/z 712 is the doubly charged ion of the compound detected at m/z 1389. Preservation at -70 ^{0}C is recommended for the solvated samples while storage at +4 ^{0}C was found to be adequate for dried samples.

4.1.3 Purity assessment

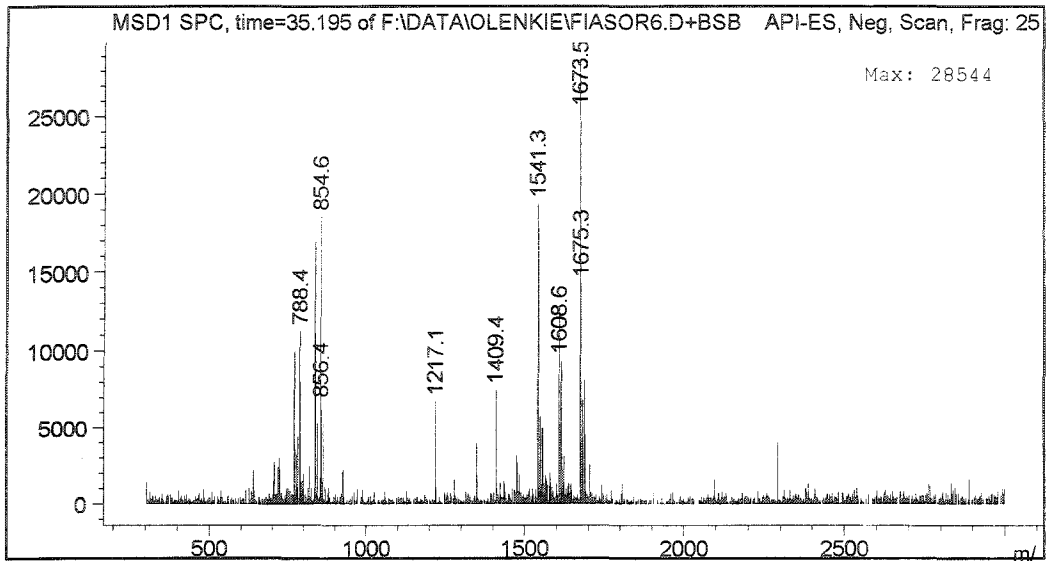
The purity of the separated fractions could be determined by a number of techniques, including, TLC, re-injection into an analytical HPLC column alone or equipped with a mass spectrometer or mass spectrometry alone. The latter method of purity assessment was used because it was the quickest and most accurate of these methods. For example the MS spectrum shown in Figure 15 shows that the fraction analyzed is clearly impure while the samples analyzed in Figures 13 and 14 are shown to be of relatively much higher purity.

4.1.4 Determination of the chemical nature of the components.

The chief constituents of the bark of *Quillaja* saponaria Molina tree are saponins, which together with other extractable material (in water, ethanol or water ethanol mixtures) constitute 20-25% of the weight of the bark. The saponins themselves are estimated to constitute 5% of the bark weight and 20% of the extractable material from the freshly prepared extract of the bark. The rest of the

(cont. on p. 120)

a)



b)

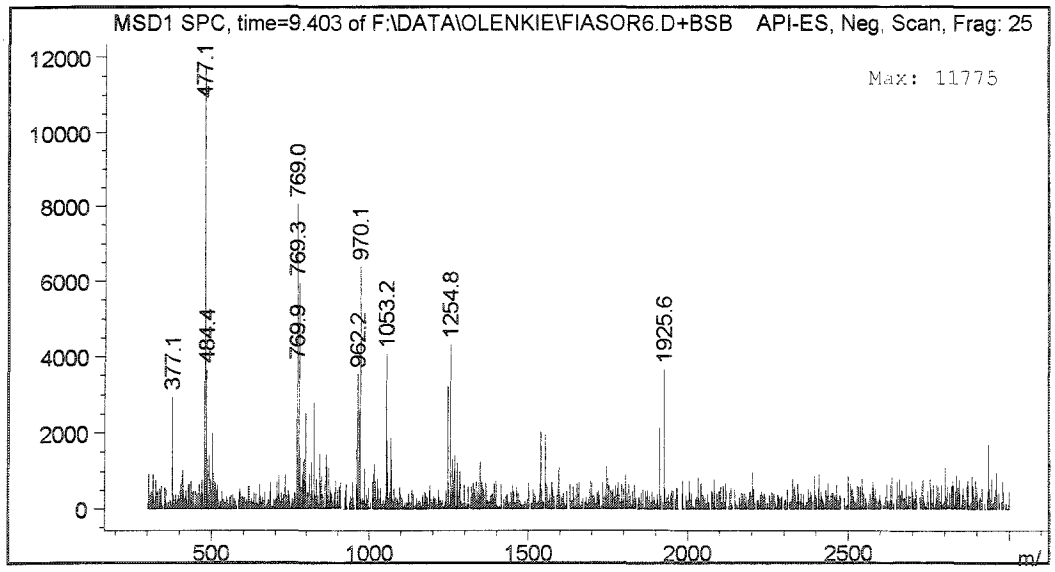


Figure 15: Impure analyzed fractions: a) fraction QF-35

b) fraction QF-10.

extractables are tannins and phenolic compounds which are separable from the saponin extract of Quil-A via dialysis, gel filtration and dial filtration (Kensil *et al.*, 1996). In our research we have employed SPE to eliminate these non-saponin materials from Quil-A. However, we have abandoned this procedure because it is not known if these are active or inactive immunologically, as it has been purported that the immunological activity of Quil-A may very well be due to its non-saponin constituents as well as its saponin ones (Campbell and Peerbaye, 1992). We accordingly decided to collect fractions from all of the elution profile in case we become interested in including these components in our research (these are labeled QF-1 to QF-10).

Quil-A extract also has been shown to contain proteins - 0.5%, fat - 4%, fibre - 0.3%, ash - 2.5%, moisture- 5.5%, and other carbohydrates, including starch and sugars - 70%. Calcium oxalate and uronic acid may also be found in the bark extract. Upon hydrolysis of the saponins the principal sapogenins yielded are quillaic acid and gypsogenin (Wallis, 1967; Plato *et al.*, 1986; Trease and Evans, 1972;1996; Anagaude, 1999) .

Interest has been focused on analyzing fractions found to contain high molecular weight compounds (showing singly charged ions at 800-3000 m/z) as demonstrated by mass spectrometry because these are highly likely to be saponins. Consistently, fractions that will exhibit a considerable amount of frothing upon a slight agitation will contain saponins. For example, the fraction R6 T9 obtained

during the sixth run showed frothing upon agitation and was shown to contain the compound designated QF-9, showing a molecular ion at m/z 955, in pure form (Figure 16). This compound is of high molecular weight and shows frothing, therefore it is considered a saponin and indeed a compound with this molecular ion has been identified as a *Quillaja* saponin and structurally characterized by So *et al.*, (1997) and named QS-L1. Inspection of the diode array detector signal of the LC/ESI-MS spectrum at the region of retention time nine minutes, (which also corresponded to the retention time of nine minutes at which a molecular ion of 955 m/z was identified in the MS detector signal), showed this compound possessed a UV absorption characteristic found to be typical of saponins as shown in Figure 17, which is comparable to Figure 7 (p. 101). On the other hand, fractions collected in vials R6T5, R6T19, and R6T40 did not show any frothing upon agitation; mass spectral analyses of the fractions showed them to contain compounds of lower molecular mass and inspection of the LC-MS spectrum in their area of collection showed them to have non-saponin UV absorption characteristics as shown in Figures 18 and 19. The conventional tests for carbohydrates may prove not very useful in this case as some non-saponin materials are carbohydrate in nature and these tests may not be able to discriminate between the saponin and non-saponin carbohydrates.

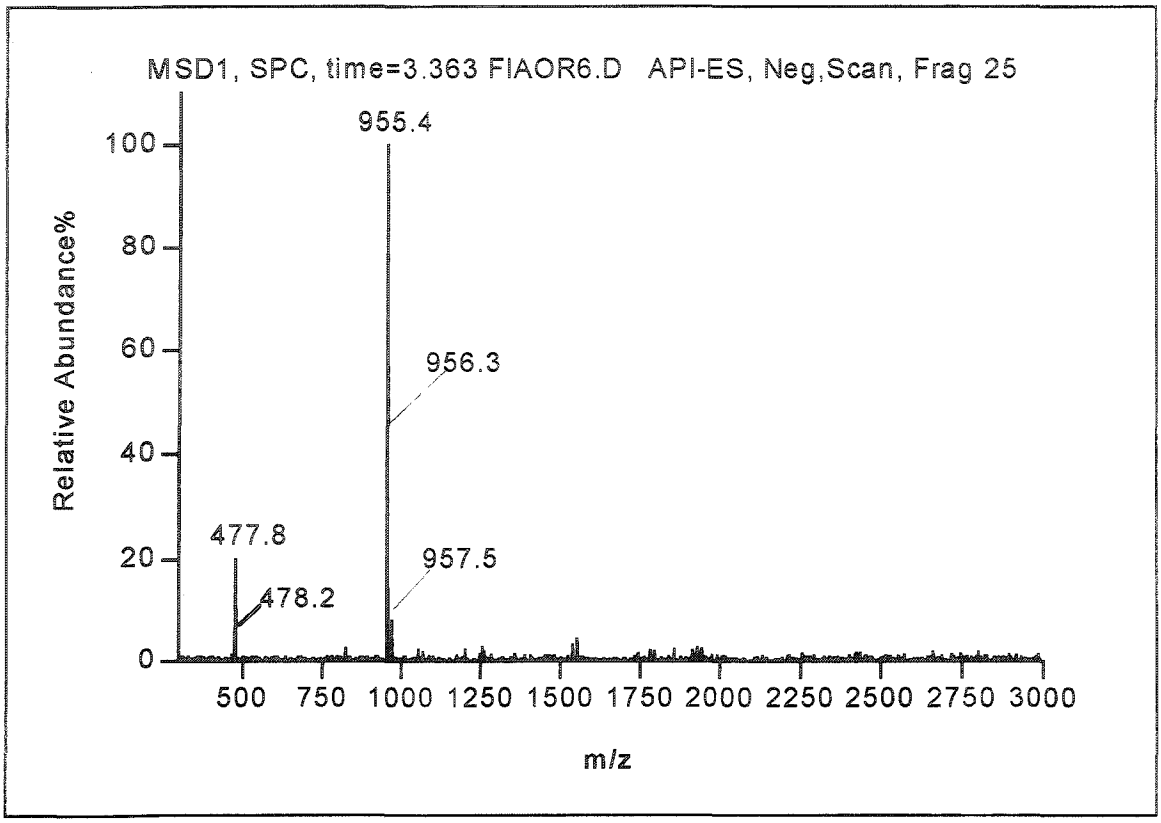


Figure 16: Pure compound Mwt 955 Daltons. ESI-MS of pure fraction QF-9 isolated by means of semi-preparative RP-HPLC. The ion of the molecule of this compound is detected at m/z 955.4, 956.3 and 957.5. Signals observed at m/z 477.8 and 478.2 correspond to the doubly charged ions of this molecule. This compound has been previously isolated, characterized and named QS-L1 by So *et al.*, (1997).

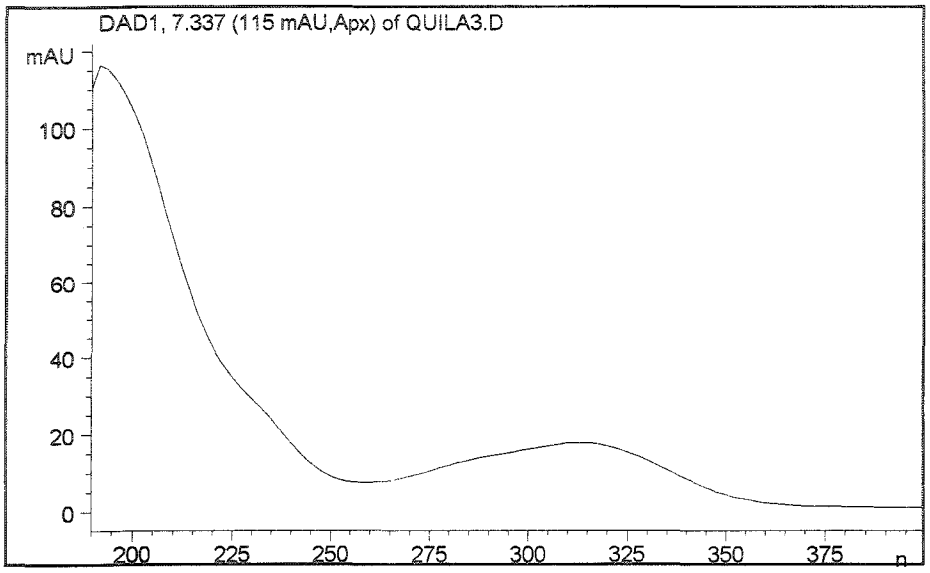
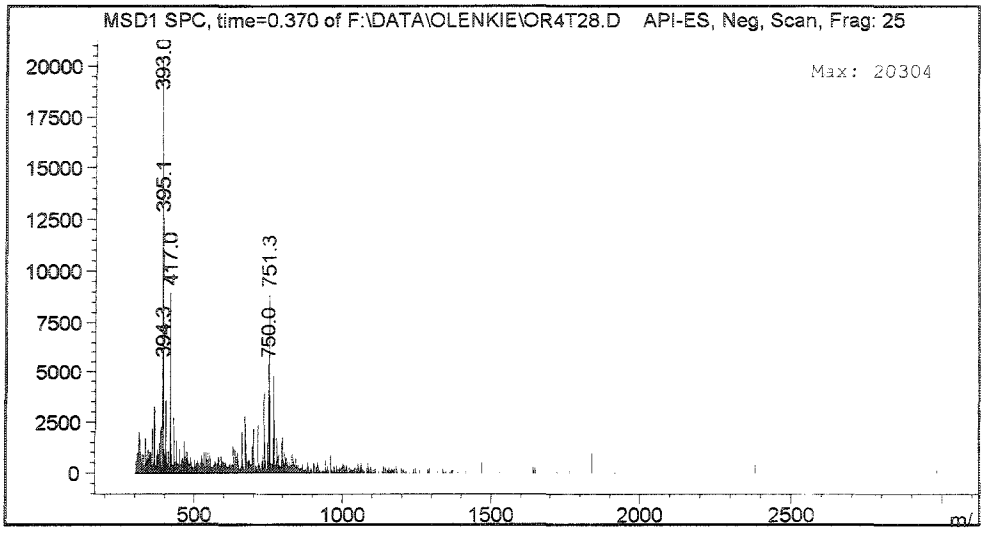


Figure 17: UV absorption characteristic of fraction QF-9, detected at molecular ion 955 m/z, found to be typical of saponins as shown previously in Figure 7.

a)



b)

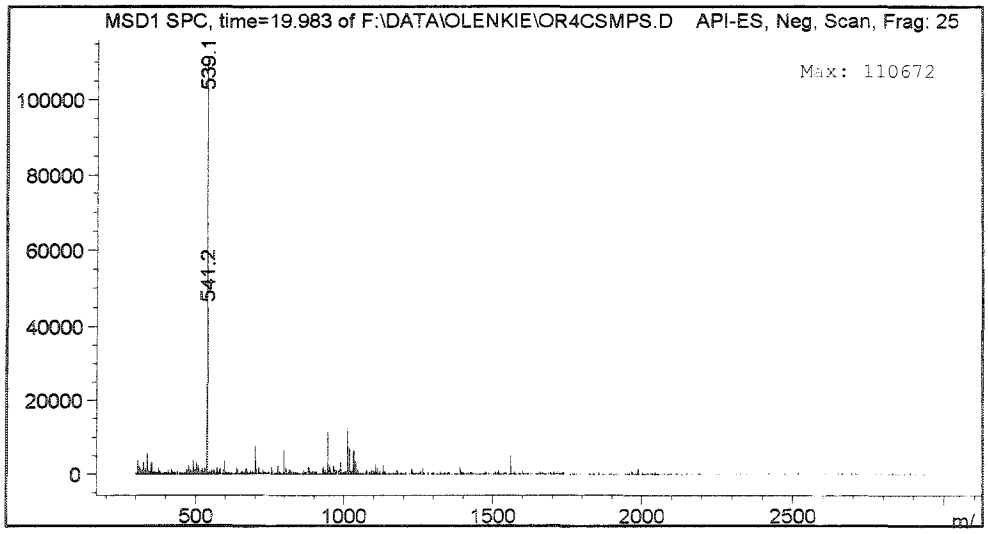


Figure 18: ESI scans of some fractions of lower molecular weight

(Continued on page 125)

c)

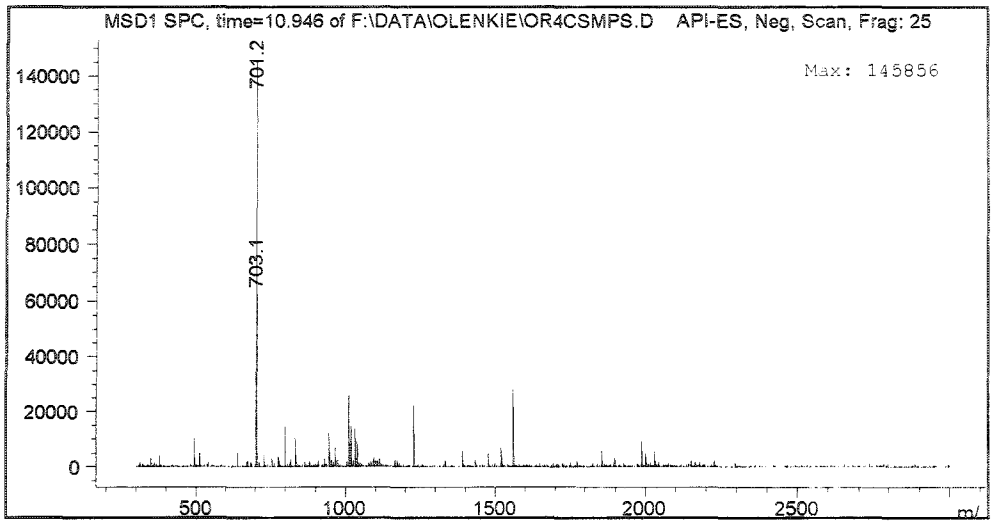
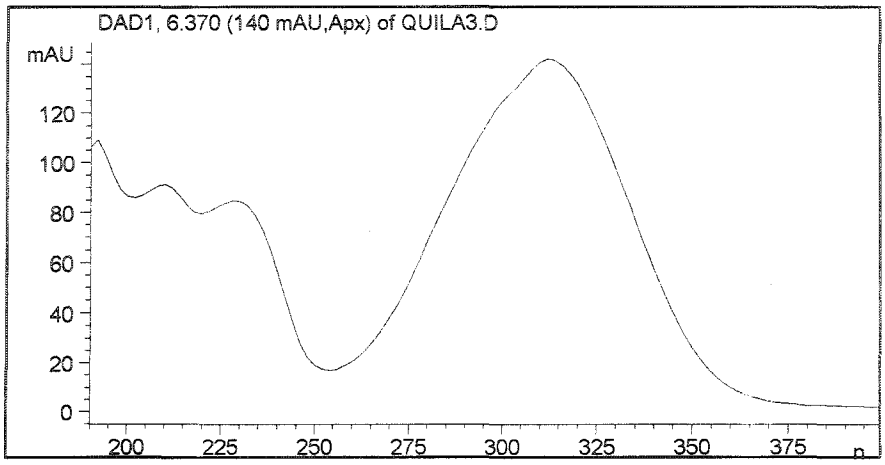


Figure 18: ESI scans of some fractions containing lower molecular weight compounds that were shown to be non-saponin and that produced no frothing upon slight agitation. **a)** = QF-2, **b)** = QF-19 and **c)**= QF-12

a)



b)

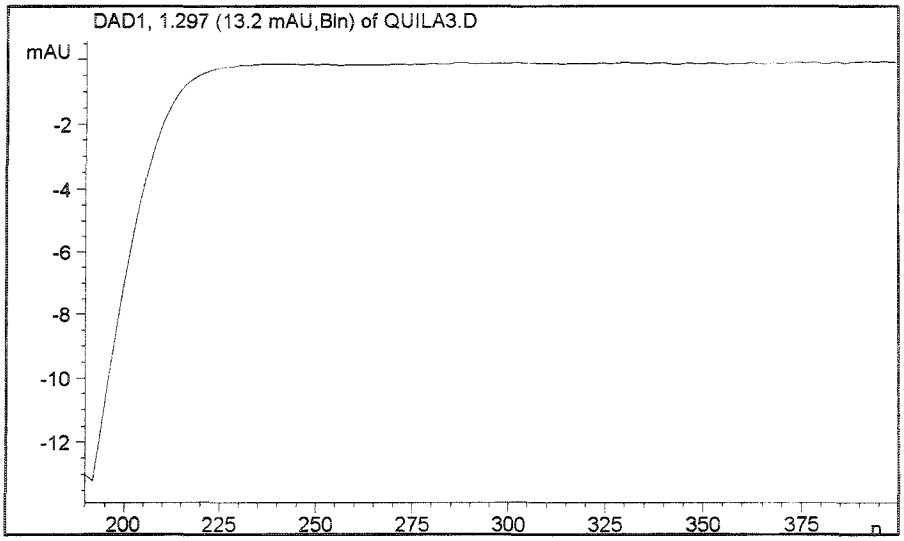


Figure 19: UV absorption characteristics of some non-saponin compounds (compare with Figure 7, p. 101-102). The compounds eluted at 6.37 minutes a) have their UV absorption maximum at 310 nm. While those eluting at 1.297 minutes b) absorb maximally at about 214 nm.

4.1.5 Solid Phase Extraction (SPE)

The chromatogram obtained after solid phase extraction appeared as shown in Figure 20. This chromatogram shows that the material eluted in the first 43 minutes of a 95 minute run was removed by SPE (compare with Figures 4, 6, and 11). Other differences in the chromatograms are due to the batch to batch differences in the Quil-A material. This procedure (SPE) was introduced with the hope of eliminating non-saponin material from Quil-A as it appeared to have detrimental effects on the column that led to the shortening of column life. In addition, simplification of the HPLC separation process was anticipated. However, this idea was abandoned after the results of the LC/MS of whole Quil-A showed the presence of high molecular weight compounds being eluted at a very early stage. Some of these high molecular weight compounds are likely to be saponins, as the vials in which they were collected showed frothing upon agitation. The inspection of the diode array signal of the LC/MS spectrum in the corresponding area of collection of these compounds showed them to possess a characteristic UV absorption for saponins (Figure 21). And indeed, fractions QF-9 (QS-L1), containing a compound having molecular mass m/z 955 (Figure 16, p. 122), and QF-11, having molecular mass of m/z1257 (Figure 22, p.132), were collected from this area. These compounds may also be biologically active.

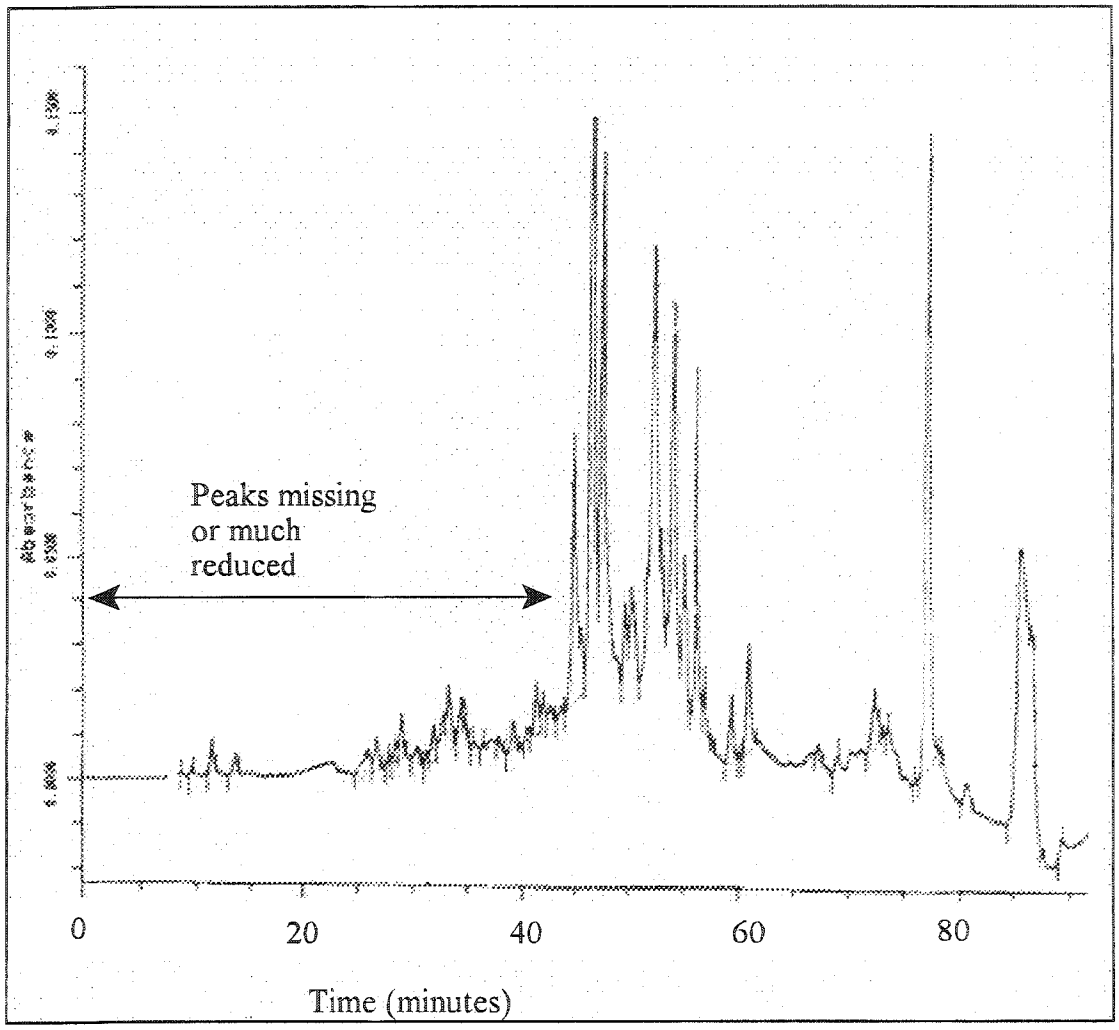
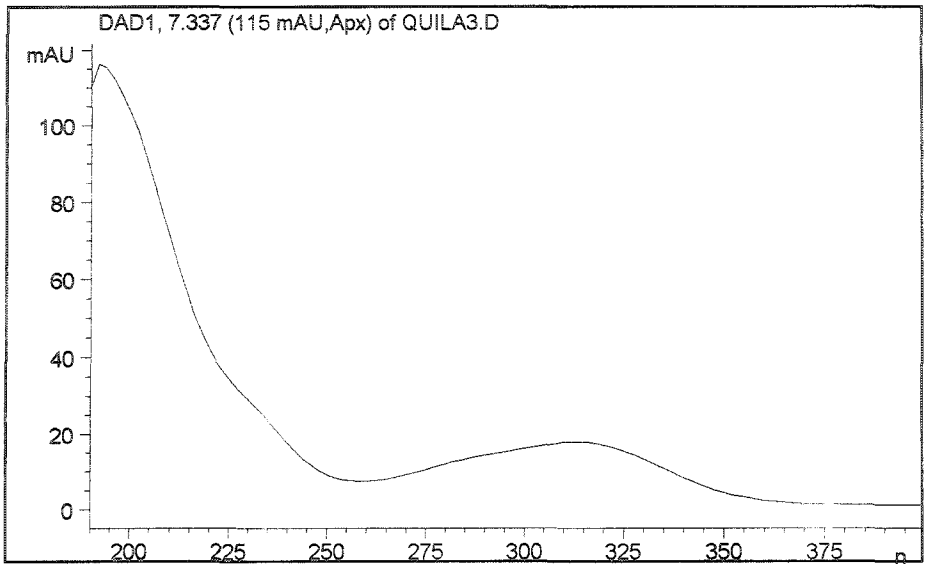


Figure 20: Elution Profile of Quil-A after solid phase extraction. Most of the material eluted in the first 40 minutes is eliminated. Compare with Figures 4 and 16.

a)



b)

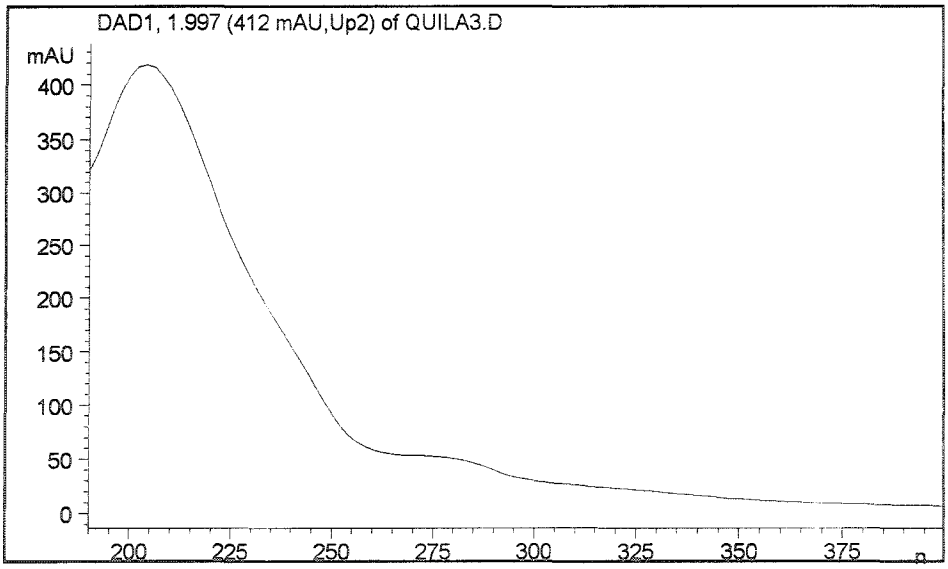


Figure 21: UV absorption characteristics for **a)** QF-9 (QS-L1), having molecular mass m/z 955 and **b)** QF-11, having molecular mass of m/z 1257 collected from the early area of the elution profile.

Consequently, a decision was made to collect samples from all sections of the chromatogram. More so because there is the possibility that the non-saponin components of Quil-A may also be responsible for some of its immunological activity. Of the 50 mg crude Quil-A used in the SPE experiment only 16.1 mg were retained.

4.1.6 Mass Spectral analysis

This technique was employed to assess the purity of the fractions separated and for the purpose of structural elucidation of the isolated components. The techniques that were used included electrospray ionization-mass spectrometry (ESI-MS), cone voltage induced dissociation - mass spectrometry (CVID-MS) and collision induced dissociation-tandem mass spectrometry (CID- MS MS). Mass spectrometric analysis was carried out on some samples suspected to be of higher purity than the others (based on the observations made on the beginning and the ending of the peaks from which they were obtained during manual collection). Six compounds were obtainable in pure or near pure form. Figures 12, 13, 14, 16, 22, 23, 24, 25, and 26 show mass spectral analysis of some of these compounds collected from semi-preparative HPLC runs. Four of the compounds collected in pure form are novel. Table 8 gives the molecular weights of the compounds obtained in pure form.

Table 8: Molecular weights of the compounds obtained in pure form.

Fraction	m/z	Comments
QF-9	955	QS-L1
QF-11	1217(1257)	Novel
QF-15	1353(1389)	Novel
QF-21	1856	Novel
QF-22	1989(2012)	QS-21
QF-23	1560	Novel

The numbers in parenthesis represent the same compound with its potassium or sodium salt (differences of 39 or 23 in the molecular weights). Indeed one or the other molecular ion was found in corresponding vials of the HPLC runs.

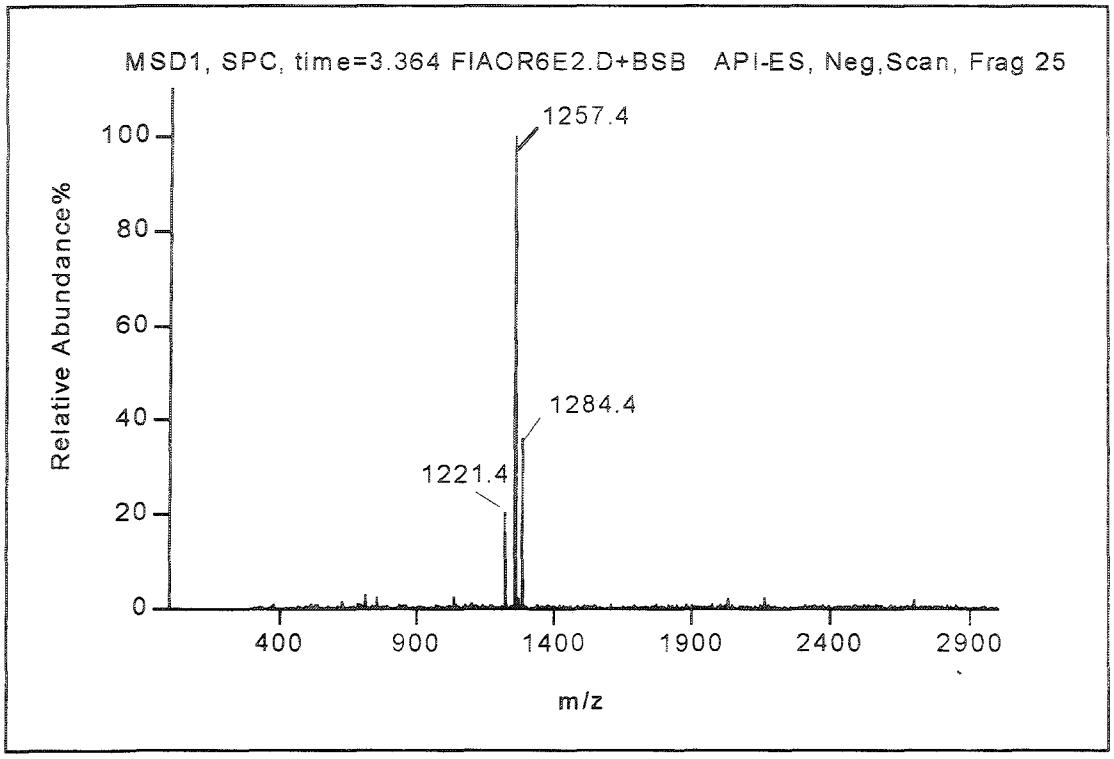


Figure 22: ESI- MS of pure novel compound QF-11 with the ion of the molecule of its potassium salt detected at m/z 1257. The molecular weight of this compound is 1218 Daltons and the ion of this molecule is detected at 1221 m/z. The peak observed at 1284 m/z represents the sodium salt of the potassium salt. All three major peaks represent the same compound.

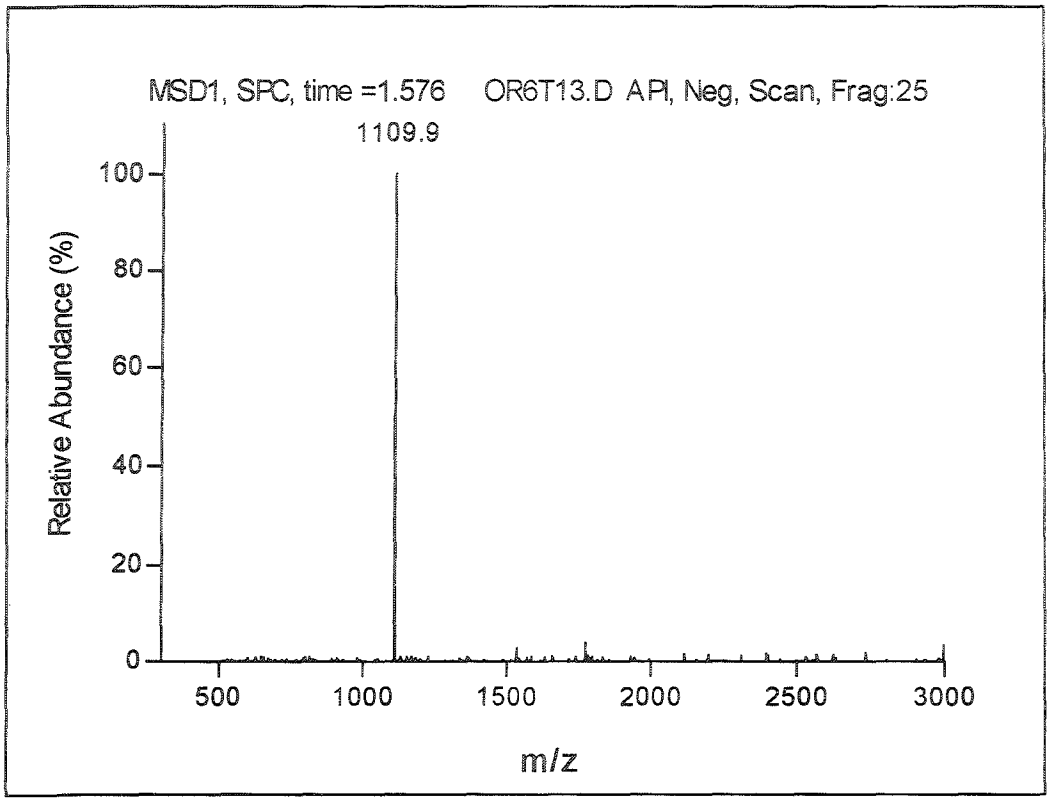


Figure 23: ESI-MS scan of QF-1. Molecular ion of compound QF-1 is detectable at m/z 1109.9

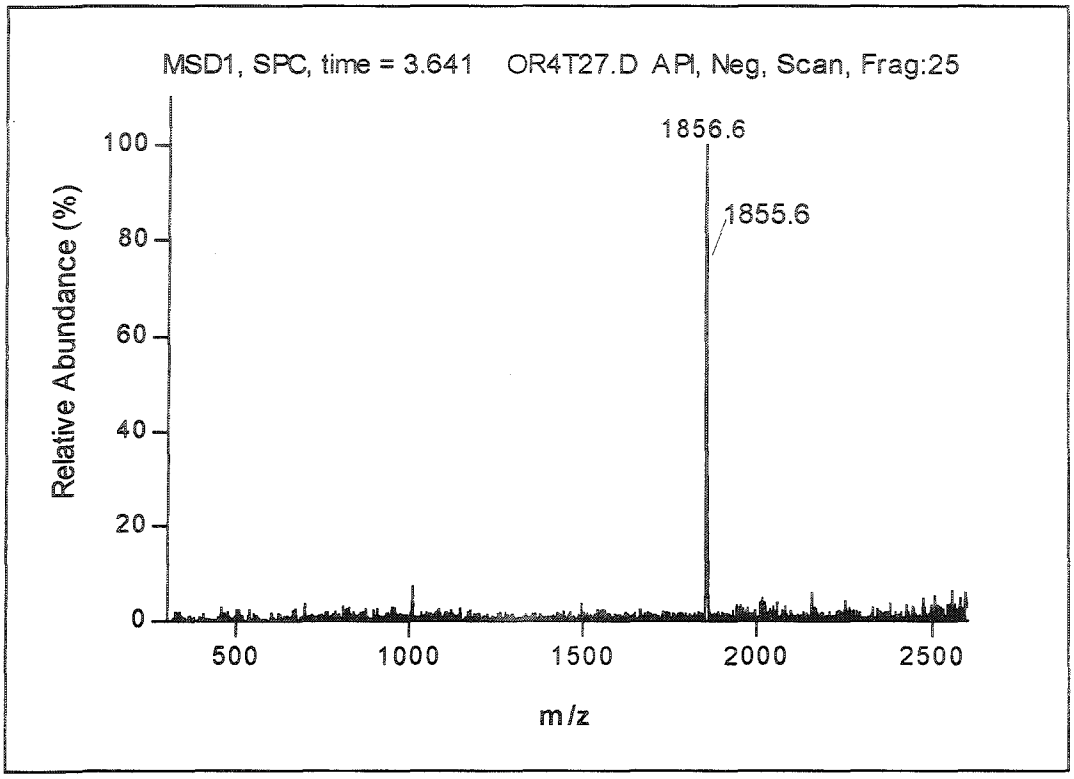


Figure 24: ESI scan of fraction QF-21, ion of this compound QF-21 is detectable at 1856 m/z

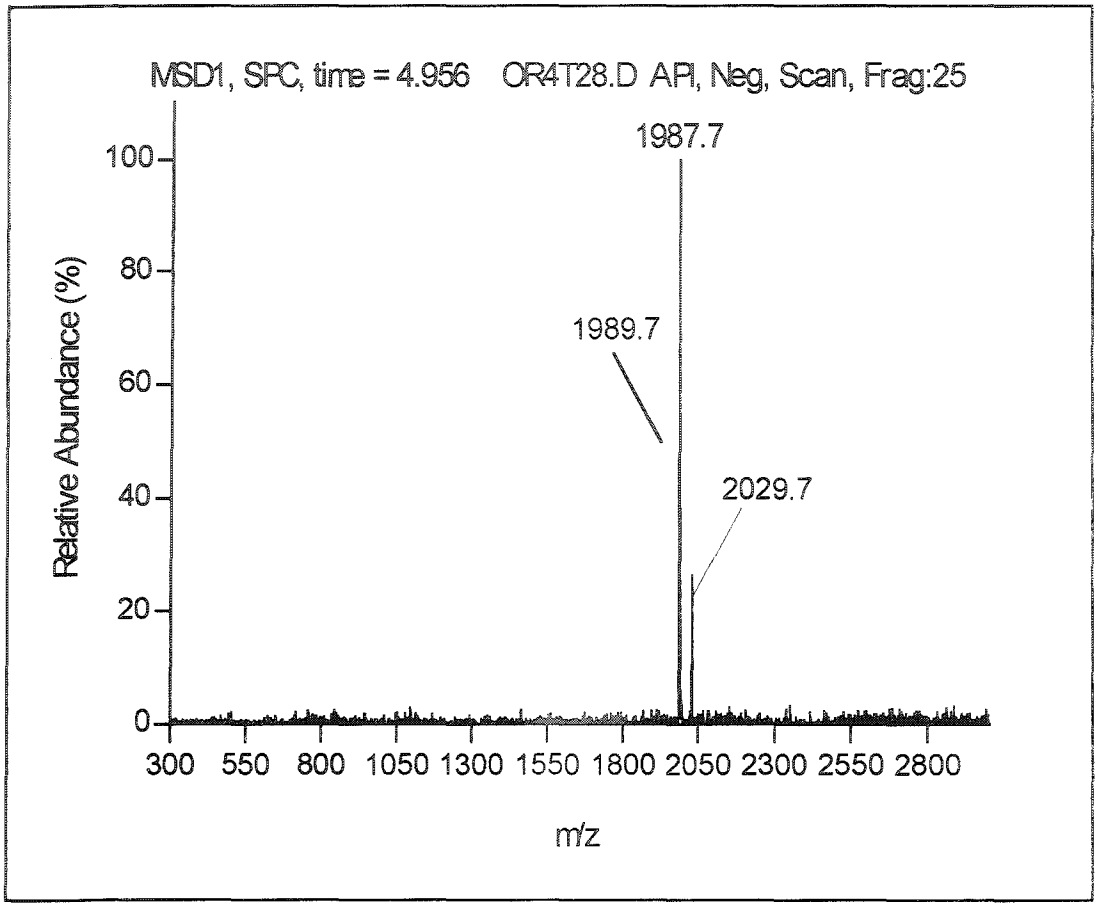


Figure 25: Pure compound QF-22 (QS-21), molecular ion m/z 1989. Ion detectable at 2029.7 is of the potassium salt of this compound.

QF-23 was the purest fraction collected as it produced a spectrum with only one major peak at m/z 1560, Figure 26, and because of that was chosen for further structural evaluation

i) Cone voltage dissociation of QF-23

Cone voltage dissociation was carried out on the ion of the molecule of QF-23. This involved the fragmentation of the ion at different cone voltages ranging from 50 to 200kV. A series of daughter ions were observed as probable daughter ions at m/z 629, 715, 773 and 895 of compound QF-23 (Figure 27).

ii) Collision induced dissociation tandem mass spectrometry (CID-MS/MS)

Collision induced dissociation tandem mass spectrometry was also carried out on QF-23. This experiment involves the fragmentation of a selected ion. The ion is selected from the first mass analyser, fragmented by means of collision with an inert gas, usually argon or xenon (in our case argon was used), in a collision cell. The fragmented daughter ions are then analyzed in another mass analyser, hence MS/MS. Probable daughter ions of QF-23 were observed in the CID-MS/MS carried out on QF-23 at 25 *kV* cone voltage and collision energy of 180 *eV*. Ions of importance were detected at m/z 671, 715, 833 and 894 (Figure 28).

Figure 26: ESI spectra of compound QF-23 acquired in the negative ion mode. Spectrum obtained with cone voltage 50 *kV*. **a)** is very clean and is without any noise. The ion detected at m/z 779 in spectrum. **b)** is the doubly charged ion $[M-2H]^{2-}$ of the compound.

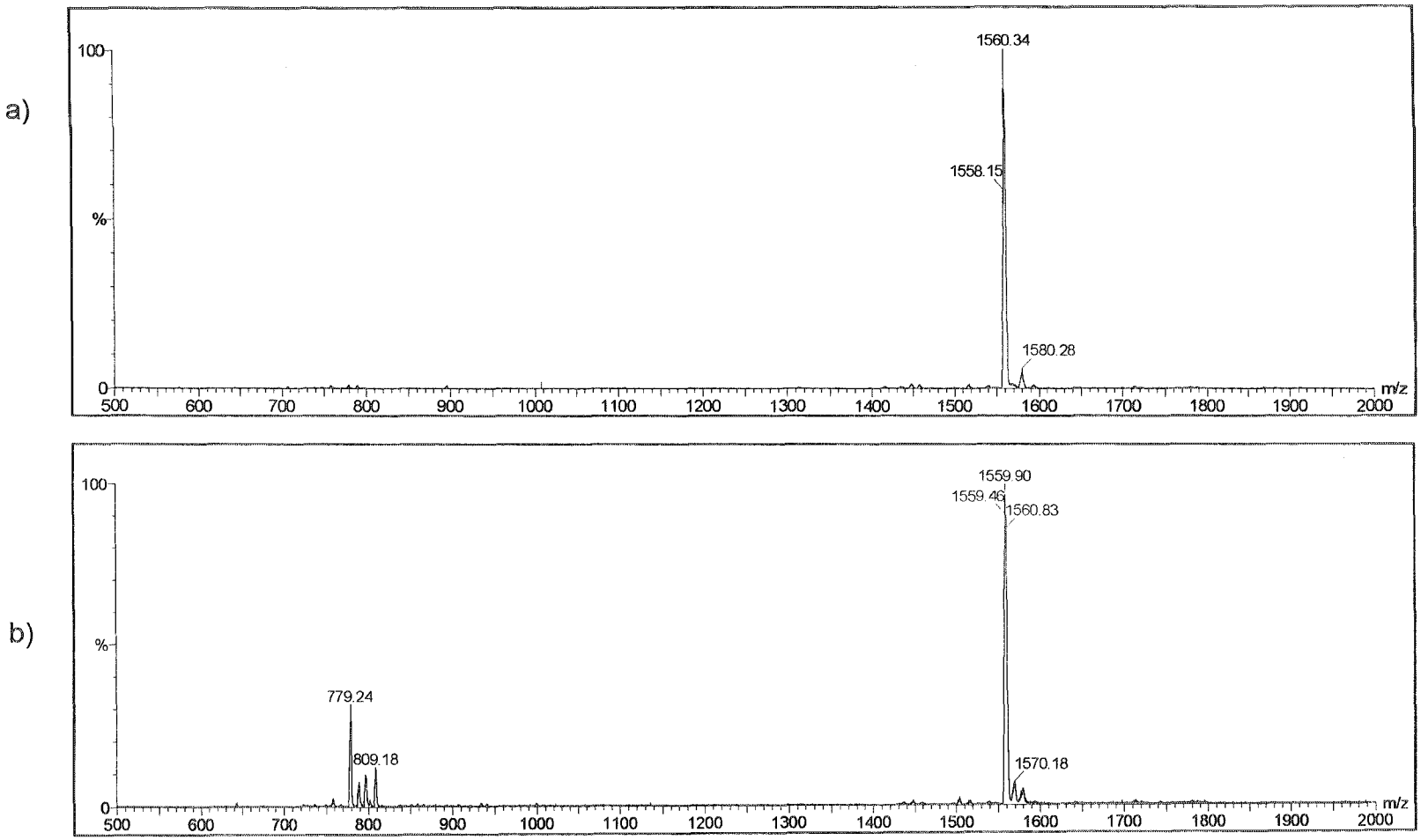


Figure 26: ESI spectra of compound QF-23

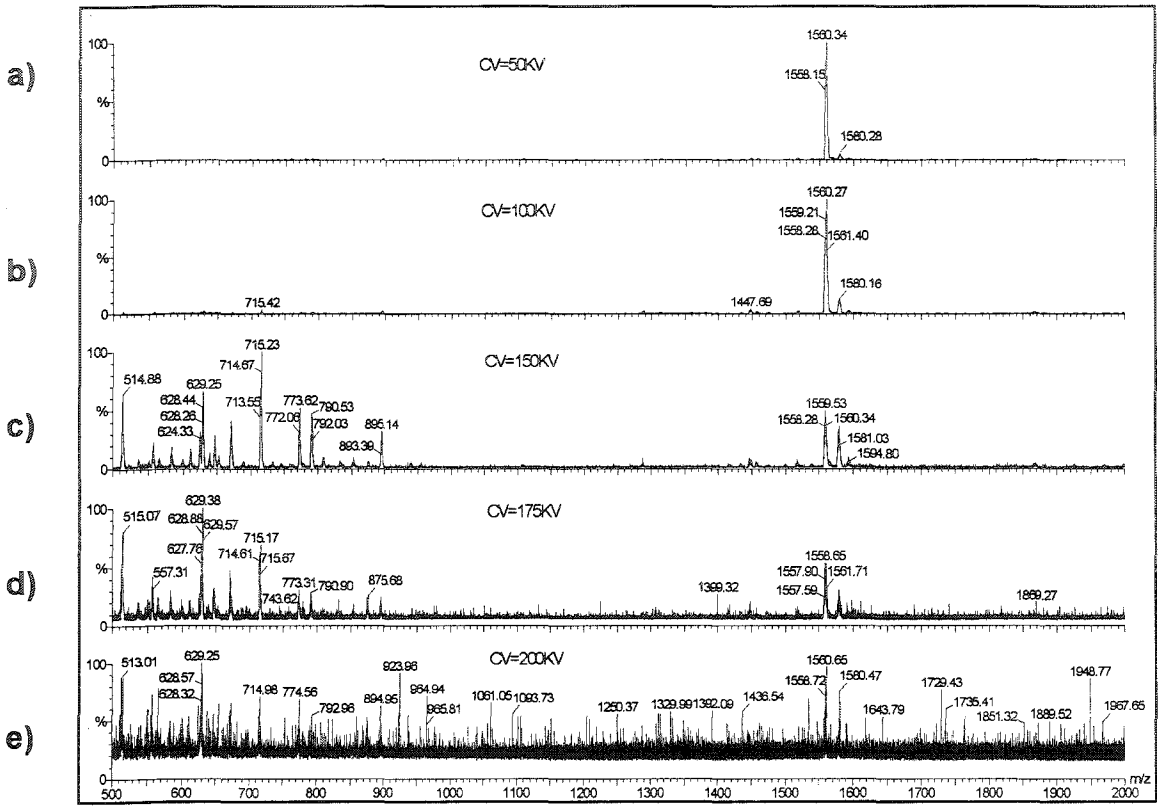


Figure 27: Cone voltage induced dissociation of fraction QF-23. Possible fragmented daughter ions of the parent molecule are observable at m/z 629, 715, 773 and 895 in spectra **c)**, **d)** and **e)** and the presence of the daughter ions at m/z 715 is visible in **b)** at minute abundance. As the cone voltage is increased and the fragmentation of the molecular ion at 1560 is increased the magnitude of this peak become diminished as compared to **a)** and **b)** where there has been no or very little fragmentation. This provides additional evidence that the fragments are indeed from the compound with the molecular ion at 1560 m/z.

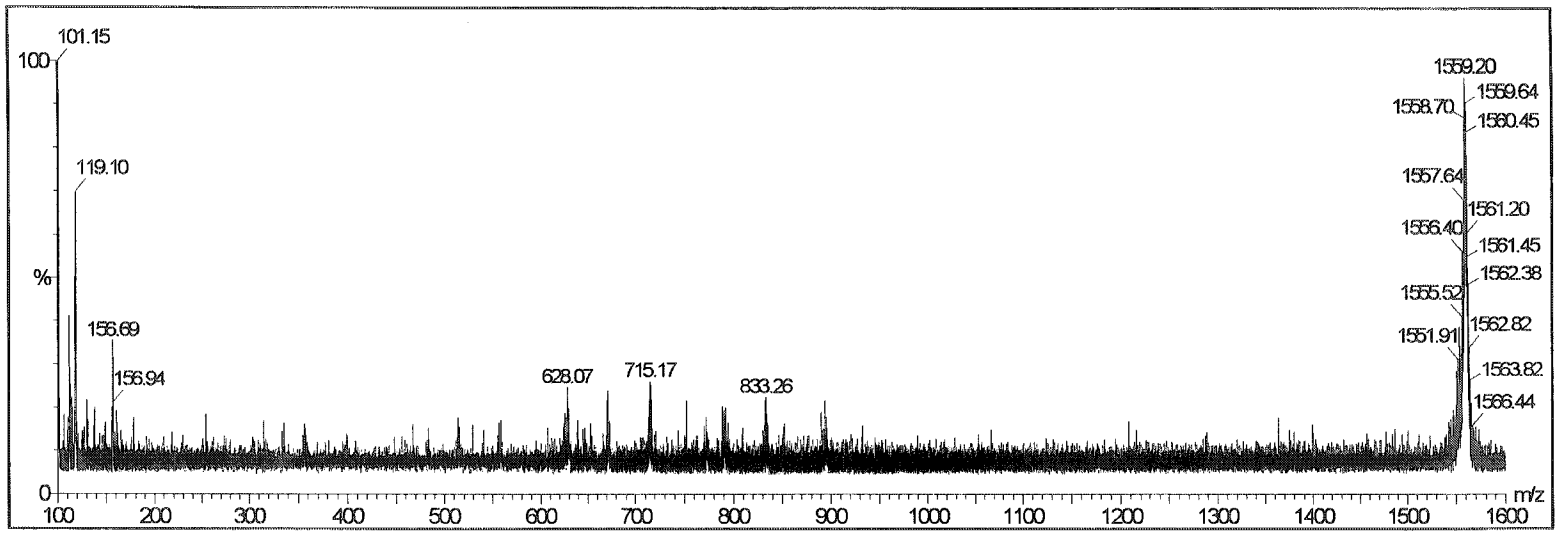


Figure 28: Collision induced dissociation tandem mass spectrometry analysis of the molecular ion of the compound QF-23 detected at m/z 1560. The fragmentation of this ion was achieved at a collision energy of 180 *eV* and cone voltage of 25 *kV*. A series of daughter ions are observed at m/z 628, 715 and 833 corresponding to plausible fragmentation products of QF-23

This information gave much insight into the fragmentation pattern of this molecule, and formed the basis of the proposed molecular structure shown in Figures 5, 31, and 43. Figure 31 on page 143 describes how the daughter ions identified in these experiments correspond to the different parts of the molecule of the compound QF-23.

4.1.7 Cleavage reactions

In addition to the experiments mentioned above, QF-23 was subjected to alkaline hydrolytic cleavage reactions. This reaction was carried out on the pure saponin compound to selectively cleave the molecule at positions that will leave the naked aglycone or the prosapogenin (that is the saponin molecule minus any 28-O bound acyl moiety). Figure 29 shows the ESI-MS of the chemical derivatives of the alkaline hydrolysis of fraction QF-23 which resulted in the appearance of a peak at 411 m/z representative of the ion formed by the aglycone quillaic acid. Thus, confirming the identity of QF-23 as a *Quillaja* saponin. The chemical structure of the ion formed from quillaic acid aglycone, detected by negative ion ESI-MS scan of the alkaline hydrolysis products of QF-23 is as shown in Figure 30.

Evaluation of the scans observed after the CID-MS/MS and the voltage induced dissociation of the molecule of QF-23 led to the proposal of the following structure and fragmentation patten of QF-23 (Figure 31).

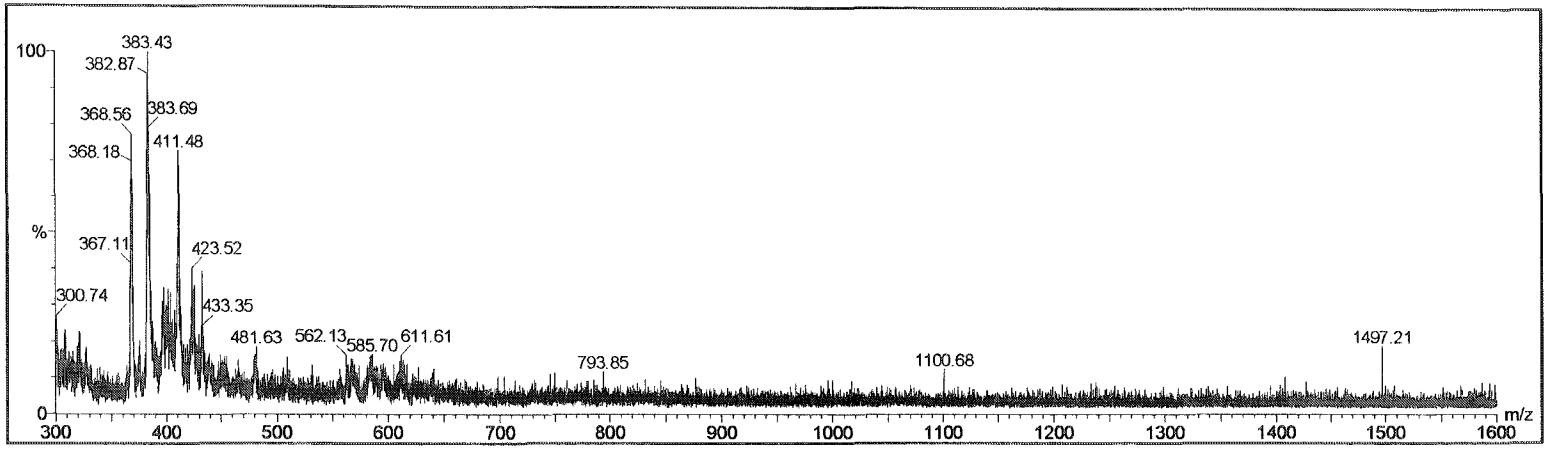


Figure 29: ESI-MS scans of the alkaline hydrolysis products of QF-23. The scan was acquired using cone voltage 25 *kV*. The ion detected at m/z 411 is suspected to have been liberated from the quillaic acid aglycone during alkaline hydrolysis (see Figure 30). The presence of this ion supports the identity of QF-23 as a *Quillaja* saponin.

Figure 30: Structure of the ion liberated from the aglycone of the *Quillaja* saponin QF-23 during alkaline hydrolysis. This ion is detected at m/z 411.48 in the ESI-MS scan of alkaline hydrolysis products of this compound shown in Figure 29. The molecule of the compound was cleaved at the highly reactive positions of C-3 and C-28 to liberate quillaic acid which then was oxidized to yield the quillaic acid ion detected at m/z 411.48. The rearrangement of the aglycone quillaic acid during isolation has been reported before (Kubota *et al.*, 1969 in van Setten and van de Werken 1996). The presence of this ion in the ESI-MS scan confirms the identity of this compound as a *Quillaja* saponin.

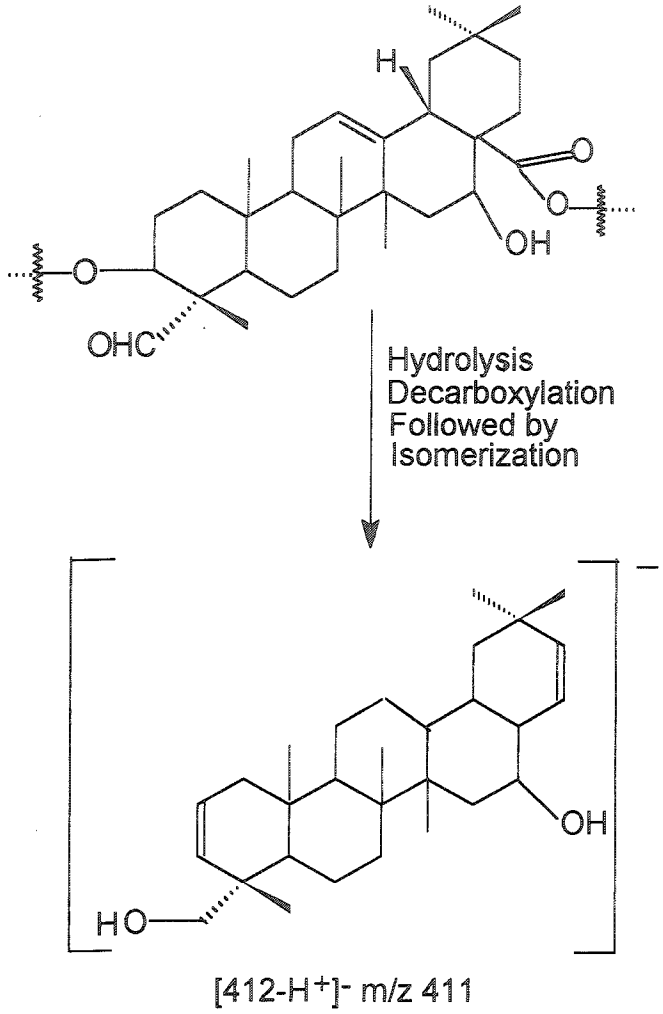
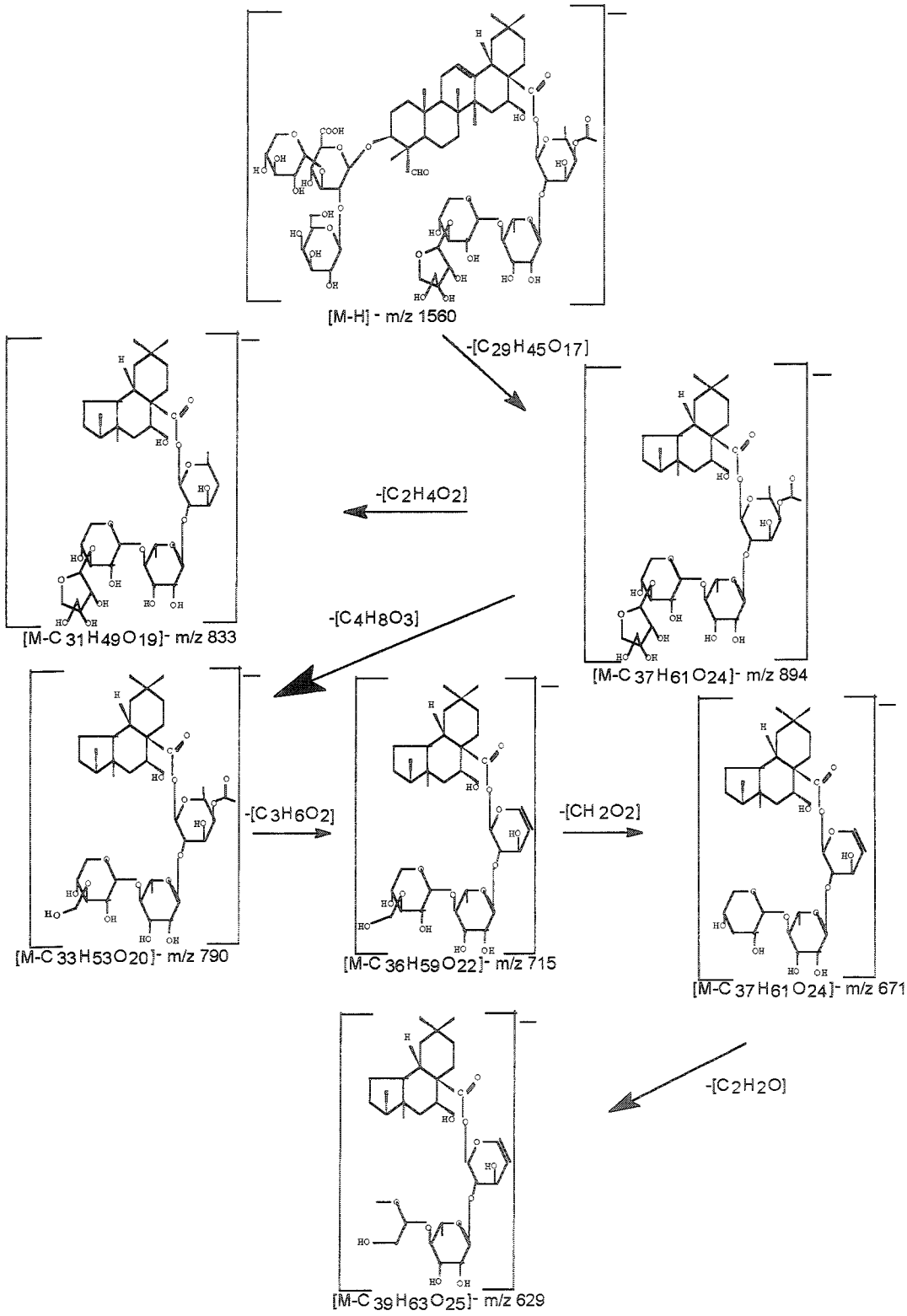


Figure 30: Structure of aglycone liberated from the pro-sapogenin during alkaline hydrolysis.

Figure 31: Proposed fragmentation pattern and structure for the deprotonated precursor ion of the molecule of QF-23 at m/z 1560. The detection of the daughter ions at m/z 629, 671, 715, 790, 833 and 894 in the scans obtained after cone voltage dissociation of the ion of the molecule of QF-23 (Figure 27), CID-MS/MS of this ion (Figure 28) and the ESI-MS scan of the alkaline hydrolytic products of this compound (Figure 29) led to the proposal of this fragmentation pattern which is based upon the "X-type sugar fragmentation" principles outlined by Domon and Costello (1988).


[M-H]- m/z 1560
-[C29H45O17]
-[C2H4O2]
[M-C37H61O24]- m/z 894
[M-C31H49O19]- m/z 833
-[C4H8O3]
-[C3H6O2]
-[CH2O2]
[M-C33H53O20]- m/z 790
[M-C36H59O22]- m/z 715
[M-C37H61O24]- m/z 671
-[C2H2O]
[M-C39H63O25]- m/z 629

4.1.8 Gas Chromatography/Mass Spectrometry (GC/MS)

This experiment was aimed towards determining the exact configuration and binding sites (linkages) of the sugar moieties that make up the molecule of the saponin QF-23. The GC/MS experiment required a permethylation reaction to be carried out on the saponin molecule to be analyzed in order to break it up into smaller molecules (including the sugars) which were then volatilized and investigated by this technique. When fraction QF-23 was subjected to GLC and GC/MS, the results showed the compound to contain the following sugars as listed in Table 9 and the linkages to be as indicated in Table 10.

This compound did not show the presence of either glucose, or arabinose molecules unlike QS-7 (Kensil *et al.*, 1998) and QS-21 (Kensil *et al.*, 1991).Taking into consideration the structural elements that are common to all *Quillaja* saponins that have been characterized to date (Figure 2, Figure 3 and Table 1), the presence of rhamnose in a 1:1 ratio and the presence of xylose in a 2:1 ratio, with one of the xylose molecules as a terminal sugar was confirmed. This evidence, in conjunction with one of the recent publications on structural analysis of some *Quillaja* saponins (Nord and Kenne, 1999) suggests the sugar arrangement for QF-23 shown in Diagram 2.

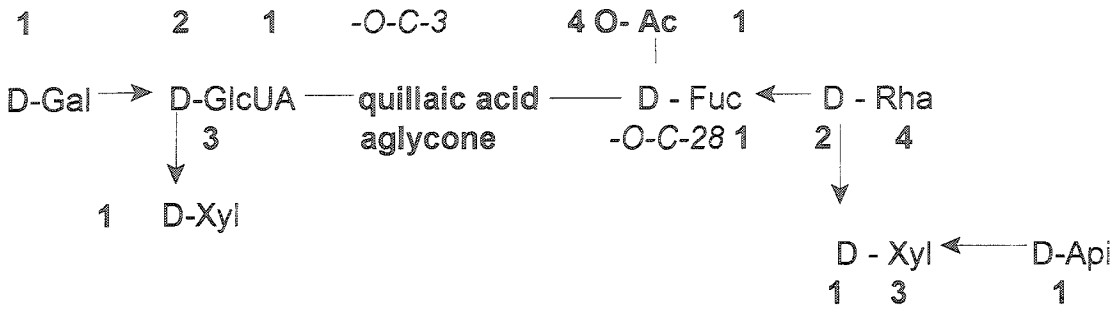


Diagram 2: Sugar arrangement in QF-23 Numbers represent the glycosidic linkages in the structure. GlcUA = glucuronic acid, Xyl= xylose, Gal = galactose, Fuc = fucose, Rha = rhamnose, Api = Apiose and D= dextrorotatory

Table 9 Sugar Analysis

D- Apiose, L- Fucose ,D- Xylose, L- Rhamnose, D-Galactose and D-Glucuronic acid;

1: 1: 2: 1: 1: 1

Table 10 Methylation Analysis

A. Three terminal non- reducing sugars were detected;

1. 1,5- di - O - Acetyl - 2,3,4,6 - tetra - O - Methyl - D - galactitol

2. 1,5- di - O - Acetyl - 2,3,4, - tri- O - Methyl - D - xylositol

3. 1,4- di - O - Acetyl - 2,3,5 - tri - O - Methyl - D - apiositol

B. Other sugars were found to be glycosylated

	Sugar	**glycosylation positions**
4. 1,2,3,5,6 - penta - O - Acetyl - 4 - O - Methyl - D - glucuronitol;	GlcUA:-	C-2, C-3
5. 1,3,5 - tri - O - Acetyl - 2,4 - di O - Methyl - D - xylositol;	2nd Xyl:-	C-3.
6. 1,4,5 - tri - O - Acetyl - 2,3 - di - O - Methyl - L - rhamnositol;	Rha:-	C-4
7. 1, 2,4,5 - tetra - O - Acetyl - 3 - O - Methyl - L - fucositol;	Fuc:-	C-2, C-4

4.1.9 Nuclear Magnetic Resonance (NMR) spectroscopy.

Three NMR experiments were carried out on two of the separated components QF-23 and QF-22. The experiments carried out were: **1)** a one dimensional proton acquisition experiment (^{1}H NMR), **2)** an inverse chemical shift correlation experiment, Heteronuclear Multiple Quantum Correlation (HMQC) that gives the signal of ^{1}H coupled directly to ^{13}C, and **3)** a long range chemical shift correlation experiment ; Heteronuclear Multiple Bond Correlation (HMBC) that is suitable for long range ^{1}H - ^{13}C connectivities. This experiment detects all the ^{13}C even those that are not directly bonded to ^{1}H. This experiment has a higher sensitivity than the more familiar COLOC (COrrelation LOng -range Coupling) since it is an inverse experiment. The advantage of inverse chemical shift techniques (Akitt and Mann, 2000; Rouessac and Rouessac, 2000) is that they detect insensitive nuclei such as ^{15}N, or ^{13}C through the detection of a very high - sensitivity nucleus, typically the proton. These techniques are most suitable for large biomolecules in which the sensitivity of ^{13}C is greatly diminished by the very often low % by weight of the carbon element (^{13}C) in the molecule, making it necessary to have very large amounts of pure compound in order to perform a direct ^{13}C acquisition experiment. Moreover because of the large size of these molecules they have very long relaxation time and as such ^{13}C acquisition experiments may require a very long time to run. Nevertheless, an inferred ^{13}C NMR

was obtainable form the two dimensional experiments. NMR spectra were recorded for saponins dissolved in deuterated methanol (CD_3-OD) at room temperature.

The ^1H NMR spectrum of QF-22 which was obtained using deuterated methanol appeared to match the spectrum of QS-21 that was acquired under similar conditions and found in the literature (Nord and Kenne, 1999) (Figure 32). Thereby, confirming the data obtained from the molecular weights that QF-22 was in fact QS-21, a well known Quillaja saponin (Kensil *et al.*,1991).

The results of the three experiments revealed the presence of an aldehyde (9.5 ppm) and an olefinic proton (5.6 ppm) in the molecules of both the compounds tested as well as two carbonyl groups in the molecule of QF-23, while the number of the carbonyl groups detectable in the molecule of QF-22 was three. A signal corresponding to a carboxyl group was also identified in each of these molecules. Figure 33 a) shows the aldehyde and the olefinic proton while Figure 33 b) shows the carbonyls and the carboxyl groups as detected in the NMR spectra of the molecule of QF-23. These findings are indeed in agreement with our proposed structure of QF-23 and the known structure of QS-21 (Kensil *et al.*, 1991).

Further analysis of the data obtained from the NMR experiments is summarised in Figure 34 and Table 11 that show the configuration at the anomeric carbon atom of the sugars (hemiacetals) that make up the molecule of QF-23 and the assignment of the ^1H NMR chemical shifts in ppm for the residues in the quillaic acid portion and the oligosaccharides of the this molecule.

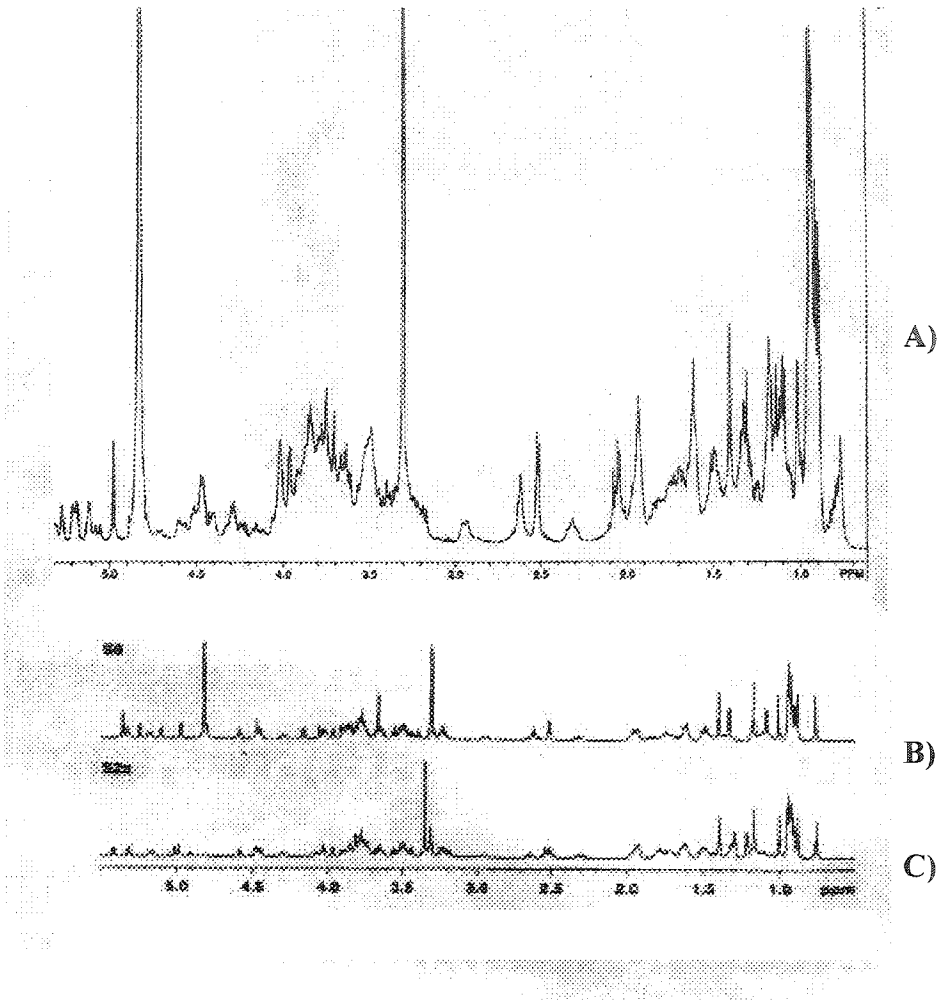
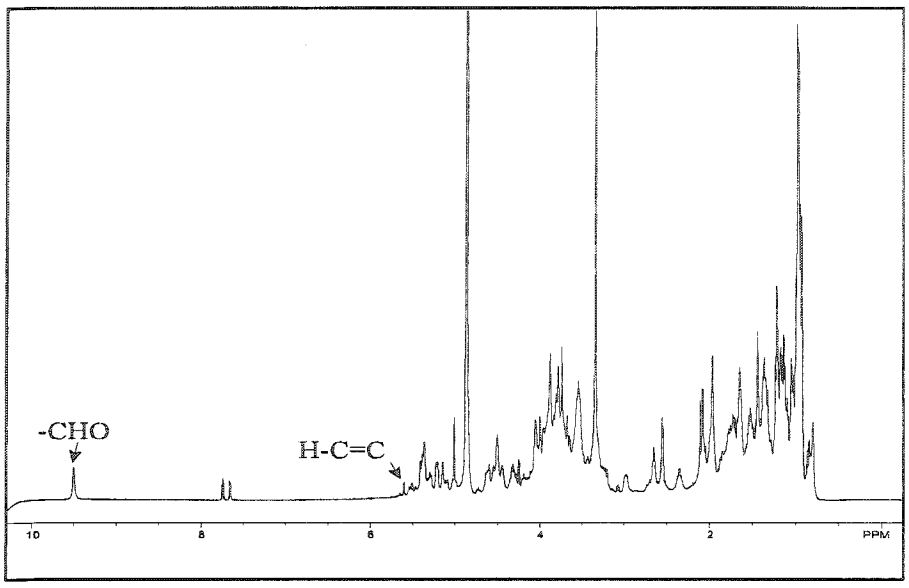


Figure 32: **A)** ^{1}H NMR spectrum of QF-22, **B)** ^{1}H NMR spectrum of compound S6 and **C)** ^{1}H NMR spectrum of compound S2a. The ^{1}H NMR spectrum of QF-22, **A),** matches that of compound S6 (Nord and Kenne, 1999), spectrum **B)**, which is QS-21, a Quillaja saponin of known molecular structure (Kensil *et al.*, 1991): Confirming that QF-22 is equivalent to QS-21. The spectrum shown in **C)** is quite different from that obtained from QF-22, showing that the compound S2a (Nord and Kenne, 1999) is clearly different from QF-22. All three spectra were acquired under similar conditions.

Figure 33: **a)** ^{1}H NMR spectrum of QF-23; **b)** HMBC spectrum of QF-23.

In **a)** the signal due to he aldehyde proton is visible as a singlet at 9.5 ppm while that of the olefinic proton is seen at 5.6 ppm also as a singlet. **b)** shows the carbonyl and the carboxylic acid signals at the proton chemical shifts of 1.16 and 1.19 ppm respectively.

a)



b)

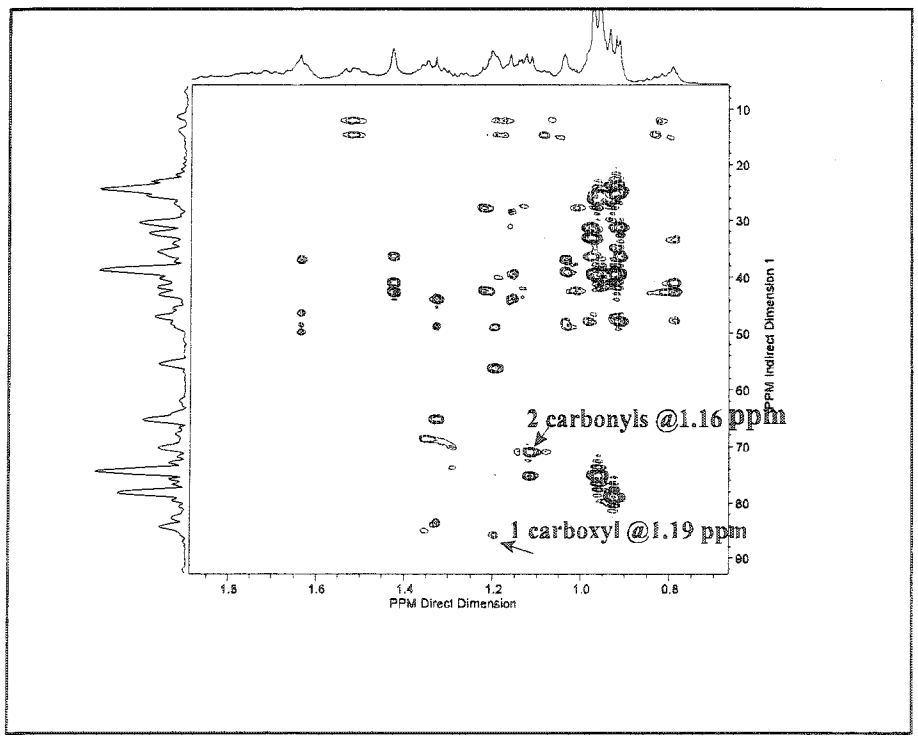
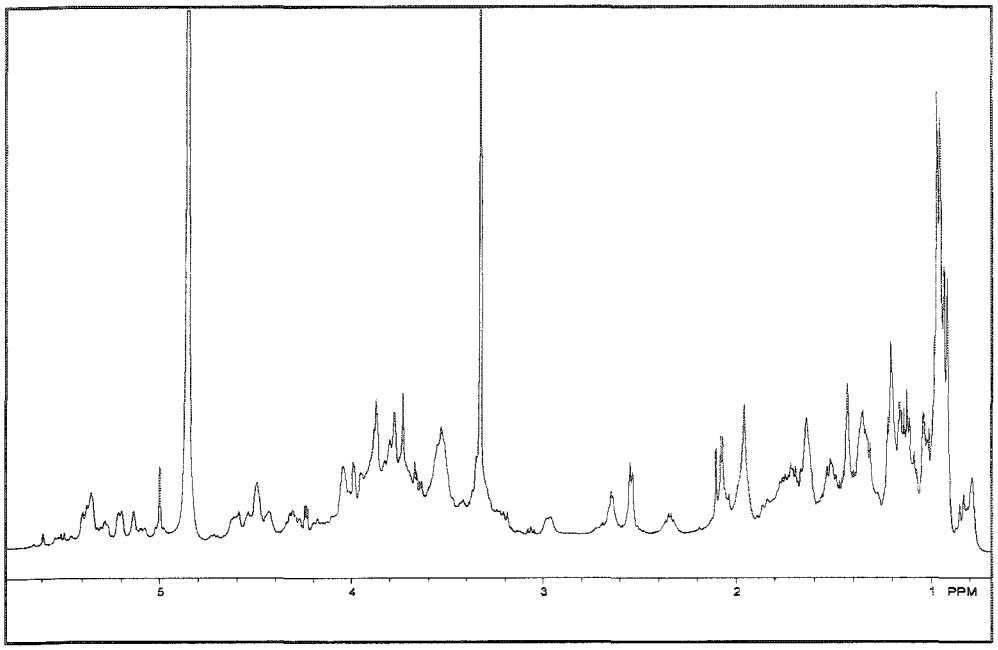


Figure 33: a) ^{1}H NMR spectrum of QF-23; b) HMBC spectrum of QF-23

Figure 34: a) ^{1}H-NMR spectrum of QF-23 from 1-6 ppm b) ^{1}H-NMR spectrum of QF-23 from 1-6 ppm showing the anomeric carbon atoms of the sugar molecules and the coupling constant calculations used in determining the sugar configurations (Table 11). Qa-1 and Qa-3 represent the carbon atom residues in the quillaic acid portion of the molecule. GlcUA = glucuronic acid, Xyl= xylose, Gal = galactose, Fuc = fucose, Rha = rhamnose, and Ara = arabinose

a)



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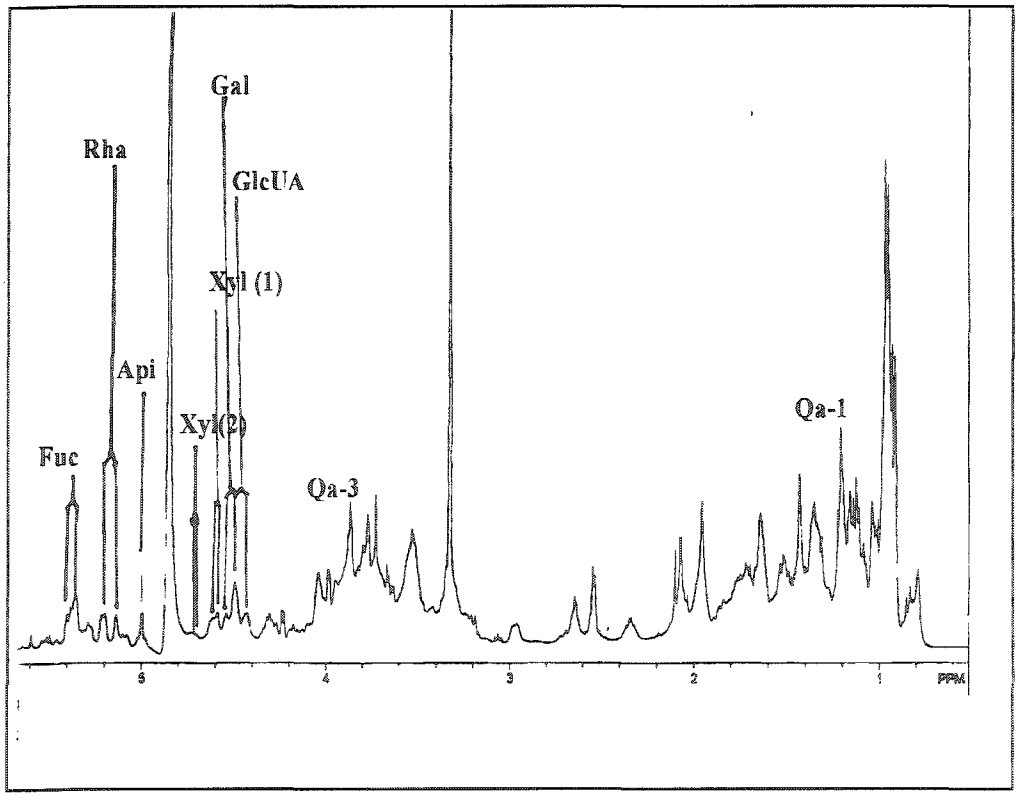


Figure 34 ^{1}H-NMR spectra of QF-23

Table 11: Assignment of the ^{1}H NMR chemical shifts in ppm and configuration for residues in QF-23. The data in this table is obtained from Figure 34. **1.** Qa-1 represents the carbon number in the quilliac acid (aglycone) portion of the molecule; Qa-3 represents the third carbon atom in the aglycone. GlcUA-1 to Api-1 represents the first carbon atom in the respective sugar molecules in the compound. **2.** Figures represents the chemical shifts observed for the hydrogen atoms attached to the respective carbon atom residues as documented in the literature (Higuchi *et al.*, 1987; Guo *et al.*, 1997; Nord and Kenne, 1999). **3.** Represents chemical shifts for the protons attached to the respective carbon atoms as observed from the ^{1}H NMR of QF-23 (Figure 34). **4.** All the J couplings are calculated in Hz; d and s represent doublet and singlet, respectively. **5.** Configurations of the anomeric carbon atoms were assigned conventionally in accordance with the J coupling figures. D = Dextrorotatory; L = levorotatory; p = pyranoside; f = furanoside. Sugar abbreviations are as explained in Figure 34.

Table 11: Assignment of the ^{1}H NMR Chemical Shifts in ppm and configuration for residues in QF-23

Residue[1]	Lit. Ref [2]	QF-23[3]	$^{1}J_{H-H}$ couplings[4]	Sugar Configuration[5]
Qa-1	1.11/1.72	1.11/1.72	-	-
Qa- 3	3.86	3.86	-	-
GlcUA-1	4.46	4.48	d J= 6.24	β -Dp
Xyl(1)-1	4.58	4.59	d J= 1.59	α-Dp
Gal-1	4.46	4.48	d J= 6.10	β-Dp
Fuc-1	5.34	5.34	d J= 6.24	α-Lp
Rha-1	5.02	4.99	d J= 6.29	α-Lp
Xyl(2)-1	4.69	4.72	d J= 1.59	α-Dp
Api -1	5.27	5.02	s J = 0	β-Df

4.2 Biological Testing

The second leg of this research project involved the determination of the *in vitro* biological activity of the isolated fractions. The biological investigations that were carried out on the purified saponins included hemolytic studies, toxicity studies and cytokine (IL-1α) induction. Figure 35 shows the fractions that were investigated for biological activity. Not all biological testing were performed on all of the fractions shown due to lack of availability of sufficient quantities.

4.2.1. Evaluation of the hemolytic activity of the fractions

The hemolytic activity of two saponin fractions was determined by an *in vitro* spectrophotometric analysis on rat's (Sprague-dawley) red blood cells (RBC) utilizing the detergent NP40 as a positive control and the dilution solution PBS as the constitutive control. The results of this experiment obtained for fractions QF-23 and QF-11 are as shown in Figure 36 (page 155). Fraction QF-11 appeared to have lower hemolytic activity than QF-23 at lower concentrations with an estimated HI_{50} of 400 µg/mL as compared to that of 333 µg/mL (estimated) for QF-23.

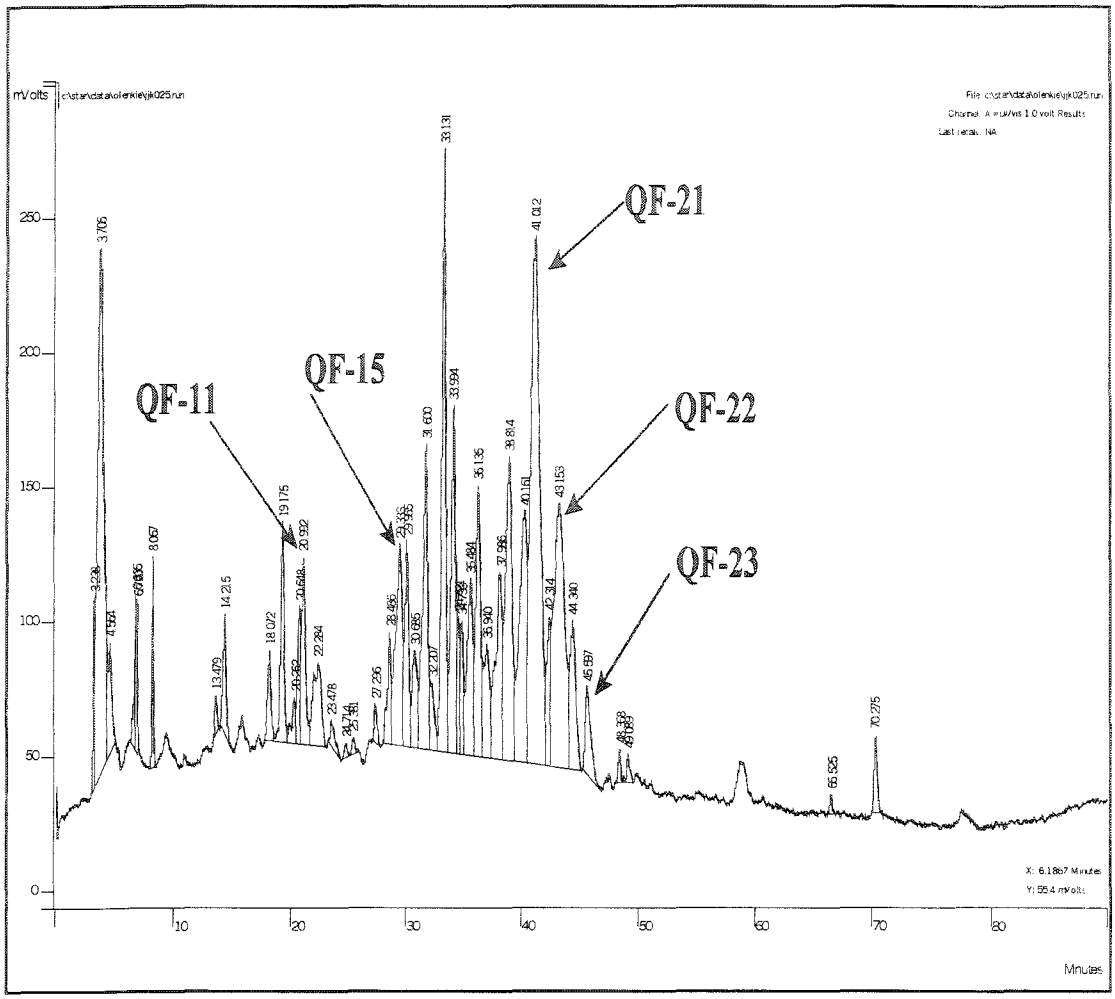
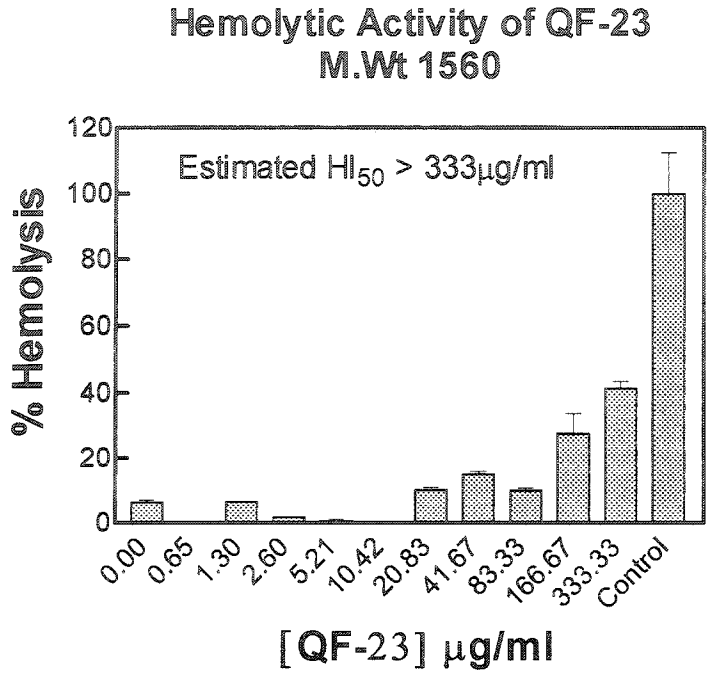


Figure 35: A trace of semi-preparative RP HPLC showing fractions which were used to estimate biological activity of saponins.

Figure 36 : Hemolytic activity of some *Quillaja* saponins. Hemolysis is estimated graphically as HI_{50} (Hemolytic Index Fifty) which is the concentration of the drug that gives 50 % hemolysis. QF-11 (HI_{50} = 400 µg/mL) appear to be less hemolytic than QF-23 (HI_{50} = 333 µg/mL). Mean values ± SD of the values obtained in triplicate (n=3) were plotted.

a)



b)

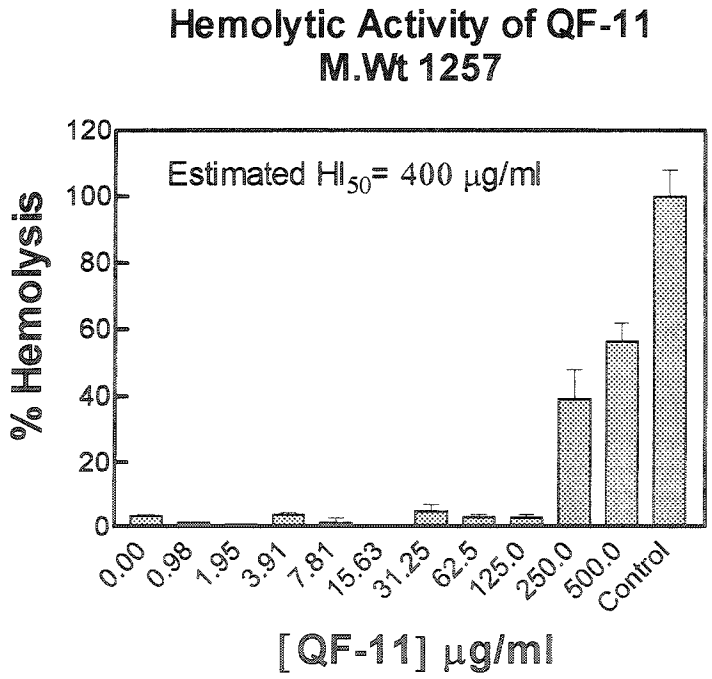


Figure 36 : Hemolytic activity of some *Quillaja* saponins

4.2.2. Toxicity of saponins to murine monocytic cell line J774A

The toxicity of the saponins was determined *in vitro* using the MTT method as developed by Ford *et al.* (1989). Toxicity of four chromatographically pure samples, QF-23, QF-22 (QS-21), QF-21 and QF-15 was assessed. QF-23 was found to have similar *in vitro* toxicity on murine monocytes as that of QF-22 (QS-21) (TC_{50} = 2.96 µg/mL for QF-23 and 3.981 µg/mL for QF-22 (QS-21). Compound QF-15 (Mw 1389) exhibited much lower toxicity (TC_{50} = 25.12 µg/mL) than the other fractions tested (Figure 37 and Table 12). Toxicity of QF-21 was found to be 1.51 µg/mL. The fractions chosen for toxicity studies were samples from various parts of the elution profile as shown in Figure 35.

4.2.3. *In vitro* cytokine production determination as a measure of adjuvant activity

Assessment of the adjuvant capability of any compound can be performed by measurement of a number of immunological parameters. These parameters are, induction of antibodies, T-cell proliferation, production of cytotoxic T-lymphocytes, and induction of cytokine by cells of the immune system including antigen presenting cells (APC). These parameters can be determined *in vivo* and in some circumstances *in vitro*.

(cont on p. 159)

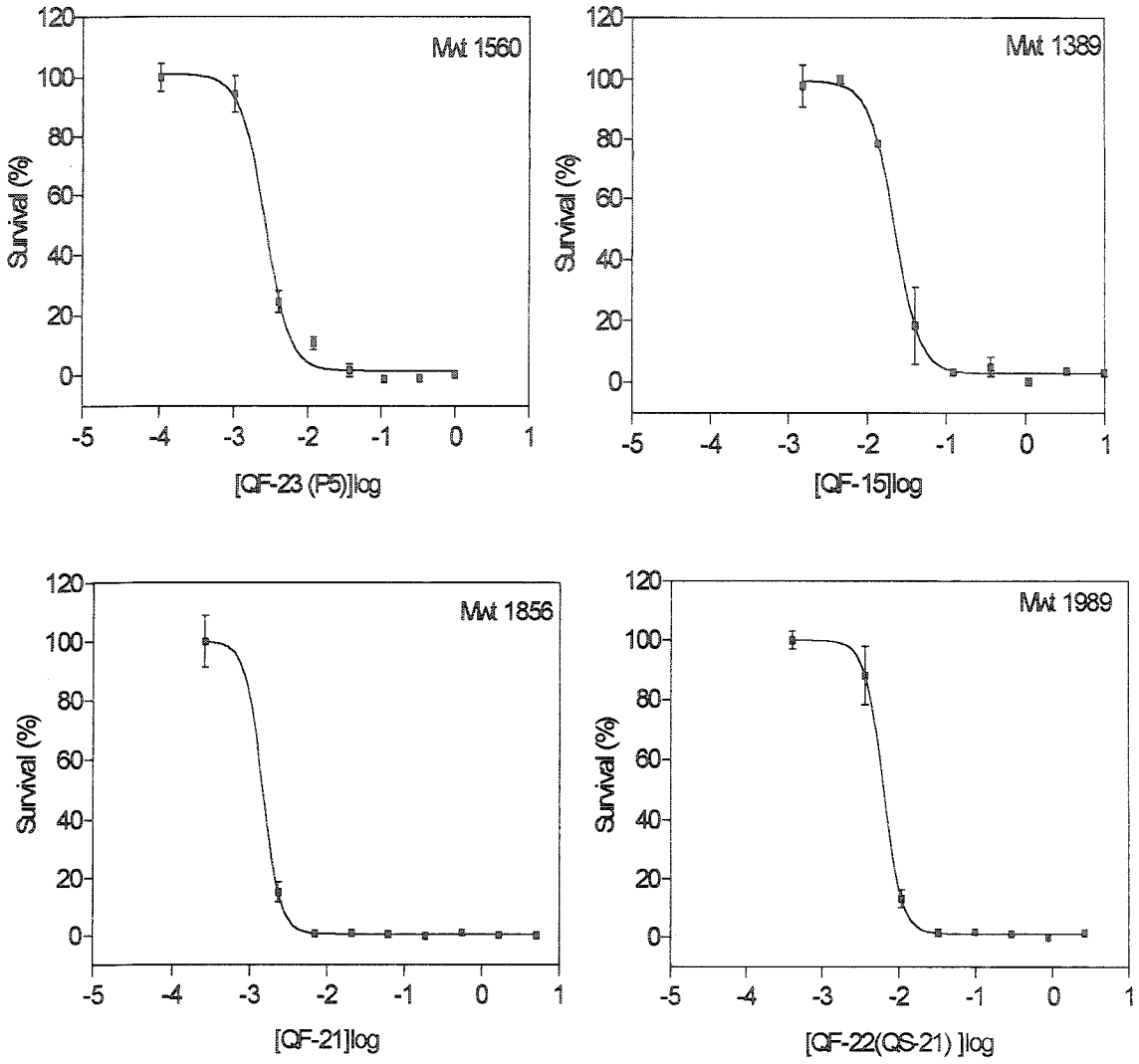


Figure 37: Results of the toxicity studies of *Quillaja* saponin fractions. Toxicity measurements in terms of TC_{50} are given in Table 12. The toxicity of QF-21, QF-22 and QF-23 is somewhat comparable, with QF-22 (QS-21) being the less toxic of the three. Values are means ± SD and each point is an average of four readings. Survival curves were drawn using Graph Pad Prism, fitting the values to a sigmoidal curve.

Table 12: TC_{50} for tested *Quillaja* saponin fractions

Fraction	Molecular Weight (Daltons)	TC_{50} (µg/ml)
QF-15	1389 (1353)[1]	25.117
QF-21	1856	1.514
QF-22 (QS-21)[2]	1989 (2012)[3]	3.981
QF-23[4]	1560	2.955

1. Corresponding vials contained compound of either molecular weight. Compound having a Mwt of 1389 is the potassium salt of the compound with Mwt 1353 (the difference in masses is approximately 39).

2. Fraction QF-22 is equivalent to the well known QS-21.

3. One compound (ion mass 2012) is the sodium salt of the other (difference in masses is 23).

4. Compound QF-23 is the compound with a characterized structure.

We assessed the adjuvant capability of one of the saponins *in vitro* through measurement of cytokine induction by cells for the following reasons: a) the results of these experiments give a general indication of the adjuvant capability of the saponin, and b) the induced cytokine measured, IL-1α (a Th-1 associated cytokine) is known to have direct effects on B-cell and T-cell proliferation and APC activation. IL-1α measurement is therefore indicative of the adjuvant potential of compounds tested. *Quillaja* saponins have been found to have a potent stimulation of both these immune responses. The first step in immune recognition by cells is initiated following uptake of antigen by APC, which then produce IL-1, a cytokine which activates the immune processes such as T cell and B cell proliferation and macrophage phagocytosis. The IL-1α produced in our assay following cell treatment with *Quillaja* fractions was measured by ELISA. See Diagram 3 (p. 160).

The cell line used in this investigation was the Murine J774, a monocytic cell line which we are using as an APC. This cell line can be stimulated with mitogens such as LPS to produce IL-1α and other cytokines as shown in Diagram 3. The ELISA protocol for quantifying the IL-1α production required considerable pre-test optimization to establish assay conditions; a substrate dilution of 1:4000 and an incubation time of between 20 - 50 minutes after addition of substrate to plates, and the plates were best read at 405 nm.

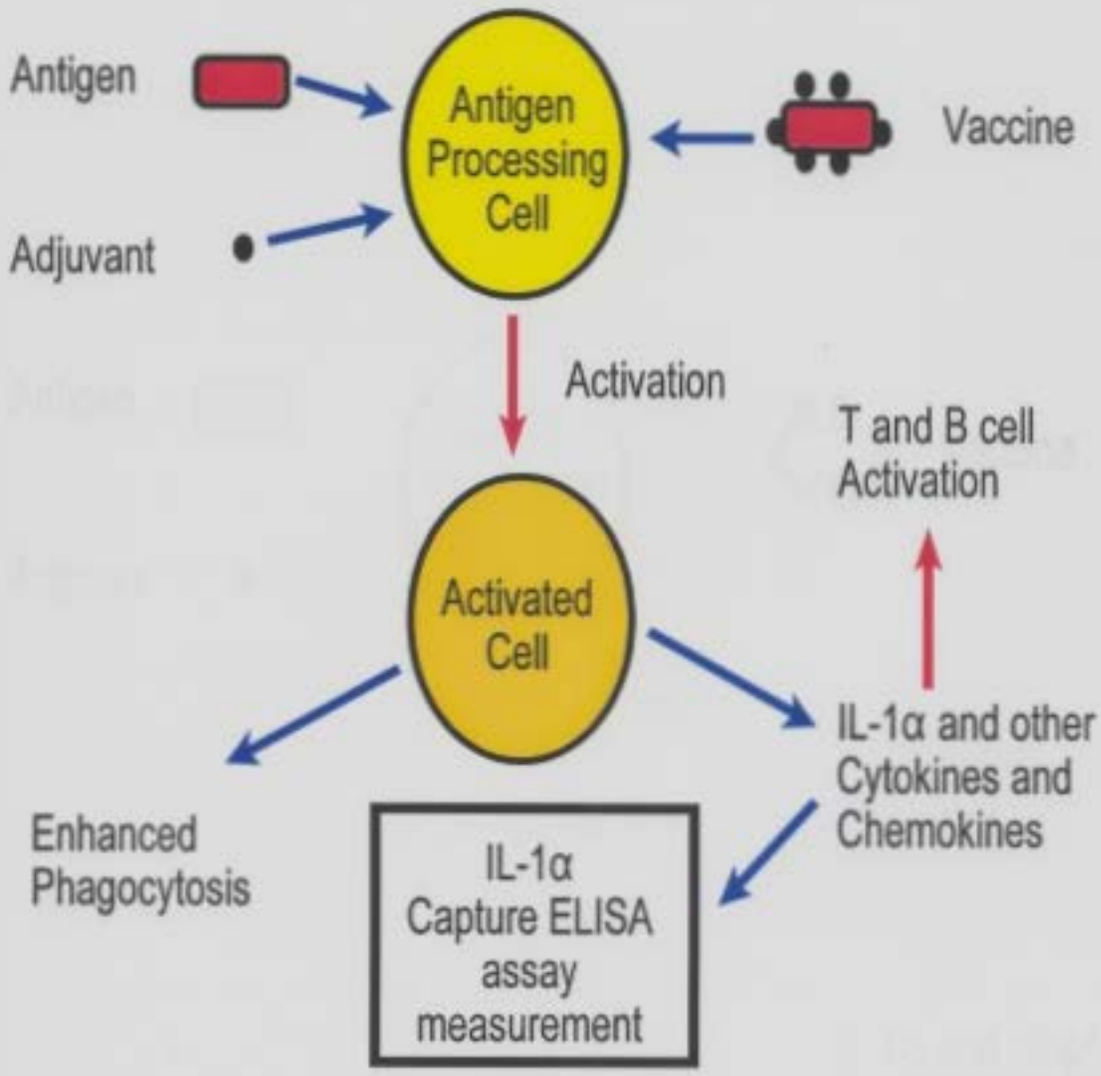


Diagram 3: Schematic representation of immune recognition of antigen.

The results obtained under these conditions were found to produce a better standard curve than other conditions investigated. (FigureS 38,39 and 40). The maximum measurable standard concentration of murine IL-1α was determined to be 1.5 ng/mLl. Optimum production of IL-1α (1.031 ng/mL/10^5/48h) following LPS stimulation was found to occur by 48 hrs, when 10^5 cells/well were stimulated with 0.5 µg/mL of LPS (Figure 41). This time point was chosen to analyse the IL-1α produced following exposure to various saponins. The cells were found to have a constitutive IL-1α production of about 10 - 50 pg/mL/10^5/48h. QF-23 was found to stimulate J774 cells to produce the cytokine IL-1α as shown in Figure 42 between 0.25 µg/mL and 1µg/mL QF-23. The level of IL-1α produced was concentration dependant, and ranged from 80-230 pg/mL IL-1α/10^5/48h. Although much lower than the levels produced by LPS stimulation, they were significant (P= 0.0004 - 0.0336). We concluded that QF-23 was therefore, a relatively good adjuvant capable of activating the monocytic cells and perhaps other APCs, if combined with a vaccine antigen. It was also noted that the effective concentration of this compound was well below its toxic concentration of 3 µg/mL established for QF-23 in this cell line see (Figure 37 and Table 12 on pages 157 and 158) .

Figure 38: ELISA protocol optimization showing variations in the color of developer avidin peroxidase. Plates for the experiments to construct standard curves for IL-1α determination were prepared as outlined in Chapter 3 (3.3.2.4), pages 69 to 72. Different avidin peroxidase concentrations ranging from 1 in 2000 (in diluent) to 1 in 10 000 were used in the detection stage of these experiments. In other experiments the highest concentrations of the standard IL-1α used was also varied (either 1.5 ng/ml or 3.0 ng/ml were used). These experiments were conducted because it was found out that the color development after the addition of the substrate took place too quickly to permit the acquisition of OD readings necessary to construct a standard curve.

Av2T= Avidin peroxidase diluted to 1:2000; Smx = S= 1.5 ng/mL (or 3.0 ng/mL) = maximum concentration of the standard solution used was 1.5 ng/mL, 7 min, 2 min = the time that the measurements were carried out after addition of substrate; 405 nm is the wavelength at which the optical density (OD) measurements were obtained. None of the above conditions appeared to produce a reliable standard curve. Readings per point were taken in triplicates and plotted values are mean values plus or minus standard deviations.

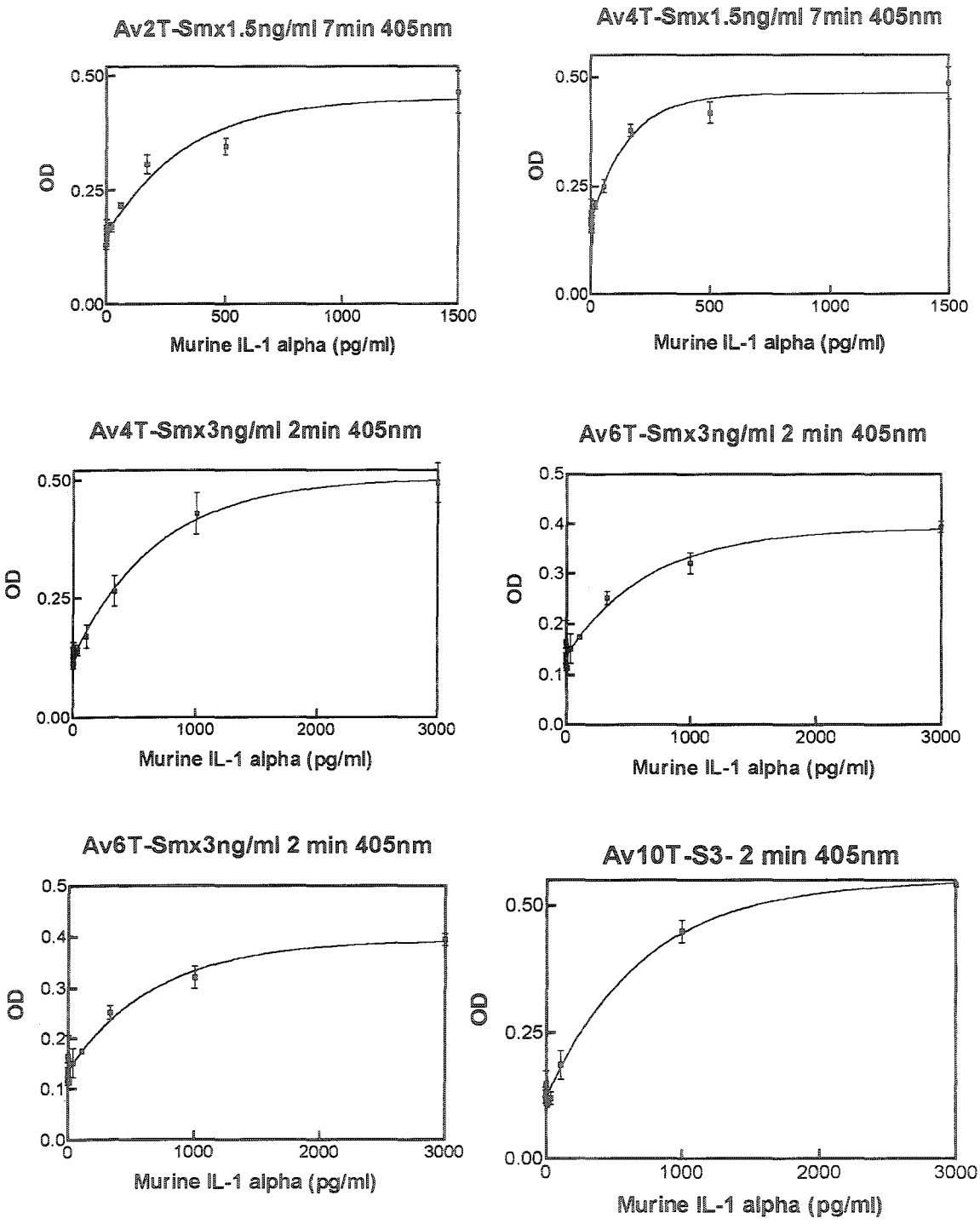


Figure 38: ELISA protocol optimization

Figure 39: Standard curves at different time variations. Optimization of the experimental conditions for ELISA in the determination of the cytokine IL-1α production by QF-23. In these experiments optical density readings were obtained at different time intervals after the addition of the substrate for color development. The avidin peroxidase enzyme concentration was maintained at 1 in 4000.

Sm=Smx = maximum concentration of the standard IL-1α . The rest of the notations are as explained in Figure 38. Although the standard curve obtained using the avidin peroxidase dilution of 1:4000 and measuring the OD after 50 minutes with the highest standard concentration at 1.5 ng/mL and the curve obtained by measuring the OD after 8 minutes with the maximum concentration of the standard curve at 3 ng/mL appear to be as equally good as the one obtained at 36 minutes with 1.5 ng/mL highest concentration of standard, the latter was chosen because it was more convenient and in agreement with the recommended time of the ELISA kit manufacturer. Readings per point were taken in triplicates and plotted values are mean values plus or minus standard deviations.

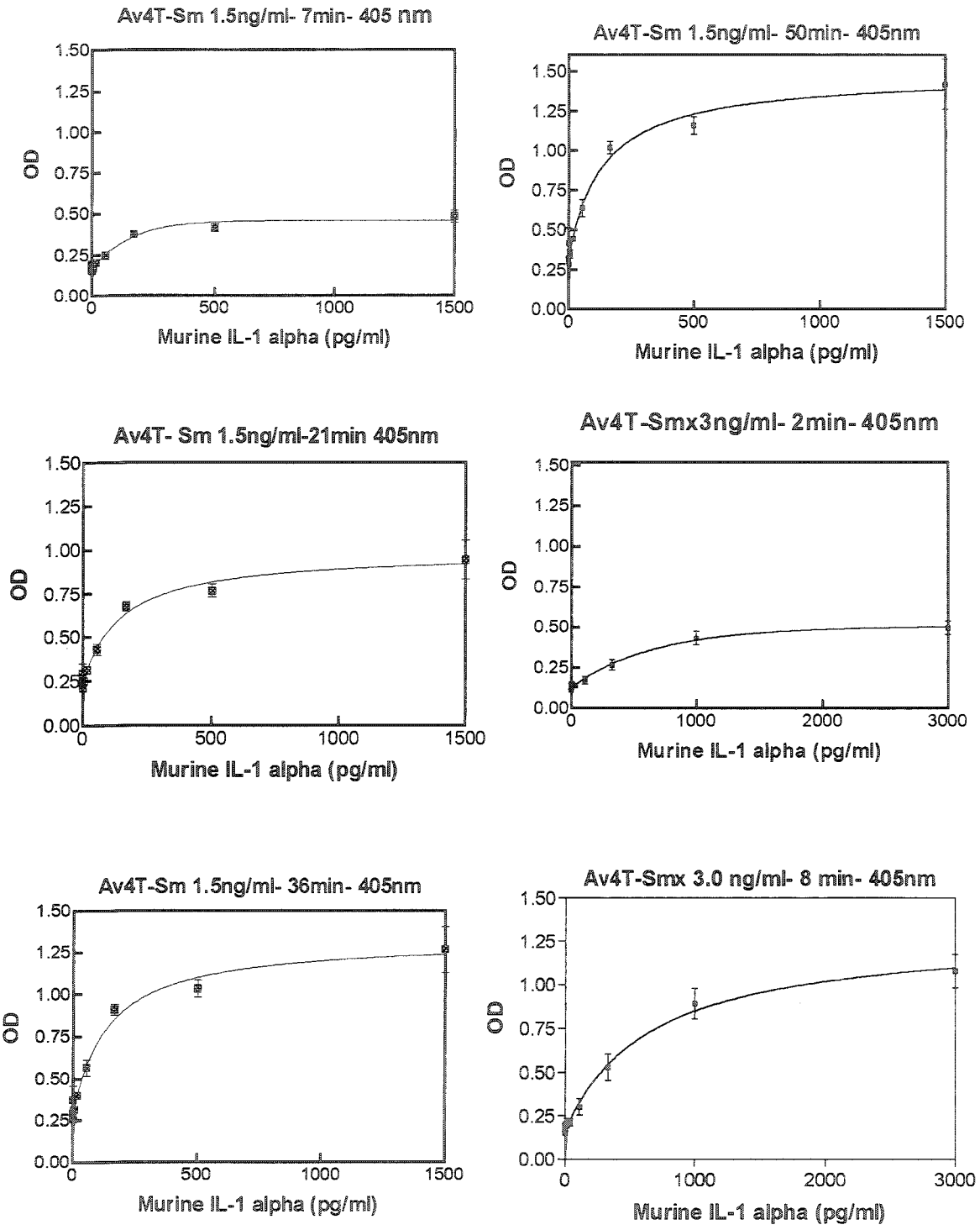


Figure 39: Standard curves at different time variations

Figure 40: Standard curves obtained for the ELISA experiments using OD readings obtained at different wavelengths. Graph labeling is as explained in Figure 38. The following conditions of; avidin peroxidase dilution factor, 1:4000, maximum standard concentration of 1.5 ng/mL and OD measurements obtained 36 minutes after addition of substrate at 405 nm, were chosen as optimum experimental conditions for subsequent IL-1α determinations. These specifications produced a desirable standard curve under as close to recommended conditions as possible. Readings per point were taken in triplicates and plotted values are mean values plus or minus standard deviations.

In conclusion, 36-50 minutes were best times for obtaining OD reading, the best wavelength for measurement of OD readings was 405 using 1.5 ng/mL of mIL-1α as the maximum standard concentration.

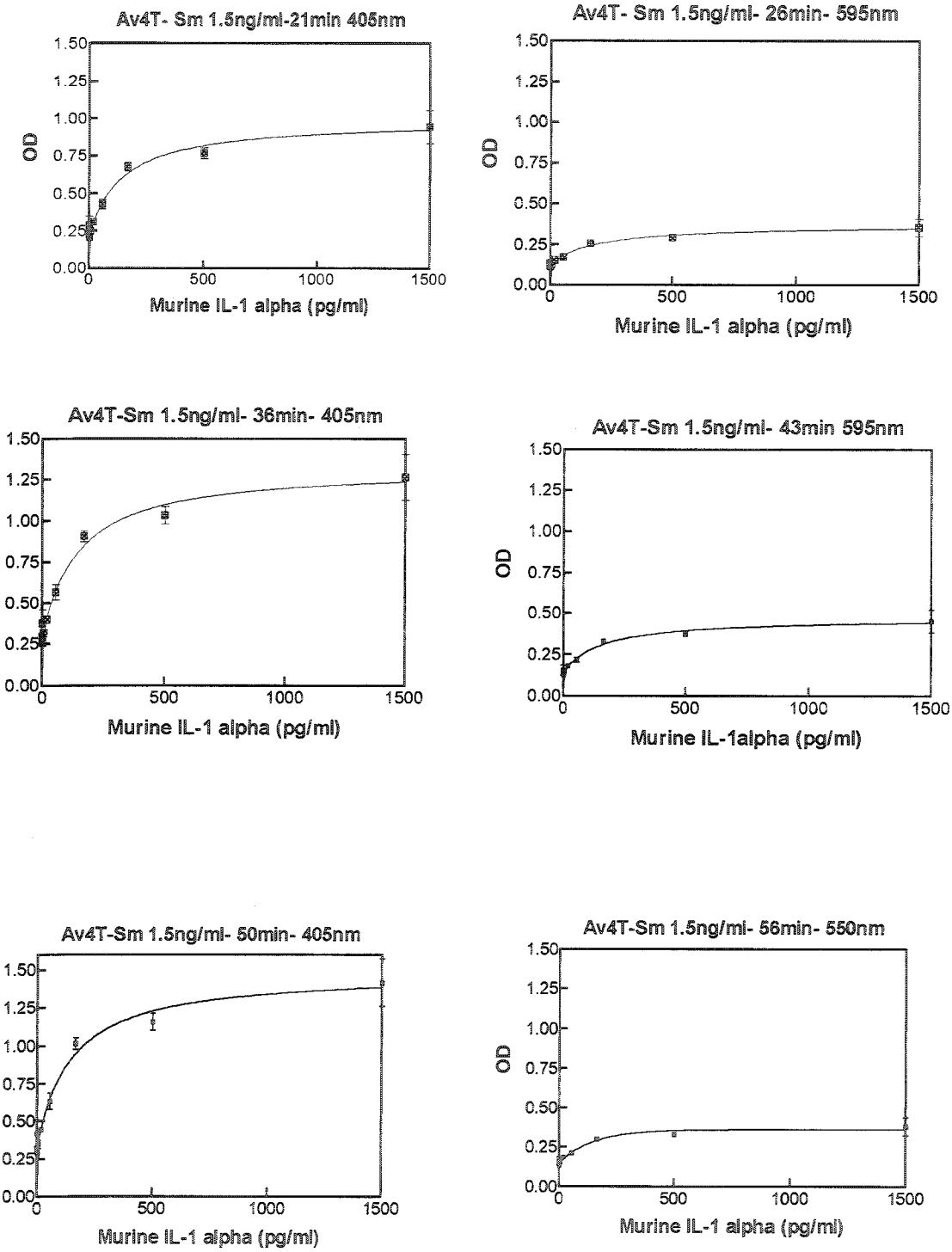


Figure 40: Standard curves obtained for the ELISA experiments *(cont. on p.165)*

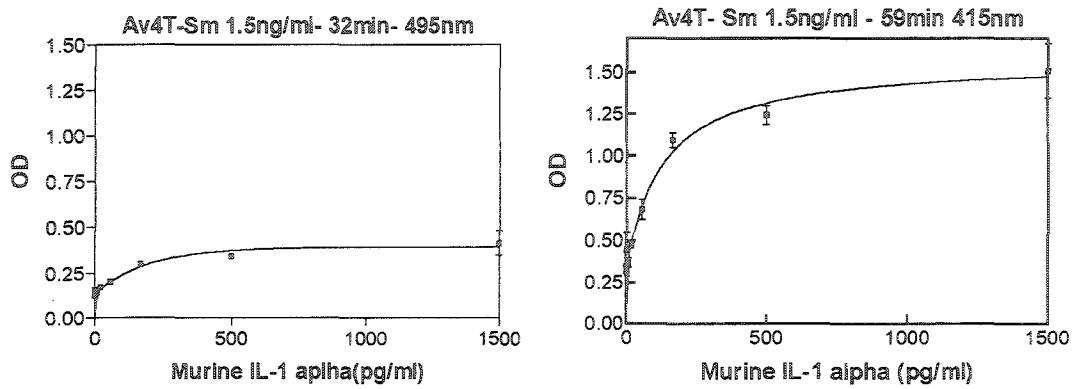


Figure 40: Standard curves obtained for the ELISA experiments *(from p.164)*

(end of Figure 40)

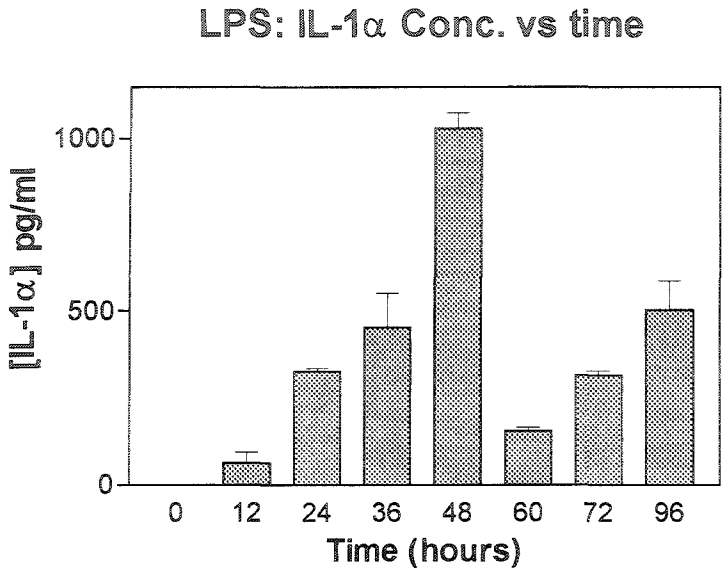


Figure 41: Kinetics of cytokine Production Studied using LPS 0.5 µg/mL, and 10^5 cells/well. Amounts of IL-1α produced by J774 murine monocytic cells incubated with an LPS solution for different time periods were measured and plotted against time. Maximum IL-1α production was found to occur after 48 hrs of incubation. Afterwards the cytokine production appear to become greatly diminished, probably due to cell death. Readings were taken in triplicates per time. Plotted values are mean values plus or minus standard deviation.

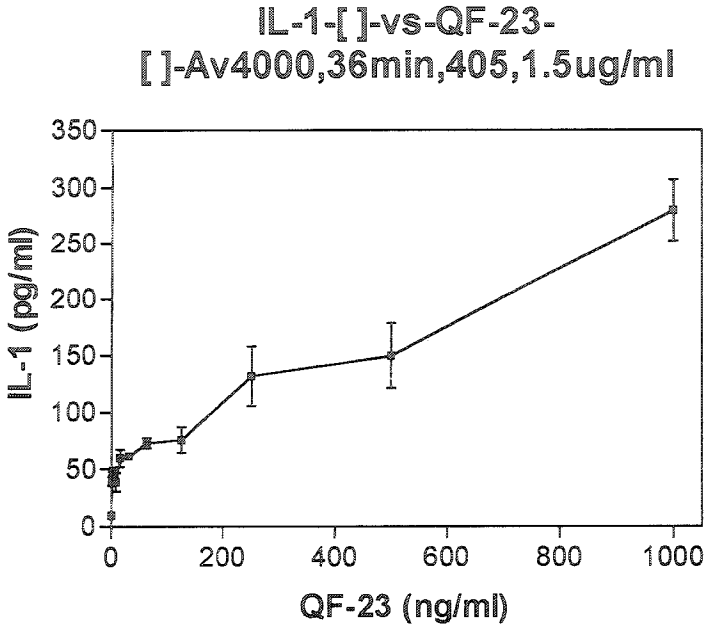


Figure 42: Cytokine Induction by QF-23. Concentrations of IL-1α produced by J774 murine monocytes treated with different concentrations of the saponin QF-23 were measured. The determination was carried out at the optimum ELISA conditions for the ideal standard curve determined earlier; avidin concentration used 1:4000, measurements carried out 30 minutes after addition of substrate color developer and the highest concentration of standard solution being 1.5 ng/mL. Mean values ± SD of four readings (n=4) per point were plotted. Values obtained at saponin concentrations of 250, 500, and 1000 ng/mL were found to be significantly different from the control (P = 0.0336, 0.0181 and 0.0004, respectively). This is the results of one out of three independent experiments. The results of two of the experiments were found to be similar. While one gave an insignificant value at the saponin concentration value of 500 ng/mL, probably due to experimental error.

4.3 Summary of Findings

1. The complex mixture of *Quillaja* saponins have a tendency to co-elute due to similar separation properties. However, a few pure saponin fractions were isolated and screened for biological activity.
2. Ions of molecules much higher than those reported in the literature have been detected in the LC/ MS experiment some of them eluting early in the HPLC separation process.
3. Eight compounds have been separated in pure or near pure form having molecular weights 955 (QF-9),1217 and1257 (QF-11), 1353 and 1389 (QF-15),1560 (QF-23), 1856 (QF-21), and 1989 (QF-22). At least four of them are novel and were not published structures (QF-11, QF-15, QF-21 and QF-23). The compounds with the molecular weights of 1217 and 1257 could be the same as well as the compounds with the molecular weights of 1353 and 1389. The difference between the two being a potassium ion molecular weights might arise from one being the potassium salt of the other.
4. The structure of compound QF-23, molecular weight 1560 Figures 5 and 43) was proposed from data obtained from mass spectrometry and CID-MS/MS. This structure has since been supported by NMR and GC/MS data.

5. Fraction QF-22 having molecular weight 1989 has been positively identified as QS-21 (a well established fraction of *Quillaja saponaria* Molina).
6. Toxicity studies carried out on four of the separated fractions revealed that a novel compound QF-15 with molecular weight 1389 the lowest toxicity and that the toxicity of QF-23 and QS-21 is more or less equivalent.
7. QF- 23 appears to have immune stimulating activity (IL-1α) and possible adjuvant activity in our model system.

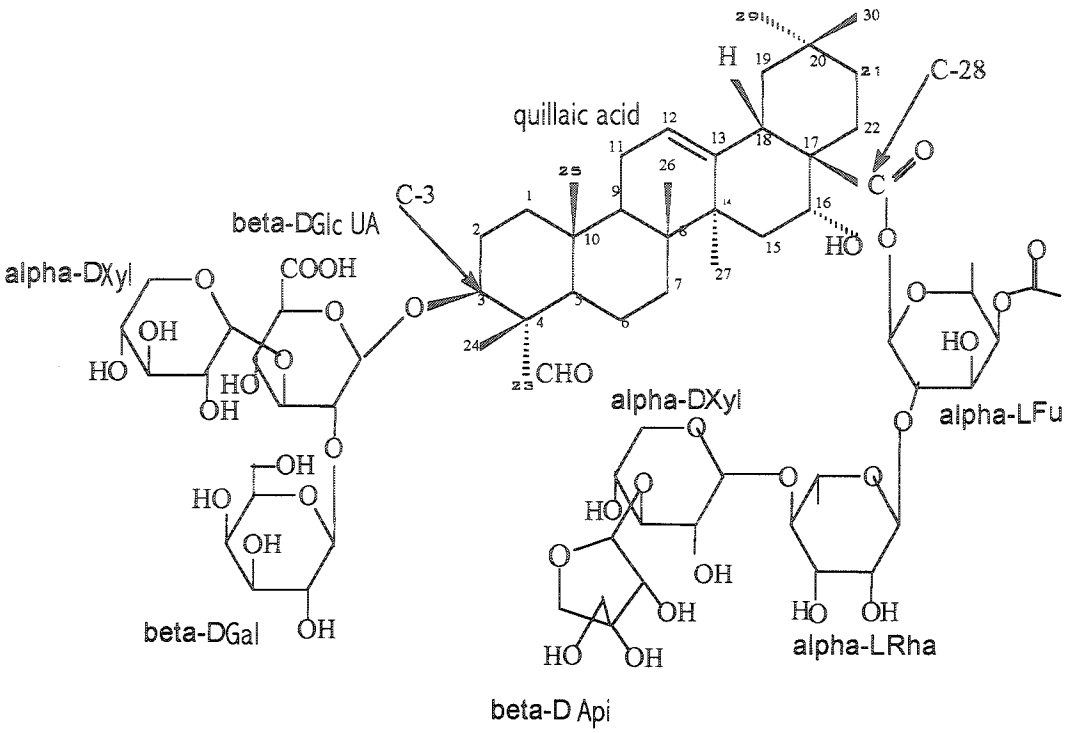
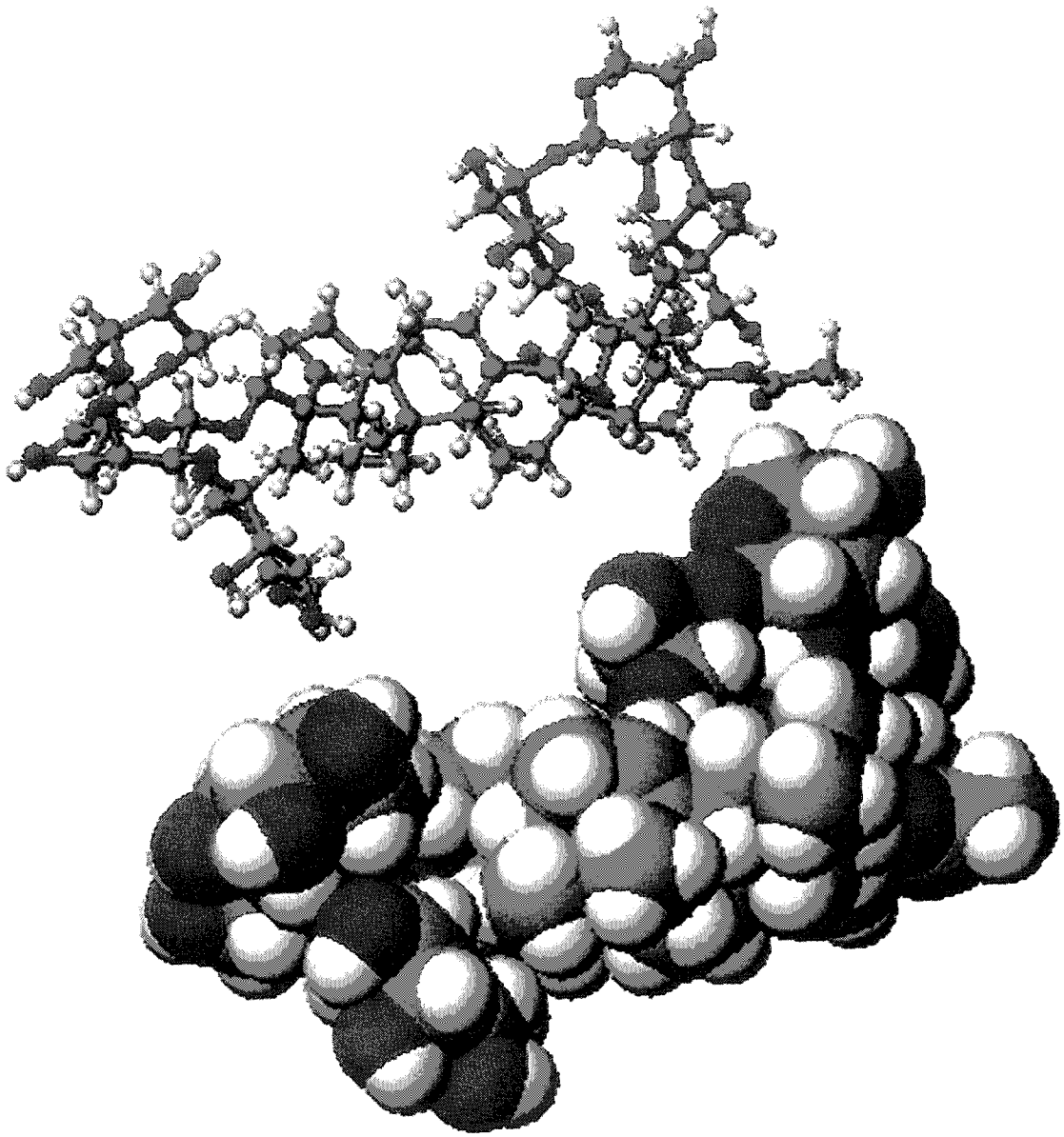


Figure 43: Structure of compound QF-23, Molecular weight 1560 Daltons

We propose the above structure for QF-23. This structure has been elucidated from a combination of molecular weight, fragmentation pattern, sugar analysis, and NMR data. It differs from previously reported structures in that the fucose is substituted at the fourth hydroxyl group with an acetyl group rather than being un-substituted as in the compounds DS-1 and DS-2 (Higuchi *et al.,* 1987). The fucose is sometimes substituted with a long chained hydrocarbon as in the compounds QS-17, 18 and 21 (Kensil *et al.*, 1991). QF-23 retains similarity with the reported structures of other compounds (e.g. QS-17,18 and 21) in having an apiose attached to the xylose in the C-28- *O* bound glycosidic moiety. It also has a similarity with QS-21 and DS-1 in not having a glucose molecule attached to the rhamnose.



Structure QF-23

Chapter 5: Conclusions

Quil-A has been found to be a complex mixture of triterpenoidal saponins and other substances from the bark of *Quillaja saponaria* Molina as previously reported by other researchers (Daaslgard, 1970; 1974; Higuchi *et al.*, 1887; 1988; Kensil *et al.*, 1991; van Setten and van de Werken, 1995). Over a hundred molecular ions, which may or may not be related, were detected in the first five minutes of the LC/MS investigation of this material. A great deal of evidence lead to the conclusion that contrary to earlier reports (Kensil *et al.*, 1991; van Setten and van de Werken, 1995) some of the material which was eluted in the first ten minutes of the analytical separation was saponin in nature (high molecular weight compounds that showed saponin like UV absorption characteristics, and whose solutions exhibited a substantial amount of frothing with slight agitation). However, most of the material that was considered non-saponin in nature was found to be more concentrated in the first ten minutes of the separation than anywhere else. The LC/MS experiment has enabled us to collect information which can be used to pinpoint (more or less) the exact position (retention time) of any given ion (or compound) of interest from the components of the bark extract of *Quillaja saponaria* Molina, Quil-A. Some of the data obtained from this investigation also provides valuable preliminary information regarding the fragmentation pattern of these

compounds. Several compounds of very high purity were also separated and isolated in sufficient quantities to enable the use of further specialized techniques to determine with adequate precision the structure of at least one of the separated fractions QF-23 and to evaluate some of its biological activities, together with that of some of the other components.

A number of challenges were encountered and most have been overcome in this research project. The most difficult and time consuming task was found to be the separation of the highly complex bark extract. The components were found to be poorly separable and had a tendency to co-elute. In addition, the *Quillaja* saponins appeared to irreversibly adsorb to the column and this caused loss of resolution after several separations. This necessitated the acquisition of several columns during the course of the research project. In an attempt to combat this problem the Quil-A material was absorbed with a solid phase extraction technique (which was later abandoned). The literature described a method of droplet counter current chromatography (DCCC) as an excellent method for preparative separation of saponins when combined with silica column separation (Nakamura *et al.*, 1994; Higuchi *et al.*, 1988). However, we lacked the necessary equipment for this particular technique and thus we could not achieve such a separation.

A suitable drying method was identified only after elaborate and labour intensive attempts with other methods that included the design and construction of a specialized piece of drying equipment (for cryo-crystallization). The drying of the

separated fractions after semi-preparative HPLC posed a very difficult problem due to the organic/aqueous solvent and the very low percentage of the solid material in a large amount of liquid that was more organic than aqueous at certain instances along the gradient of elution: resulting in the formation of a mixture with an eutectic point which made freeze-drying impossible with the equipment that we had.

The LC/ESI-MS experiment has thrown much light in the elution characteristics of Quil-A, a *Quillaja* saponin. No other research group known to us has as yet reported having undertaken this venture. Maillard and Hostettmann (1993) developed a method of online mass analysis of crude plant extracts, which has been applied by Wolfender *et al.* (1993) and Perret *et al.* (1999) on phenolic and terpene glycosides including those of *Rogeria adenophylla* J, *Gentiana dasyantha* Gilg. and *Phytolacca dodecandra* L'Hérit but not as yet on *Quillaja saponaria* Molina. Moreover the method we applied is different from the one they developed. They used thermospray mass spectrometric analysis while we engaged the slightly more advanced API technique of ESI-MS. The analysis and publication of this LC/MS data could be a very interesting undertaking that may also require a tremendous amount of time. The adjuvant activity of the compound QF-23 revealed in the *in vitro* examinations means that this compound is worth investigating further. Equally important is the further investigation of the chemical nature and the immunological adjuvant capability of the novel compound QF-15 that was found to

have a considerably lower toxicity to the murine monocytic cell line used in this investigation than other saponins tested.

Thus far, a very efficient and precise procedure for semi-preparative separation has been developed (albeit, not without problems, which contributed to the length of time it took to complete this project). This method can even be further refined by the use of specialized equipment as directed earlier in the results and discussion section (page 109). By means of this separation, adequate amounts of the following fractions, QF-1, QF-9, QF-11, QF-15, QF-21, QF-22 and QF-25 were successfully obtained in pure form. Some of these fractions are still available while others have been consumed during the course of this study but can still be easily obtained.

Chapter 6: Prospects and Future Directions

Although the primary objectives of this project were achieved (viz, separation of components in pure or near pure form and in sufficient quantities to enable further investigations, structural elucidation of one of the components and investigation of the biological characteristics of several fractions), much work is still required to further characterize the *Quillaja* saponins and their adjuvant activity. Several areas of concern became apparent. Separation, purification and characterization of the saponin constituents of this plant extract still remain an area of great importance. The large number, over 80 saponin components detectable by means of LC/ESI-MS from the commercial extract, Quil-A, still require considerable identification and analysis. Knowledge of the molecular structures, adjuvant activity, toxicological effects, and other biological activity of the individual saponins, may result in the discovery of less toxic, non-hemolytic saponins possessing immune-stimulating properties that may be suitable for use in humans. Structure-function activity studies and research on the mechanism of action of the saponins may help us identify regions of the molecule responsible for adjuvant activity, hemolytic activity or toxicity. If no naturally occurring saponin compound with all the properties of an ideal adjuvant exist, then, potentially, one could synthesise or modify naturally occurring saponins and test their properties. The

information necessary to determine these structures is still elusive, and further work is still necessary.

In future expansions of this project further biological and chemical characterization of these already separated components can be undertaken, and the spectrum of investigations already effected to other fractions may even be broadened to include X-ray crystallography in the structural evaluations of the compounds (preliminary investigation of which has shown the saponins to crystallize in a manner that is suitable to enable the conduction of this experiment) and effects of the fractions on cell division, *in vivo* toxicological and adjuvant activity examinations, and studies of the fractions in ISCOM formulations, to be included in the spectrum of the biological evaluations carried out on these saponins. Further investigation of a number of different columns and or different stationary phases for the purpose of obtaining better HPLC separation of the components of the extract of *Quillaja saponaria* Molina (Quil-A) may also be a useful future endeavour.

The immune stimulating characteristics of the *Quillaja* saponins needs to be investigated further to gain insight into the possibility of manipulating adjuvant concentrations and/or composition to obtain a particular required immune response, for example. In actuality a great deal of benefit can be acquired from the studies of the type and amount of immune responses obtainable with the different kinds of antigens and the *Quillaja* saponins as adjuvants. A comparative study of the activity

of the saponins as adjuvants to that of the existing adjuvants can assist in establishing the exact position of these saponins as adjuvants.

In vitro screening for adjuvant activity may not be the ideal way to determine the activity of saponins. *In vivo* screening of the isolated components is also necessary to compare known adjuvant fractions such as QF-22 (QS -21) with the newly characterized QF-23. Animal models, also, are only indicative of possible adjuvant activity in humans but are a necessity in the development of therapeutic and prophylactic agents used in humans, including vaccines, for a variety of reasons, such as to determine the safety and efficacy of the agents before their use in humans. Such studies employing isolated *Quillaja* saponins in ISCOM technology, shown to be the most efficient formulation of saponin adjuvants, will also be necessary.

Appendix

Parts of the report of the LC/ESI-MS experiment carried out on the material Quil-A (Referred to in Table 5, pages 89-99)

Note:

1. The pages in the appendix are representative of the computer generated data from which Table 5 was constructed.
1. Mostly those peaks for which data relating to other ions detected in that peak have not been entered in Table 5 are included in the appendix.
2. Peak numbers corresponding to peaks included in the appendix are given in brackets in Table 5.
3. Entry # 's on the top of the pages in the appendix refer to the entry numbers in Table 5.
4. Peaks are arranged in the appendix in the order in which they are entered in Table 5 and mass detector (MSD) signal peaks are placed together (MSD signal sheets on top) with their corresponding peaks from the diode array detector(DAD) signal. The MSD signal sheets that do not have matching DAD data sheets could not be matched to any data produced in the DAD

because of the reasons given in the legend to Table 5 (pages 89-91).

5. DAD signal pages in the appendix have matching peak elution times to their corresponding entries in Table 5.

Annotation to Appendix

The following information refer to all of the pages to be found in the appendix. There are two categories of data sheets presented in this appendix **1)** data generated from the MSD signal and **2)** data generated from the DAD signal. To aid with the comprehension of the data, two sample pages have been provided, one for each category, and are labeled sample explanation page numbers 1 (page 180) and 2 (page 183) for MSD and DAD signals respectively.

Sample Explanation Page # 1 (page 180) for MSD signal pages

Figure A - TIC for Quil-A separated on analytical RP-HPLC C5 column.
Shows the total ion chromatogram (TIC); gives a complete trace of the spectrum.

i) x-axis - retention time in minutes

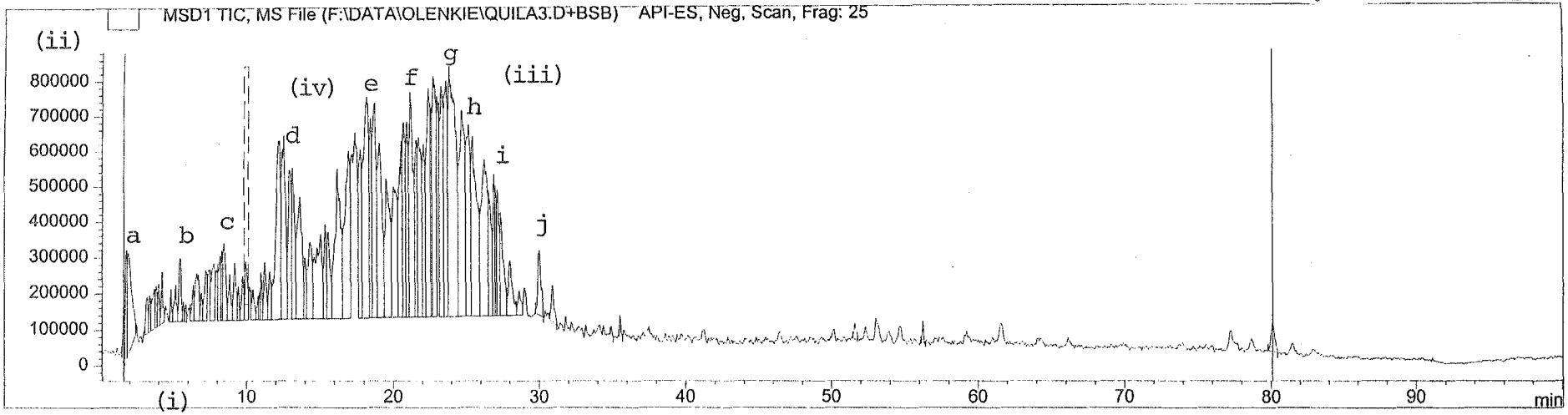
ii) y-axis - Total abundance of ions in a particular peak given in counts.

iii) a trace of all the peaks detected

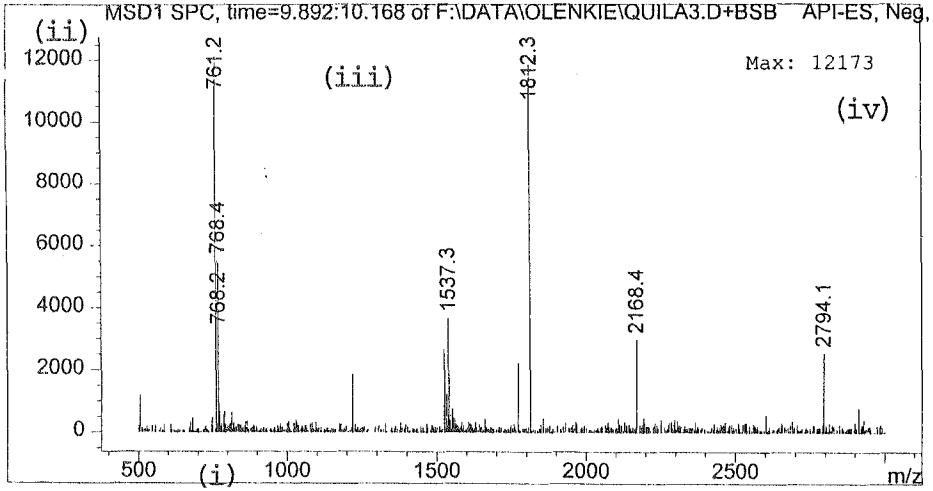
iv) a peak at a given retention time, being integrated.

Sample Name: Quillaja
Entry Number in Table 5 = 41

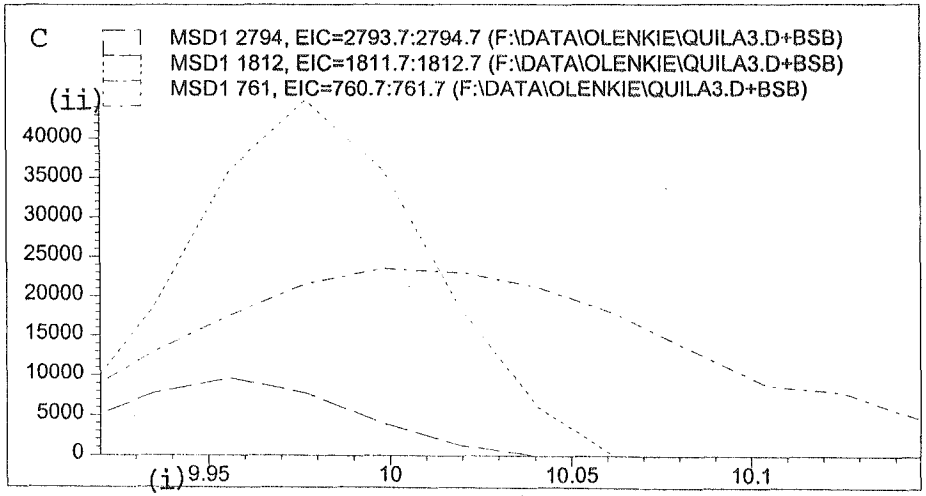
A



B



C



D Peak #27 at 9.997 min (9.892 to 10.175 min)
-> The analysis found 8 components, indicating an impure peak. <-

E
Component 1: Peak at Scan 465.0. Top ions are 2794
Component 2: Peak at Scan 466.0. Top ions are 1812
Component 3: Peak at Scan 467.3. Top ions are 761
Component 4: Peak at Scan 468.0. Top ions are 1524
Component 5: Peak at Scan 469.0. Top ions are 1523
Component 6: Peak at Scan 471.0. Top ions are 1770
Component 7: Peak at Scan 472.0. Top ions are 2168
Component 8: Peak at Scan 473.1. Top ions are 768

180

MSD = mass detector signal

TIC = total ion chromatogram

API-ES = ionization mode; atmospheric pressure ionization - electron spray

Neg.= ionization polarity ; negative

frag:25 = fragmentation voltage; 25 kV

Regions: a , b, c, d, e, f, g, h, i, j are apparent and have been investigated and shown to contain the following major ions given in m/z: a-1110; b-1996; c-956 (QS-L1; So *et al.*, 1997); d-1673; e- 1512 (DS-1; Higuchi *et al.,* 1987) and 1696 (DS-2; Higuchi *et al.*, 1987 and van Setten *et al.,* 1995); f-1193; g-1759 and 2012 (QS-21; Kensil *et al.,* 1991); h-1856 (van Setten *et al.,* 1995), 1987 (QS-21; Kensil *et al.,* 1991) and 2190; i-1560 (QF-23); j-1856.

Figure B

Shows the Apex of mass spectra of peaks detected at a specific retention time (shows the ions detected in that peak).

i) x-axis - ion masses in mass per charge (m/z)
ii) y- axis abundance of each ion detected (in counts)
iii) spectrum of all ions detected in that peak
iv) actual abundance of the most abundant ion detected in that peak (in counts)

SPC = spectrum of peak chromatogram

time = retention time of peak being examined (minutes)

Figure C

Is the extracted ion chromatogram (EIC) and shows the spectrum of the ions detected in that peak, provides a close up of the elution characteristics of each ion.

i) x-axis - retention time in minutes

ii) y-axis - abundance, in counts.

Section D

Specifies the Peak number for the peak being integrated (the peak is numbered upon integration), shows the time range of integration, the number of components detected in that peak and gives an assessment of the purity of that peak.

Section E

Gives the ions detected in each component

Sample Explanation Page # 2 (page 183) for DAD signal pages

Figure F

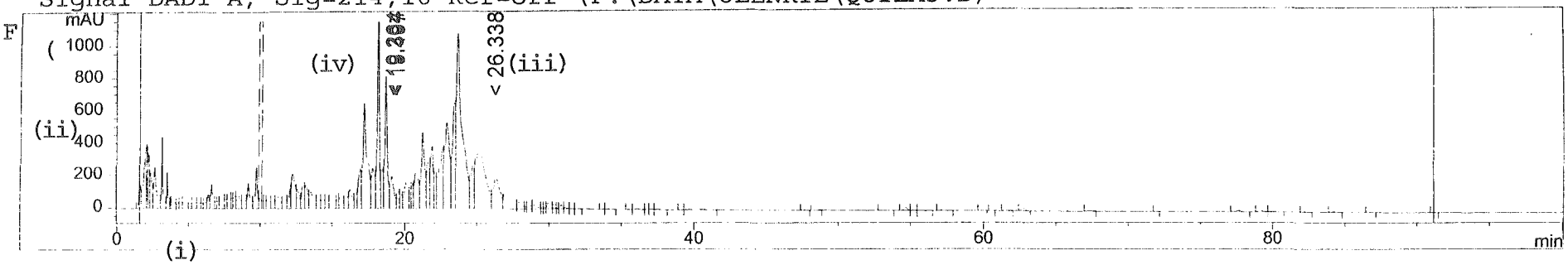
Shows the chromatogram obtained from the diode array detector (DAD) signal

i) x-axis - retention time in minutes

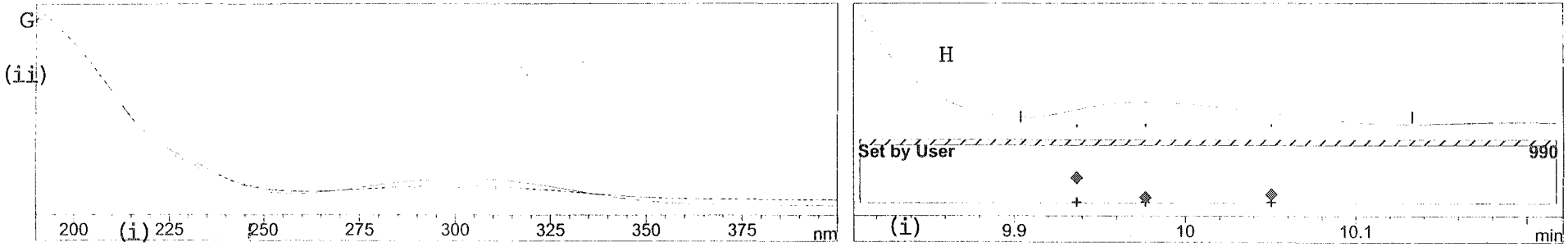
ii) y-axis - absorbance in milli absorbance units (mAU)

Sample Name: Quillaja
Entry Number in Table 5 = 41

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



Peak :52 at 9.976 min Name : ?



I -> The purity factor is within the threshold limit. <-

Purity factor : 997.948 (3 of 3 spectra are within the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 3 (Selection automatic, All)

183

iii) absorbances of all the peaks

iv) peak to be examined, eluted at a given time

DAD = diode array detector (signal)

Sig: 214.16 = gives wavelength at which peaks are being detected in nano meters (nm)

Figure G

Shows absorption characteristics of the components being detected in a specific peak designated by a peak number (given at time of intergration)

Figure H

Graphical representation of the the peak purity assessment

Section I

Interpretation of the peak purity: threshold 990.000 is the instrument default

Method employed in the analysis

LC Instrument Parameters

DAD Lamp used: UV lamp

Injection Volume: 20 µL

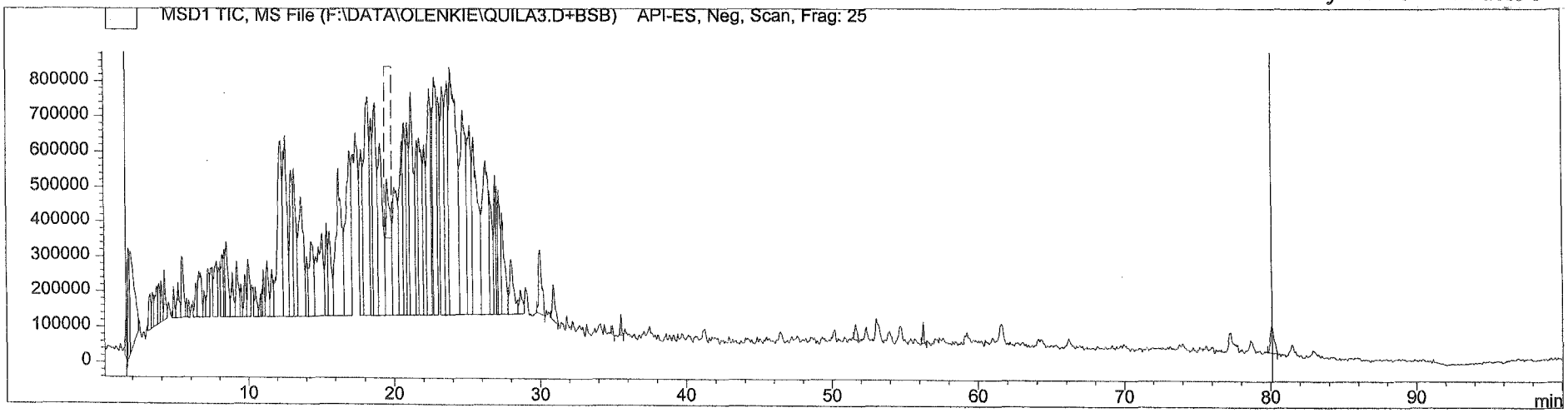
LC Instrument Conditions	At Start	At Stop	
Column Temp. (Left)	35.0	35.0	°C
Column Temp. (right)	35.0	35.0	°C
Pressure	187.4	53.3	bar
Flow	1.5	0.0465	mL/min

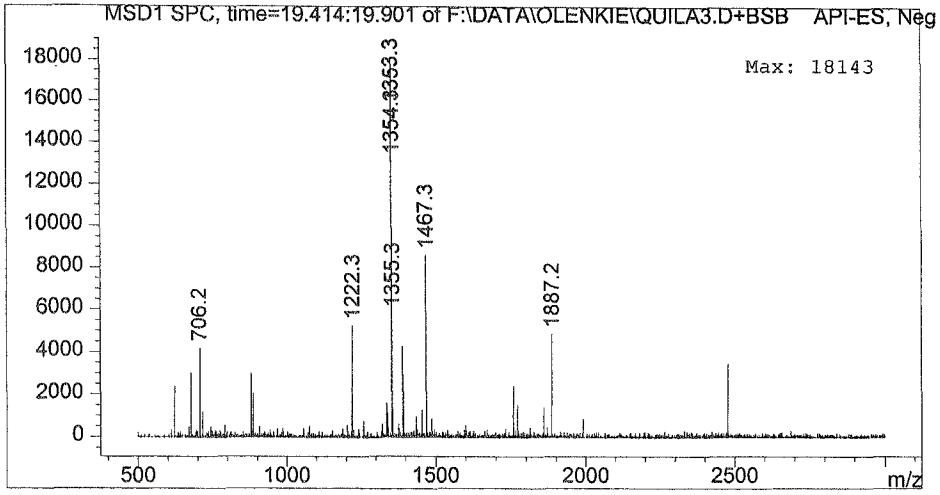
MSD Instrument Parameters

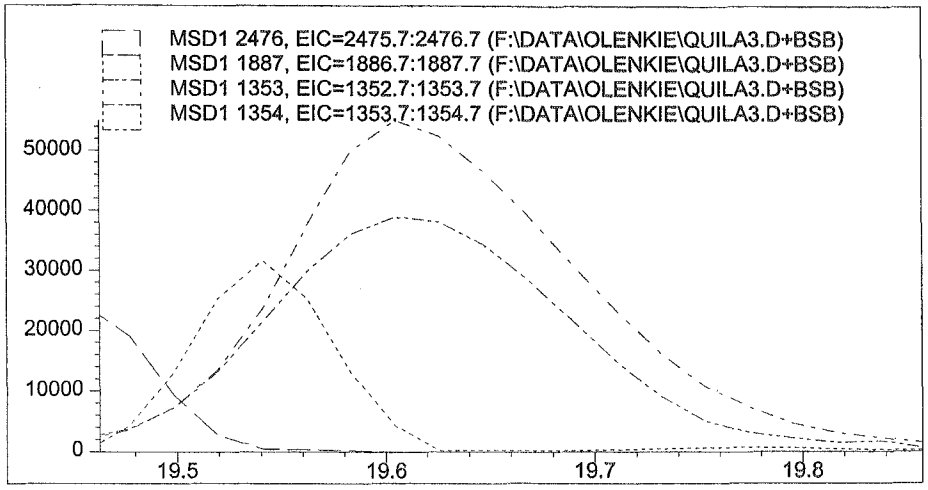
ionization polarity: negative

ionization mode: API-ES

MSD Instrument Conditions	At Start	At Stop	
Quad Temp.	99	99	°C
Gas Temp.	350	350	°C
Rough Vacuum	2	2	Torr
High Vacuum	4.6×10^{-6}	4.6×10^{-6}	Torr
Capillary Current	47	2000	nA
Cham Current	2	8	µA
Drying Gas (Flow rate)	12	12	L/min
Neb Pressure	50	50	psig





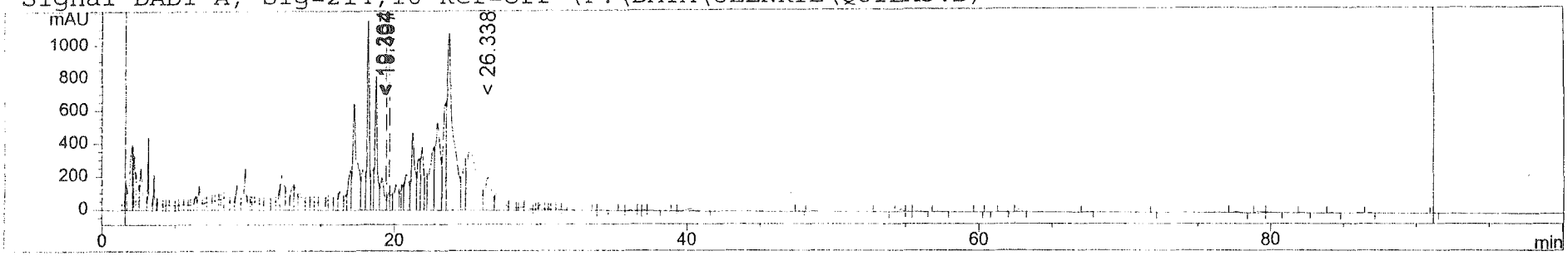


186

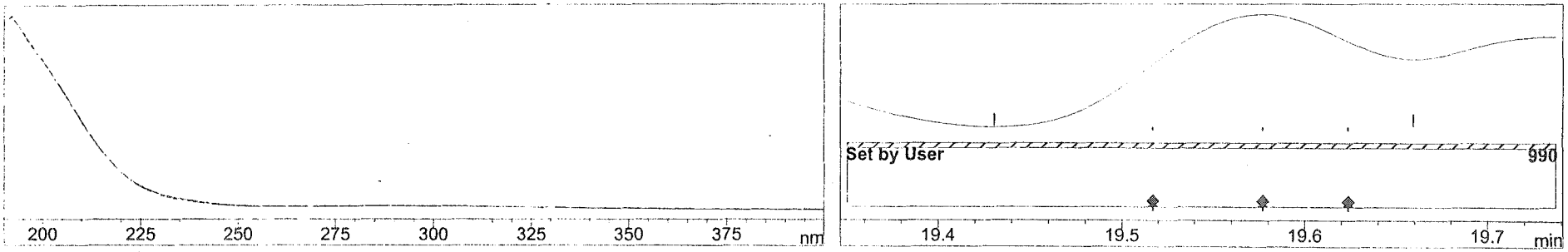
Peak #51 at 19.587 min (19.414 to 19.907 min)
-> The analysis found 7 components, indicating an impure peak. <-

Component 1: Peak at Scan 913.0. Top ions are 2476
Component 2: Peak at Scan 917.0. Top ions are 1887
Component 3: Peak at Scan 920.1. Top ions are 1353 1354 706
Component 4: Peak at Scan 921.0. Top ions are 1467 1468 1389
Component 5: Peak at Scan 921.8. Top ions are 676 1391
Component 6: Peak at Scan 929.0. Top ions are 1222
Component 7: Peak at Scan 929.9. Top ions are 1221

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



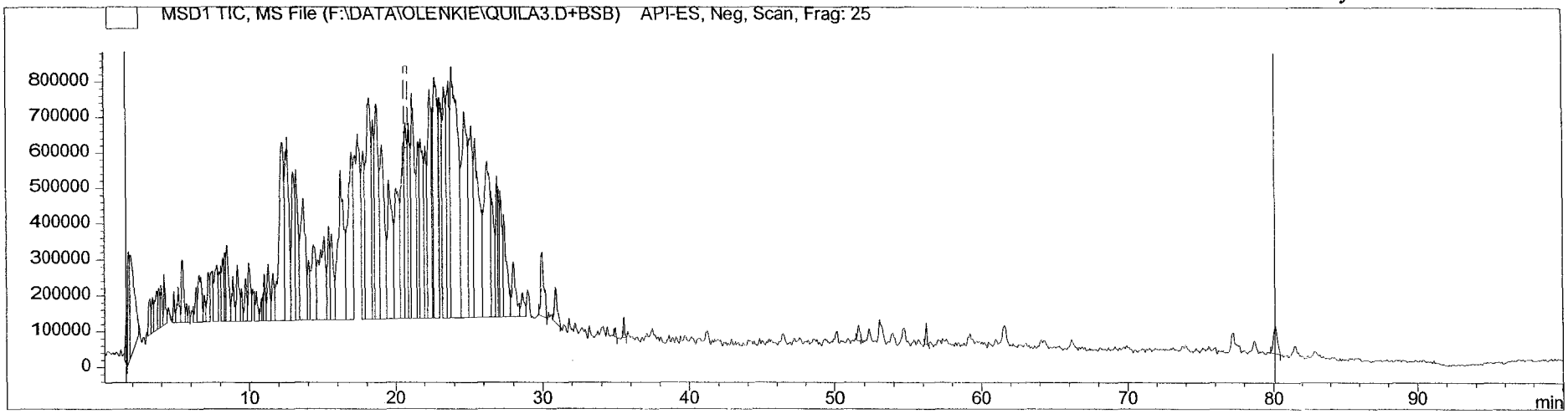
Peak :95 at 19.577 min Name : ?

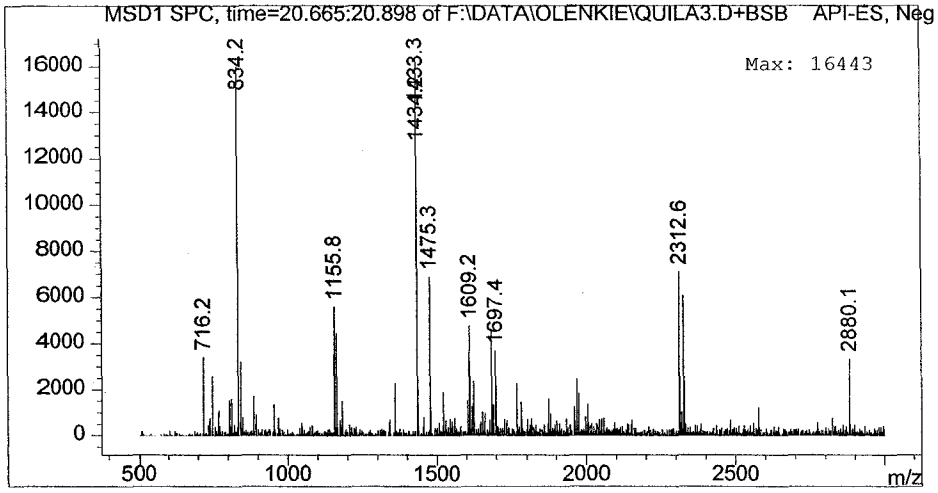


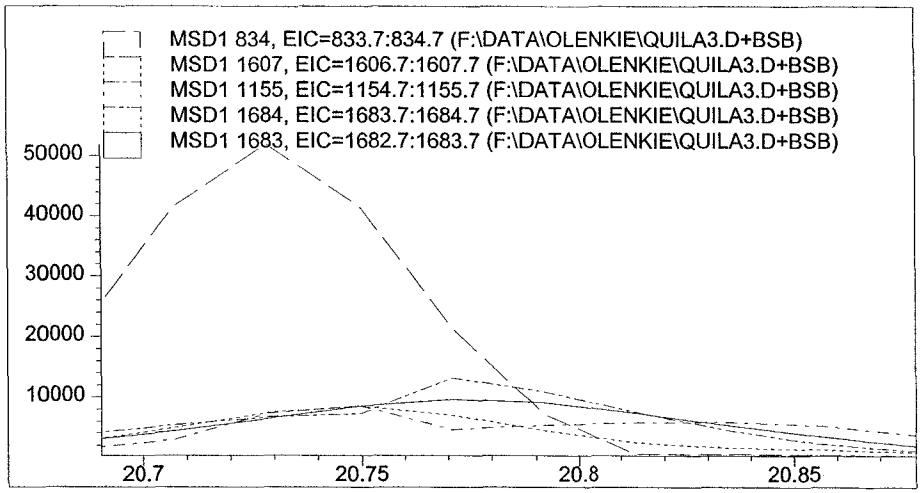
-> The purity factor is within the threshold limit. <-

Purity factor : 999.923 (3 of 3 spectra are within the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 3 (Selection automatic, All)

187



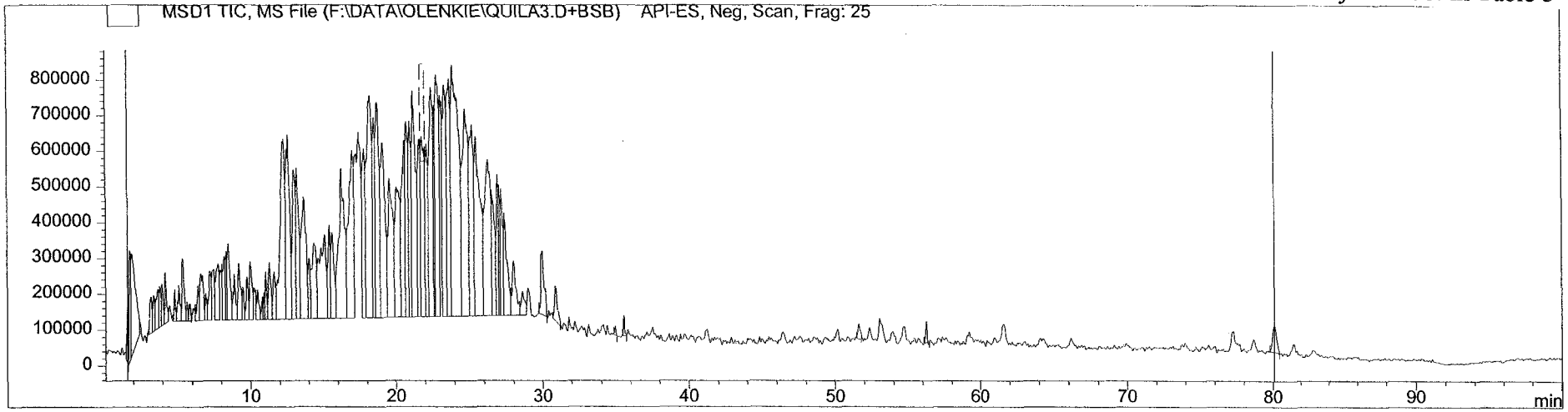


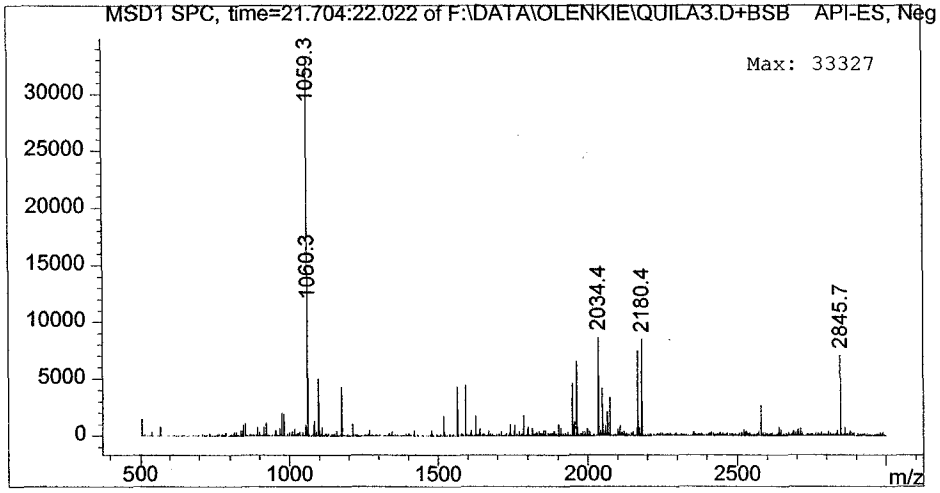


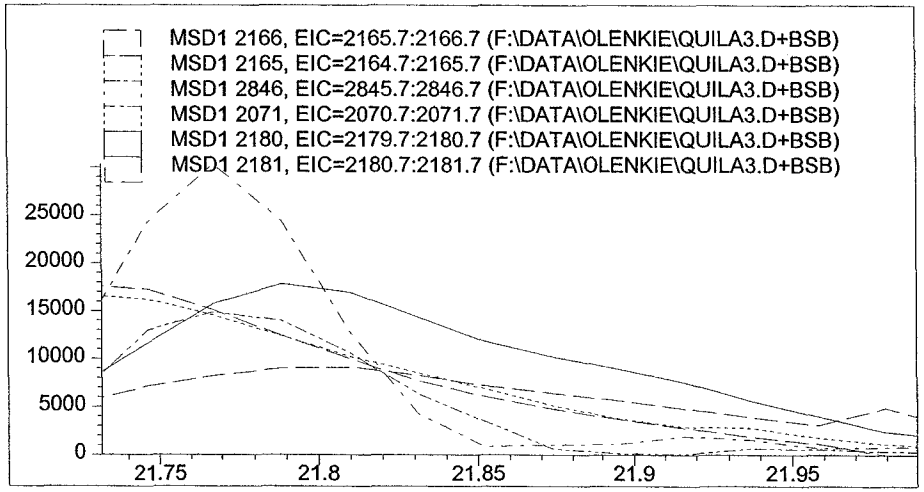
188

Peak #54 at 20.743 min (20.667 to 20.902 min)
-> The analysis found 7 components, indicating an impure peak. <-

Component 1: Peak at Scan 973.0. Top ions are 834
Component 2: Peak at Scan 973.9. Top ions are 1607 1155 1608
Component 3: Peak at Scan 975.2. Top ions are 1684 1683
Component 4: Peak at Scan 976.4. Top ions are 2880 2312 1156
Component 5: Peak at Scan 977.7. Top ions are 2311 1968
Component 6: Peak at Scan 979.0. Top ions are 1163 2325
Component 7: Peak at Scan 980.3. Top ions are 2327





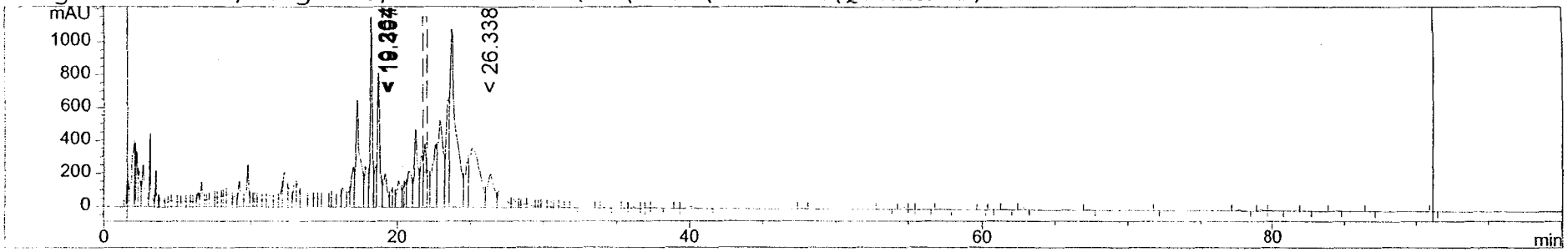


189

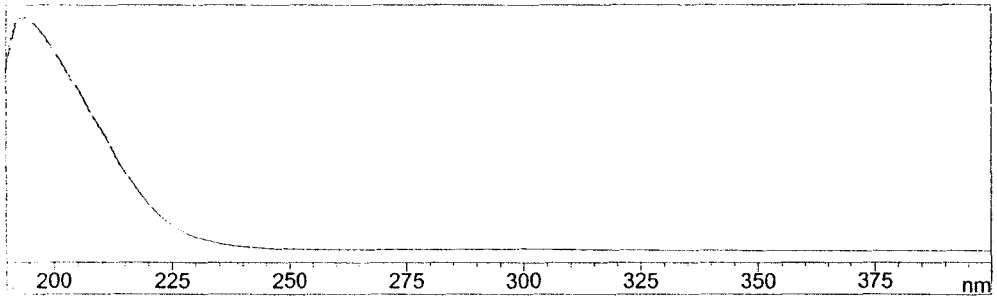
Peak #58 at 21.794 min (21.699 to 22.022 min)
-> The analysis found 8 components, indicating an impure peak. <-

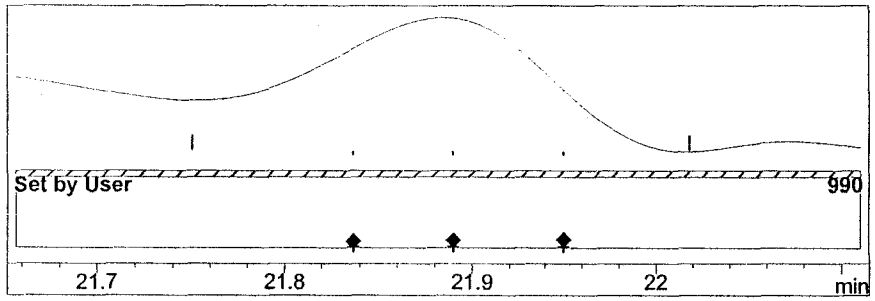
Component 1: Peak at Scan 1020.1. Top ions are 2166 2165
Component 2: Peak at Scan 1022.0. Top ions are 2846 2071 1961
Component 3: Peak at Scan 1023.4. Top ions are 2180 2181
Component 4: Peak at Scan 1024.1. Top ions are 1962 2034
Component 5: Peak at Scan 1026.9. Top ions are 2579
Component 6: Peak at Scan 1029.2. Top ions are 1059 1060 1061
Component 7: Peak at Scan 1030.0. Top ions are 1592
Component 8: Peak at Scan 1031.2. Top ions are 1173

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



Peak :106 at 21.884 min Name : ?

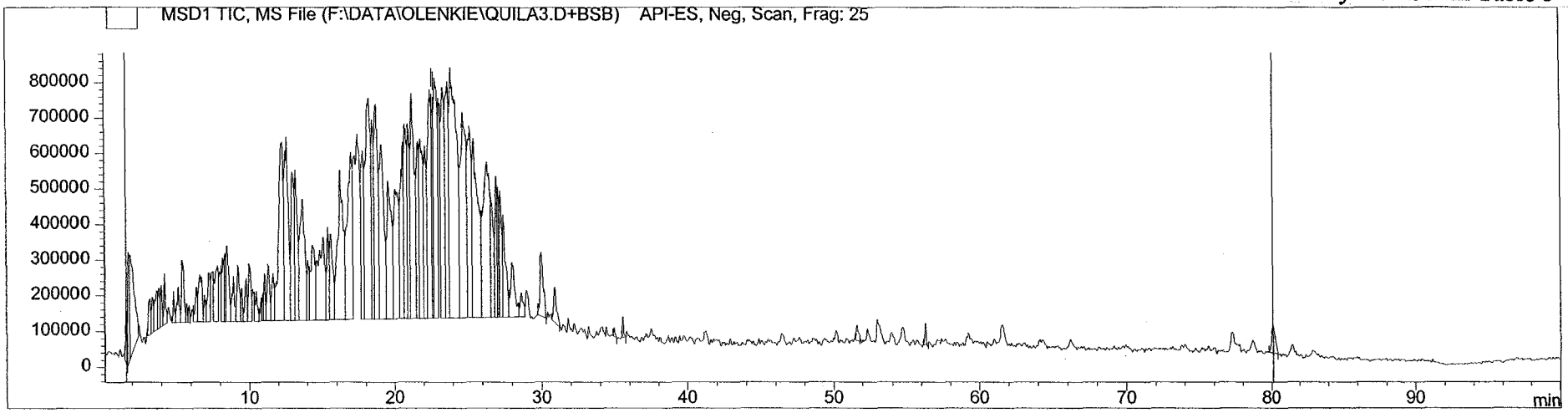


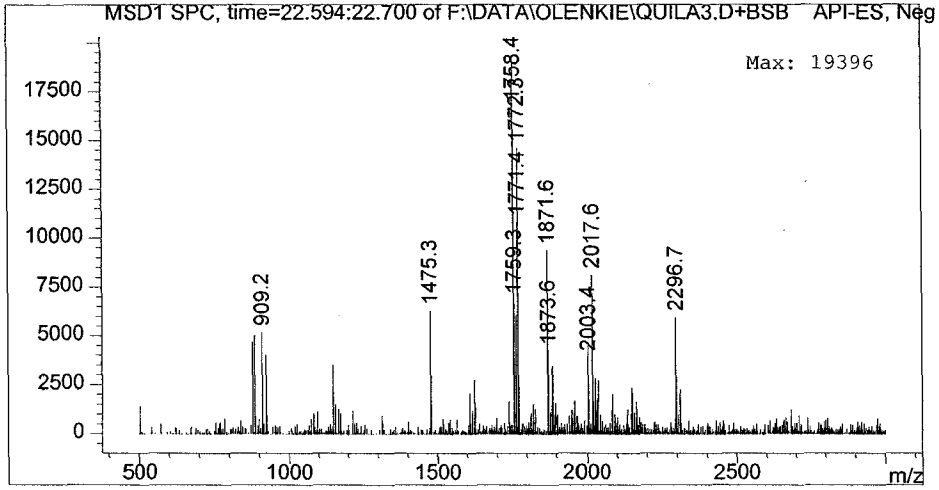


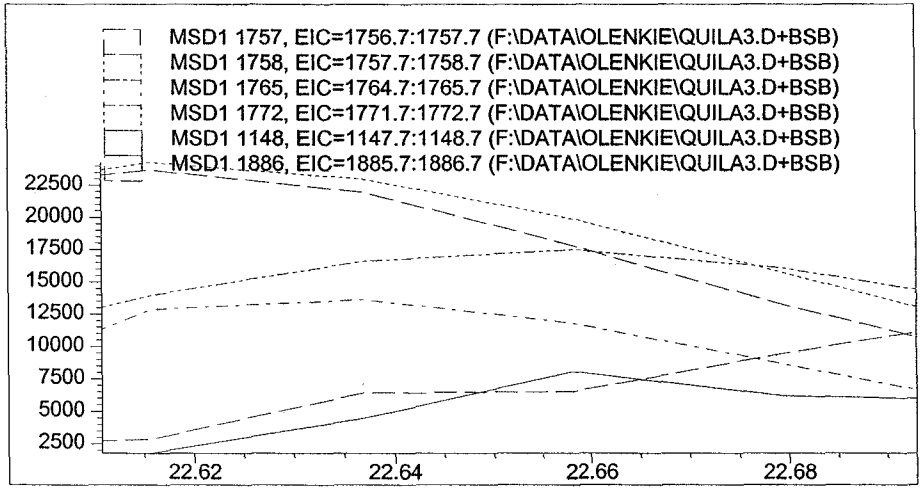
190

-> The purity factor is within the threshold limit. <-

Purity factor : 999.848 (3 of 3 spectra are within the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 3 (Selection automatic, All)





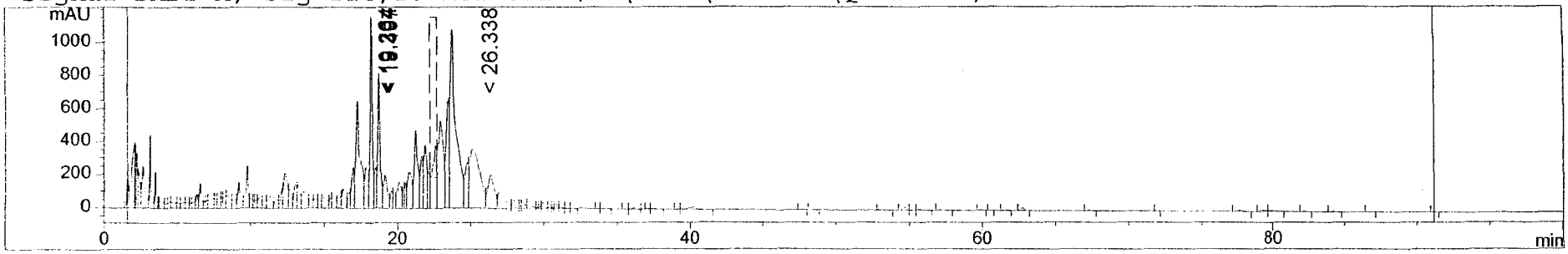


191

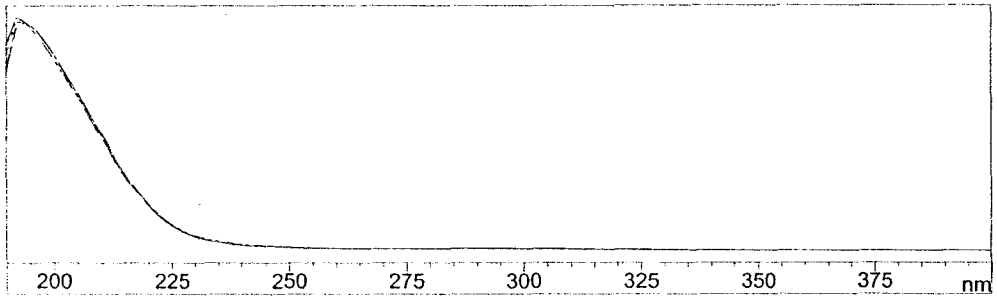
Peak #61 at 22.659 min (22.600 to 22.703 min)
-> The analysis found 4 components, indicating an impure peak. <-

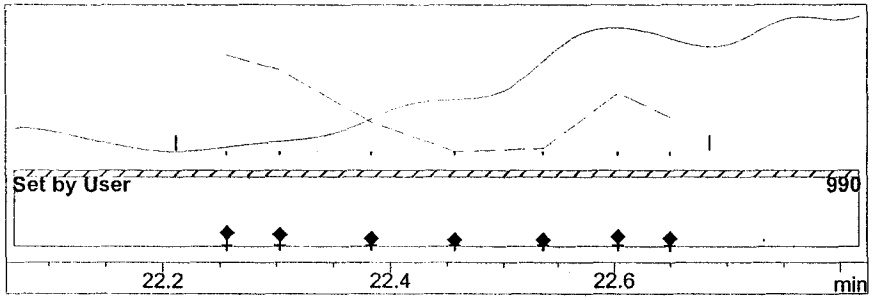
Component 1: Peak at Scan 1062.1. Top ions are 1757 1758 878
Component 2: Peak at Scan 1063.4. Top ions are 1765 1772 2017
Component 3: Peak at Scan 1064.4. Top ions are 1148 1886 1871
Component 4: Peak at Scan 1065.2. Top ions are 1885 2035 1147

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



Peak :110 at 22.602 min Name : ?

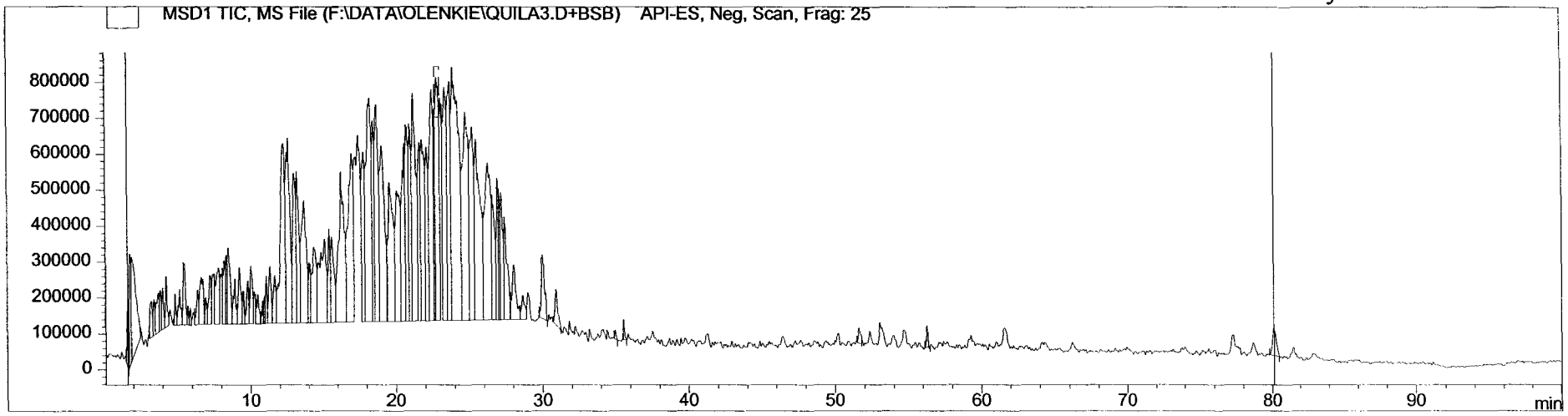


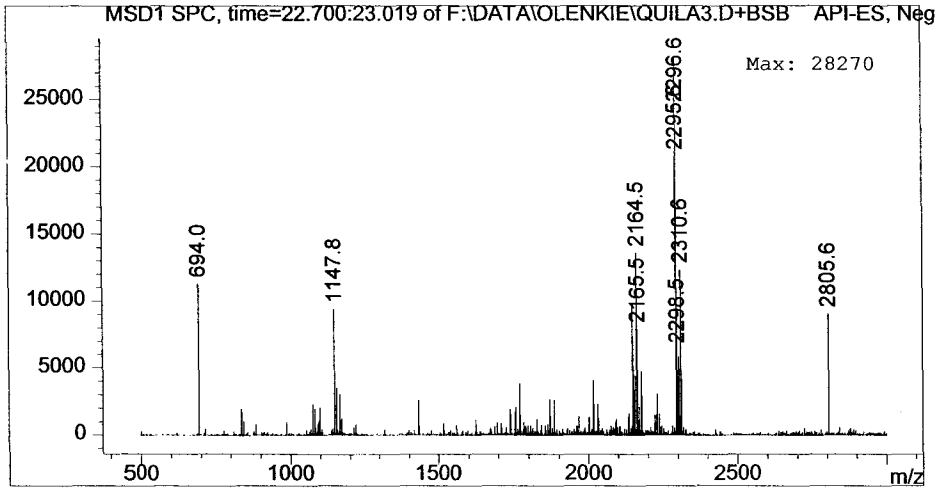


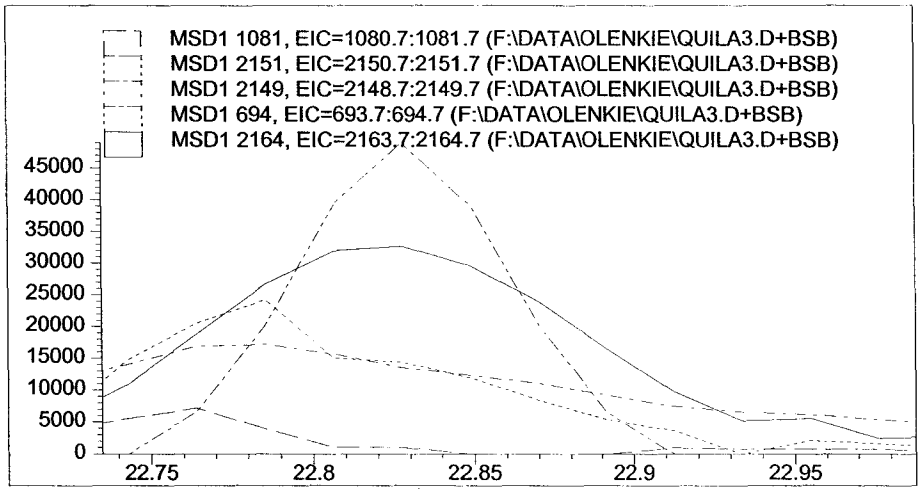
192

-> The purity factor is within the threshold limit. <-

Purity factor : 999.437 (7 of 7 spectra are within the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 7 (Selection automatic, All)

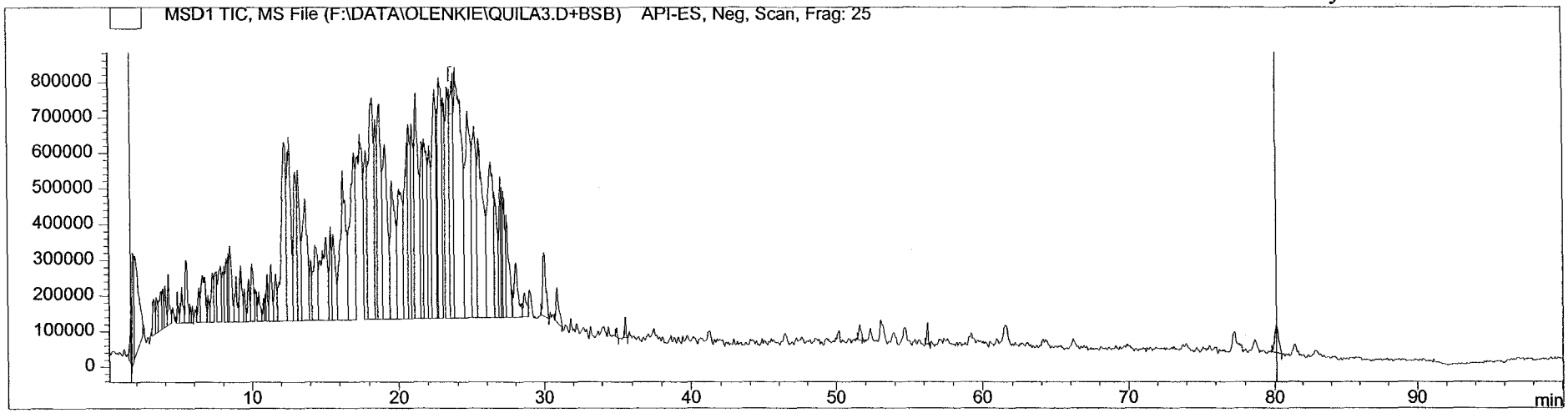


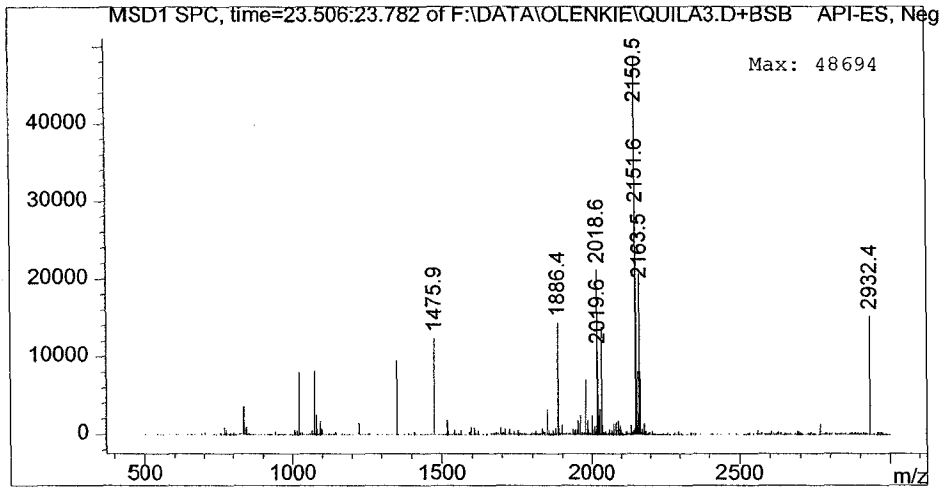


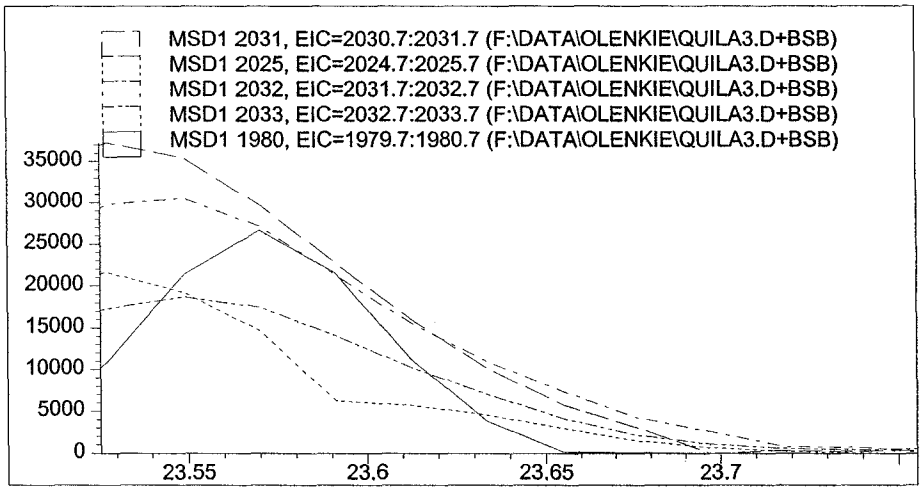


Peak #62 at 22.823 min (22.703 to 23.020 min)
-> The analysis found 9 components, indicating an impure peak. <-

Component 1:	Peak at Scan 1068.8.	Top ions are	1081
Component 2:	Peak at Scan 1069.9.	Top ions are	2151 2149 2150
Component 3:	Peak at Scan 1071.6.	Top ions are	694 2164 2163
Component 4:	Peak at Scan 1073.0.	Top ions are	2178 2177 2179
Component 5:	Peak at Scan 1075.5.	Top ions are	2224
Component 6:	Peak at Scan 1077.1.	Top ions are	2805 2298 1166
Component 7:	Peak at Scan 1078.2.	Top ions are	1148
Component 8:	Peak at Scan 1078.8.	Top ions are	2296 2304
Component 9:	Peak at Scan 1079.8.	Top ions are	2303 2295 2297





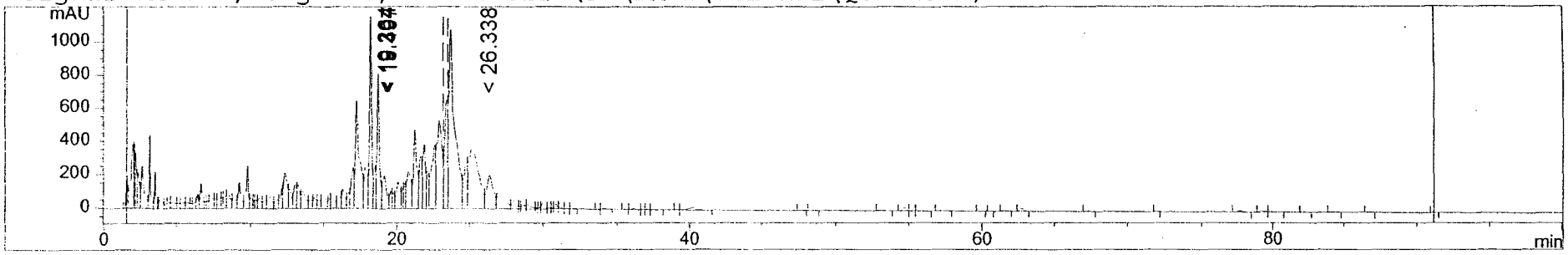


194

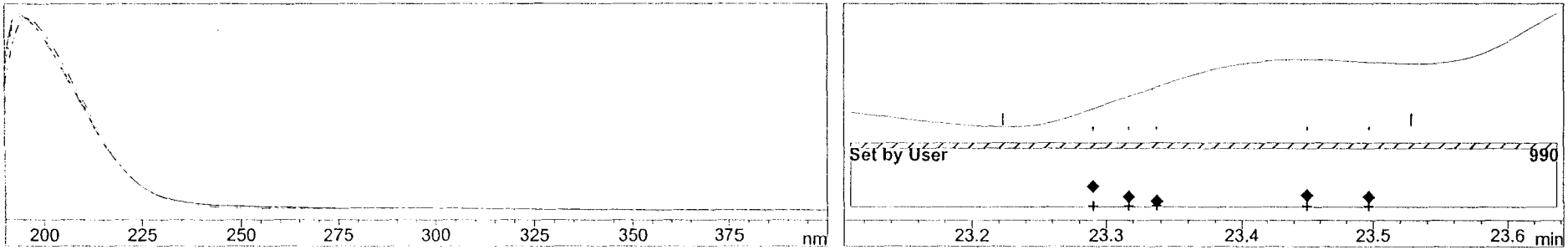
Peak #64 at 23.697 min (23.497 to 23.784 min)
-> The analysis found 9 components, indicating an impure peak. <-

Component 1:	Peak at Scan 1105.0.	Top ions are	2031	2025 1850
Component 2:	Peak at Scan 1106.0.	Top ions are	2032	2033 2021
Component 3:	Peak at Scan 1107.0.	Top ions are	1980	
Component 4:	Peak at Scan 1108.6.	Top ions are	1885	1886 1887
Component 5:	Peak at Scan 1110.0.	Top ions are	1074	
Component 6:	Peak at Scan 1112.0.	Top ions are	2932	
Component 7:	Peak at Scan 1113.1.	Top ions are	2149	2152 2158
Component 8:	Peak at Scan 1114.0.	Top ions are	2150	1022
Component 9:	Peak at Scan 1115.0.	Top ions are	1476	1351 2151

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



Peak :115 at 23.448 min Name : ?

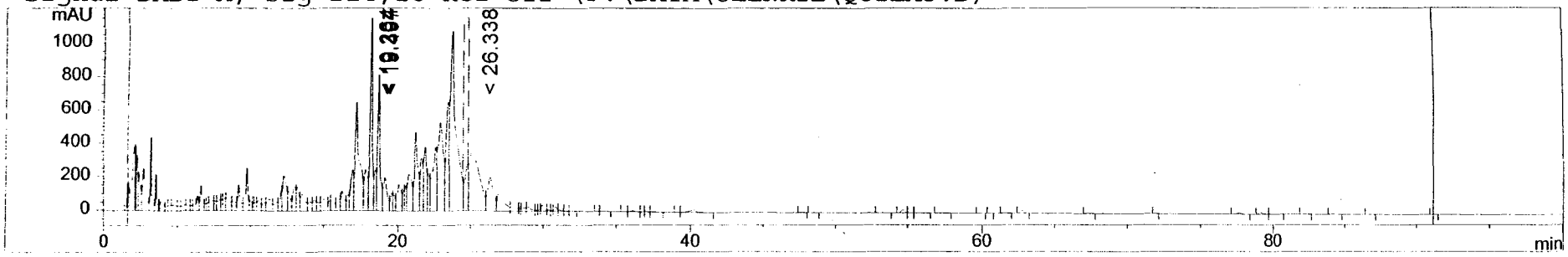


-> The purity factor is within the threshold limit. <-

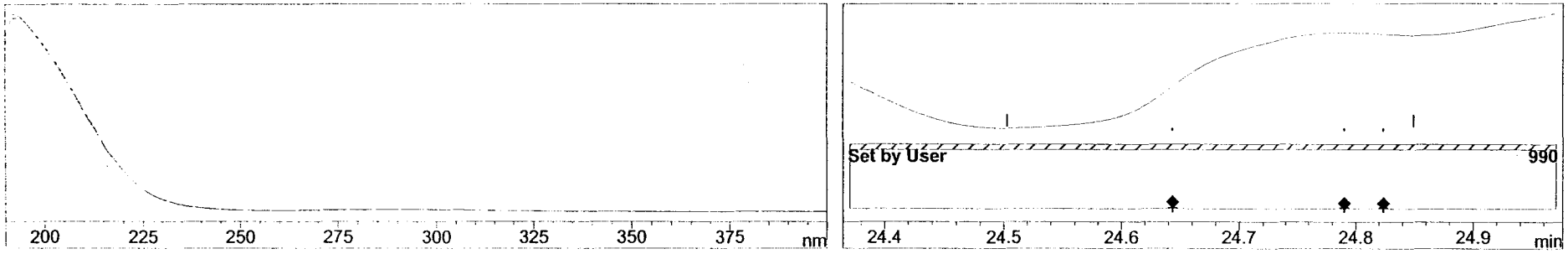
Purity factor : 998.657 (5 of 5 spectra are within the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 5 (Selection automatic, All)
Warning : Spectral absorbances > 1000 mAU (see help for more information)

195

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



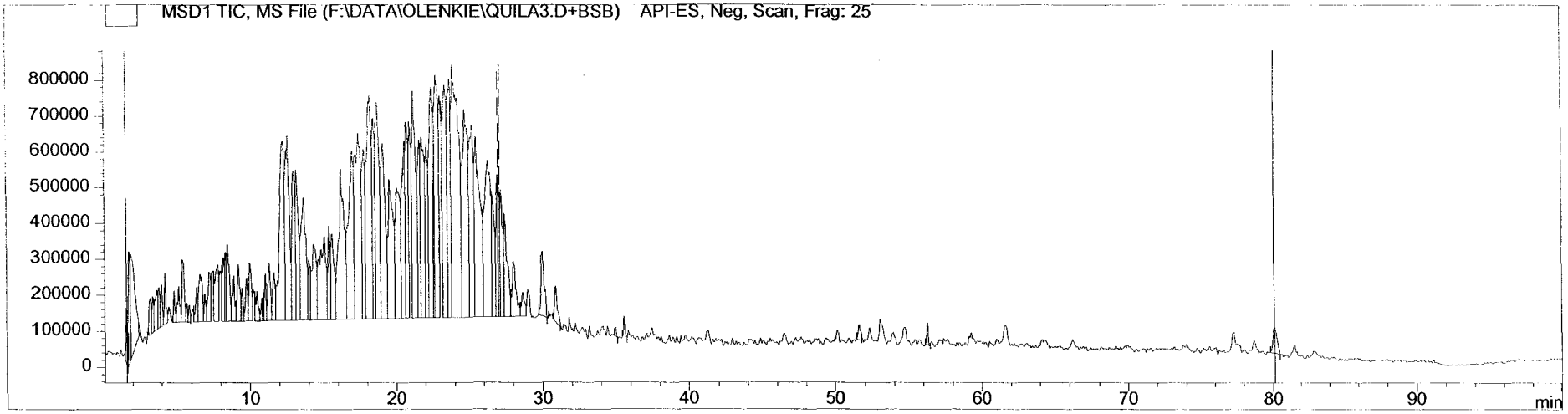
Peak :119 at 24.791 min Name : ?

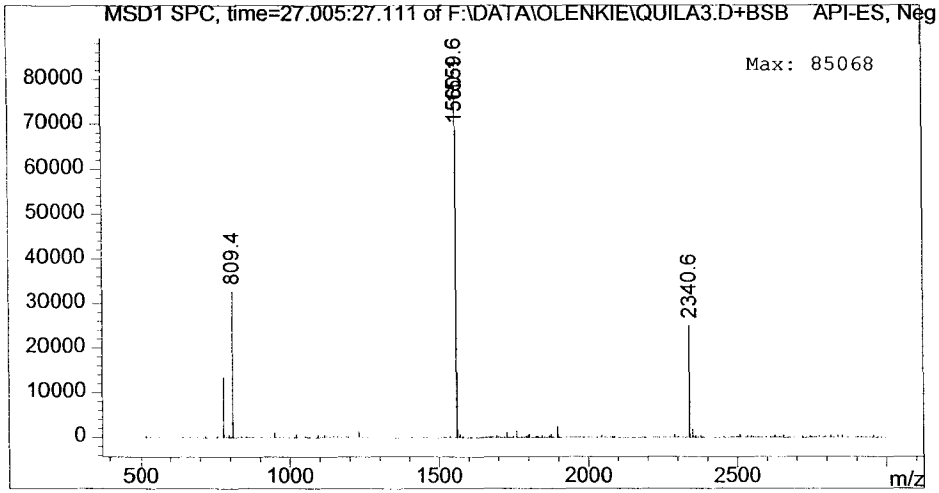


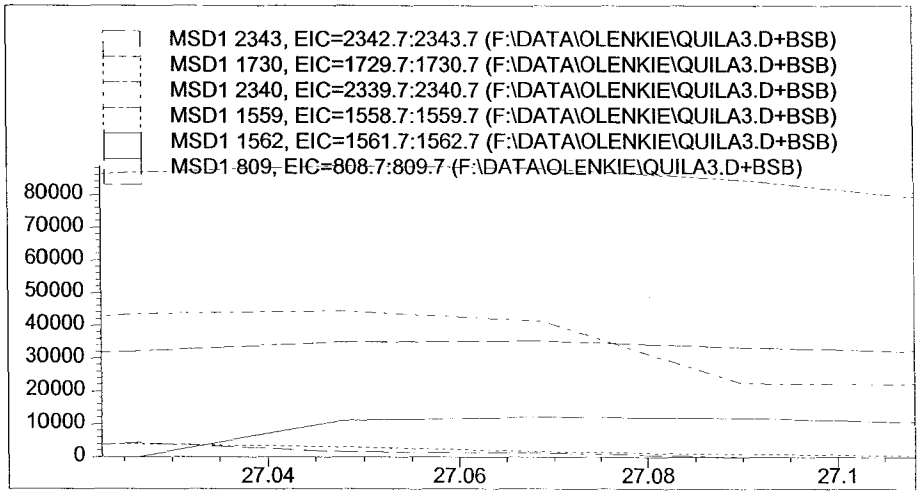
-> The purity factor is within the threshold limit. <-

Purity factor : 999.876 (3 of 3 spectra are within the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 3 (Selection automatic, All)

196





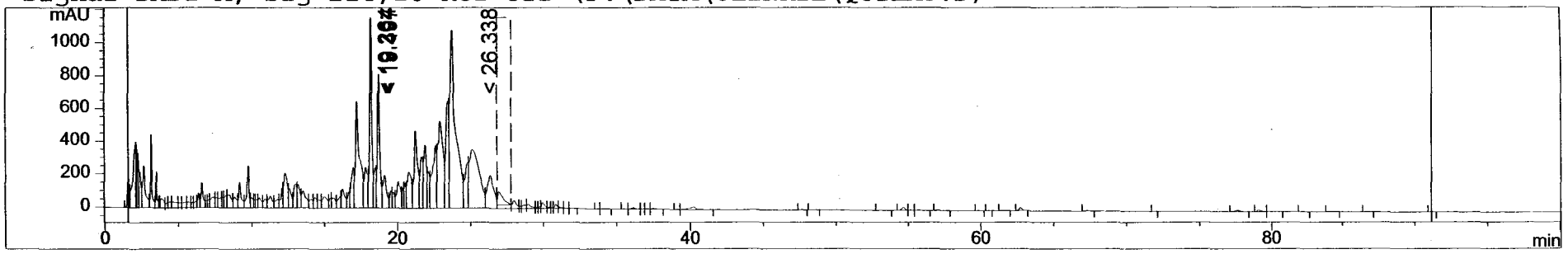


197

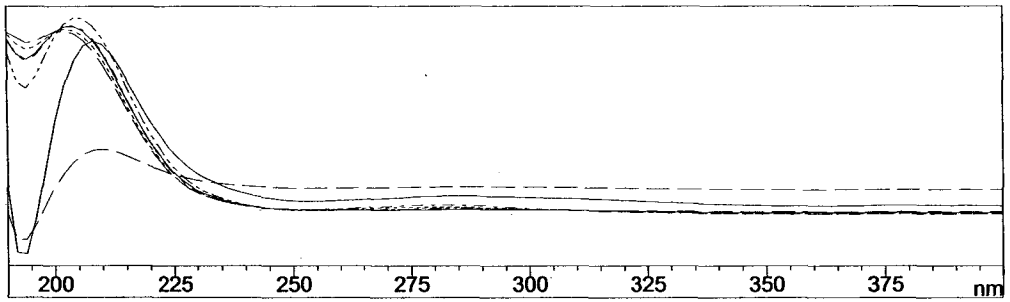
Peak #72 at 27.051 min (27.012 to 27.119 min)
-> The analysis found 3 components, indicating an impure peak. <-

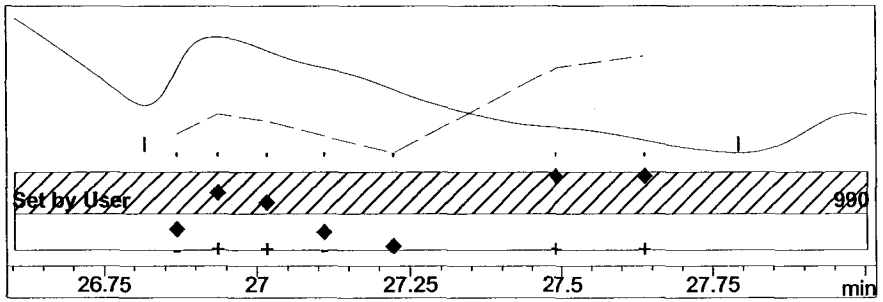
Component 1: Peak at Scan 1270.0. Top ions are 2343 1730 1231
Component 2: Peak at Scan 1271.0. Top ions are 2340 1559
Component 3: Peak at Scan 1272.1. Top ions are 1562 809 1897

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



Peak :126 at 26.936 min Name : ?

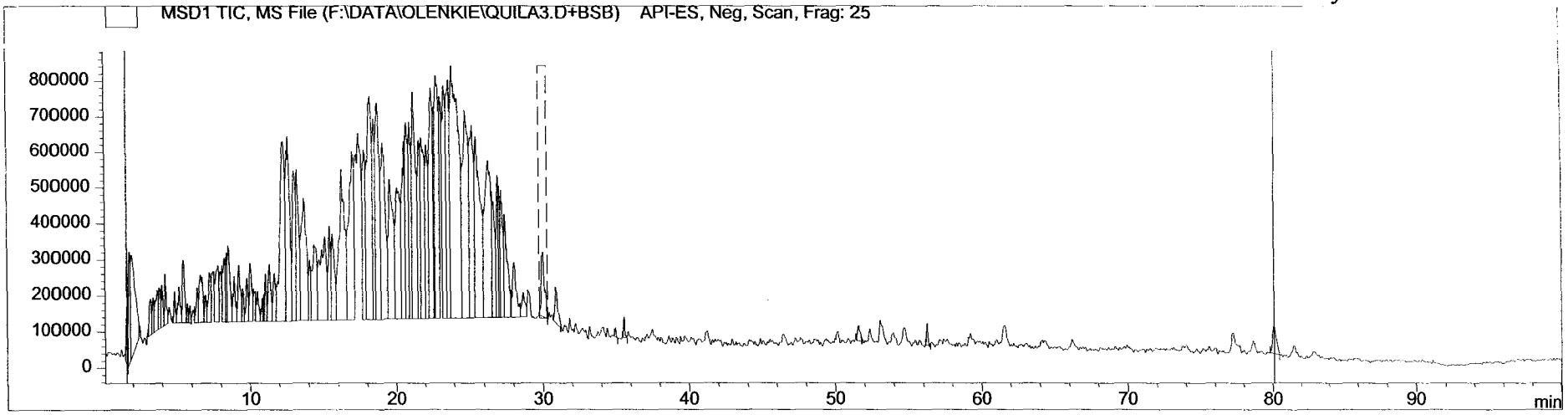


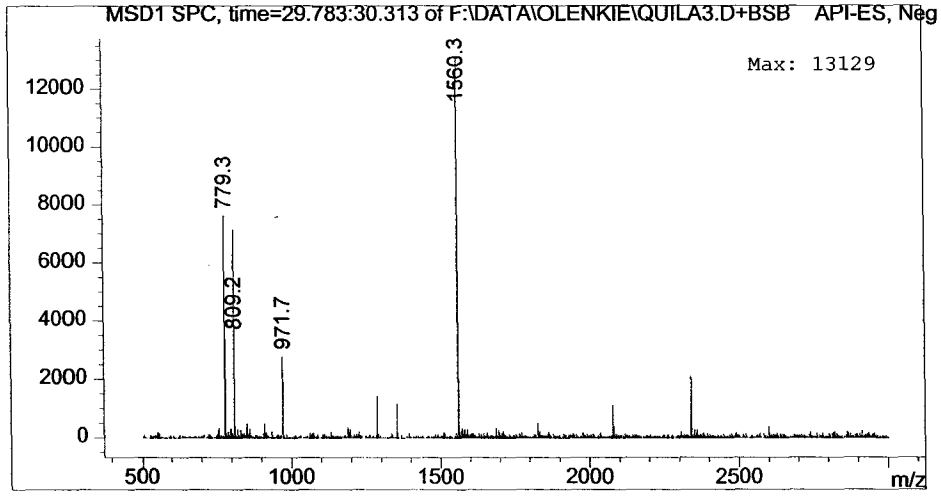


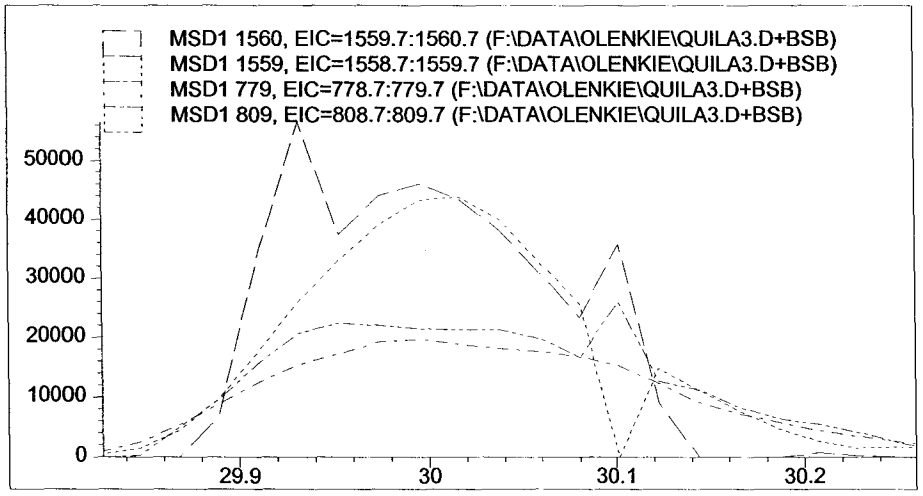
-> The purity factor exceeds the threshold limit. <-

Purity factor : 643.362 (4 of 7 spectra exceed the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 7 (Selection automatic, All)

198





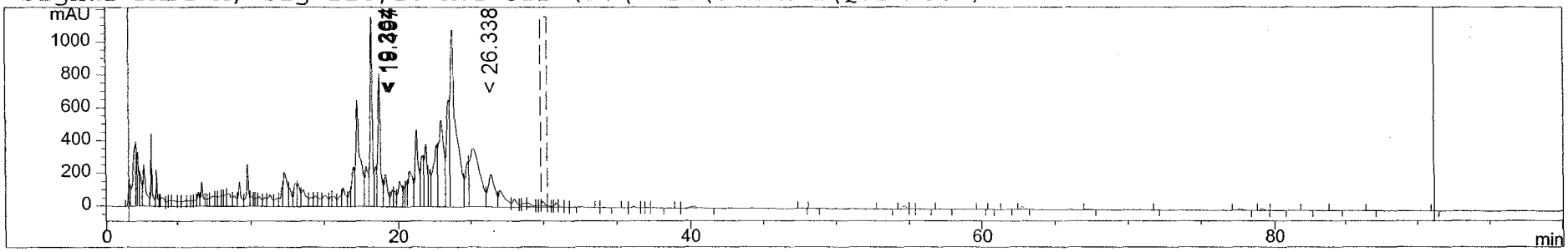


199

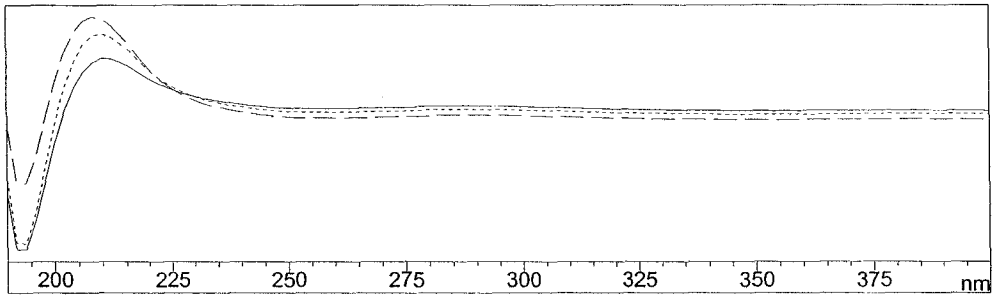
Peak #77 at 30.007 min (29.774 to 30.313 min)
-> The analysis found 7 components, indicating an impure peak. <-

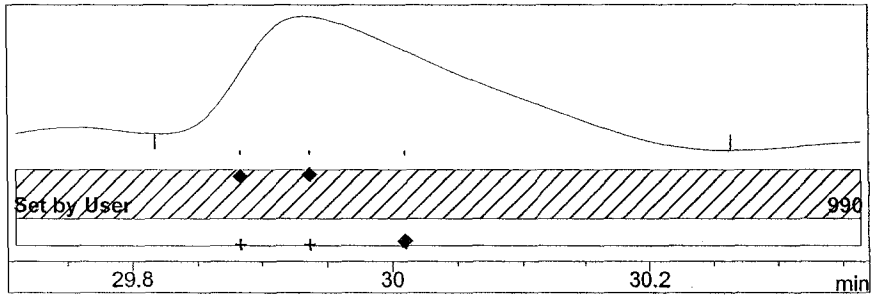
Component 1: Peak at Scan 1407.0. Top ions are 1560
Component 2: Peak at Scan 1410.3. Top ions are 1559 779 2341
Component 3: Peak at Scan 1414.9. Top ions are 809
Component 4: Peak at Scan 1416.1. Top ions are 1561
Component 5: Peak at Scan 1420.0. Top ions are 971
Component 6: Peak at Scan 1421.0. Top ions are 2077
Component 7: Peak at Scan 1422.0. Top ions are 1287

Signal DAD1 A, Sig=214,16 Ref=off (F:\DATA\OLENKIE\QUILA3.D)



Peak :138 at 29.936 min Name : ?





-> The purity factor exceeds the threshold limit. <-

Purity factor : 849.505 (2 of 3 spectra exceed the threshold limit.)
Threshold : 990.000 (Set by user)
Reference : No Reference
Spectra : 3 (Selection automatic, All)

200

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