STUDIES ON POLYSACCHARIDES OF *PUNICA GRANATUM* AND *TINOSPORA CORDIFOLIA*

THESIS
SUBMITTED TO THE UNIVERSITY OF CALICUT
IN PARTIAL FULFILMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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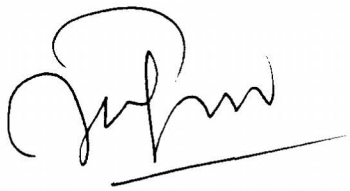
2000

DECLARATION

I, M. Jahfar, do hereby declare that the research work entitled : **STUDIES ON POLYSACCHARIDES OF *PUNICA GRANATUM* AND *TINOSPORA CORDIFOLIA***, is an original research work done by me in the department of Chemistry, University of Calicut and that it has not previously been submitted for the award of any degree, diploma, associateship, fellowship or other similar titles or recognition of any University, Institution or Board.



Calicut University

M. JAHFAR

CERTIFICATE

This is to certify that this thesis is an authentic record of the work carried out by Mr. M. Jahfar, under my supervision, guidance and preceptorship, and that no part thereof has been presented before for any other degree, diploma or fellowship of this or any other University.

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PREAMBLE

This thesis is an attempt to present the structural charecterisation of two polysaccharides isolated from plants widely used in folk medicine. In the field of biopolymer research, studies on water soluble polysaccharides of plant origin and their useful pharmacological activities forms a major thrust area. For the past two decades, many pharmacologically active polysaccharides have been isolated which includes antiinflammatory, antiulcer, antitumour and immunomodulatory compounds. The aquous extracts of the chosen plant materials have given encouraging results. These compounds were found to be polysaccharides. Many water soluble polysaccharides with promising pharmacological activities reported earlier contain glucose, galactose, mannose, rhamnose, xylose, arabinose etc., as their monomer units. To understand the biological activity and functions of complex carbohydrates, information about their primary structure is essential. Hence the glycosyl composition and glycosyl linkage analyses of the polysaccharides were carried out.

The GC-MS study of the trimethylsilylated and partially methylated alditol acetates are presented. All the spectra are attached herewith. Since these facilities are not available in our lab I have to depend Complex Carbohydrate Research Center, USA.

The polysaccharides were analysed for several biological activities such as blast transformation in human lymphocyte culture, and immunomodulatory activities; the results are promising. Biological studies were carried out at Regional Cancer Center, Thiruvananthapuram.

CONTENTS

CHAPTER - I : INTRODUCTION

INTRODUCTION

Carbohydrates are, in terms of their quantity, the largest group of organic compounds on earth and are implicated in every form of living activity in plants, animals and microorganisms. They are one of the most important food constituents, the raw materials for human clothing and the raw materials of paper and for many biotechnological processes, such as production of beverages and antibiotics. It is obvious that methods are necessary to identify the individual species and to determine them quantitatively, either alone or as a mixture of more than one component in the presence of other compounds. A recent report of the U.S. Department of Energy Workshop on Complex Carbohydrates suggests that understanding the roles played by complex carbohydrates is the next greater frontier in the advancement of molecular biology. Evidence is rapidly growing on the key roles these molecules play in a broad range of biological recognition and regulatory phenomena- cellular communication, gene expression, immunology, organism defense mechanisms, growth and development. Carbohydrates are some of the most important chemical compounds of the biological world and are the key substances of life since they are the primary products of photosynthesis. They occur in nearly all living systems either as high molecular skeletal substances, as high or low molecular

energy resource compounds as well as key intermediates in human, animal and plant metabolism. In combination with proteins or lipids, they give highly physiologically active glycoproteins (e.g. blood group substances) or glycolipids. A lot of commercial products consists of carbohydrates, or contain carbohydrates, particularly most foods, and especially those of plant origin (cereals, pulses, fruits, and vegetables) or are products of daily use such as paper and textiles. It should be noted that sugar itself with the chemical name sucrose, is the most widespread chemical in the world produced in the highest purity.[1]

Historically, the field of complex carbohydrates had been a difficult and not a very popular area of research compared to nucleic acids, proteins and lipids. The major reasons for these were the structural complexity of these molecules which complicates their analysis and synthesis, as well as the ignorance of the true functions of these molecules, and consequently the wrong perception that they were not as important as other biomacromolecules for the process of life. The study of carbohydrates and their derivatives have helped to understand the role of the molecular shape and conformations in chemical reactions[2]. It has led to the discovery of enzymatic control mechanisms and has contributed significantly to the understanding of many fundamental biological processes, such as the interaction of cells with their environment. It has provided a basis for the

recognition of the enzymic defects of several genetic disorders. A number of specific uses of carbohydrates in pharmaceutical industry have come up as a result of developments in genetic engineering and microbial technology. Recently purified polysaccharides of bacterial origin have been prepared for use as vaccines against meningococcal and pneumococcal infections. Due to the ability of certain polysaccharides to cross react, with other antisera they could provide immunity against other infections and are used as antiallergy antigens.

Complex carbohydrates are favourable candidates for encoding biological information because of the large number of structures possible in relatively short oligosaccharide sequences. A variety of complex carbohydrate structures exist in nature with ample diversity to serve as receptors in recognition phenomena. They exist either in free form as oligo or polysaccharides or in conjugation with lipids or proteins as glycolipids, glycoproteins and proteoglycans (glycoconjugates). The complexity of these molecules arises from the fact that the component monosaccharides are multifunctional and exhibit stereoisomerism. Polysaccharides have two ways of generating diversity mechanisms not available to proteins or nucleic acids, one is the formation of α- and β-anomeric linkages and the other is linear or branched chain formation or both, of the monosaccharide units in the formation of macromolecules. Thus, even three monosaccharide units can

form 1056 different trisaccharides. This diversity of structures carries with it the enormous potential for saccharides to function as informational molecules.

To understand the biological activity and functions of complex carbohydrates, information about their primary structure is essential. This task is much more complicated than the case in elucidating the structure of the other two biopolymers, (proteins and nucleic acids) where sequence alone will give ample information on the primary structure. For saccharides, in addition to the sequence of monosaccharides, position of linkage, anomeric configuration of the linkages and branching patterns must be determined. Because of these reasons the structural analysis of saccharides is slow and laborious. Moreover, due to the inherent heterogeneity of the saccharide components of glycoconjugates and the existence of a number of glycoforms, extensive fractionation is needed and hence only minute quantities of pure forms are obtainable.

It has been established that, polysaccharides as compounds of glycoconjugates and in free form are involved in a myriad of important functions and pharmacological activities. At both molecular and cellular levels, the saccharides serve as informational and recognition molecules, blood group antigens and as receptors for viruses, toxins, hormones etc.

They control vital events such as fertilization, implantation in the endometrium, embryonic development and targeting of aging cells for removal and destruction. When a cell undergoes malignant transformation, state of the surface-carbohydrate profile is drastically altered.[3] Thus, majority of cancer-associated antigens are glycoconjugates, many of which are finding clinical application in the management of cancer patients. A major thrust area of research in pharmacology and pharmaceutical chemistry is on the development of saccharide drugs for treatment of rheumatism, and the role of carbohydrate as immunomodulatory and anticomplimentary agents.

Earlier methods of analysis of carbohydrates deal with gravimetric, titrimetric and colorimetric procedures which enable only the qualitative and quantitative analyses of different groups of carbohydrates, but not the individual compounds. The major breakthrough began fifty years ago, with the developments of newer methods. Considerable progress has occurred in this field, in the last four decades, dealing with the new chromatographic and electrophoretic methods, such as thin layer chromatography (tlc), gas chromatography (gc), high pressure liquid chromatography (hplc), high voltage capillary electrophoresis, and the developments of new enzymatic methods. These procedures enable the qualitative and quantitative analysis of single species in complicated mixtures of carbohydrate compounds.

In the field of biopolymer research, studies on water-soluble polysaccharides of plant origin and their useful pharmacological activities forms a major thrust area. During the past few years, many pharmacologically active polysaccharides have been isolated and structural characterization have been accomplished. These include anti-inflammatory, antiulcer, antitumour and immunomodulatory compounds[4-10]. Most of these molecules are found to be copolymers of glucose, galactose, xylose, arabinose, rhamnose and mannose while some are even homopolymers of glucose with different degree of polymerization and molecular mass. Whistler *et al*[11] have reported that water-soluble β-D-glucans with a preponderance of, or long stretches of (1→3) linkages in the main chain are active antitumour agents. It is well known that molecules exhibiting anticomplementary and immunomodulatory properties will usually be active as antitumour agents.

Over 7,500 species of medicinal plants in India are being used in the traditional system of medicine called *Ayurveda* – the indigenous system of medicine, dating back to the vedic ages – (1500 – 800 B.C) which has been an integral part of Indian culture – for preventive, promotive and curative properties for various human and veterinary health problems. The Indian Medical Heritage can be broadly classified into two separate but inter-related streams of traditional knowledge – the oral tradition or folk medicine and the codified tradition or the classical system of medicine. In

the codified medical texts of *Ayurveda*, a recent study enumerates that only about 1,700 species of plants that are fully documented in terms of their biological properties, actions and drug formulations. So, despite its efficiency in handling complex diseases, traditional medicine hasn't been accepted worldwide because of lack of documentation and authentication backed up by scientific data. The present work is for providing some information supporting the medicinal value of two of the widely used plants in *Ayurveda* for various ailments.

It is believed that the medicinal properties in plants are mainly due to the presence of 'secondary metabolites' which express in the plants only when they grow in their natural environments under particular conditions of stress and threat of predators etc. It is therefore doubtful if these under mono-culture would still express the same medicinal qualities, as in nature.

The plants selected for the present study are *Punica granatum* L. (Punicaceae) and *Tinospora cordifolia* (Willd) Miers ex. Hook F. & Thoms. (Menispermaceae). According to the literature[12,13] these plants are used in folk medicine for various purposes. *Punica granatum* has long been esteemed as food and medicine and as a diet in convalescence after diarrhoea. The officinal part, rind of the fruit, is astringent, digestive, cardiotonic, stomachic and is highly effective in chronic diarrhoea and dysen-

tery, dyspepsia, colitis and uterine disorders. The powered drug boiled with buttermilk is an efficaceous remedy for infantile diarrhoea. Recent investigations have indicated that a substance derived from *Punica granatum* can attack certain viruses and bacteria with unprecedented efficiency and it could become a vital force against the spread of many viruses. It is a glabrous shrub or a small tree widely cultivated throughout India; also reported in central and West Asia, and southern Europe. Its branchlets often spinescent, leaves simple, opposite, short-petioled, narrowly elliptic or lanceolate obtuse, entire glabrous to 8×2.3 cm, flowers bright red, solitary, axillary, short-pedicelled, calyx tube funnel shaped, thick, coriaceous, orange coloured, adenate to the ovary below, lobes 5-7, triangular, persistent on fruit; petals 5-7, stamens numerous, ovary inferior, style long, stigma capitate, fruit a large globose berry – yellowish red when ripe, up to 10 cm. across, with a thick coriaceous rind crowned with the persistent calyx lobes.[14]

Tinospora cordifolia (Willd.) Miers ex Hook f.& Thoms (Menispermaceae) is glabrous, succulent, climbing shrub, often attaining a great height and sending down long thread-like aerial roots. The plant seems to be particularly fond of climbing up the trunks of large trees. The bark is gray or creamy-white in colour, deeply cleft, the space between the clefts being usually dotted with large rosette-like linticels. The wood is white,

soft and porous, and the freshly cut surface soon assumes a yellow tint on explosure to air. The branches bear smooth heart-shaped leaves and bunches of red berries. The sap is viscous and light yellow in colour having a peculiar slimy odour and a nauseating bitter taste.[15]

It is indigenous and found distributed throughout most parts of India. It is a fairly common wild plant of the deciduous and dry forests growing over hedges and small trees. It is commonly called *Amrta* in Malayalam. *Amrta* is a Hindu mythological term which refers to the heavenly 'elixir' which saved celestial people from senescence and kept them eternally young. This term is attributed to this drug in *Ayurveda* in recognition of its capacity to impart youthfulness, vitality and longevity to the consumer.[14] *T. cordifolia* attracted the attention of European medical men in India and has been favourably spoken of by them as a tonic, antiperiodic and diuretic and is effective in dyspepsia and other conditions. The drug itself as well as tincture prepared from it are now official in the Indian Pharmacopoeia. Inspite of the fact that this plant is so extensively used in folk medicine, the pharmacological action of the active principles isolated has not been worked out.[16]

Plate I

1. *Punica granatum* fruit


7

Plate II

2. *Tinospora cordifolia*


2

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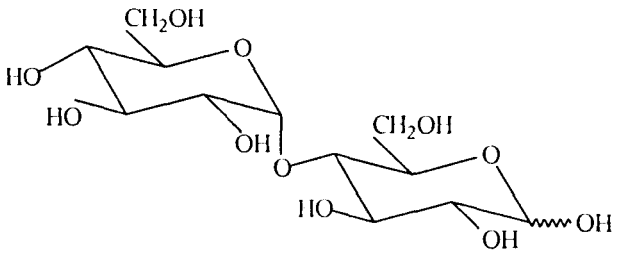
CHAPTER - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Polysaccharides are natural macromolecules occurring in almost all living organisms, constituting one of the largest groups of natural compounds classified thus far, and functions either as an energy source or as structural units in the morphology of the living material in which they are endogenous. As one of the main sources of energy for living organisms, certain polysaccharides form part of the central pathway of energy in most cells. The starches and glycogens, long-chain polymers of D-glucose, are the media for energy storage in plants and animals respectively. The most common disaccharides prepared by hydrolysis of polysaccharides are maltose (fig. 1) and cellobiose (fig. 2) obtained from starch and cellulose respectively. Polysaccharides also perform more specific roles, such as being responsible for the type specificity of pneumococcal polysaccharides. Other natural macromolecules, which are not composed entirely of sugar units, contain blocks of monosaccharide units as part of the molecular structure, and contribute extensively to the production and maintenance of living tissues of animals. The blood group substances constitute a group of glycoproteins in which the arrangement of monosaccharide residues in the carbohydrate subunits contribute towards the blood-group specificity

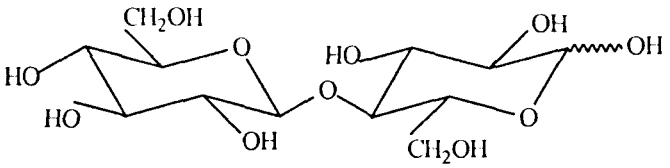
of the overall molecule. There is, at present, no proof of the existence of polysaccharides which contain more than about six different types of monosaccharide unit.



α-D-Glc *p*-(1→4)-D-Glc *p*

Figure 1

The most common constituents are the pentose and hexose monosaccharides, and monosaccharides derived from them – for example, hexuronic acids, deoxy hexoses etc., these usually form a regular repeating unit throughout the polymer.



β-D-Glc *p*-(1→4)-D-Glc *p*

Figure 2

This repeating unit is usually found in any chemical representation of the

polysaccharide but, with our increasing knowledge of polysaccharide structure obtained by physicochemical methods, such repeating units are known to represent the average case, minor differences in degree of substitution of derivatives, chain length and composition of minor components exist, because the many varied reactions which form the biosynthetic processes are not fully complimented. Such phenomena lead to minor structural deviations, known as microheterogeneity; this does not affect the overall structure but does cause differences between samples of the same polysaccharide from different sources[1].

Complex carbohydrates are often subjected to different chemical modifications in order to gain information on sequences and anomeric configurations. Investigations are being carried out in many laboratories around the world to explore new bioactive polysaccharides from micorbial, animal and plant origin with high potential activity. The role of polysaccharides in immunology was demonstrated first in 1917 by Dochez and Avery[2]. They showed that a specific substance secreated by pneumonococci during growth, precipitated antibodies to the bacterium of the pneumonococcus type from which it was derived. Subsequent studies by Herdelbergen *et al,*[3] showed that the specific soluble substances were complex carbohydrates. This was the first time that any material other than protein had been shown to be antigenic[4]. In recent years, the

use of polysaccharides as antigens and immunogens has contributed greatly to the classification and identification of bacteria, to a better understanding of the immune response, to the definition of the active site in antigen-antibody interactions and to the detection and prevention of human disease caused by invasive micro organisms.

A polysaccharide designated as MVSIVA, isolated from the seeds of *Malva verticillata* has anticomplementary and hypoglycemic activities. It showed remarkable reticuloendothelial system potentiating activity in the carbon clearance test – stimulating the phagocytic activity of cells. Chemical and spectroscopic studies demonstrated that the polysaccharide is rich in α-L-arabino furanose residues and possesses mainly an α-1,5-linked L-arabino-β-3,6-branched D-galactan structure accompanied with rhamnogalacturonan type units. Both α-1,3-linked L-arabinopyranose and β-1,4-linked D-xylopyranose residues were also identified as component units[5]. A glycoprotein isolated from jack bean (*Canavalia ensiformis*) was found to be a protease inhibitor, equally active on bovine and porcine trypsins[6]. From *Rosa davurica*, a traditional Chinese medicine, methyl-3-O-β-glucopyranosyl gallate was isolated[7].

Antitumour activity of polysaccharide fractions from the brown sea weed, *Saragassum kjellmanianum* was reported. Iizima-Mizui *et al* in

1985[8]. Antitumour activity of grifolan NMF-SN, a β-1→3-glucan obtained from mycelia of *Grifolan frondosa* was examined and found to have antitumour activities in allogenic and syngeneic tumour systems[9]. The polysaccharide fractions from the rhizome of *Trichosanthus kirilowii* showed marked antitumour and cytotoxic activity with immunopotentiating activity. It was composed of glucose, galactose, mannose and xylose, and a small amount of protein[10]. Polysaccharide fractions from Panax ginseng have also been studied for their antitumour and immunomodulatory activities [11]. An alkali soluble glucan isolated from a crude fungal drug 'Leiwan' (*Omphalia lapidescens*) showed potent antitumour activity against the solid form of sarcoma 180 in ICR mice [12]. Methylation analysis suggested that it was a (1→3) -β-O -glucan with approximately one branch at every three main chain glycosyl units at each C-6 position. (Fig. 3).

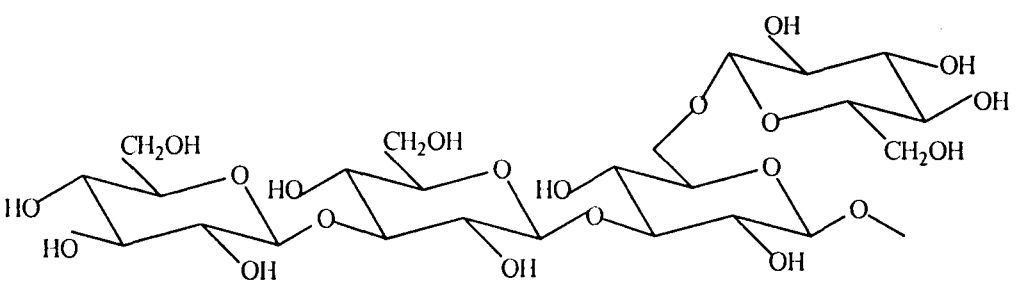


Figure 3

Carbohydrates that also function as immunomodulating agents have been isolated from several medicinal plants in recent years. Polysaccharide K

(PSK), a protein bound polysaccharide extracted from *Basidiomycetes* fungus was found to have potent immunomodulatory activity[13,14]. A higher cumulative survival rate and better results in immunological parameters such as counts of peripheral lymphocytes and skin reactions to PPD and PHA were observed after the administration of PSK. The chemical structure of PSK is however, unknown.

Polysaccharides isolated from *Tamarindus indica* was found to have different biological activities such as mitotic inhibition, preventing cell proliferation of peripheral blood lymphocytes in cultures, leukocyte migration inhibition and phagocytic enhancement[15]. Another polysaccharide isolated from *Mangifera indica* has haemagglutinating activity[16]. Other useful pharmacological properties are also exhibited by polysaccharides. Heparin, a carbohydrate polymer consisting of sulphated D-glycosamine and D-glucuronic acid unit, has been in use as an anticoagulant since 1936. Dextran is yet another example which is used therapeutically and as a tool in preparative and analytical chemistry. Lentulose is a glucan which is used in ulcer therapy.

A water-soluble polysaccharide–1,4 linked xylose and rhamnose units with glucuronic acid attached to rhamnose as end groups, was isolated from a green seaweed, *Spongomorpha indica*[17]. Another water-soluble

polysaccharide has been extracted from *Pinus densiflora* [18]. Spectroscopic and methylation analysis indicated that amylose, amylopectin, glucomannan, arabinan and arabino-glucuronoxylan are the major components of the polysaccharide. Crude fungal extracts composed mainly of β-D-(1→3) linked D-glucose (fig. 4) and small proportions of D-galactose and D-mannose have been reported to be active against solid tumours.[19,20]

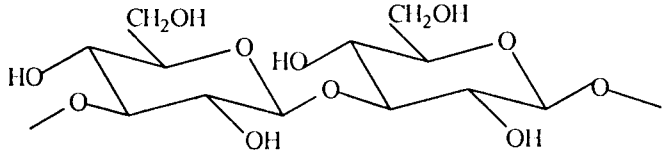


Figure 4

Oka *et al* had reported the antitumour activity of some plant polysaccharides[21]. In 1971 Haumuro *et al* isolated a new water-soluble polysaccharide, carboxymethyl pachymaran with marked antitumour activity[22]. Lentinan, a β-1→3-glucan with β-1→6 branches, (fig. 5) purified completely from Lentinus edodes, an edible mushroom in Japan, has also been found to have marked antitumour activity.

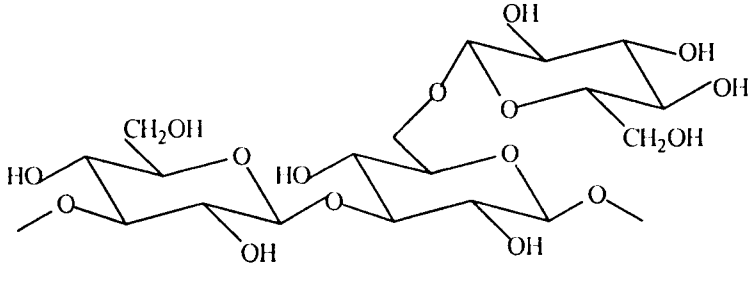


Figure 5

A water soluble galactomannan consisting of D-galactose and D-mannose in the molar ratio 3:2 was isolated from the seeds of *Cassia angustifolia*. Structural studies revealed the presence of (1→4) and (1→6) glycosidic bonds. The main chain of the galactomannan was found to consist of (1→4)-linked mannopyranosyl units having β-glycosidic bonds, while (1→6)-linked α-glycosidically bonded galactopyranosyl units form the branching points[23]. (fig. 6).

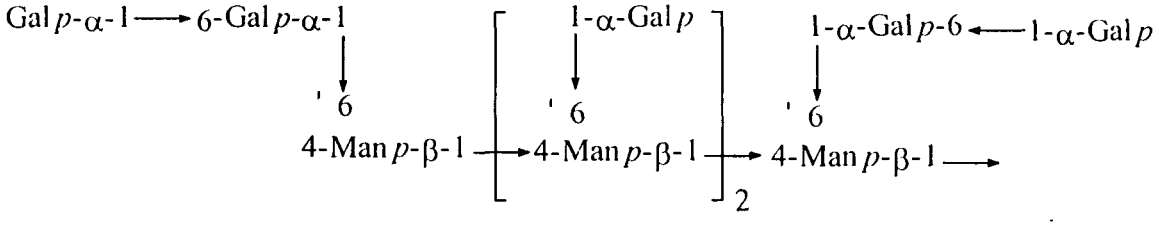


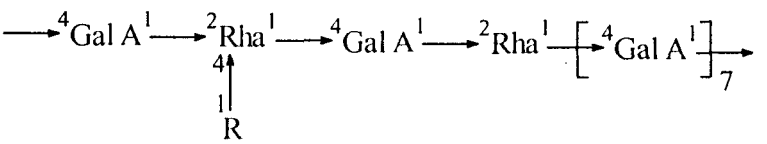
Figure 6

Another water soluble polysaccharide isolated from *Plantago lanceolate* leaves was found to be composed of L-arabinose (20%), D-galactose (8%), D-glucose (6%), D-mannose (2%), L-rhamnose (4%), D-galacturonic acid (31%), D-glucuronic acid (7%) and minor amounts of D-xylose. Analysis of component sugars, chemical and spectroscopic studies made it possible to suggest the structural repeating units for the polysaccharide [24].

The polysaccharides from the leaves of *Artemisia princeps* Pamp showed a potent anticomplementary activity,[25] and structural characterization was also made[26]. Structural characterizations of the anti-complementary

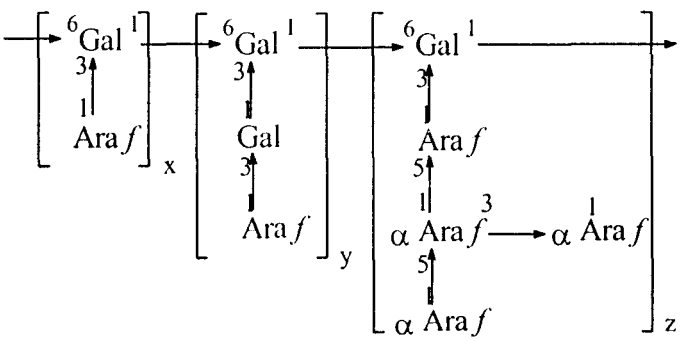
acidic heteroglycan AAF II b-2 and II b-3, obtained, revealed that they consist of rhamnose, xylose, arabinose, galactose, glucose and uronic acids (glucuronic acid and galacturonic acid) in the molar ratio of 7.6:7.6:13.0:10.9:3.0:57.9 and 3.9:2.6:24.7:19.7:2.6:46.5 respectively. The basic structure of AAF IIb-2 is similar to that of IIb-3 but IIb-3 has a higher arabino galactan content. The suggested partial structural fragment of AAF II b-2 and II b-3 are given (fig. 7)

A



R = arabino-3,6 galactan and 4-galactan

B



A = A possible partial structural fragment of AAF II b-2 and II b-3.

B = A possible structural fragment of arabino-3,6–galactan moiety in AAF II b-2 and II b-3.

Figure 7

Structure determination of polysaccharides in *Aloe arborescens* var *natalensis* was done by Yagi *et al*[27]. They isolated a linear polymer of a (1→6)–O–linked α-D-glucopyranose, a brached polymer of an arabinogalactan (molar ratio of galactose to arabinose 1:1.5) with two principal linear chains of (1→2)–O–L–arabinopyranose and (1→2)–O–D–galactopyranose at O–2 and O–6 of the D–galactopyranose residue; a linear polymer of a (1→4)–O–linked β–D–mannopyranose (fig. 8) with 10% acetyl group.

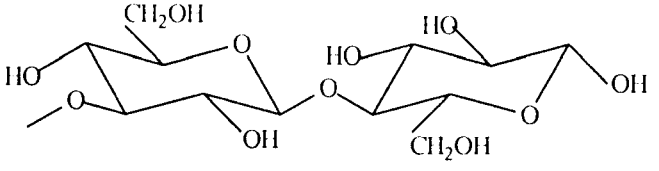


Figure 8

It was shown that oral administration of *Aloe* extract showed efficacy for chronic bronchial asthmatics of various ages as well as intrinsic types. The linear chain of (1→4)–O–linked β–D–mannopyranose with 10% acetyl groups and the glycoprotein present in the extract enhanced phagocytocis in adult bronchial asthmatic. Japanese workers have isolated a rhamnogalacturonan with (1→4)-linked galacturonic acid and (1→2)- linked rhamnose and arabino-3,6 and 4–galactan composition, which also acts as an anticomplementary agent[28]. A number of herbal drugs have been studied for their hypoglycemic activity and quite a few have been proven to be

potent hypoglycemic agents. Structural studies by Hikino *et al* [29] and Tomada *et al* [30] showed that the activity is associated with glycans and proteoglycans. The major acidic polysaccharide named Saponhnikovia A, was isolated from the roots and rhizomes of *Saponhnikovia divericata* [31]. It showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test. It was composed of L-arabinose, D-galactose and D-galacturonic acid in the molar ratio 6:15:10, and its molecular weight was estimated to be 54,000 D. About 35% of the D-galacturonic acid residues exist on the methyl esters. Methylation analysis, ^{13}C NMR and controlled Smith degradation studies indicated that the polysaccharide has the α–(1→4)–linked D–galcturonan backbone bearing α–(1→5)–linked L–arabino–β–3,6 branched D–galactan side chains. The chemical composition of polysaccharide fractions from *Strychnos nux-vomica* and *S. innocua* and comparison with those from *S. potatorum* were reported[32]. The structural studies of the galactomannans from these three *Strychnos* species were also carried out, with a view to examining chemotaxonomic relationships.

Thus many kinds of biologically active polysaccharides have been isolated from various sources, notably compounds having antitumour activity. It is possible that this activity is due to a host-mediated action of the polysaccharides; they may activate the depressed functions of

phagocytes in tumour-bearing hosts[27]. Isolation and characterization of a Mucous polysaccharide, Narcissus-T-glucomannan, from the bulbs of *Narcissus tazetta* var. *chinensis* was carried out by Tomoda *et al*[33]. Similarly another polysaccharide, Lycoris-R-glucomannan was isolated from the bulbs of *Lycoris radiata* HERBERT (*Amaryllidaceae*), which has been used as a crude drug with expectorant and emetic properties.[34] Another polysaccharide Lycoris-S-glycomannan, was isolated from the bulbs of *Lycoris squamigera* MAXIM, and structural analysis was carried out.[35]

The two plant sources chosen for the present study are also reported to have medicinal properties. *Punica granatum* is at the centre of some extra ordinary pioneering research at British Universities.[36] It could become a vital force against the spread of many viruses. Investigations have indicated that a substance derived from it can attack certain viruses and bacteria with unprecedented efficiency. Two tannins–punicalagin and punicalin–were isolated from the rind of the fruit and their structures determined (fig 9)[37]. Pectin isolated from fruits contained mannose, galactose, rhamnose, arabinose and glucose in the ration 1:1.3:2.1:4.4:6.2; principal sugar acid was galacturonic acid.[38]

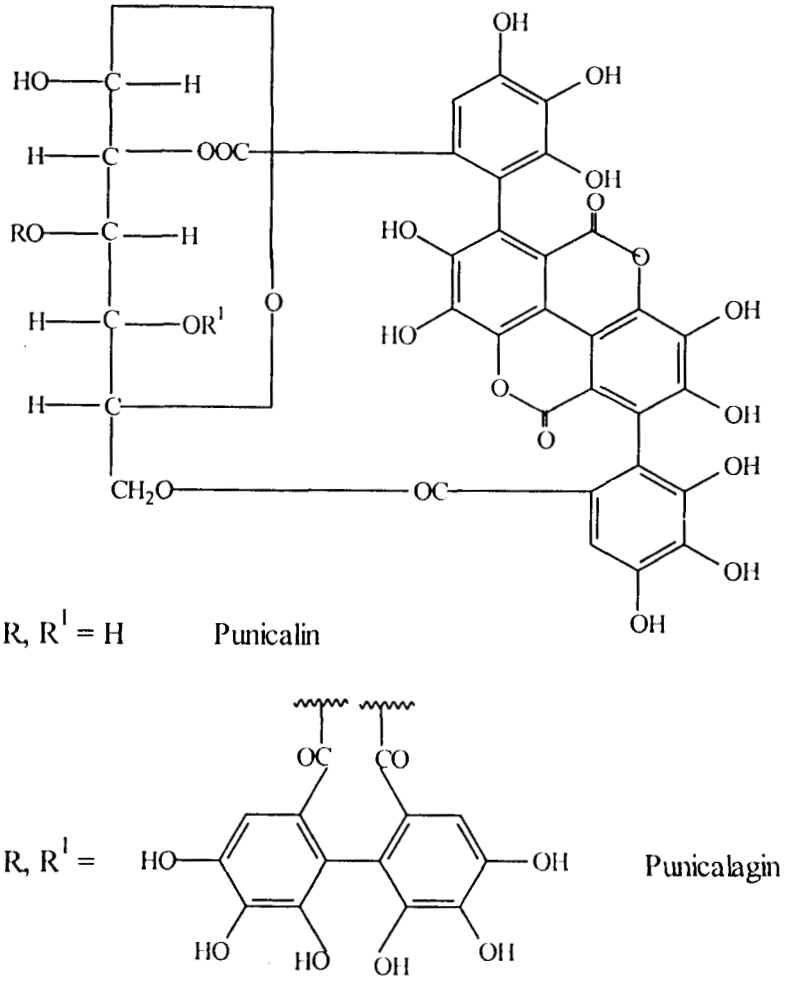
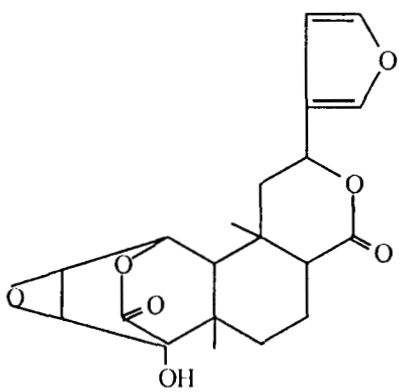


Figure 9

Tinospora cordifolia has been extensively used in Ayurveda. Clinical and pharmaceutical studies have revealed that an aquous extract of the stem helps in reducing the blood sugar in alloxan induced hyperglycaemic rats and rabbits.[39] The aquous extract was also found to be an effective remedy for different types of rheumatic afflictions[40]. The antipyretic and

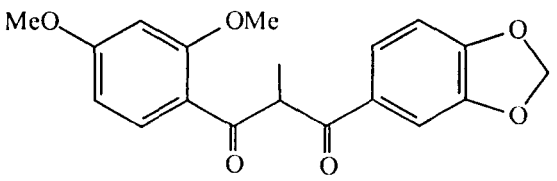
anti-inflamatory actions of the aquous extract have been studied[41]. *T.cordifolia* decoction was administered orally in the dose level of 1ml/100 g body weight in Albino rats to screen their antipyretic activity. The pyrexia was induced in rats by injecting subcutaneously TAB vaccine. The extract showed significant antipyretic activity. The anti-inflamatory action of the aquous extract was studied by Gulati and Pandey[42]. Detailed pharmacognostic studies on this plant have been made by Khosla and Prasad[43]. Tinosporide, a diterpene isolated from the fresh stems of *T. cordifolia* is reported to be $C_{20}H_{22}O_7$ and its structure was elucidated (fig . 10)[44].



Tinosporide (Magnoflorine 0.07%)

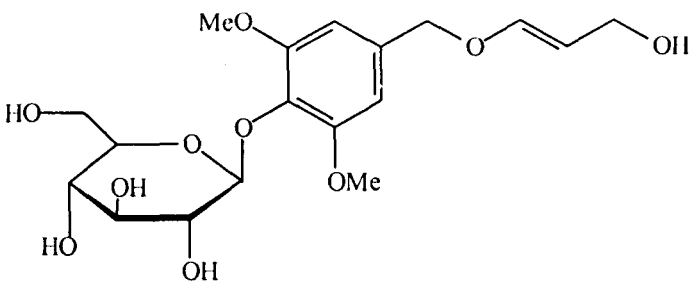
Figure 10

Other compounds isolated include Tinosporinone (fig 11)[45] and a phenolic glucoside, Tinotuberide[46] (fig 12)



Tinosporinone

Figure 11



Tinotuberide

Figure 12

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CHAPTER - III

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

CHEMICAL STUDIES

Two different polysaccharides were isolated from *Punica grantum* (Pg) and *Tinospora cordifolia* (Tc). Standard procedure was followed for the isolation of polysaccharides[1]. The powdered fruit rind of Pg and shade-dried stem pieces of Tc were treated with water at room temperature for 48 hours with stirring. From the aquous extract, polysaccharides were precipiated by addition of 3 volumes of 95% Ethanol. Solutions were concentrated at or below 60°C with rotary flash evaporator under reduced pressure. The precipitate was collected by centrifugation at 20,000 rpm, 15 min, dissolved in water, and dialyzed. The collected polysaccharides were dried over fused calcium chloride under reduced pressure. The desiccated polysaccharides were redissolved in water. The proteins from the solution of polysaccharide was removed by Sevag method.[2, 2a] The proteins are denatured by shaking in a chloroform emulsion, after which they form a gel which remains in the water-chloroform interface, and were removed. To facilitate the denaturation a pH 4 – 5 buffer was used instead of water and small quantities of 1-butanol was added. Since this technique removed only small quantities of protein, it was repeated four times and significant losses of polysaccharides resulted. The clear aqueous layer

was again treated with 95% Ethanol to precipitate the polysaccharides, dialyzed against distilled water for 48 hours in the cold. The solution in the dialysis bag was concentrated under reduced pressure and lyophilized.

Homogeneity of the polysaccharides was ascertained by purification by gel filtration chromatography on Sephadex G 200 and Ultrogel AcA–44. Elutions of the polysaccharide fractions were monitored by the absorbance of effluents at 490 nm using the phenol-sulphuric acid method[3]. The purified samples of polysaccharides from the two plant sources showed apparent homogeneity by giving single bands in zone electrophoresis experiments in polyacrylamide gel.

The total carbohydrate and uronic acid contents were determined by the phenol-sulphuric acid method[3] and m-hydroxydiphenyl method[4] respectively, using arabinose and galcturonic acid as the respective standards. Anthrone assay for hexoses was also carried out[5]. The percentage of carbohydrate in each of the crude sample was estimated as 10% and 76% for Pg and Tc respectively.

Molecular weights were determined by gel filtration studies on Sephadex G-200 using a series of dextrans of different molecular sizes as reference standards. From the data obtained in these experiments the average molecular weights were calculated by linear correlation between the

logarithm of the molecular weights of the standards and ratios of their elution volumes to the void volume of the column[6]. Molecular weight of the polysaccharides from Pg and Tc were determined to be approximately 1,10000 D and 40,000 D respectively.

III. 1. Hydrolysis

Complete hydrolysis of the homogeneous polysaccharides was done according to Kram and Franz[7] by 1M H_2SO_4 for 6 hours in sealed tubes in a boiling waterbath. Graded hydrolysis was carried out with 25 mM H_2SO_4 at 100° C- Sulphate ions and charged sugars were removed from the hydrolyzate by passage through a column of Dowex 50-X4 200–400 mesh (H^+ form) coupled to a column of Dowex 1-X8, 200– 400 mesh (formate form).[8] [The Dowex 1-formate resin was prepared from the chloride form of the resin by passage of 3N Sodium formate through it until a negative test for chloride is obtained, followed by extensive wash with distilled water].

Thin layer chromatography (t.l.c) and paper chromatography (p.c) of their hydrolysates after 15, 30, 45, 60, 90 and 120 minutes indicate the early release of D-glucose, D-galactose, D-galacturonic acid followed by D-mannose. These were identified by co-chromatography with authentic samples.

Partial acid hydrolysis followed by characterization of the product is often used in structural carbohydrate chemistry. The method is of particular value when a polymer contains a limited number of acid-labile glycosidic linkages, which may be cleaved without significant hydrolysis of the other glycosidic linkages. Such acid-labile linkages are generally associated with furanosidic sugars or deoxy sugars.[9]

TLC of the sugars and uronic acid was done on silica gel and cellulose with different solvent systems. Paper chromatography (p.c) was conducted using the descending technique on (31 ET Chr) Chromatographic paper and Whatman No.1 filter paper with the following solvent mixtures.

(A)	1- butanol/ethanol/water	5:1:4[10]
(B)	1- butanol/2-propanol/water	11:6:3[11]
(C)	ethyl actate/pyridine/water	10:4:3[12]
(D)	ethyl acetate/pyridine/water	2:1:2[13]
(E)	1-butanol/ethanol/water	31:11:9[14]

Detection of sugar spots was achieved by aniline–diphenylamine – H_3PO_4[15], anthrone and alkaline $AgNO_3$[16] and uronic acid with p-anisidine hydrochloride[17].

In recent years, classical methods of structure determination in carbohydrate chemistry have been supplemented by newer techniques.

The application of gas chromatography (g.c) to carbohydrate derivatives introduced a convenient method for the examination and small-scale separation of complex mixtures, such as those resulting from hydrolysis of polysaccharides.[18] Methyl ethers, acetates and trimethylsilyl ethers (TMSi) of carbohydrates have been shown to be amenable to this method of analysis. Trimethylisilyl ethers appear to be the most suitable, both with regard to their case of preparation and to the excellent separations which have been achieved. The successful use of TMSi derivatives in mass spectral investigations of hydroxy steroids[19-21] and the demonstrations of the utility of TMSi ethers in the investigation of complex carbohydrate-containing antibiotics [22-26] and nucleotides and related substances,[27] suggest that the technique might generally be applied to the field of carbohydrate chemistry. In addition, the requirement of submicrogram amounts of material for the determination of mass spectrum and the possibility of analysis with a gas chromatograph directly coupled to a mass spectrometer present obvious advantages for the study of trimethylsilyl ethers[28]. The exact mass measurement studies as well as deuterium labelling in TMSi groups have also been applied in many reported compounds to verify the results.

Mass spectroscopy plays a crucial role in the glycosyl composition and linkage analyses of polysaccharides-Most underivatized mono and oligo saccharides are thermally unstable and non-volatile and are therefore

unsuitable for mass spectrometric analyses. Eventhough the electron impact (EI) mass spectrum often fails to give the molecular ion of these compounds, it gives most valuable information on the structural features of the molecule from the major fragmentation patterns.

Analyses of carbohydrates by gas–liquid partition chromatography must be carried out with volatile derivatives of the substance. Extensive studies have been made of chromatographic behaviour of trimethylsilyl derivatives and excellent separations were obtained. The volatilities of the derivatives were generally sufficient for gas chromatography (g.c) under a wide variety of operating conditions. The reaction yielding a derivative must be rapid and quantitative with any carbohydrate, preferably it should be carried out at room temperature, and it must be suitable for use over a wide range of concentration of the starting material. Non polar derivatives are usually less reactive than polar ones, and are therefore preferred for quantitative determinations. For preparative gas chromatography, a further requirement is that the derivative will subsequently yield a quantitative recovery of free carbohydrate by a mild hydrolytic procedure. The TMSi derivatives of carbohydrates are virtually ideal in these respects, and therefore are probably the most satisfactory form for general analytical studies.[29]

When TMSi derivatives are made from free sugars, more than one product can be obtained, due to the presence of various anomeric and ring forms. Combined GC-MS is used to record the mass spectra of the components of such reaction mixtures. The combination of GC retention behaviour with mass spectral data provides a powerful tool for structural elucidation of carbohydrates on the submicrogram scale.

III. 2. Glycosyl composition analysis

The samples were freeze-dried prior to methanolysis. The polysaccharide was hydrolyzed using freshly prepared 1M methanolic - HCl–for 16 hours at 80^0 C. The released sugars were treated with 5ml of anhydrous pyridine (Reagent grade pyridine dried over KOH pellets), 1ml of hexamethyldisilazane (HMDS) and 1ml of trimethylchlorosilane (TMCS). The reaction was carried out in a stoppered tube. The mixture was warmed for 20 minutes at 75–85^0C. It was shaken vigorously for about 30 seconds and then allowed to stand for 5 minutes at room temperature. The solutions became cloudy on addition of trimethylchlorosilane owing to precipitation, presumably of ammonium chloride. No attempt was made to remove this, which in no way interfered with the subsequent gas chromatography. The resulting reaction mixture was used for injection into the gas chromatograph. The derivatized samples were analyzed by GC-MS using a SP 2330 supelco column. Myo-Inositol was also added as an internal standard.

III. 3. Analysis of Trimethylsilylated products.

The relative retention times of the products and their main fragments in the mass spectra are listed for Pg in Table I and Tc in Table II. Response factor and peak areas of Pg and Tc alongwith myo-inositol, used as internal standard, are given in Table III and Table IV. Table V shows the mole % of glycosyl residues of Pg and Tc.

TABLE I

Retention Times (min) on GC and Main Fragments in MS of Trimethylsilyl derivatives of **Pg**

Sugar	Retention Times	Main fragments (m/z)
Arabinose	11.26	57, 58, 59, 60, 61, 69, 71, 73, 74, 75, 76, 77, 88, 89, 98, 101, 103, 105, 115, 116, 117, 119, 129, 130, 131, 133, 134, 135, 142, 146, 147, 148, 149, 159, 189, 204, 205, 206, 217, 218, 219
	11.50	76, 89, 98, 101, 103, 116, 117, 129, 131, 133, 134, 142, 146, 147, 148, 149, 189, 204, 205, 217, 218, 219
Rhamnose	12.00	57,59,61,71,73,74,75,77,83,89,99,101,103,115,117,129,130,131,133,134,142,146,147,148,149,189,191,204,205,206,217,218
Xylose	13.78	59, 69, 71, 73, 74, 75, 89, 101, 102, 103, 116, 129, 131, 133, 147, 191, 204, 205, 217, 218
Galacturonic acid	16.56	53, 55, 57, 59, 60, 61, 71, 73, 74, 75, 76, 77, 82, 84, 89, 90, 91, 99, 101, 102, 103, 104, 105, 112, 114, 115, 117, 119, 129, 130, 131, 133, 134, 135, 143, 144, 145, 146, 147, 148, 149, 157, 159 ,160, 161, 163, 169, 171, 173, 175, 187, 189, 191, 217, 218, 219, 220, 227, 234, 235, 236, 243, 245, 246, 247, 248, 249, 261, 277, 278, 288, 319
	17.84	59, 60, 61, 70, 71, 73, 74, 75, 89, 90, 101, 103, 105, 116, 117, 129, 130, 131, 133, 134, 143, 145, 146, 147, 149, 157, 159, 160, 161, 169, 187, 217, 218, 219, 234, 245, 247, 261, 277
	20.05	53, 55, 57, 59, 60, 61, 71, 73, 74, 75, 76, 77, 82, 84, 89, 90, 91, 99, 101, 102, 103, 104, 105, 112, 114, 115, 117, 119, 129, 130, 131, 133, 134, 135, 143, 144, 145, 146, 147, 148, 149, 157, 159 ,160, 161, ,163, 169, 171, 173, 175, 187, 189, 191, 192, 199, 201, 204, 205, 206, 207, 208, 215, 217, 218, 219, 220, 227, 231, 234, 235, 236, 243, 245, 247, 248, 257, 259, 260, 261, 272, 274, 304, 318, 331, 332, 333
	20.36	57, 58, 59, 60, 61, 71, 73, 74, 75, 76, 83, 85, 89, 90, 91, 99, 101, 103, 105, 111, 113, 115, 116, 117, 119, 127, 129, 131, 132, 133, 134, 135, 143, 145, 146, 147, 148, 149, 159, 160, 161, 163, 169, 171, 175, 187, 189, 190, 191, 199, 204, 205, 206, 207, 208, 217, 218, 219, 234, 235, 247, 259, 279
Mannose	18.03	59,73,74,75,89, 01,103,115,116,117,129,131,133,134,146,147,148, 149, 157, 189, 191, 204, 205, 206, 217, 218, 231
Galactose	19.34	54, 59, 61, 69, 71, 73, 74, 75, 81, 89, 101, 103, 115, 116, 117, 129, 130, 131, 133, 134, 135, 143, 147, 148, 149, 159, 189, 191, 203, 204, 205, 206, 207, 217, 218, 219, 242
	20.52	59, 73, 74, 75, 89, 103, 117, 129, 131, 133, 134, 147, 148, 149, 191, 204, 205, 206, 217, 218
Glucose	21.44	52.5, 54, 55, 56, 57, 58, 59, 60, 61, 69, 71, 73, 74, 75, 76, 77, 81, 85, 87, 89, 90, 99, 101, 103, 104, 105, 111, 113, 115, 116, 117, 118, 119, 129, 130, 131, 133, 134, 135, 143, 146, 147, 148, 149, 155, 157, 159, 161, 163, 169, 189, 191, 192, 204, 205, 206, 207, 208, 217, 218, 219, 221, 231, 232, 233, 242, 247, 265, 279, 291, 305, 319
	22.3	52.5, 54, 55, 56, 57, 58, 59, 60, 61, 67, 71, 73, 74, 75, 76, 77, 81 ,85, 87, 89, 90, 99, 101, 102, 103, 104, 105, 111, 113, 115, 116, 117, 118, 119, 129, 130, 131, 133, 134, 135, 142, 143, 145, 146, 147, 148, 149, 155, 157, 159, 161, 163, 169, 171, 174, 176, 189, 190, 191, 192, 197, 204, 205, 206, 207, 217, 218, 219, 221, 231, 232, 242, 246, 265, 279, 291
Myo-Inositol	30.06	53, 55, 59, 73, 74, 75, 82, 84, 101, 102, 103, 104, 110, 129, 130, 131, 133, 134, 135, 142, 147, 148, 149, 191, 192, 193, 204, 205, 206, 217, 218, 219, 221, 265, 266, 291, 292, 305, 306, 307, 308, 318, 319, 320, 369, 392, 431, 432

TABLE II

Retention Times (min) on GC and Main fragments in MS of Trimethylsilyl derivatives of **Tc**

Sugar	Retention Times	Main fragments (m/z)
Arabinose	11.27	57, 58, 59, 60, 61, 69, 71, 73, 74, 75, 76, 77, 88, 89, 98, 101, 103, 105, 115, 116, 117, 119, 129, 130, 131, 133, 134, 135, 142, 146, 147, 148, 149, 159, 189, 204, 205, 206, 217, 218, 219
	11.50	76, 89, 98, 101, 103, 116, 117, 129, 131, 133, 134, 142, 146, 147, 148, 149, 189, 204, 205, 217, 218, 219
Rhamnose	12.00	57, 59, 61, 71, 73, 74, 75, 77, 83, 89, 99, 101, 103, 115, 117, 129, 130, 131, 133, 134, 142, 146, 147, 148, 149, 189, 191, 204, 205, 206, 217, 218
Xylose	13.78	59, 69, 71, 73, 74, 75, 89, 101, 102, 103, 116, 129, 131, 133, 147, 191, 204, 205, 217, 218
	14.36	59, 69, 71, 73, 74, 75, 89, 101, 102, 103, 116, 129, 131, 133, 147, 191, 204, 205, 217, 218
Mannose	18.03	59, 73, 74, 75, 89, 101, 103, 115, 116, 117, 129, 131, 133, 134, 146, 147, 148, 149, 157, 189, 191, 204, 205, 206, 217, 218, 231
Galactose	19.37	54, 59, 61, 69, 71, 73, 74, 75, 81, 89, 101, 103, 115, 116, 117, 129, 130, 131, 133, 134, 135, 143, 147, 148, 149, 159, 189, 191, 203, 204, 205, 206, 207, 217, 218, 219, 242
Glucose	21.67	52.5, 54, 55, 56, 57, 58, 59, 60, 61, 69, 71, 73, 74, 75, 76, 77, 81, 85, 87, 89, 90, 99, 101, 103, 104, 105, 111, 113, 115, 116, 117, 118, 119, 129, 130, 131, 133, 134, 135, 143, 146, 147, 148, 149, 155, 157, 159, 161, 163, 169, 189, 191, 192, 204, 205, 206, 207, 208, 217, 218, 219, 221, 231, 232, 233, 242, 247, 265, 279, 291, 305, 319
	22.62	52.5, 54, 55, 56, 57, 58, 59, 60, 61, 67, 71, 73, 74, 75, 76, 77, 81 ,85, 87, 89, 90, 99, 101, 102, 103, 104, 105, 111, 113, 115, 116, 117, 118, 119, 129, 130, 131, 133, 134, 135, 142, 143, 145, 146, 147, 148, 149, 155, 157, 159, 161, 163, 169, 171, 174, 176, 189, 190, 191, 192, 197, 204, 205, 206, 207, 217, 218, 219, 221, 231, 232, 242, 246, 265, 279, 291
Myo-Inositol	30.06	53, 55, 59, 73, 74, 75, 82, 84, 101, 102, 103, 104, 110, 129, 130, 131, 133, 134, 135, 142, 147, 148, 149, 191, 192, 193, 204, 205, 206, 217, 218, 219, 221, 265, 266, 291, 292, 305, 306, 307, 308, 318, 319, 320, 369, 392, 431, 432

Table III
Trimethylsilyl derivatives of Pg

RES (peak)	Response Factor	PEAK AREA	Weight (μg)	nmol CHO / mg Sample
Ara		1359		
Ara		595		
	0.476895324		3.99.97502	44.31463138
Rha		805		
Rha				
	0.444900041		1.762426536	17.8890229
Xyl		273		
Xyl				
	0.669726642		0.397047677	4.408701725
Gal UA		3186		
Gal UA		1251		
Gal UA		9261		
Gal UA		2672		
	0.576703386		27.64863954	237.4088918
Man		1017		
	0.62878024		1.575431977	14.57114296
Gal		1462		
Gal		658		
Gal				
Gal				
	0.74630763		2.766913407	25.59113399
Glc		16504		
Glc		6684		
	0.864628336		26.12230019	241.6047002
μg INOS =	20	20533		
mg Sample=	0.6			
mg CHO =	0.064263735			
% CHO =	10.71062251			

16

Table IV
Trimethylsilyl derivatives of Tc

RES (peak)	Response Factor	PEAK AREA	Weight (μg)	nmol CHO / mg Sample
Ara		813		
Ara		366		
	0.476895324		2.786565191	37.1294459
Rha		802		
Rha				
	0.444900041		2.031844134	24.74840601
Xyl		1802		
Xyl		.784		
	0.669726642		4.352205683	57.99074861
Gal UA				
Gal UA				
Gal UA				
Gal UA				
	0.576703386		0	0
Man		822		
	0.62878024		1.473504272	16.35409847
Gal		1134		
Gal				
Gal				
Gal				
	0.74630763		1.7126698	19.00854384
Glc		16560		
Glc		113991		
	0.864628336		369.641042	4102.564284
ug INOS =	20	17744		
mg Sample=	0.5			
mg CHO =	0.381997831			
% CHO =	76.39956621			

17

Table V

Glycosyl residues	Mole %	
	Pg	Tc
Arabinose	5.4	0.5
Rhamnose	1.5	0.2
Xylose	0.5	0.8
Galacturonic acid	33.5	---
Mannose	1.7	0.2
Galactose	4.6	0.3
Glucose	52.8	98.0

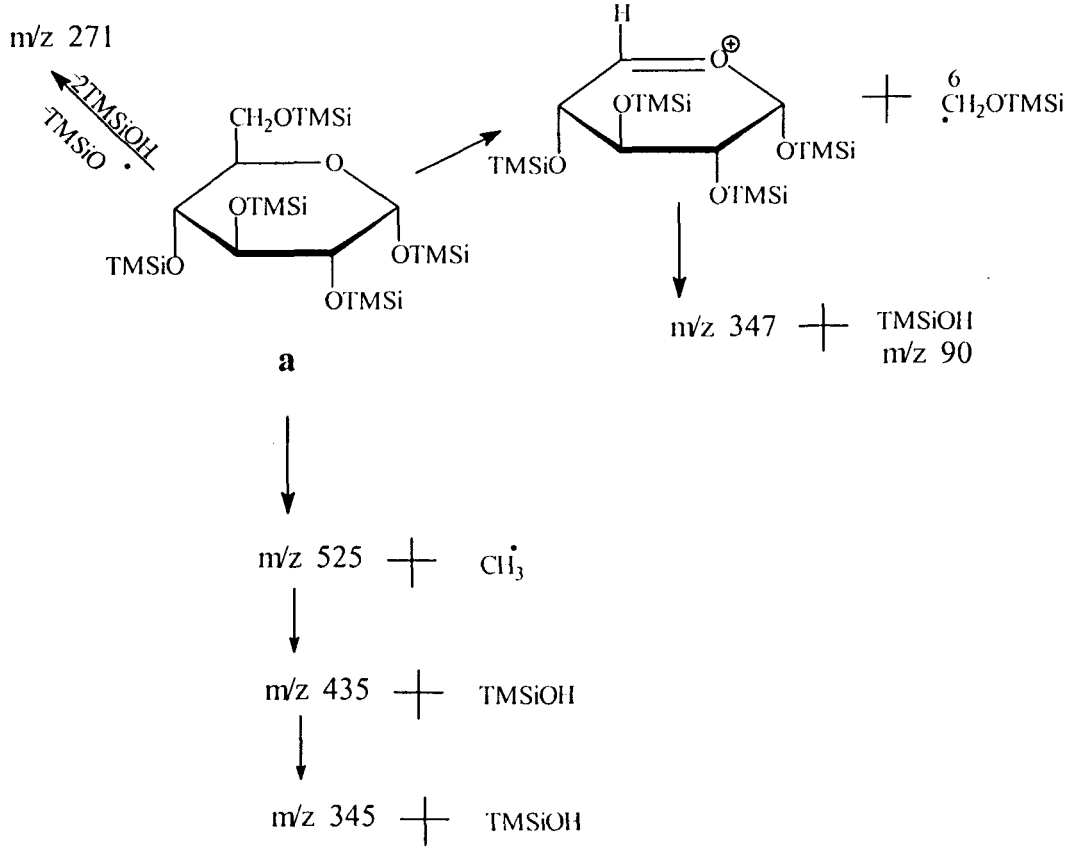
III. 4. Structural assignments for the ions

Mass spectrometry has been widely applied in the field of carbohydrate research.[30] The mass spectra of trimethylsilylated glycosides have been investigated in great detail try using high resolution mass spectrometer. The molecular ion was weak, but stronger fragments appeared at $(M-CH_3)^+$ and $(M-CH_3 - Me_3 SiOH)^+$. The major fragmentation pathways were analogous to those of permethylated glycosides.[30a] It was found that the spectra contained additional fragmentation series due to losses of a methyl or trimethyl silyloxy ($Me_3 SiO$) group from anion radicals. The relative intensities of molecular ion peaks are very low. Generally, a high sample pressure has to be maintained in the ion source, if a molecular ion is to be seen.

"M–15" ion fragments by a stepwise elimination of two molecules of trimethylsilanol (TMSiOH). A trimethylsilyloxy radical and trimethylsilanol are also lost from the molecualr ion. Scheme I shows the possible fragmentation of penta–O–trimethylsilyl–α–D–glucopyranose (**a**) – similar to penta–O–trimethylsilyl–α–D–galactopyranose and penta–O–trimethylsilyl–α–D–mannopyranose. A series of low intensity peaks results following the loss of C–6 and its substituent followed by TMSiOH. Fragments retaining one, two, three and four carbon atoms of the ring are

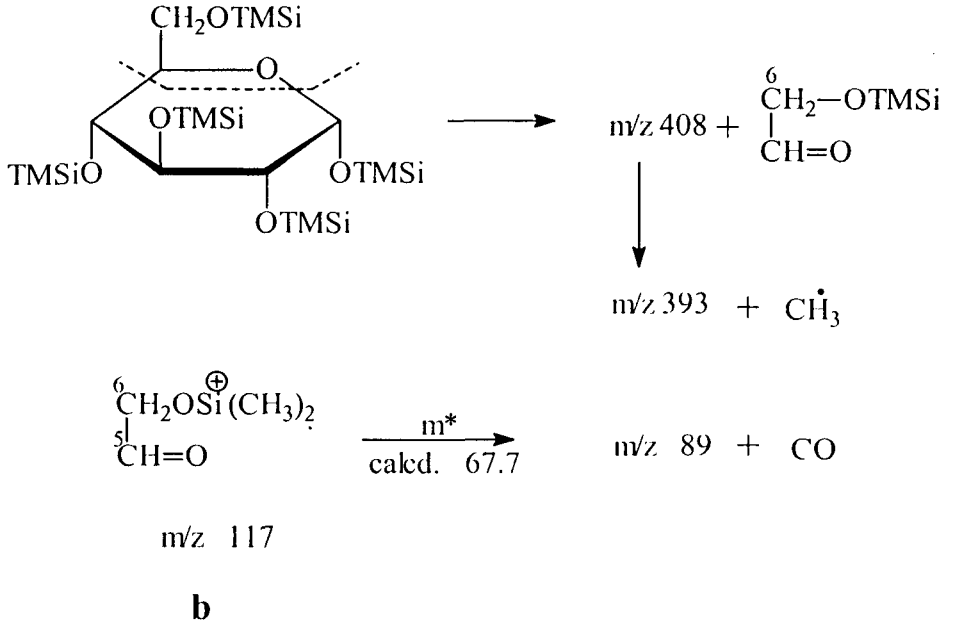
prevalent in the mass spectrum of **a**.

Scheme I



A peak at m/z 408 is possible due to the cleavage of the molecule into a fragment retaining C–1 through C–4. A further loss of $CH_3\cdot$ corresponds to a peak at m/z 393. Scheme II illustrates which part of the molecule is eliminated in the formation of m/z 408.

Scheme II

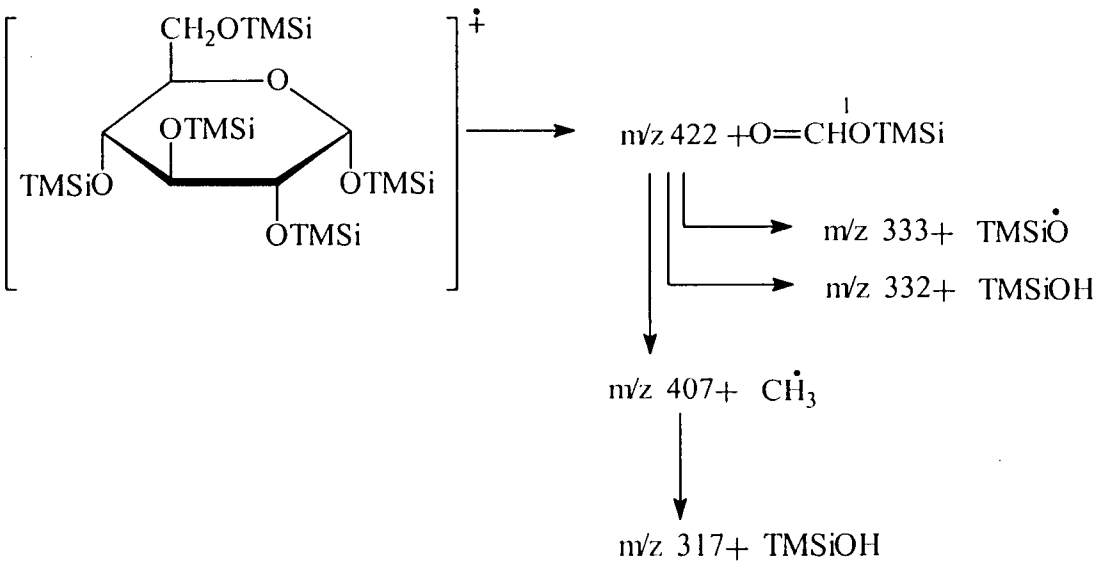


b

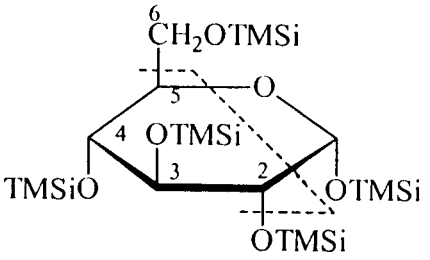
A fragment retaining C–5, C–6 and the ring oxygen and losing C–1 through C–4 is found at m/z 117. Carbon monoxide is subsequently expelled from m/z 117, a transition supported by a metastable peak.

The ring also fragments by elimination of C–1 and the ring oxygen. Structure **c** illustrates the part of the molecular ion which is lost. In addition, peaks are present for prior or subsequent eliminations of $CH_3\cdot$ and of TMSiO· and TMSiOH. (Scheme III)

Scheme III

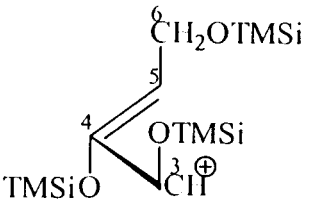


The fragment at m/z 319 retains four carbon atoms of the glucose molecule and three TMSiO groups. Structures **c** and **d** summarize two possibilities for this peak. Also, the peak at m/z 319 might result from a fragmentation pathway involving loss of TMSiO· from m/z 408 **e**. Labeling data in similar cases do indicate that C–1 and C–6 are not present. Thus **c** is the major contributor to m/z 319.



m/z 319
C 2 — C 5

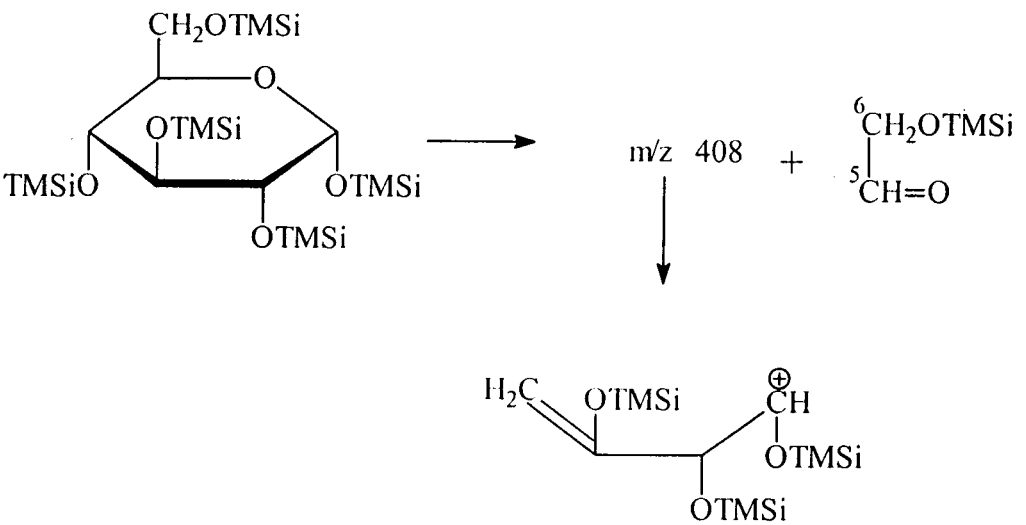
c



m/z 319
C 3 — C 6

d

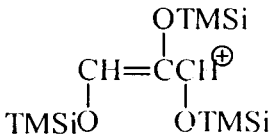
Scheme IV



m/z 319
C 1 — C 4

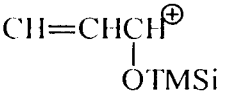
e

Fragments at m/z 305 and 217 are common from trimethylsilyl derivatives of carbohydrates.[31, 32]. These peaks retain C–2, C–3 and C–4 mainly. However, their general occurrence suggests that they can be formed from other portions of the molecule, sometimes with rearrangement



m/z 305

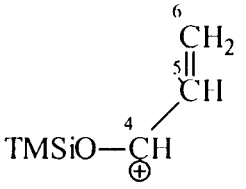
f



m/z 217

g

The formation of m/z 129 may be due to two different fragmentation pathways. [High resolution measurements on m/z 129 show that it is a doublet, approximately two-thirds of which is a $C_6H_{13}OSi$ ion and the remaining $C_5H_9O_2Si$ ion]. The presence of C–6 and four of the hydrogens of the glucose molecule can be deduced for this fragment. Structure **h** represents the part of the original molecule which comprises this ion.

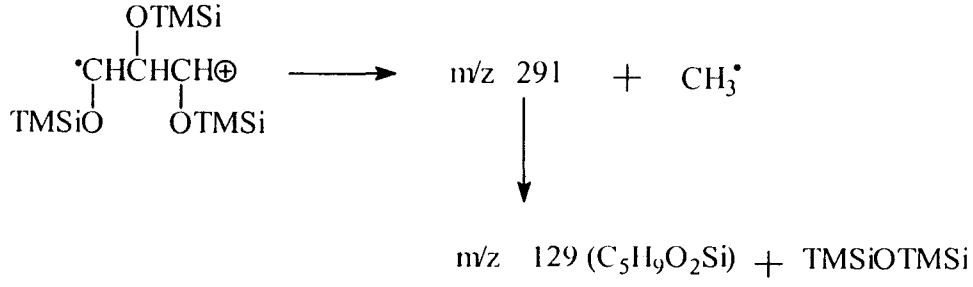


m/z 129 ($C_6H_{13}OSi$)

h

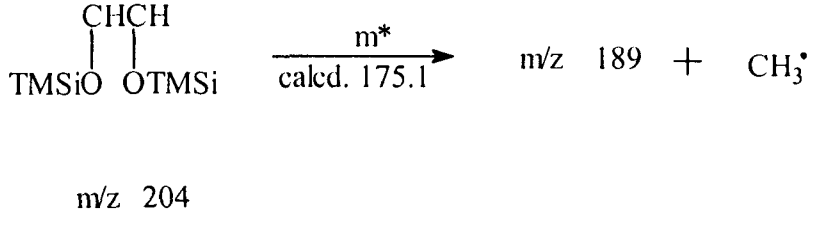
Other pathway is from an isotope of m/z 305. i.e. m/z 306 (Scheme V)

Scheme V



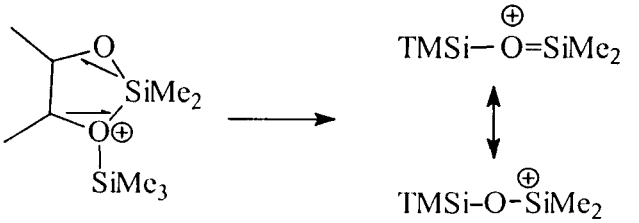
No metastable peaks were found for these transitions and no other important pathways were found to involve elimination of TMSiOTMSi. Therefore this assignment is tenuous.

The two-carbon fragment, m/z 204, is a common characteristic fragment from TMSi ethers of carbohydrates[33]. It originates, for the most part, from C-2 – C-3 and C-3 – C-4. A metastable peak is present for its further fragmentation to m/z 189.



Rearrangements are common in the mass spectra of trimethylsilyl ethers of carbohydrates–a factor which must be taken into account when interpreting their spectra. The fragment m/z 147 is expelled by way of a

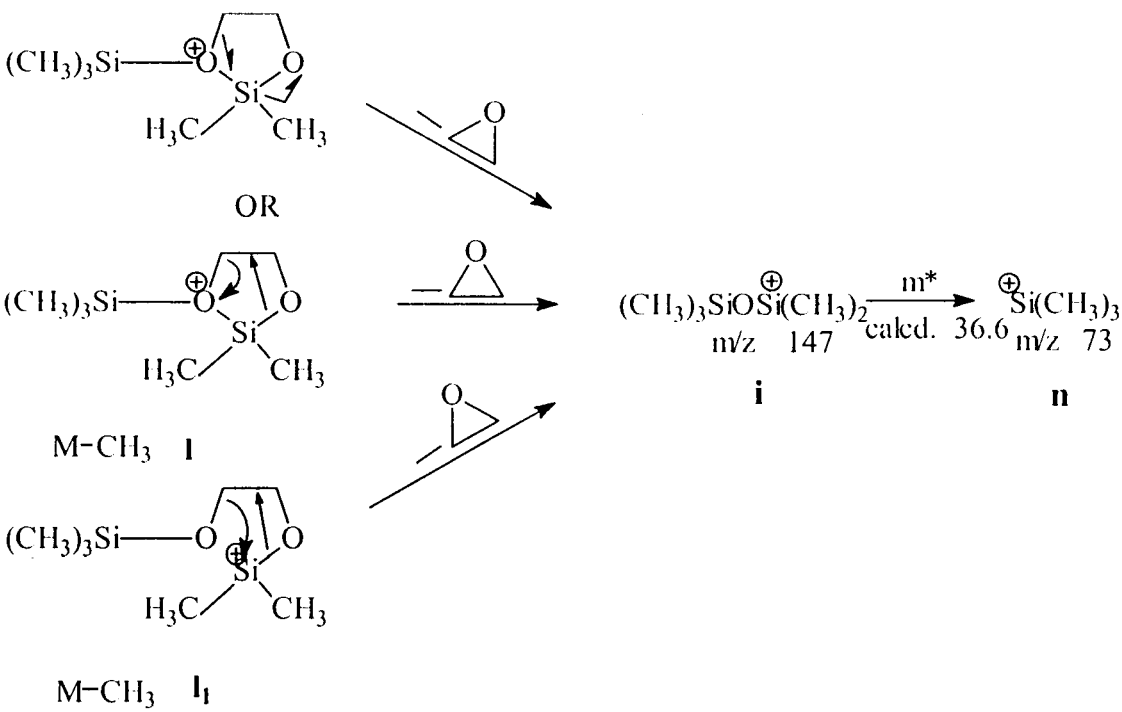
cyclic intermediate[34]. This ion is present in the mass spectra of all TMSi derivatives of carbohydrates. **i** seems to be the most plausible representation.



i

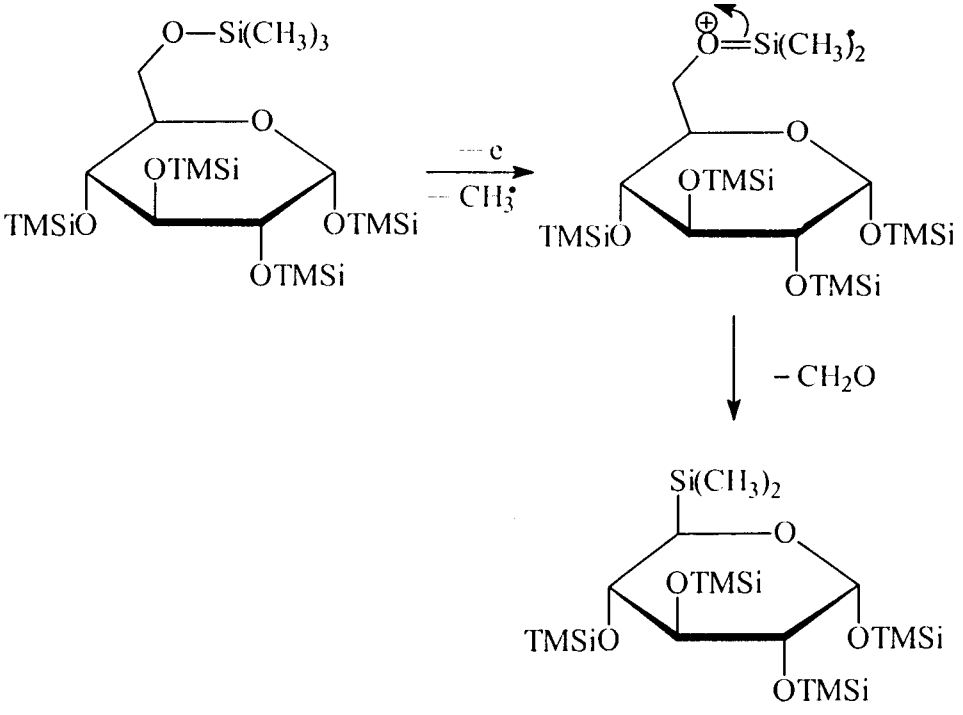
In view of the results presented by J.Dickman *et al*[34], the m/z 147 ion is formed by collapse of the cyclic oxonium ion form of an M–CH_3, **l** and $\mathbf{l}_1$, precursor with elimination of the elements of propylene oxide. (Scheme VI). It was reported by the same authors that in every case of Scheme VI, a metastable ion was observed for the formation of **i** from an (M–CH_3) progenitor, and it seemed likely that the silicon–oxygen bond was already formed in the precursor. Also a 1,4 shift in $\mathbf{l}_1$ seems as likely as the formation and collapse of **l**.

Scheme VI



Several electron-impact induced skeletal rearrangements in which sequential loss of a methyl radical and of formaldehyde is possible (Scheme VII).

Scheme VII

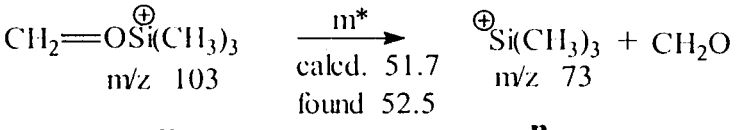


Another fragment which is formed via a rearrangement is found at m/z 191. It is composed of two TMSiO groups and one C–H of the glucose molecule. [About 90% of m/z 191 retains C–1. One of these TMSiO groups originates from C–3 and is rearranged to C–1][30]. A metastable peak will be present for the decomposition of m/z 191 to 73

$$(CH_3)_3SiO\overset{1}{C}H{=}\overset{\oplus}{O}Si(CH_3)_3 \xrightarrow[\text{calcd. } 27.9]{m^*} (CH_3)_3SiOCH{=}O + \overset{\oplus}{S}i(CH_3)_3$$

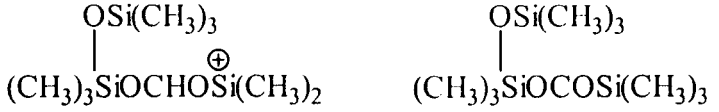
m/z 73

The peak at m/z 103 retains one carbon atom of the glucose molecule. This fragment could arise directly by cleavage of C-5 – C-6 with charge retention on C–6. Another possibility is by rearrangement of a hydrogen atom to one of the carbons of the ring. A metastable peak indicates that m/z 73 arises by elimination of formaldehyde from m/z 103.[35]



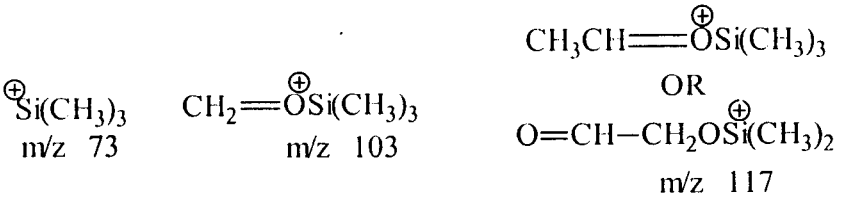
m **n**

Possible structures for another set of fragments having m/z 265 and m/z 279 formed by rearrangement are given as **j** and **k**. Both contain only one carbon atom of the sugar.



j **k**

The most intense peak occurs at m/z 73 **n** and the α–cleavage fragments **m** and **b** are also of appreciable abundance.

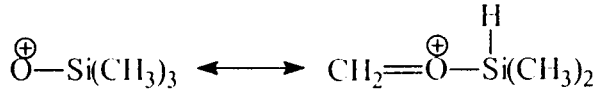


n **m** **b**

Metastable peaks at m/z 51.7 (calcd. $\frac{73^2}{103}$ =51.7) and 36.6 (calcd $\frac{73^2}{147}$ =36.6) provide evidence for the genesis of **n** m/z 73 from the α-cleavage ion **m** m/z 103 and the rearrangement ion **i** m/z 147. Another common abundant peak in the mass spectra occurs at m/z 89. This fragment could be depicted either in terms of **p** or **q**.

m/z 89

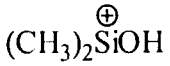
p



m/z 89

q

In all cases a moderately strong peak at m/z 75 for **r** is seen

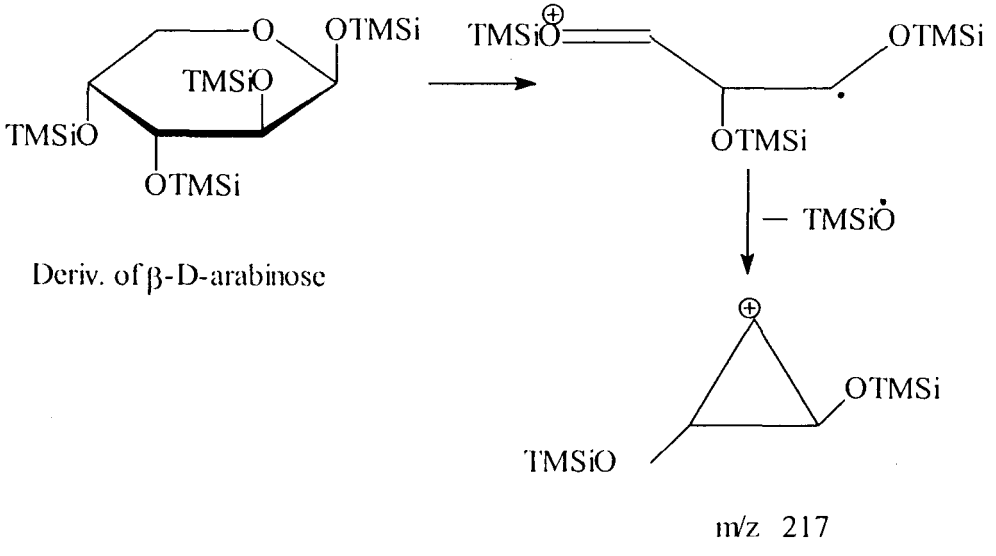


m/z 75

r

The per (trimethylsilyl)ated D-arabinose and L-xylose give practically identical mass spectra, and the fragments obtained originate by the same pathways as above. The abundance of m/z 217 can be explained by Scheme VIII

Scheme VIII



III. 5. The composition of equilibrium mixtures

It has been shown by gas-liquid partition chromatography that two major components result from trimethylsilylation of D-glucose and these have been assigned to α and β anomers by comparison of their retention times with those of the TMSi ethers of pure α and β-D-glucose. The mass spectra of penta–0-trimethylsilyl-α and β-D- glucopyranoses are identical[30]. The compositions of the equilibrium anomers calculated on the basis of the retention times and relative areas of the peaks are given in Table VI.[36] This agreed well with the known compositions of the equilibrium solutions established by optical rotations. It is generally agreed that aqueous equilibrium solutions of sugars consist almost entirely of α and β anomers with at most small amounts of "γ" sugars (probably free aldehydes or furanose modifications).

Table VI
The composition of Equilibrium mixtures
as determined by Gas chromatography of Trimethylsilyl derivatives.

	α	β	γ
Arabinose	50.8	43.8	5.4
Rhamnose	-	-	-
Xylose	41.3	55.2	3.4
Galacturonic acid	-	-	-
Mannose	72.0	28.0	
Galactose	31.9	62.6	5.4
Glucose	39.8	60.2	

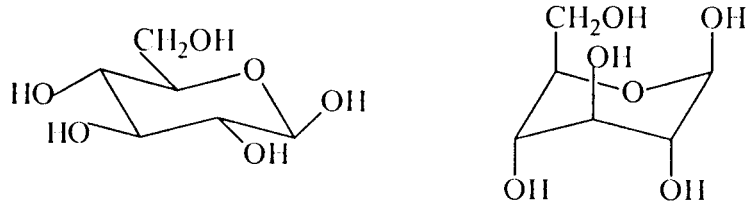
It was proposed by Bishop *et al* that "for any methylglycopyranoside, the anomer in which the C–1 methoxy group exists in *trans* relationship with the C–2 substituent, will have the lower retention volume except when the C–2 OH is unsubstituted, in which case the order is reversed."[37] But Sweeley *et al* suggested that this generalization cannot be extended to tetratrimethysilyl ethers of the pentoses nor to the pentatrimethylsilylethers of the hexoses.[38] For example, in glucose and galactose, the *trans* relationship is found in the slower moving β-anomers. Furthermore, the generalization does not hold true with the TMSi derivatives of the methyl glycosides. They revealed another possible generalization for the TMSi derivatives. "When the normal conformation of the sugar is one of the chair forms, $^4C_1$ or $^1C_4$ (Reeves notation C1 or 1C), and when there is no evidence of conformational instability, that anomer in which the anomeric OH group is equatorial has the longest retention time". For the following D-sugars of C_1 conformation, the β-anomer moves more slowly on polar and non polar columns; glucose, galactose mannose and xylose. Similarly, for D-arabinose of $^1C_4$ conformation (Reeves notation 1C), the α-anomer with the equatorial OH group has the longer retention time. For equatorial anomeric ether of arabinose, there is little difference in relative retention times[39]. It has a single axial OH group at C–4. This generalization is also true for both polar and non polar columns.

Similarly with the hexose sugars nearly identical retention times are observed with β-mannose and β- galactose, with one axial OH group at C–2 and C–4, respectively (relative retention times are 1.06 and 1.08).

In reality, the open-chain configuration of aldoses and ketoses exist only as minute quantities in solution. The main forms are the cyclic pyranosides and furanosides derived from the hypothetical "acetal" of the open-chain form by dehydration. This "hemiacetal" formation provides a new chiral centre at C-1, forming two additional diastereoisomers, the α- and β-form of such aldoses. In the Haworth projection of the structures, the hydroxyl group at C-1 of the D-sugars is below the pyranoside or furanoside plane in the α-form and above in the β-form. The configuration of these pyranosides and furanosides is not planar. The preferred structure of the pyranose compounds is the chair conformation which also exists in two different forms due to the positions of the single hydroxyl groups, these being either axial or equatorial according to their thermodynamic stabilities.[39a]

Molecules with the C-4 carbon in the upper position of the chair conformer are named as having $^{4}C_{1}$ conformation (C1 in Reeves notation), whilst molecules with the C-4 carbon in the lower position of the conformer are said to have $^{1}C_{4}$ conformation (1C in Reeves notation) as shown in the

case of β-D-Glucopyranose (fig X). For most pyranoses, the $^4C_1$ conformation is preferred due to the lower interaction between the single hydroxyl groups.



Conformation of β-D-Glucopyranose

Figure X

Reeves used the terms C1 and 1C to define the ring shapes, but it can be shown that the enantiomeric (D-and L-) forms of a sugar in the same conformation (that is, with the same axial-equatorial arrangement) have different symbols, and must therefore always be linked to the absolute configuration of the sugar. Therefore, β-D-glucopyranose in the $^4C_1$ (D) conformation has all its substituents in equatorial positions, at the greatest distance from other substituents; hence a possible reason why D-glucose is a very common monosaccharide in nature – it has a preferred conformational structure containing little strain. Hexoses can, under certain conditions exist in rings other than pyranose. The next most common ring is the furanose ring which is adopted by some hexoses and pentoses. This

ring is only slightly puckered and exists in two forms, the more common envelope (E) [fig. Y] and the less common twist (T) [fig. Z]. For the envelope forms, the conformation is defined by the one atom that is above, or below, the plane described by the other atoms. Thus in fig. Y, the conformation is ^{3}E since C-3 is above the plane described by C-1, C-2, C-4 and O-4.

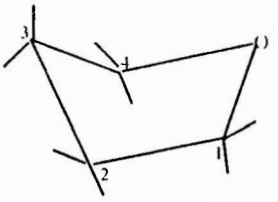


Figure Y

The defined plane for the twist conformation is described by C-1, C-4 and O-4 and the conformation of fig Z is therefore $^{3}T_{2}$

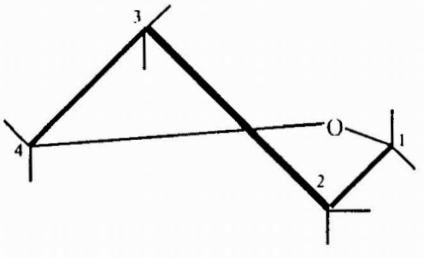
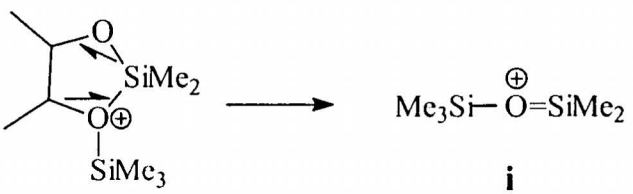


Figure Z

In order to investigate the stereochemical differences in the monosaccharides, Computer–aided Mass spectrometric Identification of stereoisomeric monosaccharides, is also being used.[40] The mass spectra

of the trimethylsilyl (TMSi) derivatives of the aldohexopyranoses, α–D–glucose, α–D–galactose, β-D-glucose, β-D- mannose and α-L- mannose turned out to be very similar, as might be expected on account of the uniform carbon skeleton. For stereoisomeric monosaccharides, a close investigation of the peak intensities shows minor but definite reprodicible differences.

Another approach to obtaining stereochemical information was suggested[41]. The fragment m/z 147 **i** is expelled from anomeric forms of per–O–trimethylsilyl derivatives of D–glucopyranose, D-galactopyranose, and D–mannopyranose, by way of a cyclic intermediate. The relative intensities of this ion could be predicted on stereochemical grounds.



i

III. 6. Glycosyl linkage analysis

Complex carbohydrates are often subjected to different chemical modifications in order to gain information on sequences and anomeric configurations. Methylation analysis has proved to be valuable for following the results of such modifications. A manual on methylation analysis, giving relative retention times on Gas chromatography of partially methylated alditol

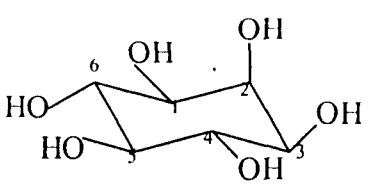
acetates, as well as a collection of computer--drawn bar graph mass spectra of these derivatives has been published.[42] A minor modification in the procedure is that the time for the acetylation of the partially methylated alditols with pyridine–acetic anhydride is extended from 10 minutes to 3 hours. The reason for this is that the secondary hydroxyl groups in some derivatives, e.g., 2,3,5,6–tetra–O–methyl–D–galactitol, was only partially acetylated during the shorter reaction time. Analyses by GC–MS, of partially methylated alditol actates are then performed. More information is obtained when the methylated polysaccharide is subjected to partial hydrolysis, reduced with a deuterated reagent, acetylated and the mixture analyzed by GC–MS[43].

The samples were methylated using the NaOH / MeI method[44]. To a solution of the carbohydrate sample (4–5 mg) in Me_2SO (0.3–0.4 mL) were added finely powdered NaOH (20 mg) and methyl iodide. (0.1 mL). Each mixture was stirred (100 rpm) for 6 minutes in a closed vial at 25°C. Water (1mL) and chloroform (1mL) were then added, and the chloroform layer was washed with water (3 × 10 mL) and dried (Na_2SO_4).

The method is characterized by short reaction times, high yields and clean gas chromatograms. It is proved to be simple and rapid for the permethylation of carbohydrates, and is superior over procedures described

by Hakomori[45], Lindberg[46] and Finne[47] *et al.* In the Hakomori method, the $CH_3SOCH_2^-$ anion, generated from methyl sulphoxide and sodium hydride, is considered to be the effective basic agent[48]. In spite of the low yields (0.3 mol of permethylated derivative per mol of sugar) the Hakomori method has been used extensively in structural investigations of carbohydrates. Potassium tertiarybutoxide when used instead of NaH improved the stability of the reagent but did not substantially increase the yield of permethylated product.[49]

The methylated samples were hydrolyzed in 2M TFA at 121^{0}C for 2 hours and the hydrolyzed carbohydrate was reduced with Sodiumborodeuteride at room temperature. The product was acetylated using acetic anhydride at 120^{0}C for 3 hours. The derivatized samples were analyzed by GC–MS using SP 2330 supelco column. Internal standard (myo-inositol) was added (25mg) to each sample prior to the reduction step.



myo-Inositol

The polysaccharide (Pg) was also methylated under different conditions in order to obtain the galacturonic acid linkage. The sample

was pre-reduced with superdeuteride for 3 hours at room temperature prior to the 2M TFA hydrolysis and reduction step.

The polysaccharide (Tc) was difficult to dissolve in dimethyl sulphoxide. The first attempt of methylation analysis of the sample produced about 60% undermethylation which results from some of the sample not dissolving well in the first stage of permethylation of the carbohydrate. Undermethylation is very common in large polymers.

The derivatized samples were subjected to GC–MS analysis using Sp 2330 Supelco column. Myo-Inositol was added as internal standard.

The recorded mass spectra were compared with the reference spectra in the mass spectral database of the associated computers using the probability based matching (PBM) search algorithm supplied by the manufacturer. The response factors, relative to the internal standard, myo-inositol, are determined empirically by injecting the standards and determining the peak areas for each sugar derivative. Peak areas give the percentage of different carbohydrate residues in Pg and Tc; [Table VII and Table VIII].

Table VII
Percentage of carbohydrate residues in Pg

Carbohydrate Residues	% present
Terminal-rhamnose	3.4
Terminal-arabinose (furanose)	7.2
Terminal-arabinose	0.8
Terminal-xylose	1.7
2-arabinose (furanose)	1.3
Terminal mannose	1.7
Terminal-glucose	31.6
Terminal-galactose	2.1
4-arabinose or 5-arabinose (furanose)	6.0
4-xylose	2.2
3-glucose	3.4
2-mannose	0.6
2-glucose	2.5
3-galactose	1.5
6-glucose	6.0
4-galactose	2.0
4-glucose	21.7
4,6-glucose	3.0
3,6-galactose	1.3

The sample (Pg) also contained galacturonic acid, that was tested in a separate experiment. The galacturonic acid was 4–galacturonic acid.

Table VIII
Percentage of carbohydrate residues in Tc

Carbohydrate residues	% present
Terminal-glucose	8.4
4-xylose	2.3
4-glucose	79.2
4,6-glucose	7.0
2,3,4,6-glucose	3.1

This polysaccharide (Tc) seems to be a polymer of 4-glucose with about 8% terminal glucose showing the 1-glucose linkage.

The mass spectra of the peracetyl derivatives of the pyranose and furanose ring forms of monosaccharides have been systematically investigated and a detailed interpretation of their fragmentation processes presented.[50] Mass spectrometry is sensitive to structural differences such as ring size and hexoses v/s pentoses v/s deoxy hexoses, whereas epimers and anomers exhibit very similar mass spectra except for minor relative-intensity differences.

In the method of forming alditolacetate derivatives of the glycosyl residues of the polysaccharide, each glycosyl component affords a single derivative, producing simple chromatograms that are easily interpreted. This method will not detect glycosyluronic acids unless the carboxyl groups of the glycosyluronic acids have been reduced.[51] These residues are subject to elimination reactions during the methylation procedure. In general, it is best to reduce glycosyluronic acids to the corresponding 6,6–dideuterio–glycosyl residues before methylation analysis. This has two advantages : it minimizes β-elimination reactions and allows glycosyluronic acid residues to be detected as PMAA.

III. 7. Identification and Quantitation of PMAA

The positions of O-acetyl and O–methyl groups on the PMAA are determined by GC–MS. GC–MS analysis is performed with a fused–

silica, 30 meter (0.25 mm i.d) capillary column (Supelco SP 2330) in a splitless mode. The following temperature program is used. Two minutes at an initial temperature of program is used: Two minutes at an initial temperature of 80^0, increased to 170^0 at 30^0/minute, then to 240^0 at 4^0 / minute, and held for 5 minutes at 240°.

The electron impact fragmentation patterns of the mass spectra of the PMAA are well known.[52] Some of the rules that can be used to determine the position of O-methyl and O-acetyl groups are reviewed here. These rules are illustrated for 1, 5–di–O–acetyl–1–deuterio– 2,3,4,6–tetra–O–methyl glucitol and 1,3,5–tri–O–acetyl–1–deuterio–2,4,6–tri–O–methyl glucitol. Fig **s**.

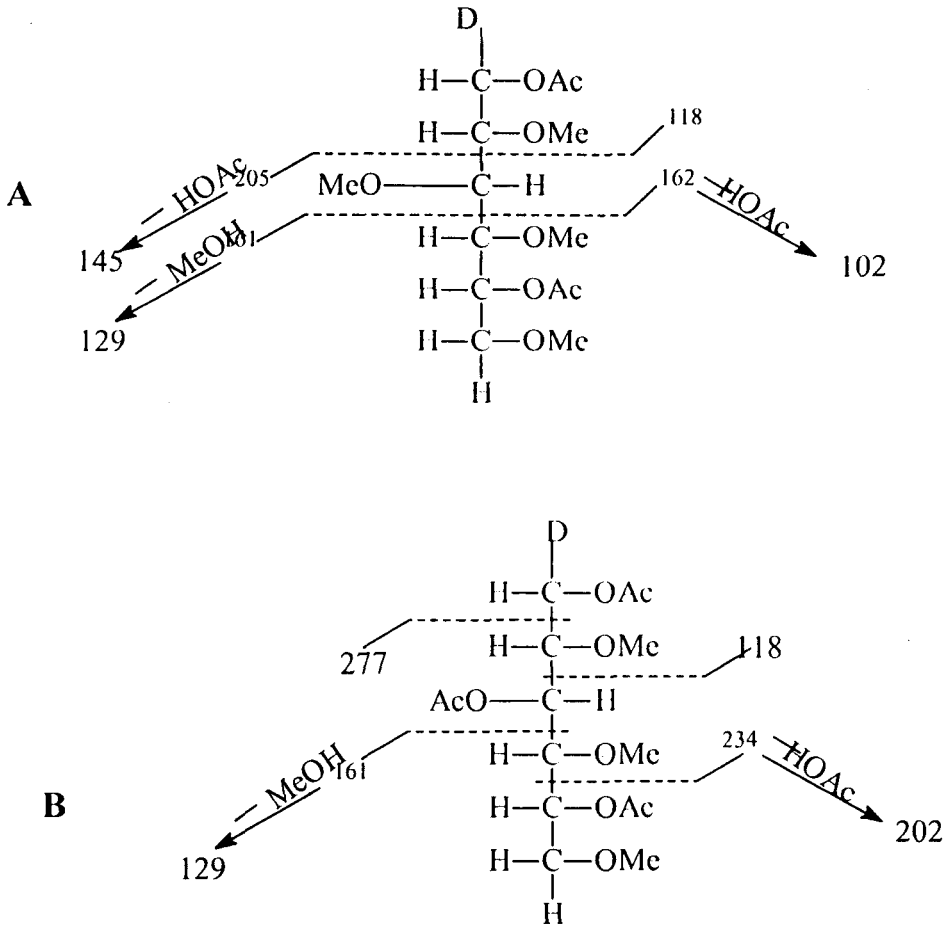


Figure s

Fragmentation of 1,5–di–O–acetyl–1–deuterio–2,3,4,6–tetra–O–methyl glucitol **A** and 1,3,5–tri–O–acetyl–1–deuterio–2, 4, 6–tri–O–methyl glucitol **B** during EI mass spectrometry.

Rule 1: Primary fragments are formed by cleavage of the alditol backbone.

Rule 2: The charge always resides on the fragment with a methoxy–bearing carbon atom adjacent to the cleavage point.

Rule 3: Fragmentation between two adjacent methoxy–bearing carbon atoms is favoured over fragmentation between a methoxy–bearing carbon atom and an acetoxy–bearing carbon atom, which itself is highly favoured over fragmentation between two acetoxy bearing carbon atoms.

Rule 4: Secondary fragment ions are produced by the loss of methanol or acetic acid. The loss of the substituent on the carbon β to the carbon bearing the charge is strongly preferred [See fig **s**].

Rule 5: When the PMAA are labeled at C–1 with a deuterium atom, the (nominal) charge–to–mass ratio (m/z) of a fragment ion that contains C–1 is even, whereas m/z of a fragment ion that does not contain C–1 is odd.

These fragmentation rules allow the rearrangement of the O-acetyl and O–methyl groups of most of the common PMAA to be readily determined. Two derivatives (1,2,3,4,–tetra–O–acetyl–1–deuterio–5–O–methyl pentitol and 1,2,3,4,5–penta–O–acetyl–1–deuterio–6–O methyl hexitol) however afford electron–impact mass spectra that are not readily interpreted by these rules. The spectra are illustrated in Fig. (**t**).

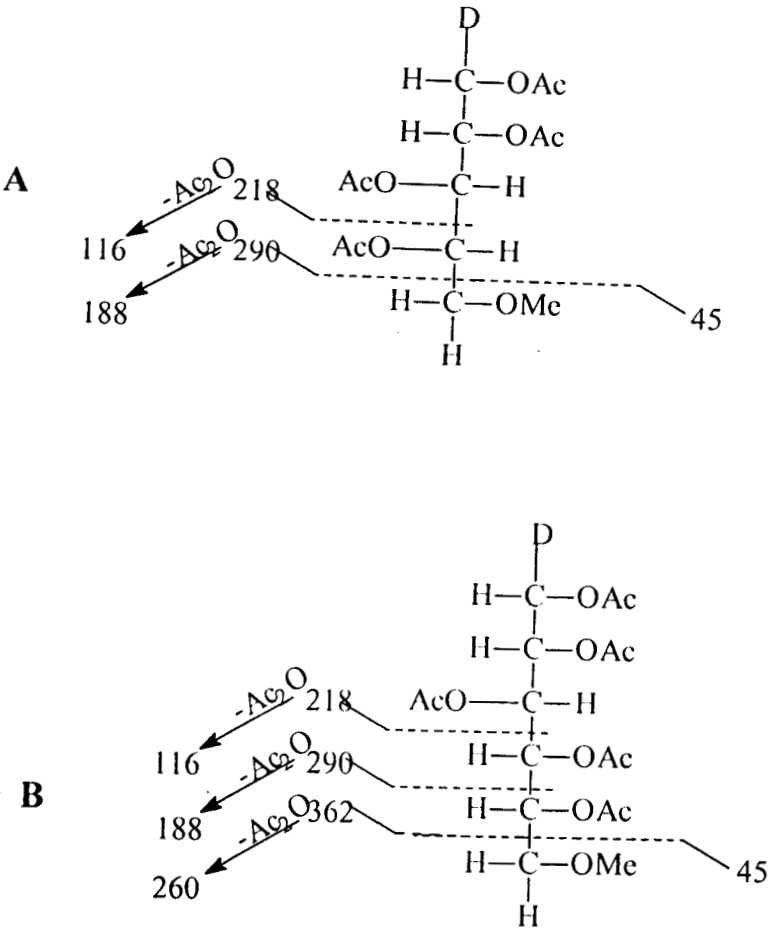


Figure t

Fragmentation of 1,2,3,4–tetra–O–acetyl–1–ducterio–5–O–methyl arabitol **A** and 1,2,3,4,5,–penta–O–acetyl–1–deuterio–6–O methyl glucitol **B** during EI–MS.

Different stereoisomers of PMAA (e.g: glucitol v/s galactitol) that have the same arrangement of O–acetyl and O–methyl groups give

indistinguishable electron-impact mass spectra. However, such stereoisomers can be distinguished. The most common method to determine the stereochemistry of the PMAA is to compare its GC retention time with the retention times of standard compounds. The positions of O-acetyl and O–methyl groups can be determined by GC–MS, and since a pure glycoside was used as starting material, the GC retention time of each PMAA is unambiguously established. The retention times of PMAA prepared from Pg and Tc are compared to the retention times of appropriate standard PMAA.

GC analysis is used both to identify and to quantitate PMAA. The peak areas corresponding to each of the PMAA are divided by the appropriate response factor and the resulting quotients are normalized to 100%. Response factors are calculated by the effective carbon–response method as decribed.[53] See Table IX and Table X.

Table IX

Pg

RES (peak)	Retention time (min.)	Peak area
Terminal-rhamnose	10.79	2691
Terminal-arabinose (furanose)	11.19	4833
Terminal-arabinose	12.16	3079
Terminal-xylose	12.39	28794
2-arabinose (furanose)	13.65	8680
Terminal mannose	13.81	3777
Terminal-glucose	13.96	159442
Terminal-galactose	14.78	2124
4-arabinose or 5-arabinose (furanose)	15.3	5821
4-xylose	16.09	5539
3-glucose	16.77	27804
2-mannose	16.9	4084
2-glucose	17.06	3671
3-galactose	17.35	4725
6-glucose	17.95	5165
4-galactose	18.08	4902
4-glucose	18.39	16739
4,6-glucose	21.80	4734
3,6-galactose	22.25	3672
Myo-Inositol	28.33	33408

Table X
Tc

RES (peak)	Retention time (min.)	Response factor	Peak area
Terminal glucose	13.93	0.8	9951
4-xylose	16.03	0.7	2769
4-glucose	18.45	0.8	93282
4,6-glucose	21.81	0.8	8274
2,3,4,6-glucose	26.8	0.8	3399
Myo-Inositol	28.35		32359

A summary of the response factors used for the common derivatives resulting from methylation analysis of polysaccharides is presented in Table XI.

Table XI
Response Factors for PMAA commonly produced during analysis of polysaccharide

Type of derivative	Number of O-acetyl groups*				
	2	3	4	5	6
Pentitol	0.60	0.66	0.7	0.75	
Hexitol	0.70	0.74	0.8	0.84	0.89
Deoxyhexitol	0.70	0.75	0.79	0.84	

* The remaining positions of the alditols are substituted with O-methyl groups. The arrangement of the O-methyl and O-acetyl groups has such a small effect on the response factors that it need not be considered. Thus, for example, the Response factor for all hexitols containing two O-acetyl groups is 0.7, regardless of the arrangement of O-acetyl and O-methyl groups.

The carbohydrate analyses presented here (colourimetric analyses, glycosyl composition analyses and glycosyl-linkage composition analyses) are sufficient to determine the identity and purity of plant polysaccharides. Clearly these analyses are not sufficient to determine the complete primary structure of most polysaccharides. More detailed structural characterizations of polysaccharides such as glycosyl–residue sequencing is possible by means of other techniques,[54,55] combined liquid chromatography–mass spectrometry[56] and nuclear magnetic resumence (NMR) spectroscopy.[57]

III. 8. Biological studies

Carbohydrates from Pg and Tc were found to inhibit cell proliferation of cultured peripheral blood lymphocytes and the human leukaemic cell line K_{562}. It acted at the early stages of M phase (prophase) and prevented most of the cells from further progression in division. This resulted in several morphological changes in prophase. Lymphocyte proliferation studies (Blast transformation) were carried out in human lymphocyte culture, using polysaccharides from Pg and Tc, and both gave encouraging results. See Table XI a and XI b.

Table XI a
Percentage of Blast transformation in human lymphocyte culture using **Pg** polysaccharide.

	Sample	Lymphocyte	Blast
Cultured (72 hrs).	Control	78	22
	Trial-1	38	62
	Trial-2	41	52

Table XI b
Percentage of Blast transformation in human lymphocyte culture using **Tc** polysaccharide.

	Sample	Lymphocyte	Blast
Cultured (72 hrs).	Control	76	24
	Trial-1	16	84
	Trial-2	18	82

The mode of action of the polysaccharide on cell proliferation could be better understood from cytological investigations on the bone marrow preparations of Swiss inbred mice which were given intraperitoneal injection of three different concentrations of the polysaccharide. The results are summarized in Table XII. The carbohydrate from Pg caused cell division arrest mostly at prophase.

Mitotic index, percentage of aberrations and percentage of aberrations in cells undergoing mitosis in the normal bone marrow and that from animals treated with various concentrations of the polysaccharide are given in Tables XIII and XIV.

Table XII

Effects of the polysaccharide from Pg on mice bonemarrow

Drug concentration	Normal interphase	Abnormal interphase	Normal prophase	Abnormal prophase	Normal metaphase	Abnormal metaphase	Normal anaphase	Abnormal anaphase	Normal telophase	Abnormal telophase
Normal control	200	10	300	-	80	-	20	-	390	-
1 μg/ml	140	130	40	650	-	30	-	10	-	-
10 μg/ml	120	180	-	690	-	10	-	-	-	-
100 μg/ml	110	185	-	705	-	-	-	-	-	-

Table XIII

Effect of Pg polysaccharide on mice bone marrow

Sample	Concentration μg/ml	Percentage of aberration
Pg Polysaccharide	1	82.0
	10	88.0
	100	89.0

(% Aberration in the control was taken as 1).

Table XIV

Effect of Pg polysaccharide on mitotic index and aberrations on mice bone marrow

Drug concentration	Mitotic index	% of aberrations	%of aberrations in cells undergoing mitosis
Normal controls	79	1	-
1 μg / ml	73	82	69
10 μg / ml	67.5	88	70
100 μg / ml	67	89	70.5

Polysaccharide from Pg lowered mitotic index in treated bone marrow. Also there was a high incidence of mitotic aberrations. Stickiness of chromosomes at prophase, metaphase and anaphase leading to pycnotic irregular nuclei was also observed. [Plate III Fig 3-6 and Plate IV Fig 1-3].

Plate III

3. Bonemarrow preparation after treatment with 100 µg/ml *Punica granatum* polysaccharide. Mitotic arrest even at G_2 resulting in the picnosis of chromatic segments within the nucleus.

4. A bonemarrow preparation showing mitotic arrest at various stages resulting in picnotic irregular nucleus with a prominent central vacoule (arrow) is also seen in the picture – 100 µg/ml *Punica granatum* polysaccharide

5. Mitotic arrest after chromosome condensation resulting in picnotic irregular nuclei – 100 µg/ml *Punica granatum* polysaccharide

6. Chromosome stickiness at metaphase leading to rosette like formation. The chromosome number appears to be less. Picnotic nuclei were also seen in the same figure – 10 µg/ml *Punica granatum* polysaccharide.

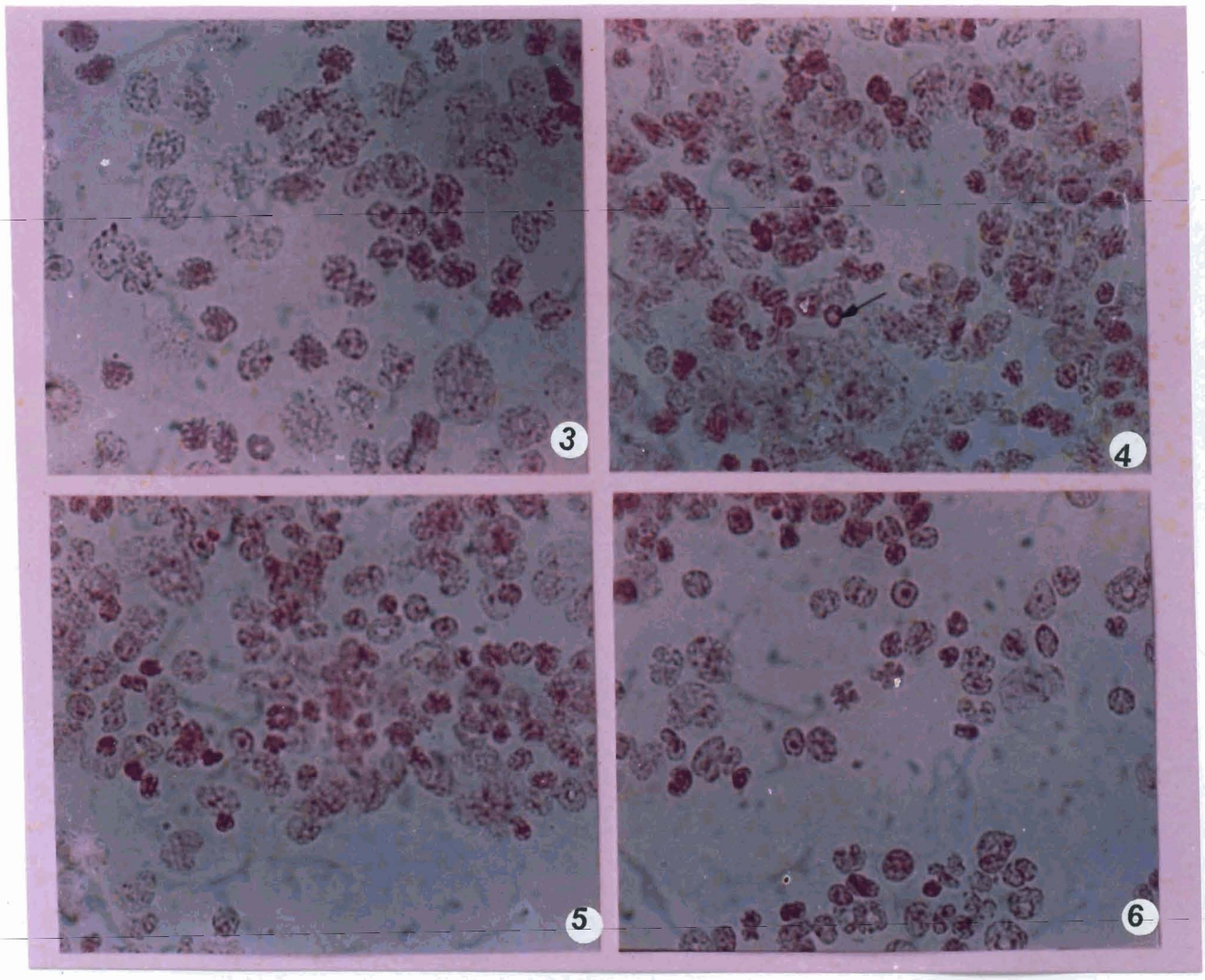

3
4
5
6

Plate IV

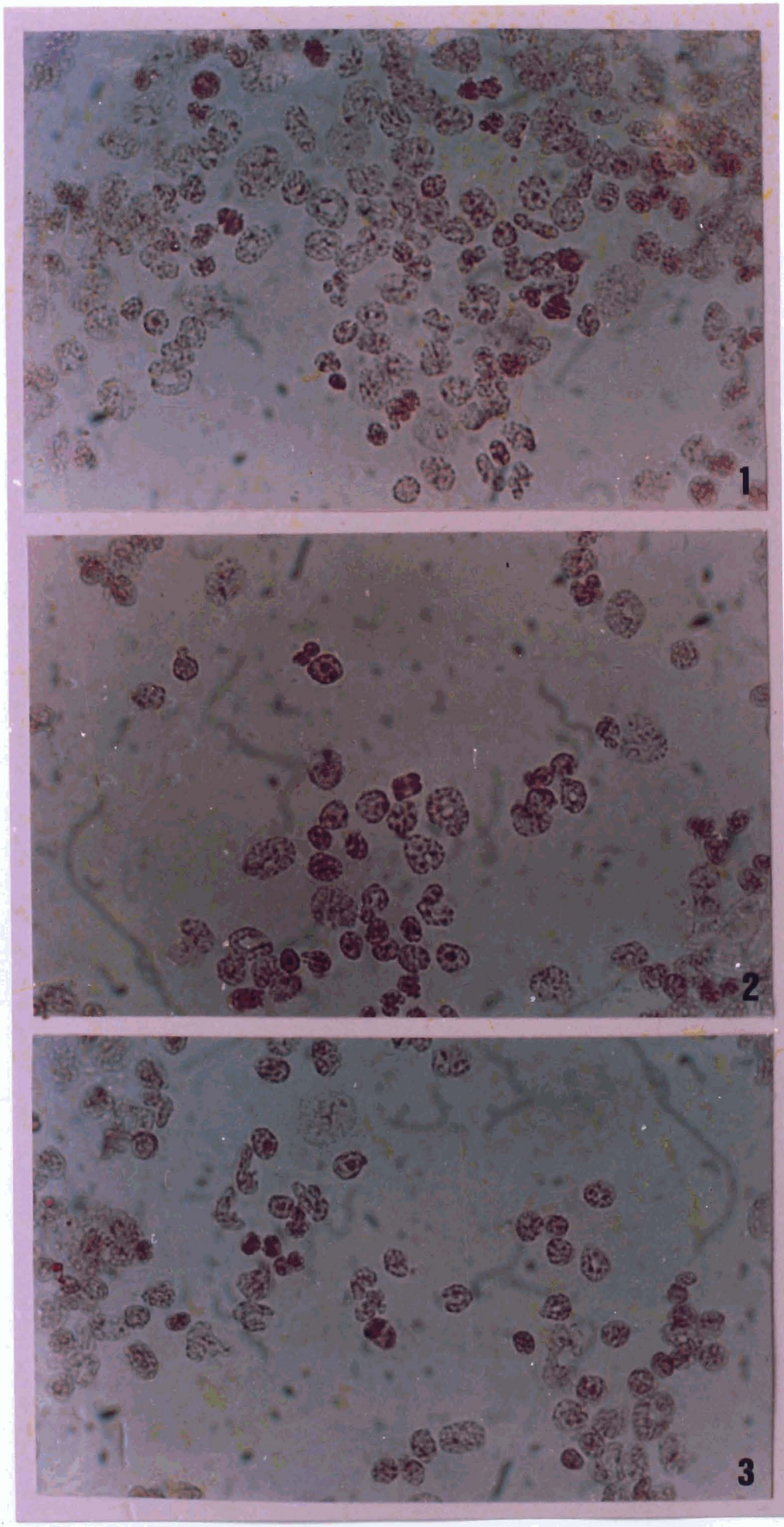
Bonemarrow preparations after treatment with 10 μg / ml *Punica granatum* polysaccharide

1. Mitotic arrest at prophase, metaphase and anaphase leading to picnotic irregular nuclei.

2. A bonemarrow cell at anaphase with stickness

3. Arrested anaphases, three neighbouring cells

85A



22

Administration of very low quantity of the polysaccharide of Pg in normal human leukocytes and also human leukaemic cell line K_{562} in culture caused in the inhibition of cell proliferation, as described in the results.[58] Also, it was found that the polysaccharide from Pg blocked the cell division even from very early prophase.

Results from the cytological investigations on the bone marrow samples in Swiss inbred mice, which where given intraperitoneal injection with three different concentrations. The study revealed the mode of action of the polysaccharide. The mitotic index was lowered with increase in drug concentration. The percentage of mitotic arrest and mitotic abnormalities increased with increasing concentrations.

In the case of cultured normal leukocytes and leukaemic cell line K_{562}, the polysaccharide from Pg blocked cell division at prophase. Clumping and stickiness of chromosome at prophase and metaphase was a salient feature of treated bone marrow cells. Structural aberrations of chromosomes and micronuclei were observed only rarely. These effects qualify the polysaccharide to be in the group of antimitotic substances described by several authors.[59,60] Nagl has expressed the view that endoreduplication, endomitosis and C–mitosis occur as a result of blockage to the cell cycle at G_2, early prophase and metaphase respectively.[61]

Blockage of the cell cycle at G_2 phase results in the lengthening of the synthetic phase (S-phase) giving more chance for extra replication of chromatin which results in endoreduplication.

It is to be noted that the polysaccharide is also capable of causing stickiness of chromosome, spindle abnormalities and arrest of cell cycle at anaphase or early telophase stages. Deysson stated that several antimitotic substances share these properties.[60]

In the light of the present results, it will be desirable to subject these polysaccharides to further drug screening programmes. These polysaccharides are not toxic to animals or human beings.

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CHAPTER - IV

EXPERIMENTAL

EXPERIMENTAL

IV. 1. Plant materials

The plant materials used in this investigation were collected from Malappuram district of Kerala, India and were authenticated by Dr.A.K.Pradeep, of Botany department, University of Calicut where voucher specimens have been deposited. The fruit rind of *Punica granatum* and stem pieces of *Tinospora cordifolia* were shade dried and powdered, 500g each of the powdered sample were taken for extraction.

IV. 2. Methods

All the chemicals were purchased from E.Merck, Sigma Chemicals Company, Pharmacia Fine Chemicals, Qualigen, BDH, Kasei Kogyo Co. Ltd.(Tokyo) and Pierce Chemical Company (USA)

Names and structures of the sugars are given according to IUPAC rules of Carbohydrate nomenclature. (1997).[1]

(a) GC of sugar derivatives was carried out using a Varian 3700 instrument (30 W,0.25 mm/30 m) with a WCOT OV 225 capillary column (Durabond fused–silica column) and a temperature programme: 175° - 205° (1°/min) 205° isothermal (15 minutes)[2]

(b) GC/MS analyses were performed with Quadrex and Hewlett-Packard model. GLC-MS HP 5970 equipped with HP 9825 B-100% methyl silicon-and a printer HP 2671 G, with a fused-silica, 30 meter (0.25 mm i.d) capillary column (Supelco Sp 2330) in a splitless mode. The following temperature programme is used: Two minutes at an initial temperature of 80°, increased to 170° at 30°/ min, then to 240° at 4° /min. and held for 5 min at 240°– scanning every 2 seconds for 5 minutes and every 1 second for 40 minutes (50-650) and every 1 second for 40 minutes (30-600). The response factors, relative to the internal standard, myo-inositol are determined empirically by injecting the standards and determining the peak areas for each sugar derivative.

The recorded mass spectra were compared with the reference spectra in the mass spectral data base of the associated computers using the probability based matching (PBM) search algorithm supplied by the manufacturer.

(c) Lyophilization was carried out using SAVANT (USA) RVT 400 speed vac. SC 110 concentrator and freeze drier.

(d) Ultracentrifugation was performed on analytical centrifuge, KUBOTA KR 20000T (Japan) machine with RA-2 12 ml×12 tubes at 10°C.

(e) Ion-exchange resin chromatography was carried out using a column of Dowex 50-X4 200-400 mesh (H+ form) coupled to a column of Dowex 1-X8 200-400 mesh (formate form). The Dowex 1-formate resin was prepared from the chloride form of the resin by passage of 3N sodium formate through it until a negative test for chloride is obtained, followed by extensive wash with distilled water.

(f) Column chromatographic separation of the crude and semipurified extracts were carried out using Silica gel (60, Merck 230-400 mesh). The column was prepared as a slurry with hexane or chloroform and eluted with selected chromatographic solvents.

(g) The thin layer chromatographic analysis was performed on 1mm coated microscope slides, 5 × 20 cm, and 10 × 20 cm. Adsorbents for TLC were Silica gel G (Merck) and Cellulose powder (Sigma). Slurries were prepared in the medium, chloroform-methanol (2:1, v/v) in the proportion 35 g/100 ml with Silica gel G, and in the medium, chloroform-methanol(50:50, v/v) in the proportion 50 g/100 ml with Cellulose powder.

(h) Paper chromatography was carried out using the descending technique on 31 ET Chr 460 × 570 Chromatographic paper and Whatman No. 1 filter paper.

(i) Elution was done with the following solvent mixtures.

(A) 1-butanol/ethanol/water	5 : 1 : 4
(B) 1-butanol/2-propanol/water	11 : 6 : 3
(C) ethyl acetate/pyridine/water	10 : 4 : 3
(D) ethyl acetate /pyridine/water	2 : 1 : 2
(E) 1-butanol/ethanol/water	31 : 11 : 9
(F) 1-butanol/acetic acid/water	3 : 1 : 2

(j) Detection of sugar spots was achieved by the following reagents;

(A) Phenol –Sulphuric acid

Reagent: 3 g of phenol + 5 ml of concentrated sulphuric acid in 95 ml of ethanol

Procedure: Spray and heat 10 to 15 minutes at 110^0. Spots may be intensified by additional heating.

Carbohydrates show brown spots.

(B). Aniline phosphate

Reagent : Add 20 ml of aniline to 200 ml of water , then add 180 ml of acetic acid followed by 10 ml of phosphoric acid. Store at 4^0.

Procedure: Dilute 2 parts of the reagent with 3 parts of acetone for spraying. Heat plate at 100^0 for 2 to 5 min.

Pentoses show red-brown, aldoses yellow or yellow brown.

(C) Aniline phthalate

Reagent: 0.93 g of aniline + 1.66g of phthalic acid in 100ml of moist butanol

Procedure: Spray, then heat for 10 minutes at105^0.

(D) Diphenylamine-aniline- phosphoric acid

Reagent : Mix 4 g diphenylamine,4 ml aniline and 20 ml syrupy (80%) ortho phosphoric acid in 200 ml acetone. This is to be prepared as required and used fresh. If kept at 0^0, the reagent appears to be satisfactory for 1-2 weeks.

Procedure: Spray, then heat at 80^0 for 4 minutes. Grey-brown spots for D-glucose, D-galactose, D-mannose. Bright red-brown for D-galacturonic acid and orange spot for L-rhamnose.[3]

(E) Anthrone

Reagent: (i) 10% sulphuric acid

(ii) 1% (w/v) anthrone in benzene

Procedure: Spray with (i) followed by (ii). Heat at 100^0 for 10 minutes. Colours are not stable and disappear on cooling. Reappear on reheating.

(F) p-Methoxy benzaldehyde

Reagent: 1 ml of p-methoxy benzaldehyde and 1ml of sulphuric acid in 18 ml of ethanol.

Procedure : Spray reagent and heat plate to 110^0.

Sugars show green, blue and violet spots in 10 min.

(G) Naphthoresorcinol

Reagent: 200 mg naphthoresorcinol in 100ml of ethanol + 10 ml of phosphoric acid.

Procedure: After spraying , heat plates to 110^0 for 5 to 10 min

(H) Anisidine phthalate

Reagent : 0.1 M solution of p-anisidine and phthalic acid in 96% ethanol.

Hexoses show green, pentoses red-violet and uronic acids brown.[4]

(k) Gel filtration chromatography

In GFC, the stationary phase is solvent-swollen hydrophilic gel in the form of porous beads; and mobile phase is an aquous solvent. The liquid outside the beads (the mobile phase) and that inside the beads is the same except that the liquid inside is immobilized. Small molecules penetrate the gel particles and are retarded and eluted later than large molecules which will move only in interstitial volume.

Sephadex G-200 (Pharmacia Fine Chemicals) was used for gel filtration in aquous media. It was allowed to swell in water for 3 days at room temperature. Ultrogel AcA-44 (LKB) is also used.

(l) Phosphate buffer (0.025 M)

3.40 g of KH_2PO_4 and 3.55 g of Na_2HPO_4 (dried for 2 hours at 110- 130^0) were dissolved in CO_2 free water and diluted to 1 litre. The solution is stable when protected from undue exposure to the atmosphere.

pH	6.88	at 20^0
pH	6.86	at 25^0
pH	6.85	at 30^0

Phosphate buffered saline (PBS)

2.56 g $NaH_2PO_4.2H_2O$, 11.94 g Na_2HPO_4 and 87.66 g NaCl were dissolved in CO_2 free water and diluted to 1 liter.

pH	7.2 - 7.4	at 25^0

IV. 3. Isolation of polysaccharides

The powdered plant material (500 g) was extracted with petroleum ether (boiling point 60-80^0) at room temperature for 72 hours (800 ml × 3) followed by methanol, (800 ml × 3) filtered and dried. This was then suspended in cold water (2000 ml) and kept

overnight with occasional stirring. After filtration, the filtrate was concentrated (500 ml) using a rotory flash evaporator at 50^0 under reduced pressure. To this 95% ethanol (1500 ml) was added slowly and stirred for 6 hours. It was then allowed to stand at 4^0 for 24 hours; the precipitate was collected by ultracentrifugation (20,000 rpm at 10^0 for 15 min). The supernatant liquid was removed and the residue obtained was dissolved in a minimum quantity of distilled water (300 ml). This solution was again precipitated by treating with 95% ethanol (1000ml) and the precipitated polysaccharide was collected by ultracentrifugation at 20,000 rpm. The process was repeated 3 times and the residue was collected and dissolved in minimum quantity of distilled water (200 ml) followed by dialysis against distilled water for 48 hours at several changes of water. The collected polysaccharide was dried over fused calcium chloride under reduced pressure. The desiccated polysaccharide was dissolved in water and shaken well with chloroform (100ml) in a separating funnel, the denatured protein in the form of gel which collected at water/chloroform interphase, was removed.[5,6] The procedure was repeated four times to get the polysaccharide free from protein, when the water/chloroform interphase became clear. To facilitate the denaturation a pH 4-5 buffer was used instead of water and small quantities of 1-butanol was added. The clear aquous layer was again treated with 95% ethanol (500 ml) to precipitate the polysaccharide. This was then collected by ultracentrifugation (20,000 rpm at 10^0 for

15 min) and dissolved in water (200 ml), dialyzed against distilled water for 48 hours in the cold; freeze-dried using a Savant Lyophilizer for 6 hours. The yield of polysaccharide from each plant material was as follows:

Punica granatum 900 mg

Tinospora cordifolia 1300 mg

IV. 4. Purification of polysaccharides

The polysaccharides were purified by gel filtration chromatography using Sephadex G-200 and Ultrogel AcA-44 (LKB), 0.001 M Phosphate buffered saline (PBS) was used as the eluent. 500 mg lyophilized crude sample was suspended in the buffer and chromatographed through a column of Sephadex G-200 (2.5 cm × 75 cm) equilibrated with the buffer. 3 ml fractions were collected in test tubes and monitored by the absorbance of the eluent at 490nm using phenol-sulphuric acid. For Pg saccharide, a single peak was obtained for fractions 4 to 36, by plotting absorbance as abscisa and fraction number as ordinate. This was pooled and concentrated under vacuum (10 ml). The concentrate thus obtained was again chromatographed using Ultrogel Ac A-44 column (2.5 cm × 75 cm) and PBS as eluent. This process also gave a single peak. The pooled fraction containing the polysaccharide was lyophilized and stored at 4^0 for analyses (yield 400 mg)

In the case of Tc polysaccharide, two peaks were obtained, a major peak for fractions 12 to 30 and a minor one for fractions 38 to 42, with an area ratio 5:1 in the chromatography on Sephadex G-200 column (2.5 × 75 cm). The major peak fractions were pooled and concentrated under vacuum (10 ml) and were then subjected Ultrogel AcA–44 column chromatography (2.5 cm × 75 cm) to yield only a single peak. The volume under the peak was collected and lyophilized and stored at 4^0 for analyses (yield 300 mg).

IV. 5. Zone electrophoresis

To test the homogeneity of the purified polysaccharides, sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed.[7] A 10% gel at pH 7.2 was used.

For the preparation of 10% gel : Working solution (a) contained 30 g acrylamide and 0.735 g bis –acrylamide. (b) 0.05 M phosphate buffer pH 7.5 and (c) ammonium persulphate 10 mg/ ml

Pipette out 2 ml (a) and 3.5 ml (b) into a conical flask. 50 µl TEMED was added to the conical flask followed by 4.5 ml (c). Immediately after the addition of ammonium persulphate, gel is transferred to the tube. A constant and rapid flow must be required. Over that a little distilled water is added to avoid miniscus formation of gel. Kept 45 min at room temperature. After removing the upper layer of water, wells were cut using the appropriate comb.

Samples were prepared in buffer containing 1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and kept at 100^0 for 3-4 minutes before application. The preparation of the polysaccharide samples were then applied to the gel and electrophoresis was carried out at 3 mA current per gel the bromophenol blue marker the other end of the gel. The gel was then treated with a solution of isopropanol, acetic acid, water (25:10:65) (2 times, 1 hour each) in bromosilicate tubes to fix the polyacrylamide. This was then placed in a solution of thymol (0.2 % w/v) for 3 hours, decanted and drained to remove the solution completely. A solution of concentrated sulphuric acid- absolute alcohol (80: 20 v/v 10 ml) was added to the tube and shaken for about 3 hours at 35^0 until the opalescence disappeared. Zones containing polysaccharides stained red, with an yellow colour for the rest of the gel portion. Single bands were obtained for the two polysaccharides.

IV. 6. Molecular weight estimation

Average molecular weights of the purified polysacharide samples were estimated from gel filtration chromatographic experiments using Sephadex G-200 column (2 cm × 75 cm). It was eluted with 0.001M PBS at a flow rate of 20 ml per hour . A series of dextrans 10, 20, 40, 70 and T- 500 (Pharmacia Fine Chemicals) was used as reference standard and molecular weights were estimated from the linear correlation between the logarithm of the molecular

weights of the standards and the ratios of their elution volumes to the void volume of the column[8,9].

The following molecular weights were obtained

Punica granatum	(Pg)	110,000 D
Tinospora cordifolia	(Tc)	40,000 D

IV. 7. Colourimetric method

2 ml of sugar solution containing between 10 and 70 μg is pipetted into a colourimetric tube, and 1 ml of 5% phenol in water is added. Then 5 ml concentrated H_2SO_4 is added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to get good mixing. The tubes are allowed to stand 10 minutes, then they are shaken and placed for 10 to 20 min in a water bath at 25^0–30^0 before readings are taken. The colour is stable for several hours. The abosorbance of characteristic yellow orange colour is measured at 490 nm for hexoses and 480nm for pentoses and uronic acid. Blanks are prepared by substituting distilled water for sugar solution. The amount of sugar are then determined by reference to the standard curve previously drawn for the particular sugar under examination.[10] The percentage of sugar in each of the sample was estimated as 10% and 76 % for Pg and Tc respectively.

IV. 8. Anthrone assay

To a 16 × 125 – mm test tube containing less than 20 μg of hexose in 500 μl of water, carefully added 1 ml of 0.2% anthrone in concentrated H_2SO_4. Mixed thoroughly, heated in boiling-water bath for 5 minutes. Cool and read abosorbance at 620nm.[11]

IV. 9. Orcinol assay for pentoses and uronic acid

To a 16 × 125 – mm test tube containing less than 10μg of pentose and /or uronic acid in 0.5 ml water, added 67 μl of 6% orcinol in 95% ethanol. Added 1 ml of 0.1% $FeCl_3.6H_2O$ in conc. HCl. Mixed thoroughly, heated in a boiling-water bath for 20 min., and are allowed to cool. Read absorbance at 665 nm.[12]

IV. 10. Meta-hydroxy biphenyl assay

To a 16 × 125 – mm test tube containing less than 20μg of uronic acid in 200 μl of water, carefully added 1.2 ml of ice- cold borate solution (4.77 g $Na_2B_4O_7.10\ H_2O$ per litre of conc. H_2SO_4), mix thoroughly, heat in boiling- water bath for 5 min., and immediately cool in an ice-water bath. Add 20 μl of 0.15% m – hydroxy biphenyl in 0.5% NaOH and mix thoroughly. Allow colour to develop for 5 min. and read absorbance at 520 nm.[13]

IV. 11. (a) Hydrolysis

The polysaccharide sample (20mg) was hydrolyzed with 1M H_2SO_4 (20 ml) at 100^0 in sealed tubes for 6 hours. After cooling, the excess acid was removed by passage over a small column

(1 cm × 50cm) of Dowex 50-X4, 200-400 mesh (H+ form) coupled to a column of Dowex 1-X8, 200 – 400 mesh (formate form), anion exchange resin and eluted with deionized water. The eluate was concentrated (to 10 ml) in a rotory flash evaporator.

The hydrolysates (0.5 µl) were applied on TLC plate cellulose (Sigma, 1 mm thick 10 cm × 20 cm) and the chromatogram was developed using 1-butanol / acetic acid/water (3:1:2) and other solvent mixtures, given earlier. For identification of spots, aniline-diphenyl amine-phosphoric acid[3] spray reagent and other spray reagents given earlier were used. Constituent saccharides were identified by comparison with Rf values of authentic samples and also by co-chromatography with authentic samples.

IV. 11 (b) Graded hydrolysis

50 mg each of the polysaccharide were hydrolyzed with 50 mM H_2SO_4 (10 ml) in sealed tubes for 4 hours at 100^0. Paper chromatography (pc) of their hydrolysates after 15, 30, 45, 60, 90 and 120 minutes indicated the early release of D-glucose, D-galactose, D-galacturonic acid followed by D-mannose. These were identified by co-chromatography with authentic samples.

IV. 12. Timethylsilylation

100 mg each of the freeze dried polysaccharide samples were transferred to 13 × 100-mm test tubes. 250 µl of 1 M HCl in methanol is added, and the resulting solutions are heated at 80° for

16 hours. [The 1M HCl in methanol can be prepared either by slowly adding acetyl chloride to methanol (special care is to be taken as this is a very exothermic reaction) or by bubbling HCl gas into methanol, determining the HCl concentration by titration, and then diluting to proper concentration]. This converts the polysaccharide into a mixture of methyl glycosides and methyl ester methyl glycosides of the glycosyluronic acids. The methanolic HCl is removed by adding 100 µl of t-butyl alcohol and then evaporating with a stream of air at room temperature.[14] The methyl glycosides and methyl ester methyl glycosides are silylated using 5 ml of anhydrous pyridine (reagent grade pyridine dried over KOH pellets), 1 ml of hexa methyldisilazane (HMDS) and 0.5 ml trimethylchlorosilane (TMCS), which is purchased conveniently in these proportions as Tri-Sil (Pierce Chemical company, USA). The samples are heated to 80° for 20 min., and the silylating reagent is gently evaporated at room temperature. The solutions became cloudy on addition of timethylchlorosilane owing to precipitation, presumably of ammonium chloride. No attempt was made to remove this, which in no way interfered with the subsequent gas chromatography. The derivatives are redissolved in hexane (10 ml) and insoluble salts are allowed to settle. The supernatant is transferred to a clean test tube and carefully evaporated. The residue is dissolved in 100 µl of hexane, and 1µl of this solution is analyzed by GC.

IV. 13. Methylation, Hydrolysis, Reduction and Acetylation

The polysaccharides were methylated using NaOH/MeI method.[15] To a solution of the carbohydrate sample (5 mg) in dimethyl sulphoxide (0.5 ml) were added finely powdered NaOH (20 mg) and methyl iodide (0.1 ml). Each mixture was stirred (100 rpm) for 6 minutes in a closed tube at 25°. [13 × 100-mm test tube fitted with a Teflon-lined screwcap]. Water (1 ml) and chloroform (1 ml) were then added, and the chloroform layer was washed with water (3 × 10 ml) and dried (Na_2SO_4).

The methylated polysaccharides are then hydrolyzed to form free glycoses as follows: 2M trifluroacetic acid (TFA) [250 µl containing myo-inositol (25 µg) as an internal standard] is added to each sample, including the standard. The samples are placed in a heating block at 121° for 2 hours. The 2M TFA is then evaporated at 40° under a stream of air. When the tube appears dry, 250 µl of isopropyl alcohol is added and evaporated at room temperature. The evaporation of isopropyl alcohol results in more complete removal of the TFA. The resulting partially O-methylated glycoses are reduced to be corresponding partially O-methylated alditols by dissolving them in 95% ethanol (220 µl) and adding aqueous $NaBD_4$ (200 µl of 10 mg/ml in 1M NH_4OH). The test tube is closed with Teflon-lined screwcap and kept for 1 hour at room temperature. Acetic acid (50 µl) is then added to the tube to convert the excess borodeuteride into

borate. Acetic acid-methanol (1:9 v/v, 200 µl) is added to the tube, its contents are mixed, and the solvents are evaporated with air at room temperature. Three more evaporations of 1:9 (v/v) acetic acid-methanol (200 µl) are followed by two evaporations of methanol (200 µl).

The partially O-methylated alditols are O-acetylated as follows: Acetic anhydride (50 µl) is added to the test tube containing the partially O-methylated alditols. The tube is sealed and heat for 3 hours at 120°. The tube is then allowed to cool to room temperature and water (500 µl) is added. Solid Na_2CO_3 is added a small amount (25 mg) at a time, until effervescence ceases. If all of the Na_2CO_3 does not dissolve, more water can be added. Dichloromethane (500 µl) is then added to the tube, and the contents of the tube are mixed. The organic and water phases are separated by centrifugation at low speed. The methylene chloride phase is removed, transferred to a fresh tube and carefully evaporated. Great care must be taken in evaporating the methylene chloride to prevent loss of some of the more volatile partially O-acetylated, partially O-methylated alditols. These products are analyzed by GC and by gas chromatography-mass spectrometry (GC-MS)

IV. 14. Biological studies

Sterile precautions were taken while carrying out these experiments.

(a) Leukocyte migration inhibition (LMI)

Leukocytes were collected from normal controls and prepared at a concentration of 1×10^8 cell/ml. LMI was carried out by the method of Roklin (1976)[16].

$$\text{Calculation of migration index} = \frac{\text{Area of migration in test}}{\text{Area of migration in control}}$$

Tissue culture medium (TC 199) used alone, served as the control.

(b) Phagocytosis

The reaction mixture consisted of approximately 1.2×10^6 neutrophils in 1ml of Hank's balanced salt solution and 5% fetal calf serum and the polysaccharide (5 μg in 50 μl). This mixture was preincubated in a water bath at 37° for 7 min. A suspension of yeast particles was diluted with saline solution to 25×10^7 particles per ml and heated at 100° for 30 min. To the preincubated reaction mixture, 0.1 ml of the suspension of the yeast particles was added and incubation continued at 37° for 60 min. The number of yeast particles phagocytosed by neutrophils was counted after staining with 5% Fuchsin in phenol solution.[9]

$$\text{Phagocytic index} = \frac{\text{Number of yeast phagocytosed by 100 neutrophils in test}}{\text{Number of yeast phagocytosed by 100 neutrophils in test control}}$$

0.1 M Phosphate buffered saline (PBS pH 7.2) was used as the control

(c) Anti-complimentary activity

The method of Yamada *et al*[17] was used. Gelatin-Veronal-buffered saline (pH 7.4) containing 500 μM magnesium chloride and 150μm calcium chloride ($GVBP^{2+}$) was prepared and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the sample in the mixtures were pre-incubated at 37° for 30 min and 350 μl of $GVBP^{2+}$ was added. The residual hemolytic complement (TCH_{50}) was determined. NHS was incubated with water to provide a control.

(d) Toxicity profile

The toxicity studies on the plant extracts in mice were carried out. The purified polysaccharides were administered and the animals were observed continuously for 2 hours, 4 hours and finally over night.

(e) Cytogenetical studies

The experiments were in mice, in human leukemic cell line K_{562} and also in human perpheral blood lymphocytes.

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CHAPTER - V

SUMMARY

SUMMARY

Carbohydrates are some of the most important chemical compounds of the biological world and are the key substances of life. Understanding the roles played by the carbohydrates is one of the greater frontiers in the advancement of molecular biology. It has been established that, polysaccharides are involved in a myriad of important functions and pharmacological activities. In recent years, many pharmacologically active plant polysaccharides have been isolated and structural characterization have been accomplished.

Extracts of *Punica granatum* and *Tinospora cordifolia* have been extensively used in folk medicine for various ailments. Biological studies of the polysaccharides isolated from these plant sources gave encouraging results, which in turn suggest that the active principles were polysaccharides. The chemical and structural characterization of these macromolecules are presented in this study.

For the isolation and purification of the polysaccharides, standard procedures for water soluble polysaccharides were followed. Final purification was carried out by gel permeation chromatography. The homogeneity of the purified compounds were tested using sodium dodecylsulphate

polyacrylamide gel electrophoresis and found be homogeneous. Molecular weights were determined by gel filtration chromatography using Sephadex G-200 with a series of dextrans as standard. The molecular weights obtained were as follows:

Punica granatum	1,10,000 D
Tinospora cordifolia	40,000 D

For charecterization, various chemical and spectroscopic analyses were carried out. Complete hydrolysis, graded hydrolysis and thin layer chromatographic analysis and co-chromatography using standard sugars revealed the constituent monosaccharides to be glucose, galactose, mannose, rhamnose, arabinose, xylose and galacturonic acid.

Glycosyl composition analysis of the purified polysaccharides were carried out in the following manner:

The samples were freeze dried prior to methanolysis. They were hydrolyzed using freshly prepared 1 M methanolic – HCl for 16 hours at 80^0 C. The released sugars were treated with anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane, and converted to trimethylsilyl derivatives. The derivatized samples were analyzed by GC-MS studies. Myo-inositol was added as an internal standard. The relative retention times of the products and their main fragments in the mass spectra, response factors and peak areas gave the mole % of glycosyl residues

of the polysaccharides from *Punica granatum* and *Tinospora cordifolia.*

Polysaccharide from *Punica granatum* was found to contain glucose (52.8%), arabinose (5.4%), galactose (4.6%), mannose (1.7%), rhamnose (1.5%), xylose (0.5%) and galacturonic acid (33.5%). Polysaccharide from *Tinospora cordifolia* was completely different and is mostly a glucose polymer; glucose (98%), xylose (0.8%), arabinose (0.5%), galactose (0.3%), rhamnose (0.2%) and mannose (0.2%).

Complex carbohydrates are often subjected to different chemical modifications in order to gain information on sequences and anomeric configurations. Methylation analysis has proved to be valuable for following the results of such modifications. Here, the glycosyl linkage analyses were carried out in the following manner:

The samples were methylated using the NaOH/MeI method. The methylated samples were hydrolyzed in 2 M TFA at 121^{0}C for 2 hours and the hydrolyzed carbohydrate was reduced with sodium borodeuteride at room temperature. The product was acetylated using acetic anhydride at 120^{0}C for 3 hours. The partially methylated alditol acetates were analyzed by GC-MS using Sp 2330 Supelco column. Internal standard, myo-inositol, was added to each sample prior to reduction step.

Polysaccharides from *Punica granatum* was also methylated under different conditions in order to obtain the galacturonic acid linkage. The

sample was pre-reduced with superdeuteride for three hours at room temperature prior to the 2 M TFA hydrolysis and reduction step.

The recorded mass spectra were compared with the reference spectra in the mass spectral database of associated computers using the probability based matching (PBM) search algorithm supplied by the manufacturer. The response factors, relative to the internal standard, myo-inositol, are determined emperically by injecting the standards and the peak areas for each sugar derivative are determined. Peak areas gave percentage of different carbohydrate residues in *Punica granatum* as follows.

Carbohydrate residues	% Present
Terminal-rhamnose	3.4
Terminal-arabinose (furanose)	7.2
Terminal-arabinose	0.8
Terminal-xylose	1.7
2-arabinose (furanose)	1.3
Terminal mannose	1.7
Terminal-glucose	31.6
Terminal-galactose	2.1
4-arabinose or 5-arabinose (furanose)	6.0
4-xylose	2.2

3-glucose	3.4
2-mannose	0.6
2-glucose	2.5
3-galactose	1.5
6-glucose	6.0
4-galactose	2.0
4-glucose	21.7
4,6-glucose	3.0
3,6-galactose	1.3

The sample also contained galactronic acid that was tested in a separate experiment. The galactronic acid was 4-galactronic acid.

The polysaccharide from *Tinospora cordifolia* was difficult to dissolve in dimethyl sulphoxide. The first attempt of methylation analysis of this sample produced about 60% undermethylation which results from some of the sample not dissolving well in the first stage of permethylation of the carbohydrate. Undermethylation is very common in large polymers. This sample seems to be a polymer of 4-glucose with about 8% terminal glucose showing the 1-glucose linkage.

Carbohydrate residues	% Present
Terminal glucose	8.4

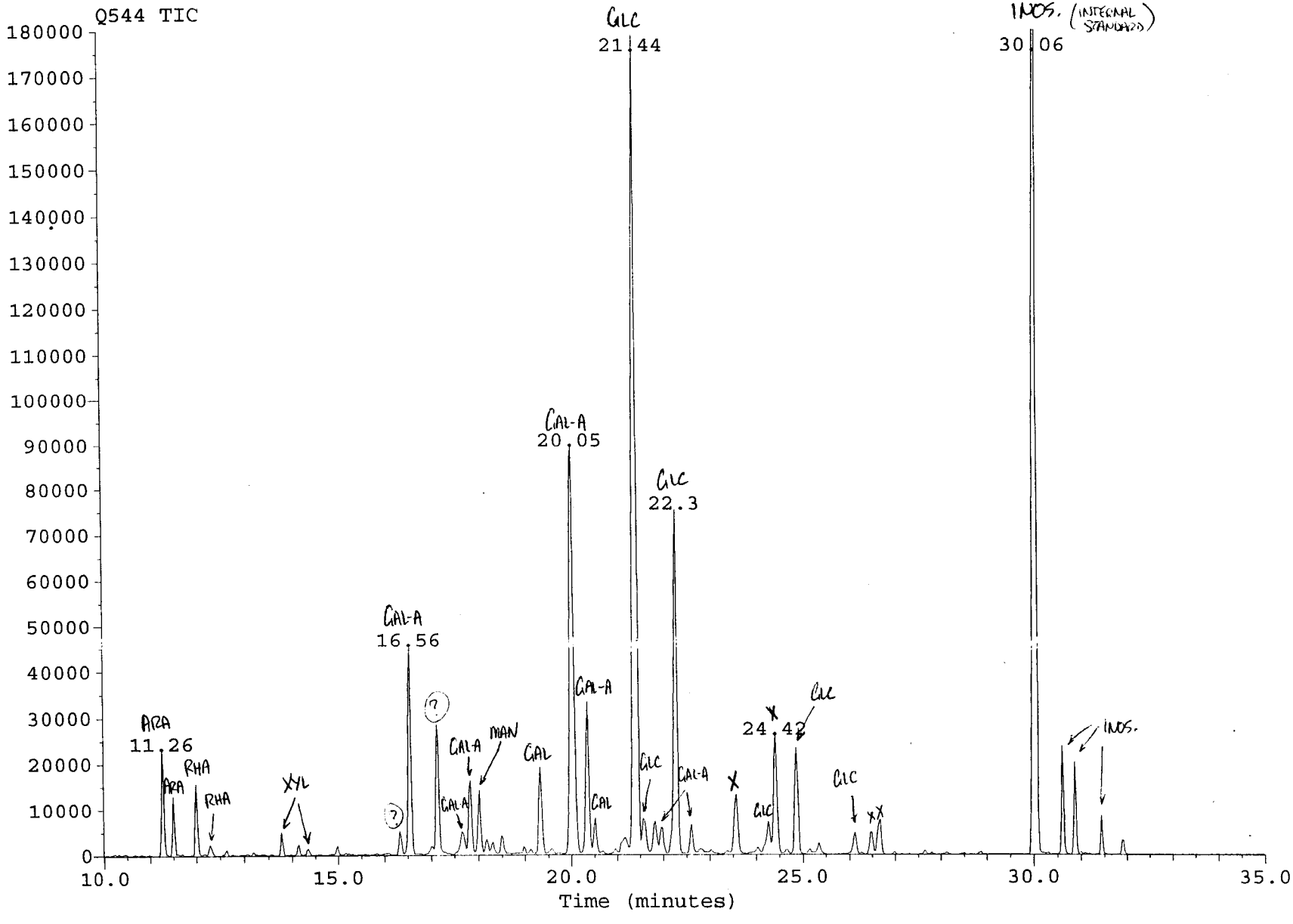
4-xylose	2.3
4-glucose	79.2
4,6-glucose	7.0
2,3,4,6-glucose (undermethylation)	3.1

Biological studies were carried out at Regional Cancer Center, Thiruvananthapuram, India.

SPECTRA

COMPOSITION ANALYSIS

MJ-1 TMS



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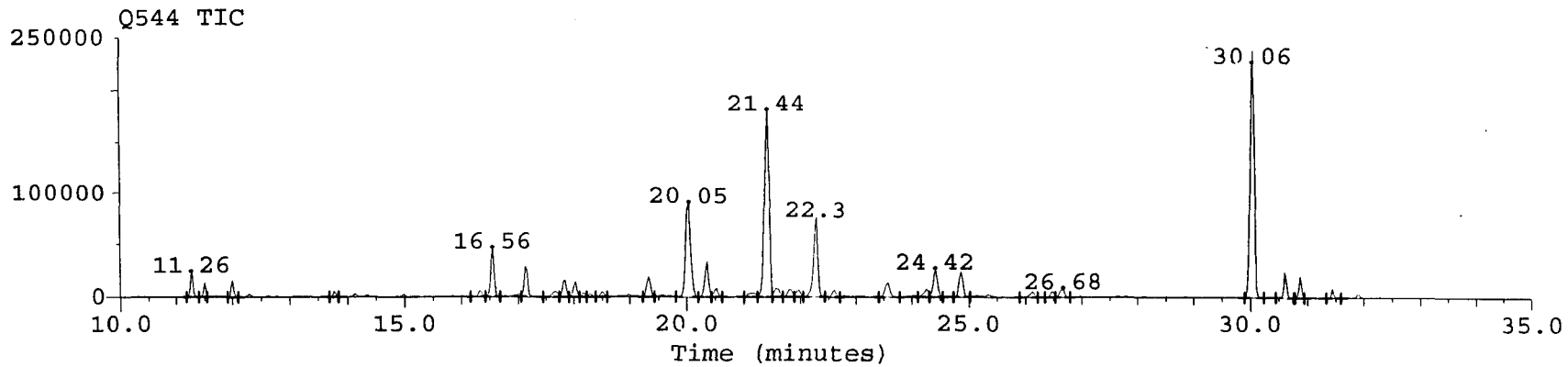
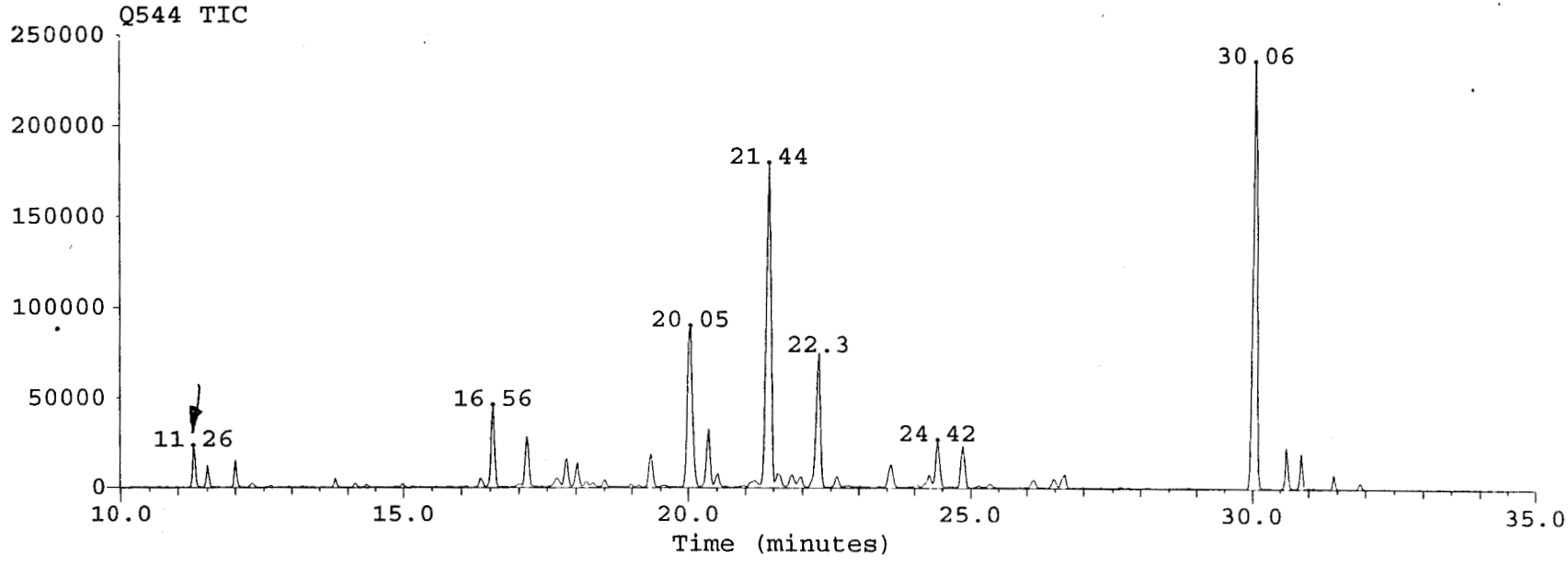
Instrument Parameters:
    StartInjection
Scan Parameters:
    SCAN every 2 secs for 5 min
    SCAN every 1 secs for 40 min
      50-650

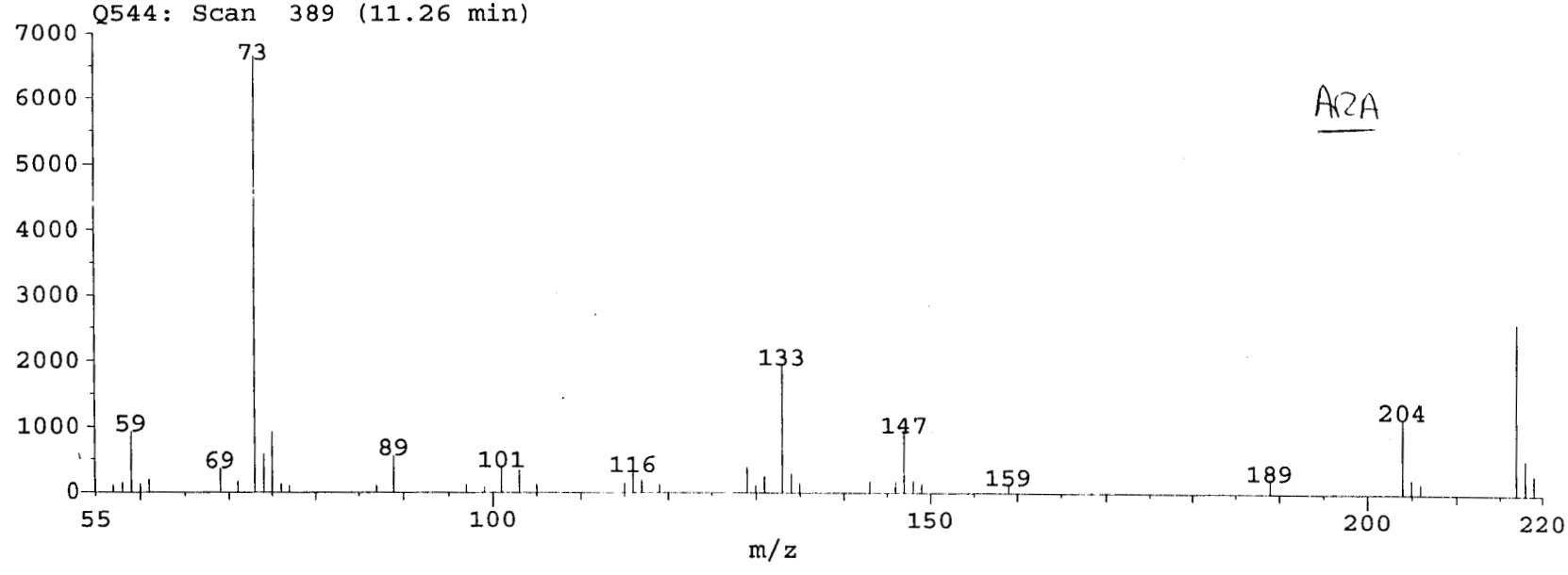


RT	AREA		HEIGHT	%T AREA	%S AREA
11.26	1359.233	ARA	22973	1.61	6.62
11.50	594.569	ARA	13017	0.71	2.90
12.00	804.791	RHA	15600	0.96	3.92
13.78	273.448	XYL	5213	0.32	1.33
16.32	373.808		5273	0.44	1.82
16.56	3185.977	GAL-A	45519	3.78	15.52
17.14	2241.682		28534	2.66	10.92
17.66	582.551		5069	0.69	2.84

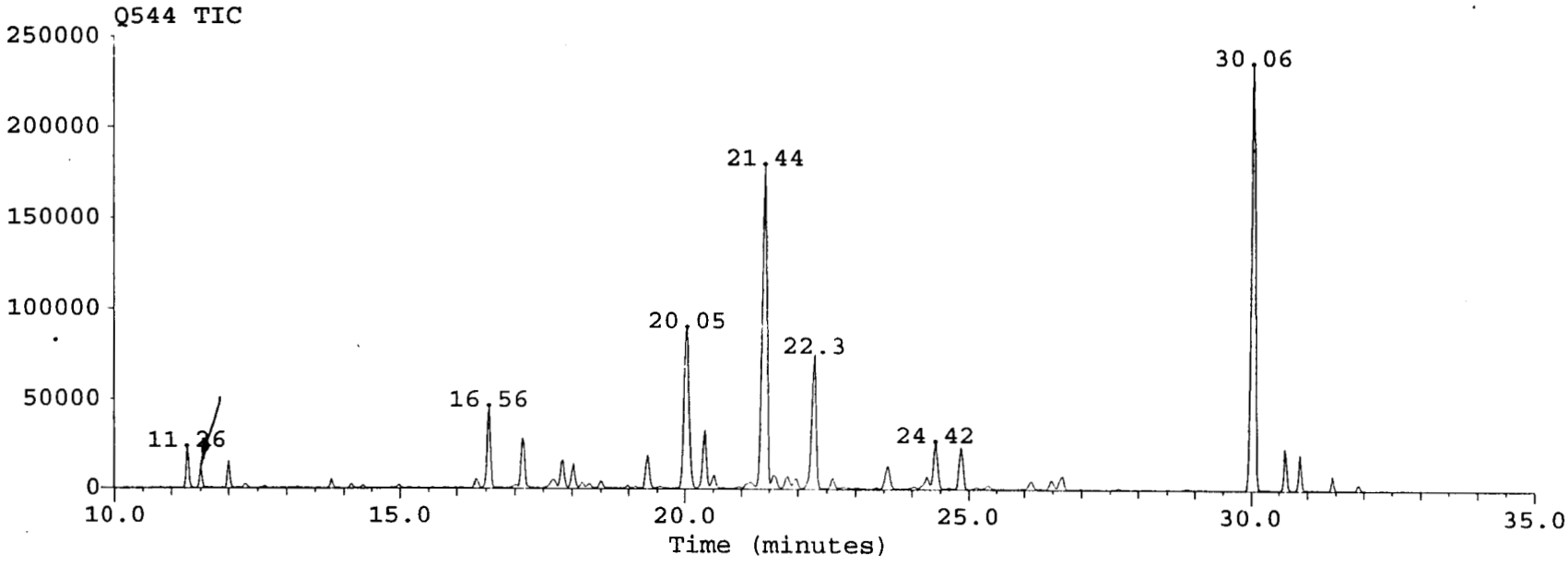
MJ-1 TMS

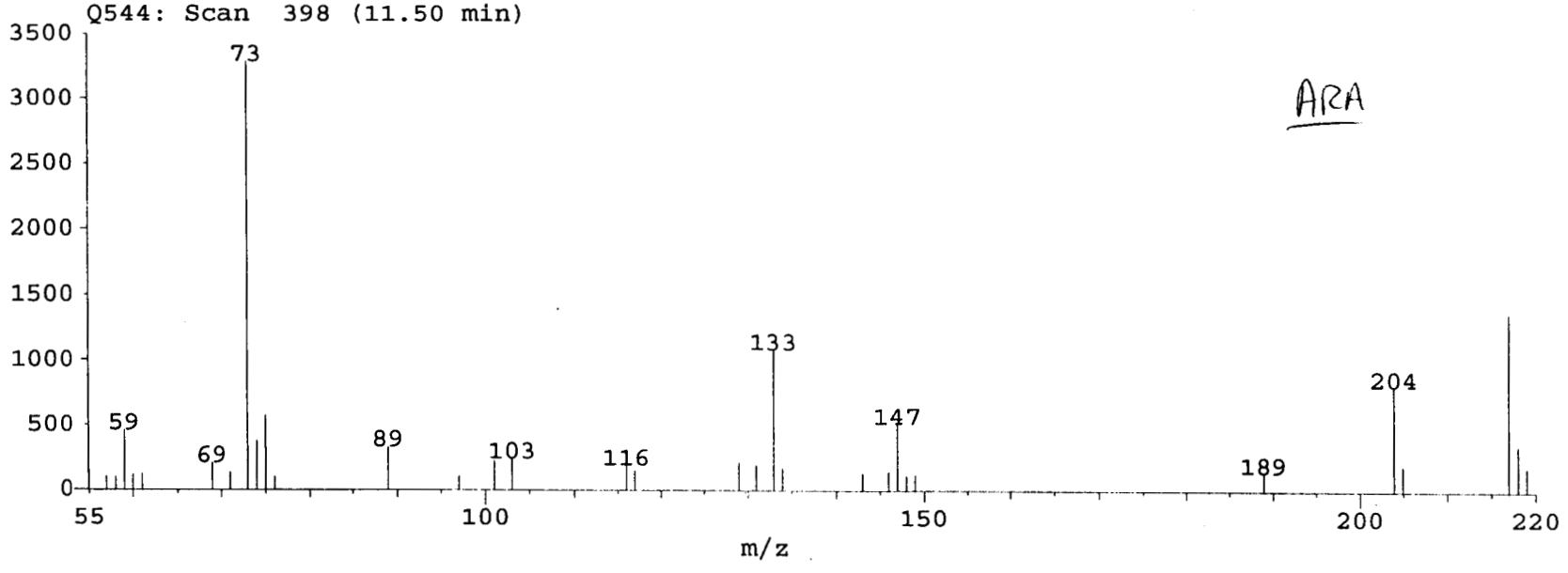
17.84		1251.179	GALA	16223	1.49	6.09
18.03		1017.318	MAN	14136	1.21	4.95
18.19		281.112		3419	0.33	1.37
18.50		341.036		4158	0.41	1.66
19.34		1461.670	GAL	19188	1.74	7.12
20.05		9260.678	GALA	89449	11.00	45.10
20.36		2671.950	GAL-A	33331	3.17	13.01
20.52		657.621	GAL	8064	0.78	3.20
21.17		581.895		3694	0.69	2.83
21.44		16503.625	GIC	178766	19.60	80.38
21.57		861.201		7975	1.02	4.19
21.83		726.719		7239	0.86	3.54
21.96		538.201		5984	0.64	2.62
22.30		6683.781	GIC	75368	7.94	32.55
22.62		565.002		6586	0.67	2.75
23.59		1372.496		13355	1.63	6.68
24.27		718.461		7318	0.85	3.50
24.42		2326.983		26094	2.76	11.33
24.87		1936.004		23404	2.30	9.43
26.13		438.318		4808	0.52	2.13
26.47		441.664		4908	0.52	2.15
26.68		705.008		7650	0.84	3.43
30.06	S	20533.271	INS.	236474	24.39	100.00
30.61		1366.961		23793	1.62	6.66
30.87		1120.574		20335	1.33	5.46
31.45		420.337		8624	0.50	2.05


Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Q544: Scan 389 (11.26 min)
7000
6000
5000
4000
3000
2000
1000
0
73
59
69
89
101
116
133
147
159
189
204
55
100
150
200
220
m/z

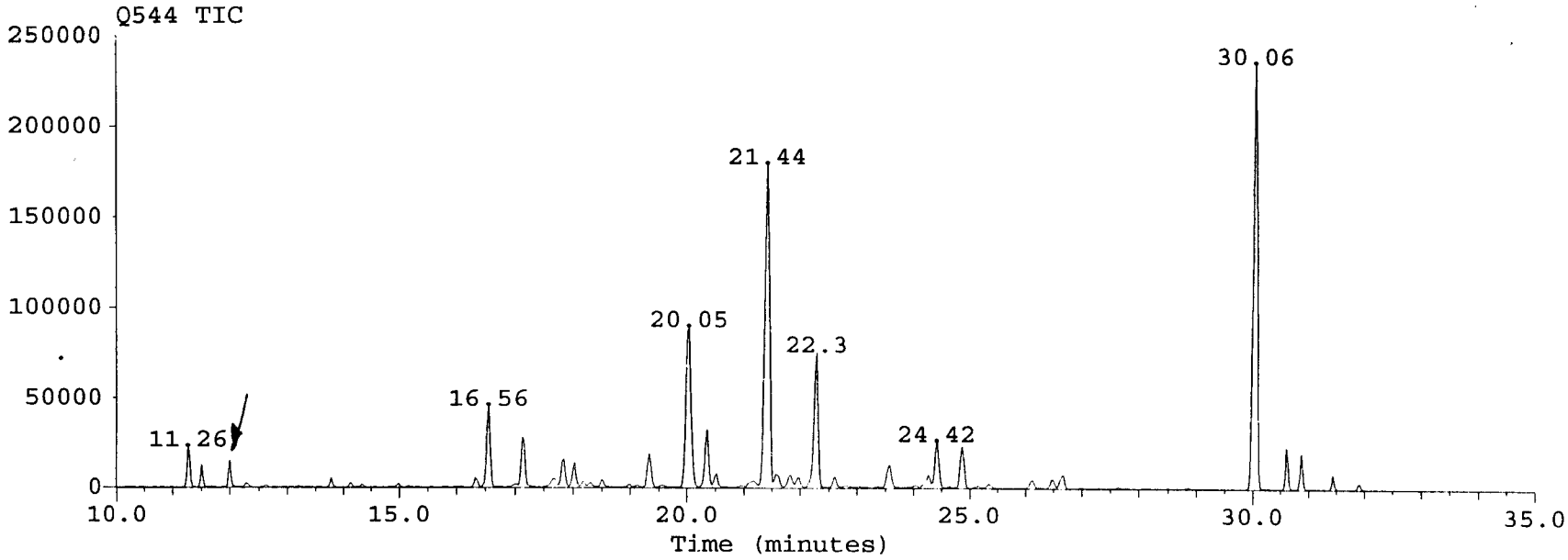
A12A

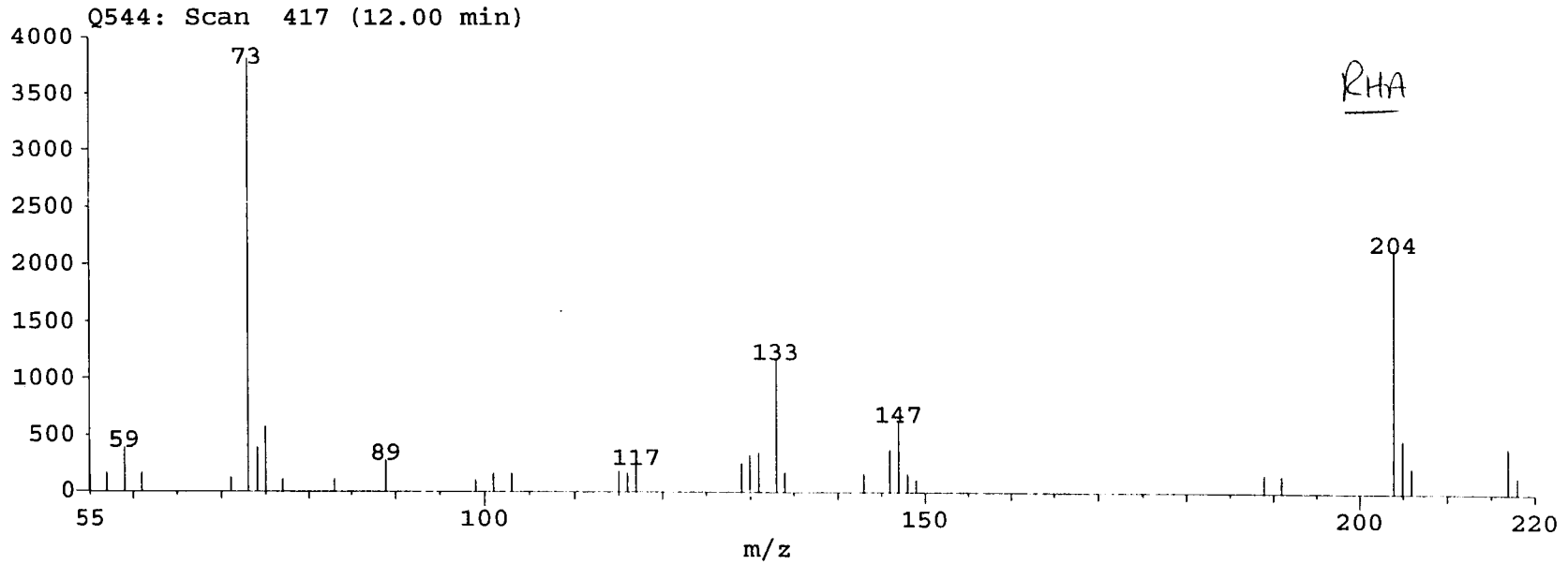

Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Q544: Scan 398 (11.50 min)
3500
3000
2500
2000
1500
1000
500
0
73
133
204
147
59
69
89
103
116
189
55
100
150
200
220
m/z

ARA

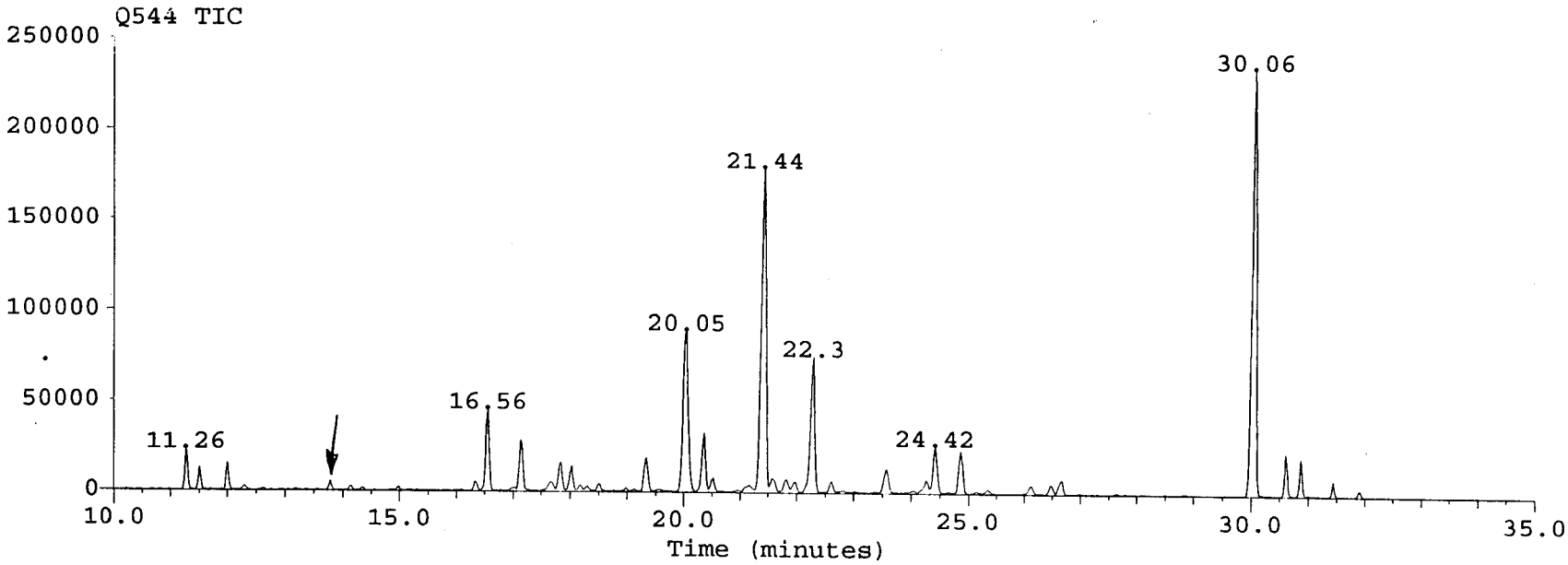
3,

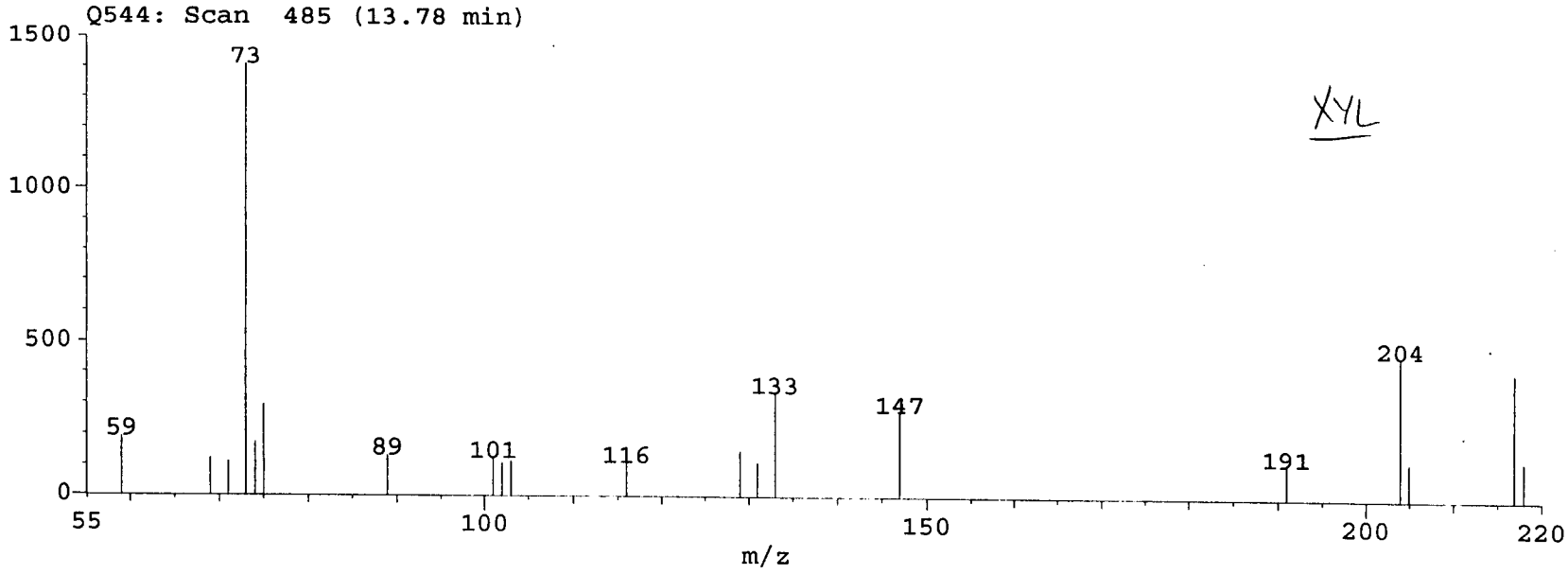

Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Q544: Scan 417 (12.00 min)
4000
3500
3000
2500
2000
1500
1000
500
0
73
204
133
147
59
89
117
55
100
150
200
220
m/z

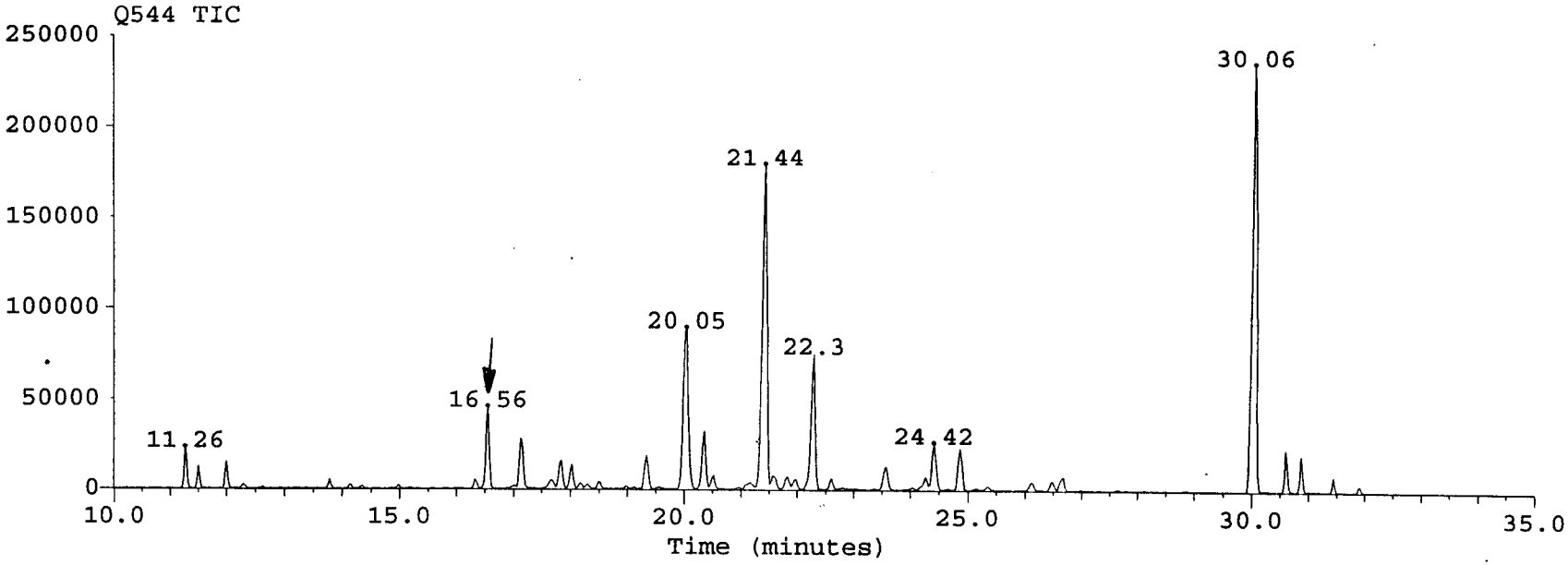
RHA

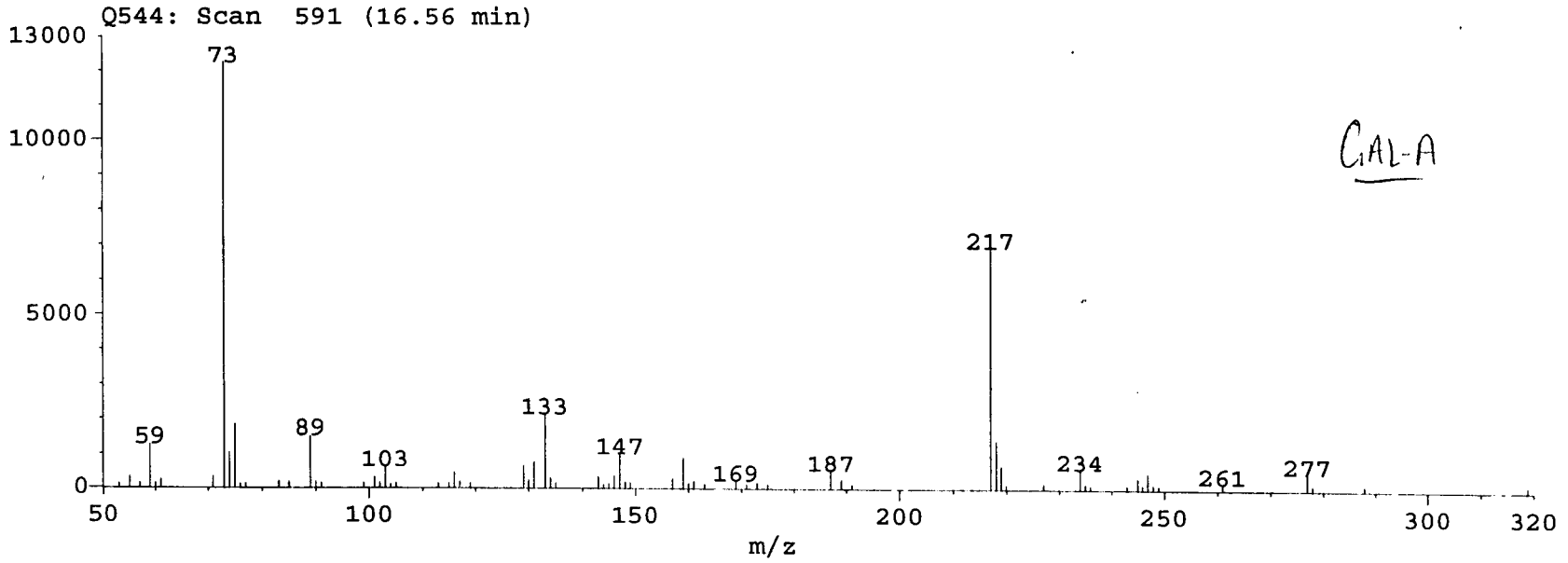
5


Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

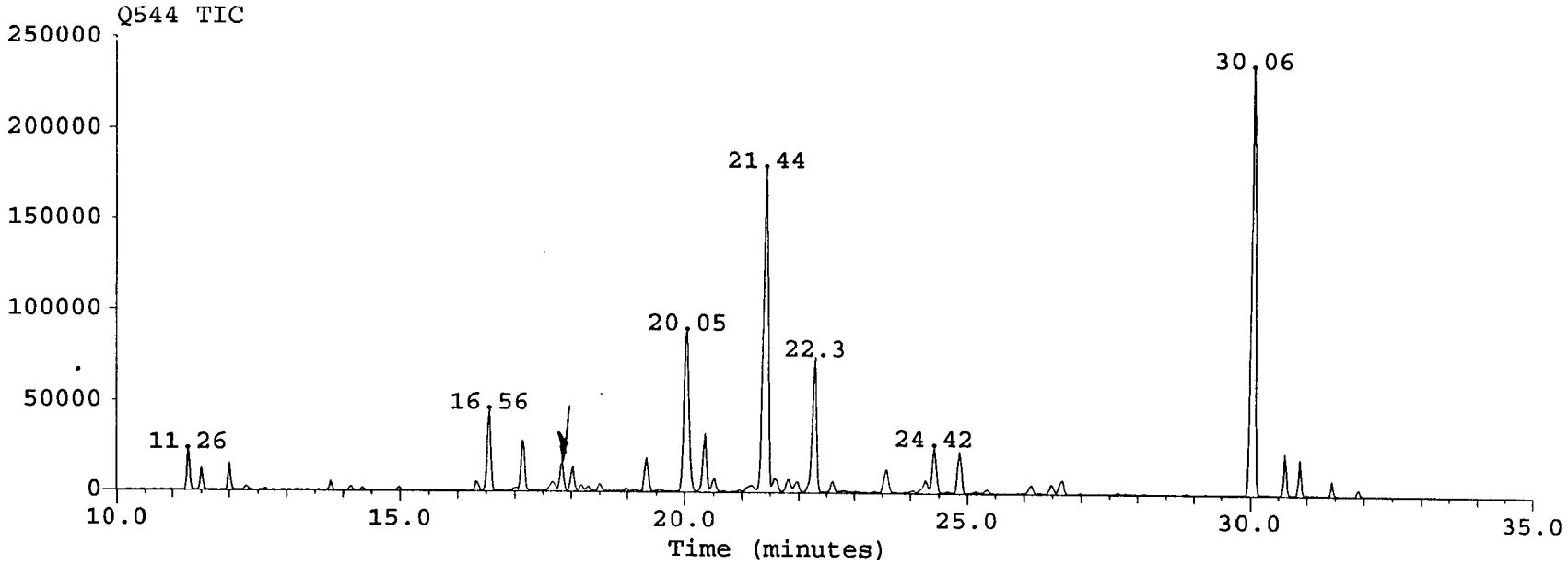

Q544: Scan 485 (13.78 min)
1500
1000
500
0
73
59
89
101
116
133
147
191
204
55
100
150
200
220
m/z

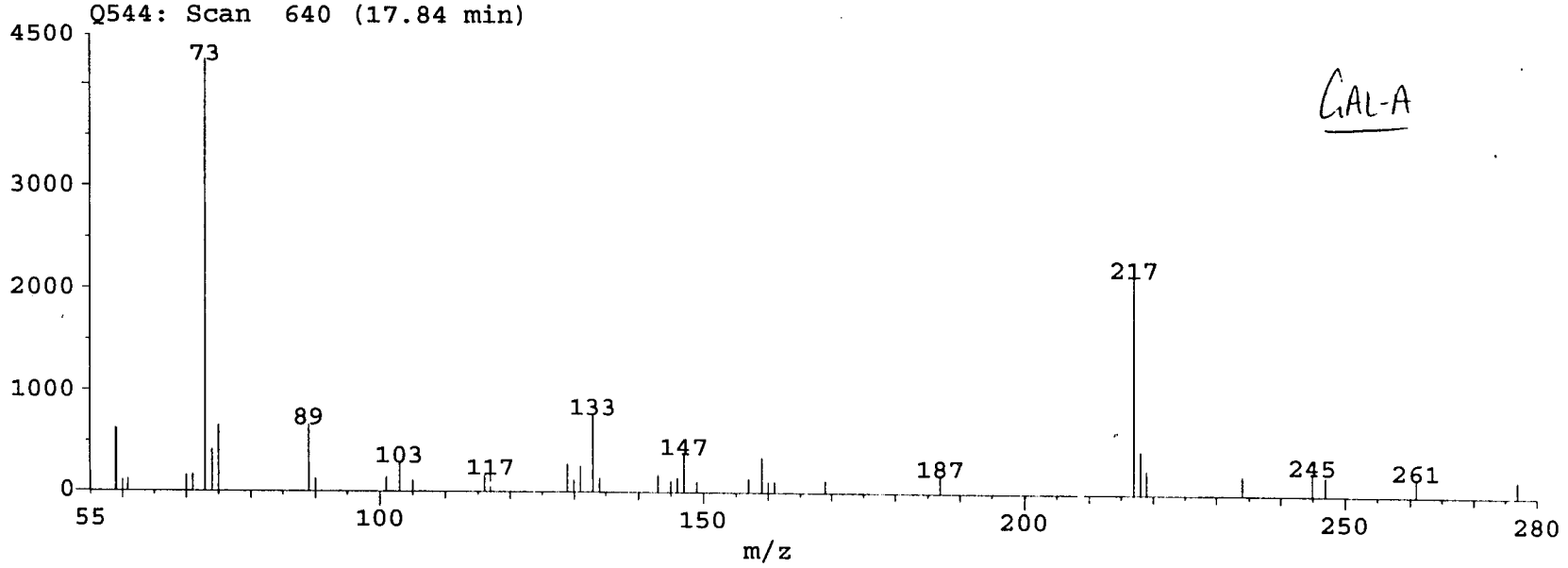
XYL


Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

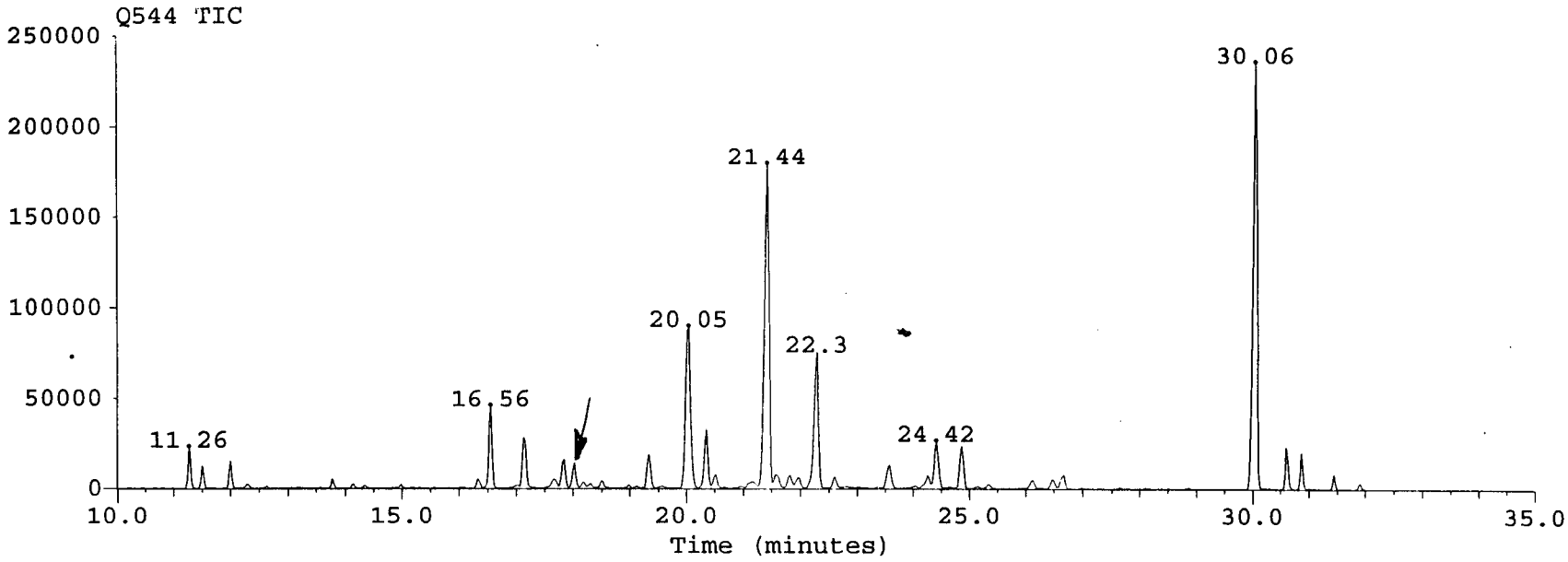

Q544: Scan 591 (16.56 min)
13000
10000
5000
0
73
217
133
59
89
103
147
169
187
234
261
277
50
100
150
200
250
300
320
m/z

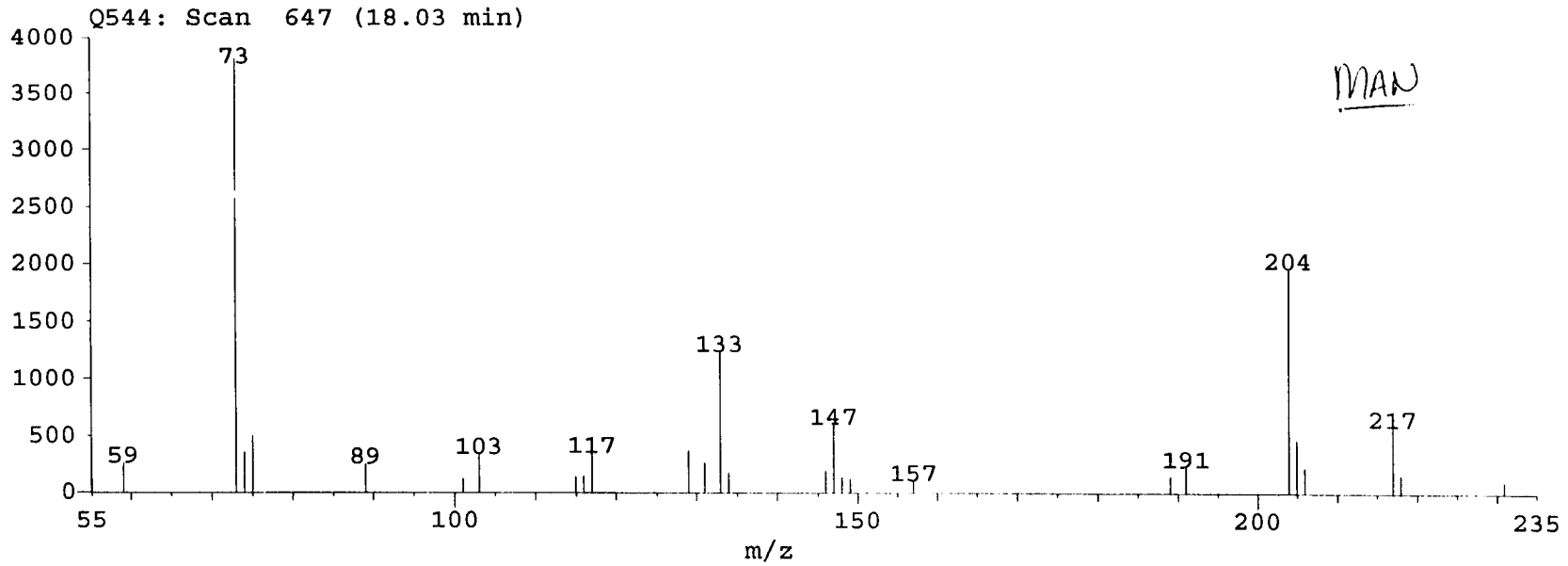
CIAL-A


Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

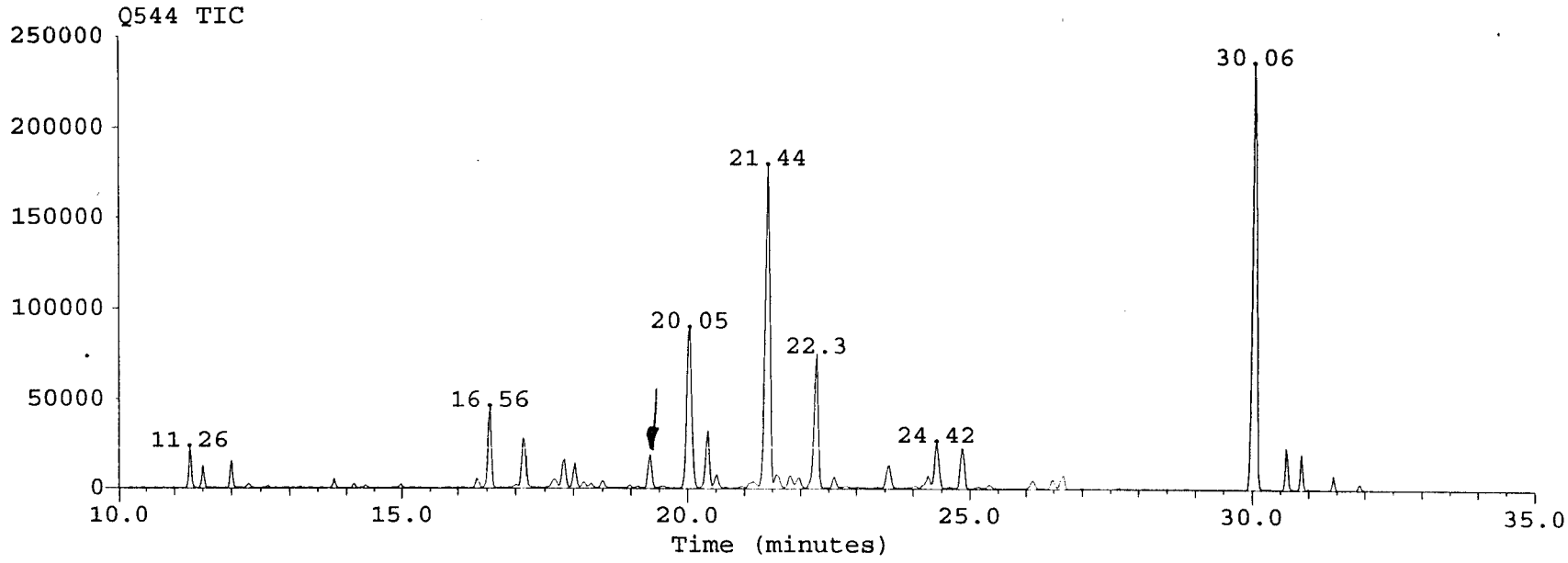

Q544: Scan 640 (17.84 min)
4500
3000
2000
1000
0
73
89
103
117
133
147
187
217
245
261
55
100
150
200
250
280
m/z

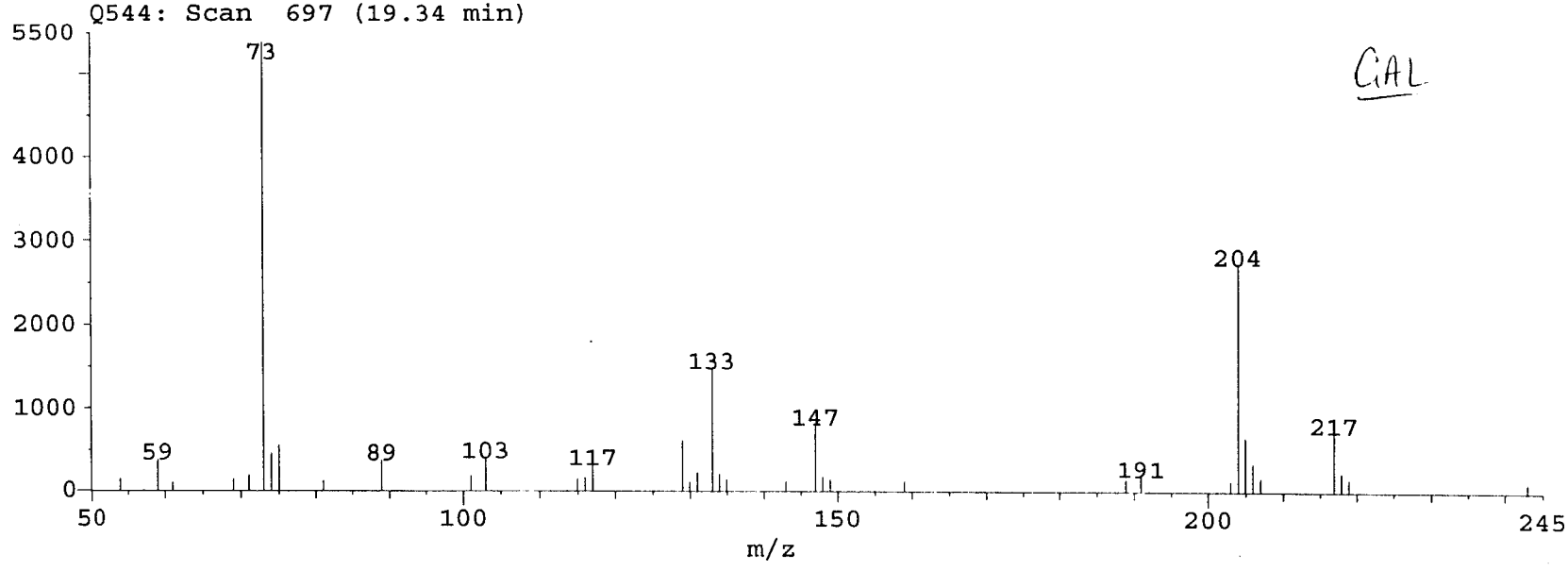
CAL-A


Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

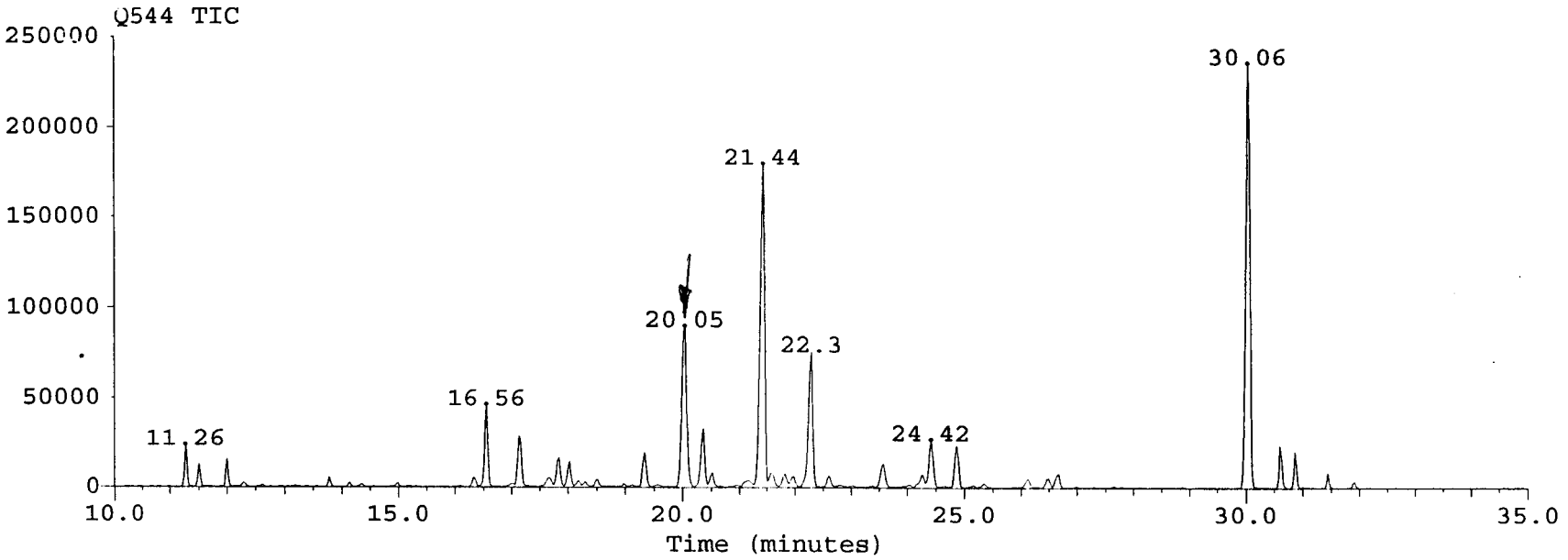

Q544: Scan 647 (18.03 min)
4000
3500
3000
2500
2000
1500
1000
500
0
73
59
89
103
117
133
147
157
191
204
217
55
100
150
200
235
m/z

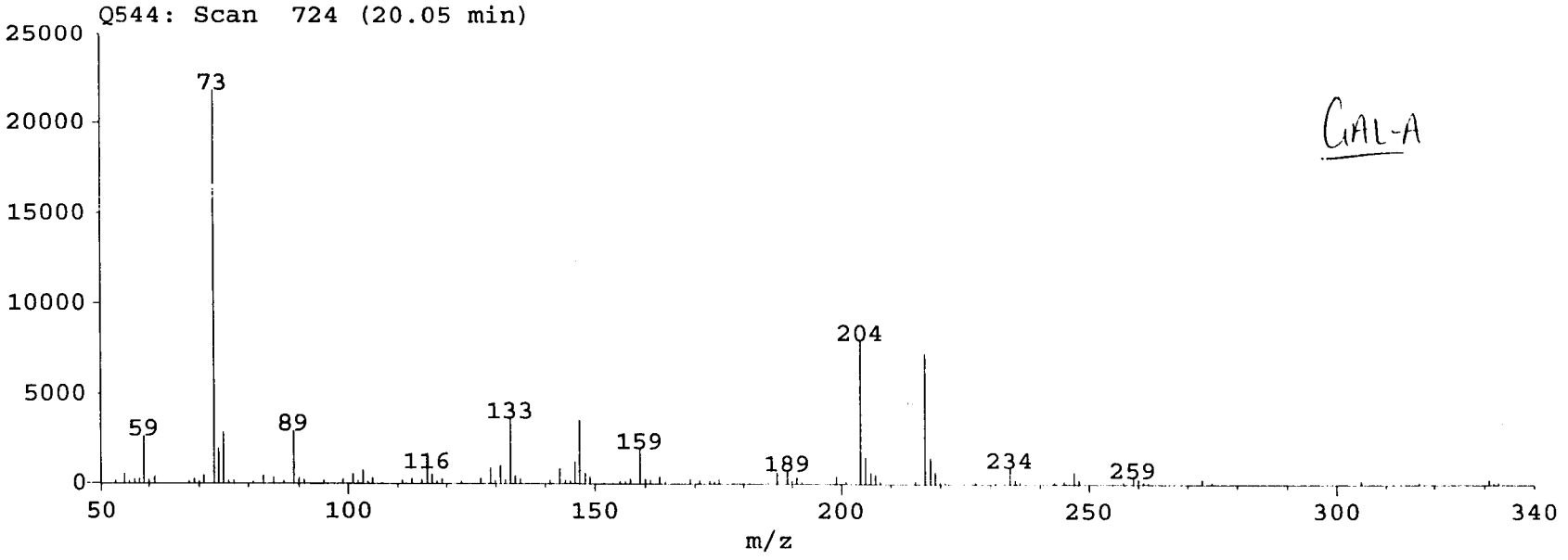
MAN


Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

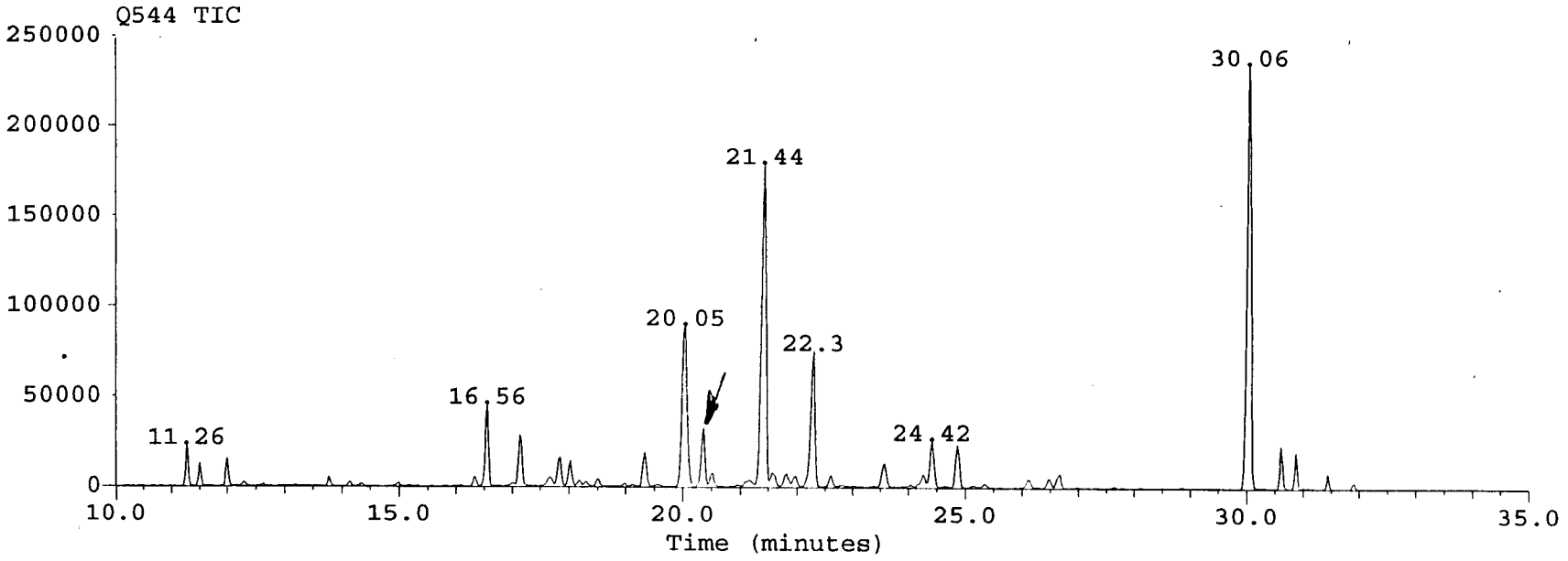

Q544: Scan 697 (19.34 min)
5500
4000
3000
2000
1000
0
73
59
89
103
117
133
147
191
204
217
50
100
150
200
245
m/z

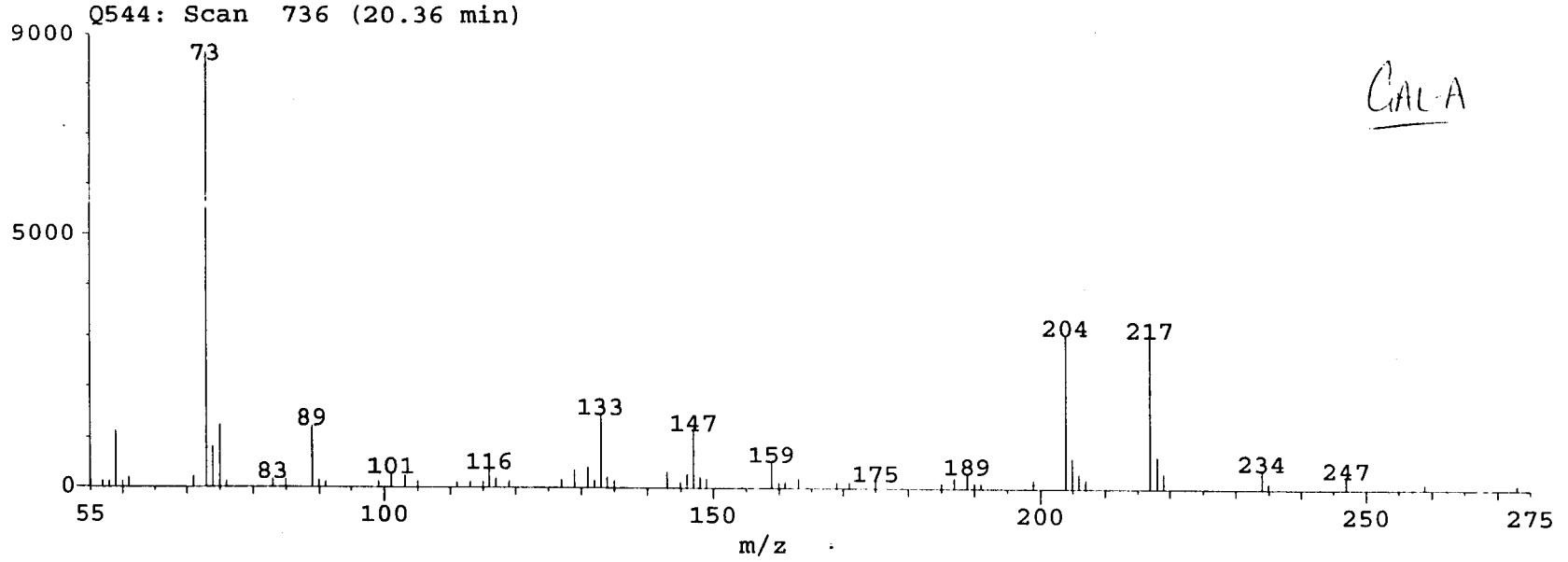
CiAL


Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

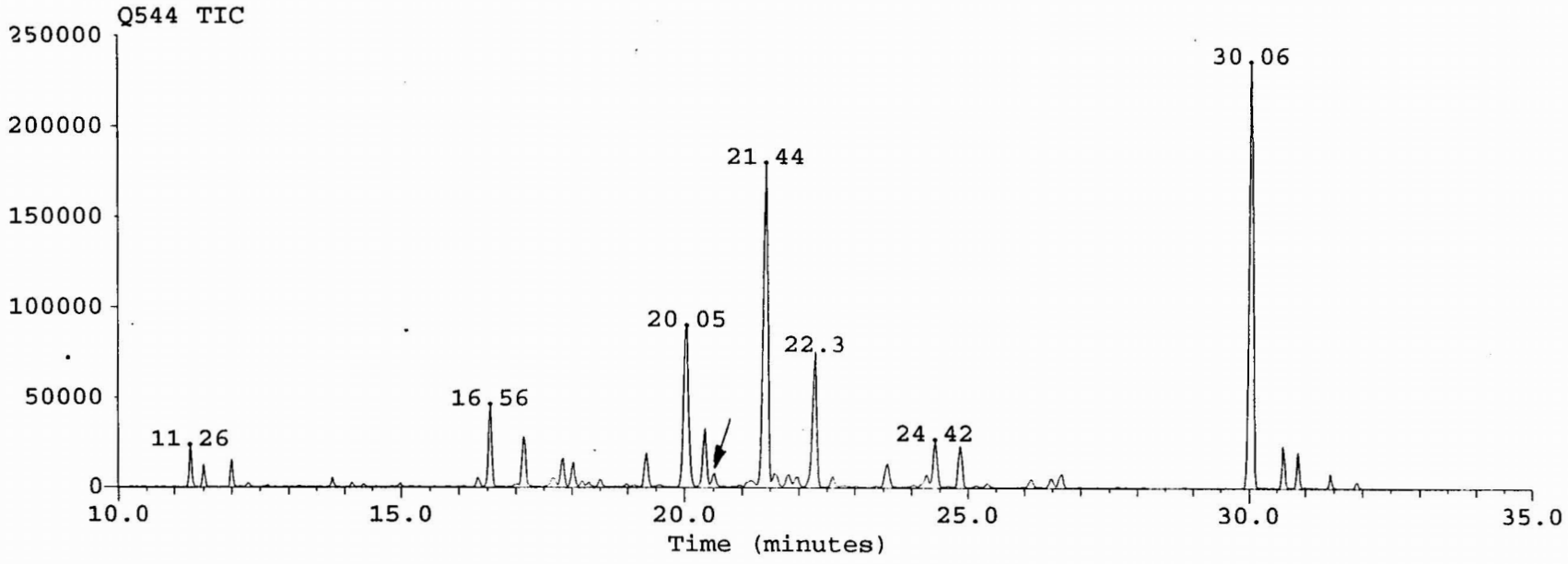

Q544: Scan 724 (20.05 min)
25000
20000
15000
10000
5000
0
73
59
89
116
133
159
189
204
234
259
50
100
150
200
250
300
340
m/z

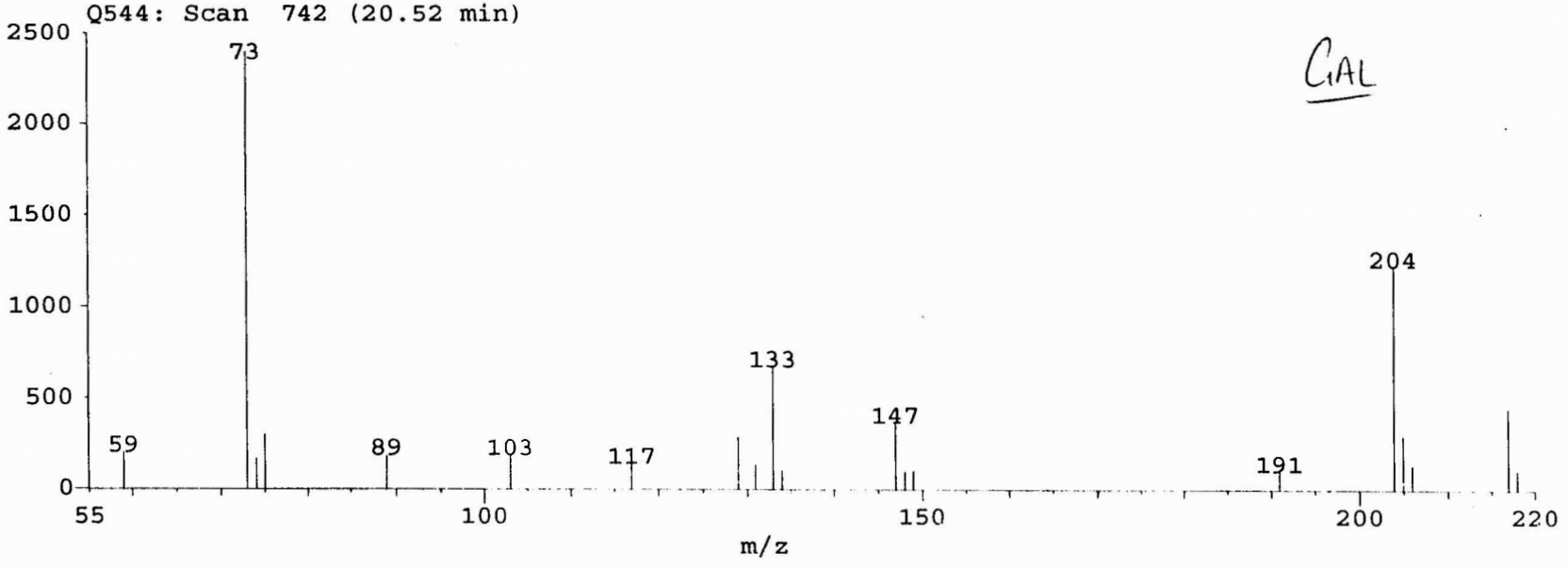
GAL-A


Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

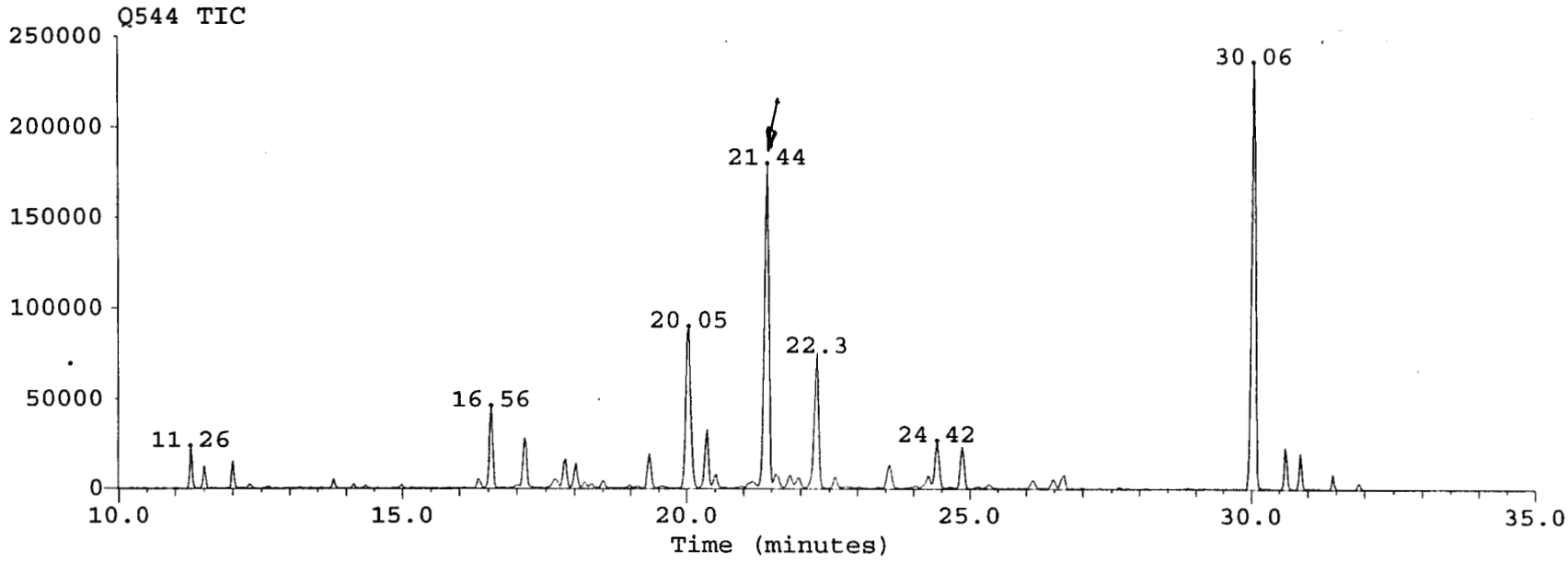

Q544: Scan 736 (20.36 min)
9000
5000
0
73
83
89
101
116
133
147
159
175
189
204
217
234
247
55
100
150
200
250
275
m/z

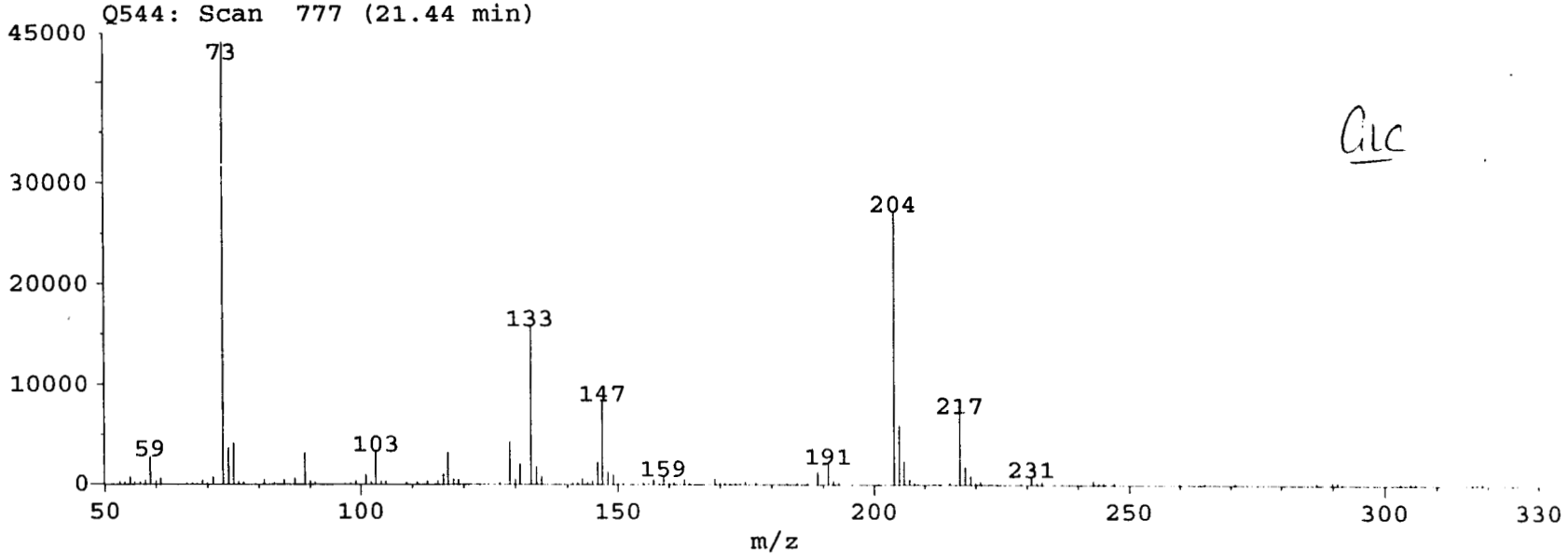
CAL-A


Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

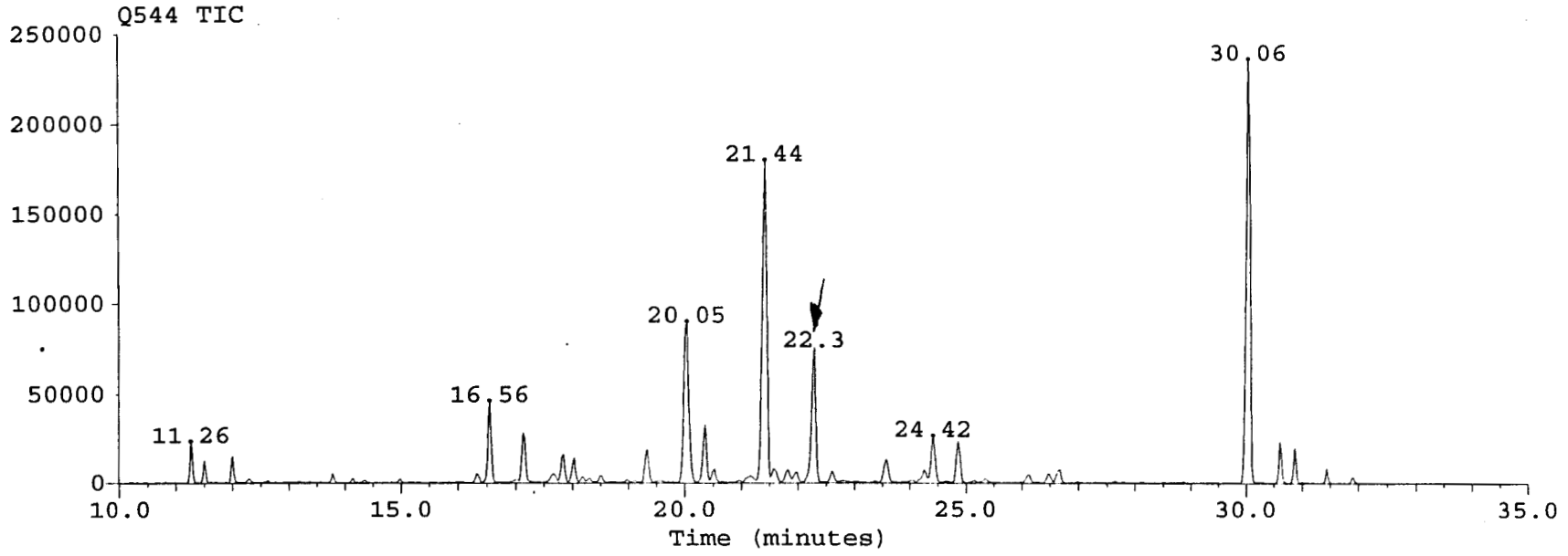

Q544: Scan 742 (20.52 min)
2500
2000
1500
1000
500
0
73
59
89
103
117
133
147
191
204
55
100
150
200
220
m/z

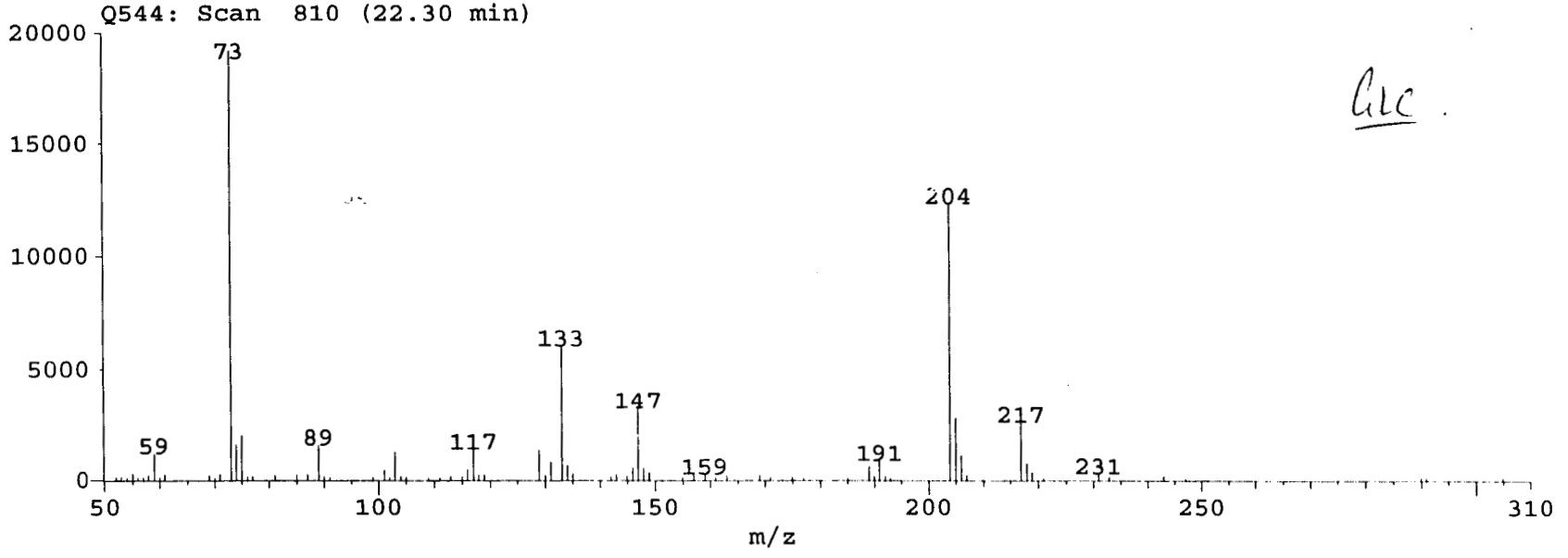
CiAL

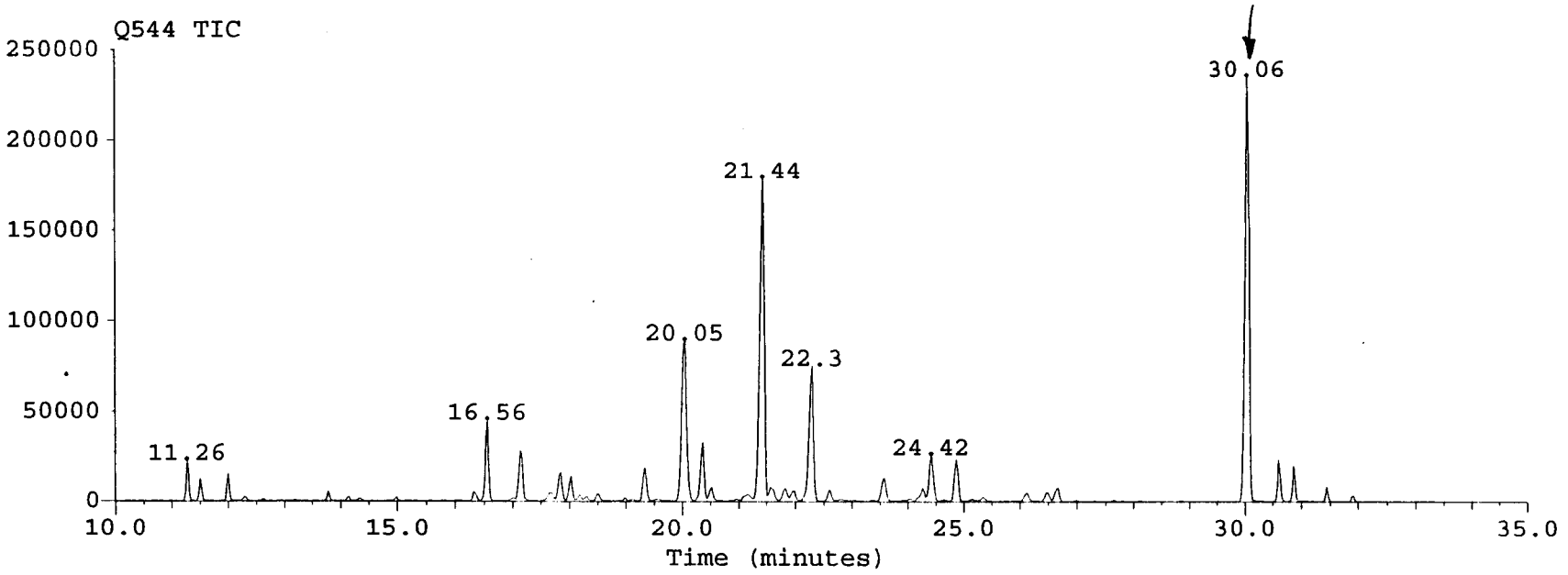

Q544 TIC
250000
200000
150000
100000
50000
0
11.26
16.56
20.05
21.44
22.3
24.42
30.06
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

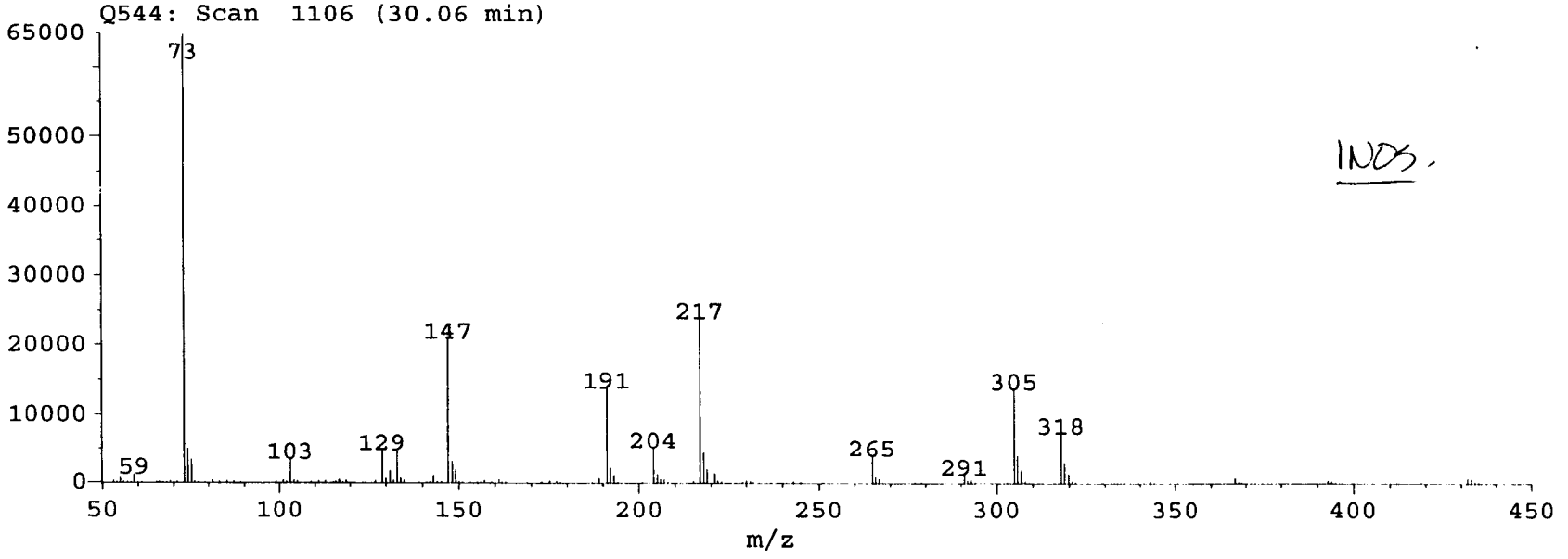

Q544: Scan 777 (21.44 min)
45000
30000
20000
10000
0
73
59
103
133
147
159
191
204
217
231
50
100
150
200
250
300
330
m/z

GLC


Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

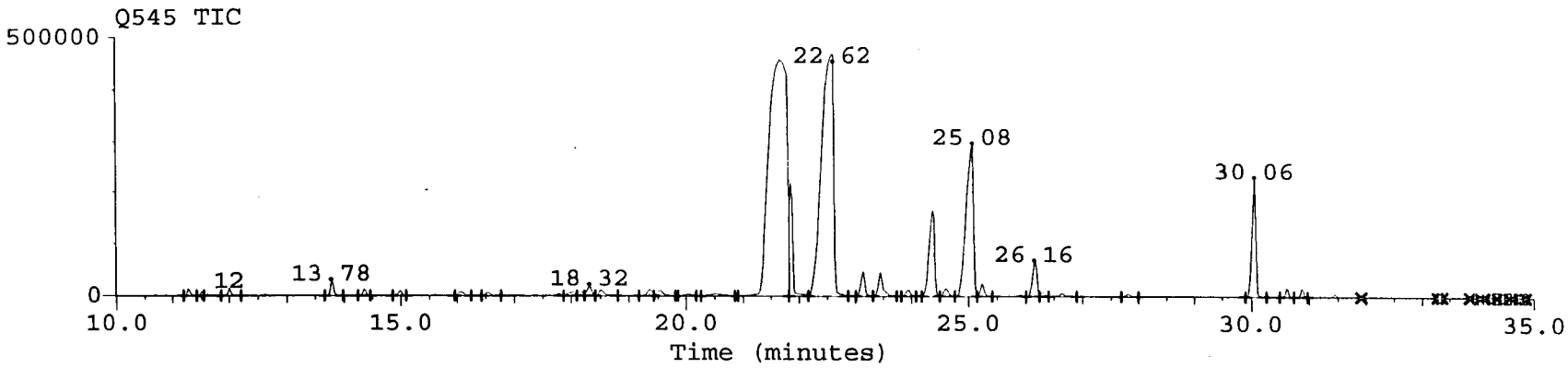

Q544: Scan 810 (22.30 min)
20000
15000
10000
5000
0
73
204
133
147
217
59
89
117
159
191
231
50
100
150
200
250
310
m/z


Q544 TIC
250000
200000
150000
100000
50000
0
30.06
21.44
20.05
22.3
16.56
11.26
24.42
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Q544: Scan 1106 (30.06 min)
65000
50000
40000
30000
20000
10000
0
73
147
217
191
305
318
204
129
103
265
291
59
50
100
150
200
250
300
350
400
450
m/z

INDS.

Quadrex
C:\VECTOR2\DATA\INST1\Q545.TKF
Acquired on Tue Aug 31 12:39:37 1999
Sample mj-2, Vol 2 Dil 1 Group 0
tms
Instrument Parameters:
    StartInjection
Scan Parameters:
    SCAN every 2 secs for 5 min
    SCAN every 1 secs for 40 min
      50-650



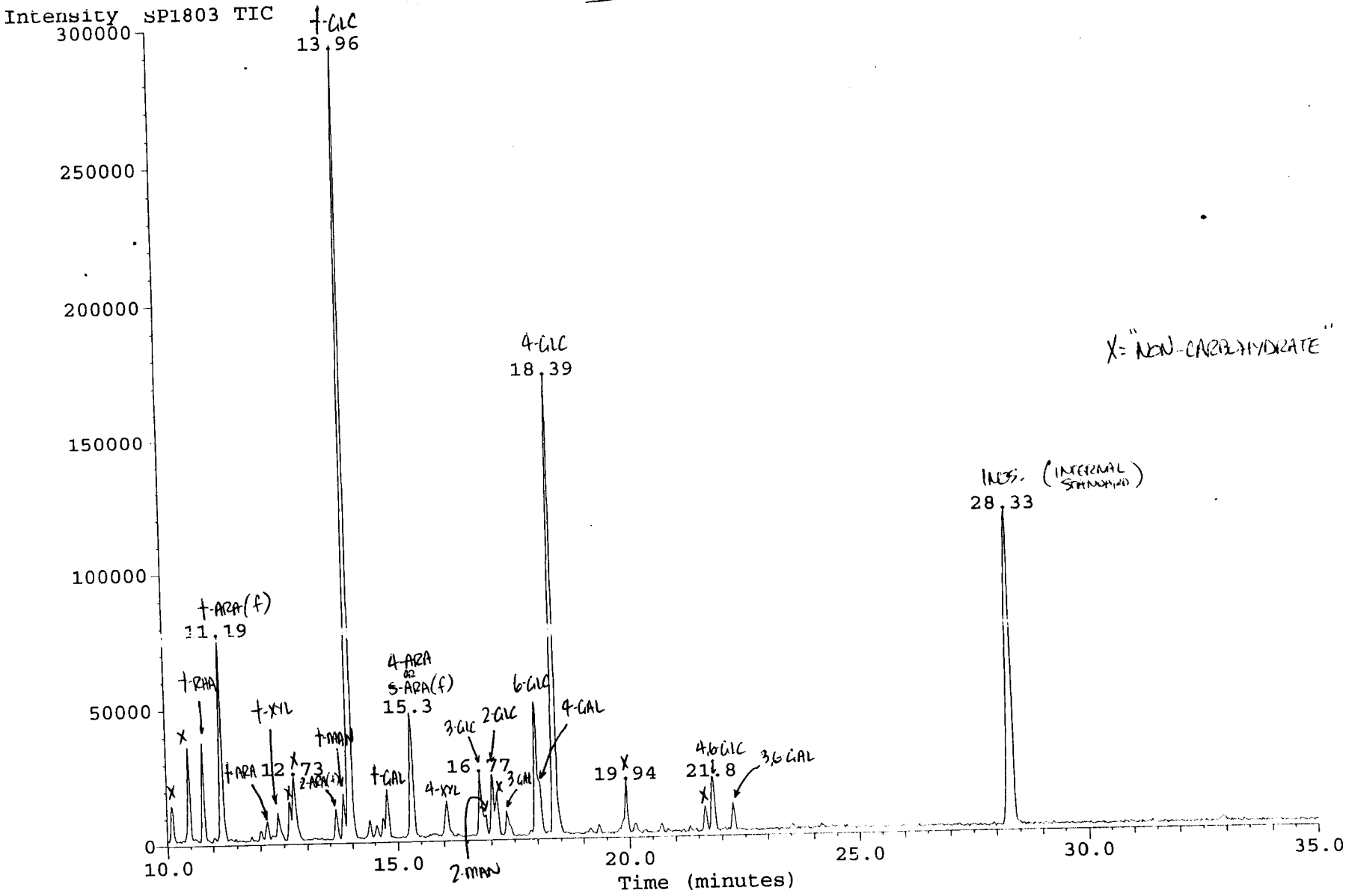
RT	AREA		HEIGHT	%T AREA	%S AREA
11.27	813.193	ARA	11897	0.20	0.48
11.50	366.436	ARA	6821	0.09	0.22
12.00	801.623	RHA	14219	0.20	0.47
13.78	1801.855	XYL	27683	0.44	1.06
14.36	783.958	XYL	11813	0.19	0.46
14.99	584.711		8827	0.14	0.34
16.06	716.574		6944	0.17	0.42
16.53	568.122		6218	0.14	0.34

MJ-2 TMS

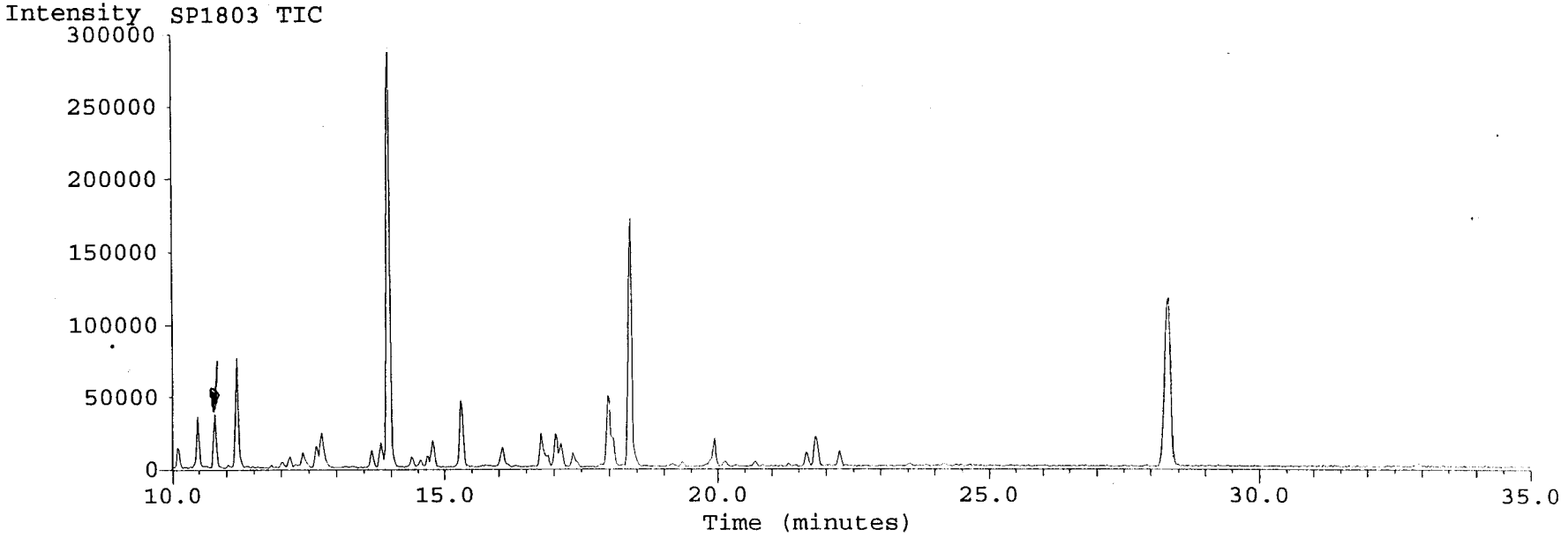
18.03		822.469 MAN	6227	0.20	0.49
18.19		540.470	6576	0.13	0.32
18.32		1674.528	19004	0.41	0.99
18.53		1080.146	9893	0.26	0.64
19.37		1133.555 CAL	12217	0.28	0.67
19.55		1228.271	10040	0.30	0.72
20.02		385.484	2736	0.09	0.23
20.55		791.699	3092	0.19	0.47
21.67	S	169559.658 CAL	454683	41.25	100.00
21.86		13868.237	217404	3.37	8.18
22.62		113990.889 CAL	464754	27.73	67.23
23.14		3902.545	47142	0.95	2.30
23.46		4427.769	45226	1.08	2.61
23.95		1026.286	10824	0.25	0.61
24.37		17996.025	163922	4.38	10.61
24.61		1339.819	14792	0.33	0.79
25.08		41473.953	293373	10.09	24.46
25.24		1947.675	23976	0.47	1.15
26.16		5998.551	66045	1.46	3.54
26.31		600.970	7310	0.15	0.35
26.65		637.123	5885	0.15	0.38
27.81		457.492	5491	0.11	0.27
30.06		17743.911 ILOS.	228430	4.32	10.46
30.61		1035.020	17049	0.25	0.61
30.87		952.503	16405	0.23	0.56

LINKAGE ANALYSIS

MJ-1 PMAA (NaOH)



X = "NON-CARBOHYDRATE"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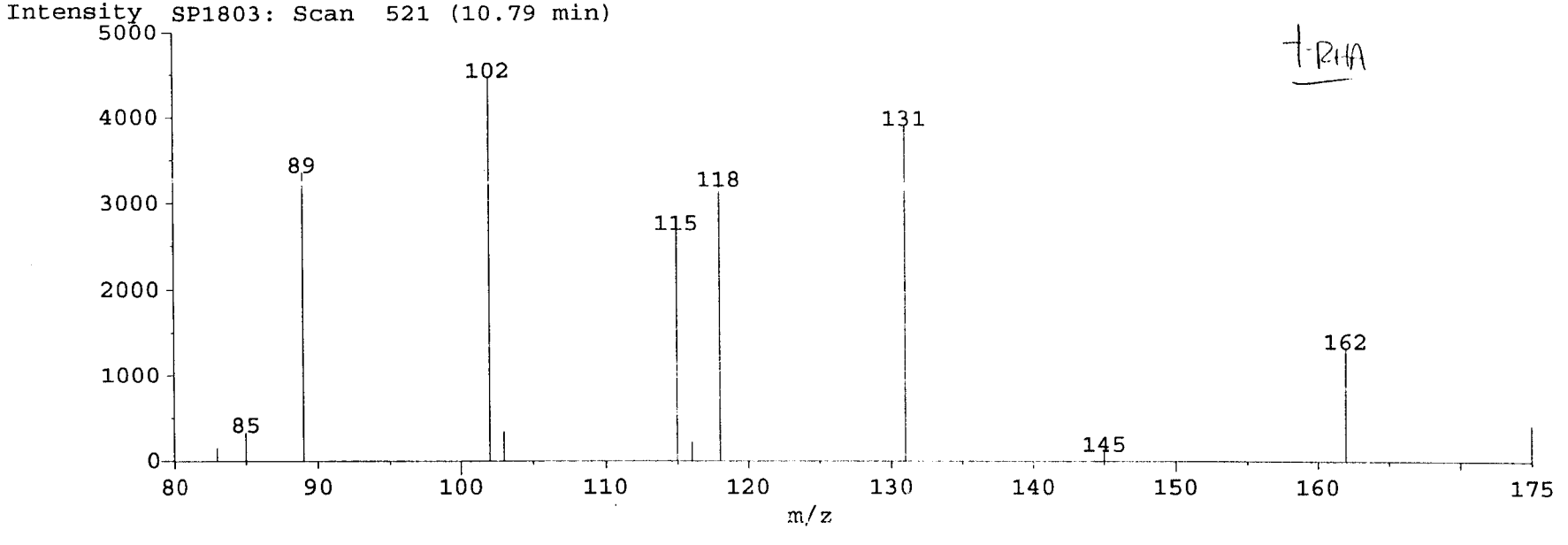
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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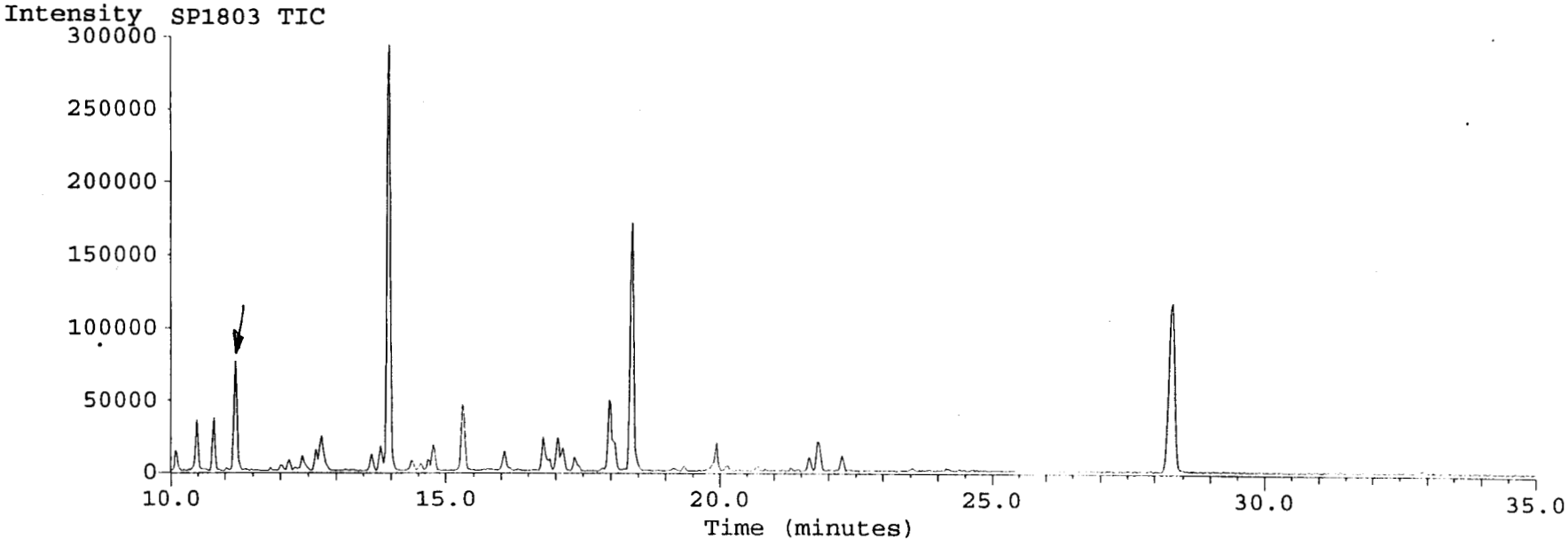
5000
4000
3000
2000
1000
0
102
89
118
115
131
162
85
145
80
90
100
110
120
130
140
150
160
175
m/z

T-RHA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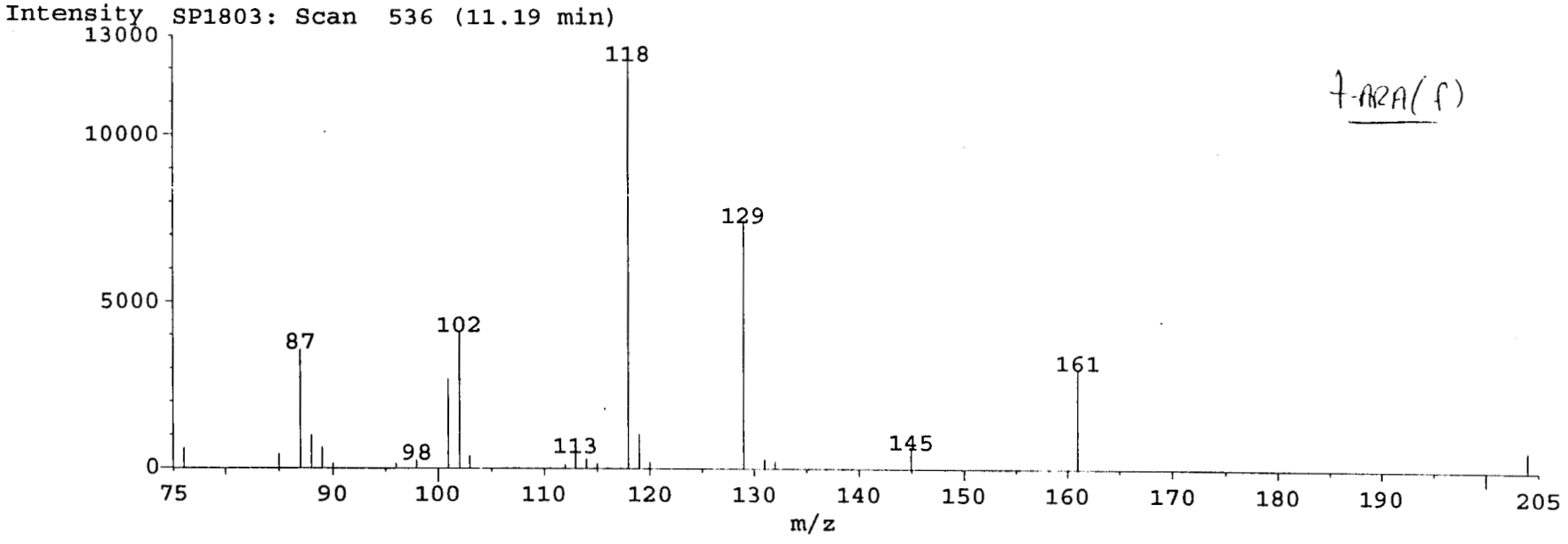
35.0
Time (minutes)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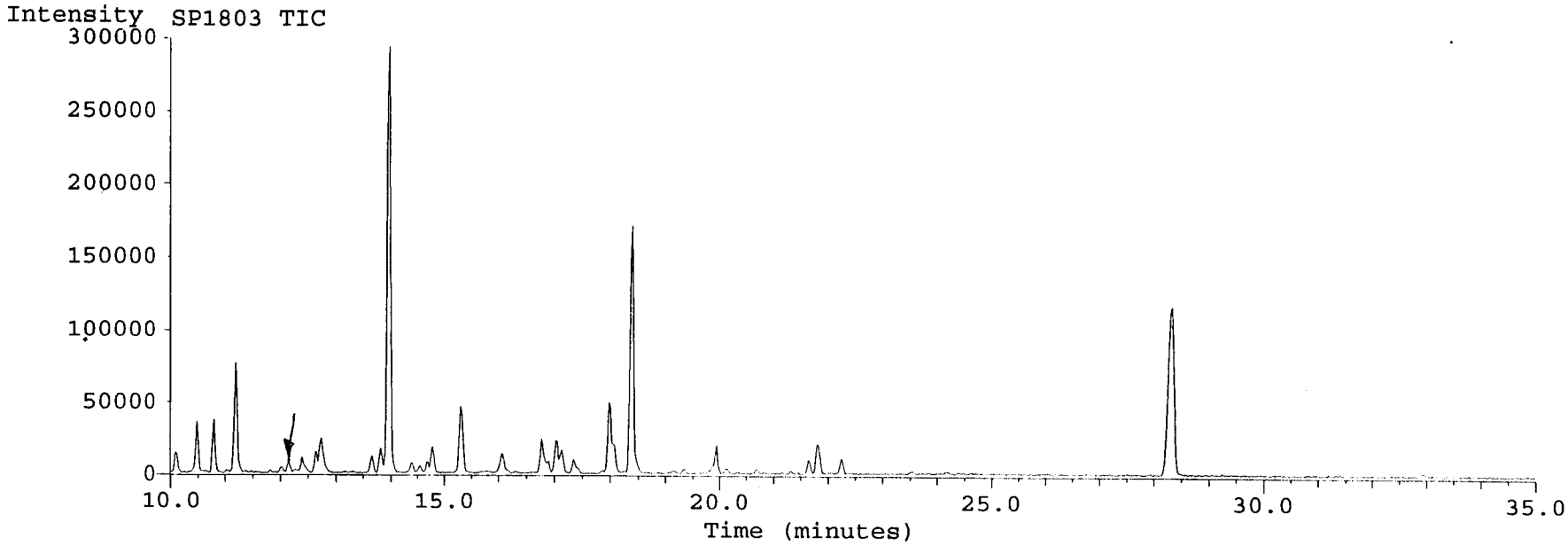
13000
10000
5000
0
118
129
87
102
161
98
113
145
75
90
100
110
120
130
140
150
160
170
180
190
205
m/z

7-AR2A(F)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300000
250000
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100000
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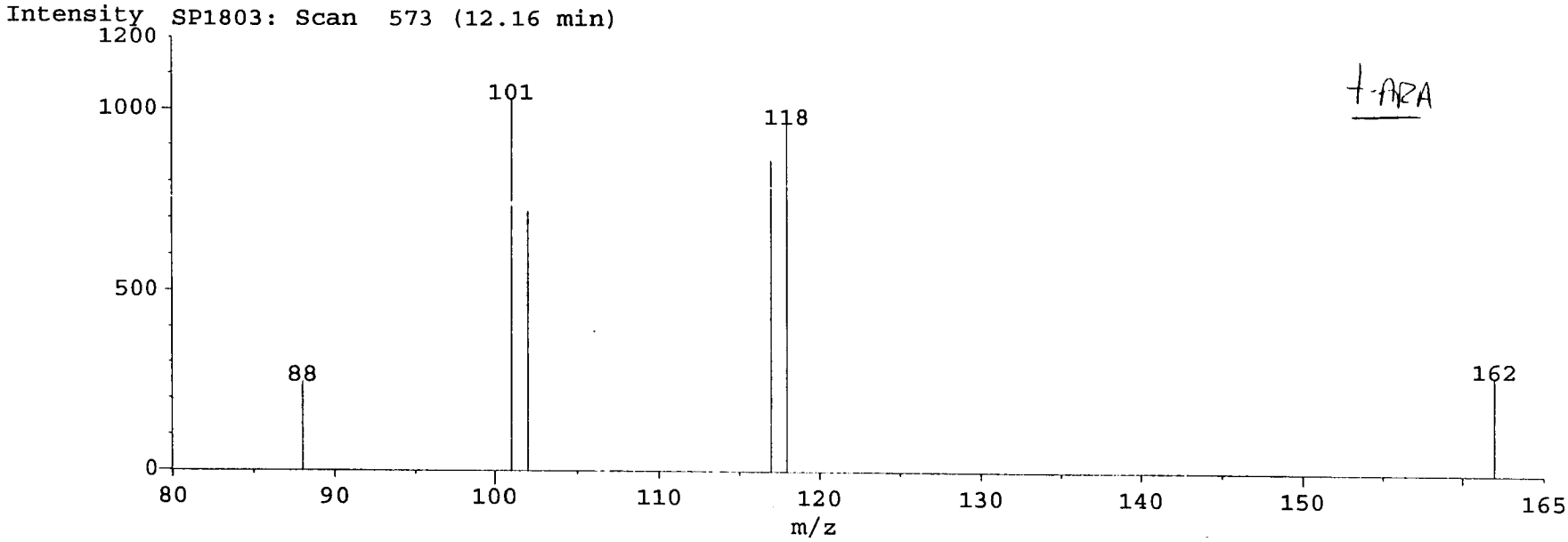
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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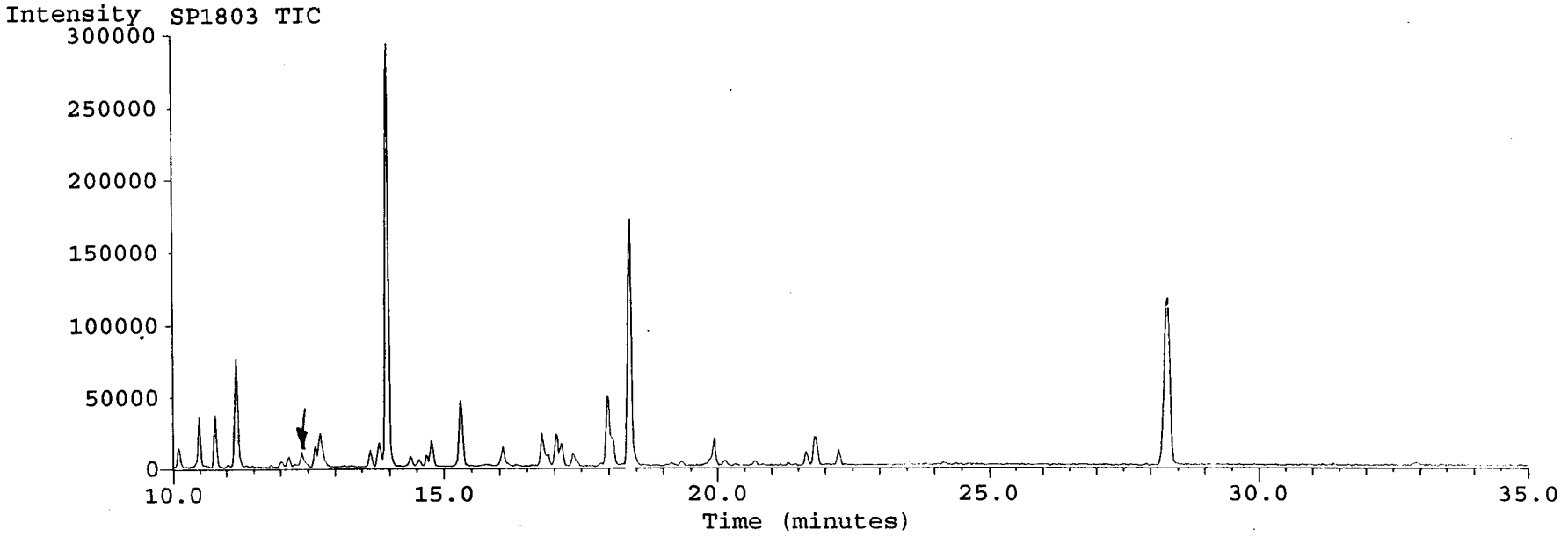
1200
1000
500
0
101
118
88
162
80
90
100
110
120
130
140
150
165
m/z

4-ARA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250000
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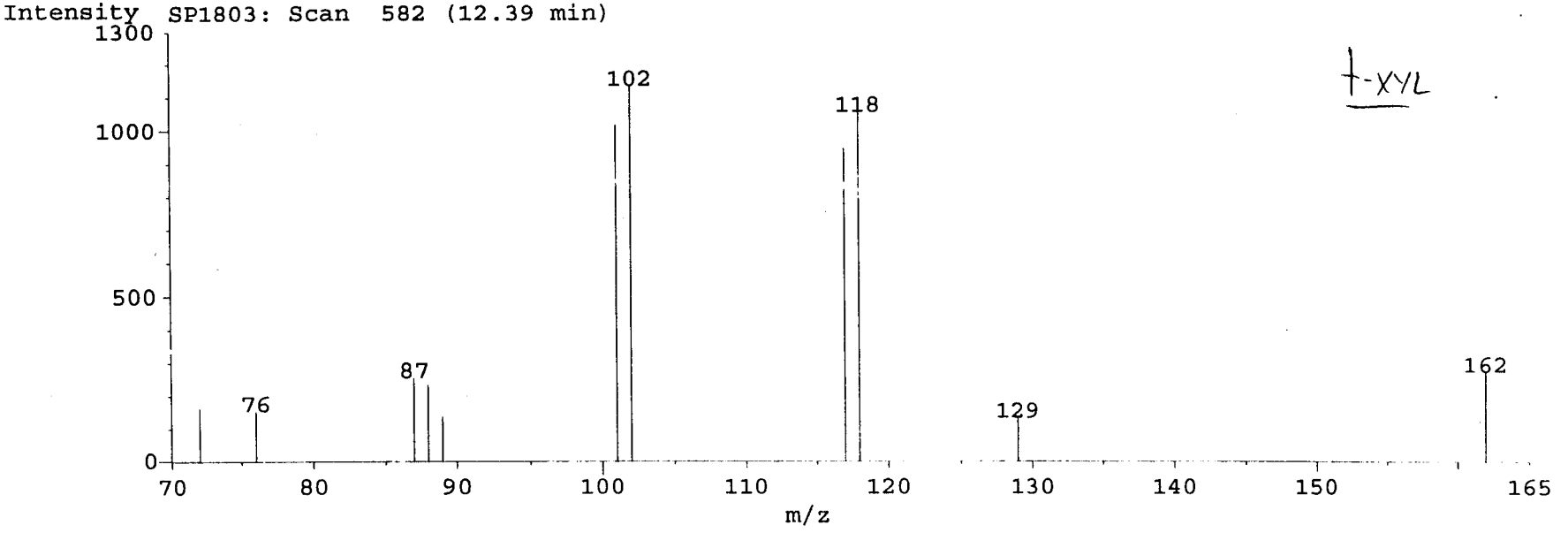
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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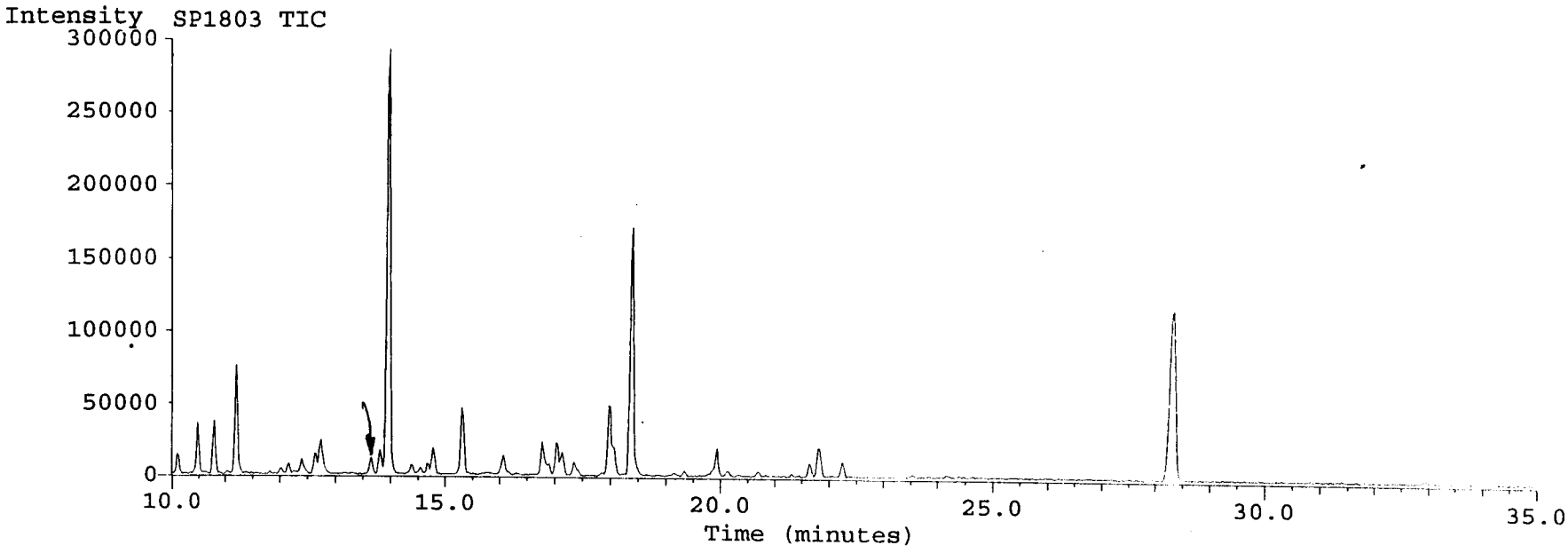
1300
1000
500
0
102
118
87
76
129
162
70
80
90
100
110
120
130
140
150
165
m/z

t-XYL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300000
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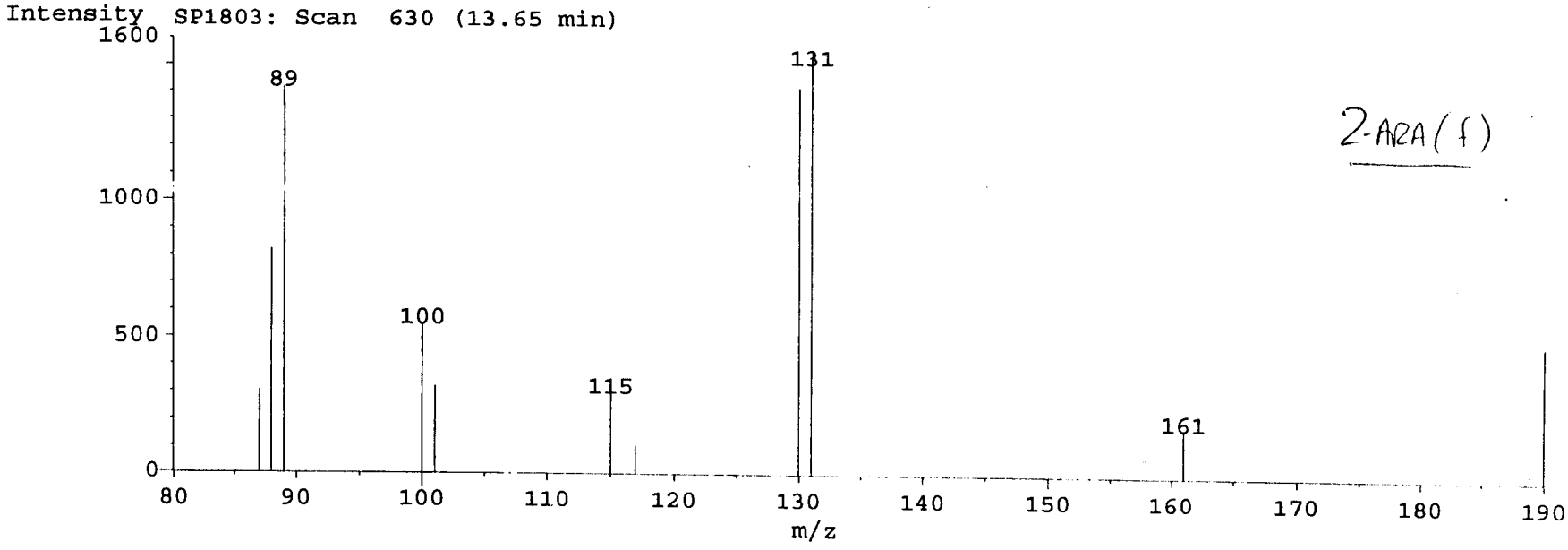
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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1600
1000
500
0
89
100
115
131
161
80
90
100
110
120
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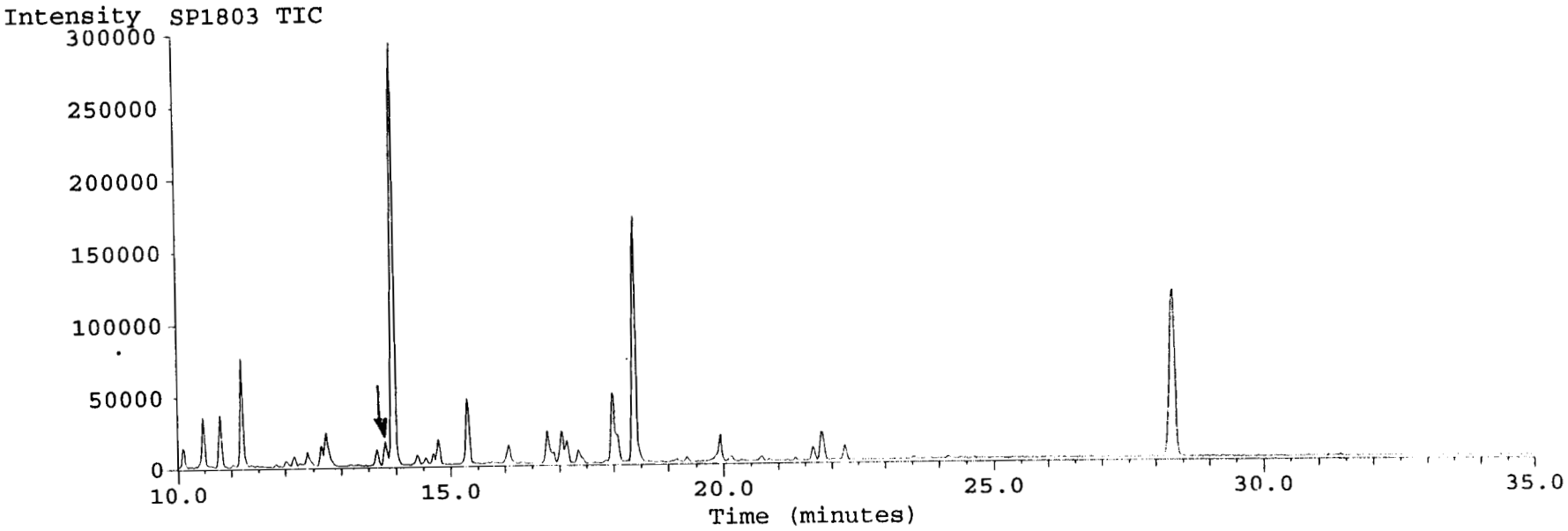
140
150
160
170
180
190
m/z

2-ARA(f)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300000
250000
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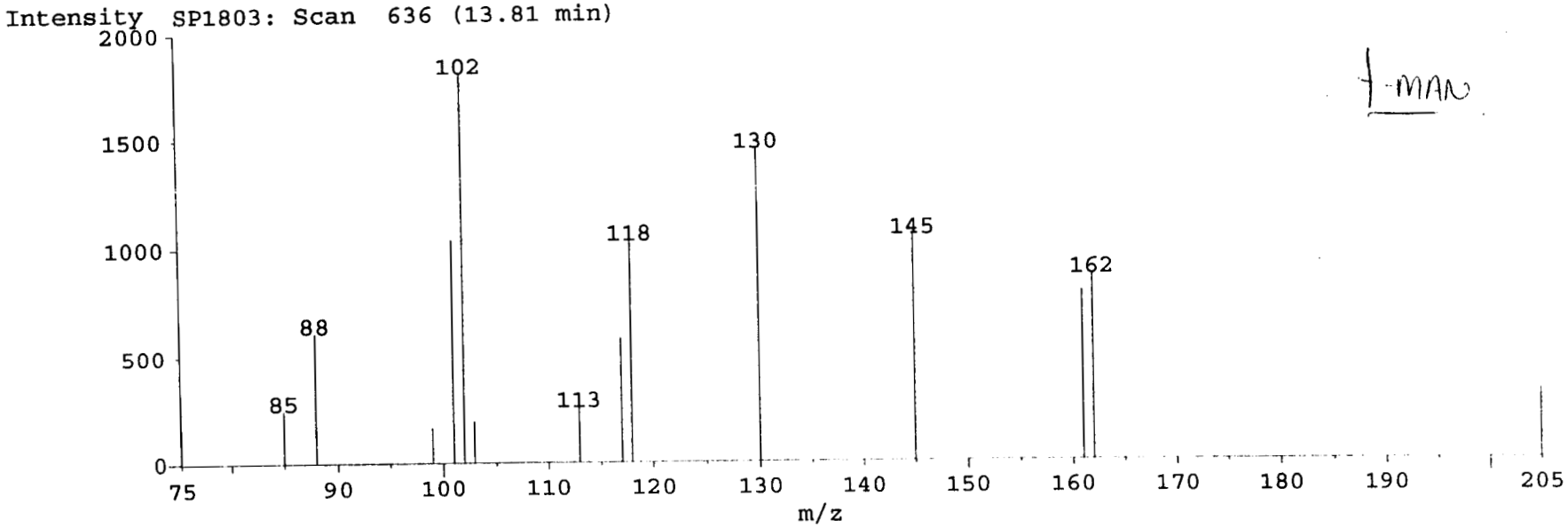
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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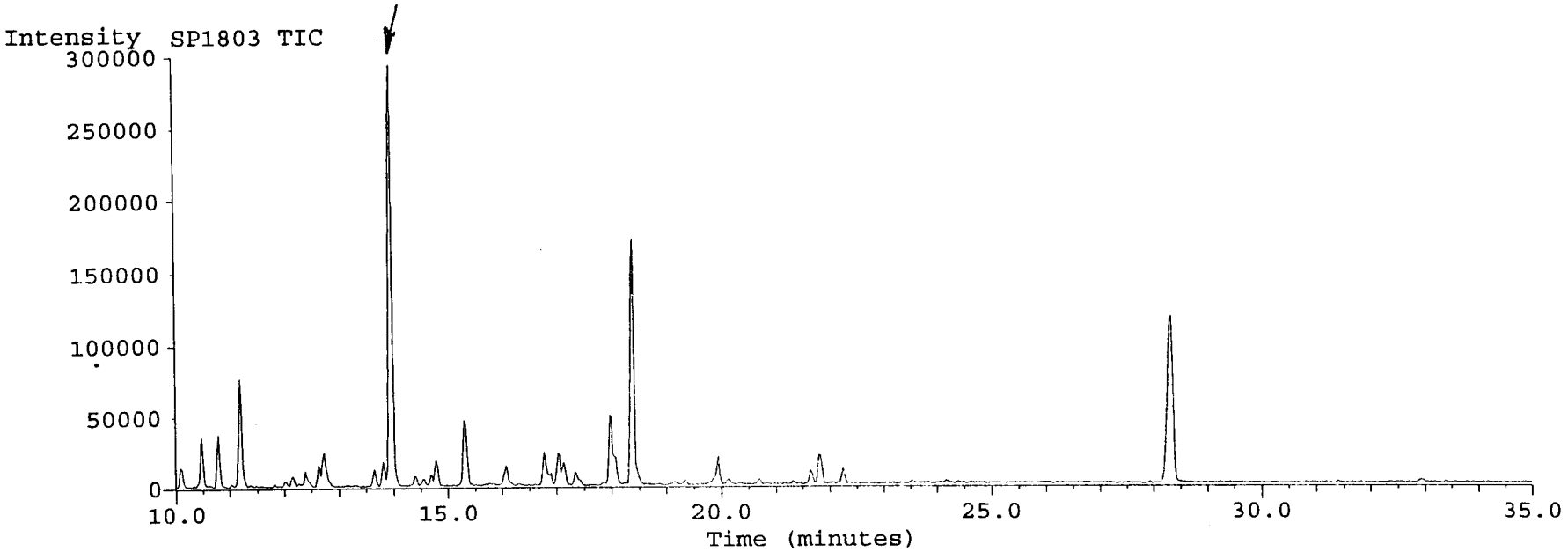
2000
1500
1000
500
0
102
130
118
145
162
88
85
113
75
90
100
110
120
130
140
150
160
170
180
190
205
m/z

4-MAN


Intensity SP1803 TIC
300000
250000
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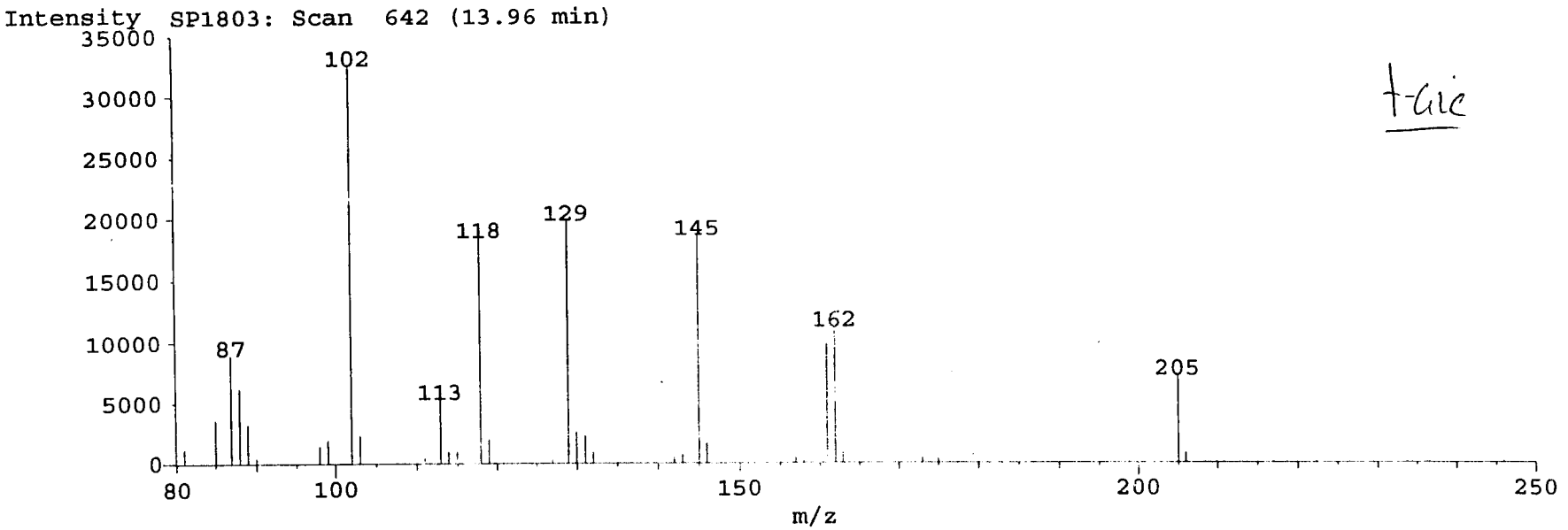
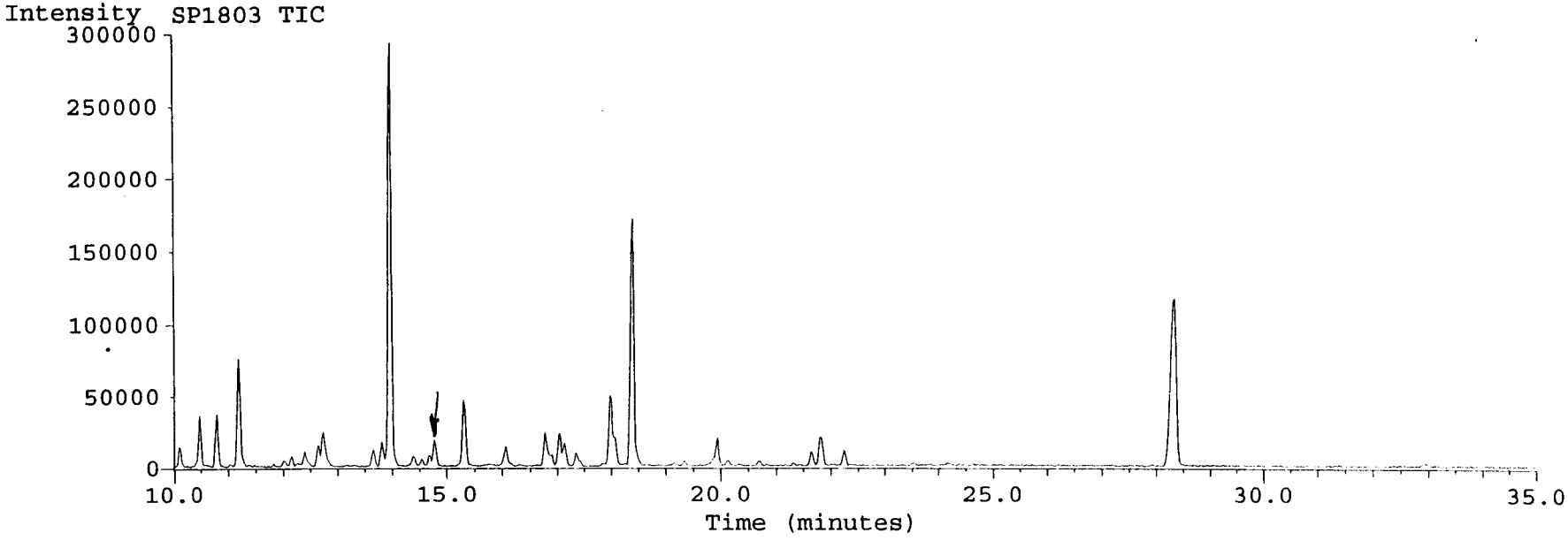
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 642 (13.96 min)
35000
30000
25000
20000
15000
10000
5000
0
102
87
113
118
129
145
162
205
80
100
150
200
250
m/z

t-aic


Intensity SP1803 TIC
300000
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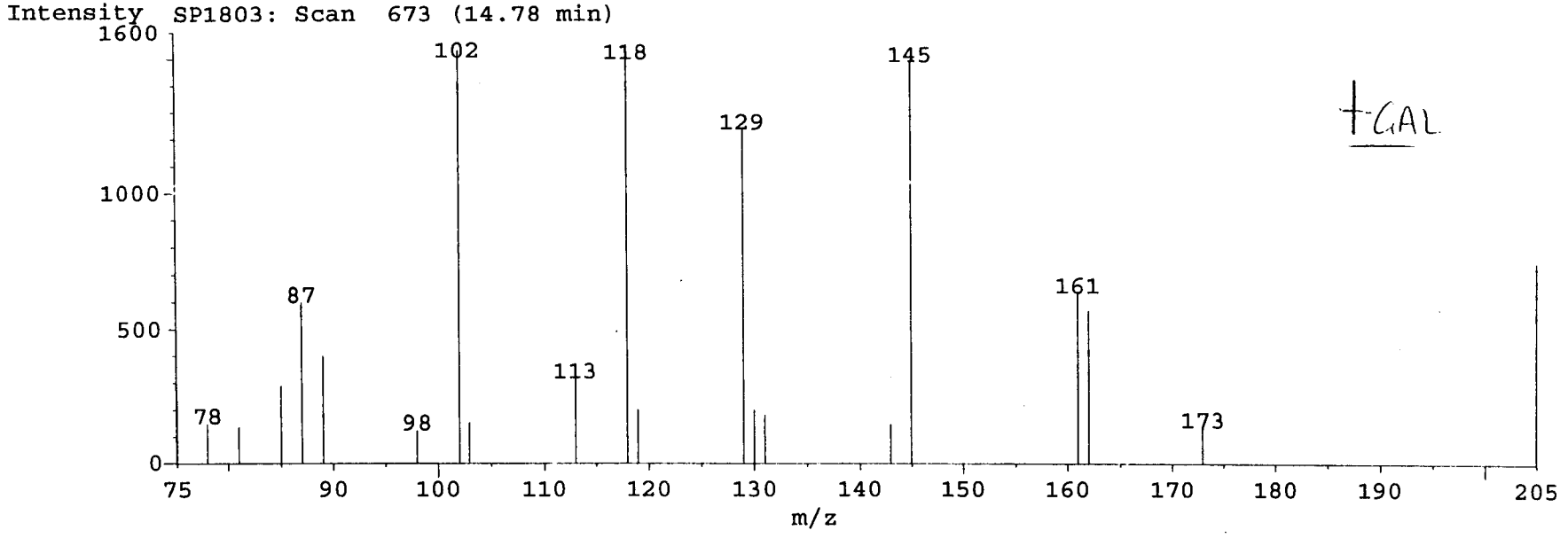
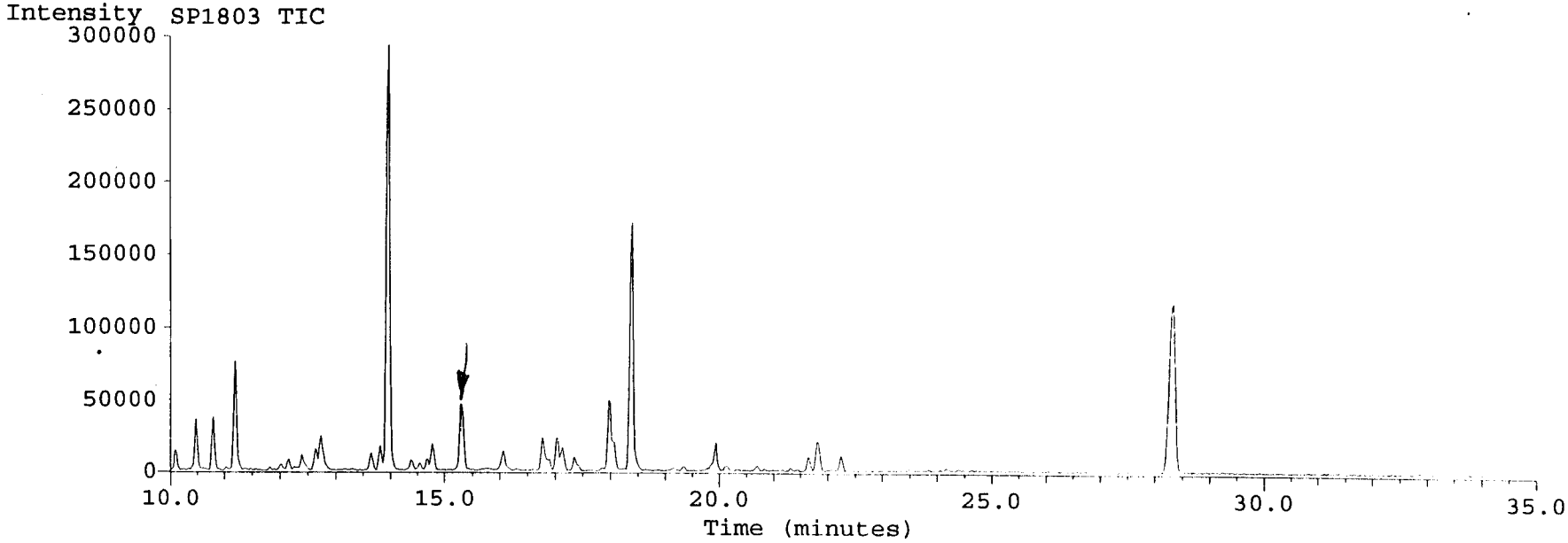
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 673 (14.78 min)
1600
1000
500
0
102
118
145
129
87
161
113
78
98
173
75
90
100
110
120
130
140
150
160
170
180
190
205
m/z

+GAL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200000
150000
100000
50000
0
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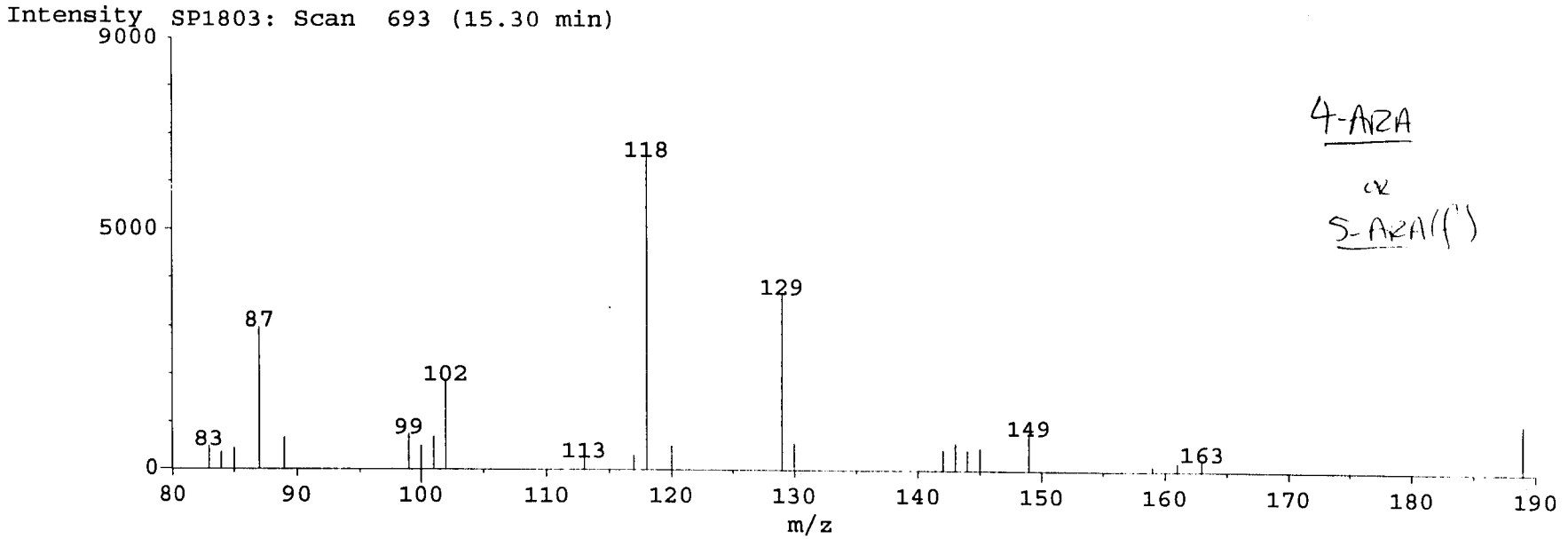
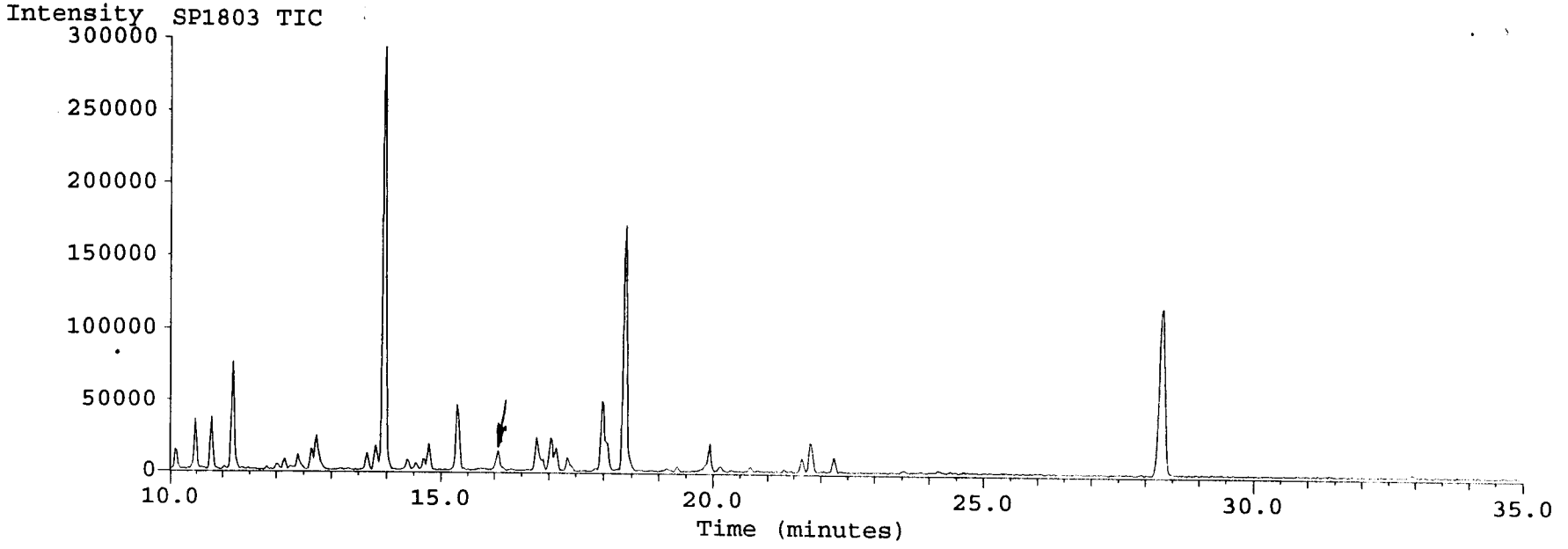
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 693 (15.30 min)
9000
5000
0
118
129
87
102
99
83
113
149
163
80
90
100
110
120
130
140
150
160
170
180
190
m/z

4-AZA
or
5-AZA(?)


Intensity SP1803 TIC
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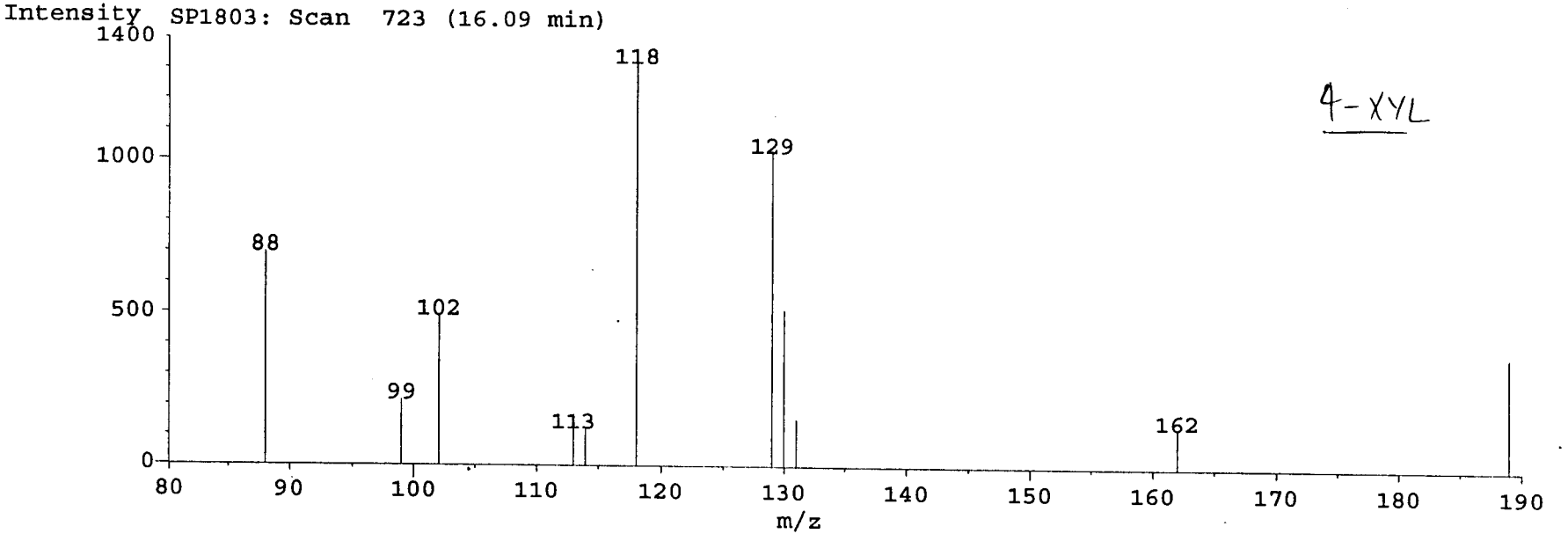
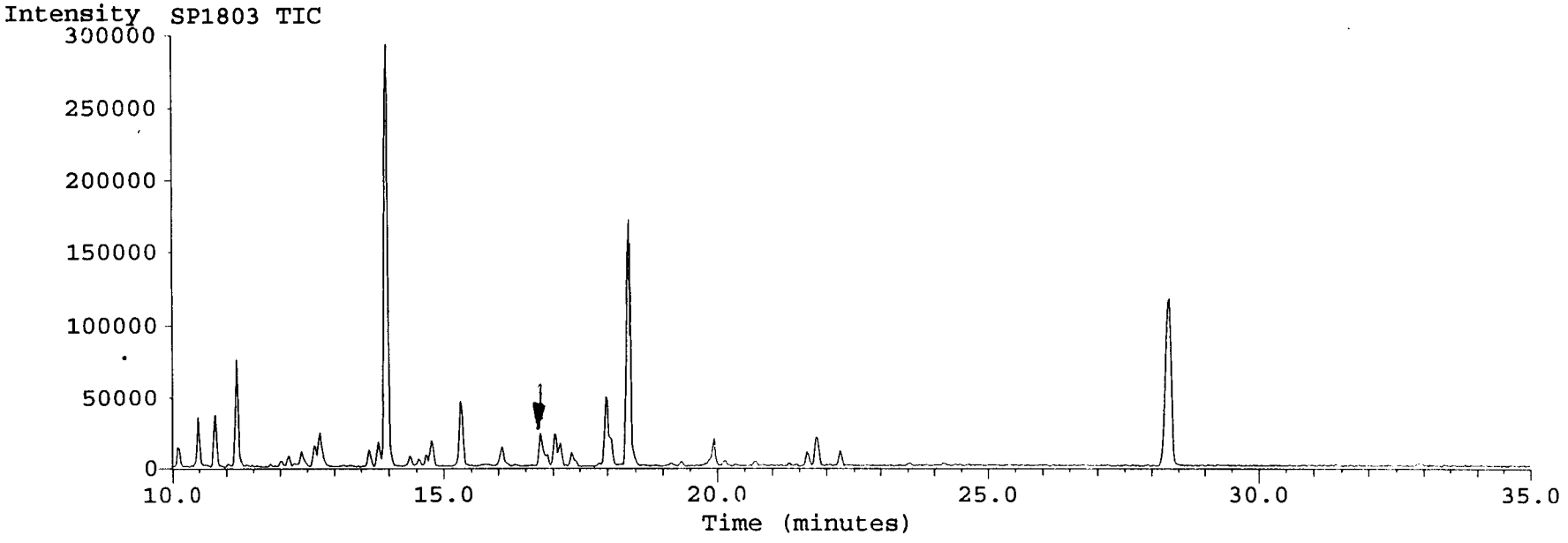
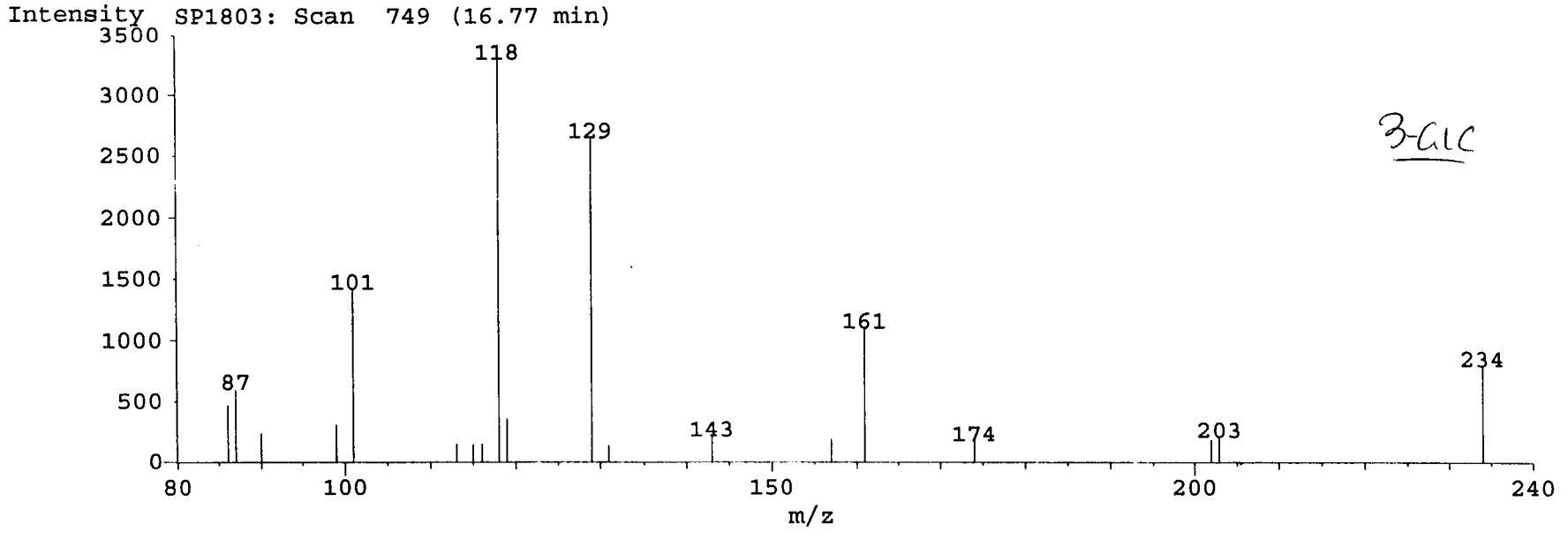
200000
150000
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)

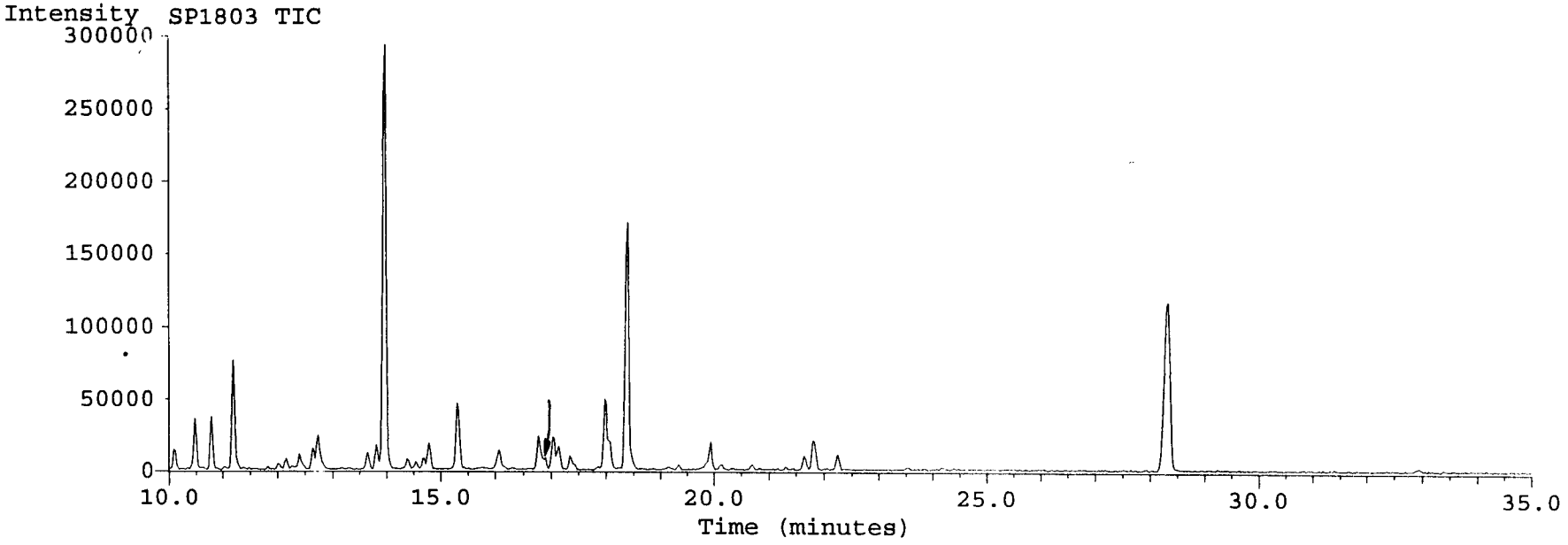

Intensity SP1803: Scan 723 (16.09 min)
1400
1000
500
0
118
129
88
102
99
113
162
80
90
100
110
120
130
140
150
160
170
180
190
m/z

4-XYL


Intensity SP1803 TIC
300000
250000
200000
150000
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 749 (16.77 min)
3500
3000
2500
2000
1500
1000
500
0
118
129
101
161
87
143
174
203
234
80
100
150
200
240
m/z

3-CAC


Intensity SP1803 TIC
300000
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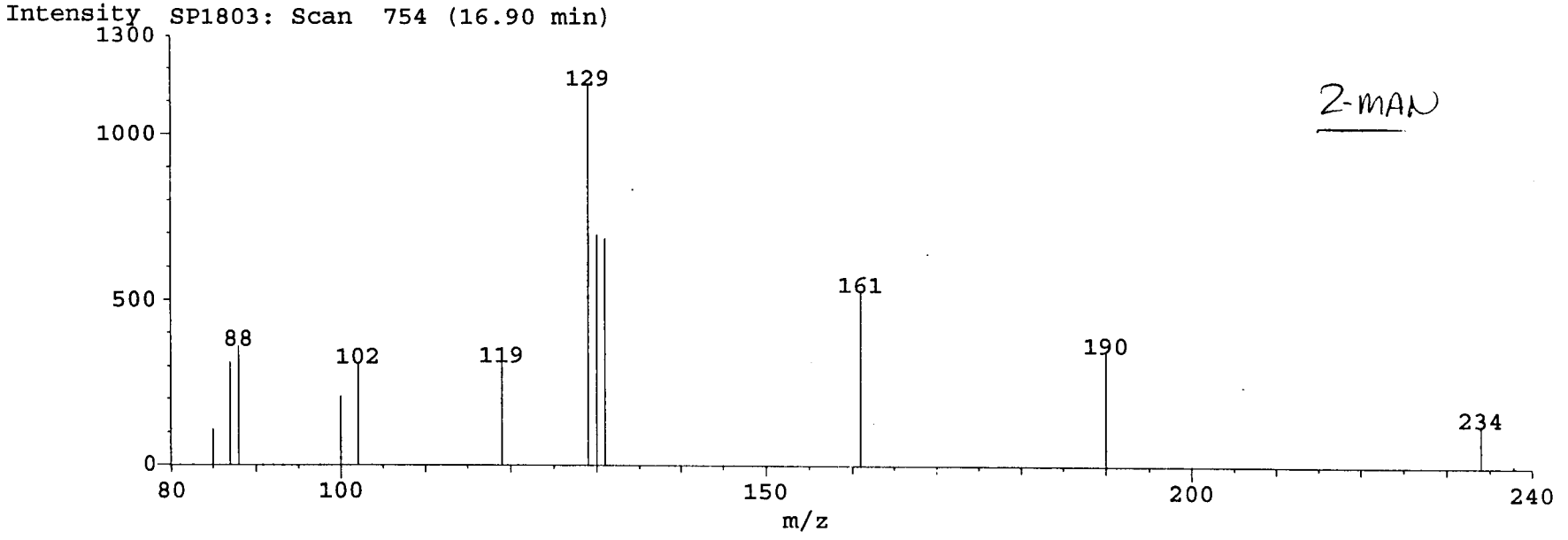
200000
150000
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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1000
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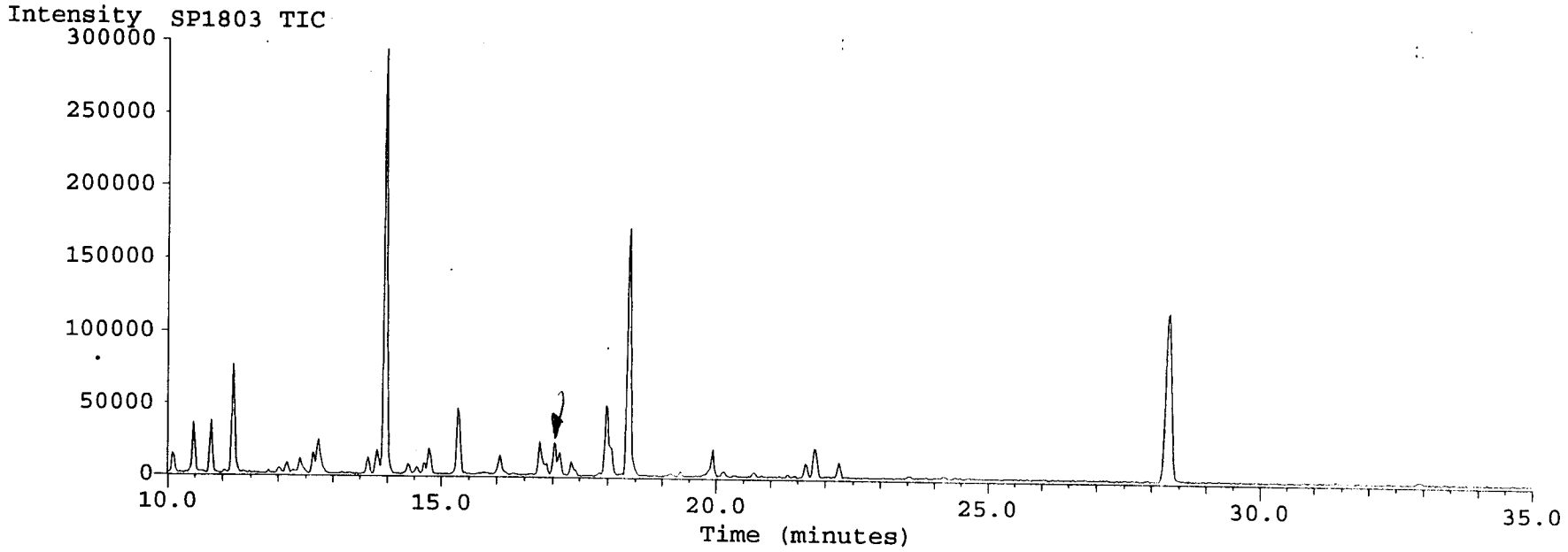
0
129
161
88
102
119
190
234
80
100
150
200
240
m/z

2-MAN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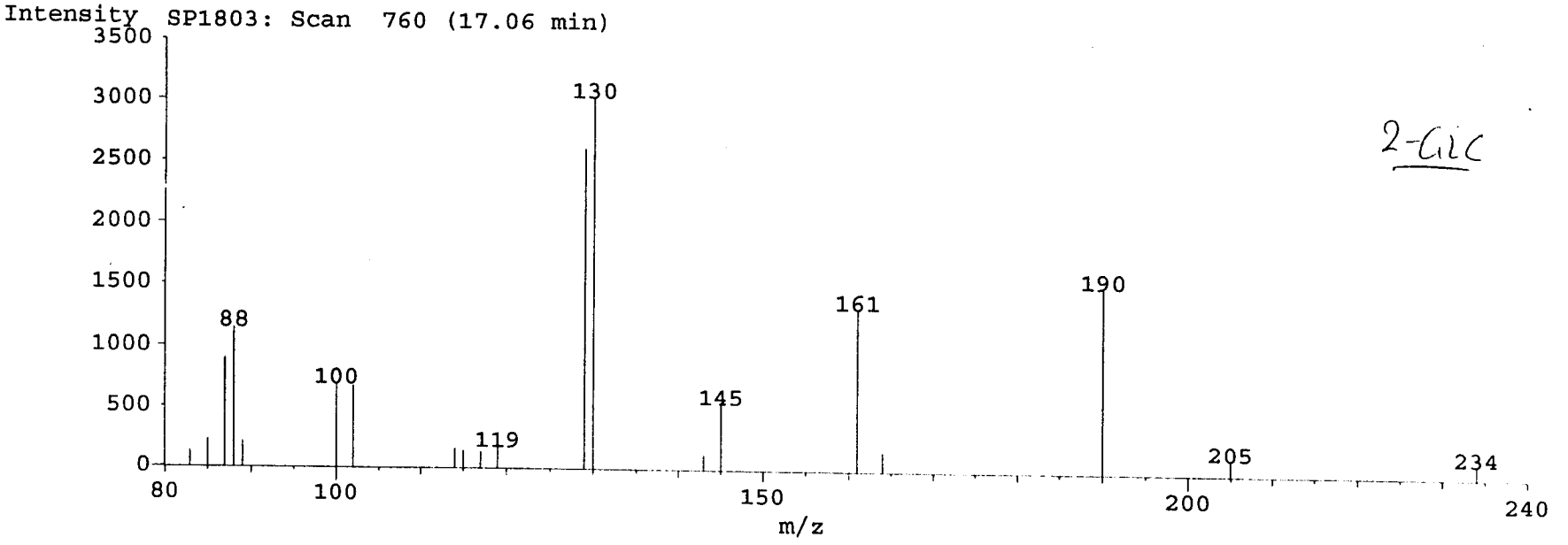
150000
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)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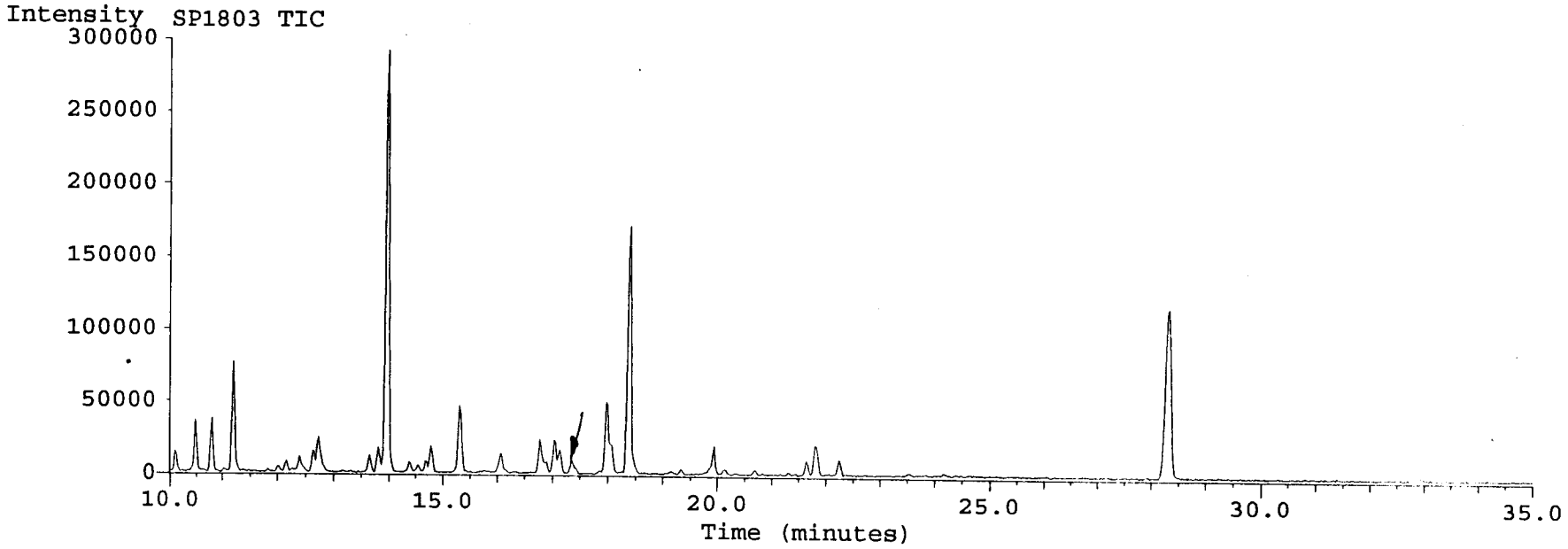
3000
2500
2000
1500
1000
500
0
88
100
119
130
145
161
190
205
234
80
100
150
200
240
m/z

2-GIC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250000
200000
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100000
50000
0
10.0
15.0
20.0
25.0
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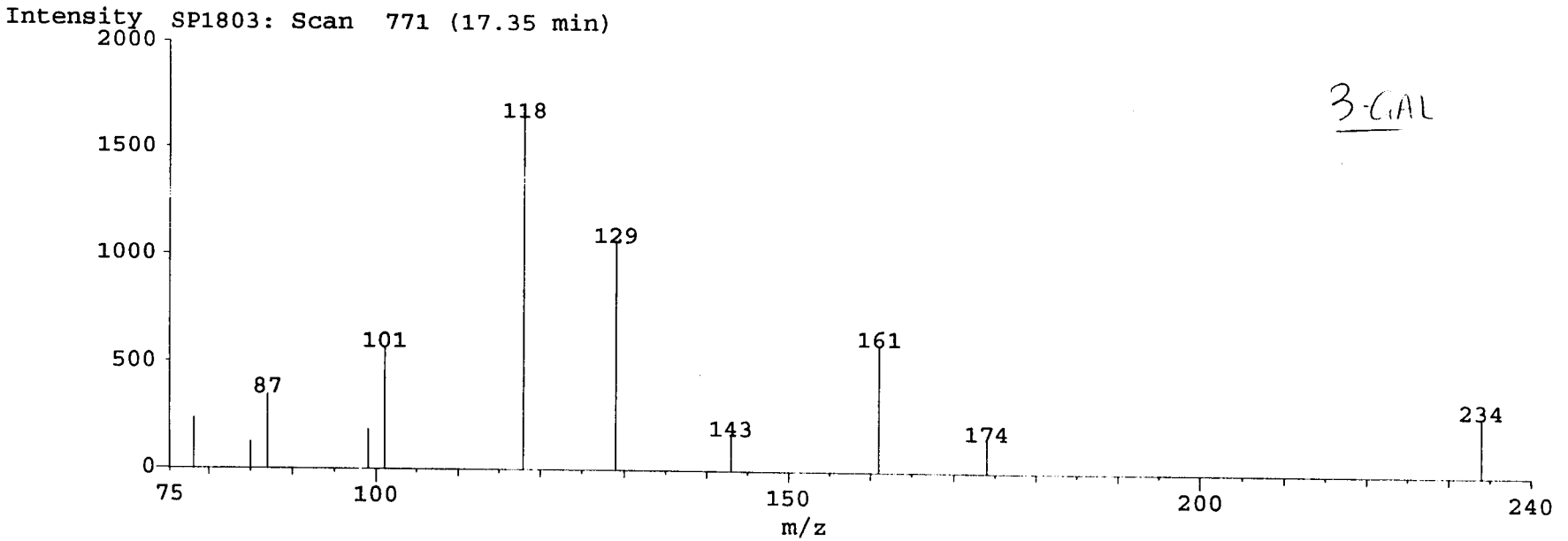
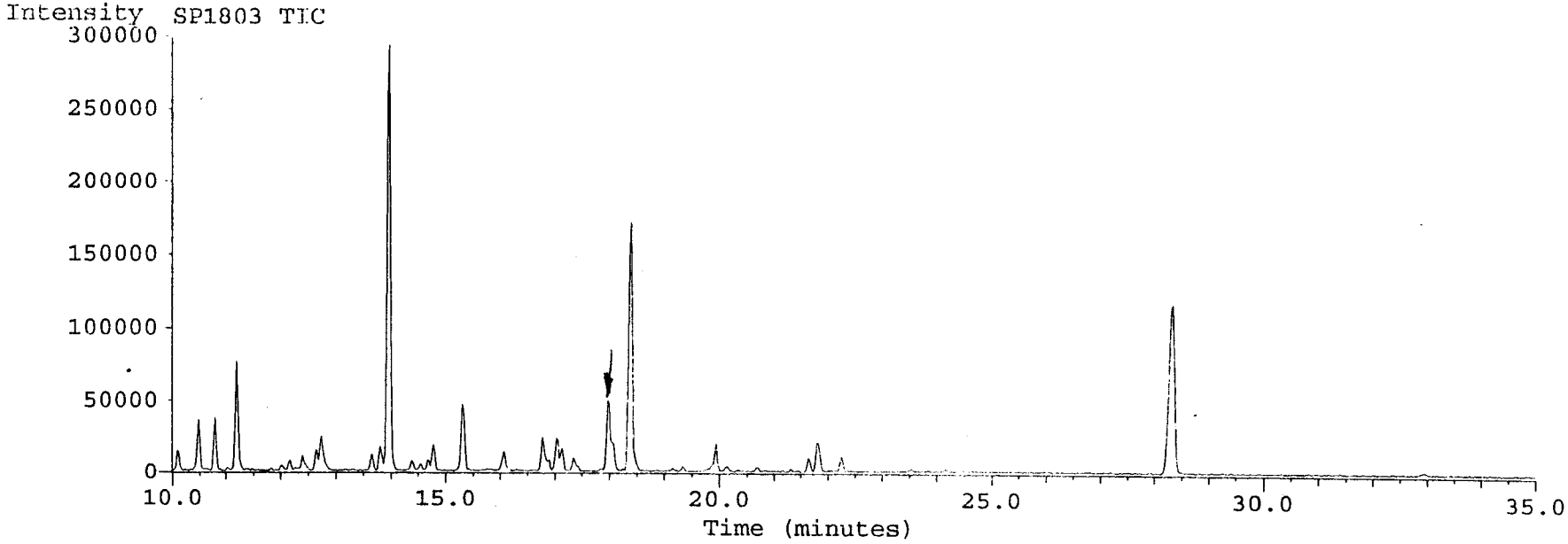
35.0
Time (minutes)


Intensity SP1803: Scan 771 (17.35 min)
2000
1500
1000
500
0
118
129
101
87
143
161
174
234
75
100
150
200
240
m/z

3-CAL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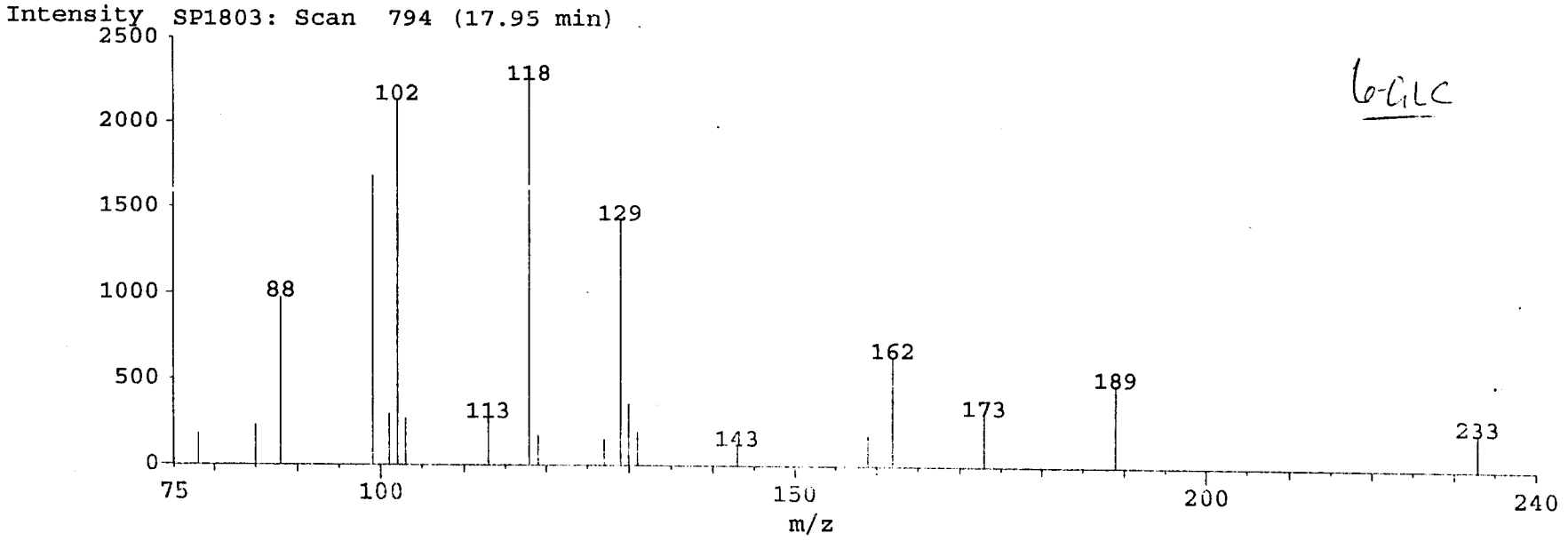
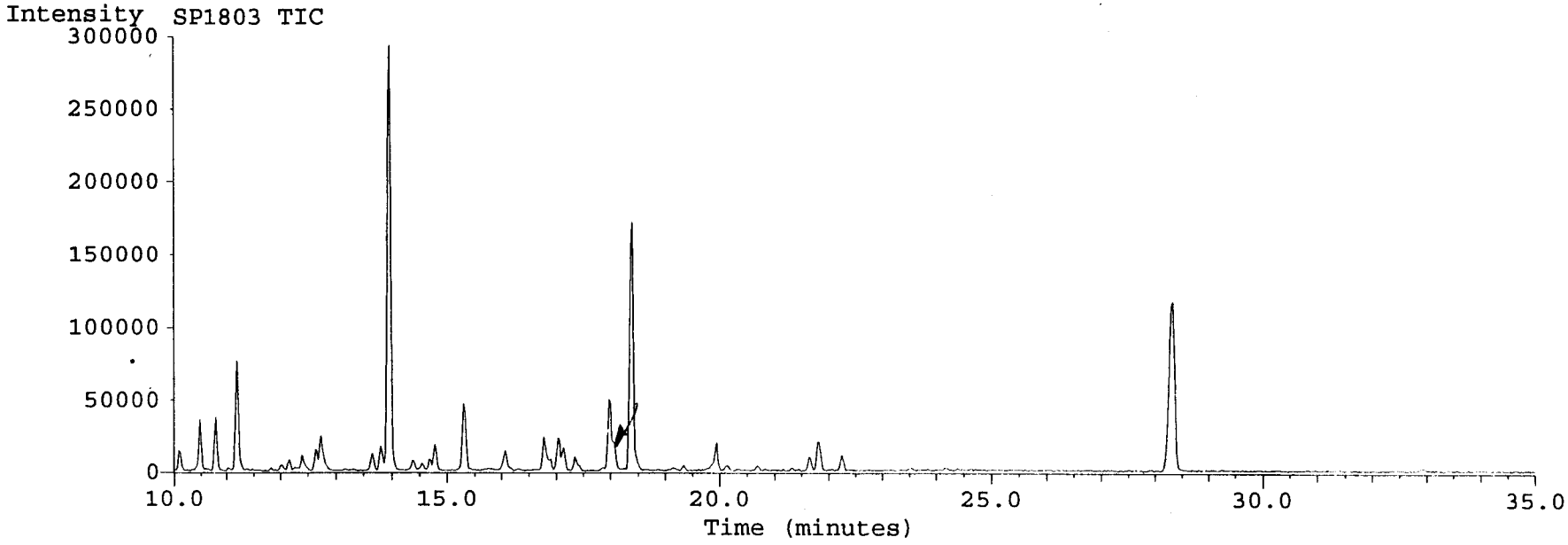
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 794 (17.95 min)
2500
2000
1500
1000
500
0
118
102
129
88
162
189
173
113
143
233
75
100
150
200
240
m/z

6-GLC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300000
250000
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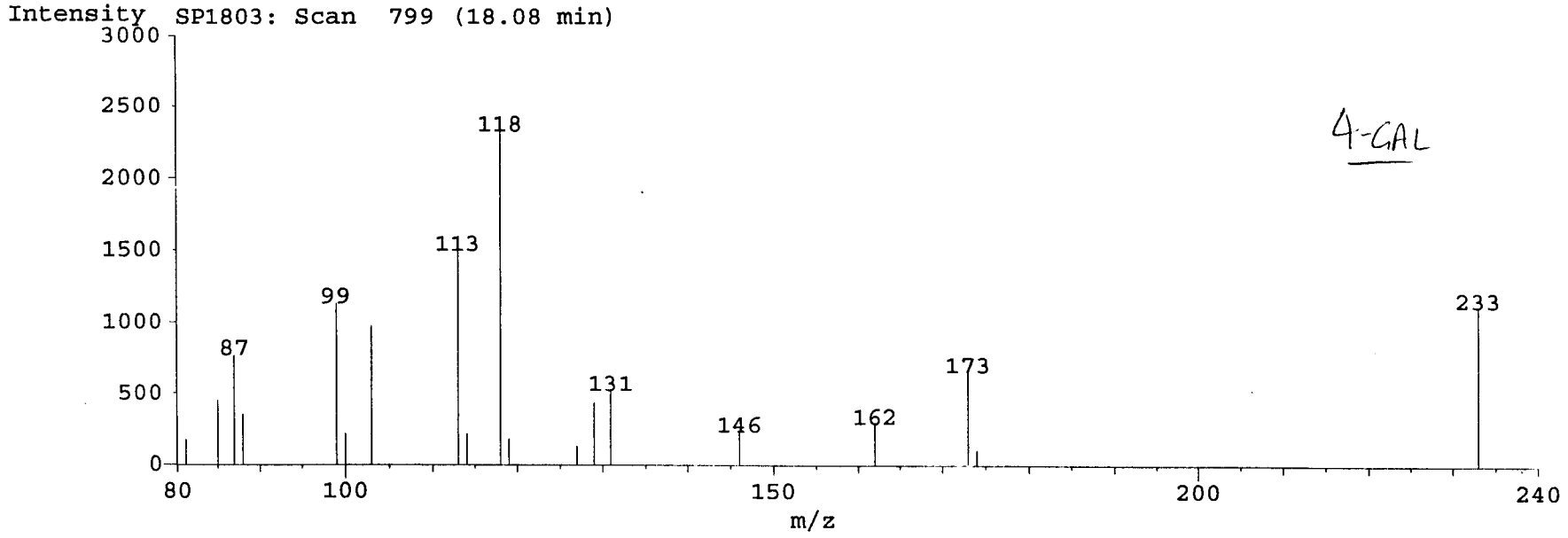
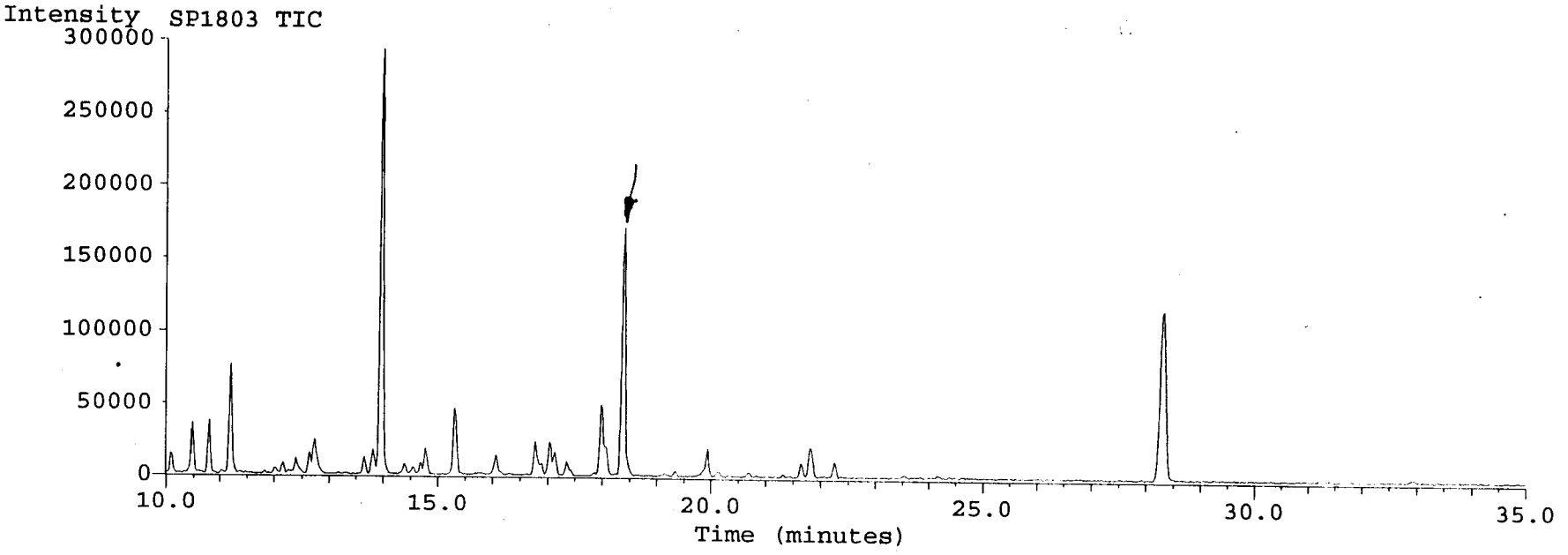
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 799 (18.08 min)
3000
2500
2000
1500
1000
500
0
80
100
150
200
240
m/z
118
113
99
87
131
146
162
173
233

4-CAL


Intensity SP1803 TIC
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50000
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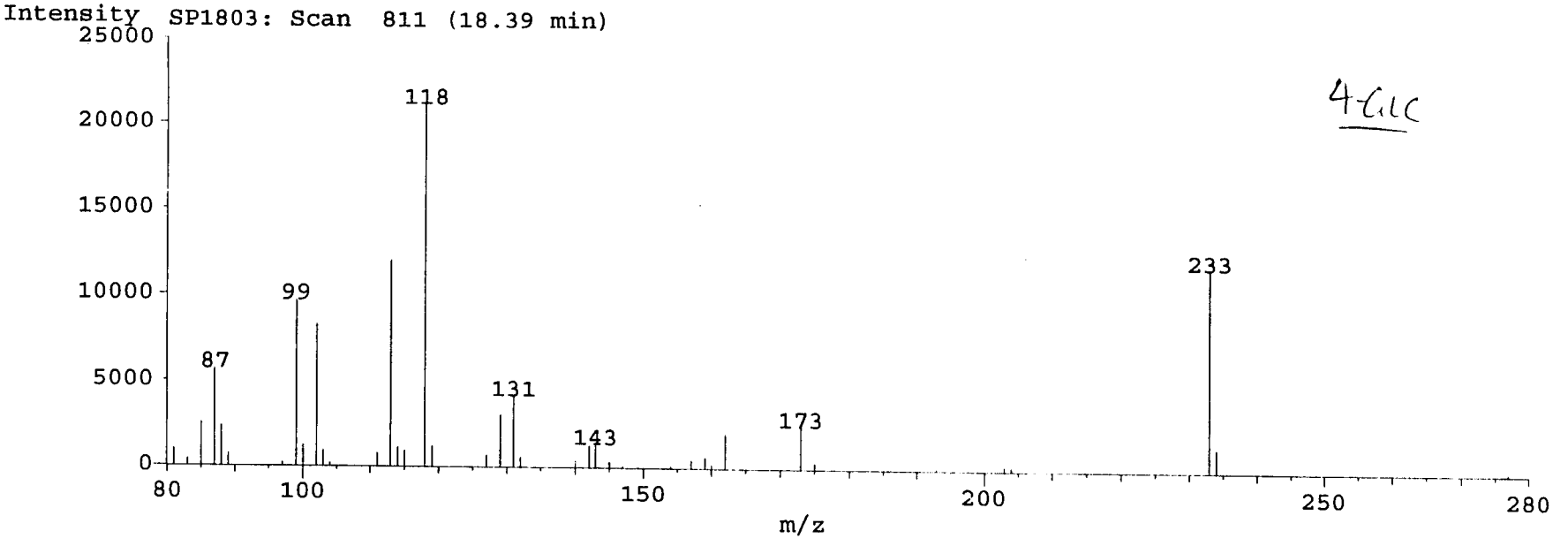
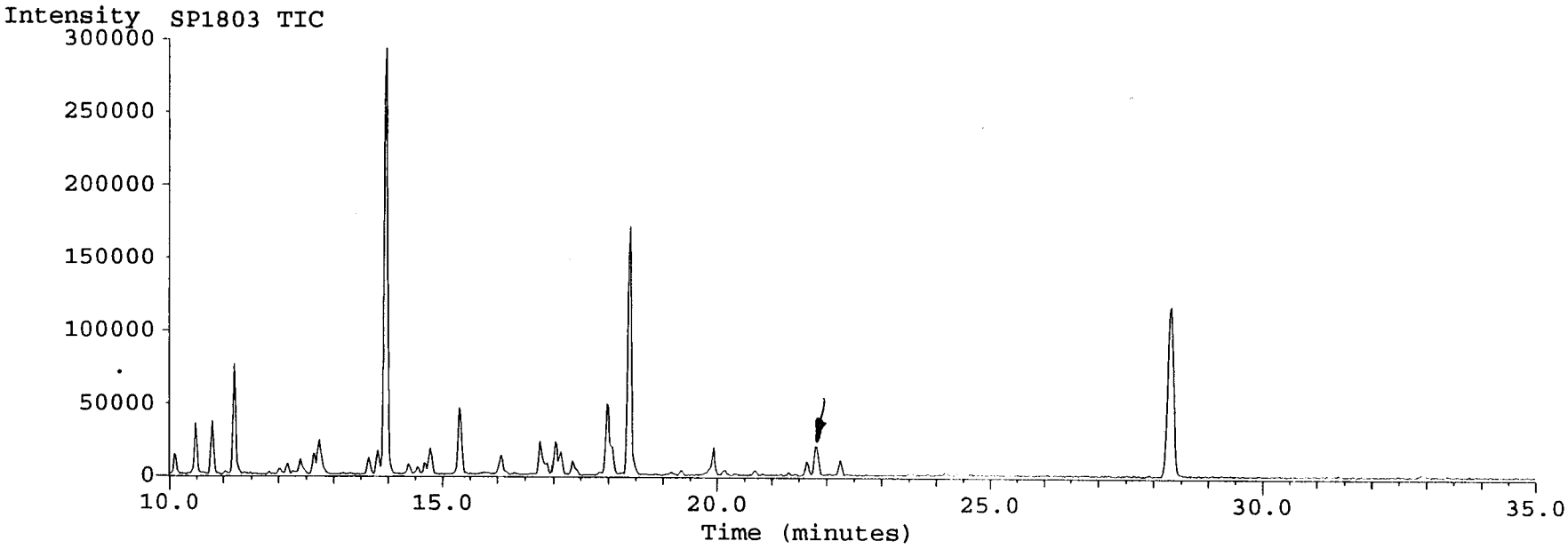
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 811 (18.39 min)
25000
20000
15000
10000
5000
0
118
99
87
131
143
173
233
80
100
150
200
250
280
m/z

4-Cl C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50000
0
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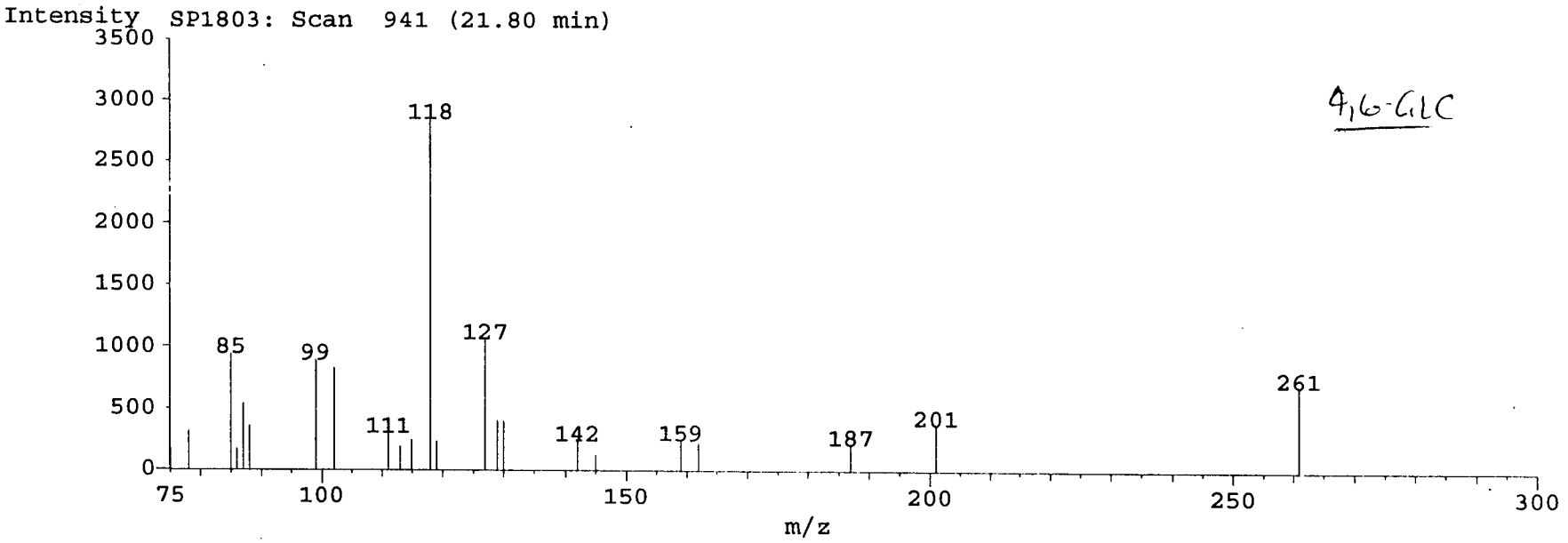
15.0
20.0
25.0
30.0
35.0
Time (minutes)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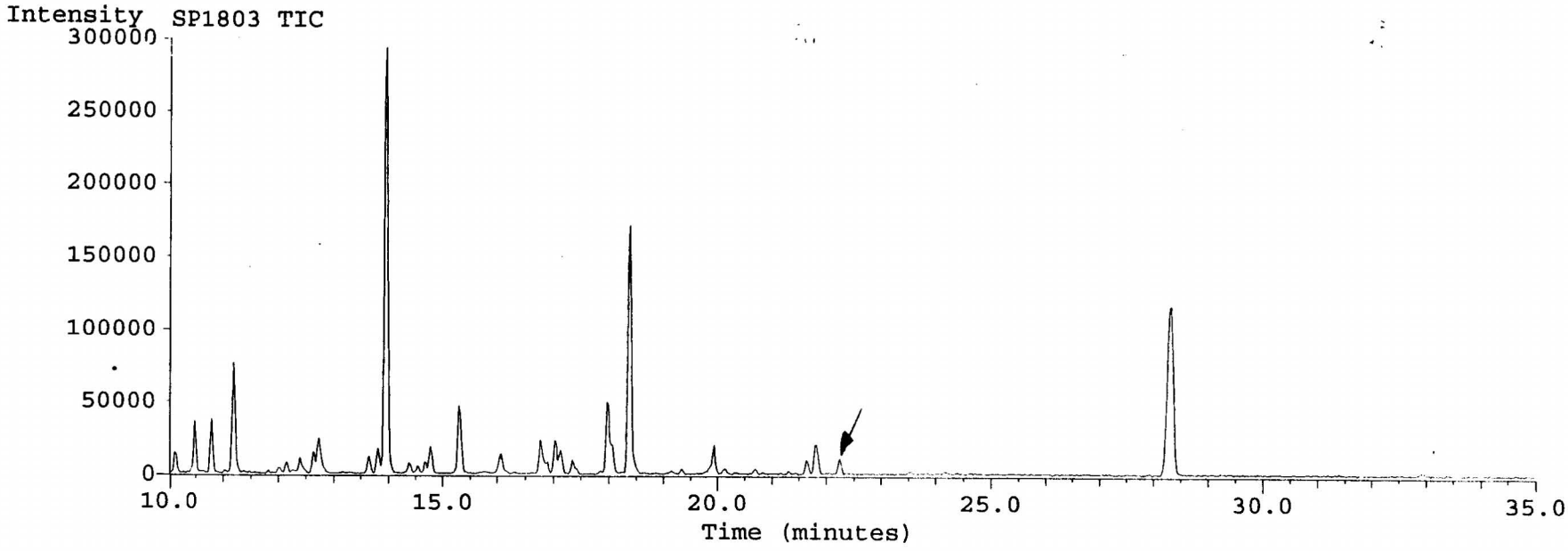
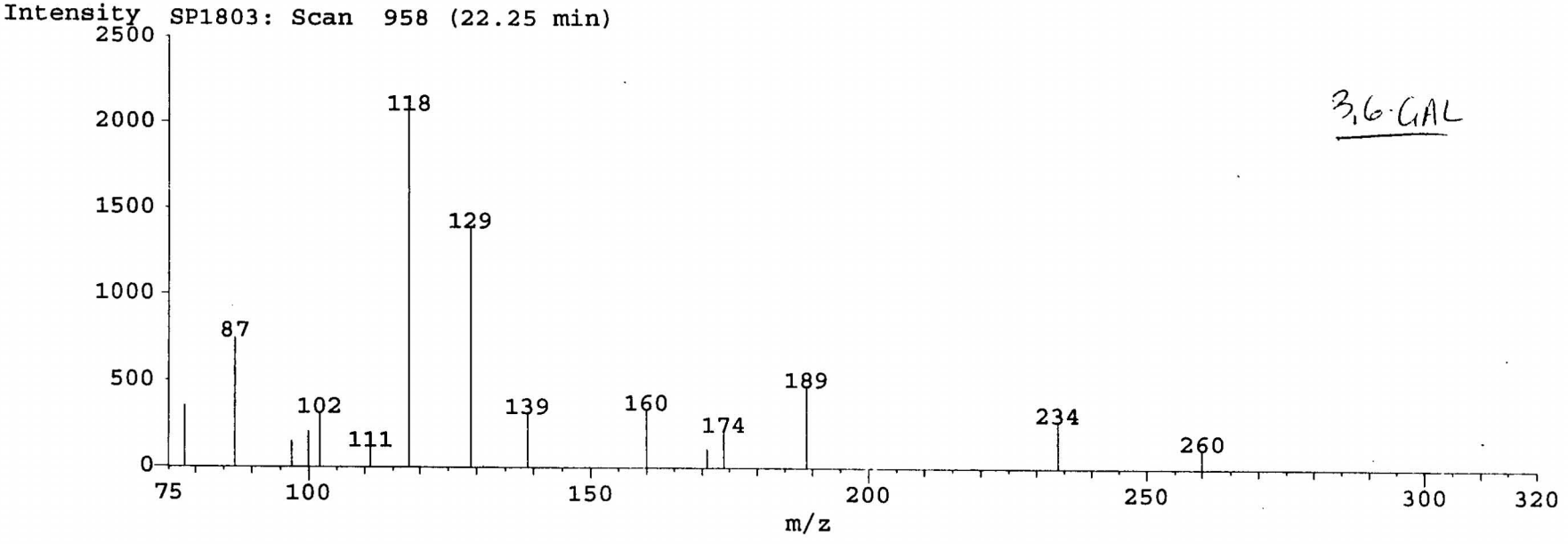
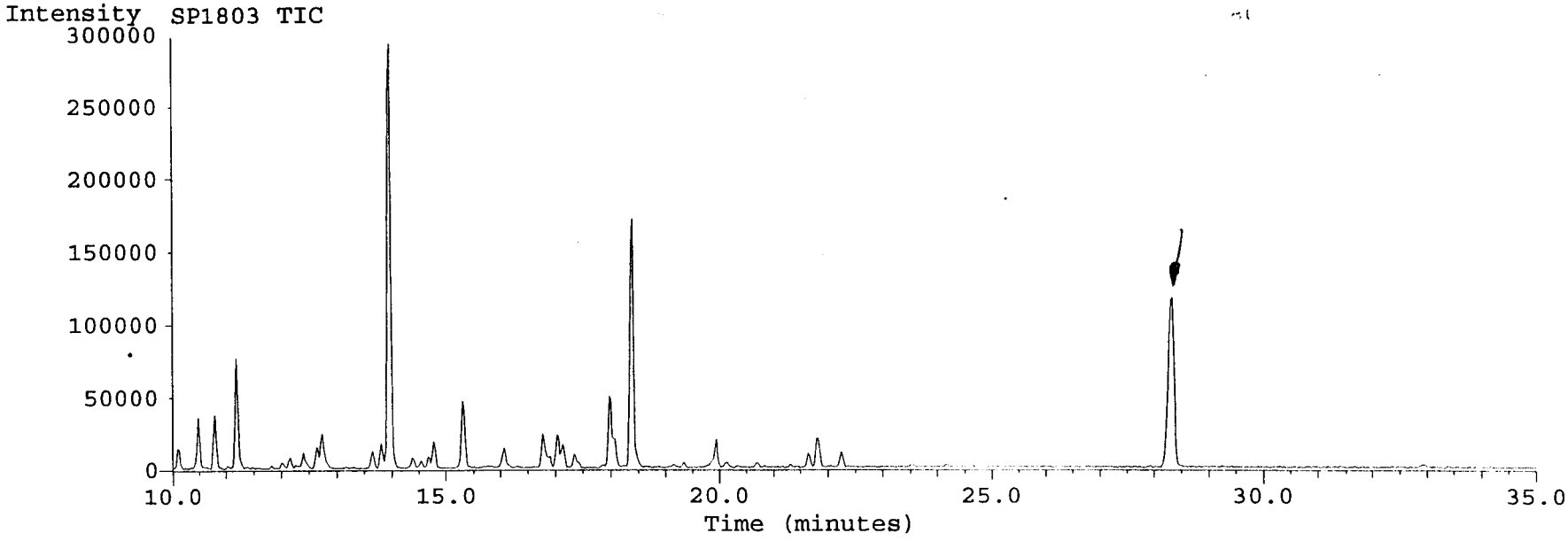
3500
3000
2500
2000
1500
1000
500
0
118
85
99
127
111
142
159
187
201
261
75
100
150
200
250
300
m/z

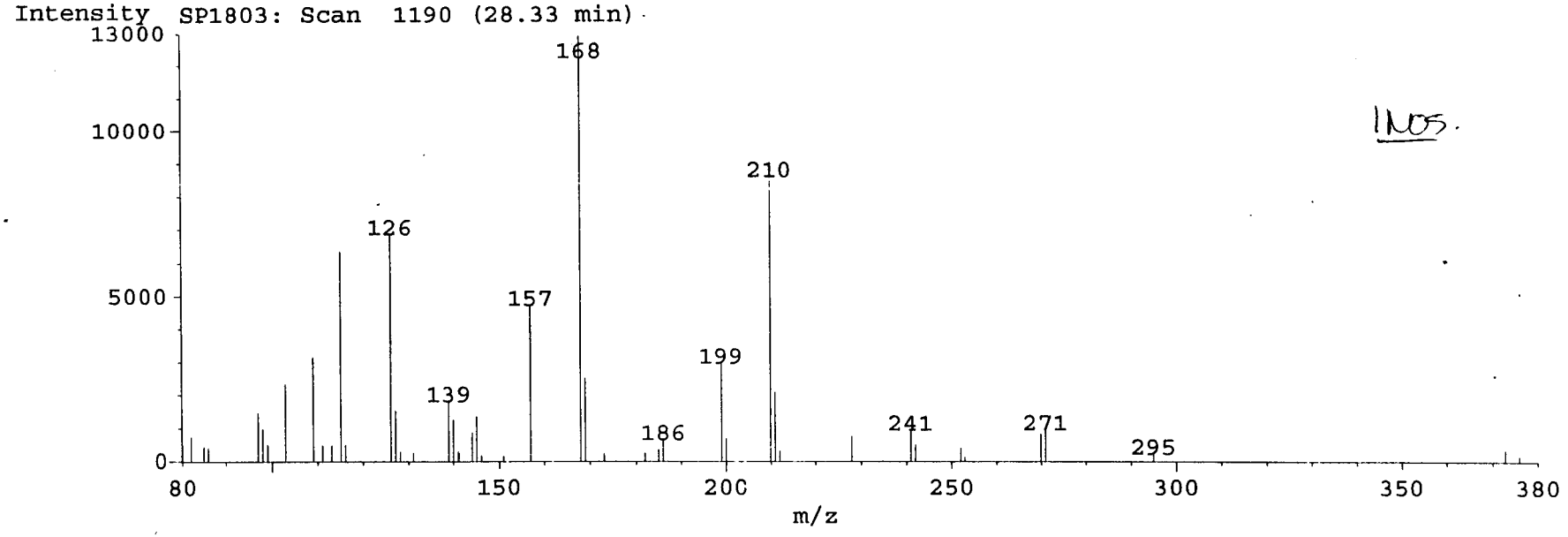
4,6-CLC


Intensity SP1803 TIC
300000
250000
200000
150000
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 958 (22.25 min)
2500
2000
1500
1000
500
0
118
129
87
102
111
139
160
174
189
234
260
75
100
150
200
250
300
320
m/z

3,6-CAL

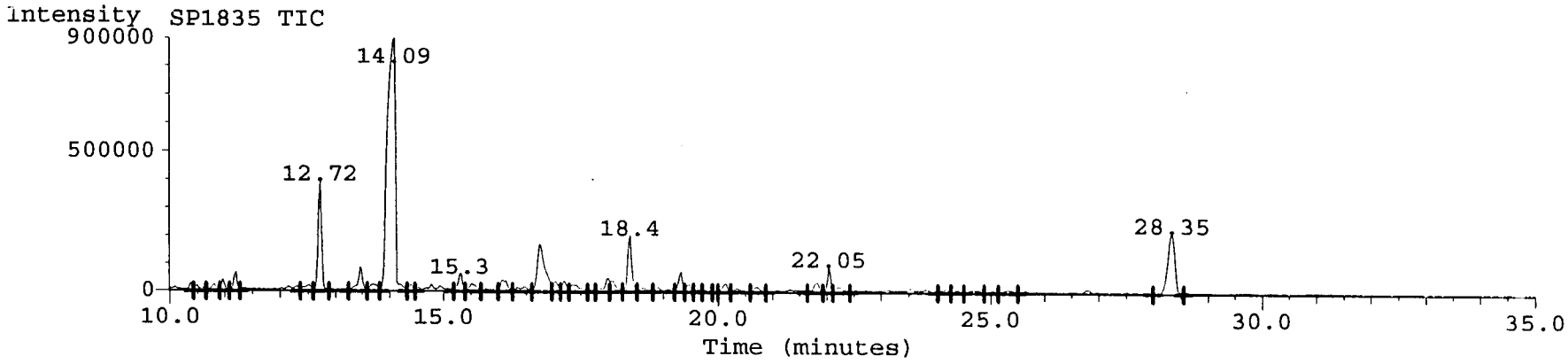

Intensity SP1803 TIC
300000
250000
200000
150000
100000
50000
0
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1803: Scan 1190 (28.33 min)
13000
10000
5000
0
168
210
126
157
199
139
186
241
271
295
80
150
200
250
300
350
380
m/z

INOS.

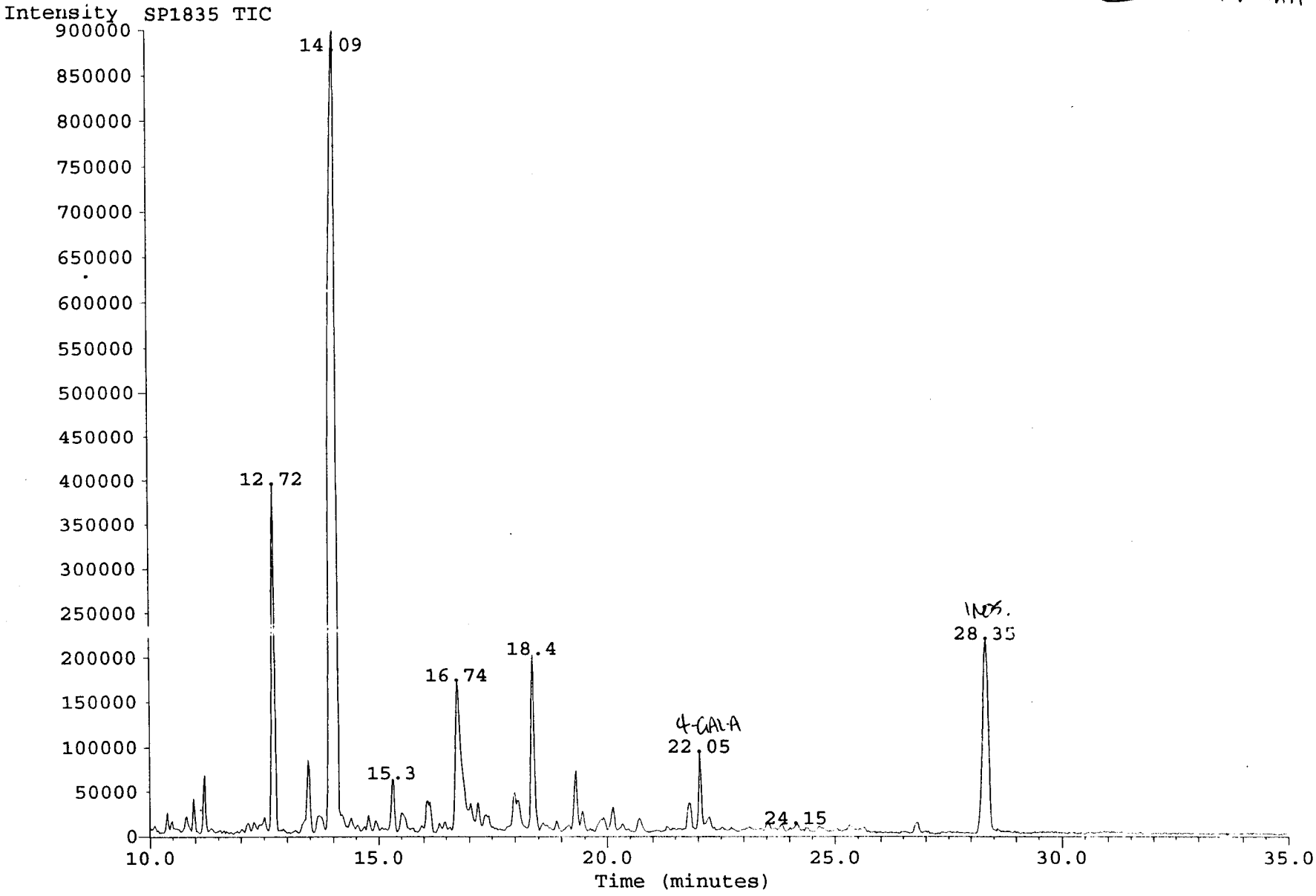
HP5970 SP2330 column Log Dev14
Data: (Thu Sep 30 12:07:52 1999) C:\VECTOR2\DATA\inst2\SP1835.TKF
Method: (Tue Sep 28 15:00:26 1999) C:\VECTOR2\METHODS\INSTR2\PMAA.MTH
Cal: C:\VECTOR2\METHODS\INSTR2\EI.CAL
Sample mj-1, Vol 1 Dil 1 Group 0
ua/pmaa
Instrument Parameters:
    StartInjection
Scan Parameters:
    SCAN every 1 secs for 5 min
    SCAN every 1 secs for 35 min
      30-600

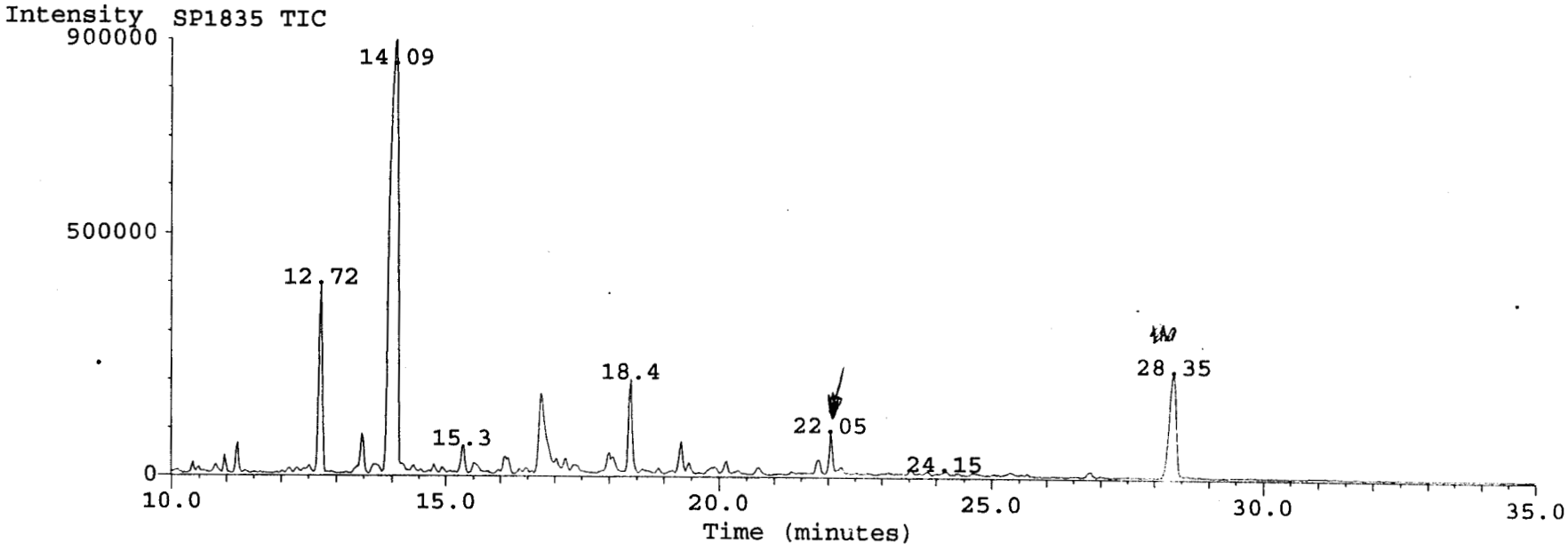
MJ-1 UA/PMAA

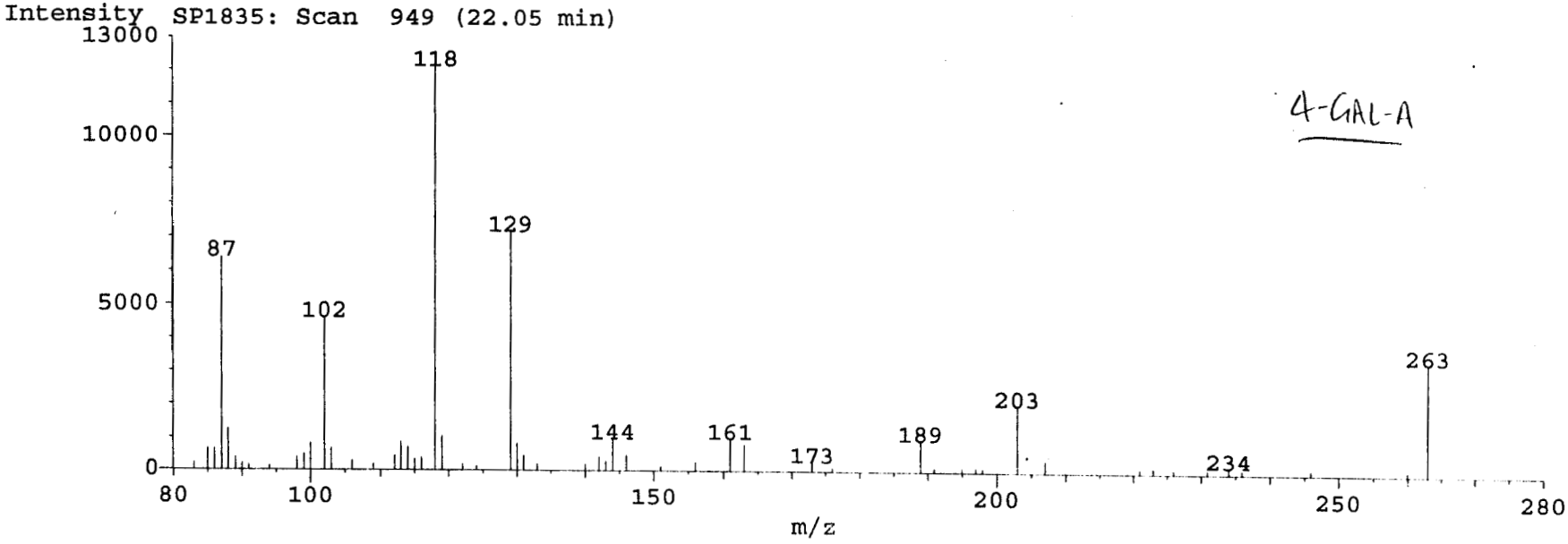


Linkage analysis (GalA linkage).

MS-1 UA/GalAA




Intensity SP1835 TIC
900000
500000
0
14.09
12.72
15.3
18.4
22.05
24.15
28.35
10.0
15.0
20.0
25.0
30.0
35.0
Time (minutes)


Intensity SP1835: Scan 949 (22.05 min)
13000
10000
5000
0
118
129
87
102
144
161
173
189
203
234
263
80
100
150
200
250
280
m/z

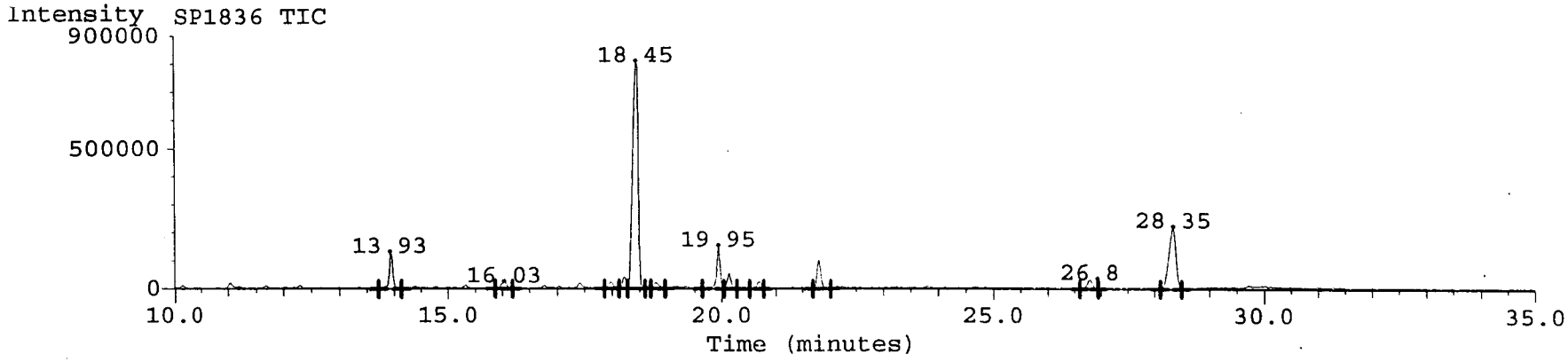
4-GAL-A

RT		AREA	HEIGHT	%T AREA	%S AREA
6.76		4299.001	36280	1.09	2.70
7.71		4161.642	68747	1.06	2.61
9.15		3595.297	32213	0.91	2.25
9.60		4340.407	50195	1.10	2.72
10.49		2307.853	16951	0.59	1.45
10.81		2691.128	22114	0.68	1.69
10.96		2728.001	41482	0.69	1.71
11.20		4833.119	68472	1.23	3.03
12.51		3079.066	21081	0.78	1.93
12.72		28794.238	394064	7.31	18.06
13.46		8679.600	85835	2.20	5.44
13.69		3776.524	22552	0.96	2.37
14.09	S	159442.369	897538	40.46	100.00
14.40		2123.863	20082	0.54	1.33
15.30		5820.886	63145	1.48	3.65
15.51		4185.915	25262	1.06	2.63
16.08		5538.831	39063	1.41	3.47
16.74		27804.148	172138	7.06	17.44
17.03		4084.235	35912	1.04	2.56
17.19		3671.029	36343	0.93	2.30
17.37		4725.329	22893	1.20	2.96
18.00		5165.068	48375	1.31	3.24
18.08		4902.449	38862	1.24	3.07
18.40		16738.554	202091	4.25	10.50
18.61		2842.135	14241	0.72	1.78
19.32		6615.894	73407	1.68	4.15
19.45		2673.378	27115	0.68	1.68
19.87		2227.954	17818	0.57	1.40
20.13		3478.019	31732	0.88	2.18
20.71		2841.505	18996	0.72	1.78
21.81		4733.675	35517	1.20	2.97
22.05		7194.450	92312	1.83	4.51
22.26		3672.115	19954	0.93	2.30
24.15		2042.072	12009	0.52	1.28

24.65	2292.018	9370	0.58	1.44
25.33	2584.586	11421	0.66	1.62
28.35	33408.455 Nos,	218279	8.48	20.95

HP5970 SP2330 column Log Dev14
Data: (Thu Sep 30 13:04:41 1999) C:\VECTOR2\DATA\inst2\SP1836.TKF
Method: (Tue Sep 28 15:00:26 1999) C:\VECTOR2\METHODS\INSTR2\PMAA.MTH
Cal: C:\VECTOR2\METHODS\INSTR2\EI.CAL
Sample mj-2, Vol 1 Dil 1 Group 0
doub. pmaa
Instrument Parameters:
StartInjection
Scan Parameters:
SCAN every 1 secs for 5 min
SCAN every 1 secs for 35 min
30-600

MJ-2 Doub. meth / RVINA



Linkage analysis

MS-2 DUB.METH./PMAA