Université de Montréal

Application of Gas Chromatography and Mass Spectrometry for Analysis of Propolis from Different Geographic Regions

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The thesis is presented to the Faculty of Graduate Studies for obtaining a Ph.D. degree in Chemistry

April 2004

Université de Montréal

Faculty of Graduate Studies

This thesis entitled:

Application of Gas Chromatography and Mass Spectrometry for Analysis of Propolis from Different Geographic Regions

Presented by:

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ABSTRACT

The main goal of this work is to investigate the chemical composition of propolis from different geographic origin. This can be achieved using the powerful combination of gas chromatography and mass spectrometry. Defining propolis main constituents will allow for their quantification and further studies of feasible biological activity and possible plant sources. With all the results obtained a method for standardization of propolis may be proposed and a decision can be made for its main applications.

A simple and reliable methodology for the analysis of propolis, which can be applied to all samples regardless of their origin, was used. Thus, the main components of propolis "balsam" from samples from Egypt, Brazil, The Canary Islands and Canada were determined using GC-MS. The results obtained revealed that their chemical compositions were extremely complex and completely different from that of the European type propolis.

A new method for studying propolis chemical composition based on metastable atom bombardment ionization mass spectrometry was developed. This ionization technique is applied for the first time in natural product chemistry and appeared to be highly beneficial for compound identification, structure elucidation and accurate mass measurements.

A method for simultaneous quantification of the main propolis phenolic constituents in Bulgarian propolis was developed based on capillary gas

chromatography. The method was applied for standardization and quality control of a veterinary preparation based on propolis.

A new method of studying propolis main phenolic constituents was developed based on capillary gas chromatography with electron capture detection.

Volatile oils obtained from propolis samples originating from different geographic and climatic regions were also analyzed by GC-MS. Significant variations in the chemical composition were observed related to the origin of the sample. In different samples, many new for propolis compounds, mainly monoterpenes, were identified.

Different propolis samples were investigated for their activity against pathogenic bacteria, fungal strains and viruses. It was found that in spite of the great differences in the chemical composition of propolis from different geographic locations, all samples exhibit significant antibacterial and antifungal (and most of them antiviral) activity.

Key Words: Propolis, Geopropolis, lignans, Terpenoids, Polyphenols, Volatiles, GC, ECD, MS, GC-MS, MAB

RÉSUMÉ

Le but de cette étude est de déterminer la composition du propolis de diverses origines. Ceci est fait en couplant la chromatographie gazeuse à la spectrométrie de masse obtenant ainsi une technique d'analyse plus performante. La détermination des constituants principaux des propolis permettra la quantification, l'étude de l'activité biologique et possiblement l'origine végétale de ceux-ci. Avec les résultats obtenus, une méthode de standardisation des propolis ainsi que des applications possibles pourront être proposées.

Une technique à la fois fiable et simple a été utilisée pour l'analyse de tous les échantillons indépendamment de leur origine. La composante principale du propolis, le balsam, a été caractérisée pour les échantillons d'Égypte, du Brésil, des Iles Canaries et du Canada à l'aide de la GC/MS. Les résultats obtenus ont révélé une extrême complexité ainsi qu'une grande différence des compositions chimiques des échantillons provenant d'Europe.

Une nouvelle technique basée sur la spectrométrie de masse par ionisation à bombardement d'atomes métastables a été développée pour l'étude de la composition chimique du propolis. Cette technique d'ionisation semble particulièrement adaptée à l'identification des espèces présentes, de leur structure ainsi que la mesure de masses précises.

Une autre technique basée sur la chromatographie gazeuse capillaire avec détection par capture d'électron a été développée pour l'étude de la composante principale phénolique du propolis.

Une méthode pour la quantification simultanée des composés phénoliques du propolis bulgare a été développée. Cette méthode a été appliquée pour la standardisation et le contrôle de qualité d'une préparation vétérinaire de propolis.

Les huiles volatiles obtenues des échantillons de propolis provenant de différents climats et régions ont aussi été analysés par GC/MS. Des différences importantes dans les compositions chimiques ont été observées par rapport aux différentes origines des échantillons. Dans plusieurs échantillons, de nouvelles espèces pour le propolis, principalement des monoterpènes, ont été identifiées.

L'activité des échantillons face aux bactéries pathogènes, aux virus et aux champignons a été étudiée pour plusieurs propolis. Il a été démontré que malgré de grandes différences dans les compositions chimiques, les échantillons de toutes les régions montrent des propriétés antibactériennes et antifongiques importantes. La majorité montre aussi des propriétés antivirales.

Key Words: Propolis, Geopropolis, lignans, Terpenoids, Polyphenols, Volatiles, GC, ECD, MS, GC-MS, MAB

Acknowledgments

I would like to take the opportunity and to express my deep gratitude to the people who supported me throughout the completion of the present study:

Prof. Michel Bertrand, my thesis supervisor, who accepted me in his team. He introduced to me new horizons of mass spectrometry and gave me continuous assistance in times of need.

I am grateful to the Department of Chemistry and especially Prof. D'Amboise for giving me the opportunity to continue my research. In addition, I am indebted to Prof. Jim Wuest, Prof. Beauchamp, Prof. Zargarian, Prof. Sauvé and Prof. Lafleur for their significant understanding and support during the last years.

I cannot forget my former scientific supervisors from the Institute of Organic Chemistry, Bulgarian Acadamy of Sciences, Prof. V. Bankova for her profound support and friendship during the last 15 years, and Prof. S. Popov who not only introduced me into the field of mass spectrometry but also, with his sometimes strange ideas, into the ocean of natural product chemistry.

Na Vassya

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List of Abbreviations

APCI	- atmospheric pressure chemical ionization
BSTFA	- N, O-bis(trimethylsilyl)trifluoroacetamide
cGC	- capillary gas chromatography
ECD	- electron capture detection (detector)
EI	- electron ionization
ESIMS	- electrospray ionization mass spectrometry
EtOH	- ethanol
FAB	- fast atom bombardment
FID	- flame ionization detector
GC-MS	- gas chromatography – mass spectrometry
HPLC	- high performance liquid chromatography
IE	- ionization energy
LSIMS	- liquid secondary ion mass spectrometry
MAB	- metastable atom bombardment
TIC	- total ion current
TLC	- thin layer chromatography
TMS	- trimethylsilyl

1. Introduction

Propolis, also known as the bee glue is a resinous or sometimes wax-like product collected by honey bees from different plant sources. Bees use this material to seal hive walls and its entrance and to strengthen the border of the combs. They also use it as an "embalming" substance to cover hive invaders, which bees have killed but cannot transport out of the hive. It has been suggested that propolis is in fact responsible for the lower incidence of bacteria and moulds within the hive as compared to the atmosphere outside (1).

Propolis has been used by man since ancient times as a remedy in folk medicine. Nowadays it is used worldwide as a constituent of pharmaceuticals, "biocosmetics", "health food", etc. (2,3).

The interest in the commercial use of propolis in pharmacology has showed a steady increase, leading to a growing activity in the chemical research on bee glue. In the last 20 years a large number of chemical studies on propolis have been published. These studies have revealed its extremely complex composition (1, 4-7). The presence of numerous low molecular substances has been demonstrated, such as phenolics, sesquiterpenes, sterols, fatty acids, amino acids, sugars, etc.

A significant result from these studies is the conclusion that in different geographic regions the chemical composition of propolis is different because of the specificity of the local flora. In the Temperate climatic zone the main source

of propolis is the resinous bud exudates of different poplar species (*Populus*). The samples originating from these locations are characterized by a common qualitative composition, the main components being flavonoid aglycones, phenolic acids and their esters (8, 9, 11-13). However, significant quantitative variations appear.

In the last years, there is an evidence for a gradually increasing demand for propolis worldwide. However, the supply of this natural product has significantly grown mainly from tropical countries, especially Brazil. Obviously the chemical composition and the plant sources of this propolis differ from those of the "poplar" propolis since poplars are not present in tropical flora (14 – 18). Unfortunately, very little is known about its chemistry, plant origin and biological activity.

The previously mentioned significant quantitative variations of samples originating from the temperate climatic zone affect the standardization of the active components even of "poplar" propolis, which is still an open question. Such standardization is strongly needed because of the various applications of propolis. The creation of a modern standardization procedure in the near future, however, is possible only following an extensive accumulation of data about propolis chemistry. Any wide-ranging accumulation of data would inevitably require the deployment of an array of powerful modern methods for the analysis of bee glue because of its very complex chemical composition. The present work is another confirmation that gas chromatography and mass spectrometry are such proven powerful methods.

2. Aims and Scope of the Study

The main aim of the present study is to continue and to enlarge the investigations on chemical composition, plant origin and biological activity of propolis from different geographic locations. The Bulgarian propolis, as a typical representative of the European "poplar" type propolis, is used as a comparison for the samples originating from other geographic regions. An attempt will be made to clarify the possibilities of developing a propolis standard relating on its plant origin.

In achieving these aims, the present work will study the following main themes:

1. Gas-chromatographic investigation on the main components of Bulgarian propolis.

1.1. Quantification of the main phenolics in Bulgarian propolis.

1.2. Development of a new procedure for rapid qualitative analysis of phenolics in propolis.

2. Investigation of polar components of propolis from different geographic regions using GC-MS.

2.1. Propolis from Egypt.

2.2. Propolis from Brazil.

2.3. Geopropolis from Brazil.

2.4. Propolis from the Canary Islands.

2.5. Propolis from Canada

3. Development of a new method of studying propolis chemical composition using metastable atom bombardment (MAB) ionization mass spectrometry

4. Investigation of propolis volatiles from different geographic locations.

3.1. Propolis from Bulgaria, Albania and Mongolia.

3.2. Propolis from Brazil.

3.3. Geopropolis from Brazil.

3.4. Propolis from the Canary Islands.

3. Review of the Literature

3.1. Chemical Composition of Propolis

Until 1960 little was known about the chemical composition of bee glue. It was claimed to contain up to 30% beeswax, up to 20% mechanical impurities, 40 - 60% resins and balsam and up to 5% volatile oils. The information concerning individual compounds was very limited; only cinnamic alcohol, cinnamic acid, vanillin and chrysin were identified (1). The development of modern chromatographic and spectral methods allowed systematic investigations on the chemical composition of propolis. Such investigations started about 1964 - 1965 in France and Russia. From the early 1980's, chemistry and pharmacology of propolis became the subject of increasing interest in many European countries and in the last decade in Japan and South America.

The chemical investigations of propolis revealed its complex composition. The presence of compounds belonging to different structural types was reported. These include mainly phenolics (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, coumarines), as well as sesquiterpenoids, sterols, fatty acids, amino acids, sugars, etc. The literature data about propolis composition are presented in Table I (page 18).

As already mentioned, the main constituents are phenolics; they comprise 30 - 50% of the weight of raw propolis from European origin (66, 67). It is important to note, however, that the concentrations of many compounds, mentioned in Table I, are less than 1% of the raw sample.

Some of the compounds mentioned in Table I, mainly flavonoid aglycones, phenolic acids and their esters, have been isolated using chromatographic techniques and identified by spectral methods. Recently, many components have been identified only by gas chromatography-mass spectrometry (GC-MS). In this respect chalcones deserve special attention. The GC-MS analysis requires sample derivatization to convert the non-volatile phenolics into volatile substances. The most commonly used procedure is the conversion of the phenols into trimethylsilyl (TMS) ethers. Under the reaction conditions, flavanones (which are among the major propolis components) can be partially converted into chalcones and the latter appeared as peaks in the mass chromatogram while actually not present in the original sample (11). For this reason, in Table I only those chalcones, which have been isolated from the original sample and identified as individual substances, are listed (4).

Besides the low molecular compounds, proteins have also been found in bee glue in concentration of about 2% (68, 69).

Many microelements were identified as well: Mg, Ca, Fe, Mn, Cu, Co, Mo, Zn, F, K, Na, Al, Sn, Si, As, Se, Ti, V, Cr, Ni, Be, Zr, Sb, Ag (70, 71).

It is very important to note that the composition of propolis from different geographic and climatic zones is different, so that a particular sample never

contains all the substances listed in Table I. On the other hand, every individual sample has a complex composition, e.g. more than 150 individual compounds have been identified in one sample (5).

3.2. Plant Origin of Propolis

Studies on bee behaviour as well as chemical data support the plant origin of propolis. At the beginning of the 20th century two opinions were formed concerning the plant sources of propolis. Kuestenmacher (39) assumed that bee glue is the result of the digestion of pollen by the bees. Other authors (19, 72) supported the view that bees collect it from the resinous buds of some trees. As a matter of fact, almost all propolis constituents are typical secondary metabolites of higher plants. However, the identification in propolis for example of phenylvinyl ether, p-methoxyphenylvihyl ether and cyclohexyl benzoate (38) must be treated with some caution. These substances are often present in products made of polymer materials and it is possible that they are not genuine components of bee glue.

The second hypothesis, that bees collect propolis from the resinous buds of some trees, is nowadays generally accepted because of the numerous and unambiguous proofs confirming it. One of the largest monographs dealing with beekeeping and bee products (4) lists more than 30 plants regarded as propolis

sources in different geographic regions. The most often mentioned sources are the resinous bud excretions of poplars, birches, aspens, willows, and chestnuts.

The qualitative and even quantitative similarity of chemical composition between phenolics from poplar buds (*Populus nigra)* and propolis has been pointed out in France, Hungary, Great Britain, Bulgaria, Mexico, Southern Russia, Albania, New Zealand (8, 9, 11, 44, 50, 66, 73, 74). In Russia propolis and birch buds have shown similar composition (66). In some regions in Ukraine propolis and *Populus tremula* buds (66) compositions were also found similar. It has been proven by chemical analyses that the source of bee glue in Mongolia is the only poplar species growing there, i.e. *P. suaveolens* (41). In Canada the source plants were American poplar species: *P. deltoides, P. fremonii, P. maximoviczi* (13).

It is obvious that the most preferred plant source in the temperate climatic zone are poplar buds. By contrast, there are little data about propolis origin in tropical regions. Only *Clusia* species have been identified to play this role in the tropical regions of Venezuela and Cuba (14, 15).

The question arises whether bees perform chemical changes of some propolis components after taking them from the plants (1, 75). The published data comparing poplar bud exudates and propolis from the same location do not give any indications that such changes occur (8, 11, 67).

The full characterization of propolis plant sources is of important interest because it is related to its biological activity and could be used as a basis for its standardization. Such characterization may also offer deeper understanding of

the interaction between bees and their environment. It is important to beekeepers that their bees have the proper plants in their flight range. Colonies suffer when they cannot collect propolis. Bees are even said to use "propolis substitutes" like paints, asphalt and mineral oils, which could severely threaten pharmaceutical uses of bee glue (76).

3.3. Biological Activity of Propolis

The most popular, well-studied and documented activity of propolis is the antibacterial one. The first systematic investigation was carried out by Kivalkina in 1948 and since then several articles dealing with this subject are published every year (1, 6). All investigations demonstrated that Gram-positive microorganisms are very sensitive to propolis, whereas Gram-negatives are often resistant (6, 77, 78). Some comparative studies demonstrated that propolis was weaker in comparison to most antibiotics, but some samples were as efficient as sulphonamides (1, 79). Propolis extracts enhanced the action of some antibiotics (1, 80, 81). The presence of propolis prevented the formation of resistant *Staphylococccus* strains when antibiotics were used (77). The antibacterial activity of propolis is attributed to flavonoids, aromatic acids and their esters (6, 82).

Propolis also possesses anti-fungal activity (83-85). Metzner, Schneidewind and other authors (27, 85, 86) proved that the active components are the flavanones pinocembrin and pinobanksin, benzyl *p*-coumarate and caffeic acid esters.

There are some reports describing the antiviral activity of bee glue (87-90). The active components were phenolics again: some flavonoids (92), and especially caffeic acid and its esters (88-90). In Brazilian propolis, anti-HIV active triterpenes were found recently (91).

Cytostatic activity of propolis has been reported in the literature (93-95). The substances involved in this activity turned out to be phenolics (96, 97), mainly the caffeic acid phenethyl ester (CAPE) (48, 98).

Recently, antioxidative activity of propolis attracted the attention of scientists. The most important antioxidants in bee glue were found to be phenolics from different plant origins (99-101).

Many other pharmacological properties of propolis have been described by different authors: tissue regenerative (102-104), local anaesthetic (53), hepatoprotective (105-107), immunomodulating (108-110), choleretic and antiulcer (1, 111), radioprotective (115), etc. Propolis extracts inhibited caries (tooth decay) formation in rats (112), showed antileishmaniosis (113) and antitrypanozomic action (114), inhibited dihydrofolate reductase (116).

Propolis is generally regarded as being harmless and non-toxic (1). However, some authors reported side effects, namely contact dermatitis, caused by propolis preparations and attributed to prenyl caffeates (1, 117-122).

Obviously, the biological activity of bee glue cannot be connected to one chemical compound or even to a group of compounds with related structures. The versatile activities could be explained with the presence of a large number of substances belonging to different structural classes. It seems that its chemical properties are not only beneficial to the bees. Propolis also possesses general pharmacological value as a natural mixture taken as a whole rather than as a source of new powerful biologically active individual compounds. Further cooperation of chemists and biologists is required for the better understanding and usage of this valuable natural product.

3.4. Practical Applications of Propolis

In the last 20 years there are hundreds of applications dealing with propolis that are subject to different patents all over the world. Most of the preparations patented are for medical use, mainly to be applied in stomatology, othorynolaringology, ophthalmology, etc. Some preparations have found application in clinical practice (6).

A smaller number of patents describe the so-called biocosmetics, such as face creams, lotions, shampooing, tooth pastes, deodorants (6).

Propolis and its extracts have been applied not only in medicine and cosmetics but in the food industry as well. Because of its antioxidative

properties (1, 123-125), it was used as a preservative for stored fish and sunflower oil (1). Alcohol extracts of propolis were used as a supplement to the basal diet of chickens and pigs, which leads to an increase of weight up to 10% (1, 126-127). This might be attributed to the prevention of digestive disorders, one of the possible applications of the bee glue.

Recently propolis has been widely used in Japan as a "health food" supplement (17).

Propolis has also been used for a long time in polishes and varnishes, especially in violin varnish (128-130).

These diverse applications of propolis have led to an increased interest concerning its chemistry and possible further standardization.

3.5. Analysis and Standardization of Propolis

The standardization of propolis is a complicated and still unsolved problem. As we have pointed earlier, it possesses a complex and a variable chemical composition and also has numerous applications. However, the knowledge of the active principles is far from being complete. For this reason some authors even recommend its use only in products like "health food" but not in medicines and cosmetics (17).

For the attempts of propolis standardization, in the available literature, many different procedures have been described. Regrettably, no one could be recommended as generally acceptable. Some authors have proposed standardization based on characteristics, which have no direct connection to biological activity, e.g. iodine number, discoloration time of 0.1 N potassium permanganate solution or some combination of such methods (1).

A number of published spectrophotometric procedures have been used to determine total phenolics or total flavonoids (1, 43, 66, 131-135). In some cases the spectrophotometry is combined with thin-layer chromatography (TLC) or paper chromatography in order to indicate the presence of some biologically active individual components, such as flavonoids and/or aromatic acids (66, 136).

The recent development of chromatographic techniques led to their increased use in analysis and quality control of propolis. The substances to be determined were flavonoid aglycones and aromatic acids as main active constituents. TLC with densitometry (75, 137), high performance liquid chromatography (HPLC) (138-140) and combinations of both methods (66, 75, 141) have been used for quantification of one or few of the main components of the bee glue. Qualitative analytical procedures based on gas chromatography (GC) after silylation of the alcohol extract have also been described (66, 141, 142). Recently, capillary electrophoresis was used for quantification of the main phenolic constituents of propolis. The procedure was especially effective for the analysis of cinnamic acids (143, 144).

The quantification of one or several of the main phenolic components is a promising approach. It is interesting to note however, that different authors have chosen different compounds, obviously the ones, which predominated in their samples.

Most importantly, it is known that samples from different geographic origins very often demonstrate similar biological activity. This fact has led some researchers to assume that biological tests are the best approach to the standardization and evaluation of bee glue. Such tests based on measuring the enzymatic activities in the presence of propolis (1, 145) were connected mainly to the antioxidative effect of propolis. The latter is related to anti-inflammatory activity but not to the antibacterial one.

According to this brief review of the literature, it seems impossible to develop a simple standardization procedure for propolis based on a single chemical or biological test only. Some combinations of both biological and chemical assays have been published (1, 146, 147). One of the significant attempts to standardize propolis was published by Vanhaelen & Vanhaelen-Fastre (21). They developed 6 analyses for evaluation of propolis samples: calcination residue; residue insoluble in water and in organic solvents; saponification number; chromatographic identification of five phenolic acids and three flavonoid aglycones (using retention times in GC and Rf values in TLC); microscopic analysis of the insoluble residue; antibacterial test. The identification of some of the main active components and the biological test are advantages to the procedure. However, no quantification was performed and

this might be of great importance with respect to the variability of propolis composition.

A modern standardization of the bee glue has to be based on a quantification of the main propolis components possessing proven pharmacological activity. Characteristics of the purity, like percentage of beeswax, insoluble residue, etc., must be involved, as well. Obviously, one or more biological tests are needed to characterize the usefulness of every individual sample. Such future standardization will enable the wide use of standardized propolis preparations in medicine and cosmetics.

All the above-mentioned investigations, related to the evaluation of propolis, are dealing with bee glue from the temperate zone, and its main components being the typical "poplar" phenolics. However, bees collect propolis even in places where no poplars grow. For this reason in 1977 Popravko (66) proposed a totally different approach to the problem. He noticed that propolis could be easily characterized using its plant source, which might be established by simple TLC comparison: birch, birch and poplar, birch and aspen, poplar. As the composition of the corresponding bud exudates is known (66, 148), this method gives information about the qualitative composition of the sample.

This idea is current again in the publications on tropical propolis (14, 16). Obviously much more investigation on the chemical composition of propolis from tropical and subtropical regions is needed in order to find out if bees in these areas have a preferred propolis plant source or sources. After that, it should be possible to define a limited number of local propolis standards such

as European, one or few tropical standards, etc. Once again, we believe that the accumulation of data on propolis chemistry will contribute to the solution of this problem and this is one of the goals of the present work.

3.6. Methods Used for Investigation of Propolis Chemical Composition

Propolis is a mixture of secondary plant metabolites and beeswax and its chemical investigation is performed by means of the usual phytochemical methods. This means isolation and structural characterization of its constituents using chromatographic and spectral techniques. This approach in most cases leads to the identification of the main bee glue components.

As already mentioned, propolis composition is very complex and varies depending on the geographic region. For this reason, the above mentioned approach is troublesome and inconvenient if one wants to investigate and compare a large number of samples. However, such investigations are obviously needed.

For serial analyses, TLC and HPLC have been applied (14, 140, 141, 149), which appeared to be particularly suitable for flavonoid aglycones, especially HPLC with diode array detector. These techniques however do not possess a resolving power high enough to separate more than 50 individual

components in one single sample. More recently, HPLC-ESIMS was also applied for propolis analysis (150, 151)

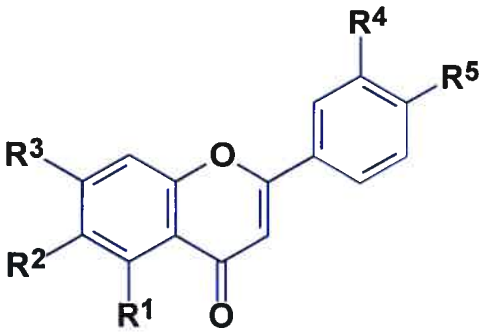
In this respect, the combination gas chromatography-mass spectrometry has proven to be very beneficial. It joins the high resolution, accuracy and reproducibility of the capillary gas chromatography (cGC) with the identification power of the mass spectrometry. This is of special importance in cases when a complex mixture of compounds, (such as propolis), belonging to different structural classes, has to be analyzed. This method makes it possible to identify some microcomponents, which are important for the investigation of the biological activity and plant origin of the bee glue (152).

Both methods are also proven to be highly accurate and can be used for the quantification of the main propolis constituents. This, we believe is one of the most important steps for the creation of a reliable standardization procedure.

TABLE I

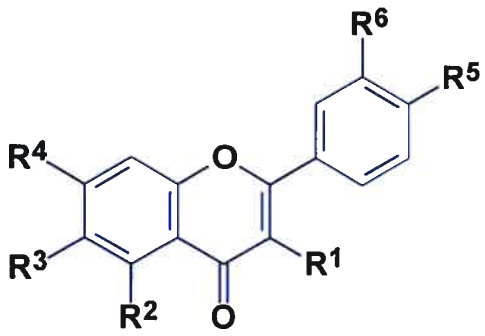
Chemical composition of propolis (literature data)

I. Flavones



	R^1	R^2	R^3	R^4	R^5
Chrysin (19-23)	OH	H	OH	H	H
Tectochrysin (8, 11, 20, 21, 24, 25)	OH	H	OMe	H	H
Acacetin (20-23, 66)	OH	H	OH	H	OMe
Apigenin (1, 20-23, 25)	OH	H	OH	H	OH
Apigenin-7-methyl ether (25, 26)	OH	H	OMe	H	OH
Apigenin-7,4'-dimethyl ether (24)	OH	H	OMe	H	OMe
Pectolinarigenin (20, 27)	OH	OMe	OH	H	OMe
Xantomicrol (9)	OH	OMe	OMe	OMe	OH
Hispidulin (4, 14)	OH	OMe	OH	H	OH
Eupatorin (14)	OH	OMe	OMe	OH	OMe
- (14)	OH	H	OMe	OMe	OMe
- (14)	OH	OMe	OMe	OMe	OMe
- (28)	OMe	H	OH	H	H

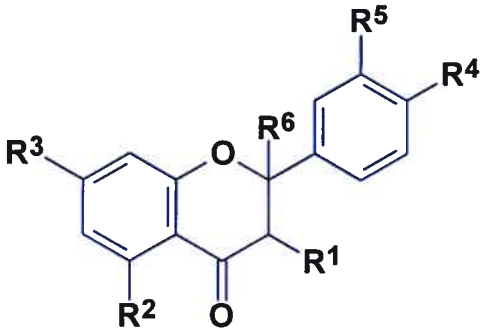
II. Flavonols



	R^1	R^2	R^3	R^4	R^5	R^6
Galangin (11, 20-23, 29)	OH	OH	H	OH	H	H
Galangin-3-methyl ether (8, 11, 23, 25, 30)	OMe	OH	H	OH	H	H
Galangin-5-methyl ether (31)	OH	OMe	H	OH	H	H
Isalpinin (8, 11, 20, 23, 24, 30)	OH	OH	H	OMe	H	H
Kaempferol (1, 8, 20-23, 25)	OH	OH	H	OH	OH	H
Kaempferide (8, 20, 21, 25, 66)	OH	OH	H	OH	OMe	H
Rhamnocytrin (23, 25, 66)	OH	OH	H	OMe	OH	H
Kaempferol-3-methyl ether (25)	OMe	OH	H	OH	OH	H
Ermanin (66)	OMe	OH	H	OH	OMe	H
Kumakatekin (32)	OMe	OH	H	OMe	OH	H
Kaempferol-7,4'-dimethyl ether (25)	OH	OH	H	OMe	OMe	H
Betuletol (20)	OH	OH	OMe	OH	OMe	H
Alnusin (4)	OH	OH	OMe	OH	H	H

	R^1	R^2	R^3	R^4	R^5	R^6
- (4)	OMe	OH	OMe	OH	OMe	H
Quercetin (20-22, 25, 66)	OH	OH	H	OH	OH	OH
Rhamnetin (8, 20-23, 25)	OH	OH	H	OH	OMe	OH
Isorhamnetin (11, 20, 22, 23)	OH	OH	H	OH	OH	OMe
Rhamnasin (20)	OH	OH	H	OMe	OH	OMe
- (27)	OMe	OH	H	OH	OH	OMe
- (20)	OH	OH	H	OMe	OMe	H
- (33)	OMe	OH	H	OH	OH	OH
- (33)	OMe	OH	H	OMe	OH	OH
- (8, 25, 33)	OMe	OH	H	OMe	OH	OH
- (33)	OMe	OH	H	OH	OMe	OH
- (33)	OMe	OH	H	OH	OMe	OH

III. Flavanones and dihydroflavonols



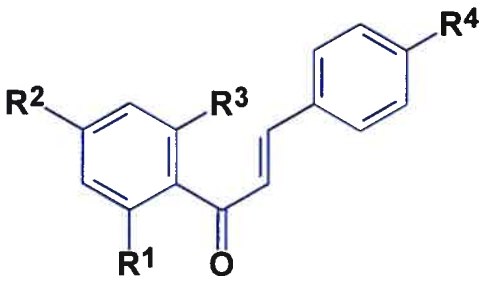
	R^1	R^2	R^3	R^4	R^5	R^6
Pinocembrin (20, 22, 24, 25, 30, 34)	H	OH	OH	H	H	H
Pinostrobin (20, 25, 30, 34, 66)	H	OH	OMe	H	H	H
Sakuranetin (20, 25, 27, 30, 34)	H	OH	OMe	OH	H	H
Isosakuranetin (35)	H	OH	OH	OMe	H	H
- (66)	H	OH	OMe	OMe	H	H
Pinobanksin (8, 25, 27, 30)	OH	OH	OH	H	H	H
Pinobanksin-3-(8, 23, 25, 27,30)	OAc	OH	OH	H	H	H
Pinobanksin-3-propanoate (25, 30)	OPro	OH	OH	H	H	H
Pinobanksin-3-butyrate (25)	OBut	OH	OH	H	H	H
Pinobanksin-3-pentenoate (25)	OPnt	OH	OH	H	H	H
Pinobanksin-3-pentanoate (25, 30)	OPtn	OH	OH	H	H	H
Pinobanksin-3-hexanoate (25)	OHx	OH	OH	H	H	H
Pinobanksin-3-methyl ether (25,30)	OMe	OH	OH	H	H	H

	R^1	R^2	R^3	R^4	R^5	R^6
- (22)	OH	OMe	OH	H	H	H
- (22)	H	OH	OMe	H	H	OH
Naringenin (25)	H	OH	OH	OH	H	H
Hesperetin (23)	H	OH	OH	OH	OMe	H

Legend:

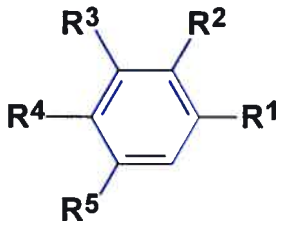
Pro = C_2H_5CO; But = C_3H_7CO; Pnt = C_4H_9CO; Hx = $C_6H_{11}CO$

IV. Chalkones



	R^1	R^2	R^3	R^4
- (2,6-OH-4-OMe) (4)	OH	OH	OMe	H
- (2,6,4'-OH-4-OMe)(4)	OH	OH	OMe	OH

V. Derivatives of benzylalcohol, benzaldehyde and benzoic acid



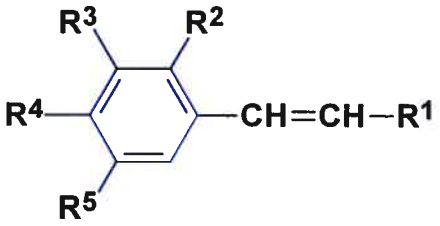
	R^1	R^2	R^3	R^4	R^5
Benzylalcohol (36)	CH_2OH	H	H	H	H
3,4,-dimethoxybenzyl alcohol (35)	CH_2OH	H	OMe	OMe	H
Benzyl acetate (36)	CH_2OAc	H	H	H	H
Benzaldehyde (29)	CHO	H	H	H	H
p-hydroxybenzaldehyde (11)	CHO	H	H	OH	H
Vanillin (21)	CHO	H	OMe	OH	H
Isovanillin (66)	CHO	H	OH	OMe	H
Protocatechuic aldehyde (11)	CHO	H	OH	OH	H
Benzoic acid (36)	CO_2H	H	H	H	H
Salicic acid (21)	CO_2H	OH	H	H	H
p-hydroxybenzoic acid (20)	CO_2H	H	H	OH	H
Anisic acid (20)	CO_2H	H	H	OMe	H
Vanillinic acid (66)	CO_2H	H	OMe	OH	H
Veratric acid (35)	CO_2H	H	OMe	OMe	H
Protocatechuic acid (20)	CO_2H	H	OH	OH	H

	R^1	R^2	R^3	R^4	R^5
Gallic acid (20)	CO_2H	H	OH	OH	OH
Gentisinic acid (21)	CO_2H	OH	H	H	OH
Benzyl benzoate (37)	CO_2Bn	H	H	H	H
Methyl benzoate (25)	CO_2Me	H	H	H	H
Ethyl benzoate (25)	CO_2Et	H	H	H	H
Methyl salicilate (25, 30, 38)	CO_2Me	OH	H	H	H
4-hydroxybenzyl bezoate (5)	CH_2OBz	H	H	H	H
Benzyl saliciclate (4)	CO_2Bn	OH	H	H	H
Benzyl 2-methoxybenzoate (5)	CO_2Bn	OMe	H	H	H
Cyclohexyl benzoate (38)	$CO_2C_6H_{11}$	H	H	H	H

Legend:

Bn = $CH_2C_6H_5$; Bz = C_6H_5CO

VI. Derivatives of cinnamic alcohol, cinnamic aldehyde and cinnamic acid



	R^1	R^2	R^3	R^4	R^5
Cinnamic alcohol (2, 11, 39)	CH_2OH	H	H	H	H
p-coumaric alcohol (5)	CH_2OH	H	H	OH	H
Coniferyl alcohol (66)	CH_2OH	H	OMe	OH	H
Cinnamic aldehyde (37)	CHO	H	H	H	H
p-coumaric aldehyde (66)	CHO	H	H	OH	H
Coniferyl aldehyde (66)	CHO	H	OMe	OH	H
E-cinnamic acid (1, 11, 20, 21, 25, 40)	CO_2H	H	H	H	H
Z-cinnamic acid (5, 41)	CO_2H	H	H	H	H
E-*p*-coumaric acid (11, 20, 21, 25, 41, 42)	CO_2H	H	H	OH	H
Z-*p*-coumaric acid (5, 42)	CO_2H	H	H	OH	H
3,5-diprenyl-4-hydroxycinnamic acid (16)	CO_2H	H	C_5H_9	OH	C_5H_9
3-prenyl-4-dihydrocinnamoyloxycinnamic acid (16)	CO_2H	H	C_5H_9	ODhc	

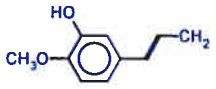
	R^1	R^2	R^3	R^4	R^5
m-coumaric acid (11, 20, 21, 25, 41)	CO_2H	H	OH	H	H
o-coumaric acid (11, 20, 21, 25, 41)	CO_2H	OH	H	H	H
4-methoxycinnamic acid (11, 25, 41)	CO_2H	H	H	OMe	H
Caffeic aicd (11, 20, 21, 25, 41)	CO_2H	H	OH	OH	H
Ferulic acid (11, 13, 20, 21, 25, 32, 41, 42)	CO_2H	H	OMe	OH	H
Isoferulic acid (11, 13, 20, 21, 30, 43, 44)	CO_2H	H	OH	OMe	H
3,4-dimethoxycinnamic aicd (11, 13, 25, 30, 41, 43)	CO_2H	H	OMe	OMe	H
Sinapic acid (39)	CO_2H	H	OMe	OH	OMe
Cinnamyl benzoate (11, 25)	CH_2OBz	H	H	H	H
Cinnamyl cinnamate (5)	CO_2Cyn	H	H	H	H
p-coumaryl benzoate (45)	CH_2OBz	H	H	OH	H
Coniferyl benzoate (45)	CH_2OBz	H	OMe	OH	H
p-coumaryl vanillate (66)	CH_2O_2CAr	H	H	OH	H
Benzyl cinnamate (37)	CO_2Bn	H	H	H	H
Benzyl *E*-*p*-coumarate (11, 25, 66)	CO_2Bn	H	H	OH	H
Benzyl *Z*-*p*-coumarate (5)	CO_2Bn	H	H	OH	H
3-methyl-3-butenyl *p*-coumarate (5)	$CO_2C_5H_9$	H	H	OH	H
3-methyl-3-butenyl *p*-coumarate (5)	$CO_2C_5H_9$	H	H	OH	H

	R^1	R^2	R^3	R^4	R^5
2-methyl-2-butenyl *p*-coumarate (5)	$CO_2C_5H_9$	H	H	OH	H
2-phenylethyl *p*-coumarate (11, 13, 25, 30)	CO_2CH_2Bn	H	H	OH	H
Cinnamyl *p*-coumarate (11, 25)	CO_2Cyn	H	H	OH	H
Coniferyl *p*-coumarate (66)	CO_2Con	H	H	OH	H
Benzyl ferulate (11, 25, 41, 66)	CO_2Bn	H	OMe	OH	H
3-methyl-3-butenyl ferulate (13, 25, 30, 41)	$CO_2C_5H_9$	H	OMe	OH	H
3-methyl-2-butenyl ferulate (13, 25, 30)	$CO_2C_5H_9$	H	OMe	OH	H
Coniferyl ferulate (66)	CO_2Con	H	OMe	OH	H
Benzyl isoferulate (11, 25)	CO_2Bn	H	OH	OMe	H
3-methyl-3-butenyl isoferulate (13, 25, 30)	$CO_2C_5H_9$	H	OH	OMe	H
3-methyl-2-butenyl isoferulate (11)	$CO_2C_5H_9$	H	OH	OMe	H
2-methyl-2-butenyl isoferulate (30)	$CO_2C_5H_9$	H	OH	OMe	H
Phenylethyl isoferulate (11, 13, 25, 30)	CO_2CH_2Bn	H	OH	OMe	H
Cinnamyl isoferulate (11, 25)	CO_2Cyn	H	OH	OMe	H
Benzyl 3,4,-dimethoxycinnamte (11,25)	CO_2Bn	H	OMe	OMe	H
Benzyl caffeate (8, 11, 13, 25, 30)	CO_2Bn	H	OH	OH	H
Ethyl caffeate (41)	$CO_2C_2H_5$	H	OH	OH	H

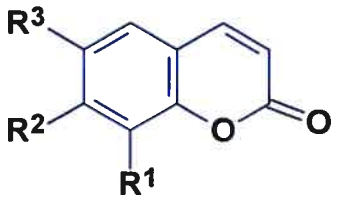
	R^1	R^2	R^3	R^4	R^5
Butyl caffeate (11, 13, 25, 30)	$CO_2C_4H_9$	H	OH	OH	H
Butenyl caffeate (11, 25, 41)	$CO_2C_4H_7$	H	OH	OH	H
Pentyl caffeate (41)	$CO_2C_5H_{11}$	H	OH	OH	H
Pent-4-enyl caffeate (25, 30)	$CO_2C_5H_9$	H	OH	OH	H
3-methyl-3-butenyl caffeate (13, 25, 30, 41)	$CO_2C_5H_9$	H	OH	OH	H
3-methyl-2-butenyl caffeate (13, 30)	$CO_2C_5H_9$	H	OH	OH	H
2-methyl-2-butenyl caffeate (11, 25)	$CO_2C_5H_9$	H	OH	OH	H
Phenylethyl caffeate (8, 13, 25, 30)	CO_2CH_2Bn	H	OH	OH	H
Cinnamyl caffeate (11, 25, 41)	CO_2Cyn	H	OH	OH	H
Diprenyl (geranyl) caffeate (5)	$CO_2C_{10}H_{18}$	H	OH	OH	H

Legend

Cyn = $C_6H_5CH{=}CHCH_2$; Ar = 3-hydroxy-4-methoxyphenyl

Con = 

VII. Coumarins



	R^1	R^2	R^3
Esculetin (1)	H	OH	OH
Scopoletin (1)	H	OMe	OH
Daphnetin (46)	OH	OH	H

VIII. Phenolic triglycerides

1,3-diferuloyl-2-acetylglycerol (47)

1,3,-di-*p*-coumaroyl-2-acetylglycerol (47)

1-feruloyl-2-acetyl-3-*p*-coumaroylglycerol (47)

IX. Other aromatic compounds

styrene (11, 25)

acetophenone (37)

methylacetophenone (37)

p-hydroxyacetophenone (11)

dihydroxyacetophenone (41)

2-phenylethanol (47)

dihydrocinnamic acid (11)

4-methoxydihydrocinnamic acid (5)

dihydrocoumaric acid (5)

anetol (48)

eugenol (49)

hydroquinone (32)

3,5-dimethoxy-4'-hydroxystilbene (pterostilbene) (35)

3,5-dihydroxystilbene (pinosilvin) (4)

naphtalene (66)

xanthorhoeol (35)

5-phenyl-*E,E*-2,4,-pentadienoic acid (31)

5-phenyl-*E*-3-pentenoic acid (50)

vinylphenyl ether (38)

vinyl-*p*-meythoxyphenyl ether (38)

polyprenylated benzophenones (14)

X. Monoterpenes

borneol (48)

limonene (51)

1,8-cineol (51)

p-cymene (51)

α-pinene (52)

β-pinene (52)

γ-terpinene (52)

linalyl acetate (5)

XI. Sesquiterpenes

α-acetoxybetulenol (5)

β-bisabolol (54)

caryophyllene (37)

β-eudesmene (37)

guaiene (37)

guaiol (37)

β-eudesmol (37)

farnesol (55)

nerolidol (55)

dihydroeudesmol (55)

α-copaene (11, 25)

β-bisabolene (55)

patchoulane (55)

β-bourbonene (4)

selinene (4)

aromadendrene (4)

calarene (4)

calamenene (4)

β-patchoulene (4)

XII. Diterpenes

17-hydroxyclerod-3,13 *E*-dienolic acid (56)

isocupressic acid (57)

acetylisocupressic acid (57)

imbricatoloic acid (57)

communic acid (57)

XIII. Triterpenes

lanosterol (55)

canophyllal (28)

XIV. Sterols

cholesterol (58)

stigmasterol (58)

fucosterol (58)

dihydrofucosterol (58)

chalinasterol (58)

XV. Carbohydrates

D-ribose (59)

D-fructose (59)

D-glucose (59)

D-gulose (59)

D-glucytol (59)

tallose (59)

sucrose(59)

sorbitol (5)

XVI. Aliphatic alcohols, aldehydes, ketones, acids and esters

isobutenol (25)

3-methyl-3-buten-1-ol (5)

3-methyl-2-buten-1-ol (5)

1-tetracosanol (5)

glycerol (5)

mio-inositol (5)

hexanal (51)

hex-2-enal (51)

6-methylhept-5-en-2-one (5)

6,10,14-trimethyl-2-pentadecanone (4)

2-heptadecanone (4)

4-hexanolactone (5)

but-2-enoic acid (5)

2-methylbut-2-enoic acid (5)

fumaric acid (25)

succinic acid (5)

2,3,4-trihydroxybutanoic (treonic) acid (5)

isobutyl acetate (5)

isopentyl acetate (5)

2-methylbutyl acetate (5)

isobutyl isobutirate (5)

3-methyl-3-bunenyl acetate (5)

3-methyl-2-bunenyl acetate (5)

isobutyl butanoate (5)

α-glycerophosphate (11, 25)

glycerol monoacetate (30)

2,3-dihydroxypropanoic acid (5)

2-hydroxybutanedioic (mallic) acid (5)

citric acid (5)

2,4-hexadienoic (sorbic) acid (5)

1,5-pentandiol monobenzoate (4)

hexadecyl acetate (4)

acetic acid(5)

butyric acid (5)

isobutyric acid (5)

2-methylbutyric acid (5)

methylpentanoic acid (5)

octanoic acid (5)

nonanoic (pelargonic) acid (5)

dodecanoic (lauric) acid (60)

tetradecanoic (myristic) acid (60)

hexadecanoic (palmitic) acid (60)

octadecanoic (stearic) acid (60)

eicosanoic acid (60)

docosanoic (behenic) acid (60)

tetracosanoic (lignoceric) acid (60)

hexacosanoic (cerotic) acid (60)

octacosanoic (montanic) acid (11, 30)

oleic acid (11)

linoleic acid (11, 60)

14-hydroxypalmitic acid (5)

15-hydroxypalmitic acid (5)

17-hydroxystearic acid (5)

tetracosyl hexadecanoate (60)

hexacosyl hexadecanoate (60)

octacosyl hexadecanoate (60)

triacontyl hexadecanoate (60)

dotriacontyl hexadecanoate (60)

tetratriacontyl hexadecanoate (60)

tetracosyl *Z*-octadec-9-enoate (60)

hexacosyl *Z*-octadec-9-enoate (60)

octacosyl *Z*-octadec-9-enoate (60)

triacontyl *Z*-octadec-9-enoate (60)

dotriacontyl *Z*-octadec-9-enoate (60)

tetratriacontyl *Z*-octadec-9-enoate (60)

3-octadecyloxy-1,2,-oleiloxypropane (60)

3-eicosyloxy-1,2,-oleiloxypropane (60)

methyl 2,8-dimethylundecanoate (4)

phenylmethyl 14-methylpentadecanoate (4)

ethyl palmitate (4)

XVII Hydrocarbons

henicosane (61)

tricosane (61)

pentacosane (61)

hexacosane (30)

heptacosane (61)

nonacosane (61)

hentriacontane (61)

tritriacontane (61)

doeicosane (61)

tetracosane (61)

hexacosane (61)

octacosane (61)

triacontane (61)

dotriacontane (61)

tripentacontane (62)

Z-9-tricosene (61)

Z-9-pentacosene (61)

Z-9-heptacosene (61)

Z-8-nonacosene (61)

Z-9-nonacosene (61)

Z-8-hentriacontene (61)

Z-10-hentriacontene (61)

Z-8-tritriacontene (61)

8,22-hentriacontadiene (61)

9,23-tritriacontadi4ene (61)

XVIII. Amino acids

alanine (63)

β-alanine (63)

α-aminobutyric acid (63)

δ-aminobutyric acid (63)

arginine (63)

asparagine (63)

aspartic acid (63)

cysteine (63)

cistine (63)

glutamic acid (63)

glycine (63)

histidine (63)

hydroxyproline (63)

leucine (63)

isoleucine (63)

lysine (63)

methionine (63)

ornithine (63)

phenylalanine (63)

proline (63)

pyroglutamic acid (63)

sarcosine (63)

serine (63)

threonine (63)

tryptophane (63)

tyrosine (63)

valine (63)

XIX. Other substances

polysaccharides, proteins, vitamins (64, 65)

4. Experimental

4.1. Gas Chromatographic Investigations of the Main Components of Bulgarian Propolis

4.1.1. Quantitation of Phenolics in Bulgarian Propolis

4.1.1.1.Propolis extraction

1 g of propolis (a commercial Bulgarian sample) was cut into small pieces and extracted with 20 ml of solvent (See Table II.) overnight at room temperature. The extracts were then evaporated to dryness.

Table II

Extraction of propolis with different solvents

No	Solvent	Extract (% of native propolis)	Note
1	70% ethanol	58	minimum waxes
2	90% ethanol	64	
3	Hexane, followed by acetone	64 (acetone extract)	
4	Acetone	81	

4.1.1.2. Silylation

The silylation of the standard mixtures, the model mixture and the propolis extract (with 70% ethanol) was performed with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 65^{o} for 30 min in a screw-capped vial. About 1.5 mg propolis extract was silylated with 95 µl of BSTFA. The large excess of BSTFA ensured reproducible results. The resulting derivatives were stable for at least 24 h. BSTFA and all the organic compounds in this study were obtained from Merk Darmstadt, Germany and Sigma-Aldrich Canada Ltd.

4.1.1.3. Gas chromatography

A 6m x 0.25 mm I.D., 0.25 µm film thickness fused silica capillary column with SE-54 as a stationary phase was used. The linear velocity of the carrier gas (nitrogen) was 9 $cm.s^{-1}$ and the split ratio was 1:100. The injector temperature was 300^{o}C. The column temperature was programmed from 80 to 280^{o}C at 20^{o}C. min^{-1} then from 280 to 300^{o}C at 2^{o}C. min^{-1} with a 10 min hold at 300^{o}C. A flame ionization detector was used at 320^{o}C. The sample volume was 1 µl.

4.1.1.4. Quantitative analysis

Quantitative analysis was performed by the internal standard method, using *n*-pentacosane (*n*-$C_{25}H_{52}$). For each of the components analyzed a calibration graph was constructed (see Table IIIA, p. 42A). For this purpose, four standard mixtures were prepared containing pinocembrin, galangin, caffeic acid and β-phenylethyl caffeate in proportions 10:4:1:2. These proportions were chosen to be similar to those in propolis. The concentrations of the standard mixtures (Table III) were chosen in scope to cover the known range of relative concentrations of the corresponding compounds in Bulgarian propolis (22, 41, 44) referring to their peak areas. The concentration of the internal standard in each standard mixture was 1.2 $mg.ml^{-1}$.

Table III

Concentrations of standard solutions used for the calibration graphs.

Compound	Concentration ($mg.ml^{-1}$)			
	Solution 1	Solution 2	Solution 3	Solution 4
Pinocembrin **(1)**	8.33	3.81	2.61	1.01
Galangin **(2)**	5.50	1.36	1.03	0.32
Caffeic acid **(3)**	1.66	0.43	0.26	0.10
Caffeate **(4)**	3.16	0.76	0.36	0.21

Table IIIA

Parameters of calibration graphs.

Compound	*b*	S.D.	ε	ε/*b* . 100 (%)	r
Pinocembrin **(1)**	0.58	0.02	0.04	6.8	0.99
Galangin **(2)**	0.59	0.02	0.04	6.7	0.99
Caffeic acid **(3)**	0.91	0.02	0.04	4.3	0.99
Caffeate **(4)**	0.67	0.02	0.04	6.0	0.99

b - slope of the calibration graph (response factor of the detector to the sample component relative to the internal standard).

S.D. – standard diviation of *b*;

ε - mean error of *b*;

(ε/*b*).100 – relative error (%) of *b*;

r - correlation coefficient.

4.1.1.5. Analysis of Propolis Extract

A 1.50 mg amount of dry propolis extract (obtained with 70% ethanol) was dissolved in 95 µl BSTFA and heated at 65°C for 30 min in a screw-capped vial. After cooling, 4 µl of internal standard solution (300 $mg.ml^{-1}$ in hexane) were added and the sample was injected three times into the gas chromatograph.

4.1.2. Development of a New Procedure for Rapid Qualitative Analysis of Phenolics in Propolis.

4.1.2.1. Propolis Extraction

Propolis was collected in Southern Bulgaria near Plovdiv. Propolis (1g) was grated after cooling and refluxed with 15 ml of methanol for 1 h. The hot extract was filtered, diluted with water and extracted successively with light petroleum (b.p. 40 - 60°C) (3x), and diethyl ether (3x). The ether extracts were combined and evaporated to dryness. This extract (1 mg) was dissolved in 100 µl of acetone, and 1 - 2 µl of this solution was injected into the gas chromatograph.

4.1.2.2. Derivatization

A 1 mg of the model mixture or the ether extract of propolis was silylated with 50 μl BSTFA at 65° for 30 min in a screw-capped vial; 1 - 2 μl of this solution were injected into the gas chromatograph.

4.1.2.3. Gas Chromatography

GC analysis was carried out on a Perkin-Elmer 8700 instrument. The separation was accomplished on a 6m x 0.25mm I.D. SE-54 fused silica capillary column with a film thickness of 0.25 μm. The linear velocity of the nitrogen carrier gas was 9 $cm.s^{-1}$ (split ratio 1:25). The temperature program was as follows: 80 - 280°C at rate $20°C.s^{-1}$, 280 - 300°C at $2°C.s^{-1}$ and a 10 min hold at 300°C. The injector temperature was 320°C and the detector temperature was 350°C. At the end of the column the gas flow was split in a ratio 1:1 using two 10 cm x 0.25 mm, 0.25 μm film thickness SE-54 capillaries, the first of them going into the flame ionization detector and the other into the electron-capture detector.

4.2. Investigations of the Polar Components of Propolis from Different Geographic Origins

4.2.1. Propolis

Propolis samples were collected, as follows:

- Egyptian propolis **Egy** - in Bani Swaief, near Giza.
- Brazilian propolis - **Br-1** near Rio Claro, Sao Paulo State; **Br-2** near Prudentopolis, Parana State; **Br-3** near Pacajus, Ceara State, **Br-4** near Limera, Sao Paulo State.
- Brazilian geopropolis - **G-1** near Picas, Piaui State (gathered by *Melipona compressipes*), **G-2** near Prudentopolis, Parana State (gathered by *Tetragona clavipes*), **G-3** near Prudentopolis, Parana State (gathered by *Melipona quadrifasciata antidioides*).
- Propolis from the Canary Islands - **K-1** near San Mateo, **K-2** near Telde, both on Grand Canaria.
- Albanian propolis - **Alb** near Tirana.
- Bulgarian propolis - **Bg** near Rousse, North Bulgaria.
- Mongolian propolis - **Mong** near Ulan Bator.

- Canadian propolis – the samples were collected near Sidney, in the region of Victoria International Airport, Vancouver Island, British Columbia, and at St-Claude, in the region of Richmond, Quebec.

4.2.2. Extraction procedure

Propolis (1g) was ground and extracted with 10 ml 70% ethanol at room temperature for 24h. The extract was filtered and evaporated to dryness.

4.2.3. Silylation procedure

About 2.5 mg of dry alcohol extract were dissolved in 20 μl dry pyridine, 40 μl BSTFA were added and the mixture heated at 80°C for 20 min in a screw-capped vial.

4.2.4. Gas chromatography-mass spectrometry

For the analysis of Egyptian sample **Egy**, Brazilian geopropolis samples **G-1** - **G-3**, and samples from Canary Islands **K-1** and **K-2**, a 30 m x 0.2 mm I.D. HP-5 fused silica capillary column, 25μm film thickness, was used in a Hewlett-

Packard 5890 gas chromatograph with a HP 5972 MSD detector. The samples were introduced *via* an all-glass injector working in the split mode, with helium as the carrier gas, linear velocity 32cm.s^{-1}. Temperature program: 80 - 240°C at 8°C. min^{-1}, 240 - 300°C at 12 °C. min^{-1} and a 20 min hold at 300°C, injector temperature 300°C.

For the analysis of Brazilian samples **Br-1**, **Br-2**, **Br-3**, **Br-4**, a 25m, 0.2mm I.D., 0.2µm film thickness OV-101 fused silica capillary column was used in a JEOL JGC-20K gas chromatograph directly coupled to a JEOL JMS D-300 mass spectrometer. The samples were introduced *via* an all-glass injector working in the split mode, with helium as a carrier gas, and a temperature program 150 - 280°C at 3°C. min^{-1}.

For the Canadian samples the GC-MS analysis was performed with a Fisons 8060 gas chromatograph connected with Autospec-TOF magnetic sector MS system (Micromass, England). GC conditions: a 25m, 0.2mm I.D.,0.25µm film thickness DB-5MS capillary column was used, splitless injection mode (40s), injector temperature 300°C, and temperature program: initial temperature 80°C (1 min hold) and up to 300°C (6°C/min) with 15 min hold. Column interface T 280°C and ionization source T 250°C. Ionization voltage 70eV.

4.2.5. Identification of Compounds

The identification was accomplished using computer searches on commercial libraries. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass-spectral fragmentation. Reference compounds were co-chromatographed where possible to confirm GC retention times and mass spectral characteristics.

4.2.6. Isolation of the Main Lignans from Propolis from Canary Islands

The main lignan components of propolis from Canary islands, sample K-2, were isolated by separation of the dry EtOH extract (1.1 g) on a silica gel column using hexane - methyl ethyl ketone mixtures with increasing polarity. A Brucker 250 NMR instrument was used to obtain ^{13}C spectra. Four pure substances were isolated as follows:

1a sesamin: EIMS *m/z* (rel. int.): 354 (M^+, 19), 161 (24), 149 (100), 135 (39). ^{13}C-NMR (62.9 MHz, $CDCl_3$): δ 147.9 (C-3' and C-3"), 147.0 (C-4' and C-4"), 135.0 (C-1' and C-1"), 119.3 (C-5' and C-5"), 108.1 (6' and 6"), 106.4 (C-2'and C-2"), 101.0 (two OCH_2O), 85.7 (C-2 and C-6), 71.6 (C-4, C-8), 54.2 (C-1and C-5).

5a aschantin: EIMS *m/z* (rel. int.): 400 (M^+, 84), 207 (31), 195 (42), 181 (54), 149 (100), 135 (63). ^{13}C-NMR (62.9 MHz, $CDCl_3$): δ 153.3 (C-3" and C-5"), 147.9 (C-3'), 147.0 (C-4'), 137.3 (C-4"), 136.7 (C-1"), 134.9 (C-1'), 119.3 (C-5'), 109.0 (C-6'), 108.1 (C-2'), 102.5 (C-2" and C-6"), 71.9 (C-4 or C-8), 71.6 (C-4 or C-8), 60.8 (4"-OCH_3), 56.1 (3"-OCH_3 and 5"-OCH_3), 54.3 (C-1 or C-5), 54.2 (C-1 or C-5).

8a yangambin. EIMS *m/z* (rel. int.): 446 (M^+, 53), 207 (60), 195 (62), 181 (100). ^{13}C-NMR (62.9 MHz, $CDCl_3$): δ 153.3 (C-3', C-3", C-5' and C-5"), 148.5 (C-4' and C-4"), 137.3 (C-1' and C-1"), 102.4 (C-2', C-2", C-6' and C-6"), 77.5 (C-2 and C-6) 71.9 (C-4 and C-8), 60.8 (4'-, 4"-OCH_3), 56.1 (3'-, 3"-, 5'-, 5"-OCH_3), 54.4 (C-1 and C-5).

9a sesartemin: EIMS *m/z* (rel. int.): 430 (M^+, 69), 207 (46), 195 (44), 191 (35), 181 (67), 179 (100), 165 (70). ^{13}C-NMR (62.9 MHz, $CDCl_3$): δ 153.4 (C-3" and C-5"), 149.1 (C-3'), 143.6 (C-5'), 137.4 (C-4"), 136.7 (C-1"), 135.7 (C-1'), 134.6 (C-4'), 105.6 (C-2'), 102.8 (C-2" and C-6"), 101.4 (OCH_2O), 100.0 (C-6'), 85.9 (C-4 or C-8), 85.7(C-4 or C-8), 60.8 (4"-OCH_3), 56.7 (5'-OCH_3), 56.1 (3"- and 5"-OCH_3), 54.3 (C-1 and C-5).

4.3. Development of a New Method of Studying Propolis Chemical Composition Using Metastable Atom Bombardement (MAB) Ionization Mass Spectrometry

4.3.1. Propolis

For the whole MAB studies the sample from Victoria region was used (as described in **4.2.1.**)

4.3.2. Extraction Procedure

As described in section **4.2.2.**

4.3.3. Silylation Procedure

As described in section **4.2.3.**

4.3.4. Gas Chromatography-Mass Spectrometry

As described in section **4.2.4.**

4.3.5. MAB Source

The MAB source (MAB gun and ionization chamber) was obtained from Dephy Technologies (Montreal, Quebec, Canada). Some modifications were made for the extraction lenses and other parts of the original EI outer source for the Autospec-TOF instrument (the same used for the two Canadian samples analysis of the alcohol extract) to fit the MAB source.

4.4. Investigations of Propolis Volatile Oils from Different Geographic Origins

4.4.1. Propolis

Propolis samples were the same as described in section **4.2.1.**

4.4.2. Isolation of Volatile Oils

The propolis samples were grated after cooling and subjected to steam distillation for 4 hours. The collected distillates were extracted with ethyl ether/*n*-

pentane 1:1, the extracts dried over Na_2SO_4, evaporated and submitted to GC-MS analysis.

4.4.3. Gas Chromatography-Mass Spectrometry

For the GC-MS analysis of samples **Alb**, **Bg** and **Mong** a 30 m, 0.2mm I.D., 0.2µm SPB-1 silica capillary column was used in a JEOL JGC-20K gas chromatograph directly coupled to a JEOL JMS D-300 mass spectrometer. The samples were introduced *via* an all-glass injector working in the split mode (split ratio 1:80), with helium as the carrier gas and a temperature program 60 - 280°C at 6°C. min^{-1}.

For the analysis of the Brazilian samples **Br-1**, **Br-2**, **Br-3**, **Br-4** the same column and apparatus were used, temperature program 150 - 280°C at 3°C. min^{-1}.

For the analysis of Brazilian geopropolis samples **G-1** - **G-3**, a 30 m x 0.25 mm ID HP-5, film thickness 25 µm, fused silica capillary column was used in a Hewlett-Packard 5890 gas chromatograph with a HP 5972 MSD detector, with He as a carrier gas, linear velocity 32 cm/min, split ratio 1:10, temperature program 50 - 200°C at 5°C/min, 200 - 300°C at 10°C/min, injector temperature 300°C.

For the analysis of the samples from Canary Islands, a 30 m x 0.25 mm ID HP-5, film thickness 25 µm, fused silica capillary column was used with a

Hewlett-Packard 5890 gas chromatograph and a HP 5972 MSD detector, with He as a carrier gas, linear velocity 32 cm/s, split ratio 1:10, temperature program 50 - 200°C at 5°C/min, 200 - 300°C at 10°C/min, injector temperature 300°C.

4.4.4. Identification of Compounds

The identification was acomplished using computer searches on commercial libraries. In some cases, when identical spectra have not been found, only the structural type of the corresponding component was proposed on the basis of its mass-spectral fragmentation. Reference compounds were co-chromatographed where possible to confirm GC retention times.

4.4. Biological Activity of Propolis from Different Locations

The antibacterial, antifungal and antiviral tests were performed at the Institute of Microbiology of the Bulgarian Academy of Sciences, Sofia, in the laboratory of Associate Prof. Dr. Kujumgiev.

Only the Canadian samples were tested for cytotoxicity and DPPH (diphenylpicrilhydrazyl) free radical scavenging activity according to the new methodologies of Soils *et al.* (170) and Banskota *et al.* (167).

Cytotoxicity assay. Brine shrimp eggs obtained locally (Petrov, Sofia) were hatched following the procedure of Soils *et al.*, 1993 (170). *Artemia salina* (nauplii) lethality was determined using caffeic acid phenethyl ester (CAPE) as active reference compound (positive control). Concentrations of 1000, 100, 10 and 1 µg/ml were used, 10 *A. salina* per treatment plus control (blank).

DPPH free radical scavenging activity. DPPH free radical scavenging activity was measured according to the procedure described by Banskota *et al.*, (167). In brief, the extracts were dissolved in ethanol, the solutions analyzed (250 µl) were diluted to 2 ml with ethanol and 1 ml DPPH solution was added (0.02% in absolute ethanol). The resulting solution was thoroughly mixed and absorbance was measured at 517 nm after 30 min. The scavenging activity was determined by comparison of the absorbance with that of blank (100%), containing only DPPH and solvent. Caffeic acid was used as a positive control.

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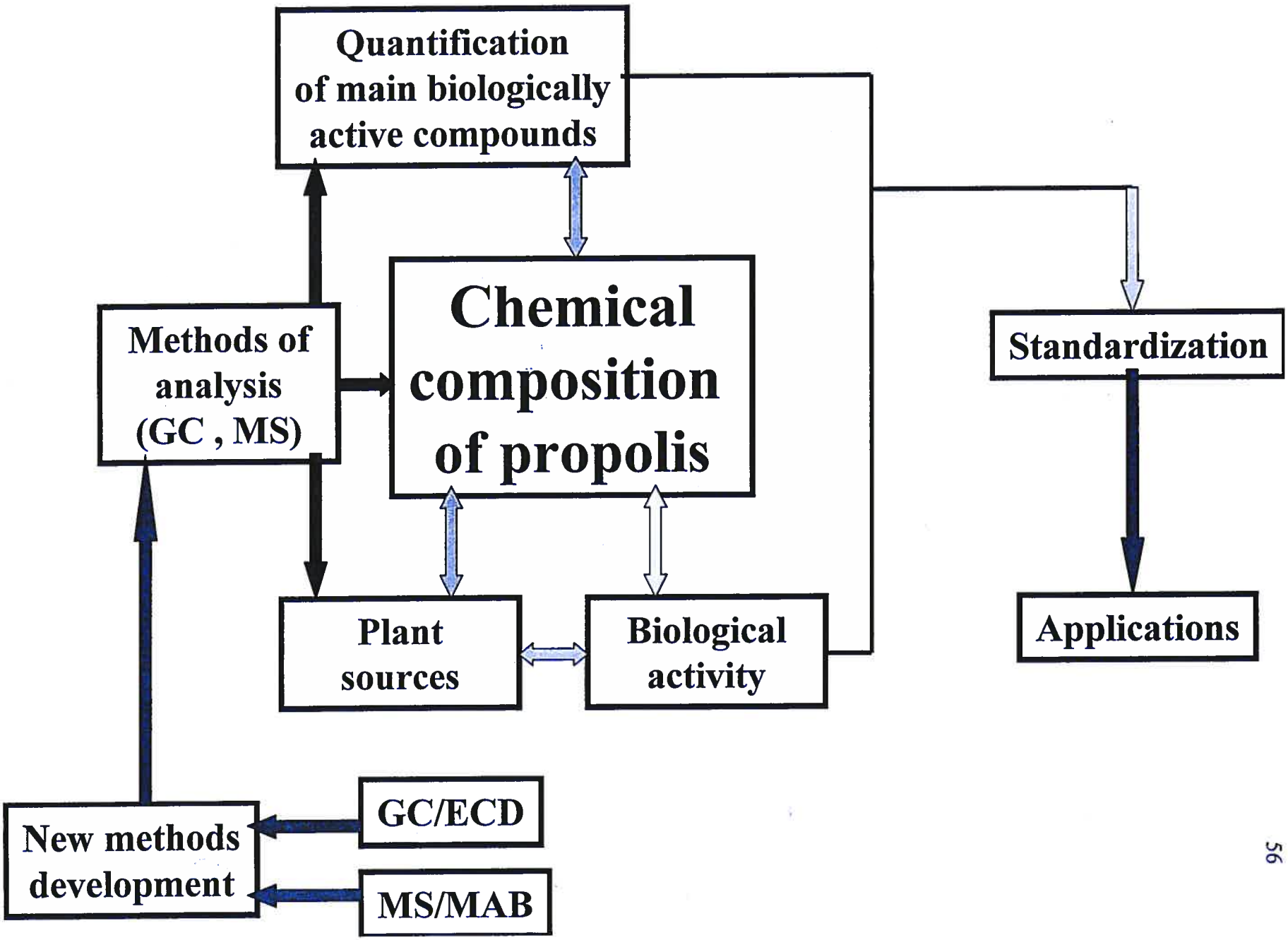
5. Results and Discussion

Based on our review of the literature we can build a simple diagram with not so simple but dynamic mutual relations between its main elements (see p. 56). This diagram will help to better understand and to solve the complex tasks of analysis, standardization and especially the application of propolis. We believe this will be the best approach for the purpose as well as for the evaluation of the present work.

Our main goal is the chemical composition of propolis. Defining its main (and of course as many as possible) constituents will allow for their quantification and further studies of feasible biological activity and possible plant sources. All this can be done with our main tools – gas chromatography and mass spectrometry with their significant potential, which also offers the opportunity for developing new methods of analysis. With all the results obtained a method for standardization of propolis may be proposed and a decision can be made on what predominantly it could be applied for.

The present work starts with quantification of the main components of Bulgarian propolis.

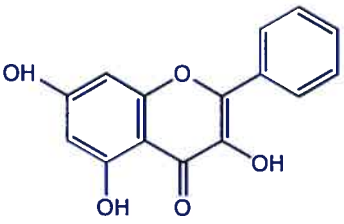

Quantification of main biologically active compounds
Chemical composition of propolis
Methods of analysis (GC , MS)
Standardization
Plant sources
Biological activity
Applications
New methods development
GC/ECD
MS/MAB

5.1. Gas Chromatographic Investigations of the Main Components of Bulgarian Propolis

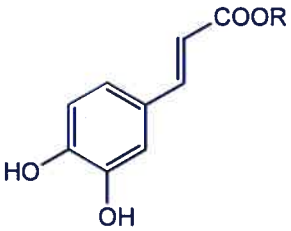
5.1.1. Quantification of the Main Phenolics in Bulgarian Propolis

The quantification of all propolis components is virtually impossible because of its complex composition. For this reason we think it is better to determine only the main representatives of each group of phenolics, which possess biological activity, characteristic for the propolis. After several year-studies of chemical composition and its variations it has been found that in Bulgarian propolis (a typical representative of European type), the main flavonoid aglycones appeared to be pinocembrin **1** and galangin **2**, and the main representatives of aromatic acids and esters are caffeic acid **3** and its β-phenylethyl ester **4**. These compounds have shown antibacterial and antifungal activity (1, 67).

1



2



3. R = H
4. R =CH_2CH_2Ph

It is known that waxes consist up to 30% of the weight of propolis (4). The GC analysis of propolis phenolics cannot be performed in the presence of waxes. Therefore, we tried some solvents for the extraction of propolis in order to prepare an extract with minimum waxes (see Experimental section 4.1.1.1, Table II). TLC showed that extraction with 70% ethanol gave the best results so this was the chosen solvent. This is also in accordance with data from other authors (11, 50).

We have already discussed the advantages and limitations of different methods of analysis of propolis. As we have mentioned so far, the best separation has been achieved by capillary GC, but it has never been used for quantification because some authors had shown that flavonoids break down under the conditions used. They are hard to be eluted from the column and produce smaller signal per mass unit than other phenolics. Thermal destruction and catalysis cause ring opening resulting in chalcones degradation products absent from the original mixture (11, 141, 153).

We found proper conditions for quantification of main propolis phenolics where the key feature was using an unusually short (only 6m) but highly efficient capillary column and conditions providing the shortest possible run time (less than 20 min). The internal standard method was used and the components were determined with *n*-$C_{25}H_{52}$ hydrocarbon (Fig. 1).

The sample was extracted with 70% ethanol (EtOH), silylated with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and subjected to GC analysis. The GC analysis of the propolis extract was repeated three times (Fig. 1), and

the concentrations of the components in the BSTFA solution were **1** = 2.33±0.02; **2** = 1.39±0.03; **3** = 0.21±0.02; **4** = 0.19±0.01 $mg.ml^{-1}$. With the proposed method the limits of detection are of **1** = 0.5, **2** = 0.2, **3** = 0.05 and **4** = 0.1 μg at S/N≥3.

The precision and accuracy of the proposed method are indicated in Table IV. It is evident that in all instances the relative error is less than 4%, which is a very good result for analysis of natural products. This is due to the prior enrichment of the phenolic mixtures and to the high efficiency of the short quartz capillary column. This is an indication that the procedure developed is suitable for analyses, control and standardization of propolis, and propolis preparations. The method has been applied by a pharmaceutical company for veterinary preparation used against post-natal infections in cows.

Table IV

Precision and Accuracy of the determination of compounds 1 – 4

Compound	Concentration ($mg.ml^{-1}$)		Precision, V (%)	Accuracy, A (%)
	Model mixture	Calculated value(x)±S.D. (n=8)		
1	5.50	5.70±0.2	3.5	3.7
2	2.20	2.17±0.2	9.2	1.4
3	0.64	0.66±0.02	3.0	3.0
4	1.20	1.18±0.05	4.2	1.7

$A(\%) = ([compound]_{actual} — [compound]_{calculated}).100/[compound]_{actual}$

$V(\%) = (S.D./x).100$

S.D. = standard deviation

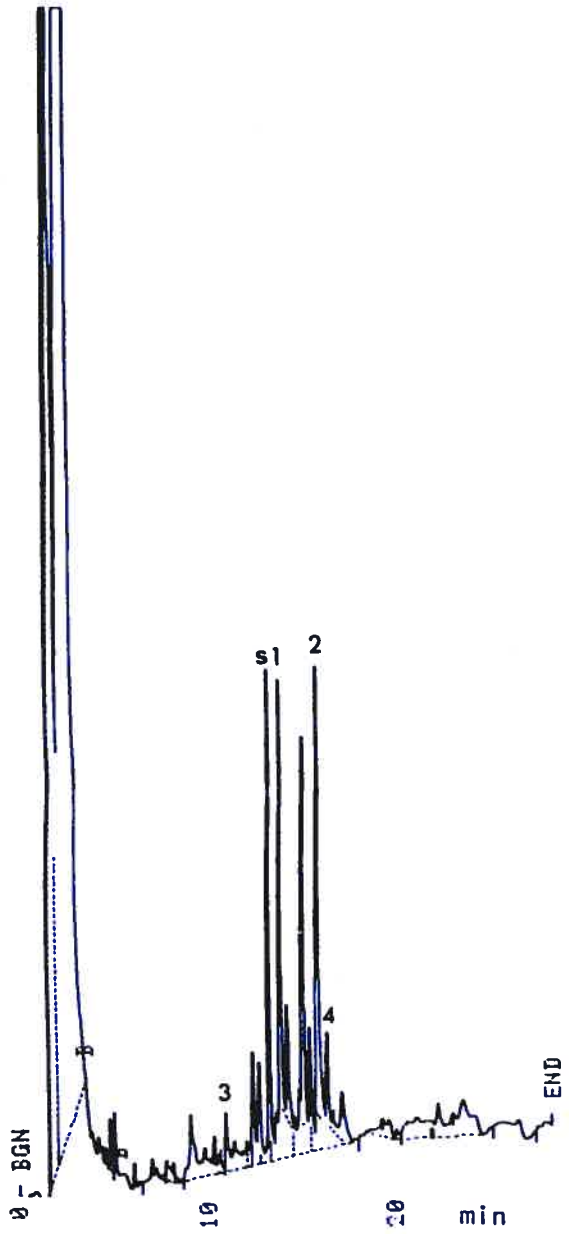


Fig. 1. Capillary GC of a wax-free propolis sample. For conditions, see Experimental section **4.1.1.3.** (p. 41). Peaks numbers correspond to compounds **1-4** from the text (p. 57), s = internal standard (n-$C_{25}H_{52}$)

5.1.2. Development of a New Procedure for Rapid Qualitative Analysis of Phenolics in Propolis.

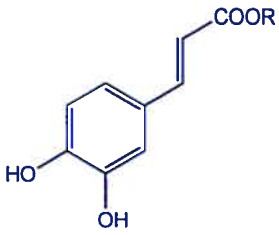
Among the various methods used for separation and analysis of complex mixtures of natural phenolics, such as propolis, the capillary gas chromatography is of major importance due to its sensitivity and resolving power. It is a common practice to prepare derivatives of phenolic compounds before GC analysis [methyl or trimethylsilyl ethers] and to use flame ionization detection (FID) (153). The derivatization is thought to be necessary to increase their volatility, but it has some disadvantages, especially when flavonoids are to be analysed (11, 141, 153) (see previous section **4.1.1.**).

Recent reports have shown that under the conditions of pyrolysis gas chromatography-mass spectrometry some flavonoid aglycones have been detected (154). This is an indication that even underivatized compounds of this type are volatile enough to be analysed by GC columns at 300 - 350^{o}C without thermal degradation.

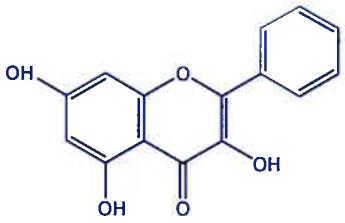
The main groups of propolis phenolics (compounds **1 – 6,** on p. 63), especially the flavonoid aglycones, are known to belong to the so-called "conjugated electrophores", which suggests that an electron might be attached and they may stabilize the negative charge by resonance. That means they might have a good response to an electron capture detector (ECD) (155).

Furthermore, for these compounds the ECD might be even more sensitive than FID.

2



1. R = H
6. R =CH_2CH_2Ph



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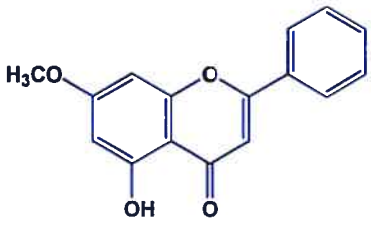


3

4

Experiments were carried out to see if the TMS ethers of propolis phenolics have a significant electron-capture response. In these experiments a model mixture of propolis phenolics (compounds **1 - 4** from the previous section, p. 57) and a propolis extract were used.

Furthermore, the same short (6 m, SE-54) fused silica capillary column was used. At the end of the column the gas flow was split (50:50) and both detectors (FID and ECD) were run simultaneously. It was shown that the electron-capture response was about one order of magnitude higher than the flame ionization response (Fig. 2).

When the injector temperature was increased (280 - 320^{o}C) higher responses were observed for both detectors because of the increased vapour pressure of the compounds analysed. An increase of the detector temperature (320 - 350^{o}C) resulted in a lower electron capture response (15 - 40% for the different compounds). This is an indication that the electron-capture process in this instance represents undissociative attachment (resonance capture) producing a stable negative molecular ion (155, 156).

The high electron-capture response of the conjugated electrophores (silylated flavonoids and cinnamic acid derivatives) encouraged us to pursue the analysis of underivatized propolis phenolic constituents by cGC with electron-capture detection. Again the same column was used for the separation of derivatized and underivatized propolis phenolic components (on p. 63, caffeic acid **1**, pinocembrin **2**, galangin **5**, chrysin **4**, tectochrysin **3**, and β-phenylethyl caffeate **6**). A satisfactory resolution (not the optimum solution) of the underivatized compounds was achieved under the same conditions used for the analysis of the TMS ethers. Therefore, these conditions were used for a comparative study (Fig. 3).

The injector temperature was 320°C; when it was increased to 350°C, only a slight increase in the relative areas of the peaks with the longest retention times (chrysin **4** and galangin **5**) was observed. The percentage of caffeic acid **1** (RT 4.5 min) in these samples was low (less than 1%) (41) and was below the limit of detection. It is interesting to note that when underivatized propolis phenolics were analysed using the two detection modes simultaneously, ECD and FID, only the largest peaks pinocembrin **2** and chrysin **4**, were satisfactorily detected by FID with an acceptable signal-to-noise ratio.

To the best of our knowledge, this is the first analysis of underivatized flavonoid aglycones by capillary GC, made possible because of the good electron-capture response of these compounds. The method proposed allows a rapid qualitative analysis of the main biologically active components of propolis (67).

The good reproducibility of the peak areas and possible further work for finding optimum conditions for GC separation may allow also their quantitative analysis.

Recently, similar studies performed by Pereira *et al.* revealed that flavanoids and other constituents of propolis could be steadily analyzed without preliminary derivatization with capillary GC with FID detection or with GC-MS. The method used, namely high temperature – high resolution GC combined with MS exhibits a significant potential for further feasible studies of analysis of propolis complex composition (192 – 196).

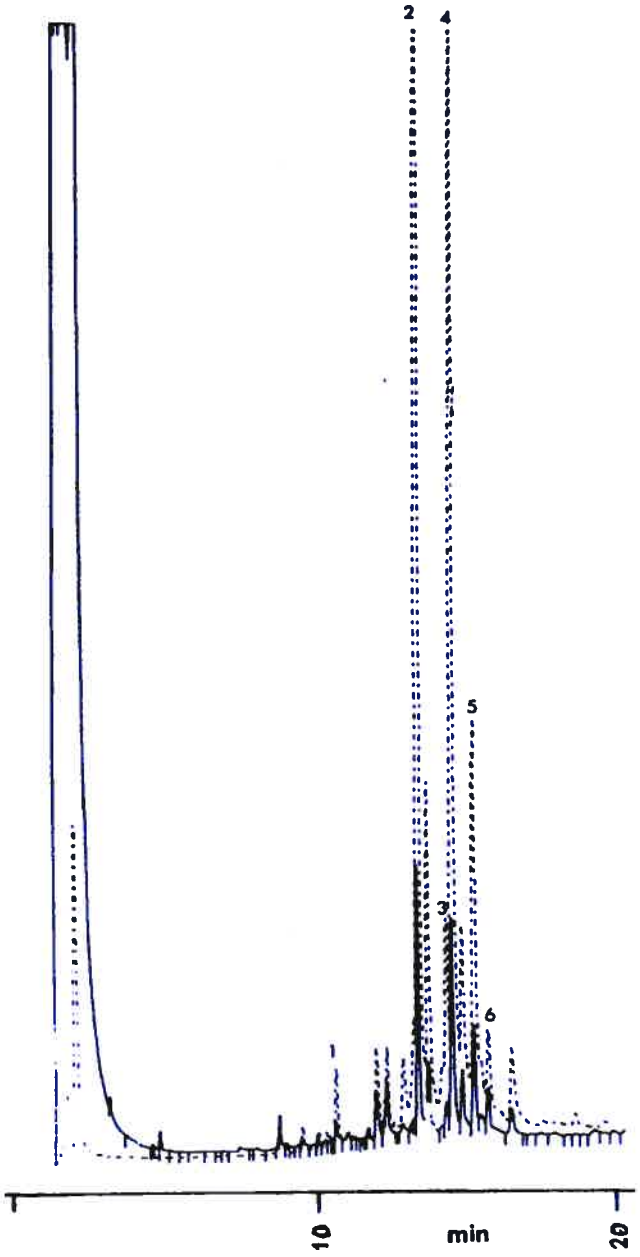


Fig. 2. Capillary GC of TMS ethers of propolis phenolic constituents.
For conditions, see Experimental section **4.1.2.3.** (p. 44). Peaks numbers correspond to compounds **1-6** from the text (p. 63), (—)-FID; (---)-ECD

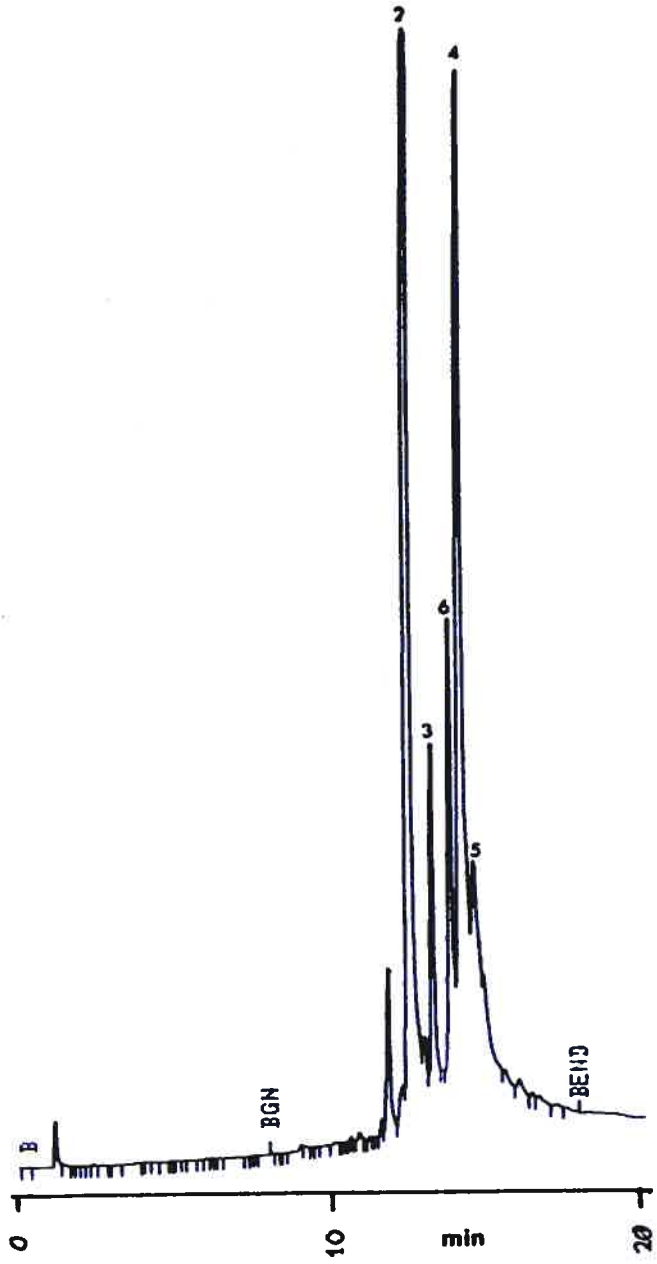


Fig. 3. Capillary GC of underivatized propolis phenolic constituent susing ECD. For conditions, see Experimental section **4.1.2.3.** (p. 44). Peaks numbers correspond to compounds **1-6** from the text (p. 63).

5.2. Investigations of the Polar Components of Propolis from Different Geographic Origins

As mentioned above all the studies we have done so far (quantification and analysis without preliminary derivatization of its constituents) were with propolis from the Temperate zone, and its main components being the typical "poplar bud" phenolics. However, bees collect propolis even in places where no poplars grow, for instance in the tropics. Obviously the chemical composition and plant sources of propolis from the tropics will differ from those of "poplar" propolis, because of the specificity of the local flora (14, 16, 17).

In fact, very little is known about tropical propolis, its chemistry, plant origin and biological activity and it is unclear what kind of substances, if any, could be its typical ones. Studies (none with detailed GC-MS data) published on tropical propolis from Venezuela and Brazil, showed, as expected, that the typical "poplar phenolics" are entirely absent and substantial amounts of prenylated derivatives of benzophenones and cinnamic acid were found (14, 16). These studies also revealed the remarkable variability of tropical propolis and the investigated samples showed large differences in their chemical composition depending on the collection site.

The investigations of propolis from locations outside the Temperate zone are of great importance because they could help to answer the question

whether it is possible to work out some chemical standardization procedure for propolis, different from the "poplar" one.

Following our diagram (p. 56), our main goal was to accumulate data about propolis chemistry in order to define the most typical substances, if any. The study was focused around samples from different climatic regions, their plant origin, biological activity and possibly further creation of a modern standardization procedure.

Based on our and other previous experience we developed a simple and reliable standard procedure (a methodology) for analysis of propolis, which can be applied to all samples regardless of their origin. Briefly, it includes extraction with 70% EtOH (for the extract to contain minimum waxes), filtration, evaporation to dryness, derivatization (silylation with BSTFA) and analysis by GC/MS.

The identification of compounds was based on comparison with mass spectra of authentic samples (computer search on commercial libraries or our own reference mass-spectrometry data and other published by different authors). In some cases when such spectra have not been available only the partial structure, the structural type of the corresponding compound was proposed based on the mass spectral fragmentation observed.

All the identified components of different samples studied are presented in tables, with numbers (in bold), which correspond to the peaks of the corresponding total ion current (TIC) GC/MS chromatogram.

5.2.1. Propolis from Egypt

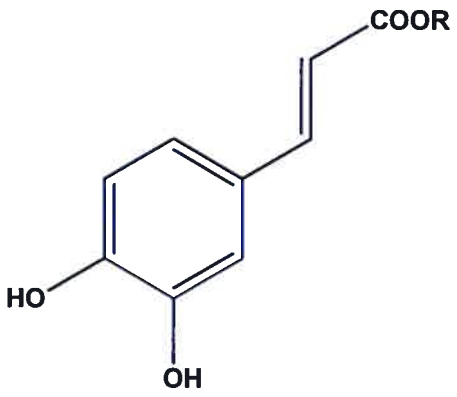
Until now, there are only some preliminary investigations on the chemical composition and biological activity of African propolis performed in Egypt (157, 158). Even though, in this country there are some poplars, the subtropical and tropical climate and the associated specific flora, could affect the chemical composition of Egyptian propolis.

The preliminary investigation of the alcoholic extract by TLC showed similarity with the European propolis: the spots of flavonoids and esters of phenolic acids have been observed, but the amount of the esters was much larger than in European samples.

In order to investigate the chemical composition of the alcoholic extract as completely as possible, it was silylated and subjected to GC/MS analysis (Fig. 4). The results obtained are summarised in Table V. The literature data concerning a Bulgarian sample, originating from *Populus nigra* (41) and a British sample, originating from various *Populus species* (11), is also given as a comparison to the Egyptian propolis.

From the results obtained, it is evident that Egyptian propolis has a complex chemical composition and several groups of compounds were identified. As in the European propolis the main components appeared to be phenolics: phenolic acids, their esters and flavonoids. Phenolic acids concentrations were lower than their corresponding esters, as it was found in

European samples. Most of the identified acids and some of their esters are characteristic for European bee glue: benzoic acid **3**, *p*-coumaric **9**, 3,4-dimethoxycinnamic **12**, ferulic **14** and caffeic acid **15**, as well as three esters of caffeic acid: isopentenyl caffeate **20**, dimethylallyl caffeate **21** and benzyl caffeate **25**. The main components of this group appeared to be four new compounds, tentatively identified as esters of caffeic acid with long-chain alcohols: dodecyl **32**, tetradecyl **33**, tetradecenyl **34** and hexadecyl **35** caffeates. The exact structures of the alcohols remain unknown and their determination needs a further isolation of the esters in pure state.



32. $R = C_{12}H_{25}$
33. $R = C_{14}H_{27}$
34. $R = C_{14}H_{29}$
35. $R = C_{16}H_{33}$

The flavonoid composition of Egyptian propolis resembles that of the European one. In both cases flavanones predominated, but their amount is significantly lower in the Egyptian sample **22, 23, 24, 27, 29, 30** (Table V). Moreover, 1-octadecylglycerol was identified for the first time in propolis.

Contrary to European propolis, the Egyptian sample contained some triterpene alcohols in significant amounts. Two of the major propolis components **37, 38** have very similar spectra and are undoubtedly isomeric pentacyclic triterpenic alcohols from the amyrine type, one of them identified as widely spread in plants β-amyrine. Analogous compounds have been found recently in Brazilian propolis (160) but never in European samples.

In European propolis, some phytosterols have been identified, which are normal for higher plants (58). Surprisingly, in the Egyptian propolis we did not find the above-mentioned sterols. Instead, we found their biogenetic precursors: lanosterol **36** (in low concentration) and cycloartenol **39**, the latter being one of the main propolis constituents. Cycloartenol was found for the first time in propolis.

The comparison of the chemical composition of the investigated sample with the previously studied Egyptian propolis showed significant differences (158). Both samples contained different flavonoids and in the sample, we investigated no chalcones were present. These results confirm the variability of the chemical composition of tropical propolis known from the literature. The explanation could be the complex origin of Egyptian propolis, which must be gathered from more than one plant source. One of the plant sources has to be

some poplar species, probably the most widely distributed in Egypt and especially at the collection site, poplar *P. nigra*. The presence of substances unusual for poplar buds, such as sterol precursors, amyrines, are an indication that there could be other plant sources of propolis in Egypt. In order to solve this problem, propolis from different regions of Egypt has to be investigated, especially these without poplars in the vicinity of the hives. Also, Egyptian plants possessing resinous exudates must be studied as probable sources of propolis.

Table V

Chemical composition (% TIC)[a] of 70% ethanolic extract of propolis from Egypt, compared to European samples.

Compound	Egy	Bg[b]	Brit[c]
Acids (aliphatic)			
Palmitic acid **13**	3.0	<1	-
Stearic acid **18**	0.9	tr	-
Oleic acid **17**	4.0	-	-
Tetracosanoic acid **31**	1.6	-	-
Succinic acid **5**	0.3	-	-
Lactic acid **1**	1.3	-	-
Piruvic acid[e] **2**	0.3	-	-
Acids (aromatic)			
Benzoic acid **3**	0.2	-	2.7
Trans-p-coumaric acid **9**	0.5	<1	6.1
Caffeic acid **15**	0.3	2	2.9
Ferulic acid **14**	0.2	<1	0.1
Dimethoxycinnamic acid **12**	0.4	<1	0.6

Compound	Egy	Bg[b]	Brit[c]
Esters			
Ethyl palmitate **11**	0.5	-	-
Ethyl oleate[e] **16**	1.2	-	-
Isopentenyl caffeate **20**	0.9	5	-
Dimethylallyl caffeate **21**	1.3	6	7.1
Dodecyl caffeate[d,e] **32**	1.1	-	-
Tetradecyl caffeate[d,e] **34**	3.1	-	-
Tetradecenyl caffeate[d,e] **33**	0.3	-	-
Hexadecyl caffeate[d,e] **35**	4.7	-	-
Benzyl caffeate **25**	0.6	3	6.9
Phenylethyl caffeate	-	7	2.1
Sugars			
D-glucose **8**	6.1	-	7.7
Sorbose **7**	3.1	-	-
Fructose **6**	3.1	-	7.0
Sucrose **28**	1.6	-	0.5
Mannitol **10**	0.2	-	-

Compound	Egy	Bg[b]	Brit[c]
Flavonoids			
Pinocembrin **23**	1.1	23	11.8
Galangin **30**	0.7	6	5.0
Chrysin **29**	0.8	4	4.8
Pinostrobin **22**	0.6	tr	-
Pinobanksin **24**	0.3	7	-
3-O-acetylpinobanksin **27**	1.1	6	-
Triterpenic alcohols			
Lanosterol **36**	1.2	-	-
Cycloartenol[e] **39**	7.1	-	-
Triterpenic alcohol of amyrine type[d] **37**	4.8	-	-
β-amyrine[e] **38**	4.7	-	-
Others			
Phosphoric acid **4**	2.7	-	-
Tricosane **19**	0.5	-	-
Glycerol octadecyl ether[d,e] **26**	1.8	-	-

[a]The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

[b] Data from (41)

[c] Data from (174)

[d] Tentatively identified by analysis of mass spectrum

[e] For the first time in propolis

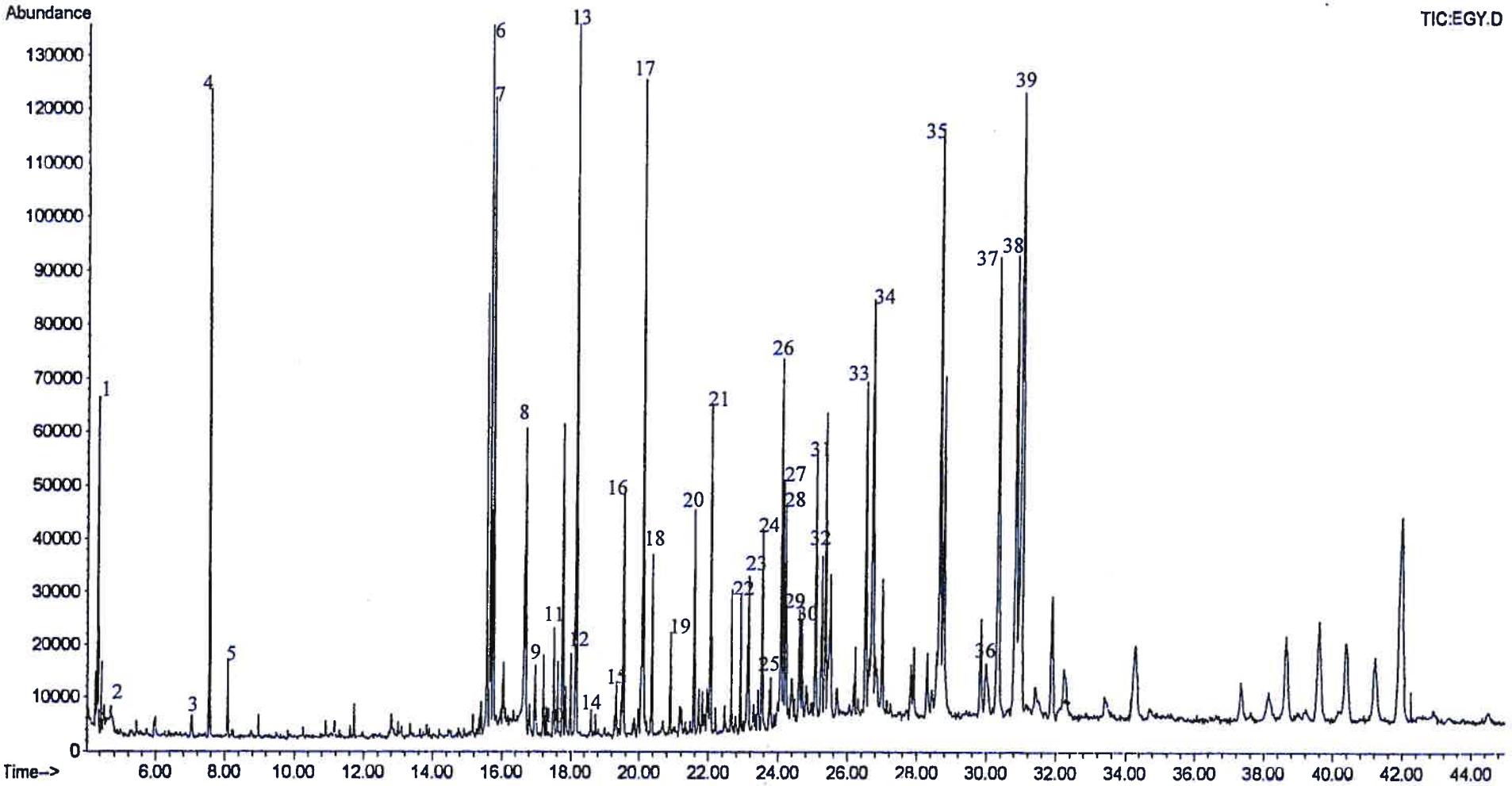


Fig.4. TIC chromatogram of EtOH extract of propolis from Egypt. Peak numbers correspond to compounds in Table V. For conditions, see Experimental section **4.2.4.** (p. 46)

5.2.2. Propolis from Brazil

Studies published on tropical propolis from Venezuela and Brazil, showed, as expected, that the typical "poplar phenolics" are entirely absent (14, 16). These studies also revealed the remarkable variability of tropical propolis.

Using GC-MS, we also studied the chemical composition of four samples of Brazilian propolis, which have been collected from different locations, every one of them characterized by some type of predominant trees or shrubs. Sample **Br-1** was collected from hives in an *Eucalyptus* forest in Sao Paulo state; sample **Br-2** in a native forest in Parana state; sample **Br-3** in a cashew plantation in Ceara state and **Br-4** in an orange plantation in Sao Paulo state. The results obtained are summarized in Table VI.

The GC/MS analyses showed that samples **Br-1** (Fig. 5) and **Br-2** have almost identical chemical composition, independently from the different collection sites and plant environments. For this reason we included in the Table data for **Br-1** only. Samples **Br-1** and **Br-4** were collected in Sao Paulo state but showed differences in their composition.

The composition of the "balsam" (extract with 70% ethanol) in all investigated samples appeared to be unusual for propolis and only few of the peaks were identified. All compound identified (besides *m*-coumaric acid in samples **Br-3** and **Br-4**) have been found earlier in European propolis, originating from poplar buds. However, these compounds are widespread in nature and must have some other origin in the Brazilian bee glue since no

poplars grow in the tropical regions of South America. Some of these compounds (e.g. hydroquinone **2**, *p*-hydroxybenzoic acid **5** and especially dihydrocinnamic acid **3**) appeared in much higher concentration in the Brazilian propolis than in the material from the temperate zone.

Flavonoids are the main constituents of propolis in the temperate zone. In most South American samples investigated until now, flavonoids have not been found. In some samples from Venezuela (14) only traces of highly methylated 6-oxygenated flavones were identified.

In sample **Br-4** we found trace amounts of two dihydroxydimethoxyflavones and in **Br-1** dihydroxydimethoxyflavanone with both hydroxyl groups in ring A. Their identification requires larger amounts of propolis.

It is evident from Table VI, that the compositions of the "balsam" in **Br-1** and **Br-4** are similar. By contrast, in **Br-3** besides oleic and palmitic acids, originating probably from bees wax, we identified only the unusual *m*-coumaric acid.

We can conclude that Brazilian propolis is characterized by very low concentration of flavonoids and esters of phenolic acids. The results obtained confirm the suggestion that the chemical composition of Brazilian propolis is substantially different from that of propolis from the temperate regions because of the different plant sources.

In our opinion now, the above study on Brazilian propolis is the biggest failure in our methodology for analyses of unknown propolis samples. It could

be because of the extraction procedure, or because of our identification capabilities or simply because of the nature of the samples. It is most likely to be a cumulative effect of all these factors. Surprisingly, the results obtained have become a good starting and reference point for many studies of different scientific teams dealing with samples not only from Brazil but also all over the world. Our studies showed for the first time that propolis with completely different chemical composition, containing mainly the above-mentioned compounds, plus as we will see later some prenylated acetophenones has biological activity similar to other samples originating from other climatic zones.

Recently, the extensive studies of Pereira *et al.* confirmed the remarkable variability of the chemical composition of the Brazilian propolis. Samples from diverse regions of Brazil have been analyzed and different classes of compounds have been identified, amongst them flavonoids, triterpenoid alcohols and esters, high molecular weight esters of fatty acids, saccharides, etc. (194, 195, 197, 198). Once again, as we have found before, it has been showed that propolis possesses biological activity irrespective of its chemical composition.

Table VI

Chemical composition (% TIC)[b] of 70% EtOH extract of propolis from Brazil.

Compound	Samples		
	Br-1	Br-3	Br-4
Acids (aliphatic)			
Palmitic acid **7**	2.0	3.0	2.8
Oleic acid	—	2.4	—
Acids (aromatic)			
Benzoic acid **1**	1.7	—	1.1
p-hydroxybenzoic acid **5**	1.5	—	0.5
m-hydroxybenzoic acid	—	—	0.5
Meyhoxybenzoic acid **4**	1.2	—	—
Dihydrocinnamic acid **3**	14.4	—	5.4
p-coumaric acid **6**	9.4	—	—
m-coumaric acid[a]	—	2.4	2.9
Caffeic acid **8**	2.7	—	3.3
Others			
Ethyl caffeate	—	—	0.6
Hydroquinone **2**	1.1	—	0.8

[a]For the first time in propolis

[b]The ion current generated depends on the characteristics of the compound concerned and it is not a true quantification.

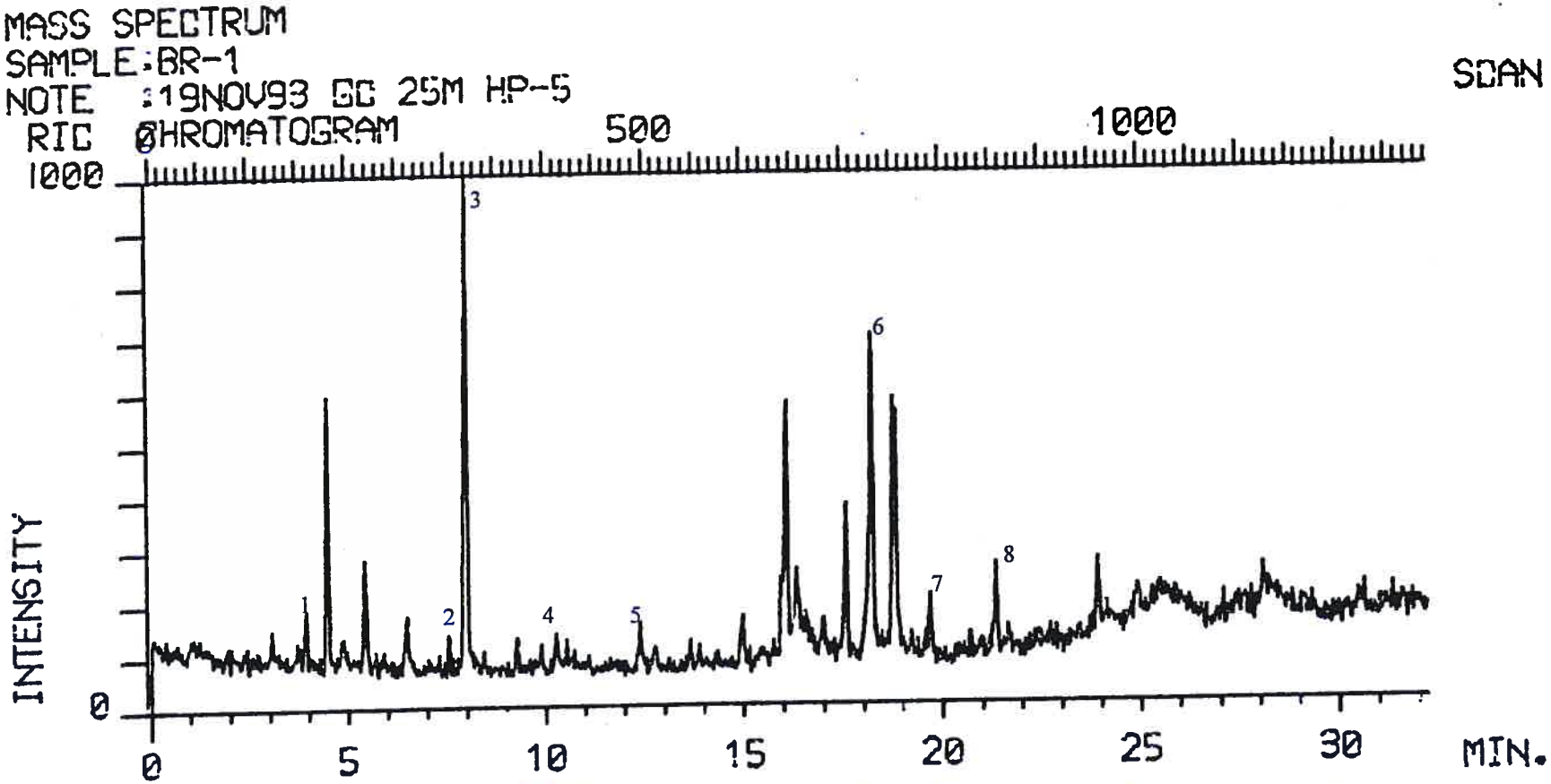


Fig.5. TIC chromatogram of EtOH extract of propolis from Brazil, sample **Br-1**. Peak numbers correspond to compounds in Table VI. For conditions, see Experimental section **4.2.4.** (pp. 46, 47)

5.2.3. Geopropolis from Brazil

In tropical South America there are some indigenous stingless bee species, which collect resinous material from plants, mixing it with beeswax and soil to form the so-called *geopropolis*. Very little is known about its chemical composition. Only one investigation has been published on the phenolic constituents of propolis from 5 species of South American stingless bees in Venezuela (14), including some *Melipona* species. We investigated geopropolis collected by three different bee species widespread in Brazil: *Melipona compressipes* (sample **G-1**), *Melipona quadrifasciata anthidioides* (sample **G-2**) and *Tetragona clavipes* (sample **G-3**). The samples **G-2** and **G-3** originated from the same region.

The preliminary TLC investigation of the alcohol extracts showed significant differences between the three samples. In order to perform a complete analysis of geopropolis and compare the results obtained with Brazilian propolis from *Apis mellifera* (honey bee) the total alcohol extracts were silylated and subjected to a GC/MS investigation (Fig. 6). The results are summarised in Table VII.

It is evident that all geopropolis samples have a complex chemical composition. Part of the GC/MS peaks remained unidentified because of lack of authentic samples and library spectra of corresponding compounds. We identified more than 50 compounds of which the main group being non-

phenolic acids. All three samples contained significant amounts of lactic **1** acid and phosphoric **5** acid, as well as long-chain fatty acids (stearic **18**, palmitic **14**, myristic **11**), usually found in propolis. Two odd numbered acids, 15:0 and 17:0 (margarinic acid), were identified in samples **G-2** and **G-3**.

Analogous to all other propolis samples investigated until now, geopropolis contained the following aromatic compounds: acids, aldehydes and alcohols. However, they were different in kind in the three samples, and their concentrations were relatively low as. Only cinnamic acid **6** was common for all the three samples. Surprisingly, dihydrocinnamic acid, which appeared to be characteristic for Brazilian propolis (see **5.2.2**), is absent in geopropolis (only traces of it have been identified in **G-1**).

While prenylated benzophenones were found to be typical for propolis gathered by indigenous bees in Venezuela (incl. *Melipona compressipes*) (14), no such substances were present in Brazilian geopropolis. We identified only *p*-hydroxyacetophenone in sample **G-1**.

In all samples investigated diterpenic acids were found **19, 20, 21, 22, 24**, their amounts being more prominent in samples **G-2** and **G-3**. Compounds of this type have been identified earlier in Brazilian propolis (56, 57) but never in propolis from the temperate zones. The similarity between their mass spectra and lack of library spectra and reference samples made their identification tentative and only the structural type was defined. Dehydroabietic acid, accompanied by its isomers and analogues were found in the

investigated samples (mainly in **G-1**) but most of them remained unidentified. In **G-2** we found also the diterpenic hydrocarbon kaur-16-ene **15**.

In sample **G-2** a number of pentacyclic triterpenoid alcohols were identified again only by mass spectra (160). β-Amyrine **30** is among the main components accompanied by four other triterpene alcohols **25, 26, 27, 28** most likely amyrines isomers (they show very small differences in their retention times and in the mass spectral peaks intensities). In this sample we also identified the pentacyclic triterpene friedooleanan-3-one **29** and probably some of its isomers **31**. Contrary to **G-2**, in the other two samples only traces of triterpene alcohols were identified. Triterpenic alcohols of amyrine type were recently found in Egyptian and Brazilian propolis (see section **5.2.1**) (160).

Flavonoids are among the main components of propolis from the temperate zones (1). In samples **G-2** and **G-3** flavonoids were practically absent. Only in **G-1** significant amounts of two flavonoids were present. One of them identified as pinobanksin, while partial structure of the second one is trihydroxymethoxy flavone.

Tomas-Barberan *et al.* (14) have shown that the composition of propolis from South American stingless bees does not depend on the bee species, and that propolis from *Apis mellifera* and indigenous bees has a similar composition. Our results do not support this conclusion. All three samples possess different chemical composition. They also differ from Brazilian hive bee propolis (see section **5.2.2**). While the specificity of sample **G-1** could be explained by the different geographic location, samples **G-2** and **G-3** were

collected at the same location and the differences observed might be associated with the bee species. Obviously the composition of **G-3** is simpler than this of **G-2**. In the latter much more terpenic compounds and especially triterpenes were found, while only traces of triterpenes were identified in **G-3**. Our findings indicate that both geopropolis samples have different plant sources - evidently *Tetragona clavipes* makes use of a specific propolis source, rich in triterpenes. Another difference between **G-2** and **G-3** is the presence of aromatic aldehydes only in the latter; *Melipona quadrifasciata anthidioides* probably takes these aldehydes from a plant, which is not visited by *Tetragona clavipes*. More investigations are needed to answer the question whether different indigenous bee species have any preferred propolis plant sources or whether the constitution of the local flora is mostly important for propolis chemical composition.

New studies on propolis collected by stingless bees (Apidae, Meliponinae) native to South-Eastern Brazil showed that it contained high concentrations of pentacyclic triterpenes like lupeol, lupeol acetate and α- and β-amyrines (198, 199). It is worth noting that propolis gathered by two different bee taxa (Meliponinae and Hymenoptera) from the same region showed, with slight variations, no differences in their chemical composition. This fact is probably again related to the specificity of the local flora.

Table VII

Chemical composition (%TIC)[a] of 70% EtOH extract of Brazilian geopropolis.

Compound	Samples		
	G-1	G-2	G-3
Acids (aliphatic)			
Lauric acid **8**	---	0.1	0.2
Myristic acid **11**	0.4	0.7	0.2
Pentadecanoic acid [b]	—	—	0.9
Palmitic acid **14**	2.5	3.2	3.8
Palmitoleic acid [b] **13**	0.8	0.2	1.0
Margarinic acid [b] **16**	—	0.2	0.4
Stearic acid **18**	0.8	0.9	1.4
Oleic acid **17**	1.8	1.3	1.9
Arachidonic acid **23**	—	0.2	—
Lactic acid **1**	0.9	0.7	2.2
Hydracrylic acid [b] **2**	—	0.1	—
Acids (aromatic)			
Benzoic acid **4**	—	0.4	0.2
p-Hydroxybenzoic acid **7**	0.1	0.3	—
Gallic acid	0.1	—	—

Compound	Samples		
	G-1	G-2	G-3
Vanillinic acid	—	—	0.4
Cinnamic acid **6**	1.2	0.3	0.5
cis-*p*-Coumaric acid	0.8	—	—
trans-*p*-Coumaric acid **12**	3.0	0.6	—
Dihydroferulic acid [b]	—	—	0.1
Phenols and aromatic alcohols			
Benzyl alcohol	0.1	—	—
p-Vinylphenol [b] **3**	—	0.2	—
Hydroquinone	0.2	—	—
p-Coumaric alcohol	-	—	1.1
3-(2-Hydroxyphenyl)-propanol	0.2	—	—
3-(4-Hydroxyphenyl)-propanol	0.3	—	—
Aromatic aldehydes and ketones			
p-Hydroxybenzaldehyde	0.2	—	—
Vanillin	—	—	1.2
Coniferylaldehyde	—	—	2.0
o-Hydroxyacetophenone	1.7	—	—

Compound	Samples		
	G-1	G-2	G-3
Vanillinic acid	—	—	0.4
Cinnamic acid **6**	1.2	0.3	0.5
cis-*p*-Coumaric acid	0.8	—	—
trans-*p*-Coumaric acid **12**	3.0	0.6	—
Dihydroferulic acid [b]	—	—	0.1
Phenols and aromatic alcohols			
Benzyl alcohol	0.1	—	—
p-Vinylphenol [b] **3**	—	0.2	—
Hydroquinone	0.2	—	—
p-Coumaric alcohol	-	—	1.1
3-(2-Hydroxyphenyl)-propanol	0.2	—	—
3-(4-Hydroxyphenyl)-propanol	0.3	—	—
Aromatic aldehydes and ketones			
p-Hydroxybenzaldehyde	0.2	—	—
Vanillin	—	—	1.2
Coniferylaldehyde	—	—	2.0
o-Hydroxyacetophenone	1.7	—	—

Compound	Samples		
	G-1	G-2	G-3
Sugars			
Glucose	0.8	—	—
Pentose **9**	—	0.2	—
C-5 sugar alcohol **10**	—	0.1	—
Flavonoids			
Pinobanksin	5.2	—	—
Dihydroxymethoxyflavanone	5.0	—	—
Diterpenes			
Kaur-16-ene [b] **15**	—	0.6	—
Dehydroabietic acid [b]	1.4	—	—
Diterpenic acid (M=302 RT[c] - 21.27) **19**	0.3	1.1	0.7
Diterpenic acid (M=302 RT - 21.79) **20**	1.0	2.5	8.1
Diterpenic acid (M=302 RT - 21.88) **21**	—	0.6	—
Diterpenic acid (M=302 RT - 22.11) **22**	—	0.3	0.3
Diterpenic acid (M=304)	—	—	1.1
Hydroxyditerpenic acid (M=320) **24**	—	1.3	—

Compound	Samples		
	G-1	G-2	G-3
Tritrpenes			
β-Amyrine **30**	—	2.5	—
Triterpene alcohol of amyrine type (RT - 28.57) **25**	—	11.1	traces
Triterpene alcohol of amyrine type (RT - 28.89) **26**	—	4.9	traces
Triterpene alcohol of amyrine type (RT - 30.33) **28**	—	8.3	—
Triterpene alcohol (RT - 29.98) **27**	—	9.8	—
Friedooleanane-3-one [b] **29**	1.4	7.2	—
Triterpene ketone **31**	—	3.8	—
Others			
Phosphoric acid **5**	0.9	0.5	1.1
Methyl *p*-coumarate	0.2	—	—
Coumaran (pesticide)	0.1	—	0.2
Benzothiazole (pesticide) [b]	0.3	—	—

[a] TIC - total ion current. The ion current generated depends on characteristics of the compound and is not a true quantitation.

[b] For the first time in propolis

[c] RT - retention time (min)

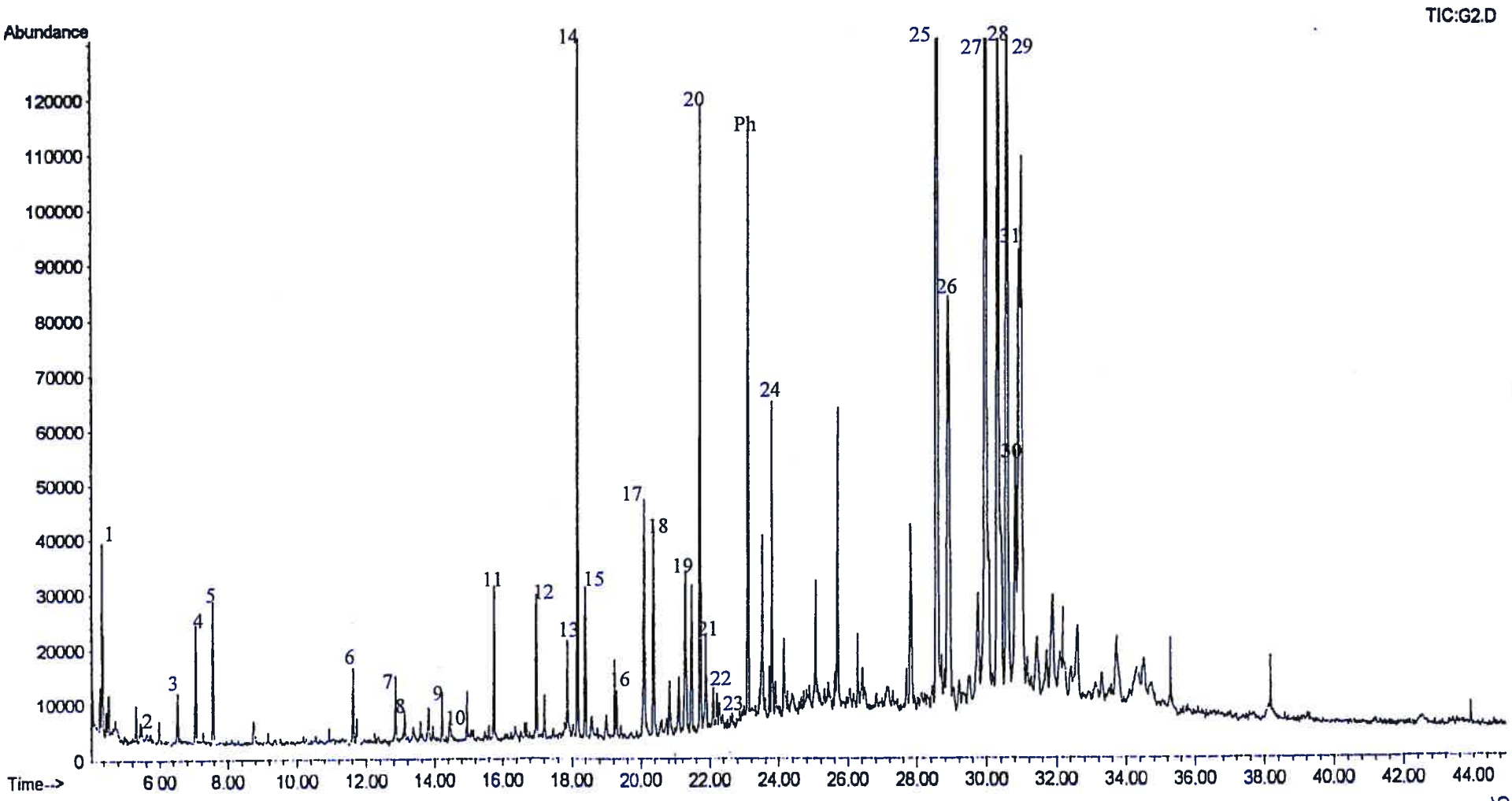


Fig.6. TIC chromatogram of EtOH extract of geopropolis from Brazil, sample **G-2**. Peak numbers correspond to compounds in Table VII, Ph – phtalate. For conditions, see Experimental section **4.2.4**. (p. 46)

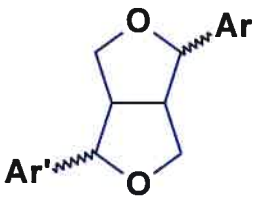
5.2.4. Propolis from the Canary Islands

The investigations on tropical propolis have shown significant differences in the chemical composition of samples originating from different geographic locations (see sections **5.2.2** and **5.2.3.**). Continuing our studies, we also analyzed 2 samples from the Canary Islands. The bee glue from that region might be of special interest because of the climatic differences from Europe and tropical South America, as well as the absence of poplars in this area. This fact implies other source(es) of propolis.

Both samples were collected at the Island of Gran Canaria. Preliminary analysis by TLC showed a significant similarity in their chemical composition. Only quantitative differences existed. The extracts with 70% alcohol were investigated by GC-MS (Fig. 7) and the results obtained are summarised in Table VIII.

Besides some low molecular mass organic acids including phosphoric acid, characteristic for propolis from different regions, the investigated samples contained mainly carbohydrates and phenolics. Sample **K-1** was very rich in carbohydrates: pentoses, hexoses and disaccharides. The main compounds of this group, identified as mannose **26**, glucose **28**, fructose **23** and sucrose **35**, characteristic for honey and propolis, were found in significant amounts. Some polyalcohols as xylitol and mio-inositol etc. were also detected. The same compounds have been found in sample **K-2**, however, in lower concentrations.

The phenolic compounds in both samples appeared to be identical, but their concentrations in **K-2** were much higher. Contrary to bee glue samples from the temperate zones, the typical "propolis phenolics" were now absent. Instead, two of the significant components of sample **K-2** were identified with 97% probability match of computer mass spectrometry library as episesamin **1** and methyl xanthoxylol **2**. These substances belong to an unusual (for propolis) group of plant phenolics, lignans, and are both of the furofuran type (2,6-diaryl-3,7-dioxabicyclo[3,3,0]octanes). The mass spectral fragmentation of furofuran lignans produces a few very typical fragments as shown in Fig. 8 (161). The peaks in the mass spectra of the identified components **1** and **2** were confirmed by this fragmentation.



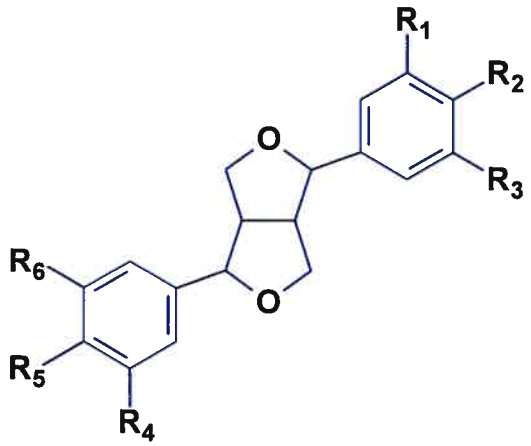
$M^{+\cdot}$

$ArCHO^{+\cdot}$	$ArCO^{+}$	$ArCH{=}CHCH_2^{+}$	$ArCH_2^{+}$
$Ar'CHO^{+\cdot}$	$Ar'CO^{+}$	$Ar'CH{=}CHCH_2^{+}$	$Ar'CH_2^{+}$

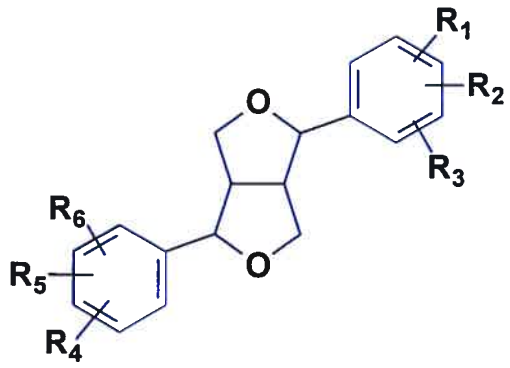
Fig. 8. Mass-spectral fragmentation of furofuran lignans according to (161)

Careful analysis of the mass spectra allowed us to propose the tentative structures of 11 other members of this class of compounds, **3 - 13**, present in both propolis samples from the Canary Islands. The molecular mass and the masses of fragment ions shown in Fig. 8 enabled to determine the type and the number of substituents in every aromatic nucleus but not their exact positions. The fragmentation cannot give information about the stereochemistry of the molecule (162). So substances **12** and **13** possess mass spectra identical to **5** and **6** respectively but different retention times (t_R). Probably they are positional isomers of **5** and **6** (Fig. 9).

Fig. 9. Lignans found using GC-MS



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	OCH_2O		H	OCH_2O		H
2	OMe	OMe	H	OCH_2O		H



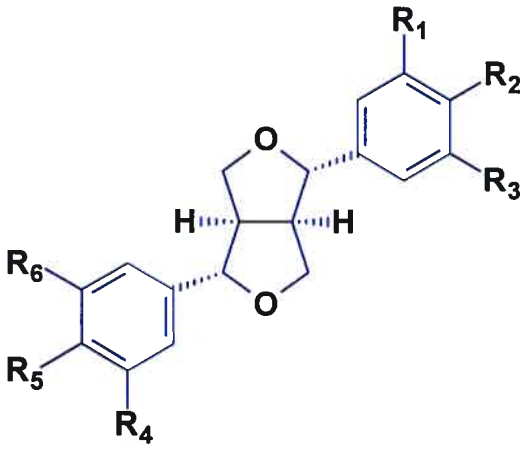
Compound	R_1	R_2	R_3	R_4	R_5	R_6
3	OMe	OMe	H	OMe	OMe	H
4	OH	OH	H	OCH_2O		H
5, 12	OMe	OMe	OMe	OCH_2O		H
6, 13	OMe	OMe	H	OMe	OMe	OMe
7	OMe	OH	H	OMe	OMe	OMe
8	OMe	OMe	OMe	OMe	OMe	OMe
9	OMe	OMe	OMe	OCH_2O		OMe
10	OMe	OMe	H	OH	OH	H
11	OMe	OMe	OMe	OH	OH	H

According to its mass spectrum, substance **10** has two methoxy groups in one of the aromatic ring and two hydroxy groups in the other one; while in **11** one of the ring bears three methoxy groups, and the other one two hydroxy groups. To the best of our knowledge, furofurans with such distribution of substituents have not been reported from natural sources until now. We are not able to give their exact constitution and stereochemistry, but evidently they are new natural compounds.

Until now, only one lignan was found in propolis in small amounts (159), belonging to the benzofurane type. The discovery of the lignans can give information about the origin of the propolis from the Canary Islands. The source has to be a plant species producing resinous exudate rich in lignans of the furofuran type. According to the data we obtained, there could be a second plant source from which most of the sugars, besides glucose and fructose, originate.

The stereochemistry of the lignans is of great importance with respect to the elucidation of the plant source since different stereoisomers were found in natural sources. Thus, we tried to isolate the main furofuran lignans from sample **K-2** using column chromatography on silica gel. Four individual substances were isolated and characterized by mass and ^{13}C NMR spectra as the known compounds **1a** sesamin, **5a** aschantin, **8a** yangambin and **9a** sesartemin (Fig. 10) (163, 164).

Fig. 10. Isolated lignans



Compound	R_1	R_2	R_3	R_4	R_5	R_6
1a	OCH_2O		H	OCH_2O		H
5a	OMe	OMe	OMe	OCH_2O		H
8a	OMe	OMe	OMe	OMe	OMe	OMe
9a	OMe	OMe	OMe	OCH_2O		OMe

The propolis samples from Gran Canaria turned out to be very different from all other samples investigated until now, including tropical ones. This is another confirmation of the thesis that much more data are needed about the chemistry of propolis from tropical regions in order to better understand its origin and potential application.

Table VIII

Chemical composition (%TIC)[a] of EtOH extracts of propolis from Canary Islands (Gran Canaria)

Compound	K-1	K-2
Acids		
palmitic acid **29**	0,9	0,5
Stearic acid **33**	0,1	0,1
Oleic acid **32**	1,1	1,0
Methylmalonic acid [b] **16**	<0,1	<0,1
Lactic acid **14**	0,3	0,3
Malic acid **17**	0,2	0,1
Dimethoxybenzoic acid	<0,1	—
Phosphoric acid **15**	1,5	0,9
Sugars		
D-ribofuranose **21**	0,5	0,1
D-xylopiranose [b] **22**	0,2	0,1
D-mannopyranose [b] **26**	13,0	2,0
D-sorbopyranose **24**	9,5	2,1
D-galactose [b] **25**	1,2	0,4
D-fructose **23**	5,6	1,7

Compound	K-1	K-2
β-D-glucopyranose **28**	10,4	2,0
Sucrose **35**	1,6	0,7
Lactose [b]	0,5	—
Maltose [b] **36**	2,4	0,3
Melibiose [b] **37**	0,5	0,2
Sugar alcohols and acids		
Erytritol [b] **18**	0,1	0,1
Xylitol [b] **20**	0,1	<0,1
Inositol [b] **27**	0,2	0,1
myo-inositol [b] **31**	0,1	0,1
Erytraric acid [b]	0,1	—
2-deoxyerytropentaric acid [b] **19**	0,1	0,1
Tetronic acid [b]	<0,1	—
Glucuronic acid [b] **30**	0,3	0,1
Lignans		
Isosesamin [b] **1**	2,0	7,4
Methyl xantoxylol [b] **2**	3,1	13,5
3 [b]	0,1	0,6
4 [b]	0,1	0,4

Compound	K-1	K-2
5 [b]	0,2	1,1
12 [b]	4,5	20,3
6 [b]	0,4	1,8
13 [b]	1,4	6,4
7 [b]	0,2	1,0
8 [b]	2,8	13,5
9 [b]	1,8	7,4
10 [b]	0,1	0,4
11 [b]	0,1	0,4
Others		
Diterpenic acid **34**	0,1	0,1

[a] The ion current generated depends on the characteristics of the compound concerned and it is not a true quantification.

[b] For the first time in propolis.

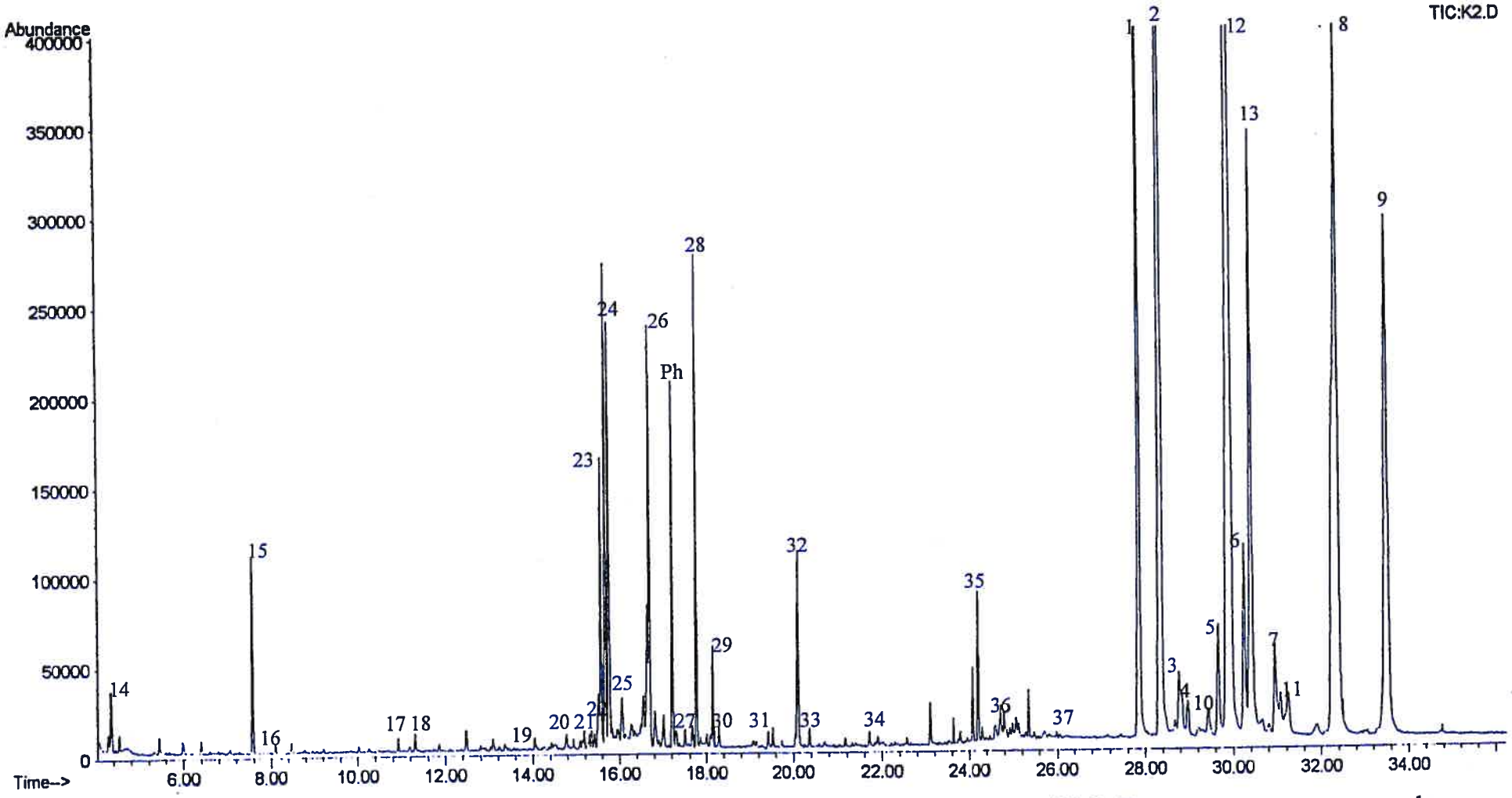


Fig.7. TIC chromatogram of EtOH extract of propolis from Canary Islands, sample **K-2**. Peak numbers correspond to compounds in Table VIII, Ph – phtalate. For conditions, see Experimental section **4.2.4.** (p. 46)

5.2.5. Propolis from Canada

As we have noticed several times, bud exudates of poplar trees are the main source of bee glue in the Temperate zone (7) and the chemical data show a clear preference to *Populus* species belonging to the section *Aigeiros* (10, 25, 30, 67). However, propolis from the northern regions, where *Aigeiros* poplars are absent, has received little attention. In Northern Russia, birch (*Betula verrucosa* Ehrh.) and trembling aspen (*P. tremula* L., sec. *Leuce*) are documented as propolis plant sources (66). In Canada, only bee glue from Sydenham, Ontario, has been analyzed and found to originate from poplars of section *Aigeiros*: *P. deltoides* Marsh, *P. fremontii* Wats. or *P. maximoviszii* Henry (13). We wanted to study the chemical composition and biological activity of bee glue from regions in Canada that lay outside the area of distribution of *Aigeiros* poplars: Boreal forest (near Richmond, Quebec) and Pacific coastal forest regions (near Victoria, British Columbia).

The chemical composition of the ethanol extracts of both samples was investigated by GC-MS after silylation (Fig 11). The results obtained showed distinct chemical profiles of the two specimens (Table IX).

What are the most characteristic things for them? For both the main aromatic acids are benzoic, cinnamic and *p*-coumaric (peaks **2**, **9**, **16** for Victoria; and **2**, **6**, **15** for Richmond). The main esters for Richmond sample are benzyl-E-*p*-coumarate **22** and benzyl ferulate **28**, but this propolis does not

contain benzyl hydroxybenzoate **17** and benzyl methoxybenzoate **18**, which are the main representatives from this group in the propolis from Victoria.

The sample from Victoria contains only a few low abundant flavonoids (**27**, **28**, **34**) compared to the Richmond sample (**23**, **24**, **26**, **27**, **30**). Surprisingly, we found significant amounts of flavonoid biogenetic precursors instead, namely 5 dihydrochalcones. Amongst them, 2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone **29**, 2',4',6'-trihydroxy-4-methoxydihydrochalcone **30** and 4,2',6'-trihydroxy-4'-methoxydihydrochalcone **32** are observed for the first time in propolis. Dihydrochalcones are considered to be characteristic of poplars of Section *Tacamahaca* but not of Section *Aigeiros* and have been found in propolis samples only rarely and in low concentrations (25). Obviously, the plant source of this sample was a poplar of Section *Tacamahaca*. Two species of this section are widespread throughout Canada: *P. trichocarpa* Torr. et Gray and *P. balsamifera* L. (177). The black cottonwood *P. trichocarpa* is regarded as the Pacific coastal species of poplar (178). The major components of *P. trichocarpa* exudates have been found to be *p*-hydroxyacetophenone (also the major component in our sample, peak **8**), benzyl hydroxybenzoate and cinnamic acid (12). These compounds were the main components of the sample from the region of Victoria. Thus its plant source is definitely *P. trichocarpa*. And this represents the first report of bee glue collected from a poplar tree from Section *Tacamahaca*.

As we pointed out the sample from Richmond region was characterized by large amounts of *p*-coumaric and cinnamic acids, while acetophenones and

dihydrochalcones were completely absent. This sample, as expected, also lacked the typical compounds of section *Aigeiros* bud exudates: series of pinobanksin 3-O alkanoates and caffeic acid derivatives (25). The high concentration of cinnamic and *p*-coumaric acid and the low concentration of flavonoids are typical of poplars from section *Leuce*, subsection *Trepidae*, such as *P. tremula* (67). A representative of this subsection in North America is the widespread aspen *P. tremuloides* Michx. Obviously, like *P. tremula* in the European Boreal forests (66), its close relative *P. tremuloides* can serve as propolis source plant in the Canadian Boreal forests.

Evidently, in the absence of poplars of section *Aigeiros*, bees have found other poplar trees to be suitable as propolis sources, which resulted in varying chemical composition of the bee glue.

As mentioned above, our samples originate from two distinct vegetation regions of Canada: the Boreal forest to the northeast of Montreal, and the Pacific coastal forest in British Columbia (179). In both zones poplars of section *Aigeiros* are not present. Nonetheless, the bees have chosen the most widespread *Populus* species in the corresponding regions to collect bud exudates. These results demonstrate that honey bees are able to find suitable plant sources of bee glue in the absence of their most preferred propolis source, *P. nigra* L., just like they do in tropic habitats (7). Obviously, Northern type propolis is a promising source of biologically active substances and deserves further investigations.

Table IX

Chemical composition of EtOH extracts of Canadian propolis (%TIC)[a]

Compound	Victoria	Richmond
Aromatic acids		
Benzoic acid	1.6 **2**	9.7 **2**
Dihydrocinnamic acid	0.4 **5**	0.3 **4**
Z-cinnamic acid	0.3 **7**	-
E-cinnamic acid	10.3 **9**	9.1 **6**
3-phenyl-3-hydroxypropanoic acid	1.4 **10**	-
Methoxyphenylpropanoic acid	0.6 **12**	-
4-hydroxybenzoic acid	0.6 **11**	-
Z-*p*-coumaric acid	-	0.6 **9**
E-*p*-coumaric acid	3.4 **16**	18.8 **15**
Ferulic acid	1.0 **20**	3.1 **17**
Caffeic acid	-	0.8 **18**
Other aromatics		
Benzyl alcohol	0.1 **1**	0.3 **1**
4-hydroxybenzaldehyde	0.3 **3**	-
Hydroquinone	0.6 **4**	-
Cinnamyl alcohol	0.4 **6**	0.1 **5**
Hydroxyacetophenone	16.8 **8**	-

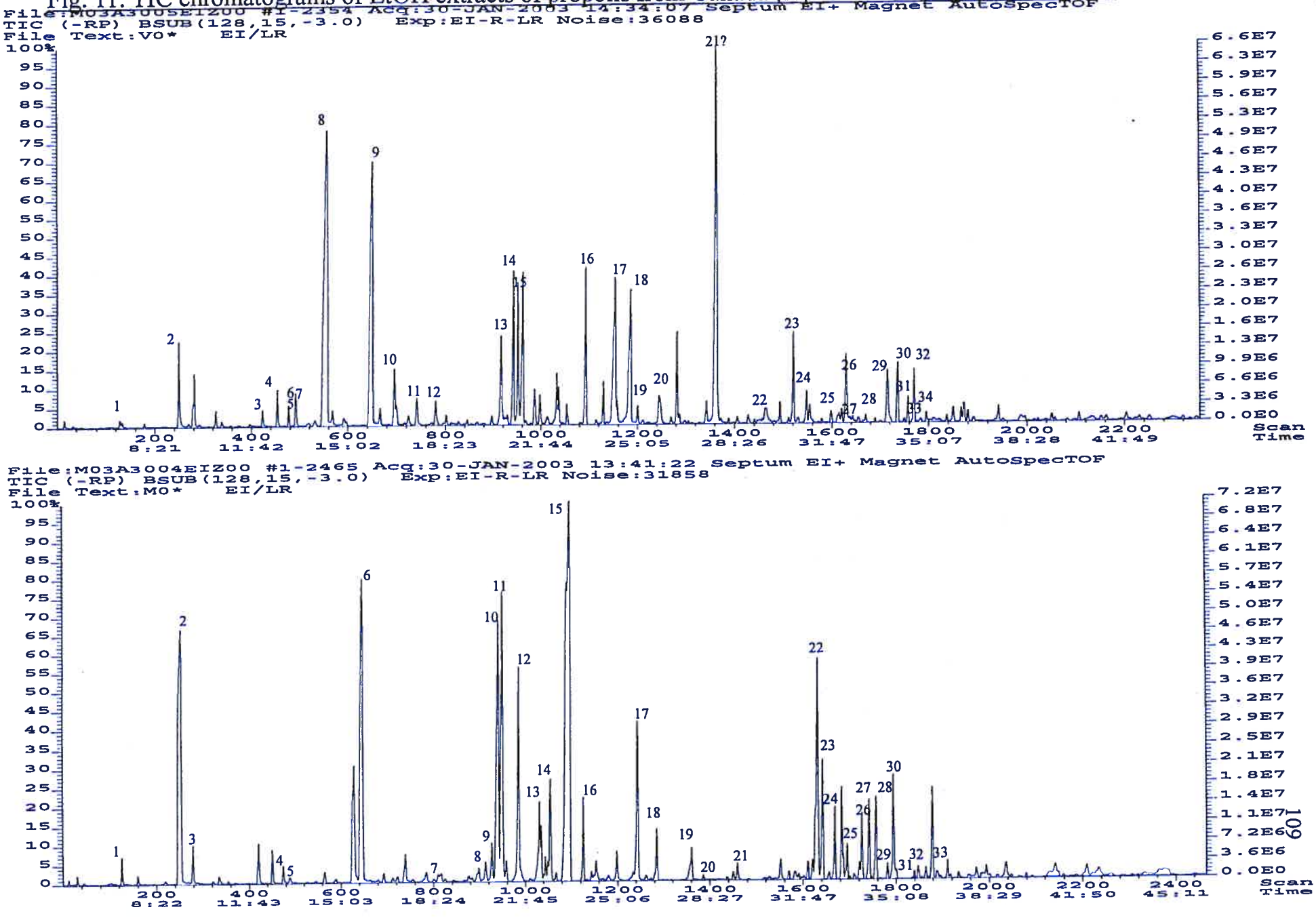
Compound	Victoria	Richmond
Fatty acids		
Palmitic acid	0.3 **19**	-
Oleic acid	-	0.7 **19**
Stearic acid	-	0.1 **20**
Esters		
Benzyl benzoate	2.4 **13**	0.3 **8**
Benzyl methoxybenzoate	5.0 **17**	-
Benzyl hydroxybenzoate	5.0 **18**	-
Benzyl-Z-*p*-coumarate	0.2 **22**	0.3 **21**
Benzyl-E-*p*-coumarate	0.8 **26**	5.4 **22**
Phenethyl *p*-coumarate	-	0.6 **25**
Benzyl ferulate	0.5 **31**	1.5 **28**
Benzyl caffeate	0.1 **33**	0.3 **29**
Phenethyl caffeate	-	0.1 **31**
Flavones and flavanones		
Pinostrobin chalcone	0.3 **25**	-
Pinocembrin	0.1 **27**	2.4 **23**
Pinobanksin	0.2 **28**	1.2 **24**
Sakuranetin	-	1.1 **26**
Pinobanskin 3-O-acetate	-	1.4 **27**
Galangin	0.2 **34**	2.0 **30**
Isosakuranetin	-	0.2 **32**

Compound	Victoria	Richmond
Alpinone	-	0.1 **34**
Dihydrochalcones		
2',6'-dihydroxy-4'methoxy - dihydrochalcone	1.9 **23**	-
2',4',6'-trihydroxydihydrochalcone	0.6 **24**	
2',6'-dihydroxy-4,4'-dimethoxydihydrochalcone[b]	1.6 **29**	-
2',4',6'-trihydroxy-4-methoxydihydrochalcone[b]	1.3 **30**	-
4,2',6'-trihydroxy-4'-methoxydihydrochalcone[b]	1.0 **32**	-
Others		
Glycerol	-	0.5 **3**
Hexoses	7.9 **14, 15**	26.5 **10,11, 12,13, 14,16**
Sesquiterpene	-	0.2 **7**
Unidentified	14.0 **21?**	-

[a] The ion current generated depends on the characteristics of the compound concerned and is not a true quantification.

[b] For the first time in propolis

Fig. 11. TIC chromatograms of EtOH extracts of propolis from Canada (top – Victoria region, bottom – Richmond region)



5.3. Development of a New Method of Studying Propolis Chemical Composition Using Metastable Atom Bombardment (MAB) Ionization Mass Spectrometry

Now we will further exploit another element of our diagram (see p. 56). The goal is again new methods development, however, this time from mass spectrometry direction, in particular the use of metastable atom bombardment (MAB) ionization MS for the analysis of propolis.

So far, for all our studies of propolis chemical composition we used the classical Electron Ionization (EI) MS. Like all the other ionization techniques used in mass spectrometry, EI also has both advantages as well as limitations. An abundant number of spectra have been accumulated through the years, which now allows fast and reliable computer library search for identification or structural elucidation of the compounds analyzed. The EI source itself is very sensitive, stable, easy to operate and gives reproducible results for both qualitative and quantitative analyses.

One of its biggest disadvantages, however, comes from the fact that very often it deposits relatively large amounts of energy into the molecule resulting in extensive fragmentation. Thus, the molecular ion ($M^{+\cdot}$), if observed, can be of very low intensity in the mass spectrum, and no conclusion about the analyte molecular mass can be made. Therefore, EI is not appropriate for relatively large, polar, nonvolatile and thermally labile compounds.

Coincidently, these limitations are valid to some extent for gas chromatography as well, and that might create some additional difficulties when a particular analytical problem has to be solved.

We have already underlined that for our purposes (analysis of complex natural mixture, such as propolis) GC gives the best results because of its high sensitivity, efficiency and resolving power. That brings some additional limitations. We are not able to use some soft ionization methods like Fast Atom Bombardment and the currently most sophisticated Atmospheric Pressure Ionization (Electrospray or Atmospheric Pressure Chemical Ionization) because they can not be coupled with gas chromatography.

On the other hand, Electrospray can be coupled with liquid chromatography (HPLC), but the latter does not have enough resolving power to separate more than 50 compounds in one propolis sample. Moreover, let us consider for instance the propolis from the Temperate zone with its characteristic flavonoid aglycones. These compounds' structure is not a typical example for easy protonation of the molecule regardless of the soft ionization technique used. (One possible suggestion is that negative mode of operation could be performed; see also section **5.1.2.**)

An excellent opportunity to resolve such complex tasks is offered by the MAB source of ionization. First, it allows coupling with GC. Second, the internal energy imparted to the molecular ion can be controlled to some extent.

In MAB a beam of metastable species (atoms or small molecules) generated outside the ionization volume is used to bombard molecules in gas phase leading to Penning ionization. In this ionization process a metastable species **A*** collides with a neutral molecule **BC** (Fig.12). One of the electrons from the molecular orbitals of **BC** (ϕ_{BC}) attacks the vacant orbital of the metastable species (χ_a). Simultaneously, an electron from the outer shell of **A*** is ejected into a continuum mode (γ_e) leading to ionization. The ejected electron can take a range of kinetic energy (E_k), which is defined by the species involved.

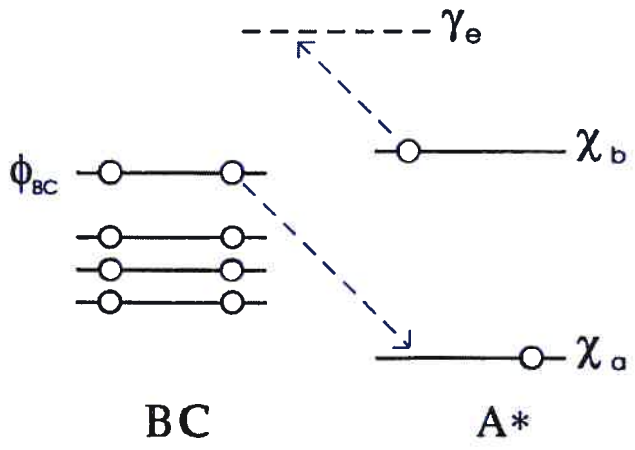


Fig.12. The electron-transfer process in Penning ionization

The ionization can be nondissociative, in which case a stable molecular ion (M^+) is formed,

$$A^* + BC \rightarrow A + BC^{+\cdot} + e^-$$

and dissociative, in which case fragment ions are formed.

$$A^* + BC \rightarrow A + B^{+\cdot} + C + e^-$$

These reactions basically occur if the ionization energy (IE) of **BC** is lower than the excitation energy (E*) of **A***. However, they are even possible when the excitation energy of the metastable species is lower than the IE of the molecule through associative complex formation (182).

$$A^* + BC \rightarrow ABC^{+\cdot} + e^-$$

The energy of the metastable beam is quantized and can easily be varied in the range of 8-20 eV by changing the nature of the metastable species (Table X). Thus, the technique is universal for the analysis of volatile organic compounds and allows selective ionization and controlled fragmentation.

Table X

Some characteristics for given metastable species (200, 201)

Gas	Metastable state	Energy (eV)	Lifetime (s)	Approximate Population(%)	E_{int} (IP ≈ 8eV)
Xenon	$^3P_0$	9.45	7.8×10^{-2}	< 7	
	$^3P_2$	**8.32**	**1.5×10^{2}**	**> 93**	**0 – 0.32**
Krypton	$^3P_0$	10.56	4.9×10^{-1}	< 10	
	$^3P_2$	**9.92**	**8.5×10^{1}**	**> 90**	**0 – 1.92**
Argon	$^3P_0$	11.72	4.5×10^{1}	14	
	$^3P_2$	**11.55**	**5.6×10^{1}**	**86**	**0 – 3.55**
Neon	$^3P_0$	16.72	4.3×10^{2}	20	
	$^3P_2$	**16.62**	**2.4×10^{1}**	**80**	**0 – 8.62**
Helium	$^1S_0$	20.61	2.0×10^{-2}	10	
	$^3S_1$	**19.82**	**2.0×10^{-2}**	90	**0 – 11.82**
Nitrogen	**E $^3\Sigma_g^+$**	**11.88**	**2.0×10^{-4}**	**< 15**	**0 – 3.88**
	w $^1\Delta_u$	**9.02**	**$1 - 5 \times 10^{-4}$**	**> 85**	**0 – 1.02**
	a $^1\Pi_g$	**8.67**	**$1 - 1.5 \times 10^{-4}$**		**0 – 0.67**
	a' $^1\Sigma_u$	**8.52**	**1.4**		**0 – 0.52**
	W $^3\Sigma_u$	7.32	17	no data	
	A $^3\Sigma_u$	6.17	1 – 2.6	available	

According to the data from this table, Xe followed by N_2 will produce the softest ionization, leading to stable molecular ions with very little or no fragmentation and He and Ne will produce hard ionization leading to extensive fragmentation. It should be noticed that the second higher energy level of N_2 metastables (≈15%) will contribute to the ionization process with more energy deposition to the molecule, thus making the fragmentation more prominent.

The internal energy deposited to the ion (E_{int}) in the ionization process is given by the difference of the excitation energy of the metastable species (E^*), the ionization energy of the molecule (IE) and the kinetic energy taken by the ejected electron (E_k).

$$E_{int} = E^* - IP - E_k$$

If $E_k \rightarrow 0$, then the maximum internal energy (E_{int}^{max}) of the ion can be determined by E^* - IE, i.e., by the choice of the metastable species (E^*), thus allowing a control of the fragmentation. It should be pointed out, however, that the energy taken by the ejected electron is not controlled and depends on the dynamics of the ionization process. As a result, there is a distribution of the kinetic energies taken by the electrons in a range from 0 to E^* - IE, and the internal energy transferred to the analyte will be affected by this distribution, varying from E_{int}^{max} to 0. The energy distribution range will be only 0.32eV with Xe^* and the largest (11.82 eV) with He^*. Faubert *et al.* (183), have shown that

despite the large transfer of energy to the analyte using He^* and Ne^*, an intense molecular ion still appears in the mass spectra because the ejected electron takes most of the excess energy from the above reaction.

Thus, for a particular compound only an upper limit of internal energies can be chosen and there is no precise control on the energy spread in this ionization reaction. In contrast, the internal energy of the analyte ion obtained for instance by ion-molecule reactions (proton transfer or charge transfer) is more accurately known (190). As a final point, there is no simple relation between the energy of metastables and the extent of fragmentation of the analyte upon Penning ionization.

In practice, when $E^* >> IP$, e.g. with Ne as a reagent gas, the fragmentation can be made extensive, while for similar values, when $E^* \approx IP$, the fragmentation will be negligible or absent (when Xe or N_2 is used). This means, that to some extent, it can be controlled. Similarly, within a mixture the ionization can be performed selectively simply by choosing a value of E^* which is bellow the IE of some classes of compounds present in it.

Both selective ionization and controlled fragmentation were checked in the behaviour of the propolis sample originating from Victoria, Vancouver Island, British Columbia.

To date, MAB Ionization MS has been used for environmental analysis (184-186) and chemotaxonomy (187), but never in natural product chemistry characterization especially for such complex natural mixtures.

Our discussion of the results obtained will be focused only on the compounds identified unambiguously, based on their EI mass spectra (see section **5.2.5**, Fig 11 and Table IX).

The results from all MAB studies with different gas reagents of the propolis sample chosen revealed several positive aspects of this ionization technique. The first important thing that should be underlined is the low operational temperature of the MAB source. Experiments showed that the lowest possible operational temperature of the source is 140 ºC without affecting the peak shape of any of the components from the sample. This was independent of the maximum programming temperature of the capillary column, which in this case was 300 ºC. It is a direct consequence of the specificity of the Penning ionization process and the subsequent source geometry design. Such design allowed the tip of the capillary column to be put directly into the metastable beam. By contrast, the corresponding temperature of the EI source was 220 ºC. Such a difference will of course contribute additionally to the extension of the fragmentation process.

MAB analyses of the propolis sample using Ne, Ar and Kr resemble that observed when using EI ionization. With N_2, (Fig. 13) some slight differences in the relative peak intensities begin to appear; and with Xe (Fig. 14) these differences are substantial. Some peaks are quite diminished (**9**), (**14**, **15**), (**21**), others like (**16**) and all in the dihydrochalcone and flavonoid region (**29** – **32**) are enhanced, more than 5 times.

Fig. 13. TIC chromatogram of propolis sample from Victoria region (top - EI, bottom - MAB/N_2)

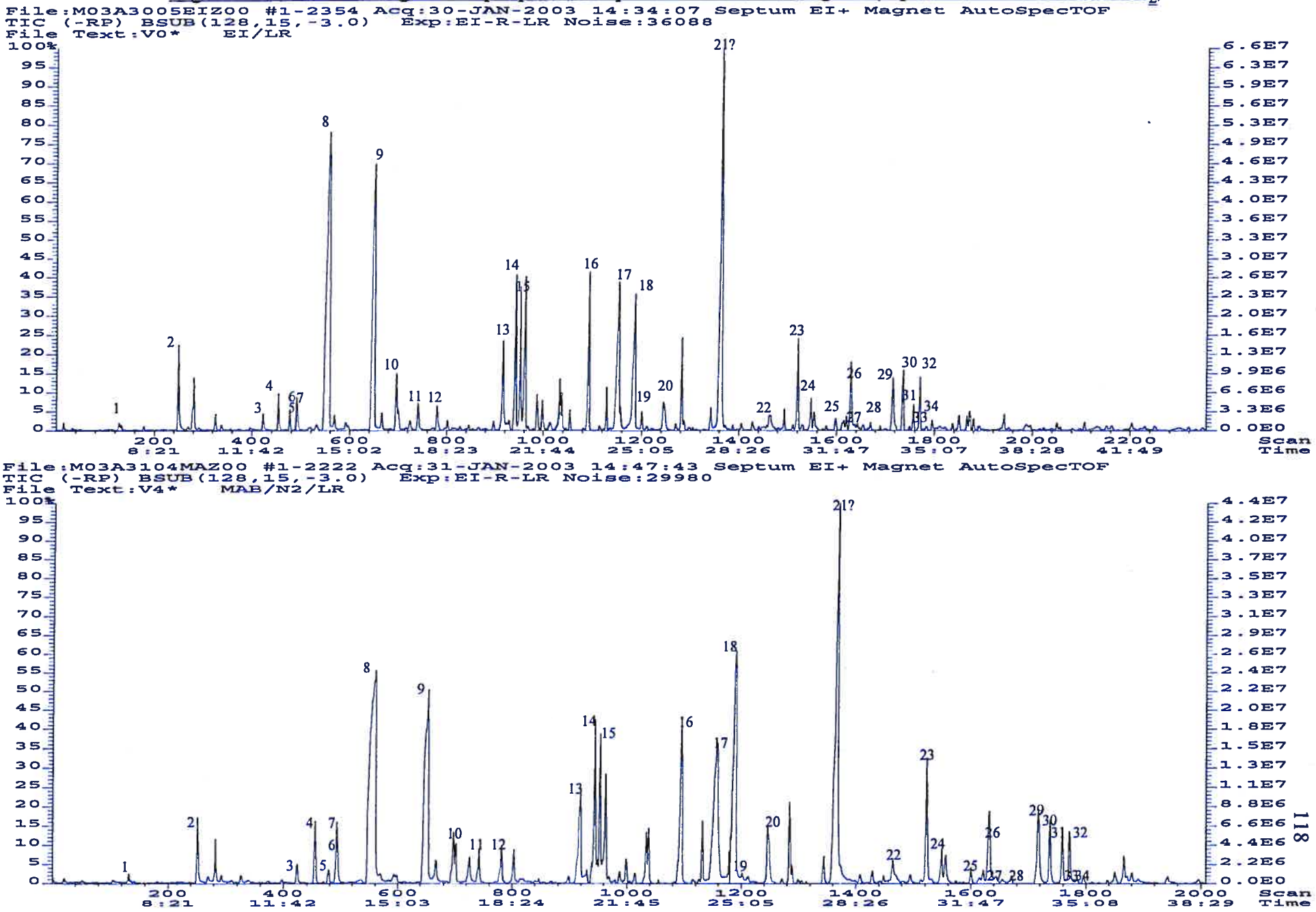
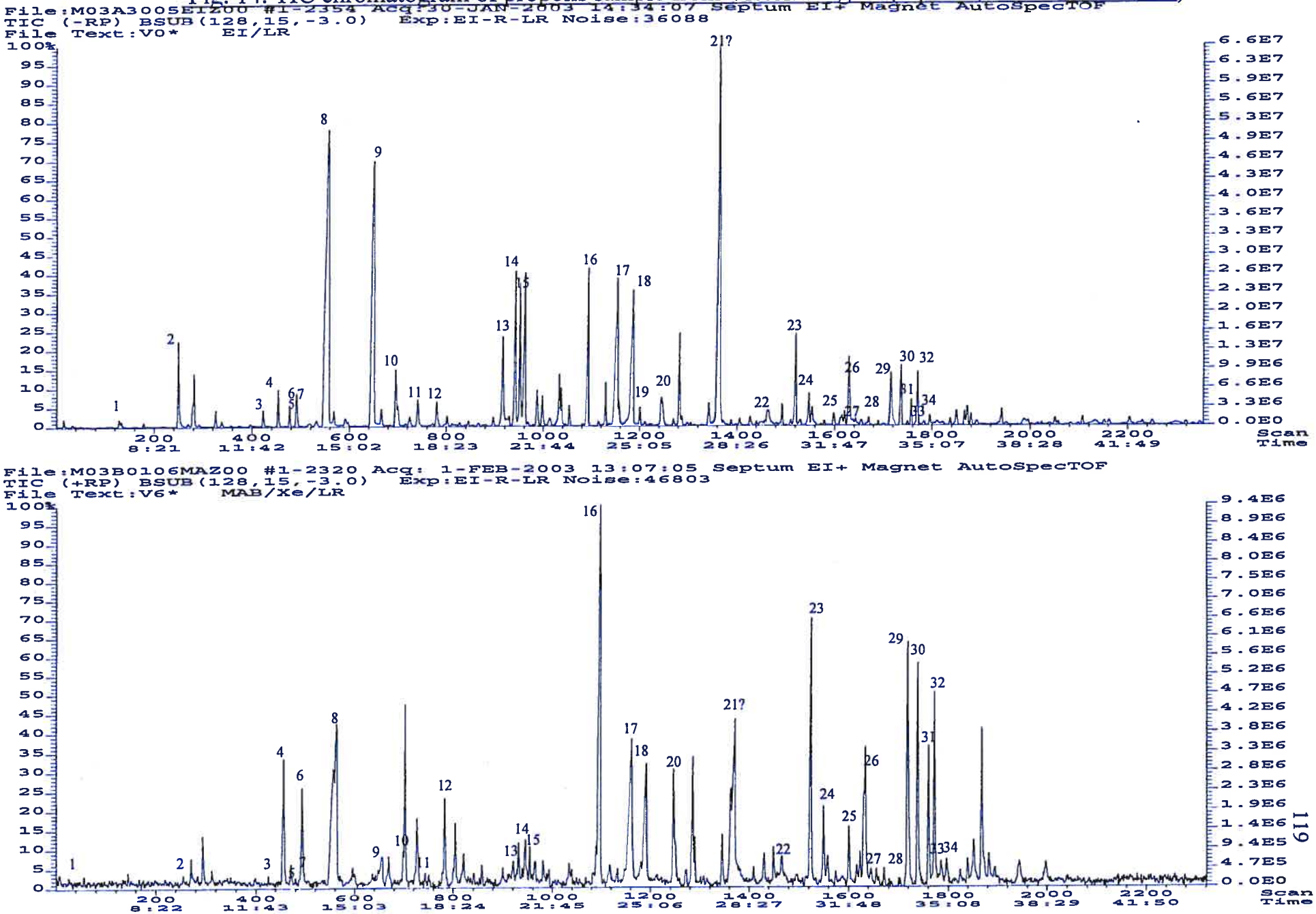


Fig. 14. TIC chromatogram of propolis sample from Victoria region (top - EI, bottom - MAB/Xe)



Going from the TIC chromatogram to a particular current generated from ions in an individual compound, i.e. its mass spectrum, it can be seen that the fragmentation pattern and its intensity obtained by MAB with Ne, and to some extent with Ar, resemble those obtained by EI. The fragmentation is much less extensive with Kr where the molecular ion is predominantly observed in the mass spectrum. With N_2, mainly the molecular ion ($M^{+\cdot}$) together with a loss of CH_3–group are present. With Xe, only the molecular ion appears in the mass spectrum. That is valid for almost all of the identified compounds with few exceptions. The above can be demonstrated with some main representatives of the different groups of compounds found in the mixture (see spectra of **8** – hydroxyacetophenone, Fig. 15, **16** – E-p-coumaric acid, Fig. 16, **31** – benzylferulate, Fig. 17)

Fig. 15. Mass spectra of peak 8 – hydroxyacetophenone (EI and MAB with different gas reagents)

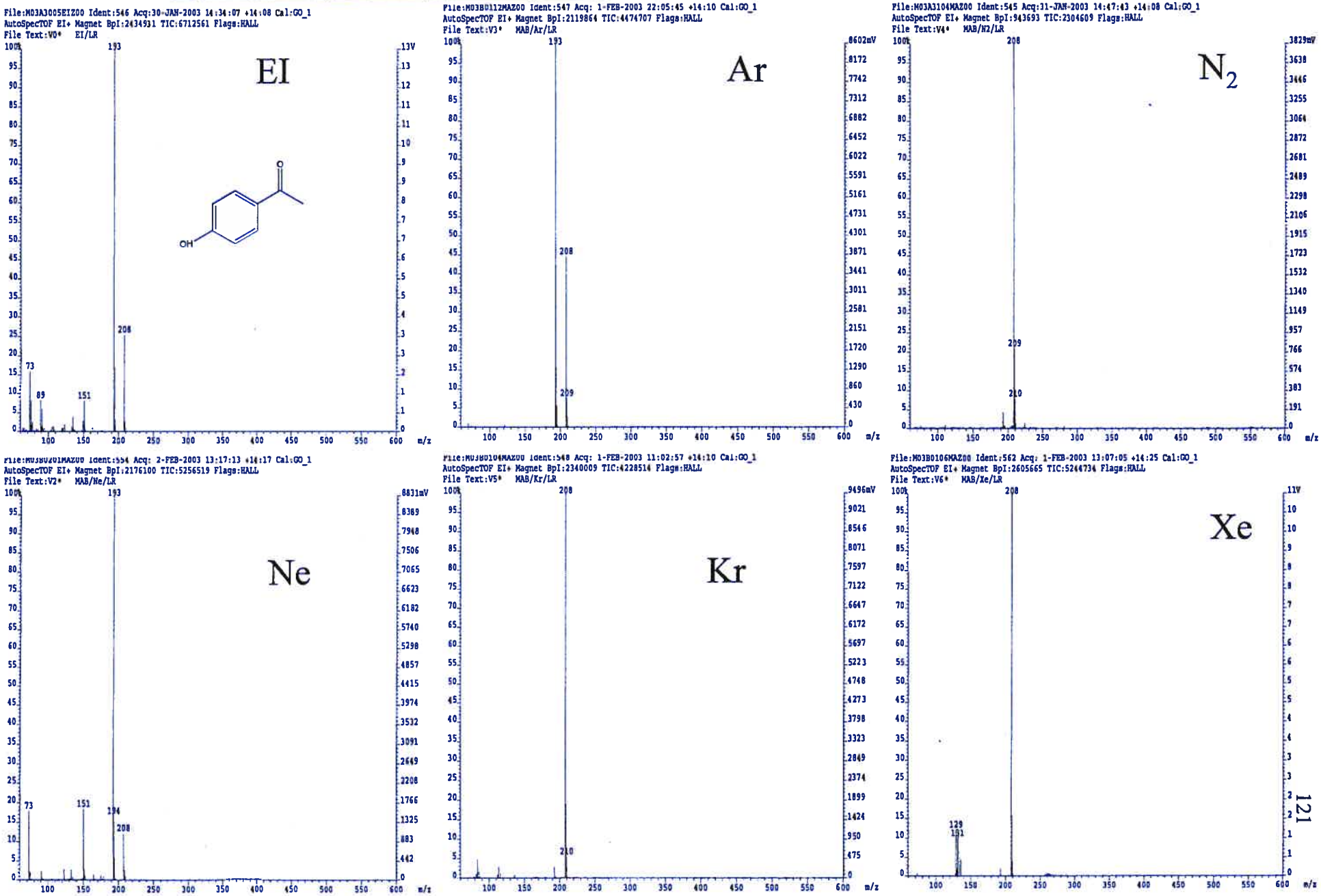
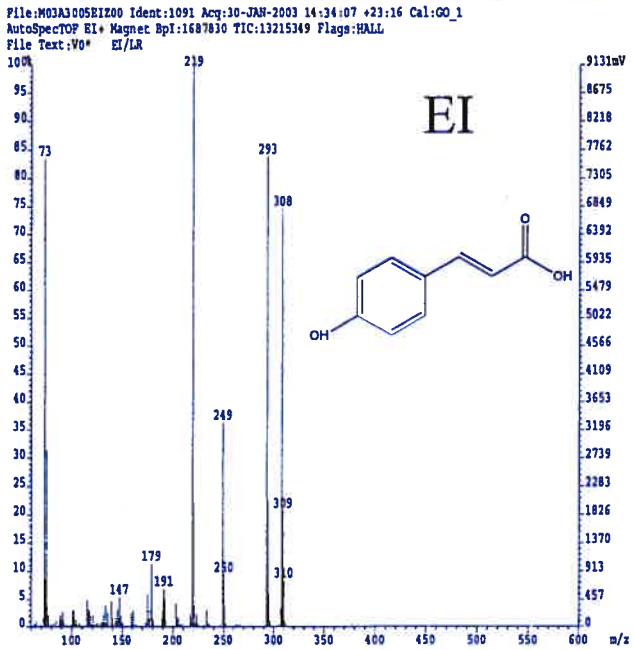
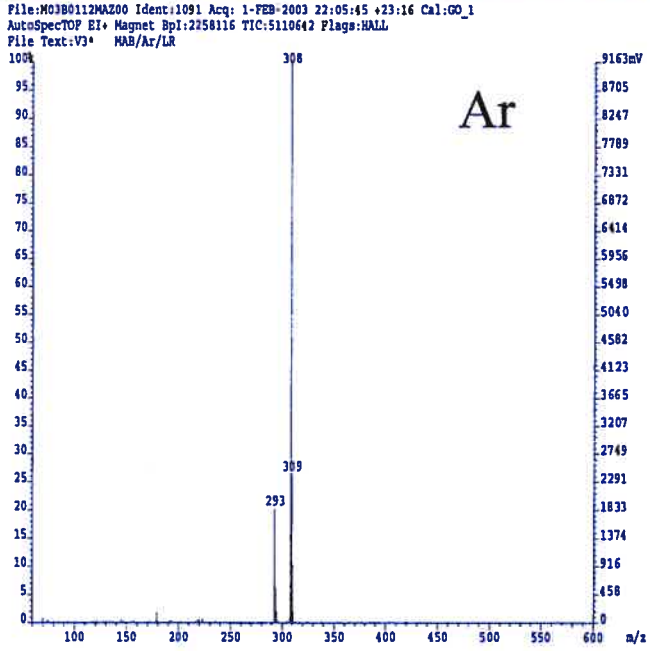
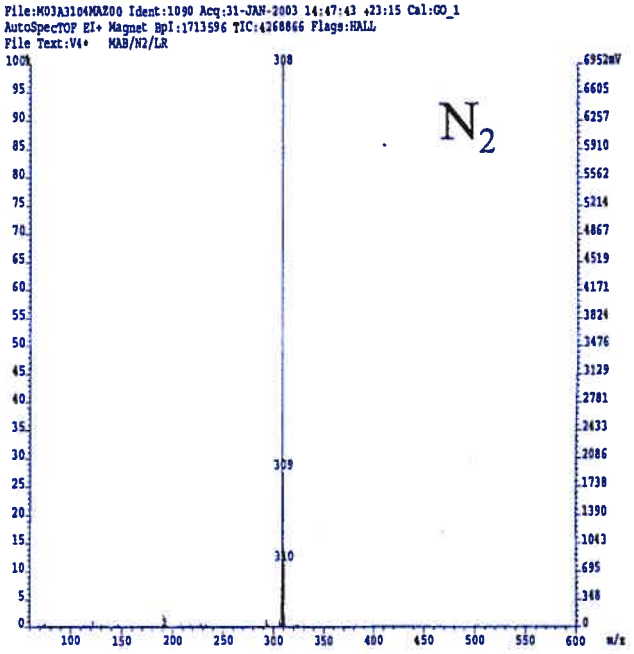
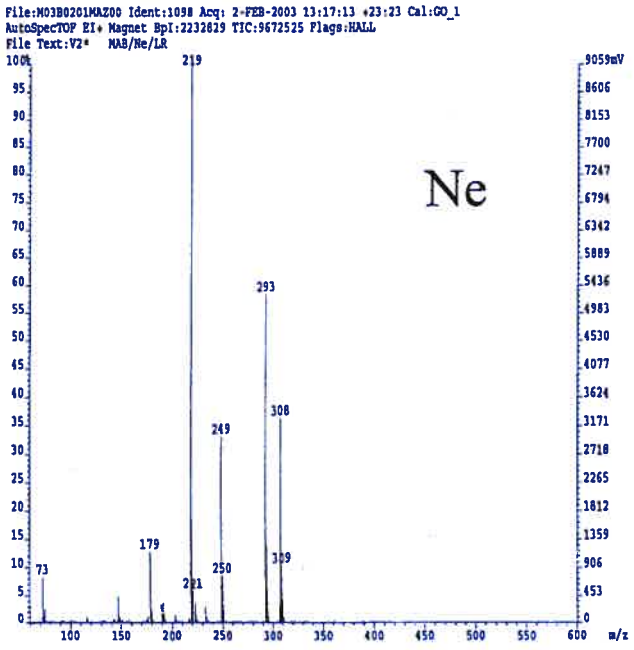


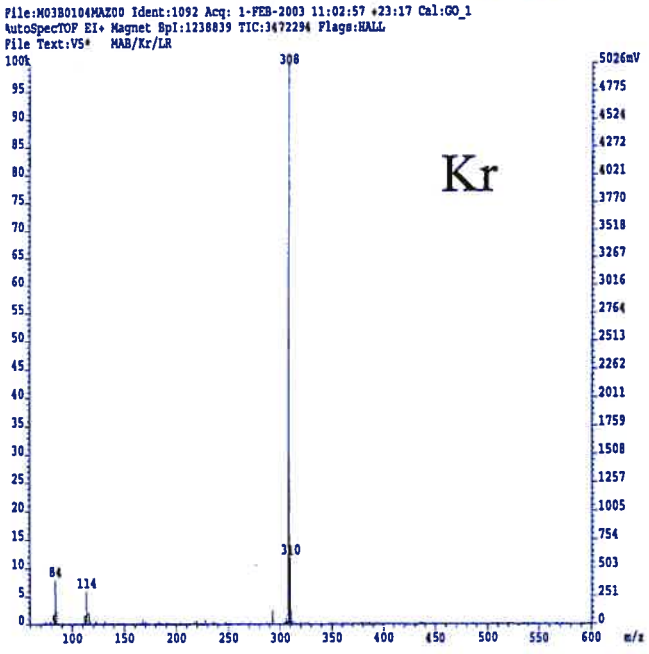
Fig. 16. Mass spectra of peak 16 – E-*p*-coumaric acid (EI and MAB with different gas reagents)











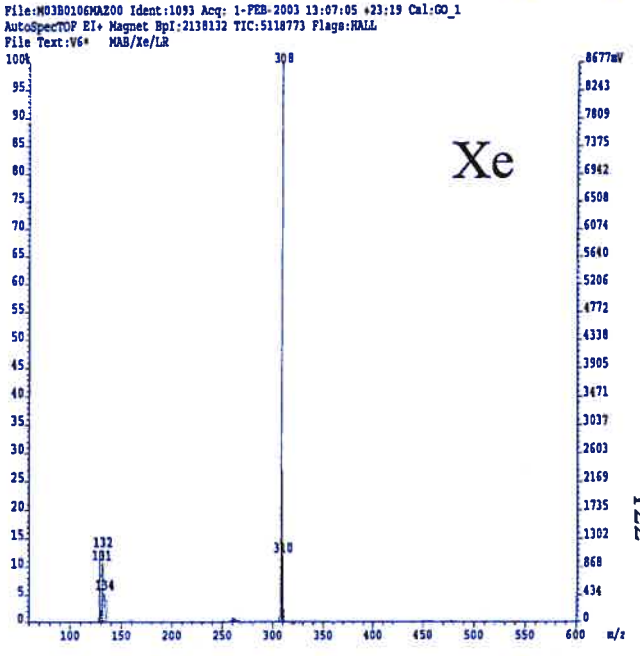
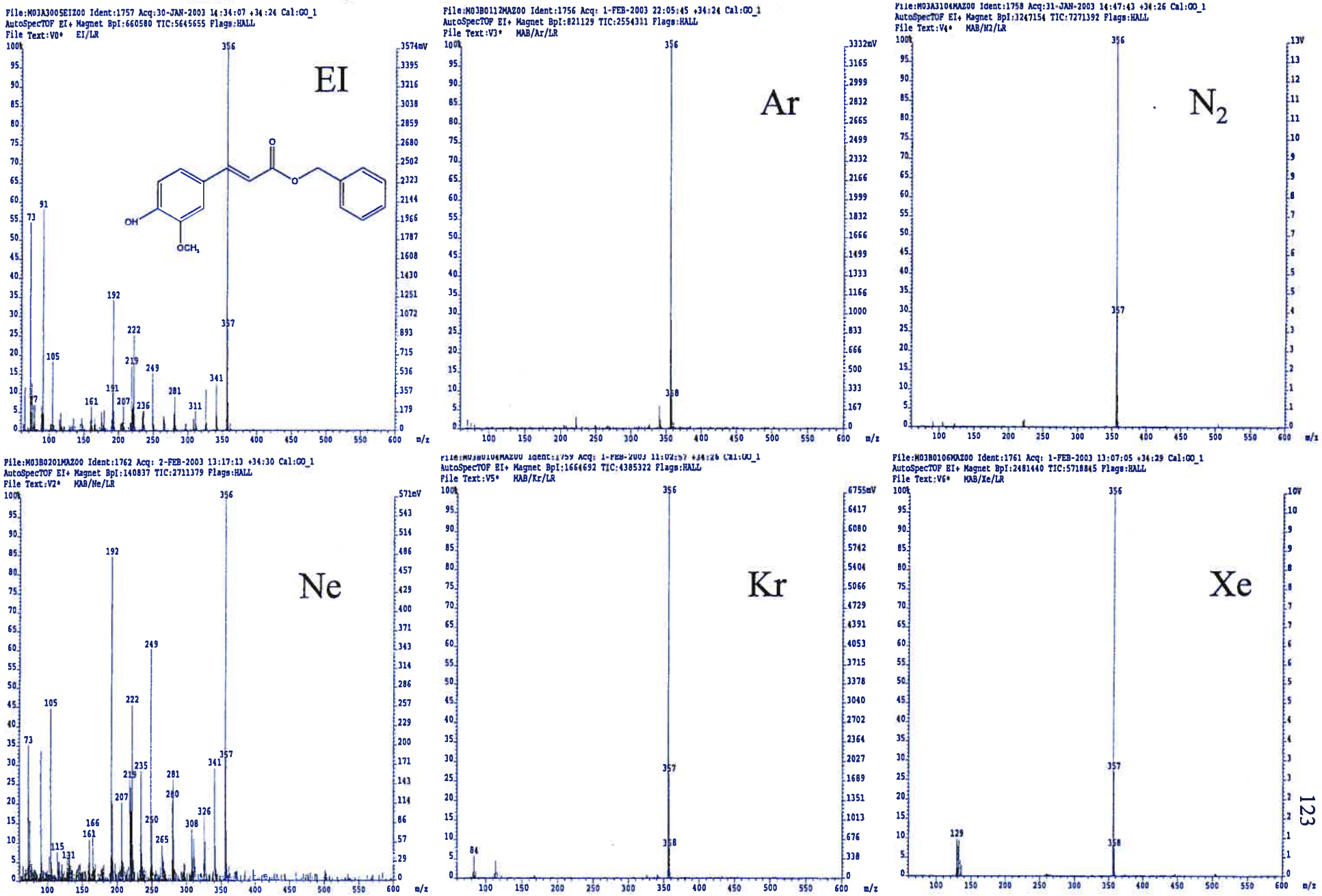


Fig. 17. Mass spectra of peak 31 – benzylferulate (EI and MAB with different gas reagents)



Probably now, at this stage, it is most appropriate to discuss one of the disadvantages of MAB. The response obtained from MAB, the signal as a TIC for the whole chromatogram or for an individual compound, is on average 10 times lower than that obtained with EI. And that is when we use the most conventional gas for MAB, N_2. A similar situation is encountered when using Ne, Ar and Kr. However, when Xe is used, the signal is 10 times lower than that from N_2. Therefore, there is a difference in two orders of magnitude in the TICs between EI and MAB with Xe. Later we will see how we can try to overcome this or even to extract some positive features from this disadvantage. For now, the most important thing for us is that the amount of an individual compound, a peak eluting from the capillary column, is enough to be reasonably detected with an adequate signal to noise ratio.

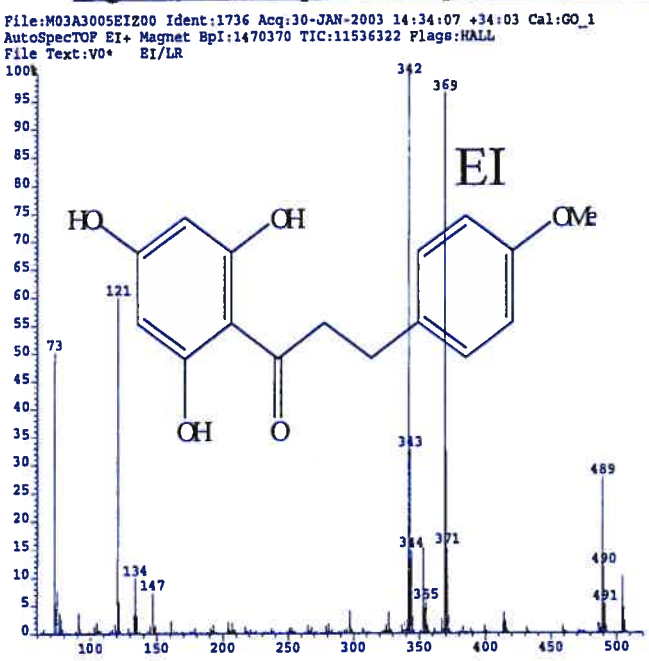
The previously mentioned relative enhancement of some peaks, namely those of the flavonoids and dihydrochalcones, is a result of the expected selectivity of the MAB ionization process and of the subsequent controlled fragmentation. Compounds having lower IE, close to the energy of Xe metastables, will be ionized selectively and will not fragment extensively. Only the molecular ion or very few fragments will appear, leading to improved signal to noise ratio and overall sensitivity. Unfortunately, the results obtained cannot be compared with those theoretically predicted due to lack of data for IEs for TMS – derivatives of the compounds analyzed.

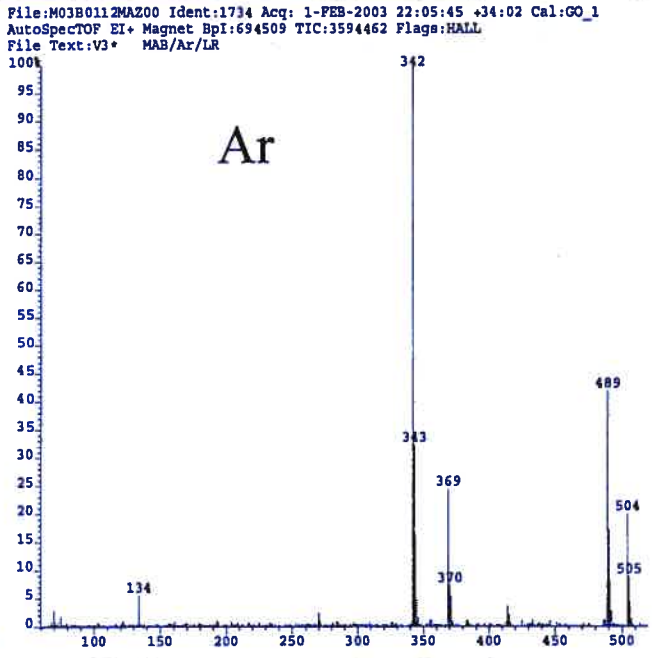
Peak **30** - 2',4',6'-trihydroxy-4-methoxydihydrochalcone (Fig. 18) is a good demonstration of controlled fragmentation. The MAB spectrum with Ne is

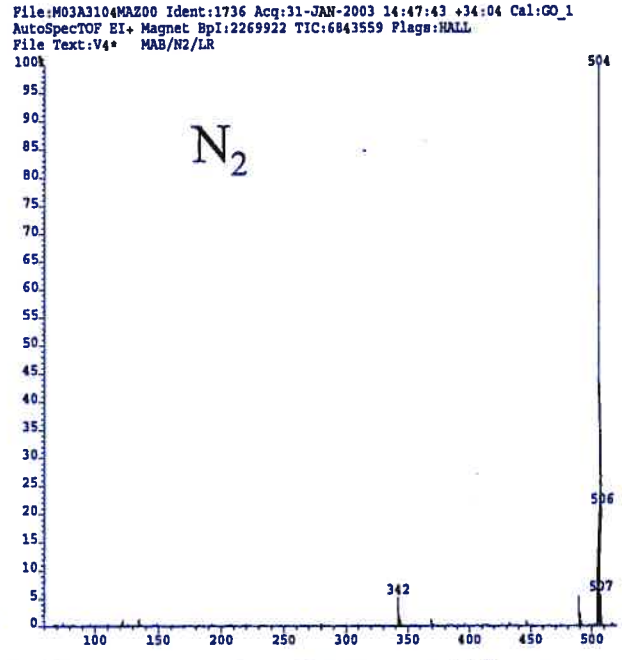
very similar to EI with molecular ion ($M^{+\cdot}$) *m/z*-504 less than 10% intensity. One of the main fragment ions is substantially decreased (*m/z*-369) as well as the ions from the low mass region when Ar is used. With Kr, $M^{+\cdot}$ becomes the base peak and there is an additional decrease of fragmentation. Using N_2, $M^{+\cdot}$ and only 2 low intensity fragments are present; and with Xe only the molecular ion with trace level fragments appear in the mass spectrum.

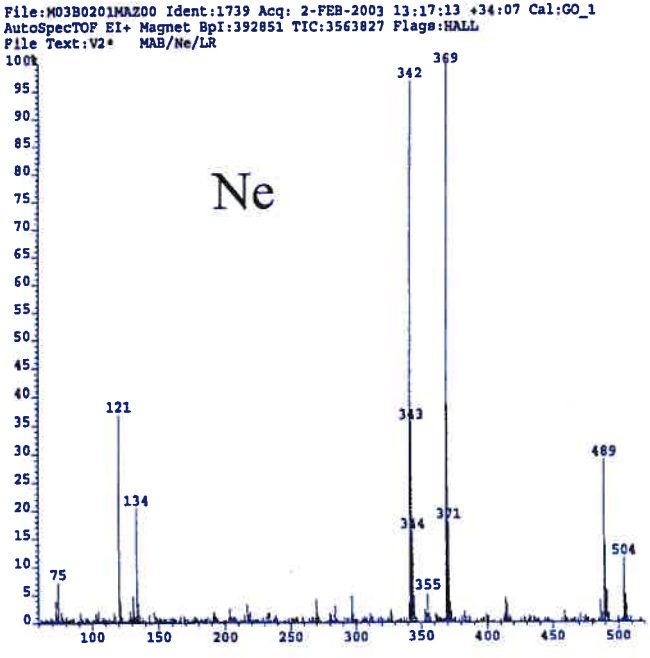
Peak **27**–pinocembrin (Fig. 19), is a good example of what we have discussed above, namely how selective ionisation and controlled fragmentation may overcome to some extent the problem of poorer sensitivity compared to EI. It is very small, not fully resolved, in the tail of peak **26** (see TIC chromatogram in Fig. 11). Silylated flavonoids do not normally show very prominent molecular ion peaks upon EI, and sometimes they are even completely absent. Instead, $[M\text{-}15\ (CH_3)]^+$ is much more intensive (in this case the base peak). The $M^{+\cdot}$ should appear at *m/z*-400, but becomes a prominent peak only using N_2, and as a base peak when using Xe. Unexpectedly, we still observe 3 intensive fragments in the spectrum (*m/z*-303, 326, 385), which is an indication that even the low internal energy imparted by Xe metastables is sufficient to cause some fragmentation. Apparently, the silylated pinocembrin molecule has a low IE and is quite labile upon Penning ionisation. A similar situation was observed with the other flavonoids, pinobanksin **28** and galangine **34,** under these conditions.

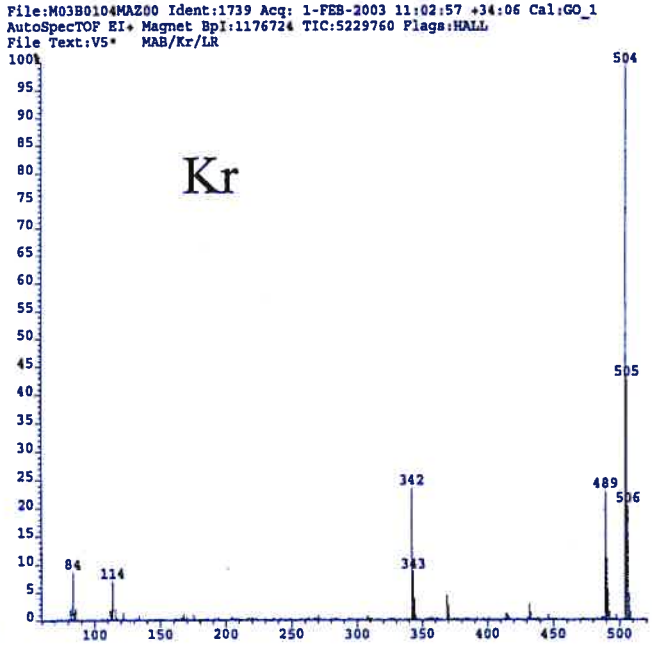
Fig. 18. Mass spectra of peak 30 - 2',4',6'-trihydroxy-4-methoxydihydrochalcone (EI and MAB with different gas reagents)











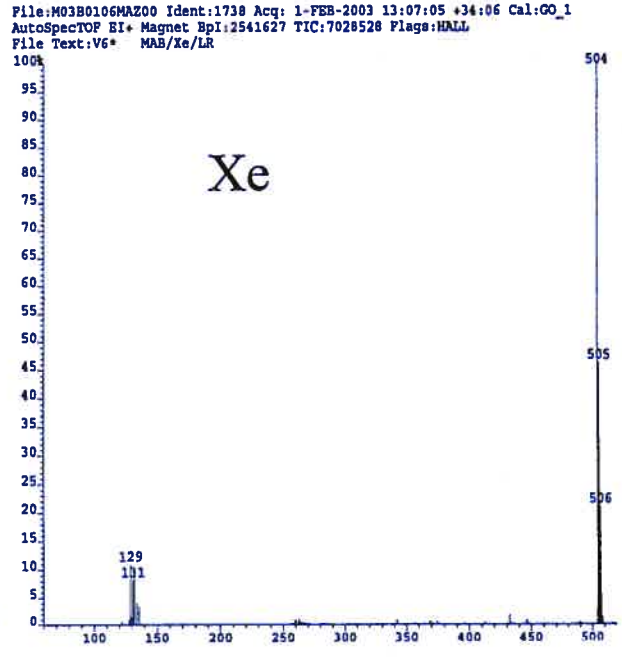
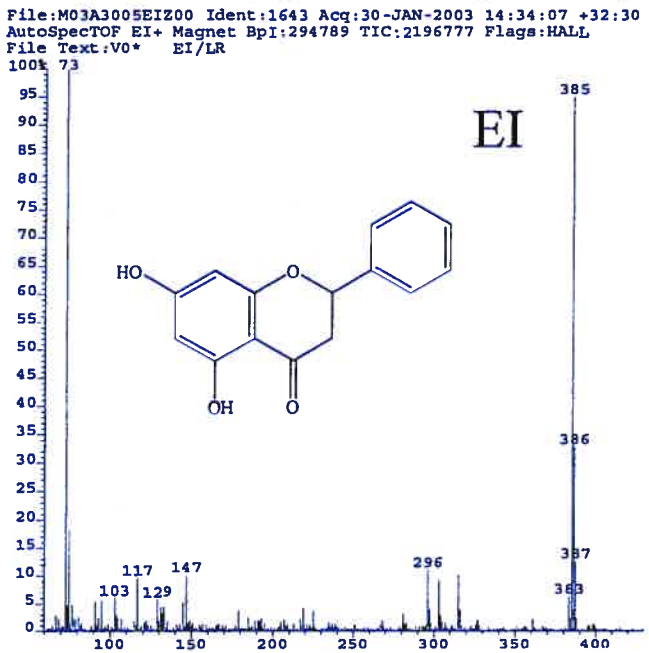
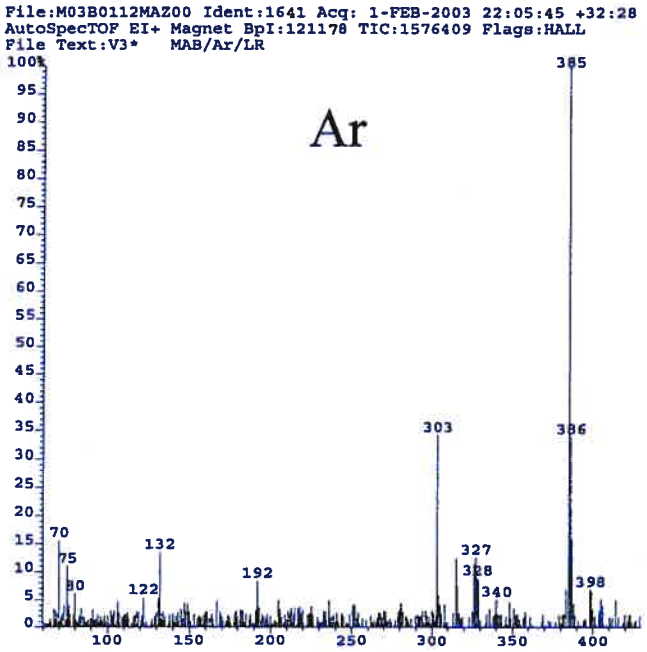
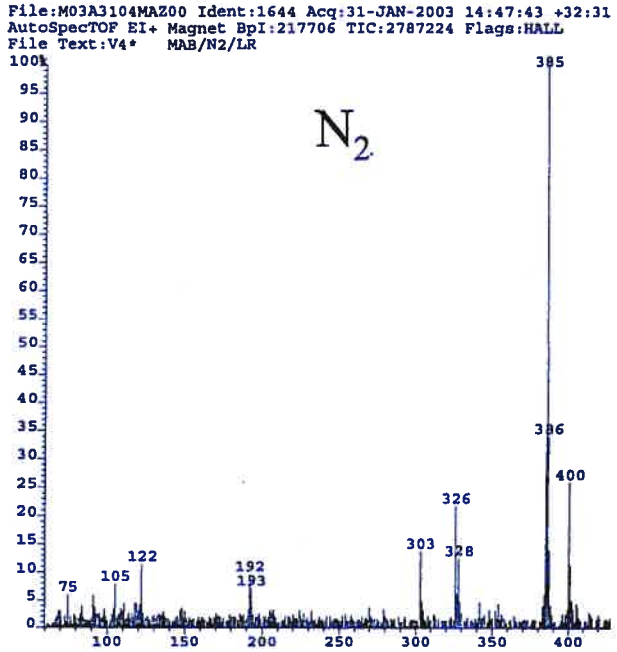
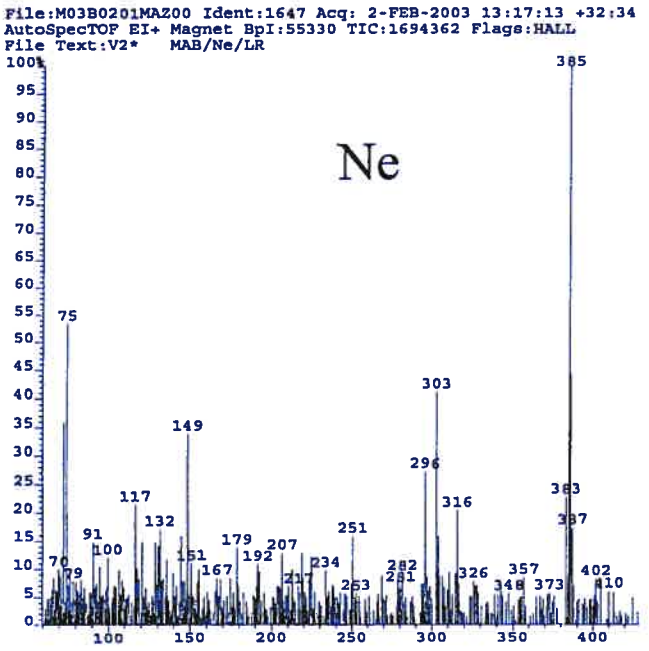


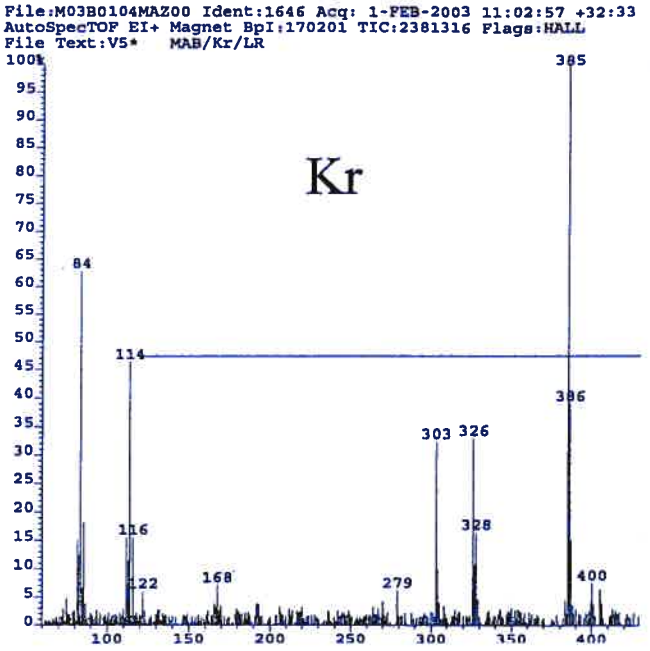
Fig. 19. Mass spectra of peak 27 – pinocembrin (EI and MAB with different gas reagents)

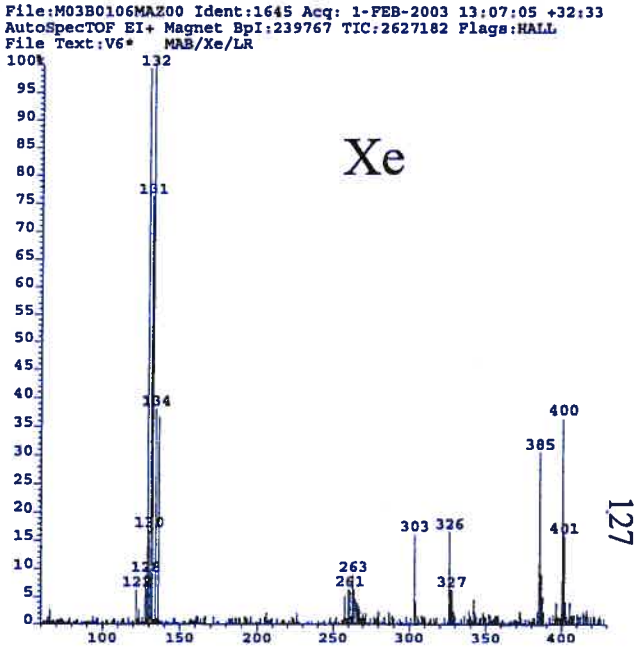












Another peculiar phenomenon noticed in the TIC chromatogram (p. 119) is the substantially decreased peak intensities of some components when using Xe, like *E*-cinnamic acid (**9**), hexoses (**14**, **15**) and peak **21**. Assuming that their IEs are lower than the energy of Xe^* metastables, we should have obtained similar peak intensities relative to the other peaks in the mixture. If their IEs are higher than the energy of Xe^* metastables, no peaks should have been observed since the ionisation process cannot occur. The only one conclusion that can be reached is that another type of ionisation may have contributed in this case. There are various reasons for this suggestion. For instance, in the mass spectrum of cinnamic acid (Fig. 20) only $M^{+\cdot}$ and a small fragment ion corresponding to the loss of a methyl group $(M\text{-}15)^+$ is observed as expected. However, its intensity is much lower than expected. Hexoses (Fig. 21, 22) also show some reasonable changes of fragment intensities with MAB ionisation going from Ne to N_2, with only trace level of $M^{+\cdot}$ (*m/z*-540) and $(M\text{-}15)^+$ even using N_2. Surprisingly, with Xe the molecular ion intensity is lower than that obtained with N_2. This means, as we have mentioned above, that even with Xe^* metastables the internal energy deposited is sufficient to cause the molecule to fragment extensively. The absence of $M^{+\cdot}$ implies that its IE is quite low. All that should have led to an intense peak, which is not observed in this case.

Fig. 20. Mass spectra of peak 9 – *E*-cinnamic acid (EI and MAB with different gas reagents)

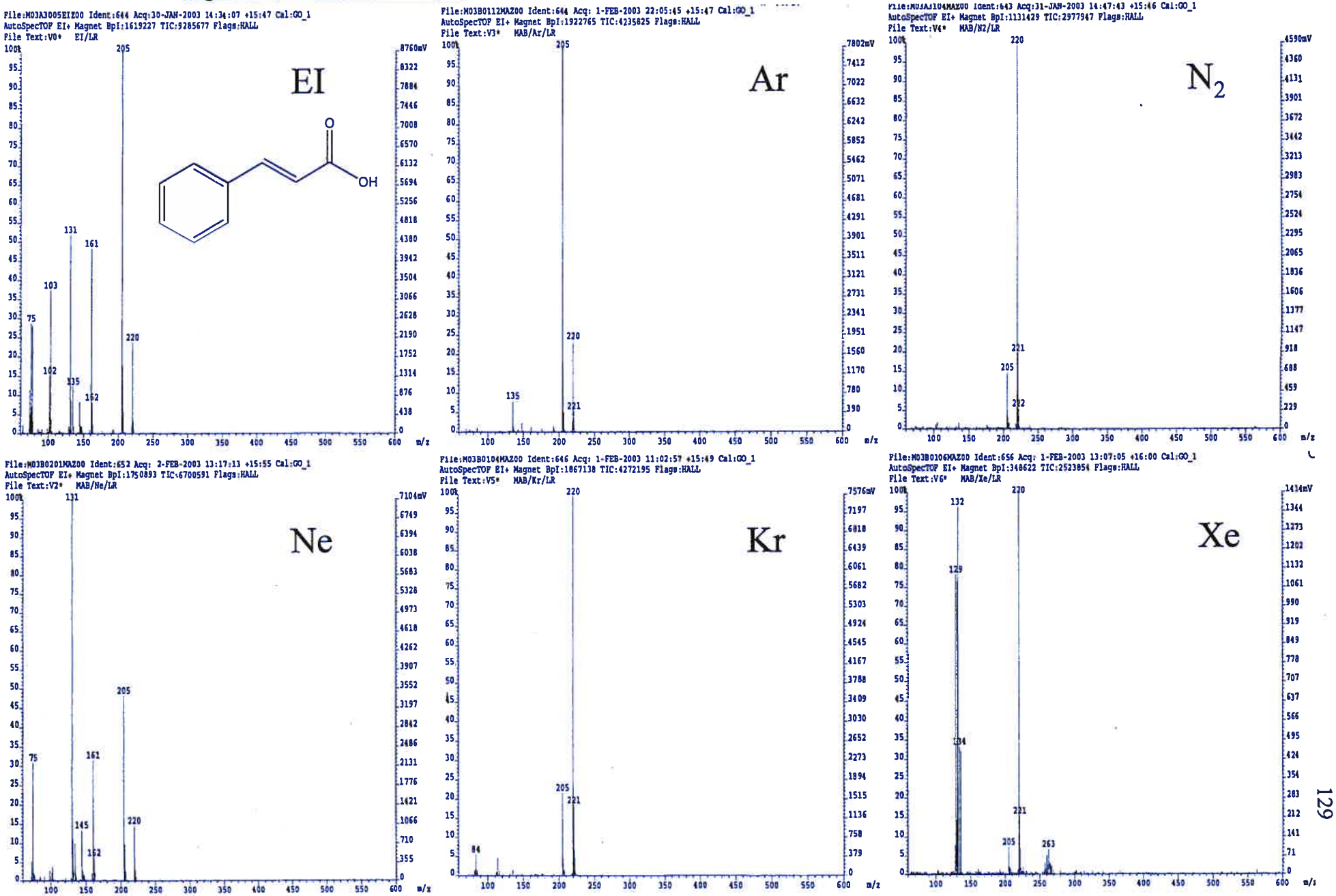


Fig. 21. Mass spectra of peak 14 – hexose (EI and MAB with different gas reagents)

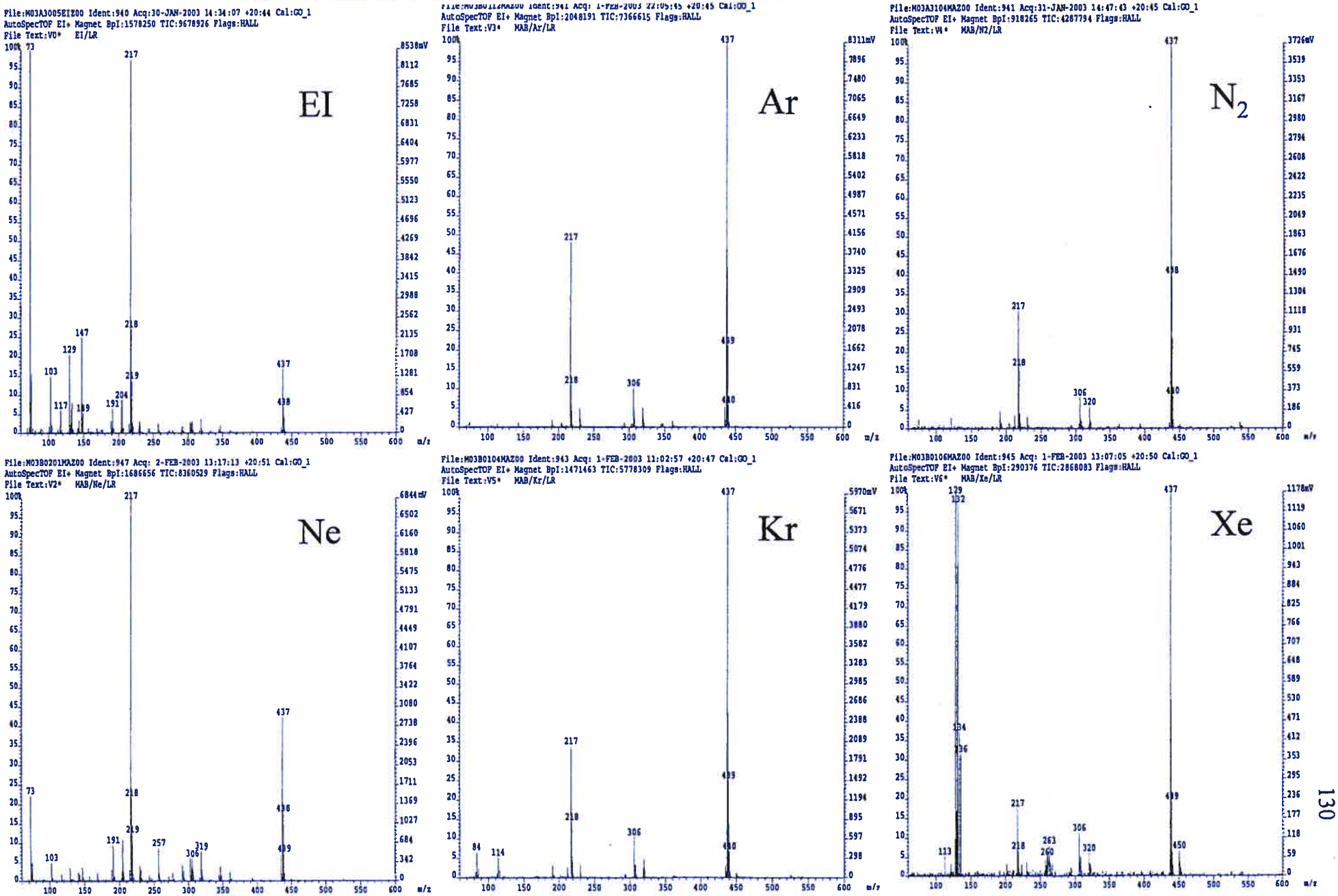
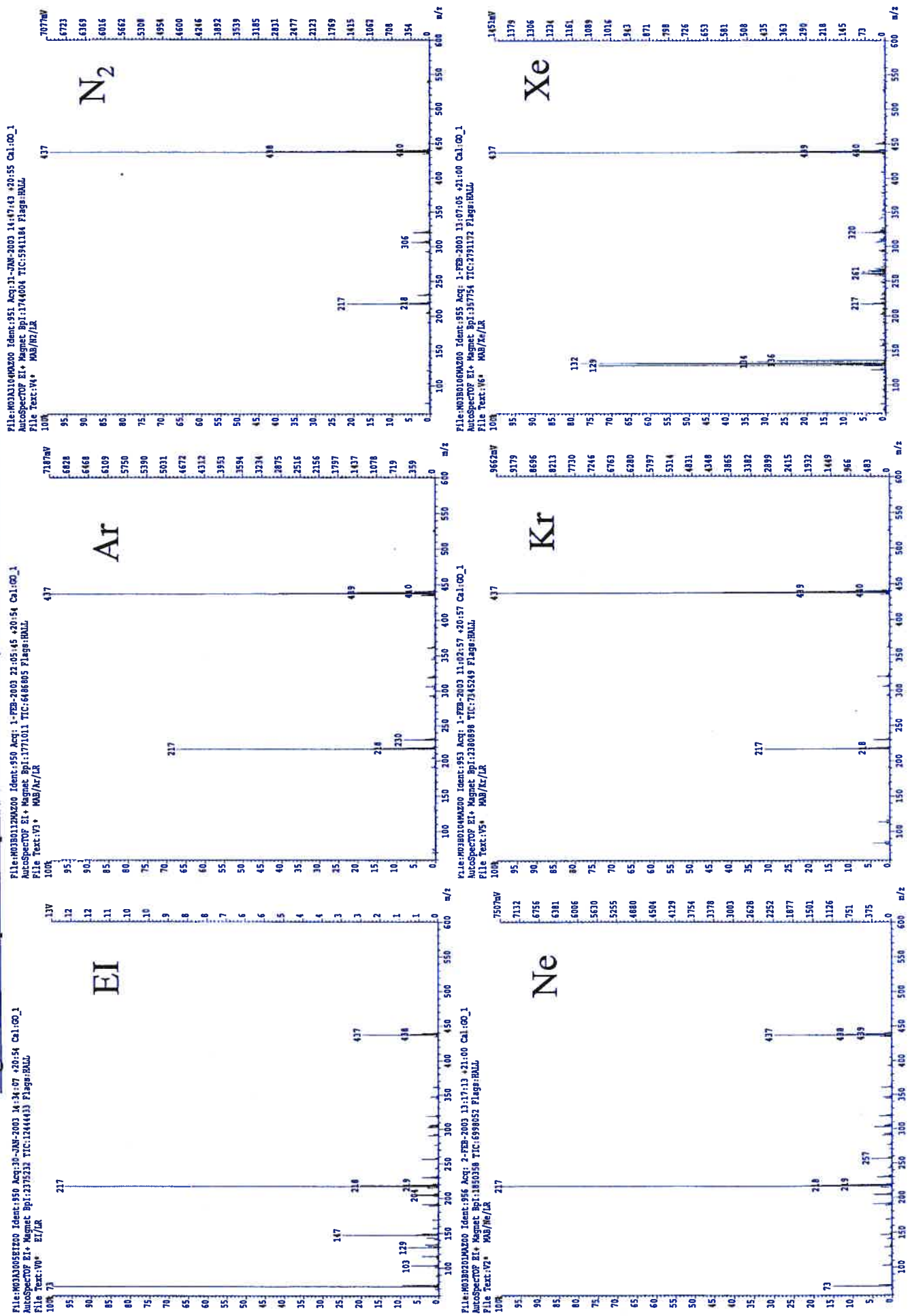


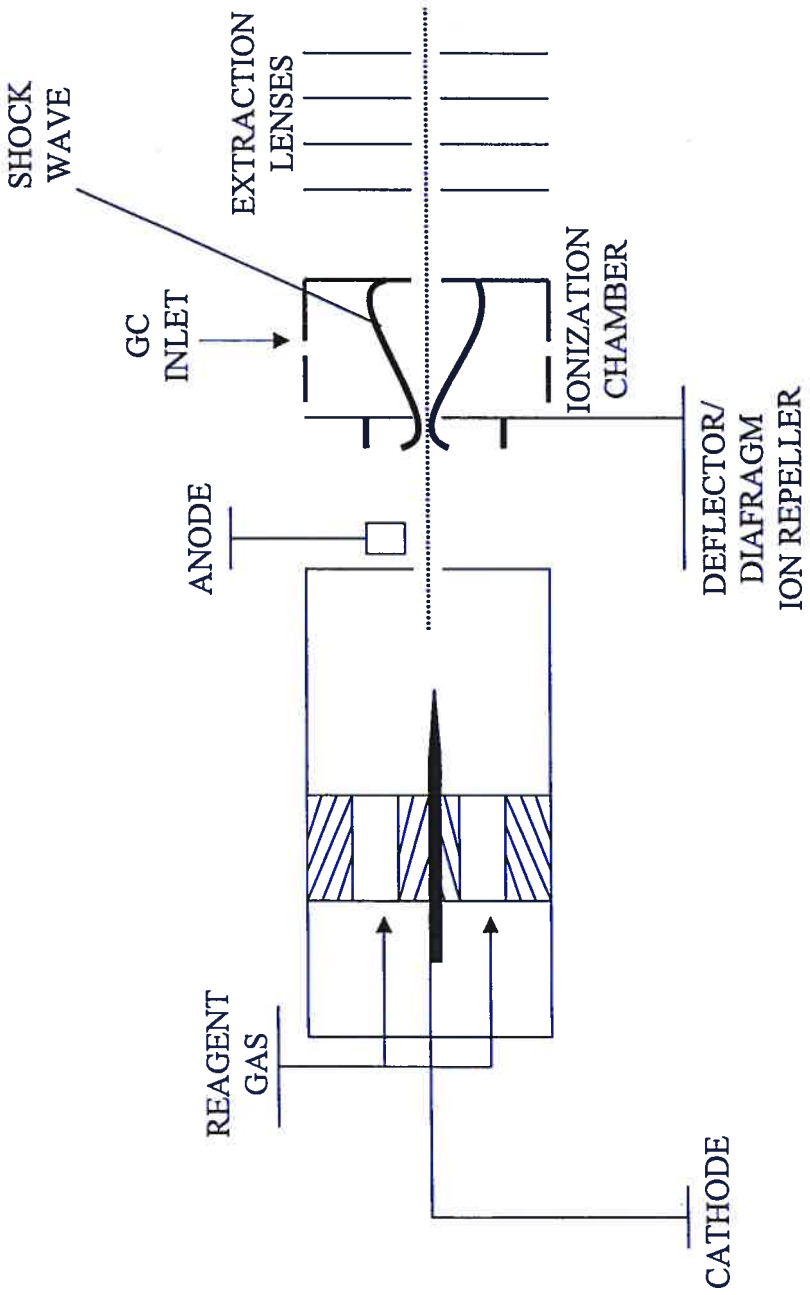
Fig. 22. Mass spectra of peak 15 – hexose (EI and MAB with different gas reagents)



According to Olney *et al.* (188), the available shape of the repeller (skimmer) plate is related to the formation of a shock wave in the supersonic flow in front of the orifice and scattering from the edges (see page 132A). The effect is stronger at higher gas densities (in our case with Xe as a reagent gas) and is also dependent on the diameter of the orifice, the distance of the plate from the gun (its position relative to the Mach disk) and the inlet pressure of the gas. As a result, the density of the metastable species is much higher in front of the skimmer and much lower behind it, causing a new expansion to occur at the skimmer tip. Finally, the density of the metastables along the centreline behind the skimmer is much lower than expected, which affects the ionisation efficiency (189).

From one side, the shock wave formed at the repeller wall may cause a recombination to occur through collisions between metastables. That will lead again to a decreased density of the metastable beam, and as we pointed out, the result is a less efficient ionisation process and a poorer response and sensitivity.

On the other hand, if all the ions and electrons from the plasma are not deflected and removed completely from the metastable beam before they reach the repeller plate, in the shock wave formed they will collide with metastables and will contribute to quenching its intensity. Furthermore, behind the repeller the electrons (now with lower energy after collisions) may participate in the ionisation process, thereby depositing less energy to the analyte molecule and thus causing less intensive fragmentation. The presence of reagent ions in the



MAB GUN AND SOURCE

spectra (see in all Kr and Xe spectra cluster ions around *m/z*-82 for $Kr^{+\cdot}$ and *m/z*-132 for $Xe^{+\cdot}$) is an indication that ionization of the analyte through charge transfer is likely to occur as well.

The discussion above is just one possible explanation of the substantial decrease in peak intensity (accompanied at the same time by far less intensive fragmentation) of an analyte having presumably IE higher than the energy of Xe metastables. In this case the analyte is ionized by either low energy electrons or by $Xe^{+\cdot}$ ions through charge transfer reactions.

Another possible explanation is that when a collision between a metastable atom and analyte molecule occurs, part of the kinetic energy of the species can be transformed into excitation energy of the analyte (approximately up to 0.10 eV) which in this particular case might be enough for an analyte molecule to be ionized upon Penning reaction. Once again, poor efficiency of the process will result in weak peak intensity.

Of course, a combined effect of all the phenomena discussed above is also possible. More detailed studies for the individual compounds are needed to confirm or reject some of these suggestions. For instance, Langmuir probe measurements of the electron density (if there are any) and $Xe^{+\cdot}$ will help answer the question about their contribution to the ionisation process.

As we mentioned earlier, the geometry of the MAB source as a whole allows the analysis to be performed at much lower temperatures than with the EI source. This geometry also allows some changes to be made in order to perform experiments for improving its overall sensitivity or to minimize some

undesirable phenomena like shock wave formation. Studies for optimization of the distance between the MAB gun, anode and repeller as well as improving the poor transmission efficiency through the skimmer are highly feasible.

Finally, the analytical potential of MAB ionisation is illustrated by the following problem. Peak **21**, one of the major components in the sample, remained unidentified. Computer library search did not provide any meaningful identification or reasonable proposals we could have relied on. After a detailed analysis of the mass spectrum (Fig. 23) we made only the suggestions that probably it is a silylated hydroxy- or carboxy- containing compound (because of the intensity of ion at *m/z*-73). And it may also contain a benzyl group (*m/z*-91). Apparently, the molecule is quite labile upon EI conditions (a lot of fragments are present) and the molecular ion either does not appear or is at a trace level in the spectrum. Assuming that it is not a nitrogen-containing compound, the highest mass peaks with reasonable intensity *m/z*-373 and *m/z*-381 cannot represent its molecular ion.

MAB with N_2 shows right away that there are at least 2 compounds in this peak in approximate ratio 1:5 (Fig. 24): 21' with $M^{+\cdot}$ (*m/z*-388) accompanied with a loss of methyl group (*m/z*-371) and 21'' with $M^{+\cdot}$ (*m/z*-396) with fragment ions corresponding to losses of methyl group (*m/z*-381) and probably of CO (*m/z*-368). MAB with Xe confirms the above proposals, but the presence of a large number of fragments again suggests that the molecule is very labile and probably has an unusually low IE.

Fig. 23. EI mass spectra of peak 21

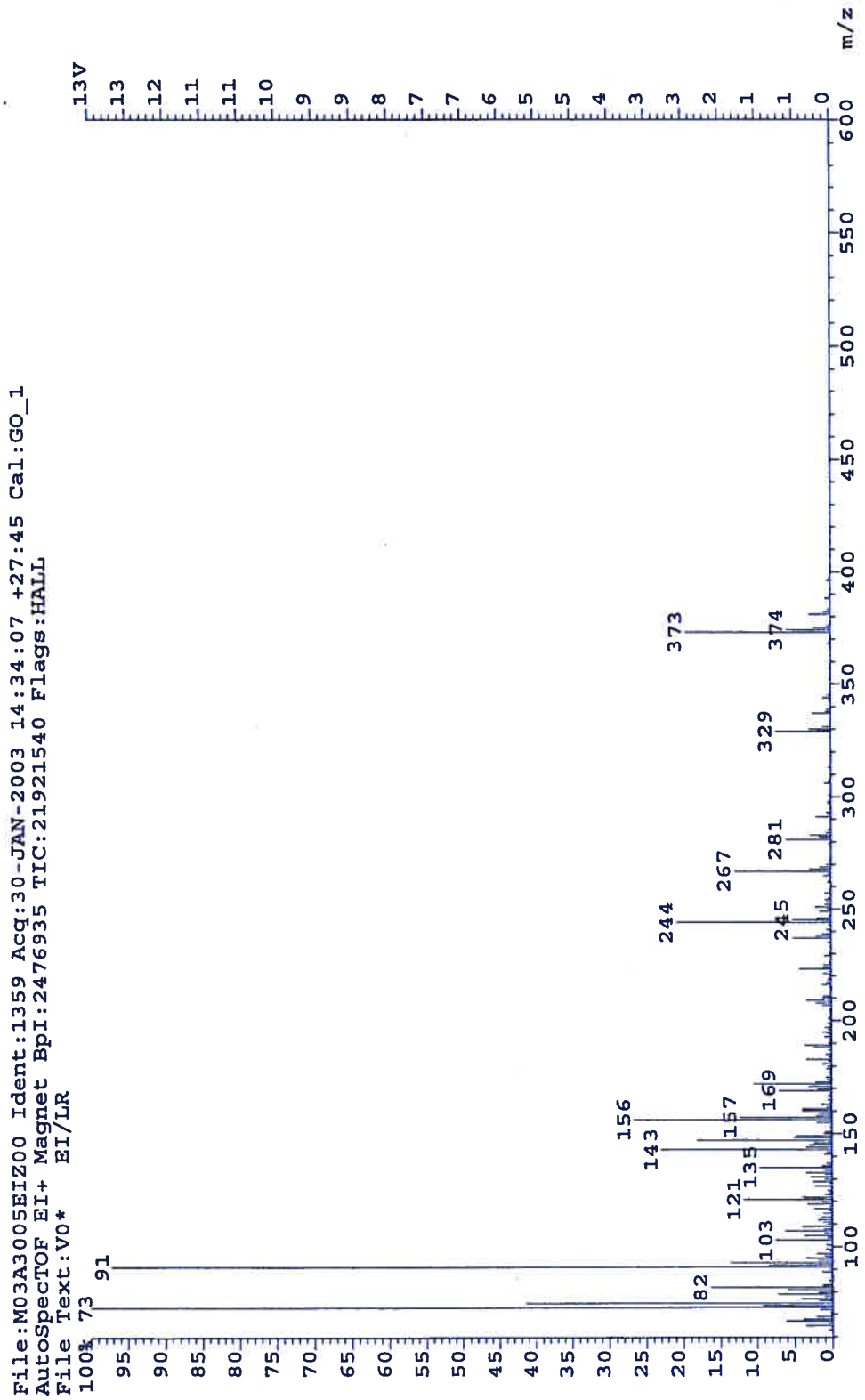
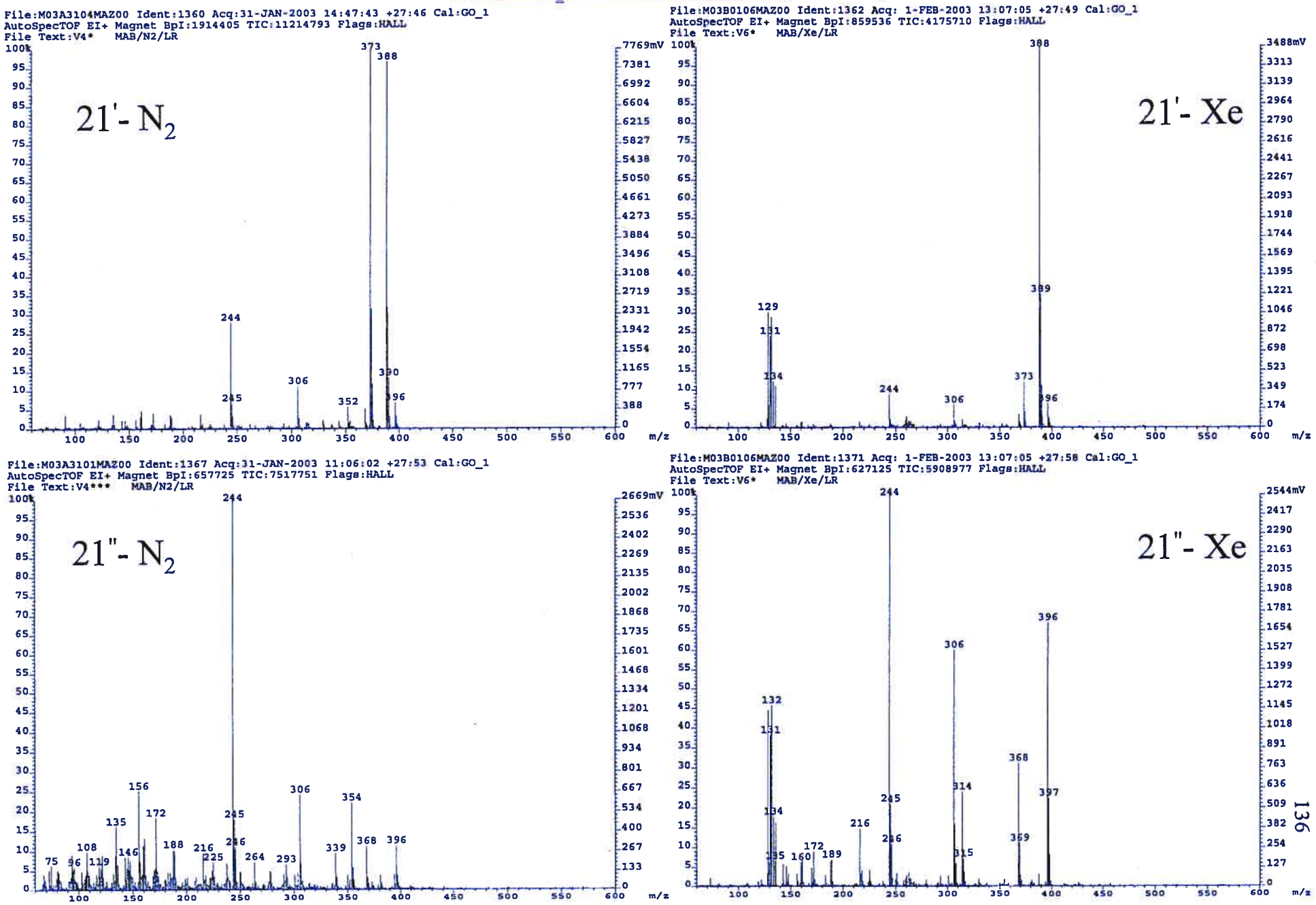


Fig. 24. N_2 and Xe MAB mass spectra of peak 21



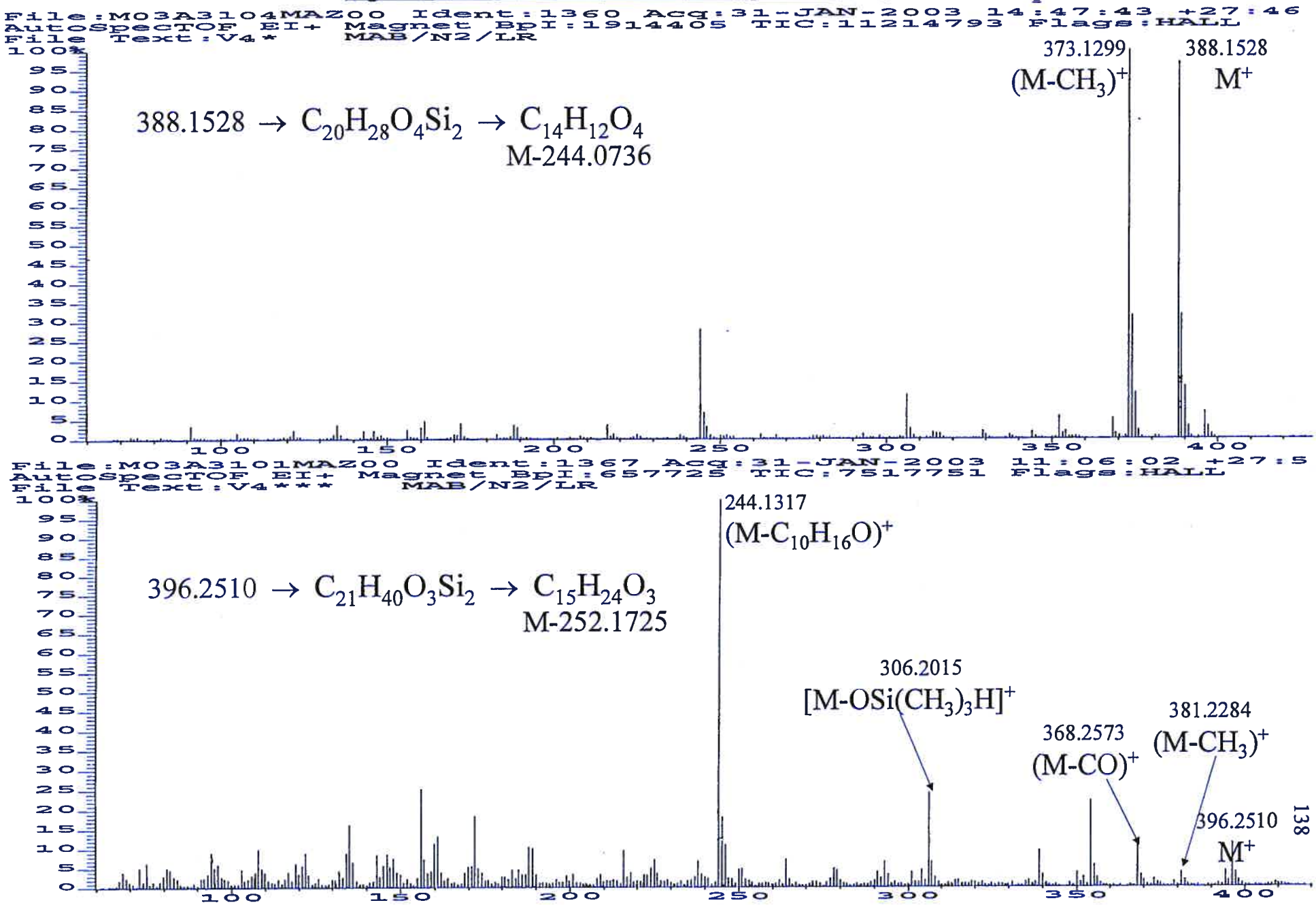
All the above fragments were verified by performing GC/MS accurate mass measurements using MAB with N_2 (Fig. 25), which appeared to be another advantage to this ionisation technique. The excellent results we obtained (in all measurements the deviation was less than 2.5 ppm) lead unequivocally to the following conclusions: the first compound can only have elemental composition $C_{20}H_{28}O_4Si_2$, which corresponds to a nonderivatized molecule $C_{14}H_{12}O_4$; the second can only have elemental composition $C_{21}H_{40}O_3Si_2$, corresponding to a nonderivatized molecule $C_{15}H_{24}O_3$.

Now, going back to the EI spectra, it can easily be recognized that the first compound is benzyldihydroxybenzoate with its fragments at *m/z*- 373, 329, 281, 267, 135. Analogous type of fragments, but 88 mass units lower, appeared in the mass spectra of previously identified benzylmonohydroxybenzoate.

The second compound needs to be isolated and fully characterized by other spectral techniques like NMR and IR. The limited amount of propolis sample we possess will make this process difficult, but we will make an attempt in the near future because this compound may contribute significantly to the overall biological activity.

It should be underlined that without the MAB ionisation we could not have reached proper identification of these analytes. Even further, an eventual attempt to isolate these compounds will be possible only if MAB is used to monitor the separated fractions coming out from the preparative column to define where the target compound is.

Fig. 25. Accurate mass measurment of peak 21 using MAB with N_2



From all the results obtained we can conclude that the MAB source of ionisation is valuable for the analysis of propolis, particularly for compound identification, structure elucidation and exact mass measurements. Further studies of propolis samples from other geographic regions containing different classes of compounds as well as studies to improve the overall sensitivity are highly desirable.

5.4. Investigations of Propolis Volatile Oils from Different Geographic Origins

Some authors suggested that the chemical composition of volatile constituents of propolis (volatile oils) could give additional information about its probable plant sources. Furthermore, volatiles are important propolis components not only because they determine its pleasant aroma but also because of their proven antibacterial activity (37, 48, 51, 173).

As an additional and distinct part of our whole study we also analyzed volatile oils of propolis samples from different geographic and climatic regions. All volatiles were obtained by steam distillation of the samples and subsequent extraction with ether/*n*-pentane (see Experimental section), and then submitted for GC/MS analysis.

5.4.1. Propolis from Bulgaria, Albania and Mongolia

Comparative investigations were performed on volatile oils from Bulgarian, (Fig. 26) Albanian and Mongolian propolis samples. These samples are of different plant origin: in Bulgaria from *Populus nigra* and to some extent from *P. italica* buds, in Mongolia from *P. suaveolens* buds and in Albania from

P. nigra buds and from some unidentified plants. The results obtained are summarized in Table XI. In the same table, data available from the literature about volatile oils of other propolis samples are presented.

According to Petri *et al.* (165), propolis could be divided into two types with respect to the volatile oils. The first one is characterized by the presence of substantial amounts of β-eudesmol, while in the second the main volatile constituent appeared to be benzyl benzoate. According to our results, the samples from Albania and Mongolia belong to the second type (41, 74), while that from Bulgaria is from the first type (41). Probably the second type does not originate from pine trees, as Petri *et al.* suggested, because all the data available about phenolic composition of Hungarian propolis indicate that its main source is *P. nigra* buds (8, 23, 146). Until now, it is not proven that pine trees can be a source of propolis, and in no case volatiles from bee glue contained typical pine terpenoids, (e.g. pinenes).

Analogous to all other propolis samples investigated, the largest amount of volatile constituents appeared to be sesquiterpenoids. Most of them have been identified in Bulgarian propolis. From Table XI it is evident that a substantial part of the identified sesquiterpenoids have not been found earlier in other propolis samples. In addition to the identified sesquiterpenoids, a large number of unidentified representatives of this group have been found. They are 11 hydrocarbons and 12 alcohols and most of them are constituents of Mongolian propolis. This can be explained with its specific source.

4-Phenyl-3-buten-2-one **5** was found for the first time in nature in Albanian and Bulgarian propolis. Its structure is close to that of cinnamic acid, whose derivatives are important propolis constituents.

From the results obtained it may be concluded that the differences between the compositions of volatile oils from propolis from different locations in the temperate zone are higher than that of their phenolic constituents.

In the samples investigated we identified some sesquiterpenoid alcohols which might possess antimicrobial and other biological activities (55). This shows that volatile oils could contribute to the propolis activity.

Table XI

Chemical composition of volatile oils (% TIC)[c] from Bulgarian, Albanian and Mongolian Propolis

Compound	Alb.	Bulg.	Mong.	Others[a]
Esters				
Benzyl acetate **1**	—	1.6	1.0	+
Benzyl benzoate	1.7	—	8.6	+
Unidentified ester of 2-phenylethanol **2**	—	1.6	—	—
Ketones, alcohols, phenols				
2-phenylethanol	0.9	—	—	+
Isoeugenol[b] **6**	1.1	0.8	—	—
Methoxyacetophenone[b] **3**	9.0	3.3	1.7	—
Methoxyacetophenone (iso)[b] **4**	—	0.6	—	—
4-phenyl-3-buten-2-one[b] **5**	1.0	1.1	—	—
Sesquiterpenes				
δ-cadinene[b] **14**	1.0	5.3	—	—
Cadinene (isomer)[b] **12**	10.5	3.4	—	—
Calamenene **15**	—	2.2	2.6	+
α-muurolene[b] **16**	0.9	2.0	1.2	—

Compound	Alb.	Bulg.	Mong.	Others[a]
γ-muurolene[b] **13**	—	4.7	1.8	—
β-selinene[b] **10**	—	1.2	—	+
β-eudesmol **18**	—	8.8	—	+
α-elemene[b] **11**	—	2.3	—	—
α-copaene **17**	—	0.9	—	+
Bulnesol[b] **19**	—	2.3	—	—
Guaiol **17**	1.3	2.9	—	+
β-caryophylene **8**	—	1.2	—	+
Hydrocarbons				
Heneicosane	3.6	—	—	+
Tricosane **20**	4.8	4.9	1.4	+
Pentacosane **21**	4.1	4.4	—	+
Heptacosane **22**	6.6	2.7	—	+
Nonacosane	5.4	—	—	+
Hentriacontane	4.0	—	—	+
3-methylindene[b]	0.8	—	—	—
Alkylbenzene (M^+=162) **9**	—	0.6	—	+

[a] Compounds, found in volatile oils from other propolis samples by other authors but absent in our samples, are not included in this table

[b] For the first time in propolis.

[c] The ion current generated depends on the characteristics of the compound concerned and it is not a true quantification.

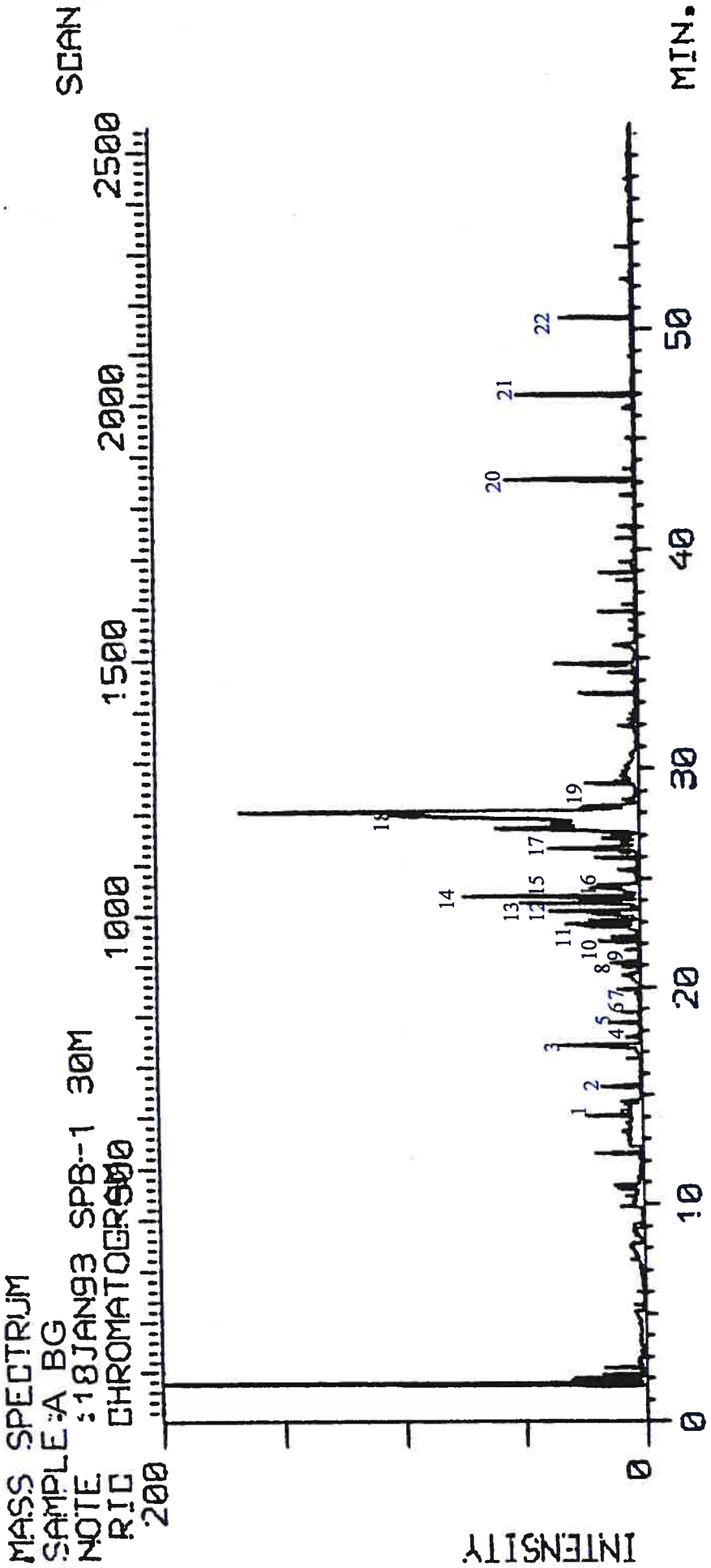


Fig. 26. TIC chromatogram of volatile oils of propolis from Bulgaria. Peak numbers correspond to compounds in Table XI. For conditions, see Experimental section **4.4.3.** (p. 52)

5.4.2. Brazilian Propolis

The detailed investigation on chemical composition of Brazilian propolis included also the analysis of the volatile oils from the same four samples described in section **5.2.2.** The results from GC-MS analysis (Fig. 27) are summarized in Table XII.

The results obtained showed that the volatiles from samples **Br-1** and **Br-2** have almost identical chemical composition in spite of the different collection site similarly to the alcohol extracts. We also found some similarities between the composition of volatiles from **Br-1** and **Br-4**, the latter containing more components. It is interesting to note that in **Br-3** besides hydrocarbons, we only found three sesquiterpenoids.

Derivatives of acetophenone are characteristic for different propolis samples. While Bulgarian samples contained only methoxy- and hydroxyacetophenones, we found in Brazilian samples mono- (peak **14**) and diprenylated (peak **16**) acetophenones, which appeared to be among the main volatile components of **Br-1** and **Br-4**. The elucidation of the exact location of the prenyl substituents needs further isolation of these compounds and additional amounts of propolis samples. In Venezuelian samples (14), prenylated benzophenones have been found, while in Brazilian propolis C- and O-prenylated cinnamic acids are among the main constituents (16).

Samples **Br-1** and **Br-4** contained significant amounts of terpenoids, only few of them being found in Bulgarian propolis. Almost all of them are sesquiterpenoids (hydrocarbons and alcohols), part of them found for the first time in propolis. The main constituents appeared to be α-terpineol **4**, 2Z, 6E-farnesol **11** and ledol **13**. Only δ-cadinene **10** was found in all investigated Brazilian and Bulgarian samples and humulene was found in the unusual **Br-3** sample.

The results obtained confirm the proposal that the chemical composition of Brazilian propolis is substantially different from that of propolis in temperate regions because of the different plant sources.

Table XII

Chemical composition of volatile oils from Brazilian propolis (%TIC)[a], compared to this of Bulgarian propolis

Compound	Br-1	Br-3	Br-4	Bulg
Acids				
Pellargonic acid **6**	0.7	—	—	—
Decanoic acid[b] **8**	4.7	—	—	—
Myristic acid **15**	2.2	—	—	—
Esters				
Benzyl acetate	—	—	—	1.6
Ethyl phenylacetate[b]	—	—	0.7	—
Methyl dihydrocinnamate[b]	—	—	1.2	—
Ethyl dihydrocinnamate[b] **5**	0.3	—	0.7	—
Alcohols, phenols				
1-phenylethanol[b]	—	—	1.2	—
2-phenylethanol	—	—	0.6	—
3-phenylpropanol[b]	—	—	3.7	—
Ethylphenol[b] **3**	0.6	—	4.6	—
Isoeugenol	—	—	—	0.8

Compound	Br-1	Br-3	Br-4	Bulg
Ketones, aldehydes				
Methoxybenzaldehyde[b]	—	—	1.5	—
Acetophenone **2**	0.7	—	2.8	—
Methoxyacetophenone	—	—	—	3.3
Methoxyacetophenone (isomer)	—	—	—	0.6
4-phenyl-3-butene-2-one	—	—	—	1.1
Prenyl acetophenone[b] **14**	3.6	—	8.2	—
Diprenyl acetophenone[b] **16**	11.1	—	1.7	—
Monoterpenes				
α-terpineol[b] **4**	1.5	—	1.6	—
Sesquiterpenes				
Farnesol **11**	17.4	—	6.1	—
δ-cadinene **10**	3.3	3.3	0.7	5.3
Calamenene	—	—	—	2.2
α-muurolene **9**	2.4	—	—	2.0
γ-muurolene	—	—	—	4.7
β-selinene	—	—	—	1.2
α-elemene	—	—	—	2.3

Compound	Br-1	Br-3	Br-4	Bulg
α-copaene	—	—	—	0.9
Bulnesol	—	—	—	2.3
Guaiol	—	—	—	2.9
β-eudesmol	—	—	—	8.8
Ledol[b] **13**	5.7	—	0.1	—
β-caryophyllene **7**	1.9	—	—	1.2
α-humulene[b]	—	1.0	—	—
Sesquiterpenoid alchohol $M^{+\cdot}$=220 **12**	12.9	—	—	—
Hydrocarbons				
Octadecane[b]	—	2.5	—	—
Nonadecane[b]	—	3.0	0.6	—
Heneicosane	—	3.8	1.3	—
Tricosane	—	5.2	2.3	4.9
Pentacosane	—	3.9	1.8	4.4
Heptacosane	—	—	2.9	2.7
Xylene[b] **1**	0.9	0.3	—	—
Others				
Coumaran (pesticide)	—	0.5	2.0	—

[a]The ion current generated depends on the characteristics of the compound concerned and it is not a true quantification.

[b] For the first time in propolis.

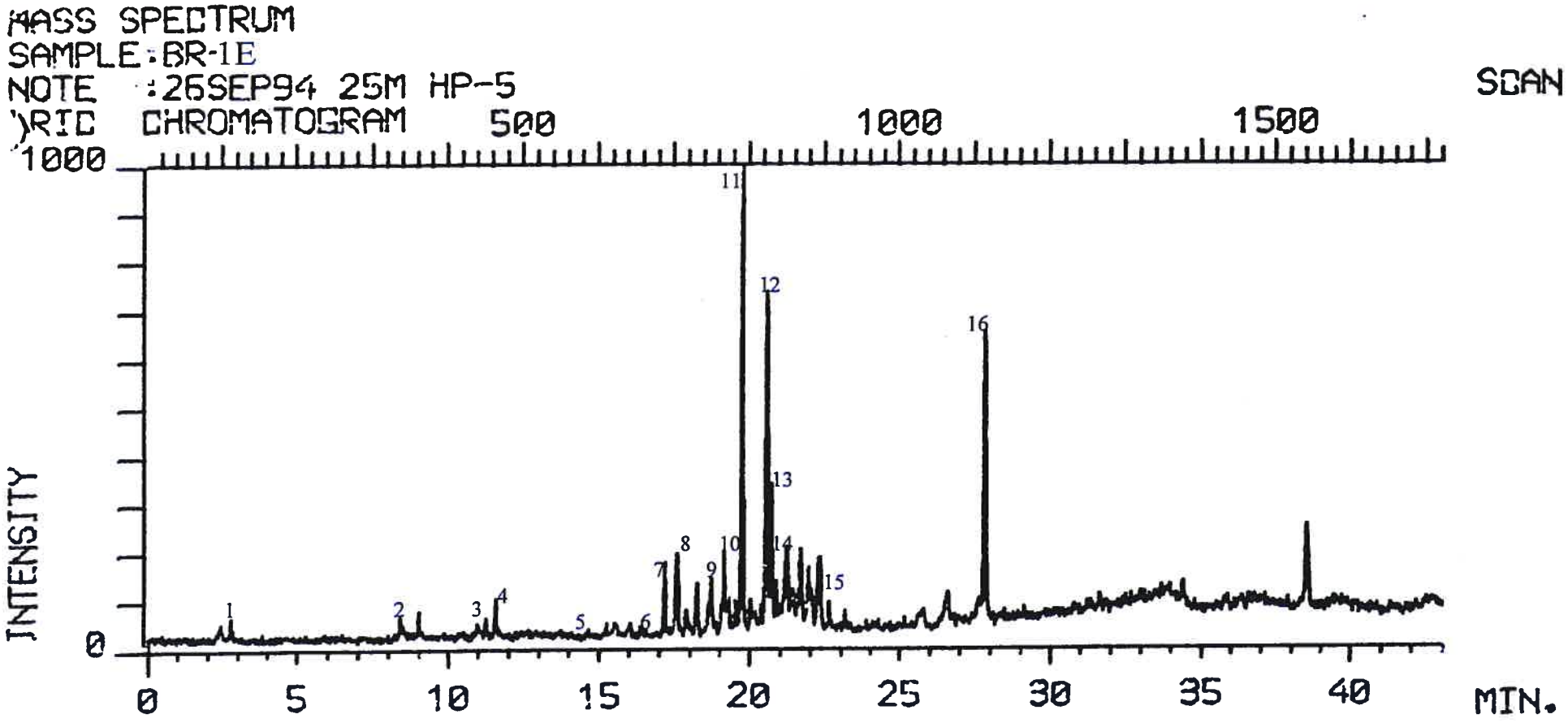


Fig.27. TIC chromatogram of volatile oils of propolis from Brazil, sample **Br-1**. Peak numbers correspond to compounds in Table XII. For conditions, see Experimental section **4.4.3**. (p. 52)

5.4.3. Brazilian Geopropolis

The volatile compounds of the same three geopropolis samples collected by three different stingless bee species have been investigated by GC/MS (Fig. 28) and the results obtained are summarized in Table XIII. In order to compare their chemical compositions we included data about volatile compounds from Brazilian propolis (**Br-1**), collected by *Apis mellifera* (honey bee) in the same region as samples **G-2** and **G-3** (see section **5.3.2.**).

The most important characteristic of geopropolis we have studied is the presence of a large amount of monoterpenoids. While in poplar propolis monoterpenoids were identified only in a few cases (6) and in Brazilian propolis from Prudentopolis, sample **Br-1,** we found only α-terpineol, here in sample **G-3** we identified 19 monoterpenoids (5 hydrocarbons, 6 carbonyl compounds and 8 alcohols). Sample **G-2** contained the same groups of monoterpenoids, but now they were mainly 6 alcohols, 4 carbonyl compounds and 2 hydrocarbons. In **G-1** we found only one monoterpenoid (α-pinene) in low concentration.

The samples contained also sesquiterpenoids (concentrated mainly in sample **G-2**) and some phenolics.

Evidently the compositions of the volatiles from samples **G-2** and **G-3** differ significantly. The samples were collected in the same region, so the available plant sources should be identical. For this reason we could conclude that different bee species collect propolis from different plants. This is in

agreement with the significant differences between geopropolis and propolis (**Br-1**) collected by *Apis mellifera* in the same region (near Prudentopolis).

Based on the results obtained, we can conclude that the composition of Brazilian geopropolis depends, as expected, on the collection site. Also, it might depend on the bee species collecting it, which is confirmed by the substantial differences in the chemical composition of volatiles and of alcoholic extracts (see section **5.2.3.**) of samples **G-2** and **G-3** collected in the same region. It is very likely that different bee species prefer different propolis plant sources and more research is needed on the topic.

Table XIII

Chemical composition of geopropolis volatile oils (% TIC)[a]

Compound	Sample			
	G-1	G-2	G-3	Br-1
Acids, esters				
Butyric acid	—	0.3	—	—
Isovaleric acid[b] **1**	—	—	2.4	—
Caproic acid[b]	0.2	—	—	—
Pelargonic acid	—	—	—	0.7
Decanoic acid	—	—	—	4.7
Myristic acid	2.0	0.9	—	2.2
Palmitic acid **9**	—	2.8	0.7	—
Cinnamic acid **34**	3.7	1.4	0.9	—
Dihydrocinnamic acid	—	3.2	—	—
Benzyl benzoate	—	1.7	—	—
Ethyl dihydrocinnamate	—	0.6	—	0.3
Acohol, phenols,aldehydes				
Hexanol[b]	0.4	—	—	—
Benzyl alcohol **10**	1.0	0.3	0.2	—
1-Phenylethanol	0.5	—	—	—
2-Phenylethanol	0.2	0.4	—	—

Compound	Sample			
	G-1	G-2	G-3	Br-1
p-Cresol[b] **11**	—	—	0.4	—
Ethylphenol	10.2	—	—	0.6
Benzaldehyde **4**	0.7	—	0.2	—
Acetophenone	—	—	—	0.7
Prenylacetophenone	—	—	—	3.6
Diprenylacetophenone	—	—	—	11.1
4-Isopropylidene - benzaldehyde[b] **26**	—	—	0.5	—
Monoterpenes				
Terpinene-4-ol[b] **20**	—	1.5	0.9	—
α-Terpineol	—	—	—	1.5
p-Mentha-1,4-diene-8-ol[b] **19**	-	0.8	2.4	—
trans-Carveol[b] **24**	I	0.4	0.7	—
Carvone[b] **27**	—	—	0.4	—
p-Cimene **8**	—	0.1	1.5	—
p-Cimene-8-ol[b] **21**	—	1.5	11.4	—
p-Cimene-7-ol[b] **29**	—	—	0.8	—
Thymol[b] **31**	—	—	1.3	—
Sabinene **9**	—	—	0.3	—
β-Thujone[b] **15**	—	—	0.3	—

Compound	Sample			
	G-1	G-2	G-3	Br-1
Umbellulone[b] **25**	—	0.3	0.9	—
2-Carene[b] **6**	—	—	1.5	—
Car-3-ene-2-one[b] **28**	—	0.3	1.1	—
α-Pinene **3**	0.3	0.2	0.7	—
β-Pinene **5**	—	—	0.2	—
trans-Pinocarveol[b] **17**	—	1.0	1.0	—
Verbenol[b] **18**	—	3.1	1.4	—
Verbenone[b] **23**	—	3.0	6.5	—
α-Campholene aldehyde[b] **16**	—	0.3	0.3	—
Sesquiterpenes				
Farnesol	—	—	—	17.4
Nerolidol	1.0	12.3	—	—
γ-Cadinene[b]	—	0.9	—	—
δ-Cadinene	—	2.0	—	3.3
α-Muurolene	—	0.6	—	2.4
α-Calakorene[b]	—	0.7	—	—
T-Muurolol[b]	0.4	3.5	—	—
β-Selinene	—	0.2	—	—
β-Bourbonene **32**	—	—	0.9	—

Compound	Sample			
	G-1	G-2	G-3	Br-1
α-Copaene	—	0.6	—	—
γ-Gurjunene[b]	—	1.6	—	—
α-Gurjunene[b]	—	0.6	—	—
Aromadendrene **35**	—	0.7	0.2	—
Alloaromadendrene[b]	—	0.5	—	—
Ledol[b]	—	3.4	—	5.7
Ledol diastereoisomer	—	1.1	—	—
Ledol diastereoisomer	—	1.4	—	—
Spatulenol **37**	0.9	10.4	1.3	—
β-Caryophylene	—	1.5	—	1.9
Caryophylene oxid[b] **38**	—	—	1.6	—
Diterpenes				
Kaur-15-ene (isokaurene)[b] **43**	—	—	0.7	—
Kaur-16-ene (kaurene) **44**	—	0.3	0.9	—
Kauran-16-ol[b] **46**	—	—	2.8	—
Kaur-16-ene-19-ol[b] **48**	—	—	1.2	—
Kaur-16-ene-18-oic acid[b] **49**	—	—	0.7	—
Aliphatic hydrocarbons				
Octane[b]	0.4	—	—	—

Compound	Sample			
	G-1	G-2	G-3	Br-1
Nonane[b] **2**	0.2	—	0.1	—
Decane[b]	0.4	—	—	—
Undecane[b] **14**	1.0	0.2	1.1	—
Dodecane[b] **22**	1.5	0.5	1.6	—
Tridecane[b] **30**	1.2	0.5	0.8	—
Tetradecane[b] **33**	1.0	0.5	1.2	—
Pentadecane[b] **36**	0.6	0.3	0.3	—
Hexadecane[b] **39**	0.9	0.8	0.6	—
Octadecane **40**	0.6	0.3	0.3	—
Henicaosane **45**	0.7	0.7	0.2	—
Docosane[b]	0.6	0.5	—	—
Tricosane **47**	0.6	1.2	0.4	—
Pentacosane **50**	1.3	1.6	0.6	—
Phytane[b] **41**	—	0.5	0.6	—
Aromatic hydrocarbons				
1-Methyl-4-isopropylbenzene[b] **12**	0.1	—	0.4	—
1-Methyl-2-isopropylbenzene[b] **13**	0.2	—	1.9	—

Compound	Sample			
	G-1	G-2	G-3	Br-1
Xylol	0.2	—	—	0.9
Coumol[b]	0.2	—	—	—
1,2-Dimethyl-4-ethylbenzene	—	—	0.2	—
7				
1-Propenylbenzene[b]	0.2	—	—	—
1-Methyl-3-propylbenzene[b]	0.4	—	—	—
1-Methylnaphtalene[b]	0.4	—	—	—
2-Methylnaphtalene[b]	0.2	—	—	—
2,6-Dimethylnaphtalene[b]	0.1	—	—	—
Others				
Ethylcyclohexane[b]	0.2	—	—	—
n-Amylcyclohexane[b]	0.1	—	—	—
Cholesterol **51**	—	—	0.3	—
Cholesta-5-ene-3-one[b] **52**	—	—	0.1	—
Cholesta-4,6-diene-3-one[b] **53**	—	—	0.1	—
Coumaran (pesticide)	1.2	—	—	—

[a] The total ion current generated depends on the characteristics of the compound concerned and is not a true quantification.

[b] For the first time in propolis.

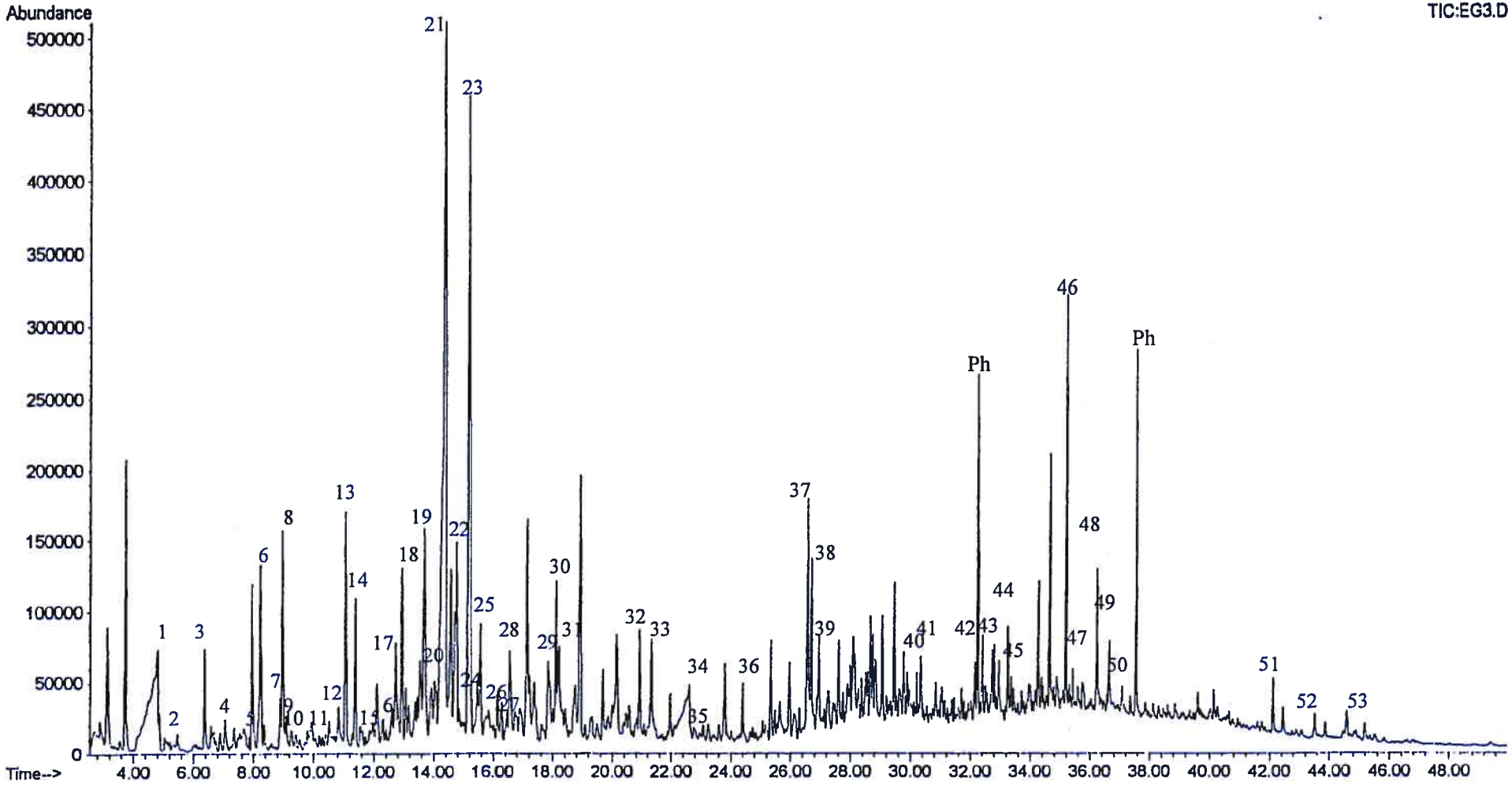


Fig.28. TIC chromatogram of volatile oils of geopropolis from Brazil, sample **G-3**. Peak numbers correspond to compounds in Table XIII, Ph - phtalate. For conditions, see Experimental section **4.4.3**. (p. 52)

5.4.4. Propolis from the Canary Islands

Volatiles were obtained from both investigated samples **K-1** and **K-2** (see section **5.2.4.**) and analyzed by GC-MS (Fig. 29). The results obtained are summarized in Table XIV. It is evident that contrary to the polar constituents that we discussed before, the composition of volatiles is more or less similar to that in propolis from other regions. The main components appeared to be terpenoids. Their concentrations were significantly higher in sample **K-2**. This is an indication that the plant source of these compounds might be the same one that gives the furofuran lignans, which predominate in the alcohol extract of sample **K-2**. Most of the terpenes were sesquiterpenoid hydrocarbons and alcohols, (analogous to all other propolis samples investigated), while monoterpenoids were in low concentrations. It must be mentioned that the characteristic for Brazilian propolis spatulenol (see Table XIII from the previous section) appeared to be the main sesquiterpene in the Canary Islands samples. Benzyl benzoate (peak **36**), but not β-eudesmol, was discovered in both samples, which is an indication that Canary Islands propolis belongs to the benzyl benzoate type (165), analogous to Brazilian propolis. Other aromatic compounds were found in low concentrations (Table XIV).

The pesticide Vanguard BT **8**, as well as m-methylstirol **4** and 2-methylnaphtalene **10** are evidently due to the pollution, which confirm our suggestion that propolis could be used as a bio-indicator of pollution.

Table XIV

Chemical composition (%TIC)[a] of volatiles of propolis from Canary Islands (Gran Canaria).

Compounds	K-1	K-2
Acids and Esters		
Miristic acid **35**	1,3	0,7
Cinnamic acid **17**	3,6	0,5
Methyl palmitate[b] **39**	0,7	0,4
Ethyl palmitate **40**	4,3	1,1
Ethyl oleate **42**	6,5	2,5
Benzyl benzoate **36**	0,7	1,2
Ethyl dihydrocinnamate **13**	0,3	0,2
Aldehydes		
Benzaldehyde **2**	0,4	0,2
Piperonal[b] **12**	0,4	0,2
Monoterpenes		
Linalyl propionate[b] **6**	—	0,5
Geraniol[b] **9**	—	0,2

Compounds	K-1	K-2
Sesquiterpenes		
Nerolidol **27**	3,2	11,0
δ-cadinene **24**	0,9	2,5
α-muurolene **23**	0,7	0,9
α-calakorene **26**	0,5	0,7
T-muurolol **33**	1,2	2,2
β-selinene **21**	0,2	0,8
Germacrene D[b] **20**	0,2	0,5
α-copaene **14**	0,5	0,2
Ledene[b] **22**	1,5	1,3
Aromadendrene **18**	0,3	2,8
Ledol **30**	1,6	3,8
Spatulenol **29**	3,2	8,4
Isospatulenol[b] **32**	1,2	0,8
Palustrol[b] **28**	0,2	0,8
β-cariophillene **16**	2,4	1,7
α-humulene **19**	0,2	1,1
Aliphatic hydrocarbons		
Nonane **1**	0,3	0,2
Decane **3**	0,7	0,2

Compounds	K-1	K-2
Undecane **5**	1,4	0,6
Dodecane **7**	1,9	0,6
Tridecane **11**	1,4	0,5
Tetradecane **15**	1,0	0,3
Hexadecane **31**	1,4	1,2
Heptadecane **34**	1,0	0,6
Octadecane **37**	0,6	0,5
Nonadecane **38**	1,5	0,7
Henicosane **41**	1,4	0,9
Docosane **43**	1,3	0,9
Tricosane **44**	1,1	1,2
Aromatic hydrocarbons		
2-methylnaphtalene **10**	0,5	0,2
m-methylstirol **4**	—	0,4
Others		
Vanquard BT (pesticide) **8**	3,7	1,5
Dodecaniene-1-ol[b] **25**	2,0	0,8

[a]The ion current generated depends on the characteristics of the compound concerned and it is not a true quantification.

[b]For the first time in propolis.

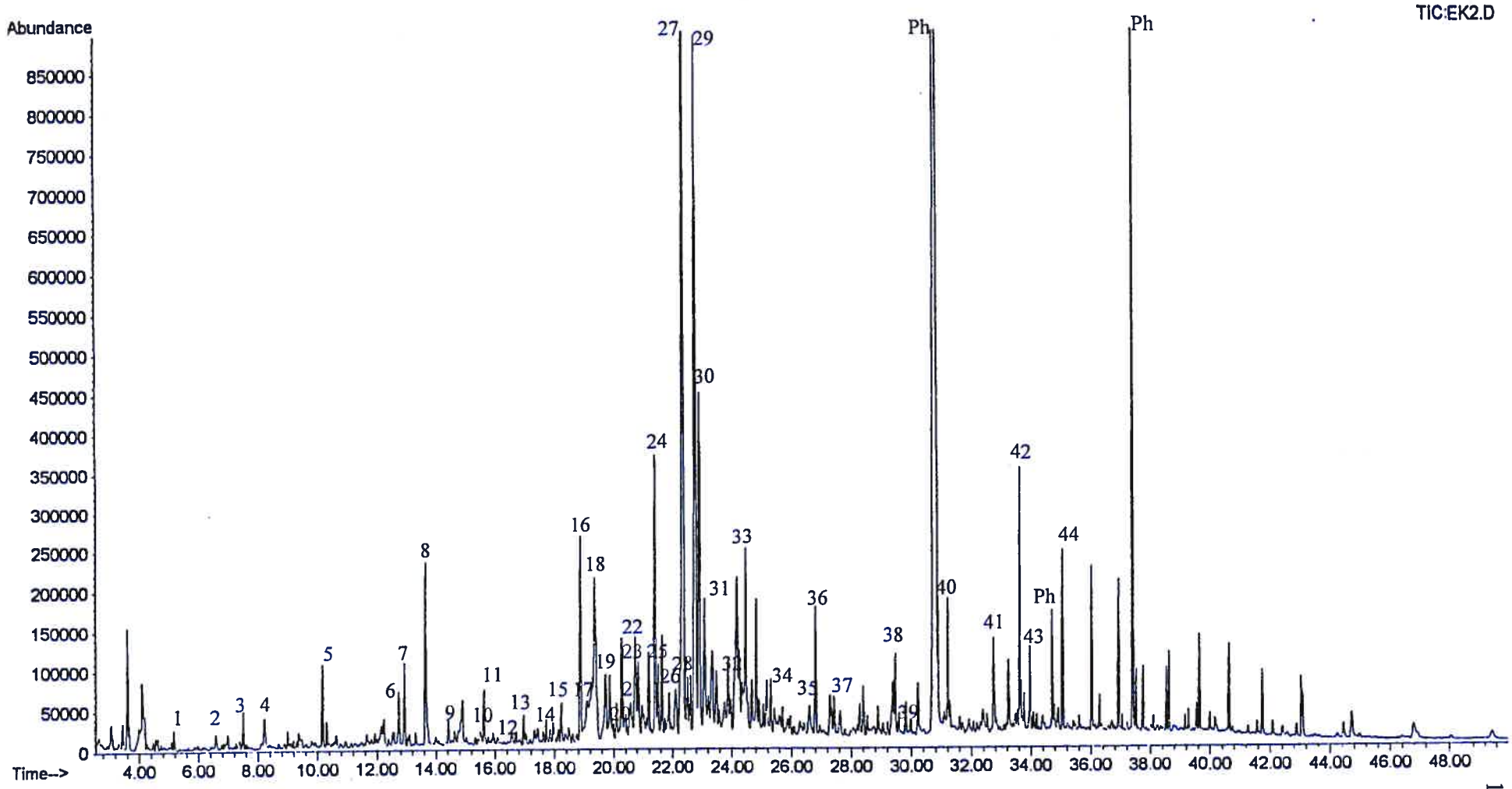


Fig.29. TIC chromatogram of volatile oils of propolis from Canary Islands, sample **K-2**. Peak numbers correspond to compounds in Table XIV, Ph - phtalate. For conditions, see Experimental section **4.4.3.** (p. 52)

5.5. Biological Activity of Propolis from Different Geographic Locations

Two kinds of materials derived from propolis were investigated: the extracts of propolis samples with 70% ethanol (the so called "balsam") most often used in folk medicine (5), as well as the volatile oils. The activity against pathogen bacterial and fungal strains and the antiviral activity were tested in the labs of the Institute of Microbiology, Bulgarian Academy of Sciences (166). The results obtained are summarized in Tables XV and XVI.

Our results present an unambiguous proof that in spite of the great differences in the chemical composition of propolis from different geographic locations, all samples exhibit significant antibacterial and antifungal (and most of them antiviral) activity. This is an expected result since propolis is thought to be bees' defence against infections.

Table XV

Biological activity of propolis samples (extracts with 70% ethanol)

Propolis sample	Antibacterial activity[a]	Antifungal activity[b]	Antiviral activity[d]
	(diameter of the inhibitory zone±stand. deviation, mm)[c]		(SI)
Bg	13.7±0.3	17.7±1.2	8
Mong	16.2±0.3	18.0±1.0	4
Alb	13.8±0.6	17.0±1.0	4
Egypt	15.3±1.5	17.3±0.4	not tested
Br1	12.0±1.0	14.3±0.6	2
Br2	11.8±0.8	17.2±1.2	4
Br3	11.0±1.0	15.7±1.0	0
Br4	11.8±0.8	18.2±0.3	4
G1	12.7±0.6	17.0±0.5	35
G3	11.2±1.0	16.2±1.0	4
K1	29.0±0.7	18.0±1.0	not tested
K2	17.3±1.2	17.0±0.7	not tested
Nystatin[e]	-	32±1	-
Streptomycin[f]	28±1	-	-

[a] Against *Staphylococcus aureus*

[b] Against *Candida albicans*

[c] Mean of three measurements

[d] Against Avian influenza virus

[e] 50 I.U.

[f] 0.1 mg in the spot

Table XVI

Antibacterial activity of volatile oils from propolis samples.

Propolis sample	Antibacterial activity[a,b]
Bg	4>
Br1	12.1±0.6
Br2	11.5±0.3
Br3	12.8±0.3
Br4	11.2±0.3
G1	21.0±2.0
G3	16.0±0.7
K1	23.0±1.3
K2	12.3±1.1
Streptomycin[c]	28±1

[a] Against *Staphylococcus aureus*

[b] Diameter of the inhibitory zone±stand, deviation, mm, mean of three measurements

[c] 0.1 mg in the spot

Further to this study and as a confirmation of the way the differences in chemical composition affect the biological properties of propolis, the two Canadian samples were tested for their toxicity against brine shrimp *Artemia salina*, and for DPPH radical scavenging activity. The results are represented in Table XVII.

Table XVII

Biological activity of Canadian propolis samples

Sample	DPPH Radical Scavenging activity ED_{50}[a]	Brine shrimp toxicity LC_{50}[b] ± SD[c]
Victoria	79.0	5 ± 3
Richmond	65.0	28 ± 17
CAPE	Not tested	0.45 ± 0.07
Caffeic acid	58.0	Not tested

[a] Concentration inhibiting 50% of the free radicals, μg/ml

[b] Concentration lethal to 50% of the *Artemia salina nauplii*, μg/ml

[c] Standard deviation, mean of three measurements

Both samples showed very good radical scavenging activity compared to the well-known antioxidant caffeic acid used as positive control. These results are in accordance with previous ones published on antioxidative activity of propolis from different geographic origin (167, 168). The presence of diverse phenolic compounds, although different in both samples, is a good explanation for this type of activity. For the propolis sample from Victoria, dihydrochalcones might be of special importance in this respect, as they are known to have significant radical scavenging activity against DPPH (169).

The toxicity against brine shrimp is usually regarded as a preliminary test for potential cytotoxicity (170). The sample from Victoria region showed remarkable toxicity, comparable to that found for propolis from European black poplar *P. nigra* (*Aigeiros*) (171). The value for the Richmond sample was somewhat less favourable. In black poplar propolis, the high cytotoxicity is due to the presence of CAPE, a typical component of *Aigeiros* poplar bud exudate (101). In Victoria propolis however, CAPE was not detected (Table IX). Its high activity could be due to the benzyl esters of methoxybenzoic and hydroxybenzoic acids. Recently, benzyl benzoate and benzyl cinnamate were found to be highly toxic to *A. salina* (172). This type of propolis is of particular interest for further bioguided chemical investigations, taking into consideration that little is known about biological activity of dihydrochalcones. The unidentified major component (14%), presumably of terpenoid nature, could be a contributing factor to the activity as well.

Our results, as well as the literature data, dealing with chemical composition and biological action of propolis cannot point out one individual substance or a particular substance class which could be responsible for this action. Obviously, the combinations of different substances are essential for the biological activity of the bee glue. It is important to note that all investigations on the antibacterial action of individual substances, isolated from propolis, showed that no single propolis component has an activity greater than that of the total extract (149, 166).

It seems that the chemical properties of propolis are not only beneficial to bees but have general pharmacological value as a natural mixture and not as a source of new powerful compounds possessing antimicrobial, antifungal and antiviral activity.

5.6. Plant Origin of Propolis from Different Geographic Locations

It is generally accepted and chemically proven that in the temperate zones including Europe (8, 11, 44, 66, 67, 71), North America (13), the non-tropical regions of Asia (41) and even New Zealand (50) the bud exudates of *Populus* species are the sources of the bee glue. In Russia, especially in its northern parts, birch buds (*Betula verrucosa*) play this role (66).

From our results it is evident that the main taxonomic markers of poplar bud exudates, prenyl caffeates, and the flavanones pinocembrin, pinobanksin and 3-O-acetylpinobanksin are present in Egyptian propolis, which is an unambiguous proof of its poplar origin. However, the presence of large amounts of β-amyrin and cycloartenol, which are unusual for poplar buds, is an indication that some other plant sources are involved.

In Canada in the absence of poplars of section *Aigeiros*, bees have found other poplar trees to be suitable as propolis sources, namely *P. trichocarpa*, Section *Tacamahaca* and probably the wide-spread in North America aspen *P. tremuloides* Michx, Section *Leuce*, subsection *Trepidae*.

In tropical regions there are no poplars and birches and obviously bees have to find new plant sources of bee glue. The chemical analyses performed in the present investigation, as well as the literature data showed that the variability in chemical composition of tropical samples is much more pronounced than the variability of samples from the temperate zone.

Prenylated coumaric acids, recently found in Brazilian propolis, prenylated acetophenones, diterpenic acids and triterpenes are typical components of the leaf exudates of shrubs belonging to the genus *Baccharis*, Asteraceae. This South American genus is characterized by a large number of species and a remarkable chemical diversity of their leaf exudates: some of them contain mainly di- and triterpenes, in others, lipophylic flavonoid aglycones predominate. In some cases different phenolics and terpenes occur in mixtures (176, 180, 181).

Our investigations on the composition of Brazilian propolis confirmed the known hypothesis that some *Baccharis* species might be the bee glue sources in Brazil. Thus, friedooleanan-3-one, one of the main components of sample G-2 was found together with other triterpenes in *B. salicifolia* (176). Pinobanksin, found in significant concentration in sample **G-1**, might come from another species, *B. oxydonta*, where it is a main constituent of the exudate (180). On the other hand, from seven *Baccharis* species investigated in Brazil four turned out to contain spathulenol as one of the main components of their essential oils (181). Spathulenol is an important sesquiterpenoid in the volatiles of our samples, as well. Parallel analyses of propolis and leaf exudates from *Baccharis* species growing in the vicinity of the hives have proved that the source plant in Sao Paolo State is *Baccharis dracunculifolia* (191).

The discovery of lignans can give information about the origin of propolis from the Canary Islands. The source has to be a plant species producing resinous exudates rich in lignans of the furofuran type. According to the data

obtained there could be a second plant source from which most of the sugars, besides glucose and fructose, originate.

Recently, based on chemical composition (comparison of polyisoprenylated benzophenones), Cuban propolis was found to originate from the floral resin of *Clusia* species (15)

The knowledge about plant sources of propolis is not only of academic interest. As already mentioned, it could be useful as a basis for its chemical standardisation. Furthermore, it is important to beekeepers to be sure that their bees have the proper plants in their flight range. It is known that colonies suffer when they cannot collect propolis, bees are even said to use "propolis substituents" like paints, asphalt and mineral oils which could severely threaten pharmaceutical uses of bee glue (76).

6. Conclusions

The results obtained from the analysis of all propolis samples in the present study (most of them already published, see the Appendix) have led to the following principal conclusions:

1. A method was developed based on capillary gas chromatography for quantification of the main phenolics in Bulgarian propolis. The analysis might be used for control and standardization purposes and was applied for quality control of a veterinary preparation produced by "Farmacia", Dupnitsa, Bulgaria.

2. A new method was developed for rapid qualitative analysis of the main phenolics in Bulgarian propolis based on capillary gas chromatography. The use of electron capture detector enables an analysis without preliminary derivatization of the phenolics.

3. The main components of propolis "balsam" (extract with 70% ethanol) from samples of different geographic origin were determined using GC-MS:

- In Egyptian propolis, 39 compounds were identified, 7 of them new for propolis. The Egyptian propolis is to some extent similar to the Bulgarian one but there are some differences, as well.

- In Brazilian propolis, gathered by honey bee *Apis mellifera* (4 samples), as well as by some indigenous stingless bee species (3 samples), 52 compounds were identified, 11 of them new for propolis. Even though there are

some substantial differences among them, these samples are completely different from the European type propolis.

- In propolis from the Canary Islands 40 compounds were identified, 26 new for propolis. Of special interest are the lignans of furofuran type, which were found for the first time in propolis. Two of these compounds turned out to be new natural products. Their tentative structures were proposed on the basis of mass-spectral data.

- In propolis from Canada (2 samples) 43 compounds were identified, 3 of them new for propolis.

4. For propolis from the Canary Islands four main lignans were isolated and fully characterized by NMR and mass spectrometry.

5. A new method was developed for studying propolis chemical composition based on MAB ionization MS. This ionization technique is applied for the first time in natural product chemistry and appeared to be highly beneficial for compound identification, structure elucidation and accurate mass measurements.

6. In volatile oils from propolis of different geographic regions the main components were identified using GC-MS. Samples from Bulgaria, Mongolia, Albania, Brazil, the Canary Islands were analyzed. Significant variations in the chemical composition were observed, related to the geographic origin of the sample. In different samples, 98 new compounds for propolis, mainly monoterpenes, were identified.

7. It was found that in spite of the great differences in the chemical composition of propolis from different geographic locations, all samples exhibit significant antibacterial and antifungal (and most of them antiviral) activity. Obviously, in different samples, different substance combinations are essential for the biological activity of bee glue.

8. The results obtained give some indication concerning the plant origin of the investigated samples:

- The Egyptian sample originates mainly from *Poplar* buds, but a second plant source, still unknown, has been involved, as well.

- One of the main sources of the bee glue in Brazil is the leaf exudates of different *Baccharis* species, the *Asteraceaen* shrubs widespread in South America.

- The plant source of propolis from the Canary Islands must be a local plant producing an exudate, rich in lignans of the 2,6-diaryl-3, 7-dioxabicyclo[3,3,0]octane.

- The plant source of one of the Canadian samples was determined as *P. trichocarpa* , Section *Tacamahaca*; while for the other sample the widespread in North America aspen *P. tremuloides* Michx., Section *Leuce*, subsection *Trepidae* is probably the original contributor.

9. The present study proves the striking variability of the chemical composition of propolis produced in tropical regions. This fact is obviously connected to the great diversity of the flora in these regions. It remains an open question whether a number of "local" standards, based on chemical analysis, could be formulated, e.g. "European", some kinds of "Brazilian", etc. To answer

this question, further investigations are needed, including systematic investigations of the chemistry of bee glue in greater regions.

In conclusion, we do believe that the adherence to the presented simple diagram (p. 56) may contribute to the eventual solution of the problems related to the future standardization and preferential applications of this valuable natural product.

The determination of the chemical composition of propolis and the subsequent quantification of its main biologically active compounds along with its main proven plant sources may give the basis for the development of a reliable standardization procedure. The specific relations among these elements in the diagram can further determine several distinct "regional" standards.

Furthermore, better knowledge of the chemically active characteristics of propolis will eventually enable classification of different reasonable applications such as in the pharmaceutical industry, bio-cosmetics, "health food" supplements, etc.

We also believe that the existing powerful analytical methods used in this work, supplemented by the analytical potential of the MAB ionization, are real proofs that further method developments are highly feasible. They will help to achieve our main goal, fast and precise determination of the complex and variable chemical composition of propolis originating from diverse geographic locations.

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