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(54) **Title:** PROCESS FOR THE PREPARATION OF SOUR CHERRY SEED EXTRACT, USE OF THE EXTRACT FOR THE PREPARATION OF PHARMACEUTICAL COMPOSITIONS AND PHARMACEUTICAL COMPOSITIONS CONTAINING SAID EXTRACT

(57) **Abstract:** The present invention relates to a process for the isolation of the components of seed of Primus cerasus (sour cherry), the components thus obtained, pharmaceutical compositions containing said components as well as the use of the components for the preparation of cardioprotective pharmaceutical compositions. The components according to the invention are especially useful for improving circulation, preventing stenosis or ameliorating ischemia-induced myocardial damages.

Process for the preparation of sour cherry seed extract, use of the extract for the preparation of pharmaceutical compositions and pharmaceutical compositions containing said extract

Technical field

The present invention relates to a process for the isolation of the components of seed of Prunus cerasus (sour cherry), the components thus obtained, pharmaceutical compositions containing said components as well as the use of the components for the preparation of cardioprotective pharmaceutical compositions.

Background art

Disorders of the cardiovascular circulation are major causes of morbidity and mortality and can result in life-long disabilities in survivors. For the 13 million people worldwide affected by heart failure and nearly 1,000 individuals succumb to sudden cardiac death in the US each day as a result of fatal ventricular arrhythmias (Pearson, 2004; Ackerman, 2004). Most of sudden deaths claim middle-aged and elderly populations. Therefore, the high morbidity and mortality of cardiovascular diseases have focused the attention of physicians and clinicians on restoring coronary blood flow to resuscitate the ischemic or hypoxic myocardium. The appropriate pharmacological interventions and therapy can facilitate the salvage of myocardium, improve cardiac function, and decrease cardiac morbidity and mortality.

According to the above there is a need for active substances, Particularly there is a need for active substances of natural origin.

Disclosure of the invention

According to the above, the aim of the present invention is to prepare active substances - possibly of natural origin - which successfully prevent, improve or reverse the above disorders and conditions.

According to the present invention the aim is achieved by obtaining said active substances from the components of sour cherry seed.

Thus, the present invention relates to a process for the preparation and isolation of the components of sour cherry seed, and the components thus obtained.

The invention relates further to the use of said components for the preparation cardioprotective pharmaceutical compositions.

The invention also relates to pharmaceutical compositions comprising the components prepared according to the process of the invention.

There are several prior art documents discussing the use of various components of sour cherry, however, no document can be found which discloses the isolation of the components of sour cherry seed, or suggest the use of the same for the treatment and/or prevention of cardiac disorders.

Surprisingly, we found that the sour cherry seed extract exerts cardioprotective activity in various biological samples. As an outstanding result, the tests sowed that the extract used do not involves any side effects.

According to the present invention, the process of the invention, after removing the wall of seed, leads to Fraction I (oil fraction) and Fraction II (solid phase) of Prunus cerasus (sour cherry) seed. The steps of separation are depicted in Figure 25.

The invention features cardioprotective effects with no adverse effects of sour cherry (Prunus cerasus) seed extract in biological samples.

The sour cherry seed contains two main fractions:

Fraction I: The sour cherry seed contains 35% of oil fraction (O) including vitamin E (alpha-tocopherol, 52 mg/100g), vitamin E-like components (delta-tocopherol, tocotrienol), unsaturated free fatty acid esters (hexa-, hepta-, and octadecane acids, aldehide (e.g., hexanal), mixtures of triglycerids including free fatty acids LLL (L: linoleic acid) LLO (O: oleic acid), LLP (P: palmitil acid). The total tocopherol content of the O fraction of sour cherry seed is about 90 mg/100g. The O fraction does not contain flavonoids, polyphenols, and cyanide components in comparison with the Fraction II (see below).

Fraction II: the solid (S) fraction of sour cherry seed includes flavonoids, rhamnetin, malvidin, delfinidin, pinocembrin, naringenin, quercetin, rezveratrol, dihydroquercetin, peonidin, apigenin, pro- and athocyanidins, glucose (e.g., feruloil-D-glucose, cumaroil-glucose, feruloil-d-glucose), stilbenes, catechins, gallic acid, gallocatechins, and other atioxidants (e.g., gallotannin). The fraction II was divided in two parts (**fraction IIa** and **fraction IIb**) according to the extraction procedure of sour cherry seeds. Thus, fraction IIa was obtained with the extraction of 70% of methanol, and fraction IIb was the product of seed extraction using methanol and hydrochloric acid mixture (9:1).

Results: Analysis of sour cherry (Prunus Cerasus) seed

Figure 1, Figure 2, and Figure 3 show the infra red (IR) spectra of O fraction. Figure 1 shows a typical unsaturated fatty acid ester component at 3020 cm^{-1}. An ester group (=O) can be detected at 1742 cm^{-1} of the spectra. Between 2500 and 2800 cm^{-1}, OH^{-} group peaks are detected indicating the components of free carbonyl acids. The long carbonyl chain components can bee seen at the ranges between 1460 and 720 cm^{-1}, and 3000 and 2800 cm^{-1}

The spectra of O of sour cherry seed was compared to the sunflower's oil (Figure 3) and many similarities were found. However, the major difference between the O fraction of the sour cherry seed and sunflower's oil is in the content of free fatty acids. Thus, free fatty acids can not be found (or in a very little amount) in the sunflower's oil. However, the O fraction of the sour cherry seed contains a relatively high amount of free fatty acids detected between 970 and 930 cm^{-1}.

The IR spectra of fractions IIa and IIb could be seen in Figure 4 and Figure 5. It is well shown that fraction IIa contains ester components indicating by the peek at 1666 cm^{-1} (carbonyl component). The peaks of IR spectra, at 3400 and 1050 cm^{-1}, indicates a substantial numbers of hydroxyl groups. The fraction IIb does not contain ester components, and this is the so called flavonoid-fraction. This is proven by the UV spectra in Figure 6 showing the peaks at 330 nm and 275 nm, respectively. The UV absorbance spectra of the fraction IIb at 430 nm (Figure 7) indicates the presence of anthocyan and proanthocyanidin components which is proven by the red color of the extract.

Figure 8 shows the gas chromatogram (GC) of O fraction in comparison with the sunflower (Figure 9) chromatogram. The O fraction of sour cherry seed extract, beside the main components, contains many minor components (Figure 8) in comparison with the analysis of sunflower oil (Figure 9). The fraction IIb (solid fraction) also contains volatile components (Figure 10). Figure 11 shows the GC results in detail obtained from Figure 10. The O fraction does not contain organic-cyanide components, however, fraction IIa contains cyanide components like amygdaline. Polyphenols and flavonoids cannot be detected in O fraction. These components (polyphenols and flavonoids) are detected in fractions IIa and IIb. The so-called Folin-Ciocelteau method indicates that fraction IIb has gallic acid-like components about 205.6 mg gallic acid components (polyphenols) in 100 g sour cherry seed extract.

The free radical scavenger activity of each sample (O, fraction IIa and IIb) was studied by galvinoxyl radical method. The results show that the O fraction and fraction IIb possess free radical scavenger activities. The use of galvinoxyl technique (UV study) indicates (Figure 12) that fraction IIb in the presence of alcohol showed UV absorption, directly supporting that fraction IIb contains flavonoids.

Figures 13-17 show the analysis of Prunus cerasus seed by mass spectroscopy (MS). Thus, the fraction IIb (Figure 13) contains dihydro-p-cumaric acid indicating by the peak at 185 m/z (M+1), ferrulic acid at 213 m/z (M+1) and, and this latter peak is overlapped by the peek of coffee acid at 213 m/z (M+1) as well. Major components of fraction II are cyanidin at 287 m/z and peonidin at 301 m/z. The peak at 301 m/z (M-1) proofs the presence of quercetin in fraction IIb. Furthermore, there is a peak of dimmer cyanidin (procyanidin) at 577 m/z giving a light/red color of the extract. The typical flavon components are pinocembrin at 257 m/z M+H), and tangeretin at 371 m/z, respectively. The peaks between 425 and 525 m/z suggest the presence of vitamin E-like compounds in fraction IIb.

The use of MALDI-TOF spectra analysis shows some high molecular weight components. Thus, the peak at 487 m/z indicates the presence of quercetin-3-glucosid M+Na) in fraction IIa (Figure 14). Furthermore, the peak at 820 m/z (M+H_2O) is corresponding with the chlorogen acid-related acetylated quercetin-3-glucosid compound (Figure 14). The peak appeared at 1141 m/z shows the presence of gallic acid-related acetylated procyanidin trimer (M+Na) (Figure 15). The peak of epicatechin-3-gallate dimer form is appeared at 859 m/z (Figure 15). Acetylated form of catechin-3-glucoside by cumarin acid is detected at 685 m/z (Figure 16). Figure 17 shows the presence of galangin acetylated by p-cumarin acid at 685 m/z. Total flavonoid concentrations of fractions IIa and IIb are about 2%.

GC-MS studies:

Cromatograms were obtained by total ion chromatography. Figures 18 and 19 show that 'O' fraction consists of mainly triglycerides including linoleic acid (LA), oleic acid (OL). However, a small amount of palmitil acid and stearin acid was also detected. Thus, the 'O' fraction contains mainly unsaturated triglyceride components. Beside triglycerides, free fatty acids such as ω-3 α-linoleic acid, hexa-, hepta-, octadecanoic acids, and aldehydes (e.g., hexanal and decadienal) can also be detected in the 'O' fraction (Figure 20). The most important components of the 'O' fraction are vitamin E and its isomers (Figures 21-24). Thus, δ-tocopherol (Figure 21), α-tocopherol (Figures 22 and 23), and δ-tocotrienol (Figure 24) are the major components. The α-tocopherol content is 52-53 mg/100g, while the total tocopherol content is 80-85 mg/100g.

Pharmacological effects of the sour cherry (Prunus cerasus) seed extract (fractions I and II)

Methods:

(i) Isolated rat and mouse heart preparations:

Male Sprague-Dawley rats (320-350 g) were used for all studies. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research prepared by the National Academy of Sciences (Publication No. 86-23, revised 1985). Rats were anesthetized with i.p. pentobarbital (60 mg/kg) and then given intravenous heparin (500 IU/kg). After thoracotomy, the heart was excised, and the aorta and left atrium were cannulated. Hearts were initially perfused according to Langendorff then preparations were switched to the working mode as previously described (Tosaki and Braquet, 1990). The isolated mouse heart preparation was carried out as described by Bak et al (2003).

(ii) Experimental time course and idices measured:

Before the onset of ischemia and reperfusion, and the isolation of hearts, rats and mice were treated orally with various doses (1 mg/kg/day, 5 mg/kg/day, 10 mg/kg/day, and 30 mg/kg/day) of the sour cherry seed extract (the components of fraction IIa and fraction IIb), respectively, for 14 days.

The extract of sour cherry seed (fractions IIa and IIb) was homogenized in 2 ml of 1% methylcellulose solution and then diluted with 0.9% of NaCl to 10 ml. Rats were orally treated daily with 10 ml/kg of the solution (containing 1 mg/kg, 5 mg/kg, 10 mg/kg or 30 mg/kg of flavonoid-rich extract, fractions IIa and IIb together) for 14 days, and no changes in the behavior and physical activities of animals were observed during the treatment. After 14 days pretreatment, hearts were isolated and subjected to 30 min of ischemia followed by two hours of reperfusion.

An epicardial ECG was recorded by a computer acquisition system throughout the experimental period by two silver electrodes attached directly to the heart. The ECGs were analyzed to determine the incidence of VF and VT. Hearts were considered to be in VF if an irregular undulating baseline was apparent on the ECG. VT was defined as five or more consecutive premature ventricular complexes, and this classification included repetitive monomorphic VT which is difficult to dissociate from rapid VT. The heart was considered to be in sinus rhythm if normal sinus complexes occurring in a regular rhythm were apparent on the ECG. Aortic flow was measured by an in-line flow rotameter. Coronary flow rate was measured by a timed collection of the coronary effluent that dripped from the heart. Before ischemia and during reperfusion, heart rate (HR), coronary flow (CF) and aortic flow (AF) rates were registered. Left ventricular developed pressure (LVDP) was also recorded by the insertion of a catheter into the left ventricle via the left atrium and mitral valve. The hemodynamic parameters were registered by computer acquisition system (PouwerLab, ADInstruments, Australia).

Determination of infarct size:

Hearts for infarct size measurement were perfused, at the end of each experiment, with 25 ml of 1 % triphenyl tetrazolium solution in phosphate buffer (Na_2HPO_4 88 mM, NaH_2PO_4 1.8 mM) via the side arm of the aortic cannula, then stored at -70 °C for later analysis. Frozen hearts were sliced transversely (Schultz et al., 1997) in a plane perpendicular to the apico-basal axis into 2-3 mm thick sections, weighted, blotted dry, placed in between microscope slides and scanned on a Hewlett-Packard Scanjet 5p single pass flat bed scanner (Hewlett-Packard, Palo Alto, CA). Using the NIH Image 1.61 image processing software, each digitalized image was subjected to equivalent degrees of background subtraction, brightness and contrast enhancement for improved clarity. Infarct zones of each slice were traced and the respective areas were calculated in terms of pixels (Dickson et al., 2001). The areas were measured by computerized planimetry software and these areas were multiplied by the weight of each slice, then the results summed up to obtain the weight of the risk zone (total weight of the left ventricle, mg) and the infarct zone (mg). Infarct size was expressed as the ratio, in percent, of the infarct zone to the risk zone.

Measurement of caspase III activity by immunocytochemistry:

The free-floating sections of the heart were first incubated with biotinylated goat anti-caspase-3 antibody (Sigma, St. Louis, MO, USA; diluted 1:1000) for 2 days at 4 °C. The immunological and immunocytochemical characteristics of antibody have been published earlier (Hatib-Al-Khatib et al., 2004). The sections were then transferred into a solution of biotinylated goat antirabbit (Vector Laboratories, Burlingame, CA, USA; diluted 1:200) for 50 min at room temperature, than avidin-biotinylated-peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA; diluted 1:100) for 4 h at room temperature, and was completed with a diaminobenzidine chromogen reaction (Hancock, 1984). Prior to the antibody treatments sections were kept in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 50 min. All incubations were performed under continuous gentle agitation, and all of antibodies were diluted in 10mM phosphate-buffered saline (PBS, pH 7.4) to which 0.1% Triton X-100 and 1% normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) were added. Sections were mounted on gelatin-coated slides and covered with Permount neutral medium (Fluka, Buchs, Switzerland).

Statistics:

The data for HR, CF, AF, LVDP, caspase-3 activity, and infarct size were expressed as the mean ± SEM. One-way analysis of variance test was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of sour cherry seed extract (fractions IIa and IIb together) treated groups were compared with those of the drug-free control group by multiple t-test followed by Bonferroni correction. For the distribution of discrete variables such as the incidence of VF and VT which follows a nonparametric distribution, an overall chi-square test for a 2xn table was constructed followed by a sequence of 2x2 chi-square tests to compare individual groups. A change of $p<0.05$ between the drug-free control and treated groups was considered to be significant.

RESULTS (pharmacological studies):

Figure 26 shows the representative picture of Prunus cerasus (sour cherry) seed extract (10 mg and 30 mg/kg) on infarct size limitation in isolated rat hearts subjected to 30 min of ischemia followed by 120 min of reperfusion. White areas represent infracted areas. Figure 26A shows infarct size in the drug-free ischemic/reperfused myocardium, and Figure 26B and Figure 26C show infarct size in hearts treated with 10 mg and 30 mg/kg of sour cherry seed extract (fractions IIa and IIb together), respectively.

Table 1 (below) shows the numerical (in each heart) values of infarct size in hearts (n=6 in each group) obtained from rats treated with various doses of sour cherry seed extract (fractions IIa and IIb together) for 14 days, and subjected to 30 min of ischemia followed by 120 min of reperfusion. The incidence of VF and VT were also detected (n=12 in each group). Comparisons were made to the values of the drug-free ischemic/reperfused control group. * $p < 0.05$. Thus, in hearts treated with 10 mg/kg and 30 mg/kg of sour cherry seed extract, a significant reduction in the infarct size, the incidence (%) of VF, and the incidence (%) of VT were reduced from their drug-free control values of 38.3% ± 1.3% (infarct size), 93% (VF), and 100% (VT) to 26.5% ± 2% (infarct size, *p<0.05, 10 mg/kg sour cherry) and 21.8% ± 1.8% (infarct size, *p<0.05, 30 mg/kg sour cherry), 50% (VF, 10 mg/kg sour cherry) and 17% (VF, 30 mg/kg sour cherry, *p<0.05), and 58% (VT, 10 mg/kg sour cherry) and 25% (VT, 30 mg/kg sour cherry, *p<0.05), respectively.

Table 1A, B, C. Effect of sour cherry seed extract (fractions IIa and IIb together) on infarct size, and incidence (%) of VT and VF. Each individual value is shown, and comparisons were made to the values of the drug-free control (Table 1A) group.

A). Drug-free control group: rats were orally treated with vehicle for 14 days then hearts were isolated and subjected to 30 min ischemia followed by 120 min reperfusion.

No. of Hearts	Infarct size (%)	Incidence (%) of VF	Incidence (%) of VT
1.	40	+	+
2.	34	-	+
3.	44	+	+
4.	38	+	+
5.	35	+	+
6.	39	+	+
7.		+	+
8.		+	+
9.		+	+
10.		+	+
11.		+	+
12.		+	+
mean	38.3	93%	100%
SD	3.3		
SE	1.3		

B). Rats were orally treated with 10 mg/kg of sour cherry seed extract (fractions IIa and IIb together) for 14 days then hearts were isolated and subjected to 30 min ischemia followed by 120 min reperfusion.

No. of Hearts	Infarct size (%)	Incidence (%) of VF	Incidence (%) of VT
1.	28	+	+
2.	22	-	+
3.	30	+	+
4.	35	+	+
5.	21	+	+
6.	23	-	-
7.		-	-
8.		-	-
9.		-	-

10.		+	+
11.		+	+
12.		-	-
mean	**26.5***	50%	58%
SD	**5.0**		
SE	**2.0**		

C). Rats were orally treated with 30 mg/kg of sour cherry seed extract (fractions IIa and IIb together) for 14 days then hearts were isolated and subjected to 30 min ischemia followed by 120 min reperfusion.

No. of Hearts	Infarct size (%)	Incidence (%) of VF	Incidence (%) of VT
1.	23	+	+
2.	28	-	-
3.	19	-	-
4.	18	-	-
5.	16	+	+
6.	27	-	-
7.		-	-
8.		-	-
9.		-	-
10.		-	+
11.		-	-
12.		-	-
mean	**21.8***	**17%***	**25%***
SD	**4.5**		
SE	**1.8**		

Figure 27 shows caspase activities (caspase III) in hearts subjected to ischemia/reperfusion and obtained from rats treated with sour cherry seed extract (fraction II) for 14 days. Caspase activity, using immunohistochemistry, was reduced in treated subjects indicating by a reduction in brown color intensity. A: nonischemic aerobically perfused heart; B: drug-free heart subjected to 30 min ischemia followed by 120 min of reperfusion; C and D: rats were treated with 10 mg/kg and 30 mg/kg of sour cherry seed extract (fractions IIa and IIb together) for 14 days, respectively, than hearts were subjected to 30 min ischemia followed by 120 min reperfusion.

The reduction in the infarct size (Fig. 1 and Table 1), the incidence of VF and VT (Table 1), and the caspase activities (Fig. 27) reflected in the "dose-response" postischenic recovery of cardiac function including CF, AF, and LVDP. Thus, lower concentrations (1 mg/kg and 5 mg/kg) of sour cherry seed extract (fractions IIa and IIb together) failed to significantly improve postischemic cardiac function (Table 2b and Table 2c) in comparison with the drug-free control values (Table 2a). However, the higher doses of sour cherry seed extract (10 mg/kg and 30 mg/kg) significantly improved postischemic recovery in CF, AF, and LVDP (Table 2d and Table 2e). These tables (Table 2a to Table 2e), beside the mean, SD, and SEM, show the individual values of HR, CF, AF, an LVDP in each heart, in each untreated and treated group

IN SUMMARY, the patent includes the pharmacological effects of the composition of the following components of 'O' phase and solid fraction (fraction II):

The oil (O) phase for ointment production in order to improve vascular circulation and prevention of arteriosclerosis. The following components of sour cherry seed extract ('O' fraction) are patented: unsaturated triglyceride components, free fatty acids such as ω-3 α-linoleic acid, hexa-, hepta-, octadecanoic acids, and aldehydes (e.g., hexanal and decadienal), and vitamin E and its isomers (δ-tocopherol, α-tocopherol, and δ-tocotrienol). The α-tocopherol content is 52-53 mg/100g, while the total tocopherol content is about 80-85 mg/100g. It is also possible (at the moment no evidence) that some stable prostaglandin derivatives are also responsible for the protective effects of the 'O' phase.

The solid phase (phase II) for capsule or tablet production in order to improve vascular circulation and improve ischemia-induced damage in the myocardium. The following components of sour cherry seed extract (fraction II), as major components are patented:

Rhamnetin, malvidin, delfinidin, pinocembrin, naringenin, quercetin, rezveratrol, kaempherol, dihydroquercetin, peonidin, apigenin, pro- and athocyanidins, stilbenes, catechins, gallic acid, gallocatechins, and other atioxidants (e.g., gallotannin).

Table 2a. Cardiac function before ISA and after RE in control ischemic/reperfused rat hearts.

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
1	310	31,0	44	17,8	280	17	8	9,6	290	19	7	11	285	18	8	9,7
2	290	24,0	51	18,7	270	19	14	11	275	18	12	11,3	270	19	11	11,1
3	340	23	48	16,6	310	14	10	8,4	285	17	9	9,5	280	16	7	8,9
4	310	28,0	57	18,2	265	20	11	12	270	21	11	10,5	270	19	13	10
5	300	27	50	17,9	295	16	7	9,2	290	17	8	9,7	290	17	9	9,9
6	285	26	52	17,2	280	15	9	9,6	275	15	10	10,0	280	16	11	8,5

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
Mean	306	26,5	50,3	17,7	283	16,8	9,8	10,0	281	17,8	9,5	10,3	279	17,5	9,8	9,7
SD	20	2,6	3,9	0,7	17	2,1	2,3	1,2	9	1,9	1,7	0,7	7	1,3	2	0,8
SE	8	1,1	1,6	0,3	7	0,9	0,9	0,5	4	0,8	0,7	0,3	3	0,5	0,8	0,3

n=6 in each group; heart rate (HR) beats/min; coronary flow (CF) ml/min;

Aortic flow (AF) ml/min; left ventricular developed pressure (LVDP) kPa; ischemia (ISA); reperfusion (RE).

Table 2b. Cardiac function in sour cherry seed extract (fractions IIa and IIb together) treated myocardium, 14 days pretreatment with a daily dose of 1 mg/kg (rat).

No. of Heart	**Before ISA**				**After 30 min RE**				**After 60 min RE**				**After 120 min RE**			
	HR	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**
1	320	28	46	18,7	310	16	7	8,4	305	15	8	7,5	300	16	9	9,0
2	295	27	53	16,4	270	17	13	10,7	275	14	12	10,4	280	16	11	11,0
3	330	31	52	17,8	300	15	10	11,3	290	19	11	9,6	295	18	13	9,1
4	300	24	57	16,6	295	16	9	12,0	300	13	9	11,0	305	14	10	10,5
5	310	26	47	17,5	280	18	8	9,2	270	18	8	10,2	270	17	8	11,2

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**
6	290	29	49	18,0	295	17	12	10	285	17	11	9,8	275	18	12	10,3
Mean	308	27,5	51	17,5	292	16,5	9,8	10,3	288	16,0	9,8	9,8	288	16,5	10,5	10,2
SD	14	2,2	4	0,8	13	1,0	2,1	1,2	11	2,2	1,6	1,1	13	1,4	1,7	0,9
SE	6	0,9	1,5	0,3	5	0,4	0,9	0,5	5	0,9	0,6	0,4	5	0,6	0,7	0,3

n=6 in each group; heart rate (HR) beats/min; coronary flow (CF) ml/min; aortic flow (AF) ml/min; left ventricular developed pressure (LVDP) kPa; ischemia (ISA); reperfusion (RE).

Table 2c. Cardiac function in sour cherry seed extract (fractions IIa and IIb together) treated myocardium, 14 days pretreatment with a daily dose of 5 mg/kg (rat).

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**
1	290	27	56	17,3	260	19	10,0	10,5	250	20	11.0	10,5	260	20	10,0	11,00
2	340	32	54	18,4	310	20	9,0	12,4	300	19	9,0	11,0	290	17	8,5	11,8
3	320	24	48	16,5	295	16	8,5	13,0	300	17	9,0	10,0	320	18	9,0	9,6
4	300	23	49	17,2	280	15	11,9	9,5	290	16	12,9	9,4	280	17	12,4	10,8
5	315	27	51	17,6	300	17	10,9	10,8	300	18	12,0	11,9	300	18	11	12,4

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
6	330	28	52	17,0	305	18	11,8	11,7	295	18	11,5	11,3	300	19	12,0	10,7
Mean	321	26,8	51,7	17,3	292	17,5	10,4	11,3	289	18,0	10,9	10,7	292	18,2	10,5	11,1
SD	20	2,9	2,7	0,6	17	1,7	1,3	1,2	18	1,3	1,5	0,8	19	1,1	1,5	0,9
SE	8	1,2	1,1	0,2	7	0,7	0,5	0,5	7	0,5	0,6	0,3	8	0,4	0,6	0,4

n=6 in each group; heart rate (HR) beats/min; coronary flow (CF) ml/min;
Aortic flow (AF) ml/min; left ventricular developed pressure (LVDP) kPa; ischemia (ISA); reperfusion (RE).

Table 2d. Cardiac function in sour cherry seed extract (fractions IIa and IIb together) treated myocardium, 14 days pretreatment with a daily dose of 10 mg/kg (rat).

No. of Heart	**Before ISA**				**After 30 min RE**				**After 60 min RE**				**After 120 min RE**			
	HR	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**
1	295	28	46	18,2	270	22,0	14,0	11,5	280	24	18	11,7	290	25	19,0	11,2
2	330	24	55	16,4	300	18,0	26,0	13,6	310	19	25,0	13,2	310	19	24	13,0
3	300	33	52	17,6	260	27	21	11,4	270	27	24,0	12,5	300	26	25,0	12,5
4	320	27	48	17,3	310	22	20	14,8	320	24	27	14,6	310	23	22	14,0
5	315	26	53	16,0	300	21	30	12,8	290	22	21,0	13,5	285	21	22	13,5

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
6	320	29	51	17,9	290	23	15	14,7	295	23	17	14,7	285	22	18,0	14,1
						*	*	*		*	*	*		*	*	*
Mean	313	27,8	50,8	17,2	288	22,0	21,0	13,1	294	23,2	22,0	13,4	297	22,7	21,7	13,4
SD	12	2,8	3,0	0,8	18	2,7	5,7	1,4	17	2,4	3,7	1,1	11	2,4	2,5	1,1
SE	5	1,1	1,2	0,3	7	1,1	2,3	0,6	7	1	1,5	0,4	4	1	1	0,4

n=6 in each group; heart rate (HR) beats/min; coronary flow (CF) ml/min;

Aortic flow (AF) ml/min; left ventricular developed pressure (LVDP) kPa; ischemia (ISA); reperfusion (RE).

Table 2e. Cardiac function in sour cherry seed extract (fractions IIa and IIb together) treated myocardium, 14 days pretreatment with a daily dose of 30 mg/kg (rat).

No. of Heart	**Before ISA**				**After 30 min RE**				**After 60 min RE**				**After 120 min RE**			
	HR	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**
1	310	24	48	16,5	265	21	26,0	12,8	270	22	28	13,6	270	22	28,0	13,2
2	290	28	53	18,2	255	25	21,0	14,9	270	25	22	14,9	280	23	21	14,0
3	280	33	47	17,4	270	28	32	13,5	285	28	32,0	14,5	280	26	30,0	14,5
4	295	30	57	17,7	290	27	34	15,7	290	26	35	16,1	290	26	26	15,2
5	330	27	52	16,4	310	25	19	12,5	300	26	20,0	13,0	320	25	22	12,2

No. of Heart	Before ISA				After 30 min RE				After 60 min RE				After 120 min RE			
	HR	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**	**HR**	**CF**	**AF**	**LVDP**
6	320	28	53	17,5	310	23	24	15,1	305	27	26	15,8	310	27	25,0	15,1
						*	*	*		*	*	*		*	*	*
	304	28,3	51,7	17,3	283	24,8	26,0	14,1	287	25,7	27,2	14,7	292	24,8	25,3	14,0
SD	17	2,7	3,3	0,6	22	2,3	5,4	1,2	13	1,9	5,2	1,1	18	1,8	3,1	1,1
SE	7	1,1	1,4	0,3	9	1	2,2	0,5	4	0,8	2,1	0,5	7	0,7	1,3	0,4

n=6 in each group; heart rate (HR) beats/min; coronary flow (CF) ml/min;
aortic flow (AF) ml/min; left ventricular developed presure (LVDP) kPa; ischemia (ISA); reperfusion (RE).

The above results clearly show that the oil phase and solid phase of the sour cherry seed possess a high cardioprotective effect.

The oil phase is suitable for preparing ointments, preventing stenosis and improvement of circulation. The present invention encompasses the potential active ingredients selected from the group consisting of unsaturated triglyceride components; free fatty acids, e.g. ω-3 α-linolenic acid, hexa-, hepta and octadecanoic acid; and aldehydes (for example hexanal and decadienal), further vitamin E and its isomers (δ-tocopherol, α-tocopherol and δ-tocotrienol). The α-tocopherol content is 52-53 mg/100g sour cherry seed, while the whole tocopherol content is approximately 80-85 mg/100 g sour cherry seed. It is believed (not proven) that some stable prostaglandin derivative also contributes to the protective effect of the oil fraction of the sour cherry seed.

The solid phase is suitable for improving circulation and reducing ischemia-induced myocardial damages. The present invention encompasses the potential active ingredients selected from the group consisting of rhamnetin, malvidin, delfinidin, pinocembrin, naringenin, quercetin, rezveratrol, kaempherol, dihydroquercetin, peonidin, apigenin, pro- and athocianidines, stilbenes, catechines, gallic acid, gallocatechines and other antioxidants (for example gallotannin).

The solid phase can also be combined with Ca-channel blockers and beta-blockers for use in connection with the indications mentioned above. Such combinations are particularly advantageous, as lower doses are possible which contribute to avoid undesired side effects caused by Ca antagonists and beta blockers.

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CLAIMS

1. Process for the preparation of solid sour cherry seed extract comprising the steps of:

i) removing the wall of the seed and grinding the inner content of the seed

ii) extracting the dry grist substance of step i)

iii) drying and filtering the extract obtained in step ii)

iv) extracting the solid fraction obtained in step iii)

v) evaporating the extract obtained in step iv)

2. Process according to claim 1 wherein Soxhlett-extraction is carried out in step ii).

3. The process according to claim 2 wherein n-hexane is used as extracting agent.

4. Process according to claim 1 wherein Soxhlett-extraction is carried out in step iv).

5. The process according to claim 4 wherein 70% methanol is used as extracting agent.

6. The process according to claim 4 wherein methanol-hydrochcloric acid mixture at a ratio of 9:1 is used as extracting agent.

7. Process for the preparation of oily sour cherry seed extract comprising the steps of:

i) removing the wall of the seed and grinding the inner content of the seed

ii) extracting the dry grist substance of step i)

iii) filtering and evaporating the extract obtained in step ii)

8. Process according to claim 7 wherein Soxhlett-extraction is carried out in step ii).

9. The process according to claim 2 wherein n-hexane is used as extracting agent.

10. Pharmaceutical composition comprising the sour cherry seed extract according to claim 1 together with other pharmaceutical excipients commonly used.

11. The pharmaceutical composition according to claim 10 which is a tablet.

12. The pharmaceutical composition according to claim 10 which is a capsule.

13. Pharmaceutical composition comprising the sour cherry seed extract according to claim 7 together with other pharmaceutical excipients commonly used.

14. The pharmaceutical composition according to claim 10 which is an ointment.

15. Use of the sour cherry extracts of claims 1 or 7 for the preparation of pharmaceutical compositions having cardioprotective effect.

16. Use of the sour cherry extracts of claims 1 or 7 for the preparation of pharmaceutical compositions suitable for improving circulation, preventing stenosis or ameliorating ischemia-induced myocardial damages.

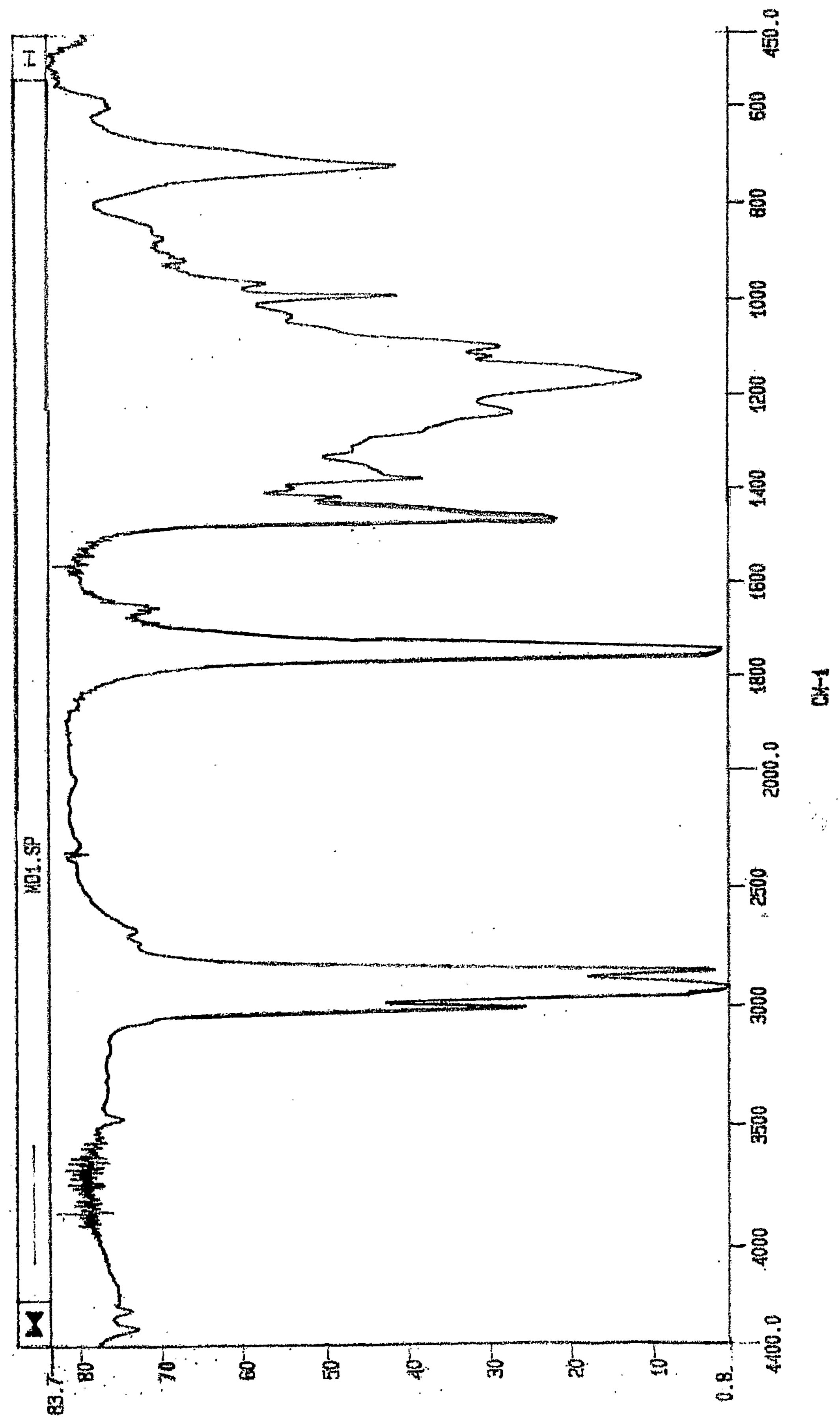

Figure 1.

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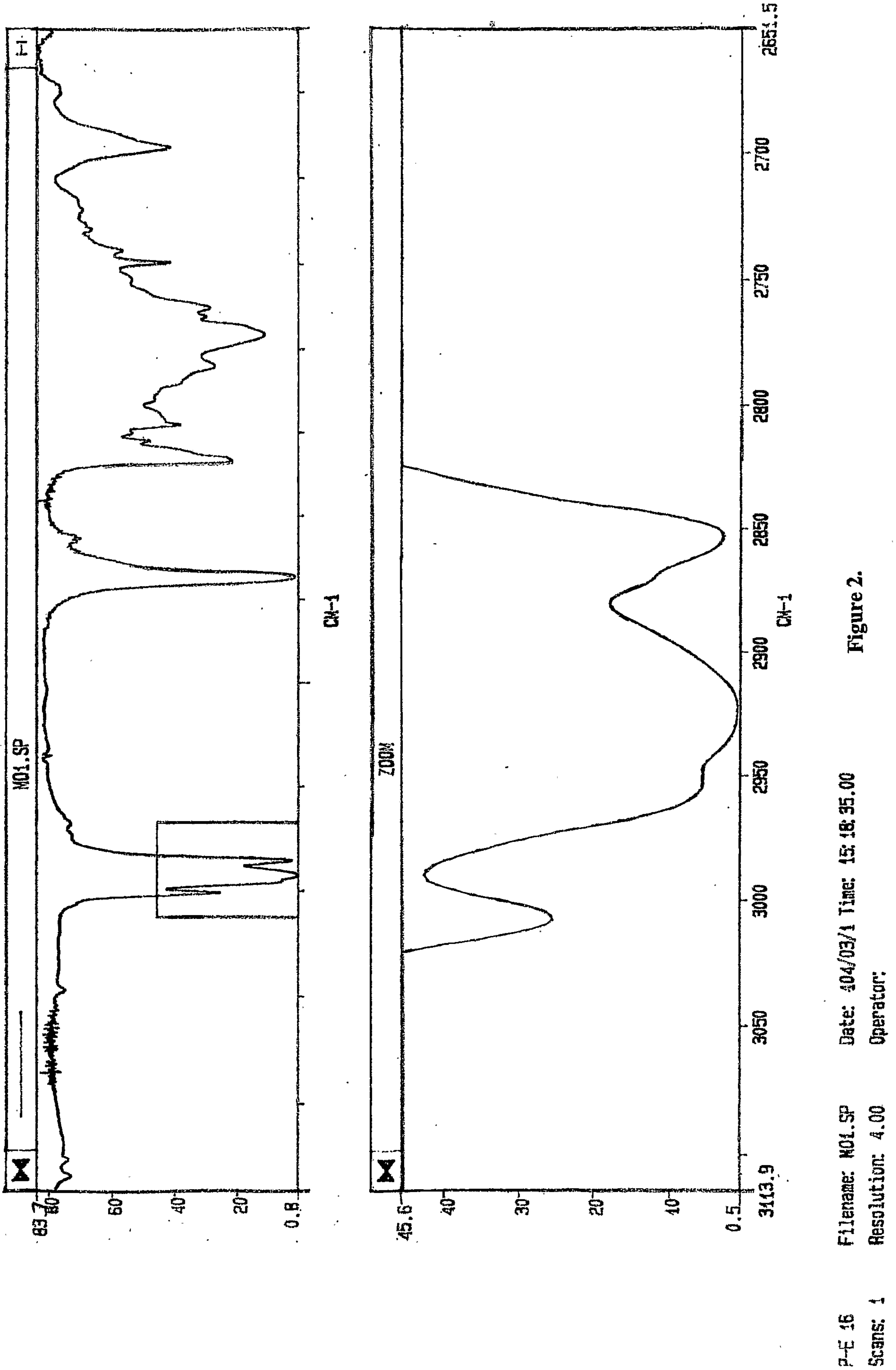
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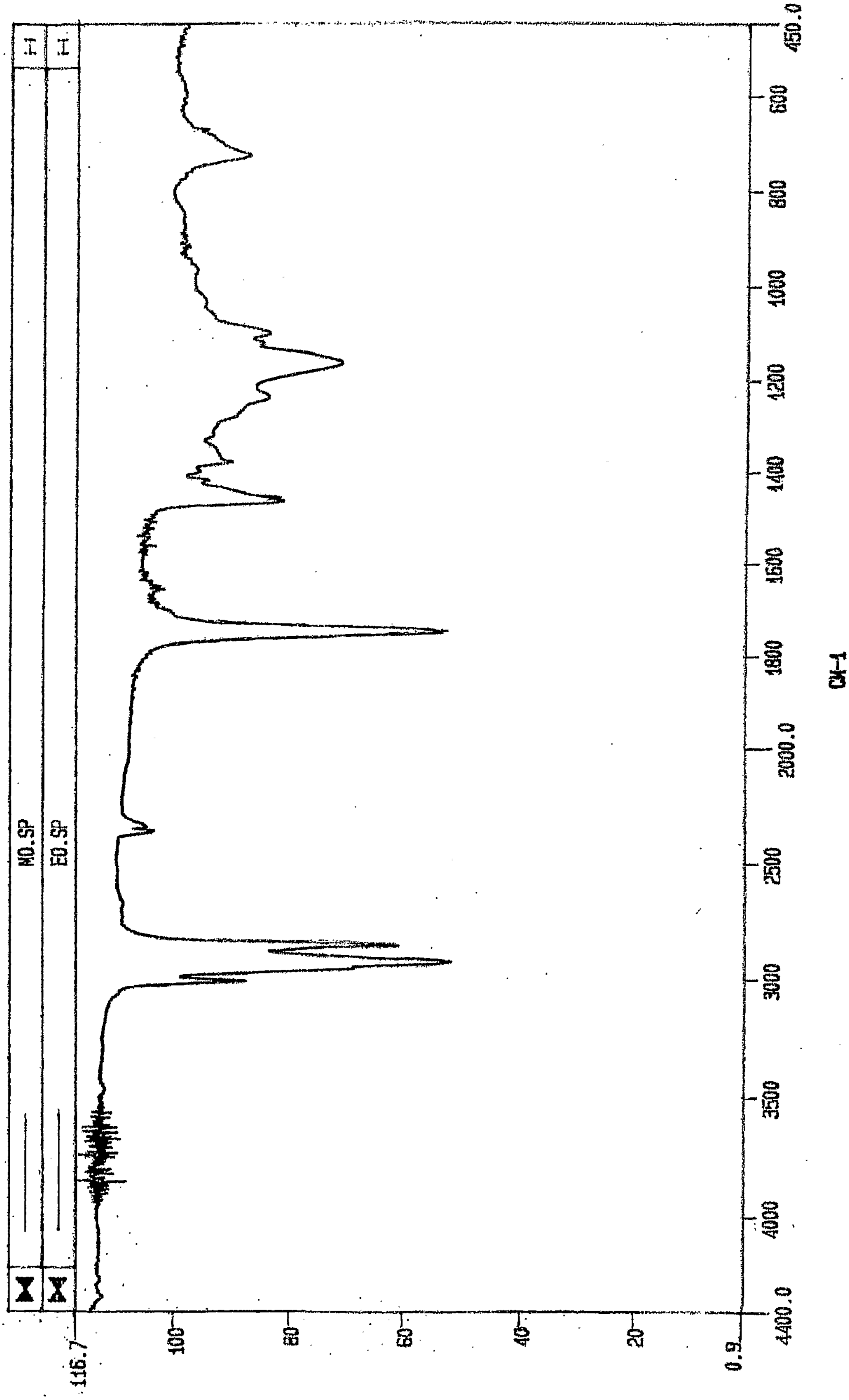
Scans: 1

Figure 2.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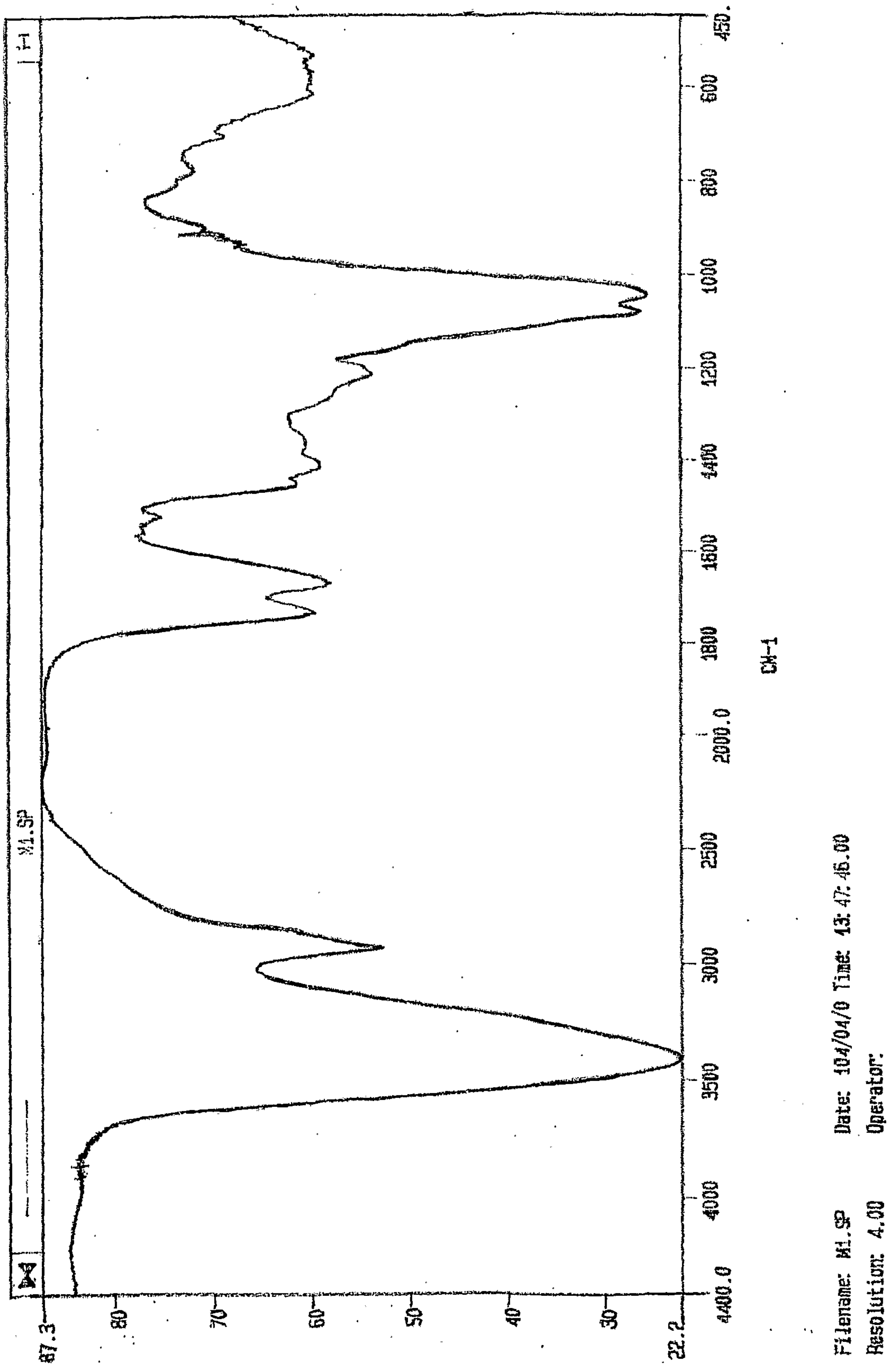
CM-1

Figure 3.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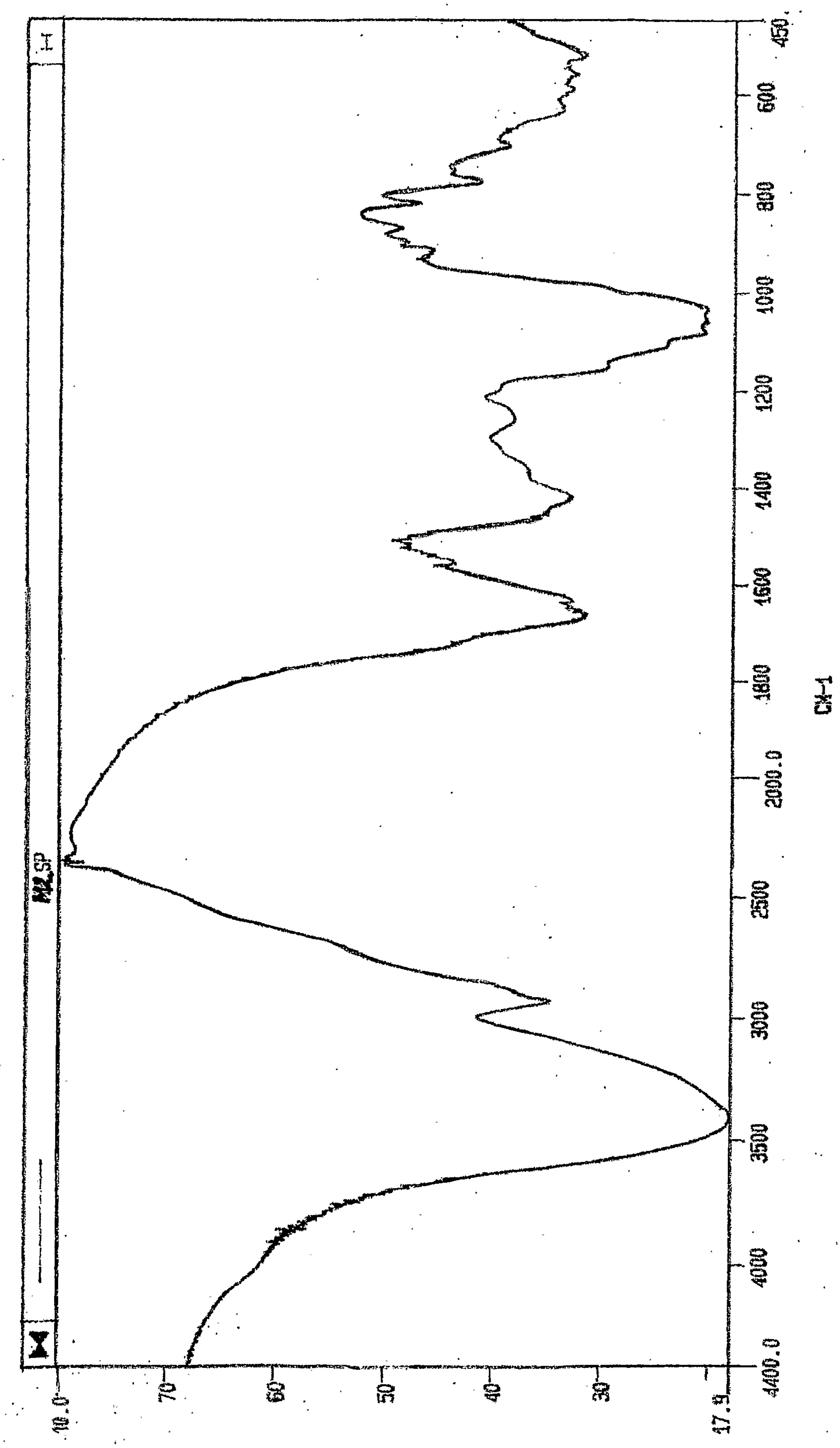
Operator:

Figure 4.



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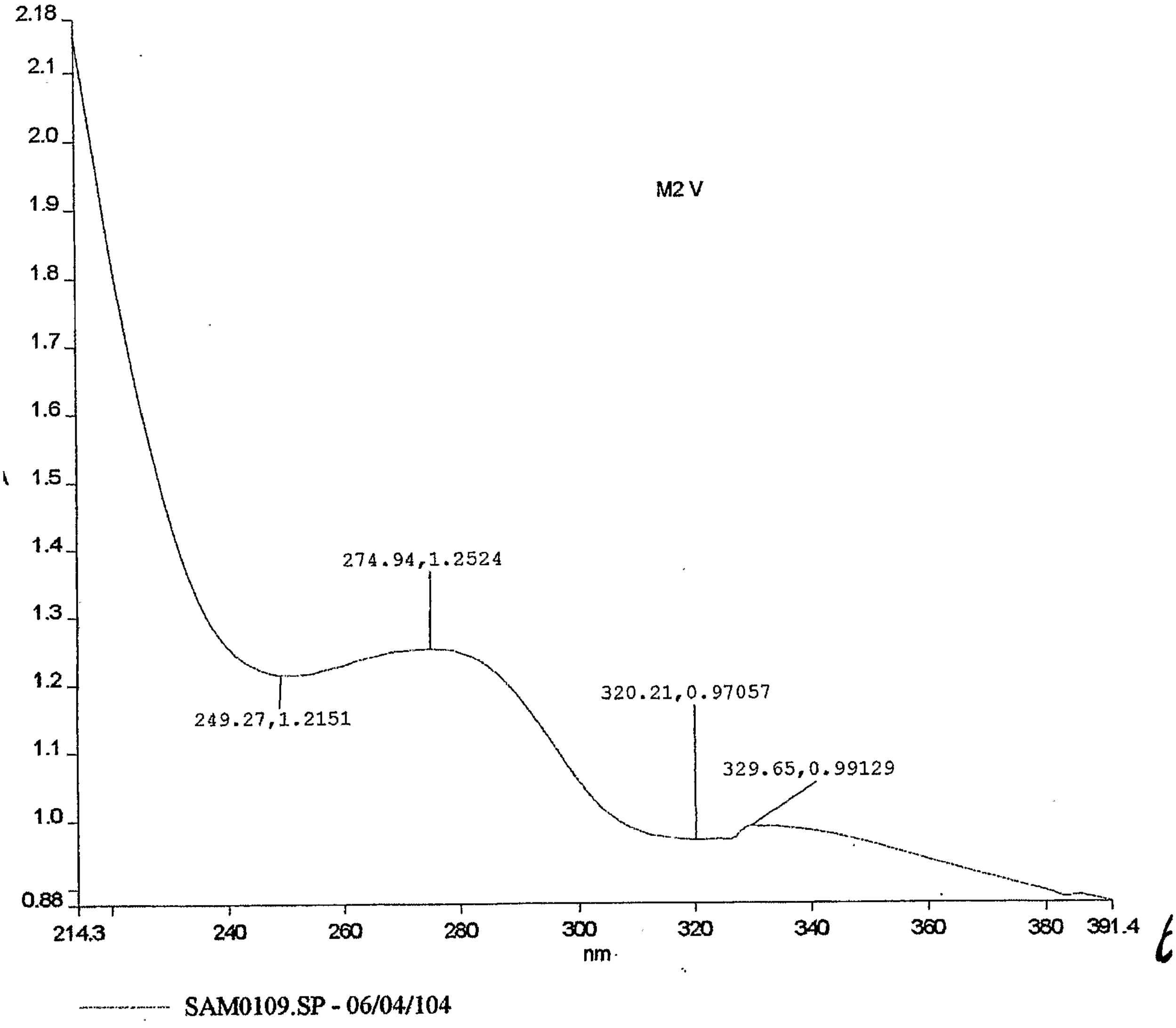
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1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
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280
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320
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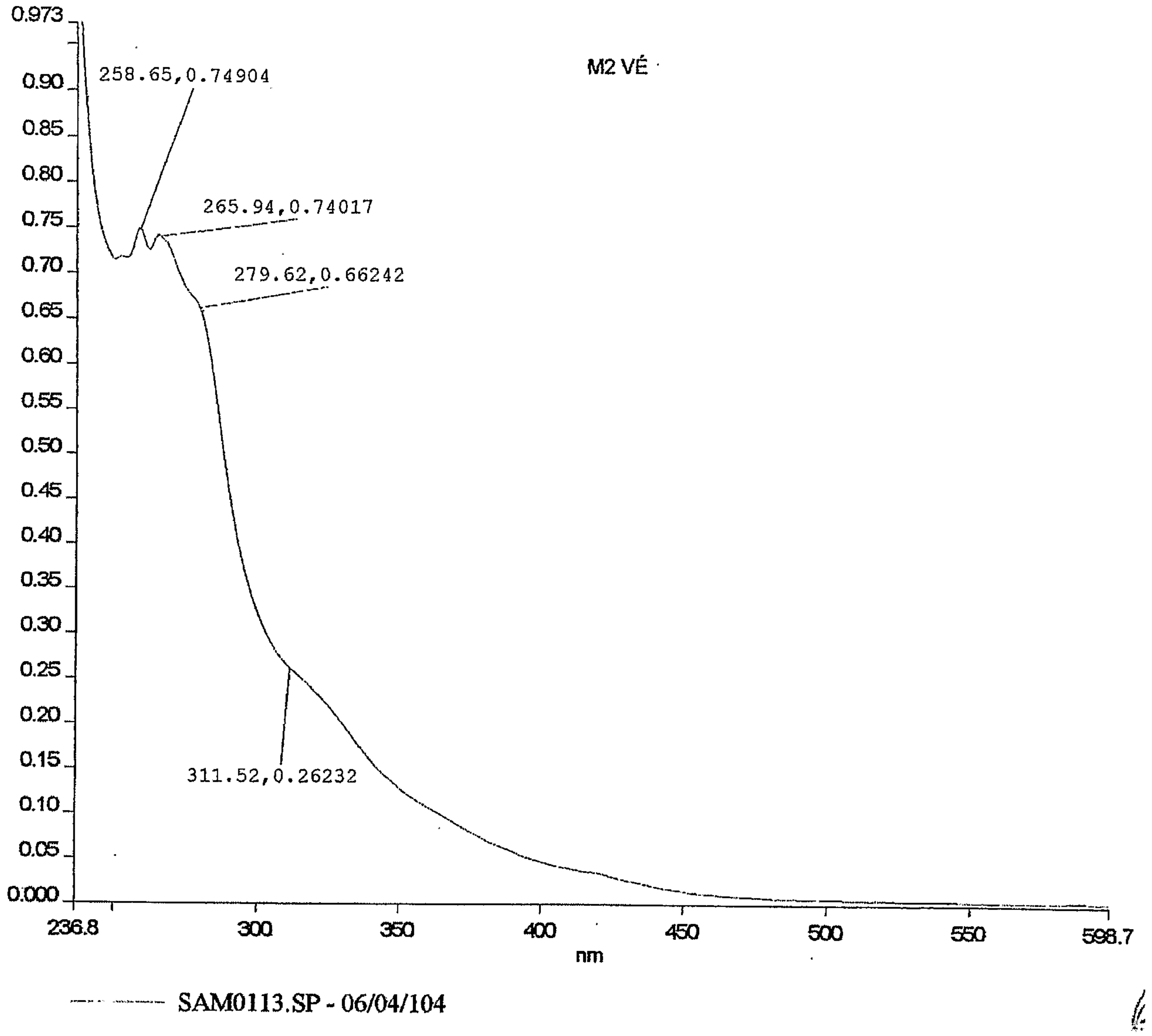
360
380
391.4
nm
SAM0109.SP - 06/04/104

Figure 6.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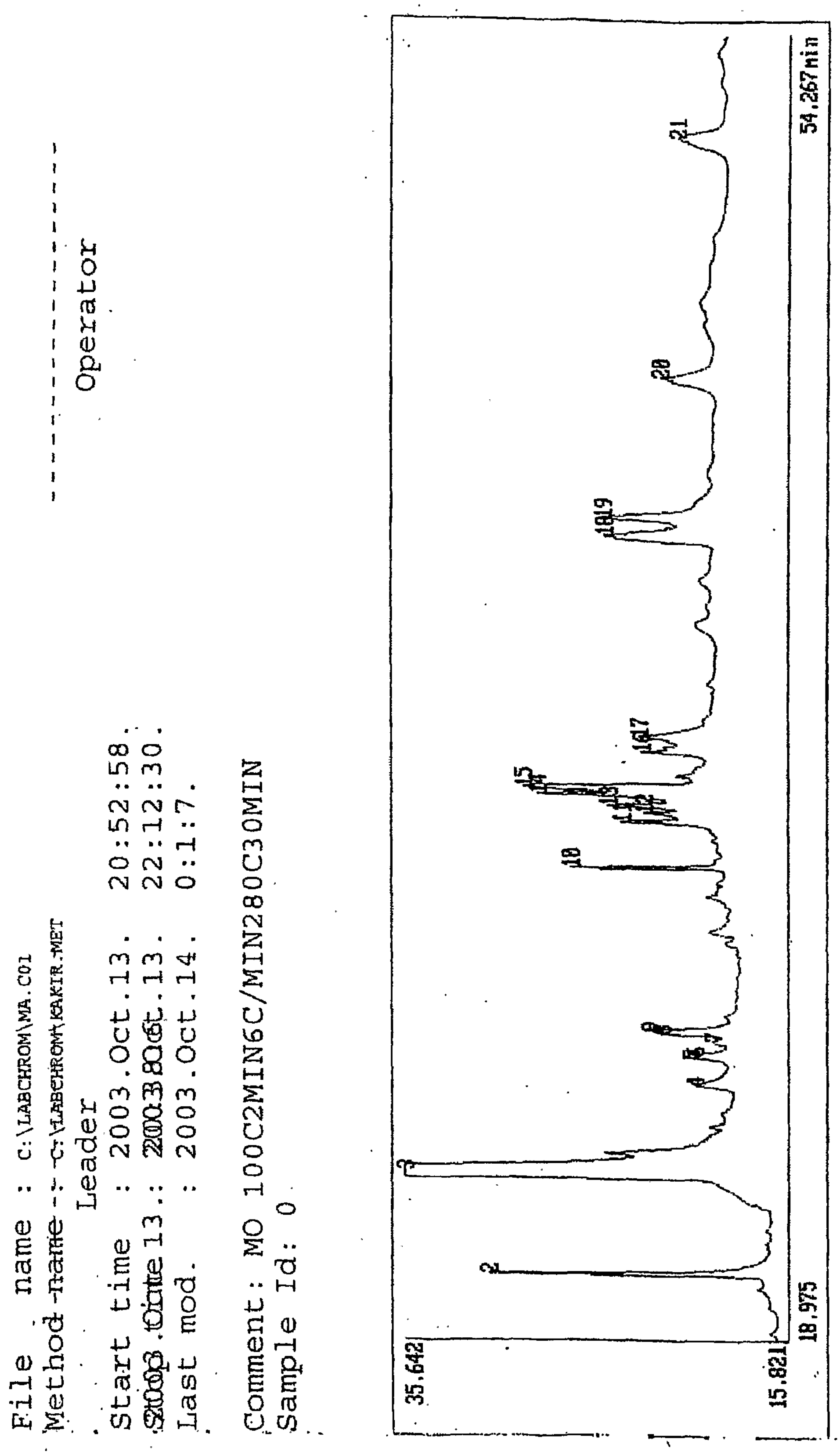
SAM0113.SP - 06/04/104

Figure 7.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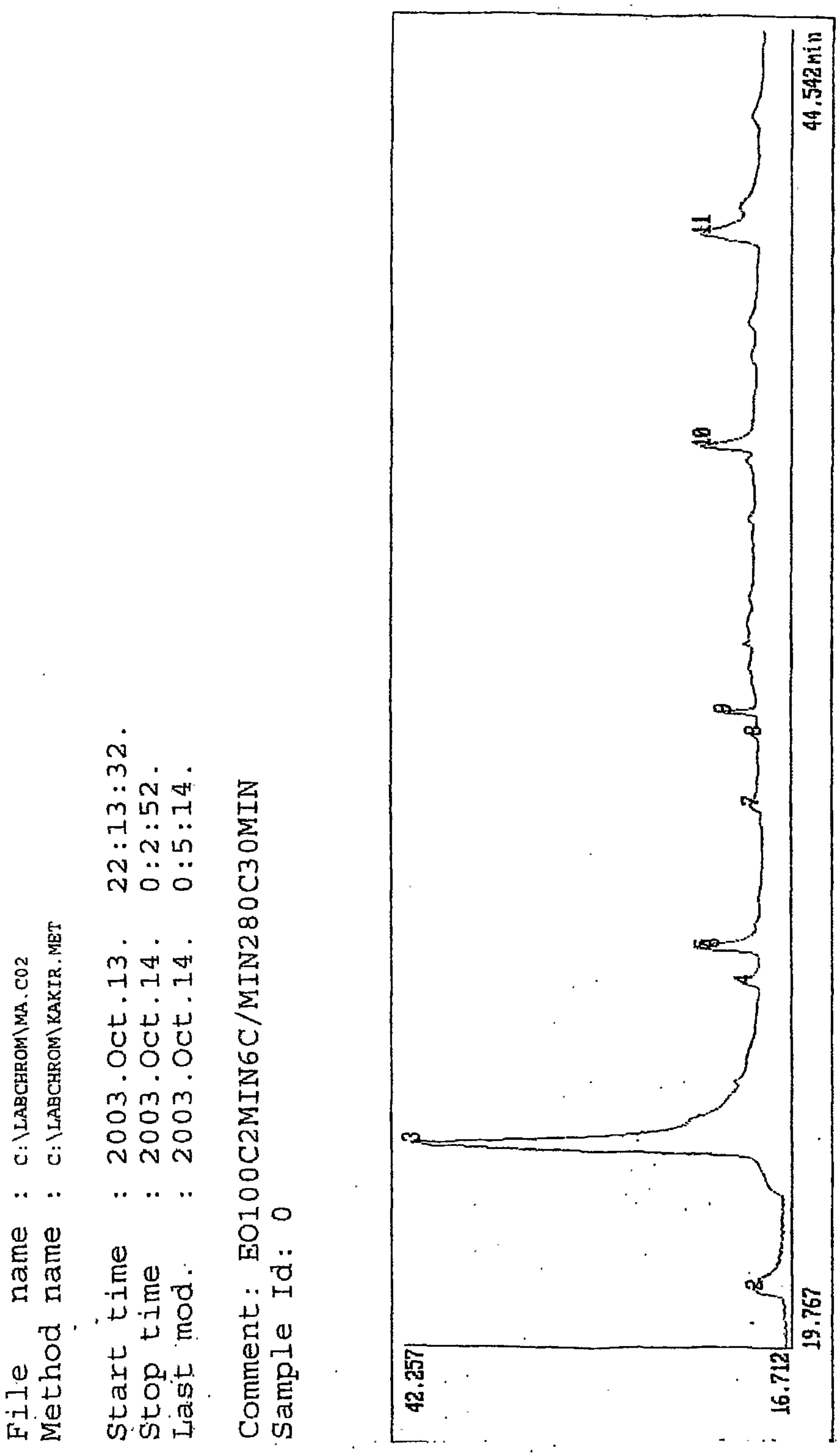
20
21

Figure 8.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3
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5
6
7
8
9
10
11

Figure 9.

LabChrom report of chromatogram
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Sample Id: 0

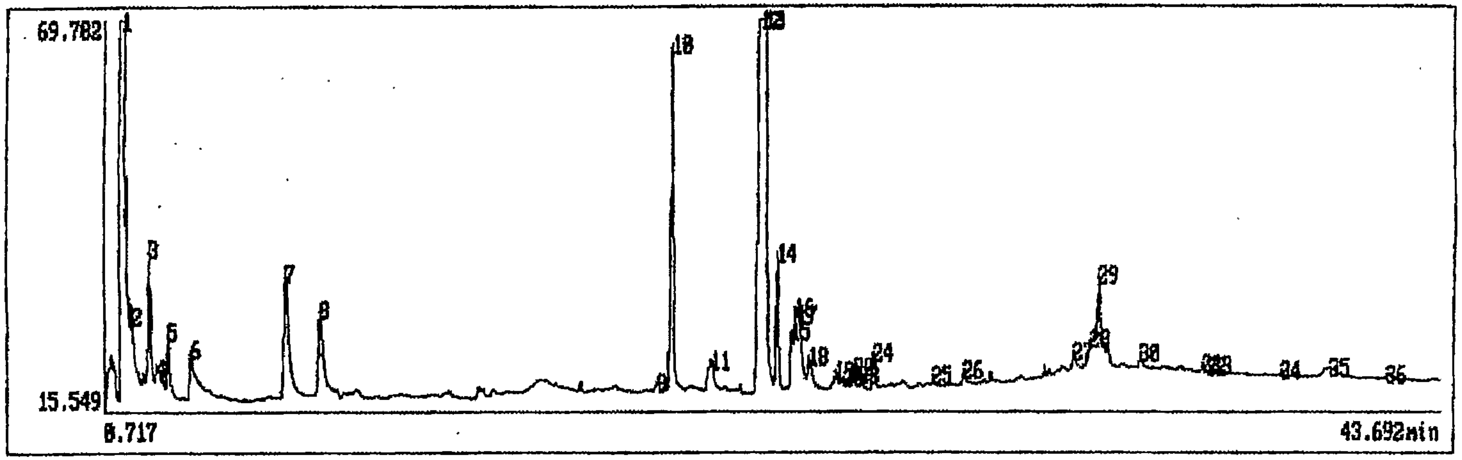
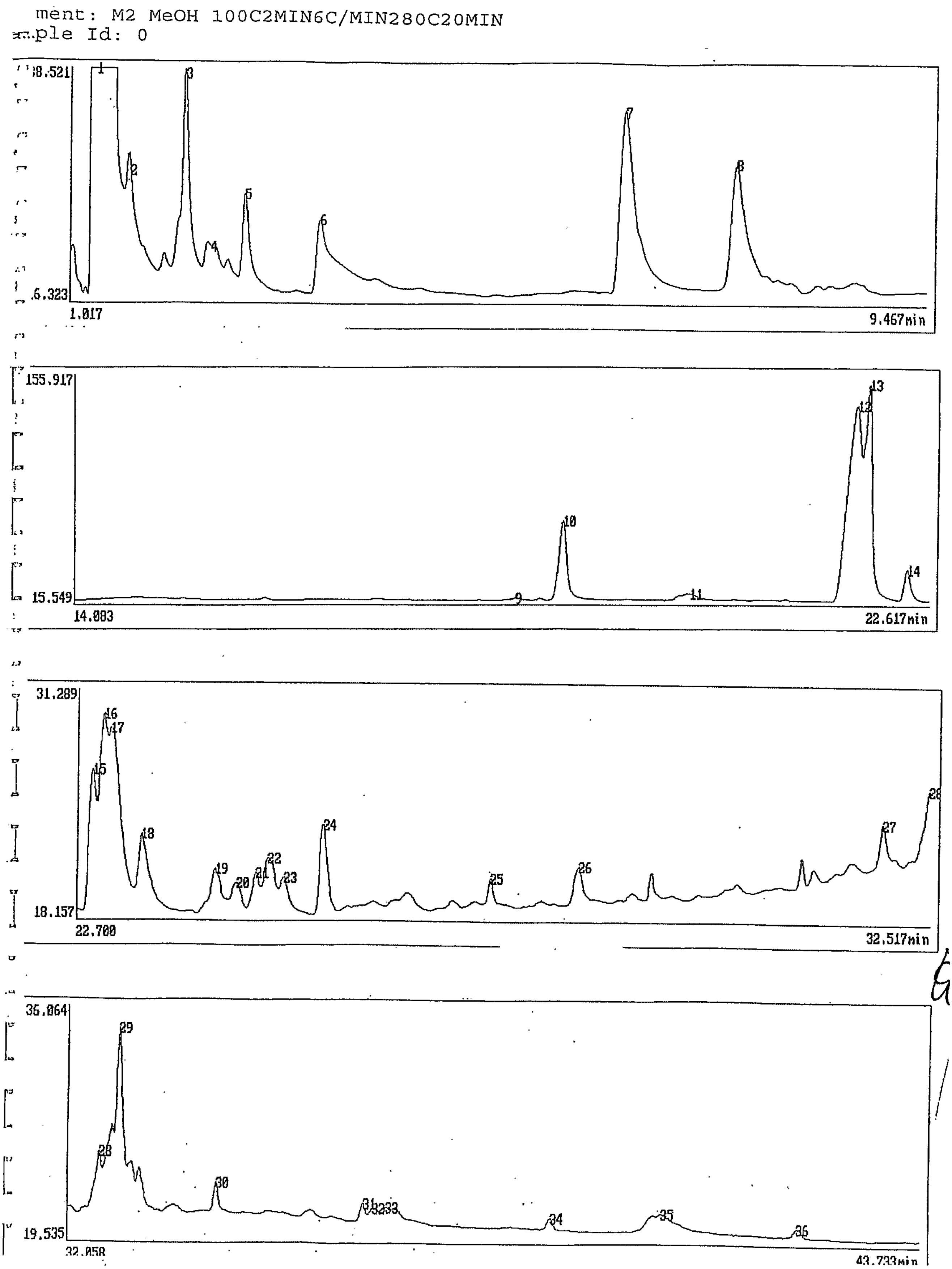


Figure 10.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155.917
15.549
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Figure 11.

08. 12. 2006

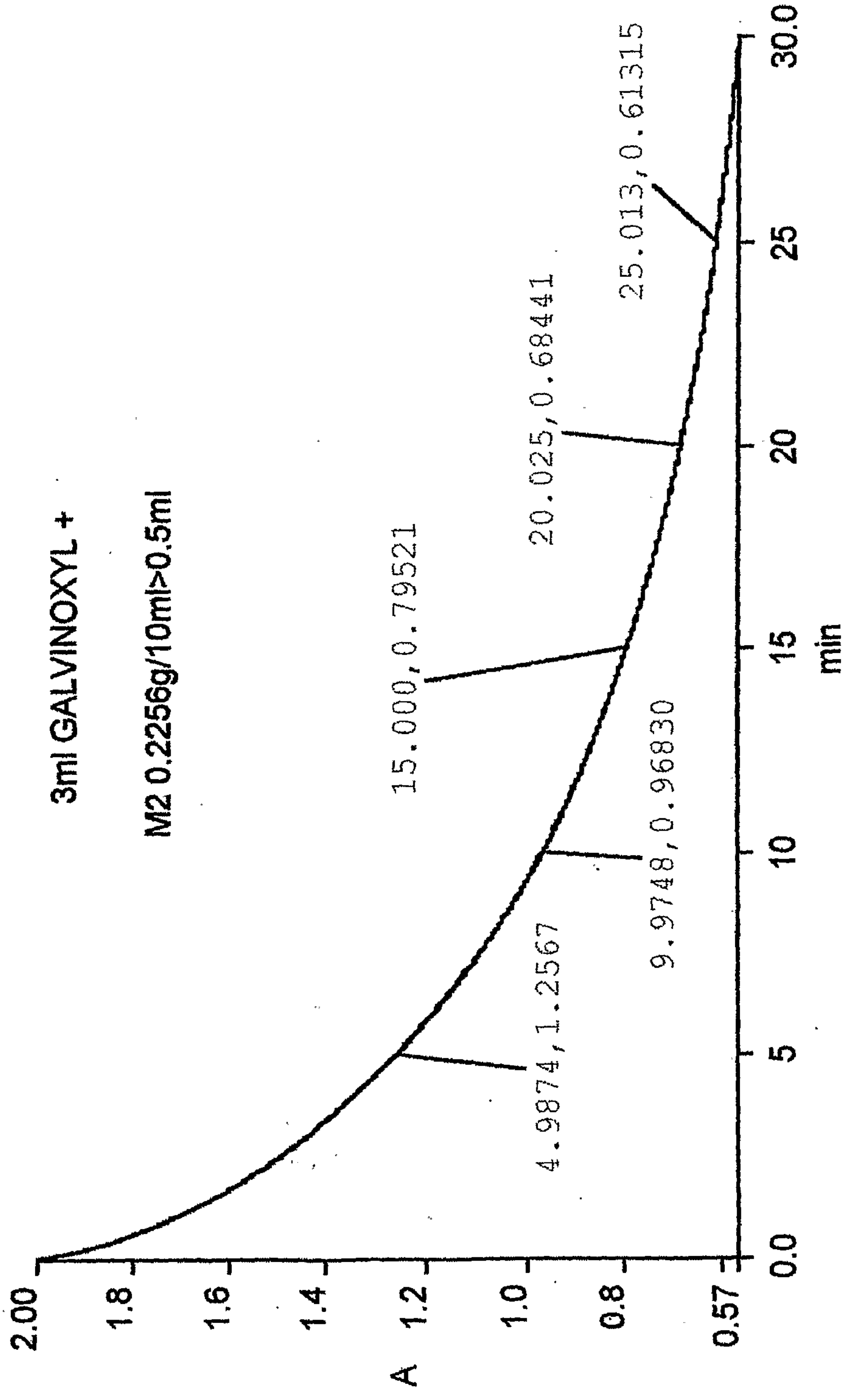
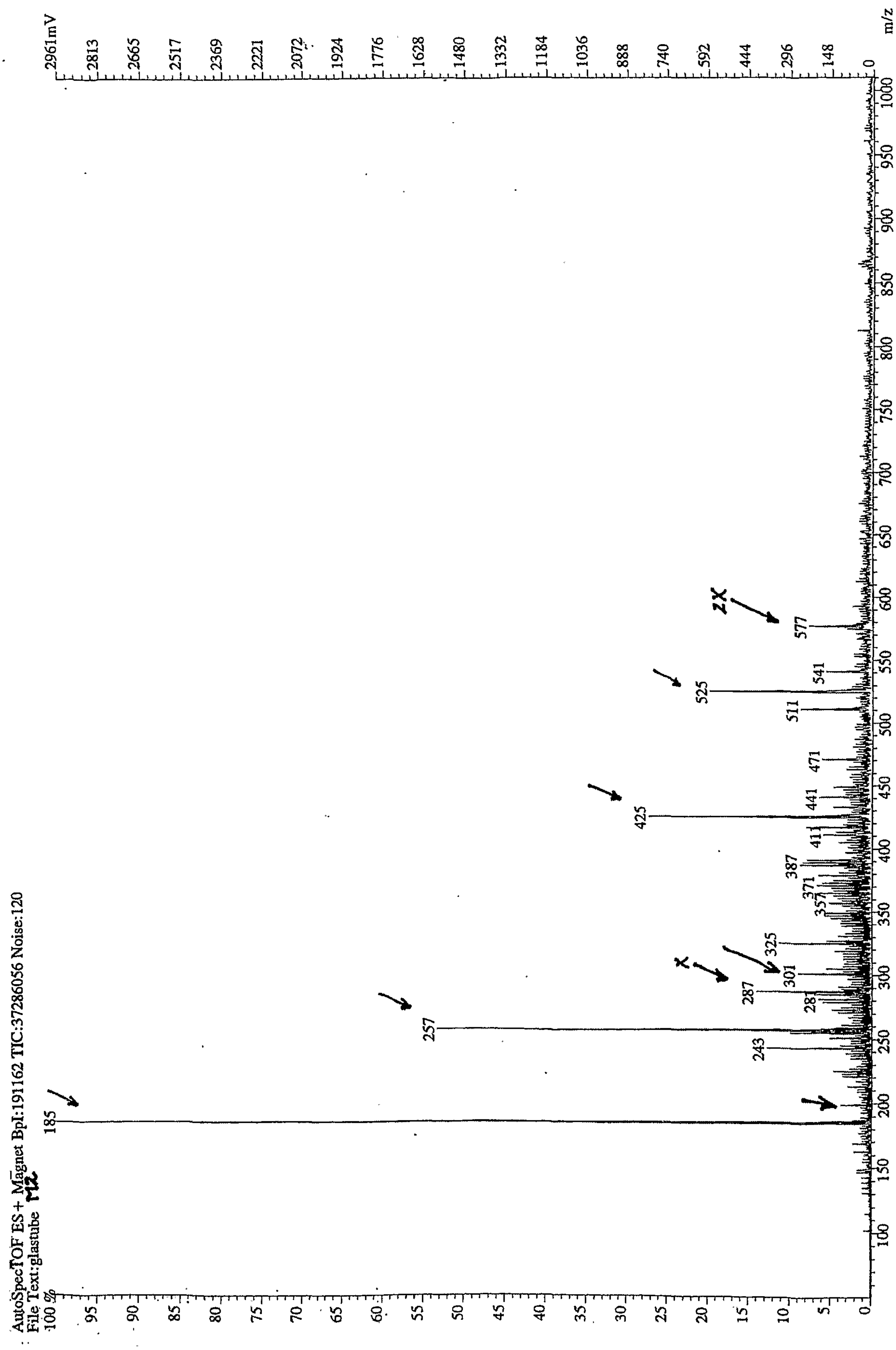


Figure 12.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281
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Figure 13.

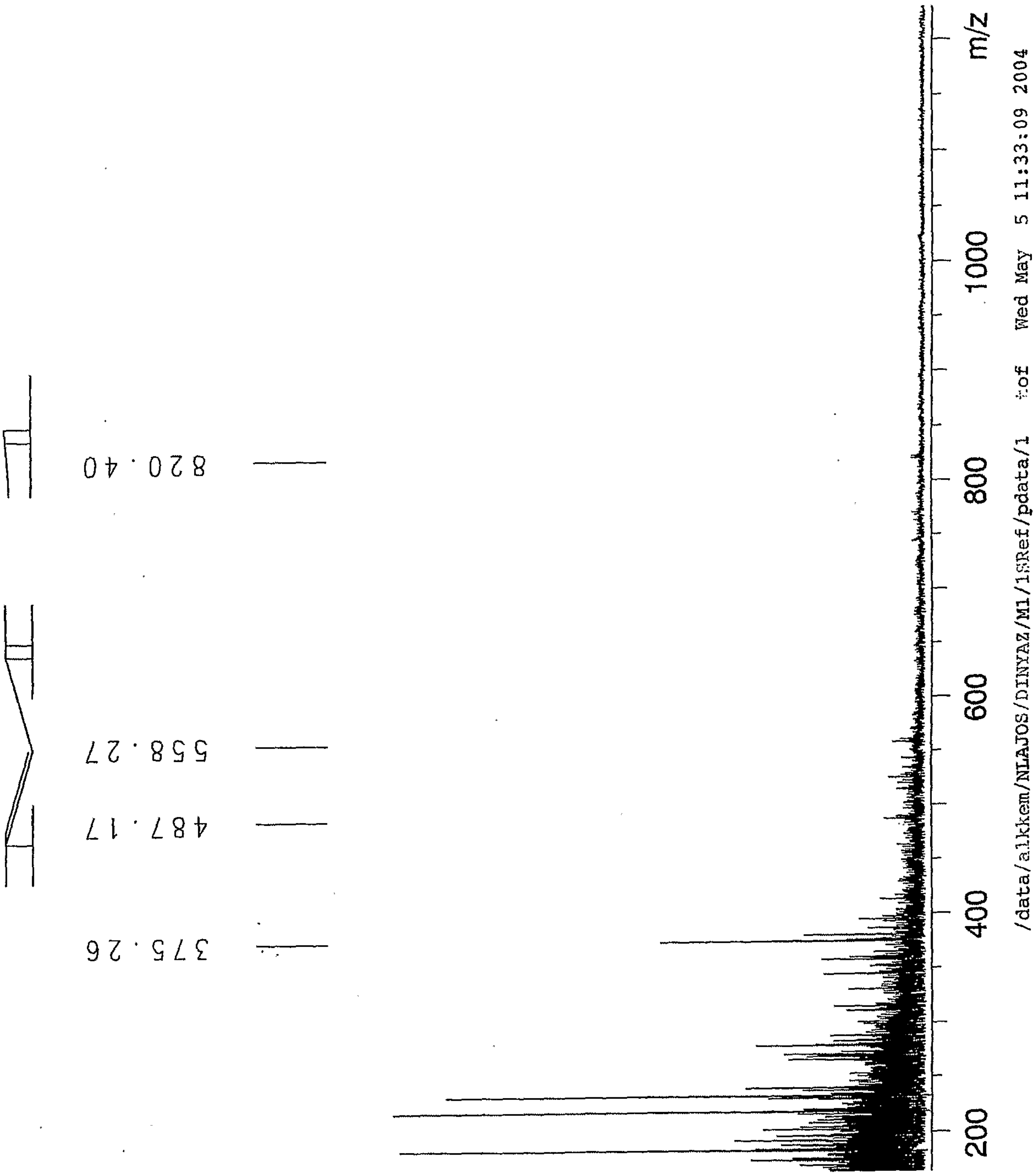
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Figure 14.

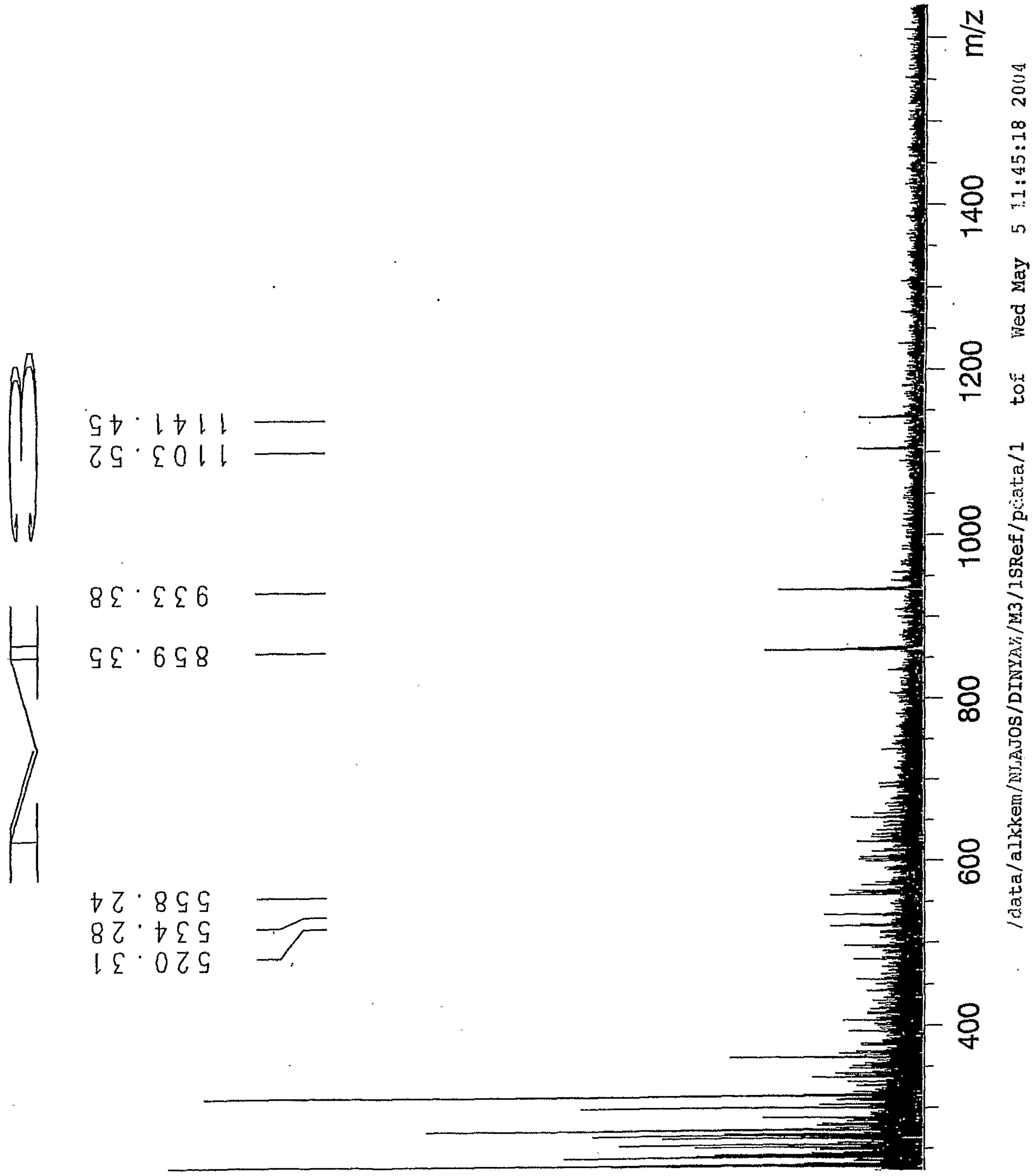
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Figure 15.

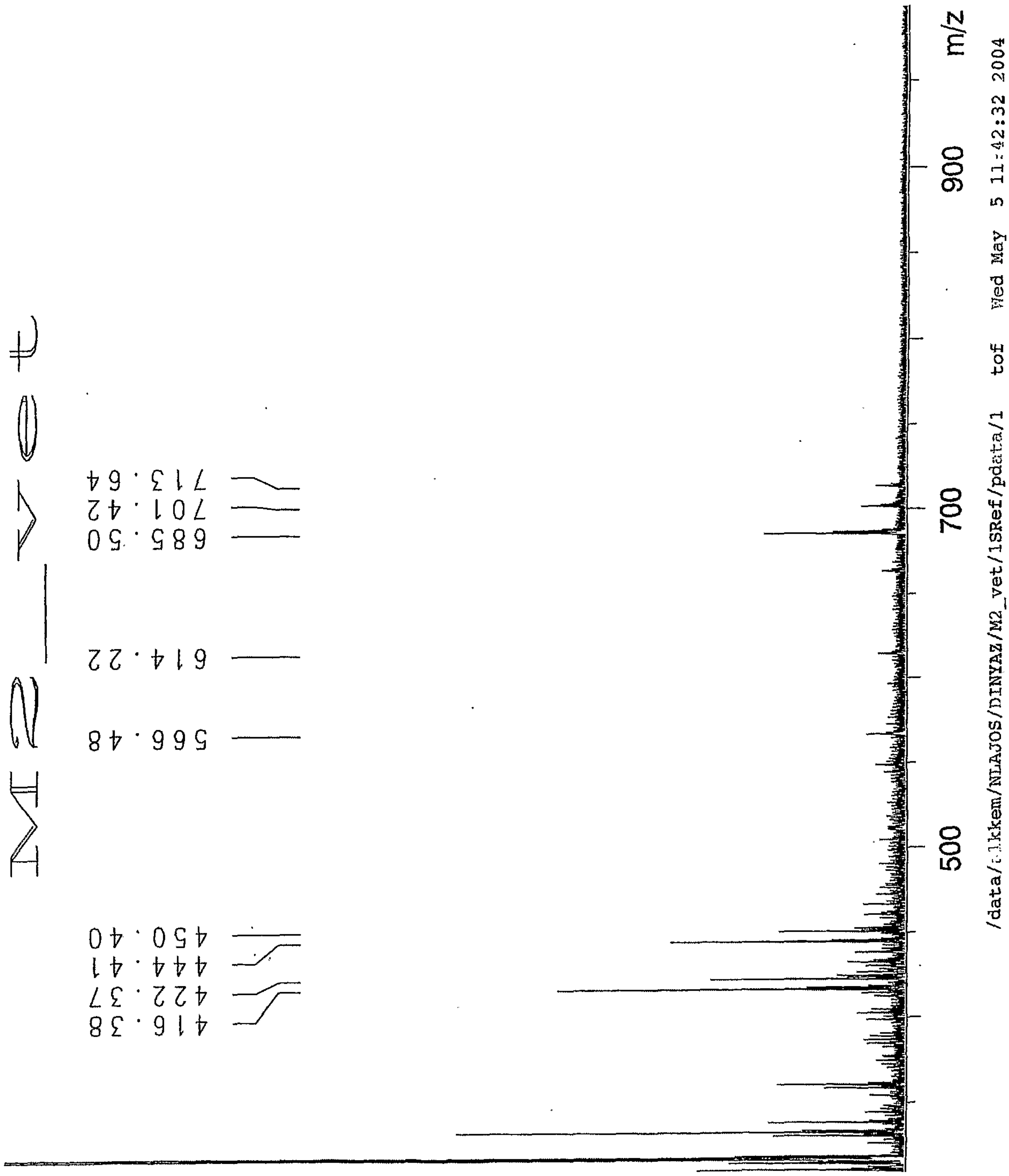
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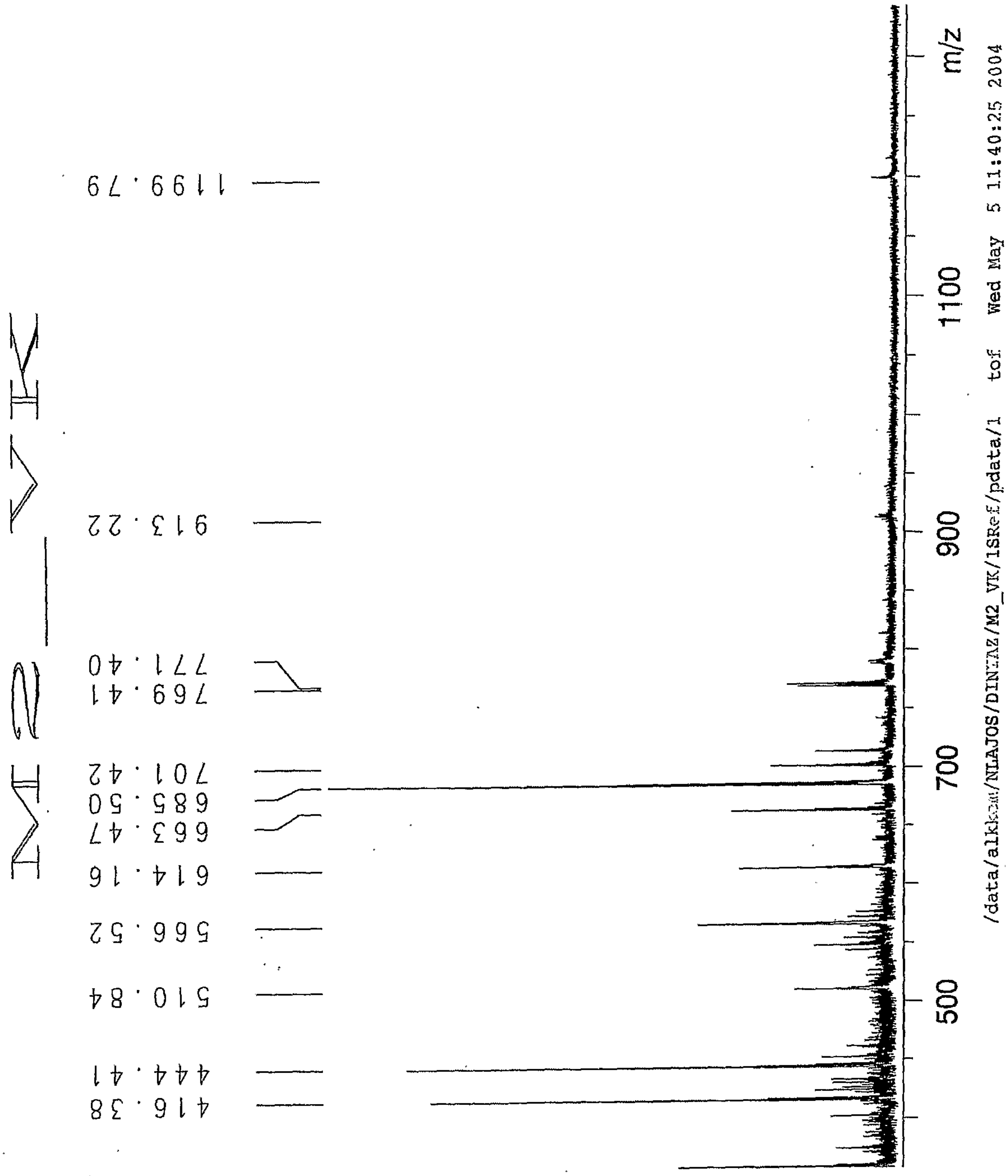
Figure 16.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416.38
500
700
900
1100
m/z
tof Wed May 5 11:40:25 2004

Figure 17.

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Vial Number: 1

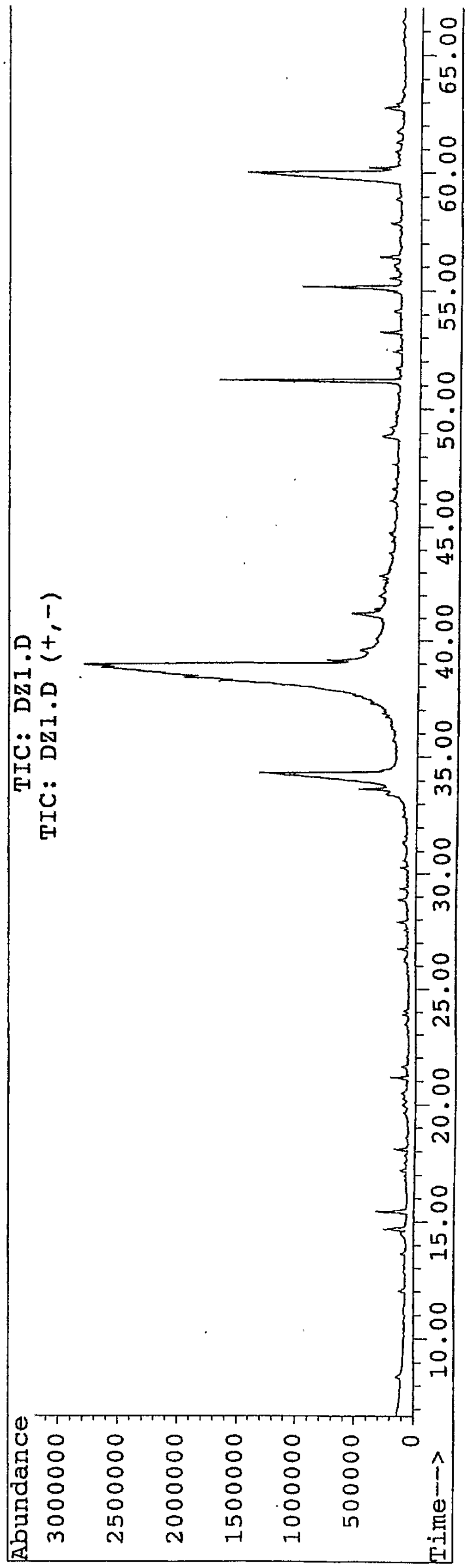
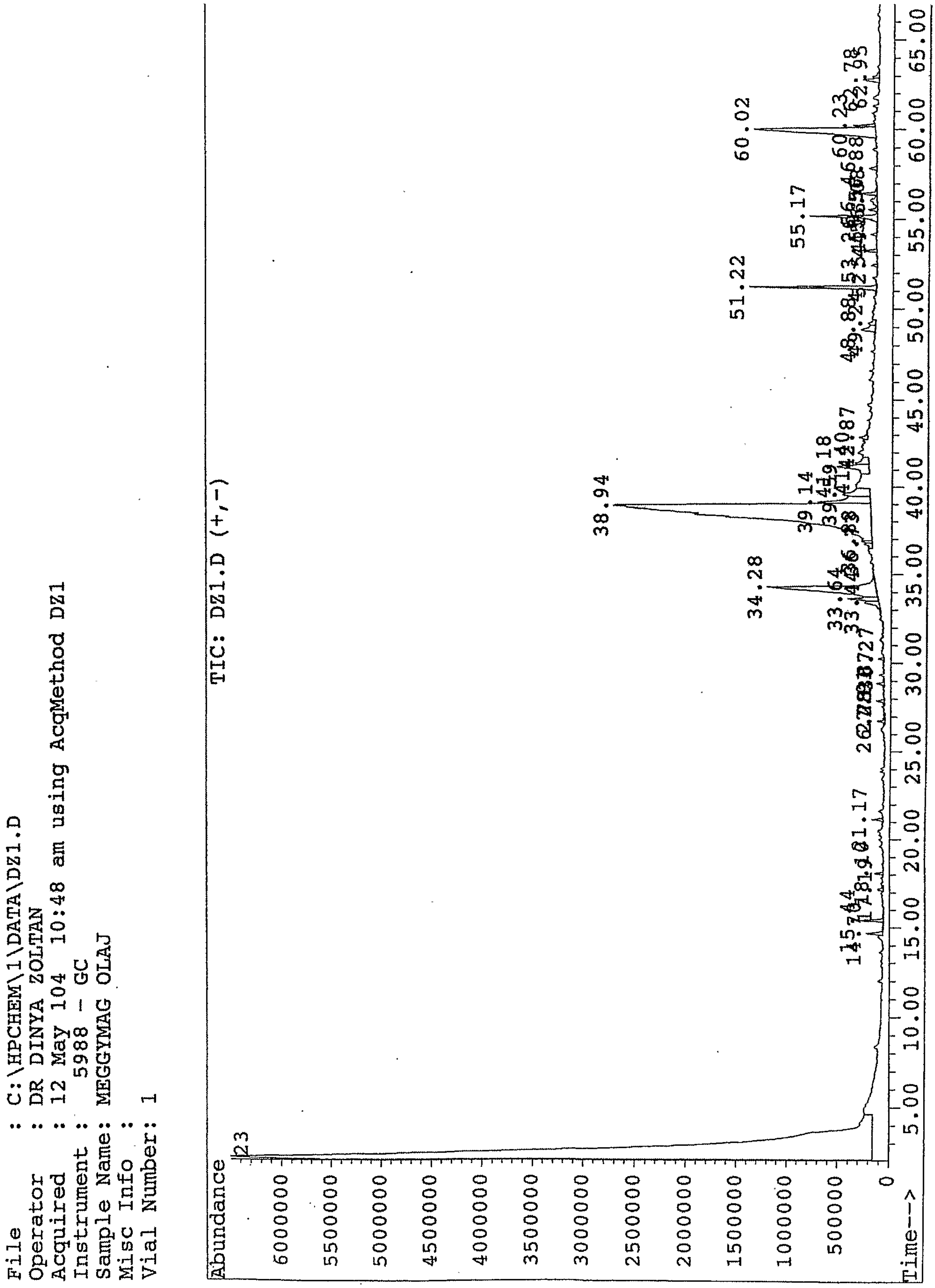


Figure 18.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TIC: DZ1.D (+,-)
Abundance
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5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Time-->
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10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
38.94
34.28
51.22
55.17
60.02
39.14
33.64
21.17
48.88

Figure 19.

```
File         : C:\HPCHEM\1\DATA\DZ1.D
Operator     : DR DINYA ZOLTAN
Acquired     : 12 May 104  10:48 am using AcqMethod DZ1
Instrument :    5988 - GC
Sample Name: MEGGYMAG OLAJ
Misc Info    :
Vial Number: 1
```

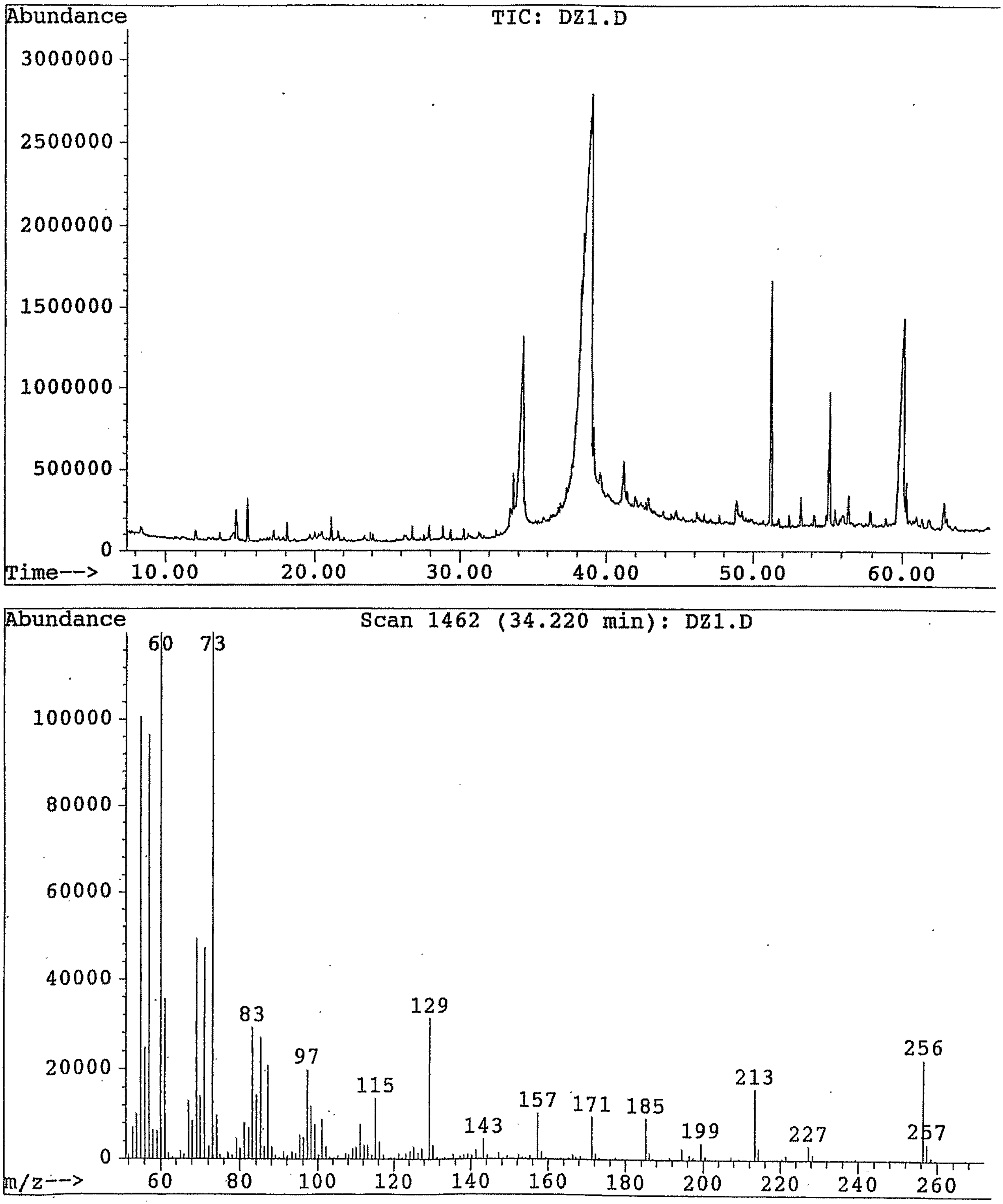


Figure 20.

File : C:\HPCHEM\1\DATA\DZ1.D
Operator : DR DINYA ZOLTAN
Acquired : 12 May 104 10:48 am using AcqMethod DZ1
Instrument : 5988 - GC
Sample Name: MEGGYMAG OLAJ
Misc Info :
Vial Number: 1

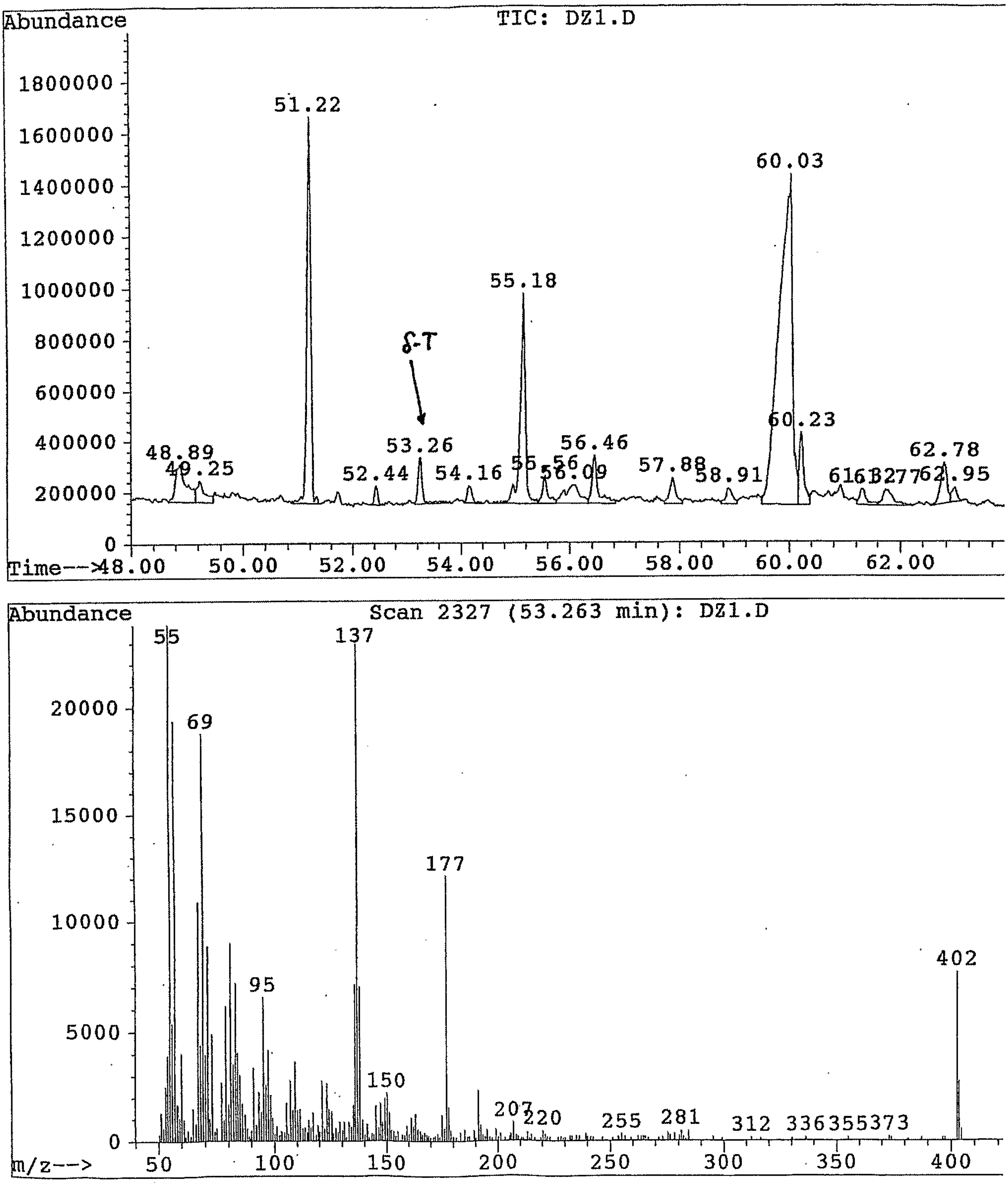
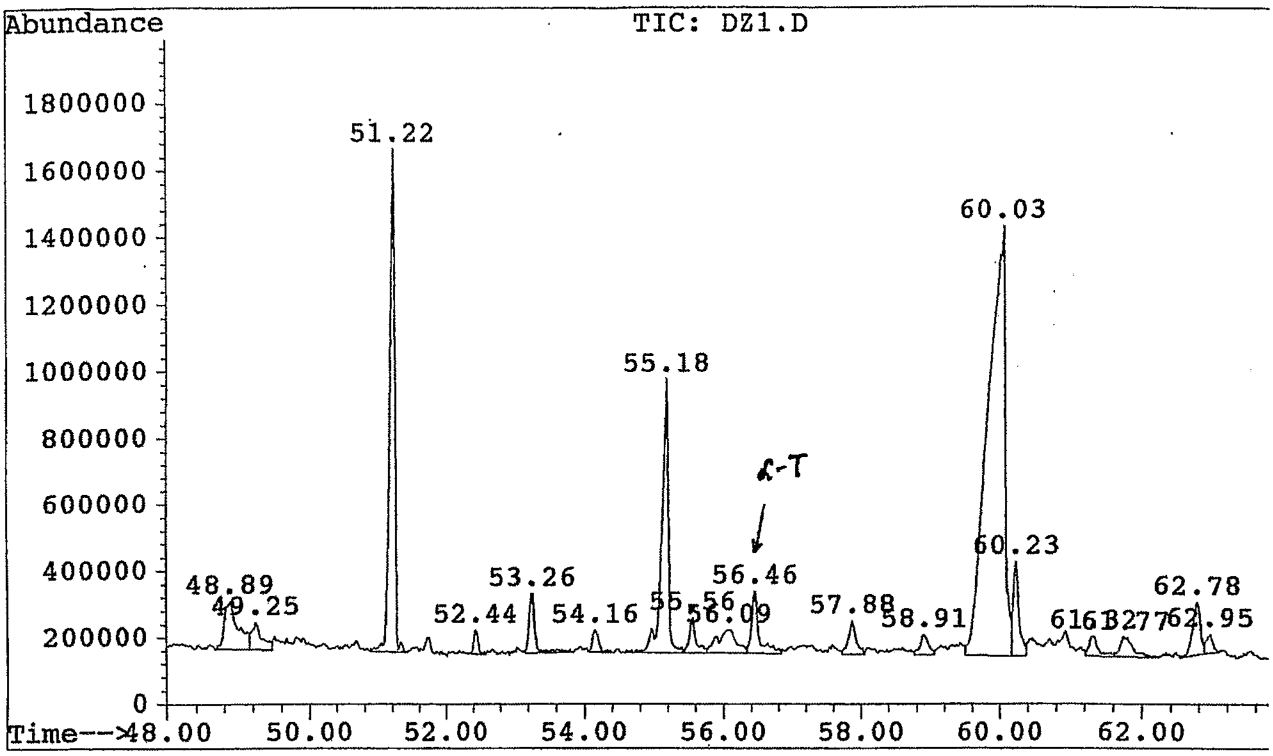


Figure 21.

```
File        : C:\HPCHEM\1\DATA\DZ1.D
Operator    : DR DINYA ZOLTAN
Acquired    : 12 May 104  10:48 am using AcqMethod DZ1
Instrument  :    5988 - GC
Sample Name: MEGGYMAG OLAJ
Misc Info   :
Vial Number: 1
```



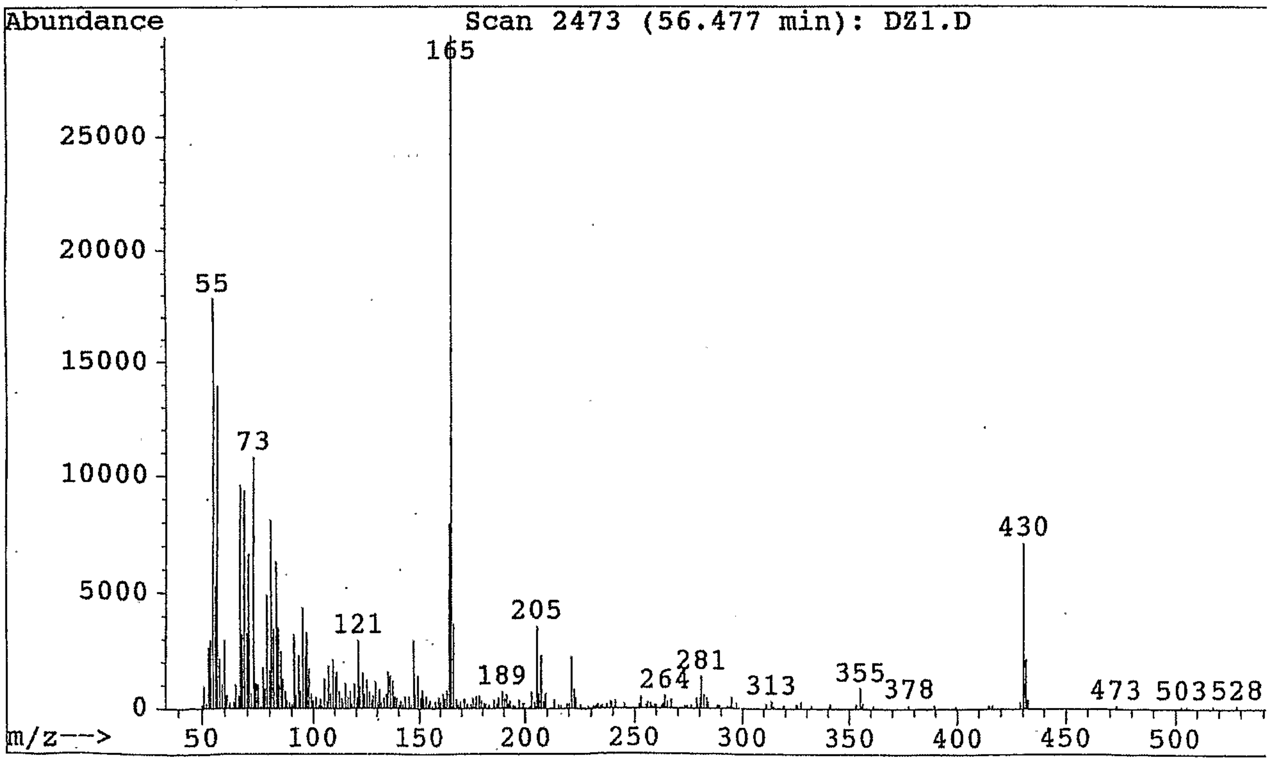


Figure 22.

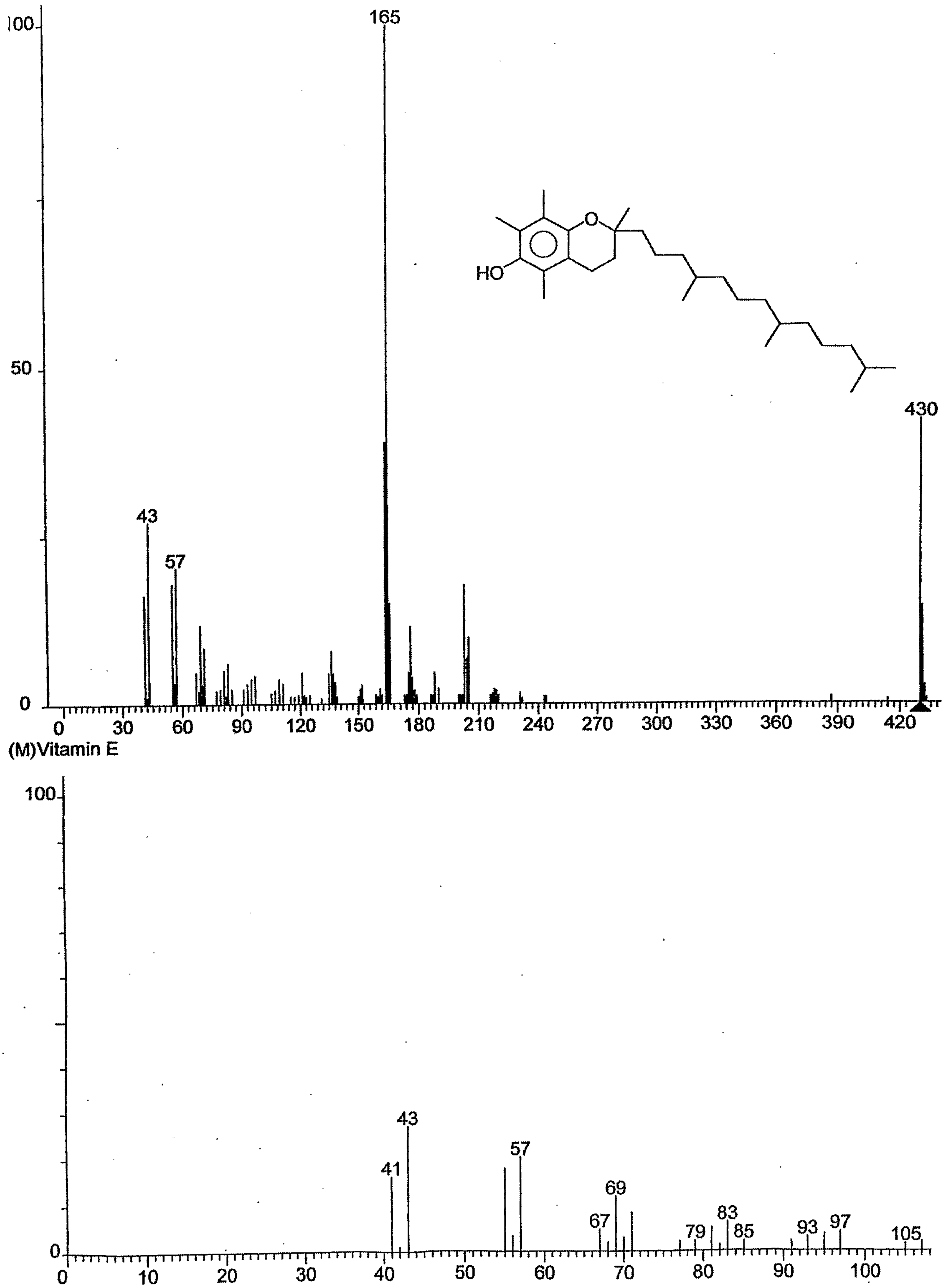

165
HO
430
43
57
(M)Vitamin E
0
30
60
90
120
150
180
210
240
270
300
330
360
390
420
100
50
41
69
67
79
83
85
93
97
105
10
20
40
70
80

Figure 23.

```
File        : C:\HPCHEM\1\DATA\DZ1.D
Operator    : DR DINYA ZOLTAN
Acquired    : 12 May 104  10:48 am using AcqMethod DZ1
Instrument  :    5988 - GC
Sample Name: MEGGYMAG OLAJ
Misc Info   :
Vial Number: 1
```

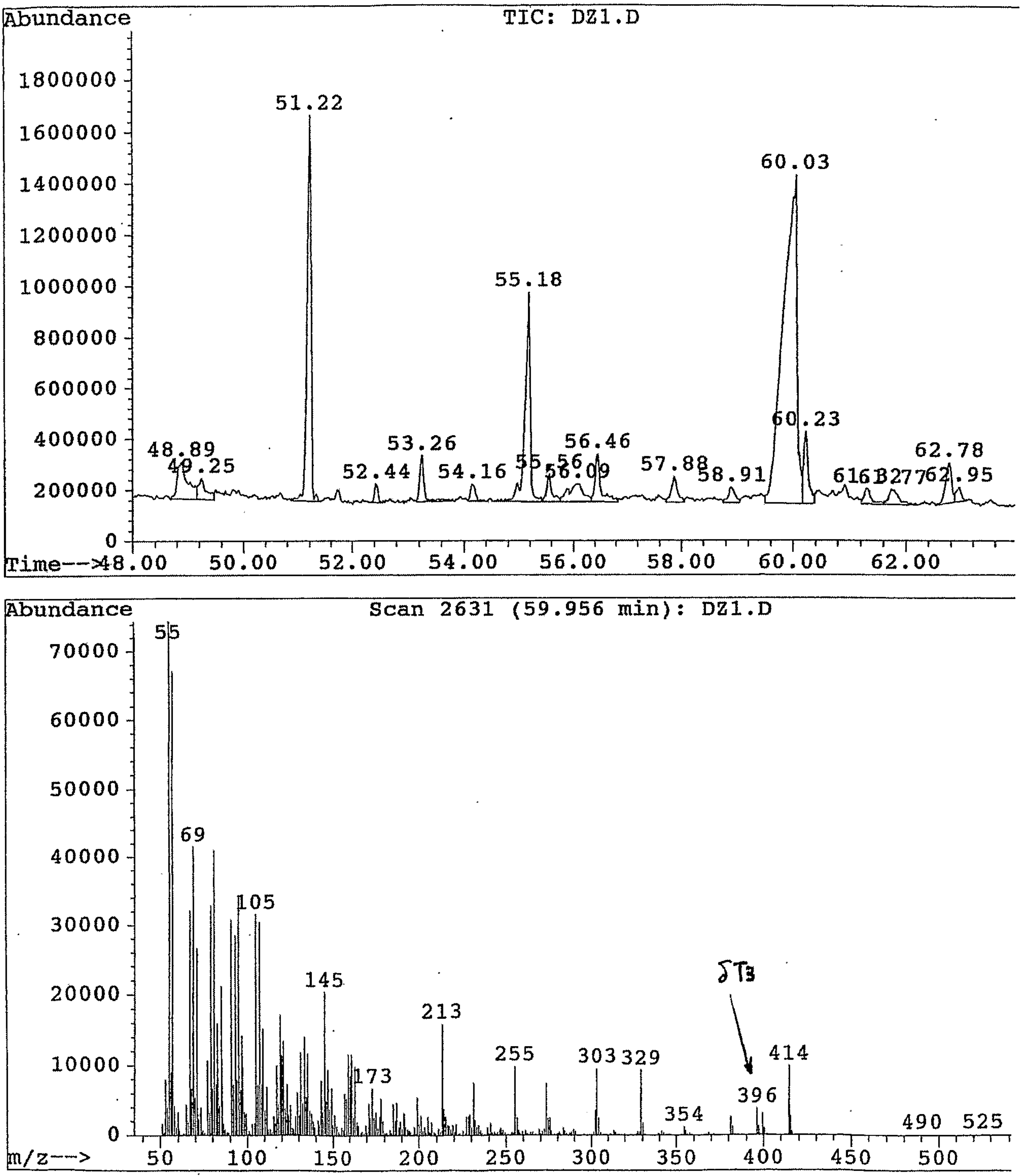


Figure 24.

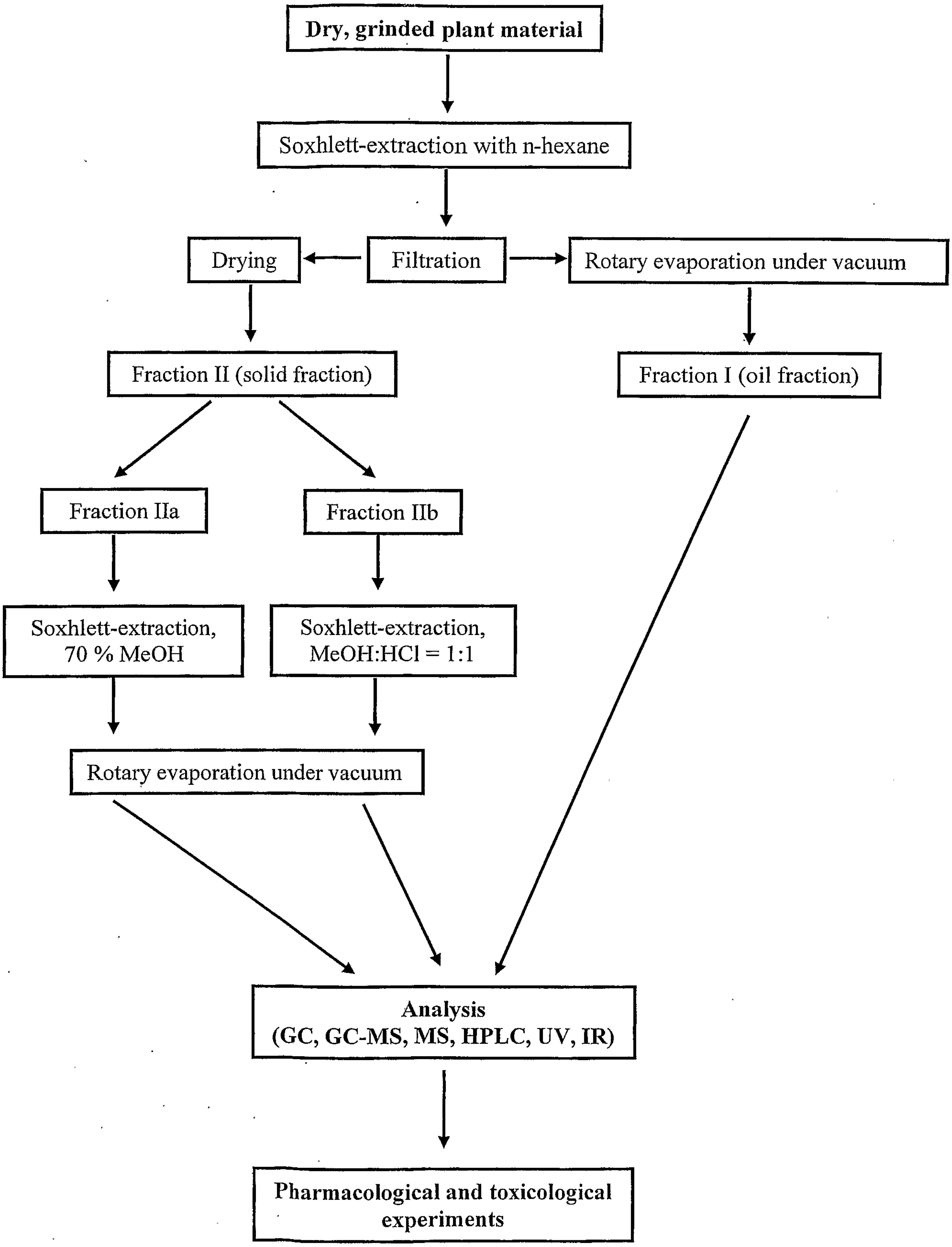

Dry, grinded plant material
Soxhlett-extraction with n-hexane
Drying
Filtration
Rotary evaporation under vacuum
Fraction II (solid fraction)
Fraction I (oil fraction)
Fraction IIa
Fraction IIb
Soxhlett-extraction, 70 % MeOH
Soxhlett-extraction, MeOH:HCl = 1:1
Rotary evaporation under vacuum
Analysis (GC, GC-MS, MS, HPLC, UV, IR)
Pharmacological and toxicological experiments

Figure 25.

A

B

C

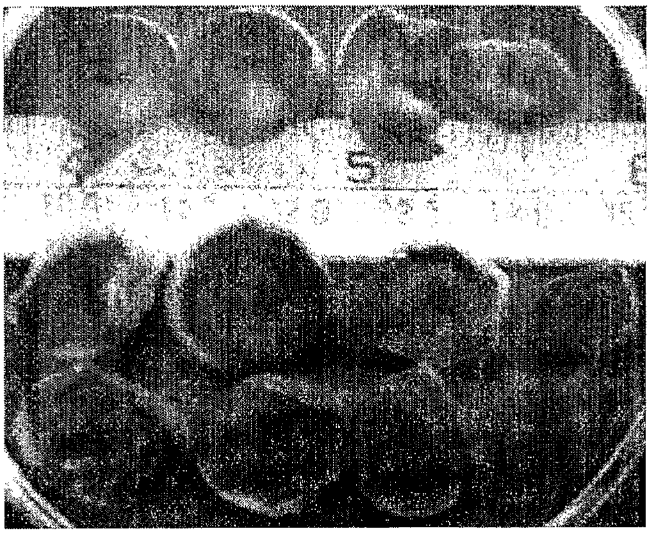


Figure 26

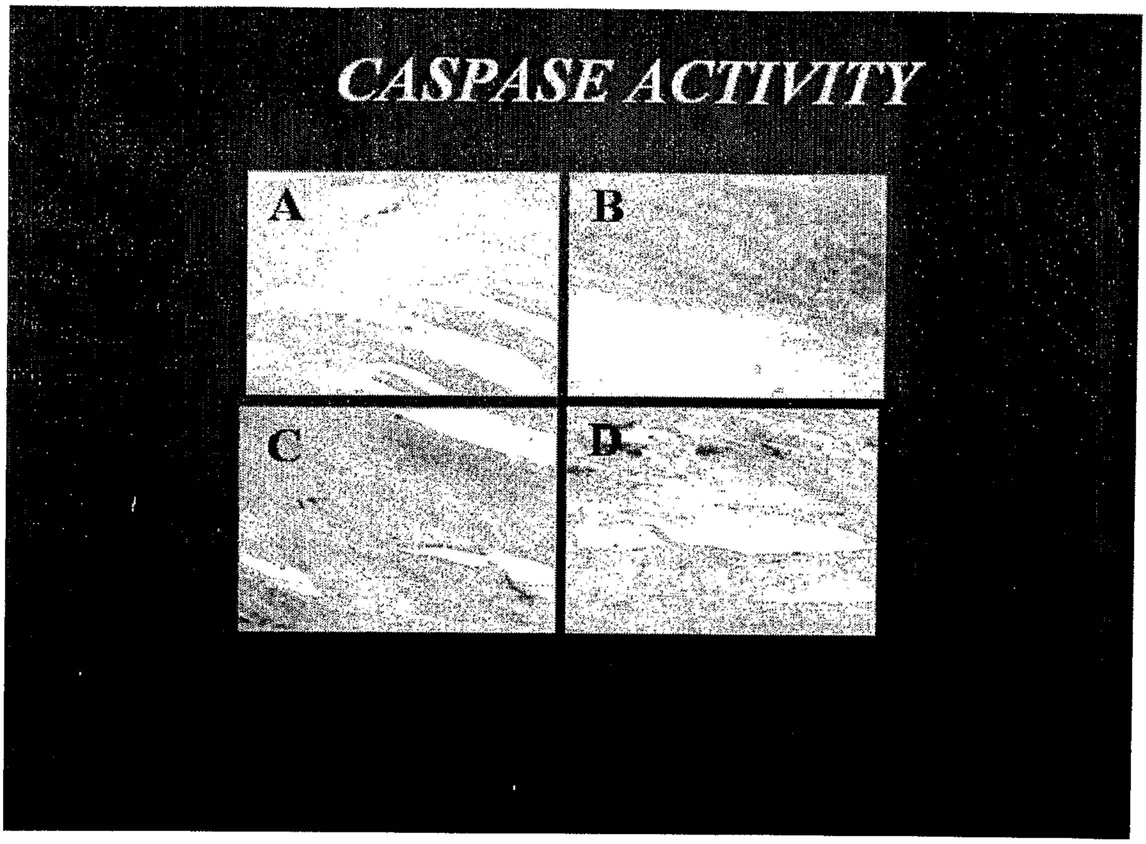


Figure 27.

INTERNATIONAL SEARCH REPORT

International application No
PCT/HU2006/000093

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/353 A61P9/10 A61K36/736

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, CHEM ABS Data, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NAGY NORBERT ET AL: "EFFECTS OF SOUR CHERRY SEED EXTRACT IN ISOLATED ISCHEMIC/REPERFUSED MOUSE HEART" JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY, XX, XX, vol. 37, no. 1, July 2004 (2004-07), page 278, XP009079168 ISSN: 0022-2828 Abstract no. C59 abstract ----- -/--	1-16

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2007	07/03/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Economou, Dimitrios

INTERNATIONAL SEARCH REPORT

International application No
PCT/HU2006/000093

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SZABO MARTA E ET AL: "HEME OXYGENASE-1-RELATED CARBON MONOXIDE AND FLAVONOIDS IN ISCHEMIC/REPERFUSED RAT RETINA" INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE, ASSOCIATION FOR RESEARCH IN VISION AND, US, vol. 45, no. 10, October 2004 (2004-10), pages 3727-3732, XP009079169 ISSN: 0146-0404 abstract page 3729, left-hand column, paragraphs 3,4	1-16
P,X	BAK ISTVAN ET AL: "CARDIOPROTECTIVE MECHANISMS OF PRUNUS CERASUS (SOUR CHERRY) SEED EXTRACT AGAINST ISCHEMIA-REPERFUSION-INDUCED DAMAGE IN ISOLATED RAT HEARTS" AMERICAN JOURNAL OF PHYSIOLOGY: HEART AND CIRCULATORY PHYSIOLOGY, THE AMERICAN PHYSIOLOGICAL SOCIETY, XX, vol. 291, no. 3, September 2006 (2006-09), pages H1329-H1336, XP009079177 ISSN: 0363-6135 the whole document	1-16
P,X	ISTVAN BAK ET AL.: "Cardioprotective mechanisms of sour cherry seed extract against ischemia/reperfusion-induced damage in isolated rat hearts" JOURNAL OF MOLECULAR AND CELLULAR CARDIOLOGY / ABSTRACTS, vol. 40, June 2006 (2006-06), pages 962-963, XP005473210 Abstract No. 116 abstract	1-16
X	US 2004/116512 A1 (NAGUIB YOUSRY M A [US] ET AL) 17 June 2004 (2004-06-17) the whole document	1-16
X	THERIAULT A ET AL: "TOCOTRIENOL: A REVIEW OF ITS THERAPEUTIC POTENTIAL" CRITICAL REVIEWS IN FOOD TECHNOLOGY, THE CHEMICAL RUBBERT CO, vol. 32, no. 5, July 1999 (1999-07), pages 309-319, XP001120035 the whole document	1-16

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INTERNATIONAL SEARCH REPORT

International application No
PCT/HU2006/000093

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TOMEO A C ET AL: "ANTIOXIDANT EFFECTS OF TOCOTRIENOLS IN PATIENTS WITH HYPERLIPIDEMIA AND CAROTID STENOSIS" LIPIDS, CHAMPAIGN, IL, US, vol. 30, no. 12, 1995, pages 1179-1183, XP008007118 ISSN: 0024-4201 the whole document -----	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/HU2006/000093

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004116512 A1	17-06-2004	AU 2003301069 A1	14-07-2004
		EP 1572195 A2	14-09-2005
		WO 2004056348 A2	08-07-2004
		US 2006093664 A1	04-05-2006