Eurasian Journal of Analytical Chemistry
Volume 3, Number 1, 2008

GC-MS and AAS in Fractionation Analysis of Herbal Supplement

Stela Georgieva, Christo Chanev, Sonja Arpadjan

1164 Sofia, bld. J. Bourchier 1, Faculty of Chemistry, University of Sofia, Bulgaria,

Abstract

An analytical scheme based on solid phase and liquid-liquid extraction was proposed for the fractionation of element species and organic constituents in a mixture (1:1) of 8 Bulgarian medicinal plants proposed as herbal nutritional supplement using GC-MS and AAS. Solid phase extraction was performed with water, 0.1 mol L^{-1} HCl, Tris-buffer (pH 7.6) and methanol. Liquid-liquid successive extractions were performed with petroleum ether, diethyl ether, $CHCl_3$ and ethyl acetate. The potential bioavailability of water and lipid – soluble compounds were discussed.

Keywords: Herbal supplement, GC-MS, AAS, fractionation

1. Introduction

Medicinal plants are widely used as home remedies, nutritional supplements or as raw materials for the pharmaceutical industry. Their observed and proven efficacy, low price and absence of toxic side effects explain the social interest in the use of herbs. Bulgarian pharmaceutical industry is going to investigate a new herbal supplement HNS8 prepared by mixing equal quantities of eight medicinal plants *Anethum graveolens, Calendula officinalis, Centaurium erythraea, Glycyrrhiza glabra, Hypericum perforatum, Linum usitatissimum, Ononis hircina; (Ononis spinosa), Pimpinella anisum*. The main known characteristics of the herbs included in HNS8 are summarized in Table 1. From such information about the total content of organic and inorganic substances, however, the nutritional, pharmacological or toxicological action of constituents in a phytomedicine cannot be readily understood, and rational dosage criteria cannot be proposed. In human nutritional sciences the concept of bioavailability was regarded as the efficiency with which nutrients are utilized [1, 2]. The solubility of nutritional supplement species in gastrointestinal tract, their absorption and delivery to circulation are determinants for their subsequent bioavailability [3, 4]. Many useful approaches for in vitro assessment of species solubility [5, 6] and absorptivity [7-9] were reported.

ISSN: 1306-3057

Table 1. Main characteristics of medicinal plants included in herbal supplement HNS8 [10-13]

Medicinal Plants	Part Used	Constituents	Medicinal Uses
Centaurium erythraea	*Herba Centaurii*	Amarogenin, Alkaloide, Eryhtramine, Erythricin, Essential oil, Vitamin C	Cholagogue; Diaphoretic; Digestive; Emetic; Febrifuge; Hepatic; Stomachic; Tonic
Pimpinella anisum	*Fructus Anisi;*	Choline; Coumarin*;* Essential oil*;* Fats; Proteins; Rutin; Saccharides*;* Stigmasterol; Vitamin C	Antiseptic; Antispasmodic; Carminative; Digestive; Expectorant; Galactogogue; Pectoral; Stimulant;
Ononis spinosa	*Radix Ononidis*	Essential oil, Flavonoide, Glycoside, Pseudoononin; Tannin, Resin, Saccharose; Saponins	Antitussive; Aperient; Diuretic; Lithontripic.
Glycyrrhiza glabra	*Radix Glycyrrhizae (Liquiritiae)*	Glucose; Glycyrrhizine; Isoflavones (phytoestrogens); Liquiritin; Saccharose;	Antiinflammatory; Antispasmodic; Demulcent; Diuretic; Emollient
Linum usitatissimum	*Semen Lini*	Carotene;); Glyceride, Glycoside; Linamarinase; Linolic **a**cids; Omega-3 fatty acids; Pectin; Phytoestrogen; Proteine; Saccharide	Analgesic; Cancer; Cardiotonic; Demulcent; Emollient; Expectorant; Laxative; Nervine; Antioxidant
Hypericum perforatum	*Herba Hyperici*	Anethole; Carveole; Carvone Coumarins; Flavonoide; Limonene; Myristicin; a- and b-Phellandrene; x-Pinene; Xanthon	Analgesic; Antiseptic; Antispasmodic; Antideprss; Astringent; Cholagogue; Digestive; Diuretic; Expectorant; Nervine;
Anethum graveolens	*Fructus Anethi*	Essential oil; Coumarins; Fellandrene; Ferulic acid; Limonene; Myristicin; Proteins; Terpinen	Carminative; Anti-spasmodic; Anti-inflammatory;
Calendula officinalis	*Flores Calendulae*	Auroxanthin, b-Carotene; Calendulin; Citroxanthin; Enzymes; Essential oil; Flavoxanthin; Rubixanthin; Triterpenoid esters	Antiphlogistic; Antiseptic; Anti-inflammatory; Antioxidants; Antispasmodic; Aperient; Astringent; Bactericide;

To investigate the therapeutic action of HNS8 it is very important to study the distribution of organic components and microelements in various fractions, modeling the potential physiological absorptivity and bioavailability of constituents included in herbal supplement. In the present work solubility studies were performed for assessment of the usefulness of the herbal supplement HNS8. Furthermore, the octanol-water system was applied to investigate the distribution of elements and organic species under stomach and intestine conditions.

2. Experimental

2.1. Instrumentation

The atomic absorption measurement techniques applied were flame AAS with an air-acetylene flame (PYE Unicam SP1950) or electrothermal AAS (Perkin-Elmer Zeeman 3030 with an HGA–600 atomizer). The light sources were hollow cathode lamps for Cr, Cu, Fe, Mn, Ni, Pb and electrodeless discharge lamps for As, Cd and Se. The spectral bandpass and the wavelengths used were as recommended by the manufacturers. Standard uncoated graphite tubes (for Cr, Cu, Fe, Mn and Ni) and uncoated graphite tubes with a platform (for As, Cd, Pb and Se) were used. Autosampler AS-60 was used for injections of 10 μL aqueous sample solutions into the graphite tube. Injection of the organic solutions (10 μL) was carried out manually. Only peak areas were used for quantification. The graphite furnace operating parameters are presented in Table 2.

Table 2. Electrothermal atomization programme for determination of microelements by ETAAS with Zeeman background correction

	Step				
Parameter	I	II	III	IV	V
Temperature/^{o}C	50	120	Variable	Variable	2600
Ramp time/s	2	10	Variable	0	2
Hold time/s	8	15	20	3	3
Read	-	-	-	On	-
Gas flow (mL min^{-1})	0	300	300	0	300

(Table 2 continued)

	Step III		Step IV
Analyte	T/^{o}C	Ramp time/s	T/^{o}C
Cd	250	5	1200
Cr	1500	15	2500
Cu	1000	10	2000
Fe	1000	10	2000
Mn	1000	10	2000
Ni	1200	12	2200
Pb	500	5	1500

The GC-MS measurements were performed on GC 6890 (Agilent) with MS 5973. GC was supplied with HP-5/MS column 30 m × 0.250 mm × 0.25 μm. The carrier gas was helium (0.8 $cm^3\ min^{-1}$ constant flow), the injection mode was pulsed split-less and the temperature program was first 50 °C for 5 min, then with 4 °C to 280 °C for 10 min. Ionization mode was electron impact (EI) at 70 eV electro beam energy.

2.2. Reagents and Materials

All reagents used were of analytical reagent grade. Doubly distilled water was used throughout. Working standard solutions were prepared by serial dilution of commercial aqueous stock solutions containing 1 g L^{-1} analyte (Merck, Darmstadt) with 0.2 M nitric acid or oil soluble stock solutions (Merck, Darmstadt) containing 0.9 g L^{-1} analyte with isobutyl methyl ketone.

2.3. Fractionation procedure

The fractionation scheme is presented in Fig. 1. For determination of the total content the samples (0.5 g) were stored with 10 ml nitric acid (65%) overnight at room temperature. After heating to remove the nitrogen oxides 1.5 mL 30% H_2O_2 were added and again lightly boiled until clarification. The sample solutions were transferred to 25 mL volumetric flasks.

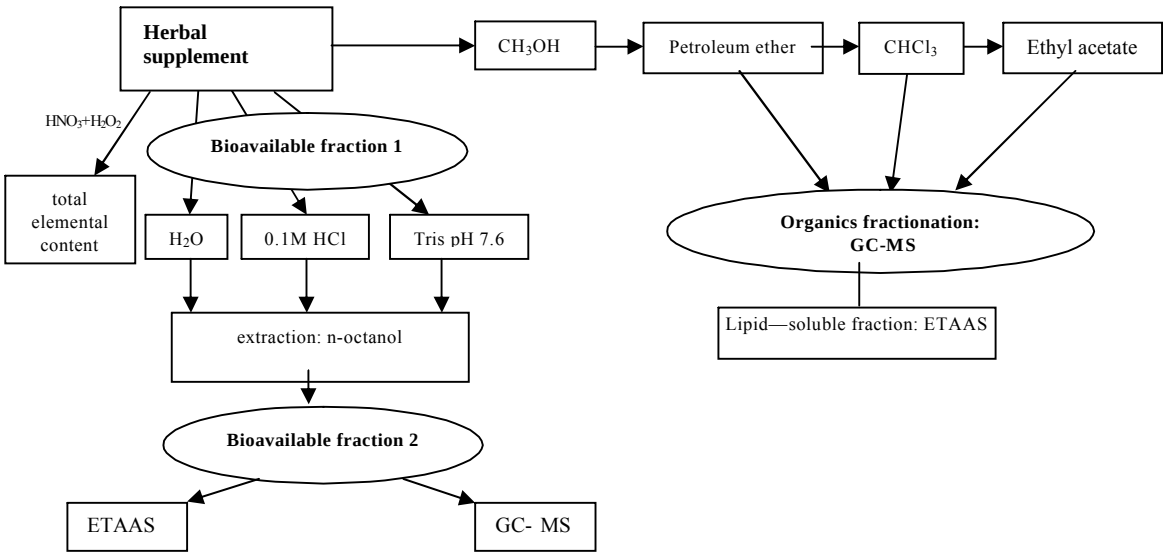


Fig. 1. Procedure for GC-MS and AAS fractionation analysis of herbal supplement HNS8

The solid phase extracts in water, in 0.1 mol L^{-1} HCl (stomach acidity) and in Tris-buffer solution with pH 7.6 (intestine acidity) were prepared in relation 1 : 10 (solid (1.0 g) to liquid phase (10 mL) after 5 min heating in water bath at about 60^{o}C and additional 10 min shaking. The prepared extracts (bioavailable fraction 1) were further used for estimation of bioavailable fraction 2 performing three-phase extraction. As third phase 2 mL octanol were added and extracted for 30 min. The octanol fraction was analysed for trace elements by ETAAS and for organic substances by GC-MS.

The organic fractions for GC-MS investigations were prepared by solid phase (10 g sample) extraction with 100 mL methanol (24 hours at room temperature). From the obtained methanol extract three fractions were prepared by consecutive liquid-liquid extraction with petroleum ether, $CHCl_3$ and ethylacetate. The extraction with each organic solvent was performed three times with (15+10+5) mL solvent. Then each organic fraction was evaporated under vacuum to 2.0 ml before analysis (GC-MS - organic fractions and ETAAS - lipid soluble element fraction).

3. Results and Discussions

3.1. Element fractionation

The total concentration of microelements in the herbal supplement is given in Table 3. The results indicate that iron is present in highest concentration, followed by Mn, Zn, Cu. The extractable part of these elements in water, 0.1 mol L^{-1} HCl (stomach acidity condition), in Tris-buffer at pH 7.6 (intestine acidity condition) presents the available for biosorption fraction of

these biometals. These three fractions are denoted as bioavailable fraction 1 presenting in what extent organically bound microelements soluble in aqueous phase are potentially bioavailable. The lipophility of the elements from bioavailable fraction 1 is assessed using extraction with n-octanol and analysis of the organic phase [9]. The octanol extractable part is denoted as bioavailable fraction 2. The results for bioavailable fraction 1 are given in Table 4 and for bioavailable fraction 2 – in Table 5. The extractability of the analytes is higher in 0.1 mol L^{-1} HCl than in water and Tris-buffer with pH 7.6. The element complexes with phytoligands are not stable at pH 1 and release labile metals in soluble form. The remarkable higher solubility of iron species under stomach conditions is important for Fe uptake by the human organism. It could be supposed that 0.15 mg Fe, 0.02 mg Cu, Mn, Zn, 0.2 μg Cr and 0.08 μg Ni from one gram nutrition supplement are potentially bioavailable. But only 5% from this amount of Fe, 1.5% from that of Cu, Zn and 20% for Mn are octanol soluble (Table 5), hence lipophilic. From the toxic elements no detectable lead was registered. Cadmium species have similar behavior as Cu and Zn. There is no substantial difference in the solubility of elements in water and in Tris-buffer as can be seen from the results in Table 4. But the species of Cd, Fe and Mn are more soluble in octanol at pH 7.6 in comparison to pure water phase (Table 5). The species dissolved under gastric acidity conditions are less octanol soluble than under intestine conditions formed species with exception of Cr. The chromium species are in all cases quantitatively extracted into octanol.

Table 3. Total element concentration in herbal supplement HNS8 (mean value ± SD, n=4)

Element	Concentration (mg kg^{-1})
Cd	0.23 ± 0.06
Cr	0.50 ± 0.04
Cu	67 ± 4
Fe	336 ± 18
Mn	97 ± 6
Ni	1.4 ± 0.2
Pb	1.3 ± 0.1
Zn	72 ± 6

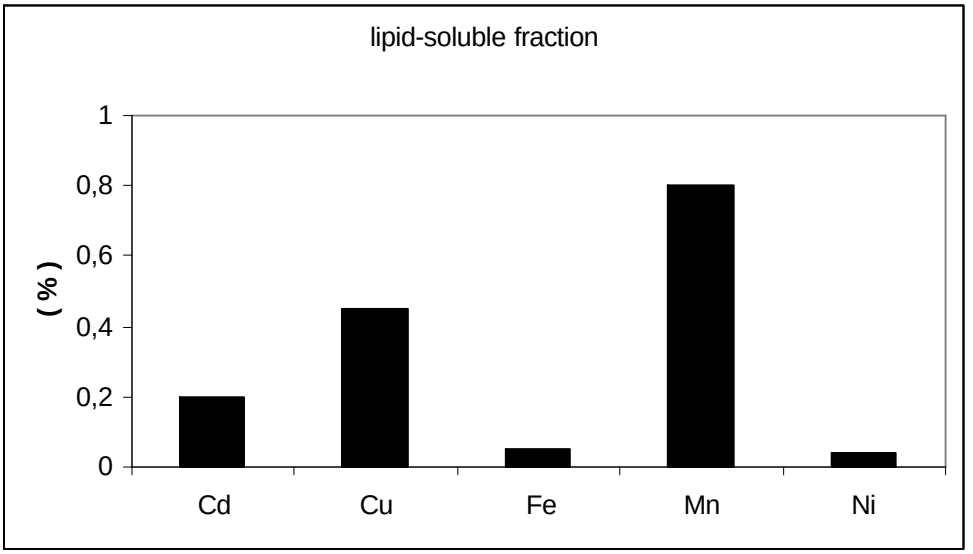
Table 4. Concentration of elements extracted in water, 0.1 mol L^{-1} HCl and Tris-buffer (pH 7.6) as percentage of their total content in HNS8 (mean value ± SD, n=3)

Element	H_2O, %	pH 1, %	pH 7.6, %
Cd	2.2 ± 0.4	17 ± 1	2.2 ± 0.4
Cr	5.4 ± 0.8	25 ± 2	5.3 ± 0.9
Cu	8.7 ± 0.8	25 ± 1	8.9 ± 0.8
Fe	5.2 ± 0.3	44 ± 3	5.1 ± 0.4
Mn	6.9 ± 0.6	26 ± 2	6.7 ± 0.5
Ni	4.8 ± 0.8	21 ± 2	4.9 ± 0.8
Zn	9.3 ± 0.7	35 ± 2	9.1 ± 0.7

Table 5. Octanol-extractable fraction of microelements as percentage of their content in water-, 0.1 mol L^{-1} HCl and Tris-buffer (pH 7.6) extracts (mean value ± SD, n=3)

Element	H_2O, %	pH 1, %	pH 7.6, %
Cd	58 ± 4	5 ± 1	92 ± 8
Cr	94 ± 4	93 ± 2	97 ± 3
Cu	3.8 ± 0.3	1.8 ± 0.1	5.5 ± 0.4
Fe	17 ± 2	5 ± 1	47 ± 6
Mn	24 ± 2	20 ± 2	92 ± 7
Zn	4.5 ± 0.6	1.4 ± 0.2	5.4 ± 0.7

The lipid soluble fractions contain relatively high concentrations of lead and chromium, whereas less than 1% Cd-, Cu-, Fe-, Mn-, Ni- and Zn species were found to be lipid soluble (Fig. 2).



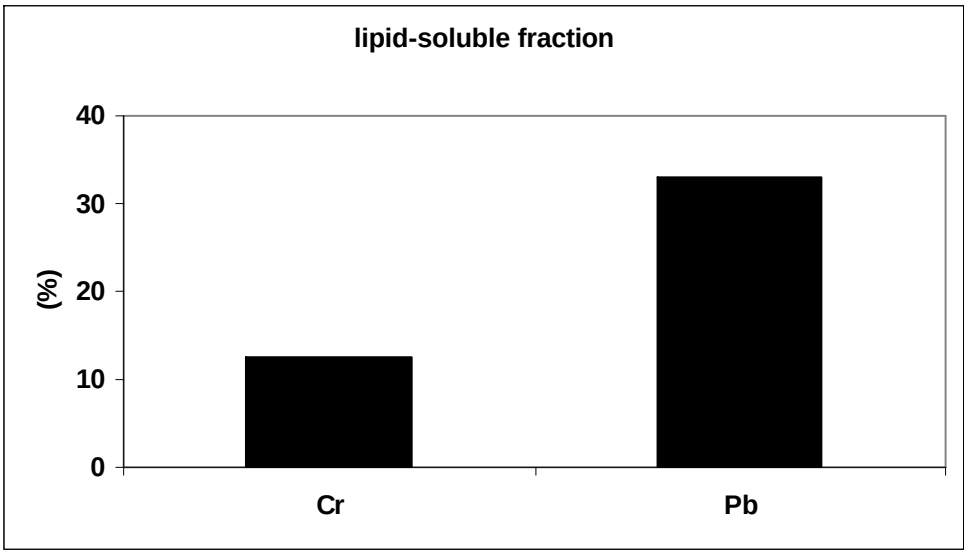
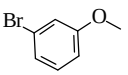
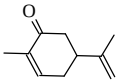
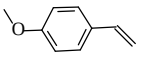
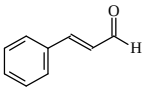
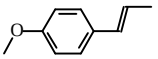
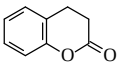
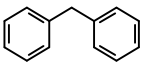
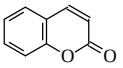
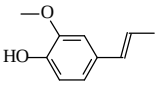
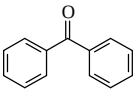
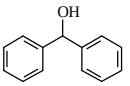
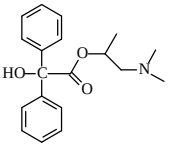
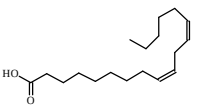
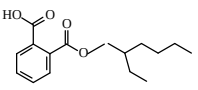


Fig.2. Lipid-soluble fraction of microelements as percentage of the total concentration

3.2. Organics fractionation

Organic fractions obtained after extraction of methanol soluble substances with petroleum ether, $CHCl_3$ and ethylacetate were investigated by GC-MS. The chromatogram for petroleum ether is presented in Fig. 3a. Some of the identified compounds are summarized in Table 6. There is no substantial difference in the composition of the petroleum ether and $CHCl_3$ fractions. Ethylacetate fraction contains the most polar compounds in considerable quantity. These three fractions contain constituents specific for essential oils.

Table 6. GC-MS identified compounds in organic fractions. PE-petroleum ether, CL-CHC_3, EA- Ethylacetate

Name	Structure	PE	CL	EA	RT
benzofuran, 2,3-dihydro-		+	+	-	**12.289**
1-Bromo-3 methoxybenzene		+	+	-	**13.453**
5-isopropenyl-2-methyl-2-cyclohexen-1-one		+	+	-	**15.650**
4-methoxybenzene-carbaldehyde		+	+	-	**16.010**
3-phenyl-2-propenal		+	+	-	**16.560**
1-methoxy-4-1-propenylbenzene		+	+	-	**17.090**
Name	**Structure**	PE	CL	EA	**RT**
2H-1-benzopyran-2-one, 3,4-dihydro-		+	+	-	**20.740**
1-benzylbenzene		+	+	-	**21.960**
2H-1-benzopyran-2-one		+	+	-	**22.420**
2-methoxy-4-[(E)-1-propenyl]benzenol		+	+	-	**24.460**
diphenylmethanone		+	+	-	**27.806**
diphenylmethanol		+	+	-	**28.120**
2-(dimethylamino)-1-methylethyl 2-hydroxy-2,2-diphenylacetate		+	+	+	**34.044**
(9Z,12E)-9,12-octadecadienoic acid		-	+	+	**41.405**
2-[(2-ethylhexyl)oxy]carbonylbenzenecarboxylic acid		-	-	+	**49.110**

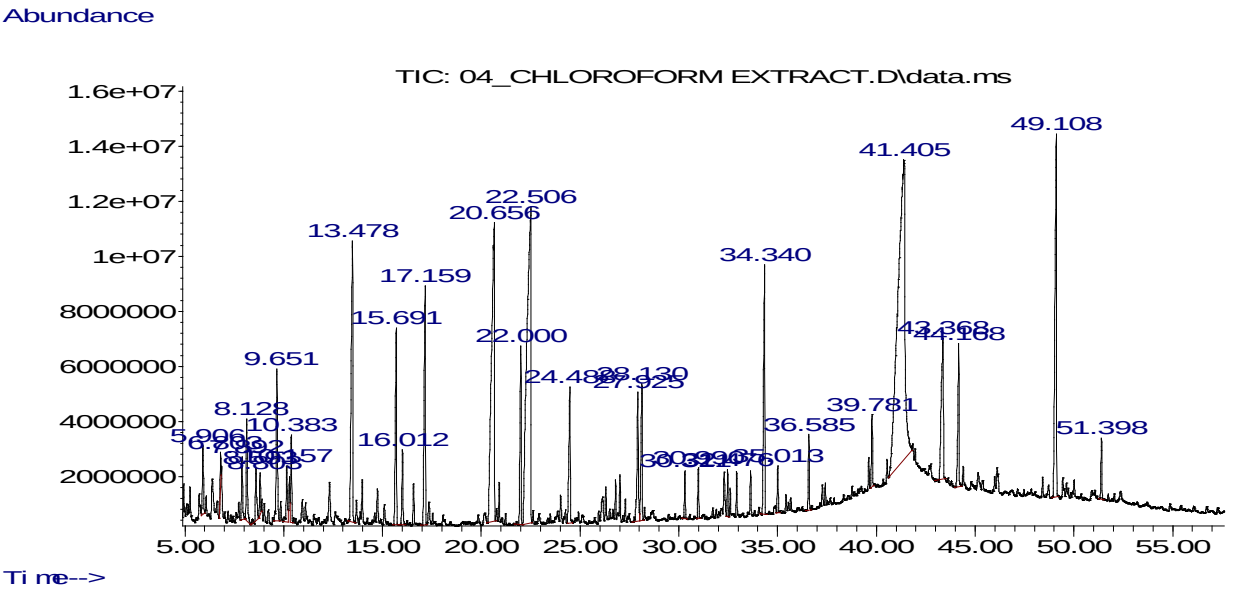


Fig. 3. (a) GC-MS TIC of chloroform extract from NS1. HP-5/MS, 30m×0.250 mm×0.25 µm ft; $T_{(oven)}$: 50°C (5 min), 4°C/min to 280°C (10 min); EI 70 eV ;

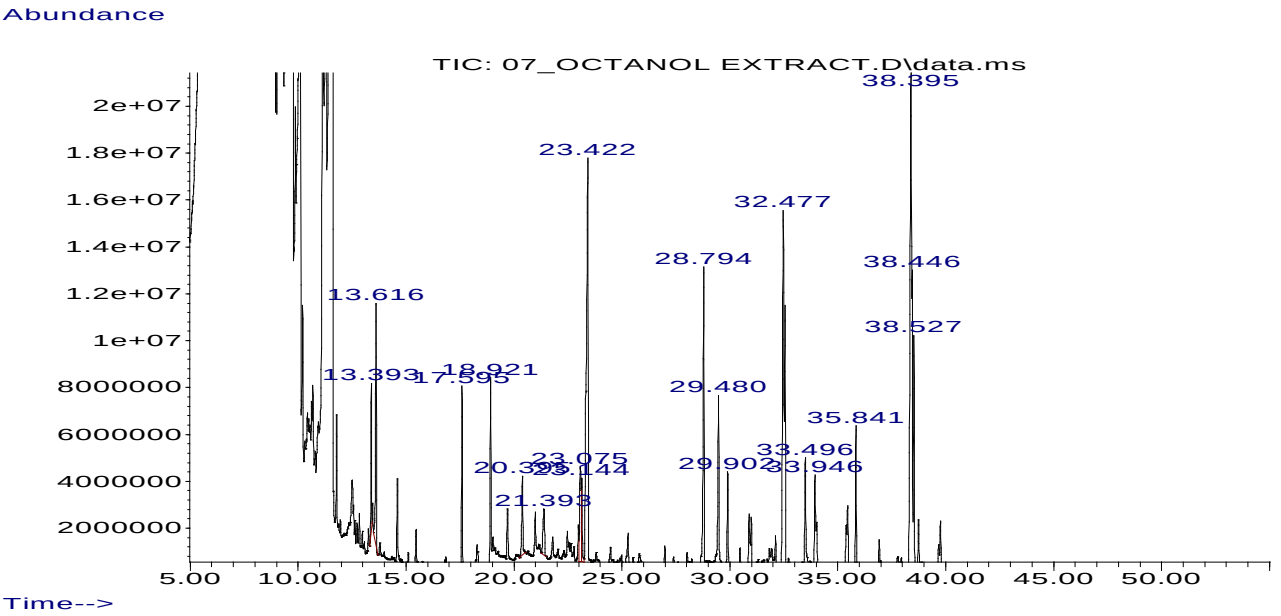


Fig. 3. (b) GC-MS TIC of octanol extract from NS1. HP-5/MS, 30m×0.250 mm×0.25 µm ft; $T_{(oven)}$: 50°C (5 min), 4°C/min to 280°C (10 min); EI 70 eV

The chromatogram for the *n*-octanol fraction is presented in Fig. 3b. Analyzing GC-MS data we uncovered in this fraction octal esters of short-chain alcanoic acids, some aromatic ethers as di-phenyl ether, di-benzyl ether, 3-phenyl-2-propenoic acid and a few free volatile organic acids.

4. Conclusion

It was found that 0.15 mg Fe, 0.02 mg Cu, Mn, Zn, 0.2 μg Cr and 0.08 μg Ni from one gram herbal supplement HNS8 are potentially bioavailable. Lead species are below the detection limit of the method. Quantitative extraction of chromium into octanol under stomach (pH 1) or intestinal (pH 7.6) conditions was established. The organic fractionation needs more detailed investigations in order to obtain a correlation with the expected therapeutic action.

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Corresponding author: Christo Chanev

Address: 1164 Sofia, bld. J. Bourchier 1, Faculty of Chemistry, University of Sofia, Bulgaria,

Phone: (+359 2 8161 249)

Fax: (+359 2 962 54 38)

E-mail: hchanev@chem.uni-sofia.bg,