Gas Chromatography in Environmental Sciences and Evaluation of Bioremediation

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1. Introduction

Crude oil and its derivatives, as the key energy-generating substances and raw materials used for production, are very widely used in all domains of work and everyday life. With the advent of oil as a fuel, there was the most intense economic growth and it can be said that the entire modern civilization is based on the utilization of oil.

However, rapid growth and development of civilization in the past two centuries with the mass use of fossil fuels has led to imbalances and distortions of natural processes. In spite of improvements in technology and equipment used for oil drilling, transport and processing by the petroleum industry, oil and oil derivatives represent a significant source of environmental contamination.

Both soil and water become contaminated by oil and oil derivatives due to accidental spills in their exploitation, transportation, processing, storing and utilization. In 2010, 3.91 billion tons of crude oil was produced (BP Statistical Review, 2011) and estimations are that annually 0.1% of produced petroleum is released into the environment (Ward et al., 2003) as a result of anthropogenic activities.

Petroleum and petroleum products are complex mixtures consisting of thousands of compounds that are usually grouped into four fractions: aliphatics, aromatics, nitrogen–oxygen–sulphur (NSO) compounds and asphaltenes. Asphaltenes are generally solvent insoluble and resistant to biodegradation. Aliphatic hydrocarbons consist of normal alkanes (n-alkanes), branched alkanes (isoalkanes) and cyclic alkanes (naphthenes). Isoalkanes, naphthenes and aromatics are much less biodegradable than *n*-alkanes (Evans & Furlog 2011; Pahari & Chauhan, 2007). The fraction of saturated hydrocarbons is the dominant fraction in most oils as compared to aromatic hydrocarbons and NSO compounds (Peters et al. 2005).

When crude oil or petroleum products are accidentally released into the environment, they are immediately subject to a wide variety of weathering process (Jordan & Payne, 1980). These weathering processes can include: evaporation, dissolution, microbial degradation, dispersion and water–oil emulsification, photooxidation, adsorption onto suspended

particulate materials, and oil–mineral aggregation. Petroleum compounds are substrates for microorganisms which can use these substances as the sole source of hydrocarbons (Head et al., 2006; Van Hamme et al., 2003). The susceptibility of hydrocarbons to microbial attack is ranked in the following order n-alkanes > branched alkanes > branched alkenes > n-alkylaromates of small molecular mass > monoaromates > cyclic alkanes > polycyclic aromates > asphaltenes (Alexander, 1999; Atlas & Philp, 2005; Singh & Ward, 2004).

Bioremediation is a technology of cleaning and remedying the soil through biological methods by means of non-pathogenic microorganisms that feed on the contaminating substances. The microorganisms are used to reduce the complexity of organic molecules (biotransformation), or for degradation to complete mineralization (biodegradation). Some defined bacterial species are able to degrade, to a limited extent, all hydrocarbons present in heavy fuel oil or oil sludge. Some of the polluting components may be dissolved only by the coupled metabolic activity of multiple genera of microorganisms. A consortium (mixed culture) of microorganisms can conduct these complex processes of degradation, while at the same time, being more resistant, on average, to changes in the ecosystem than just a single microbial species (Brenner et al., 2008).

To increase the rate of biodegradation of hydrocarbons in the ecosystem and to maximize the process in bioremediation technologies, three main approaches are applied: biostimulation, in which nutrients are added to stimulate the intrinsic hydrocarbon degraders, bioventilation which ensures the required quantity of the molecular oxygen – aeration, and bioaugmentation, in which microbial strains with specific degrading abilities are added to work cooperatively with normal indigenous soil microorganisms (Alvarez & Illman, 2006). The contaminated soil can be treated by bioremediation *in situ* or *ex situ*. As a natural cleaning process, bioremediation has been proven to be efficient in the removal of crude oil and oil derivatives (Ollivier & Magot, 2005), chlorinated solvents (Bamforth & Singleton, 2005; Gavrilescu, 2005), and even some heavy metals (Seidel, 2004).

In order to optimize bioremediation, continuous monitoring is required. One indicator that is critical for bioremediation of soil polluted with crude oil, and which should be monitored, is total petroleum hydrocarbons (TPH). According to the most widely used method, ISO 16703 (ISO 16703, 2004), TPH represents the total content of hydrocarbons ranging from C_{10} through C_{40} which originate from petroleum. Data observed are used to set the end-point of bioremediation in the fulfillment of legal and regulatory criteria, and together with microbiological indicators are used for assessing the biodegradation potential of contaminated soil.

Several review papers have been published recently about new gas chromatography (GC) techniques and their application (Cortes et al., 2009; Marriott et al., 2003). This review presents a brief overview of the GC techniques, especially gas chromatography–mass spectrometry (GC–MS) that are currently applied in differentiating and fingerprinting oil hydrocarbons, identifying oil spills in assessments of environmental impacts, and in following up the efficacy of bioremediation procedures. It is focused on up-to date results observed in differentiation and transformation studies on petroleum-type pollutants in underground and surface water from the locality of Pančevo Oil Refinerty, Serbia (River Danube alluvial formations), and trends in the analysis methods of oil hydrocarbon biomarkers for monitoring the industrial-level bioremediation process in soil polluted with oil derivatives (Beškoski et al., 2010; Beškoski et al., 2011; Gojgić-Cvijović et al., 2011;

Jovančićević et al., 1997). The application area of GC in environmental sciences in the context of this work relates to monitoring the changes of oil pollutants in natural environments and during bioremediation as a controlled process of microbiological transformation and degradation.

2. Gas chromatography as analytical method of choice in environmental sciences

A wide variety of analytical methods and techniques are currently used in the examination of environmental samples, which include GC, GC–MS, high-performance liquid chromatography (HPLC), size exclusion HPLC, infrared spectroscopy (IR), supercritical fluid chromatography (SFC), thin layer chromatography (TLC), ultraviolet (UV) and fluorescence spectroscopy, isotope ratio mass spectrometry, and gravimetric methods. GC technique is the most widely used and today it is very hard to imagine an environmental laboratory without at least a gas chromatograph (Wang et al., 1999).

2.1 Gas chromatography

Chromatography is the method of separation in which several chemicals to be separated for subsequent analyses are distributed between two phases. In GC, separation is based mainly on the partitioning between a gas mobile phase and a liquid stationary phase. It is estimated that more than 60 types of GC detectors have been developed. For analysis of samples from the environment the few most commonly used are flame ionization detector (FID), thermal conductivity detector (TCD), electron capture detector (ECD), nitrogen-phosphorous detector (NPD), flame photometric detector (FPD), photo ionization detectors (PID) and mass selective detector or mass spectrometer (MS) which also allows qualitative and quantitative analysis (Driscoll, 2004). The GC-FID technique is a routine technique for the quantitative analysis of all the non-polar hydrocarbons which are extracted by applied solvents (*n*-hexane or acetone / *n*-heptane), and it has various scopes, depending on the standard (ISO 16703, 2004; Jovančićevič et al., 1997; Jovančićević et al., 2007). It is also used for rapid semiquantitative assessments of the successfulness of bioremediation treatments of polluted environments (water, soil, sediments) or the decomposition of hydrocarbon materials originating from oil under natural conditions or historical pollution (Beškoski et al. 2011; Hinchee and Kitte, 1995; Jensen et al., 2000; Jovančićević et al., 2008; Milic et al., 2009). GC methods are sensitive, selective and can be used to determine the specific target compounds. In environmental science GC methods are mainly used for: identifying organic pollutants in recent sediments, following abiotic and biotic transformations of petroleum-type pollutants, improving our understanding of migration mechanisms of organic pollutants in soil/water/air environments, distinguishing the oil pollutant from the native organic substance of recent sediments, fingerprinting and differentiation of petroleum-type pollutants, following transformation of petroleum pollutant during soil bioremediation experiments, determining other organic pollutants such us POP's (persistent organic pollutants) and analysing petroleum biomarkers.

2.2 Gas chromatography coupled with mass spectrometry

GC-MS is routinely applied to identify the individual components of petroleum hydrocarbons. These methods have high selectivity and compounds can be authenticated by

analysing retention times and unique mass spectra. GC-MS can confirm the presence of the target analyte and the identification of untargeted analyte and also can be used for the separation of hydrocarbons into the groups. The main drawback of these methods is that isomeric compounds can have identical, and many different compounds can have similar mass spectra. Heavy fuel oil can contain thousands of components that cannot be separated in the gas chromatograph. Different compounds may have the same ions, which complicates the identification process (Jensen et al, 2000). Determination of TPH is possible using different methods - gravimetrically (DIN EN 14345, 2004), by infrared spectroscopy (ISO/TR 11046, 1994) or GC (ISO 16703, 2004). All these methods can be used for the quantitative analysis of analytes; however, when it comes to a mixture of compounds which is often the case with environmental samples, qualitative analysis can be realized only by GC-MS.

2.3 Novel GC techniques

The common techniques listed above, have now been in use for several decades but new techniques offer greater opportunities in this area. GC coupled to high-resolution time-of-flight mass spectrometry (GC–TOF–MS) has been applied for non-target screening of organic contaminants in environmental samples (Serrano et al., 2011). GC–TOF–MS has been successfully applied for screening, identification and elucidation of organic pollutants in environmental water and biological samples (Hernández et al., 2011) and also for confirmation of pollutants in a highly complex matrix like wastewater (Ellis et al., 2007). The strong potential of GC–TOF–MS for qualitative purposes comes from the full spectrum acquisition of accurate mass, with satisfactory sensitivity.

Comprehensive two-dimensional GC (GC×GC) coupled with MS has been widely applied in environmental analyses in the last decade (Ieda et al., 2011). The GC×GC–MS method has many practical advantages, e.g. high selectivity, high sensitivity, large separation power, group type separation and total profiling. Panić and Górecki reviewed GC×GC in environmental analyses and monitoring and they indicated that the main challenge in environmental analysis is that the analytes are usually present in trace amounts in very complex matrices (Panić & Górecki, 2006). In overcoming this problem, GC×GC–MS is a very powerful and attractive system that has been successfully applied for powerful identification of polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) (de Vos et al., 2011), polychlorinated biphenyls (PCBs) (Focant et al., 2004; Hoh et al., 2007), polychlorinated naphthalenes (PCNs) (Korytár et al., 2005), nonyl phenol (NP) (Eganhouse, et al., 2009), benzothiazoles, benzotriazoles, benzosulfonamides (Jover et al., 2009), Cl-/Br-PAH congeners (Ieda et al., 2011), pharmaceuticals and pesticides (Matamoros et al., 2010) in complex environmental samples.

2.4 Targets of GC and GC-MS in environmental analysis

In the fraction of saturated hydrocarbons the most predominant are *n*-alkanes and isoprenoid aliphatic alkanes. *n*-Alkanes in oil may be present in various quantities, most often as C_{10}-C_{35}, and among isoprenoids the predominant are C_{19}, pristane (2,6,10,14-tetramethyl-pentadecane) and C_{20}, phytane (2,6,10,14-tetramethylhexadecane). These molecules are used as biomarkers (Beškoski et al, 2010; Jovančićević et al., 2007; Peters et al., 2005) and their analysis is applied in organic geochemistry, environmental chemistry and studies of biodegradation (Figure 1a and 1b).
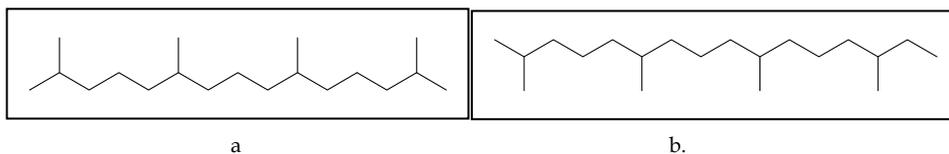
Fig. 1. a) Pristane, b) Phytane.

For the purpose of rigorous quantification of biodegradation and after the degradation of pristane and phytane, compounds of polycyclic hydrocarbons of the sterane-type (C_{27}-C_{29}) and terpane (tri-, tetra- and pentacyclic; C_{19}-C_{35}) may be used as a conserved internal standard (Figure 2a and 2b). They are most often found in much smaller quantities. Steranes and terpanes are very resistant to biodegradation and because of this feature they are exquisite biomarker compounds in comparison to which all other biodegradable compounds may be normalized. In addition, due to weak biodegradability, they are enriched in residual oils by the aging process, vaporization and biodegradation (Gagni & Cam, 2007; Jovančićević et al., 2008; 1997; Wang et al, 2005).
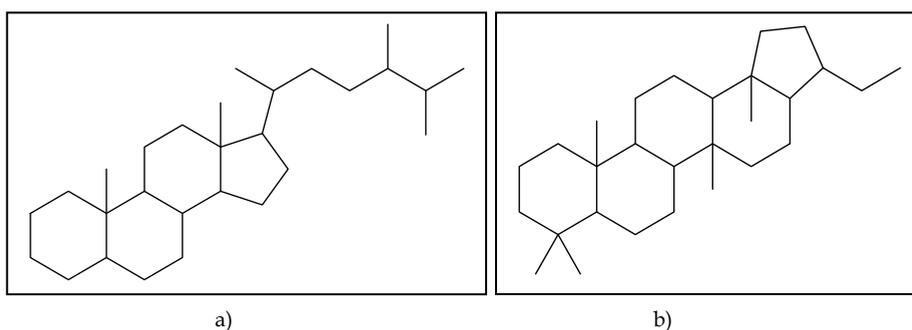

Fig. 2. a) Sterane, b) Triterpanes (hopanes)

The ratio between normal and isoalkanes depends on the degree of weathering since *n*-alkanes first undergo biodegradation.

Recently, compounds of sesquiterpane are increasingly being used as biomarker compounds. Most sesquiterpane compounds originate most probably from higher order plants and simple algae and bacteria. The introduction of so called new biomarker compounds resulted from the fact that in the case of lighter oil fractions, such as jet fuel and diesel, most of the high molecular biomarker compounds are removed during the process of refinement. Therefore, pentacyclic terpanes and steranes in these oil fractions are present in very small quantities, while sesquiterpane are concentrated in these fractions (Gagni & Cam, 2007; Wang et al., 2005).

For the analysis of oil and biomarkers, GC-MS is predominantly used in the selected ion monitoring mode (SIM). This method is applied in evaluating raw oil, in monitoring of biodegradation processes and in the process of bioremediation of oil that is released into the environment. Isoprenoids are identified from *m/z* 183, steranes from *m/z* 217 and triterpanes from *m/z* 191 selected ion chromatograms obtained from analysis in the SIM mode. Based on the abundance and distribution of biomarkers, reliable information about the origin and geological history of oil may be obtained. Therefore, the significance of these biomarkers in

organic and geochemical investigations is great. Analytical data are also successfully applied in order to make a distinction between oils from various deposit layers. On the other hand, in environmental chemistry the determination of the presence of oil-type pollutants in the environment may be useful, e.g. for the distinction between native and anthropogenic organic substances (Jovančićević et al. 1997; Jovančićević et al., 2007), to follow up oil contamination, to determine the origin of long-term oil contamination and for forensic investigations of the origin of oil spills, to monitor the transformation due to atmospheric conditions (weathering), and to monitor biodegradation processes and the stage of the aging process of oil and its derivates under various conditions (Wang et al. 2005). GC and GC-MS methods are very selective and they offer a chance for a better understanding of the procedures of guided or natural biodegradation (Wang & Fingas, 2003). However, it is found that in highly degraded asphalt pavement samples, even the most refractory biomarker compounds showed some degree of biodegradation, and that the biomarkers were generally degraded in declining order of importance diasterane > C_{27} steranes > tricyclic terpanes > pentacyclic terpanes > norhopanes ~ C_{29} αββ-steranes (Wang et al., 2001).

A typical gas chromatogram of the TPH extract of soil polluted with diesel oil is presented in Figure 3. Chromatograms of petroleum hydrocarbons usually exhibit peaks of *n*-alkanes and a typical hump of unresolved components in different sections of the chromatogram.
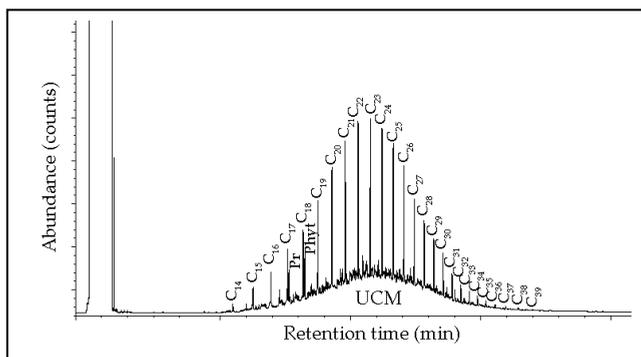

Fig. 3. Gas chromatogram of TPH of the soil recently polluted with diesel oil (Pr: Pristane; Phyt: Phytane; UCM: Unresolved Complex Mixture).

These peaks exhibit smooth distribution with one or several maxima and, possibly, alternating peak intensities of components with even and odd numbers of carbon atoms. Pristane and phytane peaks are observed in the vicinity of peaks of $C_{17}H_{36}$ and $C_{18}H_{38}$ alkanes; low intensity peaks of isoalkanes, cycloalkanes, and aromatic hydrocarbons appear between peaks of *n*-alkanes. Unresolved Complex Mixture (UCM), or ''hump'' of hydrocarbons (Rogers & Savard, 1999), is a common feature of the gas chromatograms of crude oils and certain refined products, and it is especially pronounced for weathered and biodegraded oils and oil-polluted sediment extracts. It is perhaps surprising that until recently virtually nothing was known about UCM compositions and molecular structures, even though the concentrations of these components in oils are significant (Frysinger et al., 2003; Wang & Fingas, 2003). In aged petroleum products, peaks of *n*-alkanes may be very weak against the hump or even absent from the chromatogram.

2.5 International GC standard methods

There are three major categories of GC-based US EPA methods. The first group is the US EPA methods for the determination of toxic organic compounds in air. The second group of GC-based methods was developed by the US EPA for water and wastewater, including 500 and 600 series methods. The two most important methods in the EPA 600 series are methods 624 and 625. Method 624 is a GC-MS method for purgeable organic compounds and method 625 measures semivolatile organics. If certain compounds are detected by method 625, they should then be confirmed with a GC method specific to that compound (methods 601, 602, 603, and 612 for 624 and methods 604, 606, 607, 609, 610, 611, and 612 for 625). The third group comprises approximately 27 GC-based methods, known as the 8000 series methods published in SW-846, and it concerns waste analysis.

3. GC for identification and determination of oil pollutant in the environment

For identification and determination of oils and petroleum products pollutants in the environment, the following structural features were used in GC and GC-MS:

- typical shape of chromatograms (fingerprints) of petroleum products;
- peaks of n-alkanes (C_6 through C_{40}) in chromatograms;
- measurement of total petroleum hydrocarbons;
- shape of the UCM;
- stable carbon isotope ratio ($\delta^{13}C$) is also included in many cases;
- close-to-unity ratio between n-alkanes with even and odd numbers of carbon atoms;
- presence of some isoalkanes, including pristane and phytane and in some cases farnesane, trimethyl-C_{13}, and norpristane isoprenoids;
- ratio between phytane and pristane and the closest $C_{17}H_{36}$ and $C_{18}H_{38}$ n-alkanes;
- presence of biomarkers (isoprenoids, steranes, triterpanes, etc.);
- predominance of methyl- and alkyl-substituted mono-, bi-, and polynuclear aromatic hydrocarbons over unsubstituted aromatic hydrocarbons;
- volatile hydrocarbons including BTEX (benzene, toluene, ethylbenzene, and three xylene isomers) and alkylated benzenes (C_3- to C_5-benzenes), volatile paraffins and isoparaffins, and naphthenes (mainly cyclopentane and cyclo-hexane compounds)
- distribution (profile) of polycyclic aromatic hydrocarbons (PAHs) and the petroleum-specific alkylated (C_1–C_4) homologues of selected PAHs;
- typical profile of sulfur-containing aromatic hydrocarbons;
- determination of NSO heterocyclic hydrocarbons for oil spill identification using ratio between different hydrocarbon groups (group composition);
- specific ratio between the concentration of PAHs and the background (Brodskii et al., 2002).

3.1 Environmental forensic - Fingerprinting of oil spills

Variability in chemical compositions results in unique chemical ''fingerprints'' for each oil and provides a basis for identifying the source(s) of the spilled oil. Since petroleum contains thousands of different organic compounds, successful oil fingerprinting involves appropriate sampling, analytical approaches and data interpretation strategies. Sampling is

crucial, because if the sampling is not conducted properly and the sample is not representative, it will produce a result which will not accurately reflect the situation in the field. Also, in order to forensically fingerprint oil spills, it is necessary to select source-specific target analytes.

GC is in a central part of the fingerprinting as presented in the oil spill identification protocol. Figure 4 presents the modified ''Protocol/decision chart for the oil spill identification methodology''. The final assessments are concluded by the four operational and technical defensible identification terms: positive match, probable match, inconclusive or non-match (Wang & Fingas, 2003).
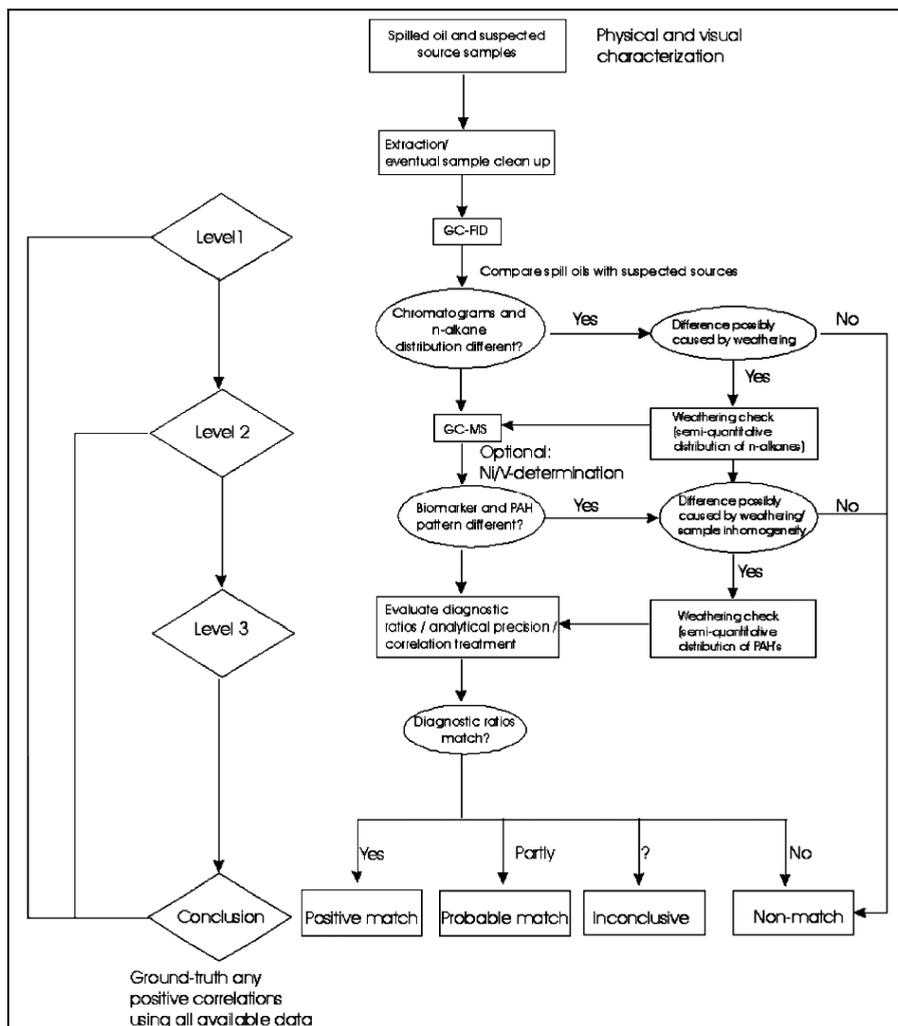
Fig. 4. Decision chart for the oil spill identification methodology (from Daling et al., 2002 as cited in Wang & Fingas, 2003).

3.2 Identification and determination of oil pollutant in the environment

GC was used for differentiation of petroleum-type pollutant and to study the fate of petroleum-type pollutants in underground and surface waters. Water and particulate matter are derived from the locality of Pančevo Oil Refinery, Serbia (River Danube alluvial formations), an area described in detail by Kaisarevic et al., (2009).

3.2.1 Determination of oil pollutant by *n*-alkane distribution and $\delta^{13}C$ analysis

Identification of petroleum-type pollutants in recent sediments, soil, underground or surface waters, requires reliable and precise differentiation of native and anthropogenically released substances. The compositional differences are mainly pronounced with *n*-alkanes, one of the most abundant fractions in soluble sedimentary organic matter. An example of typical petroleum *n*-alkane distribution (in the form of a gas chromatogram of sample I1 representing a petroleum-type pollutant found in underground water from the area of Pančevo Oil Refinery), and several examples of distributions characteristic of organic substance derived from recent sediments, typical for all other samples (B2, B4, B6, B8, B10, B11, D1 and D2) originating from different localities of the River Danube alluvial formations, are shown in Figure 5 (Jovančićević et al. 1997). *n*-Alkane fraction in sample I1 was characterized by uniform distribution of odd and even homologues and a maximum at a lower member (*n*-C_{19}). The carbon preference index (CPI) a ratio of the sum of odd-numbered hydrocarbons to the sum of even-numbered hydrocarbons was around 1. On the other hand, the distribution of *n*-alkanes in nonpolluted sediments was characterized by domination of odd homologues (CPI considerably above 1) and a maximum at some of the higher homologue members (*n*-C_{29} or *n*-C_{31}). The soluble sedimentary organic substance, called bitumen, was generally found in very small amounts. Its higher content in sediments may therefore indicate the presence of anthropogenic contaminants. The conclusion is that *n*-alkanes may successfully be used for differentiating the petroleum-type pollutants, as anthropogenic organic substances, from native organic matter in recent sedimentary formations.
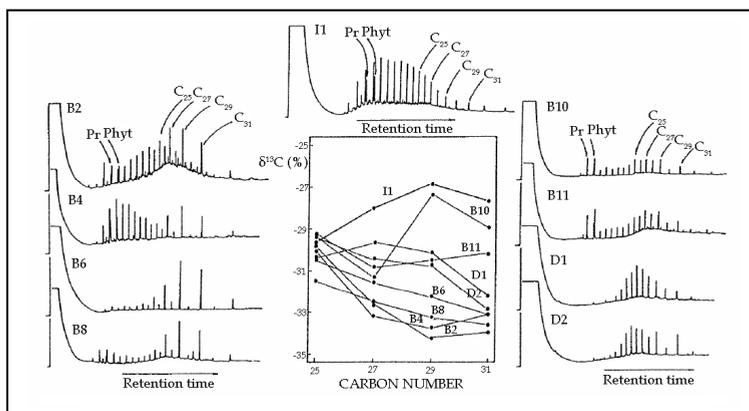

Fig. 5. Distributions of *n*-alkanes and $\delta^{13}C_{PDB}$ values of individual *n*-alkanes of recent sediments' bitumen fractions and one oil type pollutant from Pančevo Oil Refinery locality; Pr: Pristane; Phyt: Phytane; (from Jovančićević et al., 1997).

An alternative method to solve the problem may be $\delta^{13}C$ analysis. Being a mature organic substance in the geosphere, crude oil contains the greatest amount of the heavier carbon isotope, ^{13}C. Consequently, the ratio of ^{12}C and ^{13}C carbon isotopes $\delta^{13}C_{PDB}$ in petroleum is less negative compared to organic matter in recent sediments. For example, the sample I1 presented in Figure 5 (Jovančićević et al., 1997) showed less negative $\delta^{13}C_{PDB}$ with C_{25}, C_{27}, C_{29} and C_{31} *n*-alkanes compared to all other samples. As mentioned above, this particular sample was characterized by a typical petroleum *n*-alkane distribution, in contrast to all other samples demonstrating distributions typical for recent sediments. Hence, it is suggested that environmental native and anthropogenic organic substances may be differentiated based on comparison of their carbon isotopes ratios – in other words, $\delta^{13}C_{PDB}$ may be used as a tool for revealing petroleum-type pollutants in the environment. For this purpose gas chromatographic-isotope ratio mass spectrometric (GC-IRMS) analysis of single compounds is required, or the analysis of its corresponding fractions.

In the case when differentiation of native and anthropogenic origin of an organic substance based on distribution of *n*-alkanes or carbon isotope analysis becomes questionable, analysis of polycyclic alkanes of sterane and triterpane types may be helpful (Jovančićević et al., 1998; Jovancicevic et al., 2007), since the distribution of these polycyclic alkanes is typical for crude oils. The use of *n*-alkanes and polycyclic alkanes of the sterane and triterpane types can be defined as an "organic-geochemical approach" in the identification of oil type pollutants in the environment.

3.2.2 Monitoring transformation processes of petroleum type pollutants

Petroleum-type pollutants in recent sediments, soil, underground or surface waters are exposed to microbial degradation (Jovančićević et al., 2003). The degradation intensity differs depending on a great number of biological, chemical and physicochemical parameters. The process of biodegradation of petroleum type pollutants in underground waters from Danube alluvial sediments (the locality of Pančevo Oil Refinery) was followed through a period from November 1997 to February 2000 by GC analyses of isolated alkane fractions. The corresponding gas chromatograms are shown in Figure 6.

In underground waters, a petroleum-type pollutant is exposed to microbiological degradation which is manifested through relatively fast degradation of *n*-alkanes. In the period from November 1997, when the first sample was taken, to February 2000, when the fifth sample was taken, important changes in the chemical composition were obvious. The relative contribution of *n*-alkanes as compared to pristane and phytane in sample A indicated changes defined as "initial petroleum biodegradation". The abundance of C_{17} and C_{18} *n*-alkanes was somewhat smaller than the abundance of pristane (C_{19}) and phytane (C_{20}). In the period from November 1997 to May 1998 the amount of *n*-alkanes relative to isoprenoids was reduced, a phenomenon typical for biodegradation intensity in geochemical literature defined as "minimal biodegradation". In September 1998 (sample C), the amount of *n*-alkanes was still smaller. Finally, during the next year, *n*-alkanes were almost completely degraded (sample D) while pristane and phytane remained nonbiodegraded. Comparison of Pr/*n*-C_{17} and Phyt/*n*-C_{18} ratios observed in samples A–C (winter 1997–autumn 1998) confirmed that biodegradation was considerably more intensive during the summer period than during the winter or spring periods.
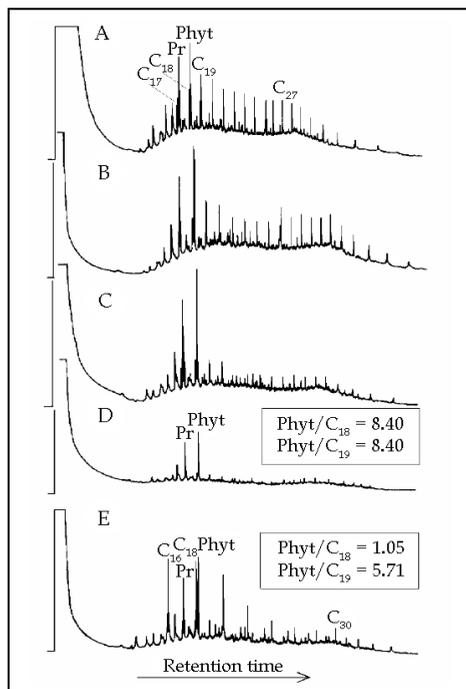
Fig. 6. Gas chromatograms of alkanes isolated from oil polluted alluvial ground waters (Pančevo Oil Refinery locality). Samples were taken in November 1997 (A), May 1998 (B), September 1998 (C), September 1999 (D) and in February 2000 (E); Pr: Pristane; Phyt: Phytane; (from Jovančićević et al., 2001).

In a relatively short period of time, from September 1999 to February 2000, the alkane fraction of the petroleum-type pollutant suffered an unexpected change. Namely, while pristane and phytane were found in the same amounts characterized by approximately the same ratios, in this fraction of the pollutant, new even carbon-number C_{16}–C_{30} n-alkanes were observed. Such a significant change raised the question of possible new contamination during this period of time, e.g., by a pollutant characterized by dominating even carbon-number n-alkanes. However, identical distributions of both steranes and triterpanes in sample D and E were excellent evidence that no new pollution occurred (Jovančićević et al., 2001). Detailed analyses of extracts obtained from samples D and E were conducted. Sample E, containing even n-alkane homologues, contained, also in the alcoholic fraction, a homologous series of even carbon-number alcohols in the C_{14}–C_{20} range and a relatively significant amount of cholesterol (Figure 7). On the other hand, sample D, which did not contain any significant amounts neither of odd nor even n-alkane homologues, also did not contain any alcohols or higher fatty acids.

Even carbon-number alcohols and fatty acids observed in sample E were taken as a proof of the presence of particular microorganisms, i.e., of unicellular algae of *Pyrrophyta* type known as "fire algae". These types of microorganisms are able to synthesize even n-alkane homologues in a suitable base such as petroleum-type pollutants.
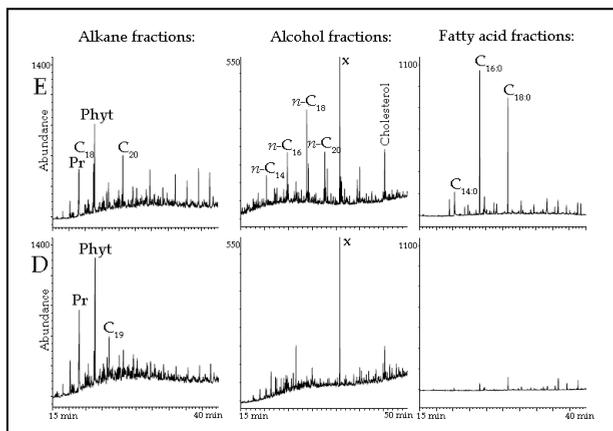
Fig. 7. Total ion chromatogram of GC-MS analyses of fractions of alkanes, alcohols and fatty acids (their methyl-esters), isolated from extracts E and D; Pr: Pristane; Phyt: Phytane; (from Jovančićević et al., 2003).

4. GC in evaluation of bioremediation

In evaluating bioremediation, GC was applied in the estimation of the bioremediation potential of microorganisms for crude oil biodegradation, and also in the monitoring of *ex situ* bioremediation of a soil contaminated by mazut (heavy residual fuel oil). In that study, GC was used for analysis of TPH and *n*-alkane composition, analysis of transformation of isoprenoids, steranes and terpanes and for determination of bioremediation effects on tricyclic aromatic hydrocarbons. In general, GC was used for analysis of TPH and biomarkers to evaluate the successfulness of applied bioremediation treatments.

4.1 Study of the bioremediation potential of microorganisms for crude oil biodegradation

The bioremediation potential of zymogenous microorganisms isolated from soil was investigated under controlled laboratory conditions using a mixture of paraffinic types of oils as a substrate (Šolević et al., 2011). The ability and efficiency of these microorganisms in crude oil bioremediation was assessed by comparing the composition of samples which were exposed to the microorganisms with a control sample which was prepared and treated in the same way, but containing no microorganisms. Biodegradation was stopped by sterilization at 120 °C for 25 min, after which samples were taken on 15, 30, 45, 60, and 75 days, while the control experiment was sampled only after 75 days and hydrocarbons were analyzed by the GC–MS.

The total ion current (TIC) chromatogram of the hydrocarbon fraction from the control and treated samples are shown in Figure 8. The dominant compounds in the hydrocarbon fraction of the control sample were *n*-alkanes and the isoprenoids pristane and phytane. These preliminary analyses, based on the TIC chromatograms showed a gradual decrease in the amount of *n*-alkanes and isoprenoids during 45 days. After 60 days of the experiment, *n*-alkanes and isoprenoids could not be observed in the TIC chromatograms indicating their

possible complete degradation. At the end of the study, the TIC chromatograms were dominated by sterane biomarkers as the most abundant compounds in the fraction of hydrocarbons. The dominant compounds in the hydrocarbon fraction of the control sample were *n*-alkanes and the isoprenoids pristane and phytane.
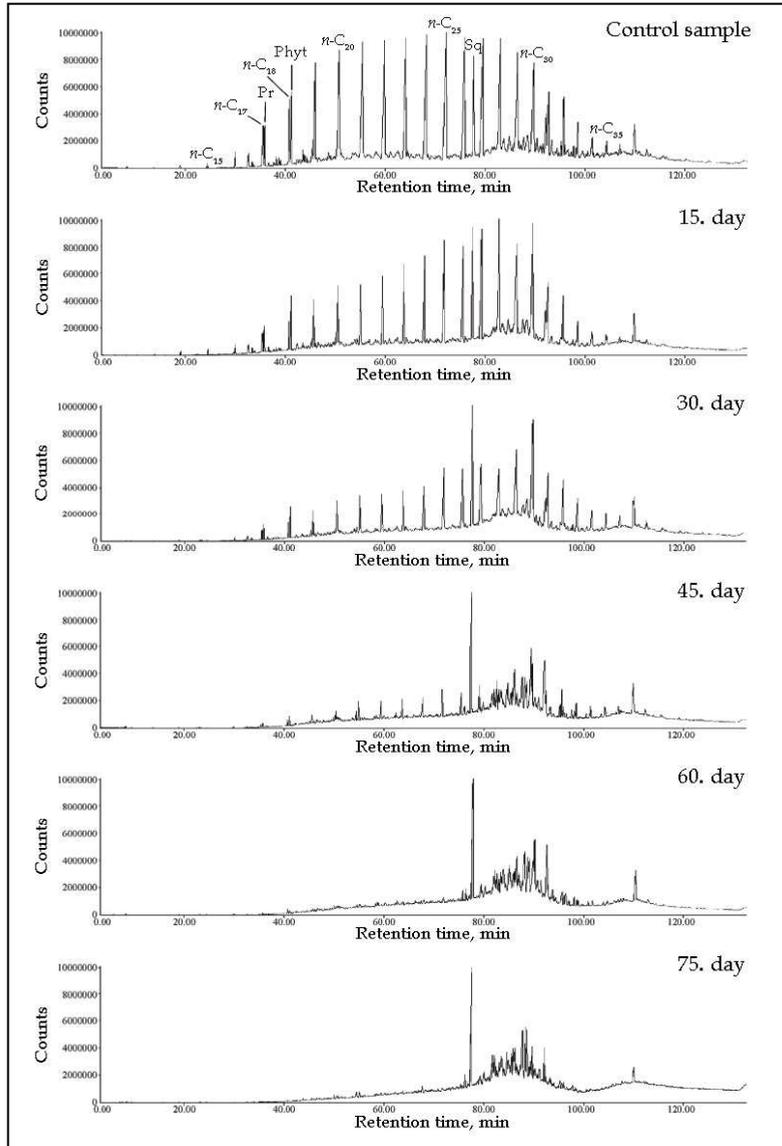

Fig. 8. Total ion chromatograms of the hydrocarbon fractions isolated from the extracts of the control sample and from the samples during the biodegradation experiment after 15, 30, 45, 60, and 75 days; Pr: Pristane; Phyt: Phytane; Sq: squalane; (from Šolevic et al., 2011).

According to GC-MS analysis by the end of the study, after 75 days of exposure to the microorganisms, the *n*-alkanes and isoprenoids had been completely degraded. The surprisingly low bioremediation potential of these microorganisms was proven in the case of polycyclic alkanes of the sterane and triterpane type and dimethyl phenanthrenes since after 75 days of the study, no significant alteration in the concentration of these compounds was observed. In the class of aromatic hydrocarbons, it was confirmed that the microorganisms had a considerably high bioremediation potential in the biodegradation of phenanthrene and methyl phenanthrenes. This fact can be surprising at first glance, but there is a reasonable explanation. Although the biodegradation of crude oil is often explained as a quasi-stepwise process in which various components are removed in a well-recognized sequence, it is well known that several compound classes are actually degraded simultaneously but at different rates, reflecting differences in the rate of their catabolism under varying conditions (Peters et al., 2005). Numerous studies have shown that aromatic compounds not only can be degraded prior or concomitantly with sterane and terpane biomarkers in reservoir oils, (Huang et al., 2004), but also can be degraded in the environmental conditions during oil spills (Wang & Fingas, 2003).

4.2 *Ex situ* bioremediation of a soil contaminated by mazut (heavy residual fuel oil)

Previously, it was reported that the oil pollutant mixture in the soil treated by *ex situ* bioremediation behaves in a complex way: different degradation rates and time evolutions are observed for fractions of the hydrocarbon mixture characterized by different molecular weights and structures (Beškoski et al., 2010; Jovančićević et al., 2008a, 2008b). Also, a stable microbial community had been formed after initial fluctuations, and the microorganisms which decompose hydrocarbons were the dominant microbial population at the end of the *ex situ* bioremediation process, with a share of more than 80% (range 10^7 colony forming units g^{-1}) (Milic et al., 2009).

While there is significant information in the literature about the microbiological degradation of defined individual hydrocarbons (Singh and Ward, 2004) there is significantly less data about the biodegradability of some commercial petroleum products, including mazut and heavy residual fuel oil (Iturbe et al., 2004). Mazut is a low quality, heavy (chain length 12–70 C atoms) residual fuel oil (ASTM D396-09a, 2009.; ISO 8217, 2005), blended or broken down with the end product being diesel in Western Europe and US and used as a source of heating fuel in Eastern Europe.

A field scale study was designed and conducted in order to evaluate the possibility of using bioremediation for treating a soil contaminated with heavy residual fuel oil such as mazut and mazut waste material (Beškoski et al., 2011). The mazut-polluted soil was excavated contaminated soil from an energy power plant which, due to a break-down, had been polluted with mazut and sediment from a mazut reservoir for a year. The bioremediation biopile contained approximately 600 m³ soil polluted with mazut, mixed with un-graded river sand and softwood sawdust. GC-FID and GC-MS were used for analysis of the process. The techniques of biostimulation, bioventilation and reinoculation of a microbial consortium were performed during a period of 150 days. A part of the materials (10 m³) prepared for bioremediation was set aside uninoculated, and maintained as an untreated control pile subjected to natural bioremediation process.

4.2.1 Change in TPH and *n*-alkane composition

The content of TPH in the soil was extracted as described in ISO 16703 (ISO 16703, 2004) and determined gravimetrically in accordance with DIN EN 14345 (DIN EN 14345, 2004). The contamination level of TPH at the start was found to be 5.2 g kg^{-1} of soil. With application of bacterial consortium and nutrients, the TPH level was reduced to 2.1, 1.3 and 0.3 g kg^{-1} of soil after 50, 100, and 150 days, respectively, meaning 60%, 75% and 94% of the TPH were biodegraded. The analysis of change in the group composition evidenced that the average rate of decrease during the biodegradation of mazut was 23.7 mg kg^{-1} day^{-1} for the aliphatic fraction, 5.7 mg kg^{-1} day^{-1} for the aromatic fraction and 3.3 mg kg^{-1} day^{-1} for the NSO-asphaltene fraction (Beškoski et al., 2010).

The chromatograms (Figure 9) gave qualitative and semiquantitative information on the changes in the composition of hydrocarbons in the samples. As judged from GC, the abundance of *n*-C_{17} and *n*-C_{18} *n*-alkanes at time zero was somewhat smaller than the abundance of pristine (C_{19}) and phytane (C_{20}). This indicates that the hydrocarbons left in the soil were already degraded to some extent during the natural biodegradation process. Around 50% of *n*-alkanes in the size range of C_{29}–C_{35} were biodegraded during the first 50 days. *n*-Alkanes in the range of C_{14}–C_{20} were degraded completely by 100 days, followed by
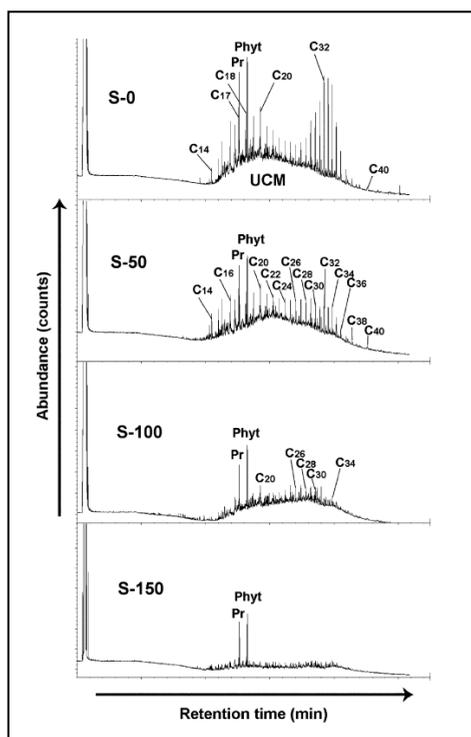

Fig. 9. Chromatograms of the TPH extracts of the treated samples during the bioremediation experiment after 0, 50, 100 and 150 days; Pr: Pristane; Phyt: Phytane; (from Beškoski et al., 2011).

complete degradation of C_{20}–C_{36} by 150 days. After 100 days the GC trace also revealed a significant reduction in the UCM.

The applied zymogenous microbial consortium biodegraded and ''consumed'' all components of the complex mass of hydrocarbons, although different rates of degradation were observed, as shown in Table 1.

Indicator	S-0	S-50	S-100	S-150
Dominant n-alkane	C_{32}	C_{20}	C_{20}	-
Pristane/n-C_{17}	1.47	1.42	2.16	3.8
Phytane/n-C_{18}	1.75	1.70	2.9	4.17
Biodegradation of TPH, [%]	0	60.2	74.9	94.4
Biodegradation of Pristane, [%]	0	31.8	50.9	56.8
Biodegradation of Phytane, [%]	0	32.0	42.0	55.8

Table 1. Indicators of bioremediation from gas chromatography (from Beškoski et al., 2011).

The ratios of pristane/n-C_{17} and phytane/n-C_{18} can be used to differentiate physical weathering and biodegradation (Wang et al., 1998). As the volatility of n-C_{17} and pristane and n-C_{18} and phytane are similar, weathering should be attributed to a reduced concentration of these substances over time, if their ratio remains constant. An increase in the pristane/n-C_{17} and phytane/n-C_{18} ratios over time is likely to be a consequence of bioremediation, since n-C_{17} and n-C_{18} degrade more rapidly than pristane and phytane, respectively. Biodegradation of pristane and phytane, as found in Beškoski et al., (2011), shows that these compounds are not suitable as markers for following a bioremediation process. In fact, comparison of the content of other hydrocarbons in relation to pristane and phytane could underestimate the degree of their biodegradation.

4.2.2 Transformation of isoprenoids, steranes and terpanes

Isoprenoid aliphatic alkanes, pristane and phytane, and polycyclic alkanes of sterane and triterpane types in saturated hydrocarbon fractions were analysed by GC–MS. Isoprenoids were identified from the m/z 183, steranes from m/z 217 and triterpanes from m/z 191 selected ion chromatograms obtained from analysis in the SIM mode. The most relevant peaks were identified based on organic geochemical literature data (Peters et al., 2005), or based on total mass spectra, using mass spectra databases (NIST/EPA/NIH Mass spectral library NIST2000, Wiley/NBS Registry of Mass spectral Data 7th Ed., electronic versions).

In the chromatogram m/z 183 of all the samples, the pristane and phytane peaks are clearly differentiated, being the most intensive in the sample S-0 (Figure 10). In this sample, individual peaks originating from the homologue chain > C_{20} isoprenoids are also observed, while in sample S-150 they are biodegraded.

The chromatogram of terpane in the initial sample S-0 (SIM, m/z 191, Figure 11) is dominated by peaks originating from C_{19}–C_{28} tricyclic terpanes, C_{24} tetracyclic terpane and C_{27}–C_{29} pentacyclic terpanes, with a distribution not typical for raw heavy fuel oil (data not shown), but rather which is typical for samples that had been exposed to weathering and biodegradation over extended periods of time (Peters et al., 2005). During the bioremediation process, the quantity of all tricyclic and tetracyclic terpanes decreased and in

the sample S-150, the quantity of tricyclic (C_{19}, C_{24}, C_{25}, and C_{28}) and tetracyclic terpanes (C_{24}), is according to their intensity virtually no different from the peaks overlapped by the background noise. In relation to them, the quantity of pentacyclic terpanes of the hopane type also decreased at an intensity that conforms to degradation of oil and oil derivatives under natural conditions.
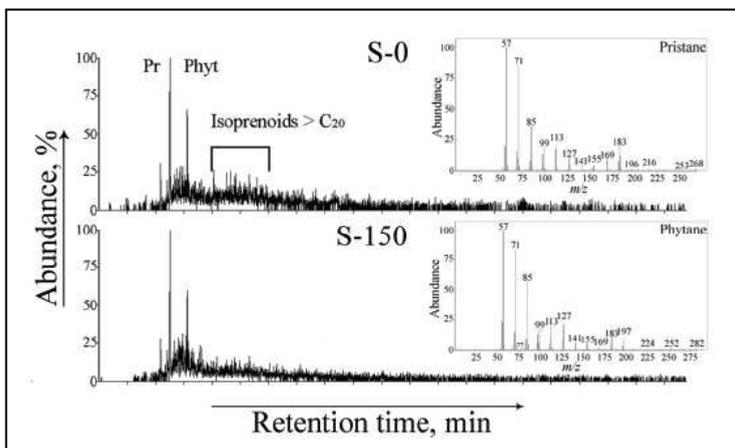

Fig. 10. Selected ion chromatograms of isoprenoids (SIM, *m/z* 183) of alkane fractions in samples S-0 and S-150 (full mass spectra corresponding to peaks of pristane C_{19} and phytane C_{20} for S-0 sample are also presented); Pr: Pristane; Phyt: Phytane; (from Beškoski et al., 2010).
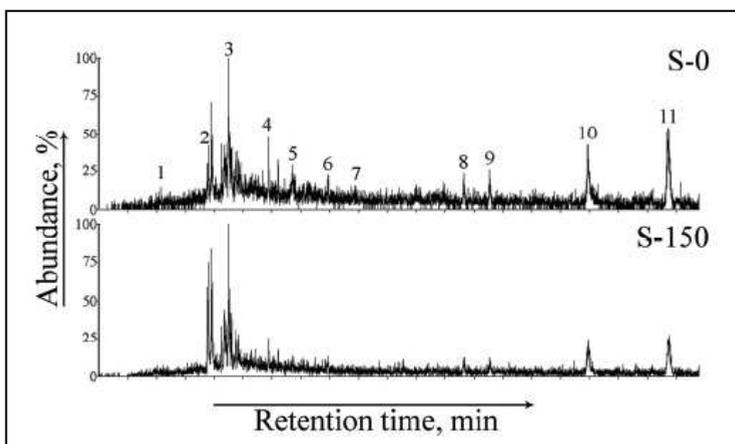

Fig. 11. Selected ion chromatograms of terpanes (SIM, *m/z* 191) of alkane fractions in samples S-0 and S-150. 1: C_{19}-tricyclic terpane; 2: C_{21}-tricyclic terpane; 3: C_{22}-tricyclic terpane; 4: C_{24}-tricyclic terpane; 5: C_{25}-tricyclic terpane; 6: C_{24}-tetracyclic terpane; 7: C_{28}-tricyclic terpane; 8: C_{27}-18α(H)-22,29,30-trisnorhopane (Ts); 9: C_{27}-17α(H),18α(H),21β(H)-25,28,30-trisnorhopane; 10: C_{29}-17α(H),21β(H)-hopane; 11: C_{29}-18α(H),21β(H)-30-norneohopane (from Beškoski et al., 2010).

Steranes and diasteranes are polycyclic alkanes that are degraded more quickly and easily than terpanes. Over the course of the bioremediation experiments conducted, the fate of steranes and their more stable structural isomers, diasteranes, C_{27}–C_{29}, is similar to the fate of the terpanes (Figure 12), and biodegradation, decrease and loss of resolution of the individual signals, were observed in the sample S-150.

Polycyclic alkanes, steranes and diasteranes, as well as terpanes, were biodegraded in the sample S-150 and did not differ from peaks overlapped by the background noise (Beškoski et al., 2010).
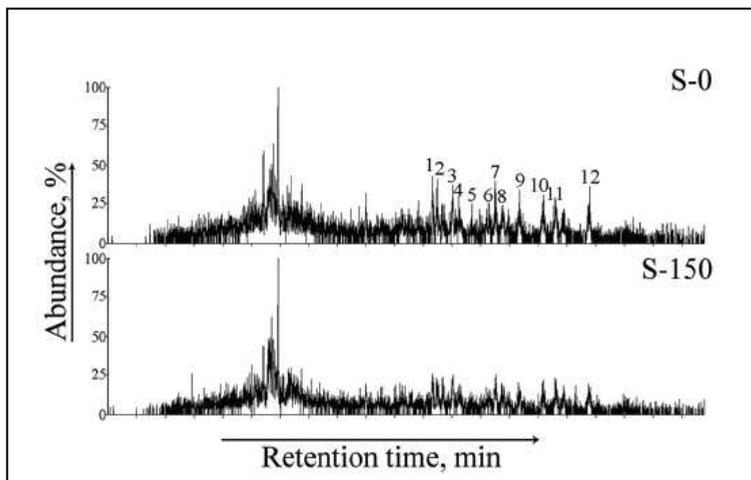

Fig. 12. Selected ion chromatograms of steranes (SIM, *m/z* 217) of alkane fractions in samples S-0 and S-150. 1: C_{27}-13β(H),17α(H) diasterane (20S); 2: C_{27}-13β(H),17α(H)-diasterane (20R); 3: C_{27}-13α(H),17β(H) diasterane (20S); 4: C_{27}–13α(H),17β(H) diasterane (20R); 5: C_{28}–13β(H),17α(H) diasterane (20R); 6: C_{28}-13α(H),17β(H) diasterane (20S) + C_{27}-14α(H),17α(H) sterane (20S); 7: C_{29}-13β(H),17α(H) diasterane (20S) + C_{27}-14β(H),17β(H) sterane (20R); 8: C_{27}-14β(H),17β(H) sterane (20S) + C_{28}-13α(H),17β(H) diasterane (20R); 9: C_{29}-13β(H),17α(H) diasterane (20R); 10: C_{29}-13α(H),17β(H) diasterane (20R) + C_{28}-14β(H),17β(H) sterane (20R); 11: C_{28}-14α(H),17α(H) sterane (20R); 12: C_{29}-14α(H),17α(H) sterane (20R) (from Beškoski et al., 2010).

4.2.3 The effect on tricyclic aromatic hydrocarbons

During *ex situ* bioremediation of soil contaminated by mazut the changes in the distribution of phenanthrene and its methyl isomers were investigated (Novaković et al., 2011). The results of bioremediation of soil were compared with the results of biodegradation in untreated control biopile. During the process of natural microbial degradation an expected trend was observed: the concentration of phenanthrene is reduced in relation to methyl-, dimethyl- and especially to trimethyl-phenanthrenes. However, during the process of "stimulated biodegradation" a different sequence was observed: there was a uniform increase in the relative abundance of phenanthrene compared to its methyl isomers, especially relative to trimethylphenanthrenes.

Mass selected ion chromatograms of phenanthrene, methyl-phenanthrenes, dimethyl-phenanthrenes and trimethyl-phenanthrenes obtained by GC-MS analysis of aromatic fractions isolated from extracts of samples M1 and M5, from bioremediation biopile, are shown in Figure 13a. Selected ion chromatograms of control tests (samples M1k and M5k, from untreated controle biopile) are shown in Figure 13b.
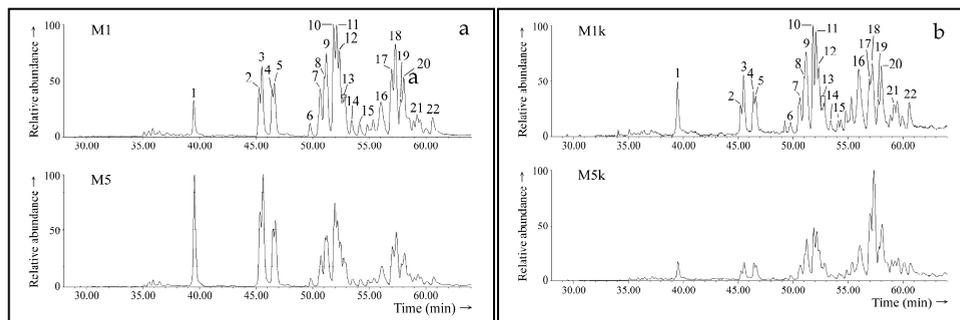

Fig. 13. Selected ion chromatograms of phenanthrene (P) (m/z 178), methyl-phenanthrenes (MP) (m/z 192), dimethyl-phenanthrenes (DMP) (m/z 206) and trimethyl-phenanthrenes (TMP) (m/z 220), obtained by GC-MS analysis (using the single ion monitoring, SIM method) of (a) aromatic fractions isolated from M1 and M5 bioremediation biopile extracts and (b) aromatic fractions isolated from untreated control biopile extracts (M1k and M5k); 1: P; 2: 3-MP; 3. 2-MP; 4. 9-MP; 5. 1-MP; 6. Et-P (Ethyl-phenanthrenes); 7. EtP + 3,6-DMP; 8. 2,6-DMP; 9. 2,7-DMP; 10. 2,7-DMP; 11. 1,6 + 2,9 + 2,5-DMP; 12. 1,7-DMP; 13. 2,3 + 1,9 + 4,9 + 4,10-DMP; 14. 1,8-DMP; 15. 1,2-DMP; 16. 2,6,10-TMP; 17. 1,3,6-TMP; 18. 1,3,7 + 1,3,9 + 2,7,10-TMP; 19. 2,3,6-TMP; 20. 1,3,8 + 2,3,7 + 2,8,10-TMP; 21. 2,3,10 + 3,8,10-TMP; 22. 1,2,3 + 1,2,8 + 1,7,10-TMP; (adapted from Novaković et al., 2011).

There is a possibility that the observed changes occurred as a result of demethylation (Huang et al., 2004) through the process of oxidative decarboxylation. In this way, an increase in the absolute concentration of phenanthrene could have occured at the expense of degradation of methyl-, dimethyl-and trimethyl-phenanthrenes. However, demethylation is a thermodynamically less favorable process, and it is more likely that the biostimulation process favored those bacterial strains in the consortium that decompose methyl-isomers (first of all trimethyl-phenanthrenes), as a consequence of better interaction of reactive methyl groups with the active centers on the surface of bacterial cells, and, thus promoted decomposition of methyl-phenanthrene derivatives (Lamberts et al., 2008).

It can be concluded that higher availability of phenanthrene and its methyl derivatives to microorganisms increases the degradability of methyl-phenanthrenes compared to phenanthrene and that the degree of degradability depends on the number of methyl groups, i.e. on the level of alkylation.

5. Conclusions

GC is an indispensable method in environmental chemistry, biotechnology, organic geochemistry and in practical aspects of environmental cleaning, protection and

preservation (Evans & Furlog, 2011; Scott, 1998; Singh & Ward, 2004). With its inherent high sensitivity and high separating power GC is one of the more commonly used techniques in the analysis of environmental samples. For fundamental works in this field and for the development of biotechnologies and techniques in environmental sciences, the combination of GC with MS in all techniques is often of essential importance for the success of research and development procedures. The use of the said instrumental methods is particularly relevant in the case of oil and oil derivatives due to countless varieties of their composition and their general presence in the environment, either through their widespread usage or their occurrence as pollutants.

Our research experience in the field of fundamental and applied aspects of petroleum pollutants in the environment and their remediation by microorganisms especially by *ex situ* bioremediation methods, confirms the prominent place of GC and techniques that are based on GC among analytical methods which are commonly applied to good effect in environmental chemistry and environmental biotechnology.

Also, primarily because of the quantities of substances, the use of inert gases, and the possibility of replacing conventional organic solvents with solvents which have much smaller negative impacts on the environment, such as the use of carbon dioxide as a supercritical fluid, GC can be considered as a green analytical method. Further technical advances can be expected in the development of column fillings, probably in the area of nano-materials, and in terms of separation efficiency, as well as application of polar "green" solvents, such as water and ethanol.

6. Acknowledgments

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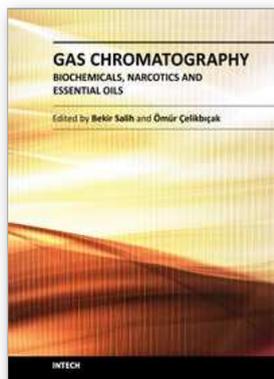
Gas Chromatography - Biochemicals, Narcotics and Essential Oils

Edited by Dr. Bekir Salih

Gas Chromatography involves the study of various vaporizable molecules in chemistry and the other related research fields. This analytical method has a number of features and advantages that make it an extremely valuable tool for the identification, quantification and structural elucidation of organic molecules. This book provides detailed gas chromatography information to applications of biochemicals, narcotics and essential oils. The details of the applications were briefly handled by the authors to increase their comprehensibility and feasibility. This guide should be certainly valuable to the novice, as well as to the experienced gas chromatography user who may not have the enough experience about the specific applications covered in this book. We believe this book will prove useful in most laboratories where modern gas chromatography is practiced.

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