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AN ALTERNATIVE TO TRIAL-ERROR METHODOLOGY IN SOLID PHASE EXTRACTION: AN ORIGINAL AUTOMATED SOLID PHASE EXTRACTION PROCEDURE FOR ANALYSING PAHs AND PAHs-DERIVATIVES IN SOOT

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KEYWORDS: High Performance Liquid Chromatography (HPLC), Normal-Phase HPLC, Hydrophylic Interaction Chromatography (HILIC), Polycyclic Aromatic Hydrocarbons, Oxy-Polycyclic Aromatic Hydrocarbons, Nitrated-Polycyclic Aromatic Hydrocarbons, bio-kerosene.

ABSTRACT

This paper introduces the generalization of Reverse-Phase HPLC fundamentals to Normal-Phase Liquid Chromatography and Automated Solid Phase Extraction (A-SPE). This paper upholds that the same fundamentals and principles of reverse-phase HPLC are also applicable in Normal Phase HPLC and A-SPE. Based on these fundamentals, we could overlook the error-trial method and accomplish a rational and fast selection of the most suitable SPE procedure for analyzing aromatic hydrocarbons and polar-aromatic-hydrocarbons in soot from methyloctanoate-kerosene blends. These fundamentals and procedure could be applied to any sample. Application to soot samples was carried out for demonstrating the high efficiency of the procedure to very complex matrices.

The analytical methodology introduced here consists of: (i) Solution of the soot sample into hexane, (ii) extraction of the target analytes by filtration twice through PTFE filters, (iii) A-SPE fractioning through silica column by using an HPLC pump and hexane at flow rate of 0.05 ml/min as mobile phase, and (iv) analysis of the fractions collected by Reverse-Phase HPLC with Photodiode Array Detector (PDA). The methodology developed was successfully applied to the identification of 56 PAHs and 44 PAHs-derivatives (belonging to 14 different chemical classes) in soot from methyloctanoate-kerosene blends in a reproducible, simple, and environmental friendly way. The aforementioned procedure can be implemented in any lab having an HPLC system.

1. INTRODUCTION

HPLC is the most widely used analytical technique for the separation, identification and quantification of a huge variety of compounds in biomedical[1], forensic[2], environmental, [3,4] wastewater[5], pharmaceutical,[6] phytochemical,[7] and food[8] samples. However, in spite of the advantages of HPLC, before a successful application of chromatographic methods, clean-up and fractioning are crucial and frequently required in order to separate analytes from the interfering matrix components and separate them into families.[9] Particularly, complexity of soot samples hampers their direct injection in instrumental systems and makes clean-up and fractioning crucial and mandatory steps of their pre-treatment. Both of these procedures (clean-up and fractioning) are also some of the most time and solvent-consuming steps out of the analytical procedure.[10] Moreover, a significant amount of the target analytes can be lost during this stage and any mistake occurring in collecting and processing of an analytical sample could lead to a substantial error in the final results regardless the excellent performance of the analytical technique applied subsequently.[11]

SPE is the most commonly used method for the extraction from matrix, clean-up, concentration and fractioning of organic compounds from clinical, biological, industrial, environmental and food samples.[12-17] However, the SPE procedures reported in literature are still poorly developed with little consideration to the physics involved in the process and are described as a largely empirical, labour intensive and time consuming trial and error processes.[11] In the aim of overcoming the limitations aforementioned and achieving some kind of systematization, we addressed the following tasks:

a) To gain deeper knowledge on the Normal-Phase and A-SPE principles and fundamentals. Studying if the fundamentals and principles published by our group on the Reverse-HPLC are also applicable to normal phase HPLC and A-SPE.[18,19]
b) Rational application of the qualitative information obtained to the development of new optimization strategies of SPE methodologies dismissing the traditional trial-error procedure. The SPE methodology developed should demonstrate great efficiency for cleaning-up and fractioning of PAHs and PAHs derivatives in soot.

The analytical methodology developed consists of the following processes: a) filtration of the soot extracts twice through PTFE membranes, b) A-SPE for isolation and fractioning the target analytes and c) analysis of the fractions collected by HPLC. This analytical methodology turns out being an efficient, time-, cost- and solvent-saving procedure for analysing PAHs and PAHs-derivatives in soot from methyloctanoate-kerosene blends. Other advantages of the method developed are the simplicity, the

reliability and reproducibility and certainly its applicability because it can be implemented in any lab having an HPLC. No expensive facilities are needed for accomplishing the procedure. Moreover, the entire procedure can be monitored (with PDA detector) and the volume of solvents used is small and safe (hexane at flow rate of 0.05 mL/min is used as mobile phase in the fractioning procedure). The silica column used for clean-up and fractioning the samples can be reused up to 25 times, which makes the procedure environmentally friendly. And finally, the procedure might be easily adapted and applied to analyse PAHs and PAHs-derivatives in other samples.

To the best of our knowledge, this methodology is totally original, because although some previous fractioning processes relying on an HPLC pump have already been reported in the literature, the optimization strategy and the experimental conditions were totally different. Namely, Bamford and coll. (2003)[20] applied SPE followed by chromatographic separation for analysis of nitro-PAHs. These authors employed an amino/cyano column combined with a moderately mobile phase consisting of 20% dichloromethane in hexane at high flow rate (5 ml/min) for separating PAH and Nitro-PAHs. By applying the aforementioned procedure, a mono-nitroPAH fraction followed by a di-nitroPAH fraction was obtained. In this case, each chromatographic run took about 35 minutes, which implies that a volume of 160 ml of mobile phase (32 mL out of these 160 mL are dichloromethane, a toxic solvent) is consumed and consequently the dilution of the target analytes is high. On the other hand, our methodology allowed us to fraction 14 different families of compounds in soot samples within 70 minutes, by using only 3.5 mL of hexane as mobile phase (flow rate of 0.05 ml/min) and silica as stationary phase, which leads to save solvents and improve the sensibility of the method.

2. EXPERIMENTAL SECTION

2.1. Material and reagents

A mixture of 18 PAHs (2000 $\mu g mL^{-1}$ each component in dichloromethane: benzene (1:1)) from Sigma–Aldrich was used for preparation of standards by dilution. Components in the mixture were: acenapthene, acenaphthylene, anthracene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene, benzo[a]pyrene, chrysene, dibenzo[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, 1-methylnaphthalene, 2-methylnaphthalene, naphthalene, phenanthrene and pyrene. Solid coronene was also supplied by Sigma–Aldrich. Final solutions into hexane or isopropanol were prepared by dilution.

HPLC grade hexane, water, 2-propanol (isopropanol) and acetonitrile were purchased from Sigma Aldrich, whereas acetic acid glacial was purchased from Carlo Erba.

Kerosene used for soot generation was a commercial Jet-A1. A 80/20 mixture of Jet-A1 and methyloctanoate was used as bio-kerosene.[21] Methyloctanoate was used as a simple surrogate of rapeseed oil methyl ester (RME) consisting of a complex mixture of C_{14}–C_{22} methyl esters with highly saturated carbon chains.

0.2 μm sealed hydrophobic PTFE membrane filters with PTFE housing from Alltech and Durobax glass syringes of 5 mL from Poulten & Graf were used for filtering soot extracts before A-SPE. A Spherisorb Phenyl column (12.5x4.6 mm ID, 10 μm) from Waters and an Ascentis silica column (25 cm x 2.1 mm ID, 5μm) from Supelco were employed for A-SPE. Empty stainless-steel casing pipes were also used for hosting different stationary phases in the preliminary studies.

2.2 Production of soot samples

Methyloctanoate-kerosene (20:80, v/v) blends were burned under well characterized and controlled conditions using a flat-flame burner. A simplified scheme and a detail description of the soot production and collection system have been presented elsewhere.[22-24] The soot was collected on the outer surface of a Pyrex rod tube at height 4 cm above the head of the burner and removed mechanically from the glass tube. The mass of soot (15.8 mg/2ml n-hexane) was measured with a high accuracy mass balance, diluted in 2 ml of n-hexane and driven for further analysis.

2.3 Extraction of the target analytes from the soot matrix

Selection of the methodology for extraction of PAHs and PAHs derivatives from soot is a crucial issue. The thermal instability of most polar-PAHs (namely nitro-PAHs), makes methodologies such as thermal extraction, not suitable for pulling out target analytes from premixed-flames soot even though this procedure seems to be needed for extraction of analytes from diesel soot samples.[25]

On the other hand, we found out that up to 25% of some PAHs can be destroyed by the probe during assisted-ultrasound extraction (one of the most used methodology for extraction PAHs and PAHs-derivatives from soot, Table S1). As a consequence, extraction procedure was simplified and only filtration twice through two PTFE filters was accomplished in order to remove the soot particles and extract the aromatic material from the soot matrix.

2.4 Selection of stationary and mobile phase for purifying and fractioning the soot extracts

As families of PAHs and PAHs derivatives differ mainly in polarity, their fractioning should be accomplished as a function of this physical property rather than as a function of their polarizability as in reverse-phase HPLC occurs.

In the aim of simplifying the SPE methodology and gaining comprehension on this issue, besides fundamentals published in previous papers,[19,26] some additional preliminary essays were carried out.

Briefly, in the aforementioned papers the following statements have been published:

1. Retention times of target analytes depend on the difference between the relative permittivity (also called dielectric constant) of the stationary and the mobile phases. The greater this difference, the greater the retention times for the probe molecules. When a great $\Delta\varepsilon$ occurs, such in the case of Reverse-HPLC, compounds elute mostly as a function of their medium polarizability.
2. In Reverse-Phase HPLC, the higher the pressure, the higher retention times for very polarizable compounds and the lesser the retention times for non polarizable compounds. Retention times of dipoles (such as nitro-PAHs) are more dependent on pressure than neutral molecules, and under very low pressure they elute before other compounds more polarizable.

Could these achievements be generalized to Normal-Phase HPLC and A-SPE procedures?

For answering this question, some preliminary studies were carried out combining different mobile phases and different stationary phases. The empty stainless steel casing pipe (250x4.6 mm ID) was successively filled with silica, alumina and phenyl commercial phases. Amino-packings are discarded because the primary amino groups can easily form Schiff-bases (imines) with aldehydes and ketones under typical normal-phase chromatographic conditions. On the other hand, cyano columns are quite stable in non-polar solvents but often exhibit the curious phenomenon of bed collapse when exposed to solvents of intermediate polarity like neat acetonitrile, THF or methanol.[27,28] Methanol, n-hexane, isopropanol, acetonitrile, acetone, dichloromethane and water were selected and tested as mobile phases at different flow rates. Dependence of the Retention Times on pressure (related to dimensions of the column) and $\Delta\varepsilon$, were investigated.

Hundreds of experiences (combining different stationary and mobile phases at different flow rates) confirmed that the retention times of the target analytes (PAHs) exhibit the same dependence on $\Delta\varepsilon$ in Normal-Phase HPLC and in HILIC (Hydrophilic Interaction Chromatography) and in SPE as in Reverse-Phase HPLC.[19] Thus, under the experimental conditions aforementioned, retention time of light PAHs in the silica column was 76 min; whereas in phenyl column was about 49 min. In the phenyl column

case, retention is lesser (smaller $\Delta\varepsilon$, $\sim\Delta\varepsilon_{crit}$), whereas in silica column higher retentions were achieved for light and for heavy PAHs (higher $\Delta\varepsilon$). Also alumina was used for filling with the same steel casing pipe. In this case retention time was higher for PAHs, especially for light PAHs. This matches with the fact that relative permittivity is higher for alumina than for silica (here the size particle of stationary phase is very important). Besides, when silica is combined with solvents exhibiting high relative permittivity (such as acetonitrile or dichloromethane), retention times of PAHs increase. As a conclusion, under the same backpressure, the higher the difference between the relative permittivity the higher the retention of the analyte, as in the Reverse-Phase HPLC occurs.

All the results are summarized in Figures 1 and 2 where:
$\Delta\varepsilon$ is the difference between the relative permittivity of mobile phase and that of the stationary phase, and
$\Delta\varepsilon_{crit}$ is the critical value of $\Delta\varepsilon$, below which no retention occurs.

Figure 2 depicts as polarizability gains importance in the case of high $\Delta\varepsilon$, but solubility accounts for the elution of most of compounds when the value of $\Delta\varepsilon$ falls down. On the other hand, repulsion forces (those among dipole molecules as nitro-PAHs) are important for weak values of $\Delta\varepsilon$ and also for very high $\Delta\varepsilon$ values when pressure falls down dramatically.

Another very important parameter for efficient fractioning is the backpressure. In this case, best experimental conditions might be selected according to the following information:

a) If $\Delta\varepsilon < \Delta\varepsilon_{crit}$ (for example, C_{18}-hexane), then, no matter the backpressure or the flow rate, compounds cannot be eluted separately. Retention is very low.
b) If $\Delta\varepsilon \sim \Delta\varepsilon_{crit}$ (for example phenyl phase-hexane), then the following options can be observed:
    b.1) High Backpressure. In this case, compounds elute together. No discrimination is possible.
    b.2) Medium Backpressure. Compounds elute as a function of their solubility although polarizability exhibits some relevance.
    b.3) Low backpressure. Compounds elute mainly as a function of their polarity (that is, their solubility in the mobile phase).
c) If $\Delta\varepsilon > \Delta\varepsilon_{crit}$ (as in the case of silica column, 250 mm length, 2.1 ID, 5 µm size particle), then the following options can be observed:
    c.1) Low backpressure (lesser than 4 bar). In this instance, elution takes a very long time.
    c.2) Medium backpressure (about 5 bar). Solubility accounts for most of compounds but there are some analytes as phenalen-1-one, hydroxy-acids

of naphthalene and di-acids of naphthalene eluting as a function of their polarizability rather than as a function of their solubility (or polarity).

c.3) High backpressure (>5 bar). Some groups of compounds elute together. Efficiency of the columns for fractioning gets poorer.

d) If $\Delta\varepsilon >> \Delta\varepsilon_{crit}$ (for example silica-alumina column as stationary phase and acetonitrile/water as mobile phase, or C_{18} as stationary phase and acetonitrile/water as mobile phase). Two possibilities can be seen:

d.1) Low backpressure (under 11 bar). In this case repulsion forces take on importance for neutral dipole molecules (such as nitro-PAHs)[19]

d.2) High backpressure (above 11 bar, as usual in Reverse-HPLC). In this case only polarizability takes on importance and influence.

What is considered backpressure depends on the dimensions of column selected. For instance, for a column of 10 cm length x 4.6 mm I.D. and 10 µm of size particle, more than 2 bar can be considered high backpressure. So, for achieving a high backpressure, small diameters, long column and small particle size are recommended.

Therefore, for eluting the target analytes as a function of their polarity rather than their polarizability, a medium $\Delta\varepsilon$ value is desirable. For achieving a medium value of $\Delta\varepsilon$, two different stationary phases (phenyl and silica) and n-hexane as mobile phase were selected. As home-made columns provide a poor reproducibility, commercial columns were preferred from now on. We selected two different commercial columns:

a) A commercial phenyl phase column. Dimensions: 125 mm length, 4.6 mm I.D, 10 µm particle size.
b) A commercial silica column. Dimensions: 250 mm length, 2.1 mm ID, 5 µm particle size.

Dimensions were selected based on the backpressure expected to develop inside them (very low in the first case and high in the second one) and on their dead volume (for phenyl column, it is estimated to be approximately 2.4 times that for silica column). Note that the $\Delta\varepsilon$ value for phenyl column combined with hexane is lower than that of silica combined with hexane. Consequently, lower backpressure will be required inside the phenyl column; otherwise, no separation of compounds would be achieved. The small length of the phenyl column, combined with large inner diameter and large particle size, makes the backpressure developed by using this column very low. In this case, compounds are expected to elute strictly in order of their polarity. On the contrary, the length of the silica column and its very small inner diameter makes the backpressure developed in this column higher than in the phenyl column. In this case, polarizability gains importance (Figures 1 and 2), and consequently some very polar compounds such as hydroxy-acids and small di-acids might be easily eluted, whereas they could not be

eluted from phenyl phases, where the elution occurs strictly in order of polarity (these compounds are not soluble into hexane).

2.5 Pretreatment of the extracts after A-SPE

Unburned ester from the combustion of methyloctanoate-kerosene as well as aromatic acids and hydroxy-acids (see Table 2) found in the extracts of soot samples could impair the determination of PAHs and PAHs-derivatives in our samples unless the extracts were conveniently pre-treated. For unburned ester, because this compound co-elutes with the aromatic fraction from the fractioning columns, fractions collected must be pre-treated before injection into the HPLC column by adding some µL of acetic acid glacial. In acidic medium, esters undergo hydrolysis and convert into acids and alcohols, which easily elutes from the C_{18} column. Otherwise, if esters are injected along PAHs into the C_{18} column, they quench the spectroscopic signals of PAHs and prevent their efficient identification and quantification.
Moreover, pre-treatment of fractions containing aromatic acids and hydroxy-acids is mandatory because of the insolubility of some of these compounds into water. In this case, acetic acid (added to the fraction) is also efficient for facilitating the elution of these compounds from C_{18} column during the HPLC analysis of fractions.

2.6 Chromatographic Analysis

Chromatographic experiments were performed by using a HPLC system from Shimadzu. The instrument consisted of a system controller CBM-20A/20 Alite Prominence, a solvent delivery module LC-20AB Prominence, an autosampler SIL-20A/20AC Prominence, a column oven CTO-20A/20AC Prominence and a UV/Visible photodiode array detector SPD-M20A Prominence. A Vydac column 201TPC18, 5 µm, 250 mm length x 4.6 mm ID (Grace Davison Discovery Sciences), was used for the analysis of each fraction collected.

3. RESULTS AND DISCUSSION

Both of the columns detailed in the experimental section (phenyl column, 12.5 cm length, 4.6 mm ID, 10 µm particle size) and silica column (25 cm length, 2.1 mm ID, 5 µm particle size) combined with hexane at flow rate of 0.05 ml/min as mobile phase were studied and their efficiency for fractioning PAHs and PAHs derivatives in soot from methyloctanoate-kerosene blends and from fossil kerosene.

3.1. **Elution order from the silica column.**

As we can see from Figure 3 and from supplementary information (S1-S24), fractioning through silica column by using hexane as mobile phase is efficient and even the most

polar compounds (dicarboxylic acids) elute from the stationary phase. The elution order of analytes from silica columns is:

1. Alkanes, alkenes….
2. Light PAHs (indene, naphthalene, isomers of methyl-naphthalene, acenaphthene, acenaphthylene…).
3. Medium-size PAHs (anthracene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene…).
4. Heavy PAHs (perylene, benzo[ghi]perylene, benzo[b]fluoranthene, benzo[k]fluoranthene, anthranthrene, coronene…)+nitro-PAHs.
5. Oxa-PAHs such as xanthenes and benzoxanthenes.
6. 9-fluorenone, isomers of naphthalene-carboxaldehyde.
7. Other ketones (benzanthrone, etc)
8. 1-Naphthol
9. 2-Naphthol
10. Phenalen-1-one.
11. Quinones (9,10-anthraquinone, 9,10-phenanthrene-quinone…)
12. Naphthalen-carboxylic acids.
13. Hydroxy-acids of naphthalene.
14. Naphthalene-dicarboxylic acis.

From the observed elution order, we can affirm that, although polarizability is more important in silica columns than in phenyl columns, compounds elute mostly as a function of their polarity when hexane is used as mobile phase. Thus, although phenalen-1-one (a light ketone-PAH) elutes after 1-naphtol and 2-naphthol (much more polar than ketones) due to its higher polarizability than those of naphtols, most of compounds elute from the silica column as a function of their solubility into n-hexane.

a. Elution from phenyl column

From phenyl column the elution order is the following:

1. Alkenes, alkenes
2. Light PAHs (indene, naphthalene, methyl-naphthalene, acenaphthene, acenaphylene…).
3. Medium-size PAHs (fluoranthene, pyrene…)
4. Heavy PAHs (benz[b]fluoranthene, perylene, benzo[ghi]perylene,...)
5. Very heavy PAHs (coronene, naphtha(2′.3′:1.2)coronene,…)+nitro-PAHs.
6. Oxa-PAHs such as xanthenes and benzoxanthene.
7. 1-Naphthalene-carboxaldehyde.
8. 9-Fluorenone+2-Naphthalene-aldehyde.
9. Phenalen-1-one
10. Other ketones (heavier than phenalen-1-one) such as benzanthrone.

11. 1-naphthol, 2-naphthol, quinones.

Figure 4 shows some classes of compounds eluting along with others. The fractioning becomes less efficient than with a silica column (Figure 3). Contrary to what happens in silica columns, the elution of carboxylic-acids of naphthalene and hydroxy-carboxylic acids from phenyl columns turns out really difficult, most likely due to the very low value of $\Delta\varepsilon$ compared to that of silica column which makes elution of the target analytes dependent on the polarity (hydroxy-acids of naphthalene and di-acids are not soluble into hexane) rather than on polarizability.[19]

As shown in Figures 3 and 4 and in Table S2, in general, compounds elute in increasing order of solubility for both columns studied, although polarizability becomes more important in the case of silica column than in the case of phenyl-column. So, although the polarity of phenalen-1-one is similar to that of 9-fluorenone, the first compound elutes after hydroxy-PAHs from silica column likely because of its high polarizability ($22.2\pm0.5\times10^{-24}cm^{-3}$).[29] Furthermore the elution from silica columns depends on both polarizability and polarity, even if the later is more influential. Otherwise, compounds strictly elute in increasing order of polarity from phenyl column. So, phenalen-1-one elutes from phenyl column before other heavier PAH-ketones and far sooner than hydroxy-PAHs.

Finally for HPLC analysis of PAHs and PAHs derivatives, silica column was selected not only due to its higher efficiency compared to that of phenyl column, but also due to diffusion problems occurring in phenyl colums. In particular, silica column backpressure (5 atm) is high enough for minimizing diffusion inside the tubes driving the liquid from the column to the fraction collector. Too low backpressures as those developed in phenyl column make mass transfer inside the tubes driving the liquid to the fraction collector be significant and consequently the mixing of fractions (diffusion phenomenon).

As a very small flow rate is used, there is a gap between the instant when the UV-visible signals are detected and the moment when the compounds reach the fraction collector. This gap is about 15 min for our HPLC system; as a consequence, fractioning must start about 13 minutes after the UV-PAHs signal is detected (very concentrate samples elute a bit before more dilute ones). This is the reason why if aromatic compounds elute at 15 minute, we should start their collection at 28 minute. Reduced time intervals (about 3 or 4 minutes at the beginning) should be collected to avoid collecting PAHs and some polar PAHs together.

Based on the information aforementioned, we propose the following fractioning program:

1. 28-32 min. Alkanes, alkenes and other aliphatic hydrocarbons can be usually found in this fraction.
2. 32-35 min. Light and medium PAHs can be found in this fraction.
3. 35-38.5 min. Heavy PAHs elute now and nitro-PAHs elute in this fraction. Some oxa-PAHs (xanthenes…) elute also now.
4. 38.5-42.5 min. Some oxy-PAHs (fluorenone and other ketones, some aldehydes…) are found in this fraction.
5. 42.5-48 min. Quinones elute in this fraction.
6. 48-55 min. Hydroxy-PAHs and perinaphthone elute at this time.
7. 55-65 min. Acids, hydroxy-acids and anhydrides elute in this fraction.
8. 65-85 min. Isopropanol is passed at 0.1 ml/min for cleaning-up the column.

b. Chromatographic Analysis

Injection of the polar fractions is done in n-hexane (in this case flow rate of the mobile phase is 1.0 ml/min) whereas injection of the unpolar fractions was carried out in isopropanol and the flow rate of the mobile phase is 0.5 ml/min. The isocratic gradient was 50% acetonitrile: water for 30 min, then the linear gradient elution from 50% acetonitrile at 30 min to 100% acetonitrile at 90 min was applied followed by isocratic elution with 100% acetonitrile for 15 min to remove possible impurities adsorbed onto the column. Each run concluded with a conditioning step (50% water/Acetonitrile) for 20 min. The column oven temperature was maintained at 30 ºC throughout the analysis.

The compounds (PAHs and PAHs-derivatives) identified in soot from methyloctanoate-kerosene soot are shown in Tables 1 and 2. In summary, 100 compounds (56 PAHs and 44 PAHs-derivatives) were found and identified in the extract of methyloctanoate soot (Tables 1 and 2).

c) Validation of the methodology

Validation of the methodology was accomplished in the following way: Real samples of kerosene-methyloctanoate soot were spiked with a standard mixture of 18 PAHs (EPA PAHs, 1-methylnaphthalene and 2-methylnaphthalene) at concentration levels similar to that of real samples and submitted to the same procedure as soot samples for analyzing PAHs and PAHs derivatives. Results from Table S3 shows that recoveries of the PAHs studied are satisfactory in all the cases (within the range 93-100%), except in the case of naphthalene, 1-methylnaphthalene and 2-methylnaphthalene (whose recoveries were 75.0%, 81.6% and 83.9%, respectively, Table S3) most likely due to its high volatility. We think that part these compounds can be lost during the fraction collection of samples. All the PAHs analyzed show recoveries higher than those of manual SPE procedure for similar samples. A-SPE greatly improves the recoveries of naphthalene, acenaphthene and acenapthylene compared to those obtained by manual SPE (Andrade-Eiroa and coll., 2010a).

The main advantages of the analytical methodology introduced here are compared to manual SPE procedures and the trial-error method in Table 3. The new methodology is cost- and time-saving and much more reproducible than traditional methodologies. On the other hand, automated-SPE can be easily implemented in any lab having an HPLC, no big facilities are needed for implementing this methodology.

2. CONCLUSIONS

Based on the present results and phenomena observed, we can state that:

1. A minimum value of Δε (difference between the relative permittivity of stationary and mobile phase) is required for optimal functioning of SPE and chromatographic columns. The minimum value of Δε for fractioning organic aromatic compounds is approximately that existing between phenyl phase and n-hexane. Medium Δε (like that between silica and n-hexane) are recommended for efficient fractioning of aromatic organic material.

2. For chromatographic Reverse-phase separations, maximum Δε (like that between C_{18} and acetonitrile:water) is recommended in order to elute the compounds as a function of medium polarizability rather than in order of solubility like in SPE procedures.

3. Fundamentals published for Reverse-Phase HPLC[19] can be extended and applied to Normal-Phase HPLC, HILIC and even to A-SPE. Under the light of the present results, we can state that fundamentals of these procedures can be unified under the same theory, simplifying the optimization of SPE and chromatographic procedures.

4. Trial-error method should be disregarded in the case of automated SPE and Liquid Chromatography.

5. Even if relative permittivity data are not always available, a qualitative description of the situation is possible and useful for selecting the best SPE procedure (dimensions of the column, flow rate, stationary and mobile phases).

Acknowledgements

This research was supported by the European Research Council under the European Community's Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement n° 291049−2G-CSafe. We are also grateful to Éléonore Heitz for her efficient bibliographic assistance and to Dr. Valerie Cancellieri-Leroy from Laboratoire des Systèmes Pour l'Environnement (SPE) (CNRS-Corte, France) for her technical assistance in the development of the methodology.

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FIGURES

Δε values

a) $|\Delta\varepsilon| < |\Delta\varepsilon_{crit}|$. In this case, compounds can be neither discriminated as a function of their polarity, nor as a function of their solubility. They elute all together from the SPE column.

b) $|\Delta\varepsilon| \sim |\Delta\varepsilon_{crit}|$. In this case, compounds elute mainly as a function of their solubility. Polarizability is a non significant factor.

c) $|\Delta\varepsilon| > |\Delta\varepsilon_{crit}|$. In this case, compounds elute mainly as a function of their solubility and polarizability takes on importance.

d) $|\Delta\varepsilon| >> |\Delta\varepsilon_{crit}|$. In this case compounds elute mainly as a function of their medium polarizability. If Pressure falls dramatically, dipole molecules elute very fast (Andrade-Eiroa and coll., 2011).

Figure 1. Summary of the importance of each factor depending on the value of Δε.

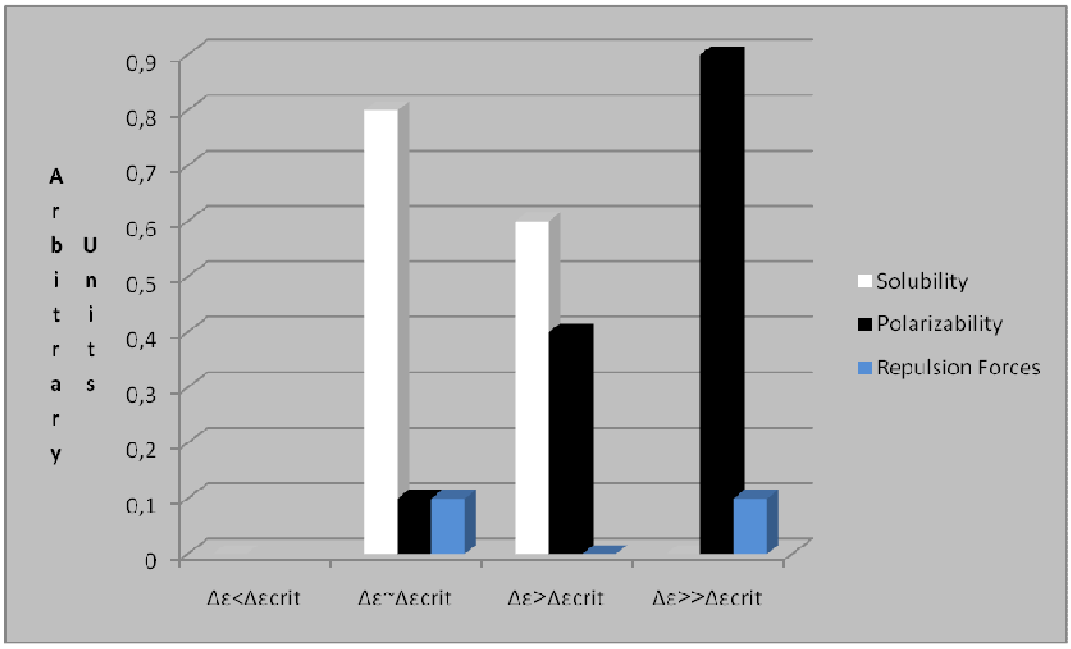
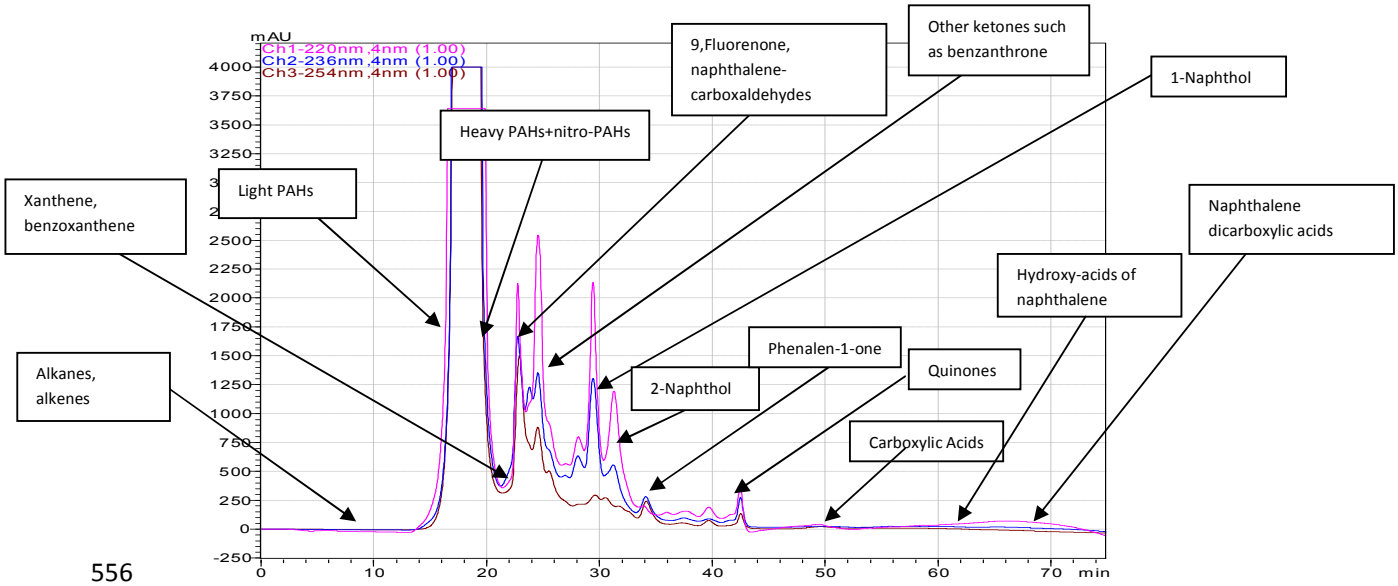


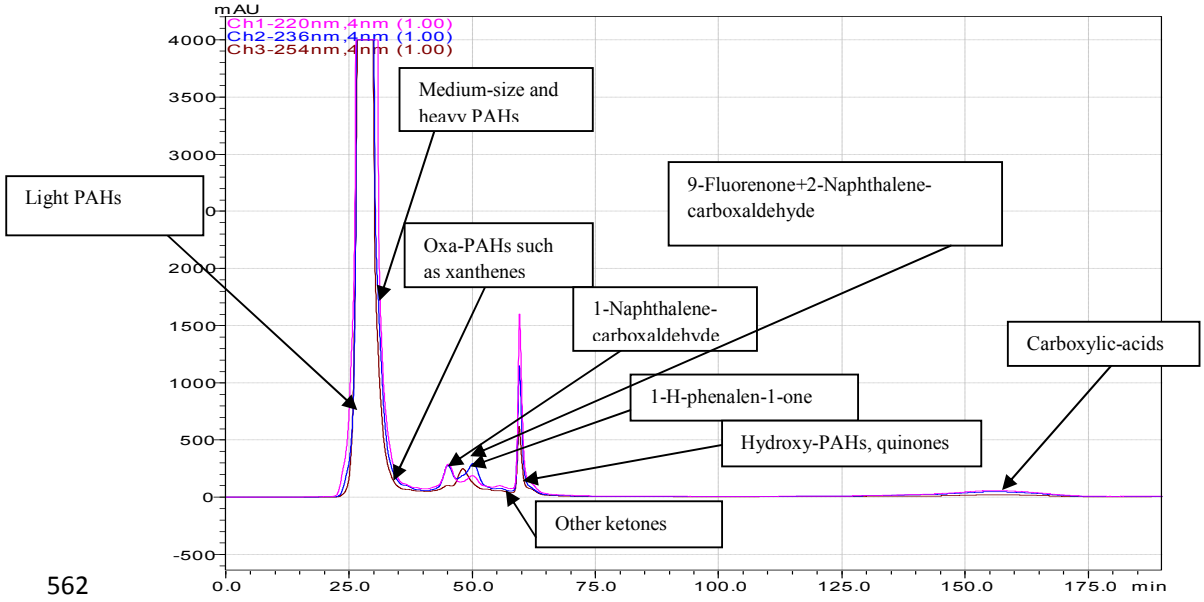
Figure 2. Relative importance of solubility, polarizability and repulsion forces on the retention times depending on Δε values.



556

557 **Figure 3. Fractioning of the methyloctanoate-kerosene (20% v/v methyloctanoate) soot extract into**
558 **hexane blends through the silica column described in the experimental part. Experimental**
559 **conditions: V_{inj}=50µL of the pure extract of soot (15.8mg/2mL). Mobile phase: 100%hexane at flow**
560 **rate of 0.05ml/min.**

561



562

563 **Figure 4. Fractioning of methyloctanoate-kerosene soot (19.3 mg soot/2mL hexane) extract through**
564 **phenyl column. Mobile phase: 100% hexane. Flow rate= 0.05 ml/min. V_{inj}=30µL.**

565

566

567 # TABLES

568 **Table 1. PAHs found in soot from methyloctanoate-kerosene soot (15.8 mg/2mL of hexane).**

PAHs
< 5 rings
Styrene
Indene
Naphthalene
1-methyl naphthalene
2-methyl naphthalene
1-ethynyl-naphthalene
Acenaphthylene
Acenaphthene
Phenanthrene
Anthracene
4,5-methylene phenanthrene
Fluoranthene
Pyrene
Acephenanthrylene
Benzo[a]fluorene
1-methylpyrene
3-methylpyrene
4-methylpyrene
Benz[a]anthracene
Chrysene
Triphenylene
1,2,3,4-tetrahydrochrysene
2-methylbenz[a]anthracene
6-methylbenz[a]anthracene
2,2'-binaphthalene
Benzo[e]indene
Benzo[f]indene
Benzo[g]indene
2-phenyl-naphthalene
5 rings
Cyclopenta[hi]acephenanthrylene
3,4-Dihydrocyclopenta[cd]pyrene
Cyclopenta[c,d]pyrene
Dibenzo[a,i]fluorene
Naphtho[1,2-a]fluorene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Benzo[e]pyrene
Perylene

Benzo[a]pyrene
Dibenzo[a,c]anthracene
Dibenzo[a,j]anthracene
6 rings
11-H-Indeno[2,1,7-cde]pyrene
Corannulene
Benzo[g,h,i]perylene
Indeno[1,2,3-cd]pyrene
Anthanthrene
Dibenzo[a,h]pyrene
7 rings and above
Dibenzo[e,ghi]perylene
1.12-2.3-dibenzoperylene
2.3,8.9-dibenzoperylene
2.3,10.11-dibenzoperylene
Coronene
3.4,9.10-Dibenzopentaphene
Naphtho-(2´.3´:1.2)-coronene
Methylcoronene

569

570

571 **Table 2. PAHs-derivatives found in soot from methyloctanoate-kerosene soot (15.8 mg/2mL of**
572 **hexane).**

hydroxy-PAHs
Phenol
1-naphthol
2-naphthol
2-hydroxyfluorene
2-hydroxy phenanthrene
1-hydroxypyrene
2-Hydroxy-9H-fluoren-9-one
3-hydroxypyrene
aldehydes/ketones
1-naphthaldehyde
2-naphthaldehyde
Benzaldehyde
p-tolylaldehyde
pyrene-3-carboxaldehyde
P-terphenyl-2,2',5',2''-tetracarboxaldehyde
9H-fluoren-9-one
1H-Phenalen-1-one
Benzanthrone
1-keto-1,2,3,4-tetrahydro phenanthrene
Carboxylic acids
1-Naphthalen-carboxylic Acid
2-naphthalene-carboxylic acid
2-hydroxy-1-naphthoic acid
[…]hydroxy-1-naphthoic acid
Naphthalene-2,7-dicarboxylic acid
Naphthalene-1,6-dicarboxylic acid
Naphthalene-1,7-dicarboxylic acid
Quinones
9,10-anthraquinone
Phenanthrene-9,10-quinone
Benzo[a]pyrene-6,12-quinone
2,9-Diketo-1,2,9,10,10a,10b-hexahydro,3,4-benzphenanthrene
Benz[a]pyrene-3,6-quinone
6-(4-methoxy)-5,12-Naphthacene-quinone
Ethers cyclics, oxides and lactones/coumarins, methoxy-
9H-xanthene
9H-xanthone
Benzo[]xanthenes
Benzo[c]cinnoline-5-oxide
Methoxy-1-naphthalen-carboxaldehyde (tentative identification)

Nitrogenated
Acridine (Acridinylium or Acridinium Ion in water/acn)
Benzo[f]quinolone
Carbazole
Benzo[c] phenanthridine
Naphtho[2,1-c]cinnoline
Naphtho[f]naphtho[2,1,c]cinnoline
Phenanthridine
Benzo[c]cinnoline

573

574

575 **Table 3. Compararison between our analytical method and conventional solid phase extraction**

Our A-SPE methodology		**Manual SPE**
Analysis time*	50 min	240 min
Reagents	a) 2.5 ml of Hex+2.5 mL for cleaning up the column=5 mL of organic solvents. b) Silica column (re-usable)	a) 4 ml ACN, 3 ml methanol or 2.5 mL of acetone and 11 ml mixture hexane:isopropanol (2:5). TOTAL: 20.5 mL of organic solvents b) SPE cartridge (not re-usable) c) Vacuum Pump (Andrade-Eiroa and coll., 2010a)
Recoveries	HIGH (most of compounds show recoveries between 93 and 100%, except naphthalene, 1-methylnaphthalene and 2-methylnaphthalene which recoveries are about 75% and 82% and 84%respectively, Table S3)	MEDIUM (although the recovery of most of the PAHs is higher than 75%, acenaphthene recovery is only 50%, and the recovery of fluorene is only 64%)[3]
Reproducibility	VERY HIGH (RSD % about 3.2 %)	VERY LOW (RSD % between 19.2 and 2.7%)
Convenience	HIGHLY CONVENIENT	NOT CONVENIENT

576 *Analysis Time: clean-up+ 18 quantification of PAHs.

577

578

579

SUPPLEMENTARY INFORMATION

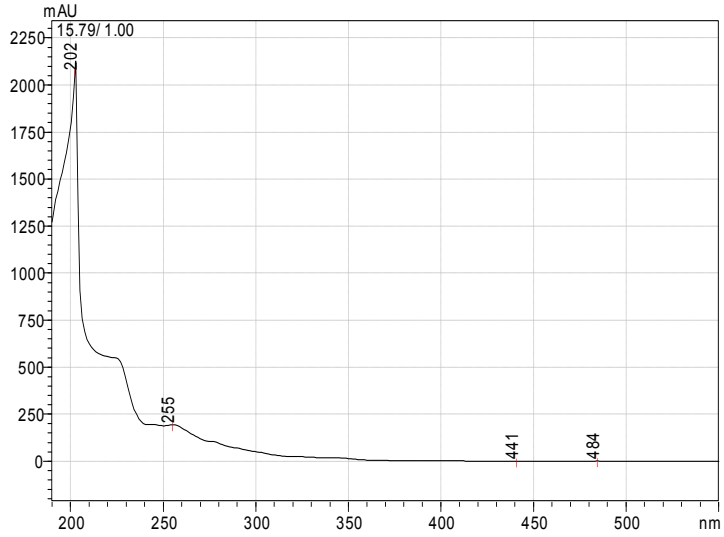


Figure S1. UV spectrum of the fraction eluting at 15.79 min. Spectral features belonging to Indene can be distinguished.

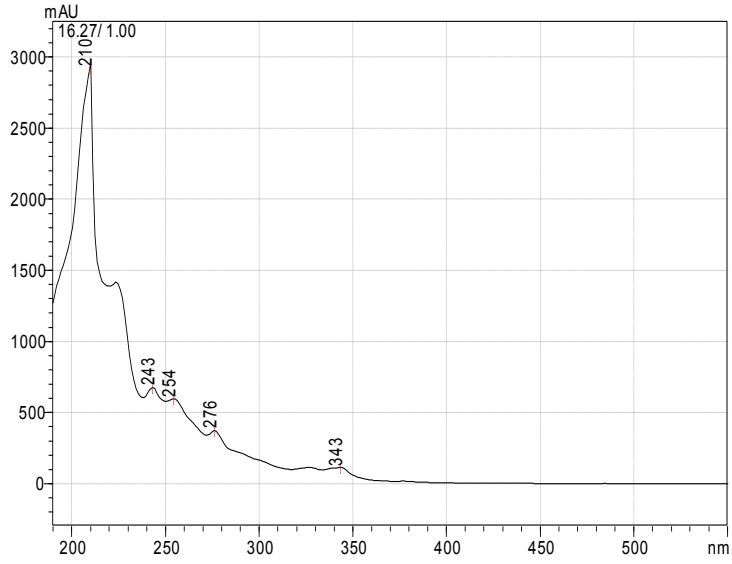


Figure S2. UV spectrum of the fraction eluting at 16.27 min. Spectral features belonging to light PAHs (i.e. naphthalene, fluoranthene and anthracene) can be distinguished.

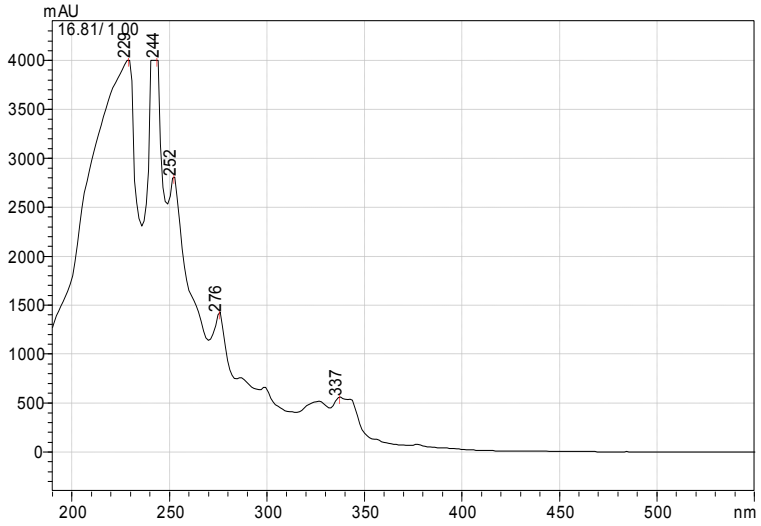


Figure S3. UV spectrum of the fraction eluting at 16.81 min. Spectral features belonging to medium-size PAHs (i.e. acenaphthene, fluoranthene, pyrene…) can be clearly distinguished.

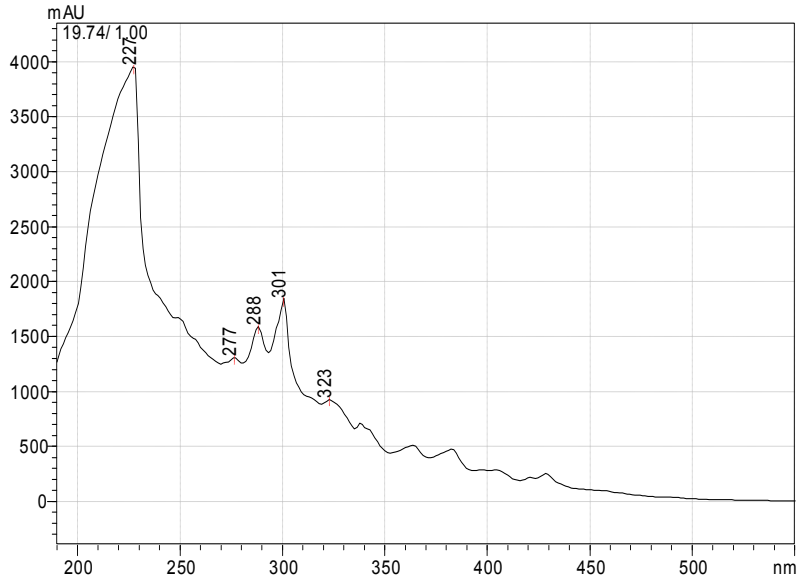


Figure S4. UV spectrum of the fraction eluting at 19.74 min. Spectral features belonging to heavy PAHs (i.e. perylene, benzo[ghi]perylene, anthranthrene, coronene) can be distinguished.

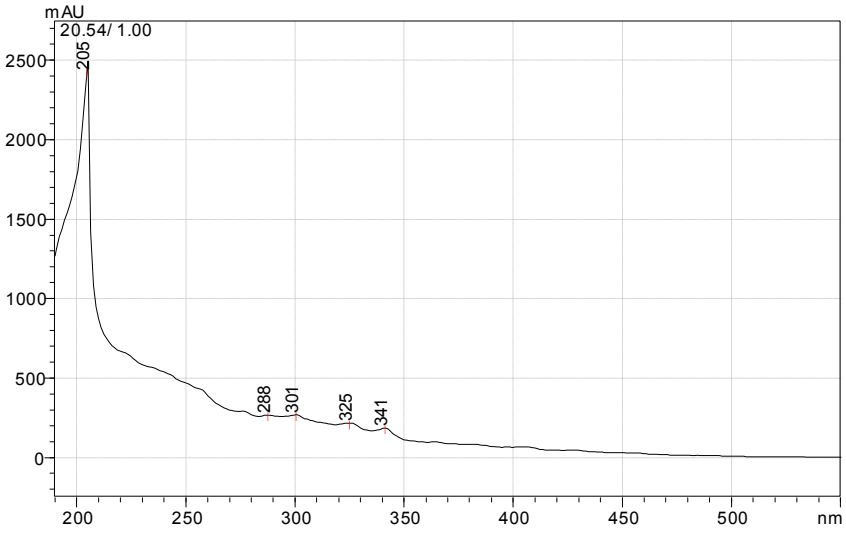


Figure S5. UV spectrum of the fraction eluting at 20.54 min. Spectral features belonging to heavy PAHs (i.e. coronene, naptho(2´.3´:1.2)-coronene) can be distinguished.

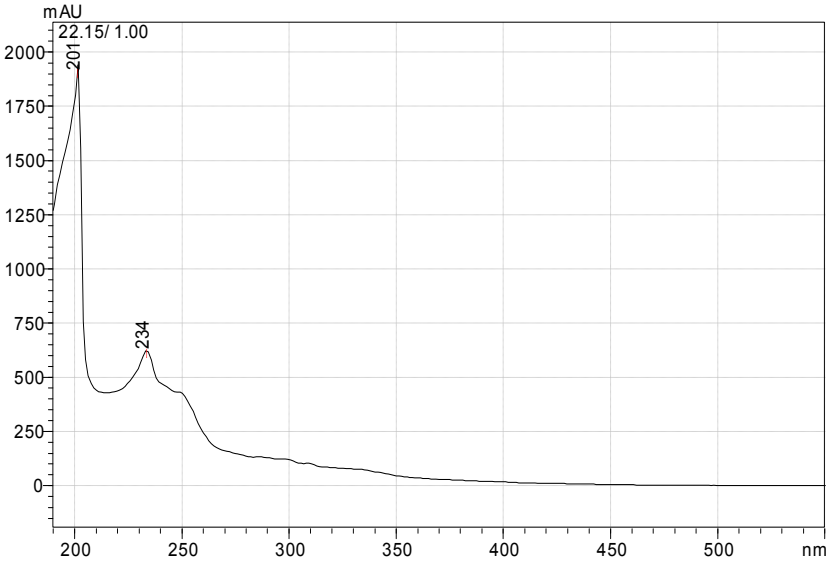


Figure S6. UV spectrum of the fraction eluting at 20.15 min. Spectral features belonging to oxa-PAHs can be clearly distinguished.

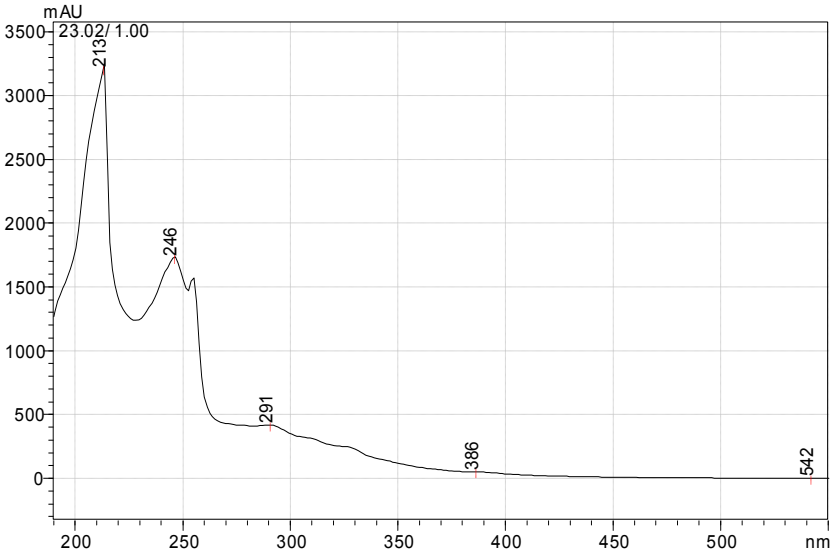


Figure S7. UV spectrum of the fraction eluting at 23.02 min. Spectral features belonging to fluorenene and 2-naphthalenecarboxaldehyde can be clearly distinguished.

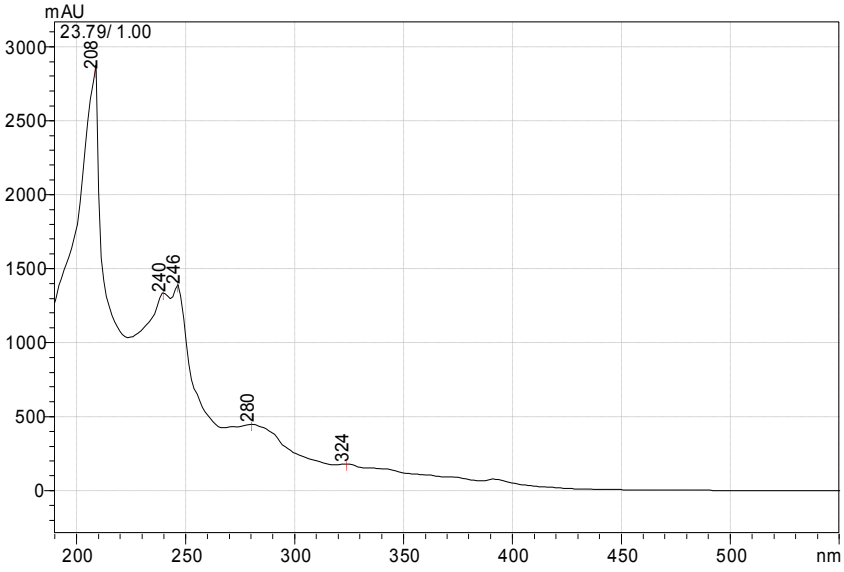


Figure S8. UV spectrum of the fraction eluting at 23.79 min. Spectral features belonging to 1-naphthalenecarboxaldehyde and 2-naphthalenecarboxaldehyde can be clearly distinguished.

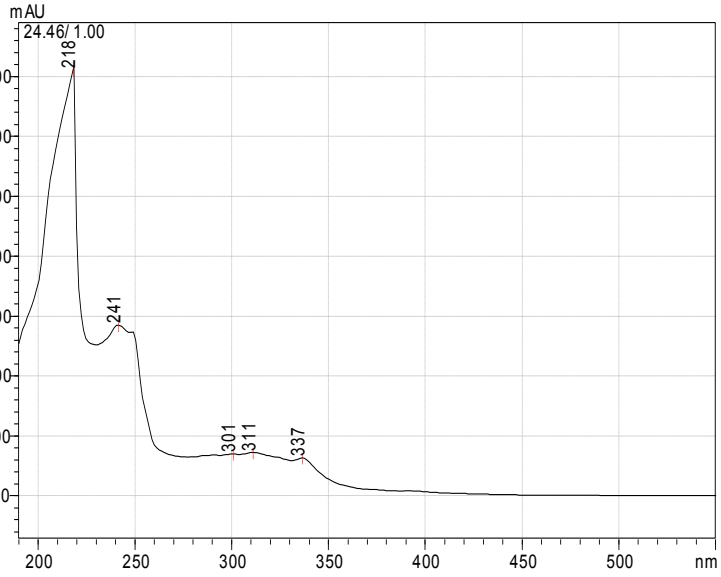


Figure S9. UV spectrum of the fraction eluting at 23.79 min. Spectral features belonging to 1-naphthalenecarboxaldehyde can be clearly distinguished.

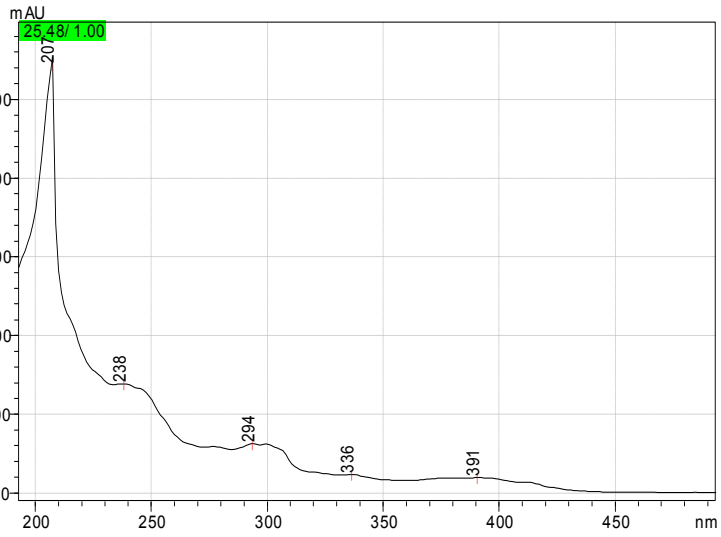


Figure S10. UV spectrum of the fraction eluting at 25.48 min. Spectral features belonging to benzanthrone can be clearly distinguished.

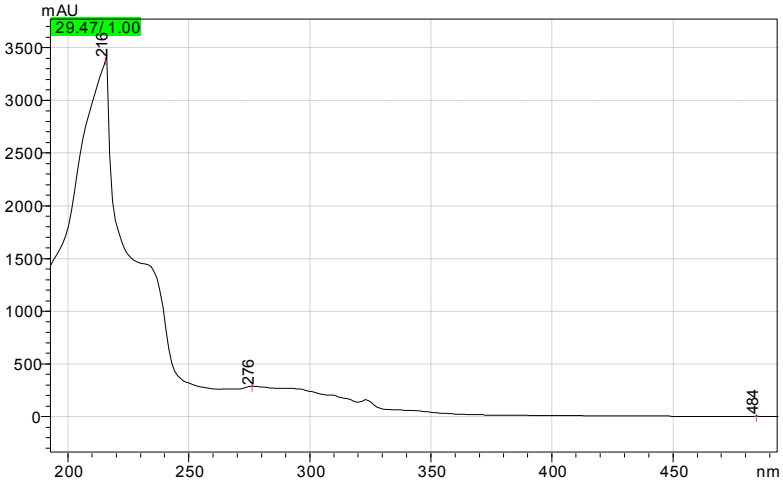


Figure S11. UV spectrum of the fraction eluting at 29.47 min. Spectral features belonging to 1-naphthol can be clearly distinguished.

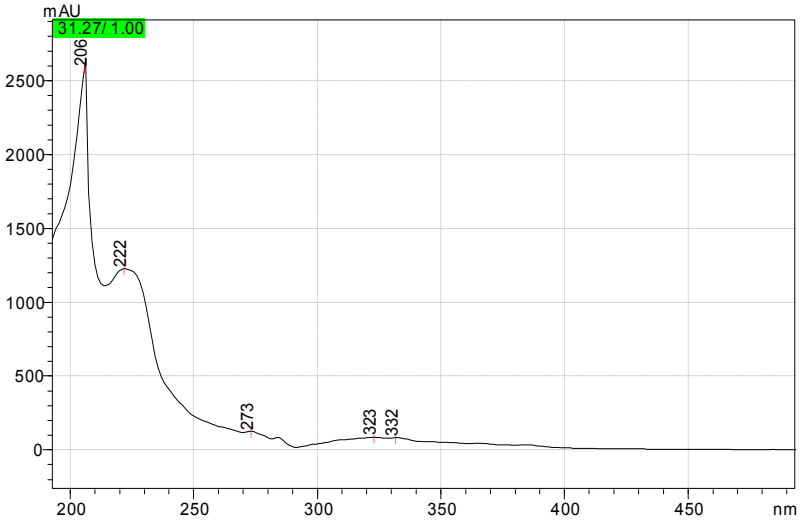


Figure S12. UV spectrum of the fraction eluting at 31.27 min. Spectral features belonging to 2-naphthalenecarboxaldehyde can be clearly distinguished.

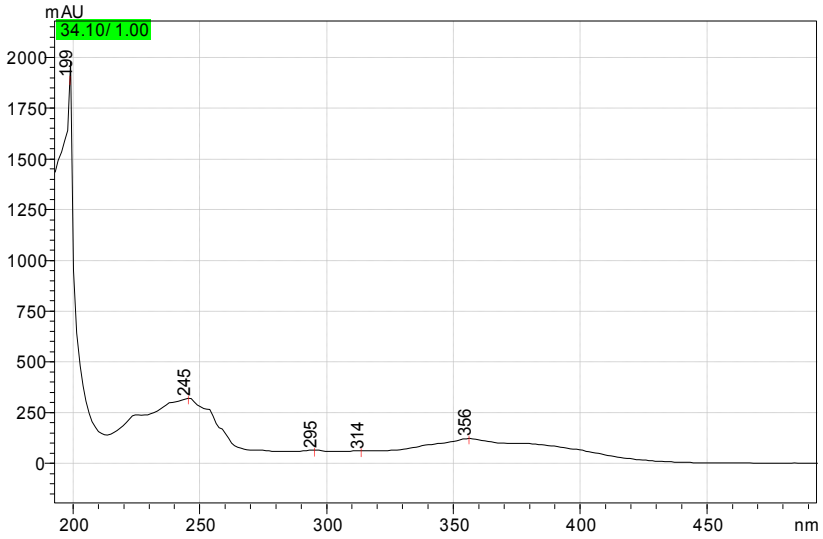


Figure S13. UV spectrum of the fraction eluting at 34.10 min. Spectral features belonging to phenalen-1-one can be clearly distinguished.

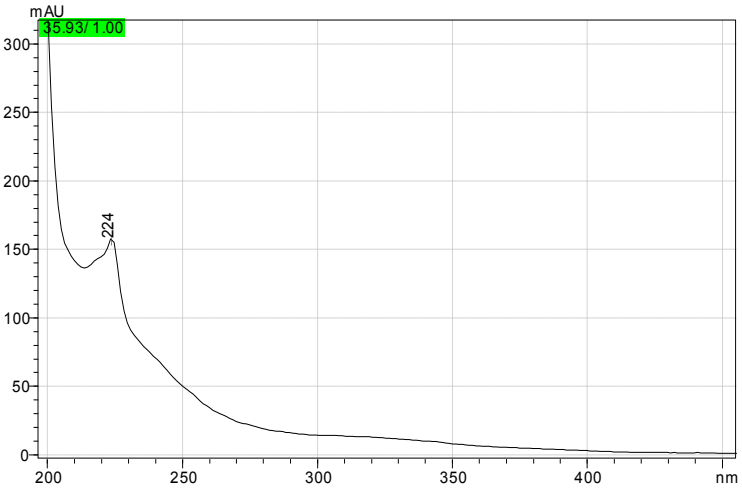


Figure S14. UV spectrum of the fraction eluting at 35.93 min. Spectral features belonging to can be clearly distinguished.

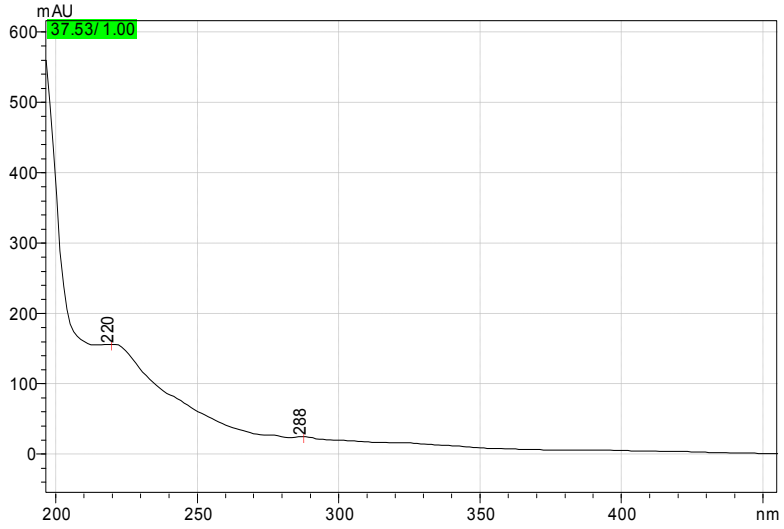


Figure S15. UV spectrum of the fraction eluting at 37.53 min. Spectral features belonging to can be clearly distinguished.

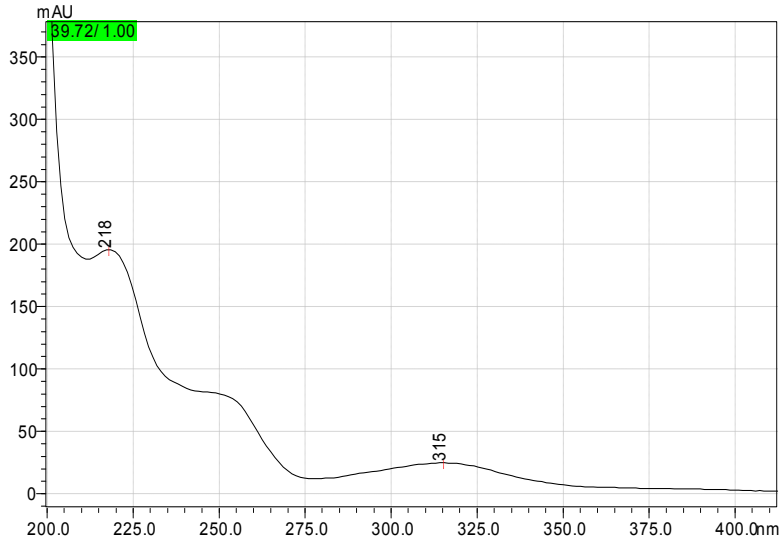


Figure S16. UV spectrum of the fraction eluting at 39.72 min. Spectral features belonging to phenanthrene-9,10-quinone can be clearly distinguished.

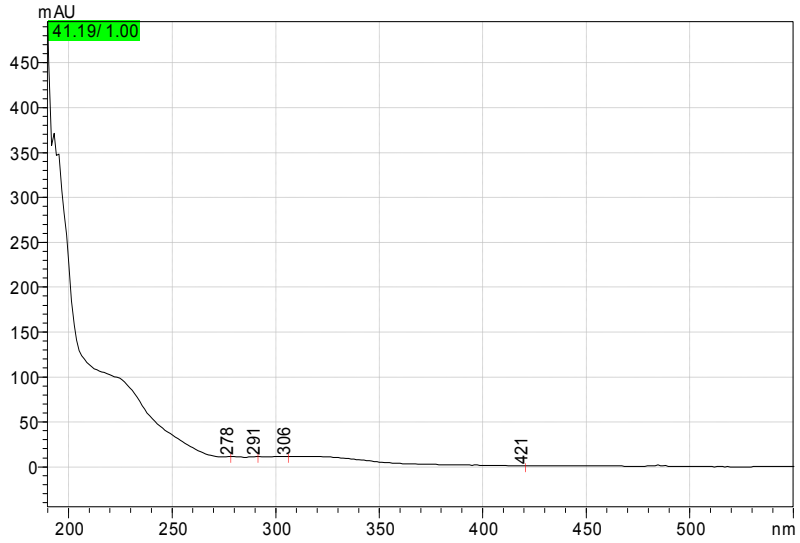


Figure S17. UV spectrum of the fraction eluting at 39.72 min. Spectral features belonging to acenaphthoquinone can be clearly distinguished.

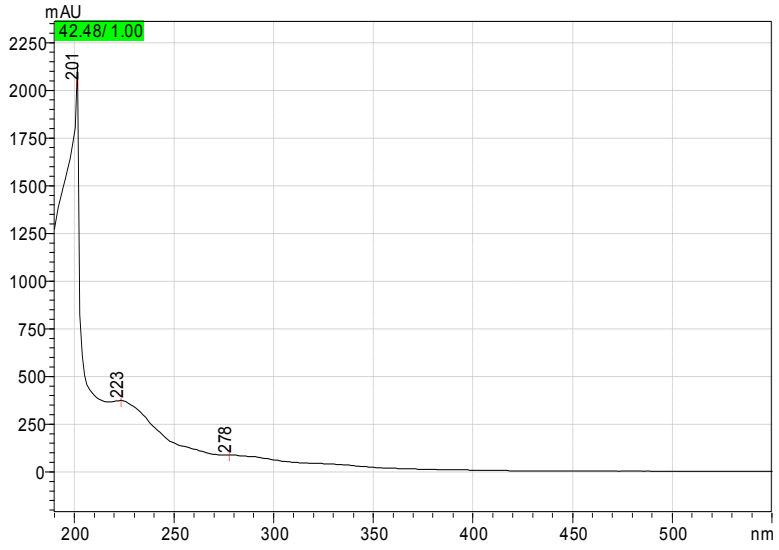


Figure S18. UV spectrum of the fraction eluting at 42.48 min. Spectral features belonging to 1-naphthalencarboxilic acid can be clearly distinguished.

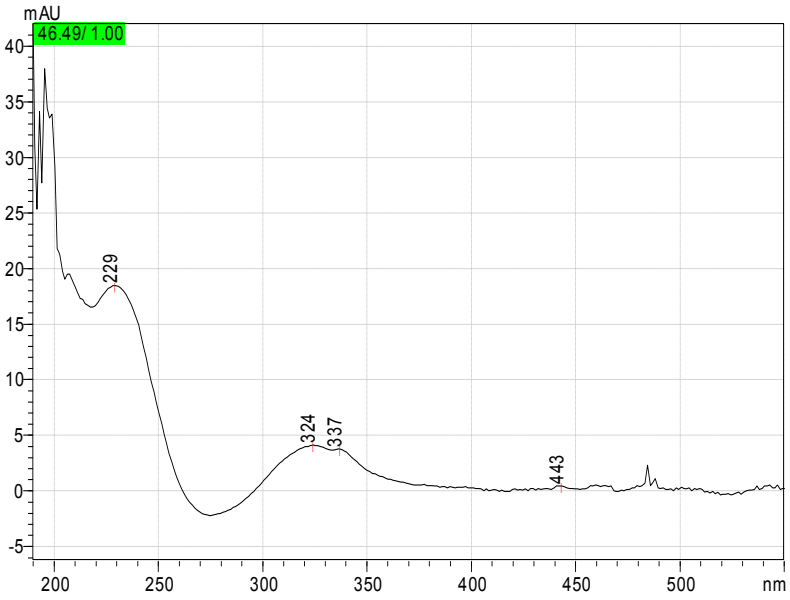


Figure S19. UV spectrum of the fraction eluting at 46.49 min. Spectral features belonging to 2-naphthalencarboxilic acid can be clearly distinguished.

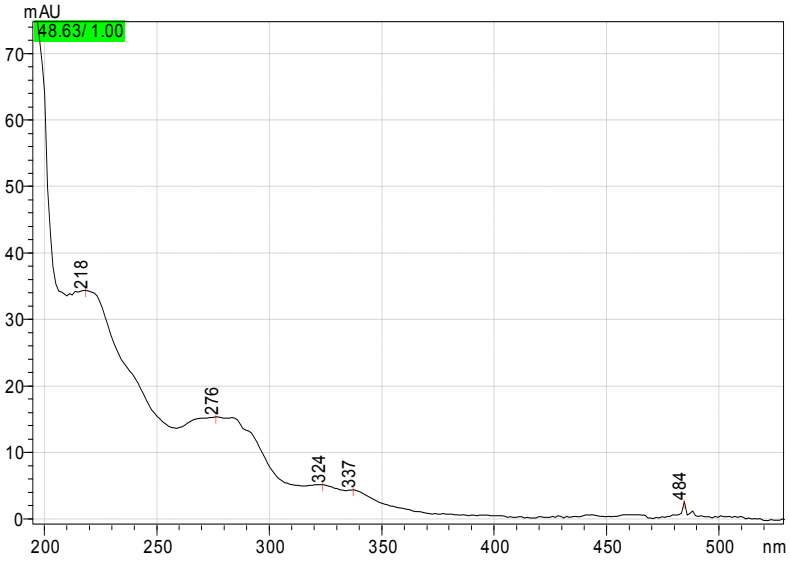


Figure S20. UV spectrum of the fraction eluting at 48.63 min. Spectral features belonging to 2-hydroxy-1-naphthoic acid can be clearly distinguished.

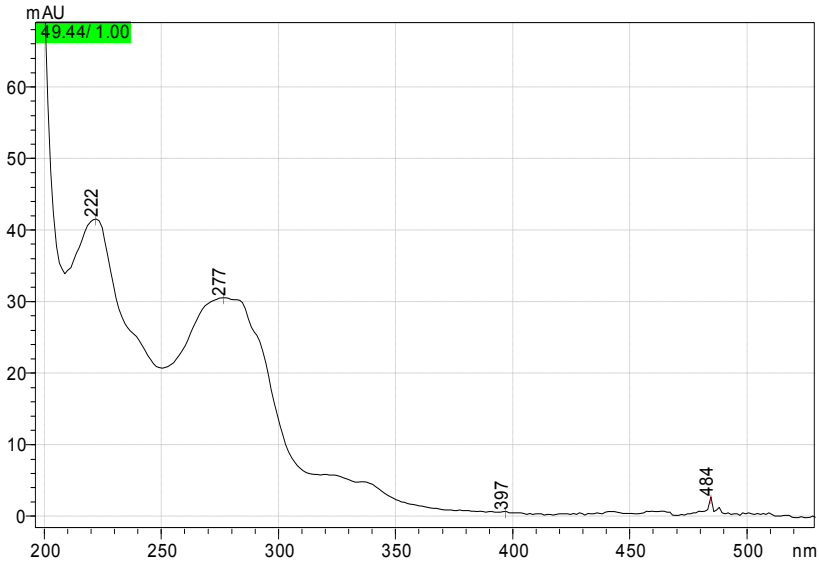


Figure S21. UV spectrum of the fraction eluting at 49.44 min. Spectral features belonging to another isomer of hydroxy-1-naphthoic acid can be clearly distinguished.

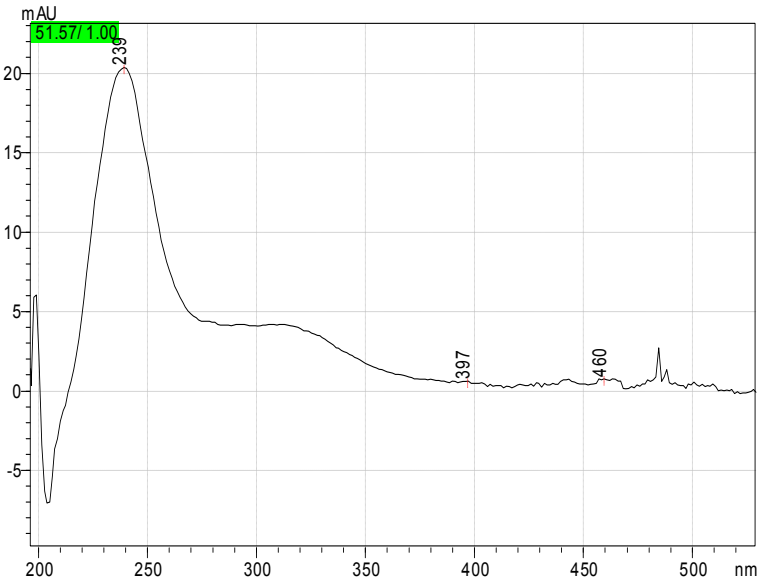


Figure S22. UV spectrum of the fraction eluting at 51.57 min. Spectral features belonging to naphthalene-1,6-dicarboxylic acid can be clearly distinguished.

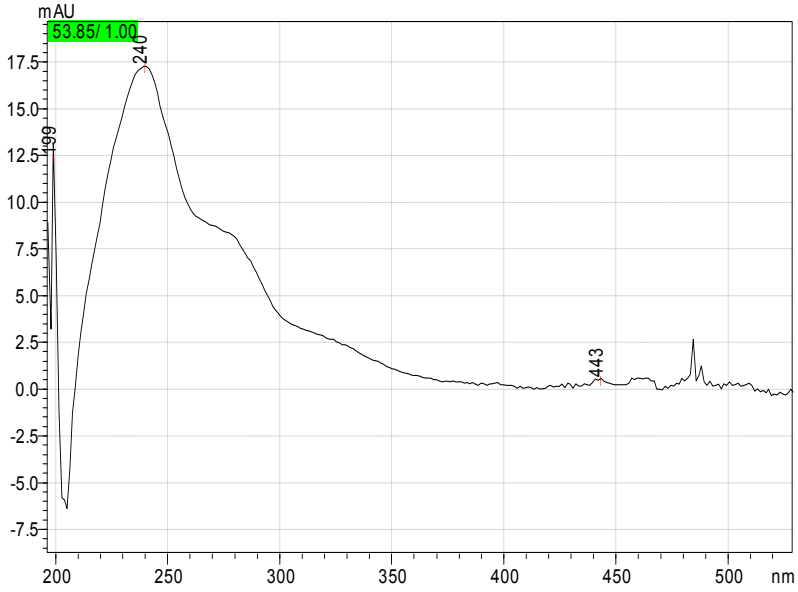


Figure S23. UV spectrum of the fraction eluting at 53.85 min. Spectral features belonging to naphthalene-2,7-dicarboxylic acid can be clearly distinguished.

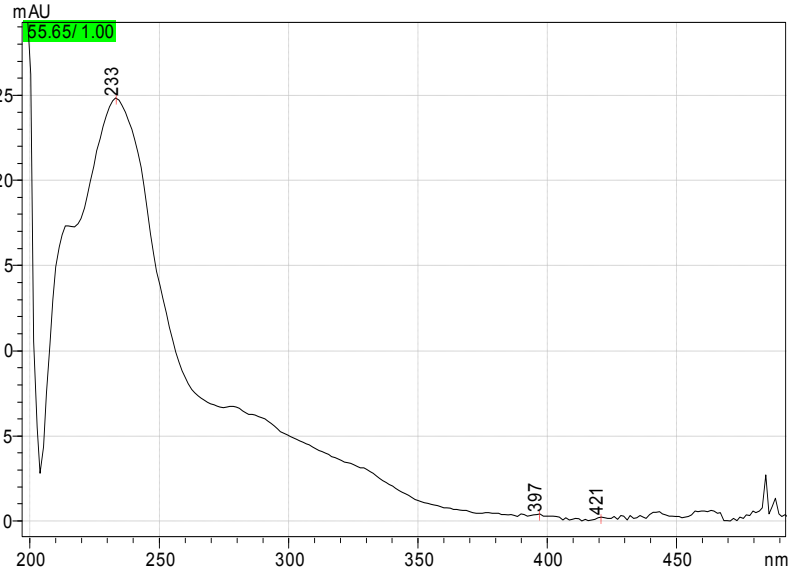


Figure S24. UV spectrum of the fraction eluting at 55.65 min. Spectral features belonging to naphthalene-1,7-dicarboxylic acid can be clearly distinguished. Ref. Davies, 1973.

709 **Table S1. Comparison of recovering percentages for several PAHs and several nitro-PAHs with**
710 **and without ultrasound probe. Sample: Kerosene soot treated with NO_2 during 1,5 hours. Mass: 8.2**
711 **mg/4 mL ACN.**

COMPOUND	λ (nm)	PDA Signals (mAU) after Ultrasound Probe UV-Visible Intensity Signal (1.5 min, 2 s on, 2 s off)	PDA Signals (mAU) Without Ultrasound Probe UV-Visible Intensity Signal	% Variation
Naphthalene+other light PAHs	220	ND	ND	-
Ethynyl-acenaphthylene	220	17.96	22.34	19.61
9,nitroanthracene+phenanthrene	254	57.8	58.42	1.96
Alkylphenanthrene	254	39.44	41.94	5.96
Nitrofluoranthenes+fluoranthene	236	158.07	175.20	9.78
1,Nitropyrene+pyrene	236	338.72	363.55	6.83
Benzo[b]fluorine	262	23.07	27.76	16.85
Cyclopenta[cd]pyrene	220	177.77	223.10	20.32
6,Nitrochrysene+Chrysene	254	64.17	72.78	11.83
Benzo[a]fluoranthene+Benzo[b]fluoranthene+ Perylene	220	17.30	19.82	12.71
Benzo[k]fluoranthene	220	8.25	9.01	8.44
Benzo[e]pyrene+Benzo[a]pyrene	254	31.97	38.91	17.84
Nitrocorannulene+Corannulene	254	25.85	28.48	9.23
Benzo[ghi]perylene+Unknown	220	85.28	105.34	19.04

Indeno[1,2,3-cd]pyrene	254	34.89	42.09	17.11
Dibenzo[a,e]pyrene	220	17.84	21.44	16.79
Coronene	300	98.77	115.59	14.55
Dibenzo[ah]coronene	300	21.31	25.94	17.85

712 ND. Not detected (below Detection Limit).

713

714 **Table S2. Polarizability data of the most representative compounds found in soot from**
715 **methyloctanoate-kerosene blends.**
716

Compound	Polarizability (cm^3)
Alkenes	$8.1\pm0.5\ 10^{-24}$
Naphthalene	$17.5\pm0.5\ 10^{-24}$
Pyrene	$28.7\pm0.5\ 10^{-24}$
1H-Phenalen-1-one	$22.2\pm0.5x10^{-24}$
9-Fluorenone	$21.6\pm0.5x10^{-24}$
2-Naphthol	$18.2\pm0.5\ 10^{-24}$
1-Naphthol	$18.2\pm0.5\ 10^{-24}$
1-Naphthalene-carboxaldehyde	$20.2\pm0.5\ 10^{-24}$
2-Naphthalene-carboxaldehyde	$20.2\pm0.5\ 10^{-24}$
1,4-chrysene-quinone	$31.2\pm0.5\ 10^{-24}$
5,6-chrysene-quinone	$30.3\pm0.5\ 10^{-24}$
1-naphthoic acid	$20.2\pm0.5\ 10^{-24}$

717

718 Table S3. **Table 4. Recoveries of 17 PAHs in soot from kerosene-methyloctanoate (20% v/v)**
719 **blends premixed flames.**

Compound	Recovery (%)
Naphthalene	58.5
Acenaphthylene	99.2
1-Methylnaphthalene	81.6
2-Methylnaphthalene	83.9
Acenaphthene	98.7
Phenanthrene	94.9
Anthracene	98.0
Fluoranthene	98.3
Pyrene	100.9
Benz[a]anthracene	86.4
Chrysene	99.8
Benzo[b]fluoranthene	97.6
Benzo[k]fluoranthene	98.9
Benzo[a]pyrene	99.2
Dibenzo[ah]anthracene	98.3
Benzo[ghi]perylene	95.1

Indeno[1,2,3-cd]pyrene	98.7

n=3, standard deviation within the range 3-7%