Optimisation of solid-state urea clathrate formation as a chemical separation method coupled to compound-specific stable carbon isotope analysis

Márton Novák, Csaba Kirchkeszer, Dóra Palya, Zsolt Bodai, Zoltán Nyiri, Norbert Magyar, József Kovács, Tamás Rikker and Zsuzsanna Eke

ABSTRACT

Solid-state urea clathrate formation (SSUCF) as a chemical separation method prior to stable carbon isotope fingerprinting of diesel fuel contaminations was studied. The stable carbon isotope ratios ($\delta^{13}$C) of $n$-alkanes in diesel fuel can be used to trace the origin of a contamination. The accurate measurement of the stable isotopic composition of individual compounds requires baseline separation from any other co-eluting compounds. For this purpose silica gel column chromatography (SGCC) and SSUCF were applied. Detailed optimisation of SSUCF was performed: different activators, clathrate formation temperatures, activator volumes, clathrate formation times and sample capacity were investigated. The main benefits of the developed method are reduced clathrate formation time and increased recoveries for lower molecular weight $n$-alkanes. The recoveries of the developed SSUCF method ranged between 63 and 100% for C10–C24 $n$-alkanes with relative standard deviation no more than 7%. The precision of the gas chromatography-isotope ratio mass spectrometry measurement was acceptable with a standard deviation of the $\delta^{13}$C values ranging between 0.08 and 0.15‰. The absence of isotopic fractionation was also investigated.

The robustness of the method was tested within a model experiment. Nine different water samples including distilled water, tap water, river water, industrial wastewaters and groundwater samples were spiked with the same diesel fuel. The water samples were extracted with $n$-hexane and after purification with both SGCC and SSUCF $n$-alkanes were measured. The $\delta^{13}$C values of $n$-alkanes were found to be similar for all samples. The importance of sample purification prior to compound-specific isotope analysis (CSIA) was also demonstrated within this model experiment by analysing samples from different stages of the sample preparation.

Our results show that the proposed method can remarkably improve the precision of compound-specific stable carbon isotope analysis of $n$-alkanes originating from diesel contamination of the aquatic environment.

CONTACT Zsuzsanna Eke eke.zsuzsanna@wirec.eu

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1. Introduction

Refined petroleum products such as diesel fuel are one of the most frequent contaminants in the aquatic environment (rivers, lakes, groundwater, coastal waters, etc.) [1,2]. To unequivocally establish the liability related to contaminations is one of the main purposes of environmental forensic investigations. Chemical fingerprinting methods are widely applied to identify the possible source and estimate the date of release to the environment [3]. The analytical methods developed for chemical fingerprinting have appreciably evolved over the past decades. However, the complexity of refined petroleum products still provides serious challenges in the field of chromatographic method development.

Compound-specific isotope analysis (CSIA) combines conventional gas chromatography with isotope ratio mass spectrometry (GC-IRMS) to determine the stable isotopic composition of individual compounds within a complex sample [4,5]. A huge number of studies published over the last two decades on the application of CSIA prove its utility in the field of environmental forensics. For a comprehensive overview, detailed review articles, textbooks and guides were published recently in the field of instrumental challenges as well as applications [6–15].

Stable isotope ratio data are especially valuable when the spilled material is a refined product, since these products may lack the sufficient quantity of the common components (e.g. high molecular weight biomarkers) used for source correlation studies [13]. Despite this fact only a handful of studies are available on the CSIA of diesel fuel contaminations. Still, chemical fingerprinting based on the stable carbon isotope ratios (δ¹³C) of individual n-alkanes has been applied successfully for source correlation studies of diesel fuel spills in aquatic environment [2,16–18].

Co-elution of any contaminant peak with the target compound can have negative effect on both accuracy and precision of the stable isotope ratio determination [4]. Since baseline separation of n-alkanes from all interfering peaks in diesel fuel is impossible with conventional gas chromatography [19], either selective sample preparation or special data processing such as background subtraction or mathematical deconvolution must be applied. The reliability of the later techniques can be influenced strongly by the ratio between the signal of the target peak and the background level or the degree of overlapping with a major interfering peak [18,20]. Ellis et al. [20] showed that in the case of crude oil the failure to apply sample purification techniques prior to CSIA of n-alkanes results in increased errors in precision. To the best of our knowledge, the importance of purification prior to CSIA of diesel fuel contaminations has not been adequately addressed or investigated previously. Source correlation studies [2,16–18] of diesel fuel contaminations were based on n-alkane isotopic fingerprinting without applying any selective purification method. In these cases it is possible that the measured stable isotope ratios are not fully compound-specific but still relevant in correlation studies. Nevertheless, the discriminatory potential obviously decreases with the decrease of accuracy and precision of isotope ratio data.

Solid-state urea clathrate formation (SSUCF) and various types of molecular sieve chromatography techniques both proved to be applicable for the separation of n-alkanes from complex hydrocarbon mixtures [20]. Nwadinigwe et al. [21] published a
laboratory-scale study on the comparison of these techniques for separating \( n \)-alkanes from crude oil. Their results showed that purification with urea has several advantages over molecular sieve techniques. For example it can separate a larger range of \( n \)-alkanes with high efficiency and it is also less time-consuming. The disadvantage of urea clathrate formation is pointed out by Ellis et al. [20]. The efficiency of the separation for lower molecular weight \( n \)-alkanes (below \( C_{20} \)) is lower for urea clathrate formation compared to that for molecular sieve techniques.

SSUCF separates \( n \)-alkanes based on their molecular shape. For a hydrocarbon to form clathrate with urea a long, unbranched chain, usually containing at least six carbon atoms is necessary under the conditions most often used (ambient temperature and atmospheric pressure) [22]. This means that in the case of diesel fuels, which are blended with biodiesel, urea forms clathrate with fatty acid methyl esters (FAMEs) as well. To address this problem Harvey et al. [23] combined urea clathrate formation with normal-phase column chromatography.

To achieve effective clathrate formation a polar reagent solvent called an activator is required. The dissolution of urea in the activator releases the molecules from their tetragonal crystal lattice and promotes forming chiral helical hollow tubes on contact with \( n \)-alkanes [24]. The resulting crystalline clathrate can be separated by filtration and then washed with a non-polar solvent. The decomposition of the crystalline precipitate and the recovery of \( n \)-alkanes are most often performed by dissolving the clathrate in warm water followed by extraction of the alkanes [23,25,26]. Although numerous methods [23,25–28] were published on the application of SSUCF following the above-mentioned process, to the best of our knowledge, the detailed optimisation of clathrate formation for analytical purposes has not yet been published.

The aim of this research was to optimise the parameters of SSUCF as a selective chemical separation method coupled to compound-specific stable carbon isotope analysis (carbon CSIA). In the course of method development different activators, clathrate formation temperatures, activator volumes, clathrate formation times and sample capacity were investigated. Our purpose was to develop a routinely applicable purification method for stable isotopic characterisation of \( n \)-alkanes in environmental samples contaminated with diesel fuel. Therefore both the poor efficiency for lower molecular weight \( n \)-alkanes and convenience (time and equipment demand) were considered throughout the method development. To verify the suitability of the developed purification method for GC-IRMS measurements, isotopic fractionation was investigated. We also aimed to demonstrate the importance of purification prior to CSIA of \( n \)-alkanes in diesel fuel. Finally, we applied our method to nine different water samples spiked with the same diesel fuel to test whether various sample matrices have any notable effect on the measurement.

2. Experimental

2.1. Chemicals and reagents

Urea (purity \( \geq 99\% \)) and anhydrous calcium chloride (purity \( \geq 98\% \)) were obtained from Molar Chemicals Ltd. (Budapest, Hungary). Silica gel cartridges (Isolute SI 5 g/25 mL with
50 µm average particle size and 60 Å nominal porosity) were purchased from Biotage (Uppsala, Sweden). Filtration of the solid-state urea clathrate was carried out using Whatman grade 50 (2.7 µm) quantitative filter paper. The filter paper was purchased from Sigma-Aldrich (Steinheim, Germany). Methanol was from LGC Promochem GmbH (Wesel, Germany) and was of Optigrade® quality. Propan-1-ol and propan-2-ol were from Merck (Darmstadt, Germany) and were of LiChrosolv® quality. Ethanol (96% purity) was purchased from Thomsaker Finomvegyszer Ltd. (Budapest, Hungary). N-Hexane and n-pentane were obtained from Sigma-Aldrich (Steinheim, Germany) and were of at least 99% purity. Nitrogen (purity 4.5) was purchased from Messer Group GmbH (Bad Soden, Germany). The C_{10}–C_{24} n-alkane standards (purity ≥ 99%) were purchased from Sigma-Aldrich, the n-alkane stock solutions were prepared in n-hexane. The FAME mix was purchased from Sigma-Aldrich (Steinheim, Germany). Stable carbon isotope ratio measurements were calibrated using in-house caffeine (δ^{13}C = −42.37‰ vs. VPDB) and phenacetin (δ^{13}C = −26.65‰ vs. VPDB) standards. In-house standards were calibrated by elemental analysis coupled to IRMS against IAEA-600 caffeine standard (δ^{13}C = −27.77‰ vs. VPDB). The reference carbon dioxide gas (Alphagas isotope) was purchased from Messer (Bad Soden, Germany). Diesel oil was purchased from a local OMV petrol station in Budapest, Hungary.

2.2. Instrumentation

In the course of sample preparation an IKA HS 250 B compact flat orbital shaker, an IKA RCT Basic heated magnetic stirrer and a Hermle Z206A centrifuge were used. The development of the sample preparation method was performed on an Agilent 6890N gas chromatograph equipped with a flame ionisation detector (FID) and an Agilent 7683B autosampler. Carbon CSIA analyses were performed using an Agilent 6890N gas chromatograph equipped with a 7683B autosampler and a FID detector coupled to a GV Instruments Isoprime™ isotope ratio mass spectrometer.

2.3. Gas chromatographic analysis

Chromatographic runs during the optimisation of the SSUCF were carried out on a 10 m long RTX-5 column with 0.1 mm internal diameter and 0.1 µm film thickness. The carrier gas was hydrogen at a constant flow rate of 0.5 mL min\(^{-1}\). Injections of 1 µL were made in split mode into a 280°C inlet with a split ratio of 1:50. The oven was programmed at 40°C for 2 min, then heated up at 12°C min\(^{-1}\) to 290°C and held at this final temperature for 3 min. The FID was operated at 300°C.

2.4. Compound-specific stable carbon isotope analysis

The separation of individual n-alkane compounds was performed on an HP-5 column (30 m × 0.32 mm i.d. × 0.25 µm). The carrier gas was helium at a constant flow rate of 2.0 mL min\(^{-1}\). One µL aliquots were injected in splitless mode with 1.5 min splitless time to an injection port held at 280°C. Oven temperature was programmed at 40°C and held for 2 minutes and then ramped at 5°C min\(^{-1}\) to 260°C followed by a 40°C min\(^{-1}\) ramp to 300°C. This final temperature was held for 5 minutes. For stable
carbon isotope measurements separated n-alkanes were combusted to carbon dioxide and water using a microfurnace filled with copper-oxide operated at 850°C. Water was removed by a cryogenic trap operated at −100°C. Precise isotope ratio measurement was obtained using Alphagas isotope (δ13C = −25.1‰ vs. VPDB) as the carbon dioxide reference gas and Micromass reference gas injector system. In each sample run five pulses of reference gas were introduced to the IRMS before and one after the n-alkane peaks eluting from the capillary column. All samples were injected five times. The five injections were performed on at least two different days. Stable carbon isotope ratios were reported as delta (δ) notations, which is determined using the following equation:

\[ \delta^{13}C_{i} = \left( \frac{R_{i} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000, \]  

where \( R_{i} \) is the absolute \( ^{13}C/^{12}C \) isotope ratio of the target compounds or an internationally accepted reference standard (\( R_{\text{standard}} = 0.01122372; \) (VPDB)) [10]. Accuracy of the measurement was checked daily by measuring the isotopic composition of the in-house standards with a known isotopic composition.

2.5. Working solutions

Optimisation of SSUCF required a relatively large amount of \( C_{10} \sim C_{24} \) n-alkane working solution. To keep costs down, working solutions were prepared from diesel fuel using silica gel column chromatography (SGCC) and SSUCF as purifying methods. The silica gel column was dried at 110°C for 24 hours. For conditioning, 500 mL of \( n \)-hexane was used prior to application. A total of 20 mL of diesel fuel was fractionated using 200 g silica gel (with 50 µm average particle size and 60 Å nominal porosity). The aliphatic fraction was eluted with 600 mL \( n \)-hexane and evaporated to 40 mL final volume using a gentle stream of nitrogen. SSUCF was performed based on a previously described method by Lappas et al. [29]. A total of 120 g urea and 250 mL methanol were added to the concentrated aliphatic fraction. The mixture was placed in a 1000 mL volume closed bottle and stirred for 30 min at 65°C using a heated magnetic stirrer at 1200 rpm. The mixture was further stirred at room temperature for 90 min and then cooled down to 10°C and stirred for an additional 30 min. The solid clathrate was isolated by filtration using a Büchner funnel and filter paper, and then washed with 250 mL \( n \)-hexane. The purified solid-state urea clathrate was dissolved in 1000 mL of 70°C distilled water; then the \( n \)-alkanes were extracted three times with 100 mL \( n \)-hexane. The three extracts were combined. The exact concentration of \( n \)-alkanes in the obtained solution (WS 3) was measured with GC-FID (see conditions in Section 2.3) using external calibration. The other working solutions (WS 1; WS 2; WS 4; WS 5) were prepared from WS 3 by either dilution in \( n \)-hexane or evaporation under a gentle stream of nitrogen (Table 1).

2.6. Optimisation of SSUCF

During optimisation, the following parameters were kept uniform. Every experiment was carried out using WS 3 except for the investigation of the sample capacity. Six grams of
urea and 2 mL working solution were used to ensure the large excess of urea. Since clathrate formation is an equilibrium process, this facilitated the clathrate formation to fulfilment. The isolation of the solid clathrate was achieved by centrifugation at 2000 rpm (410 rcf) for 5 min. The clathrate was washed twice with 10 mL \( n \)-hexane. After each washing step centrifugation (2000 rpm (410 rcf) for 5 min) was performed to separate the solvent. The purified clathrate was dissolved in 50 mL of 70°C distilled water. The \( n \)-alkanes were extracted two times with 8 mL \( n \)-hexane. The intense shaking of the urea mixture was performed at room temperature for 45 min at 300 cycles \( \text{min}^{-1} \) shaking speed.

The effect of temperature on clathrate formation was tested at four different values: 10°C, room temperature (approx. 22–25°C), 45°C and 65°C. The experiments were carried out using a heated magnetic stirrer operated at 1200 rpm. The activator was 12 mL methanol. The SSUCF was performed by stirring the mixture at the desired temperature for 45 min. Those samples where 45°C or 65°C was applied were cooled down to 10°C and stirred for further 15 min after the clathrate formation. Four different activators were tested: methanol, ethanol, propan-1-ol and propan-2-ol. The volume of the activators was 12 mL. The volume of the activator was tested using 6 mL, 9 mL, 12 mL and 15 mL of methanol. The clathrate formation time was tested at six different values: 1 min, 5 min, 15 min, 30 min, 60 min and 90 min. The capacity was tested at five different \( n \)-alkane concentration levels (see Table 1. WS 1–5).

At each parameter set five parallel sample preparations were performed. The recoveries for the \( n \)-alkanes were calculated by dividing the peak areas of the parallel measurements with the peak areas measured form the initial working solution used.

### 2.7. Characterisation of the main sample preparation steps

To evaluate the possible isotopic fractionation during SGCC, 2 mL of WS 6 (see Table 1.) was applied to a 5 g silica gel column. The silica gel column was dried at 110°C for 24 hours. For conditioning 15 mL of \( n \)-hexane was used prior to application. The \( n \)-alkanes were eluted using 20 mL \( n \)-hexane. The solution was concentrated under a gentle stream of nitrogen to the final volume of 2 mL.

| \( n \)-alkane | \( \mu \text{g mL}^{-1} \) |
|---------------|------------------|
| \( C_{10} \)  | 8.5              |
| \( C_{11} \)  | 15.0             |
| \( C_{12} \)  | 16.6             |
| \( C_{13} \)  | 18.4             |
| \( C_{14} \)  | 19.4             |
| \( C_{15} \)  | 18.8             |
| \( C_{16} \)  | 16.6             |
| \( C_{17} \)  | 15.2             |
| \( C_{18} \)  | 13.1             |
| \( C_{19} \)  | 11.0             |
| \( C_{20} \)  | 8.8              |
| \( C_{21} \)  | 7.0              |
| \( C_{22} \)  | 5.4              |
| \( C_{23} \)  | 3.8              |
| \( C_{24} \)  | 2.5              |
| Total         | 180              |

Table 1. The concentration of \( C_{10} \)-\( C_{24} \) \( n \)-alkanes in the working solutions (WS 1–6).
The investigation of the SSUCF-induced isotopic fractionation was carried out using 6 g urea activated with 12 mL methanol in a 40 mL EPA vial. Two mL of WS 6 was added and the vial was closed tightly. The mixture was shaken at 300 cycles min\(^{-1}\) shaking speed for five minutes at room temperature. The crystalline urea clathrate was then isolated by centrifugation of the mixture for 5 min at 2000 rpm (410 rcf). The liquid phase was decanted and the clathrate washed two times with 10 mL \(n\)-hexane. After each washing step centrifugation was used to separate the solvent. The clathrate was then dissolved in 70°C distilled water and the \(n\)-alkanes were isolated by extracting two times with 8 mL \(n\)-hexane. The extracts were united and evaporated under a gentle stream of nitrogen to a final volume of 2 mL.

For both SGCC and SSUCF, five parallel sample preparations were carried out separately. Each sample and the working solution were measured five times with GC-IRMS. The precision of the recoveries and the \(\delta^{13}C\) values of \(n\)-alkanes were determined by evaluating the relative standard deviation (RSD) and the standard deviation of the parameters, respectively.

### 2.8. The analysis of water samples spiked with diesel fuel

We applied our method to nine different water samples spiked with the same diesel fuel to test whether various sample matrices have any notable effect on the carbon CSIA measurement. The samples were distilled water, tap water, river water, three different types of industrial wastewater, confined water and two different types of groundwater. Basic water-quality parameters (pH, electrical conductivity, dissolved oxygen, oxygen saturation, chemical oxygen demand and concentrations of ammonium ion, nitrite ion, nitrate ion and orthophosphate) characterising these samples are available in the supporting information (see Table S1).

A total of 500 mL of each water sample was spiked with 1 mL diesel fuel. The spiked samples were left under a fume hood for 24 hours in an open bottle shaken at 80 cycles min\(^{-1}\) shaking speed. The samples were extracted two times with 20 mL \(n\)-hexane. The extracts were united and dried with anhydrous calcium chloride, then evaporated under a gentle stream of nitrogen to 2 mL final volume. These concentrated primary extracts were purified with SGCC. The resulting aliphatic fractions were further purified with SSUCF to separate the \(n\)-alkanes. The SGCC and SSUCF were carried out as described in Section 2.7. The two steps were connected by processing 2 mL aliquot of the unconcentrated aliphatic fraction. The purified solutions, containing the \(n\)-alkanes, were measured with GC-IRMS.

For one groundwater sample (Sample ID: GW2), the primary extract and the aliphatic fraction were also measured with GC-IRMS. The \(\delta^{13}C\) profiles of these samples were compared with the \(\delta^{13}C\) profile of the urea purified \(n\)-alkane solution to demonstrate the importance of peak purity in carbon CSIA. The diesel oil used for these experiments was purified as well. The resulting solution containing the \(n\)-alkanes was measured with GC-IRMS to compare the \(\delta^{13}C\) values measured from the spiked water samples and the contaminating diesel fuel.
2.9. Uni- and multivariate statistical analysis

Besides the calculation of the descriptive statistics of the data sets, uni- and multivariate statistical methods (T-test, cluster analysis and discriminant analysis) were applied to evaluate the $\delta^{13}$C data sets. The significant differences ($p = 0.05$) between the $\delta^{13}$C values of $n$-alkanes in the samples were investigated using a T-test. F-test was applied to compare the variances of the samples. To answer the question of which prepared samples were similar to each other, hierarchical cluster analysis was used with Ward’s method [30] and squared Euclidean distance. The accuracy of the cluster results was verified using linear discriminant analysis, which separates the observations with linear planes resulting in the percentage of correctly classified cases [31,32]. The statistical analyses were performed using R Software 3.1.2.

3. Results and discussion

3.1. Optimisation of SSUCF

In order to maximise the recovery of $n$-alkanes and minimise the time and effort required by the sample preparation, detailed optimisation of SSUCF was carried out. The temperature of the $n$-alkane–urea clathrate formation was tested in the first step. As previously described by Lappas et al. [29] and Marquart et al. [33], higher clathrate formation temperatures (55–60°C) increase the recovery of higher molecular weight $n$-alkanes by increasing the solubility in the activator-urea phase. For improving the recoveries of the lower molecular weight compounds Marquart et al. [33] suggested the inclusion of an additional, low-temperature (0°C) period at the end of the clathrate formation. Lappas et al. [29] also used a three-stage clathrate formation procedure starting at 55–60°C and finishing at 10°C to obtain the highest recoveries for both higher and lower molecular weight $n$-alkanes. However, these papers provide no information on either the precision of the recoveries at different temperatures or the extent by which the recoveries of the higher molecular weight $n$-alkanes are affected by omitting the high-temperature (55–60°C) stage.

Hence, we decided to test two multistage procedures starting at elevated temperatures (45°C and 65°C) and finishing at 10°C and two one-stage versions: room temperature and constant 10°C. The recoveries obtained at these clathrate formation temperatures are shown in Figure 1.

Indeed, elevating the temperature to 65°C gave the highest recoveries for higher molecular weight $n$-alkanes (96–100% for $C_{16}$–$C_{24}$ $n$-alkanes). However, heating up to this temperature resulted in progressively worse recovery and precision for lower molecular weight $n$-alkanes (58–94% for $C_{10}$–$C_{15}$ $n$-alkanes with RSD ranging between 6 and 42%) despite the 10°C period at the end of the clathrate formation. The recovery and precision values obtained at 10°C, room temperature and with the 45°C/10°C temperature programme are similar to each other: 60–100% for $C_{10}$–$C_{24}$ $n$-alkanes with RSD ranging between 4 and 8%. This suggests that evaporation of the lower molecular weight compounds plays a major role in the loss of these compounds. To increase their recovery, it is better not to exceed modest heating (max 45°C) during the clathrate formation, despite the inclusion of a lower temperature period. Thus, room temperature can maximise sample throughput without any unacceptable sample fraction loss.
The nature of the activator was also tested. Our results with methanol, ethanol, propan-1-ol and propan-2-ol as activators are shown in Figure 2a. We achieved the highest recoveries (between 60–100%) and the best precision (RSD less than 7%) using methanol.

The volume of the activator was varied between 6 and 15 mL using 6 g urea and 2 mL working solution each case. The results are presented in Figure 2b. Using only 6 mL methanol gave the smallest recoveries for \( n \)-alkanes longer than dodecane. Furthermore, the difference between the recoveries obtained with 6 mL methanol and the maximum recoveries increased progressively with molecular weight. So this amount proved to be too little for optimal analyte contact with the urea for clathrate formation. On the other hand, 15 mL methanol decreases the recoveries of lower molecular weight \( n \)-alkanes (34–81% for \( C_{10} \)–\( C_{15} \) \( n \)-alkanes), likely by keeping more in solution instead of allowing them to precipitate as clathrate. At the volume of 9 mL and 12 mL the recoveries are about the same, but the precision is enhanced with 12 mL methanol. Thus the mass ratio of urea: methanol was found to be optimal at 5:8 with recoveries between 63 and 100% and RSD values ranging between 4 and 7%.

The influence of clathrate formation time was investigated in the range of 1–90 minutes at room temperature. The results are shown in Figure 2c. A duration of 1 min is not enough to reach maximum recoveries and also the precision is far worse (14–19% RSD) compared with that at longer clathrate formation times. A duration of 5 min resulted in recoveries (62–99%) near the maximum with good precision (4–8% RSD). Further increase in the clathrate formation time does not bring notable improvement, either in recovery or in precision.

The capacity of SSUCF was tested by examining the recoveries as a function of the amount of \( n \)-alkanes. The same volume (2 mL) of working solutions (WS 1–5) containing...
different amounts of \( n \)-alkanes was used. The total \( n \)-alkane concentration of these working solutions varied from 180 µg mL\(^{-1}\) to 18 mg mL\(^{-1}\). (The concentrations of the individual compounds are presented in Table 1.) As demonstrated in Figure 2d the recoveries are independent of the total concentration of \( n \)-alkanes in the range of 180–9005 µg mL\(^{-1}\). A definite decrease in the recoveries of higher molecular weight \( n \)-alkanes was observed when the most concentrated solution (WS 5) was used. This phenomenon is likely attributed to the limited solubility of higher molecular weight \( n \)-alkanes at the conditions used in our experiments (e.g. temperature, activator, etc.). Nevertheless, the capacity of our SSUCF method exceeds the concentration range necessary with regard to the linearity of the IRMS instrument.

All further parameters of the sample preparation were optimised with a focus on maximising sample throughput. A key factor to achieve shorter clathrate formation times is the intense shaking or stirring of the urea mixture, which ensures the effective contact between the urea and the \( n \)-alkanes. Since temperature control proved to be unnecessary, a simple flat orbital shaker was used at the highest shaking speed available, namely 300 cycles min\(^{-1}\). The separation and the washing of the solid clathrate were carried out using centrifugation instead of vacuum filtration. Only two washing steps were applied to remove any interfering compounds from the surface of the crystalline clathrate. Also the extraction after the dissolution of the clathrate was carried out in only two steps. All these settings provide a fast and convenient purification method, which ensures increased recovery for lower molecular weight \( n \)-alkanes (80–88% for \( C_{13} \)–\( C_{16} \) \( n \)-alkanes, compared to 3–60% for \( C_{13} \)–\( C_{16} \) \( n \)-alkanes as described by Ellis \textit{et al.} [20] for example).
3.2. Characterisation of the main sample preparation steps

The absence of complete recovery introduces compound fractionation and the possibility of stable isotope fractionation. Recoveries as well as the isotopic fractionation effect were investigated separately for SGCC and SSUCF. The results are presented in Table 2. The SGCC failed to completely recover n-alkanes with less than 17 carbon atoms because of the evaporative loss during the concentration of the aliphatic fraction. Still its recoveries are better than those of the SSUCF for all compounds. In the case of SGCC the lowest values were obtained for decane and undecane, 86% and 89%, respectively. The lower recoveries of the SSUCF are the combined effect of evaporative loss and the chain length dependence on the stability of the n-alkane–urea clathrate [22].

The average $\delta^{13}$C values of n-alkanes for WS 6 before treatment and the five parallel solutions treated either with SGCC or SSUCF are also presented in Table 2. The precision is acceptable in all cases; standard deviation values do not exceed 0.15‰ for any of the n-alkanes. Hierarchical cluster analysis was used to examine the possible isotopic fractionation effect. For both SGCC (Figure 3a) and SSUCF (Figure 3b) six groups were distinguished. The parallel samples did not form separated groups either in the case of SGCC or for SSUCF. The five parallels measured to each SGCC or SSUCF treated and the untreated sample belonged to a minimum of three groups in all cases. This grouping indicates that no fractionation occurred, the $\delta^{13}$C values of n-alkanes measured before and after the sample purification methods are not different. The absence of isotopic fractionation enables the application of the developed sample purification method for the carbon CSIA of n-alkanes.

3.3. The importance of purification prior to carbon CSIA of n-alkanes in diesel fuel

In order to demonstrate the importance of purification prior to carbon CSIA different stages of the sample preparation were further investigated for one groundwater

Table 2. Recoveries of n-alkanes with silica gel column chromatography (SGCC) and solid-state urea clathrate formation (SSUCF) and the measured $\delta^{13}$C values before and after SGCC or SSUCF treatment.

|          | SGCC Av. Rec. (%) | RSD% (n = 5) | SSUCF Av. Rec. (%) | RSD% (n = 5) | Before treatment $\delta^{13}$C (%) | 6 (n = 5) (‰) | After SGCC $\delta^{13}$C (%) | 6 (n = 5) (‰) | After SSUCF $\delta^{13}$C (%) | 6 (n = 5) (‰) |
|----------|-------------------|--------------|-------------------|--------------|-----------------------------------|--------------|--------------------------------|--------------|--------------------------------|--------------|
| C_10     | 86                | 2            | 63                | 7            | −42.95                            | 0.13         | −43.00                         | 0.12         | −42.87                         | 0.08         |
| C_11     | 89                | 2            | 70                | 7            | −32.51                            | 0.13         | −32.50                         | 0.12         | −32.44                         | 0.10         |
| C_12     | 91                | 2            | 76                | 6            | −28.08                            | 0.13         | −28.13                         | 0.12         | −28.09                         | 0.09         |
| C_13     | 93                | 2            | 80                | 6            | −29.09                            | 0.11         | −29.15                         | 0.13         | −29.07                         | 0.09         |
| C_14     | 94                | 2            | 84                | 5            | −27.90                            | 0.12         | −28.00                         | 0.13         | −27.86                         | 0.08         |
| C_15     | 96                | 2            | 87                | 5            | −30.49                            | 0.13         | −30.55                         | 0.14         | −30.46                         | 0.10         |
| C_16     | 97                | 2            | 88                | 5            | −27.89                            | 0.14         | −27.91                         | 0.13         | −27.87                         | 0.09         |
| C_17     | 98                | 2            | 89                | 5            | −23.48                            | 0.12         | −23.53                         | 0.13         | −23.45                         | 0.10         |
| C_18     | 99                | 2            | 90                | 5            | −32.03                            | 0.15         | −32.07                         | 0.13         | −31.98                         | 0.10         |
| C_19     | 99                | 2            | 90                | 5            | −32.20                            | 0.13         | −32.16                         | 0.13         | −32.15                         | 0.08         |
| C_20     | 99                | 2            | 90                | 5            | −31.85                            | 0.14         | −31.98                         | 0.14         | −31.79                         | 0.15         |
| C_21     | 99                | 2            | 91                | 5            | −35.84                            | 0.12         | −35.81                         | 0.14         | −35.77                         | 0.08         |
| C_22     | 99                | 2            | 92                | 4            | −32.69                            | 0.15         | −32.78                         | 0.13         | −32.62                         | 0.15         |
| C_23     | 98                | 2            | 94                | 4            | −32.60                            | 0.14         | −32.69                         | 0.14         | −32.52                         | 0.12         |
| C_24     | 98                | 2            | 100               | 4            | −32.85                            | 0.10         | −32.85                         | 0.14         | −32.62                         | 0.13         |
sample (Sample ID: GW2): the primary extract and the aliphatic fraction were also measured with GC-IRMS along with the urea purified \textit{n}-alkane solution. Figure 4 shows the \textit{m/z} 44 chromatograms of the three solutions. The chromatogram of the primary extract (Figure 4a) clearly shows the complexity of a diesel fuel contamination. Several co-eluting components result in a characteristic baseline hump in the chromatogram. In the aliphatic fraction – i.e. after the SGCC – the elevated baseline still exists in the chromatogram (Figure 4b). But the FAME content of diesel fuel has been removed. The chromatogram obtained after the purification with SSUCF (Figure 4c) shows that clathrate formation was highly effective in removing the numerous co-eluting compounds. The absence of the baseline hump resulted in pure \textit{n}-alkane peaks.

The stable isotope ratios of \textit{n}-alkanes measured in the primary extract, the aliphatic fraction and the urea purified \textit{n}-alkane solution are presented in Table 3. The urea purified solution provided the best precision for the $\delta^{13}$C values for each compound. For this solution the average standard deviation for all the investigated \textit{n}-alkanes was 0.10‰. For both the primary extract and the aliphatic fraction the average standard deviation was 0.39‰.

The application of \textit{T}-test revealed that the three stages of the sample preparation gave significantly different $\delta^{13}$C values for five \textit{n}-alkanes ($C_{12}$; $C_{15}$; $C_{17}$; $C_{18}$; $C_{21}$) between
the primary extract and the urea purified solution and for four n-alkanes (C_{16}; C_{17}; C_{18}; C_{24}) between the aliphatic fraction and the urea purified n-alkane solution. These significant differences originate in co-elution of both major and minor interfering compounds. In the case of minor interfering compounds the interfering peak or peaks are small with respect to the peak area of the target compound; often they are even not clearly visible in the chromatogram. Still they can significantly change the measured $\delta^{13}$C values as

![Figure 4. The m/z 44 chromatograms and a cut-out of the m/z 45/44 ratios of the primary extract (PE), the aliphatic fraction (AF) and the urea purified n-alkane solution (UP).](image)

Table 3. $\delta^{13}$C values of n-alkanes measured from the three main sample preparation steps of the contamination model experiment using a groundwater (GW 2) sample.

|                | Primary extract | Aliphatic fraction | Urea purified n-alkane solution |
|----------------|-----------------|--------------------|---------------------------------|
| $\delta^{13}$C | $\delta^{13}$C  | $\delta^{13}$C     | $\delta^{13}$C                  |
| (‰)           | (‰)             | (‰)               | (‰)                             |
| C_{11}         | 32.14           | 32.15              | 32.07                           |
| C_{12}         | 32.45           | 31.94              | 32.67                           |
| C_{13}         | 31.98           | 31.92              | 31.60                           |
| C_{14}         | 31.25           | 31.26              | 31.56                           |
| C_{15}         | 30.85           | 31.06              | 31.58                           |
| C_{16}         | 31.46           | 31.22              | 31.64                           |
| C_{17}         | 28.24           | 30.93              | 31.65                           |
| C_{18}         | 30.55           | 30.32              | 31.61                           |
| C_{19}         | 31.44           | 31.72              | 31.35                           |
| C_{20}         | 31.52           | 31.55              | 31.36                           |
| C_{21}         | 32.01           | 31.34              | 31.21                           |
| C_{22}         | 31.19           | 31.57              | 31.18                           |
| C_{23}         | 31.60           | 31.47              | 31.15                           |
| C_{24}         | 32.05           | 31.85              | 31.15                           |
well as the precision of the measurement. The effect of major co-eluting compounds is clearly illustrated in Figure 4 which also shows the $m/z$ 45/44 ratio chromatogram in the range of C$_{17}$–C$_{21}$ n-alkanes in each $m/z$ 44 chromatogram. Figure 4a demonstrates that without any purification pristane co-elutes with C$_{17}$ n-alkane, phytane with C$_{18}$ n-alkane and FAME compounds with C$_{21}$ n-alkane. Owing to the chromatographic isotope effect (i.e. the earlier elution of the molecules containing the heavier stable isotopes), the δ$^{13}$C value of C$_{17}$ and C$_{18}$ n-alkanes increase significantly, because pristane and phytane elute after the n-alkanes. For C$_{21}$ n-alkane the δ$^{13}$C value is an average for the C18:0; C18:1; C18:1(b) and C18:3 FAME and the n-alkane with the influence of the later eluting isotopically lighter molecules of C18:2 FAME.

Hierarchical cluster analysis was used to compare the whole δ$^{13}$C n-alkane profile of the three sample preparation stages. For this data set three groups were distinguished based on the dendrogram (Figure 5). As expected, the parallels belonging to the urea purified solution are forming a well separated group. This demonstrates that the carbon CSIA of n-alkanes in diesel fuel can be remarkably improved using a selective purification method to obtain accurate and precise stable isotope ratio data.

3.4. Application of the purification method to water samples spiked with diesel fuel

To test the robustness of the proposed method with regard to different water matrices we analysed nine water samples spiked with diesel fuel. Beside distilled water, tap water, river water, three different types of industrial wastewater, confined water and two different types of groundwater samples and also the diesel fuel used for spiking were prepared and analysed with GC-IRMS. The $m/z$ 44 chromatograms are presented in the supporting information in Figures S1–S10. In all cases the removal of the characteristic baseline hump is unmistakable; the absence of the elevated baseline and any co-eluting compounds allows the accurate isotope ratio measurement.

Table 4 presents the δ$^{13}$C values of individual n-alkanes for each water sample and the contaminating diesel fuel. Data are not given for C$_{10}$ n-alkane because the peak intensities for this compound were below the linearity range of the GC-IRMS instrument (1–10 nA for $m/z$ 44). The low intensities were caused by the relatively low concentration of this compound in the diesel fuel and the evaporative loss.

Figure 5. The dendrogram obtained by hierarchical cluster analysis for the δ$^{13}$C values of n-alkanes in the primary extract (PE), the aliphatic fraction (AF) and the urea purified n-alkane solution (UP) for groundwater sample 2 (GW 2).
Table 4. $\delta^{13}$C values of $n$-alkanes in the different water samples and the contaminating diesel fuel.

|            | Distilled water | Tap water | River water | Industrial Wastewater 1 | Industrial Wastewater 2 | Industrial Wastewater 3 | Confined water | Groundwater 1 | Groundwater 2 | Diesel fuel |
|------------|-----------------|-----------|-------------|-------------------------|-------------------------|-------------------------|----------------|----------------|----------------|-------------|
| $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) | $\delta^{13}$C (‰) |
| C<sub>11</sub>      | -31.99          | 0.17      | -31.97      | 0.11                    | -32.10                  | 0.15                    | -32.14                  | 0.08                    | -32.07                  | 0.10                    | -32.06                  | 0.13                    | -32.04                  | 0.10                    | -32.07                  | 0.09                    | -32.07                  | 0.13                    | -32.13                  | 0.09                    |
| C<sub>12</sub>      | -31.69          | 0.13      | -31.61      | 0.14                    | -31.71                  | 0.17                    | -31.69                  | 0.05                    | -31.74                  | 0.15                    | -31.64                  | 0.10                    | -31.63                  | 0.10                    | -31.66                  | 0.09                    | -31.60                  | 0.10                    | -31.74                  | 0.14                    |
| C<sub>13</sub>      | -31.73          | 0.13      | -31.69      | 0.10                    | -31.74                  | 0.09                    | -31.74                  | 0.07                    | -31.78                  | 0.12                    | -31.78                  | 0.12                    | -31.76                  | 0.09                    | -31.64                  | 0.08                    | -31.64                  | 0.10                    | -31.68                  | 0.10                    |
| C<sub>14</sub>      | -31.58          | 0.19      | -31.60      | 0.17                    | -31.69                  | 0.11                    | -31.67                  | 0.12                    | -31.68                  | 0.17                    | -31.63                  | 0.10                    | -31.60                  | 0.11                    | -31.64                  | 0.11                    | -31.60                  | 0.09                    | -31.67                  | 0.10                    |
| C<sub>15</sub>      | -31.57          | 0.13      | -31.54      | 0.14                    | -31.62                  | 0.16                    | -31.66                  | 0.13                    | -31.71                  | 0.17                    | -31.66                  | 0.15                    | -31.66                  | 0.14                    | -31.61                  | 0.06                    | -31.56                  | 0.06                    | -31.67                  | 0.12                    |
| C<sub>16</sub>      | -31.64          | 0.13      | -31.65      | 0.10                    | -31.68                  | 0.18                    | -31.69                  | 0.08                    | -31.70                  | 0.12                    | -31.68                  | 0.11                    | -31.60                  | 0.10                    | -31.63                  | 0.07                    | -31.58                  | 0.07                    | -31.63                  | 0.14                    |
| C<sub>17</sub>      | -31.67          | 0.13      | -31.63      | 0.12                    | -31.64                  | 0.10                    | -31.72                  | 0.12                    | -31.70                  | 0.18                    | -31.67                  | 0.13                    | -31.71                  | 0.09                    | -31.63                  | 0.09                    | -31.64                  | 0.07                    | -31.74                  | 0.11                    |
| C<sub>18</sub>      | -31.68          | 0.12      | -31.64      | 0.10                    | -31.74                  | 0.15                    | -31.72                  | 0.12                    | -31.70                  | 0.09                    | -31.75                  | 0.08                    | -31.64                  | 0.07                    | -31.65                  | 0.07                    | -31.65                  | 0.13                    | -31.69                  | 0.16                    |
| C<sub>19</sub>      | -31.60          | 0.11      | -31.63      | 0.10                    | -31.63                  | 0.16                    | -31.65                  | 0.10                    | -31.62                  | 0.13                    | -31.58                  | 0.10                    | -31.55                  | 0.10                    | -31.54                  | 0.10                    | -31.61                  | 0.10                    | -31.53                  | 0.13                    |
| C<sub>20</sub>      | -31.41          | 0.14      | -31.46      | 0.10                    | -31.45                  | 0.14                    | -31.47                  | 0.11                    | -31.50                  | 0.10                    | -31.44                  | 0.12                    | -31.41                  | 0.12                    | -31.42                  | 0.15                    | -31.35                  | 0.13                    | -31.42                  | 0.09                    |
| C<sub>21</sub>      | -31.43          | 0.10      | -31.43      | 0.10                    | -31.40                  | 0.10                    | -31.44                  | 0.15                    | -31.43                  | 0.09                    | -31.38                  | 0.14                    | -31.34                  | 0.13                    | -31.33                  | 0.09                    | -31.36                  | 0.10                    | -31.33                  | 0.10                    |
| C<sub>22</sub>      | -31.30          | 0.11      | -31.35      | 0.12                    | -31.28                  | 0.15                    | -31.34                  | 0.16                    | -31.26                  | 0.16                    | -31.25                  | 0.10                    | -31.24                  | 0.06                    | -31.22                  | 0.08                    | -31.21                  | 0.12                    | -31.23                  | 0.11                    |
| C<sub>23</sub>      | -31.23          | 0.11      | -31.24      | 0.12                    | -31.24                  | 0.15                    | -31.26                  | 0.15                    | -31.26                  | 0.17                    | -31.23                  | 0.11                    | -31.15                  | 0.13                    | -31.15                  | 0.10                    | -31.17                  | 0.14                    | -31.22                  | 0.22                    |
| C<sub>24</sub>      | -31.19          | 0.09      | -31.20      | 0.08                    | -31.18                  | 0.09                    | -31.25                  | 0.12                    | -31.27                  | 0.12                    | -31.21                  | 0.10                    | -31.14                  | 0.13                    | -31.17                  | 0.10                    | -31.15                  | 0.14                    | -31.19                  | 0.08                    |
during the model experiment. Precision of the GC-IRMS measurement was acceptable for all the samples with standard deviations ranging between 0.05 and 0.22‰ for all the n-alkanes with an average of 0.12‰.

T-test was applied to assess the significant differences between the $\delta^{13}C$ values of the contaminating diesel fuel and the water samples. From the whole data set only one n-alkane from the tap water sample (Sample ID: TW) gave significant difference. For all of the other samples none of the $\delta^{13}C$ values differ significantly from that of the contaminating diesel fuel.

Hierarchical cluster analysis was also applied for the assessment. Ten groups of the parallel samples were distinguished (Figure 6). The five parallels belonging to each sample fall into minimum three groups, which suggest that every sample is similar to each other.

Thus we can conclude that the different water matrices tested in our model experiment had no effect on the $\delta^{13}C$ values of n-alkanes measured after SGCC and SSUCF.

4. Conclusions

SSUCF-based purification methods tend to give poor recovery for the lower molecular weight n-alkanes (below $C_{20}$) and are often time-consuming to perform. The present study provides a thorough optimisation of SSUCF aiming at a routinely applicable chemical separation method that can be coupled with compound-specific stable carbon isotope analysis of n-alkanes in diesel fuel. The proposed method is improved in many aspects. The clathrate formation proved to be sufficiently effective at room temperature without any temperature control. The clathrate formation time was reduced to 5 minutes while the recovery and precision remained maximal. The elimination of high temperatures from the clathrate formation and the appropriate urea: methanol ratio improved the recovery for lower molecular weight n-alkanes notably compared to previous methods [20,28].

Figure 6. The dendrogram obtained by hierarchical cluster analysis for the $\delta^{13}C$ values of n-alkanes from different water samples: distilled water (DW), tap water (TW), river water (RW), three different types of industrial wastewater (IWW 1, IWW 2 and IWW 3), confined water (CW), two different types of groundwater (GW 1, GW 2) and a contaminating diesel fuel (DF) sample.
The developed SSUCF method was combined with SGCC to remove the FAME content of diesel fuel prior to clathrate formation. The characterisation of the two methods proved that both of them are free from isotopic fractionation. The recovery and the precision were found to be satisfactory for C10–C24 n-alkanes. The precision of the measured δ13C values for the sample preparation parallels and the precision of the GC-IRMS measurement were also satisfying.

The determination of the isotopic composition at three different stages of sample preparation (primary extract, aliphatic fraction and urea purified solution) demonstrated the importance of selective purification prior to carbon CSIA of n-alkanes in a diesel contamination. Major differences were found in the δ13C values of certain n-alkanes at the different stages of sample preparation. Moreover, the precision of the GC-IRMS measurement was reduced in the analysis of the primary extract and the aliphatic fractions compared with the combined SGCC/SSUCF approach.

The applicability of the developed method as a fingerprinting approach to characterise diesel fuel contaminations in aquatic environment was further investigated with spiked water samples. The δ13C values measured from the contaminating diesel fuel used for spiking and the different water samples were completely similar to each other. This model experiment demonstrated the robustness of the proposed sample preparation method with regard to different types of water samples.

Our results show that the proposed sample preparation method can remarkably improve the precision of carbon CSIA of n-alkanes originating from the diesel contamination of aquatic environment. Thus, it can become a fundamental element of the chemical fingerprinting method used to characterise such contaminations. Owing to the reduced labour and time intensity of the SSUCF, the method may prove especially useful when a great number of samples are being examined (e.g. contamination model experiments).

Disclosure statement
No potential conflict of interest was reported by the authors.

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