Usability of gas chromatography techniques (GC-FID, GC-MS and GC-IRMS) for identification of maritime paraffin wax

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This document describes the outcome of an initial intercalibration study investigating possibilities of environmental forensic analysis for source identification of paraffin spills using and enhancing methods regularly applied in oil spill environmental forensics. This study was part of the R&D project "Untersuchung über verölte Seevögel und Strandverölungen (Investigations concerning oiled birds and oil contamination on beaches)", realised by Schleswig-Holstein's Agency for Coastal Defense, National Park and Marine Conservation (LKN.SH) (2013-2016) and financed by the Federal Maritime and Hydrographic Agency (BSH), Hamburg, Germany. It is a working document displaying the current state of discussion (January 2016).

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Scope of this study: the research and development project

The presented study is part of the ongoing research and development project "Untersuchung über verölte Seevögel und Strandverölungen (investigations concerning oiled birds and oil contamination on beaches)", realised by Schleswig-Holstein Agency for Coastal Defense, National Park and Marine Conservation (LKN.SH) and financed by the Federal Maritime and Hydrographic Agency (BSH), Hamburg, Germany.

The project, which started in May 2013 and proceeds until June 2016, focusses on the monitoring of drift lines along the German's North Sea coast (including most of the Friesian islands and holms) for mineral oil and for oil-derived products (paraffin wax, lubrication oil, etc.). Apart from plastics, all kinds of apparently oil-like persistent floaters are investigated. It can be assumed that most of those substances are remnants of tank washing activities from commercial vessels.
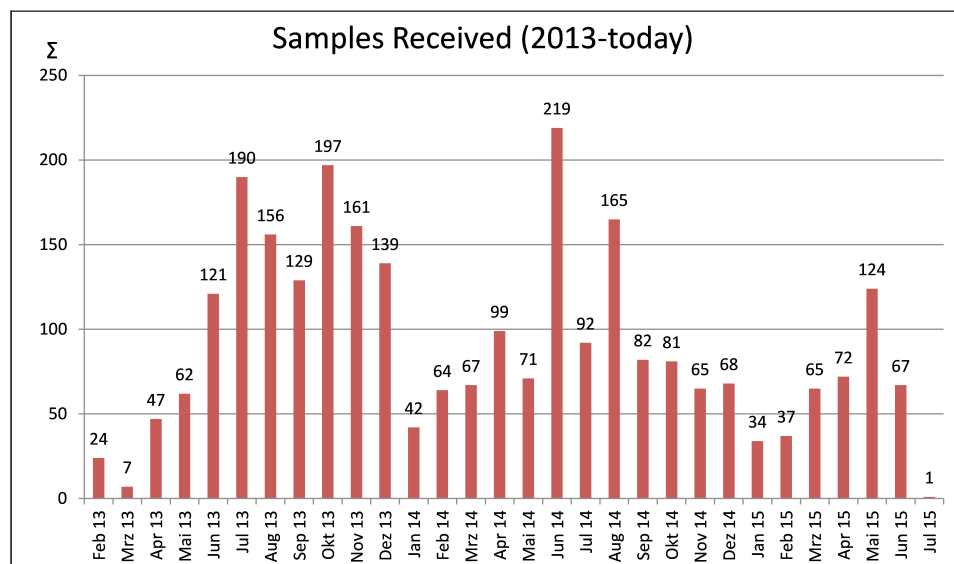
Fig. 1: Monthly load of samples arriving in the laboratory.

Sample collection is substantiated through cooperation with a number of non-governmental organisations (NGOs) and local institutions on the coast which provide the manpower to monitor such a wide-stretched area.

So far (July 2015), the BSH laboratory received 2750 samples for analysis. The variability in the monthly load (see Fig. 1) is mainly due to the activity of the sampling personnel and does not necessarily reflect the actual abundance of target substances on the coast during this time.

Chemical analyses were performed on 2239 samples so far. After a first categorisation, it turned out that paraffin wax is by far the most abundant kind of persistent floater (see Fig. 2). The predominance of paraffin wax emphasises the significance of this substance which can be considered to replace the classic oil pollution in numbers, since today, the latter is heavily regulated.
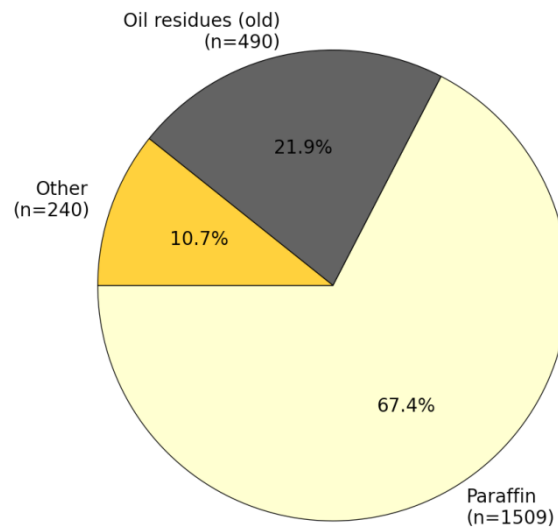
Oil residues (old)
(n=490)

21.9%

Other
(n=240)

10.7%

67.4%

Paraffin
(n=1509)

Fig. 2: Composition of sample type according to the analysis.

Background

In the 1980s and early 1990s, coastal oil pollution resulting from oil spills at sea was a common and frequent event [2]. To reduce oil spill, legislation was tightened and substantial improvements in oil spill forensics were sought. Based on the NORDTEST method (NORDTEST 1991), experts of the OSINet (Oil Spill Identification Network of experts within the Bonn-Agreement) developed a common methodology for oil identification work which was published as a CEN standard by the European Committee of Standardisation, the CEN/TR 15522-2:2012 [3], in the following abbreviated with CEN2012. With regulations and effective enforcement tools, which allow the successful prosecution of polluters, in place, the number of oil spills declined drastically over the last decades [9][10][11][12].

In recent years, however, numerous major incidents involving paraffin wax were observed along the North Sea coast from Denmark to the Netherlands [4, 5]. In March 2014, for example, more than 50 tons of paraffin wax was washed ashore on the island of Sylt (Germany) [6].

The sources for such spills are transport vessels which perform tank washing procedures.

One side of the problems related to such spills are the costs for beach closure and clean up. The Central Command for Maritime Emergencies (CCME) does extensive remediation work for spills succeeding a certain threshold which turns them into "complex costal pollution incidents". This threshold consists of a spill involving more than 30 m³ of material, or when at least 10 km of polluted shorelines [4] are observed. The majority of paraffin wax spills however are blow this definition and the concerned communities of the beaches have to pay for the clean-up, facing economic challenges with no perspective of governmental support.

The other side of paraffin wax spills are toxicological implications for human health and the environment. While pure paraffin wax is not considered hazardous to humans, tank ships however never carry paraffin wax with medical or cosmetic grade, but an industrial product with a rather low level of purity. Some of the impurities can be polyaromatic hydrocarbons (PAH), originating from the extraction process. PAHs are known to be carcinogenic and may occur in concentrations that pose a threat to humans and the environment [5].

To date, tank washing under certain circumstances conforms to IMO's MARPOL regulations [7]. Nevertheless, these incidences contribute to the ongoing debate if tank cleaning activities of vessels at sea should be further restricted.

If such restrictions would enter into force, an immediate demand for analytical methods and techniques for proper source identification of paraffin spills would arise. The presented study can therefore contribute to the aim of developing a method for paraffin spill identification which provides the same confidence CEN2012 is providing for oil spill identification.

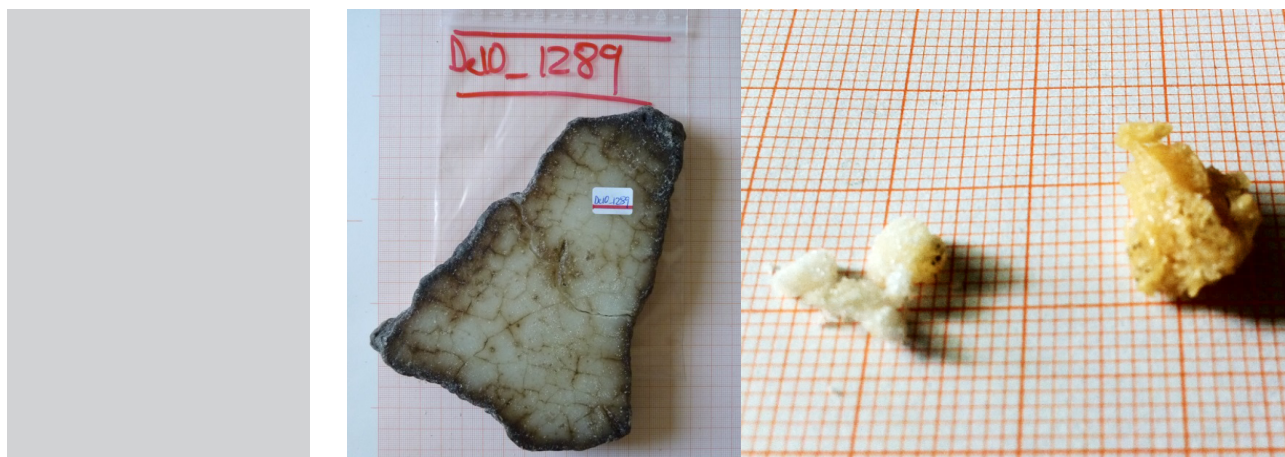# Status of GC-FID-generated data and handling

Fig. 3: different sizes, shapes and colors of paraffin wax findings on the beaches. From small, brittle flakes (top, 3a) to bigger and denser chunks (bottom, 3b).

Paraffin wax collected from the beaches displays a great variety of size, shape and colour. Sizes range from tiny flake-like portions to larger chunks with diameters up to 20 cm. Most samples show a whitish colour but shades of 'dirty'-grey to intensely yellow are recorded as well (Fig. 3). The material properties observed range from a hard and brittle texture to ductile and viscose conditions.

The variety in both texture and colour of the retrieved paraffin wax samples is reflected in the GC-FID chromatograms. Looking at a GC-FID chromatogram of a paraffin wax, the peak heights of every n-alkane (more or less) resemble a bell shape. As can be seen in Fig. 4, shape and position of the n-alkane bell shape and of the unresolved complex mixture (UCM), also known as the "hump") differ greatly.

Note that the chromatogram on the right side shows a paraffin wax containing higher boiling n-alkanes ("hba", up to C-50). In such cases, the applied GC-FID method (CEN2012) is clearly reaching its limits as higher components cannot be separated sufficiently. However, investigations using a high-temperature GC-FID device (HTGC-FID) confirmed that those waxes are showing a bell-shaped appearance of n-alkanes as well.
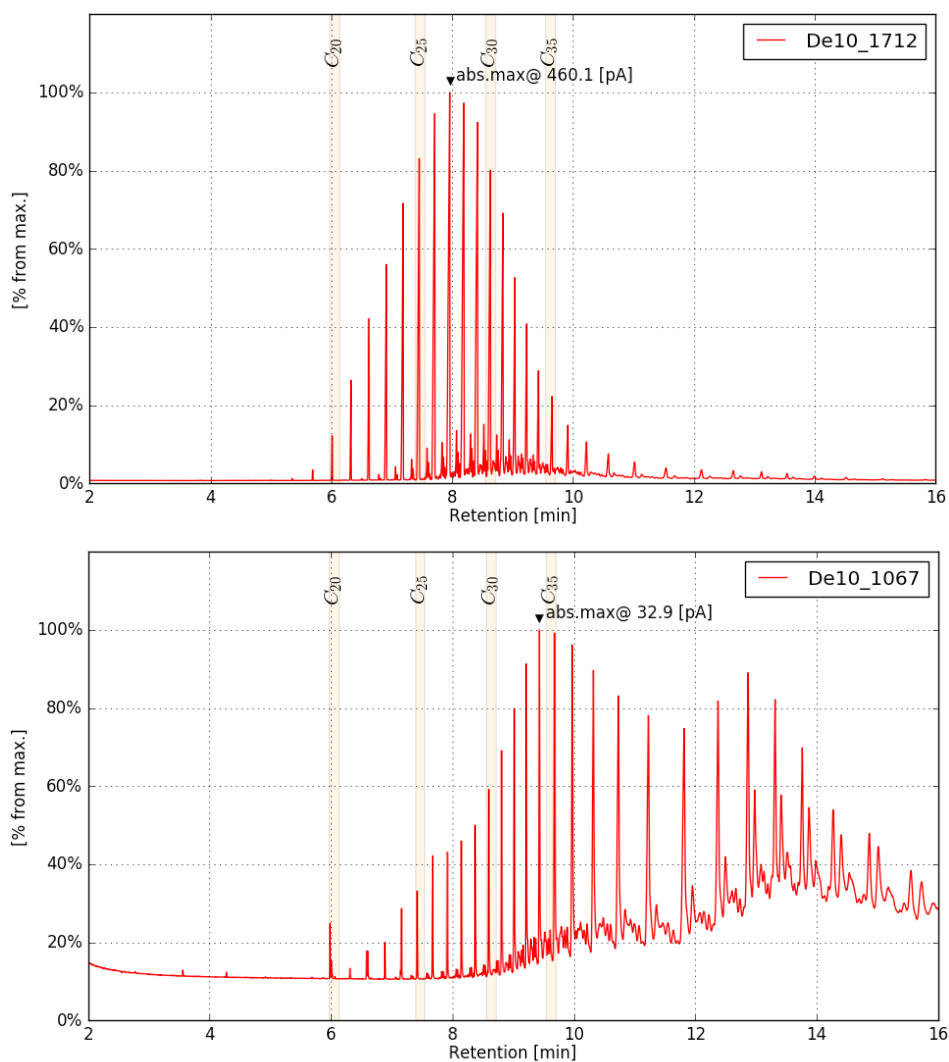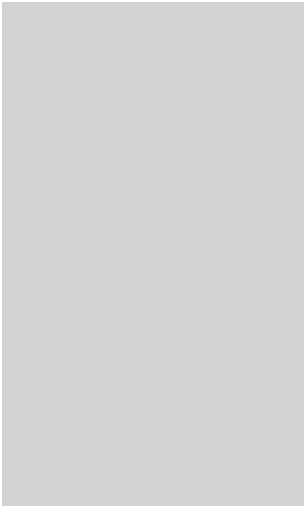
Fig. 4a (top), 4b (bottom): GC-FID Chromatograms of differently appearing paraffin wax samples (maximum-.normalized and expressed as relative percentage). Note that it is the CENT/Tr's GC-FID method here. This method is not sufficient to deal with paraffin waxes containing the higher boiling n-alkanes as sample DE10_1067 (bottom) clearly shows.

In order to parametrise the bell-shape curve and the above-mentioned properties, peak detection and extraction of intensities (pico-Ampère, [pA]) were applied to each n-alkane present in a GC-FID-chromatogram. Consequently, each sample is represented as a data set, consisting of individual peak intensities of all the n-alkanes present in the corresponding chromatogram.

Similar as it is in case of oil, it can be assumed that there is always a homologue series of n-alkanes. This means once the starting n-alkane is successfully identified the following identification is easier since there is a predictable pattern to follow and absolute retention times are not as critical as in other analytical approaches.

```
+---------+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
|De10_1712| C18 | C19 | C20 | C21 | C22 | C23 | C24 | C25 | C26 | C27 |
+---------+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
| raw [pA]| 5.6 |15.7 |56.0 |121.3|193.9|257.6|329.6|382.3|435.2|460.1|
+---------+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
| norm_max|0.012|0.034|0.122|0.264|0.423|0.560|0.716|0.831|0.946|1.000|
+---------+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+
```

Tab. 1: A cutout of the data processing. The last row (norm_max) represents the vector for further processing.

To make data sets more comparable, normalisation of each peak's intensity to the maximum peak (maximum-normalisation) is done for every sample. It was observed that more than 80 % of the samples consist of n-alkanes in the range from C18 to C45. Therefore, every paraffin wax sample is represented by a vector of normalised intensities of those 27 n-alkanes. This vector-like representation of n-alkanes is taken as the basis for the correlation, whitening and clustering of the samples (Tab. 1).

Limitations of GC-MS analysis for paraffin wax

The workflow of oil spill identification according to CEN2012 starts with a GC-FID analysis to characterise the mineral oil types or the mineral oil products. The decision to further proceed to GC-MS analysis is based on the findings of characteristic similarities between the investigated samples. Characterisation of biomarkers based on GC-MS analysis is an essential part in oil spill identification and in CEN2012. However, paraffin wax mostly is a refined mineral oil product, which has undergone thermal and other physical processes. Therefore it is not just a distilled fraction of the source oil but is of a considerably altered chemical composition. Therefore, biomarker analysis by GC-MS according to CEN2012 provides generally no reliable and meaningful possibility of discrimination between samples (Fig. 5).
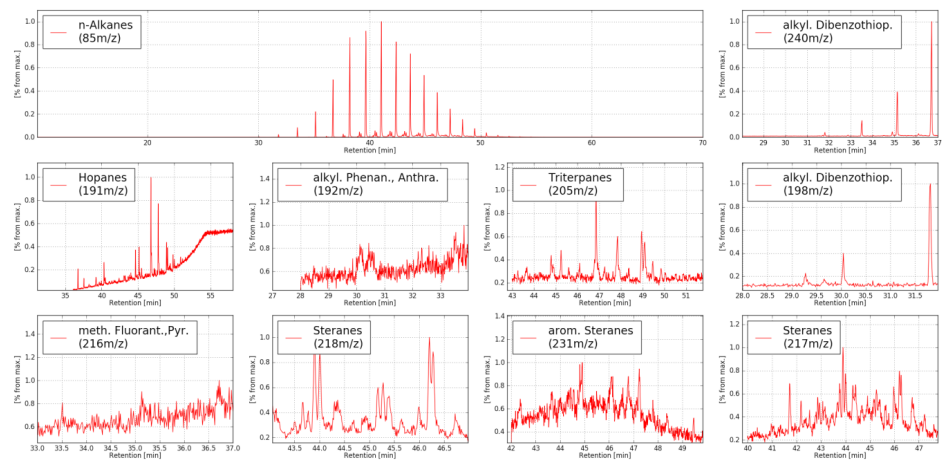
Fig. 5: Overview of biomarkers in paraffin wax. Presented are the corresponding ion-chromatograms of important biomarkers, showing very low abundance. Only hopanes and steranes seem to be analysable.

Therefore, it becomes clear that the elegant way of the oil identification method according to CEN2012 which relies strongly on calculating ratios of the biomarkers to form a highly individual fingerprint of the oil is of very limited use in case of paraffin wax.

Thus, it is obvious that there is need for a different way to identify similarities and differences of paraffin wax samples.

As GC-MS might not be able to provide the necessary data the focus shifts to the question if the results of GC-FID analyses executed according to CEN2012 can be used in an extended way for further paraffin identification.

Furthermore, the application of isotope ratio mass spectrometry (IRMS) provides additional parameters for the identification of paraffin wax.

Isotope Ratio Mass Spectrometry (IRMS)

The IRMS can be considered as a special type of mass spectrometry where the content of stable isotopes is measured. In the case of carbon, it is ^{12}C and ^{13}C, however, analysing ^{1}H and ^{2}H might also be an option.

^{13}C content is expressed in per mil as the ratio $^{13}C : {}^{12}C$, normalised to the ratio of a standard and formed as the difference (δ to this double ratio of the standard itself):

$$\delta^{13}C = \left[\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{PDB}} - 1\right] \times 10^3$$

The PDB (Pee Dee Belemnite)-Standard is a marine fossil limestone with an extraordinary high content of ^{13}C. As most of the other materials on earth have lower ^{13}C contents, their values according to this equation turn out to be negative. In geological sciences, these calculations are used to classify and categorise materials according to their origin as shown in Fig. 6.

As paraffin wax is a product of mineral oil, or "petroleum", a δ ^{13}C value in the same range (-35 to -20) is expected (Fig. 6).

Consequently, comparing isotopic signatures of paraffin wax samples might provide meaningful information regarding a common source of two samples.
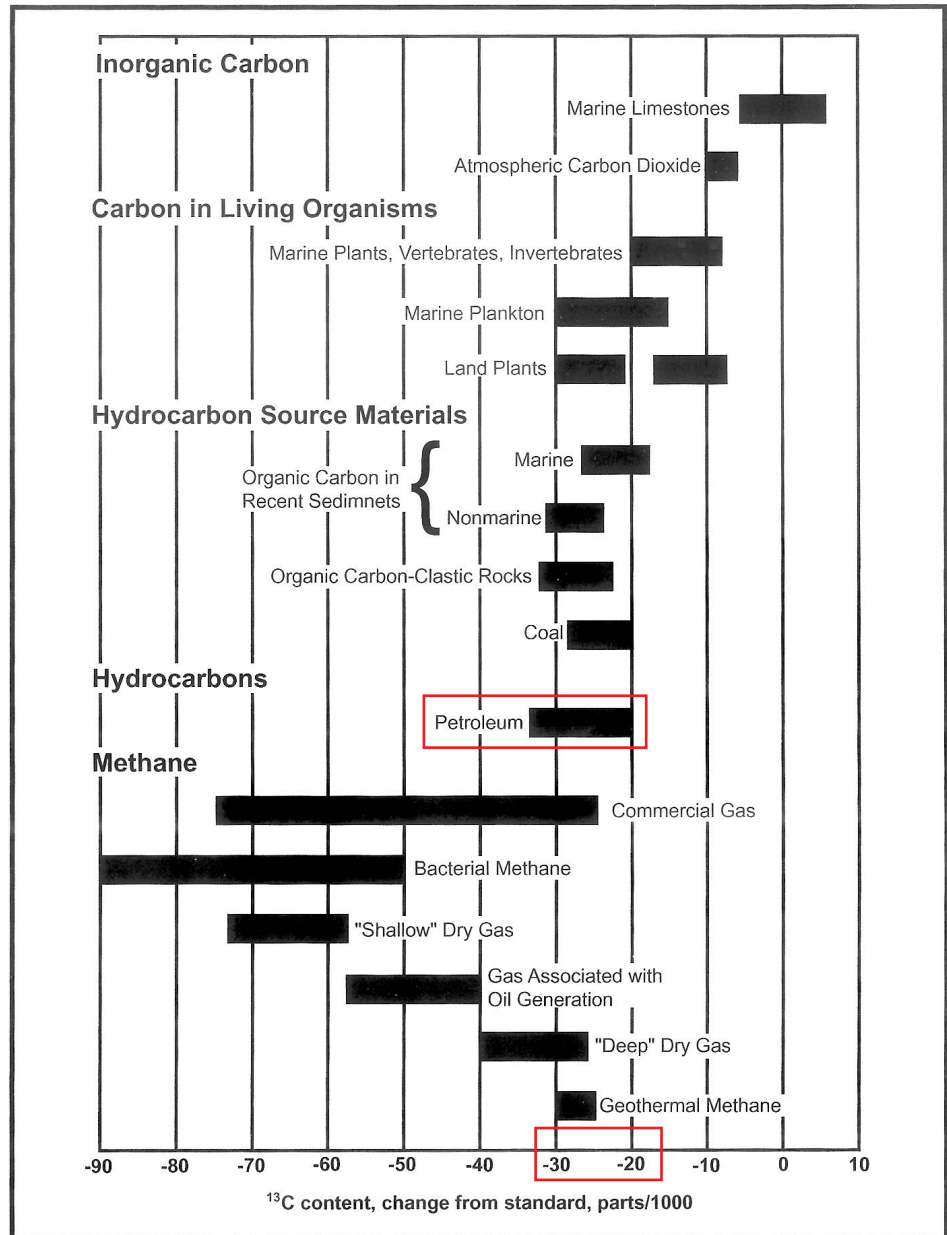
Fig. 6: d13C Contents of different materials [8].

The scope and concept of this mini Round Robin (miniRR)

In a first approach on evaluating different analytical methods for paraffin identification, an intercalibration study (mini Round Robin) with a small group of environmental laboratories was set up which investigated the possibilities of environmental forensic analysis for source identification of paraffin spills using and enhancing methods regularly applied in oil spill environmental forensics.

The overall question of the miniRR was to what extend paraffin wax samples can be distinguished from each other or stated as a positive match, using the techniques at hand (GC-FID, GC-MS and GC-IRMS).

For this purpose a collection of samples was sent to the participants.

As the samples from the R&D-project are all from the German coast, having samples from a very distant origin might decrease the probability to be exactly the same product. Thanks to David Francois (Ministére de la Défense, France), we were able to use additional samples from France, where paraffin wax is beached on the coast lines as well. After GC-FID analysis, the samples were compared with the pool of project samples. The collection of samples used for the miniRR finally consisted of samples with a) a high probability to be the same product, b) a low probability and c) a sample in duplicate. The assessment of similarities was done through cross correlation based on the vector-like representation. In addition to the method presented before (normalised on maximum, see above), a second approach was used. This second approach is based on "next-a-neighbour" normalisation, whereat each peak's intensity (peak height) was normalised to the following n-alkane in the homologous series. This method, suggested by Paul Kienhuis (Rijkswaterstaat-Laboratory, RWS), is used to better compensate for instrumental differences.

As a result, there were two different types of data generated from the same compilation of samples. Cross-correlation using these two numerical representations led to different results, meaning different best matches.

Finally, three sets of samples were put together, grouping each of the best matches from the two different approaches in one set. A fourth set was added, consisting just of one sample, the duplicate of sample 1 set 1. This setup resulted in a total of ten samples.

set	origin/date	Approach 1		Approach 2	
		origin/date	r	origin/date	r
1	Fr, 05-2013	Amrum, Ger /05-2013	0.9977	Ockholm, Ger/12-2013	0.9980
2	Fr, 2010	Trischen, Ger/05-2013	0.9949	Hooge, Ger/08-2014	0.9609
3	Buesum,Ger, 08-2013	Buesum, Ger/ 07-2013	0.9991	HaHaKoog Ger/07-2013	0.9910
4	duplicate				

Set 1 consisted of samples which came from different countries but were found within a relatively short time span.
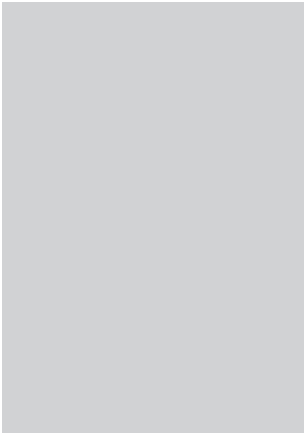
Set 2 consisted of samples which were again retrieved from very distant origins. However, in terms of the bell shapes (GC-FID), they were quite dissimilar to the first set and were separated temporarily by three years (samples were found 2010 and 2014).

Set 3 had the function of a control group. These samples were found at the North Sea coast within close spatial and temporal proximity (07/08–2013). The data suggested that these samples stem from the exact same paraffin-wax bulk.

Set 4 was a duplicate (from the same stock solution) of the very first sample from Set 1 (from France).

Solutions of the dissolved paraffin samples were shipped to the participants. Participants were asked to analyse the samples in duplicate and in exactly the order given above with their available instrumentation. This way we have a duplicate analysis of the same sample at the beginning and at the end of a sequence to monitor analytical performance.

Participating laboratories

The international group of participants and the instrumentation used in this mini-RR:
- Nederlands Forensisch Instituut, **NFI** (GC-FID, GC-IRMS)
- Rijkswaterstaat-Laboratory, **RWS** (GC-FID, GC-MS)
- International Atomic Energy Agency, **IAEA**
  Marine Environmental Studies Laboratory (GC-FID, GC-IRMS)
- Federal Maritime and Hydrographic Agency, **BSH** (GC-FID, GC-MS)

Thanks to the effort of the participants, we received:
- 4 sets of GC-FID data
- 2 sets of GC-MS data
- 2 sets of GC-IRMS data

Method of data evaluation

The key issues to be addressed within this miniRR were to find conclusive indications if two paraffin-wax samples are statistically similar or not. Therefore, two peak-based features, "relative standard deviation" (RSD) and the "critical difference" (CD) are used for each analytical parameter (= peak) of a sample and are used for comparison. Thus, the number of RSD- or CD-features depends on the number of peaks on which a comparison is based on.

These features have the advantage of being well known by people conducting analysis according to CEN2012; moreover, CEN2012 provides statistically derived thresholds of 5 % and 14 % for RSD and CD, respectively. If these values are exceeded, dissimilarity of the samples could be concluded.

To generate the data regarding the two desired features, peak heights of the target compounds in the chromatograms of the samples are extracted and maximum-normalised, allowing for a concentration-independent handling.

The second step is to calculate the two features of RSD and CD for every individual peak comparison. Each feature with the established threshold can be applied directly and might respond positively or negatively, meaning it is below (matching) or above (non-matching) this threshold.

In a third step, the results of every feature-test can be further summarised by building another ratio, the so called "matching-ratio", showing the number of positive responses to the total number of features tested. A sample's set (all peak comparisons) of matching-ratios results in the conclusion matrix. Thus, a matching-ratio can be based on RSD- or CD-features. The following assumption was made for the final comparison of samples in this study: A match is concluded when at least half of the values in step 2 exceed their thresholds of 5 % and 14 %, respectively, resulting in a matching-ratio of ≥ 50 % (step 3). Ratios below that 50 %-limit were believed to be dissimilar, as the majority of RSD- or CD-features considered are not fulfilling their criterion in the first place. Those are coloured red in the conclusion matrix (Step 3).

The following example illustrates this comparison process (the two samples to be compared consist of three peaks each (peak 1–3)):

Step 1 (raw data and ratios)				Step 2 (features)			Step 3 (conclusion matrix)	
peak	raw [pA]	norm**			RSD	CD	RSD	CD
1	193.9	0.242		1 on 1	5.1%	10.2%	33%	100%
2	257.6	0.322		2 on 2	4.8%	9.6%		
3	329.6	0.412		3 on 3	5.2%	10.3%		
1	214.7	0.268						
2	283.6	0.355						
3	365.5	0.457		Criterion	< 5%	< 14%	≥ 50%	

** Normalized on virtual maximum of 800 [pA]

Step 1: The extracted heights are normalized on a hypothetical maximum of 800 pA. In order to narrow the number of features, a pre-sorting was conducted: Every peak smaller than 10 % of the maximum was not considered in following computations. In our example above, all normalized peaks a larger than 0.1, so they were all used to calculate features.

Step 2: Creation of the two features of RSD and CD. A green background indicates that the result of this feature is below criterion limits, and therefore statistically similar. If these features exceed their criterion, the background is coloured in red. This means that the compared peaks of the samples are statistically dissimilar.

Step 3: The amount of features showing matches are compared to the number of total features and displayed as ratios.

The first step of extracting peak intensities and normalisation to the maximum should not need further explanation.

Based on the normalised ("norm") peak values of step 1, for every peak comparison the RSD- and CD-features are created. Looking at the RSD column (step 2) we see that only the RSD of the second peak's comparison is below 5 % (green background). Since this is only one feature out of three, this results in a matching-ratio of 33 % (step 3). In contrast, for CD, step 2 shows that all three peak comparisons are below the threshold value of 14 %, which leads to a 100 % matching-ratio in step 3 for CD[1].

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[1]   Note: the nature and usability of both of these features (RSD and CD) and how to proceed and handle data in case of contradicting results, is not part of this investigation. Anyway, in the following, both values are presented.

Performance and results

GC-FID data

The first object of data analysis was the bell shape comparison, meaning the form of the distribution of n-alkenes among the homologues. As described above, sample 1 (Set 1) and sample 10 (Set 4) were duplicates and because of the duplicate analysis of each samples, this resulted in at least four individual measurements of the same sample.

FID-Paraffin-Bell-Shape-Comparison
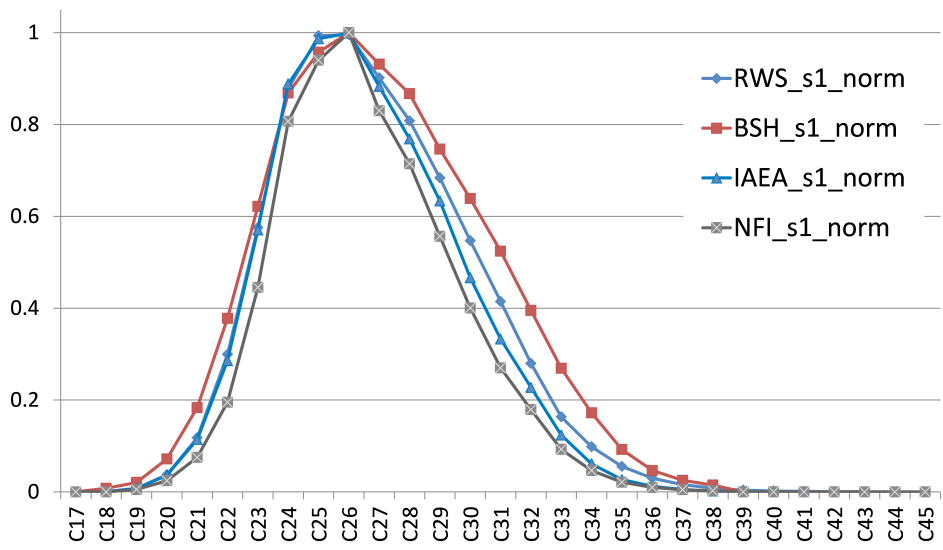**Mean of sample_1 and 10 (n=4), normalized on maximum**

Fig. 7: Mean of peak heights from GC-FID measuring of every participant.

For the data of each participant, mean calculation and normalisation from those four analyses were performed. The plotted results (Fig. 7) show in general a fair accordance between the data sets.

However, especially on the descending side of the bell shape, larger differences between the different data sets are found. One reason might be an effect of mass discrimination as higher boiling n-alkanes are known to cause such difficulties.

Another reason for these differences might be the quality of the chromatographic separation. If peak broadening appears, absolute peak height might be decreased which influences the results accordingly. Therefore, an area-based analysis of the peaks might be appropriate.

The laboratory performance is expressed as the relative standard deviation (repeatability) based on the raw data of the four analyses per participant. Fig. 8 shows that repeatability decreases with the lower concentrated n-alkanes.

For unknown reasons, the IAEA's data show elevated RSDs for C18 and for C28–C38. Closer investigation of the IAEA's raw data (not shown here) might indicate some drift problem. Further aspects need to be investigated in order to clearly pinpoint the underlying problem.

Laboratory Performance RSD (*n*=4)
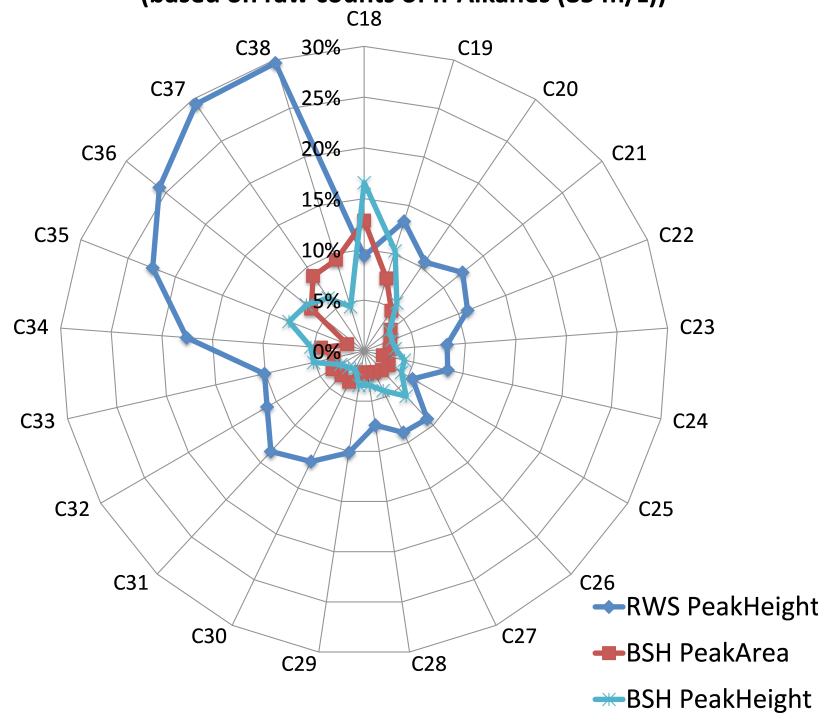### (based on raw counts of n-Alkanes (85 m/z))

Fig. 8: RSD for individual n-alkanes for each participant's data set.

Processing the GC-FID data from each participant as presented in "Method of data evaluation" leads to the results in the following "conclusion matrix" (see Tab. 2).

Consider the discussion points to this section, DP-1 and DP-2 on page 31.

		RWS		BSH		IAEA		NFI	
		RSD	*CD*	*RSD*	*CD*	*RSD*	*CD*	*RSD*	*CD*
Set_1	1 on 2	71%	71%	47%	60%	62%	69%	58%	75%
	1 on 3	57%	71%	60%	80%	69%	69%	83%	83%
	2 on 3	64%	71%	53%	60%	69%	69%	50%	67%
Set_2	4 on 5	45%	45%	35%	48%	53%	59%	29%	35%
	4 on 6	20%	15%	43%	52%	24%	47%	24%	29%
	5 on 6	25%	20%	30%	39%	29%	29%	12%	18%
Set_3	7 on 8	65%	100%	94%	94%	75%	60%	100%	100%
	7 on 9	53%	65%	67%	89%	58%	87%	64%	71%
	8 on 9	59%	82%	83%	94%	75%	67%	71%	71%
Dup	1 on 10	100%	100%	100%	100%	100%	100%	100%	100%

Tab. 2: Conclusion matrix of the GC-FID data for all samples of all participants.

All FID data generated by the participants are well in accordance with each other. It can be concluded that the samples of the **first set** are quite similar. The non-match resulting from the BSH data (RSD, 1 on 1) is considered to be an outliner in this context.

The results for the **second set** point predominantly, apart from some few exceptions (BSH and IAEA), to dissimilarities: The average of the matching ratio for Set 2 is only 34 %.

The data for **Set 3** consistently point to a match between the samples.

Set 4, the duplicate analysis (sample 1 on 10) checked out with a 100 % matching ratio for every participant.

MS data on biomarkers

As presented under the topic "Limitations of GC-MS analysis for paraffin wax", there are some biomarkers present in paraffin wax. The two most abundant ones (with nonetheless still very low intensities) were the groups of hopanes and steranes. Consequently they are looked at in more detail:

Patterns in the analyses of BSH and RWS do match visually all right; mass chromatograms are, at least from this point of view, fairly comparable (see Fig. 11).
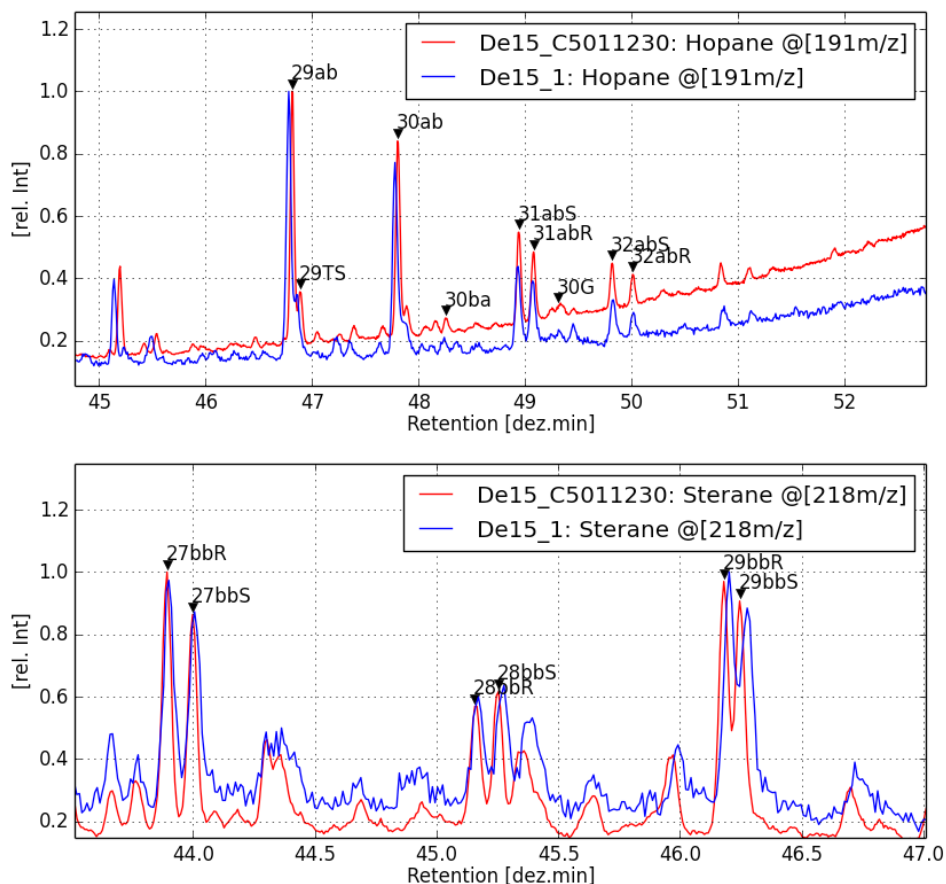
Fig. 11: Overlaid mass chromatograms (Set 1, sample 1) of hopanes (top) and steranes (bottom) from RWS (red) and BSH (blue).

Of the hopanes, nine representative substances were considered in this investigation as well as six of the steranes. Normalisation was performed to the highest peak in each group (hopanes: 29ab, steranes: 27bbR). As can be seen in the upper chromatogram in Fig. 11, both analyses are impacted by an increasing baseline (probably due to column bleeding). The further processing of the peak heights was done without any compensation for that influence, which might have a negative effect on the CD- or RSD-features for the final comparison. Considering areas instead of peak heights, again, might be an option to resolve this problem.

Anyway, absolute abundance of both groups of biomarkers is very low and consequently high RSDs are to be expected.

The radar chart Fig. 12 clearly shows that the hopanes are more stable in terms of repeatability than the steranes, as the latter seem to be more difficult to analyse.

Laboratory Performance RSD (*n*=4)
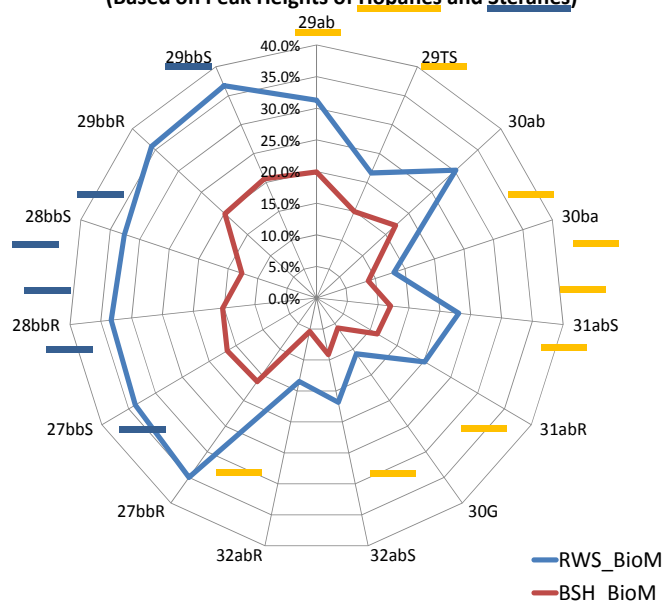### (Based on Peak Heights of Hopanes and Steranes)

Fig. 12: RSD of the biomarker groups hopanes and steranes, based on the peak heights (raw counts).

The RSD values in the radar chart are based on raw counts, expecting that normalisation compensates for variance if the main impact is of a systematic nature. Consider discussion point DP-3 on page 31.

Nevertheless, looking at the conclusion matrix (Tab. 4), it is surprising that matching ratios from the group of the steranes are succeeding those of the hopanes by far.

On the other hand, positive matches are suggested for samples of set 2 from both, RWS and BSH analysis, which contradicts the results from FID and MS-85m/z conclusions. This observation continues into the duplicate analyses, where hopanes show a non-matching tendency and steranes suggest a 100 % match.

These results lead to the conclusion that the biomarkers are of very limited use for this purpose, without further preparation or concentration of the samples only leads to inconclusive and erratic results.

		RWS Hopanes		RWS Steranes		BSH Hopanes		BSH Steranes	
		RSD	*CD*	*RSD*	*CD*	*RSD*	*CD*	*RSD*	*CD*
Set_1	1 on 2	0%	13%	100%	100%	38%	13%	100%	100%
	1 on 3	13%	38%	100%	100%	100%	100%	100%	100%
	2 on 3	63%	100%	100%	100%	100%	75%	100%	100%
Set_2	4 on 5	13%	13%	60%	100%	13%	13%	60%	40%
	4 on 6	63%	63%	20%	40%	88%	88%	80%	40%
	5 on 6	13%	13%	60%	60%	13%	13%	60%	60%
Set_3	7 on 8	100%	100%	100%	100%	100%	100%	100%	100%
	7 on 9	0%	0%	60%	60%	0%	0%	80%	80%
	8 on 9	0%	0%	60%	60%	0%	0%	100%	80%
Dup	1 on 10	13%	13%	100%	100%	75%	38%	100%	100%

Tab. 4: Conclusion matrix of the peak heights of hopanes and steranes as the most abundant biomarkers found in paraffin wax.

IRMS data

Laboratory Performance RSD
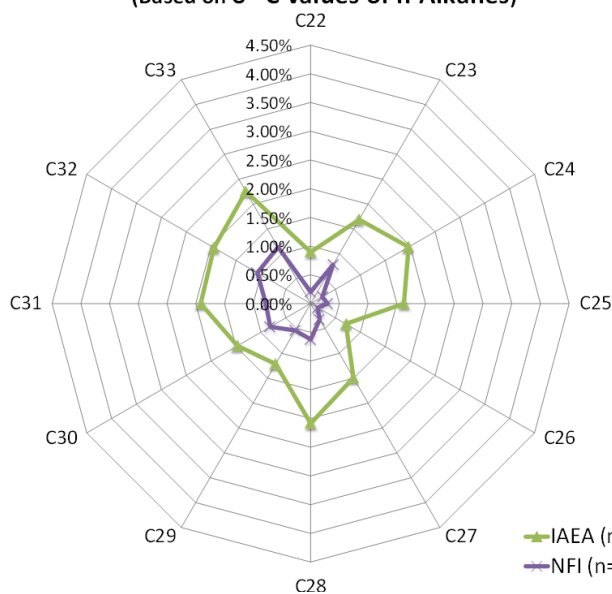(Based on $\delta^{13}C$ values of n-Alkanes)

Fig. 13: Compound specific RSD based on d13C measurements of n-alkanes.

In compound specific $\delta^{13}C$- isotope ratio mass spectrometry, one criterion commonly used is 1‰ (per mil PDB). This is partly given by the instrumental measuring uncertainty of around 0.5‰. This means, if one sample's measuring is within the range of 1 per mil of another sample, the $\delta^{13}C$- content of the material can be considered the same, or cannot be further distinguished.

Apart from the laboratory performance where the RSD is used to describe the repeatability (Fig. 13), the defining feature here is the absolute difference of $\delta^{13}C$ with a threshold of 1‰.

The NFI followed a straightforward approach and performed analysis, in duplicates, on the solutions as received without fractionation or similar preparative techniques.
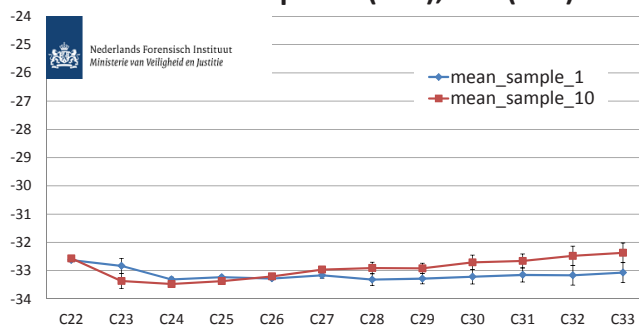
Means of sample #1 (n=1), #10 (n=2)

Fig. 14: d13C-means of sample 1 and 10 with standard deviation as error margin Measured by NFI.
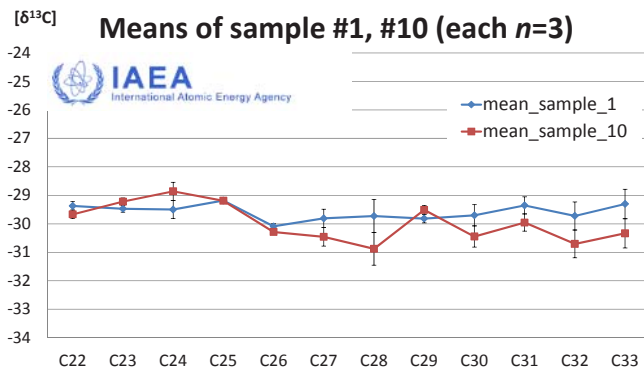
Means of sample #1, #10 (each *n*=3)

Fig. 15: d13C-means of sample 1, 10 from IAEA with standard deviation as error margin. IAEA measured in triplicates (n=3).

IAEA performed analysis in triplicates and used a more elaborated methodology compared to the NFI, with following steps involved:
- Preparation of a linear chained hydrocarbon fraction of the paraffin wax mixture
- Reference CO_2, calibrated against PDB, was introduced five times at the beginning, three times at the end of every isotopic GC determination.
- Calibration standards of n-alkanes were introduced every 4 samples.

For the duplicate analysis, the results of both laboratories show that the $\delta^{13}C$-content of each individual n-alkane is within the range of 1‰ (Fig. 15 and Fig. 14). In case of IAEA's duplicate analysis (Fig. 15), sample 10 shows unstable ^{13}C-contents along the series of n-alkanes. Especially the sudden increase (< 1 per mil) from C28 to C29 does not correspond to the average composition of the neighbouring n-alkanes.

However, there was an outlier in the NFI's duplicate run of the first sample, resulting in increased RSD (repeatability) and very low matching-ratios (< 10 % for comparison of set 1 "1 on 2" and "1 on 3" and >59 % for "1 on 10"). This was very obvious even without the application of any statistical test (e. g. Grubbs, etc.). After eliminating the outlier by ignoring the whole run and using just the data from the second analysis (instead of the mean, only values from the second run were considered, n=1), the comparison turned out to be very well in accordance (> 83 % for comparison of set 1 "1 on 2" and "1 on 3" and 100 % for "1 on 10").

		IAEA	NFI
		< 1	< 1
Set_1	1 on 2	100%	83%
	1 on 3	100%	100%
	2 on 3	100%	100%
Set_2	4 on 5	8%	31%
	4 on 6	0%	8%
	5 on 6	0%	0%
Set_3	7 on 8	92%	92%
	7 on 9	77%	92%
	8 on 9	85%	92%
Dup	1 on 10	83%	100%

Tab. 5: IRMS-Conclusion matrix

Considering the results of the duplicate analysis, the data consistency within the laboratories measurements appears therefore to be good. Comparing both laboratories, using absolute values however, reveals a rather different situation: Looking at both ^{13}C-content averages we observed an absolute discrepancy of 3‰. Consider discussion point DP-2 on page 31.

In the context of paraffin-wax samples one could conclude that this discrepancy of actually identical samples originate from different products which are derived from different raw materials (oil)/or different sources. Thus, in case of spill identification, this could be contradicting the otherwise solid results from GC-FID and GC-MS analysis. We therefore conclude that it might be possible that samples which appear to be similar in terms of GC-FID results (bell shape), nonetheless might be derived from different materials and consequently do not stem from the same source.

At this point it becomes obvious that there is some need to compare methodical and instrumental properties in order to increase inter-laboratory accuracy.

As shown in Tab. 5 is a high discrimination power between the samples using IRMS. From the matching ratios, one can derive a high probability for samples of set 1 to originate from the same bulk or educt material. This is of course a necessity for having samples of the same composition. However, it is also possible that the samples consist of two different products or batches, which were produced from the same origin material.

To sum up, this technique is undeniably a valuable addition to spill identification investigations, but it has to be interpreted in context of results from e. g. GC-FID.

Consider discussion points DP-5 and DP-6 on page 31.

Advanced Statistics

The statistical methods of correlation and clustering can be helpful in comparing samples to find possible matches. As shown above, correlation was applied to find suitable matches (see "Method of data evaluation"), whitening and clustering however are going to be looked at in more detail during further investigations. The following section focuses on an unconventional but very interesting approach of sample characterisation, which was suggested by René der Bruyn (NFI). This approach uses the GC-FID data and condenses the individual bell shape and its position within the chromatogram into a set of 2D data.

Looking at a GC-FID chromatogram of a paraffin wax, the peak heights of every n-alkane (more or less) resemble a bell shape (compare Fig. 16). This line, described by each peak-intensity, can be seen as an analogy to a distribution curve. Considering the n-alkanes abundancies under this curve as relative frequencies of a discrete probability density function, it can be used to derive the standard deviation (describing the variability) and the mean (as a weighted average, the location of the geometric balance point). These two values now represent the very unique properties of the paraffin wax composition. Calculation of these values can be performed following the common formulas (Formula 1).
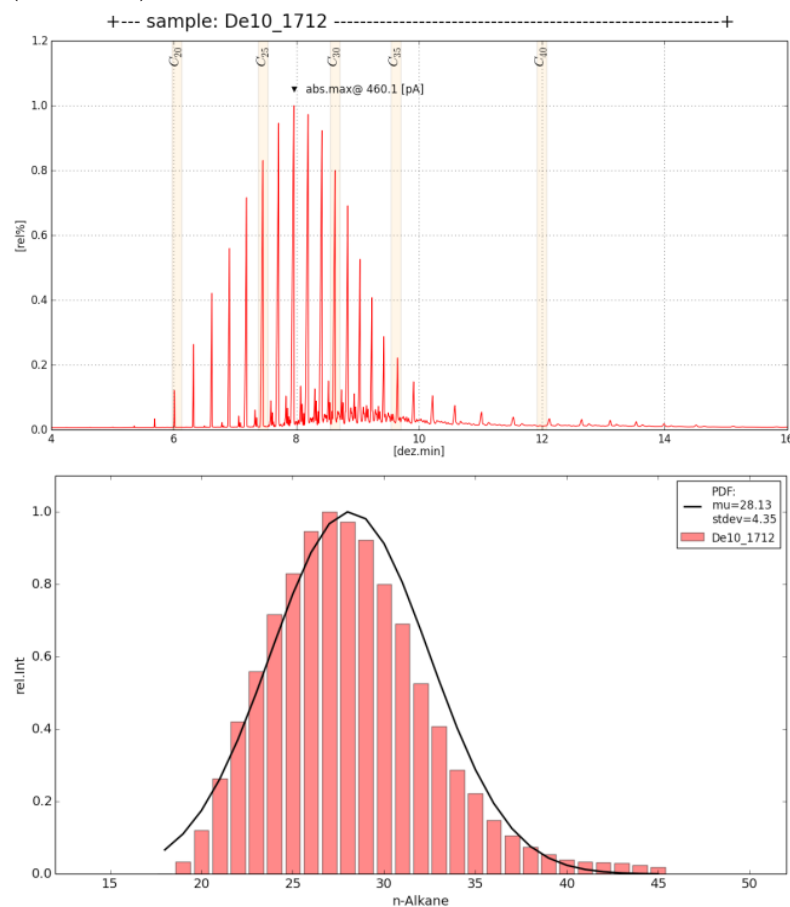

Fig. 16: Transformation of the n-alkane profile derived from GC-FID analysis to a discrete probability density function (PDF).

$$\mathrm{Var}(X) = \sum_{i=1}^{n} p_i \cdot (x_i - \mu)^2,$$

$$\mu = \sum_{i=1}^{n} p_i \cdot x_i$$

Formula 1: Top: variance, bottom: weighted average.

- The chain length is the random value x_i
- p_i represents the normalised intensities of n-alkanes.
- μ is the weighted average (wa)
- $\sigma = \sqrt{(variance)}$ the standard deviation (stdev)

Similar samples show identical bell shapes as n-alkane intensities describe, ideally, the same curve at the same position in the chromatogram. Consequently, those derived values are similar as well. In Fig. 5, standard deviations from all samples are plotted against their corresponding weighted averages.
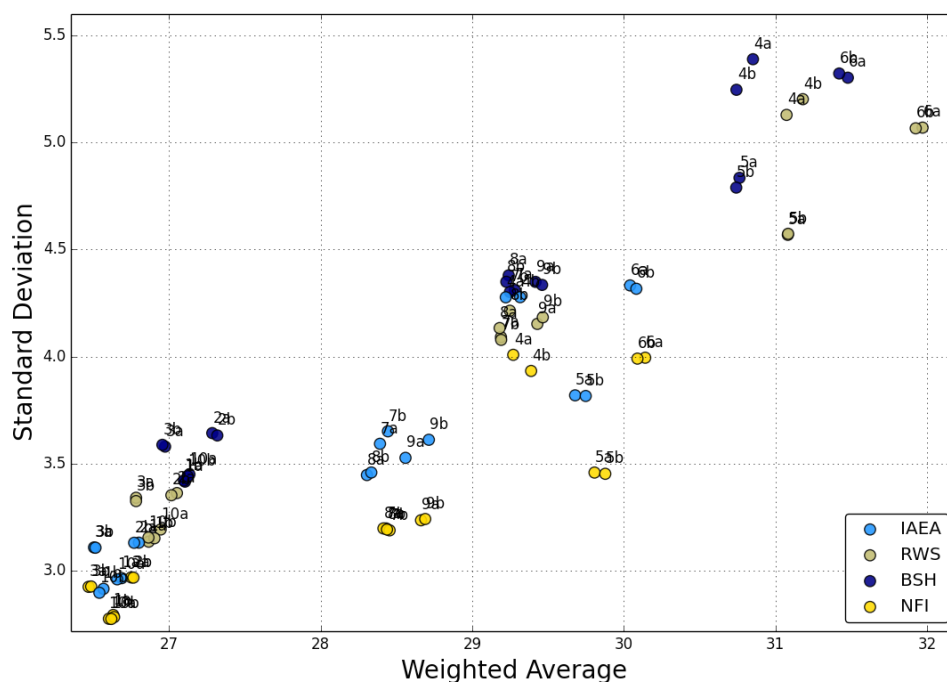
Fig. 17: Weighted average plotted against standard deviation for all samples.

As can be seen at first glance, the majority of the a/b pairs overlap or are located in very close proximity to each other. This confirms the similarity of the duplicates. Samples 1 to 3 (first set) from all participants are located in the lower left corner of the plot (ranges from 2.5 - 3.8 and 26 – 27.5 for standard deviation and weighted averages, respectively).

However, there are larger differences in the data among the second and third set. One reason for this discrepancy might be the nature of the paraffin wax samples of this set, as the content of higher boiling n-alkanes (hba) is, especially in set 2,

significantly higher, compared to the samples of the other sets. The GC-FID method used (CEN2012) is not very suitable for these high boiling compounds, the separation of components above C40 shows an irregular pattern (RT-intervals are increasing, decreasing height through peak broadening, etc.).

The data from the second set (sample 4, 5 and 6) of both RWS and BSH are assembled in the upper right-hand corner. The data from the same samples acquired by IAEA and NFI, however, form a second group at around 4/29.7 (stdev/wa).

Comparison of sample 6, set 2
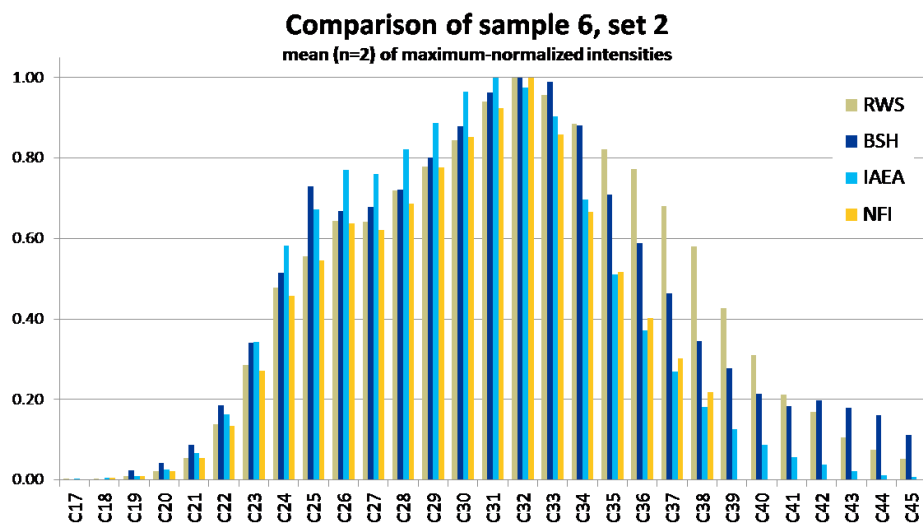
mean (n=2) of maximum-normalized intensities

Fig. 18: Comparison of normalized peak intensities of the second set's sample #6, from all participants.

Due to this way of compressing the curve's properties into 2-D data, the range and relative intensity of the considered n-alkane species are of great influence. Fig. 18 shows a comparison of the average means of the intensity of sample 6 from all participants. Especially for higher n-alkanes, larger discrepancies occur. As mentioned above, these discrepancies might be related to chromatographic issues (resolution) or mass discrimination of the targeted substances. A comparatively high similarity between RWS and BSH is revealed as both participants had a higher gain of the hba (C35–C45). Even though the NFI provided data just up to C38, the resulting weighted averages and variances are still corresponding very well with IAEA's data. One reason for this is that the relative intensities of the data of both laboratories are quite alike from C32 to C38, leading to the mentioned similarities.

As described above, the values of the duplicates of set 1 (Fig. 19, sample 1 and sample 10, marked with red circles) are in close vicinity to each other, indicating a strong bell-shape similarity. Closer investigation reveals a typical pattern between the samples of this set, which appears in similar form for every participant's data: the samples 1, 2 and 3) are arranged in a triangular constel-

lation (marked in green). Again, the relations based on similarity (Fig. 18) are reflected in the sample positions in Fig. 19: NFI and IAEA on the bottom left-hand corner, BSH and RWS upper right-hand corner.
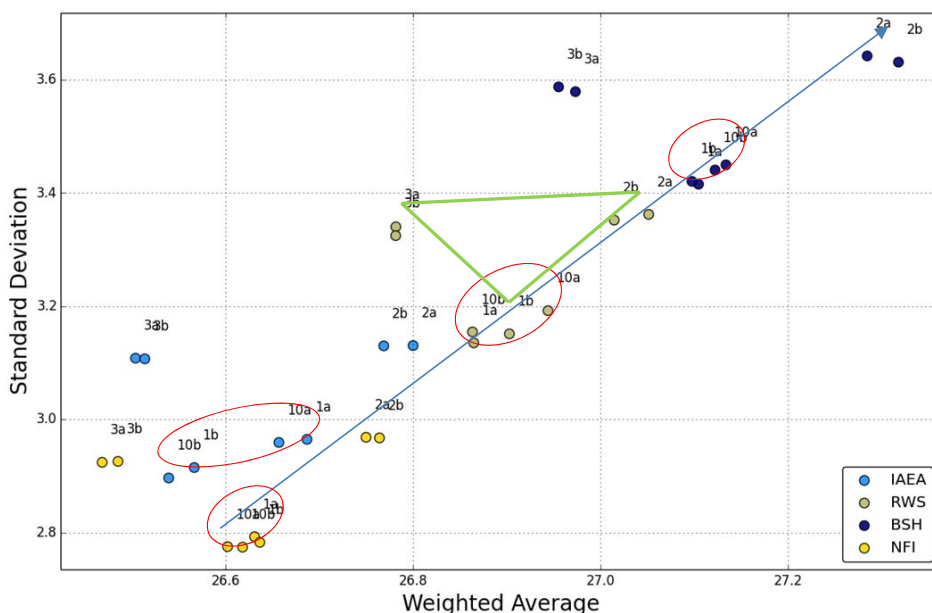

Fig. 19: Zoom into the cluster of the samples of the first set. Red circles indicate results of duplicates (sample 1 and 10), green triangle frames the pattern of sample 1 to 3. Blue arrow indicates higher peak intensities in the higher boiling area.

As this triangular pattern of the samples is repeating itself among the results, the relative difference between the data is somehow constant and might be of significance. It is to be remembered that shifts in standard deviation and weight average are strongly dependent on the number of individual homologues of n-alkanes present and their intensity. As identical samples were used, the differences are mainly caused by the analysis itself. Consequently, in such a comparison, shifts in standard deviation and weighted averages indicate increased or decreased detection of higher n-alkanes (direction of the blue arrow in Fig. 19).

Generally, for the assessment of the analysis of paraffin-wax samples within one laboratory (meaning analysis run by the same analytical inventory), the above-mentioned method presents a solid way to spot similarities and dissimilarities. Once a Euclidean distance (in terms of standard deviation and weighted average) or threshold value for the certainty of discrepancy is established (comparable to the 5 % and 14 % for RSD and CD, respectively), a match or no match could be concluded.

The inter-laboratory comparison conducted in the presented study has the additional benefit of monitoring differences of analytical performance as well.

Discussion Points

DP-1 for Fig. 8 and its data, I. Tolosa: From my point of view, calculating the RSD using the raw counts is not correct, because the raw counts will depend on the concentration (or dilution) of the sample. I meant that gradual evaporation of the solvent from the vials seating on the GC-auto sampler will cause an increase on the raw counts with time (increase in the absolute height value of the peak) and therefore normalized values to a fixed compound within the sample should be taken in account for calculating the RSD. In this sense, I suggest to add the same Fig based on normalized counts as it is in new Fig. 8.

DP-2 for Fig. 8 and its data, I. Tolosa: Drift problems are minimized by normalization to the maximum peak intensity in each individual sample. The higher RSD for the high boiling hydrocarbon is mainly due to the mass discrimination occurring in the injection port and the use of peak height instead of peak area.

DP-3 for Fig. 10 and Fig. 12 and their data, I. Tolosa: Similar comment as before; RSD should be based on normalized data and not raw counts. Moreover, here it is shown that the peak area provides lower RSD values than the peak height, at least for the higher n-alkanes with a high boiling point.

DP-4 for paragraph 10 in section *IRMS data*, I. Tolosa: It is obvious that IRMS laboratories participating in the same intercalibration exercise should use the same certified standard reference to avoid and minimize this discrepancy. Traceability to the same reference is essential for comparability of the results. More details on the calibrated 13C standards used for the measurements is needed to understand the discrepancy of 3 per mil.

DP-5 for section *IRMS data*, C. Blaga: Not "very" surprising the discrepancies in absolute results as the instruments are not always calibrated in the same manner in between labs. Different standards used, columns, lab conditions in general. We haven't been measuring steadily on the GC-IRMS and this gave also so stability issues. The standard mixtures I have mentioned refer to an "in-house" made mixture. The alkanes are commercially available in powder form and from each a certain amount was taken and dissolved in hexane to obtain the mixture used. The powders were previously measured with EA-IRMS to have an initial value for the isotopes and further the mix analyzed on the GC-IRMS (with the expected off-set, and less expected variability in time due to the low frequency in use of the instrument).

DP-6 for section *IRMS data*, C. Blaga: As a reference PDB is exhausted hence only VPDB is available. During each analysis, at least two pulses of reference CO_2 gas from the cylinder are introduced. This CO_2 is previously calibrated against other VPDB calibrated international standards, themselves calibrated against international IAEA standards. The reference CO_2 gas can be additionally calibrated against in-house standards but as such standards are not existent and frequently tested among labs this has not been done in our case.

Summary and Conclusion

The presented study focussed on investigating possibilities of environmental forensic analysis for source identification of paraffin spills using and enhancing methods regularly applied in oil spill environmental forensics.

The results show that GC-FID analysis provides data which are quite usable for categorizing and characterizing paraffin wax samples, as the bell shape formed by the n-alkanes in the chromatograms are of excessive uniqueness. Considering instrumental variations, the peak-based features of RSD (relative standard deviation) and CD (critical difference) are to be determined in order to confirm a matching of samples or tell them apart.

The analysis of biomarkers by GC-MS, which is efficiently used in oil spill identification, is of very limited use for paraffin-wax characterisation, since in paraffin-wax, the biomarker signature is too much altered or almost completely depleted. This renders a direct analysis useless without the processing of analytical data or enhanced sample treatment.

Two paraffin wax samples from the same product mixture or from the same tank should be, per definition, of a similar composition. If this is the case, the raw material from which the wax was produced should consequently be of the same origin as well. This can be very reliably confirmed by GC-IRMS analysis. Compound-specific or bulk analysis should both confirm such a relation.

In a case however, where paraffin waxes have very similar bell shapes but are of different origin (which can easily occur with such refined products), GC-FID and -MS would be unable to detect this difference and would lead to a false-positive match. Only IRMS analysis provides this crucial information. Such a difficult scenario highlights the valuable augmentation of IRMS to the standard techniques of GC-FID and -MS.

Nevertheless, IRMS is an expensive and labour-intensive technique compared to GC-FID or –MS systems. More so, the technique needs considerable harmonisation efforts before it can be reliably applied: The comparison of the absolute values of the 13C-Content of identical samples measured by the IAEA and by NFI lead to a difference of more than 3‰. Consequently, this material would be considered to be of a different origin and as a result as not being the same. However, even with its use in routine analysis being currently unlikely, IRMS represents an option to be considered in difficult cases and might provide to be a powerful parameter.

The all-over conclusion matrix shows a good accordance between the used techniques (Tab. 6).

		FID								MS						IRMS	
		RWS		BSH		IAEA		NFI		RWS Height		BSH Area		BSH Height		IAEA	NFI
		RSD	CD	RSD	CD	RSD	CD	RSD	CD	RSD	CD	RSD	CD	RSD	CD	1 ‰	1 ‰
Set_1	1 on 2	71%	71%	47%	60%	62%	69%	58%	75%	64%	71%	45%	64%	50%	50%	100%	83%
	1 on 3	57%	71%	60%	80%	69%	69%	83%	83%	86%	86%	73%	82%	42%	67%	100%	100%
	2 on 3	64%	71%	53%	60%	69%	69%	50%	67%	71%	71%	55%	64%	50%	67%	100%	100%
Set_2	4 on 5	45%	45%	35%	48%	53%	59%	29%	35%	45%	25%	22%	30%	28%	44%	8%	31%
	4 on 6	20%	15%	43%	52%	24%	47%	24%	29%	30%	25%	17%	22%	11%	28%	0%	8%
	5 on 6	25%	20%	30%	39%	29%	29%	12%	18%	35%	30%	9%	13%	17%	28%	0%	0%
Set_3	7 on 8	65%	100%	94%	94%	75%	60%	100%	100%	100%	100%	100%	100%	36%	71%	92%	92%
	7 on 9	53%	65%	67%	89%	58%	87%	64%	71%	59%	71%	64%	79%	29%	50%	77%	92%
	8 on 9	59%	82%	83%	94%	75%	67%	71%	71%	65%	82%	57%	79%	64%	79%	85%	92%
Dup	1 on 10	100%	100%	100%	100%	100%	100%	100%	100%	86%	71%	100%	100%	91%	82%	83%	100%

CD = Critical Difference (14%)
RSD = Relativ (Mean) Standard Deviation (5%)

Tab. 6: Total conclusion matrix of all analytical approaches (GC-FID, GC-MS, GC-IRMS).

The statistical method suggested by René der Bruyn (NFI), which compares standard deviation vs. weighted average, might work best for the assessment of data derived from a single laboratory. The results of the presented study show differences in inter-laboratory comparison which are most likely related to very individual properties within different GC-FID systems as the patterns of data sets from different laboratories are comparable, but having an offset on both axes. To make results more comparable between different laboratories, an additional normalisation on a common standard (paraffin-wax) might be beneficial.
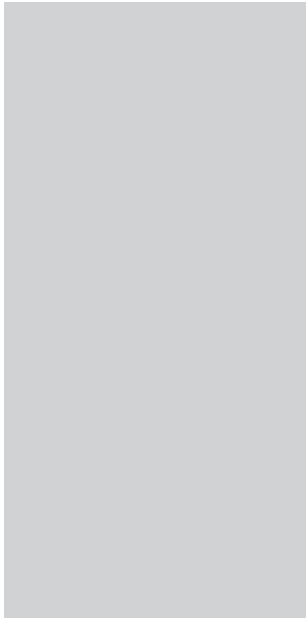
Outlook

The techniques used and the statistics applied so far are focused on the bell shape of the n-alkanes within a paraffin-wax mixture. However, many samples contain isomeric compounds as smaller peaks between the n-alkanes or have a more pronounced "unresolved complex mixture" (UCM). These characteristics are probably of the same uniqueness or individuality as the bell-shape. So it might be beneficial to look into a pattern-describing computation to map them as well.

To reduce the analytical effort while still benefiting from the IRMS' discrimination power between samples, it might be valuable to look into bulk analyses instead of using a method based on single compounds.

As this miniRR was based on real-life samples instead of the usage of standardised reference material, accuracy of the analytical results (defined as correctness and precision) is unclear. Therefore, a repetition of the miniRR with pre-defined standard material could be used to enhance the analytical precision so that the data of all participants would match even better.

Some paraffin samples used for this miniRR contained higher boiling n-alkanes. The boiling points of these compounds exceed the temperature program requested by CEN2012 and they are therefore not covered by the standard analysis. Thus, BSH conducted some investigations with a high-temperature GC-FID (HTGC-FID) and was able to separate compounds properly up to C70. It turned out that most of the higher boiling n-alkanes within the paraffin-waxes also display a proper bell-shape, and can therefore be analysed with the same statistical methods as presented above. Further investigations will explore the meaningfulness of such work for paraffin spill identification.

Acknowledgement

Abbreviations

2D	two-dimensional
µ, also: wa	weighted average
27bbR	5α (H),14β(H),17β(H), 20R-cholestane
29ab	17α(H),21β(H)-30-norhopane
BSH	Federal Maritime and Hydrographic Agency (German: Bundesamt für Seeschifffahrt und Hydrographie)
C	carbon
CD	critical difference
CEN2012	The technical report of the European Committee for Standardisation (2012), CEN/TR 15522-2:2012
CO_2	carbon dioxide
DP	discussion point
EA-IRMS	elemental analyser-isotope ratio mass spectrometry (EA-IRMS)
GC-FID	gas chromatography coupled with flame ionization detector
GC-IRMS	gaschromatography isotope ratio mass spectrometry
GC-MS	gas chromatography–mass spectrometry
hba	higher boiling n-alkanes
HTGC	high-temperature GC
IAEA	International Atomic Energy Agency
IMO	International Maritime Organization
IRMS	isotope ratio mass spectrometry
LKN-SH	The Schleswig-Holstein Agency for Coastal Defence, National Park and Marine Conservation (German: Landesbetrieb für Küstenschutz, Nationalpark und Meeresschutz Schleswig-Holstein)
m/z	Unit in mass spectrometry, where m=mass of ion, z=charge. number of ions. In mass analysis, an electron is taken from molecules to create single charged ions. If two electrons are removed, double charged ions are produced. The number of electrons removed is the charge number (for positive ions). m/z represents mass divided by charge number and the horizontal axis in a mass spectrum is expressed in units of m/z. Since z is almost always 1 with GCMS, the m/z value is often considered to be the mass.
MARPOL, also: MARPOL 73/78	International Convention for the Prevention of Pollution from Ships, 1973 as modified by the Protocol of 1978
NFI	Netherlands Forensic Institute (Dutch: Nederlands Forensisch Instituut)
NGO	non-governmental organization
norm	normalized
OSINet	Oil Spill Identification Network of experts within the Bonn-Agreement
pA	pico-Ampère

PAH	polycyclic aromatic hydrocarbon (also: polyaromatic hydrocarbon)
PDB	Pee Dee Belemnite
per mil	parts per thousand (also: per mille)
p_i	normalised intensities of n-alkanes
PUI	Peaceful Uses Initiatives program
R&D	research and development
Retention	Time for a chemical compound to pass through a chromatographic system
RR	Round Robin (also: intercalibration study)
RSD	relative standard deviation
RWS	Rijkswaterstaat
s, also: stdev	standard deviation
UCM	unresolved complex mixture
UEG	Independent Group of Environmental Experts „Consequences of Pollution Accidents" at the Central Command for Maritime Emergencies (German: Unabhängige Umweltexpertengruppe „Folgen von Schadstoffunfällen" beim Havariekommando)
VPDB	"Vienna Pee Dee Belemnite", an analytical standard for δ^{13}C-Analysis
x_i	Random variable. Here: chain length of n-alkane
δ	Mathematical symbol or a difference of tow values
δ^{13}C	^{13}C content expressed as difference to a standard

A look at the hardware

GC-FID-Conditions

		BSH	IAEA	NFI	RWS
Hardware and Operation					
	Instrument	Agilent 7890A	Agilent 7890A	Agilent 6890N	Agilent 7890A
	Detector	FID	FID	FID	FID
Carrier gas	He, H2, other	H2	He	N2	He
Injection					
	Volume [µL]	1	1	1	1
	Injection temp. [°C]	320	300	300	325
	Pre-inject. delay [sec]	1	0	0	5
	Injector type	solvent vent*	spiltless	split, 20:1	splitless
Column					
	Name	J&W DB-5	HP-5	HP Ultra-1	J&W DB-1
	Length [m]	10	30	25	15
	Diameter [mm]	0.1	0.25	0.2	0.25
	Film [µm]	0.1	0.25	0.33	0.25
	Const. flow [mL/min]	0.92	1.2	0.4	1.1
Oven program					
	Start [°C]	40	60	60	35
	Hold_start [min]	0.71	1	1	1.5
	Rate_1 [°C/min]	30.2	6	30	10
	Rate_1_end [°C]	300	310	150	325
	Hold_1 [min]	2.5	-	0	-
	Rate_2 [°C/min]	15	-	5	-
	End [°C]	325	310	320	325
	Hold_end [min]	3.6	15	12	10
	Run time [min]	17.0	57.7	16.0	40.5
FID-Conditions					
	Heater [°C]	340	300	250	375
	H2 [mL/min]	30	35	30	40
	Air [mL/min]	320	350	320	300
	Makeup [mL/min]	30	28.09	30	25

*= The "Solvent Vent Mode" is a setting unique to PTV-injectors. It is a special type of splitless injection but allows the handling of large volumes when needed.

As can be seen from the table above, GC-FID conditions vary a lot between the participants. Carrier gas, column type and separation conditions can therefor contribute to the systematic differences observed.

IRMS-Conditions

		IAEA	NFI
Hardware and Operation			
	GC	Trace GC	Agilent 7890A
	IRMS-Instrument	GC-Isolink-Conflo IV	Thermo MAT 253
	Detector	Delta V advantage	
Carrier gas	He, H2, other	He	He
Column			
	Name	DB5	HP5
	Length [m]	30	30
	Diameter [mm]	0.25	0.32
	Film [µm]	0.25	0.25
		non-polar, 5m, 0.53 mm i.d.	
Injection	Volume [µL]	1	1
	Injection temp. [°C]	60	300
	Pre inject. delay [sec]	-	0
	Injection type	on column	?
Oven program	Start [°C]	60	60
	Hold_start [min]	-	1
	Rate_1 [°C/min]	10	30
	Rate_1_end [°C]	100	150
	Hold_1 [min]	-	0
	Rate_2 [°C/min]	3	5
	End temperature [°C]	310	320
	Hold_end [min]	19	32

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