Automated and solvent-free microextraction techniques for the GC-MS analysis of food and environmental samples

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Summary

Sample preparation is one of the most time-consuming steps in analytical chemistry, whose purpose is the transfer of analytes into solution, the removal of interfering matrix compounds, the enrichment of analytes for trace analysis, and sometimes the derivatization to a more suitable structure for successful separation or detection. Automation, reliability, minimizing the use of toxic solvents and increasing the sensitivity, are some of the demands for sample preparation. Microextraction techniques fulfill these demands and are valuable alternatives for conventional used extraction techniques like liquid-liquid extraction (LLE) or solid-phase extraction (SPE). The aim of the thesis was to investigate, validate and expand the use of fully automated and solventless microextraction techniques to show new alternatives for the analysis of water and food samples. Therefore, gas chromatography mass spectrometry (GC-MS) based methods were developed, optimized and validated, using in-tube extraction dynamic headspace (ITEX-DHS) and solid phase microextraction (SPME) Arrow. Chemometric methods were used for the method optimization (design of experiments, DoE) and for data evaluation (linear discriminant analysis, LDA).

ITEX-DHS is a dynamic extraction approach, in which the headspace is pumped multiple times through a sorbent bed. As ITEX-DHS is only applied for headspace analysis, only volatile analytes can be analyzed, but matrix interferences can be avoided. Therefore, more complex matrices like viscous, oily or sticky matrices could be analyzed without problems. In this thesis, ITEX-DHS based methods in two areas of food analysis were developed and validated. In the first method, ITEX-DHS was optimized for the analysis of 21 volatile organic compounds (VOCs), commonly present in extra virgin olive oil (EVOO). The monitoring of EVOOs is of special interest, as it is one of the most important targets in food fraud. 31 EVOOs from five different geographical origins were measured and LDA was used for classification. The limits of detections (LODs) ranged from 0.1 to 68.6 µg kg^{-1} and thus were much smaller than shown in previous studies using SPME. The second method shows the use of ITEX-DHS for the analysis of honey samples. Honey is often mislabeled and has a high potential for food fraud. ITEX-DHS allows a quick, easy and robust analysis of VOCs, which are responsible for the aroma of honey and which can be linked to the botanical and geographical origin of honeys. Therefore, 14 VOCs were quantified with ITEX-DHS with method detection limits ranging from 0.8-47 ng g^{-1} and repeatabilities shown as relative standard deviations of below 8 %. 38 honey samples were measured to show the applicability of the method and to give an overview how this method could be used for future work.

SPME is one of the most famous microextraction techniques. However, the fiber is very fragile, and the mechanical stability of the device is not very satisfactory. To overcome these drawbacks, the SPME Arrow was developed. Its mechanical stability is increased by an Arrow shaped tip and the sorbent material is coated around a stainless-steel rod. Furthermore, the larger sorbent volume leads to more sensitivity. In comparison to ITEX-DHS, not only headspace sampling is possible, as the SPME Arrow can be placed into the aqueous sample for direct immersion sampling. Both options are presented in this thesis, and in total, three different SPME Arrow based methods were investigated, optimized and validated for a more sensitive analysis of the target analytes. The first presented use of SPME Arrow is the automated derivatization of fatty acids to fatty acid methyl esters directly on the sorbent material of the SPME Arrow. The fatty acids were extracted from acidified water via direct immersion sampling. Subsequently, the SPME Arrow was moved to another vial, containing a mix of methanol and sulfuric acid. The derivatization takes place on the fiber material and the fatty acids are methylated and afterwards thermally desorbed in the GC injector. Unfortunately, validation was not successful as the fiber material decomposed during the derivatization step. For the analysis of taste and odor compounds in water, a SPME Arrow headspace method was optimized using DoE, and then validated. The achieved LODs were below the thresholds of the target analytes and varied from 0.05-0.6 ng L^{-1} with satisfactory repeatabilities (RSDs < 11 %). Compared with conventional approaches, this method showed a significant enhancement in sensitivity, and outstanding robustness and stability. Furthermore, the required sample volume was reduced in comparison to other methods, and the method was able to detect seven analytes at very low concentrations. To show the option of direct immersion sampling, phosphorous flame retardants were analyzed directly from different water samples, using SPME Arrow. Again, the method was first optimized using DoE and was then validated. The limit of quantification (LOQ) ranged from 0.2-1.2 ng L^{-1} and thus, showed again great sensitivity for the application of SPME Arrow. Furthermore, the analysis was carried out completely automated, resulting in a more time-efficient method than LLE or SPE, which are very labor-intensive. This method showed to be the most sensitive analytical approach for the determination of phosphorous flame retardants in water.

All in all, this thesis presents five new options for the use of ITEX-DHS and SPME Arrow, as examples for modern, solvent-free and fully automated microextraction techniques. It demonstrates the potential of microextraction techniques for routine analysis as robust, sensitive and non-hazardous alternatives for commonly used extraction techniques, which often deal with large amounts of organic solvents.

Zusammenfassung

Die Probenvorbereitung ist einer der zeitaufwendigsten Schritte in der analytischen Chemie, dessen Zweck die Überführung von Analyten in Lösung, die Entfernung störender Matrixverbindungen, die Anreicherung von Analyten für die Spurenanalyse und die Derivatisierung in eine leichter detektierbare Struktur ist. Automatisierung, Zuverlässigkeit, Minimierung von toxischen Lösungsmitteln und Erhöhung der Empfindlichkeit sind einige der Anforderungen an die Probenvorbereitung. Mikroextraktionstechniken erfüllen diese Anforderungen und sind wertvolle Alternativen für konventionell angewandte Extraktionstechniken wie Flüssig-Flüssig-Extraktion (*liquid-liquid extraction*; LLE) oder Festphasenextraktion (*solid-phase extraction*; SPE). Ziel der Arbeit war es, durch den Einsatz vollautomatischer und lösungsmittelfreier Mikroextraktionstechniken neue Alternativen für die Analyse von Wasser- und Lebensmittelproben aufzuzeigen. Daher wurden auf der Gaschromatographie-Massenspektrometrie (GC-MS) basierende Methoden entwickelt, optimiert und validiert, wobei die dynamische In-Tube Extraction (*in-tube extraction dynamic headspace*; ITEX-DHS) und die Festphasenmikroextraktion (*solid-phase microextraction*; SPME) Arrow verwendet wurden. Chemometrische Methoden wurden für die Methodenoptimierung (*Design of Experiments*, DoE) und für die Datenauswertung (lineare Diskriminanzanalyse, LDA) eingesetzt.

ITEX-DHS ist ein dynamischer Extraktionsansatz, bei dem der Gasraum mehrfach durch ein Sorbensbett gepumpt wird. Da ITEX-DHS nur für die Headspace-Analyse verwendet wird, können nur flüchtige Analyten analysiert werden, jedoch werden Matrixinterferenzen vermieden, wodurch komplexere Matrices problemlos analysiert werden können. In dieser Arbeit werden ITEX-DHS basierende Methoden für zwei Beispiele der Lebensmittelanalytik entwickelt und validiert. In der ersten Methode wurde ITEX-DHS für die Analyse von 21 flüchtigen organischen Verbindungen (*volatile organic compounds*; VOCs) optimiert, die üblicherweise in extra nativem Olivenöl (*extra virgin olive oil*; EVOO) vorkommen. Die Überwachung von EVOOs ist von Interesse, da sie eines der häufigsten Ziele bei Lebensmittelbetrug sind. Es wurden 31 EVOOs aus fünf Ländern gemessen und anschließend wurde eine LDA genutzt, um die EVOOs nach ihrem geographischen Ursprung zu klassifizieren. Die Nachweisgrenzen (*limit of detections*; LODs) reichten von 0.1 bis 68.6 µg kg^{-1} und waren damit kleiner als in früheren Studien mit Hilfe von SPME gezeigt werden konnte. Die zweite Methode zeigt die Verwendung von ITEX-DHS für die Analyse von Honigproben. Honig wird häufig falsch etikettiert und hat ein hohes Potential für

Lebensmittelbetrug. ITEX-DHS ermöglicht eine schnelle, einfache und robuste Analyse von VOCs, die für das Aroma des Honigs verantwortlich sind und mit der botanischen und geographischen Herkunft des Honigs in Verbindung gebracht werden. Daher wurden 14 VOCs mit ITEX-DHS quantifiziert, wobei die LODs der Methode zwischen 0.8 und 47 ng g^{-1} lagen.

SPME ist eine der bekanntesten Mikroextraktionstechniken, dessen Faser jedoch sehr zerbrechlich ist. Um diesen Nachteil zu überwinden, wurde der SPME Arrow entwickelt. Seine mechanische Stabilität wird durch eine pfeilförmige Spitze erhöht. Außerdem führt das größere Sorptionsmittelvolumen zu einer höheren Empfindlichkeit. Im Vergleich zu ITEX-DHS ist nicht nur eine Headspace-Probenahme möglich, da der SPME Arrow zur direkten Immersionsprobenahme in die wässrige Probe eingebracht werden kann. Die erste hier vorgestellte Nutzung des SPME Arrows ist die automatisierte Derivatisierung von Fettsäuren zu Fettsäuremethylestern direkt auf dem Sorbensmaterial. Die Fettsäuren wurden mittels direkter Immersionsprobenahme aus einer wässrigen Probe extrahiert. Anschließend wurde der SPME Arrow in ein anderes Vial mit einer Mischung aus Methanol und Schwefelsäure gebracht. Die Derivatisierung findet direkt auf dem Fasermaterial statt und anschließend werden die Fettsäuremethylester im GC-Injektor thermisch desorbiert. Jedoch konnte keine Validierung durchgeführt werden, da das Phasenmaterial zu instabil war und sich zersetzte. Für die Analyse von Geschmacks- und Geruchsstoffen in Wasser wurde eine SPME-Arrow-Headspace-Methode mittels DoE optimiert und validiert. Die erreichten LODs lagen unter den Schwellenwerten der Zielanalyten und variierten von 0.05-0.6 ng L^{-1}. Im Vergleich zu konventionellen Ansätzen zeigte diese Methode eine signifikante Steigerung der Empfindlichkeit sowie eine hervorragende Robustheit und Stabilität. Um die Möglichkeit der direkten Immersionsprobenahme zu zeigen, wurden phosphorhaltige Flammschutzmittel aus verschiedenen Wasserproben mit dem SPME Arrow analysiert. Auch hier wurde die Methode zunächst mittels DoE optimiert und anschließend validiert. Die Bestimmungsgrenzen (*limit of quantification*, LOQ) lagen im Bereich von 0.2-1.2 ng L^{-1}. Die Methode erwies sich als empfindlichster Ansatz für die Bestimmung phosphorhaltiger Flammschutzmitteln in Wasser.

Insgesamt werden in dieser Arbeit fünf verschiedene Optionen für den Einsatz von ITEX-DHS und SPME Arrow als Beispiele für moderne, lösungsmittelfreie und vollautomatische Mikroextraktionstechniken vorgestellt. Sie demonstriert die potenziellen Mikroextraktionstechniken für die Routineanalyse als robuste, empfindliche und ungefährliche Alternativen für häufig verwendete Extraktionstechniken, die oft mit großen Mengen organischer Lösungsmittel arbeiten.

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1 Introduction

1.1 Sample preparation in instrumental analytical chemistry

Sample preparation is one of the most time-consuming steps in analytical chemistry. Nevertheless, it is one of the most important steps, as errors made in sample preparation falsify the analysis and thus lead to incorrect data. However, the progress in the development of sample preparation techniques is rather slow in comparison to the sophisticated development of chromatographic and mass spectrometric instrumentations [1]. Even though the instruments get more sensitive with each development, sample preparation cannot be neglected, as the purpose of sample preparation is the transfer of analytes into solution, the removal of interfering matrix compounds, the enrichment of analytes for trace analysis, and sometimes even the derivatization to a more suitable structure for successful separation or detection [1, 2]. Gas chromatography (GC) coupled to mass spectrometry (MS) offers good separation efficiency and highly sensitive detection, although the development in more sensitive detectors is still proceeding. Nevertheless, errors made in sample preparation cannot be corrected regardless of separation power, selectivity or sensitivity. Besides, many sample preparation protocols rely on classical techniques like liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Typically used sample volumes are rather large as well as high amounts of organic solvents are used as extraction phases. The samples often require further concentration by evaporation or distillation and/or clean-up steps and thus a large amount of wastes is produced. Additionally, this results in time consuming protocols which are also prone to analyte losses or sample contamination, as many steps are required. The human workload and the corresponding risk of human error call for the automation of sample preparation. This goes hand-in-hand with the need to miniaturize sample preparation, as most autosamplers cannot handle huge amounts of reagents, solvents or samples [1].

1.2 Microextraction techniques

Miniaturization is an important part of green analytical chemistry (GAC). GAC was introduced by Anastas and Warner (1998) and describes the approach to minimize organic solvent use, sample size, consumption of energy and the generation of waste. Furthermore, the goal of GAC is to substitute hazardous substances with less harmful substances to protect the user and the environment [3, 4]. Combining all these considerations, the need for rapid, accurate and miniaturized sample preparation methods is increasing and lead to the use of

microextraction techniques, which are often solventless, require less sample volume and mostly work fully automated [1]. Automation of workflows is of great interest, because the sample throughput can be increased, as the autosamplers can work nonstop. However, the amount of time required for automated sample preparation protocols remains similar to the manual protocols, as the autosampler has to do all steps sequentially and cannot really work in parallel. The advantage is that the autosampler can prepare the next sample during the GC runtime, so that each sample is freshly prepared and extracted and no time due to the overlapping of preparation and separation and detection is lost. Additionally, less sample volume and solvent volumes are required, as most applications are miniaturized, and less errors occur. Furthermore, automated solutions are more reproducible than manual sample preparation, as the autosampler performs each task always in the same way and is less prone to errors.

Microextraction techniques handle small volumes of extraction medium and samples, and are influenced by a huge variety of parameters, like extraction time, temperature, pH, salt content, and stirring, which might even correlate with each other. Many microextraction techniques work completely solventless and their applications can be fully automated, which make them favorable in comparison to classical extraction approaches [5, 6]. Microextraction techniques have a large potential to replace more traditional sample preparation protocols in many application areas, such as analysis of environmental samples [7], food samples [8], forensic samples [9, 10] and biological samples [11, 12], but they are yet not part of routine analysis [13].

Most used representatives of microextraction techniques are solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), liquid-phase microextraction (LPME), solid-phase dynamic extraction (SPDE), microextraction in a packed syringe (MEPS) and in-tube extraction dynamic headspace (ITEX-DHS) [5-7, 14]. Most of them developed out of the most common conventional extraction techniques SPE and LLE [8]. Thus, microextraction techniques can be divided into those, that are based on sorbents (like SPE in classical extraction techniques), and those, that are based on solvents (like LLE in classical extraction techniques) [15]. Microextraction techniques can be divided into static and dynamic extractions, as well as into solvent or solventless techniques. The extraction is performed in either headspace (HS) or in direct immersion (DI) mode, which is shown in a scheme in Figure 1-1.

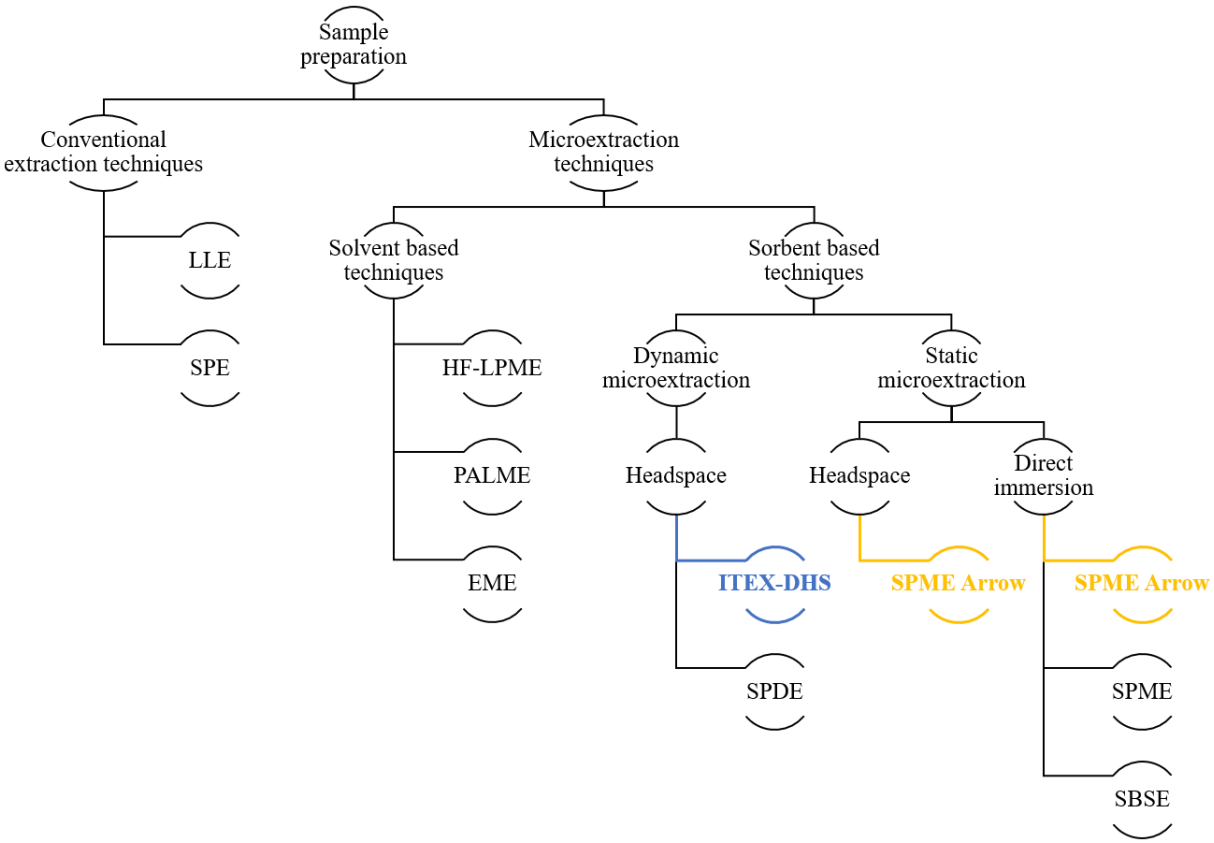

Figure 1-1: Overview of sample preparation techniques with a focus on microextraction. The blue and yellow highlighted techniques are the ones used within this thesis. LLE: liquid-liquid extraction. SPE: solid phase extraction. HF-LPME: hollow fiber-liquid phase microextraction. PALME: parallel artificial liquid membrane extraction. EME: electromembrane extraction. ITEX-DHS: in-tube extraction-dynamic headspace. SPDE: solid phase dynamic extraction. SPME: solid phase microextraction. SBSE: stir bar sorptive extraction.

As this thesis focusses on solventless microextraction techniques, only representatives of these techniques will be discussed in the following sections, divided into static microextraction techniques (Chapter 1.3), covering SPME, PAL SPME Arrow and SBSE, and dynamic microextraction techniques (Chapter 1.4), including SPDE and ITEX-DHS. A comparison of the different techniques is shown in Figure 1-2 for the application in water analysis. The sampling preparation procedure is similar in all the techniques, with a few adaptations explained in the according chapter. In general, the sampling preparation can be divided into four steps: incubation, extraction, thermal desorption and cleaning. Incubation of the sample is required for HS sampling, as the equilibrium between the sample and the headspace should be achieved prior to extraction.

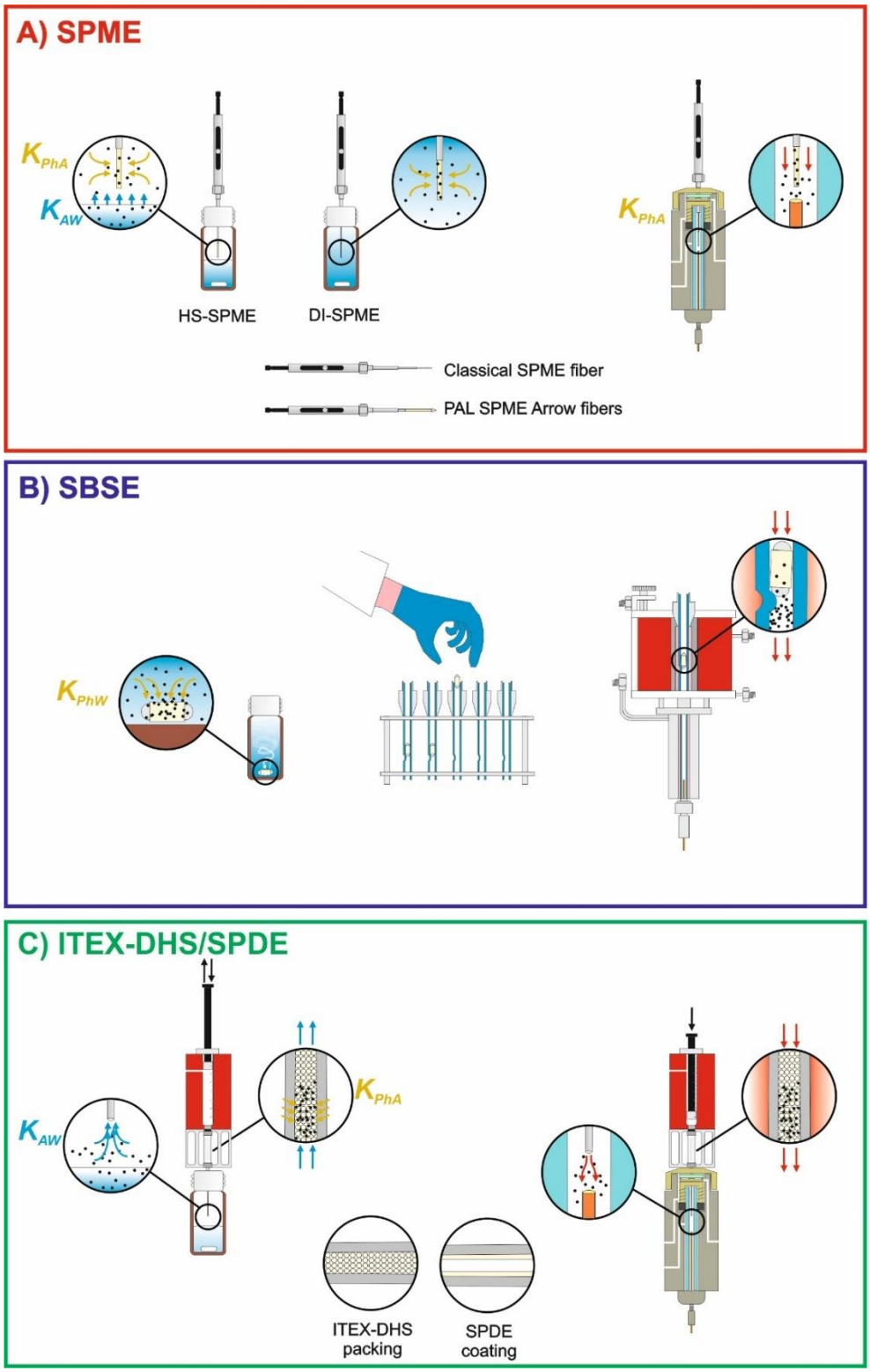

Figure 1-2: Graphical comparison of the microextraction techniques discussed in this thesis. A) SPME/PAL SPME Arrow, B) SBSE, C) ITEX-DHS/SPDE. Sorption depends on the partitioning constants of the analytes between sorbent and water, K_{PhW}, for direct immersion and between air and water sample, K_{AW}, and the sorbent phase and air, K_{PhA}, for headspace sampling [13].

1.3 Static microextraction techniques

In static microextraction, the sample is extracted once for a defined amount of time. All analytes are extracted based on partitioning constants between sample and extraction phase, either via HS sampling or with DI. For water samples, the HS sampling relies on the partitioning constant K_{PhA}, which describes the partitioning between the sorbent phase and air, and furthermore, on the partitioning between water and air, K_{WA}. For direct immersion, the partitioning constant between water and the sorbent phase, K_{PhW}, is the important factor. With other samples, other partitioning constants become important. A scheme is shown in Figure 1-3. SPME and SBSE, as widely used techniques, are presented in the following sections, as well as the recently developed PAL SPME Arrow.

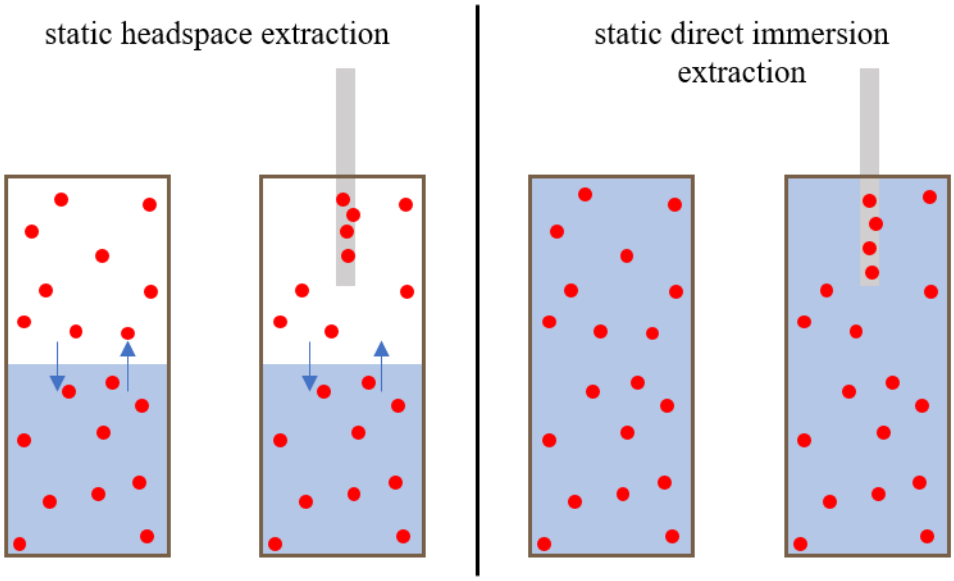

Figure 1-3: Scheme of static extraction. On the left side of the figure, headspace extraction is shown and on the right side of the figure direct immersion extraction is shown. The grey rod symbolizes the extraction phase. Water is shown in blue, air in white. The red dots represent the analytes.

1.3.1 Solid-phase microextraction

SPME is probably the best known microextraction technique, which was first introduced in 1990 by Arthur and Pawliszyn and was developed to eliminate problems, which were associated with SPE, such as the use of toxic reagents and long extraction times [16]. Additionally, the reduction in sample volume, the low operation costs, the high sensitivity, and the option for automation make it an appealing alternative in comparison to SPE and LLE [17-19].

The set-up consists of a thin fiber made of silica, which is coated with the sorbent material [5]. Commercially available coating materials are polydimethylsiloxane (PDMS), divinylbenzene (DVB), carboxen (CAR), polyethylene glycol (PEG) and Carbowax (CW). Furthermore, the combination phases PDMS/DVB, PDMS/CAR, CW/DVB and DVB/CWR/PDMS are available [5, 18]. The recent trend in the SPME research is to find more selective and specific coatings, including triazine-based structures, metal-organic frameworks, ionic liquids and many more [20-24].

Using SPME, the sample preparation is improved by combining sampling, extraction, enrichment and sample introduction within a two-step process (Figure 1-2 A). In the first step, the fiber is exposed to the sample (either HS or DI) and the analytes partition between the sample and the extraction phase, where they sorb to the fiber material, either via adsorption or absorption, depending on the sorbent phase. The second step is the thermal desorption in the GC injector [25-27].

However, SPME comes with some drawbacks. The set-up of the fiber is very fragile and thus very unstable. The coatings tend to strip or break (Figure 1-4), the fiber itself bends which leads to a significant reduction in their overall lifetime [28, 29]. Another problem is the batch-to-batch variation of the fiber coatings [5], and the small extraction volume [30, 31]. Despite these limitations, SPME is widely used as it is a simple and versatile technique. There are hundreds of publications for environmental [24, 32-35], food [36-41], biological and medical applications [42-44], as well as several books and reviews [6, 13, 28, 45-47].

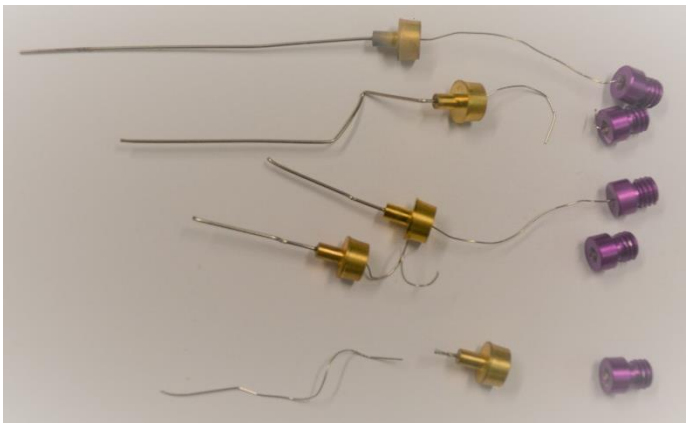

Figure 1-4: Example of physical damage of classical SPME fibers.

1.3.2 Stir-bar sportive extraction

SBSE was developed in 1999 by Baltussen et al. as a version of SPE using PDMS as sorbent material [48]. It is based on the same principle as SPME but instead of a fiber, a coated stir bar is used [49, 50] which overcomes the drawback of the small extraction phase volume in SPME, as it is increased from about 0.5-1 µL to 100 µL [49, 51]. This leads to a higher sample capacity and thus a higher enrichment factor, which is favorable for trace analysis. However, it leads to longer extraction times, as equilibrium is reached later [19]. Additionally, only PDMS coating and a PDMS/Ethylene glycol coating are available which leads to a limited spectrum of accessible analyte polarities, as mainly low polarity compounds can be extracted [7]. Furthermore, matrix effects play an important role during extraction [5], and the lifetime of the stir bars is limited to around 20-50 extraction, depending on the application [49].

For sampling, the coated stir bar is directly placed into the aqueous sample and stirred for a certain extraction time, during which the analytes sorb to the stir bar (Figure 1-2 B). After the extraction time, the stir bar is removed from the sample and dried [50]. This step is the main drawback of this technique, as it cannot be fully automated [51]. The stir bar is then placed in a thermodesorption tube, which is then placed on an autosampler, and the thermodesorption can be proceeded automatically [5, 19, 51]. The whole process can also be carried out for HS sampling. One big advantage is, that the stir bar can be applied for on-site sampling without loss of analytes during transport to the laboratory [19].

SBSE is often applied to environmental samples [7, 52-56], but also for biological samples [57-60] and food analysis [61-64], as the preconcentration factor is an important step in these field. Furthermore, there are several reviews published covering SBSE [13, 50, 65-70].

1.3.3 Solid-phase microextraction Arrow

PAL (Prep and Load) SPME arrow was developed to combine the advantages of SPME and SBSE while solving their disadvantages [51]. It consists of a stainless-steel rod, which is coated with the extraction phase. It ends in an arrow shaped tip, which closes the steel tube, when the device is not in use and the fiber is withdrawn [71]. This tip shape does not only allow smoother penetration through septa, and thus increased mechanical stability, as the pressure is more evenly distributed and the septa are cut instead of pierced, it also ensures protection of the sorption phase during transfer processes by completely enclosing the sorption phase within the sheath [51]. SPME fibers are proven to be more fragile and require replacement after 100

injections [72]. PAL SPME Arrow shows better mechanical stability, not only because of the arrow like tip, but also by the increased diameter of the outer capillary, which is 1.5 mm for PAL SPME Arrow and only 0.7 mm for SPME [51]. The sorbent volume is larger in comparison to classical SPME (~ 3.8 µL instead of 0.6 µL) and thus leads to better sensitivity [6, 29, 51] as the extraction capacity is higher and less competition occurs for adsorption sites [51]. A close-up of the PAL SPME Arrow tips, once closed and once opened, is presented in Figure 1-5.

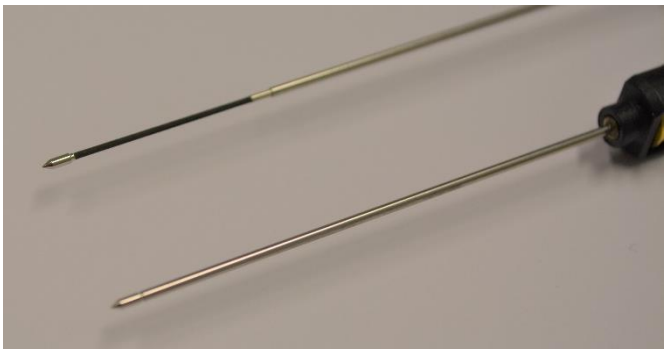

Figure 1-5: SPME Arrow close-up of the typical tip. The arrow-like tip completely encloses the extraction phase.

For PAL SPME Arrow, currently five different sorption phases exist commercially: PDMS, PA, CWR/PDMS, DVB/PDMS and DVB/CWR/PDMS [29]. The extraction principle is the same as for SPME and is described in Figure 1-2 A. The analytes partition between the sample and the sorbent phase for DI, and between sample, headspace and sorbent phase for HS extraction.

As with all techniques, PAL SPME Arrow comes also with drawbacks. As the mechanical stability was improved by the larger outer diameter, the injector openings of GCs are too narrow. This results into the need for injector modifications prior the use of PAL SPME Arrow [51], which can be done by the user itself by drilling, or some modified GC inlets are commercially available from the manufacturers [29]. Furthermore, newer systems do not require these changes anymore, as the injector openings were adapted. The same applies to the automation. The new generation of xyz-autosamplers, like PAL3 system, fully support the SPME Arrow with the corresponding modules and tools from CTC Analytics AG (Switzerland). For other autosamplers, or older versions of PAL autosamplers, SPME Arrow can still be used, but with some modifications [29].

In recent years, PAL SPME Arrow gained in importance and more and more applications on its use are published. Volatile organic compounds (VOCs) belong to the most frequently analyzed compounds with applications for environmental, biological and food

samples [73-81]. Further applications cover environmental [51, 71, 82-84], and food analysis [85, 86]. Table 1-1 summarizes exemplary applications, including analyte, matrix, sorbent phase, extraction mode and condition, limit of detection (LOD), limit of quantification (LOQ) and the linear range.

Table 1-1: Applications of PAL SPME Arrow.

Analyte	Matrix	Phase	Extraction conditions	LOD / ngL^{-1}	LOQ / ngL^{-1}	Linear range / ngL^{-1}	Ref
Aliphatic amines	Air Urine	MCM[a,b]	15 min incubation, HS extraction for 20-30 min at 40 °C, 250 rpm	0.01-3[e]	0.03-10[f]	5-500 10-10000	[82]
Amines	Waste water	ZIF-8[b,c]	5 min incubation, HS extraction for 5 min at 40 °C, 750 rpm, 40 % (w/w) NaCl	NR[d]	1[g]	1-500	[83]
Aroma compounds	Chinese liquor	DVB/ CAR/ PDMS 120 µm	5 min incubation, HS extraction for 50 min at 45 °C, 250 rpm, 2 g NaCl	NR	0.08-57800[f]	NR	[85]
Musk fragrances	Fish	PDMS 100 µm	1 min incubation, HS extraction for 45 min at 100 °C, 750 rpm	0.5-2.5[h]	2.5-5[i]	2.5-250	[86]
PAHs	Water	PDMS 250 µm	10 min incubation, DI extraction for 70 min at 35 °C, 1500 rpm	0.1–0.8[k]	NR	NR	[51]
Taste and odor	Water	DVB/ PDMS 250 µm	10 min incubation, HS extraction for 30 min at 60 °C, 1500 rpm, 30 % (w/w) NaCl	0.05-0.6[e]	NR	1-1000	[84]
Volatile amines	Waste water	DVB/ CAR/ PDMS 50/30 µm	10 min incubation, HS extraction for 30 min at RT, 1400 rpm, 40 % (w/w) NaCl	NR	130 10000[f]	130-1300 1000-5000	[71]
VOCs	Air	DVB/ PDMS 250 µm	HS extraction for 35 min	NR	NR	NR	[74]
	Air	CWR/ PDMS 120 µm	HS extraction for 35 min	NR	NR	NR	[75]

Analyte	Matrix	Phase	Extraction conditions	LOD / ngL^{-1}	LOQ / ngL^{-1}	Linear range / ngL^{-1}	Ref
VOCs	Bacteria	CWR/ PDMS 120 µm	HS extraction for 5 min	NR	NR	NR	[76]
		CWR/ PDMS 120 µm	HS extraction for 5 min	NR	NR	NR	[78]
	Brown rice vinegar	CAR/ PDMS 75 µm	HS extraction for 30 min at 50 °C	NR	NR	NR	[79]
	Fish sauce	CWR/ PDMS 120 µm	HS extraction for 30 min at 60 °C	NR	NR	NR	[81]
	Parasitic insect	CWR/ PDMS 120 µm	HS extraction for 5 min at 28 °C	NR	NR	NR	[77]
	Soy sauce	PA 85 µm	5 min incubation, HS extraction for 60 min at 50 °C	NR	NR	NR	[80]
	Water	PDMS 100 µm	10 min incubation, HS extraction for 20 min at 60 °C, 500 rpm	0.9-4.8[k]	NR	1-10000	[73]

[a]MCM = mesoporous silica materials
[b]self-made PAL SPME Arrows
[c]ZIF = Zeolite imidazolate frameworks
[d]NR = not reported
[e] LOD determined with signal to noise (S/N) = 3
[f] LOQ determined with signal to noise (S/N) = 10
[g] LOQ determined with three times standard deviation at the lowest calibration point
[h] MDL as average signal of the blanks plus three times standard deviation of blank samples
[i] MQL lowest point of the calibration curve
[k] MDL calculated with 99 % confidence interval and relative standard deviation of sevenfold measurement according to EPA

1.4 Dynamic microextraction techniques

Dynamic microextraction is mostly used for HS sampling, as direct immersion leads to problems with water being trapped in the sorbent materials, which is difficult to get rid of. Furthermore, the water may damage the sorbent during the thermal desorption process or may enter the GC column, where it can cause problems during separation and detection [13]. During sampling, the headspace above the sample (can be solid or liquid) is partially removed and analytes are trapped in/on the sorbent multiple times. This changes the analyte's concentration

in the HS, which then reestablishes again and again [87]. A scheme is presented in Figure 1-6. The automated DHS from Gerstel, as well as all purge & trap setups are not considered in this chapter, as they include flow systems and purging of the sample solution, which setups were not considered in this thesis.

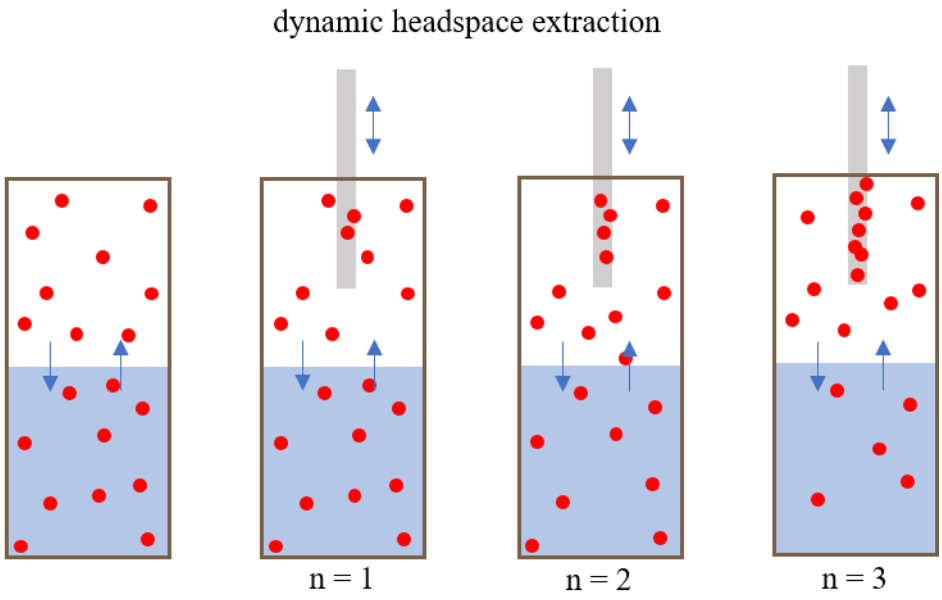

Figure 1-6: Scheme of dynamic headspace extraction. The analytes from the headspace are trapped multiple times (n) to enrich more analytes than with static extraction. The grey rod symbolizes the extraction phase. Water is shown in blue, air in white. The red dots represent the analytes.

The dynamic extraction takes place by moving the syringe plunger up and down, multiple times. The extraction flow rate and the extraction volumes are parameters, which can be optimized for each application. During the pumping of the plunger, the analytes are enriched to the sorbent material. After a certain number of extraction cycles, the tool is moved to the GC injector, where the needle penetrates the injector and the analytes are thermally desorbed. Lastly, the sorbent material is flushed with nitrogen at a chosen temperature, to clean the trap and syringe prior the next sample [88]. In comparison to the static microextraction techniques, dynamic extraction is more complex, as more parameters influence the sensitivity of this method. In all four steps, different parameters can be optimized, and thus method optimization might require more time, than for other techniques [89].

1.4.1 Solid phase dynamic extraction

SPDE was developed in 2001 by Lipinski to overcome the drawbacks of SPME, especially the fiber fragility and the low sorption volume [90]. SPDE is a packed-needle extraction technique, in which the inside of a needle is coated with an extraction phase, comparable to the coating of a GC column [90, 91]. The volume of this coating is four to six times bigger than for SPME [91]. There is a good selection of sorbent materials, which is distributed by Chromtech GmbH (Germany): PDMS, PDMS/AC (active charcoal), Carbowax (PEG), CT 5 (5 % diphenyl/ 95 % dimethylsiloxan), 1701 (14 % cyanopropyl/ 86 % dimethylsiloxan) and OV 225 (25 % phenyl/ 25 % cyanopropyl-methylsilicon/ 50 % PDMS). Another advantage of SPDE is the robustness of the needle in comparison to the fragile SPME fibers [91].

Even though direct immersion sampling can be done with SPDE, it is not recommended, as the coating inside the needle can be damaged, due to residual water [13, 91]. The enrichment is based on the equilibration constants between sorbent and sample and can be conducted completely automated with xyz-autosamplers (Figure 1-2 C). The sample is first incubated, then the coated needle, which is connected to a gas-tight syringe, is inserted into the HS. The extraction is performed by aspirating the syringe multiple times with the sample's HS. After multiple extraction cycles, the syringe is moved to the injector, where a certain gas volume is injected into the GC, while the analytes are thermally desorbed from the needle coating, inside the heated injector from the GC. Afterwards the needle is flushed with inert gas to avoid carry-over [92-94].

SPDE is a commonly used technique for dynamic HS analysis and is applied to different kinds of samples and matrices, including environmental [90, 95-98], biological [91, 93, 99, 100] and food samples [101-103].

1.4.2 In-tube extraction dynamic headspace

ITEX-DHS is a relatively new dynamic extraction technique, which is completely automated on the PAL-type autosamplers. It was first described in 2008 by Jochmann et al. for the enrichment of volatile organic hydrocarbons from aqueous samples. The setup consists of a trap, which is a stainless steel needle, packed with a sorbent material, and a gastight syringe, to which the trap is connected [88]. As the sorbent material is packed within the needle, the volume of the extraction phase is significantly increased, compared to other extraction

setups [6]. An additional heater encloses the upper part of the ITEX needle, to enable thermal desorption, as the outer diameter is too large to be injected into a GC injector. This is a big advantage in comparison to other microextraction techniques, as the desorption can take place independent from the temperature profile within the GC injector [88]. ITEX-DHS is only applied to headspace samples and the sampling is done as previously described in 1.4. Additionally, an external heater is installed around the ITEX syringe and trap and for desorption, the ITEX trap is heated, and a fixed volume of helium is injected with the desorbed analytes [88]. For method optimization, Laaks et al. created a flowchart, which might help optimizing the method faster, and summarized possible error sources and the most important parameters, which influence the extraction efficiency the most [104].

The variety of applications for ITEX-DHS is not as wide, as for other techniques, despite a large variety of commercially available sorbent materials (Carbopack C, Tenax GR, Tenax TA, Carboxen, Carbosieve S III, Molecular sieve 5A, PDMS, PDMS blue). Most applications focus on the analysis of VOCs from environmental samples [73, 88, 89, 105-110]. There are a few publications as well on the analysis of food samples [111-115] and biological samples [116-118]. An overview with some details like extraction conditions, LOD, LOQ and linear range is given in Table 1-2.

Table 1-2: Application overview of ITEX-DHS.

Analyte	Matrix	Phase	Extraction conditions	LOD / μgL^{-1}	LOQ / μgL^{-1}	Linear range / μgL^{-1}	Ref
Aliphatic hydro-carbons	Petroleum source rock	Tenax TA	Incubation 15 min at 90 °C, 150 extraction cycles with 100 μLs^{-1} and 1 mL, desorption of 1000 µL with 10 μLs^{-1} at 300 °C	NR[a]	NR	NR	[105]
BTEX	Water	Tenax TA	Incubation 15 min at 50 °C, 20 extraction cycles with 100 μLs^{-1} and 1 mL, desorption of 700 µL with 10 μLs^{-1} at 170 °C	0.028-0.036[d]	NR	0.028-360	[88]
Diamon-doids	Petroleum source rock	Tenax TA	Incubation 15 min at 90 °C, 150 extraction cycles with 100 μLs^{-1} and 1 mL, desorption of 1000 µL with 10 μLs^{-1} at 300 °C	NR	NR	NR	[106]

Analyte	Matrix	Phase	Extraction conditions	LOD / μgL^{-1}	LOQ / μgL^{-1}	Linear range / μgL^{-1}	Ref
Halogenated hydrocarbons	Water	Tenax TA	Incubation 15 min at 50 °C, 20 extraction cycles with 100 μLs^{-1} and 1 mL, desorption of 700 µL with 10 μLs^{-1} at 170 °C	0.041-0.8[d]	NR	0.047-121.8	[88]
Mercury	Water	Tenax TA	Incubation 10 min at 75 °C, 40 extraction cycles with 100 μLs^{-1} and 1.25 mL, desorption of 1000 µL with 50 μLs^{-1}	0.0008-0.001[e]	NR	NR	[107]
	Petroleum Hydrocarbons	Tenax TA	Incubation 10 min at 75 °C, 40 extraction strokes with 1300 µL, desorption of 700 µL at 260 °C	0.002-0.061[f]	NR	NR	[108]
PAHs	Water	Fullerene	Incubation 15 min at 70 °C, 71 extraction cycles with 54 μLs^{-1} and 1 mL, desorption at 257 °C	0.01-0.31[d]	NR	0.01-500	[109]
VOCs	Air	10 % PAN[b,c]	30 °C, 30 min with 56 $mLmin^{-1}$, desorption of 800 µL with 100 μLs^{-1} at 240 °C	NR	0.00003-0.0001[g]	0.00003-0.0194	[110]
	Beer	PDMS blue	Incubation 20 min at 70 °C, 65 extraction cycles with 100 μLs^{-1} and 1 mL, desorption of 500 µL with 50 μLs^{-1}	0.3-13[d]	NR	0.5-1800	[111]
	Berry juice	Tenax TA	Incubation 20 min at 60 °C, 30 extraction cycles	NR	NR	NR	[112]
	Blood	Tenax TA	Incubation 5 min at 60 °C, 30 extraction cycles with 150 μLs^{-1} and 1.5 mL, desorption of 2000 µL with 20 μLs^{-1} at 230 °C	0.9-100[f]	30[g]	NR	[116]

Analyte	Matrix	Phase	Extraction conditions	LOD / μgL^{-1}	LOQ / μgL^{-1}	Linear range / μgL^{-1}	Ref
VOCs	Plants	Tenax GR	Incubation 15 min at 80 °C, 60 extraction cycles with 150 μLs^{-1} and 1 mL, desorption of 500 µL with 100 μLs^{-1} at 260 °C	NR	NR	NR	[118]
		Tenax TA	Incubation 5 min at 60 °C, 20 extraction cycles with 100 μLs^{-1} and 1 mL, desorption with 200μLs^{-1}	NR	NR	NR	[117]
	Tap water	Tenax TA	Incubation 10 min at 60 °C, 60 extraction cycles with 50 μLs^{-1} and 1 mL, desorption of 500 µL with 25 μLs^{-1} at 300 °C	0.001-0.07[d]	NR	0.002-5.8	[73]
	Tea	Tenax TA	Incubation 12 min at 92 °C, 36 extraction cycles, desorption of 500 µL	NR	NR	NR	[115]
	Tomatoes	Tenax TA	Incubation 20 min at 60 °C, 30 extraction cycles	NR	NR	NR	[112]
	Water	Tenax TA	Incubation 5 min at 60 °C, 60 extraction cycles with 100 μLs^{-1} and 1 mL, desorption of 500 µL with 10 μLs^{-1} at 300 °C	0.0009-0.9[d]	NR	0.001-10	[89]
	Wine	Tenax TA	Incubation 5 min at 35 °C, 32 extraction cycles with 160 μLs^{-1} and 0.5 mL, desorption of 500 µL with 50 μLs^{-1} at 280 °C	0.01-15[f]	NR	10-150	[114]

[a] NR = not reported
[b] PAN = Polyacrylonitrile
[c] selfmade ITEX packing
[d] MDL calculated with 99 % confidence interval and relative standard deviation of sevenfold measurement according to EPA
[e] three times standard deviation of the blank
[f] LOD determined with signal to noise (S/N) = 3
[g] LOQ determined with signal to noise (S/N) = 10

1.5 Comparison, choice and application of microextraction techniques

As there are many different microextraction techniques, the selection of the optimal microextraction technique for a specific analytical problem can be difficult. The advantages and disadvantages, as well as the analytes' characteristics and the analyst's question need to be considered [13]. For a comparison of the five presented techniques, Table 1-3 summarizes important parameters for each technique.

Table 1-3: Comparison of the discussed microextraction techniques [13].

	SPME	SBSE	PAL SPME Arrow	SPDE	ITEX-DHS
Sampling mode	static	static	static	dynamic	dynamic
Extraction mode	HS/DI	(HS)[a]/DI	HS/DI	HS/(DI)[b]	HS
Phase volume / µL	0.026-0.612	24-126	3.8-11.8	~4.5	~160
Average number of measurements	50-100	50-100	500	500	500
Commercial sorbent materials	7	2	5	6	9
Fully automated	✓	✗	✓	✓	✓
Instrument modification required	✗	✓[c]	✓[d]	✗	✗
On-site sampling	✓	✓	(✓)	✗	✗

✓ = Yes, ✗ = No
( ) Extraction mode / On-site sampling not commonly used
[a] For HS-SBSE a special magnet is required
[b] DI-SPDE is not recommended due to residual water in the needle
[c] For SBSE a thermal desorption unit is needed
[d] For PAL SPME Arrow the injector port needs to be widened

For a successful analysis, the behavior of the analyte needs to be understood. Microextraction techniques are applied to analytes, which are present in their neutral form when GC-MS is used for separation and detection. Furthermore, the partitioning constants help to predict if the extraction should be performed in DI or HS mode. If the analytes are sufficiently volatile, HS mode is preferred over DI, as matrix interferences are excluded, the sorbent material is protected and thus its lifetime is prolonged. All the presented techniques can perform in HS mode, however, ITEX-DHS would be the technique of choice, as its dynamic mode allows faster extraction and the desorption is independent from the GC injector's temperature profile. For DI, PAL SPME Arrow is recommended, as its larger phase volume in comparison to SPME helps to increase the method's sensitivity. SBSE achieves better sensitivity than PAL

SPME Arrow, but it normally requires longer extraction times to reach equilibrium, and furthermore, it cannot operate completely automated. Consequently, in the context of this thesis both ITEX-DHS and PAL SPME Arrow have been further utilized (see chapter 2).

As previously mentioned, microextraction techniques can be used for a wide range of applications, but they are not yet widespread in routine analysis due to the lack of standardization of these techniques. There are several substances which are of toxicological concern, and which are regulated on a worldwide scale, for example by the US Environmental Protection Agency, the European Parliament and Council and the World Health Organization, like VOCs, phenols and pesticides. The agencies and organizations define thresholds for dangerous substances and specify priority pollutants for example in the manner of drinking-water guidelines [13]. Microextraction techniques can be applied to food samples as well and thus can be used as an effective tool against food fraud. Food fraud can be divided into replacement, addition and removal of food and food ingredients or the mislabeling of food packaging. Food fraud is always intentional and economically motivated. The different types of food fraud pursue different goals. The replacement of food ingredients, either complete or partial, is normally done with a less expensive substitute and leads to the dilution of the authentic ingredients and thus to the adulteration of the final product. Adulteration includes also the contamination with pathogens, which can lead to public health risks. A famous representative is the addition of melamine to baby milk powder. Furthermore, replacement also includes the false declaration of origin or production process. There, the motivation lies in evading taxes for the import of, for example, catfish from Vietnam. Another type of food fraud is the addition of non-authentic substances, for example with color additives to enhance color of poor-quality spices. The removal of authentic substances in a food product helps the retailers to hamper the determination of the real botanical or geographical origin of the product, so that mislabeling is not as easily detectable [119]. The authenticity of food products is thus gaining in importance and needs to be monitored [120]. The most involved foods in food fraud are olive oil, fish and seafood, milk and milk products, honey and natural sweeteners, fruit juices, coffee, tea and spices [119]. Different analytical methods are applied to the control of food fraud. Nuclear magnetic resonance methods are widely accepted for food analysis, for example for the analysis of coffee [121, 122], honey [123], wine [124] and spices [125]. Spectroscopic methods, like visible spectroscopy, near infrared spectroscopy and mid-infrared spectroscopy, are often used for the control of meat and dairy products from cattle and sheep [126].

1.6 Chemometric tools for data handling

With the large amount of data and the big variety of optimization parameters for the optimal sample preparation protocol, new challenges appear and chemometric approaches can help to find new ways of handling data or managing the workload for the optimization experiments.

The optimization of a workflow based on different parameters, like extraction time, extraction temperature, pH, salt content etcetera, often requires many experiments if the one-factor-at-a-time (OFAT) optimization approach is used and furthermore, does not always lead to optimal conditions, as correlations of the different parameters are not considered. Chemometric tools like design-of-experiment (DoE) can help to solve this problem [127]. DoE is used to plan the experiments in a very efficient way to find the optimal parameters for a method, by taking interactions between different parameters into account, and reducing the number of experiments, as well as reducing time, effort and resources [128]. Therefore, the simultaneous evaluation of different parameters at different levels is possible [129]. There are several designs that can be selected depending on the question behind the experiment. A full factorial design is used when only a few factors are studied. The fractional factorial design is used, when screening variables. If there is already some knowledge of the process, and the most important factors are known, response surface designs are used to find the optimal levels of these factors [130]. The central composite design is one of the most popular designs of the response surface designs and combines a full or fractional factorial design with a star design and a center point [131].

As data sets of real samples can get big and complex very easily, chemometric methods can be used to identify differences or similarities between the different samples. There are multiple approaches like, for example, classification approaches. Classification approaches are divided into unsupervised and supervised approaches. The unsupervised approaches, like principal component analysis (PCA), do not require a labelling of groups for the used data sets. In supervised approaches, like linear discriminant analysis (LDA), more information is required and class labels are considered [132-135].

The main goals of PCA are to find relationships between observations and to extract the most important information of the data. Therefore, the maximum variance of the data is searched and represented by the PCA space [136].

The principle of LDA is to find the maximum variation between the different groups but keeping the variance within the group as small as possible. Before LDA is performed, samples must be categorized into groups. Dissimilarities among these predefined groups are then discovered and a model that predicts the correct group for unknown samples is created [137].

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2 Scope of this thesis

The main aim of this thesis was to investigate and expand the use of fully automated and solventless microextraction techniques in sample preparation of samples ranging from fatty foodstuffs to water. Furthermore, new solutions for complex sample preparation protocols were studied. Two different microextraction techniques were considered: the dynamic extraction from the headspace (ITEX-DHS) for honey and olive oil samples, and the static extraction for aqueous samples (PAL SPME Arrow). Chemometric methods were used for method optimization (DoE) and data evaluation (linear discriminant analysis; LDA). The work was divided into five main chapters, which are visualized in Figure 2-1.

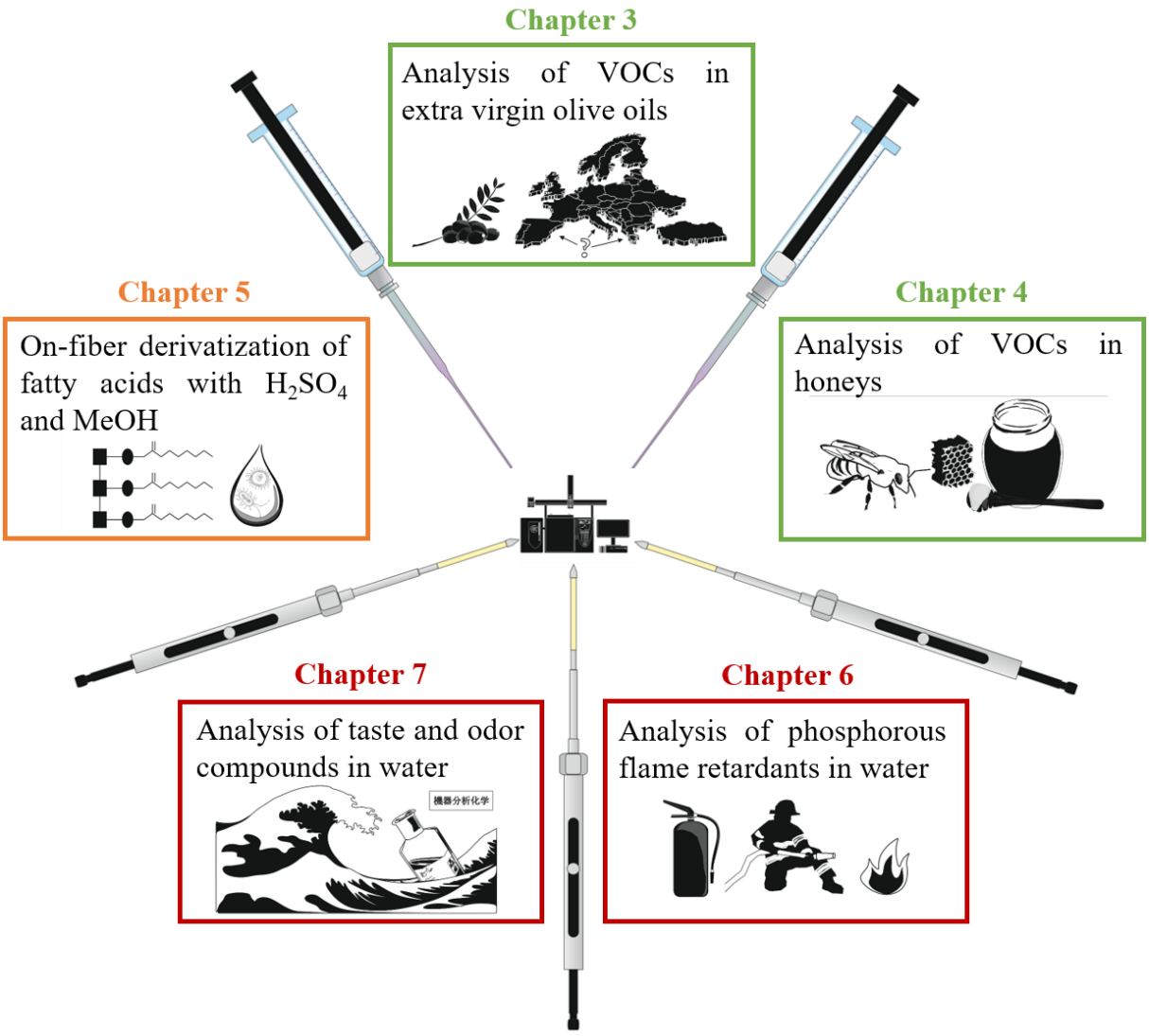

Figure 2-1: Graphical summary of the presented thesis. Chapters marked in green deal with ITEX-DHS applications. Chapters marked in red comprise PAL SPME Arrow applications. Chapter marked in orange uses PAL SPME Arrow for derivatization.

In the analysis of food samples, sample preparation is often time-consuming and complex. In contrast, ITEX-DHS gives a very simple solution of analyzing volatile compounds directly from the headspace above the sample. In **Chapter 3**, ITEX-DHS was applied to the analysis of 31 extra virgin olive oils (EVOO) from different countries (Spain, Italy, Greece and Portugal). First the method was optimized using the OFAT approach, and secondly it was validated. Through LDA, a prediction model was created to identify the origin of the EVOO with the analysis of 21 VOCs. **Chapter 4** focuses on the ITEX-DHS analysis of 38 honey samples. 32 samples were used to create the prediction model based on three different botanical origins: acacia honey, blossom honey and forest honey. The botanical origin of six honeys was not defined and was then predicted using the created LDA.

As a static microextraction technique, which requires little sample preparation, PAL SPME Arrow can be used. It can be applied in headspace analysis as well as for extractions directly from an aqueous phase. In the context of this thesis, both extraction modes were shown for the analysis of water samples. **Chapter 5** does not only show the use of PAL SPME Arrow for the extraction of analytes, but furthermore, presents a way of derivatization directly on the sorbent material. The analytes of choice were fatty acids, which are better analyzed as their according methyl ester when GC-MS is used. As they are important analytes in water samples, a quick and easy method for their derivatization was desired. In this chapter it is described, how the analytes are first extracted directly from the water sample onto the PAL SPME Arrow, secondly derivatized in the headspace of the derivatization agent and lastly thermally desorbed into the GC system. The method was developed using DoE. In **Chapter 6**, the use of PAL SPME Arrow for the analysis of phosphorous flame retardants (PFRs) is optimized using DoE for the extraction of PFRs directly from the aqueous phase. Afterwards, the method was validated and applied. **Chapter 7** discusses the use of PAL SPME Arrow for the analysis of taste and odor compounds in water. In this chapter, the use of PAL SPME Arrow for headspace sampling is presented. The method was optimized using OFAT and was afterwards validated.

Finally, **Chapter 8** gives an overall conclusion from this thesis and an outlook on future tasks and challenges.

3 In-tube dynamic extraction for analysis of volatile organic compounds in extra virgin olive oils to identify their geographical origin using linear discriminant analysis – A proof of principle

This chapter was adapted from: *W. Kaziur-Cegla, L. Wykowski, K. Molt, A. Bruchmann, T.C., Schmidt, M. A., Jochmann, In-tube dynamic extraction for analysis of volatile organic compounds in extra virgin olive oils to identify their geographical origin using linear discriminant analysis – A proof of principle, Journal of Chromatography A (submitted)*

Abstract

As extra virgin olive oil (EVOO) is one of the most important targets in food fraud, its monitoring is of special interest. To analyze the volatile organic compounds of EVOO, an automated, robust and sensitive in-tube extraction dynamic headspace (ITEX-DHS) GC-MS method was developed, optimized and validated. 21 volatile organic compounds (VOCs), typically appearing in EVOOs, were used to develop the method. The extraction procedure was optimized for incubation time, incubation and extraction temperature, number of extraction cycles and desorption volume. Repeatability was satisfying (<10 %) for all analytes, except octanal, and the recovery samples showed good recovery (84-118 %). The ITEX-DHS method allowed VOC analysis in EVOO at much smaller concentration than in previous studies using solid phase microextraction, with limits of detections from 0.1 to 68.6 µg kg^{-1}. A linear discriminant analysis (LDA) was conducted including 31 EVOO samples from five different geographical origins, which led to 90.3 % correct predictions.

3.1 Introduction

Food fraud is a widely occurring offense, in which the producer, groceries or food processors mainly intend to profit financially from the use of lower quality products or low-priced substitutes, for example by mixing with a cheaper product. The improvement in food quality control is counterbalanced by new forms of adulteration and fraud. Most fraud is done with high value products such as extra virgin olive oil (EVOO) [1, 2]. EVOO quality can be related to its geographical origin, which resulted in a regulation of the European Union, which introduced the protected designation of origin (PDO) concept for olive oils. For olive oils classified as a PDO product the physical and chemical characteristics and the defined cultivar name are known and verified [3]. Therefore, quality control is a very important tool to ensure food safety standards [2].

Volatile organic compounds (VOCs) are responsible for the aroma of olive oils. Their composition defines whether an olive oil is defined as virgin olive oil (VOO) or EVOO [4, 5]. EVOOs have sensory descriptors, which are appreciated by consumers, like fruitiness, and no defective sensory descriptors, like rancid or vinegary [6]. Aldehydes, alcohols, esters, ketones, furans and hydrocarbons are important groups of VOCs playing a role for the olive oil aroma. During oil extraction, the lipoxygenase (LOX) pathway results in formation of C_6 aldehydes, alcohols and their esters, which can be related to the “fresh green fruit odor” [7, 8]. The final aroma of olive oils is thus related to the activity of the enzymes present in the LOX pathway [9, 10]. Hexenal, hexanol and hexenylacetate are compounds produced during the LOX pathway. They are responsible for a fruity and bitter taste, which are positive attributes for olive oils. Hexanol is normally present in concentrations in high ppb to low ppm range, whereas hexenal normally occurs in lower concentrations in the range of ppb [11-13]. C_5 compounds play also an important role in olive oil aroma. They are produced in another branch of the LOX pathway, resulting in C_5-alcohols, which can be further converted into C_5-carbonyl compounds via enzymatic oxidation [14, 15]. Fatty acid and amino acid metabolism lead to the production of alcohols, acids, esters and ketones, and fermentation processes from sugars form ethyl alcohol, ethyl acetate and methyl or ethyl butyrates. Furthermore, ethyl acetate, ethanol and acetic acid play an important role for the winey-vinegary taste of some olive oils. Phenolic compounds, like phenolic acid, are responsible for the bitter taste of oils and are important for the nutrition, as they have beneficial effects on the health [16]. High concentrations of C_7-C_{10} monounsaturated aldehydes and C_5 branched aldehydes and alcohols lead to negative notes in olive oil aroma. They occur due to oxidation and exogenous enzymes [11].

The complexity of EVOO aroma, not only due to the many VOCs, but also to the great differences in their concentrations (from µg kg^{-1} to mg kg^{1}), leads to problems during analysis [17]. Often, VOCs can be lost during sample preparation steps and some VOCs in trace amounts cannot be detected. Furthermore, the composition of VOCs, which defines the aroma of the olive oil, can be influenced by many factors, ranging from agronomic aspects, like region, cultivar, ripeness, to technological ones, like storage time, processing methods and extraction procedures [11]. Some VOC concentrations increase during storage, as they are transformation products due to light exposure. They are responsible for a rancid taste and odor. Heptanal and nonanal can be associated with rancidity and thus can be used as markers to show ageing of the oil during storage [18]. This study uses 21 exemplary analytes, based on the previous explanations and published studies, where these analytes were also considered [13, 19-21].

Headspace-solid phase microextraction (HS-SPME) in combination with gas chromatography (GC) coupled to either mass spectrometry (MS) or flame ionization detection (FID) are the most frequently applied methods for the analysis of VOCs from olive oils [2, 5, 6, 10, 13, 15, 19, 22-25]. They are used for the classification of EVOOs regarding cultivar or geographical origin [2, 5, 23, 24]. Oliver-Pozo et al. investigated a dynamic headspace approach, modifying the SPME setup, to overcome competition phenomena among volatiles during static headspace sampling. The new application was used to distinguish between different olive oil qualities [19]. Volpe et al. showed, that principal component analysis (PCA) can be used to discriminate between different extraction methods during olive oil production by using HS-SPME GC-MS [24].

Despite these successful applications of SPME for olive oil analysis, it has also some drawbacks, including instability of the fibers, which leads to a short lifetime and a recommended use from 50 to 100 samples only [26-28].

In-tube extraction (ITEX) is a solvent-free dynamic headspace (DHS) approach, which was first described in 2008 [29]. In comparison to SPME, the lifetime of an ITEX needle is more suitable for high-throughput analysis, as up to 500 measurements can be done with one sorbent trap, without a loss in reproducibility or physical damage. Furthermore, the desorption is independent of the GC injector temperature gradient, as the syringe and the trap are heated externally [27]. Despite its advantages, ITEX-DHS applications in food analysis are so far limited, mostly dealing with the analysis of beer, wine, juice and tea [30-33].

As data sets of real samples can get quite big and complex, chemometric methods can be a helpful tool to identify differences or similarities between the real samples. Classification approaches can be divided into unsupervised approaches and supervised approaches. The unsupervised approaches, like principal component analysis (PCA), do not require a labelling of groups for the used data sets. In supervised approaches, like linear discriminant analysis (LDA), class labels are considered [34-37]. The principle of LDA is to find the maximum variation between the different groups but keeping the variance within the group as small as possible. Before LDA is performed, samples must be categorized into groups. Dissimilarities among these predefined groups are then discovered and a model that predicts the correct group for unknown samples is created [38].

This study focusses on the analysis of VOCs in EVOO using ITEX-DHS in combination with LDA, as the tested EVOOs were of different geographical origins, and thus classes could be defined. ITEX-DHS was chosen as a robust, sensitive, and fully automated technique for headspace sampling directly from the samples, without the need for further sample preparation. To that end, the method was developed, optimized and validated. Subsequently, LDA was tested to discriminate the geographical origin of the EVOO. The usability of the method was demonstrated on the analysis of 31 EVOO samples originating from four countries (Portugal, Spain, Italy and Greece) as well as mixed samples from these countries.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Methanol (99.8 %) from Fisher Scientific (Loughborough, UK) was used to prepare stock solutions. Milli Q water was used from a water purification system (Purelab ultra, Elga, High Wycombe, UK). Benzaldehyde (99 %), benzoic acid (99.5 %), ethanol (98 %), 1-heptanal (95 %), 2-heptanone (analytical standard), 1-hexanol (analytical standard), *trans*-2-hexenal (analytical standard), *cis*-3-hexenyl acetate (98 %), hexyl acetate (99 %), limonene (97 %), nonanal (analytical standard), octanal (99 %), octanoic acid (96 %), 1-pentanol (99 %), 3-pentanone (99 %), 1-penten-3-ol (analytical standard), phenyl acetic acid (99 %) and styrene (99 %) were purchased from Sigma-Aldrich (Steinheim, Germany), 2-butanol (99 %) from Fluka (Darmstadt, Germany), acetic acid (110 %) from VWR Chemicals (Darmstadt, Germany), and ethyl acetate (99 %) from Merck Chemicals (Darmstadt, Germany).

3.2.2 Stock solutions and standard mixture

Stock solutions of all 21 analytes were prepared by weighing 10 mg of the pure substance in a 10-mL volumetric flask and diluting it with methanol to a final concentration of 1 g L^{-1}. A standard mix of the 21 analytes was prepared with a concentration of 25 mg L^{-1} by transferring 125 µL of each analyte's stock solution into a 5-mL volumetric flask and diluting it with methanol to the final volume. The standard mix was prepared monthly. All solutions were stored in the refrigerator at 4 °C in 10-mL amber glass vials sealed with magnetic screw caps with butyl rubber/PTFE septa (BGB Analytik, Rheinfelden, Germany).

3.2.3 Sample preparation

For the optimization of the ITEX-DHS procedure, 1 g of olive oil were weighed into a 20-mL amber glass vial and were spiked with 50 µL of the standard mix, resulting in a concentration of 250 µg kg^{-1}. To ensure complete mixing, magnetic stir bars (12 x 4.5 mm; VWR International GmbH, Darmstadt, Germany) were added to each vial, before being closed with magnetic screw caps with butyl rubber/PTFE septa.

3.2.4 Validation

Olive oils possess a viscous matrix so that a matrix adjustment for an external calibration is necessary. Several oils where tested according to their VOC contents. The validation revealed that neutral oil, namely Miglyol® 812 (Caesar & Loretz GmbH; Hilden, Germany) was VOC clean and was used as calibration matrix. A calibration curve was measured with six levels evenly distributed between 50 and 900 µg kg^{-1}. Each concentration was prepared five times, by diluting the standard mixture in 1 g of the neutral oil. The method detection limit (MDL) as defined by the U.S. Environmental Protection Agency as the minimum concentration of an analyte that can be reported greater than zero with a confidence of 99 % [39] was determined. It was measured at 100 µg kg^{-1} with n = 7 and was calculated using the relative standard deviation multiplied by the t-factor. Repeatability and recovery were measured at 100 µg kg^{-1} (n = 7).

3.2.5 Olive oil samples

In this study, 31 different extra virgin olive oils from five different origins (Italy, Greece, Spain, Portugal and a mixture of Spanish, Greek and Italian oil) were investigated. Each EVOO was measured with the optimized and validated method. 1 g of the EVOO was weighed into a

20-mL amber glass vial with a stir bar and was closed with a magnetic screw cap with butyl rubber/PTFE septa. Each oil was measured in five replicates.

3.2.6 ITEX-DHS

A PAL RTC autosampler (CTC Analytics, Zwingen, Switzerland) was used for sample extraction and injection. The autosampler was equipped with a tray holder for 20-mL headspace vials, a parking station, holding the ITEX-DHS tool, and an IKA magnetic stirring plate (RCT basic; Staufen, Germany). The ITEX-DHS tool provided a 1.3 mL gastight syringe with a side port (CTC Analytics) attached to a Tenax TA ITEX trap (80/100 mesh; BGB Analytik). The autosampler was controlled using Chronos (V. 4.9.1.; Axel Semrau, Sprockhövel, Germany) and the extraction was performed by custom-made macros. The optimized ITEX procedure, which was used for validation and sample analysis, is presented here; more details regarding method optimization are given under results and discussion.

Prior to extraction, the samples were incubated on the magnetic stir plate at 50 °C for 15 min stirring with 1500 rpm. During this incubation time, the ITEX trap was preconditioned by being heated to 250 °C and flushed with nitrogen for 13 min. After the trap temperature was set back to 50 °C, the sample was extracted performing 55 extraction cycles of 1 mL with a flow of 100 μLs^{-1}. Thermal desorption took place in the split/splitless injector, aspirating the syringe with 500 µL helium, heating the trap and the syringe to 250 °C and injecting it with 100 μLs^{-1}. Afterwards, a post-conditioning of the trap was conducted for 10 min in the same way as the preconditioning. The decision to perform pre- and post-conditioning was made considering the overall sample preparation time. In this way, the conditioning was always performed in parallel to another task. The whole sample preparation procedure for each sample (incubation, preconditioning, extraction, desorption and postconditioning) takes about 38 min and is performed in parallel to the GC analysis. This means that the GC temperature program is the time-determining step for the overall analysis time.

3.2.7 GC-MS measurements

All samples were measured using a GC-2010 coupled to a single quadrupole GCMS-QP2010plus (Shimadzu, Japan). The injection temperature was set to 240 °C in splitless mode with a column flow of 1.5 $mLmin^{-1}$. Helium (99.999 %, AirLiquide, Krefeld, Germany) was used as carrier gas. Separation of the analytes was performed on a ZB-FFAP capillary column (50 m length, 0.32 mm inner diameter, 0.5 µm film thickness) from Phenomenex (Aschaffenburg, Germany). The initial temperature of the GC oven was set to 35 °C for 5 min

and was then raised to 110 °C with a rate of 5 °Cmin^{-1}, was held for 2 min, and heated further at 10 °Cmin^{1} to 240 °C with a hold time of 6 min before being raised to the final temperature of 250 °C with a rate of 10 °Cmin^{1} and a hold time of 8 min. The MS transfer line was set to 240 °C and the ion source temperature to 250 °C. Electron ionization (EI), with an ionization energy of 70 eV, was used in full scan mode in the range of *m/z* 40-250. Instrument automation, data acquisition and data processing were done with GCMSsolution (Shimadzu, Japan). Table 3-1 presents the information used for detection and identification.

Table 3-1: Chromatographic and mass spectrometric information: Retention time, retention indices I on ZB-FFAP as well as quantifier and qualifier ions.

Analytes	**Chromatographic information**		**Mass spectrometric detection**	
	retention time / min	retention index I	quantifier ion m/z	qualifier ions m/z
Ethyl acetate	6.2	1458	43	45, 61
Ethanol	7.8	1558	45	46, 43
3-Pentanone	9.0	1670	57	86, 43
2-Butanol	10.6	1772	45	59, 44
1-Penten-3-ol	15.1	2042	57	41, 43
2-Heptanone	15.9	2091	43	58, 71
Heptanal	16.0	2105	44	70, 57
Limonene	16.3	2120	68	67, 93
trans-2-Hexenal	17.2	1946	41	55, 69
1-Pentanol	18.0	2210	42	55, 41
Styrene	18.4	2238	104	78, 51
Hexyl acetate	18.7	2256	43	56, 55
Octanal	19.3	2299	44	43, 41
cis-3-Hexenyl acetate	20.1	2347	43	67, 44
1-Hexanol	21.1	2407	56	43, 55
Nonanal	22.7	2486	57	41, 43
Acetic acid	24.4	2630	43	45, 60
Benzaldehyde	26.3	2745	77	106, 105
Octanoic acid	33.5	3272	60	73, 43
Benzoic acid	38.0	3687	77	45, 105
Phenolic acid	39.7	3832	91	65, 92

3.2.8 Linear discriminant analysis

To perform statistical interpretation of the data, LDA was performed. LDA was achieved using the free software R 3.6.2 (The R Foundation for Statistical Computing, www.r-project.org) using the lda-function from the R-package "MASS"[40]. The peak areas of all 21 analytes were used as explanatory variables for the LDA and the 31 EVOO samples were divided into five groups according to their geographical origin.

3.3 Results and discussion

3.3.1 Method optimization

For ITEX-DHS, several parameters, like incubation time and temperature, pH value, ionic strength, extraction temperature, extraction volume and speed, number of extraction cycles and desorption volume, temperature and speed, can influence the extraction efficiency, and thus all three steps (extraction, injection and trap conditioning) give room for optimization [30]. In this study, four parameters were optimized namely the incubation time, the extraction temperature, the number of extraction cycles and the desorption volume. For the other parameters, recommended values were used [41]. The sample size of 1 g olive oil was not optimized.

Incubation/extraction temperatures were investigated in 10 °C intervals within a range from 40 to 70 °C. As Figure S 3-1 reveals, all analytes showed increasing extraction yields with an increase in temperature. However, standard deviations increase at temperatures higher than 60 °C. A closer look into the chromatograms revealed multiple peaks overlaying each other and the targeted peaks. These peaks were detected and influenced the automated data evaluation of the peak integration software. Thus, peaks eluting at a similar retention time, but showing different m/z ratios in the mass spectrum, were integrated by mistake, resulting in higher peak areas than for previous conditions. Best results in terms of peak area and precision were reached for an incubation and extraction temperature of 50 °C.

The choice of the optimal incubation time must be investigated carefully. It should not be too long, as this would prolong the overall sample preparation time, but at the same time it should ensure equilibration between sample and headspace before the dynamic extraction. Incubation times from 15 to 45 minutes with 10-minute intervals were tested, showing that 15

minutes was already the time which ensured equilibrium. Shorter times were not investigated, as the trap conditioning took place at the same time and required thirteen minutes.

Looking at the number of extraction cycles, tested from 45 to 85 cycles with intervals of 10, it becomes clear that as the number of extraction cycles increases, the peak areas of the analytes will increase until saturation occurs. However, an increase in extraction cycles leads to the increase of the overall sample preparation time. The results show that, for most analytes, the peak areas increase with increasing number of extraction cycles (Figure S 3-2). The slope of the curves flattens towards the highest number of extraction cycles, as the influence of the increasing number of extraction cycles diminishes when approaching saturation of the sorbent material. Heptanal, nonanal benzoic acid and octanoic acid showed a decrease in peak area after 45 cycles. This behavior can be explained with sorption competition taking place on the sorbent trap. Tenax TA is a porous organic polymer, whose active sites enable the adsorption of a wide variety of compounds. [42, 43]. In Figure S 3-2 it becomes clear, that after 55 extraction cycles, there is only a slow increase of extraction efficiency and thus 55 extraction cycles were set as the optimal number.

The last parameter, which was optimized, was the desorption volume. As the injection was operated in splitless mode and without cryo-focusing, all desorbed analytes were directly introduced onto the capillary column. The used desorption volumes were 500 µL and 1000 µL. The detected peak areas showed for nearly all analytes comparable thermodesorption efficiencies, only the peak shapes showed small differences (Figure S 3-3). For some analytes injected with the larger injection volume, the peak broadened in comparison to the smaller injection volume. This appears, as a larger desorption volume requires more time to be injected than a smaller one. Analytes enter the column with a time shift, which leads to peak broadening, as low boiling analytes are not refocused on the GC oven starting temperature of 35 °C. Cryo-focussing can help with these problems, but as the larger injection volume did not result in a significant improvement of the extraction efficiency, the desorption volume of 500 µL was chosen. An exemplary chromatogram is presented in Figure 3-1. It shows spiked olive oil measured with the optimized ITEX-DHS procedure. With this procedure, a high number of peaks can be detected. In this study, only 21 peaks were identified with reference standards and used for optimization, validation and LDA. Additionally, peaks only identified using the NIST library could be added into the data evaluation, but this was not done in this study. Nevertheless, the chromatogram shows the high peak capacity of this method.

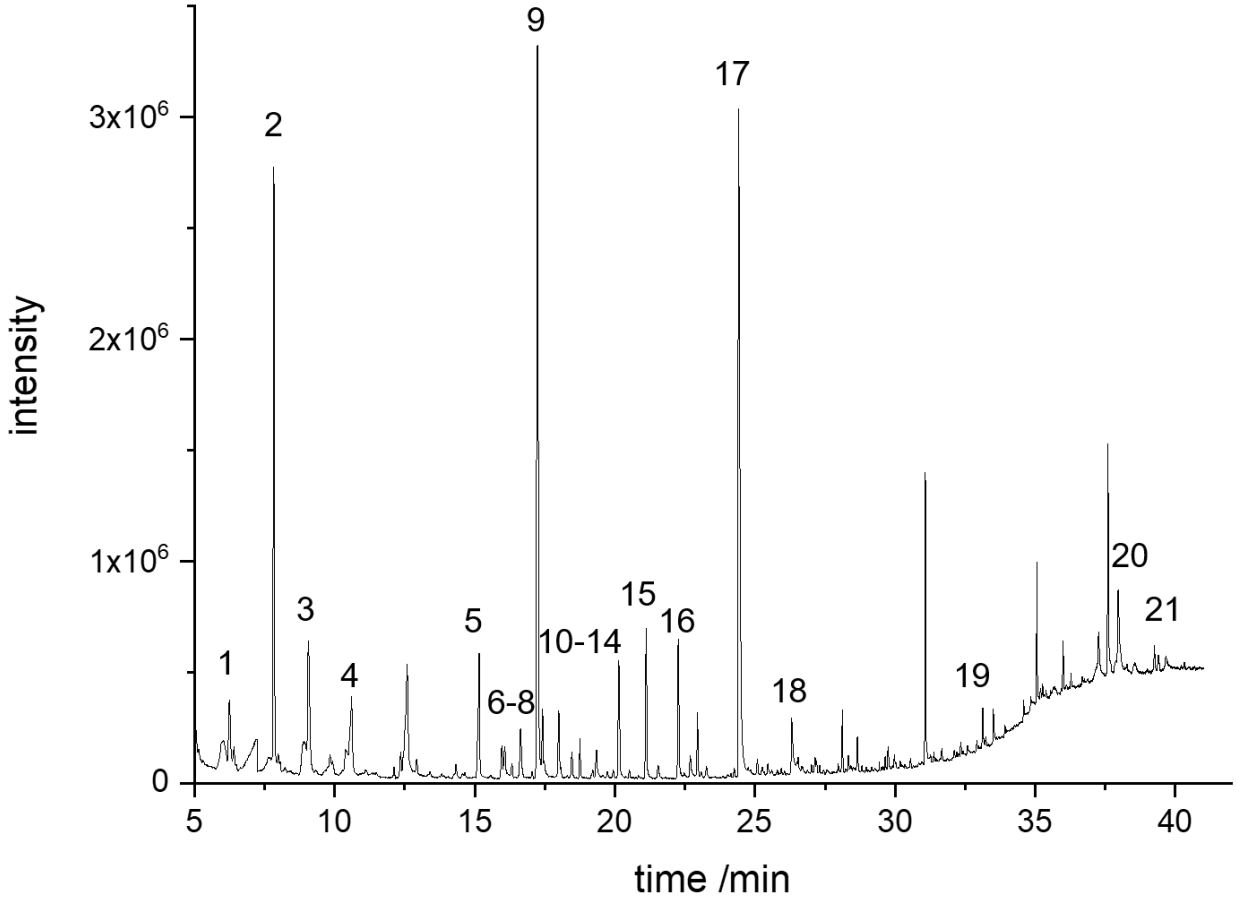

Figure 3-1: Chromatogram of olive oil spiked with the 21 analytes at a concentration of 250 µg kg^{-1} and measured with the optimized ITEX-DHS method. 1: Ethyl acetate. 2: Ethanol. 3: 3-Pentanone. 4: 2-Butanol. 5: 1-Penten-3-ol. 6: 2-Heptanone. 7: Heptanal. 8: Limonene. 9: trans-2-Hexenal. 10: 1-Pentanol. 11: Styrene. 12: Hexyl acetate. 13: Octanal. 14: cis-3-Hexenyl acetate. 15: 1-Hexanol. 16: Nonanal. 17: Acetic acid. 18: Benzaldehyde. 19: Octanoic acid. 20: Benzoic acid. 21: Phenolic acid.

3.3.2 Method validation

MDL, limit of detection (LOD), repeatability and recovery were determined using the optimized ITEX procedure and spiked samples as described in 3.2.4. The validation was carried out in a neutral oil, to match the matrix without the requirement of spiking each sample at multiple concentration levels. The results are summarized in Table 3-2.

As in previous studies [30], the MDL was calculated based on the procedure of the U.S. Environmental Protection Agency [30, 39], as it can be used as a good alternative to LOD. The MDL gives a good impression of the reliability of the sensitivity of a method as it is calculated based on the relative standard deviation and thus presents the lowest concentration at which the

method works with a good repeatability. Additionally, for better comparison with literature, the LOD was determined by using the signal to noise ratio with a factor of three (S/N = 3). Therefore, the noise area was picked in 0.5 min intervals directly in front of the according peak, and the signal-to-noise value was calculated from the software (GCMS analysis, Shimadzu). The repeatability is shown as the relative standard deviation of a seven-fold measurement at a concentration of 100 µg kg^{-1} and the recovery was measured with spiked neutral oil.

Table 3-2: Method validation parameters of the optimized ITEX-DHS GC-MS method and comparison with limits of detections (LODs) with two publications by Fortini et al. (2017) and Oliver-Pozo (2019).

Analytes	**repeatability / %**	**recovery / %**	**MDL / µg kg^{-1}**	**LOD[a] / µg kg^{-1}**	**LOD[a] [13] / µg kg^{-1}**	**LOD[b] [19] / µg kg^{-1}**
Ethyl acetate	5	84	15.4	24.9	19	-
Ethanol	4	110	13.4	68.6	-	-
3-Pentanone	2	107	7.0	13.2	35	-
2-Butanol	3	118	10.7	0.3	14	-
1-Penten-3-ol	2	115	7.6	0.1	14	-
2-Heptanone	2	111	5.6	1.1	14	-
Heptanal	9	104	27.9	12.3	16	-
Limonene	8	102	24.0	9.2	24	-
trans-2-Hexenal	6	104	20.0	19.5	-	110
1-Pentanol	2	122	6.4	0.5	15	-
Styrene	1	108	3.2	1.2	-	-
Hexyl acetate	7	125	22.9	1.5	26	-
Octanal	11	95	36.0	23.7	104	-
cis-3-Hexenyl acetate	8	137	25.0	4.3	56	-
1-Hexanol	3	103	8.8	1.3	190	-
Nonanal	6	85	28.0	18.4	251	1570
Acetic acid	7	116	22.9	3.2	-	1430
Benzaldehyde	1	103	4.5	0.4	-	-
Octanoic acid	6	102	19.8	0.9	-	-
Benzoic acid	3	112	10.7	17.1	-	-
Phenolic acid	7	117	22.6	10.2	-	-

[a] LOD calculated based on S/N = 3; [b] Informations on calculation of LOD not given

Repeatability achieved for all analytes, except octanal, were satisfactory (<10 %), with an average of 5 %. Recoveries for almost all analytes samples were in an acceptable range (84-118 %). Only 1-Pentanol, hexyl acetate and *cis*-3-Hexenyl acetate were found with recoveries higher than 120 %.

When the MDLs are compared to the LODs (calculated with S/N = 3), some differences become clear. Especially for ethanol, the LOD and the MDL differ with a factor of four to five, with the MDL being the more sensitive one. It would have been expected that the MDL is higher than the LOD for all analytes, but this is not the case especially for the first three analytes. The analytes are presented in the order of retention time; thus, it can be concluded (and confirmed by looking at the chromatograms) that the noise in the chromatogram decreases with time after injection. After 3-pentanone eluted after 9 min, the LODs get better in comparison to the MDLs and show increased sensitivity by, on average, a factor of nine. Benzoic acid, at the end of the chromatogram, again shows a worse sensitivity than with the MDL which can again be related to increasing noise in the chromatogram caused by the increasing baseline at increasing column temperature. As the comparison between LODs and MDLs is difficult, the determined LODs were used for comparison with literature. When the LODs are compared to the LODs of a SPME method using a similar set of analytes, the ITEX method shows higher sensitivity than HS-SPME for all analytes except ethyl acetate [13] (see Table 3-2). Ethyl acetate was one of the analytes which was negatively influenced by the noise of the injection and where the MDL showed a more sensitive limit. The lower LODs using Tenax TA as trap material in the ITEX-DHS setup is caused by the larger sorbent volume of ITEX compared to the phase volume of a SPME fiber (usually between 0.026 and 0.612 µL) [27] and the dynamic extraction process. Oliver-Pozo et al. [19] showed that applying a DHS method instead of a static sampling like SPME improves sensitivity. They used a modified SPME setup to establish dynamic conditions, but the basis for the reported LOD values was not described, thus, a direct comparison is not possible. Nevertheless, values for the three common analytes in both studies suggest that ITEX-DHS is much more sensitive than the modified SPME setup [19]. The better sensitivity will help to analyze low concentrated VOCs. For the VOCs, which are responsible for off-flavors, like nonanal, heptanal, ethanol, ethyl acetate and acetic acid, the lower MDLs are very important, as these VOCs are significant for the quality control of the EVOO. This applies as well for positive attributes, like hexenal, which is often present in the low ppb range [11], and thus the achieved LOD of 19.5 ppb in comparison to 110 ppb in the study of Oliver-Pozo [19], is satisfactory.

3.3.3 Quantitative analysis

All 21 analytes were measured in the 31 measured EVOOs. Not all analytes were found in each sample and the concentration ranges were different for each analyte as well. An overview of the detected concentration ranges and the number of samples, in which they were detected, are presented in Table 3-3. In Table 3-4, the average concentrations of the analytes of the different sample groups are given. The average concentrations are based on the samples, in which the analyte was found. A sample showing the analyte in a concentration smaller than the MDL was not used. Thus, some average concentrations might only represent one sample and are thus marked in the table with an asterisk. For more details of the concentrations for all analytes in the 31 EVOO samples information is provided in Table S 3-1 - Table S 3-5.

Benzaldehyde and octanoic acid were not detected in any of the real EVOO samples, as the signals were always below the MDL. 2-Butanol was not detected in the Italian EVOOs, in the Portuguese EVOOs no styrene was found, as well as no benzoic acid could be found in the Spanish EVOO samples. The tested linear range was only up to 900 µg kg^{-1} and was exceeded for ethyl acetate, ethanol, 3-pentanone, limonene, *trans*-2-Hexenal, hexyl acetate, *cis*-3-hexenyl acetate, 1-hexanol, nonanol, acetic acid and phenyl acetic acid. Only eight analytes were present in all 31 olive oil samples: ethyl acetate, 3-pentanone, 1-penten-3-ol, limonene, *trans*-2-hexenal, *cis*-3-hexenyl acetate, 1-hexanol and acetic acid. Ethyl acetate is a typical flavor component, which is responsible for the winey-vinegary taste of olive oil as well as acetic acid. Hexenal and hexanol are the typical products from the LOX pathway and give a fruity and bitter taste. If the average concentrations of the analytes are compared between the different EVOO varieties some differences can be detected. The Spanish oils have the smallest concentrations for ethyl acetate, 2-butanol, 1-penten-3-ol, 2-heptanone, 1-pentanol and phenyl acetic acid. Furthermore, they do not have the highest concentration for any analyte when compared to the other varieties. The Portuguese oils have more than double the amount of *trans*-2-hexenal when compared to Greek, Spanish and the mixed oil (s), as well as the concentration of acetic acid is a lot higher. The Portuguese EVOOs and the Italian EVOOs differ mainly in the concentrations of octanal and heptanal. As the concentrations often exceed the end of the tested calibration range, the given concentrations must be handled with care, as the extrapolation is rather big. The quantification of analytes was not the focus of this work and thus no bigger range was tested for linearity. Consequently, for LDA, the detected peak areas of the analytes were used instead of the concentrations.

Table 3-3: Lowest and highest detected analyte concentrations, mean, median and number of samples in which the analytes have been detected.

Analytes	**lowest c** **µg kg^{-1}**	**highest c** **µg kg^{-1}**	**Mean** **µg kg^{-1}**	**Median** **µg kg^{-1}**	**samples** **(n)**
Ethyl acetate	60	1800	740	720	31
Ethanol	1200	29000	8100	6200	27
3-Pentanone	80	2200	540	430	31
2-Butanol	15	70	40	50	7
1-Penten-3-ol	150	900	430	400	31
2-Heptanone	6	70	30	20	13
Heptanal	30	300	100	40	22
Limonene	60	3100	200	80	31
trans-2-Hexenal	500	22000	8200	8300	31
1-Pentanol	8	260	60	40	22
Styrene	6	280	40	10	14
Hexyl acetate	50	3000	750	510	30
Octanal	40	500	100	70	25
cis-3-Hexenyl acetate	50	12000	3800	3000	31
1-Hexanol	60	4000	1600	1600	31
Nonanal	130	15000	2800	780	19
Acetic acid	1300	38000	15000	14000	31
Benzaldehyde	nd	nd	nd	nd	0
Octanoic acid	nd	nd	nd	nd	0
Benzoic acid	180	680	300	240	6
Phenyl acetic acid	50	4000	1000	690	9

Table 3-4 Number of samples in which the analytes have been detected and average concentrations of analytes in the different EVOO varieties (Mix from Spain, Greece and Italy (s), Greece (G), Spain (S), Italy (I) and Portugal (P)).

Analytes	**samples (n)**	**Mean (s) µg kg^{-1}**	**Mean (G) µg kg^{-1}**	**Mean (S) µg kg^{-1}**	**Mean (I) µg kg^{-1}**	**Mean (P) µg kg^{-1}**
Ethyl acetate	31	1000	250	670	1100	1000
Ethanol	27	13300	3700	9000	6400	6100
3-Pentanone	31	370	500	300	1000	700
2-Butanol	7	50*	50	10	nd	50*
1-Penten-3-ol	31	340	480	250	600	550
2-Heptanone	13	30*	30	10*	50*	80*
Heptanal	22	30	180	50	100	40*
Limonene	31	100	420	80	130	70
trans-2-Hexenal	31	4500	7300	8800	11000	1700
1-Pentanol	22	40	80	20	80	30
Styrene	14	10	30	30	100	nd
Hexyl acetate	30	270	1400	550	250	900
Octanal	25	70	110	70	150	50
cis-3-Hexenyl acetate	31	2100	5700	4100	1100	9000
1-Hexanol	31	1200	830	2000	2700	2400
Nonanal	19	1800	900	6100	2700	7500*
Acetic acid	31	19000	6500	15000	21500	29000
Benzaldehyde	0	nd	nd	nd	nd	nd
Octanoic acid	0	nd	nd	nd	nd	nd
Benzoic acid	6	240	200*	nd	230	670*
Phenyl acetic acid	9	1000	2000	400*	670	800*

*only one sample was used for the mean concentration, as only in one sample of the group this analyte was detected

3.3.4 Linear discriminant analysis

The 31 EVOOs were divided into five groups with different numbers of samples within the group. The number of linear discriminant functions (LD) is limited to that number of groups minus one. Therefore, four LDs were found, and their proportions of trace, which are the percentage separations achieved by the LDs, are shown in Figure 3-2. It describes the proportion of the between-class variance. The first two LDs describe about 85 % of the between-group

variance and should provide a good separation of the analyzed EVOO samples. The different loadings on the LDs are presented in Table S 3-6. They describe the correlation of the different analytes with the according LD, which can be positive or negative values. The main loadings on LD1 were given to 2-heptanone in negative direction and to octanal in positive direction. For LD2, the main loadings are given to heptanal in negative direction and, like for LD1, to octanal in positive direction. When the first two discriminants (LD1 and LD2) are used for prediction, the proportion of correct predictions is 90.3 %. Only three samples are wrongly predicted, and all are between Greece (G), Spain (S) and the mix from Greece, Spain and Italy (s). Table 3-5 shows these results.

To get a better visual understanding of these numbers, a scatter plot based on LD1 and LD2 is presented in Figure 3-3. The dotted lines show the boundaries between each EVOO origin. Portuguese EVOO and Italian EVOO are clearly separated from the rest, as well as the Greek EVOO. The EVOOs with the mixed origins s lie between the Italian and the Greek EVOOs, and the Spanish EVOOs are very close to the Greek ones. The misclassification took place in exactly this region, which shows, that the mixture leads to some errors in the LDA, but as the correct prediction is 90 %, the model works very nicely for the analyzed EVOOs.

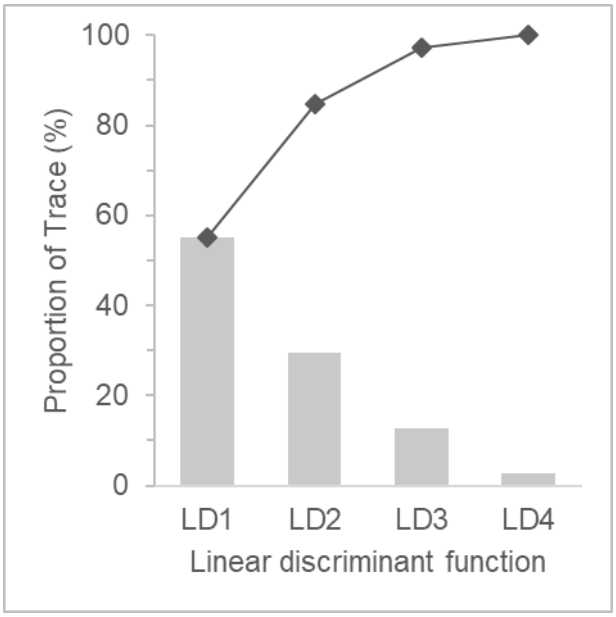

Figure 3-2: Proportion of trace of the four different linear discriminant functions (light grey bars). The dark grey line shows the cumulative proportion.

Table 3-5: Predicted results for the analyzed samples (mixture of Greek, Spanish and Italian EVOO (s), Greek EVOO (G), Spanish EVOO (S), Portuguese EVOO (P) and Italian EVOO (I)).

variety	predicted variety					correct predictions (%)
	s	G	S	P	I	
s (n = 8)	7		1			87.5
G (n = 10)		9	1			90.0
S (n = 5)	1		4			80.0
P (n = 2)				2		100.0
I (n = 6)					6	100.0
Total						90.3

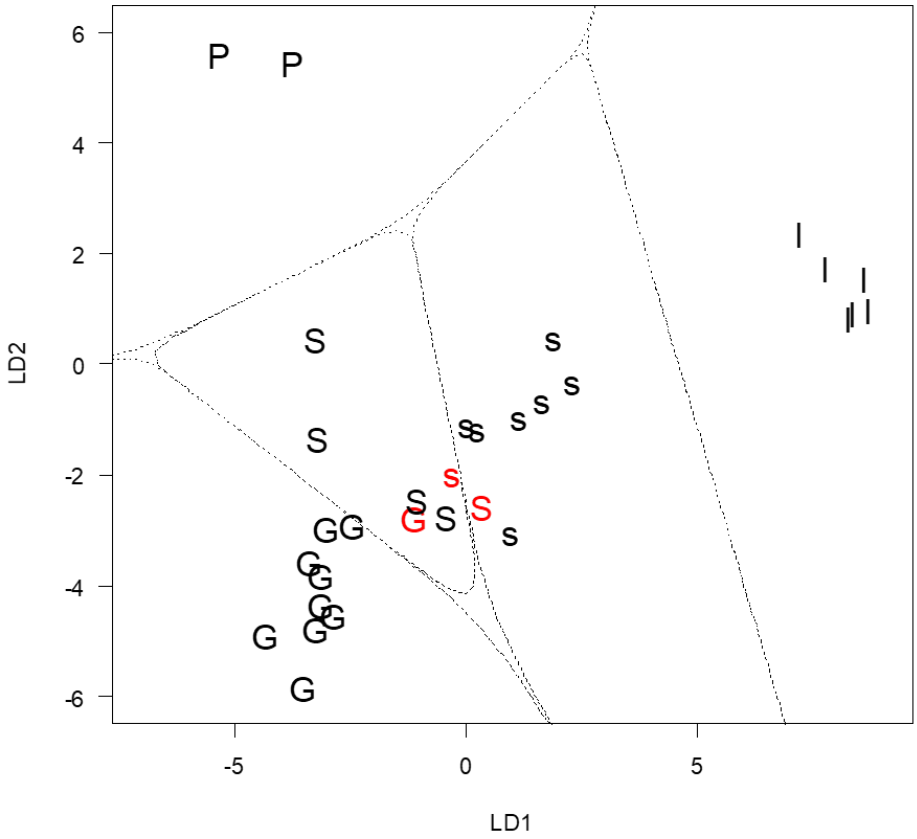

Figure 3-3: Scatterplot of the first two linear discriminant functions (LD) with the boundaries for each EVOO origin. Falsely classified samples are shown in red. P: Portuguese EVOO, I: Italian EVOO, S: Spanish EVOO, G: Greek EVOO, s: Mixture of Spanish, Greek and Italian EVOO.

3.4 Conclusion

The developed ITEX-DHS GC-MS method was appropriate for a robust and sensitive analysis of EVOO samples. The obtained MDLs were more sensitive compared with existing SPME methods, and the ITEX-DHS set-up allows a more robust and mechanical stable analysis than SPME. LDA as a chemometric model was successfully applied to 31 analyzed EVOO samples from five different geographical origins. It was possible to discriminate between the different origins and the few misclassifications could be explained due to the mixture of origins in some samples. As the correct predictions are 90 % it would be possible to use this model for quality control screening of EVOOs to check the correctness of the declared origin.

3.5 Supporting information

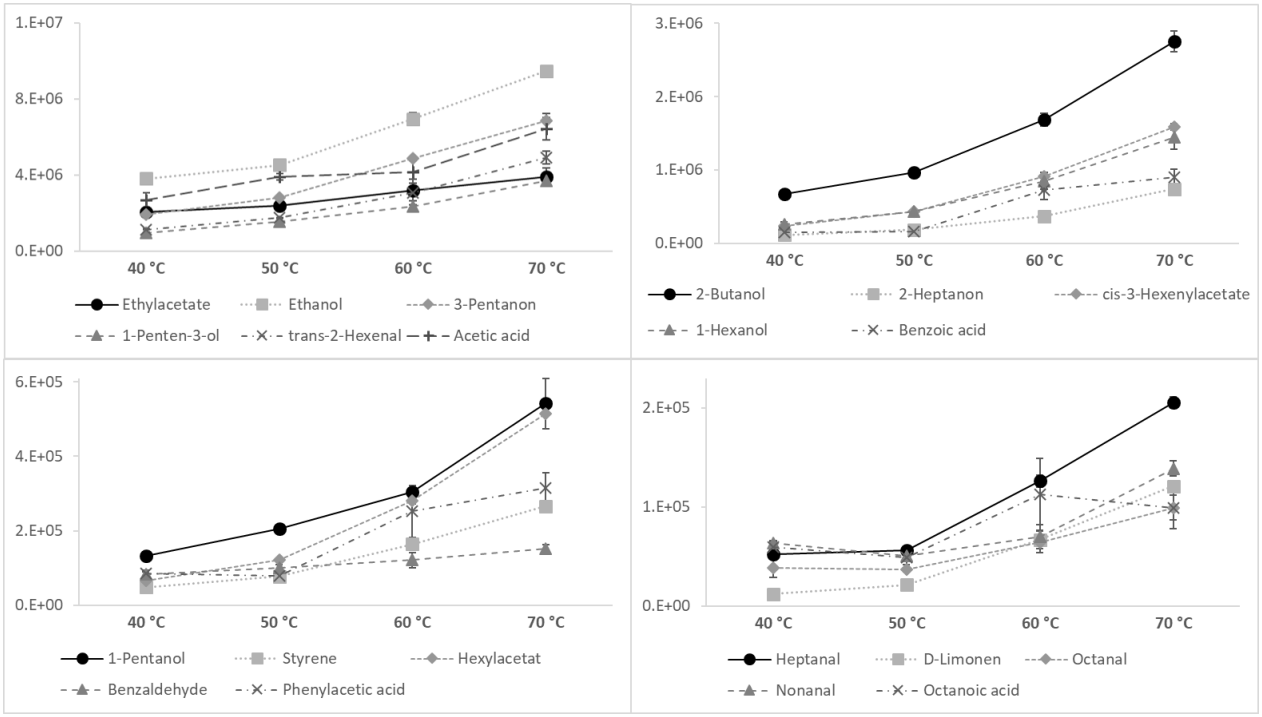

Figure S 3-1: Graphical presentation of the results for the optimization of extraction temperature for ITEX-DHS of extra virgin olive oil.

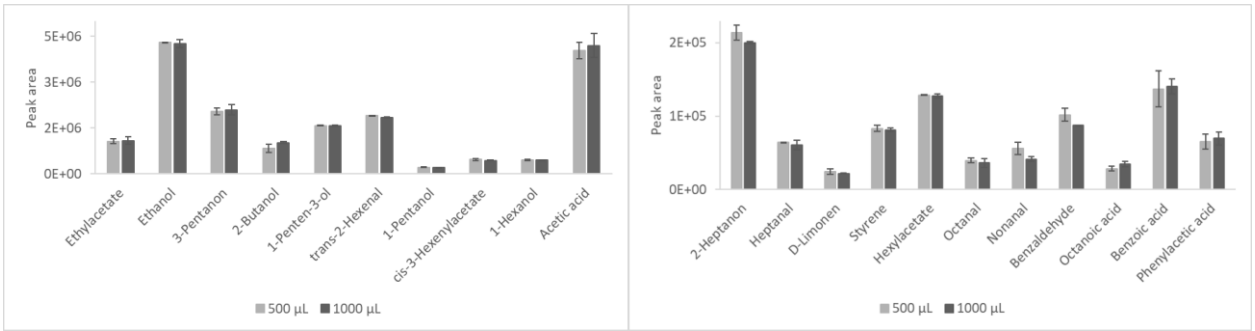

Figure S 3-2: Graphical presentation of the results for the optimization of desorption volume for ITEX-DHS of extra virgin olive oil.

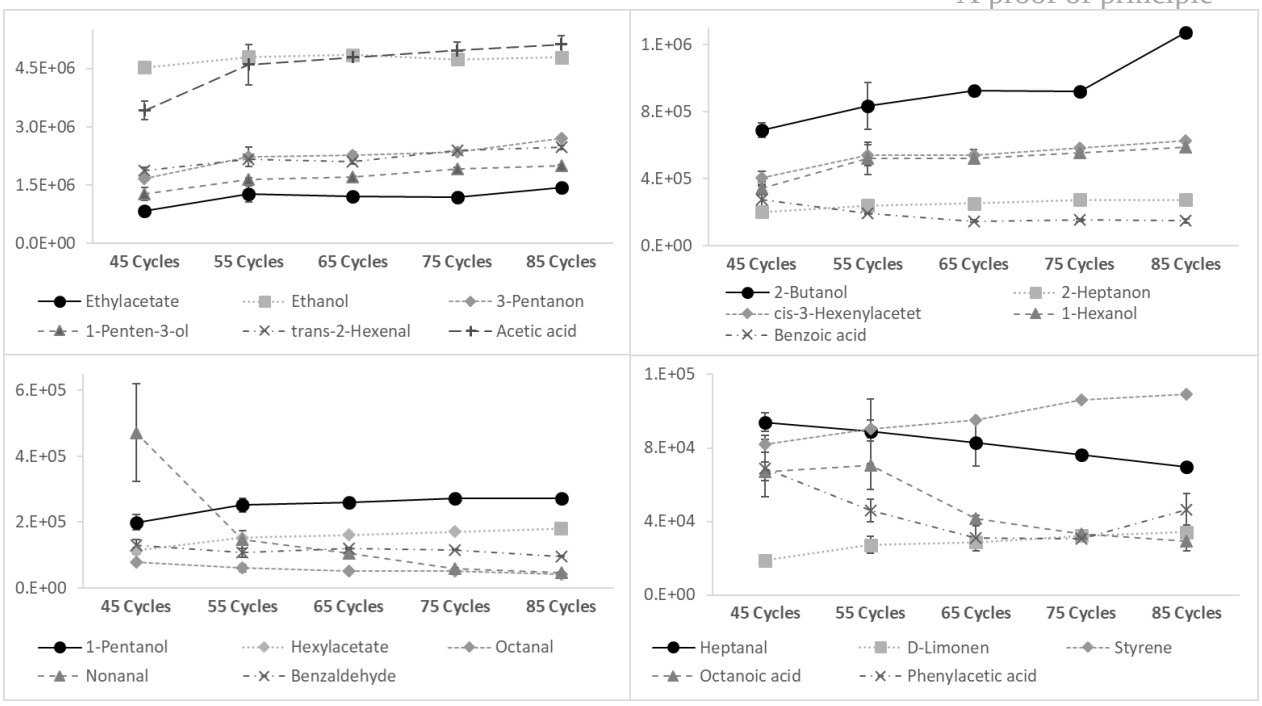

Figure S 3-3: Graphical presentation of the results for the optimization of number of extraction cycles for ITEX-DHS of extra virgin olive oil.

Table S 3-1: Concentrations of analytes detected in the EVOOs from the mix of Spain, Greece and Italy.

Analytes	concentration in µg kg^{-1}							
	s-01	**s-02**	**s-03**	**s-04**	**s-05**	**s-06**	**s-07**	**s-08**
Ethylacetate	911	1391	1845	726	629	401	506	1634
Ethanol	2818	8485	14329	10523	14676	12570	14501	28650
3-Pentanon	431	399	635	489	248	237	292	179
2-Butanol	46	nd	nd	nd	nd	nd	nd	nd
1-Penten-3-ol	341	382	510	541	286	271	239	175
2-Heptanon	32	nd	nd	nd	nd	nd	nd	nd
Heptanal	37	nd	29	nd	32	33	34	47
D-Limonen	91	108	220	73	70	72	68	66
trans-2-hexenal	20688	2865	3423	2231	1612	1488	2794	1266
1-Pentanol	nd	29	52	71	nd	nd	18	nd
Styrene	nd	9	nd	6	nd	nd	nd	nd
Hexylacetate	291	182	715	568	53	169	95	139
Octanal	91	62	96	nd	nd	nd	44	nd
cis-3-Hexenylacetate	2582	1602	3865	3795	1192	1118	865	1470
1-Hexanol	3522	1323	1666	989	530	525	802	409
Nonanal	5609	1420	nd	nd	271	387	1084	nd
Acetic acid	27145	24836	26135	24593	13940	6511	6384	19159
Benzaldehyde	nd	nd	nd	nd	nd	nd	nd	nd
Octanoic acid	nd	nd	nd	nd	nd	nd	nd	nd
Benzoic acid	nd	nd	295	188	nd	nd	nd	nd
Phenylacetic acid	nd	nd	1266	683	nd	nd	nd	nd

Table S 3-2: Concentrations of analytes detected in the Greek EVOOS.

Analytes	concentration in µg kg^{-1}									
	G-01	**G-02**	**G-03**	**G-04**	**G-05**	**G-06**	**G-07**	**G-08**	**G-09**	**G-10**
Ethylacetate	254	268	326	194	63	353	281	121	401	251
Ethanol	1620	nd	3164	2551	nd	1960	6163	nd	8674	1662
3-Pentanon	619	869	692	396	598	465	546	357	205	270
2-Butanol	68	nd	73	19	nd	nd	nd	nd	nd	nd
1-Penten-3-ol	533	634	715	404	296	553	685	435	231	276
2-Heptanon	24	52	8	12	52	10	18	43	nd	12
Heptanal	322	217	151	150	340	78	41	162	nd	nd
D-Limonen	161	175	119	183	3105	101	122	77	70	77
trans-2-hexenal	9722	16770	13490	8276	9691	3394	4645	3104	2412	1117
1-Pentanol	8	26	nd	9	260	62	42	175	nd	33
Styrene	20	22	8	48	83	7	nd	nd	nd	nd
Hexylacetate	755	1733	2151	1102	48	2999	2167	1132	538	1795
Octanal	147	132	78	100	330	89	65	135	45	47
cis-3-Hexenylacetate	4912	9063	12456	7319	58	6694	6827	3235	1773	4195
1-Hexanol	1181	2093	1649	981	59	753	564	195	515	312
Nonanal	306	247	4080	126	586	nd	653	304	nd	nd
Acetic acid	6585	10480	6150	13914	8090	5365	2981	8166	1726	1320
Benzaldehyde	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Octanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzoic acid	nd	nd	nd	nd	nd	216	nd	nd	nd	nd
Phenylacetic acid	nd	nd	nd	nd	nd	3949	nd	nd	49	nd

Table S 3-3: Concentrations of analytes detected in the Spanish EVOOS.

Analytes	concentration in µg kg^{-1}				
	S-01	**S-02**	**S-03**	**S-04**	**S-05**
Ethylacetate	745	253	993	1036	315
Ethanol	5384	2839	21327	6329	nd
3-Pentanon	434	162	83	327	464
2-Butanol	20	15	nd	nd	nd
1-Penten-3-ol	230	155	144	280	462
2-Heptanon	6	nd	nd	nd	nd
Heptanal	28	84	39	nd	nd
D-Limonen	81	77	81	71	70
trans-2-hexenal	10067	20230	487	8295	5079
1-Pentanol	nd	nd	17	16	24
Styrene	53	7	25	nd	nd
Hexylacetate	300	1114	110	893	352
Octanal	89	45	82	nd	46
cis-3-Hexenylacetate	4283	4905	805	7669	2993
1-Hexanol	2307	3169	431	1885	2025
Nonanal	8751	14993	265	nd	781
Acetic acid	21602	7526	23509	18436	4138
Benzaldehyde	nd	nd	nd	nd	nd
Octanoic acid	nd	nd	nd	nd	nd
Benzoic acid	nd	nd	nd	nd	nd
Phenylacetic acid	nd	nd	nd	417	nd

Table S 3-4: Concentrations of analytes detected in the Italian EVOOS.

Analytes	concentration in µg kg^{-1}					
	I-01	**I-02**	**I-03**	**I-04**	**I-05**	**I-06**
Ethylacetate	778	795	1745	920	1420	1185
Ethanol	11522	1224	4189	5091	3100	13344
3-Pentanon	2235	391	1104	616	1135	408
2-Butanol	nd	nd	nd	nd	nd	nd
1-Penten-3-ol	852	403	603	518	662	507
2-Heptanon	nd	49	nd	nd	nd	nd
Heptanal	nd	347	44	nd	33	37
D-Limonen	82	125	94	70	236	172
trans-2-hexenal	6487	11410	13527	13674	13075	10567
1-Pentanol	141	119	97	46	51	21
Styrene	276	8	8	nd	nd	nd
Hexylacetate	181	330	494	115	94	nd
Octanal	nd	493	73	62	47	46
cis-3-Hexenylacetate	1184	1844	2771	48	821	228
1-Hexanol	2460	2381	3922	2148	2873	2693
Nonanal	2909	nd	nd	2500	nd	nd
Acetic acid	8921	20501	38047	18712	19157	23705
Benzaldehyde	nd	nd	nd	nd	nd	nd
Octanoic acid	nd	nd	nd	nd	nd	nd
Benzoic acid	nd	274	180	nd	nd	nd
Phenylacetic acid	nd	276	1418	nd	nd	297

Table S 3-5: Concentrations of analytes detected in the Portuguese EVOOS.

Analytes	concentration in µg kg^{-1}	
	P-01	**P-02**
Ethylacetate	1168	906
Ethanol	3057	9052
3-Pentanon	629	733
2-Butanol	54	nd
1-Penten-3-ol	374	717
2-Heptanon	nd	76
Heptanal	35	nd
D-Limonen	75	60
trans-2-hexenal	21592	11589
1-Pentanol	nd	33
Styrene	nd	nd
Hexylacetate	1148	648
Octanal	41	55
cis-3-Hexenylacetate	11477	6608
1-Hexanol	2798	2030
Nonanal	7484	nd
Acetic acid	31731	25727
Benzaldehyde	nd	nd
Octanoic acid	nd	nd
Benzoic acid	nd	674
Phenylacetic acid	nd	815

Table S 3-6: Compounds used for linear discriminant analysis with their loadings on LD1, LD2, LD3 and LD4.

Compound	**LD1**	**LD2**	**LD3**	**LD4**
Ethylacetate	-5.1E-07	-5.1E-07	-1.7E-07	7.5E-08
Ethanol	5.4E-08	1.3E-07	2.6E-08	2.7E-09
3-Pentanon	2.5E-07	9.7E-07	2.1E-08	9.2E-07
2-Butanol	-7.3E-06	7.3E-06	-5.9E-06	-6.2E-06
1-Penten-3-ol	2.8E-06	8.2E-07	-1.7E-06	8.3E-07
2-Heptanon	-2.6E-04	4.1E-05	-1.1E-05	1.2E-05
Heptanal	1.2E-04	-3.8E-04	7.0E-05	1.1E-04
D-Limonen	-8.6E-06	-5.8E-07	-9.7E-06	8.5E-06
trans-2-hexenal	2.3E-06	5.8E-06	-4.6E-06	-4.0E-06
1-Pentanol	3.5E-06	2.0E-05	-1.4E-05	-7.4E-06
Styrene	1.7E-05	-4.1E-05	-3.2E-05	-6.3E-05
Hexylacetate	8.5E-06	-9.6E-06	-1.9E-05	-6.9E-06
Octanal	2.7E-04	4.2E-04	1.7E-04	-2.5E-04
cis-3-Hexenylacetate	-7.0E-06	1.5E-06	4.8E-06	4.4E-07
1-Hexanol	4.0E-06	7.8E-06	1.2E-05	-7.8E-06
Nonanal	1.2E-07	-3.4E-06	8.6E-06	9.5E-06
Acetic acid	8.0E-07	4.2E-07	-3.9E-07	5.4E-07
Benzaldehyde	-1.3E-05	-2.6E-05	3.0E-05	-1.7E-05
Octanoic acid	9.0E-05	-2.0E-04	8.9E-05	1.3E-04
Benzoic acid	2.0E-07	5.3E-08	-7.6E-08	-4.7E-09
Phenylacetic acid	-6.2E-06	4.7E-06	2.3E-06	3.3E-06

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4 In-tube dynamic extraction for analysis of volatile organic compounds in honey samples

Abstract

The composition of a honey is associated with its botanical and geographical origin. Honey is often mislabeled and has a high potential for food fraud. Thus, quick and easy analyses are required. Volatile organic compounds (VOCs) are important aroma components of honey and linked to the botanical or geographical origin. To analyze VOCs directly from the sample an in-tube extraction-dynamic headspace (ITEX-DHS) method was developed for 14 different VOCs. The analytes were extracted from the headspace with 65 extraction cycles and then thermally desorbed into the gas chromatograph (GC). Repeatability was shown for two concentration levels (0.1 ng g^{-1} and 10 ng g^{-1}) as the relative standard deviation of a seven-fold measurement. For both levels, the repeatability was below 13 % and 9 %, respectively. Satisfying recoveries were reached as well (83-110 %), except for octanal, where only 62 % revovery was achieved. The method detection limit (MDL) varied from 0.8-47 ng g^{-1}, depending on the analyte. 38 honey samples were measured after method validation, four acacia honeys (A), six forest honeys (F) and 22 blossom honeys (B). The type of six honeys was not known (U) but could be predicted with the help of a linear discriminant analysis (LDA). The LDA was carried out with the three groups (A, B, F) leading to a proportion of correct predictions of 91 %.

4.1 Introduction

Honey is a natural product which can be used by humans without any processing [1]. Regarding to the Commission of the European Union honey consists essentially of different sugars as well as other substances such as organic acids, enzymes and solid particles derived from honey collection. The color of honey varies from nearly colorless to dark brown and its consistency ranges from fluid via viscous to partly or entirely crystallized. Honey shall not have any food additives and it should be prevented from heat, as heat destroys or inactivates the natural enzymes present in the honey [2]. Flavor and aroma derive from the plant's origin, as well as soil and climate can cause melliferous flora [1, 2]. Honey can be distinguished into two groups: monofloral honey and honey blends or multifloral honeys. The latter are produced in areas where no flower predominates [3]. Thus, the composition of honey is tightly associated to its botanical and geographical origin, as some honey components come from the plants, some from the honeybees and some are due to the biochemical reactions which take place during honey maturation [1, 4]. Honey is one of the most involved foodstuffs in food fraud. Food fraud is intentional and economically motivated. Mostly, food ingredients are replaced by a less expensive substitute and thus leads to a dilution of the authentic ingredients and the adulteration of the product [5]. Therefore, the monitoring of authentic samples is important for the product's quality [6].

The traditional analysis of the botanical origin of honey is done by melissopalynology, which is the analysis of the pollen present in honey using a microscopic examination. The main drawbacks of this technique are that it is time consuming and requires experienced analysts. Furthermore, it is dependent on the expert's interpretation [7].

Another approach to identify the honey's origin is to analyze the volatile organic compounds (VOCs) present in the honey [8]. VOCs, such as aldehydes, hydrocarbons, alcohols, ketones, acids and esters, can derive from the plant or nectar source, from the transformation processes of plant compounds, from heating or from microbiological or environmental contamination [9, 10]. The aroma depends on the different VOCs present in the honey, which results in the influence of taste and odor. Benzaldehyde is a compound which is related to a fragrant, aromatic and sweet odor of honeys, as well as linalool, which is additionally refreshing and lemony. Carvarol on the other hand is associated with a pungent odor and dimethyl sulfide with a sulfuric, gasoline-like smell [11, 12]. As there are not many marker compounds known for the different monofloral honey types, the adulteration of honey is hard to detect and thus, it is one of the most frequently adulterated food products. Most often the botanical source is

declared as monofloral, when mixed with cheaper honey blends. Therefore, several studies have been carried out to identify marker components for specific monofloral honeys [2, 3, 13-17].

As volatile compounds play an important role in assessing the botanical origin of honey [18], microextraction techniques like headspace solid phase microextraction (HS-SPME) can be used prior to gas chromatography coupled to a mass spectrometer (GC-MS). One of the main advantages of headspace analysis is that it can be carried out on untreated samples almost regardless of its composition and viscosity [19]. HS-SPME is a well implemented technique for the analysis of VOCs in honey samples [8, 13, 16, 17, 19-21].

In-tube extraction (ITEX) is a dynamic headspace (DHS) approach, which is fully automated for PAL-type autosamplers and uses a sorbent filled trap with a fixed steel needle attached to a gastight syringe, which can be heated for thermal desorption. Enrichment of analytes takes place by repeated pumping of the sample's headspace trough the sorbent trap by aspirating and dispensing of the syringe. The injection takes place in the GC injection port by heating the trap to the set desorption temperature and aspiration of a gas, either a portion of the sample's headspace or a carrier gas. Afterwards the trap is cleaned thermally whilst flushing it with nitrogen through the syringe side-port hole [22, 23].

The aim of this study was to show for the first time the applicability of the dynamic headspace approach ITEX-DHS and to create a quantitative method for the analysis of VOCs in honey. In this initial study, 38 exemplary honey samples were analyzed with ITEX-DHS GC-MS. Additionally, a linear discriminant analysis (LDA) was conducted, to show a way of grouping the different honey types and to cluster unknown honey samples.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Methanol (99.8 %) from Fisher Scientific (Loughborough, UK) was used to prepare stock solutions. Milli-Q water was used from a water purification system (Purelab ultra, Elga, High Wycombe, UK). 2-Phenylethanol (99 %), linalool oxide (97 %), benzoic acid (99.5 %), carvacrol (98 %), octanoic acid (96 %), ethanol (98 %), octane (99 %), octanal (99 %), benzaldehyde (99 %), dimethyl sulfide (98 %), nonanol (98 %) and nonanoic acid (99.5 %) were purchased from Sigma-Aldrich (Steinheim, Germany), thymol (99 %) from Honeywell Riedel-de Haën AG (Seelze, Germany), phenylacetic acid (99 %) from Alfa Aesar (Karlsruhe,

Germany) and 2-Butanol from Fluka (Darmstadt, Germany). Compound related CAS numbers, logarithmic water−air (log K_{wa}), Setschenow (salting out with NaCl, K_s) constants and used quantifier and qualifier ions can be found in Table 4-1. Log K_{wa} and K_s were calculated using PP-LFERs database [24]. Sodium chloride (> 99.5 %, NaCl) purchased from Bernd Kraft (Duisburg, Germany) was used for salting out and to minimize sample composition effects on transfer into the headspace. Therefore, a 25 % (w/v) NaCl solution was prepared weekly by dissolving 125 g of NaCl in 500 mL Milli-Q water.

Table 4-1: Physicochemical properties of used compounds.

	CAS No.	**Purity %**	**log K_{wa} 25°C**	**Setschenow constant K_s**	**quant. Ion m/z**	**qual. Ions m/z**
Alcohols						
Ethanol	64-17-5	98	3.54	0.12	45	46
2-Butanol	78-9-2	>99	3.35	0.17	45	59, 74
Nonanol	143-08-8	98	2.77	0.29	55	56
Linalool	60047-17-8	97	2.95	0.29	59	54, 11, 132
Phenylethanol	60-12-8	99	5.02	0.19	91	92, 122
Aldehydes						
Benzaldehyde	100-52-7	>99	3.12	0.18	77	105, 106
Octanal	124-13-0	99	1.85	0.27	55	56, 84
Alkanes						
Octane	111-65-9	99	-1.98	0.32	43	57, 85, 114
Acids						
Benzoic acid	65-85-0	99.5	5.25	0.17	77	105, 122
Octanoic acid	124-07-2	96	4.13	0.25	43	55, 73
Nonanoic acid	112-05-0	99	4.01	0.27	57	60, 73
Thymol	89-83-8	99	4.27	0.24	91	135, 150
Carvacrol	499-75-2	98	4.64	0.24	91	135, 150
Dimethyl sulfide	75-18-3	98	1.18	0.16	47	61, 62

4.2.2 Stock solutions and standard mixtures

Stock solutions of all 14 analytes were prepared by weighing 10 mg of the pure substance in a 10-mL volumetric flask and diluting it with methanol to a final concentration of 1 gL^{-1} in 10 mL. A standard mix of the 14 analytes was prepared with a concentration of 50 mg/L by transferring 250 µL of each analytes stock solution into a 5-mL volumetric flask and diluting it with methanol. The standard mix was prepared monthly. All solutions were stored in 20-mL amber glass vials sealed with magnetic screw caps with butyl rubber/PTFE septa (BGB Analytik, Rheinfelden, Germany) at 4°C in the refrigerator. Lower concentrations for method validation were prepared likewise by dilution with 25 % (w/v) NaCl to the required concentration levels.

4.2.3 Honey samples

38 honey samples were purchased from supermarkets and local beekeepers. Three different types of honey were covered with the selection: Acacia honey (4), blossom honey (22), forest honey (6). Additionally, there were six honeys, where the type was not defined (U). The geographical origin of most of the honeys was defined as a mixture of honey from EU-countries and non-EU countries. Exceptions were as follows: One blossom honey was from Tostedt, Germany, one from Bausendorf, Germany, one from Essen, Germany, one from Niedersachsen and Schleswig-Holstein, Germany, and one from Asiago, Italy. One forest honey was produced in Asiago, Italy and one in Westerkappeln, Germany.

Honey samples were prepared by weighing 1 g honey into 20-mL amber screw cap glass vials (BGB Analytik GmbH (Böckten, Switzerland). PTFE laminated 8 × 3 mm magnetic stir bars (VWR International GmbH, Darmstadt, Germany), as well as 10 mL of 25 % (w/v) NaCl solution were added to each vial. The vials were closed by magnetic screw caps with rubber/PTFE septa (BGBAnalytik AG, Boeckten, Switzerland) and placed onto the autosampler tray for ITEX-DHS.

4.2.4 ITEX-DHS

For extraction of the VOCs from the headspace a Tenax TA ITEX trap (BGB Analytik, Adliswil, Switzerland) and a 1.3-mL Headspace syringe (CTC Analytics, Zwingen,

Switzerland) were used. The Tenax TA material (polydiphenylene oxide) has a particle size of 80/100 mesh with a surface area of 35 m^2g^{-1}. The maximum operation temperature is 350°C.

The samples were incubated at 70°C for 30 min while stirring at 500 rpm. Meanwhile the ITEX trap was cleaned for 10 min with a trap temperature of 300 °C. After incubation, the ITEX needle was injected into the headspace of the vial with syringe and trap temperatures of 70 °C. Extraction was performed with 65 extraction cycles with an extraction volume of 1 mL and a speed of 100 µL/s. Desorption of 1 mL took place in the GC-injection port with a trap temperature of 300 °C and a flow of 100 µL/s.

4.2.5 Instrumentation

All samples were measured using a Trace GC Ultra (S+H Analytik, Mönchengladbach, Germany) coupled to a single quadrupole mass spectrometer (DSQ II, S+H Analytik). The GC was equipped with a PAL Combi-xt autosampler (Axel Semrau, Sprockhövel, Germany), a split/splitless injector (SLL), and an Optic 3 injector (Axel Semrau) with a cryofocussing unit. The ITEX2 option for the autosampler consisted of a heatable syringe holder, a 1.3-mL syringe with a side port, and a trap heater (CTC Analytics). Additionally, a single magnet mixer (SMM) was attached to the autosampler. Data acquisition and processing were carried out using XCalibur Data System (Version 2.2, ThermoFisher Scientific), ITEX2 procedures were controlled with PAL cycle composer (CTC Analytics) and the temperature for the Optic 3 was controlled with ATAS evolution workstation. For the separation of the analytes an Optima FFAPplus fused-silica capillary column from Macherey-Nagel (60 m x 0.32 mm I.D., 0.5 µm film thickness, Macherey-Nagel, Düren, Germany) was used.

The injector was used in splitless mode at a temperature of 300 °C and was equipped with a 2.0 mm I.D. deactivated splitless liner (Restek, Bellefonte, USA). The cryotrap temperature was set to -20 °C with a hold time of 2 min. After the transfer time, the split was opened at 50 mL/min and the cryotrap was heated to 250 °C with a rate of 50 °C/s. Constant column flow of 1.5 mL He 5.0 (Air Liquide, Oberhausen, Germany) was set. The initial GC oven temperature was set to 35 °C and held for 5 min. Then the temperature was raised to 110 °C with a ramp of 7 °C/min and held for 2 min. Afterwards temperature was raised to 200 °C with 5 °C/min and held for 4 min, before being raised with a ramp of 10 °C to the final temperature of 230 °C which was held for further 2 min, resulting in a total GC run time of nearly 45 min.

The temperatures for the transfer line and the ion source were set to 260 °C and 230 °C, respectively. The MS was operated in electron impact ionization mode (EI) at 70 eV. Full-scan mode (m/z = 40-200, 500 amu/s) was used for all measurements.

4.2.6 Linear discriminant analysis

To perform the LDA, the free software R 3.6.2 (The R Foundation for Statistical Computing, www.r-project.org) was used, including the R-package "MASS" [25]. The peak areas of the 14 analytes were used as the explanatory variables for the LDA. The 38 honey samples were divided into four groups: acacia honey (A, 4), forest honey (F, 6), blossom honey (B, 22) and unknown honeys (U, 6). The first three groups were used to train the linear function and to find a discrimination between the different groups. The group with the unknown samples was not used in the training set, as there the honey type was not defined and should be revealed by applying the samples to the model. Thus, these samples were used as the testing set.

4.3 Results and discussion

4.3.1 Method validation

For the validation of the method, the method detection limit (MDL), the repeatability and the recovery were used. The MDL is defined by the U.S. Environmental Protection Agency as the minimum concentration of an analyte that can be reported greater than zero with a confidence of 99 % [26]. Repeatability is shown at two concentration levels (0.1 ng g^{-1} and 10 ng g^{-1}) as the relative standard deviation of a seven-fold measurement. All results are summarized in Table 4-2. The validation experiments were done in 10 mL 25 % (w/v) NaCl solution, as later, the honey samples are diluted as well in 10 mL of the salt solution, for two different reasons. Firstly, the dilution adapts the matrix, so that the matrix of the honey becomes nearly negligible, and secondly, the salt within the solution helps to further facilitate the transfer from the aroma compounds into the headspace, where they are dynamically extracted [27].

The repeatability at the lower concentration level showed already satisfying results with an average of 9 %, but with six analytes above 10 %. The results for the higher level showed an average of 8 % and no analyte above 10 % which shows good precision at this concentration level. The recovery was measured at 10 ng g^{-1} and showed good results for nearly all analytes. Only octanal showed poor recovery with only 62 %. On average, the recovery was 92 %. The

MDL varies a lot between the different analytes (0.8-4.7 ng g^{-1}). The MDL was used because it is not directly dependent on the noise around the peak but rather is a measure of repeatability, and thus shows the lowest concentration, at which the analyte can be detected with an acceptable repeatability. Odeh and collaborators [28] used a HS-SPME method for the identification of different honeys. They showed low limits of quantification (LODs, < 8.4 ng/ g honey) by using a signal-to-noise ratio of three. As the calculations of the detection limits were done in different ways, the comparison is difficult. Nevertheless, they are in the same order of magnitude. Four analytes can be compared with the ITEX-DHS method, namely octanoic acid, nonanoic acid, benzaldehyde and phenylethanol. Overall, sensitivities of the HS-SPME and the ITEX-DHS method were comparable, but repeatability and reproducibility were superior with the presented ITEX-DHS method.

Table 4-2: Method validation results.

Analytes	**repeatability at 0.1 ng g^{-1} / %**	**repeatability at 10 ng g^{-1}/ %**	**recovery at 10 ng g^{-1} / %**	**MDL / ng g^{-1}**
Dimethyl sulfide	6	8	83	2.0
2-Butanol	3	6	97	2.5
Octane	10	9	86	0.8
Ethanol	7	9	94	2.2
Octanoic acid	9	6	86	2.0
Octanal	13	9	62	3.0
Linalooloxide	11	8	100	1.5
Benzaldehyde	4	4	110	4.6
Benzoic acid	9	9	86	3.4
Nonanol	9	9	96	4.7
2-Phenylethanol	11	9	100	2.7
Nonanoic acid	12	6	96	4.3
Thymol	12	7	98	1.6
Carvacrol	8	9	96	1.0

4.3.2 Quantitative analysis

For quantification, all 14 analytes were investigated in the 32 honey samples with known botanical origin (acacia (A), blossom (B) and forest (F)). Detected concentrations of the target analytes ranged from nanogram per gram to microgram per gram. For each group of honey type, a mean was calculated. Table 4-3 summarizes for all 14 analytes the minimum, maximum, mean and median concentration, and the number of detections above the MDL. In addition, the mean concentrations of the analytes in the different groups is given.

Table 4-3: Lowest and highest detected analyte concentration, mean, median, number of samples in which the analytes have been found and average concentration of analytes in the three different honey varieties (acacia (A), blossom (B) and forest (F)).

	lowest c ng g^{-1}	highest c ng g^{-1}	Mean ng g^{-1}	Median ng g^{-1}	samples (n)	ng g^{-1}		
						Mean (A)	Mean (B)	Mean (F)
Dimethyl-sulfide	nq	nq	nq	nq	nq	nq	nq	nq
2-Butanol	135	2580	760	920	32	1450	845	860
Octane	0.9	1.6	1.1	1.1	32	1.3	1.1	1.2
Ethanol	3.2	690	50	160	6	nd	185	110
Octanoic acid	220	1255	500	635	11	590	835	530
Octanal	4.5	4.5	4.5	4.5	1	nd	4.5[+]	nd
Linalool-oxide	2.4	90	31	40	29	25	47	25
Benzaldehyde	7.5	120	28	34	32	47	32	30
Benzoic acid	12	450	120	130	28	38	150	150
Nonanol	6.0	3960	490	930	19	660	870	1370
2-Phenyl-ethanol	170	4515	1320	1535	32	1960	1450	1570
Nonanoic acid	24	1615	390	400	30	540	310	690
Thymol	1.8	4.8	2.2	2.8	6	2.7	2.8	nd
Carvacrol	1.1	5.6	2.0	2.7	8	nd	2.9	1.1[+]

[*]nq = not quantified, as the signals were not in the calibration curve range
[+]only one sample was positive in this group and represents the mean

Dimethyl sulfide was not quantified in the real samples. The calibration curve for dimethyl sulfide had a high y-intercept, as the detected peak areas even at the lowest level were very high (Figure S 4-1). The first measured concentration in the calibration curve was

50 µg kg^{-1}. Therefore, the measured peak areas of the real samples were in the extrapolated areas, which might lead to wrong results, as it might have been imprecise. The chosen calibration range did not show a high slope, which could indicate the beginning of the saturated area. The impact is shown in Figure S 4-2, where the regression was forced through zero. To show the presence of dimethyl sulfide in the honey samples, an example is presented in Figure 4-1.

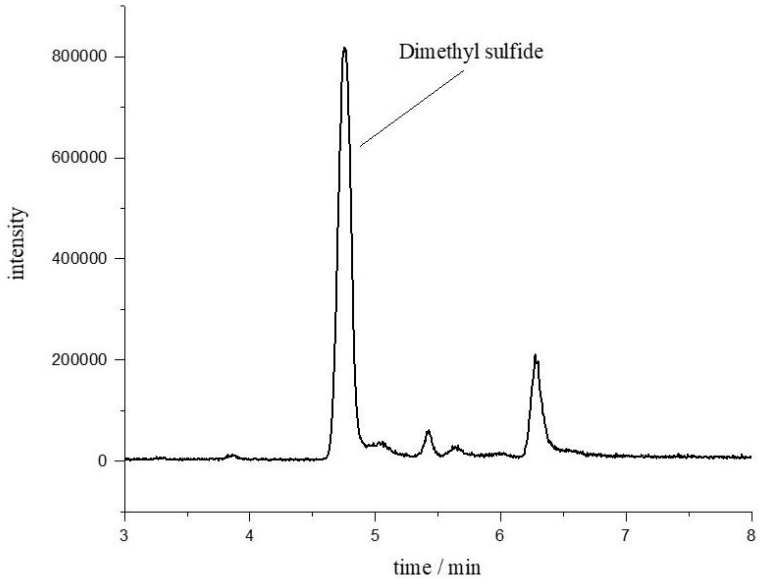

Figure 4-1: Chromatogram detail of dimethyl sulfide detected in a blossom honey sample.

Ethanol is often identified in different honey types which leads to the idea, that it indicates fermentation processes and is not related to a specific honey type [19]. It was found in six different samples, but not in acacia honey. 2-Butanol, octane, benzaldehyde and 2-phenylethanol were found in all 32 samples. Benzaldehyde, octane and 2-phenylethanol are known to be ubiquitous, so they were expected to be found in all samples [29]. Octanal was only found in one of the blossom honeys. For all other real samples, the detected concentrations were below the MDL. Some analytes do not show a big variation among the botanical groups, as for example octane, linalooloxide, benzaldehyde, thymol and carvacrol. On the other hand, analytes like 2-phenylethanol, nonanol, octanoic acid and 2-butanol vary a lot among the groups and can thus help to find differences between honey varieties. The forest honey shows the highest concentrations in nonanol and nonanoic acid, which is typical for forest honeys, whereas the acacia honey shows the highest concentration in 2-phenylethanol and 2-butanol, which are especially present in acacia honeys [29]. Octanoic acid was present in high

concentrations in the blossom honeys. The comparison of the quantitative data to other publications is difficult, as often only qualitative analysis was done, and the chromatograms, which were achieved by different methods, were used for fingerprinting approaches [18, 30]. Ciotlaus et al. measured chromatographic profiles of volatiles of multifloral and unifloral honeys using HS-SPME. Validation results were not presented, so that the method sensitivity and repeatability cannot be compared. Furthermore, they did not quantify the identified components in the honeys and only presented their percentage amount [17]. It was not stated what the percentage amount represents. It does not become clear from the publication, if the percentage amount is calculated on the relative abundance of the peak or if it is calculated based on detected VOCs or something else. Anyhow, they found multiple components, including some of the analytes used in this study. Dimethyl sulfide was found in a small percentage of 1.8 % in multifloral honey, but not in unifloral honeys. Benzaldehyde, linalool oxide and nonanoic acid were found in both, multifloral and unifloral honey in similar percentages. This matches the result of this study, as all three analytes were found in all groups. Benzaldehyde and linalool oxide did not vary much in quantity, which matches the results of Ciotlaus et al., but nonanoic acid showed higher concentrations for forest honey samples in this study. Nonanol was only found in the multifloral honeys, which does not match the results of this study. Nonanol was also found in acacia honeys in this study, which is a monofloral honey. Octanoic acid was found in multifloral honey as well as in acacia honey, in small quantities. Here, octanoic acid was found in all three studied honey groups but showed a higher concentration for the blossom honeys. Thymol was only present in a very small quantity in the multifloral honeys, sunflower honey and linden honey. The latter two honey types were not investigated in this study and thus, no comparison can be done for them. Anyhow, in this study, thymol was found in blossom honey in small concentrations, which matches the result of the multifloral honeys. Additionally, it was detected in acacia honeys in very low concentration, which is a difference to Ciotlaus et al., who were not able to identify this compound for acacia honeys. This might be explained by the increased sensitivity of the ITEX-DHS method, but as no validation results were presented by Ciotlaus et al., this is only an assumption, and cannot be proven. The last compound which overlaps in the two studies, is ethanol. It was detected in rape honey, which was not investigated with ITEX-DHS. However, both methods could not detect ethanol in acacia honey, which leads to the conclusion, that ethanol is not present in this honey type. Karabagias et al. studied different monofloral honeys, as well as a multifloral honey, using HS-SPME. They quantified based on an internal standard, so that the quantities are difficult to compare. Only four analytes overlap with our study, and for all, the concentrations differ a lot.

Benzaldehyde, Octanol and octane were found in higher concentrations (280, 240 and 1860 ng g^{-1}, respectively) in the study of Karabagias et al., whereas nonanol showed a smaller concentration (70 ng g^{-1}) [21]. Ouradi et al. studied different types of honey from morocco, including different monofloral honeys as well as some mutlifloral honey, using HS-SPME. Ethanol was found in all monofloral honeys, but not in the mutlifloral ones. This does not match with the results found using ITEX-DHS, as in the blossom honeys, ethanol was detected. Ouradi et al. presents only four analytes per honey type, as these were found in the largest quantities. For the multifloral honeys, only linalool oxide overlaps with the analytes studied with ITEX-DHS. In the monofloral honey samples, benzoic acid, nonanoic acid and octanoic acid were detected, but no acacia honey was studied and thus the comparison to the here presented honey samples is not possible [20].

4.3.3 Linear discriminant analysis

LDA is a commonly used chemometric tool to classify groups. The LDA performs a calculation, finding the smallest variance within a group, but the biggest variances among the different groups [31]. The LDA is first performed with a training data set, where the groups are known. Afterwards, the test data can be loaded, and unknown samples can be identified. For the LDA the peak areas of the detected analytes were used and not the quantified amounts. Even though dimethyl sulfide was not quantified, it was still detected. It was not necessary to get the concentrations of the different analytes to perform LDA, as there, also peak areas can be used. It is only important, that all used data points are in the same unit. Therefore, all fourteen analytes were used for the LDA.

The number of linear discriminant functions (LD) is limited to the number of groups minus one, leading to only two discriminant functions in this application. The LDs can be used for prediction, resulting here in a proportion of correct predictions of 91 % for the training set. The different loadings on the LDs are presented in Table S 4-1.

The real groups and the predicted groups are presented in Table 4-4. 96 % of group B is correctly grouped, as well as 100 % of group A. Only in the forest honeys, the correct predictions are only 66.7 %, as two honeys are wrongly classified.

For visualization of the group classification, a scatter plot is shown in Figure 4-2, which is based on LD1 and LD2. The dotted lines show the boundaries between the different honey types. The three misclassifications are shown in red. The unknown samples, which were added

as a test set, are presented in blue. As they are added into the trained model, they are assigned to the three different groups. Two of the unknown honeys are predicted to be forest honeys and four of them to be blossom honeys.

Table 4-4: Predicted and real varieties of the different samples.

variety	predicted variety			correct predictions (%)
	A	B	F	
A (n = 4)	4			100.0
B (n = 22)		21	1	95.5
F (n = 6)	1	1	4	66.7
total				90.6

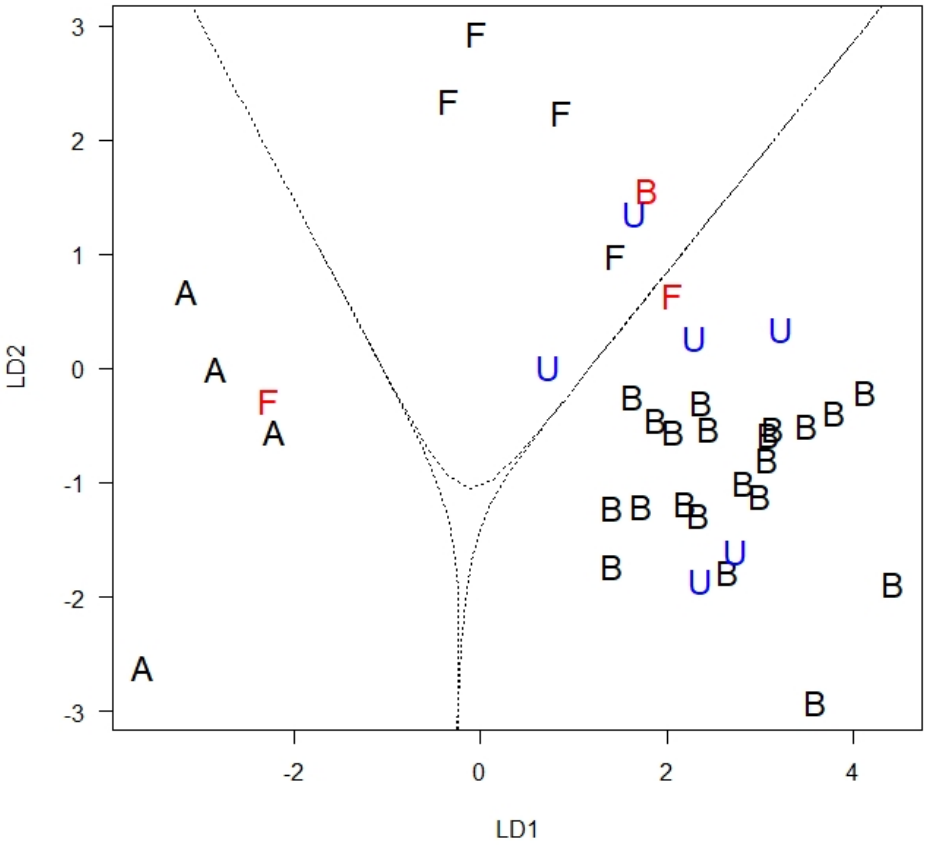

Figure 4-2: Scatterplot of the first two linear discriminant functions (LD) with the boundaries for each honey type. Falsely classified samples are shown in red. Unknown samples for prediction are shown in blue. A: acacia honey, B: blossom honey, W: forest honey, U: unknown honey.

4.4 Conclusion

The results show that ITEX-DHS was successfully applied to the quantitative analysis of VOCs in honey. The reproducibility of the method was satisfying, as well as its recovery. ITEX-DHS is a quick, fully automated and very robust method, not only with regard to the excellent reproducibility, but also in terms of mechanical stability and lifetime. As an example, 32 honeys were measured and used as a basis of LDA for the classification into three honey types. Further six unknown samples were classified using the prediction model. As here only exemplary honey samples were used, it would be of interest to increase the number of honey samples and monofloral honey types.

4.5 Supporting information

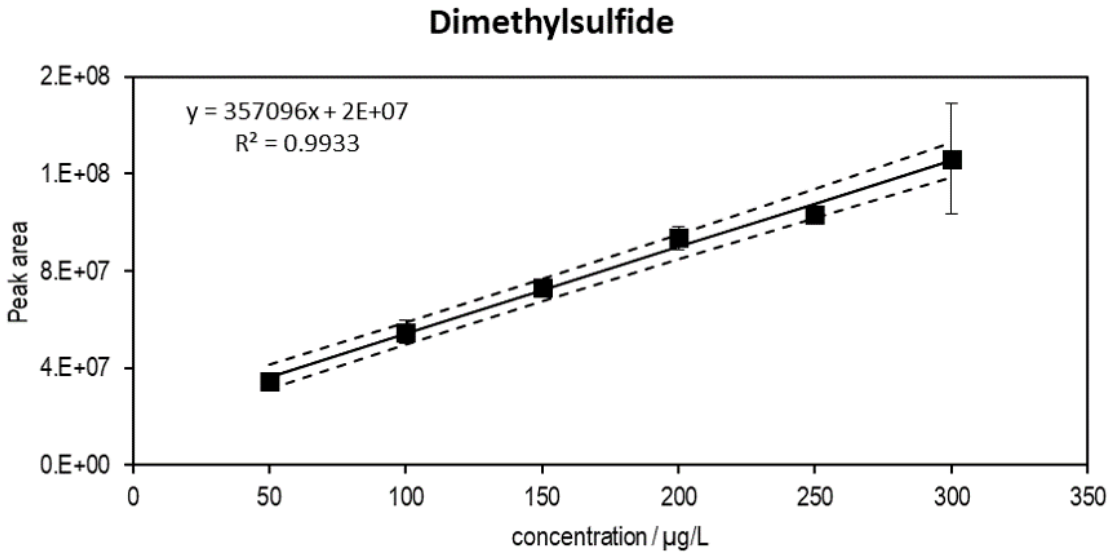

Figure S 4-1: Calibration curve of dimethyl sulfide.

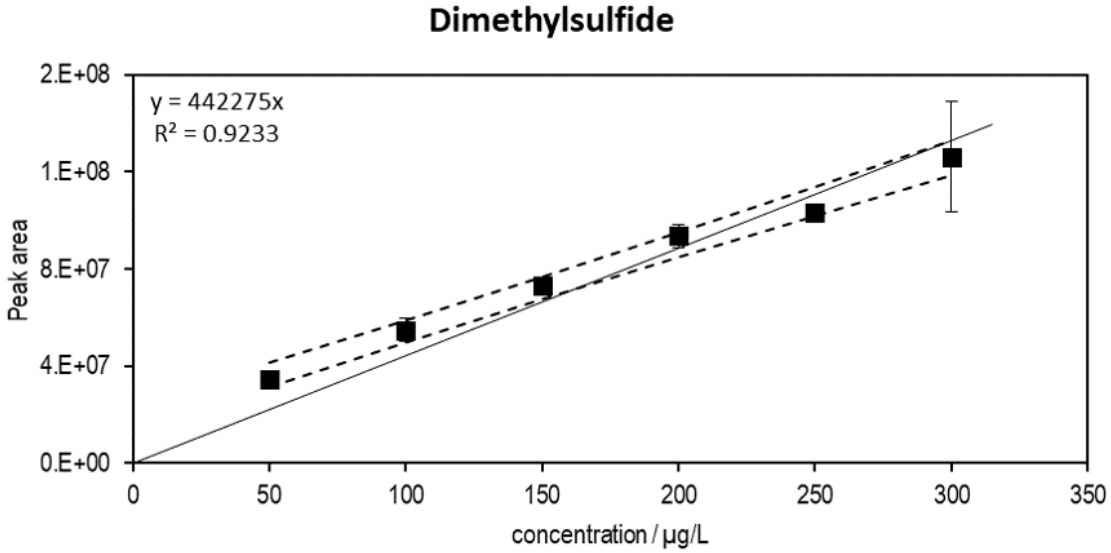

Figure S 4-2: Calibration curve of dimethyl sulfide with the linear regression forced through zero.

Table S 4-1: Main loadings of LD1 and LD2.

Compound	LD1	LD2
Dimethylsulfide	1.60E+06	8.1177E-08
2-Butanol	-2.66E-06	-6.97E-06
Octane	2.61E-07	1.506E-07
Ethanol	2.737E-07	-1.08E-06
Octanoic acid	-1.54E-06	8.652E-07
Octanal	-2.23E-06	-1.31E-06
Linalooloxide	3.409E-07	-4.56E-07
Benzaldehyde	1.355E-07	-2.2E-08
Benzoic acid	2.351E-06	2.608E-07
Nonanol	2.983E-06	6.648E-07
2-Phenylethanol	-8.97E-07	6.728E-07
Nonanoic acid	1.243E-06	1.798E-06
Thymol	-1.45E-06	-1.37E-06
Carvacrol	1.76E-06	1.28E-07

4.6 References

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5 Fully automated extraction coupled with on-fiber derivatization for the analysis of fatty acids from water samples using PAL SPME Arrow and GC-MS

Abstract

As fatty acids are important in many biological processes, their analysis is of special interest. However, their separation and detection by gas chromatography-mass spectrometry is rather difficult due to their poor thermal stability and their low volatility. The derivatization into less polar derivatives is therefore preferred. Alkylation is a commonly used derivatization technique, where the fatty acids are converted into the according methyl esters. Derivatizations are normally very time-consuming and labor intensive. Therefore, on-fiber derivatization with solid phase microextraction (SPME) Arrow was introduced in this study as a fully automated approach for the analysis of fatty acids from aqueous samples. The method was successfully optimized using three designs of experiments for the three different steps: extraction, derivatization and desorption. The validation was carried out with a calibration curve from 1-100 µg L^{-1}, the determination of the detection and quantification limits, as well as the measurement of the repeatability at two different concentration levels. Problems with the repeatability occurred, as the SPME Arrow was decomposed due to the acidic conditions during the derivatization. Nevertheless, a real sample from a composting facility was measured and all saturated fatty acids were found in the mg L^{-1} concentration range. This shows the potential of the method, but further improvements regarding repeatability and SPME Arrow lifetime are required.

5.1 Introduction

Fatty acids play an important role in many biological processes. They occur in natural fat, which is used for energy storage in living organisms. Furthermore, fatty acids are common biological intermediates which are found in the environment, body liquids, food and bioreactors [1-5]. In the environment, they occur as biomass or excrements of bacteria, plants or animals. Fatty acids can be used as biomarkers, for example, to identify bacterial diseases in clinical diagnosis, where the fatty acid profiles can be used as indicators for specific bacteria or bacterial communities [3, 6]. This information can as well be used for biofuel production to find organisms with the most suitable fatty acid profile for a successful and efficient biofuel production [7-9]. However, separation and detection of fatty acids by gas chromatography-mass spectrometry (GC-MS) is challenging due to their low volatility and thermal stability. Derivatization of fatty acids into less polar derivatives leads to improved chromatographic properties and thus to a more sensitive method using GC-MS [10]. A commonly used derivatization for fatty acids is alkylation, where the fatty acid is transformed into an ester [11]. The reaction is normally carried out in organic solvent and different derivatization reagents can be used. Boron trifluoride (BF_3) in combination with ethanol or methanol forms respective esters under the release of hydrogen fluoride (HF) [12]. Another approach is to conduct the reaction with acidic or basic agents, for example with sulfuric acid in alcohol or with dimethyl sulfate [12-14]. Often, aqueous media are problematic during alkylation, but Wang et al. showed the successful application of the combination of sulfuric acid and methanol in aqueous samples [15]. As manual alkylation is labor and time consuming, the automation of the extraction and derivatization is beneficial.

To extract fatty acids from aqueous samples, liquid-liquid extraction (LLE) is a very commonly used technique. However, LLE requires large volumes of organic solvents, which are not only hazardous to humans, but also have negative impacts on the environment. Additionally, extensive clean-up processes during LLE lead to sample losses as well as it requires a lot of time and labor [10]. Solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE) are very commonly used techniques in the analysis of environmental samples [16-21]. Nevertheless, both come with some disadvantages, like small sorbent volume and mechanical instability for SPME [22, 23], and no complete automation as well as limited range of sorbent material for SBSE [24, 25]. Therefore, PAL (Prep and Load) SPME Arrow was developed by keeping the advantages of SPME and SBSE, but furthermore to overcome their drawbacks [24]. The technical composition and set-up of PAL SPME Arrow are described in

detail in literature [23, 24]. The main advantages are the increased sorbent volume, and thus the possibility for a more sensitive analysis, as well as the improved mechanical stability and lifetime [23, 24, 26]. The extraction principle is the same as for SPME. The analytes partition between the sample and the sorbent phase for direct immersion sampling (DI) and between sample, headspace and sorbent phase for HS extraction. Martos and Pawliszyn introduced on-fiber derivatization using the classical SPME fiber in 1998 for the analysis of formaldehyde in gas samples [27]. Since then, many different applications for different analytes were reported [3, 28-31]. With this approach, the extraction (either by direct immersion or headspace sampling) is coupled directly to the derivatization and can be automated completely. The sample preparation starts with the extraction of the analytes onto/into the fiber. Subsequently, the fiber is exposed to the derivatization agent for a specific time, and lastly, the derivatized analytes are thermally desorbed into the GC system. With this setup, all steps of the sample preparation are combined and fully automated, which saves labor and time.

Here, the new PAL SPME Arrow was used for the automated extraction coupled to on-fiber derivatization of fatty acids to fatty acid methyl esters from aqueous samples. The method was first optimized by design of experiment (DOE) using a commercially available SPME Arrow. The optimized method was then validated, and finally real environmental samples were analyzed using the proposed method.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Methanol (99.9 %) from VWR (Fontenay-sous-Bois, France) was used to prepare the fatty acid stock solutions, as well as the stock solutions of the used internal standards. MilliQ water was utilized from a water purification system (Purelab ultra, Elga, High Wycombe, UK). Decanoic acid (99.5 %), dodecanoic acid (99 %), tetradecanoic acid (99.5 %), hexadecenoic acid (99 %), *cis*-9-hexadecenoic acid (98.5 %), heptadecanoic acid (98.5 %), octadecanoic acid (98.5 %) and *cis*-6-octadecenoic acid (98.5 %) were purchased from Sigma Aldrich (Steinheim, Germany) and sulfuric acid (95 %) from Fisher Scientific (Loughborough, UK). Hydrochloric acid was purchased from VWR.

Glyceryl triundecanoate (98 %),1-tetradecene (99.8 %) and methyl nonanoate (99.8 %) from Sigma-Aldrich were used as internal standards to check for saponification and complete esterification. Palmitic acid-d_{31} (98 %) and Methyl heptadecanoate-d_{33} (97.5 %) from Sigma-Aldrich were used as isotope-labelled standards to test for system stability.

5.2.2 Stock solutions, standard mixtures and derivatization agent

Stock solutions of the eight fatty acids were prepared by weighing 10 mg of the pure substance into a 10-mL volumetric flask and diluting it with methanol to the final concentration of 1 g L^{-1}. A standard mix of the eight analytes with a concentration of 50 mg L^{-1} was prepared by transferring 250 µL of each analyte's stock solution into a 5-mL volumetric flask and diluting it with methanol to the final volume. Internal standards were equally prepared with concentrations of 1 g L^{-1} and merged within one mix with the concentration of 50 mg L^{-1}. The analyte mix and the internal standard mix were prepared monthly. All solutions were stored in the refrigerator at 4 °C in 10- or 20-mL amber glass vials with magnetic screw caps with butyl rubber/PTFE septa (BGB Analytik, Rheinfelden, Germany).

For the derivatization of fatty acids to fatty acid methyl esters, a mixture of sulfuric acid and methanol was used. It was prepared every two weeks and kept in the refrigerator at 4 °C in a 10 mL amber glass vial. The reagent was prepared by adding 100 µL sulfuric acid to 9.9 mL cooled methanol, leading to a concentration of 1 % (v/v).

5.2.3 Sample preparation

For the optimization experiments, samples were prepared with a concentration of 100 µg L^{-1}. The high concentration was chosen to ensure the detection of positive and negative effects of different sample preparation conditions. For the optimization of the derivatization step, all samples were prepared by adding the standard analyte mix and the internal standard mix to ultrapure water, which was adjusted to pH 2.6 by the addition of HCl. 18 mL of the samples were then transferred into 20-mL amber glass vials and closed with magnetic screw caps with silicone/PTFE septa (BGB Analytik, Rheinfelden, Germany). 2.7 % (w/v) NaCl were added to enhance the extraction. For the optimization of the extraction, the influence of the pH value and the salt content were studied, so that the samples contained different amounts of salt (0-8.3 % (w/v)) and were adjusted to different pH values (2.6-6.2). Again, the two standard

mixes were diluted in the according water and 18 mL of the samples were transferred into the amber glass vials. For the optimization of the desorption, the samples were prepared with the optimized values. The pH was adjusted to 2.6 and 8.3 % (w/v) of NaCl were added to the samples.

For validation, the samples were prepared in ultrapure water with a pH of 2.6. The vials contained 8.3 % (w/v) salt and 18 mL of the samples were added. The sample concentrations for the calibration curve ranged from 1 to 100 µg L^{-1} with five levels (1, 25, 50, 75, 100 µg L^{-1}) and were prepared in triplicates. For repeatability measures, seven samples with the concentration of 25 µg L^{-1} and seven samples with the concentration of 75 µg L^{-1} were prepared and measured.

The measured real sample was a leachate from a composting facility and was provided, already centrifuged and filtrated, from the department of Prof. Widmann from the University of Duisburg-Essen. The sample was expected to contain fatty acids, as personal communication led to the information that specific microorganisms were added to the system, which should result in the production of long chain fatty acids. The sample was diluted with ultrapure water, as high concentrations were expected. Additionally, matrix interferences were minimized with this step. The sample was diluted 1:1000 and 1:500, as the fatty acids were present in different concentration and the sample had to be adjusted to the calibrated range. The pH was adjusted to 2.6 and 8.3 % (w/v) salt were added in the vials.

5.2.4 Extraction and on-fiber derivatization

A PAL RTC autosampler (CTC Analytics, Zwingen, Switzerland) was used for sample extraction and injection. The autosampler was equipped with a tray holder for 20-mL headspace vials, a parking station, holding the SPME Arrow tool, a Heatex stirrer module, a SPME Arrow conditioning module and an IKA magnetic stirring plate (RCT basic; Staufen, Germany). The SPME Arrow tool was equipped with a Divinylbenzene/Polydimethylsiloxane (DVB/PDMS; 120 µm) SPME Arrow. The autosampler was controlled using Chronos (V. 4.9.1.; Axel Semrau, Sprockhövel, Germany) and the extraction was performed by custom-made macros. The optimized extraction and on-fiber derivatization, which was used for validation and sample analysis, is presented here. The optimization was done using a central composite design for the individual steps (extraction, derivatization, desorption) and is explained in more detail in the results and discussion.

The automated sample preparation can be divided into three parts: the extraction, the on-fiber derivatization and the thermal desorption (Figure 5-1). The extraction started with 10 min incubation of the sample at 80 °C and stirring of 800 rpm on the heating plate. The stirring rate was not optimized, as previous experiments showed, that a stirring of 800 rpm for direct immersion is a good value [32]. At the same time, the derivatization reagent vial was already transported to the heatex stirrer (70 °C) and the SPME Arrow was conditioned for 8 min at 280 °C using the conditioning station. After the incubation time, the SPME Arrow penetrated the vial septum and extracted the analytes via direct immersion for 30 min. Afterwards, the SPME Arrow was withdrawn and moved to the derivatization agent vial. There, the on-fiber derivatization was conducted by exposing the SPME Arrow to the headspace above the derivatization agent (85 µL) so that the analytes were derivatized on the fiber for 60 min. Finally, the SPME Arrow was again withdrawn and then moved into the GC injector for the thermal desorption at 245 °C for 4 min.

The sample preparation time is longer than the GC runtime. Therefore, the overlapping function of the Software was used, which enables that the following sample is already prepared, whilst the GC measures the latest sample (Figure 5-2). Due to this overlapping of process steps, analysis time per sample was 104 min.

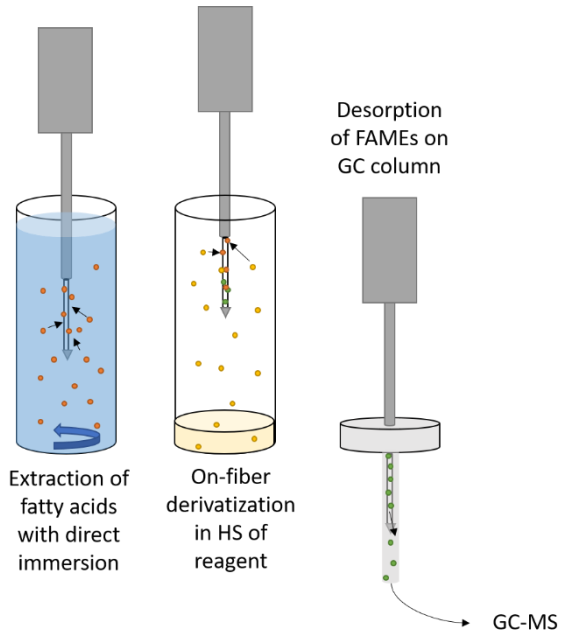

Figure 5-1: Visualization of the automated on-fiber derivatization which can be divided into three steps: extraction, derivatization and desorption.

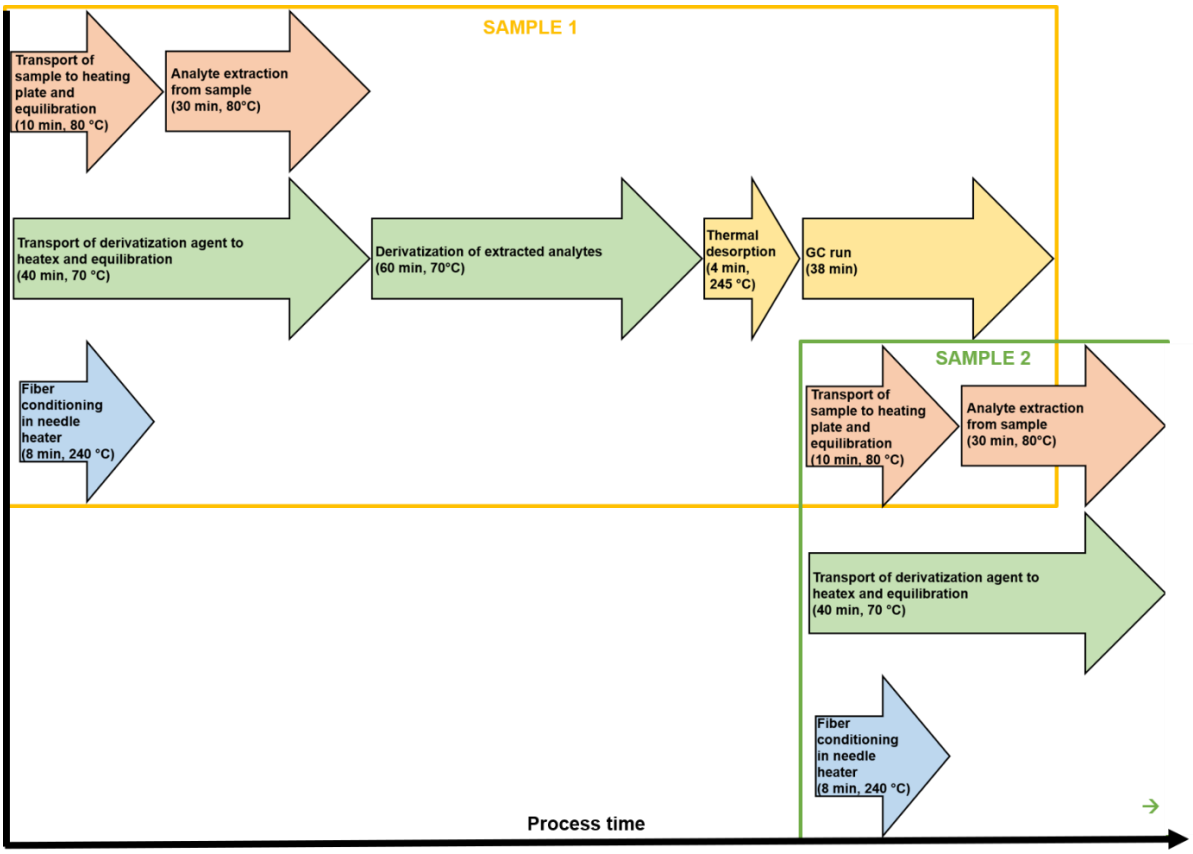

Figure 5-2: Visualization of the autosampler tasks and the overlapping schedules.

5.2.5 Design of experiment

For the optimization of the on-fiber derivatization, a rotatable (axial distance = 1.68179) CCD, with 20 runs (2^3 factorial + 6 star points and involving 6 center points) was used based on the derivatization time, derivatization temperature and volume of used derivatization agent, as quantitative factors (Table S 5-1). A rotatable (axial distance = 2) central composite design, CCD, with 31 runs (2^4 factorial + 8 star points and involving 7 center points) was selected for the optimization of the extraction procedure, based on extraction time, extraction temperature, salt content of the samples and pH value of the samples, as quantitative factors (Table S 5-2). Finally, for the optimization of the desorption, a rotatable (axial distance = 1.141421) CCD, with 13 runs (2^2 factorial + 5 star points and involving 4 center points) was selected, based on desorption time and temperature, as quantitative factors (Table S 5-3). Minitab® (V. 18.1) was used for design of the experiments and processing the data. After performing the optimization experiments, the peak areas of the analytes were normalized (Microsoft Excel®) and transferred into the software, together with the geometric mean of the normalized data. The normalization process was carried out by dividing the peak area of each

individual analyte by its maximum value throughout the entire set of (CCD) experiments. The simultaneous extraction of the analytes was optimized based on the geometric mean, as the main response.

5.2.6 GC-MS measurements

All samples were measured using a GC-2010 coupled to a single quadrupole GCMS-QP2010plus (Shimadzu, Japan). The injection temperature was set to 245 °C in splitless mode with a column flow of 1.5 mLmin^{-1}. Helium (99.999 %, AirLiquide, Krefeld, Germany) was used as carrier gas. Separation of the analytes was performed on a ZB-FFAP capillary column (50 m length, 0.32 mm inner diameter, 0.5 µm film thickness) from Phenomenex (Aschaffenburg, Germany). The initial temperature of the GC oven was set to 60 °C for 5 min, was then raised to 75 °C with a rate of 12 °C min^{-1}, then to 100 °C with a rate of 14 °C min^{-1}, and then to 150 °C with a rate of 12 °C min^{-1}. The oven was then further heated with 6 °C min^{1} to 170 °C, then with a rate of 4 °C min^{-1} to 210 °C and finally to 240 °C with a rate of 12 °C min^{-1} where it was held for 10 min. The MS transfer line was set to 240 °C and the ion source temperature to 240 °C. Electron ionization (EI), with an ionization energy of 70 eV, was used in full scan mode in the range of *m/z* 50-350. Instrument automation, data acquisition and data processing were done with GCMSsolution (Shimadzu, Japan). Table 5-1 summarizes the information used for detection and identification of the analytes.

Table 5-1: Retention time, quantifier, qualifier ions and dissociation constants (pk_a) for the used analytes. C14:1, C9 and C11 were used as internal standards (IS).

Analytes	retention time / min	quantifier ion m/z	qualifier ions m/z	pk_a of according fatty acid
C14:1 (IS)	12.7	55	56, 57, 69	n.a.
FAME C9 (IS)	13.3	74	55, 59, 87	4.95 [33]
FAME C10	14.7	74	55, 59, 87	4.88 [34]
FAME C11 (IS)	16.1	74	55, 59, 87	n.a.
FAME C12	17.6	74	55, 59, 87	5.3 [35]
FAME C14	21.1	74	55, 57, 87	4.9 [36]
FAME C16:1	23.7	55	59, 74, 83	n.a.
FAME C16	25	74	55, 57, 87	4.75 [37]
FAME C18:1	25.7	55	59, 69, 74	n.a.
FAME C17	26.8	74	55, 57, 87	4.78 [37]
FAME C18	28.3	74	55, 57, 87	5.2 [38]

5.3 Results and discussion

5.3.1 Method optimization

Optimization of on-fiber derivatization

For the optimization of the on-fiber derivatization, the extraction of the fatty acids was carried out at 60 °C for 45 min, as the extraction was optimized afterwards. The optimal conditions for the individual fatty acids and for the geometric mean were calculated with Minitab and are presented in Table S 5-1. The optimal solution of the geometric mean, and thus the best condition for all fatty acids as a mix, was 120 min derivatization at 70 °C with 82 µL of the derivatization agent. To get a better understanding of this result, and to see if a reduction of time would be possible, the contour plots were studied. As there are three parameters, and the contour plots can only show two dimensions, the third one is set to a constant value. The contour plots of the geometric mean for the on-fiber derivatization are shown in Figure 5-3.

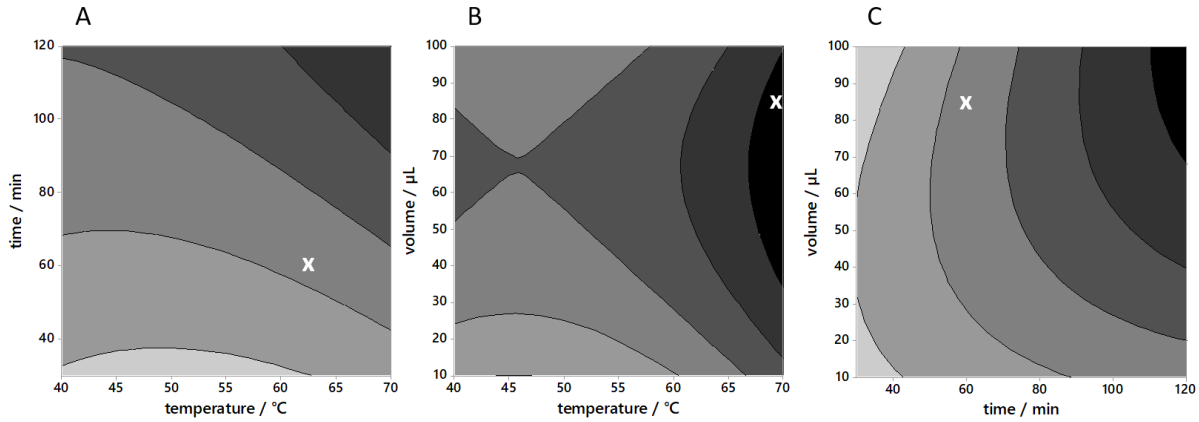

Figure 5-3: Contour plots of the optimization of the on-fiber derivatization. A: time vs temperature, hold value of derivatization agent was 85 µL. B: volume vs temperature, hold value of the derivatization time was 60 min. C: volume vs time, hold value of the derivatization temperature was 70 °C. The color ranges from light grey to black, with light grey meaning low response and black high response. The white cross shows the chosen derivatization conditions.

The contour plots show that the high temperature of 70 °C is important for the success of the on-fiber derivatization. The optimal calculated amount of 82 µL of derivatization agent was increased to 85 µL for better handling. The time has a big influence on the efficiency, but it can be seen, that a reduction to 60 min, still leads to an acceptable response. The idea behind the time reduction is to save more time in compromise with losing some of the sensitivity. During the CCD, the chosen time range was chosen in a range from 30 to 120 min, as Cha et al. used an extraction time of 90 min [3]. Only with these settings, it was able to check, whether

longer extraction times would increase the sensitivity. As the SPME Arrow enables great sensitivity, the loss in the response can be compensated. The saving of time, in this case 60 min per sample, leads to a big increase in sample throughput. Therefore, the on-fiber derivatization was carried out at 70 °C for 60 min with 85 µL of derivatization agent for the following experiments.

Optimization of extraction

With the optimized on-fiber derivatization conditions, the extraction procedure was investigated. Therefore, the salt content, the pH, the extraction temperature and the extraction time were studied. The CCD led to the optimal conditions of 60 min extraction at 80 °C with pH 2.6 and a salt content of 8.3 % (w/v) for the geometric mean. The optimal conditions for the single analytes are shown in Table S 5-2. The optimal result for pH 2.6 can be explained by the dissociation constants of the fatty acids (Table 5-1) and was previously shown by Cha et al. [3]. The salt content was studied as it is known that the addition of salt helps to improve the extraction efficiency [39]. The calculated derivatization time was 60 min, but again, it can be shortened with the compromise of a small loss in sensitivity. The contour plots used for this decision are shown in Figure 5-4. The contour plots show the three different parameters in relation with the extraction time, to check whether it can be reduced. For the temperature in relation with the time it becomes clear, that if the temperature is high (80 °C), the extraction time can be reduced to 30 min without loss in response. For the salt content a small loss in response appears if the time is reduced to 55 min, but then the response is constant between 30 and 55 min, if the salt content is chosen at its maximum with 8.3 % (w/v). The pH value shows that the decrease of extraction time to 30 min results in a small loss in response. The difference in response between 30 min and 60 min is expected not to be substantial, so that the saving of time is again favorable to increase the sample throughput by keeping the overall sample preparation time per sample as short as possible.

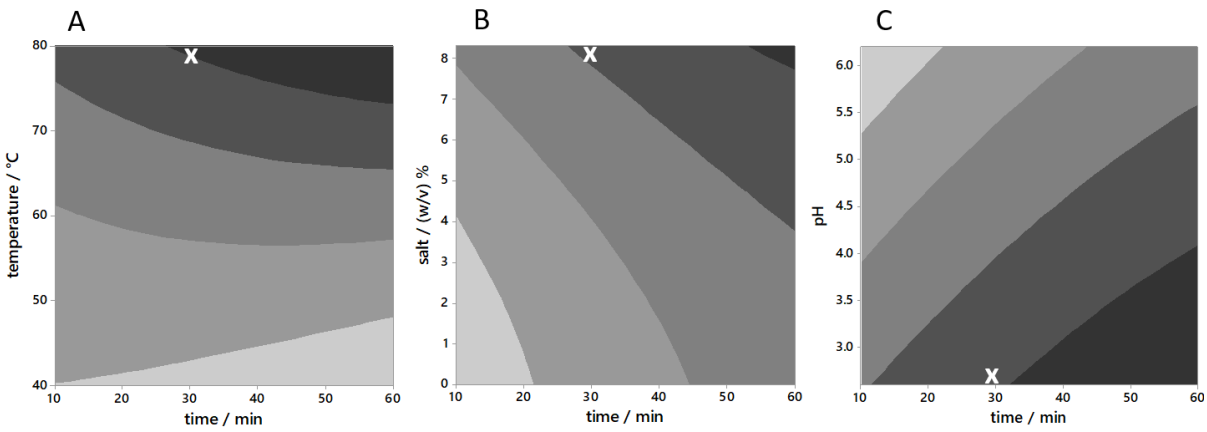

Figure 5-4: Contour plots of the optimization of the extraction. A: temperature vs time, hold value of pH 2.6 and salt content of 8.3 % (w/v). B: salt content vs time, hold value of the pH 2.6 and temperature 80 °C. C: pH vs time, hold value of the temperature 80 °C and salt content 8.3 % (w/v). The color ranges from light grey to black, with light grey meaning low response and black high response. The white cross shows the chosen extraction conditions.

Optimization of desorption

The desorption was the last part of the sample preparation which was optimized using CCD. There were only two factors which needed to be optimized, namely the desorption time and the desorption temperature. The calculated optimal conditions of the single fatty acids and the geometric mean are shown in Table S 5-3. The optimal condition for the geometric mean is again visually checked using the contour plot (Figure 5-5). The plot shows, that the optimal condition is at a high temperature and when desorption is performed longer than 3.5 min. As optimal desorption conditions 245 °C and 4 min were further used.

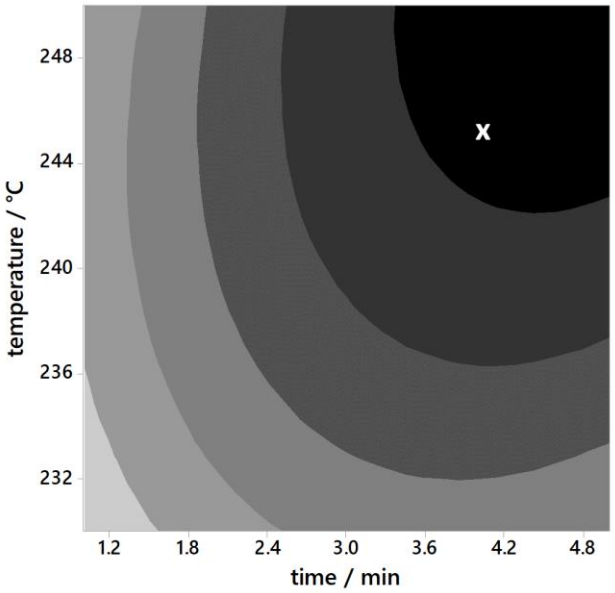

Figure 5-5: Contour plot of the optimization of the desorption. The color ranges from light grey to black, with light grey meaning low response and black high response. The white cross shows the chosen desorption condition.

5.3.2 Internal standards

Two sets of internal standards were used in this study. The first set contained Glyceryl triundecanoate, methyl nonanoate and 1-tetradecene. Suter et al. [41] showed that these three standards can be used to check for complete transesterification reaction and whether saponification occurred. To control if the transesterification was complete, glyceryl triundecanoate is used, as it forms the respective C11 fatty acid methyl ester during the reaction. In the case of the fatty acid samples, it is not as important to control the complete reaction from the glyceryl to the fatty acid methyl ester, as the analytes were already present as fatty acids. Nevertheless, for food samples, this technique could be used to check for complete transesterification. Methyl nonanoate, which is the methyl ester of the C9 fatty acid, checks for saponification. Both are set into relation with 1-tetradecene, which is used as an inert standard. The peak areas of these three internal standards are measured and then compared with each other (Figure 5-6). An incomplete transesterification of triglyceride leads to an insufficient amount of the according C11-FAME, as well as the reduced peak area of the C11-FAME can also indicate saponification. Saponification can also be noticed by the reduction of the FAME-9 peak area [41]. The ratio between the glyceryl triundecanoate and the 1-tetradecene must be above 75 % for complete transesterification. If the ratio is below 75 %, either the transesterification was not complete, or saponification occurred already. The latter can be

checked with the ratio of the peak areas of the methyl nonanoate and the 1-tetradecene. If this ratio is above 67 % no saponification occurred [40]. These ratios were checked during the design of experiment and showed that the transesterification was successful without any saponification occurring in most cases (see Table S 5-4 to Table S 5-6).

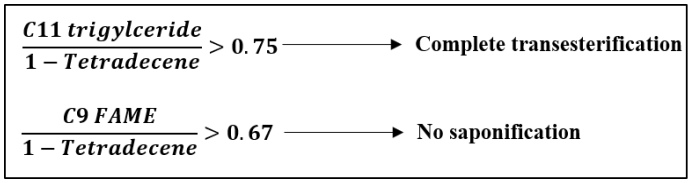

Figure 5-6: Scheme of used internal standards and their relation to each other to check whether saponification or complete transesterification occurred.

The second used set of internal standards were two deuterated standards. $C_{16}D_{31}HO_2$ and $C_{18}D_{33}H_3O_2$. These standards should improve the accuracy and precision of the used method. The chosen internal standards are chemically similar to the compounds, as one was a deuterated fatty acid, and the other one a deuterated fatty acid methyl ester. However, it was not possible to use these standards, as they showed increased RSDs, and thus a normalization of the detected peak areas of the analytes did not increase the robustness of the method. These two standards were only used after the method was optimized. During the validation, new problems with the fiber material occurred, which is discussed in the following chapter. Due to the decomposition of the fiber material during the measurements, it is not clear if this led to the high RSDs of the internal standards, or if there is another problem which negatively influenced the use of these standards.

5.3.3 Method validation

To examine the performance of the developed method, a validation was performed including linear range, limit of detection (LOD), limit of quantification (LOQ) and repeatability. The LODs and LOQs were calculated based on the signal to noise ratio of three and ten, respectively. The results are presented in Table 5-2.

The calculated LODs can be compared with a study of Cha et al., where a self-made SPME fiber was used with a sol-gel derived butyl methacrylate/hydroxy-terminated silicone oil

coating, which is not commercially available. In comparison to that study, the present method shows lower detection limits for all analytes.

Table 5-2: Validation results, including linear range, R^2, LOD, LOQ and repeatability at two concentration levels of the optimized on-fiber derivatization of fatty acids. Additionally the LODs of a comparable published study [3] are presented.

Analytes	Linear range / µg L^{-1}	R^2	LOD[a] / µg L^{-1}	LOD[a] [3] / µg L^{-1}	LOQ[b] / µg L^{-1}	Repeatability[c] / %	
						c =25 µg L^{-1}	c =75 µg L^{-1}
FAME C10	1-100	0.9561	2.0	-	6.8	28	11
FAME C12	1-100	0.9155	2.1	150.4	7.0	30	22
FAME C14	1-100	0.9608	1.6	12.8	5.4	37	46
FAME C16:1	-		3.1	-	10.5	41	36
FAME C16	1-100	0.9324	2.2	3.6	7.3	14	50
FAME C18:1	1-100	0.8807	5.4	-	17.9	50	55
FAME C17	1-100	0.9069	3.3	8.2	10.9	31	44
FAME C18	1-100	0.8750	2.4	4.78	8.0	58	49

[a] LOD calculated with S/N = 3
[b] LOQ calculated with S/N = 10
[c] Repeatability shown as relative standard deviations in %

With FAME C16:1, no linear range could be found, all other analytes showed linearity in the range of 1-100 µg L^{-1}, but the R^2-values were not satisfactory. The trend can be seen, but the variations among the replicates were too large. This behavior becomes clear as soon as the repeatability is considered. The repeatability at both concentration levels is rather poor. It was noticed, that the SPME Arrow decomposed throughout the measurements of one whole calibration curve (30 measurements). A decrease in peak areas was visible, the more samples were measured. Thus, the SPME Arrow could not reliably extract and derivatize the samples, as the phase material was destroyed from sample to sample, which explains the poor repeatabilities and R^2-values. This behavior of the SPME Arrow was already noticed at the beginning of the project, as higher concentrations (up to 10 % (v/v)) and higher amounts (up to 1mL) of the derivatization agent were preliminary tested. BF_3-MeOH was initially tested as derivatization agent as well, but with this agent, the complete sorbent phase of the SPME Arrow was degraded. As the sample matrix is water, HF can be released, when BF_3 is used [12, 42]. HF leads to the degradation of polysiloxanes, which are a part of the DVB-PDMS structure [43]. Therefore, BF_3-MeOH was excluded from further experiments. Another problem which

is discussed in literature [44] is the instability of silica-based sorbents at extreme pHs (pH < 2 or pH >8). The extraction of the fatty acids was conducted at pH 2.6, which is already near that reported limit. Additionally, the used temperature of 80 °C, increases the pH effect and thus, is even more aggressive towards the DVB-PDMS material. To check if the phase was already damaged in this step, the procedure was once stopped after the extraction, to see if the sorbent phase was swollen, which would indicate a possible reason for the instability of the SPME Arrow. As no swelling was observed, the more reasonable explanation for the fiber's degradation is the derivatization step. In this step, the conditions are very acidic and additionally a high temperature is applied. The combination of both might be the reason for the occurring problems. Nevertheless, these conditions are necessary for a successful derivatization of the fatty acids to fatty acid methyl esters.

5.3.4 Real samples

Even though the SPME Arrow starts to degrade after a few measurements, and thus the validation failed, a real sample was analyzed to check if it would be possible to extract and derivatize fatty acids from aquatic samples per se. Of course, the quantification must be treated with care, as the calibration only showed R^2-values of 0.88-0.96. Nevertheless, it was possible to detect all saturated fatty acids, which were used in this study, in high concentrations. The two unsaturated fatty acids, C16:1 and C18:1 could not be detected. The calculated concentrations are presented in Table 5-3. To match the real sample with the calibration range, the real sample was diluted, as the fatty acids were present in high concentrations. The benefit of diluting the sample is, that the matrix is diluted as well and thus scavenging effects are minimized.

Additionally, more peaks than the studied eight fatty acids were found in the chromatogram. It was possible to identify the peaks using the NIST library of the GCMS solution software from Shimadzu. The additional found fatty acids were C6, C7, C8, C13 and C15. This shows that the method works as well for the short chained fatty acids, but they would need to be confirmed by reference standards.

Table 5-3: Concentrations and measured dilutions of the different fatty acids in the leachate sample.

Analytes	Dilution	Concentration / mg L^{-1}
C10	1:500	1.6 ± 0.4
C12	1:1000	2.9 ± 0.1
C14	1:1000	7.5 ± 1.0
C16	1:500	16.3 ± 2.9
C17	1:1000	20.5 ± 7.1
C18	1:5000	10.1 ± 0.3

5.4 Conclusion

The idea of an on-fiber derivatization for aquatic fatty acid samples was successfully implemented in this study. A real sample confirmed that the successfully optimized method enables the quantification of fatty acids by a completely automated on-fiber derivatization. However, problems with the stability of the sorbent phase of the SPME Arrow occurred, which led to problems with the reproducibility of the method and which drastically shortened the SPME Arrow lifetime. Therefor it would be of special interest to either test further derivatization agents, as for example diazomethane, trimethylsulfonium hydroxide or dimethylsulfate, or to test new SPME Arrow coatings, which are not commercially available yet. The demands for these coatings would be the stability at high temperatures as well as the high tolerance for acidic conditions. Absorptive particles as DVB or activated carbon attached with polyimide glue could be a solution for the temperature problem. The research in new fiber materials or coatings is ongoing and the alternatives often provide increased surface areas, increased thermal stability and an improved wettability. Carbon nanotubes and metal organic frameworks are examples for new fiber materials [45]. Furthermore, chemical robustness should be another benefit. A completely different idea would be to automate an in-situ approach, where the derivatization takes place by addition of a derivatization agent to the aqueous sample. The derivatized fatty acids could then be extracted from the sample's headspace, which would increase the SPME Arrow lifetime, if the conditions were less acidic than in the here presented approach. The successful use of the combination of sulfuric acid and methanol in aqueous samples was shown by Wang et al. [15].

All in all, the idea of the on-fiber derivatization is promising, but further improvements are required. Changing the set-up to an in-situ derivatization followed by an extraction might be another promising option for a fully automated fatty acid analysis.

5.5 Supporting information

Table S 5-1: Factor levels and optimum solutions of the CCD for the optimization of fatty acid derivatization.

		Factor		
		Derivatization time (min)	**Derivatization temperature (°C)**	**Derivatization agent volume (µL)**
CCD levels	**-alpha[a]**	30	40	10
	-1	48	46	28
	0	75	55	55
	1	101	64	82
	alpha	120	70	100
Optimum solutions[b]	FAME C10	120	70	100
	FAME C12	120	59	100
	FAME C14	120	70	100
	FAME C16:1	120	70	10
	FAME C16	120	70	100
	FAME C18:1	120	70	100
	FAME C17	120	40	10
	FAME C18	120	40	10
	GeoMean	**120**	**70**	**82**

[a] alpha = 1.68179
[b] Calculated with normalized peak areas

Table S 5-2: Factor levels and optimum solutions of the CCD for the optimization of fatty acid extraction.

		Factor			
		Extraction time (min)	**Extraction temperature (°C)**	**Salt content (%, w/v)**	**pH value**
CCD levels	**-alpha[a]**	10	40	0	2.6
	-1	22.5	50	2	3.5
	0	35	60	4.2	4.4
	1	47.5	70	6.25	5.3
	alpha	60	80	8.3	6.2
Optimum solutions[b]	FAME C10	60	80	0	2.6
	FAME C12	11	80	8.3	2.6
	FAME C14	32	80	8.3	2.6
	FAME C16:1	60	80	8.3	2.6
	FAME C16	60	80	8.3	2.6
	FAME C18:1	60	64	0	6.2
	FAME C17	60	80	0	6.2
	FAME C18	60	80	8.3	2.6
	GeoMean	**60**	**80**	**8.3**	**2.6**

[a] alpha = 2

[b] Calculated with normalized peak areas

Table S 5-3: Factor levels and optimum solutions of the CCD for the optimization of fatty acid methyl ester desorption.

		Factor	
		Desorption time (min)	**Desorption temperature (°C)**
CCD levels	**-alpha[a]**	1	230
	-1	1.6	233
	0	3	240
	1	4.4	247
	alpha	5	250
Optimum solutions[b]	FAME C10	2	243
	FAME C12	2.9	243
	FAME C14	4.8	249
	FAME C16:1	5	244
	FAME C16	5	230
	FAME C18:1	2.8	240
	FAME C17	5	250
	FAME C18	5	230
	GeoMean	**4.9**	**250**

[a] alpha = 1.41421
[b] Calculated with normalized peak areas

Table S 5-4: Results of internal standard check for complete reaction or saponification during the CCD of the derivatization. Numbers presented in bold do not pass the test.

Time /min	Volume / µL	Temperature / °C	Complete reaction (>0.75)	No saponification (>0.67)
48	28	46	**0.35**	34.17
102	28	46	**0.25**	10.15
48	82	46	**0.47**	12.23
102	82	46	1.33	26.98
48	28	64	3.81	56.64
102	28	64	0.77	17.16
48	82	64	7.21	17.19
102	82	64	28.27	19.98
30	55	55	**0.56**	20.36
120	55	55	20.60	22.02
75	10	55	0.79	20.57
75	100	55	7.39	36.32
75	55	40	1.52	33.17
75	55	70	4.15	17.34
75	55	55	82.80	498.05
75	55	55	3.55	52.81
75	55	55	7.33	63.42
75	55	55	**0.44**	19.30
75	55	55	0.91	13.06
75	55	55	0.33	16.22

Table S 5-5: Results of internal standard check for complete reaction or saponification during the CCD of the extraction. Numbers presented in bold do not pass the test.

Time /min	Temperature / °C	Salt c / % (w/v)	pH	Complete reaction (>0.75)	No saponification (>0.67)
35	60	4.2	4.4	2.01	39.58
35	40	4.2	4.4	1.33	34.77
22.5	50	2.0	3.5	15.60	23.08
47.5	50	6.25	3.5	8.29	105.21
22.5	70	6.25	5.3	5.60	198.66
47.5	70	6.25	3.5	3.62	17.56
47.5	50	2.0	3.5	1.05	30.06
35	60	4.2	4.4	**0.46**	11.98
22.5	50	6.25	5.3	1.05	11.61
35	60	4.2	4.4	**0.14**	3.67
10	60	4.2	4.4	13.01	141.59
35	60	4.2	2.6	7.70	49.72
47.5	50	6.25	5.3	1.01	6.12
22.5	70	6.25	3.5	2.82	28.10
35	60	0.00	4.4	**0.39**	7.74
22.5	70	2.0	3.5	1.74	23.23
35	80	4.2	4.4	0.38	15.44
35	60	8.3	4.4	4.96	21.63
47.5	50	2.0	5.3	7.29	**0.16**
35	60	4.2	4.4	**0.11**	1.19
47.5	70	6.25	5.3	0.86	25.09
35	60	4.2	4.4	5.63	15.50
22.5	50	2.0	5.3	0.90	12.36
47.5	70	2.0	3.5	1.25	17.40
22.5	70	2.0	5.3	1.63	1.39
60	60	4.2	4.4	0.75	27.06
35	60	4.2	4.4	1.66	34.60
35	60	4.2	4.4	0.96	12.98
47.5	70	2.0	5.3	0.93	17.32
35	60	4.2	6.2	4.65	81.92
22.5	50	6.25	3.5	11.58	54.82

Table S 5-6: Results of internal standard check for complete reaction or saponification during the CCD of the desorption. Numbers presented in bold do not pass the test.

Time /min	Temperature / °C	Complete reaction (>0.75)	No saponification (>0.67)
4.00	232.9	2.36	36.15
3.00	240.0	0.78	26.60
3.00	250.0	0.76	30.50
3.00	240.0	**0.53**	21.69
3.00	240.0	**0.57**	24.13
2.00	247.1	**0.47**	24.81
4.00	247.1	0.80	31.05
3.00	230.0	18.64	68.48
5.00	240.0	3.78	53.41
1.00	240.0	0.80	37.28
2.00	232.9	0.81	24.24
3.00	240.0	0.89	41.90
3.00	240.0	**0.54**	31.31

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6 Optimization and Validation of Automated Solid-Phase Microextraction Arrow Technique for Determination of Phosphorus Flame Retardants in Water

This chapter was adapted from: *W. Kaziur-Cegla, A. Salemi, M.A. Jochmann, T.C. Schmidt, Optimization and validation of automated solid-phase microextraction arrow technique for determination of phosphorus flame retardants in water, Journal of Chromatography A 1626 (2020) 461349.*

Abstract

In the present work, a very sensitive and fully automated direct immersion PAL SPME Arrow method, coupled with GC-MS, has been developed and validated for the determination of nine phosphorus flame retardants in different types of water samples (river, drinking and rainwater). PDMS-DVB was selected among three commercially available SPME Arrows, since it resulted in the best sensitivity. Three experimental parameters were optimized via a central composite design response surface methodology and resulted in an extraction time of 65 min, an extraction temperature of 80 °C and an added salt concentration of 19 % (w/v). The optimized method showed linear response over the calibration range (2 – 500 ng L^{-1}), with R^2-values higher than 0.9937. The precision (RSD %) measured by replicate analyses (n = 7) was estimated at 2 and 100 ng L^{-1} and was less than 29 % and 21 %, respectively. The LOQ of PAL SPME Arrow, calculated as S/N = 10, was between 0.2 and 1.2 ng L^{-1} (for triphenyl phosphate and tris-(1-chloro-2-propyl) phosphate, respectively) with extraction efficiencies between 5.9 and 31 % (for tris-(1,3-dichloro-2-propyl) phosphate and tri-n-butyl phosphate, respectively). To assess the performance of the developed technique for real samples, two river water samples, tap water from two regions and a rainwater sample were analyzed. Most of the target analytes were observed in the river samples with concentrations of 1.0 – 250 ng L^{-1} and the obtained recoveries at 50 ng L^{-1} ranged between 60 and 107 %. Considering the figures of merit of the optimized method, PAL SPME Arrow-GC-MS showed to be the most sensitive analytical approach for the determination of phosphorus flame retardants in water, with satisfying precision and accuracy, compared with conventional SPME-NPD, LLE-GC-MS and SPE-LC-MS/MS.

6.1 Introduction

Organic derivatives of phosphoric acid have gained widespread applications as flame retardants, antifoaming agents and plasticizers, and their usage has increased even more after the limitations exerted on polybrominated diphenyl ethers (PBDEs), due to their adverse environmental and health effects [1, 2]. Although in a general view phosphorus flame retardants (PFRs) are considered to be less harmful than PBDEs, adverse biological effects, such as endocrine disruption, hemolytic and reproductive effects, have been observed [3-6]. In many applications, PFRs are not chemically bonded to other components of the formulation, such as in electronic devices, building construction material and polymer foams. As a result, these additives can leach out over time and thus be released into the environment [7]. A wide range of log K_{ow} (from -9.8 to 10.6) has been reported for PFRs [8], and hence, while some of the PFR members could be easily dissolved and transported by water, other compounds act completely hydrophobic and tend to exit aqueous media, through adsorption and/or absorption processes. This diversity of physicochemical properties has also been observed for gas phase related physicochemical parameters of PFRs, such as their Henry's law constants and vapor pressures [7]. Therefore, different PFRs may be detected in various environmental media, regarding the dominant equilibrium (distribution) process in each matrix. Studies have shown the presence of PFRs in various water samples [9-14], among those different drinking water samples could be mentioned, with concentrations up to 192 ng L^{-1} [11].

Various gas chromatography instrumentations have been reported for determination of PFRs, using different detectors, such as flame photometric (FPD) [15], nitrogen-phosphorus (NPD) [16-18], mass spectrometric (MS) [13, 14, 19] and tandem mass spectrometric (MS/MS) [12, 20] detection systems. GC separation of the analytes has been normally performed on non-polar stationary phases, mainly on polydimethylsiloxane with 5 % phenyl replacement. Liquid chromatography-mass spectrometry (LC-MS) [21], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [11, 22, 23] and liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS) [24], have also been successfully used for this purpose, using non-polar C8 and C18 analytical columns. Several sample preparation and extraction techniques have been developed and applied for PFRs, depending on the sample type (gas, solid, aqueous, etc.) and the analytical instrumentation (GC or LC). However, considering the aim of the present study, the following examples have been restricted to the aqueous samples. Successful application of classical liquid-liquid extraction (LLE) [25-27] and solid-phase extraction (SPE) [9, 10, 28, 29] has been reported for PFRs analysis. However, some

disadvantages, such as the need for high volumes of sample and also, the fact that only a small fraction of the extract is normally introduced into the chromatographic system, show the importance of using microextraction techniques, such as solid-phase microextraction (SPME) [17].

The most impressive advantages of the SPME technique are its capability to be operated in a solvent-free manner of operation, as well as its considerable sensitivity due to the direct desorption of all extracted analytes into the analytical (usually GC) system. However, fragility of the sorbent containing fibers of the conventional SPME is a significant drawback. Also, small dimensions and low volume of the sorbent material can limit the sensitivity of the method. To overcome such drawbacks, a PAL (Prep And Load solution) SPME Arrow, was developed and presented [30, 31]. In brief, PAL SPME Arrow can provide at least one order of magnitude better sensitivity, due to the larger volume of the sorbent. In addition, the design and structure of this extraction device significantly improve its robustness and mechanical resistance. The arrow-like tip penetrates more easily through the vial and GC septa, as the pressure is more evenly applied. Furthermore, the diameter of the PAL SPME Arrow is larger than the classical SPME (1.5 mm in comparison to approximately 0.7 mm), which leads to less bending, when the sample is agitated and extracted at the same time [30]. The analytical performance of PAL SPME Arrow coupled with GC-MS has been recently demonstrated for determination of sub-ng L^{-1} concentration of taste and odor compounds in surface water samples, [32] and has also gained interest in a few further applications for various matrices and analytes [33-40].

However, PAL SPME Arrow as a rather new solvent-free microextraction technique has only been studied for a limited number of analytes. Therefore, finding its optimum behavior, its potential capabilities for trace analysis, and the effects of its significant improvements (in terms of sorbent volume and mechanical stability) in comparison with the classical SPME, are all adequate reasons for further scientific investigation. In the present study, an optimized fully automated PAL SPME Arrow-GC-MS method was developed for determination of nine PFRs in water sample. The most important experimental parameters that could have significant effects on the performance characteristics of the entire method (i.e. Arrow type, extraction time and temperature and added salt concentration) were optimized using combination of one-at-a-time and response surface methodology, to find the best set of conditions for simultaneous analysis of the target PFRs. Finally, real water samples were investigated in order to evaluate the precision and accuracy of the optimized method in real matrices and also for checking the possible presence of the analytes.

6.2 Materials and methods

6.2.1 Reagents and materials

Tri-*n*-Butyl phosphate (TnBP, >99 %), triphenyl phosphate (TPP, 99 %), tris-(1,3-dichloro-2-propyl) phosphate (TDCPP, >95 %), tris-2-ethylhexyl-phosphate (TEHP, >97 %), tris-m-cresyl phosphate (mTCP, >97 %) and tris-o-cresyl phosphate (oTCP, >97 %) were purchased from Sigma-Aldrich (Steinheim, Germany), tri-*iso*-butylphosphate (TiBP, >90 %) from Merck (Darmstadt, Germany), and tris-(1-chloro-2-propyl) phosphate (TCPP, 99 %) and tris-p-cresyl phosphate (pTCP, >98 %) from TCI (Eschborn, Germany). Methanol (99.8 %, Fisher Scientific, Loughborough, UK) was used to prepare stock solutions of 1 g L^{-1} of each analyte. A mixed standard solution of all 9 analytes was prepared by mixing and diluting the single stocks with methanol to a final concentration of 50 mg L^{-1} and was used to construct spike samples in water. Stock solutions were stored at 4 °C. Pure water, used to prepare spiked standard solutions for the optimization and method validation experiments, was produced by a PURELAB Ultra analytic water purification system (ELGA, LabWater, Germany). Sodium chloride (> 99.5 %) for adjusting the ionic strength of the sample solutions was from Bernd Kraft (Duisburg, Germany).

6.2.2 GC-MS instrumentation

Separation and quantification of the analytes was conducted using a Shimadzu GCMS-QP2010 Ultra (Shimadzu Deutschland GmbH, Germany). The injector was modified to enable SPME Arrow injection and was equipped with a 2 mm i.d. × 5 mm o.d. × 95 mm length splitless liner from BGB Analytik (Böcken, Switzerland). The regular GC septum retainer nut had also a wide orifice to permit penetration of SPME Arrow into the septum. Thermal desorption was performed in splitless mode for 3 min at 270 °C, after which the inlet was switched to split mode (1:10). A SGE BPX5 column (30 m × 0.25 mm × 0.25 µm; Trajan, Australia) was used for analyte separation, using helium (5.0, AirLiquide, Oberhausen, Germany) as carrier gas at a constant flow rate of 0.95 mL min^{-1}. The temperature program started at 100 °C (3 min hold time) and was raised to 180 °C with a rate of 20 °C min^{-1} and then to 280 °C with a rate of 5 °C min^{-1}, where it was held for another 4 min. MS transfer line and ion source temperatures were set to 280 °C and 250 °C, respectively. Quantitative analyses were performed in selected ion monitoring (SIM) mode. Selected ion fragments for detection and quantification of the analytes are listed in Table S 6-1.

6.2.3 Extraction procedure

A PAL RTC autosampler (CTC Analytics AG, Zwingen, Switzerland), equipped with a parking station, a tray holder, a heating plate (IKA-Mag RCT basic; IKA-Werke GmbH & Co KG, Staufen, Germany), a SPME Arrow Conditioning module and three SPME Arrow Tools was used for the extraction of the analytes (see "Optimization procedure" for description of the Arrows). The extraction procedure started by transferring the 20-mL septum capped sample vial, containing 18 mL of the sample, onto the heating/stirring plate, where it was conditioned for 10 min to reach thermal equilibrium and complete dissolution of the added salt (0-30 % w/v), as described in the following section (optimization procedure). The temperature was manually set according to the design of experiments (40-80 ^{o}C) and magnetic stirring was kept constant at 800 rpm for all samples. After conditioning time, the SPME Arrow penetrated the vial septum and extracted the analytes via direct immersion for a predetermined time (6-74 min). Afterwards, the Arrow was withdrawn, washed by immersing the fiber for 2 min in a vial containing pure water and was then thermally desorbed in the GC-MS inlet. Subsequently, the SPME Arrow was conditioned in the conditioning module at 270 °C for 8 min to ensure a clean phase for the next sample.

6.2.4 Optimization procedure

Selection of the best SPME Arrow was performed using a one-factor-at-a-time strategy. Arrows with polydimethylsiloxane/carbon wide range (PDMS/CWR), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and divinylbenzene/ polydimethylsiloxane/carbon wide range (DVB/PDMS/CWR) sorbents, all from BGB Analytik, were selected for this purpose. All Arrows were of the same dimensions, with diameter of 1.1 mm, film thickness of 120 µm, sorbent volume of 11.8 µL and surface area of 62.8 mm^2. An aqueous sample of the analytes at 10 µg L^{-1} concentration was extracted (five replicates with each Arrow) at 40 °C for 30 min and the resulting peak areas were used for comparison.

A rotatable (axial distance = 1.68179) central composite design, CCD, with 20 runs (2^3 factorial + 6 star points and involving 6 center points, d.f. = 10) was selected for the optimization of the extraction procedure, based on extraction time, extraction temperature and salt content of the samples, as quantitative factors (Table S 6-2). Design-Expert® (Stat-Ease, Min, USA) was used for design of the experiments and processing the data. After performing

the optimization experiments, the peak areas of the analytes were normalized (Microsoft Excel®) and transferred into the software, together with the geometric mean of the normalized data. The normalization process was carried out by dividing the peak area of each individual analyte by its maximum value throughout the entire set of (CCD) experiments. The simultaneous extraction of the analytes was optimized based on the geometric mean, as the main response.

6.2.5 Real water samples

Surface water samples were grabbed from Ruhr River, Wupper River and a local small stream (Velbert-Neviges), all in North Rhine-Westphalia, Germany. Two samples of tap water were collected from Velbert-Neviges and Essen, both in North Rhine-Westphalia, Germany. The rainwater sample was also collected in Essen. All samples were analyzed for the presence and quantity of the target analytes. To estimate the accuracy of the method and evaluate the effect of the sample matrix, the relative recoveries of the optimized method were calculated for target analytes. The sample was divided in two portions and one portion was spiked with the individual analytes, at 50 ng L^{-1}. Two portions were extracted and analyzed in duplicate, without filtration. The reported detected and recovery values for the developed method were calculated by comparing the results obtained from analysis of this sample with the calibration curve.

6.3 Results and discussion

6.3.1 Optimization of the extraction procedure

General considerations

In many cases, as in the present study, a wide range of volatilities of the set of targets, although members of the same class of compounds, limits the analyst's choices, especially between headspace (HS) and direct immersion (DI) modes of extraction. This fact has been thoroughly studied for PFRs [17] and therefore, a direct immersion mode of extraction has been selected in the present work. Also, since high concentrations of the added salt (NaCl, up to 30 %, w/v) have been tested during the optimization, a washing/clean up step was added to the extraction procedure to prevent deposition of solid salt residues on the fiber and to avoid desorption disruption.

It is well known that stirring the sample solution has a significant effect on shortening the time needed for equilibrium between aqueous sample and the sorbent. However, preliminary studies showed that high stirring rates would lead to poor reproducibility of DI-SPME, with RSDs up to 40 % at a concentration level of 10 µg L^{-1} (detailed data not shown). Further examination of the case revealed that at high stirring rates the SPME Arrow was not completely immersed in sample solution, even when the extraction vial was filled to the maximum (18 mL) and the maximum penetration depth (of the automated extraction setup) was implemented. In fact, a part of the SPME Arrow length remained out of the sample solution (in the vortex region). So, a slower stirring rate (800 rpm) was set for the extraction, to keep the Arrow completely in contact with the sample, which resulted in an improved precision of the replicate analyses.

Then, as described below, SPME Arrow (sorbent) type, extraction time, sample temperature and salting out effect were studied to find the optimum experimental conditions of PAL SPME Arrow extraction of the target analytes.

Selection of SPME Arrow sorbent

The effect of the SPME sorbent type on the extraction performance was studied by comparing the peak area of the analytes obtained from extraction with each Arrow. The sample was pure water spiked at 10 µg L^{-1} of each analyte and kept at 50 °C during the experiments. Figure 6-1 (columns) demonstrates the results of this study, where each column represents the mean of five replicate extractions. The mean peak area of each individual compound has been normalized based on its maximum peak area among three Arrows, to make the comparison clearer. In other words, a column height of one for a specific analyte on an Arrow, means that this Arrow yields the best extraction performance for that compound, compared with other Arrows. Therefore, it can be seen that PDMS/DVB showed the best performance for seven out of nine target analytes, as well as very good response for TCPP and TDCPP. On the other hand, PDMS/CWR and DVB/PDMS/CWR demonstrated relatively better extraction performance for the more volatile analytes (i.e. compounds that had shorter GC retention times).

A similar behavior had been previously observed for a different set of analytes [32]. Compared with other Arrows, PDMS/CWR was the best choice for TCPP, with considerable efficiency for TiBP and TnBP. Finally, DVB/PDMS/CWR Arrow provided the maximum peak area for TDCPP and good to moderate responses for the other analytes. Comparing the obtained

results, as relative peak areas (Figure 6-1), with polarity of the compounds, as octanol-water partitioning constant (Table S 6-3), showed that Arrows containing PDMS/CWR and DVB/PDMS/CWR had better performance for more polar compounds (lower logK_{ow}). In contrast, PDMS/DVB Arrow showed a more uniform extraction performance with less discrimination between compounds of different polarity.

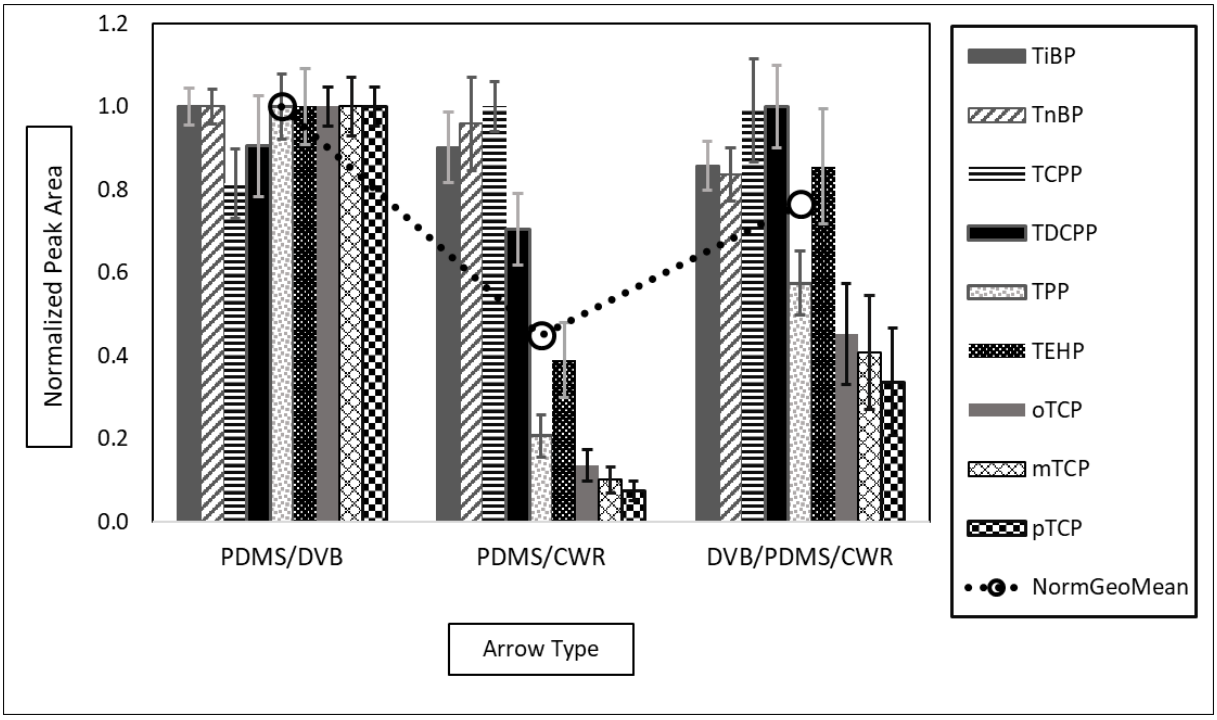

Figure 6-1:Relative extraction efficiencies of three PAL SPME Arrow fibers for individual analytes and geometric means of normalized peak areas. Error bars on geometric means demonstrate the average RSD % of the analytes for each Arrow.

The general pattern observed in Figure 6-1 and/or merely, the number of the analytes with maximum peak areas belonging to each fiber, may be enough to compare the Arrows and select the best one. However, geometric means of the normalized peak areas can provide a better measure for comparison. As has been demonstrated by the dotted line in Figure 6-1, the geometric mean of responses (normalized based on its maximum) had the highest value for PDMS/DVB, showing that this Arrow was the best choice for simultaneous extraction of the target analytes. Error bars on the geometric mean line (vertical lines) show the relative standard deviation of peak areas for each Arrow and were used as a criterion for comparing the uniformity of Arrows efficiency for different analytes. Clearly, PDMS/DVB, besides higher sensitivity and precision (calculated as the average of RSD values of the analytes for each

Arrow and shown by error bars in Figure 6-1), provided similar extraction efficiency for the entire set of analytes and hence, was selected for the rest of the study.

Central composite design

After conducting the designed experiments (20 runs), the peak area data of the compounds were normalized and transferred to the optimization software, together with the corresponding geometric mean values. In this step, it was possible to calculate the optimum set of factor levels, both for the geometric mean (simultaneous extraction) and for the individual analytes (Table S 6-2). The corresponding design models have been summarized in Table S 6-4 (with a p-value of 0.5086 for lack of fit of the model). Figure 6-2 shows the response surfaces obtained for the geometric mean of the responses and demonstrates the variation in the efficiency of the simultaneous extraction of the analytes with changes in the experimental factor levels.

Based on the calculated optimum solution, the maximum extraction efficiency was achieved at 80 °C (the maximum level). Using the model, the geometric mean of the responses was calculated at 90 °C and only a 4 % increment was observed (that was less than relative standard deviation of the central points of the design, 23.6 %), while it may decrease the working lifetime of the Arrow sorbent. So, more extreme extraction conditions, i.e. higher temperatures, were not practically investigated. The value obtained for the salt content of the sample solution was clearly a compromise of the different behavior of the selected PFRs. TCP isomers were best extracted from solutions with no added salt, while compounds like TCDPP and TCPP, needed a high ionic strength (Table S 6-2). Also, 65 min was sufficient to achieve the maximum sensitivity. It must be noted that since the response of these surfaces is the geometric mean of the individual analyte peak areas, an accurate interpretation of the response variation based on the physico-chemical properties of the compounds, is not possible. However, the optimized solution for the geometric mean of the responses, is a useful tool to find the best practically available set of experimental conditions for the maximum extraction efficiency. Thus, the rest of the experiments in this study was conducted at the calculated optimum conditions, i.e., 65 min of extraction at 80 °C from a sample containing 19 % of sodium chloride.

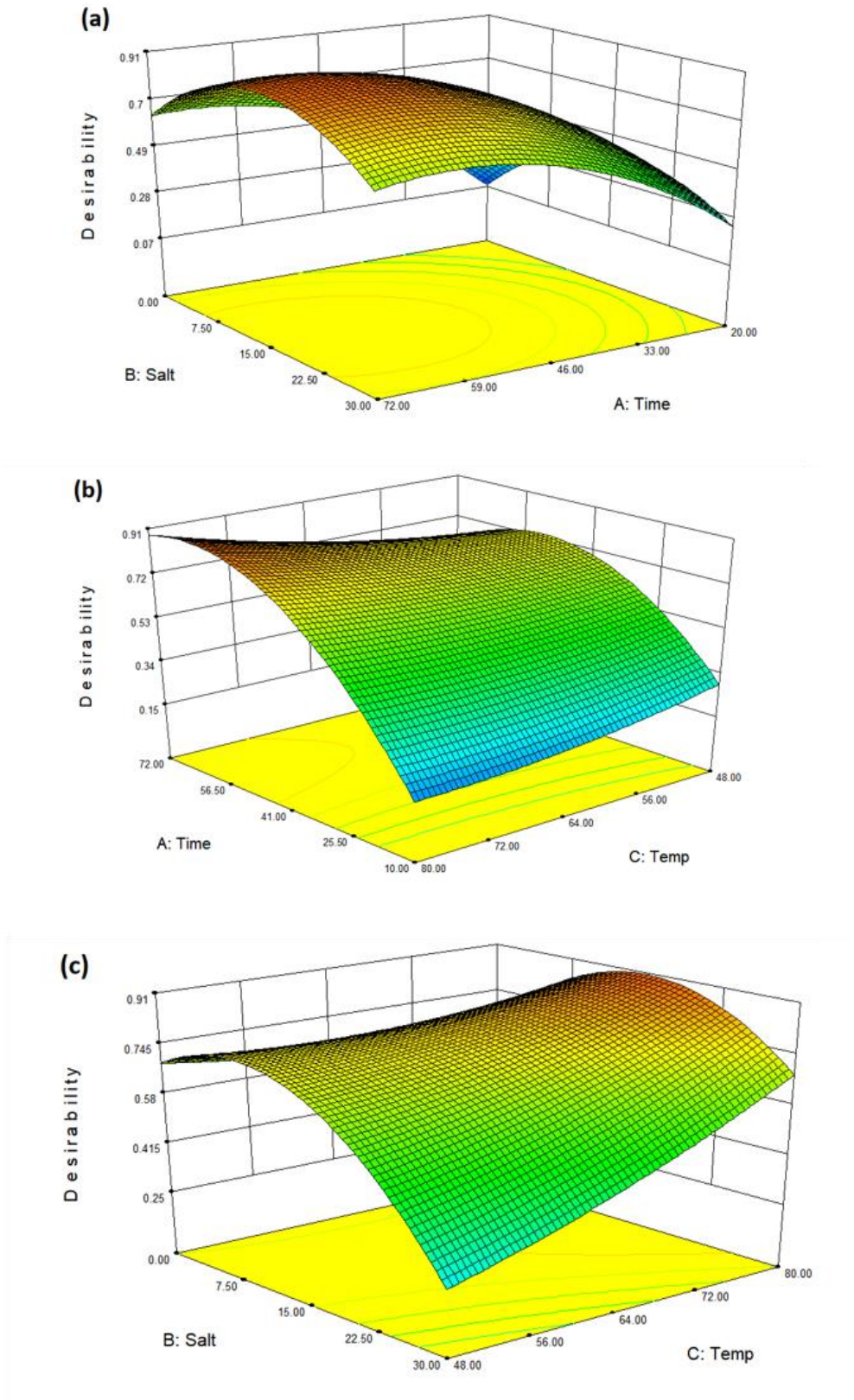

Figure 6-2: Response surfaces obtained using CCD, showing the dependence of extraction efficiency on pairs of experimental factors; (a) salt concentration and extraction time, (b) extraction temperature and time and (c) salt concentration and extraction temperature.

6.3.2 Analytical performance characteristics

Calibration curves for the quantitative analysis of the target PFRs were obtained via analysis (n = 5) of a set of calibration samples at six concentration levels ranging between 2 and 500 ng L^{-1}. Linearity of the response for all analytes was observed, as indicated in Table 6-1 (R^2 values > 0.9937). The LOQ values were calculated based on S/N = 10 (using the baseline noise in the closest practically possible time intervals) and showed the excellent sensitivity of the method for the selected PFRs with LOQs of 0.18 – 1.24 ng L^{-1} for TPP and TCPP, respectively (Table 6-1). Precision of the method was also evaluated based on replicate analysis (n = 7) of the pure water spiked at two concentration levels of 2 and 100 ng L^{-1} (with the maximum value of 29 % for mTCP at 2 ng L^{-1}) and reasonable repeatability was observed, in particular considering the low concentration levels used for this purpose.

A set of experiments was designed and conducted to estimate the efficiency of extraction (*E*), using the depletion curve method [41]. Two pure water samples were spiked at 50 ng L^{-1}, successively analyzed (n = 10) and the logarithms of peak areas were plotted against the run number, so that the slope of each trend line was equal to log (1-*E*). The calculated extraction efficiencies are reported in Table 6-1, as well as the coefficient of determination of the corresponding trend lines. The calculated values showed that at the optimum conditions of extraction, between 5.9 and 31.0 % of the analytes were removed from the sample (18 mL) after each extraction. The coefficient of determination values (R_E^2) showed that the extraction efficiency values remain almost constant along with decreasing concentration.

Table 6-1: Analytical performance characteristics of PAL SPME Arrow for PFR compounds.

	R^2 [a]	LOD[b] (ng L^{-1})	LOQ[b] (ng L^{-1})	RSD[c] (%)		Extraction efficiency		Recovery %	Surface water[f]
				2 ng L^{-1}	100 ng L^{-1}	R_E^2 [d]	E (%)	(RPD)[e]	(ng L^{-1})
TiBP	0.9972	0.11	0.35	18	13	0.9977	30.9	79 (8)	84.4
TnBP	0.9955	0.11	0.37	10	15	0.9985	31.0	60 (8)	17.0
TCPP	0.9961	0.38	1.24	6	9	0.8967	11.6	74 (8)	249.5
TDCPP	0.9939	0.10	0.33	16	12	0.8723	5.9	71 (14)	59.3
TPP	0.9947	0.06	0.18	9	16	0.9179	22.1	89 (22)	15.0
TEHP	0.9937	0.12	0.39	24	20	0.9345	23.1	96 (2)	5.6
oTCP	0.9989	0.09	0.30	23	19	0.7401	10.8	78 (36)	5.0
mTCP	0.9976	0.06	0.19	29	21	0.7758	17.4	71 (11)	N.D.
pTCP	0.9971	0.09	0.31	20	21	0.8179	10.3	107 (50)	1.0

[a] Over the range of 2 – 500 ng L^{-1}.
[b] Based on S/N = 3 for LOD and S/N = 10 for LOQ, based on the lowest point of the calibration.
[c] Based on 7 replicate analyses of spiked pure water.
[d] Correlation coefficient of the depletion curve. See 6.3.2 and reference [42] for more details.
[e] Recovery calculated based on duplicate analysis of real sample spiked at 50 ng L^{-1}. Relative percent difference values have been indicated in parentheses.
[f] Average of duplicate analysis of river Ruhr water sample.

6.3.3 Real sample analysis

The effect of sample matrix was studied by the analysis of river water sample (Ruhr River). The relative recovery values (

Table 6-2) obtained from extraction of the real sample spiked at 50 ng L^{-1}, between 71 and 107 % for mTCP and pTCP, respectively, showed satisfying accuracy of the SPME Arrow GC-MS technique, in spite of matrix interference in direct immersion mode (Figure S 6-1 and Figure S 6-2). Seven out of nine analytes were also detected in the native river sample with concentrations in the calibration range of the method. The results of analyses of other real samples, in terms of detected values and the corresponding precisions are summarized in

Table 6-2. None of the target analytes were detected in tap water samples. TiBP and TnBP were observed in the collected rain water sample with concentrations of 64.2 and 54.6 ng L^{-1}, respectively. It has been shown that the volatilization of PFRs is a major path of releasing

these compounds into the atmosphere and hence to the aquatic environments, via precipitation [42]. The second river (Wupper) water sample also showed presence of target PFRs, but compared with Ruhr River, only five analytes were observed at lower concentrations (except TnBP that had higher contamination level). Incomplete removal of PFRs during wastewater treatment processes [43] and washout of atmospheric pollution via rain and snow [42] have been reported as the main sources of surface water contamination by PFRs.

6.3.4 Comparison with other methods

Table S 6-5 compares the performance characteristics of three alternative methods [10, 17, 44], with a similar set of analytes, with the presented PAL SPME Arrow GC-MS. The two SPME techniques have used the lowest (and similar, 18 and 22 mL) sample volumes, as it is one of the advantages of SPME, regarding its sensitivity due to the (nearly) complete transfer of the extracted analytes into the analytical instrument. SPE and LLE have been calibrated over larger calibration ranges, compared with SPME and SPME Arrow method. Detailed data showed that the method precision (as RSD %) varies between different analytes, however, SPE-LC-MS/MS showed the best repeatability and the other techniques were of the same level of precision. Sensitivity, expressed as LOQ values, was the greatest advantage of the present SPME Arrow over other methods.

Table 6-2: Results of real sample analysis.

Sample	**Stream (Neviges)**		**Tap water (Essen)**		**Tap water (Neviges)**		**Rain water**		**River water (Ruhr)**		**River water (Wupper)**	
Analyte	c ng L^{-1}	RSD % [a]	c ng L^{-1}	RSD %	c ng L^{-1}	RSD %	c ng L^{-1}	RSD %	c ng L^{-1}	RPD[c] %	c ng L^{-1}	RSD %
TiBP	113.3	14.9	nd	-	nd	-	64.2	7.6	84.4	8.3	19.3	13.5
TnBP	46.6	16.3	nd	-	nd	-	54.6	22.5	17.0	8.2	46.2	17.3
TCPP	nd[b]	-	nd	-	nd	-	nd	-	249.5	8.2	nd	-
TDCPP	nd	-	nd	-	nd	-	nd	-	59.3	13.8	1.6	25.7
TPP	nd	-	nd	-	nd	-	nd	-	15.0	22.2	nd	-
TEHP	6.2	32.0	nd	-	nd	-	nd	-	5.6	1.9	8.2	8.2
oTCP	0.51	21.7	nd	-	nd	-	nd	-	5.0	35.6	0.48	23.3
mTCP	nd	-	nd	-	nd	-	nd	-	nd	-	nd	-
pTCP	nd	-	nd	-	nd	-	nd	-	1.0	49.8	nd	-

[a] Based on three replicate analyses.
[b] Not detected.
[c] Relative percent difference values of duplicate analyses.

6.4 Conclusion

An automated technique was introduced for determination of PFRs in water samples based on coupling PAL SPME Arrow with GC-MS. The optimized and validated PAL SPME Arrow technique, benefitted from a mechanically strong sorbent support structure with higher sorbent volume, compared with conventional SPME fibers that lead to excellent sensitivity of the analysis. Despite direct contact of the fiber with sample matrix (in direct immersion mode of SPME), satisfying recoveries were obtained from analysis of unfiltered river water. The entire process of extraction, consisting of conditioning, extraction and cleaning (washing the salt residue from the sorbent), took 75 min and completely overlaps the GC-MS run. Due to the full automation, the system can run 24/7 and up to 19 samples can be automatically extracted and analyzed per day.

Sensitivity of the developed method was superior compared with other reported methods and the linear calibration range covered the concentration ranges, which are frequently reported for PFRs. The small sample volume required for the analysis was another advantage of the introduced method and can significantly facilitate the sample collection, storage and transport, during the monitoring programs.

6.5 Supporting information

Table S 6-1: Selected ion fragments for analysis of PFRs.

	Ion fragment		
	1	**2**	**3**
TiBP	95	**155**	211
TnBP	95	**155**	211
TCPP	99	125	**277**
TDCPP	75	99	**191**
TPP	77	325	**326**
TEHP	57	**99**	113
oTCP	165	**368**	-
mTCP	165	367	**368**
pTCP	165	367	**368**

[a] Ion fragments shown in bold have been used for quantitation purpose.

Table S 6-2: Factor levels in the CCD optimization procedure and the resulting solutions.

		Factor		
		Extraction time (min)	**Extraction temperature (°C)**	**Salt content (%, w/v)**
CCD levels	**-alpha[a]**	6	40	0
	-1	20	48	6
	0	40	60	15
	1	60	72	24
	alpha	74	80	30
Optimum solutions	TiBP	67	72	18
	TnBP	68	78	15
	TCPP	72	40	30
	TDCPP	72	70	24
	TPP	69	52	9
	TEHP	67	40	11
	oTCP	53	80	0
	mTCP	72	40	0
	pTCP	72	40	0
	GeoMean[b]	**65**	**80**	**19**

[a] alpha = 1.68179

[b] Geometric mean of the normalized peak area

Table S 6-3: Octanol-water partitioning constant values of the analytes.

Analyte	logK_{ow}	Reference
TiBP	3.6	1
TnBP	4.00	2
TCPP	2.59	2
TDCPP	3.65	2
TPP	4.59	2
TEHP	9.49	2
oTCP	6.34	2
mTCP	6.34	2
pTCP	6.34	2

Reference 1: chemspider.com
Reference 2: pubchem.ncbi.nlm.nih.gov

Table S 6-4: Coefficients of the model for individual analytes and the geometric mean.

Factor	Intercept	A	B	C	A*B	A*C	B*C	A^2	B^2	C^2
Coefficient	**a_0**	**a_1**	**a_2**	**a_3**	**a_{12}**	**a_{13}**	**a_{23}**	**a_{11}**	**a_{22}**	**a_{33}**
TiBP	0.810	0.190	0.016	0.044	-0.002	0.063	0.063	-0.069	-0.099	-0.057
TnBP	0.780	0.180	-0.016	0.045	-0.007	0.063	0.070	-0.061	-0.088	-0.034
TCPP	0.500	0.062	0.220	-0.014	0.009	0.043	0.006	-0.021	0.027	-0.056
TDCPP	0.730	0.210	0.010	-0.019	0.030	0.021	0.081	-0.053	-0.058	-0.064
TPP	0.720	0.220	-0.130	0.024	-0.044	0.042	0.120	-0.084	-0.069	0.019
TEHP	0.620	0.160	0.017	0.030	-0.054	0.014	0.080	-0.056	-0.120	0.110
oTCP	0.480	0.054	-0.140	0.056	0.001	0.083	-0.004	-0.180	-0.027	0.080
mTCP	0.410	0.130	-0.170	-0.033	-0.065	0.001	0.027	-0.100	-0.011	0.057
pTCP	0.490	0.140	-0.160	-0.030	-0.071	-0.013	0.058	-0.120	-0.028	0.045
Geometric mean	0.700	0.170	-0.056	0.004	-0.017	0.048	0.054	-0.110	-0.088	0.018

The compound specific model for each analyte can be constructed by inserting the corresponding coefficients in the following model, where A is extraction time, B is salt content of the sample and C is extraction temperature (all in coded units).

$$\text{Response} = a_0 + a_1*A + a_2*B + a_3*C + a_{12}*AB + a_{13}*AC + a_{23}*B*C + a_{11}*A^2 + a_{22}*B^2 + a_{33}*C^2$$

Table S 6-5: Comparison of the developed PAL SPME Arrow with other methods for analysis of PFRs.

Method	Common analytes	Sample volume (mL)	Calibration range (ng L^{-1})	RSD (%)	LOQ (ng L^{-1})	Ref.
LLE-GC-MS	TCPP, TDCPP, TnBP, TiBP, TPP	No data [a]	2-10000	<27	4.9-14	[44]
SPE-LC-MS/MS	TnBP, TEHP, TDCPP, TCPP, mTCP	200	100-100000	<9	2-6	[10]
SPME-GC-NPD	TiBP, TnBP, TCPP, TDCPP, TPP, TEHP	22	50-5000	<34	10-15	[17]
PAL SPME Arrow	TiBP, TnBP, TCPP, TDCPP, TPP, TEHP, oTCP, mTCP, pTCP	18	2-500	<29	0.2-1.2	Present work

[a] Only extraction solvent volume has been reported in the referenced work as 10 mL of toluene.

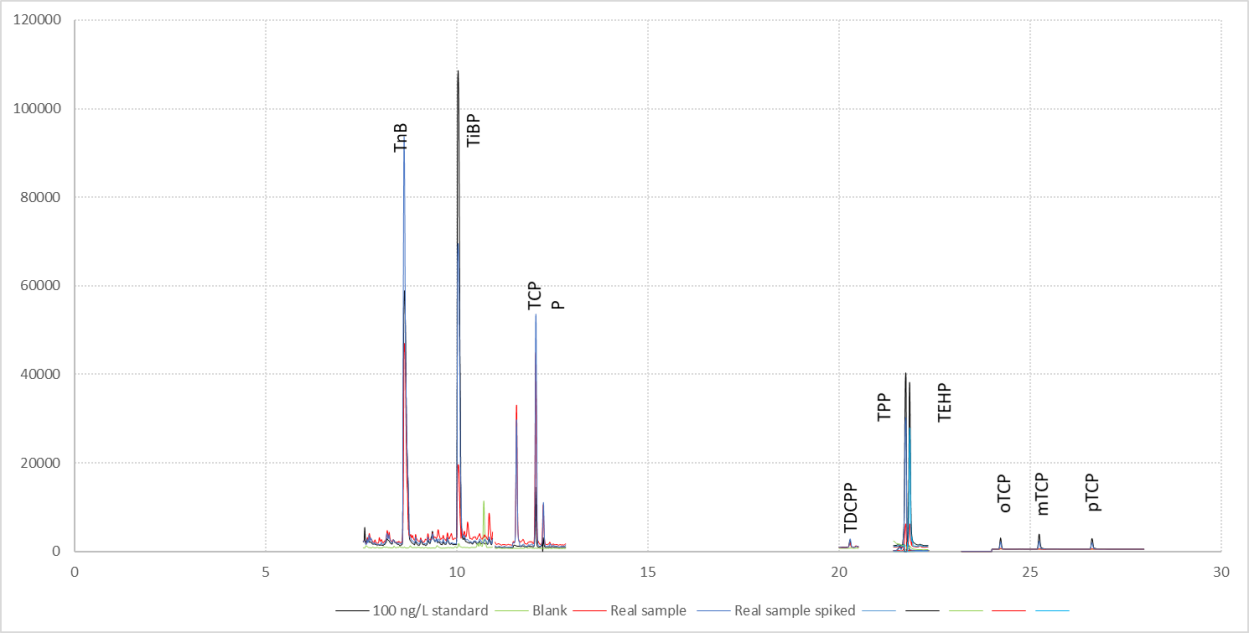

Figure S 6-1: Overlaid SIM chromatograms of the standard (100 ng L-1), blank (pure water), real sample and spiked real sample (50 ng L-1).

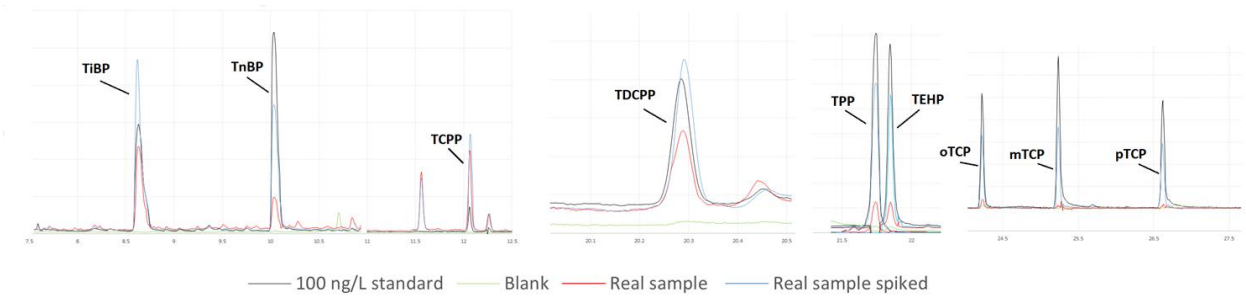

Figure S 6-2: Re-scaled overlaid SIM chromatogram sections of the standard (100 ng L-1), blank (pure water), real sample (Ruhr River) and spiked real sample (50 ng L-1).

6.6 References

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7 Automated determination of picogram-per-liter level of water taste and odor compounds using solid-phase microextraction arrow coupled with gas chromatography-mass spectrometry

This chapter was adapted from: *W. Kaziur, A. Salemi, M.A. Jochmann, T.C. Schmidt, Automated determination of picogram-per-liter level of water taste and odor compounds using solid-phase microextraction arrow coupled with gas chromatography-mass spectrometry, Analytical and Bioanalytical Chemistry (2019).*

Abstract

Taste and odor compounds are organic chemicals produced via biochemical processes. Their presence, even at low nanogram per liter concentrations, can make water useless for drinking purposes. In this work, a very sensitive and completely automated analytical procedure, based on solid-phase microextraction, has been developed and optimized for determination of seven taste and odor compounds in water media, well below their odor threshold. The selected analytes were isopropyl-3-methoxypyrazine, 2-isobutyl-3-methoxypyrazine, geosmin, 2-methylisoborneol, 2,4,6-trichloroanisole, 2,4,6-tribromoanisole and beta-ionone. Compared with a conventional approach, the recently introduced PAL SPME Arrow showed a significant enhancement in sensitivity and also outstanding robustness and stability. Three commercially available fiber coatings, as well as experimental parameters of the headspace extraction procedure, such as extraction temperature, time and ionic strength of the aqueous sample were investigated to optimize the method. The linearity of the response was assessed over a three orders of magnitude range, with R^2-values higher than 0.9914. The method was satisfactorily precise with RSDs of less than 11 % at the second lowest calibration point (10-26 ng L^{-1}). The calculated LODs (S/N = 3) were below odor thresholds of the target analytes and varied between 0.05 and 0.6 ng L^{-1}, for just 10 mL of water sample. An original and spiked river water sample was also analyzed and relative recoveries of 75-116 % were achieved. Based on these analytical performance characteristics, and compared with other published methods, the present method can be considered as the most sensitive fully automated approach for determination of taste and odor compounds in water.

7.1 Introduction

Among the wide range of water quality parameters, the presence of off-flavor compounds and the resulting taste and odor (T&O) could be directly detected by people, either in natural water resources or in drinking water [1]. T&O is usually the consumer's first measure of water safety and arises people's complains [2]. While these compounds might typically have far too low concentrations to pose adverse health effects, their presence can prohibit utilization of the contaminated water [3]. T&O is also considered as an indicator of anthropogenic water contamination [4], regarding the mechanisms of T&O production. Geosmin (trans-1,10-dimethyl-trans-9-decalol, GSM) and 2-methylisoborneol (MIB) are the most frequently detected water T&O compounds [5]. These two compounds are generally produced by cyanobacteria and actinobacteria, and considering their low odor thresholds (1.3 ng L^{-1} for GSM and 6.3 ng L^{-1} for MIB), could be sensed at extremely low concentration levels [6]. 2-isopropyl-3-methoxypyrazine (IPMP) and 2-isobutyl-3-methoxypyrazine (IBMP) are metabolites of actinomycetes and are other frequent causes of T&O in water [7] and wines [8]. Odor thresholds of as low as 2 ng L^{-1} have been reported for these two compounds in water [9]. 2,4,6-trichloroanisole (TCA) is the product of bio-methylation (detoxification) of 2,4,6-trichlorophenol [10] and is considered as one of the main causes of musty odor of water, generally detectable at concentrations less than 10 ng L^{-1} [11, 12]. In a similar way, some organisms produce 2,4,6-tribromoanisole through a defensive O-methylation reaction [13]. Beta-ionone is another cyanobacterial metabolite and a degradation product of carotenoids, which is considered to have a significant role in water T&O (with odor threshold of 7 ng L^{-1}) [14, 15].

Considering the extremely low odor thresholds of the mentioned T&O compounds, their resistance against conventional water treatment processes, and also the importance of providing reliable and valid data on their presence and production routes, highly sensitive analytical techniques of detection and quantification are required [16]. Also, the sample preparation techniques that are combined with the analytical instruments, must be able to eliminate the unwanted and sometimes unpredictable effects of the matrix constituents [17]. Another important point of the proper sample preparation-analysis method is, that regarding the relative volatility of most of the selected analytes, there is always a considerable risk of analyte loss during the procedure. Such a phenomenon could be even worse when the analytes are being determined at very low concentration levels. Therefore, a fully automatized sample preparation/introduction

procedure would be preferred to minimize the probability of analyte loss [18]. Gas chromatography-mass spectrometry has been the method of choice, due to its sensitivity and selectivity [19-23]. As an alternative for analysis of different haloanisoles, the electron capture detector (ECD) has also been successfully implemented [24, 25]. Many extraction approaches have been developed and optimized, with the main focus of enhancing the sensitivity, as well as its simplicity and speed. Closed-loop stripping analysis (CLSA), in its classical [26] or modified (hollow fiber stripping analysis, HFSA) form [27] has been one of the first and most popular techniques of extraction of T&O compounds. CLSA provides high sensitivity of analysis, as well as low volumes of organic solvent consumption, although through a time consuming and rather labor-intensive manner of operation. Alternative liquid-phase extractions have been also developed for T&O compounds in water, using the least practically possible volumes of solvents, i.e. from 6 [28] and 1 mL [29] down to few microliters in hollow-fiber liquid-phase microextraction [30] and headspace solvent microextraction [31]. The latter, in spite of using a negligible volume of solvent (1.5 μL of 1-octanol) and producing satisfactory results, was based on a rather unstable suspended drop. Also, only geosmin has been examined in the work and regarding the necessity of using a semi-volatile extraction solvent for headspace extraction, chromatographic analysis of a large set of analytes would be difficult (because of appearance of a huge solvent peak in the middle of the chromatogram). Dispersive liquid-liquid microextraction (DLLME) has also been developed for determination of GSM and MIB [32]. Via a simple and fast operational procedure, the technique provides reasonable sensitivity, although the reported limits of detection are above the odor threshold of the analytes. Additionally, there seems to be no practical way to automate the DLLME procedure. An automated purge and trap technique has been reported for the determination of five of the most important T&O compounds (GSM, MIB, TCA, IPMP and IBMP) in water [18]. The technique was able to analyze large batches of samples and detect concentrations below the odor threshold of T&O. In-tube extraction (ITEX 2) has been also developed and evaluated for analysis of GSM and MIB, along with other volatile organic compounds (VOCs), in water [33]. Although the reported detection limits for GSM and MIB are above the odor threshold of these compounds, the method showed significant advantages, such as simplicity, flexibility and affordability of the instrumentation, low sample volumes, low risk of sorbent contamination (due to the matrix constituents) and good precision and recoveries. Stir bar sorptive extraction (SBSE) is another microextraction technique that has been successfully implemented for T&O compounds analysis [34-37]. Based on the relatively large volume of the sorbent (coated on the stir bar) and thermal

desorption of theoretically all of the extracted analytes into the chromatographic instrument, SBSE is very sensitive. However, direct contact with the sample matrix and consequent interferences, can be a main drawback. In the last decade, solid-phase microextraction (SPME) has been the most frequently used sample preparation technique for T&O compounds [23, 38-42]. Ease of automation, less steps of sample preparation, solvent-free character, direct desorption into the chromatographic system and good sensitivity are among SPME advantages. Also, SPME is flexible enough to be operated in both, direct immersion and headspace mode. For T&O extraction the headspace mode has been dominant to reduce the matrix interference, and better protect the sorbent material from varied pH and ionic strength of the samples. The technique, however, suffers from two main drawbacks; fragility of the fibers and limited amount of the coated sorbent.

PAL (Prep And Load) SPME Arrow, has been recently presented to overcome these disadvantages of classical SPME [43, 44]. This new version of SPME has been thoroughly studied and compared with conventional SPME and also SBSE [43]. In summary, PAL SPME Arrow was advantageous over SBSE, based on the possibility of automation and availability of both, headspace and direct immersion modes of operation. Compared with conventional SPME, PAL SPME Arrow, with larger sorbent volume (10.2 μL vs. 0.6 μL), provided one order of magnitude better sensitivities and showed an increased mechanical robustness.

The focus of the present study was to evaluate and optimize PAL SPME Arrow for extraction of seven T&O compounds. As described earlier, high sensitivity is required to detect and analyze such compounds and most of the published works present LODs close to T&O threshold concentrations. So, the main motivation here is to develop a technique capable of quantifying T&O compounds well below their odor threshold. Availability of such a technique can be of great importance in scientific research focused on production and degradation of T&O compounds, and also provides a mean for water resource management studies and operations. Considering the trend of global temperature and the resulting increase in the frequency of harmful algal blooms [45], the importance of the methods, like the one presented here, is clearly revealed.

7.2 Material and methods

7.2.1 Reagents and materials

Standard solutions of GSM (98 %), MIB (>98 %), IPMP (>99 %), IBMP (>99 %) and TCA (>99 %), as well as pure 2,4,6-Tribromoanisole (TBA; 99 %) and beta-ionone (BIN; 97 %) were purchased from Sigma-Aldrich (Steinheim, Germany). A mixed stock standard solution was prepared using analytical-grade methanol (KMF Laborchemie, Germany) by dissolving and diluting the analytes to the concentrations of 2 mg L^{-1} for IPMP, IBMP, TCA and MIB, 1 mg L^{-1} for GSM, 1.9 mg L^{-1} for BIN and 2.6 mg L^{-1} for TBA. Pure water was produced by a PURELAB Ultra analytic water purification system (ELGA LabWater, Germany) and was used to prepare standard spiked solution during the optimization and calibration procedure.

7.2.2 GC-MS instrumentation

A Shimadzu GC-MS-QP2010 Ultra (Shimadzu Deutschland GmbH, Germany) was used for the separation and quantification purposes. The GC split/splitless inlet was equipped with a 2 mm i.d. × 5 mm o.d. × 95 mm length splitless liner from BGB Analytik (Switzerland). Desorption was performed at 250 °C for 1 min (splitless) and the inlet was switched into split mode (10:1) after 1 min. A Restek Rxi-624 sil MS analytical column (60 m × 0.25 mm × 1.4 μm), from Restek (Bad Homburg, Germany) was implemented for chromatographic separation of the analytes, using He (5.0, AirLiquide, Oberhausen, Germany) as carrier gas at constant flow rate of 2 mL min^{-1}. The column temperature program was started at 40 °C (held for 1 min), raised to 240 °C at 25 °C min^{-1} and then to 280 °C at 10 °C min^{-1} (held for 5 min) and completely separated the analytes in 18 min.

MS transfer line (interface) and ion source were set to 250 °C. Total ion current mode was used to determine the retention times of the analytes (m/z range of 40-400). Quantitative analyses were performed in selected ion monitoring (SIM) mode, using the mass fragments shown in Table 7-1.

Table 7-1: Analytical performance characteristics of PAL SPME Arrow for T&O compound.

	linear range[a] / ngL^{-1}	**Spike c[b] / ngL^{-1}**	**R^2**	**Mass fragments**	**LOD[d] / ngL^{-1}**	**LOQ[e] / ng L^{-1}**	**RSD[f] / %**	**Reco-very[g] / %**	**RDP[g] / %**	**Surface water[h] / ng L^{-1}**
IPMP	2 - 1600	20	0.9956	124, **137** [c], 152	0.09	0.31	8.2	95	9.9	ND[i]
IBMP	2 - 1600	20	0.9941	94, **124** [c], 151	0.24	0.80	8.7	92	6.8	ND[i]
MIB	2 - 1600	20	0.9914	**95** [c], 108	0.14	0.48	7.1	94	12.4	12.3
TCA	2 - 1600	20	0.9944	**195** [c], 197, 210	0.05	0.15	10.9	110	17.3	ND[i]
GSM	1 - 800	10	0.9952	**112** [c], 125	0.30	1.00	7.8	75	16.7	6.1
BIN	1.9 - 1520	19	0.9967	135, **177** [c]	0.34	1.14	11.0	116	15.3	ND[i]
TBA	2.6 - 2080	26	0.9932	331, **344** [c], 346	0.63	2.09	9.4	103	9.3	< LOQ[j]

[a] Each calibration point was analyzed in triplicate.

[b] Concentration of the second lowest calibration point, used for calculation of recovery and RSD.

[c] Quantifier ion.

[d] Based on S/N = 3.

[e] Based on S/N = 10.

[f] Based on 5 replicate analysis of pure water spiked at the second lowest calibration point (spike concentration [b]).

[g] Recovery and relative percent difference (RPD) were calculated based on duplicated analysis of real sample spiked at the second lowest calibration point (spike concentration [b]).

[h] Average of duplicated analysis of river water sample.

[i] Less than LOD and not detected

[j] The result was below LOQ.

7.2.3 Extraction procedure

For the extraction of the analytes, a PAL RTC autosampler equipped with PAL SPME Arrows of different types; CWR (Carbon Wide Range)-PDMS, DVB-CWR and DVB-PDMS (250 µm×20 mm, 10.2 µL) was implemented. The entire autosampler setup and SPME Arrows and holder were from CTC Analytics AG, Zwingen, Switzerland. 10 mL of samples, in 20-mL amber glass vials containing 0-30 % of NaCl (Merck, Darmstadt, Germany), were placed on the sample holder and kept at room temperature (23 °C) before extraction. The extraction procedure started by transferring the sample into a lab-made stirring/heating station, constructed based on an IKA-Mag RCT basic (IKA-Werke GmbH & CO KG, Staufen, Germany), to achieve temperature equilibrium and complete dissolving of the added salt. Temperature was manually set at 40-70 °C and magnetic stirring at the maximum (1500 rpm) for all samples. At the same time SPME Arrow was

conditioned at 250 °C for 8 min and then was moved to penetrate the vial septum into the sample headspace for the extraction of the analytes. After predetermined extraction time periods (10-60 min), the fiber was withdrawn and entered the GC-MS inlet, for desorption.

7.2.4 Surface water sample

A surface water sample was grabbed in an August 2018 midday, from Ruhr River, Essen, Germany. This sample was spiked with the individual analytes, at 10 - 26 ng L^{-1} (Table 7-1) and was extracted and analyzed. The reported recovery values for the developed method were calculated by comparing the results obtained from analysis of this sample with the calibration curve.

7.3 Results and discussion

7.3.1 Optimization

Two main strategies for studying the influencing factors and consequently finding the optimum (maximum) extraction efficiency and the best practically possible sensitivity, are one-factor-at-a-time (OFAT) and design of experiments (DOE). DOE provides advantages like clear identification of the factor interactions, creating a model to describe the phenomenon under study and generally reducing the number of the required experiments for achieving the optimum [46]. However, sometimes it is necessary to study significant factors in a rather large number of levels, such as while examining the effect of extraction time (to find the equilibrium) and temperature. In such cases, increasing the number of levels of each factor, necessitates a huge number of experiments to be performed and therefore, OFAT approach seems to be a better choice. This is also true for the experiments with varying factor levels and factor types (numerical/categorical).

Characteristics of the sorbent (SPME fiber type), concentration of the added salt to the sample solution, temperature and duration of the extraction are among the most important factors with significant effects on the efficiency of SPME and have been studied in the present work.

Previous studies revealed that headspace mode of extraction is preferred for T&O compounds, whenever the selection between headspace and direct immersion has been possible,

e.g. when using SPME [23, 38] and solvent microextraction [31]. Protection of the extraction phase (sorbent or solvent) from the non-volatile matrix constituent and also avoiding the unwanted effects of sample ionic strength on the solid sorbent, are among the benefits of using headspace based extractions for semi-volatile analytes [47].

Stirring rate has also been considered in the literature as an effective factor to shorten the equilibrium time [48]. But since this factor had an obvious effect and there was no limitation in increasing the stirring rate (regarding the headspace mode of operation), it was set at the maximum (1500 rpm) throughout the study.

In the present work, based on the above discussion, the experimental parameters of the extraction step, i.e. SPME fiber type, effect of added salt, extraction time and extraction temperature were optimized by an OFAT strategy. For each parameter, the goal was to achieve the maximum geometric mean of the responses, as the optimum conditions for the entire set of the analytes.

Fiber selection

The type of the sorbent was the first studied factor during the optimization procedure. Carbon wide range-polydimethylsiloxane (CWR-PDMS), divinylbenzene-carbon wide range (DVB-CWR) and divinylbenzene-polydimethylsiloxane (DVB-PDMS) were examined to find the best fiber, regarding the sensitivity of the method. Figure 7-1 demonstrates the results of the extraction of seven analytes from water. These experiments were performed through headspace extraction at 40 °C for 30 min from pure water spiked at 100 ng L^{-1}.

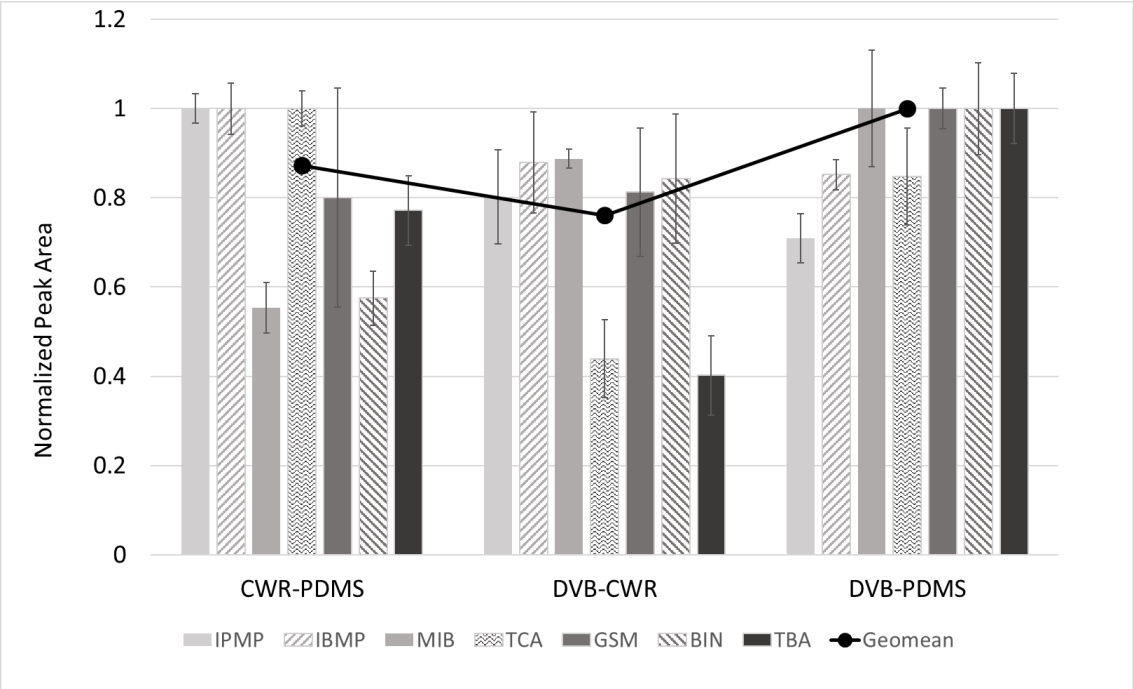

Figure 7-1: Relative extraction efficiencies of different PAL SPME Arrow fibers, for individual analytes and geometric average of the responses (Geomean).

The data in this figure have been presented as normalized peak area. In other words, the peak areas of each analyte have been normalized based on the results of the best SPME Arrow fiber for that analyte. With the normalized data, it is easier to compare the extraction efficiency of the three fibers for each individual compound, as well as the entire set of analytes (using the geometric mean of the normalized peak areas). It is clear that under the studied conditions, DVB-CWR showed the lowest efficiencies, while each of the other two fibers were suitable for a part of the set of analytes. CWR coating is usually used to extract low molecular weight and very volatile compounds and its effect is clear in the results obtained by both CWR-PDMS and DVB-CWR. In both cases, the fibers showed better extraction efficiencies for more volatile analytes. Comparing DVB-PDMS and CWR-PDMS showed that the compounds that were best extracted on CWR-PDMS (i.e. IPMP, IBMP and TCA) were relatively more volatile (except MIB) than the others, while DVB-PDMS showed to be of superior efficiency for the less volatile compounds (MIB, GSM, BIN and TBA, that had higher sorbent-air partitioning coefficients, and correspondingly longer GC retention times). To select the practically best fiber for simultaneous analysis of the targeted T&O compounds, the geometric mean of the normalized peak area (Geomean, shown by

solid line in Figure 7-1) was calculated and used as the decision tool. As result, DVB-PDMS demonstrated the best efficiency and hence, was selected for the rest of the study.

Salting out effect.

Increasing the ionic strength of aqueous samples to enhance the extraction performance (or salting out) is commonly used during extraction in method development studies [48]. Adding highly water-soluble inorganic salts, such as sodium chloride and sodium sulfate, can decrease the solubility of the organic analytes and hence, move the extraction equilibrium toward the headspace and/or the organic sorbent. In this work, three levels of salt (NaCl) concentration were examined; 0, 15 and 30 % (w/v), as it can be observed in Figure 7-2. Substantial increase in peak area was observed for individual analytes, as well as geometric mean. It is clear from the Figure that this enhancement was of different extent for individual analytes, as TCA was the least affected compound with less than 20 % of increase in peak area. This increment was 100 % for TBA and approximately 400 % or more for the other analytes. So, the rest of the experiments were carried out using the salt concentration of 30 % (w/v).

It is worth noting that during this part of the study, saturation of solution was intentionally not considered, although it could be predicted from the trend of the plot (Figure 7-2) that saturated sample solution would result in even better sensitivity. The reason for this decision was that since the effect of sample temperature was still to be examined, and also, the saturation concentration of NaCl was temperature dependent, changing the temperature would directly alter the dissolved salt concentration and make the decision making somehow complicated. So, 30 % (w/v) had been considered as the maximum salt concentration and was demonstrated to be the optimum level for this factor.

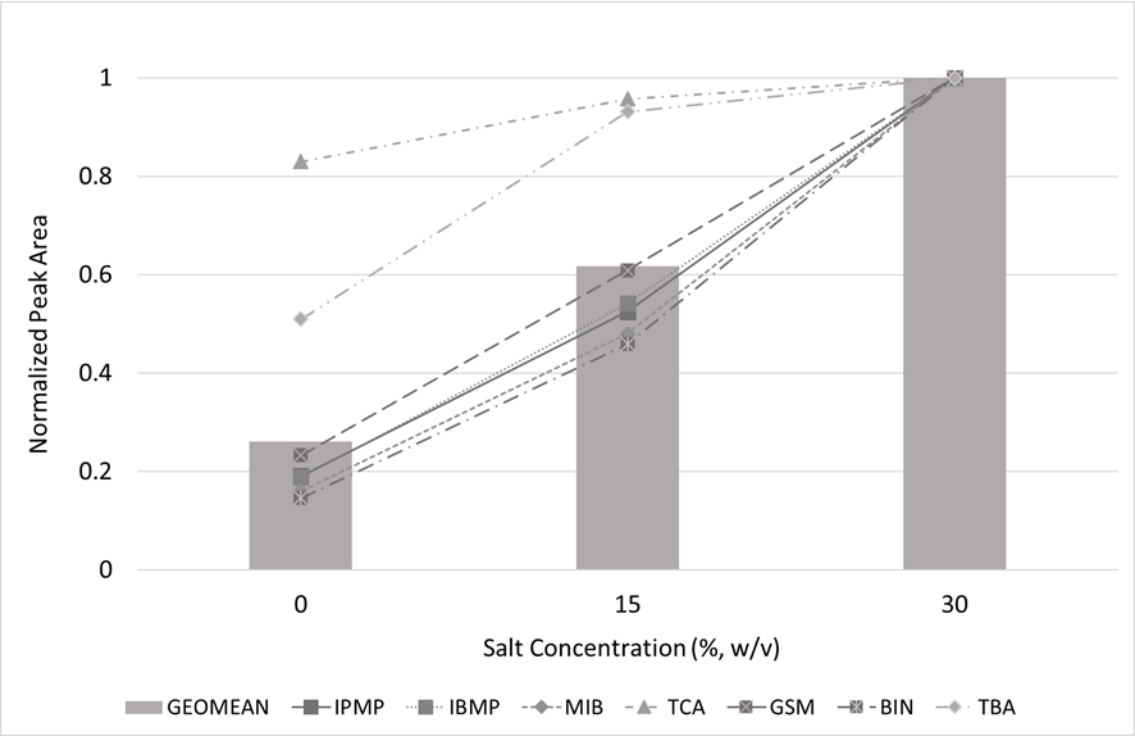

Figure 7-2: Effect of added salt concentration (%, w/v) on relative extraction efficiency of T&O compounds.

Extraction temperature.

The effect of temperature on the efficiency of headspace extraction has been thoroughly discussed in literature [49]. It has been shown that the fraction of the analytes in sample headspace (and hence, the efficiency of extraction) can be increased by increasing the air-water partitioning constant (Kaw). The latter depends on sample (and headspace) temperature and increases with temperatures, in accordance with a van't Hoff-type equation:

$$lnK_{aw} \cong -\frac{A}{T} + B \qquad (1)$$

where the compound-specific constants A and B determine the different value of the partitioning constant for each analyte. However, there will be a limitation to this phenomenon when a sorption process is going to take place in the headspace of the sample, with K_{sa} as the partition coefficient. Sorption of the analyte on SPME Arrow (as on conventional SPME) sorbent is an exothermic process and hence, increasing the temperature, via decreasing K_{sa}, reduces the transfer of the analytes from the gas phase to the sorbent. In some headspace techniques, such as ITEX, it is possible to independently control the temperature of the sorbent [50] and prevent K_{sa} decreasing in

higher extraction temperature. However, since a cooling system was not installed on the PAL SPME Arrow device, it was expected that the enhanced sample temperatures would simultaneously have a positive effect on extraction through increasing the air fraction of the analytes, and at the same time show a negative effect on the tendency of the analytes towards the sorbent. The effect of temperature on the extraction performance was studied by increasing the sample temperature from 40 up to 70 °C. Figure 7-3 shows the results of this experiment and clearly demonstrates that 60 °C was the optimum temperature for headspace extraction of T&O compounds from water and further increasing temperature would decrease overall extraction efficiency.

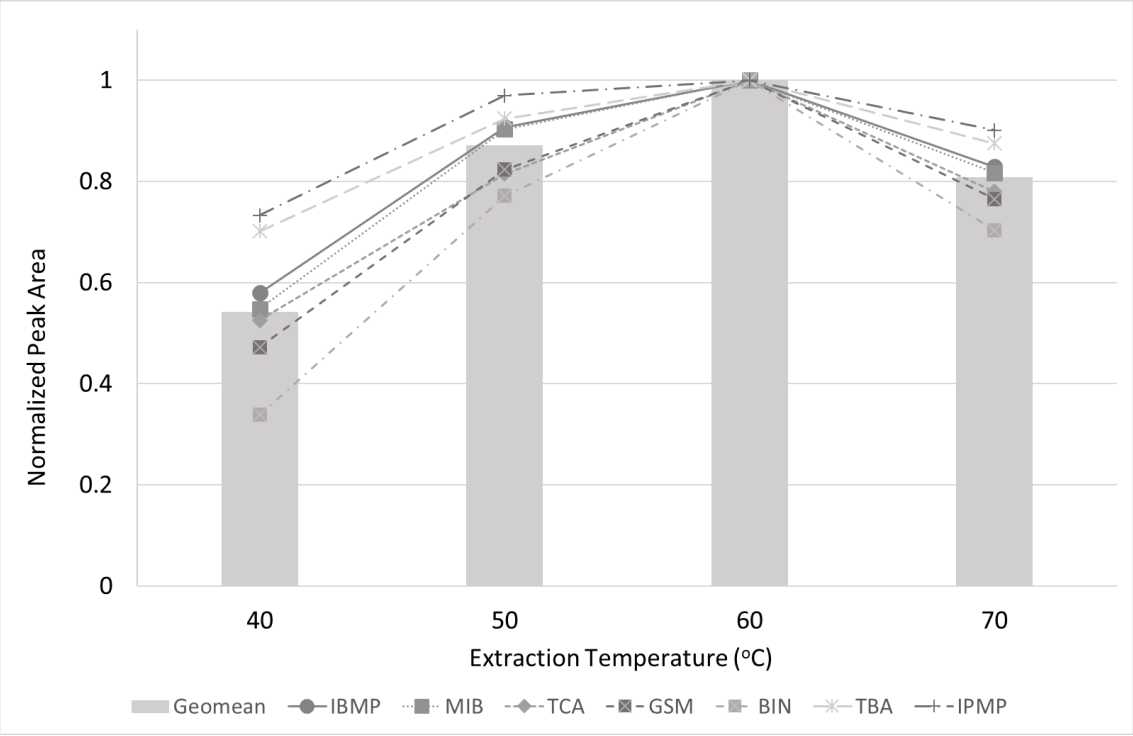

Figure 7-3: Effect of temperature on relative extraction efficiency of T&O compounds.

Extraction time.

Considering the equilibrium nature of the extraction process, it is necessary to provide enough time for the partitioning to reach an equilibrium point, if the maximum amount of the analytes shall be extracted. It can be observed in Figure 7-4 that no significant improvement occurs after 30 min of extraction, at 60 °C and from a sample containing 30 % (w/v) of sodium chloride. The variation of peak areas beyond 30 min, as is demonstrated by overlapping error bars on the

individual analytes, was not significant. So, this time period was set as the optimum extraction time in the rest of the study. It is worth noting that in case of requiring faster overall analysis, choosing a shorter extraction time would be possible. For example, an extraction time of 10 min, would result in only 35 % decrease in the geometric mean of the responses and at the same time would save 20 min in sample preparation. Thus, if the somewhat lower sensitivity suffices the requirements of the analysis, pre-equilibrium sampling could also be used to increase throughput.

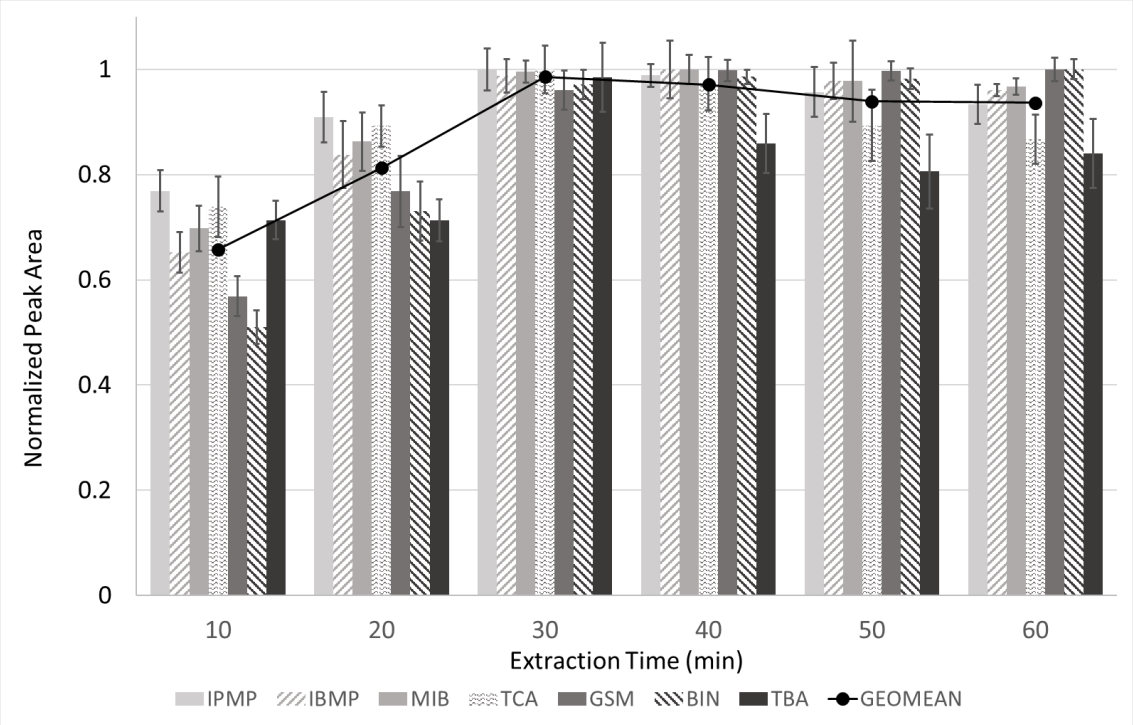

Figure 7-4: Effect of extraction time on relative extraction efficiency of T&O compounds. Standard deviation values have been represented by error bars.

7.3.2 Analytical performance characteristics

To evaluate the linearity of the results of extraction/analysis procedure and also its capabilities for quantitative analysis of T&O compounds, a set of calibrations samples, in 7 concentration levels, was prepared and analyzed in triplicate (Table 7-1). R^2 values showed that the method can produce responses with very good linearity, over a range of 3 orders of magnitude (minimum R^2 value was 0.9914 for MIB). Precision of the method was also estimated based on 5 replicate measurements of a spiked water sample with 10 ng L^{-1} of GSM, 26 ng L^{-1} of TBA and 20 ng L^{-1} of IPMP, IBMP, MIB, TCA and BIN. RSD % values, as shown in Table 7-1, were less than

11.0 % and completely satisfactory for precise analysis of these compounds. LOD values, based on S/N=3, were calculated using the lowest calibration points and were below 1 ng L^{-1}, with the minimum of 0.05 ng L^{-1} for TCA and maximum of 0.6 ng L^{-1} for TBA.

7.3.3 Real sample measurement and relative recovery

To evaluate the effect of the sample matrix constituents on the performance of the developed method, a real sample was analyzed before and after spiking at 20 ng L^{-1} with the analytes (10 ng L^{-1} of GSM, 26 ng L^{-1} of TBA) and the results were compared with spiked pure water (analyzed at the same day). Of the set of the studied analytes, MIB, GSM and TBA were detected in the river water sample, with concentrations of 12.3, 6.1 and 1.3 ng L^{-1}, respectively. Of course, the latter value (TBA) could not be considered as a statistically significant figure, since it was between LOD (0.63 ng L^{-1}) and LOQ (2.09 ng L^{-1}) values. The calculated relative recovery values range between 75 % (for GSM) and 116 % (for BIN), as depicted in Table 7-1, and were completely satisfactory, considering the low nanogram-per-liter concentrations of the spiked analytes. The precision of the method for the spiked real sample, was calculated as relative percent difference, RPD of duplicate analyses (Table 7-1), and showed that the sample matrix had no considerable effect on the precision of the method.

7.3.4 Comparison with other methods

Among the various extraction techniques developed and implemented for the analysis of T&O compounds in water, a few examples have been presented in Table 7-2 for a brief comparison with the developed PAL SPME Arrow. It is clear that the presented technique benefits from better sensitivity with the exception of SBSE, but there, 6 times more sample volume was extracted. Furthermore, the SBSE method could not be run fully automated, unlike PAL SPME Arrow, SPME and purge and trap. Solvent microextraction is obviously not as robust as PAL SPME Arrow and only GSM has been studied as the target analyte. The reported LOD is also higher than in this work (0.3 ng L^{-1}). The reported purge and trap method was studied over a smaller range of calibration and despite larger sample volume (compared with this work) and more complicated instrumentation, has been less sensitive. DLLME, although it is very simple and fast, has not been

sufficiently sensitive for T&O compounds. And finally, comparing sample volume, linear calibration range and sensitivity of PAL SPME Arrow with those of conventional SPME, reveals the superiority of the former.

Table 7-2: Comparison of the developed PAL SPME Arrow with similar methods.

Method	Analytes	Sample volume (mL)	Calibration range (ng L^{-1})	LOD (ng L^{-1})	RSD (%)	Ref.
Purge and trap	IPMP, IBMP, GSM, MIB and TCA	20	10-200	0.2-2	<8	[32]
Solvent microextraction	GSM	5	5-900	0.8	<5	[30]
SBSE	GSM, MIB, TCA	60	0.1-100	0.02-0.16	<3.7	[34]
DLLME	GSM and MIB	12	10-1000	2 and 9	<11	[31]
SPME	IPMP, GSM, MIB, TCA, BIN	40	5-100	0.2-0.5	<7	[22]
PAL SPME Arrow	IPMP, IBMP, GSM, MIB, TCA, BIN and TBA	10	1 (2.6)-1000 (2600)	0.05-0.6	<11	-

7.4 Conclusion

An automated PAL SPME Arrow technique coupled with GC-MS was developed, optimized and evaluated for determination of T&O compounds in water samples. Using PAL SPME Arrow, excellent sensitivities were achieved via a robust and mechanically strong sorbent supporting device. Considering the maximum sample preparation time of 40 min (8 min of fiber conditioning, 30 min for extraction and 2 min of desorption), which easily overlaps the 18 min chromatographic run, and also the automation of the entire procedure, a rather high throughput analysis of water sample would be at hand, for water quality assessment laboratories. Sensitivity of the developed method was satisfactorily high to produce statistically significant quantitative results below the odor threshold of the examined T&O compounds. The analytical procedure only needed very small volume of water sample to produce such sensitivity and this makes the sample gathering and

transport simple and economically favorable. Analytical performance characteristics of the optimized PAL SPME Arrow in Table 7-1 demonstrate a fast, straightforward, reliable, robust, precise and accurate technique, which can be implemented for analysis of the lowest practically relevant concentrations of T&O compounds in surface water samples.

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8 General conclusion and outlook

Demands on modern analytical chemistry develop into the direction of reliable automation, high sensitivity, repeatability and flexibility. Furthermore, the green aspect, meaning the reduction in organic solvent use, sample size, consumption of energy and generation of waste, is gaining in importance and thus, miniaturization is one of the main demands for sample preparation [1-3]. Microextraction techniques were developed as rapid, accurate and miniaturized sample preparation methods and work often solventless and fully automated [1]. Most microextraction techniques developed from liquid-liquid extraction (LLE) and solid-phase extraction (SPE) [4]. The variety of existing microextraction techniques is increasing, but the standardization of these techniques is lacking. Consequently, microextraction techniques are not yet widespread in routine analysis [5]. The most used microextraction technique is solid phase microextraction (SPME). The SPME Arrow is a further development from SPME and was studied in three different projects in this study. The other two projects focused on in-tube extraction dynamic headspace (ITEX-DHS).

Volatile organic compounds (VOCs) are an important part of food analysis. The range of components and their concentrations is and has always been challenging for analysis [6]. The VOC profile of foodstuffs or single VOCs, which can be used as marker components, can be related to the geographical origin and botanical type, but furthermore depend on seasonal variations and storage conditions. Therefore, their variation within different batches of the same food may result in a different VOC pattern. Consequently, volatilomics, meaning the analysis and quantification from biological samples, gain in importance [7]. The method of choice for VOC analysis is gas chromatography-mass spectrometry (GC-MS) [8]. Here, the combination with microextraction techniques is easily implemented, which are often reported in food analysis, as their benefits like reduced labor time and costs, the low sample volumes and the low solvent volumes make them environmentally friendly and time-efficient. The most used microextraction techniques are stir bar sorptive extraction (SBSE) and SPME [9]. The sample pretreatment, which includes freeze drying, grinding and homogenization, was not studied in this thesis, as only honey and extra virgin olive oil (EVOO) were studied as exemplary targets of food fraud [10, 11]. The extraction of the VOCs was the focus of the presented work. SPME gained acceptance for the extraction of VOCs in food analysis, but other headspace methods, like static or dynamic headspace methods, should not be excluded [7, 12]. ITEX-DHS as a dynamic headspace approach, which was already applied to some food samples [13-17], allows a rapid, robust and fully automated analysis of samples. The analysis

of EVOO and honey samples was successfully investigated and validated. Generally, ITEX-DHS shows to be an adequate tool for the analysis of aroma of food samples and therefore the idea to use it as a method against food fraught has to be considered. SPME is already well implemented in this field [18-23], but ITEX is mechanically more robust and the traps have a longer lifetime than SPME fibers [5]. Therefore, in the future, the use of ITEX-DHS is expected to increase for headspace analysis. The combination with linear discriminant analysis (LDA), as a chemometric tool for classification, enabled a great option for the monitoring of the geographical origin for EVOOs and the botanical origin of honey. The use of chemometric approaches is necessary and gains importance, as a big amount of data is produced when it comes to VOC analysis of food samples. The data handling is thus a very important part of a successful monitoring of foodstuff. Multivariate analysis is thus required. It can be divided into unsupervised approaches, which do not need a labelling of groups for the used data sets, like principal component analysis (PCA), principal component regression (PCR) and hierarchical cluster analysis (HCA), and into supervised approaches, where more information is required prior analysis. Examples for the latter group are LDA or decision trees [7, 24, 25].

This thesis does not only address food samples, but also environmental samples. All studied samples and the overall questions are related to water analysis. The development of water analysis is similar to the one in food analysis: the green analytical aspect leads to the increased use of microextraction techniques as alternative to the classical approaches. The most used microextraction techniques in this area are SPME, SBSE, solid phase dynamic extraction, ITEX-DHS and more recently SPME Arrow [5]. Even though SPME Arrow is attracting attention recently, its use in routine analysis is still missing [5, 26]. Therefore, its use for the analysis of taste and odor compounds, phosphorous flame retardants (PFRs) and fatty acids has been studied. In all studies, the benefits of SPME Arrow, namely the larger phase volume, the mechanical stability and the consequential increased lifetime, were used to find new, more sensitive and more robust ways to analyze the target analytes. The decision, which microextraction technique might be the correct one for the scientists' question, is challenging and often overwhelming, which leads to the tendency to use conventional methods, which might be not ideal and which require more time and labor. It must be considered that the properties of the analytes of interest are decisive for the choice of the most appropriate microextraction technique and if the extraction mode is headspace analysis or direct immersion [5].

For taste and odor compounds, the odor thresholds in water samples are ranging in a low ng L^{-1} range [27-32]. Existing methods like SPME, stir-bar sorptive extraction (SBSE) and purge and trap already show LODs in this range. With the developed SPME Arrow method, seven taste and odor compounds were detected in the desired LOD range. Only with SBSE, lower LODs were detected, but the used sample volume was six times higher when compared to the presented SPME Arrow method. Furthermore, SBSE is not fully automated unlike SPME, purge and trap and SPME Arrow, which makes it less attractive for routine analysis. Additionally, the small required sample volume of only 10 mL makes sampling and transport of the samples economically favorable. The same applies to the analysis of PFRs in water. Again, classical methods like SPE and LLE are well established [33, 34], but require high volumes of sample and solvent, as well as they are labor-intensive and not completely automated. The change to a fully automated, solvent-free method was thus overdue. Rodriguez and coworkers developed a SPME method for the analysis of PFRs in 2006 but showed problems in repeatability [35]. The same problem occurred with SPME Arrow but the benefit of the change from SPME to SPME Arrow is the increase in sorbent volume which enabled a more sensitive method, which can be seen by the lower LOQs. Furthermore, its mechanical robustness leads to an easier implementation in routine analysis, as the fiber lifetime is increased from 100 to 500 uses [5]. Nevertheless, the poor repeatability can be problematic regarding standardization for routine analysis. Therefore, a future task would be to optimize the setup further, in order to increase the reliability of the system. The use of internal standards could help to improve the repeatability. The problem with the addition of an internal standard for SPME Arrow or even SPME is, that the internal standard would have to be applied directly to the fiber material, so that the concentration is always fixed. The fiber or Arrow would be preloaded with the internal standard, but as soon as it is exposed to the sample matrix, desorption of the internal standard from the fiber/Arrow occurs [36]. If the standard would be added to the sample, the variation of the analytes signals and the standards signals would be into the same directions. Additionally, it could behave unpredictably as well.

Another important topic in sample preparation is the derivatization of analytes. The idea is to change the analytes properties to adapt them to the separation techniques, or to enable a more selective or more sensitive detection. There is a large variety in derivatization techniques, like alkylsilylation, acylation and alkylation [37, 38]. Automation of derivatization is of course favorable. In combination with the prior extraction of the analytes from an aqueous sample, the idea of on-fiber derivatization using SPME Arrow was created. The idea of an on-fiber

derivatization using SPME is not completely new. Martos and Pawliszyn introduced it already in 1998 for the analysis of formaldehyde in gas samples [39] and many more followed for different GC applications [40-44]. The analysis of fatty acids is of considerable interest, as they are not only found in food, but also in the environment, in body liquids or in bioreactors [44-47]. Fatty acids occur in biomass of bacteria and thus can be used as markers for bacterial diseases or as indicators for specific bacterial communities [44, 48]. Cha et al. studied the fatty acids in sputum samples as markers for tuberculosis by using the on-fiber derivatization to fatty acid methyl esters (FAMEs) with a home-made SPME fiber, which was made of sol-gel derived butyl methacrylate/hydroxy-terminated silicone oil [44]. The idea of using SPME Arrow was to increase the sensitivity and stability and furthermore to enable routine analysis with commercially available set-ups. As building the own SPME Arrow is not an option for routine analysis, the idea was transferred to a commercially available PDMS/DVB SPME Arrow in this study. BF_3/MeOH was tested as derivatization agent, which directly led to the decomposition of the SPME Arrow coating (PDMS/DVB). This could be traced back to the formation of HF due to the presence of water, which then degrades the polysiloxanes of the DVB/PDMS SPME Arrow [37, 49]. As an alternative, a mix of H_2SO_4/MeOH as derivatization reagent, was tested, but the very aggressive conditions during the derivatization step led to the slow decomposition of the phase material. Nevertheless, the analysis of a leachate sample from a composting facility was successfully done, which proofs that in principle it is possible to extract, derivatize and analyze fatty acids in aqueous samples with SPME Arrow. Now, it is of great interest to further improve the sorbent phase stability or to improve the derivatization step to less aggressive conditions, so that reliable measurements can be done, as the idea of combining the extraction and enrichment of the analytes with the derivatization within a three-step fully automated sample preparation is very promising. It would be of great interest to find a material, which is stable at high temperatures for a long time, as well as can handle acidic conditions. The development of such a phase material are steps in future research. The research interest in new fiber materials is ongoing, but the last step to commercialization tends to be difficult, or not of interest. Alternatives for the coatings often provide increased surface areas, inherent functional groups, increased thermal stability and an improved wettability. Examples are carbon nanotubes, graphitic carbon nitride, boron nitride, metal organic frameworks and porous aromatic frameworks [50]. Of course, automated derivatization does not have to be conducted with on-fiber derivatization. The easier step would be an in-situ approach, where the derivatization agent is directly added to the sample itself. Optional heating on an autosampler is easily implemented.

There are some publications in recent years [51, 52], dealing with this approach, but it was not considered for this thesis, as an extraction from a derivatized solution might lead to problems, if direct immersion would be necessary. To avoid sorption competition of the derivatized analytes with the typically applied excess of derivatization reagent, non-volatile reagents have to be chosen.

As sample preparation is an important topic of analytical chemistry, microextraction techniques, especially the solvent-free once, will gain in importance in the future. As can be seen in the number of publications, the trend towards such techniques is already present and the development from the first microextraction techniques like SPME and SBSE into further developed techniques, like for example SPME Arrow, microextraction by packed sorbents (MEPS), and dispersive solid-phase extraction (dSPE), is happening [4, 12, 26, 53]. The selection of the optimal technique is therefore rather difficult and is based on different questions, like the analytes of interest, the sample type, the extraction mode or the analytes' characteristics. This can be overwhelming and thus be the reason for the small implementation in routine analysis, or better, in standardization. This goes in hand with the problems occurring in validation of methods. For example. when viewing the literature, it is difficult to compare different techniques as often different ways to express minimum quantities of an analyte are used. The development of new sorbent materials is of course of interest as mentioned previously, as the stability for more extreme conditions needs to be increased. Additionally, further automation will become a big part of future research [53, 54]. This is does not only apply to microextraction techniques but also to fully automated versions of LLE or SPE [4, 55-57]. The digitalization of laboratories is evolving and thus is the request for fully automated methods. Sample throughput, and thus, time efficient work schedules are a key factor for routine analysis. Tool changing autosamplers like the PAL RTC, which was used in this thesis, are developing more and more. New modules and tools for more complex sample preparation protocols are developed every year to enable their full automation. Even the adaption to smart-technology is already implemented by the latest version of the PAL RTC series 2. The tools and required syringes, SPME fibers or SPME Arrows are equipped with an ID chip, which allows the precise tracking of the exact use. It can be seen, how often the tool was used or to which temperatures it was exposed. Furthermore, for SPME and SPME Arrow, the correct parameters are directly applied. This helps to prevent the fibers and Arrows from too high temperatures, which would lead to the destruction of the sorbent materials. These technologies will become more important in the upcoming years.

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9 Appendix

9.1 List of abbreviations

AC	Active charcoal
BIN	Beta-Ionone
CAR	carboxen
CCD	Central composite design
CLSA	Closed-loop stripping analysis
CT 5	5 % diphenyl/ 95 % dimethylsiloxan
CW	Carbowax
DI	Direct immersion
DLLME	Dispersive liquid liquid microextraction
DoE	Design of experiment
DVB	divinylbenzene
ECD	Electron capture detector
EME	Electromembrane extraction
EPA	United states environmental protection agency
EVOO	Extra virgin olive oil
FID	Flame ionization detector
FPD	Flame photometric detector
GAC	green analytical chemistry
GC	Gas chromatography
GSM	Geosmin
H_2SO_4	Sulfuric acid
HF-LPME	Hollow-fiber liquid phase microextraction
HFSA	Hollow fiber stripping analysis
HS	Headspace
IBMP	2-isobutyl-3-methoxypyrazine
ICP	Inductive coupled plasma
IPMP	2-isopropyl-3-methoxypyrazine
ITEX-DHS	In-tube extraction dynamic headspace
K_{aw}	Air-water partitioning constant
K_{ow}	Octanol-water partitioning constant
K_{sa}	Sorbent-air partitioning constant

LD	Linear discriminant function
LDA	Linear discriminant analysis
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LPME	Liquid-phase microextraction
MDL	Method detection limit
MeOH	Methanol
MEPS	Microextraction in a packed syringe
MIB	2-methylisoborneol
MQL	Method quantification limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometer
mTCP	Tris-m-cresyl phosphate
NaCl	Sodium chloride
NPD	Nitrogen-phosphorous detector
NT	Needle trap
OFAT	One-factor-at-a-time
oTCP	Tris-o-cresyl phosphate
OV 225	25 % phenyl/ 25 % cyanopropyl-methylsilicon/ 50 % PDMS
PAHs	Polycyclic aromatic hydrocarbons
PAL	Prep and Load
PALME	Parallel artificial membrane extraction
PBDEs	Polybrominated diphenyl ethers
PCA	Principal component analysis
PDMS	polydimethylsiloxane
PDO	Protected destination of origin
PEG	polyethylene glycol
PFRs	Phosphorous flame retardants
PFRs	Phosphorous flame retardants
pTCP	Tris-p-cresyl phosphate
RDP	Relative percent difference
RSD	Relative standard deviation

RT	Room temperature
S/N	Signal to noise ratio
SBSE	Stir-bar sorptive extraction
SIM	Selected ion monitoring
SPDE	Solid-phase dynamic extraction
SPE	Solid phase extraction
SPME	Solid-phase microextraction
T&O	Taste and odor
TBA	2,4,6-tribromoanaisole
TCA	2,4,6-trichlorophenol
TCPP	Tris-(1-chloro-2-propyl) phosphate
TDCPP	Tris-(1,3-dichloro-2-propyl) phosphate
TEHP	Tris-2-ethylhexyl phosphate
TiBP	Tri-iso-butyl phosphate
TnBP	Tri-n-butyl phosphate
TPP	Triphenyl phosphate
UK	United Kingdom
US	United States
VOC	Volatile organic compounds
ZIF	Zeolite imidazolate framework

9.2 List of Figures

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9.4 List of Tables

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9.6 List of Publications

9.6.1 Articles in Peer-Reviewed Journals as first author

Kaziur-Cegla, W., Wykowski, L., Molt, K., Bruchmann, A., Schmidt, T.C., Jochmann, M.A., In-tube dynamic extraction for analysis of volatile organic compounds in extra virgin olive oils to identify their geographical origin using linear discriminant analysis – A proof of principle, Journal of Chromatography A (submitted)

Kaziur-Cegla, W., Salemi, A., Jochmann, M. A., Schmidt, T. C., Optimization and validation of automated solid-phase microextraction arrow technique for determination of phosphorous flame retardants in water, Journal of Chromatography A 1626 (2020) 461349

Kaziur, W., Salemi, A. Jochmann M. A., Schmidt, T.C., Automated determination of picogram-per-liter level of water taste and odor compounds using solid-phase microextraction arrow coupled with gas chromatography-mass spectrometry, Analytical and Bioanalytical Chemistry 411 (2019) 2653

9.6.2 Articles in Peer-Reviewed Journals as co-author

Dobaradaran, S., Schmidt, T. C., Lorenzo-Parodi, N., Kaziur-Cegla, W., Jochmann, M. A., Nabipour, I., Lutze, H. V., Telgheder, U., Polycyclic aromatic hydrocarbons (PAHs) leachates from cigarette butts into water, Environmental Pollution 259 (2020), Article nr 113916

Akinlua, A. Jochmann, M. A., Lorenzo-Parodi, N., Sojanovic, N., Kaziur, W., Schmidt, T. C., A green approach for the extraction of diamondoids from petroleum source rock, Analytica Chimica Acta 1091 (2019) 23

N. Lorenzo-Parodi, W. Kaziur, N. Sojanovic, M. A. Jochmann, T.C. Schmidt, Solventless microextraction techniques for water analysis, TrAC Trends in Analytical Chemistry 113 (2019) 321

9.6.3 Oral Presentations

Kaziur-Cegla, W., Jochmann, M. A., Schmidt, T.C., Automated microextraction techniques: comparison, optimization and application in food and environmental analytics. 30. Doktorandenseminar des AK Separation Science, Hohenroda, Germany, 11/01-14/01/2020

Kaziur, W., Wykowski, L., Jochmann, M. A., Schmidt, T. C., Optimization of an ITEX-DHS method for the analysis of volatile organic substances from olive oil with GC-MS. Lebensmittelchemische Gesellschaft, Regionalverband NRW Arbeitstagung 2019, Wuppertal, Germany, 06/03/2019

Kaziur, W., Jochmann, M. A., Schilling, B., Schmidt, T. C., Automated microextraction techniques: comparison, optimization and application in food and environmental samples. Food Safety Analysis, Singapore, 27/11-28/11/2018

9.6.4 Poster Presentations

Kaziur, W., Jochmann, M. A., Schmidt, T. C., Analysis of volatile organic compounds in honey by ITEX-DHS GC-MS.

- Anakon 2019, Münster, Germany, 25/3-28/3/2019
- Food Safety Analysis, Singapore, 27/11-28/11/2018

Kaziur, W., Lorenzo Parodi, N., Schmidt, T. C., Automation and miniaturization of the derivatization of aromatic amines.

- 3. Mülheimer Wasseranalytisches Seminar, Mülheim, Germany, 12/9-13/9/2018
- 42nd ICC, Riva del Garda, Italy, 14/5-18/5/2018
- Analytica München, München, Germany, 11/4-12/4/2018

9.7 Declaration of Scientific Contribution

The present thesis includes work that was published in cooperation with co-authors. My own contribution declares as follows:

Chapter 3

Submitted: Kaziur-Cegla, W., Wykowski, L., Jochmann, M.A., Molt, K., Bruchmann, A., Schmidt, T.C., In-tube dynamic extraction for analysis of volatile organic compounds in extra virgin olive oils to identify their geographical origin using linear discriminant analysis – A proof of principle, Journal of Chromatography A

Experiments were planned by Wiebke Kaziur-Cegla. Experiments were implemented and data was evaluated by Lena Wykowski and Wiebke Kaziur-Cegla. The chemometric solution was done by Prof. Dr. Karl Molt and partially by Wiebke Kaziur-Cegla. The draft manuscript was written by Wiebke Kaziur-Cegla. The editorial of the manuscript was done by Prof. Dr. Torsten C. Schmidt, Maik A. Jochmann and Andreas Bruchmann. Prof. Dr. Torsten C. Schmidt and Dr. Maik A. Jochmann supervised the study.

Chapter 6

W. Kaziur, A. Salemi, M.A. Jochmann, T.C. Schmidt, Automated determination of picogram-per-liter level of water taste and odor compounds using solid-phase microextraction arrow coupled with gas chromatography-mass spectrometry, Analytical and Bioanalytical Chemistry (2019) 2653

Experiments were planned by Prof. Dr. Amir Salemi and Wiebke Kaziur-Cegla. Wiebke Kaziur-Cegla did all experiments and the data evaluation. Statistical evaluation was done by Prof. Dr. Amir Salemi. The draft manuscript was written by Prof. Dr. Amir Salemi and partially by Wiebke Kaziur-Cegla. The editorial was done by Prof. Dr. Torsten C. Schmidt and Dr. Maik A. Jochmann.

Chapter 7

W. Kaziur-Cegla, A. Salemi, M.A. Jochmann, T.C. Schmidt, Optimization and validation of automated solid-phase microextraction arrow technique for determination of phosphorus flame retardants in water, Journal of Chromatography A 1626 (2020) 461349

Experiments were planned by Prof. Dr. Amir Salemi and Wiebke Kaziur-Cegla. Wiebke Kaziur-Cegla helped with the experiments and the data evaluation. Statistical evaluation was done by Prof. Dr. Amir Salemi. The draft manuscript was written by Prof. Dr. Amir Salemi and partially by Wiebke Kaziur-Cegla. The editorial was done by Prof. Dr. Torsten C. Schmidt, Dr. Maik A. Jochmann and Wiebke Kaziur-Cegla.

9.8 Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

9.9 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

‚Automated and solvent-free microextraction techniques for the GC-MS analysis of food and environmental samples‘

selbst verfasst, keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet sind und die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, 03. September 2020 W. Kaziur-Cegla

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