Multidimensional high-performance liquid chromatography–gas chromatography (HPLC-GC) hyphenation techniques for food analysis in routine environments

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Summary

Routine monitoring of foodstuffs is an important instrument for consumer protection. Understandably, fast and robust analysis methods are necessary to cope with the modern nutritional habits of the fast-growing population. In contrast, the chemical complexity and variety of foodstuffs are challenges complicating the development of such analytical methods. Nevertheless, routine laboratories faced with thousands of samples per year need to be able to identify harmful substances and quality mismatches in a short time span.

Chromatographic methods are omnipresent in the field of food science. They are used throughout the whole analytical process either for sample preparation or for analyte separation. However, online hyphenation of sample preparation with the separation and detection processes is so far rarely observed in routine environments.

Hyphenation of liquid and gas chromatographic techniques (LC-GC), for example, provides an efficient and fast sample preparation online coupled to the chromatographic separation and detection method. Although this technique is known for almost thirty years, it is hardly used in routine laboratories. This might be related to technical difficulties in the past affecting robustness.

Therefore, aim of this work was the development of selected analytical methods by means of LC-GC hyphenation with robust hardware solutions. Each development included a validation process enlightening the suitability of the analytical method in the scope of accuracy, robustness, and sample throughput for routine environments. The selected applications involve the analysis of unwanted contaminations in food (polycyclic aromatic hydrocarbons, mineral oil contaminations), the quality assurance of edible oils and fats, as well as the determination of the vitamin D content in certain foodstuffs for nutrition analysis.

LC-GC hyphenation allowed direct injection of samples for applications typically involving time-consuming column chromatography cleanups. Methods requiring preceding sample preparation, such as saponification, derivatization, or liquid-liquid extraction, were successfully realized by coupling autosampler-based sample handling with subsequent robust LC-GC hyphenation. The determination of the sterol distribution and content in edible oils and fats was chosen as an example.

The traditional method involves a multistage sample preparation, which requires tremendous amounts of manual and error-prone work. Collaborative trials regularly show insufficient

precision among the participating laboratories. On the contrary, total automation of the sample preparation with subsequent LC-GC analysis offered a substantial increase of precision, robustness, and sample throughput. The here developed method allowed an average sample throughput of one sample per hour. The only manual step left was to weigh the sample into an autosampler vial, making this method amenable to the requirements of contract laboratories. Additionally, the identity of a previously unreported sterol specifically present in sunflower oils was enlightened by the advantages of the analytical HPLC cleanup and extension of the method with mass spectrometric (MS) detection for structure elucidation.

The determination of the vitamin D content in multiple foodstuffs is another example for a successful implementation of an LC-GC method. While vitamin D is typically detected by liquid chromatographic methods, the use of online LC-GC-MS allowed the quantitation of vitamin D also in complex foodstuffs, such as cholesterol-lowering margarines, known to be challenging for established analysis methods.

In the further course of this work, deficiencies of LC-GC hyphenation for the determination of aromatic hydrocarbons in a broad range of foodstuffs were examined. A single HPLC cleanup proved to be insufficient for a robust determination of the selected analytes, showing that LC-GC hyphenation has its limits as any other analytical tool. Increasing the dimensionality of the sample cleanup was necessary to overcome these deficiencies.

The determination of polycyclic aromatic hydrocarbons (PAHs) became feasible by the development of an easy and quick generic sample preparation protocol and employment of a second HPLC cleanup stage. This two-dimensional heart-cut LC-GC-MS method offered high sample cleanup for complex foodstuffs such as edible oils/fats, teas, coffees, or chicken eggs. Additionally, comprehensive validation of the method was performed for extra virgin olive oils proving its precision, robustness, and trueness.

Selective derivatization of interfering food matrix compounds was used for the determination of mineral oil aromatic hydrocarbons (MOAH) in edible oils and fats. While HPLC cleanup was insufficient, alteration of the food matrix polarity in conjunction with LC-GC hyphenation provided an appropriate sample cleanup. Automation and optimization of the reaction conditions, however, was essential for a robust method.

In a nutshell, this thesis shows that LC-GC hyphenation is comprehensively suited for the use in routine environments with a growing portfolio of applications. Extension of common LC-GC hyphenation with automated sample preparation techniques opened new possibilities for

challenging applications as could be shown for the determination of sterols. Besides analytical suitability, however, standardization work by national authorities or responsible working groups is needed for a widespread distribution of LC-GC methods in routine laboratories.

Zusammenfassung

Die Lebensmittelüberwachung ist ein wichtiges Instrument des Verbraucherschutzes. Die modernen Ernährungsgewohnheiten der stetig wachsenden Bevölkerung machen die Verwendung effizienter, schneller und robuster Analysemethoden notwendig. Im Gegensatz dazu stehen jedoch die Vielfalt und Komplexität vieler Lebensmittel, die eine schnelle Analytik oft erschweren. Gängige Analysetechniken umfassen neben dem eigentlichen Analyseprozess komplizierte und teils zeitaufwendige Probenvorbereitungen. Entsprechend optimierte Techniken sind daher notwendig, um diesen Anforderungen gerecht zu werden. Andernfalls wäre es für Auftragslabore, die mit mehreren Tausend Proben im Jahr konfrontiert sind, nicht möglich, gesundheitsschädliche Inhaltsstoffe oder Qualitätsdefizite eines Lebensmittels zeitnah aufzudecken.

Chromatographische Trenntechniken finden zumeist Anwendung im gesamten Probenhandling, sei es bei der Probenvorbereitung oder im eigentlichen Trennprozess. Die Onlinekopplung beider Schritte ist jedoch bisher selten für Routineumgebungen beschrieben worden. Die Kopplung flüssigkeits- und gaschromatographischer Techniken (LC-GC) ermöglicht es beispielsweise, komplexe Analysemethoden effizient und robust zu automatisieren. Dabei wird die Probenvorbereitung online direkt an den anschließenden chromatographischen Trenn- und Detektionsprozess gekoppelt. Die HPLC übernimmt hier die Aufgabe einer analytischen Probenvorbereitung, während die GC als robuste Quantifizierungstechnik verwendet wird. Obwohl die Kopplungstechnik als solche bereits seit über 30 Jahren bekannt ist, wurde sie dennoch nie in großem Maßstab in Routineumgebungen eingeführt. Ein Grund dafür mag in den technischen Anforderungen und den daraus resultierenden Stabilitätsproblemen liegen.

Aus diesen Gründen lag der Fokus bei der Erstellung dieser Arbeit auf der Entwicklung und Validierung ausgewählter Analysemethoden basierend auf einer modernen und robusten LC-GC-Kopplung. Hauptaugenmerk war dabei stets die Eignung der entwickelten Methoden in Routineumgebungen. Hoher Probendurchsatz war genauso wichtig wie eine robuste, präzise und richtige Analytik. Die ausgewählten Applikationen umfassen Beispiele aus dem Bereich der Kontaminantenanalytik, der Qualitätskontrolle von Speiseölen und -fetten sowie der Bestimmung des Vitamin D-Gehaltes ausgewählter Lebensmittel.

Die LC-GC-Kopplung erlaubt in vielen Fällen die Direktinjektion von Proben, für welche im Normalfall zeitaufwendige Säulenchromatographien notwendig sind. Methoden, die

üblicherweise vorhergehende Probenvorbereitungsschritte, wie Verseifungen, Derivatisierungen oder Flüssig-flüssig-Extraktionen, benötigen, können erfolgreich durch die Einbindung von Probenrobotern (Autosamplern) automatisiert werden. Die Bestimmung der Sterinverteilung und des Gesamtgehaltes in Speiseölen und -fetten wurde im Rahmen dieser Dissertation als Beispiel gewählt und beschrieben.

Die manuelle Standardmethode beinhaltet eine mehrstufige, zeit- und arbeitsaufwendige Probenvorbereitung. Ringversuche belegen regelmäßig, dass die erzielte Vergleichspräzision oft ungenügend ist. Im Gegensatz dazu konnte durch eine LC-GC-Methode mit vorgeschalteter automatisierter Probenvorbereitung eine erhebliche Steigerung der Präzision, Richtigkeit und des Probendurchsatzes erzielt werden. Der durchschnittliche Probendurchsatz lag bei einer Probe pro Stunde, wobei der einzige manuelle Schritt nur noch aus der Probeneinwaage bestand. Dies stellt einen signifikanten Vorteil zur Standardmethode dar, sodass die hier vorgestellte Applikation für Auftragslabore prädestiniert erscheint. Abschließend konnte durch die analytischen Vorteile einer LC-GC-MS-Methode die Identität eines vorher unbekannten Sterins aus Sonnenblumenöl näher beschrieben werden.

Die Bestimmung des Vitamin D-Gehaltes aus ausgewählten Lebensmitteln stellt ein weiteres Beispiel einer erfolgreichen LC-GC-Kopplung dar. Während sich für diese Analytik in der Vergangenheit ausschließlich HPLC-Methoden durchgesetzt haben, die für komplexe Lebensmittel, wie cholesterinsenkende Margarinen, teilweise ungeeignet sind, konnte mit einer LC-GC-MS-Methode der Vitamin D-Gehalt jener Lebensmittel effizient bestimmt werden.

Im weiteren Verlauf dieser Arbeit wurden Lösungsstrategien zur Behebung LC-GC-spezifischer Unzulänglichkeiten erarbeitet. Bei der Bestimmung aromatischer Kohlenwasserstoffe in einer Vielzahl von Lebensmitteln stellte sich eine Probenaufreinigung mittels einfacher HPLC als nicht zielführend heraus. Dies ermahnte, dass eine LC-GC-Kopplung wie jedes andere analytische Werkzeug stets an die analytische Fragestellung adaptiert werden muss. Die Verbesserung der Aufreinigungsqualität war daher eine zwingende Notwendigkeit.

Die Bestimmung polyzyklischer aromatischer Kohlenwasserstoffe (PAK) konnte effizient erfolgen, indem eine einfache und generische Probenvorbereitung mit einer zweidimensionalen heart-cut HPLC-Aufreinigung gekoppelt wurde. Die Aufreinigungsqualität für komplexe Lebensmittel, wie Speiseöle, Kaffees, Tees oder Hühnereier, konnte hierdurch signifikant gesteigert werden. Eine umfassende Methodenvalidierung auf der Matrix „Olivenöl“ belegte die Präzision, Richtigkeit und Robustheit dieses Ansatzes.

Selektive Derivatisierungsreaktionen störender Matrixbestandteile wurden zur Bestimmung aromatischer Kohlenwasserstoffe aus Mineralölen (MOAH) für die Probenmatrix „Speiseöle und -fette“ untersucht, weil sich ein HPLC-Aufreinigungsansatz als ungenügend erwies. Im Verlauf der Arbeiten stellte sich heraus, dass eine robuste Analytik nur durch eine präzise Reaktionsführung möglich war. Diese konnte durch die etablierte Autosamplerbasis mit anschließender LC-GC-Kopplung sichergestellt werden.

Abschließend zusammengefasst, wird in dieser Arbeit gezeigt, dass eine moderne LC-GC-Kopplung für den Einsatz in Routineumgebungen vom analytischen Standpunkt umfassend geeignet erscheint. Die Erweiterung dieser Technik um bekannte und bewährte Probenvorbereitungsschritte eröffnet überdies neue Möglichkeiten, wie am Beispiel der Sterinbestimmung gezeigt werden kann. Für einen flächendeckenden Einsatz der LC-GC-Technik in Routineumgebungen sind jedoch neben der analytischen Eignung ausführliche Normierungsarbeiten nationaler Behörden bzw. der verantwortlichen Arbeitsgruppen notwendig.

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1. Introduction

Conservation of human health and life by appropriate nutrition is a problem as old as humanity itself. Consequently, assurance of food safety was always of great importance since it is directly related to human health. Economic progress, however, made it necessary to analyze foodstuffs also for other reasons. For example, adulteration of foodstuffs is of big commercial interest. Therefore, also quality of food has to be ensured and monitored.

While organoleptic probing of foodstuffs was always a valuable tool for safety assurance, scientific progress allowed to investigate foodstuff compositions more comprehensively. Unwanted contaminations or allergens are only two classes of constituents that have to be identified and declared nowadays. Reaching these goals in a world, in which billions of people have to eat and drink, is only feasible with highly efficient analytics. A general overview of foodstuff analytics in routine environments is given in Fig. 1.1.

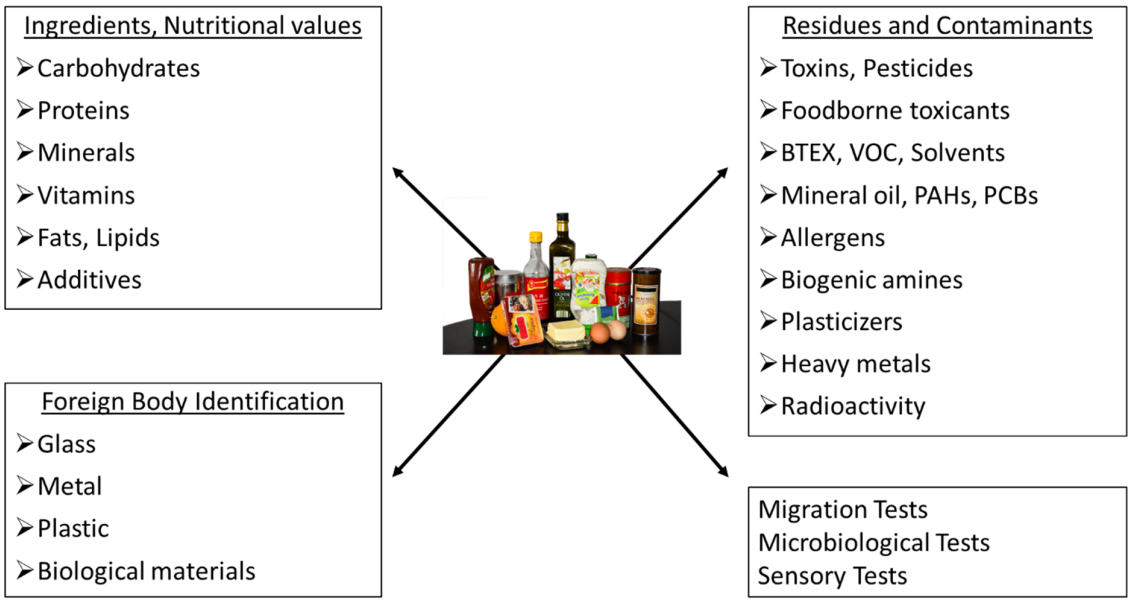

Fig. 1.1. General overview of food analytics in routine laboratories (BTEX: Benzene, toluene, ethylbenzene, xylenes – VOC: Volatile organic compound – PAHs: Polycyclic aromatic hydrocarbons – PCBs: Polychlorinated biphenyls)

Because of the complex nature of food, adaptable analytical techniques are necessary to answer emerging questions. Reliable detection of ppb-amounts (parts per billion) of an analyte in the presence of a complex food matrix is a highly demanding problem that cannot be solved in a unique way.

1.1 Chromatographic food analysis

Chromatographic methods, such as gas chromatography (GC) or high-performance liquid chromatography (HPLC), coupled to various detection systems, e.g., flame ionization detectors (FID), UV detectors, or mass spectrometric (MS) detectors, are omnipresent in food analytics. Although these techniques offer a high degree of certainty combined with high sensitivity for the detection of individual analytes, the direct injection of foodstuffs is generally not possible. Sample preparation is needed to remove the bulk of matrix beforehand. For this purpose, additional chromatographic cleanup during sample preparation is usually employed. Otherwise, rapid contamination of the analysis system and the impossibility of analyte identification would be the consequences.

Advantages in instrument designs offer steadily increasing sensitivity paired with reduction of analyses time. Sample preparation, however, is often still complicated, work-intense and error-prone, and limits overall sample throughput and precision.

Solid-phase extraction (SPE) and thin-layer or column chromatography are important tools in sample preparation protocols. Silica gel or polymeric-based materials are used for the separation of the analytes of interest from the interfering matrix. Automation of these techniques is most of the times problematic. Comparable materials are also found as stationary phases in HPLC columns. Because of highly sophisticated manufacturing processes and smaller particle sizes, HPLC columns exhibit much higher separation efficiencies than the other techniques. Additionally, automation is easily achieved.

For this reason, the use of HPLC techniques for sample preparations is desirable. While few selected analysis methods already make use of HPLC, it is still only a minor percentage in the daily routine [1]. One possible explanation could be the problematic coupling of the sample preparation to the analysis system and its rugged operation.

1.2 Hyphenation of multiple chromatographic dimensions

Coupling of an automated HPLC-based sample preparation and the subsequent analysis step can be accomplished in two different ways: Offline and online.

In offline methods the cleaned-up HPLC fractions are collected before they are used for subsequent analysis steps. Fraction collection is performed either manually or automatically. For instance, fraction collection after gel permeation chromatography (GPC) in the field of pesticide residue analysis is commonly automated [2]. One big disadvantage of this technique is the sample dilution which has to be compensated somehow afterward. Hence, time-consuming evaporation of the fraction solvent is necessary and can be the source for additional contamination. Even then, only a small fraction is injected either into HPLC- or GC-based systems.

Online methods, however, try to solve these disadvantages with additional instrumental effort. The complete analyte fraction obtained after the sample preparation is transferred directly into the analysis system. Thus, less sample is needed in the first place, higher sensitivity is observed, and less contamination is generally feasible. Furthermore, less manual work is needed, which is an important factor in routine laboratories.

1.3 HPLC-GC hyphenation

In this context, the coupling of HPLC and GC is noteworthy. The idea is not new: First publications can be found already in 1980 [3]. The link of both techniques is highly efficient. The advantages are obvious:

- High sample capacity of HPLC needed for sample preparation
- High separation efficiency/peak capacity of capillary GC
- Fast GC analysis cycles
- Orthogonal separation modes of HPLC and GC
- Automated sample preparation
- Minimized analyte loss
- Minimized memory and carryover effects
- Sensitivity increase by transfer of whole HPLC fractions
- No evaporation or solvent exchange necessary (less sample degradation and contamination)

- Recovery rates are generally higher
- Higher sample throughput

HPLC generally exhibits higher sample capacity in comparison to GC. Furthermore, HPLC allows highly efficient sample cleanups. HPLC is readily compatible with high molecular weight sample matrix while GC is struggling with the low or nonexistent volatility.

Achieving separation orthogonality by the numerous HPLC operation modes (normal phase, reversed phase, size exclusion, etc.) is more efficiently possible in comparison to simple GC-based methods. On the contrary, capillary GC exhibits significantly higher peak capacities than conventional HPLC. Furthermore, GC offers a wide range of detection systems normally not amenable to HPLC, e.g., FID or electron impact mass spectrometry (EI-MS).

In HPLC-GC coupled systems the lossless fraction transfer from HPLC to GC is one key point. The volumes often exceed 100 µL and can range up to several milliliters. In classical GC applications normally 1 µL is injected. Hence, large volume transfer techniques must be thoroughly understood and precisely controlled.

A very prominent example from the last decades is the determination of mineral oil contaminations in food and paperboard [4]. HPLC-GC hyphenation tries to solve this analytical task very efficiently. Normal-phase HPLC on silica gel sorbents is used for the extraction and purification of mineral oil contaminants from accompanying matrix, e.g., triglycerides, fatty acids, etc. [6]. Even further, mineral oil contaminations are separated into aliphatic and aromatic compounds. Quantitation is accomplished by GC-FID. A FID offers significant advantages over most other detectors. It exhibits a quasi-unity response for all compounds with similar structures. Therefore, no expensive or nonexistent quantitation standards have to be used.

1.3.1 Historical system overview

As mentioned before, the hyphenation of HPLC and GC was reported already in the 1980es. Especially Konrad Grob and his team from the Official Food Control Authority of the Canton Zürich (Switzerland) pushed the limits of this technology. Numerous international peer-reviewed publications can be found [5–7]. In 1989, the company Carlo Erba (later known as Thermo Fisher Scientific) commercialized the HPLC-GC technique under the brand Dualchrom 3000. It was the first complete solution consisting of both, hard- and software. It was the instrumental base for various peer-reviewed articles from several research groups all over Europe [8–10].

In general, the Dualchrom system consisted of an HPLC syringe pump equipped with several rotary HPLC valves, a conventional GC-system with on-column injector, as well as selectable analog GC detectors. Even today, almost 20 years later, this system is still available with only slight modifications from Brechbühler AG (Schlieren, Switzerland). Its current setup is shown in Fig. 1.2.

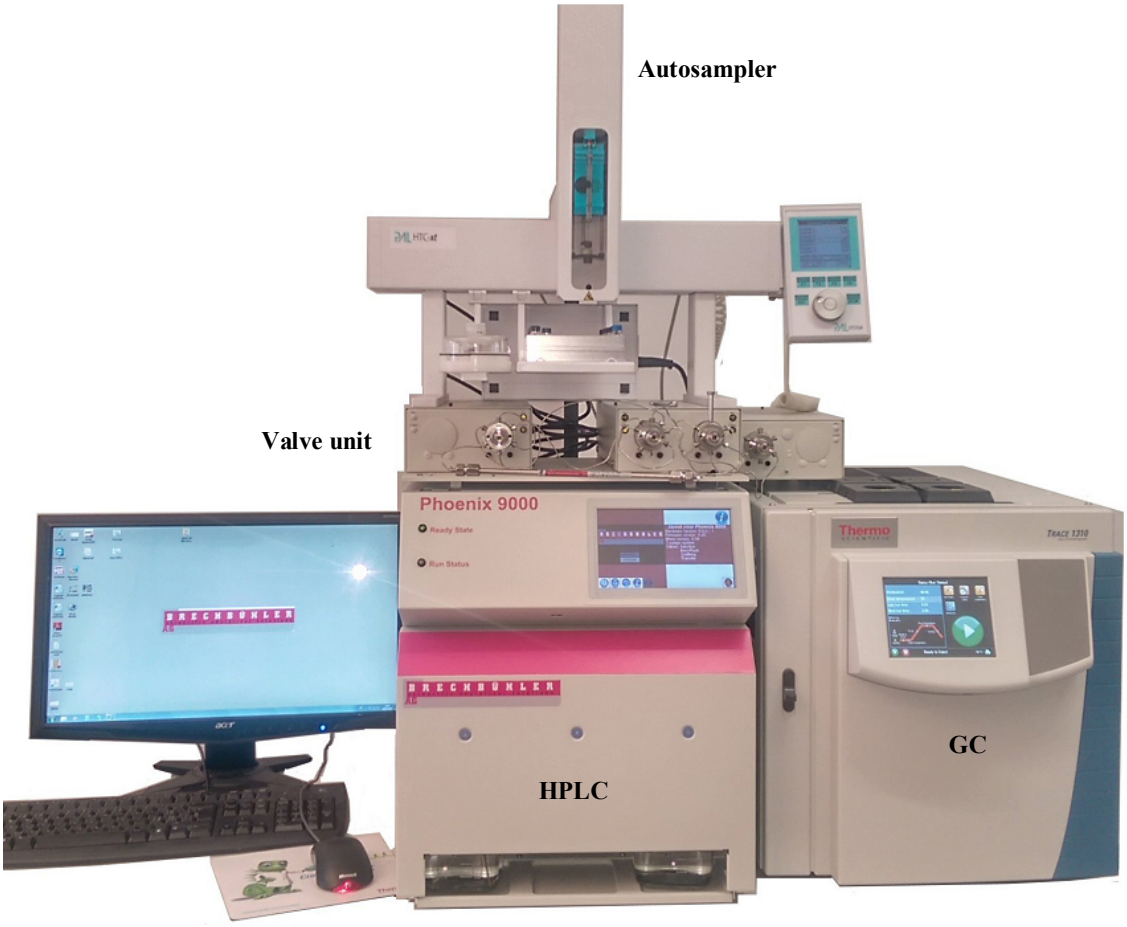

Fig. 1.2. General system overview of an LC-GC system (LC-GC 9000 from Brechbühler AG) consisting of an HPLC syringe pump, GC-FID, CTC Analytics HTC-xt PAL autosampler, and Valco HPLC valves.

After sample extraction, an aliquot is injected by an autosampler into a common HPLC valve equipped with a sample loop. First chromatographic separation takes place on a conventional HPLC column. The fraction containing the analytes of interest is guided by a transfer valve online directly into the GC. For this purpose, an appropriate liquid transfer interface able to remove the HPLC solvent without discrimination of the analytes has to be selected.

Solvent evaporation is generally realized either by large-volume on-column or PTV-based techniques (programmable temperature vaporization). However, the former one was reported more frequently in literature in the past. After removal of the majority of solvent, which will be explained in detail in subchapter 1.8, a common GC separation process including detection is performed. In Fig. 1.3, an example for an LC-GC separation is given. A small fraction is ideally obtained by HPLC, which is further separated and detected by GC-FID. During GC separation, residual matrix compounds stuck on the HPLC column are usually removed simultaneously by backflushing. After equilibration of the column, the system is ready for subsequent runs.

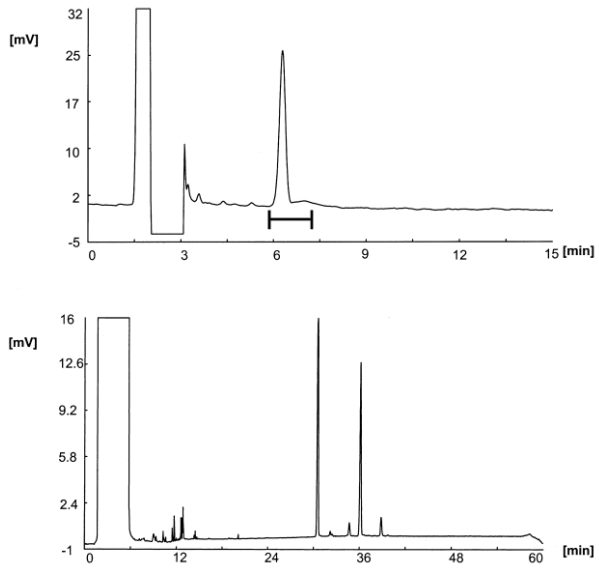

Fig. 1.3. Transfer of a fixed fraction window from HPLC to GC (Upper trace: LC-UV chromatogram, lower trace: GC-FID chromatogram of the transferred fraction [2])

1.3.2 Recent hardware developments

In routine environments, simplification of sample preparation is appreciated. High sample numbers and short analysis cycle times make the use of highly automated systems inevitable. LC-GC can comply with these requirements. However, one prerequisite had to be met before starting the work on this thesis.

Up till now the LC-GC hyphenation technique was thought to be complicated and error-prone. It is undoubted that the coupling of two powerful chromatographic techniques is challenging. Consequently, the deficiencies of existing and published LC-GC hardware solutions had to be analyzed and tried to be fixed. As mentioned, the origin of most LC-GC solutions dates back

to one common source. By improving the stability and robustness of most system parts, i.e., HPLC, GC, and software, a new LC-GC hardware approach could be designed.

Result of this work, which started in 2010, was the CHRONECT LC-GC interface available since 2014 (Axel Semrau GmbH & Co. KG, Sprockhövel, Germany). As can be seen in Fig. 1.4, the system is based on routine hardware parts already available in many routine environments. This decision was vital to consolidate the acceptance of such systems in routine laboratories.

Fig. 1.4. LC-GC system designed and used for application development in the scope of this thesis (Axel Semrau GmbH & Co. KG, Sprockhövel, Germany). The instrument is based on a conventional Agilent 1260 HPLC, Agilent 7890B GC-FID, and CTC Analytics PAL autosampler. The CHRONECT LC-GC interface module is the connecting element.

Hard- and software developments were out of the scope of this thesis focused on method developments, however, it is noteworthy that this thesis would not have been possible without them. Throughout all chapters of this work the described platform was used as a starting point.

1.4 LC-GC or LCxGC – Heart-cut vs. Comprehensive

Hyphenation of HPLC and GC is mainly performed to remove matrix interference from the analytes of interest. In simple cases, an LC fraction of several hundred microliters of volume is obtained that is directed online into the GC, in which evaporation of the solvent takes place. Large volume injection techniques are used to selectively remove the solvent. This kind of coupling is known as LC-GC or heart-cut mode.

Opposed to this, the transfer of "all" LC fractions with subsequent GC separation and detection is called comprehensive LCxGC. In this mode of operation, every bit of the LC eluate is guided into the GC. The modulation ratio, i.e., the sampling rate of each first-dimension (^{1}D) peak to the second dimension, should be at least 1.5 for major and 3 for trace compounds [11]. Reasons for this are twofold: firstly, the ^{1}D-separation should be conserved during ^{2}D-separation and, secondly, the influence of unintentional time variations (phase shifting etc.) on quantitative results should be minimized.

Consequently, a volume of approximately one third of each expected LC peak has to be continuously transferred into the GC without losing the subsequent LC eluate. Realization of such experiments is more demanding compared to LC-GC setups.

To prevent loss of LC eluate, it has to be either collected somewhere or the HPLC has to be used in the "stopped-flow" mode. This mode halts the HPLC flow during GC separation. Transfer of "all" LC fractions becomes feasible hereby.

Nevertheless, with conventional GC separation techniques LCxGC experiments require a huge amount of time. For instance, thirty 1 min LC fractions with corresponding GC runtimes of 30 min would require a total runtime of 15 h. However, ultrafast-GC separations with total runtimes below 1 min were reported recently and would drastically increase sample throughput [12]. Besides this, processing and evaluation of the obtained data is not possible by standard tools.

Brinkman et al. analyzed the FAME distribution (fatty acid methyl esters) of butter with an LCxGC approach [13]. First separation of the sample was realized by a silver coated silica gel HPLC column. Further separation and detection was performed by GC-TOF-MS. An example for an obtained chromatogram is shown in Fig. 1.5.

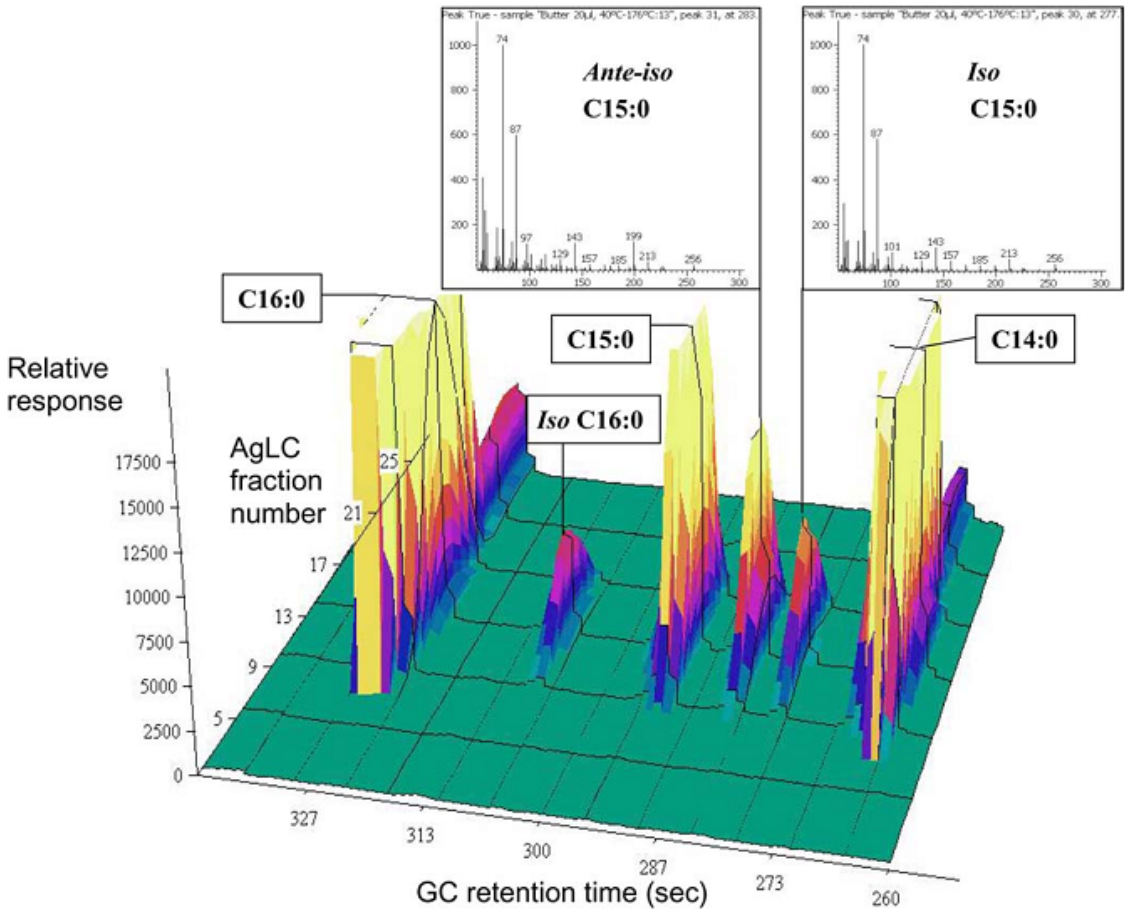

Fig. 1.5. Two-dimensional Ag-LCxGC-TOF-MS chromatogram of FAMEs found in butter [13]

1.5 HPLC as cleanup for GC

Choice of a suitable HPLC separation mode is crucial for a successful LC-GC hyphenation. The following questions arise:

- Selectivity
  - How can the analytes be separated from the sample matrix?
- Solvent compatibility
  - Is the chosen mobile phase suitable for real-time evaporation and compatible with the GC separation column and detection system?
- Sensitivity vs. capacity
  - Which HPLC column dimension is needed for the aspired detection limits?

While in classical HPLC mainly reversed-phase separation modes are used, hyphenation to GC most often is performed with normal-phase chromatography. One apparent reason for this

choice is the possibility to use non-aqueous solvents. Introduction of aqueous HPLC mobile phases into the GC is quite demanding. Liquid water attacks conventional fused silica surfaces, from which GC columns are made, thus rendering these surfaces more active. Additionally, water does not wet uncoated GC precolumns very well. Combined with the need of high evaporation temperatures, good recovery of volatile analytes becomes a challenge.

The choice of the right LC column diameter is another important aspect. While 4.6 mm allow injection of high sample amounts, the high flow rates of approximately 1 mL/min are not well suited for online evaporation ahead of a GC separation. On the other extreme, LC capillary columns (75 – 500 µm i.d.) can be run with extremely low flow rates but do not allow injection of sufficient sample material. Therefore, the use of 2.1 mm columns was established. This dimension combines sufficiently high sample amounts with appropriate LC flow rates (0.2 – 0.5 mL/min) which can be guided online into a GC without flooding the whole instrument.

Besides the solvent advantage in normal-phase chromatography, increasing retention depending on analyte polarity opposed to non-polarity as on conventional C_{18}-material, offers a well-suited cleanup prior to GC separation. If reversed-phase separation is needed, non-aqueous mobile phases should be checked. This mode of operation is known as NARP chromatography (non-aqueous reversed-phase) [14]. GPC is a third separation mode which is suited for GC hyphenation. Size exclusion is achieved in total organic solvents which can be introduced into the GC. A few successful approaches can be found in literature, e.g., removal of triglycerides for pesticide analysis [15].

Nevertheless, bare silica phases are the most popular HPLC phases used for LC-GC. Grob et al. showed that bare silica can hold high amounts of triglycerides without notable column overload [16]. This effect is the key for the solution of a lot of separation problems in fatty foodstuffs. Separation of nonpolar compounds is easily achieved and was reported, e.g., hydrocarbons or long-chain fatty acid esters [17, 18]. Additionally, bare silica phases provide a high pressure and solvent stability allowing regular backflushing of the column with various solvents for matrix removal without deterioration of the stationary phase.

Interestingly, bare silica is known to be a tricky phase. Uncontrolled adsorption of matrix compounds and unreproducible retention times were reported in the past [19]. Reason for this behavior are the active sites on the stationary phase surface. These are formed mainly by free silanol groups providing retention for increasingly polar molecules. Previous generations of bare silica HPLC columns contained rather high amounts of free metal ions which in turn were the source for uncontrolled retention and bad column-to-column reproducibility [20].

Another problem is that even slightly polar mobile phases are hardly removed from the column surface and elongate equilibration times [21]. The analytes of interest compete with the polar solvent molecules for the active adsorption sites.

The use of gradient separation is restricted to few suitable solvent combinations. Because chromatography on bare silica is mainly based on adsorption, an effect called “solvent demixing” is coming into account having a significant influence on gradient formation [22]. Adsorption of the solvent changes the composition of the mobile phase, influencing the separation.

Nevertheless, the use of bare silica HPLC phases in gradient elution is successfully applied in specific applications, e.g., the separation of aliphatic and aromatic hydrocarbons [4].

1.6 LC-GC Transfer and real-time solvent evaporation

The cleaned-up LC fraction has to be minimized in volume to become compatible with GC. Therefore, real-time evaporation of the solvent is necessary. In the last decades several attempts were reported in literature [23]. Generally speaking, the transfer of large-volume LC fractions into the GC is comparable with GC-injection of large sample volumes with a syringe. This field of research, large volume injections (LVI), was explored thoroughly by several research groups.

1.7 PTV Solvent split

PTVs are commonly found in gas chromatographs. Opposed to the classical SSL injector (split/splitless), it can be heated and cooled independently in a short time. Its construction can be taken from Fig. 1.6. The injection volume for conventional split or splitless injections is restricted to the used liner dimensions and is therefore essentially the same as for SSL injectors.

During classical 1 µL splitless injections, evaporation of the sample liquid forms gas vapors of 500 to 1000 µL [24]. If the vapor cannot be hold by the liner, expansion of the gas volume into dead volumes, such as the carrier gas or septum purge lines, is observed being the source for memory and carryover effects.

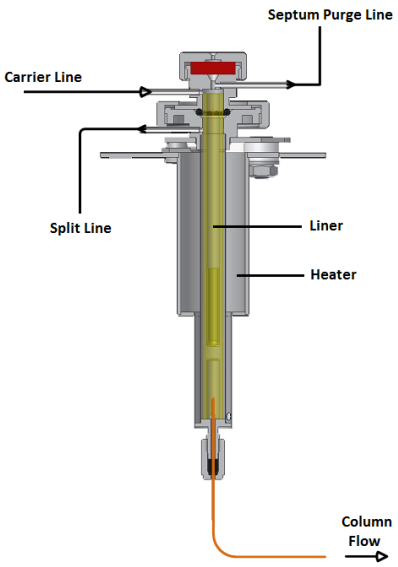

Fig. 1.6. Schematic overview of a PTV injector (OPTIC-4 Multi Mode Inlet, GL Sciences B.V., Eindhoven, The Netherlands)

A technique called "Solvent Vent" tries to circumvent the problem by precise temperature and carrier gas control during evaporation. A liner with a packing material is chosen having a high surface area supporting rapid evaporation of introduced liquid.

Instead of total evaporation of the sample at high temperatures as in splitless injections, the PTV temperature is held at a low temperature allowing selective evaporation of the solvent by means of a high carrier gas flow rate. In turn, the liner volume is not the limiting factor for the introduced sample volume anymore. Nevertheless, recovery of highly volatile compounds can be problematic because of co-evaporation. In general, this process is comparable with common nitrogen evaporators found in most laboratories.

1.7.1 Speed-controlled large volume injection

In simple cases, evaporation conditions are optimized to allow instantaneous evaporation of the introduced sample liquid. In this mode of operation, virtually no limit for the sample amount exists. Unfortunately, co-evaporation of volatile compounds renders high recovery of these compounds impossible. In terms of carbon-numbers, analytes up to $C_{18}H_{38}$ (octadecane) are lost [25].

Improving recovery is possible by PTV sub-ambient cooling although being inefficient and complicated. Another approach makes use of high retentive liner packaging, e.g., Tenax TA. Even at elevated injection temperatures generally needed for high-boiling solvents, e.g., water, recovery of volatile compounds becomes feasible [26]. Nevertheless, retention on the liner can be problematic for high-boiling compounds [25]. In this case, on-column injection can offer superior results.

Staniewski et al. investigated the physical properties needed for speed-controlled PTV injections [27]. They proposed an equation giving an estimation for the possible sample introduction rate. Only two assumptions were made: Firstly, the incoming carrier gas is totally saturated with solvent vapor when leaving the injector through the split line. Secondly, evaporation occurs under isothermal conditions.

Although neither of both assumptions is correct, fairly good starting conditions are obtained for further practical testing.

$$V_{inj.max} = V_{el} = \frac{M p_a}{\rho R T_o} \frac{p_o}{p_i} V_{t,o}$$

$V_{inj.max}$: Max. Injection speed (µL/min)

V_{el}: Evaporation speed (µL/min)

M: Molecular mass of the solvent (g/mol)

p_a: Partial pressure of the solvent, dependent on the injection temperature (Pa)

ρ: Density of the solvent (kg/m³)

p_o: Ambient pressure (Pa)

p_i: Injector pressure (Pa)

$V_{t,o}$: Total injector gas flow (µL/min)

R: Universal gas constant (J mol^{-1} K^{-1})

T_o: Ambient temperature (K)

As a consequence, increase of sample introduction speed can be accomplished by:

- Increase of injector temperature
  → Increase of the partial pressure of the solvent inside the injector
- Increase of the carrier gas flow through the injector
  → Decrease of the dew point of the solvent-carrier gas mixture
- Reduction of the injector pressure
  → Decrease of the solvent's boiling point

1.7.2 At-Once/Rapid large volume injection

Another mode of operation is called “At-once” or rapid large volume injection. Here, the total sample liquid is quickly injected into the liner. Consequently, the liner volume and packing material must be optimized to hold the injected liquid volume. An inert material with high surface area is used for this purpose, e.g., glass wool or diatomaceous earth.

Again, a high carrier gas flow rate is used at low temperature to induce evaporation. On the solid support the sample liquid spontaneously starts evaporation creating a cold spot in the liner packaging. This spot is suited for retention of highly volatile material. Before the last bit of solvent is evaporated, the split exit of the injector is closed and a conventional PTV-splitless injection is initiated.

Efficiency of this operation mode is significantly dependent on the used solvent. A low boiling point with low enthalpy of vaporization favors a rapid cooldown of the liner packaging during evaporation. For example, vaporization of *n*-hexane is more efficiently possible than water.

Using this technique for LC-GC hyphenation requires mostly a collection of the LC eluate. Typically, a syringe with appropriate fraction volume is used for this purpose. After fraction collection, a rapid large volume injection can be performed as described above. In Fig. 1.7, the general operation scheme is given.

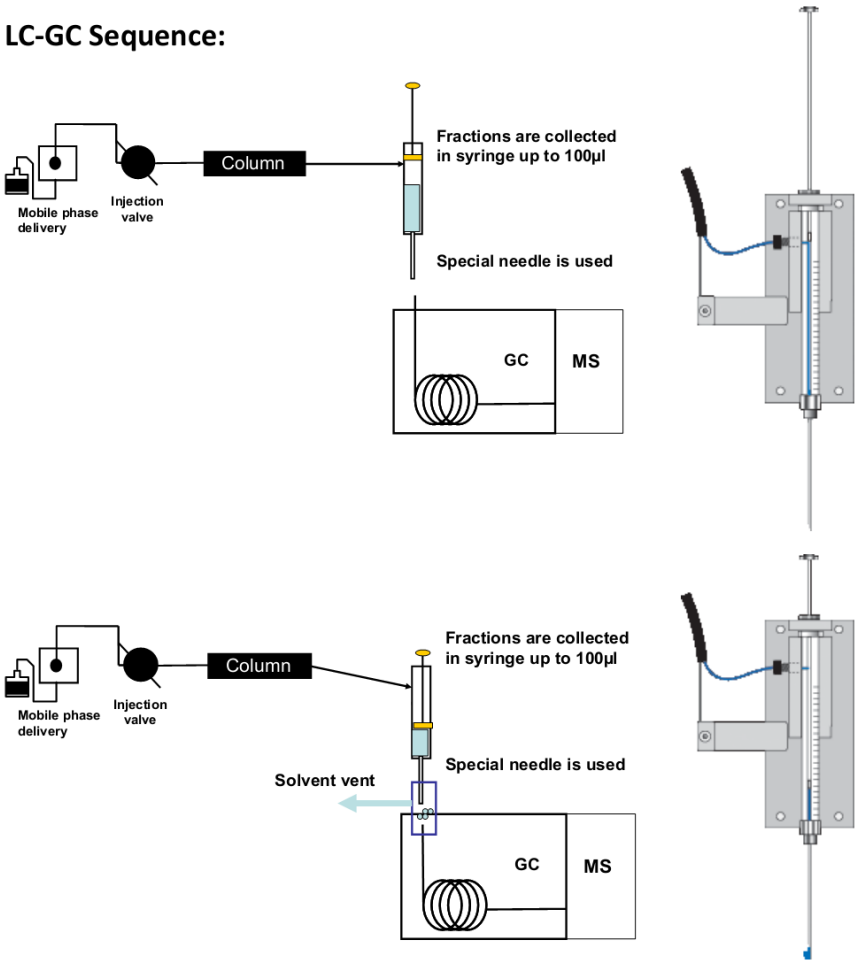

Fig. 1.7. Syringe-based LC-GC interface with a PTV and the at-once mode (GL Sciences B.V.)

1.8 On-column techniques

For large volume injections and for HPLC-GC hyphenations, in particular, the use of on-column injection techniques were also reported in the past.

Using thermal vaporizers, such as SSL or PTV injectors, the injected sample is vaporized and only the gaseous compounds are flushed onto the GC column by means of the carrier gas. In contrast, in on-column setups the sample is directly injected into the GC column. Consequently, an on-column injector is mainly a fixation for the GC column. This way, an autosampler is able to directly inject into the column (see Fig. 1.8).

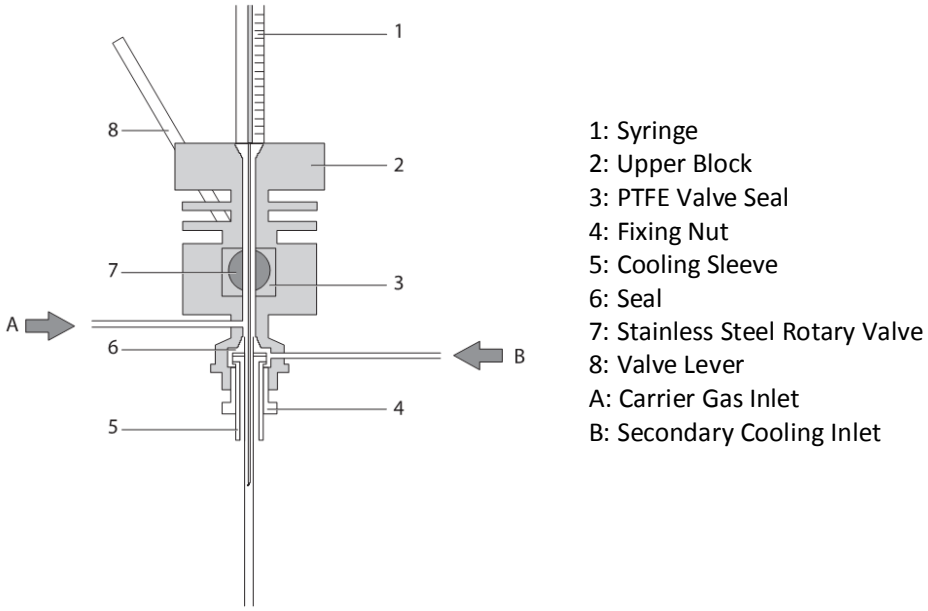

Fig. 1.8. Schematic overview of an on-column injector (Thermo Fisher Scientific, Dreieich, Germany)

Evaporation takes places inside the GC column. Thermostatting is controlled by the GC oven itself. Opposed to classic injectors, on-column injection techniques ensure the complete transfer of the sample into the GC [28]. Thermal degradation of labile compounds is minimized. Furthermore, the transfer of high-boiling material is easily achieved. Unfortunately, the transfer of non-volatile material cannot be avoided. Accumulation inside the GC column can be the source for rapid degradation and peak shape deterioration [29].

In LC-GC setups, however, the LC pre-separation step is optimized to remove non-volatile material. In these cases, on-column transfer is the method of choice to guarantee the complete transfer of LC fractions.

Coupling of HPLC and GC is normally performed by means of a 6-port rotary HPLC valve. Eluate from the LC column is guided either into waste direction or directly into the GC by a thin fused silica capillary permanently inserted into the on-column injector (see Fig. 1.9).

One problem originating from permanent installation emerges after a finished liquid transfer. In this situation, the transfer capillary is filled with LC eluate. If this residual liquid is not removed, pronounced solvent peak tailing is observed on the GC detector because of diffusion effects. Removal of this liquid (approximately 1–2 µL) is achieved by backflushing the transfer capillary with clean GC carrier gas. Therefore, a high restriction capillary is installed on the 6-port valve (see Fig. 1.9). The carrier gas is used to push residual liquid through the restriction

capillary. An inner diameter of 50 – 75 µm is sufficient to allow removal of the solvent while maintaining the carrier gas flow rate needed for GC separation. Since this approach is working like a carrier gas split, bigger diameter capillaries should be avoided.

Although the liquid is removed in this way, residual sample material is deposited inside the transfer capillary. It can be the source for carryover or memory effects in a subsequent run. Therefore, it is important to choose the right LC fraction window. The last few seconds of an LC fraction should mainly consist of pure solvent.

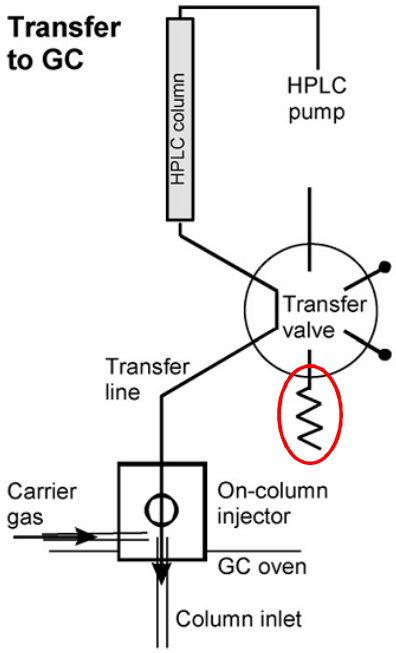

Fig. 1.9. Connection between HPLC and an on-column injector (restriction capillary encircled in red) [30]

GC columns with an inner diameter of 0.53 mm permit insertion of a typical syringe needle or fused silica transfer line. To allow the use of common GC column diameters for separation, this wide-bore column is normally connected to a classic GC column by means of a pressfit or suited metal connector. This way, the 0.53 mm (precolumn) can be used to allow sample injection whereas a classic GC column is used for separation.

These fundamentals were the base for creation of the Dualchrom 3000. Nevertheless, it was reported that a simple connection of LC and GC by an on-column injector bore the problem of significant carryover of approximately 0.5 – 3 % [30]. Biedermann et al. investigated the

sources and found the carryover in the dead volume inside the on-column injector. Therefore, they proposed the use of a dead volume-free 3-way pressfit. The so called Y-interface takes the place of the on-column injector. Carryover and memory effects were reported to be less than 0.1 % (see Fig. 1.10) [30].

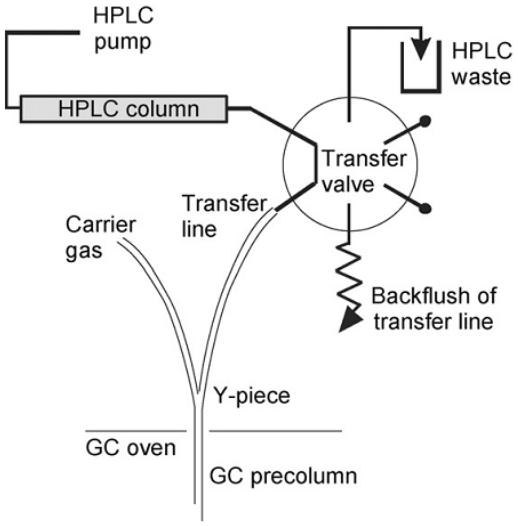

Fig. 1.10. Replacement of the on-column injector by the Y-interface offering significantly less carryover and memory effects [30]

1.8.1 Alternative on-column techniques

It is worth mentioning that in the past several other on-column interfaces were reported, e.g., a loop-type interface [31]. Nevertheless, in the past few years mainly the on-column interface mentioned above was reported in literature.

1.8.2 Solvent trapping and band broadening

The evaporation of a sample inside a GC column follows defined rules. Injecting a liquid sample directly into the column requires a GC oven temperature below the pressure corrected boiling point of the sample solvent. Otherwise, evaporation of the sample at the column head would generate a pressure surge inside the column hindering the introduction and spread-out of the residual sample.

The volume covered by the liquid sample inside the column is called “flooded zone”. The length of the flooded zone is mainly dependent on the sample size, the column dimensions, and the surface properties of the inner column walls. The process is visualized in Fig. 1.11.

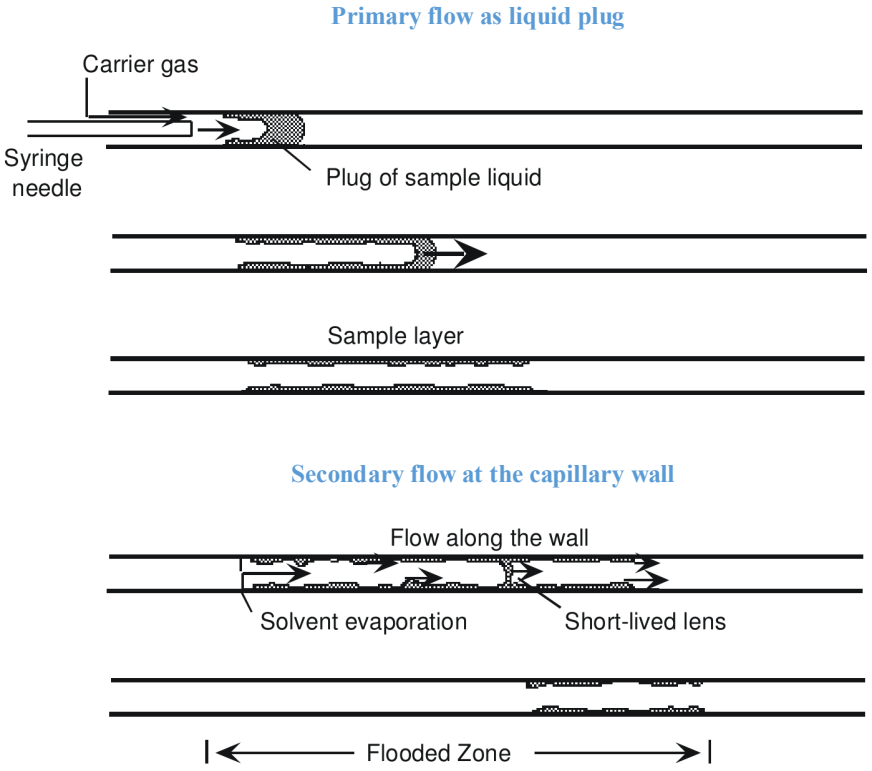

Fig. 1.11. Formation of the flooded zone at the column entry: After formation of an instable film at the capillary wall, the flooded zone is generated in secondary processes thereafter [32].

Conventional capillaries are coated with nonpolar polysiloxane polymers. Therefore, surface tension and other physical properties allow good wetting of the inner surface when nonpolar solvents, such as alkanes, are used. This way, a stable liquid film is formed on the inner capillary walls exhibiting a flooded zone of minimal length. On the contrary, if a polar solvent, e.g., methanol, is injected droplets are observed not able to wet the column surface. The observed flooded zone is significantly enlarged. A few microliters can deeply penetrate into the column.

Evaporation of the sample is performed by saturation of the carrier gas starting at the column entrance. As soon as it is saturated with solvent vapor, it flows unhindered through the residual flooded zone and column. Discharge of the formed vapors requires travelling through the total column and the installed detection system. During evaporation two observations can be made:

- Volatile analytes co-evaporate with the solvent
- High boiling compounds reside at the location inside the column where they were deposited during formation of the flooded zone

As evaporation of the flooded zone starts from the backside, volatile compounds co-evaporating with the solvent are re-trapped and enriched in the residual solvent front. This process, called "Solvent trapping', is repeated till the residual solvent amount is minimized to a few microliters. However, wetting of the column surface by the used solvent is one prerequisite for efficient solvent trapping.

High boiling material is spread all over the flooded zone after evaporation. This phenomenon is called "Band broadening in space". Depending on the initial solvent amount, this size can range from a few centimeters up to several meters. However, detection of a chromatographic peak requires an initial band width of approximately 20–40 cm when conventional capillary columns are used [34]. Therefore, re-focusing of high boiling material after evaporation is necessary for a successful detection.

Narrowing of chromatographic bands requires consideration of a few aspects. Opposed to the more common "Band broadening in time", which is observed during conventional splitless injections, band broadening in space is a consequence of direct injection into the column. While effective use of solvent trapping (=lowering the GC oven temperature) can narrow the initial band width caused by band broadening in time, this is not possible for band broadening in space.

The retention gap effect is an effective possibility to cope with large initial bands when band broadening in space is observed. An uncoated fused silica capillary is coupled to the actual GC column. If evaporation takes place in the uncoated capillary, the flooded zone is also located inside this capillary. Because of missing stationary phase, migration of analytes is possible at significantly lower GC oven temperatures compared to coated capillary columns. According to Grob et al., migration temperatures are approximately 100 °C lower [35]. Because of this, this type of capillary is also known as retention gap. As soon as the analytes reach the stationary phase of the analytical column, further migration is slowed down. Enrichment of the analytes in a small column segment is therefore possible. This effect is known as "Phase ratio focusing". As soon as the GC oven temperatures reaches temperatures suitable for migration in the presence of a stationary phase, a typical chromatographic separation is thereafter feasible.

Enrichment for both analyte groups, i.e., volatile and high boiling analytes, is visualized in Fig. 1.12.

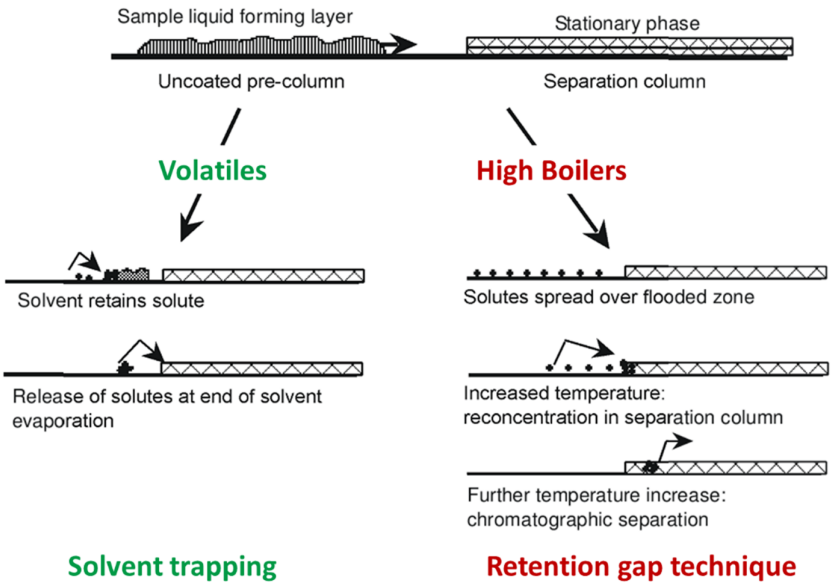

Fig. 1.12. Reconcentration of the initial bands for volatile and high-boiling compounds inside a capillary column [32]

1.9 The early solvent vapor exit (SVE)

Discharge of the solvent vapors through the total GC column is not effective. Additionally, not all detectors are compatible with high amounts of solvent vapors, e.g., ECD – electron capture detector for detection of chlorinated compounds). Effective discharge of solvent vapors is possible by using a SVE (Solvent vapor exit) [36].

Instead of direct coupling of retention gap and analytical column, an additional sidearm between both columns is installed. At the end of this sidearm an electric valve is installed which is opened against atmosphere during solvent evaporation. During analytical separation, however, the valve is closed and only a small purge flow is maintained to purge the sidearm. By using an SVE, discharge of solvent vapors is significantly increased (20 – 50 times) because flow restriction is given only by the retention gap instead of the total column system [37].

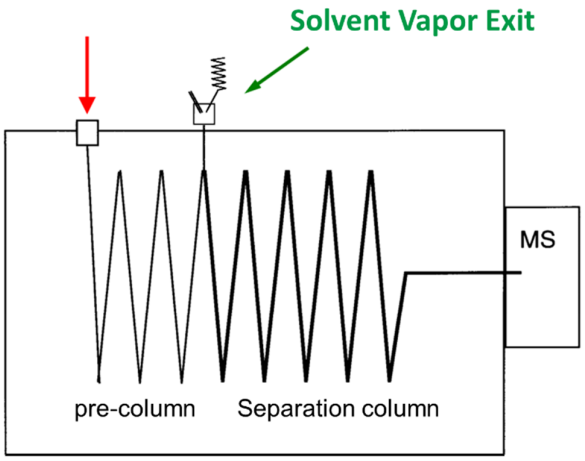

Fig. 1.13. Position of the solvent vapor exit (SVE) for the large-volume on-column technique between pre- and separation column [32]

1.9.1 Fully concurrent solvent evaporation (FCSE)

Two important kinds of sample introduction can be distinguished when using on-column injection techniques: Fully and partially concurrent solvent evaporation. The first one discharges the created solvent vapors at the speed of sample introduction through the SVE. Therefore, only a short retention gap of 30–50 cm is needed providing enough flow resistance. Since no solvent trapping is possible, highly volatile compounds are lost by solvent co-evaporation (see Fig. 1.14). Minimizing these losses is possible by use of a retaining precolumn. This is essentially a retention gap coated with a thin film of stationary phase, mostly a polysiloxane polymer. When this precolumn is exposed to high solvent vapor amounts, a swelling of the stationary phase is observed that can be used to trap volatile analytes. This effect is known as “Phase soaking” and its consequences can be seen in Fig. 1.15.

As soon as the GC oven is heated after the sample introduction, the stationary phase swelling is reversed and the trapped analytes can start chromatography. The length of the retaining precolumn is derived from the maximum tolerable initial band width of the measured analyte. Thus, approximately 30–50 cm are sufficient.

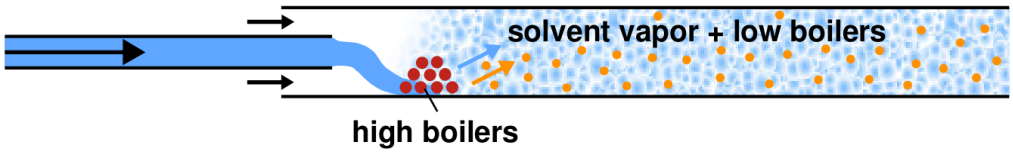

Fig. 1.14. Fully concurrent solvent evaporation for the analysis of high boiling analytes [33]

Phase soaking is most efficient when the GC oven temperature is slightly above the dew point of the introduced solvent vapor/carrier gas mixture. Recondensation of the solvent must be prevented. Lowering the dew point of the solvent vapor is possible by dilution with a higher amount of carrier gas. Therefore, increasing the carrier gas pressure (=higher dilution) allows a reduction of the GC oven temperature without observing recondensation.

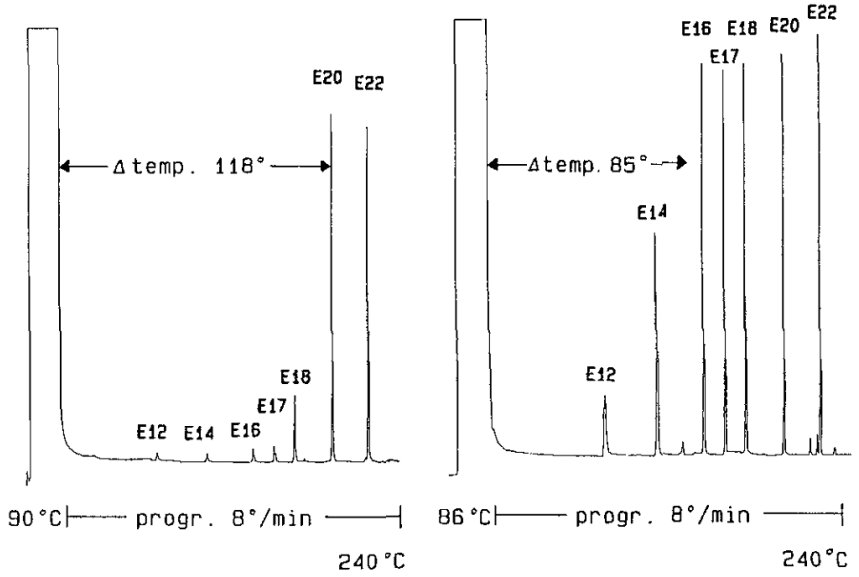

Fig. 1.15. LC-GC large-volume on-column transfer of fatty acid methyl esters by use of FCSE – Left: with a short retention gap – Right: with an additional retaining precolumn connected to the retention gap (it may be worth mentioning that a retaining precolumn can be used without a retention gap.) [18]. Phase soaking significantly increased the recovery of volatile compounds.

1.9.2 Partially concurrent solvent evaporation (PCSE)

If more volatile compounds have to be analyzed, partially concurrent solvent evaporation is the method of choice. Retention gaps of 10 m length and 0.53 mm inner diameter allow the introduction of approximately 1 mL of solvent. Retention of highly volatile compounds is possible without loss (e. g. *n*-nonane or *n*-decane when *n*-hexane is used as solvent). Efficient use of solvent trapping is the key point when working under PCSE conditions.

Discharge of the solvent vapors occurs at a speed slightly lower than the sample introduction speed. Because of this, a flooded zone can be formed inside the retention gap able to trap volatile material as explained above. After the end of transfer, the residual flooded zone is minimized by elongation of the SVE closing time. Shortly before the last portion of solvent is evaporated, the SVE is closed and the chromatographic separation is initiated.

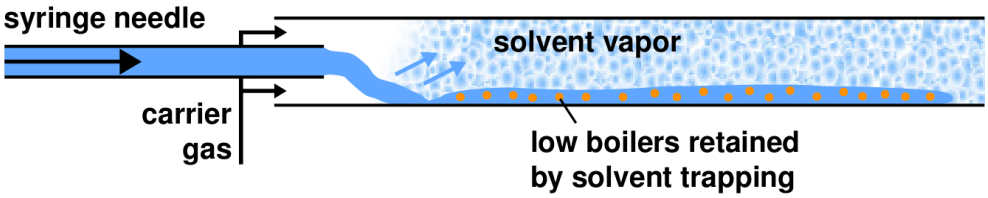

Fig. 1.16. Partially concurrent solvent evaporation for the analysis of volatile compounds [33]

1.10 References

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2. Scope of this thesis

Aim of this thesis was the generalization and expansion of the applicability of HPLC-GC techniques in the field of food science. Methods developed as part of this work should not only be of analytical value but also suited for the use in routine laboratories. Consequently, a meaningful validation was part of all method developments.

Chapter three presents a method for the determination of stigmasta-3,5-diene, a marker substance for the recognition of high temperature refining of extra virgin olive oils. This method is chosen as a first example showing the advantages of an LC-GC hyphenation in routine laboratories. Time-consuming column chromatography and manual work can be minimized to the weighing process of the sample. An analytical process, which normally took hours of work, is possible within 30 min without compromising sensitivity.

Chapter four describes the method development of an LC-GC-FID technique for the determination of sterols in edible fats and oils. Addition of standard compounds, saponification, and extraction of the samples is fully automated, showing that LC-GC is only a small part of a complex analysis system. Comparison with an established ISO method shows the suitability of the LC-GC approach in a high-throughput environment. Lastly, interfacing to a mass spectrometer is used to elucidate the structure of a previously unknown substance solely found in sunflower oils.

Chapter five deals with the quantitation of the vitamin D content in selected foodstuffs. The use of an MS detector allows significantly lower detection limits and the possibility to quantify vitamin D_2 and D_3 in one analysis cycle by means of deuterated standards. Again, the classical approach is out-performed in terms of manual work and sample throughput.

The determination of polycyclic aromatic hydrocarbons (PAH) in a broad variety of foodstuffs is the topic of chapter six. A generic sample preparation protocol is developed and combined with a powerful two-dimensional LC-LC-GC-MS method. The second LC-dimension is needed for sufficient sample cleanup demonstrating that even LC-GC hyphenation does not necessarily fit every purpose of automated sample preparation.

The final seventh chapter focuses on the determination of mineral oil aromatic hydrocarbons (MOAH) found in food. The chemical similarity to matrix-inherent constituents already enlightened in chapter six hampers the safe quantitation of MOAH in several food matrices. Separation of MOAH from these polyunsaturated hydrocarbons is not readily possible by HPLC. Instead, chemical modification of the interfering compounds is used to change their

elution behavior in the LC-GC-FID method. Usually, epoxidation is used for this purpose exhibiting several deficiencies. As will be shown in this chapter, the reaction conditions for epoxidation are of high importance for which automation is inevitable. Other derivatization reactions of the polyunsaturated hydrocarbons are discussed and tested for their suitability in the given context.

In Fig. 2.1, the key statements and relationships between the individual chapters addressed in this thesis are briefly summarized.

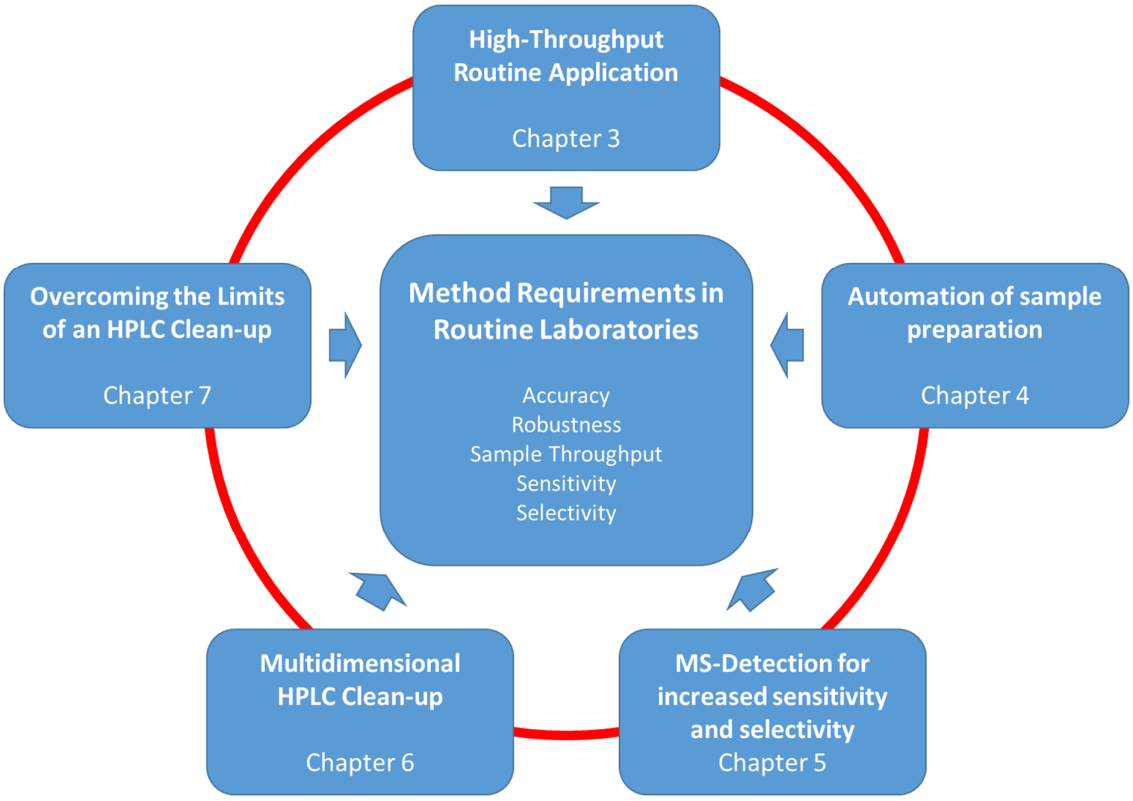

Fig. 2.1. Visualization of the scope of this thesis based on the use of LC-GC hyphenation for routine food analysis

3. Evaluation of stigmasta-3,5-diene as indicator for adulteration in extra virgin olive oil by online liquid chromatography–gas chromatography–flame ionization (LC-GC-FID)

Abstract

Detection of adulterations in high-price extra virgin olive oils is difficult and time-consuming. Several indicators are known and needed to unmask adulterations. One of them is stigmasta-3,5-diene that is formed mainly during forbidden high temperature treatments. Its detection is described by two official ISO methods. One of them involves a lot of manual work while the other is lacking selectivity, especially near the official upper limit of 0.05 mg/kg. Therefore, an adapted analytical detection method was designed based on an LC-GC workflow combining the advantages of both ISO approaches. Additionally, manual work could be reduced to the sample weighing process only. The total runtime was 30 min. This new method was compared to both ISO methods for extra virgin olive oil and aromatized truffle oil. In either case, good agreement in terms of accuracy was found. Precisions complied with the theoretical Horwitz/Thompson limits. The repeatability as indicated by relative standard deviations were 4.3 % and 2.0 % for olive and truffle oil, respectively. As a result, the designed method can be recommended as high-throughput alternative for the existing ISO methods in routine environments.

3.1 Introduction

Adulteration of high-price edible oils, such as extra virgin olive oils, is a profitable business. It is therefore not surprising that low-quality oils are either directly classified as high-class oils or are used as diluent for higher-class oils. In either case, the aim is to increase the profit margins [1]. For this reason, regular quality control has to be performed in routine environments to unmask adulterations and to maintain the high quality of edible oils.

As defined in EU regulation 1513/2001, virgin edible oils are obtained by mechanical and physical extraction only [2]. Refining processes are regularly used to extract the last portions

of oil from the corresponding fruit. Additionally, during refining, the chemical and physical properties are altered to increase the oil quality. Furthermore, properties can be specifically optimized to match other oil qualities. Nevertheless, classification as higher-class oil is forbidden and admixtures into higher-class oils have to be explicitly mentioned.

Revealing of adulterations is possible by analysis of specific chemical markers such as the fatty acid composition or the sterol profile [3]. However, use of plants with inconspicuous fatty acid compositions or desterolization, i.e., the removal of sterols during refining, can complicate the detection of adulterations [4]. Because of this, monitoring of a variety of parameters is necessary to detect suspicious oils.

In the literature, several marker substance classes are listed which are formed during various refining steps [5]. For example, sterenes (steradienes and steratrienes) are known indicators for high temperature treatments [6]. Their natural concentration in virgin oils is often negligible. They are the dehydration products of naturally occurring plant sterols (see Table 3.1 and Fig. 3.1).

The dehydration of sterols is observable at temperatures exceeding 200 °C [7]. Furthermore, it is known that bleaching earth is capable to lower the formation temperature.

Table 3.1. Main dehydration products of sterols

Sterol	Sterene
Cholesterol	Cholesta-3,5-diene
Brassicasterol	Campesta-3,5,22-triene
Campesterol	Campesta-3,5-diene
Stigmasterol	Stigmasta-3,5,22-triene
β-Sitosterol	Stigmasta-3,5-diene

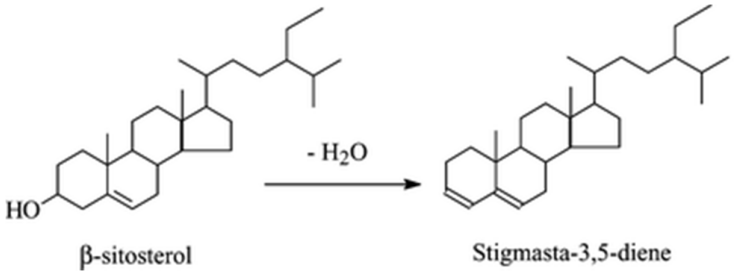

Fig. 3.1. Dehydration of sterols shown for β-sitosterol

Sterenes can be detected in oils even after desterolization. Furthermore, the sterene profile mostly resembles the original sterol profile giving the possibility to figure out which kind of oil was used for adulteration [8]. Because of this, they are a valuable marker for detection of adulterations.

Especially for olive oils, the monitoring of the sterene content seems promising. Since the total sterol content of (extra) virgin olive oils is rather low (1000 – 2000 mg/kg) and mainly consists of β-sitosterol, the presence of sterenes indicates the application of high temperature refining processes or the admixture of other (refined) oils.

After temperature treatments of olive oil, for example, high amounts of stigmasta-3,5-diene and its double bond isomers (e.g., stigmasta-2,4-diene) can be traced. They are originating from β-sitosterol (see Table 3.1), the most abundant sterol in vegetable oils. The concentrations of other steradienes in unaltered olive oils, however, are rather low compared to stigmasta-3,5-diene and can be neglected [9].

Therefore, the monitoring of stigmastadiene in olive oils with stigmasta-3,5-diene as main compound was decided by the International Olive Council (IOC). The EU regulation 1348/2013 (2568/91) regulates the allowed concentrations of stigmasta-3,5-diene for several olive oil quality grades [10, 11]. In 2013, the maximum level was set to 0.05 mg/kg for (extra) virgin olive oils. Lampante virgin olive oils are allowed to contain up to 0.5 mg/kg.

Consequently, the control of stigmasta-3,5-diene levels in olive oil has to performed in routine environments to guarantee the quality of high class olive oils such as the extra virgin ones.

3.1.1 Analytics

Two official ISO methods for the determination of stigmasta-3,5-diene are available [12, 13].

The first method (ISO 15788-1), which is recommended as reference method by the IOC, separates the steradiene fraction from other components in olive oils by saponification and subsequent silica gel column chromatography of the unsaponifiable matter. Afterward, detection and quantitation of stigmasta-3,5-diene is performed by GC-FID. Cholesta-3,5-diene is used as internal standard (ISTD).

The second method (ISO 15788-2) is relying on HPLC-UV. In contrary to the first method, the steradiene fraction is separated from the matrix by direct solid phase extraction (SPE) on silica gel without previous saponification. Afterward, detection and quantitation is performed with HPLC-UV on a C_{18} column under non-aqueous reversed-phase (NARP) conditions.

Collaborative trials revealed in the past that the first method gives more accurate results, while the second method is lacking selectivity (especially for low concentrations) because of the usage of nonselective UV detection [12, 13]. Nevertheless, it needs significantly less manual work compared to the first one and is, therefore, suited as fast screening method.

Independently, several attempts of HPLC and GC-FID hyphenation for the determination of steradienes were reported in the past [14–17]. Direct injection of edible oils after dilution was possible. Normal-phase HPLC was performed on silica gel to separate the steradiene fraction from the residual matrix. Transfer of the steradienes into the GC was generally performed by large volume on-column transfer. Limits of detection (LOD) were reported to be as low as 0.02 mg/kg [15]. Thus, suitability for the newest limits could be insufficient or restricted.

Aim of the current work was the establishment of an online LC-GC-FID method able to cope with the newest EU regulation limits for stigmasta-3,5-diene in (extra) virgin olive oils. The manual work was reduced to the weighing process. Dilution, addition of ISTD, and injection were automatically performed by an autosampler.

Comparison with the reference and screening methods was performed to check the suitability of the method in routine environments.

3.2 Experimental

3.2.1 Samples

Well-characterized extra virgin olive oil and truffle oil were available from Eurofins Analytik GmbH & Co. KG (Hamburg, Germany). They were used for method development and validation.

3.2.2 Chemicals and solutions

Dichloromethane and *n*-hexane were from LGC Promochem (Picograde quality, Wesel, Germany). Cholesta-3,5-diene (≥95 %) was from Sigma-Aldrich (Steinheim, Germany).

3.2.3 Sample preparation

Three grams of an edible oil were weighed into a 10-mL autosampler vial. The vial was placed onto the autosampler, which added 100 µL of the ISTD solution (500 ng/µL in *n*-hexane) and 6.9 mL of *n*-hexane. The vial was placed into an agitator and was shaken at a speed of 750 rpm (revolutions per minute) for one minute. Afterward, 70 µL of the solution were subjected to LC-GC-FID.

3.2.4 LC-GC-FID method

LC-GC-FID experiments were performed on a system from Axel Semrau (Sprockhövel, Germany). It consisted of a 1260 Infinity HPLC system (binary pump and variable wavelength detector by Agilent Technologies, Waldbronn, Germany), Master GC with flame ionization detector (DANI Instruments S.p.A., Cologno Monzese, Italy), and a DualPAL autosampler (CTC Analytics AG, Zwingen, Switzerland).

Three rotatory switching valves (VICI AG International, Schenkon, Switzerland) were used to guide the HPLC eluent from the HPLC into the GC. The GC was equipped with an on-column interface and a solvent vapor exit. The on-column interface, the carrier gas, and solvent vapor exit were controlled by CHRONECT LC-GC from Axel Semrau.

Seventy microliters (corresponding to 21 mg of edible oil) of the diluted sample were injected onto an Allure Si HPLC column (250 mm x 2.1 mm, 5 µm, 60 Å, Restek, Bellefonte, PA, USA)

without additional column temperature control. The mobile phase consisted of *n*-hexane and was delivered at 300 µL/min. After elution of the steradiene fraction (5.0 – 6.0 min), the column was backflushed with dichloromethane at 500 µL/min for 10 min. Afterward, the column was reconditioned with *n*-hexane at 500 µL/min for 15 min.

LC-GC transfer occurred by the retention gap technique and fully concurrent solvent evaporation (FCSE) through the Y-interface [18]. An uncoated, deactivated precolumn (MXT Hydroguard, 0.5 m x 0.53 mm, Restek, Bellefonte) was followed by a steel T-piece union connecting to the solvent vapor exit and a separation column coated with a 5 % phenyl polydimethylsiloxane film (Rxi-5Sil MS, 30 m x 0.25 mm x 0.10 µm, Restek, Bellefonte, PA, USA).

From HPLC, the steradiene fraction was transferred to the GC (resembling 300 µL) at a carrier gas inlet pressure of 80 kPa (hydrogen) in addition to an oven temperature of 80 °C. The elution window was verified by UV detection at 235 nm. The solvent vapor exit was opened 0.5 min before the elution of the sterene fraction began. Because of the high boiling points of the analytes, fully concurrent evaporation of the solvent was possible without loss of substances through the solvent vapor exit. The solvent vapor exit was closed 0.1 min after the fraction was transferred. At this time, the carrier gas inlet pressure was set to 90 kPa and maintained throughout the whole analysis. The oven temperature was programmed at 30 °C/min from 80 °C (4 min) to 170 °C and at 10 °C/min to 300 °C (3 min, total run time 23.00 min). The FID base temperature was set to 350 °C. The gas flows for air, hydrogen, and nitrogen were set to 280, 40, and 25 mL/min, respectively.

Data processing was performed with Clarity 5.5 (DataApex, Prague, Czech Republic). Quantitation was based on cholesta-3,5-diene used as ISTD. The stigmasta-3,5-diene content was calculated following the equation

$$S = \frac{A_{Stigma} * m_{ISTD}}{A_{ISTD} * m_{Sample}}$$

with S: stigmasta-3,5-diene content [mg/kg], A_{Stigma}: peak area of analyte, A_{ISTD}: peak area of ISTD, m_{ISTD}: mass of ISTD [mg], m_{Sample}: mass of test sample [kg].

3.3 Results and Discussion

The restrictions of the official ISO methods for the determination of stigmasta-3,5-diene in edible oils were conquered by the development of an LC-GC based workflow.

For separation of the steradiene fraction from the rest of the edible oil, a silica gel HPLC column with an inner diameter of 2.1 mm was chosen. According to the literature, a 2.1 mm x 25 cm column can retain up to 62.5 mg of triglycerides when *n*-hexane is used as the mobile phase [19]. Silica gel flooded by triglycerides is not able to retain other components anymore. Hence, only approximately half of the calculated capacity can be used for retention of triglycerides.

For safety reasons, only 21 mg of edible oils were injected onto the chosen column. To lower the viscosity of the injected solution, edible oils were diluted to a 30 % *n*-hexanic solution before injection by the autosampler. Consequently, a desired LOQ for stigmasta-3,5-diene of 0.05 mg/kg corresponded to an amount of approximately 1 ng on column, which was readily compatible with GC-FID detection.

Backflushing of the HPLC column after elution of the steradiene fraction was used to efficiently remove the residual oil matrix. Dichloromethane was chosen as backflushing solvent. On the one hand, its polarity was sufficiently high to remove the oil matrix; on the other hand, the HPLC column could be reconditioned with *n*-hexane afterward in approximately 15 min. Solvents exhibiting higher solvent strengths, such as ethers or alcohols, could not be removed in an appropriate amount of time [20].

Cholesta-3,5-diene was chosen as internal standard. It was suited because it was commercially available and was co-eluted with stigmasta-3,5-diene on the HPLC under normal-phase conditions [1, 16, 17].

At first, a cholesta-3,5-diene standard (15 ng on column) was injected onto the HPLC to retrieve the retention time of the steradiene fraction window (see Fig. 3.2a – blue trace). A wavelength of 235 nm was used to follow the conjugated double bond of the steradiene skeleton. Afterward, an extra virgin olive oil sample was prepared and subjected to LC-GC-FID. In Fig. 3.2 (red traces), the obtained HPLC-UV and LC-GC-FID chromatograms are shown.

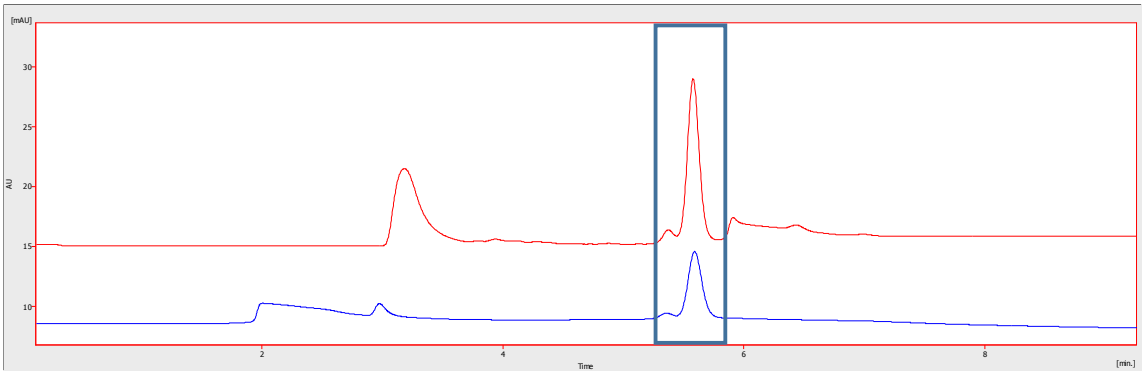

Fig. 3.2a. HPLC-UV chromatogram of an ISTD solution (blue trace) and extra virgin olive oil (red trace) (Wavelength: 235 nm)

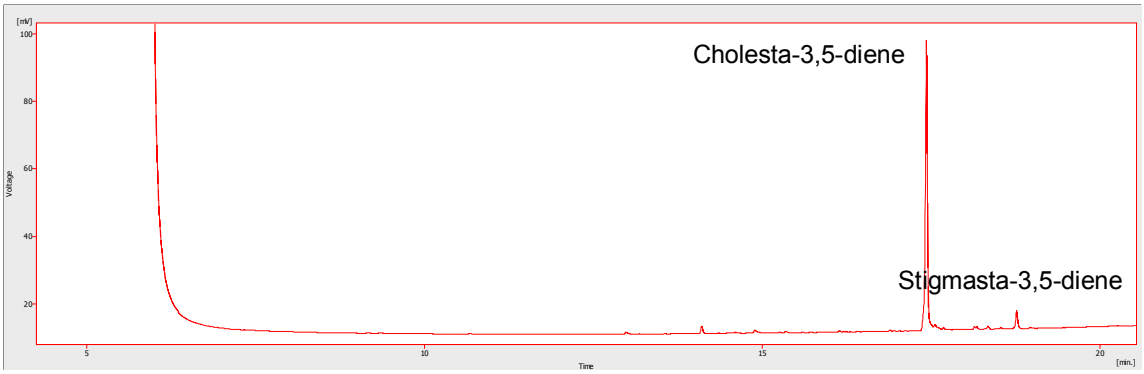

Fig. 3.2b. LC-GC-FID chromatogram of extra virgin olive oil

The LC-GC-FID chromatogram did not show any sort of chromatographic interference. Because LC elution was possible with *n*-hexane only, co-elution of squalene and other polyunsaturated compounds could be prevented. Natural concentrations of squalene in olive oil are approximately 5000 mg/kg and were reported to be the origin of chromatographic problems in the past [16, 17].

3.3.1 Precision and trueness

Precision was determined by multiple injections of extra virgin olive oil and truffle oil (aromatized refined sunflower oil). Truffle oil was chosen because of its inherent higher stigmastadiene content. Repeatability was calculated from six consecutive injections of six independently provided oils. Since no official reference material was available, the same samples were used for the estimation of trueness instead. The results are summarized in Table 3.2.

Table 3.2. Comparison of quantitative results by LC-GC-FID, GC-FID, and HPLC-UV

	Mean concentration [mg/kg]		
	LC-GC-FID[a]	**GC-FID[b]**	**HPLC-UV[b]**
Extra virgin olive oil	0.035 (± 0.002)	0.039	0.045
Truffle oil	3.06 (± 0.06)	---[c]	2.32

[a]: Mean value of six consecutive injections (n = 6)
[b]: Data were kindly supplied by Eurofins Analytik GmbH & Co. KG.
[c]: Data were not available.

The obtained relative standard deviations of repeatability were 4.3 % and 2.0 % for extra virgin olive oil and truffle oil, respectively. According to Thompson, acceptable relative standard deviations below 0.12 mg/kg under repeatability conditions are fixed at 14.7 % [21]. At 3 mg/kg, they are calculated to be approximately 9 %. Accordingly, the obtained precisions were both acceptable.

The quantified mean concentrations corresponded well to both ISO methods. In the case of truffle oil, slightly higher values were quantified compared to the HPLC-UV method.

In Fig. 3.3, the LC-GC-FID chromatogram of the truffle oil is shown. The chromatogram contains various peaks besides the ISTD and stigmasta-3,5-diene. Depending on the nature of the refined oil, additional sterol degradation products can be observed [9]. They might be responsible for the discrepancy between the LC-GC and HPLC-UV results.

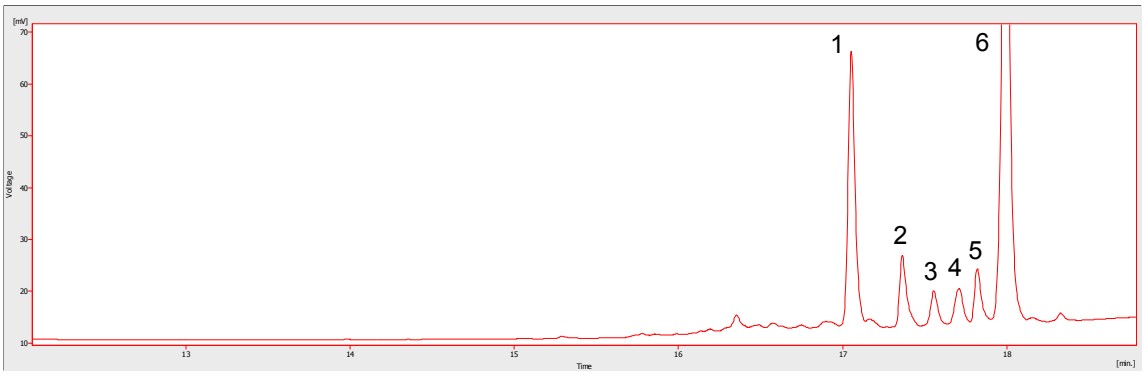

Fig. 3.3. LC-GC-FID chromatogram of the sterene fraction of truffle oil, an aromatized refined sunflower oil (1 = Cholesta-3,5-diene (ISTD), 2 = 3,5-Cyclo-6-stigmastene, 3 = Campesta-3,5-diene + Campesta-2,4-diene, 4 = Unknown Stigmastadiene + Stigmasta-3,5,22-triene, 5 = Stigmasta-2,5-diene, 6 = Stigmasta-3,5-diene + Stigmasta-2,4-diene). Nomenclature according to Grob et al. [9].

3.3.2 Sensitivity

To estimate the LOQ of the designed method, extra virgin olive oil was diluted to correspond to a concentration of 0.01 mg/kg. For this, only 6 mg of oil were injected onto the HPLC. In Fig. 3.4, the obtained LC-GC-FID chromatogram is shown. The analyte peak is still quantifiable without chromatographic uncertainties. Quantitation revealed an amount of 0.011 mg/kg, closely matching the targeted value.

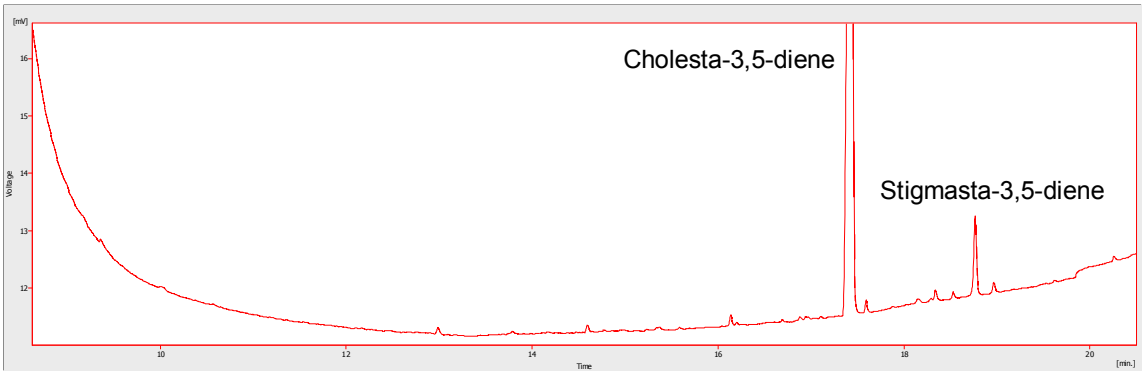

Fig. 3.4. LC-GC-FID chromatogram of extra virgin olive oil corresponding to 0.011 mg/kg stigmasta-3,5-diene. The signal-to-noise ratio was calculated to be 34:1.

Judging from the signal-to-noise ratio (SNR) of this injection, the LOQ (SNR = 10) was estimated at 0.003 mg/kg. Nevertheless, it has to be kept in mind that additional matrix components of other types of samples could increase this theoretical quantitation limit.

3.4 Conclusion

The determination of the stigmastadiene content in extra virgin edible oil was possible by an LC-GC-FID method. The limit of quantitation was estimated to be approximately 0.003 mg/kg. Therefore, the current official upper limit of 0.05 mg/kg in (extra) virgin olive oils could be safely quantified.

Opposed to the official ISO methods, which need either significant amount of manual work or are lacking selectivity, the shown method successfully combines both attempts. The final analysis result was available after approximately 30 min.

The obtained quantitative results of the available reference samples were in good agreement with both official methods. Repeatability fully complied with the Horwitz/Thompson limits. Because of this, the method can be applied without known limitations as a replacement for existing methods in routine environments.

Acknowledgements

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3.5 References

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4. Determination of the total sterol content in edible oils and fats by online liquid chromatography–gas chromatography–flame ionization detection (LC-GC-FID) with fully automated sample preparation

Abstract

Adulterations of high-price edible oils is a lucrative business. Ensuring the oil quality is therefore of great importance. One parameter used to determine the authenticity of oils is the analysis of their sterol profile. The gas chromatographic determination of sterols in edible oils and fats is described by ISO norm 12228. Extraction, purification, and detection of the sterols is time-consuming and error-prone. Collaborative trials prove this regularly. Purification by thin-layer chromatography (TLC) and robust GC determination of all regulated sterols is not straightforward. Therefore, a fully automated LC-GC-FID method was developed to facilitate the determination of sterols. The only manual step left was to weigh the sample into an autosampler vial. Saponification and extraction were performed by an autosampler while purification, separation, and detection were accomplished by online coupled normal-phase LC-GC-FID. Interlacing of sample preparation and analysis allowed an average sample throughput of one sample per hour. The obtained quantitative results were fully comparable with the ISO method with one apparent exception. In the case of sunflower oils, an additional unknown sterol could be detected generally missed by ISO 12228. The reason was found in the omission of sterol silylation before subjection to GC-FID. The derivatization reaction changed the retention time and hid this compound behind a major sterol. The compound could be identified as 14-methyl fecosterol. Its structure was elucidated by GC-MS and ensured by HPLC and GC retention times. Finally, validation of the designed method confirmed its suitability for routine environments.

4.1 Introduction

Sterols are naturally occurring compounds in animals and plants. The best-known sterol is cholesterol, which is found in animals, and stabilizes cell membranes. Similar functions can be attributed to sitosterol in plants. Among other compounds, this sterol is counted to the plant sterols (phytosterols) [1].

Sterols, in particular phytosterols, are important compounds for the nutrition and health industry. They are known to have a variety of biological effects [1]. It was reported in the past that compounds derived from phytosterols and their saturated analogues (phytostanols) have beneficial effects on the cardiologic system [2]. Foodstuffs, such as margarines, are enriched with phytosteryl esters to lower the cholesterol level in the human body. This effect is based on the structural similarities between cholesterol and phytosterols. They compete for the same absorption sites in the human organism [2]. Phytosterols are also used as emulsifiers in the cosmetic industry and are important steroidal precursors for hormone pharmaceuticals [3].

Sterols are found in the unsaponifiable matter and belong to the class of triterpenes. The synthesis pathway for all sterols is outlined in Fig. 4.1. The structure is derived from sterane hydroxylated at C-3 (see Fig. 4.2). The sterol skeleton carries a double bond at varying position, mainly found at C-5(6) ($\Delta 5$) or C-7(8) ($\Delta 7$). Compounds missing this double bond are called stanols. Additionally, C-17 contains a variable branched alkyl sidechain with possible additional double bonds. Sterols can be divided into three main classes based on the number of methyl groups at C-4, two (4,4-dimethyl), one (4-methyl) and none (4-desmethyl). The term sterol is often used as a synonym for 4-desmethyl sterols. 4,4-Dimethyl and 4-methyl sterols are metabolic intermediates transformed into 4-desmethyl sterols at the end of the pathway.

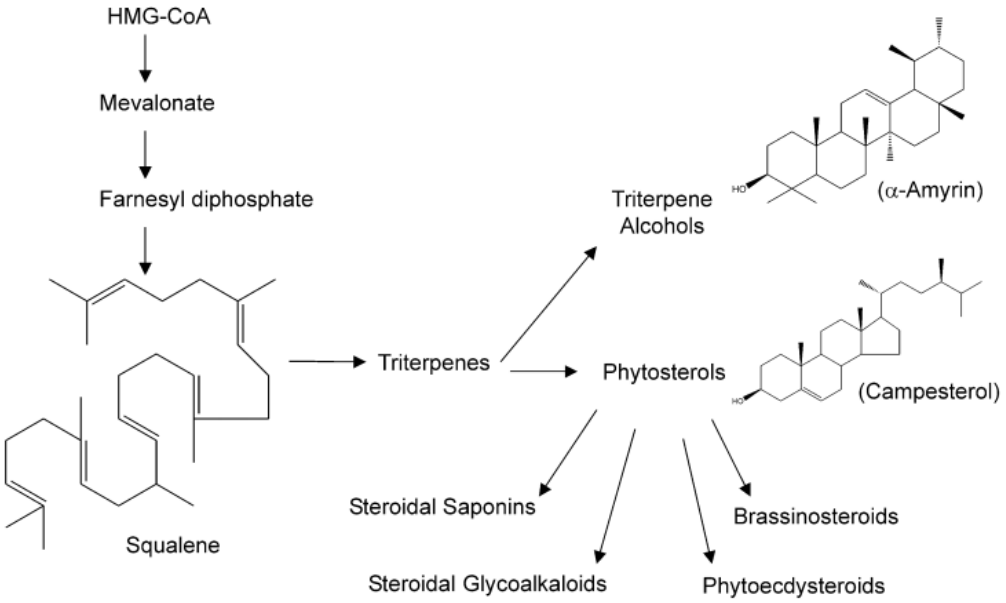

Fig. 4.1. Synthesis pathway for triterpenes and sterols (from [4] with permission)

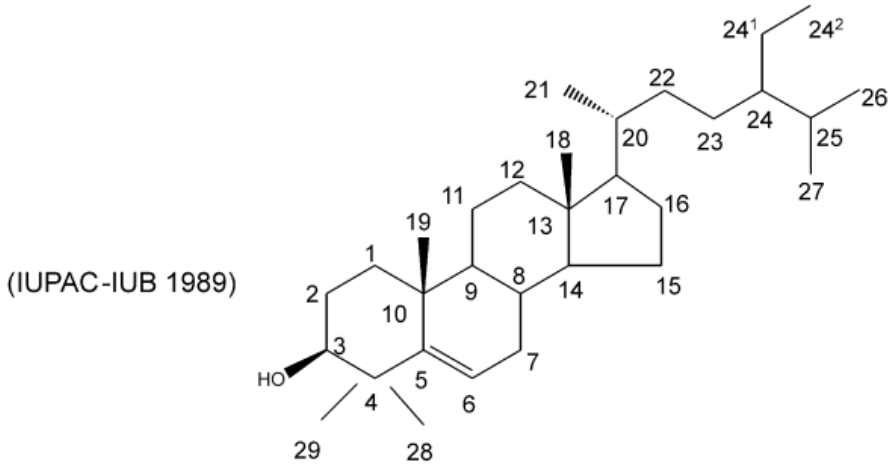

Fig. 4.2. Nomenclature of sterols (from [4] with permission)

Sterols can occur either as free alcohols or bound to other molecules, e.g., fatty acids, ferulates, or glycosides [4]. In animals, cholesterol is the most abundant sterol while in plants the most encountered phytosterols are campesterol, stigmasterol, and sitosterol [5]. In addition, numerous minor sterols with percentages less than 5 % of the total sterol content can be found. The total sterol amount in edible oils generally varies between approximately 1000 and 10,000 mg/kg [6].

4.1.1 Phytosterols as quality markers for edible oils and fats

Because of unique plant-specific compositions, phytosterols are used as quality markers for natural products such as edible oils and fats. The control of distribution and total amount of phytosterols is an important tool for ensuring the purity of high quality oils, e.g., extra virgin olive oils. ISO 12228 describes a gas chromatographic method for the determination of fifteen individual 4-desmethyl phytosterols and phytostanols in edible oils and fats [7]. In Fig. 4.3 and Table 4.1, an overview of all regulated compounds is given.

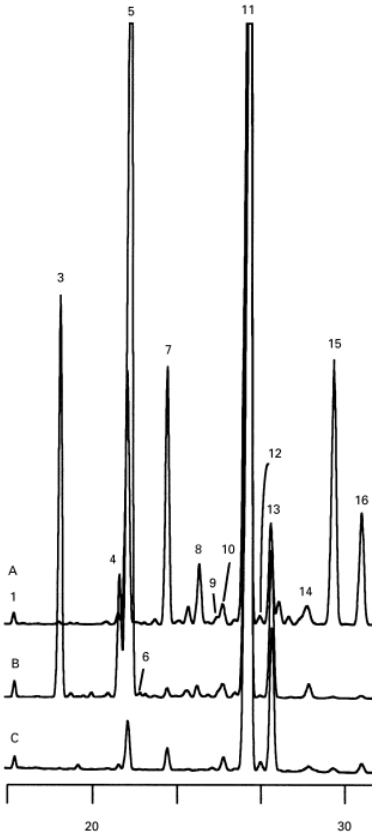

Fig. 4.3. Phytosterols and stanols regulated by ISO 12228 (adapted from [7] – A: Sunflower, B: Rapeseed, C: Olive oil – Peak allocations found in Table 4.1)

Table 4.1. Phytosterols and stanols regulated by ISO 12228 [7]

Peak no.	Compound name
1	Cholesterol
2	Cholestanol (ISTD, not shown)
3	Brassicasterol
4	24-Methylene cholesterol
5	Campesterol
6	Campestanol
7	Stigmasterol
8	Δ7-Campesterol
9	Δ5,23-Stigmastadienol
10	Clerosterol
11	Sitosterol
12	Sitostanol
13	Δ5-Avenasterol
14	Δ5,24-Stigmastadienol
15	Δ7-Stigmastenol
16	Δ7-Avenasterol

Admixtures of low-grade oils, e.g., rapeseed or sunflower oil, can be traced down to a few percent due to their prominent sterol distributions [8]. However, heating or bleaching of inexpensive oils can be used for desterolization [9]. Hence, the unique sterol distribution is destroyed. Admixtures cannot be safely detected anymore by sole analysis of the sterol profile. Because of this, numerous quality markers have to be used to ensure oil qualities.

EU regulation 1348/2013 specifies markers to ensure the quality of olive oils. Among other parameters, it regulates also the percentage distribution and upper limits of 4-desmethyl sterols [10]. Depending on the olive oil quality level, e.g., extra virgin, virgin, pomace, etc., differing limits were defined. For instance, extra virgin olive oils must contain more than 1000 mg/kg phytosterols with a sitosterol content exceeding 93 % (with regard to the total sterol content).

4.1.2 Analytics of phytosterols in edible oils and fats

Numerous publications dealing with the determination of phytosterols in edible oils can be found [11–14]. ISO 12228 is most widespread in routine environments in Europe. In general, the first step involves liberation of all bound phytosterols into their free alcohol analogues. Most of the bound phytosterols are esterified and can therefore be cleaved by alkaline saponification. Only few foodstuffs, such as tomatoes, contain acetal-bound phytosterols, which cannot be cleaved by alkaline treatments. Saponification is normally performed by addition of an alcoholic solution of potassium hydroxide (KOH) to the sample. Transesterification of esterified phytosterols is another approach described in literature [15]. After the reaction is finished, the free sterols are most commonly solvent-extracted into diethyl ether. Newer versions of ISO 12228 circumvent solvent-extraction, because formation of emulsions was regularly observed. Instead, extraction by solid-phase extraction (SPE) on aluminum oxide was established. In the following step, a chromatographic cleanup of the sterols from the rest of the unsaponifiable matter is performed by means of thin-layer chromatography (TLC). The cleaned-up sterol fraction is generally silylated (TMS-phytosterols) to improve analyte-stationary phase interactions in the subsequent GC-FID separation (gas chromatography–flame ionization detection). Due to the quasi-unity response of the FID, quantitation of all sterols is possible by a single internal standard compound. Normally, 5α-cholestanol is used for this purpose, which is added before saponification.

The extraction of 4-desmethyl sterols from edible oils is demanding. Besides the bulk of triglycerides, edible oils contain many other compounds disturbing the determination of 4-

desmethyl sterols. The unsaponifiable matter consists mainly of hydrocarbons, carotenes, tocopherols, different sterol classes, and triterpene dialcohols [16]. Because of this complex composition, a cleanup after extraction is inevitable. As already stated, silica TLC is the option described by ISO 12228. Since TLC is a complicated and time-consuming method, several alternatives were described in the past. Online coupled LC-GC-FID was one option [17–21]. The online coupling of HPLC and GC offers several advantages compared to manual methods such as reduction of manual sample preparation, decrease of cross-contamination, and increase of sample throughput.

After sterol extraction, the cleanup is performed by HPLC instead of silica TLC. The obtained fraction is online transferred into GC-FID either by use of on-column transfer techniques or programmable temperature vaporization (PTV) injectors [22]. Besides a few publications dealing with reversed-phase HPLC, normal-phase HPLC on silica gel is encountered the most. Silylation of the sterol fraction is omitted in most cases to facilitate the transfer into the GC. Hence, this might be a possible source of quantitation discrepancies when comparing LC-GC methods to ISO 12228.

The quantitation of all fifteen regulated 4-desmethyl sterols and stanols is not straightforward. Collaborative trials showed in the past that secure determination of the minor sterols (< 5 % of total sterol content) was error-prone [23]. Relative standard deviations under reproducibility conditions greater than 100 % were observed. In own studies problems due to difficulties in the TLC step and insufficient GC analyte separations were the main reasons for varying results.

Therefore, the focus of the current work was laid on automation of the sample preparation using analytical HPLC to improve accuracy. This was accomplished by the use of a versatile autosampler and an online LC-GC-FID approach.

4.2 Experimental

4.2.1 Samples

ISO 12228-1:2011 collaborative trial samples from 2012 were available and used for method development and validation. They consisted of rapeseed, safflower, and sunflower oil. Additionally, a phytosterol concentrate from a previous collaborative trial (2010) was measured.

4.2.2 Chemicals and solutions

Ethanol, methyl-*tert*-butyl ether (MTBE), *n*-hexane, and isopropanol were from LGC Promochem (Picograde quality, Wesel, Germany). 5α-Cholestanol (≥95 %), citric acid (99 %), and potassium hydroxide (≥85 %, pellets, white) were from Sigma-Aldrich (Steinheim, Germany). Sodium sulfate was from Fluka (Buchs, Switzerland). Water was supplied from a Milli-Q water purification system (Merck, Darmstadt, Germany).

4.2.3 Sample preparation

One hundred milligrams of an edible oil or fat were weighed into a 10-mL autosampler vial. The vial was placed onto the autosampler, which added 100 µL of the internal standard solution (ISTD, 1 g/L in MTBE) and 1.5 mL of an ethanolic KOH solution (1 mol/L). The vial was placed into an agitator set to 80 °C for 40 min and was shaken at a speed of 500 rpm (revolutions per minute). After cooling down, 4.9 mL of *n*-hexane and 2.5 mL of a saturated aqueous citric acid solution were added to the vial. Neutralization and extraction of the sterol fraction was accomplished by shaking the vial for 3 min at 750 rpm. Depending on the expected phytosterol content of the edible oil, an intermediate dilution step was carried out to avoid overloading of the GC column during the separation process. Reduction of the sample amount to less than 100 mg was not considered, because it would have excluded the usage of low-resolution laboratory balances. Dilution was performed in a 2-mL autosampler vial filled with a spatula tip of sodium sulfate to dry the sample. For edible oils with a phytosterol content up to 1500 mg/kg, 333 µL of the *n*-hexanic solution from the 10-mL vial were transferred into the 2-mL vial and brought up to a final volume of 1 mL with additional *n*-hexane. After shaking at 750 rpm for 1 min, 10 µL of the solution were subjected to LC-GC-FID. The whole procedure is summarized in Fig. 4.4.

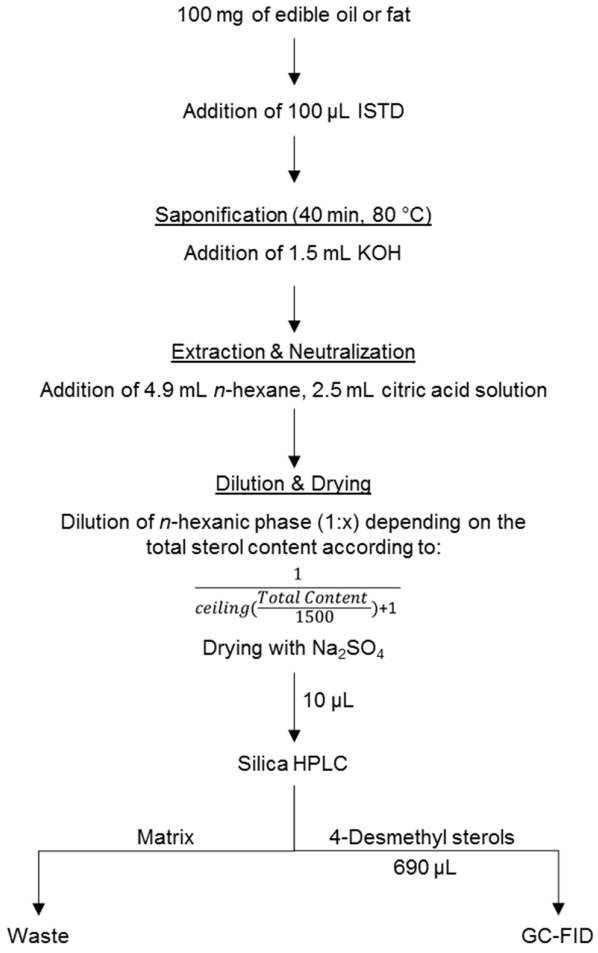

Fig. 4.4. Flowchart of automated sample preparation for edible oils and fats

4.2.4 LC-GC-FID method

LC-GC-FID experiments were performed on a system from Axel Semrau (Sprockhövel, Germany). It consisted of a 1260 Infinity HPLC system (binary pump and variable wavelength detector by Agilent Technologies, Waldbronn, Germany), Master GC with flame ionization detector (DANI Instruments S.p.A., Cologno Monzese, Italy), and a DualPAL autosampler (CTC Analytics AG, Zwingen, Switzerland).

Three rotatory switching valves (VICI AG International, Schenkon, Switzerland) were used to guide the HPLC eluent from the HPLC into the GC. The GC was equipped with an on-column interface and a solvent vapor exit. The on-column interface, the carrier gas, and solvent vapor exit were controlled by CHRONECT LC-GC from Axel Semrau.

Ten microliters of the sample extracts prepared by the autosampler were injected onto an Allure Si HPLC column (250 mm x 2.1 mm, 5 µm, 60 Å, Restek, Bellefonte, PA, USA) without

additional column temperature control. The mobile phase consisted of *n*-hexane and isopropanol (98:2, v/v) and was delivered isocratically at 300 µL/min. The 4-desmethyl sterol fraction was eluted between 9.8 to 12.1 min. Thereafter, the column was backflushed with MTBE at 500 µL/min for 5 min. Finally, the column was reconditioned with the mobile phase at 300 µL/min for 15 min.

HPLC-GC transfer occurred by the retention gap technique and fully concurrent solvent evaporation (FCSE) through the Y-interface [24]. An uncoated, deactivated precolumn (MXT Hydroguard, 0.5 m x 0.53 mm, Restek, Bellefonte) was followed by a steel T-piece union connecting to the solvent vapor exit and a separation column coated with a 5 % phenyl polysiloxane film (Rxi-5Sil MS, 30 m x 0.25 mm x 0.10 µm, Restek, Bellefonte, PA, USA).

From HPLC, the sterol containing fraction was transferred to the GC (resembling 690 µL) at a carrier gas inlet pressure of 80 kPa (helium) in addition to an oven temperature of 80 °C. The elution window was verified by UV detection at 205 nm. The solvent vapor exit was opened 0.5 min before the elution of the sterol fraction began. Because of the high boiling points of the analytes, fully concurrent evaporation of the solvent was possible without loss of substances through the solvent vapor exit. The solvent vapor exit was closed 0.1 min after the fraction was transferred. At this time, the carrier gas inlet pressure was set to 160 kPa and maintained throughout the whole analysis. The oven temperature was programmed at 25 °C/min from 80 °C (5 min) to 225 °C (20 min), at 1.5 °C/min to 265 °C and finally at 25 °C/min to 310 °C (0.74 min, total run time 60.00 min). The FID base temperature was set to 350 °C. The gas flows for air, hydrogen, and nitrogen were set to 280, 40, and 25 mL/min, respectively.

Data processing was performed with Clarity 5.5 (DataApex, Prague, Czech Republic). Quantitation was based on 5α-Cholestanol used as ISTD. Sterol distribution and total content were calculated following the equations

$$C_i = \frac{A_i}{\sum(A_i)} * 100 \qquad S = \frac{\sum(A_i) * m_{ISTD}}{A_{ISTD} * m_{Sample}}$$

with C_i: Individual sterol concentration [%], S: Total sterol content [mg/kg], A_i: Individual sterol peak area, $\Sigma(A_i)$: Sum of peak areas of regulated sterols, A_{ISTD}: peak area of ISTD, m_{ISTD}: mass of ISTD [mg], m_{Sample}: mass of test sample [kg].

For structure elucidation, the FID was replaced by a DSQ II single quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Austin, TX, USA). The ion source and transfer line temperatures were set to 200 and 320 °C, respectively. Data acquisition started after 20.0 min in full-scan mode (50 – 500 amu) at a rate of 3 spectra/s with EI ionization at 70 eV. Data processing was performed with Xcalibur 2.2 (Thermo Fisher Scientific Inc.).

4.3 Results and Discussion

The time-consuming and error-prone saponification, extraction, and TLC steps were substituted by an automated autosampler-based sample preparation followed by a normal-phase LC-GC-FID system. The only manual step during sample preparation left was to weigh the edible oil sample into an empty 10-mL autosampler vial.

Silylation of the cleaned-up sterol fraction was omitted to allow for an easy transfer into the GC. However, by omitting silylation, analyte peak shapes and separations on the GC column were significantly influenced. Thus, GC parameters had to be adapted.

4.3.1 Sample preparation

Automation of saponification and sterol extraction in closed vessels bore the problem of emulsion formation. The reason for this was obviously the production of potassium salts of fatty acids during saponification preventing a clear layer separation between the aqueous and organic phase. ISO 12228 solved this problem lately by omission of solvent extraction. Instead, an SPE step on aluminum oxide was proposed. In the current work, the problem was solved otherwise.

An aqueous citric acid solution was given to the sample after saponification, which resulted in protonation of the fatty acids. The formation of emulsions was thus prevented and a clear separation of aqueous and organic phase was observed. Extraction was carried out with *n*-hexane instead of diethyl ether. Its volatility was better suited for an automated approach. Since it is less polar than diethyl ether, co-extraction of more polar material was minimized. Nevertheless, protonation of fatty acids resulted in a change of their polarity. Protonated fatty acids are more nonpolar. Therefore, they were partially extracted by *n*-hexane, which could be verified by yellowish colored *n*-hexanic phases. However, during the HPLC cleanup on the used silica column, they could be completely separated from the 4-desmethyl sterols.

4.3.2 Influence of silylation on GC separation

As already mentioned, omission of silylation was the only significant difference between the designed LC-GC method and ISO 12228. Therefore, the influence of silylation on GC separation efficiency was investigated. Literature recommends the use of a GC separation column coated with a 5 % phenyl polysiloxane stationary phase for sterol separation [7].

The sterol distributions of several oils (rapeseed, safflower, and sunflower oil) were determined by LC-GC. Gas chromatographic separations were comparable with ISO 12228. Nevertheless, the most apparent difference between both methods was found in the sample capacity of the used GC column. Omission of silylation reduced the sample capacity of the stationary phase. It was observed that smaller sample amounts had to be injected in LC-GC analyses due to column overloading. Overloading resulted in severe fronting of major sterols and deterioration of critical peak pair separations.

Rapid overloading and peak fronting are known indicators for insufficient analyte–stationary phase interactions [25]. Low analyte solubility in the stationary phase liquid, i.e., a polarity mismatch between the analyte and the stationary phase, can be the source for this observation. In general, silylation lowers the analyte's polarity and increases therefore its partition coefficient between the nonpolar stationary and mobile phase. As a consequence, the sample capacity is increased. Although silylation would have been possible in the designed LC-GC method, it was not considered. Besides lowered sample throughput, collection of the sterol fraction, silylation, and re-injection into GC-FID would have complicated the method (additional glassware, contaminations, etc.).

The initial sample amounts (100 mg) and dilution factors (1:2) during LC-GC sample preparation were chosen to match closely the ISO method [7]. Samples containing higher sterol contents, such as rapeseed oil, showed GC column overloading in LC-GC experiments while no such observation was made for silylated ISO samples. Therefore, adaption of the dilution factor during sample preparation according to the measured sample was chosen to solve this problem (see Fig. 4.4). Reduction of the injected sample amount successfully prevented overloading. Sensitivity of the method using FID detection was not compromised, even for minor sterol percentages below 0.5 %. In Fig. 4.5, the separation of blended sunflower-rapeseed oil obtained by LC-GC-FID is shown.

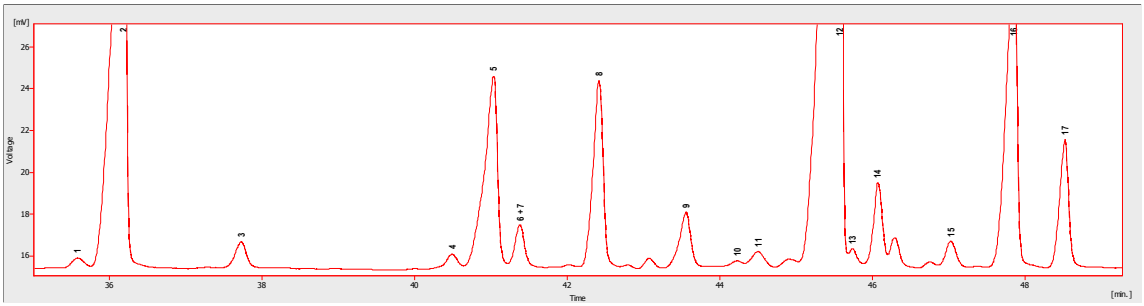

Fig. 4.5. LC-GC-FID chromatogram of blended sunflower-rapeseed oil obtained on a 5 % phenyl polysiloxane stationary phase (1 = Cholesterol, 2 = Cholestanol (ISTD), 3 = Brassicasterol, 4 = 24-Methylene cholesterol, 5 = Campesterol, 6 = Campestanol, 7 = Unknown, 8 = Stigmasterol, 9 = Δ7-Campesterol, 10 = Δ5,23-Stigmastadienol, 11 = Clerosterol, 12 = Sitosterol, 13 = Sitostanol, 14 = Δ5-Avenasterol, 15 = Δ5,24-Stigmastadienol, 16 = Δ7-Stigmastenol, 17 = Δ7-Avenasterol)

Quantitative comparisons of LC-GC and ISO 12228 revealed an overestimation of campestanol of approximately 1 % (1.5 % vs. 0.5 %) by LC-GC when sunflower oils were analyzed (see Fig. 4.5 – Peak No. 6+7). In order to clarify if this was due to the omitted silylation, sunflower oil was prepared according to ISO 12228. The obtained extract after TLC was injected silylated and non-silylated into the GC. The non-silylated extract showed the same overestimation of campestanol, confirming that LC-GC and ISO 12228 were truly equivalent. The silylated extract, however, contained the ISO-conform campestanol content. Nevertheless, a slower temperature program of the GC oven revealed a shoulder on the campesterol peak even for the silylated sample. This clearly indicated the presence of an additional unknown compound regardless of silylation. The results are shown in Fig. 4.6.

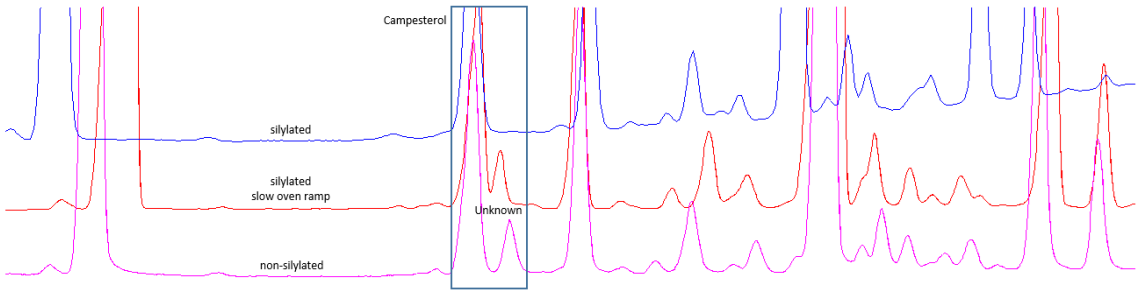

Fig. 4.6. Chromatogram overlay of a sunflower oil sample prepared according to ISO 12228 (blue trace: silylated, red trace: silylated with slowed-down oven ramp, pink trace: non-silylated)

Baseline separation of the unknown compound from the others was not possible. According to the literature, improvement of sterol GC separations is not achievable by simple change of the stationary phase type [26]. This could be verified by testing several stationary phases with higher phenyl (35, 50 %) and cyanopropyl-phenyl (14 %) content. It was found that with increasing polarity several ISO 12228 regulated sterols were co-eluted. Nevertheless, since the unknown compound seemed to be specifically present only in sunflower oils, it could be easily recognized. Although as will be shown in the validation subchapter, it was already origin for discrepancies in collaborative trials.

4.3.3 Structure elucidation of the unknown compound in sunflower oil

Normal-phase HPLC on bare silica gel offered the possibility to separate Δ5, Δ7, and other low abundant sterols. As can be seen in Fig. 4.7, the 4-desmethyl sterol fraction was separated into several sub-groups. The first peak resembled Δ5-sterols whereas the third peak corresponded to Δ7-sterols [9]. The fraction in-between contained stanols and sterols with double bond(s) in the sterol skeleton at alternating positions, e.g., pro-vitamin D derivatives (Δ5,7-sterols).

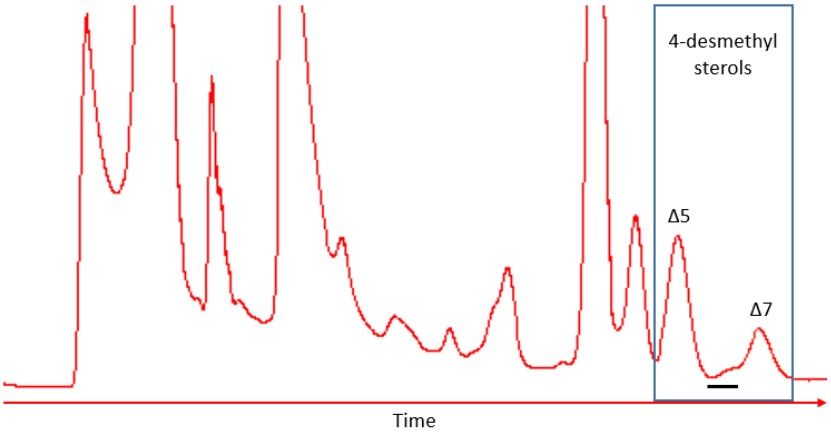

Fig. 4.7. HPLC-UV chromatogram of sunflower oil showing the separation of the 4-desmethyl sterol fraction into several sub-groups (wavelength: 205 nm, the underlined elution window corresponded to other sterols than Δ5- and Δ7-sterols)

LC-GC was used to transfer parts of the sterol fraction of sunflower oil into the GC. The unknown compound was found mainly in the intermediate fraction. The Δ5-fraction did not contain the unknown compound whereas the Δ7-fraction contained only traces of it.

The selectivity of the used HPLC column allowed a prediction about the nature of the unknown compound. Its HPLC retention time excluded a Δ5- and Δ7-sterol. Additionally, GC-MS was

used for further structure elucidation. The unknown compound was not identified as campestanol indicated by missing *m/z* 402. Instead, the unknown phytosterol was tentatively identified as 14-methyl fecosterol.

The obtained mass spectrum (see Fig. 4.8) was in accordance with the literature and the NIST reference spectra database [27]. Furthermore, the observed relative GC retention time agreed to the literature for 14-methyl fecosterol [28]. Finally, 14-methyl sterols are eluted together with 4-desmethyl sterols on bare silica HPLC phases [27].

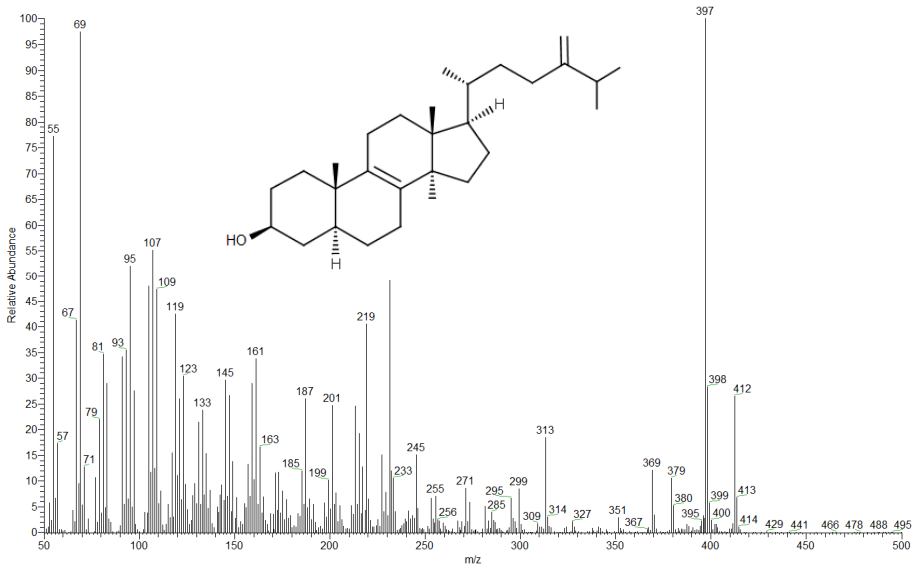

Fig. 4.8. Mass spectrum of the unknown 4-desmethyl phytosterol in sunflower oils. The spectrum matched 14-methyl fecosterol.

14-Methyl fecosterol is a 4-desmethyl Δ8-sterol with an additional methyl group at C-14 on the sterol skeleton. In plants it is a sterol originating from obtusifoliol, a 4,14-dimethyl Δ8-sterol. Obtusifoliol is also the common precursor for the three main phytosterols campesterol, stigmasterol, and sitosterol [5]. One important reaction step during sterol biosynthesis is accomplished by the sterol 14α-demethylase (CYP51), the first step in converting 14-methyl sterols to 14-desmethyl sterols. In numerous consecutive steps the final 4-desmethyl sterols are formed.

14-methyl sterols could be found in Arabidopsis (*Arabidopsis thaliana*) with deactivated CYP51 [29]. Additionally, triazole-based fungicides were shown to have the ability to inhibit the 14α-demethylase activity in fungi [30]. Thus, it is plausible to assume that sunflowers could contain inhibited CYP51 enzymes, prohibiting the C-14 demethylation step. However, no

literature could be found supporting this hypothesis. Further investigation is needed to verify or falsify this assumption.

The presence of 14-methyl sterols could be an indicator for the use of triazole-based fungicides during the cultivation of sunflowers. To test this hypothesis, virgin sunflower oil with an organic certification (EU regulation 834/2007) was analyzed. GC-MS confirmed that it contained the same peak identified as 14-methyl fecosterol as refined sunflower oil [31]. According to EU regulation 889/2008, organic labeled products must not be treated with triazole-based fungicides [32]. Therefore, other reasons for the presence of 14-methyl sterols in sunflower oils have to be evaluated.

4.3.4 Validation of the LC-GC-FID method

Since no reference material was available for the determination of sterols in edible oils and fats, collaborative trial material was used instead.

Precision was determined by multiple injections of sunflower oil. Repeatability was calculated from six consecutive injections of six independently prepared oils. Reproducibility is based on the quantitative results in duplicate on three successive days. Collaborative trial materials (rapeseed oil, safflower oil, and sunflower oil) were analyzed in triplicate to get an impression of the trueness of the LC-GC-FID method. According to Horwitz, the allowed relative standard deviation under reproducibility conditions ranges from 4.4 to 11.3 % depending on the analyte concentration (10 – 5000 mg/kg) [33].

In Table 4.2, collaborative trial and LC-GC results for sunflower oil are listed. Except for campestanol and Δ5,24-stigmastadienol, LC-GC results were in good agreement with the collaborative trial results. The observed relative standard deviations for LC-GC fully complied with the Horwitz requirements while the collaborative trial results showed insufficient precision among the laboratories.

As shown previously, the discrepancy for campestanol could be attributed to 14-methyl fecosterol. The differing results for Δ5,24-stigmastadienol could be explained by the presence of another co-eluting sterol. The retention times on HPLC and GC corresponded to Δ7,25-stigmastadienol [26].

Table 4.2. Comparison of collaborative trial (CT) and LC-GC results for sunflower oil

Compound name	CT conc. [%][a]	RSD_r	RSD_R	LC-GC conc. [%]	RSD_r[b]	RSD_R[c]
Cholesterol	0.3	34.7	60.6	0.2	3.4	9.4
Brassicasterol	---	---	---	0.1	5.7	10.8
24-Methylene cholesterol	---	---	---	0.1	2.3	6.5
Campesterol	9.0	2.0	6.6	8.1	1.1	0.8
Campestanol	0.1	24.3	1117.6	1.6	1.3	1.0
Stigmasterol	7.8	1.3	7.7	8.3	1.0	0.7
Δ7-Campesterol	2.3	8.9	15.3	2.4	0.9	1.9
Δ5,23-Stigmastadienol	0.2	14.6	73.8	0.2	5.2	5.7
Clerosterol	0.9	8.5	16.5	0.9	3.1	1.6
Sitosterol	57.4	1.0	2.7	58.3	1.0	0.3
Sitostanol	0.5	20.4	49.6	0.5	2.3	6.8
Δ5-Avenasterol	2.1	3.5	36.4	2.1	1.9	1.3
Δ5,24-Stigmastadienol	1.1	13.8	27.1	0.5	2.3	7.3
Δ7-Stigmastenol	13.2	2.5	11.1	12.6	0.9	0.9
Δ7-Avenasterol	3.8	6.2	17.2	4.1	1.1	1.6
Total sterol content [mg/kg]	3356	1.5	9.5	3098	0.9	1.4

[a]: Mean results based on individual test results ($19 < n < 29$) of 14 laboratories after elimination of outliers

[b]: Based on consecutive injections of six independently prepared samples ($n = 6$)

[c]: Based on the quantitative results in duplicate on three successive days ($n = 6$)

Rapeseed and safflower oils were analyzed by LC-GC accordingly with comparable results. The results can be found in the supporting information. Major sterols (percentage greater than 5 %) could be quantified in all cases with good precision. Minor sterols (less than 5 % of total sterol content) were sometimes troublesome. Especially for exotic types of oils such as pumpkin seed oil, selectivity even by LC-GC-FID was insufficient [34]. In these cases, GC peak integration significantly influenced quantitative results, i.e., precision was defined by the operator processing the chromatograms. Chromatographic techniques with much higher peak capacities, such as comprehensive GCxGC, would be necessary to solve this obstacle [35].

However, the question may be raised as to whether minor sterols have to be safely quantified down to a small percentage. For quality evaluation of high-price oils, the sterol distribution of

major sterols may be sufficient. From the analytical point of view, however, the elucidation of the complete sterol distribution of edible oils and fats is an interesting field of research.

4.4 Conclusions

A fully automated online LC-GC-FID method was designed increasing the robustness and precision for the determination of the sterol content in edible oils and fats. Sample preparation and analysis required approximately 2 h. Interlacing of sample preparation and analysis, however, allowed an average sample throughput of one sample per hour. On the contrary, ISO 12228 permits 6 – 12 samples per day with a tremendous amount of manual work. The LC-GC method was validated and showed results comparable with ISO 12228 in terms of trueness. Furthermore, validation data revealed good precision and robustness making this automated approach amenable to routine environments.

During the determination of the sterol content in sunflower oils, one systematic anomaly was observed. The quantified campestanol content was higher in comparison to ISO 12228. The reason was found in omission of the silylation step during sample preparation. Further investigation revealed an unknown compound usually masked by campesterol in ISO 12228. There is every indication that the unknown compound was 14-methyl fecosterol. Retention times on HPLC and GC as well as GC-MS data supported this assumption. Gas chromatographic separation of 14-methyl fecosterol from campestanol and other regulated sterols was not possible with classic GC columns.

Further investigations in this field of research will address the origin of 14-methyl fecosterol in sunflower oils. Up till now, no literature was published addressing the presence of this compound in sunflower oils. Independent from this, the suitability of comprehensive GCxGC techniques will be evaluated to further improve quantitation precision also for minor sterols.

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4.6 Supporting Information

Table S-4.1. Comparison of CT and LC-GC results for rapeseed oil

Compound name	CT conc. [%][a]	RSD_r	RSD_R	LC-GC conc. [%]	RSD_r[b]
Cholesterol	0.4	8.3	28.2	0.3	2.5
Brassicasterol	10.0	1.5	3.5	10.5	0.2
24-Methylene cholesterol	0.5	15.2	66.5	0.5	14.8
Campesterol	34.9	0.7	2.1	35.7	0.2
Campestanol	0.1	20.4	79.3	0.3	10.5
Stigmasterol	0.3	13.1	25.3	0.2	6.9
Δ7-Campesterol	0.7	11.0	40.5	0.2	0.3
Δ5,23-Stigmastadienol	0.3	16.7	44.3	0.2	5.1
Clerosterol	0.6	9.9	25.1	0.5	3.8
Sitosterol	49.1	0.7	1.5	49.0	0.5
Sitostanol	0.3	16.3	76.2	0.2	9.9
Δ5-Avenasterol	1.7	9.3	13.5	1.7	1.4
Δ5,24-Stigmastadienol	0.8	8.8	12.8	0.6	0.2
Δ7-Stigmastenol	0.2	25.8	53.3	0.1	8.9
Δ7-Avenasterol	0.1	43.4	77.7	0.1	10.4
Total sterol content [mg/kg]	7365.2	2.1	5.7	7421.8	0.5

[a]: Mean results based on individual test results ($21 < n < 29$) of 14 laboratories after elimination of outliers

[b]: Based on consecutive injections of three independently prepared samples ($n = 3$)

Table S-4.2. Comparison of CT and LC-GC results for safflower oil

Compound name	CT conc. [%][a]	RSD_r	RSD_R	LC-GC conc. [%]	RSD_r[b]
Cholesterol	0.6	10.9	68.1	0.3	2.2
Brassicasterol	---	---	---	0.2	3.6
24-Methylene cholesterol	---	---	---	0.1	1.9
Campesterol	12.0	0.7	5.4	13.0	0.2
Campestanol	0.4	10.7	47.7	0.6	1.0
Stigmasterol	5.3	2.4	6.9	5.3	1.3
Δ7-Campesterol	3.8	5.2	13.3	4.0	3.4
Δ5,23-Stigmastadienol	0.8	8.6	37.5	0.8	12.8
Clerosterol	1.1	15.0	31.1	1.0	13.4
Sitosterol	49.2	1.1	3.1	50.9	0.5
Sitostanol	3.0	4.5	17.5	2.6	3.0
Δ5-Avenasterol	1.7	9.2	65.7	1.1	5.6
Δ5,24-Stigmastadienol	3.6	6.2	30.6	1.6	5.0
Δ7-Stigmastenol	16.7	2.9	8.9	16.8	0.3
Δ7-Avenasterol	1.5	7.0	38.5	1.7	4.9
Total sterol content [mg/kg]	2447.8	3.5	12.8	2314.0	0.8

[a]: Mean results based on individual test results ($19 < n < 29$) of 14 laboratories after elimination of outliers

[b]: Based on consecutive injections of three independently prepared samples ($n = 3$)

Table S-4.3. Comparison of CT and LC-GC results for a phytosterol concentrate used in an international CT from 2010

Compound name	CT conc. [%][a]	RSD_r	RSD_R	LC-GC conc. [%]	RSD_r[b]	RSD_R[c]
Cholesterol	0.2	18.0	49.5	0.4	5.6	12.4[d]
Brassicasterol	3.6	2.8	3.6	3.7	0.5	0.8
24-Methylene cholesterol	0.7	9.5	15.8	0.6	2.5	13.8[d]
Campesterol	16.6	0.5	1.4	16.9	0.1	1.2
Campestanol	0.9	7.0	9.1	1.0	1.7	10.8
Stigmasterol	0.6	7.2	10.1	0.5	1.7	4.8
Δ7-Campesterol	0.2	38.2	62.0	0.05	13.3	33.4[d]
Δ5,23-Stigmastadienol	0.1	37.5	67.0	0.04	10.8	20.4[d]
Clerosterol	0.4	8.9	29.9	0.4	1.9	7.9
Sitosterol	67.0	0.4	1.2	67.4	0.2	0.7
Sitostanol	7.2	3.4	5.1	6.6	0.5	3.5
Δ5-Avenasterol	1.5	13.6	26.1	1.9	2.6	14.1[d]
Δ5,24-Stigmastadienol	0.3	10.1	65.2	0.2	5.7	14.4[d]
Δ7-Stigmastenol	0.4	11.8	33.7	0.3	3.8	4.2
Δ7-Avenasterol	0.3	16.4	33.4	0.2	8.6	6.5
Total sterol content [g/100 g]	6.2	1.7	11.8	5.2	1.2	3.9

[a]: Mean results based on individual test results ($15 < n < 21$) of 11 laboratories after elimination of outliers

[b]: Based on consecutive injections of six independently prepared samples ($n = 6$)

[c]: Based on the quantitative results in duplicate on three successive days ($n = 6$)

[d]: The high total sterol content (>5 %) and the apparent differences in analyte concentrations were the sources for an insufficient precision.

5. Determination of vitamins D_2 and D_3 in selected food matrices by online high-performance liquid chromatography–gas chromatography–mass spectrometry (HPLC-GC-MS)

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Abstract

An online normal-phase liquid chromatography–gas chromatography–mass spectrometry (HPLC-GC-MS) method was developed for the determination of vitamins D_2 and D_3 in selected food matrices. Transfer of the sample from HPLC to GC was realized by large volume on-column injection; detection was performed with a time-of-flight mass spectrometer (TOF-MS). Typical GC problems in the determination of vitamin D such as sample degradation or sensitivity issues, previously reported in the literature, were not observed. Determination of total vitamin D content was done by quantitation of its pyro isomer based on an isotopically labeled internal standard (ISTD). Extracted ion traces of analyte and ISTD showed cross-contribution, but nonlinearity of the calibration curve was not determined inside the chosen calibration range by selection of appropriate quantifier ions. Absolute limits of detection (LOD) and quantitation (LOQ) for vitamins D_2 and D_3 were calculated as approximately 50 and 150 pg, respectively. Repeatability with internal standard correction was below 2 %. Good agreement between quantitative results of an established high-performance liquid chromatography with UV detection (HPLC-UV) method and HPLC-GC-MS was found. Sterol-enriched margarine was subjected to HPLC-GC-MS and HPLC-MS/MS for comparison, because HPLC-UV showed strong matrix interferences. HPLC-GC-MS produced comparable results with less manual sample cleanup. In summary, online hyphenation of HPLC and GC allowed a minimization in manual sample preparation with an increase of sample throughput.

5.1 Introduction

According to the common definition, vitamins are organic compounds which cannot be synthesized in sufficient quantities by humans or animals and have to be externally supplied, typically via the daily diet. Since metabolisms differ among species, a compound can be a vitamin for a particular organism but not for the other.

The function of vitamins is not limited to a specific field of action; they can be involved in many reactions in the metabolism. For example, they can act as hormones (e.g., vitamin D), antioxidants (e.g., vitamin E), or precursors for enzyme cofactors (e.g., class of vitamin B).

Because of their variety in function, vitamins cannot be summarized into a dedicated class of compounds with fixed chemical structures. Depending on their function, the chemical structure can highly differ. Generally, vitamins are classified into water- and fat-soluble compounds.

The vitamin D class has a special standing among the vitamins. This class of fat-soluble vitamins consists of several compounds derived from 7-dehydrosterols [1]. Through photochemical ring opening and isomerization, the actual vitamin (calciferol) is formed. These reactions occur in the human body. For instance, vitamin D_3 is formed from the provitamin 7-dehydrocholesterol which is available in the human skin through daily diet. Via exposure to UV-B radiation from sunlight, the actual vitamin D_3 (cholecalciferol) is synthesized. Thus, in the narrow sense, vitamin D_3 would not be added to the vitamins for the human organism; nevertheless, historically, it is.

The chemical base structure of all vitamin D derivatives is given in Fig. 5.1. As can be seen, the particular vitamins differ in a single side chain only. Table 5.1 shows a compilation of a few prominent compounds.

Table 5.1. Derivatives of vitamin D (calciferols) [1]

Vitamin	Trivial name
D_1	1:1 mixture of ergocalciferol and lumisterol
D_2	Ergocalciferol
D_3	Cholecalciferol
D_4	22-Dihydroergocalciferol
D_5	Sitocalciferol
D_6	Stigmacalciferol
D_7	Campecalciferol

From the known derivatives, the most abundant ones are vitamin D_3 (originating from cholesterol found in animal products) and vitamin D_2 (from ergosterol originating from plants) [2, 3]. The other ones are mostly artificially created and their bioactivity is only little compared to the naturally available ones.

The role of vitamin D in the human body is strongly related to the calcium and phosphorus regulation of the metabolism and to the bone health. The biologically active form of vitamin D upon intake is achieved via hydroxylation in the liver (calcidiol) and further in the kidneys (calcitriol).

Even though sunlight may be a major source for vitamin D supplement for the population, an additional dietary intake is recommended [2]. Based on minimal sun exposure, the recommended dietary allowance for vitamin D is 15 µg/day for an adult.

Because of their lipophilicity, vitamin D_2 and D_3 are found in only a few foodstuffs in significant amounts (>0.1 µg/100 g) [3]. Vitamin D_3 is found mainly in fatty fish and milk products whereas vitamin D_2 is present, for example, in mushrooms [4]. For that reason, enriching of several foodstuffs, e.g., margarine, is allowed to ensure the population's supply with vitamin D [5]. If not explicitly declared on foodstuffs, the collective term "vitamin D" stands for vitamins D_2, D_3, or a mixture.

The deficiency of vitamin D (hypovitaminosis D) can lead to osteomalacia, osteoporosis, and other severe diseases. On the other hand, vitamin D is one of the few vitamins whose extensive intake can be toxic (hypercalcemia) [6].

5.1.1 Analytics of vitamin D in foodstuff

The analytical detection and quantitation of vitamin D in foodstuff is challenging. Extremely low vitamin D contents (few µg/100 g) have to be safely detected in the presence of other compounds in excess (fat, emulsifiers, proteins, sterols, and other fat-soluble vitamins) [7].

In routine analysis, quantitation is mainly done by high-performance liquid chromatography with UV detection (HPLC-UV) [8]. In the past, gas chromatographic methods were also evaluated [8]. Nowadays, the usage of HPLC in combination with tandem mass spectrometric detection (HPLC-MS/MS) is frequently found in literature [9]. Its use in routine analysis is reported for challenging food matrices and multivitamin determination approaches [10].

The structural relationship to other compounds derived from sterane, like sterols or hormones, is complicating the detection and quantitation processes. For these reasons, MS/MS detections can offer additional selectivity which UV detection is lacking. Because of the nonpolar chemical structure of vitamin D, atmospheric pressure chemical ionization (APCI) is usually used when MS detection is performed [11]. Although it is inherently less prone to ion suppression effects compared to electrospray ionization (ESI), these are still reported in literature [12]. Especially when plant sterol-enriched foodstuff like margarine is to be analyzed, ion suppression by high amounts of co-eluting matrix has to be taken into account [10].

Besides these effects, the high costs of HPLC-MS/MS systems still hinder their routine usage in many food control laboratories. Instead, classical HPLC-UV methods are found far more often. The missing level of selectivity has to be compensated by time-consuming sample preparation protocols involving error-prone manual work.

The classical approach for most foodstuffs involves saponification of the sample with potassium hydroxide (KOH) as the first step to remove the excess of triglycerides. The unsaponifiable matter is liquid–liquid extracted with a nonpolar organic solvent, e.g., *n*-hexane. After evaporation of the solvent and enrichment step, the organic extract is further cleaned up via solid-phase extraction (SPE) or preparative normal-phase HPLC. The eluate is evaporated once again and the analytes are separated and quantified via reversed-phase HPLC on a C_{18} material [7].

Apart from the intense manual work, the quantitation by UV detection has one additional drawback. Vitamin D_2 is usually used as internal standard (ISTD) when vitamin D_3 is quantified and vice versa. Samples containing both vitamins cannot be safely processed. MS detection solves this problem in an elegant way by the usage of isotopically labeled compounds.

As already mentioned, gas chromatography (GC) methods were reported in the past. With the advance in HPLC separation techniques, the usage of GC methods became less important. Since vitamin D is sensitive towards light and temperature, the usage of GC methods is furthermore prone to analytical problems. For instance, it is reported in the literature that during the GC injection and separation process, vitamin D isomerizes into its pyro and isopyro form at temperatures exceeding some 150 °C (see Fig. 5.1) [13]. Because of this thermo-isomerization, two peaks are detected for a single compound. Quantitation is still possible according to the literature although sensitivity might be compromised.

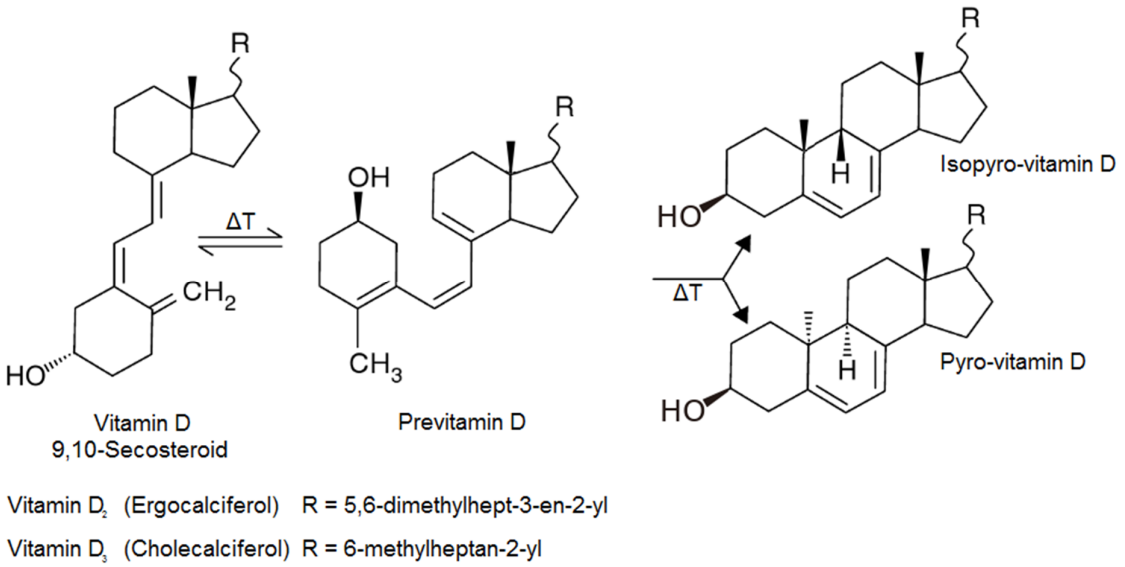

Fig. 5.1. Thermal isomerization of vitamin D into its pyro and isopyro forms (adapted from [13])

Besides this, the separation of vitamins D_2 and D_3 is quite easily achieved on a standard GC separation column, e.g., 5 % phenyl content. The loss in sensitivity can be compensated by using GC-MS detection-based systems and isotopically labeled compounds as ISTD can be applied. Furthermore, mass spectrometric detection offers an additional selectivity, which might be helpful for challenging food matrices.

Unfortunately, the direct injection of an organic extract of foodstuff containing vitamin D is not possible for GC. Sample preparation steps are inevitable to remove the vast matrix amount, e.g., fat, sterols, etc. For this reason, the coupling of a GC-MS method for quantitation to an HPLC method for sample cleanup seems favorable.

Coupling of HPLC to GC is reported in the literature for at least 30 years, especially for the determination of mineral oil originating compounds in food and paper stuff [14–16]. Nevertheless, other fields of application, like sterol or alkyl ester quantitation, were also explored [17, 18]. The hyphenation of both separation techniques allows the orthogonal coupling of well-established sample cleanup processes (HPLC) with highly efficient separation and detection techniques (GC). The online coupling offers additional advantages such as reduction of manual sample preparation, decrease of cross-contamination, and increase of sample throughput.

The aim of this work was therefore to demonstrate an online coupling of an optimized HPLC-based sample cleanup to a GC-MS method for the detection and quantitation of vitamins D_2 and D_3 in selected food matrices and dietary supplements.

5.2 Experimental

5.2.1 Samples

Food samples were obtained at local supermarkets and drugstores. They consisted of yoghurt (fat content of 2.9 g/100 g), two vitamin D_3 dietary supplements, and a plant sterol-enriched margarine (declared sterol content of 7.5 g/100 g). An internal reference instant milk powder (fat content of 1.5 g/ 100 g) was supplied by Institute Kirchhoff GmbH (Berlin, Germany). It is a commercially available product with a declared vitamin D_3 content of 10 µg/100 g. It is used as a quality control sample for quantitation of milk powders and is routinely monitored via control charts.

5.2.2 Chemicals and solutions

Dichloromethane, ethanol, *n*-hexane, and isopropanol were from LGC Promochem (Picograde quality, Wesel, Germany). Vitamins D_2 and D_3 (analytical standard quality), the corresponding deuterated 6,19,19-d_3-vitamins (97 atom % in ethanol), and potassium hydroxide (≥85 %, pellets, white) were from Sigma-Aldrich (Steinheim, Germany). Sodium sulfate was from Fluka (Buchs, Switzerland). Water was supplied from a Milli-Q water purification system (Merck, Darmstadt, Germany).´

5.2.3 Sample preparation

After homogenization, to an aliquot of the sample 10 mL of *n*-hexane and 15 mL of water/ethanol (1:1, v/v) were added. Aliquoting was based on the declared vitamin D content of the sample. To this solution, 1 mL of 50 % aqueous KOH (w/w) was added. The mixture was continuously shaken and saponified for 30 min at 60 °C in a water bath. The solution was allowed to cool down to room temperature and the separated *n*-hexane layer was washed with water/ethanol (1:1) until pH neutrality was reached. Afterward, the *n*-hexane phase was dried

over sodium sulfate. One milliliter of the *n*-hexane phase was transferred into a 2-mL autosampler vial and directly analyzed by HPLC-GC-MS.

The sample preparation for the reference instant milk powder differed slightly, because the crude extract was used also for HPLC-UV experiments. Three grams of milk powder were suspended in 30-mL water. To this suspension, 100 µL of 2.9 µg/mL vitamin D_2 were added as internal standard. Twenty milliliters of 50 % aqueous KOH solution (w/w) were added. Saponification was performed in a water bath for 30 min at 60 °C. After allowing the mixture to cool down to room temperature, the unsaponifiable matter was extracted with 200 mL of *n*-hexane. The *n*-hexane layer was separated and washed with water until pH neutrality was reached. Afterward, it was evaporated to dryness and the residue was reconstituted in 1.75 mL of *n*-hexane. This extract was directly used for HPLC-GC-MS analyses and further cleaned up for HPLC-UV experiments.

5.2.4 HPLC-UV Analysis

In case of the reference material, 250 µL of the *n*-hexane phase were injected onto a Lichrospher Si 60 preparative HPLC column (250 mm × 4.6 mm, 5 µm, 60 Å, Merck, Darmstadt, Germany). The mobile phase consisted of 2 % isopropanol in *n*-hexane (v/v). The column was operated at room temperature with a flow rate of 1.7 mL/min. UV detection was performed at 265 nm. The used system was supplied by KNAUER (Berlin, Germany). It consisted of a pump 64 and a variable wavelength monitor.

In a first run, vitamin D_2 was injected to determine its retention time. Since vitamins D_2 and D_3 elute as one peak on a normal-phase HPLC column, the retention time of vitamin D_2 could be used for locating vitamin D_3.

The vitamin D containing fraction eluted between 13 and 15 min. The collected eluate (3.4 mL) was evaporated to dryness and the residue was dissolved in 500 µL of the mobile phase of the subsequent reversed-phase HPLC step (ACN/MeOH/H_2O, 97.5:2.0:0.5, v/v/v). One hundred microliters of this solution were injected onto a Zorbax C_{18} column (250 mm×4.6 mm, 5 µm, Agilent, Waldbronn, Germany). Column oven temperature was 30 °C and flow was 1.5 mL/min.

UV detection was performed with a diode array detector (DAD) at 265 nm. Confirmation wavelengths were set to 230 and 290 nm. Vitamins D_2 and D_3 eluted after 18.5 and 19.8 min, respectively.

Calibration solutions of vitamins D_2 and D_3 were prepared between 20–300 and 10–100 ng/mL, respectively. The analyses were performed on an Agilent 1100 HPLC system. Data acquisition and processing was done with the Chemstation.

5.2.5 HPLC-MS/MS Analysis

For the sterol-enriched margarine, HPLC-MS/MS analyses were performed. HPLC-UV chromatograms showed big disturbances during the elution window of vitamin D_2/D_3 and could therefore not be evaluated. The specific sample preparation protocol is described elsewhere [10]. Shortly, 10 g of the sample were saponified and the extract was purified by SPE. The eluate was evaporated to dryness and reconstituted in *n*-hexane. Fifty microliters were injected onto a MultoHigh U-Si HPLC column (100 mm×4.6 mm, 2 µm, 120 Å, CS-Chromatographie-Service GmbH, Langerwehe, Germany). The mobile phase consisted of 3 % 1,4-dioxane and 0.3 % isopropanol in n-hexane (v/v/v). The column was operated at 20 °C with a flow rate of 1.5 mL/min.

Analyses were performed on an Agilent 1100 HPLC system coupled to an API 3200 triple quadrupole MS/MS instrument (AB SCIEX, Darmstadt, Germany). Ionization was caused by APCI and detection was performed by multiple reaction monitoring (MRM). Deuterated ISTDs were used for quantitation. Data acquisition and processing was done with Analyst

5.2.6 HPLC-GC-MS Analysis

HPLC-GC-MS experiments were performed on a system from Axel Semrau (Sprockhövel, Germany). It consisted of an Agilent 1260 Infinity HPLC system (binary pump and variable wavelength detector), Master GC and Master TOF-MS (DANI Instruments S.p.A., Cologno Monzese, Italy), and a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland).

Three rotatory switching valves (VICI AG International, Schenkon, Switzerland) were used to guide the HPLC eluent from the HPLC into the GC [19]. The latter one was equipped with an on-column interface and a solvent vapor exit. The on-column interface, the carrier gas, and solvent vapor exit were controlled by CHRONECT LC-GC from Axel Semrau.

Of the samples, 5–60 µL were injected onto an Allure Si HPLC column (250 mm × 2.1 mm, 5 µm, 60 Å, Restek, Bellefonte, PA, USA) without additional column temperature control. In order to allow automation and continuous monitoring of the system performance, ISTDs were

not added to the samples during preparation but added before injection by the autosampler. A disadvantage of late ISTD addition was that analyte losses during the sample preparation would not be covered. Because of the simple one-step sample preparation, which was virtually identical to classical workflows, the evaluation of the long-time system and method stability was favored during method development. Because of the inherent exclusion of ISTD losses, late ISTD addition allowed the recognition of analyte losses inside the system, e.g., during the HPLC cleanup or the transfer from HPLC to GC. Furthermore, it allowed a check for matrix effects during elution of the analytes in the GC dimension.

Ten microliters of a 60 ng/µL *n*-hexanic solution containing vitamin D_2/D_3-d_3 (resembling 600 pg on column) were aspirated by the autosampler syringe, 5 µL *n*-hexane for layer separation, and the actual injection volume. The optimized mobile phase consisted of 0.1 % isopropanol in dichloromethane (v/v) and was delivered isocratically at 300 µL/min. After elution of the analytes of interest, the column was backflushed with 10 % isopropanol in dichloromethane (v/v) at 500 µL/ min for 10 min. Afterward, the column was reconditioned with the mobile phase at 300 µL/min for 20 min.

HPLC-GC transfer occurred by the retention gap technique and fully concurrent solvent evaporation (FCSE) through the Y interface [20]. An uncoated, deactivated precolumn (MXT Hydroguard, 0.5 m × 0.53 mm, Restek, Bellefonte) was followed by a steel T-piece union connecting to the solvent vapor exit and a separation column coated with a 5 % phenyl-polysiloxane film (Rxi-5Sil MS, 30 m × 0.25 mm × 0.10 µm, Restek, Bellefonte, PA, USA).

From HPLC, the vitamin D-containing fraction was eluted between 18.0 to 20.0 min and transferred to the GC (resembling 600 µL) at a carrier gas inlet pressure of 100 kPa (helium) in addition to an oven temperature of 80 °C. The elution window was verified by UV detection at 265 nm. The solvent vapor exit was opened 0.5 min before the elution of the vitamin D fraction began. Because of the high boiling points of the analytes, fully concurrent evaporation of the solvent was possible without loss of substances through the solvent vapor exit. The solvent vapor exit was closed 0.1 min after the fraction was transferred. At this time, the carrier gas inlet pressure was set to 160 kPa and held for the complete analysis. The oven temperature was programmed at 15 °C/min from 80 °C (6 min) to 310 °C (6.67 min, total time 28.00 min). The Master TOF-MS ion source and transfer line temperatures were set to 200 and 320 °C, respectively. Data acquisition started after 20.0 min at a rate of five spectra/s with electron impact ionization (EI) at 70 eV. Vitamins D_3 and D_2 eluted after 23.1 and 23.3 min, respectively.

Data processing was performed with Xcalibur 2.2 (Thermo Fisher Scientific Inc., Austin, TX, USA). Quantitation was based on the corresponding deuterated ISTDs. Calibration was done by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. Calibration curves for vitamin D_2 and D_3 were created from 150 to 1800 pg in seven levels (150, 300, 600, 900, 1200, 1500, 1800 pg). Each level was measured once.

5.3 Results and Discussion

5.3.1 Optimization of HPLC conditions

As outlined in the introduction, peak detection and quantitation of vitamin D in complex matrices can be troublesome. Comparing vitamin D to sterols like cholesterol or sitosterol (all derived from sterane) reveals a big structural similarity. Chromatographic separation of vitamin D from sterols can be difficult, especially when the concentrations vary by several orders of magnitude. This can easily be the case for sterol-enriched foodstuffs like margarine. Because of this, the chromatographic properties for the separation of vitamin D from matrix compounds were optimized during development of this HPLC-GC-MS method.

A normal-phase HPLC on the base of bare silica gel was chosen for method development. Firstly, normal-phase HPLC columns use organic solvents readily compatible with large volume transfers into GC systems. Secondly, silica gel HPLC columns allow group type separation of analytes. This feature is helpful for strongly related substance classes. For instance, the separation of 4-desmethylsterols from 4-methyl- or 4,4-dimethylsterols is achieved without difficulties [21].

In the first experiments with eluents on the base of *n*-hexane and isopropanol, it was verified that vitamins D_2/D_3 and the 5-desmethylsterols could be baseline separated at equal concentrations on a silica gel HPLC column. Cholesterol was chosen as a representative for the class of 5-desmethylsterols.

Since desmethylsterols do not contain chromophoric groups and show therefore only limited UV activity even at very low wavelengths, e.g., 205 nm, the vitamin D-containing fraction from HPLC was transferred to a gas chromatography–flame ionization detection system (HPLC-GC-FID). A vitamin D_2/D_3 standard mixture (ng/µL) was doped with high amounts of cholesterol

(µg/µL) for this purpose. Inspection of the HPLC-GC-FID chromatogram revealed that the vitamin D fraction already contained significant amounts of cholesterol.

In further experiments, isopropanol as modifier was replaced with ethyl acetate and methyl-*tert*-butylether (MTBE) without improvement of separation in the HPLC dimension.

Besides *n*-hexane, literature discusses the usage of dichloromethane-based eluents [22]. In pure dichloromethane, vitamin D_2/D_3 eluted only after 25 min with a peak width of more than 3 min. To minimize the elution window of the vitamin D fraction, some percentage of isopropanol was added to the eluent. Because of the high UV cutoff of dichloromethane (>230 nm), the detection of cholesterol was no longer possible by UV detection. Transfer of the vitamin D/cholesterol standard mixture to GC-FID no longer showed evidence of cholesterol in the FID chromatogram. The isopropanol fraction of the eluent was optimized to 0.1 %. On the one hand, it allows reduction of the peak width of vitamin D_2/D_3 to less than 2 min; on the other hand, it does not significantly deteriorate the separation, which was observed at higher isopropanol percentage or with other modifiers during measurement of sterol-enriched foodstuff.

Additional testing of the optimized eluent was performed by injecting 5 µL of the extracted reference instant milk powder (resembling 8.5 mg) into the HPLC-GC-FID system.

Figure 5.2 shows FID chromatograms of injections with two eluent compositions and a vitamin D standard mixture as reference. As can be seen, transferred interferences are significantly influenced by the HPLC mobile phase. The dichloromethane-based eluent (eluent B) shows less by-products than the *n*-hexane based mobile phase (eluent A).

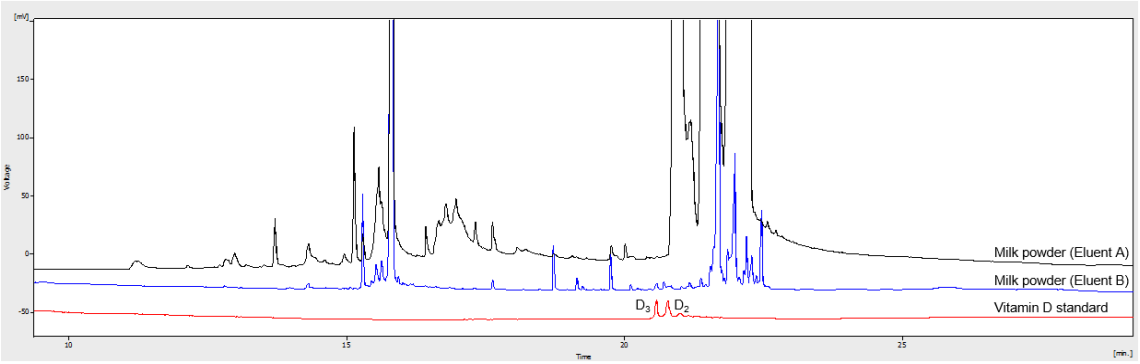

Fig. 5.2. HPLC-GC-FID chromatograms of the reference instant milk powder sample and a vitamin D_2/D_3 standard (Eluent A: *n*-hexane/isopropanol (98:2, v/v); Eluent B: dichloromethane/isopropanol (99.9:0.1, v/v))

Detection by FID did not allow the usage of isotopically labeled ISTDs. Furthermore, co-elution of the pyro and isopyro isomers of vitamins D_2 and D_3 prevented the usage of vitamin D_2 as ISTD for vitamin D_3 quantitation and vice versa. Consequently, quantitation by FID was not possible. Instead, mass spectrometric detection was chosen for further method development because of its advantage in sensitivity and selectivity.

5.3.2 Coupling of HPLC-GC to MS

The HPLC-GC system was coupled to a time-of-flight mass spectrometer (TOF-MS) for quantitation. The used TOF-MS detector showed a performance comparable to quadrupole systems operated in selected ion monitoring (SIM) mode with the advantage of full spectra data acquisition across the complete chromatogram. This way, a retrospective view on the data was possible.

HPLC-GC-TOF-MS chromatograms of vitamin D standards revealed two peaks per compound with an intermediate valley in between (see Fig. 5.3). This valley indicated a reaction of vitamin D during the GC separation process. Trapp investigated this phenomenon and used it for quantitation of reaction kinetics [23]. From the two found peaks, only the first one was used for qualification and quantitation because of its higher intensity. According to the literature, this peak resembles the pyro isomer of vitamin D [24]. Fragmentation patterns of pyro and isopyro isomers were virtually the same, but the abundance of several ions differed. Since the intensity of the isopyro isomer is not sufficient for processing purposes, it was ignored for further data analyses.

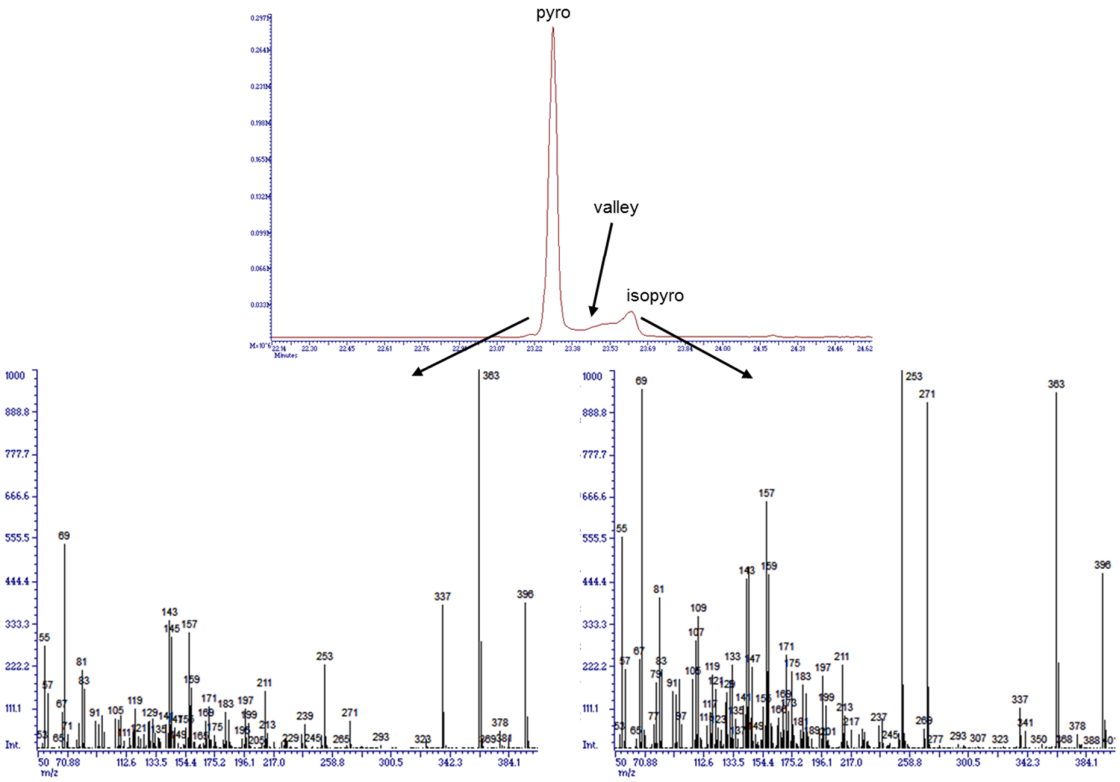

Fig. 5.3. HPLC-GC-TOF-MS chromatogram (top) and TOF-MS spectra of vitamin D_2 (bottom left: pyro isomer; bottom right: isopyro isomer)

Degradation or poor ionization efficiency due to volatility or polarity problems as described in the literature could not be observed [25]. Normally, derivatization is recommended to increase the volatility of vitamin D (boiling point >490 °C at 1 atm). It is believed that the on-column transfer of the vitamin D compounds offers a reliable and efficient way from the HPLC to the GC dimension. Figure 5.4 shows the separation of vitamins D_2, D_3, and the corresponding ISTDs, which was only possible by MS detection.

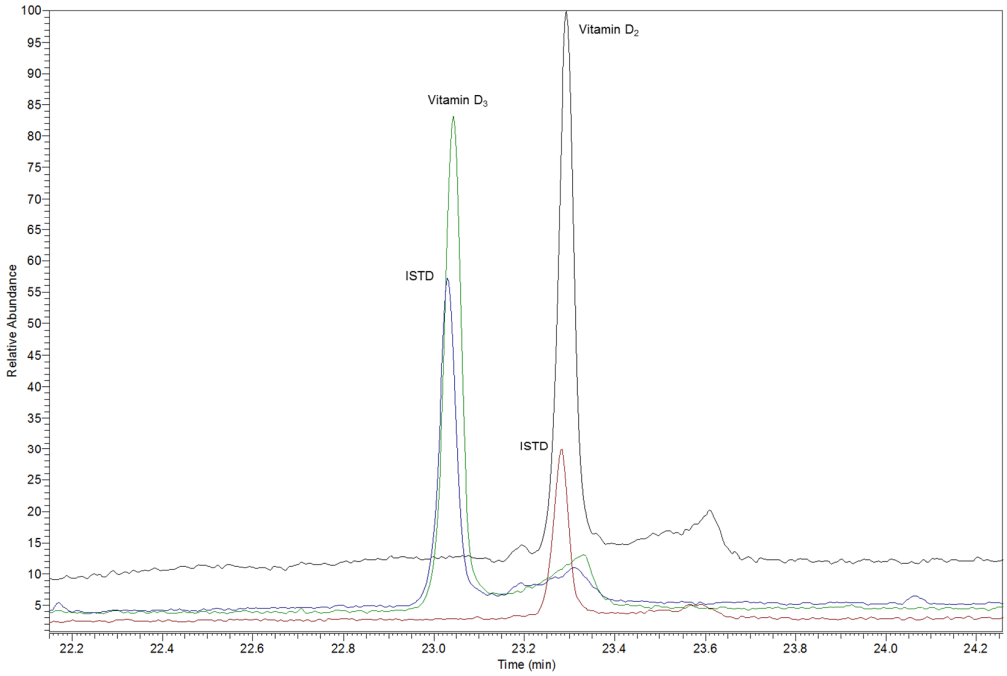

Fig. 5.4. HPLC-GC-TOF-MS extracted ion chromatogram of a standard containing vitamins D_2, D_3, and the corresponding ISTDs (see Table 5.2 for selected ions).

5.3.3 Calibration curves, LOD, and LOQ

Calibration curves for vitamins D_2 and D_3 ranged from 150 to 1800 pg on column. Table 5.2 summarizes the quantitation parameters. The available ISTDs carried only three deuterium atoms. Because of the distinct isotopic patterns of steroid compounds, analyte isotopic ions disturbed the abundance of the analogue ISTD ions to some extent (see Table 5.2). This phenomenon is known as "cross-contribution" in the literature [26]. Shortly, cross-contribution can lead to nonlinearity of calibration curves if it exceeds some percentage. Furthermore, contribution of analyte ions to the ISTD ions can lead to underestimation at high analyte concentrations. Therefore, the chosen quantifier ions were optimized as a compromise of cross-contribution and sensitivity. Measured cross-contributions were in good accordance with the theoretical calculations. Furthermore, the amount of ISTD was adjusted to minimize the effects of cross-contribution.

Table 5.2. Quantitation parameters and calibration curves

	Vitamin D_2	**Vitamin D_3**
Analyte (ISTD) quantifier ions [*m/z*]	337+396 (339+399)	325+384 (327+387)
Cross-contribution of analyte to ISTD[a] [%]	4.0	3.7
Coefficient of determination (R^2)	0.9996	0.9995
LOD[b] [pg]	42	52
LOQ[c] [pg]	128	157

[a]: Cross-contribution was calculated on the base of the theoretical isotopic distribution pattern at equal concentrations of analyte and ISTD

[b]: $3.3 * \frac{Standard\ deviation}{Slope}$

[c]: $10 * \frac{Standard\ deviation}{Slope}$

As can be seen in Table 5.2, good linearity is achieved for the chosen calibration range with coefficients of determination greater than 0.999 despite a cross-contribution of approximately 4 %. To exclude the influence of cross-contribution on the quantitative results, experimental quantitation solely based on ions not showing cross-contribution was performed (e.g., *m/z* 384/ 387 for vitamin D_3 and ISTD). Because of this, cross-contribution issues were not further taken into account for quantitation.

Additionally, the observed calibration linearity justifies the usage of the pyro isomer for quantitation of the total vitamin D content. The isomerization of the original vitamin D compound during the GC separation seems to be kinetically fast, the products seem thermodynamically stable, and the result ratio seems to be fixed. This observation is in accordance with the literature [25].

Limits of detection (LOD) and limits of quantitation (LOQ) could be derived from the calibration curves. Although EI ionization in GC-MS ion sources favors high fragmentation of steroid compounds, LOD and LOQ for vitamin D_2 were calculated as 42 and 128 pg, respectively. LOD and LOQ for vitamin D_3 were calculated as 52 and 157 pg. Usage of softer ionization techniques like chemical ionization (CI) could further lower the detection and quantitation limits [27].

5.3.4 Repeatability

Repeatability of the system was tested with a 600-pg standard which was subsequently injected six times. Table 5.3 summarizes the results. Without ISTD, the relative standard deviation (%RSD) was approximately 8 %. As can be seen by ISTD correction, the relative standard deviation could be significantly lowered to approximately 1 %.

Table 5.3. Repeatability of HPLC-GC-MS method

Compound	%RSD[a] without ISTD	%RSD[a] with ISTD
Vitamin D_2	7.8	1.0
Vitamin D_3	6.8	1.3

[a]: calculated from six injections of a 600-pg standard

5.3.5 Comparison to classical method

Figure 5.5 shows a flow diagram summarizing the elementary steps of all three described methods (HPLC-UV, HPLC-MS/ MS, and HPLC-GC-MS). As can be seen, HPLC-GC-MS allows a significant reduction of manual work steps. Although enrichment steps during a sample preparation are easily performed, they are rather time-consuming. Only a limited number of samples can be processed at once. In the classical HPLC-UV approach, the necessary normal phase HPLC cleanup step is an additional bottleneck. Because the samples have to be cleaned up sequentially, the achievable sample throughput is limited. HPLC-GC-MS does not need enrichment steps for most sample types, although they can be applied whenever needed.

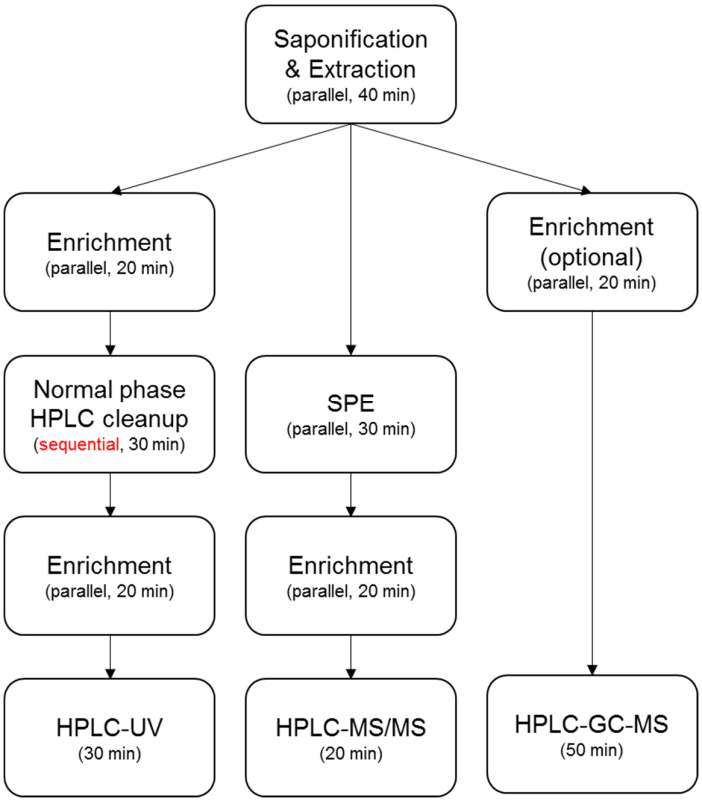

Fig. 5.5. Flow diagram of elementary steps for HPLC-UV, HPLC-MS/MS, and HPLC-GC-MS

HPLC-GC-MS was compared to the classical HPLC-UV method described in the experimental section. Comparison was performed with the reference instant milk powder. Table 5.4 and Fig. 5.6 summarize the results and compare the chromatographic characteristics. The injected sample amount could be significantly lowered for HPLC-GC-MS with adequate sensitivity. The results are in good agreement with the classical method. Thus, HPLC-GC-MS allows the reduction of the manual sample preparation and cleanup processes without compromising the analytical quality for this type of matrix.

Table 5.4. Quantitative comparison of HPLC-UV and HPLC-GC-MS for the reference instant milk powder

	HPLC-UV	HPLC-GC-MS
Injected sample amount [mg]	150	9
Vitamin D_3 concentration [µg/100 g]	10.2±0.09	10.0±0.02
Injected vitamin D_3 amount [ng]	15.2	0.9

Declared vitamin D_3 content: 10 µg/100 g
Standard deviation based on analysis in triplicate.

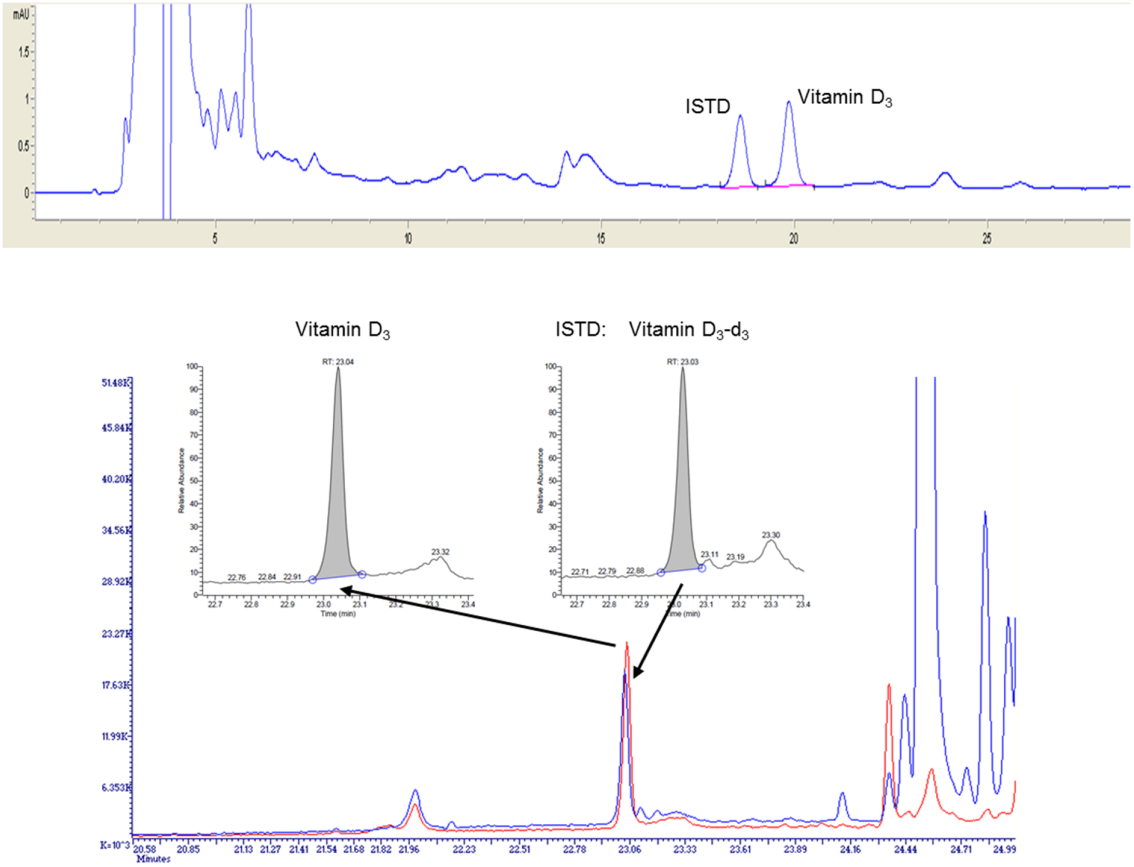

Fig. 5.6. Chromatographic comparison of HPLC-UV (top: UV-signal) and HPLC-GC-MS (bottom: extracted ion chromatogram) for the reference instant milk powder

Further matrices were tested to explore the potential of this approach. Table 5.5 summarizes the quantitative results for the selected food matrices and dietary supplements. If not stated otherwise, each sample was prepared once and analyzed in triplicate. As can be seen, the standard deviation is below 1 % for all matrices, even for a plant sterol-enriched margarine. Standard deviations of the ISTD areas during the measurement sequence were below 10 %,

which was comparable to the repeatability results shown above. The conclusion from this is that HPLC cleanup and HPLC-GC transfer were working reliably. Additionally, matrix effects during GC-MS detection could be excluded.

Table 5.5. Quantitative results of vitamin D_3 in selected food matrices via HPLC-GC-MS

Matrix	Amount	Concentration [µg/100 g]	
		Calculated[a]	Declared
Yoghurt	5 g	1.19±0.01	1.25
Dietary supplement 1	1 capsule	11.38±0.07[b]	10
Dietary supplement 2	1 capsule	747±2[b]	500
Sterol-enriched margarine	5 g	5.60±0.05	7.5

Vitamin D_2 was not detected.
[a]: Standard deviation based on analysis in triplicate; [b]: µg/capsule

The quantified results correspond well with the declaration values. For the highly concentrated dietary supplement, a 50 % higher concentration of vitamin D_3 was found than declared. The product was only available on prescription and its use was recommended for people having a vitamin D deficiency only. No analytical problems during the measurement of this matrix type could be observed. A study from 2007 revealed discrepancies of declared and measured vitamin D contents up to 45 % [28]. Vitamin D was occasionally overdosed to ensure the declared concentration for the lifetime of the corresponding product. The vitamin D content in dietary supplements is not yet regulated in the European Union [29]. Nevertheless, according to a European Food Safety Authority (EFSA) statement from 2012, the tolerable upper intake level of vitamin D for adults was set at 100 µg/day [30].

5.3.6 Comparison of HPLC-GC-MS with HPLC-MS/MS

For the sterol-enriched margarine, additional HPLC-MS/MS analyses were performed to compare the results with the ones from HPLC-GC-MS measurements. HPLC-UV chromatograms showed big disturbances during the elution window of vitamin D_2/D_3 and could therefore not be used for comparison.

The obtained results are comparable to the HPLC-GC-MS results. They are (7.7±0.1) µg/100 g and (5.6±0.1) µg/100 g for HPLC-MS/MS and HPLC-GC-MS, respectively. Standard deviations are based on analysis in triplicate. Both results were obtained independently from each other on different days from samples acquired in different local supermarkets. Figure 5.7 compares the relevant elution windows of both methods. In direct vicinity, both approaches show undisturbed analyte peaks. Since no manual sample pretreatment besides saponification was performed for HPLC-GC-MS, this method is outperforming the HPLC-MS/MS method, which needed a prior SPE and enrichment step to remove the bulk of matrix.

Inspection of the HPLC-GC-MS data of the margarine sample showed some high boiling compounds at the end of the chromatogram (elution temperature of 310 °C). TOF-MS spectra revealed the steroid structure of these compounds. They are not co-eluting with vitamins D_2 or D_3 and are therefore not disturbing their quantitation. The GC oven program was elongated to remove these by-products safely. Peak shapes as well as retention times of vitamin D_2/D_3 were not affected in subsequent injections. Nevertheless, their occurrence indicates the transfer of some unwanted material from HPLC to GC.

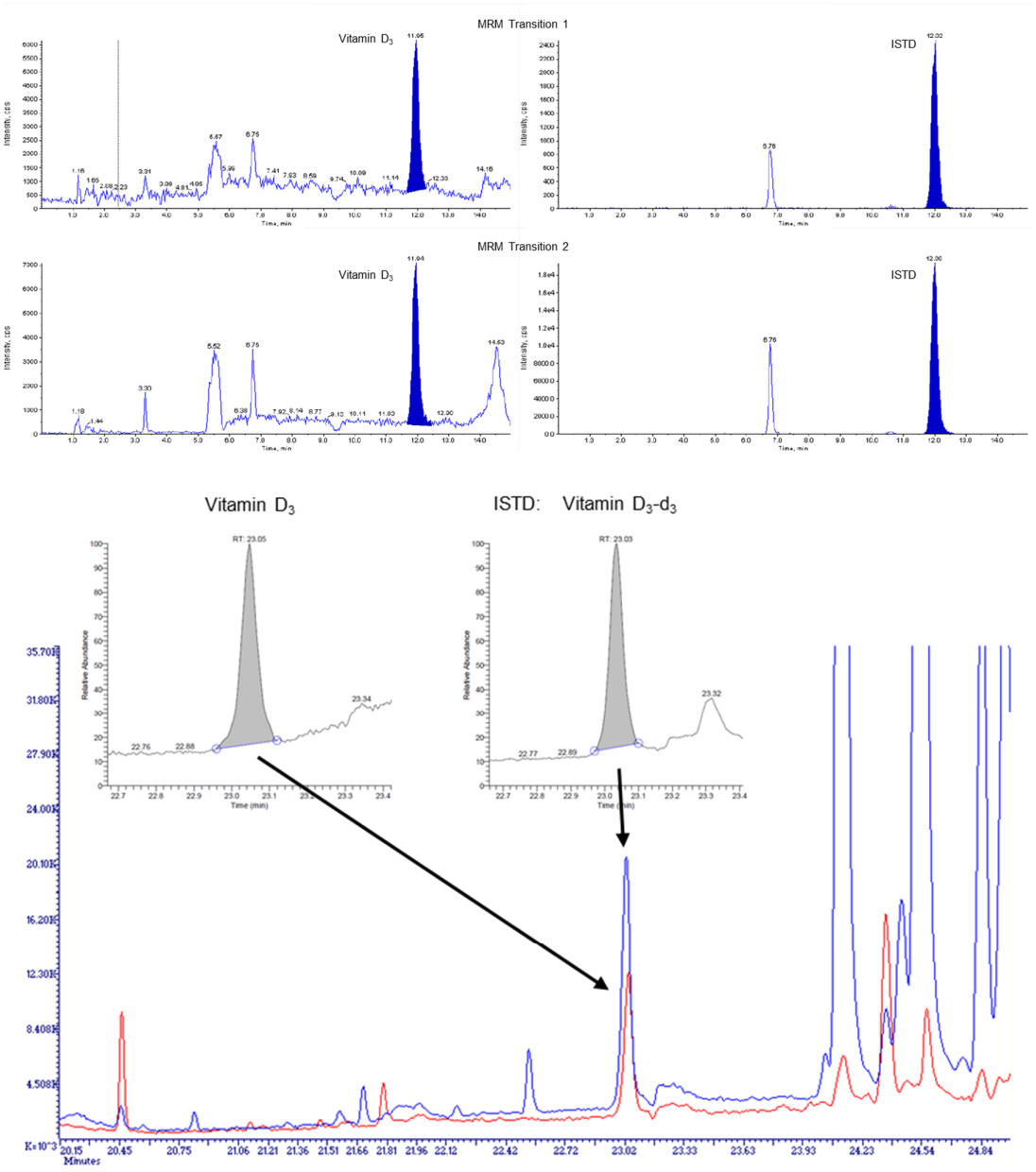

Fig. 5.7. Comparison of chromatographic conditions for HPLC-MS/MS (top: two MRM transitions for vitamin D_3 (left) and ISTD (right)) and HPLC-GC-MS (bottom: extracted ion chromatogram for vitamin D_3 and ISTD) for the sterol-enriched margarine sample. The injected amounts of vitamin D_3 for HPLC-MS/MS and HPLC-GC-MS were 6.9 ng and 0.56 ng, respectively.

5.3.7 HPLC-GC-MS suitability for detection of vitamin D metabolites

The detection of vitamin D metabolites (calcidiols, calcitriols) is an important field of research. The content of these metabolites in foodstuffs is typically even lower (<1 µg/100 g) than the vitamin D content itself [3].

HPLC-GC-MS could be suited for the detection of these metabolites. Because of the additional hydroxyl groups, the metabolites show increased polarity. Adjustments to the HPLC method are necessary [31]. GC-MS detection of vitamin D metabolites is reported in literature [25, 32]. The hyphenation of HPLC and GC-MS could be a valuable option for quantitation of the total vitamin D content in foodstuffs and its evaluation should be performed in the future

5.4 Conclusion

It could be shown that a HPLC-GC-MS based method is suitable for the detection and quantitation of vitamin D derivatives in selected food matrices and dietary supplements. Usage of the pyro isomer allows secure quantitation. The achievable LODs and LOQs are sufficiently low to process most foodstuffs without additional enrichment steps. Further increase of sensitivity can be expected by usage of chemical ionization GC-CI-MS to prohibit extensive fragmentation. Additionally, online sample enrichment via SPE prior to normal-phase HPLC will be considered. This step could be essential for the low vitamin D metabolite contents in foodstuffs.

Sample cleanup was implemented by an optimized analytical normal-phase HPLC dimension. HPLC was directly online coupled to GC-TOF-MS. This way a robust system could be designed. Manual sample preparation steps could be reduced to a minimum for the selected food matrices. Compared to classical HPLC-UV methods, the sample throughput could be significantly increased.

In the near future, online sample preparation will be realized just in time by the autosampler on top of the analytical system. Additional increase of sample throughput can be expected.

Acknowledgements

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6. Universal route to polycyclic aromatic hydrocarbon analysis in foodstuff: Two-dimensional heart-cut liquid chromatography–gas chromatography–mass spectrometry (LC-LC-GC-MS)

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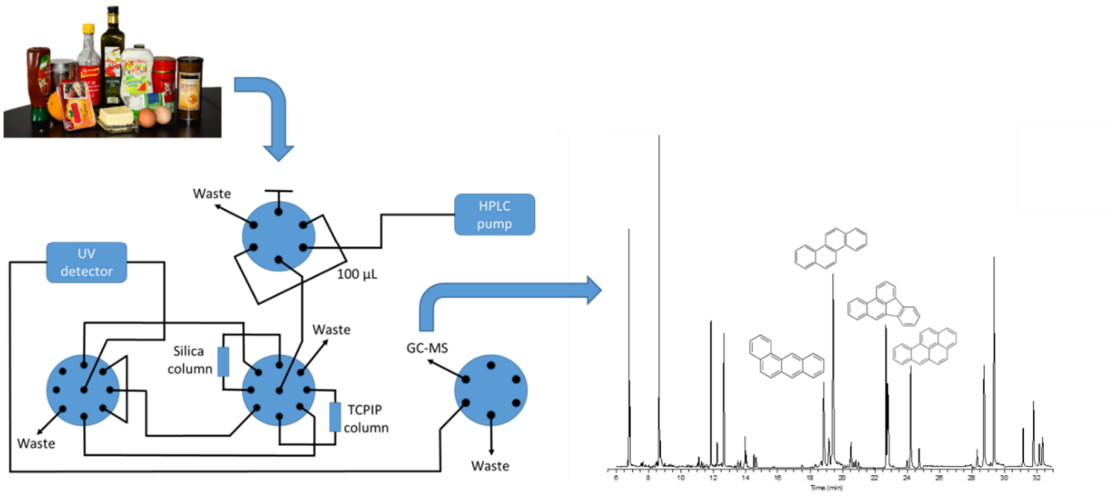

Abstract

Analysis of polycyclic aromatic hydrocarbons (PAHs) in complex foodstuff is associated with complicated and work-intensive sample preparation. Chromatographic interference has to be faced in many situations. The scope of the current work was the development of a highly efficient two-dimensional heart-cut LC-LC-GC-MS method. Detection was performed with a time-of-flight mass spectrometer (TOF-MS) to allow for a comprehensive evaluation of the obtained data in terms of cleanup efficiency. Additionally, routine detection was performed with single quadrupole MS. An easy and quick generic sample preparation protocol was realized as a first step. During method development, focus was given to optimizing HPLC cleanup for complex foodstuff. Silica-, polymeric-, and carbon-based HPLC phases were tested.

Coupling of silica gel to π-electron acceptor modified silica gel showed the best cleanup properties. A four rotary valve configuration allowed the usage of a single binary HPLC pump. Screening of several fatty and nonfatty food matrices showed the absence of unwanted matrix compounds in the cleaned-up PAH fraction down to the low picogram range using TOF-MS. Limits of quantitation (LOQ) were below 0.1 µg/kg for all EU priority PAHs. Recovery rates ranged from 82 to 111%. Validation data fully complied with EU Regulation 836/2011. Sample preparation was possible in 20 min. Interlacing of HPLC and GC allowed an average method runtime of 40 min per sample.

6.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds generally known for their toxicity, carcinogenicity, and mutagenicity. They are composed of two or more fused aromatic ring systems. Alkylated side chains increase the number of possible isomers to several millions [1, 2]. As unwanted contaminants, their exposure to the environment and the human organism should be minimized.

PAHs are the product of incomplete combustion or pyrolysis of organic matter [3]. Furthermore, PAHs are natural constituents of mineral oil fractions, coal, and tar. Urban air pollution or the processing of foodstuffs in terms of drying, roasting, or smoking can be the sources of PAH contaminations. Only few PAHs are purposefully synthesized and used in chemical industry [4].

Besides direct exposure, daily nutrition is a significant source for PAH intake of the human body. Therefore, PAH concentrations in foodstuff have to be regulated and constantly monitored [5]. Because of the variety in number, specific PAHs are chosen as representatives in the regulation process. The most important representative is benzo[a]pyrene (BaP). Its toxicity was thoroughly investigated in the past. After intake, this PAH is metabolized in the human organism and can covalently bind to DNA or proteins, altering their functionalities, which can be the origin of cancer [6].

Thus, the monitoring of foodstuffs is of big importance. In 2008, the EFSA (European Food Safety Authority) confirmed 16 (15+1) European priority PAHs that clearly showed carcinogenic and mutagenic potential. Monitoring of these compounds was recommended, although no upper limits for all compounds were available by law [7]. Three years later, in

2011, EU Regulation 835/2011 came into force, establishing the upper limits of a subset of four specific PAHs (PAH4) in various food matrices [8].

In the case of edible oils and fats, the upper limit for BaP was set to 2 µg/kg, whereas the sum of benz[a]anthracene, chrysene, benzo[b]fluoranthene and BaP was fixed to 10 µg/kg.

6.1.1 Analytics of PAHs in foodstuff

The determination of PAHs in foodstuff is challenging because of the variety in matrices in which they are found. The detection of a few micrograms per kilogram in the presence of bulk matrix compounds like triglycerides, fatty acids, proteins, etc. is demanding. It calls for optimized analytical methods. The performance criteria for the PAH4 determination are summarized in Table 6.1 created from EU Regulation 836/2011 [9].

Table 6.1. Performance criteria for the determination of PAH4 [9]

Parameter	Criterion
Precision[a] (repeatability and reproducibility)	$RSD_r < 29.3$ % and $RSD_R < 44$ %
Recovery	50 – 120 %
LOD	≤ 0.3 µg/kg for each PAH
LOQ	≤ 0.9 µg/kg for each PAH

[a]: Precision derived from Horwitz ratios, and acceptable relative standard deviations according to Thompson [10]

In general, detection of PAHs is carried out either by liquid (HPLC) or by gas chromatographic (GC) methods.

HPLC methods normally rely on optimized C_{18} columns coupled to fluorescence detection (FLD). Specific excitation and emission wavelengths allow detection limits in the low picogram (pg) range on column [11]. Unfortunately, not every PAH exhibits a fluorescence signal. For instance, cyclopenta[c,d]pyrene does not give rise to fluorescence. Because of this, additional UV detection is necessary to cope with all EU priority PAHs. UV detection is less sensitive and less specific than FLD detection. Atmospheric pressure photoionization (APPI) followed by tandem mass spectrometric detection (HPLC-APPI-MS/MS) represents another approach in PAH analytics [12].

Due to their simplicity, HPLC-based methods found their way into official methods [13]. Nevertheless, EFSA and other institutions recommend the use of methods based on gas chromatography–mass spectrometry (GC-MS) because of the increased specificity.

Compared to HPLC methods, capillary GC techniques exhibit higher peak capacities and allow, therefore, separations to be performed that were hardly possible in conventional HPLC environments [14]. MS detection is generally based on electron impact (EI) ionization coupled to single quadrupole mass analyzers operated in selected ion monitoring (SIM) mode. Because PAHs form relatively stable fragments, mainly molecular ions $M^{+\bullet}$, detection limits are in the low picogram range on column, which is comparable to that with HPLC-FLD methods. Quantitation is based on isotope dilution approaches using deuterium- or ^{13}C-labeled internal standards.

It is worth mentioning that special GC columns are needed to enable all necessary separations for the EU priority PAHs. These columns are typically based on 35 or 50 % diphenyl-dimethyl polysiloxane thin film stationary phases [15].

6.1.2 Extraction of PAHs from foodstuff

Apart from the chromatographic challenges, the isolation process of PAHs from foodstuff is of significant importance. Extraction of PAHs from the bulk of food matrix calls for specific cleanup protocols.

Depending on the food matrix, differing extraction procedures are found in literature [16]. Nonfatty food allows the use of simple solid-phase extraction (SPE) or multi-residue techniques like QuEChERS. However, isolation of PAHs from fatty food is more challenging. Co-extraction of triglycerides, emulsifiers, fat-soluble vitamins, and other matrix compounds requires more sophisticated cleanup strategies [17].

Many kinds of sample extraction and cleanup techniques can be found for PAH analytics. Liquid-liquid extraction, complexation, SPE, solid-phase microextraction (SPME), and microwave-assisted extraction are only a small number of the described methods. Alkaline saponification has been used in several cases. Chromatographic cleanup steps involve the usage of column chromatography, donor-acceptor chromatography, size-exclusion chromatography, preparative HPLC, and many more.

The bottom line is that most published methods rely on particular and specialized sample preparation methods. Very little literature is available that deals with universal protocols capable of analyzing a variety of food matrices without adaptions.

All these factors and variability increase the complexity during sample preparation. Obligatory solvent evaporation steps between cleanup stages need additional time and manual work. Because of this, extraction and detection of PAHs in foodstuff is time-consuming and error-prone.

The aim of the current work was, therefore, to find a solution for this obstacle. Hyphenation of multiple chromatographic techniques was explored to generalize and simplify PAH analytics. This decision was based on literature that deals with complex samples, e.g., edible oils, chocolate, or meat [18–20]. In these cases, multiple cleanup techniques were necessary to obtain sufficiently clean extracts, which could be injected either in HPLC-FLD or GC-MS systems.

For instance, in the official ISO method EN ISO 22959:2009, for the detection of PAHs in animal and vegetable oils and fats, an online LC-LC-FLD approach is described. It is based on a tetrachlorophthalimidopropyl (TCPIP) modified silica gel column followed by C_{18} reversed-phase HPLC [18]. Sufficient cleanup results are obtained for a high number of edible oils. Nevertheless, for foodstuffs such as olive pomace oils or algae chromatographic interferences were reported [12, 21]. Further cleanup steps or tandem MS detection were necessary.

As another approach, hyphenation of HPLC and GC was discussed in the literature.

6.1.3 LC-GC hyphenation

Coupling of HPLC to GC has been reported in the literature for at least 30 years, especially for the determination of mineral oil originating compounds in food and paper stuff [22]. Nevertheless, other fields of application, like sterol or alkyl ester quantitation, have also been explored [23–27]. The hyphenation of both separation techniques allows the orthogonal coupling of well-established sample cleanup processes (HPLC) with highly efficient separation and detection techniques (GC). Online coupling offers additional advantages, such as reducing manual sample preparation, decreasing cross-contamination, and increasing sample throughput.

Bare silica HPLC phases are the most common for the extraction and purification of nonpolar analytes from difficult food matrices [28–30]. The main reason for this choice is the ability of silica to retain high amounts of triglycerides [31]. Analytes less polar than triglycerides can be

safely separated from the bulk of matrix by using nonpolar mobile phases. A 10 cm x 2 mm silica column can retain up to 25 mg of triglycerides when *n*-hexane is used as the mobile phase. Silica flooded by triglycerides is not able to retain any other compound. Thus, flooding of the entire column must be prevented. Twenty milligrams of triglycerides flood approximately 50 % of a 25 cm x 2 mm silica column when *n*-hexane/dichloromethane (70:30, v/v) is used as the mobile phase [31]. The residual 50 % of the stationary phase is used for the chromatographic separation of analytes and other matrix compounds.

6.1.4 Detection of PAHs by LC-GC-MS

Detection of PAHs in vegetable oils was realized by an LC-GC-MS method based on a single silica cleanup [32]. Own studies revealed that vegetable oils with high squalene contents, e.g., olive oils, showed significant chromatographic interference. Biedermann et al. investigated the co-elution of squalene and aromatic hydrocarbons on silica phases [33]. They analyzed mineral oil aromatic hydrocarbons (MOAH) in foodstuffs with an LC-GC-FID system. Since MOAHs are alkylated PAHs, the elution windows of both analyte groups are virtually the same on silica phases. The authors discovered that not only did squalene elute inside the MOAH fraction but also other polyunsaturates (e.g., carotenes, steradienes, isomerized squalenes, sesquiterpenes, etc.). The last consequence of this it that silica-based LC-GC methods cannot be used for the detection of PAHs in samples containing high amounts of polyunsaturates without adaptions.

Two-dimensional heart-cut LC-LC-GC-MS was chosen to conquer this obstacle. The addition of a second HPLC dimension after a silica cleanup allowed for the selective removal of polyunsaturates. Extensive band broadening caused by the large transfer volume between the dimensions had to be prohibited. Therefore, a variety of HPLC stationary phases was probed for retention of PAHs.

During method development, GC-MS detection was performed with a time-of-flight mass spectrometer (TOF-MS). This allowed for a comprehensive evaluation of the obtained data as opposed to that with selected ion monitoring, which is normally performed with quadrupole MS. Contrary to quadrupole MS, TOF-MS allowed higher sensitivities to be achieved while maintaining complete mass spectral information. Finally, the method was combined with a minimized generic sample preparation protocol to cope with as many matrix types as possible.

6.2 Experimental Section

6.2.1 Samples

Food samples were obtained at local supermarkets. They consisted of extra virgin olive oil, refined sunflower oil, chicken eggs, tomato ketchup, mayonnaise, filter and instant coffee, drinking chocolate, chamomile tea, cream cheese, butter, and phytosteryl ester-enriched margarine. A FAPAS (Food Analysis Performance Assessment Scheme) quality control olive oil (T0656 QC) was obtained from FERA (The Food and Environment Research Agency, Sand Hutton, United Kingdom). This QC material was part of a proficiency trial and therefore statistical evaluation data were available [34].

6.2.2 Chemicals and solutions

Dichloromethane, ethanol, and *n*-hexane were from LGC Promochem (Picograde quality, Wesel, Germany). Naphthalene, naphthalene-d_8, acenaphthylene, acenaphthylene-d_8, acenaphthene, acenaphthene-d_{10}, fluorene, fluorene-d_{10}, phenanthrene, phenanthrene-d_{10}, anthracene, anthracene-d_{10}, fluoranthene, fluoranthene-d_{10}, pyrene, pyrene-d_{10}, 7H-benzo[c]fluorene, benz[a]anthracene, benz[a]anthracene-d_{12}, cyclopenta[c,d]pyrene, chrysene, chrysene-d_{12}, 5-methylchrysene, benzo[b]fluoranthene, benzo[b]fluoranthene-d_{12}, benzo[k]fluoranthene, benzo[k]fluoranthene-d_{12}, benzo[j]fluoranthene, benzo[a]pyrene, benzo[a]pyrene-d_{12}, indeno[1,2,3-cd]pyrene, indeno[1,2,3-cd]pyrene-d_{12}, dibenz[a,h]anthracene, dibenz[a,h]anthracene-d_{14}, benzo[g,h,i]perylene, benzo[g,h,i]perylene-d_{12}, dibenzo[a,l]pyrene, dibenzo[a,e]pyrene, dibenzo[a,i]pyrene, and dibenzo[a,h]pyrene in cyclohexane were purchased from Neochema (Bodenheim, Germany). Citric acid (99 %) and potassium hydroxide (≥85 %, pellets, white) were from Sigma Aldrich (Steinheim, Germany). Sodium sulfate was from Fluka (Buchs, Switzerland). Water was supplied from a Milli-Q water purification system (Merck, Darmstadt, Germany).

6.2.3 Sample preparation

The single steps of the sample preparation and the subsequent LC-LC-GC-MS analysis are summarized in Fig. 6.1.

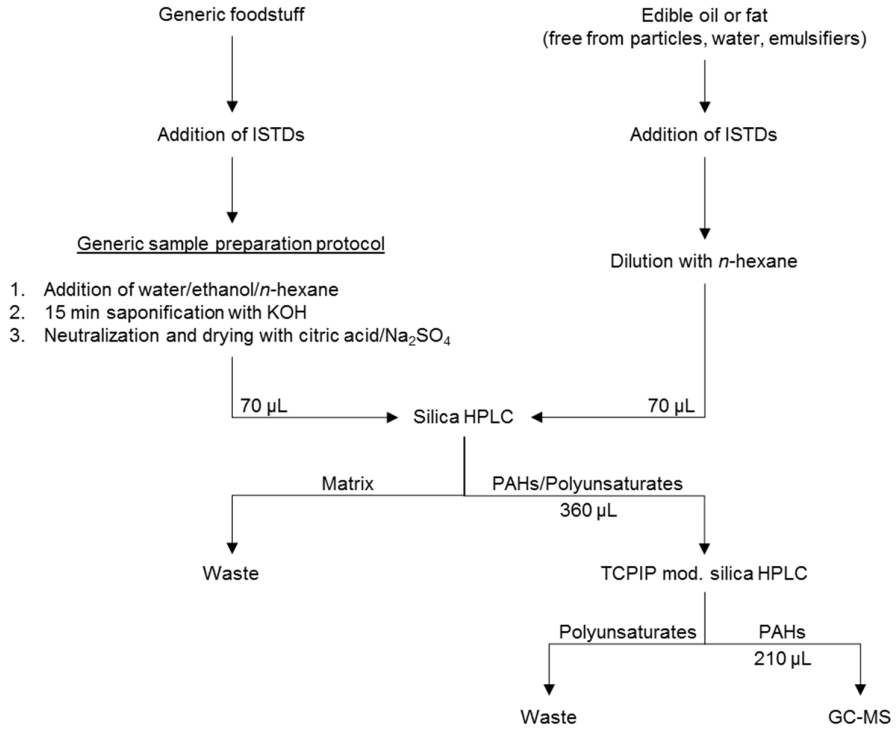

Fig. 6.1. Flowchart of a foodstuff sample subjected to LC-LC-GC-MS analysis (TCPIP: tetrachlorophthalimidopropyl).

6.2.4 Generic protocol

Three grams of the homogenized sample were weighed into a 40-mL EPA vial with a screw cap. After addition of 100 µL of the internal standard solution (ISTD, 100 pg/µL resembling 3.35 µg/kg), 15 mL of ethanol/water (1:1, v/v) and 10 mL of *n*-hexane were added. Depending on the water content of the sample, the ethanol amount was increased to maintain an ethanol-water ratio of 1:1. Less ethanol could result in problems during phase separation in the further extraction step. The sample was thoroughly shaken for two minutes. After centrifugation (5 – 10 min, 2000 g), the lower phase was discarded and replaced by 5 mL of fresh ethanol/water. If phase separation did not occur, then the aqueous layer was kept. Two-hundred fifty microliters of an aqueous KOH solution (1:1, w/w) were added. After shaking for 1 min, the solution was allowed to react for 15 min in an ultrasonic bath. If the sample contained emulsifiers, then phase separation occurred during this time. Five milliliters of the clear upper organic layer were transferred into a 20-mL headspace vial. Afterward, 1 g of a powder mixture of solid citric acid and sodium sulfate (1:2, w/w) was added to neutralize and dry the *n*-hexanic solution in a single step. After shaking for 1 min and centrifugation (1 min, 2000 g), 1 mL of the dried organic phase was transferred into a 2-mL autosampler vial.

6.2.5 Edible oils and fats

Edible oils and fats free from significant amounts of particles, water, and emulsifiers were directly processed without application of the generic protocol. In a 2-mL autosampler vial, 300 mg of the sample were mixed with 690 µL of *n*-hexane. Ten microliters of the ISTD solution (100 pg/µL) were added, resembling a PAH weight concentration of 3.35 µg/kg. Before injection, it was visually inspected and found to be clear and particle-free. In uncertain situations, a small amount of water was added. If the aqueous layer was not clearly observable and separated from the *n*-hexanic layer, then the generic protocol was applied.

6.2.6 LC-LC-GC-MS method

LC-LC-GC-MS experiments were performed on a system from Axel Semrau (Sprockhövel, Germany). It consisted of a 1260 Infinity HPLC system (binary pump and variable wavelength detector by Agilent Technologies, Waldbronn, Germany), Master GC and Master TOF-MS (DANI Instruments S.p.A., Cologno Monzese, Italy), and a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The Master TOF-MS time-of-flight detector was used for method development. Routine measurements and validation data were obtained from a DSQ II single quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Austin, TX, USA).

Four rotatory switching valves (VICI AG International, Schenkon, Switzerland) were used to guide the HPLC eluent from the HPLC into the GC. It allowed for the usage of only one binary pump in combination with an HPLC heart-cut method using two HPLC columns. The valve scheme is described in detail in the Results and Discussion.

The GC was equipped with an on-column interface and a solvent vapor exit. The on-column interface, the carrier gas, and solvent vapor exit were controlled by CHRONECT LC-GC from Axel Semrau.

Of the sample extracts, 70 µL (20 mg) were injected onto an Allure Si HPLC column (250 mm x 2.1 mm, 5 µm, 60 Å, Restek, Bellefonte, PA, USA) without additional column temperature control. The second dimension HPLC column was a Chromspher Pi (80 mm x 3.0 mm, 5 µm, 120 Å, Agilent Technologies).

The mobile phase consisted of *n*-hexane and dichloromethane. Under standby conditions, both columns were connected in series and supplied with 100 % *n*-hexane at 50 µL/min. Shortly before injection, the second column was decoupled. Starting at 100 % *n*-hexane with 300

µL/min, the mobile phase was changed to 65 % *n*-hexane in 0.5 min after injection. It was held until 4.3 min. The PAH fraction eluted from the silica column between 4.3 and 5.5 min (1.2 min). Both columns were connected in series during this time. Shortly afterward, the silica column was decoupled, and the mobile phase (100 % *n*-hexane at 500 µL/min) was delivered solely to the second column. It was maintained until 11.0 min. The mobile phase was switched to 100 % dichloromethane (300 µL/min), and the flow direction was reversed. After elution of the PAH fraction between 13.4 and 14.1 min (0.7 min), both columns were backflushed with 100 % dichloromethane at 500 µL/min for 15 min. Afterward, the columns were reconditioned with *n*-hexane at 500 µL/min for an additional 10 min in the forward direction.

HPLC-GC transfer occurred by the retention gap technique and fully concurrent solvent evaporation (FCSE) through the Y-interface [35]. A coated precolumn (Rxi-1, 0.5 m x 0.53 mm x 0.1 µm, Restek) was followed by a steel T-piece union connecting to the solvent vapor exit and a separation column coated with a modified 50 % diphenyl-dimethyl polysiloxane film (Select PAH, 15 m x 0.15 mm x 0.10 µm, Agilent Technologies).

The PAH fraction was transferred to the GC at a carrier gas inlet pressure of 80 kPa (helium) in addition to an oven temperature of 50 °C. The elution window was verified by UV detection at 230 nm. The solvent vapor exit was opened 0.5 min before the elution of the PAH fraction began. Fully concurrent evaporation of the solvent was chosen although loss of lighter PAHs through the solvent vapor exit was observed. Under these conditions, anthracene and phenanthrene were still partially lost (recoveries of approximately 70 %). Higher recoveries would have been possible, but since the focus of this work was laid on EU priority PAHs, there was no need to optimize the evaporation conditions. The solvent vapor exit was closed 0.1 min after the fraction was transferred. At this time, the carrier gas flow rate was set to 1.2 mL/min. The oven temperature was programmed at 50 °C/min from 50 °C (2.6 min) to 180 °C, at 7 °C/min to 230 °C (7 min), at 50 °C/min to 280 °C (7 min), and finally at 30 °C/min to 350 °C (3.33 min, total time 33.00 min). The Master TOF-MS ion source and transfer line temperatures were set to 200 °C and 350 °C, respectively. Data acquisition started after 6.0 min at a rate of 5 spectra/s with EI ionization at 70 eV. In contrast, the DSQ II ion source temperature was set to 230 °C. Data acquisition occurred in SIM mode. Data processing was performed with Xcalibur 2.2 (Thermo Fisher Scientific Inc.).

Quantitation was based on deuterated ISTDs. Calibration was done by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the

standards. Routine calibration curves were created from 0.1 to 5 µg/kg in six levels (2 – 100 pg on column). Each level was measured once.

6.3 Results and Discussion

6.3.1 Necessity for a two-dimensional HPLC cleanup

The retention of triglycerides on silica highly depends on the mobile phase used. Chlorinated hydrocarbons and ethers are virtually the only modifiers possible when fatty foodstuff is to be analyzed [31]. Studies with dichloromethane, chloroform, and MTBE showed that they had no influence on the separation efficiencies between polyunsaturates and PAHs. Hence, heart-cut coupling of silica to a second stationary phase was chosen. On the one hand, the superior cleanup properties of silica gel could be maintained; on the other hand, a second HPLC dimension could be used for the separation of PAHs and polyunsaturates.

Apart from silica gel, further materials for the isolation of PAHs from fatty foodstuff can be found. One type of described materials is based on polymeric polystyrene divinylbenzene (PS-DVB) [36, 37]. It is used in SPE cleanup protocols. Edible oils diluted in *n*-hexane or isooctane can be loaded onto these SPE columns. Because of hydrophobic and electrostatic quadrupole-quadrupole interactions, PAHs are retained, whereas the matrix can be removed with solvents like methanol or ethers. Afterward, PAHs are eluted with an appropriate solvent, e.g., toluene, tetrahydrofuran (THF), or dichloromethane. Similar retention mechanisms can be attributed to porous graphitic carbon (PGC) phases when used under normal-phase conditions [38].

Other types of phases are based on modified silica gel carrying ligands exposing π-electron acceptor moieties. π-electron rich molecules like PAHs can form donor-acceptor complexes with these ligands. As a consequence, they are highly retained. Elution is performed with appropriate eluents, e.g., dichloromethane or acetonitrile, releasing the PAHs from the column surface [39]. For instance, caffeine or TCPIP modified silica gel is showing this behavior [40, 41].

The usage of a single PS-DVB, PGC, or TCPIP column was not considered during the development of this LC-GC-MS method. Regarding PS-DVB, it was found that a single SPE cleanup did not remove all kinds of residual matrix compounds. For instance, extracts of vegetable oils showed significant amounts of fatty acid esters in TOF-MS total ion current (TIC)

chromatograms. Although detection of PAHs was not disturbed, a single PS-DVB cleanup was not considered for further method development.

Regarding TCPIP, the available columns are normally used with isopropanol as the mobile phase. With its high elution strength on silica, isopropanol deactivates the backbone of the column. In contrast, isopropanol shows only little elution strength on TCPIP. Therefore, analytes are not retained by the silica but only by the TCPIP ligands. LC-GC-TOF-MS experiments revealed that isopropanol continuously stripped high amounts (nanogram range) of tetrachlorophthalic anhydride and other ligand-related compounds from the column. This effect is normally of no concern when HPLC-FLD is used because of the specific excitation and emission wavelengths of PAHs. However, faster column aging definitely has to be considered. Phase stripping was not observed when *n*-hexane was used as the mobile phase. Nevertheless, elution of undesired polar compounds was no longer possible with such a nonpolar solvent.

Besides other negative side effects, which will be discussed later, PGC phases also showed retention of polar compounds when used under normal-phase conditions with nonpolar solvents.

Overall, coupling of a silica column with a PS-DVB, PGC, or TCPIP column seemed to be the most promising approach.

6.3.2 Screening of HPLC phase materials for the second dimension

The task for the second HPLC column would be the removal of remaining polyunsaturates from the fraction of the first silica column. For this, band broadening caused by the large fraction volume (>300 µL with 35 % dichloromethane) had to be prohibited. Refocusing of the PAHs at the head of the second column was necessary. Since all three types of materials seemed reasonably well-suited for this task, the use of all three was attempted.

TCPIP modified silica gel and PGC columns were commercially available. On the contrary, only a few PS-DVB columns were found. Instead, SPE bulk and cartridge material was slurry-packed in HPLC columns. As a testing procedure, HPLC-UV probing was chosen. Squalene and naphthalene were selected as probing molecules. Since naphthalene is the smallest possible PAH, it was expected that it would show the most critical separation to squalene. Both substances were diluted to a concentration of 1 µg/µL each in *n*-hexane. *n*-Hexane was also used as the mobile phase (300 µL/min). Ten microliters of both standards were consecutively

injected onto each probed column. UV detection wavelength was set to 205 nm. Capacity and selectivity factors (k and α) were calculated to quantify the separation of squalene and naphthalene. Although experimental details differ from those of the final heart-cut system, this test revealed interesting facts. The results are summarized in Table 6.2.

Table 6.2. Stationary phases probed for naphthalene (Na) retention and squalene (Sq) separation

Manufacturer	Phase[a]	Column dimension [mm]	k(Sq)	k(Na)	α(Na/Sq)
Agilent	OPT	50 x 2.1	1.00	4.14	4.14
Agilent	Plexa	50 x 2.1	1.36	4.04	2.97
Agilent	PPL	50 x 2.1	1.14	3.07	2.70
Agilent	Chromspher Pi[b]	80 x 3.0	0.48	9.09	18.75
Benson	BP-OA-Ag	125 x 4.1	0.89	1.54	1.72
Biotage	Evolute ABN	50 x 2.1	1.08	3.12	2.89
Biotage	Isolute ENV+	50 x 2.1	2.10	7.52	3.57
Hamilton	PRP-1	150 x 2.1	0.13	1.03	7.81
Hamilton	PRP-X200	150 x 2.1	0.18	1.38	7.59
Jordi Labs	Fluorinated DVB	50 x 4.6	0.71	3.08	4.34
Macherey Nagel	Chromabond Easy	50 x 2.1	1.00	5.85	5.85
Macherey Nagel	Chromabond HR-X	50 x 2.1	1.33	3.37	2.53
Merck	LiChrolut EN	50 x 2.1	0.97	6.09	6.29
Phenomenex	SDB-L	50 x 2.1	1.19	2.63	2.22
Phenomenex	Strata-X	50 x 2.1	1.35	4.32	3.19
Supelco	ENVI-Chrom P	50 x 2.1	1.68	3.88	2.31
Supelco	SupelMIP PAH	50 x 2.1	1.50	5.35	3.57
Supelco	Supel-Select HLB	50 x 2.1	1.48	4.72	3.19
Thermo	HyperCarb[c]	150 x 2.1	1.43	3.04	2.13
Waters	Oasis HLB	50 x 2.1	0.97	3.91	4.03

[a]: Materials based on PS-DVB unless stated otherwise
[b]: TCPIP modified silica gel
[c]: PGC type stationary phase
A more detailed version of this Table including particle sizes, pore sizes, and available phase compositions can be found in the Supporting Information.

TCPIP modified silica gel showed the highest retention ($k \approx 9$) of naphthalene paired with the best separation to squalene ($\alpha \approx 19$). Therefore, TCPIP modified silica gel (Agilent Chromspher Pi) was chosen for the second dimension.

Despite having differing particle sizes, pore sizes, and polymer compositions, most PS-DVB phases showed comparable retention of naphthalene. In single cases, peak shapes of squalene and naphthalene showed strong tailing, influencing their separation. Capacity factors were calculated at the peak apexes, so selectivity factors do not reflect peak asymmetries. In the Supporting Information, Table 6.2 is extended by including peak widths, asymmetries, and chromatographic resolutions.

It is noteworthy that the tested PS-DVB phases were also suited for the retention of PAHs under LC-LC-GC-MS conditions. Nevertheless, smaller PAHs, in particular cases, up to fluoranthene and pyrene, were not quantitatively retained. TCPIP exhibited higher retention for these PAHs. PGC showed an undesired side effect not found on other materials. Heavy PAHs (6 ring dibenzopyrenes) could not be quantitatively eluted even with the strongest solvents (dichloromethane or toluene). Column heating to temperatures exceeding 75 °C was necessary to allow elution to take place. Furthermore, unpreventable carryover effects rendered the usage of PGC impossible.

6.3.3 Two-dimensional heart-cut HPLC method

Heart-cut HPLC techniques require dedicated valve switching solutions. A special one that allows for the usage of only one binary HPLC pump was developed during the current work. Both columns could be supplied with the mobile phase, stand alone or in series, in either direction. If a column was not used, then it could be placed under stopped-flow conditions. The valve scheme and the sequence of method's actions are given in Fig. 6.2 and Table 6.3, respectively.

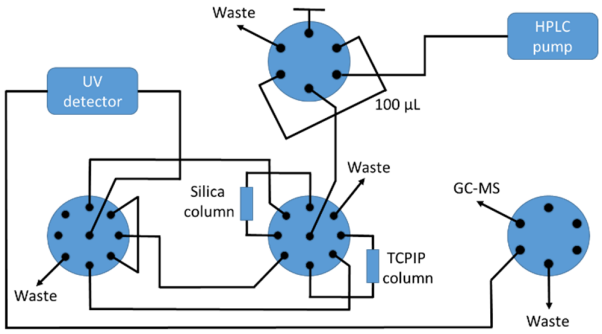

Fig. 6.2. Valve scheme for an LC-LC-GC-MS configuration with a single binary HPLC pump. A detailed version of this scheme can be found in the Supporting Information.

Table 6.3. Sequence of method's actions during an LC-LC-GC-MS run

Time [min]	Action
-0.20	Silica and TCPIP column connected in series during standby
-0.10	TCPIP column switched to stopped-flow condition
0.00	Injection on silica column
4.30 – 5.50	Heart-cut from silica to TCPIP column
5.60	Silica column switched to stopped-flow condition
11.00	Begin of backflush of TCPIP column
13.40 – 14.10	Elution of PAH fraction from TCPIP column into GC-MS
14.20 – 20.00	Backflush of TCPIP column for matrix removal
20.10 – 30.00	Backflush of silica column for matrix removal
30.10 – 40.00	Re-equilibration of silica and TCPIP column in series in forward direction

The silica column was used with *n*-hexane/dichloromethane to maximize its retention for triglycerides. As already examined, this type of column was able to purify a sample and leave behind a fraction containing only PAHs and polyunsaturates. A 25 cm x 2.1 mm column was chosen, which was able to retain 20 mg of triglycerides. Elution of the PAH/polyunsaturated fraction was possible in approximately 360 µL. Removal of polyunsaturates from the TCPIP column was achieved with *n*-hexane in forward direction, whereas elution of PAHs occurred with dichloromethane in backward direction.

Backflushing of both columns was important. On the one hand, it was needed to remove the matrix left behind on the silica column; on the other hand, backflushing the TCPIP column was

necessary to elute the PAH fraction in a small volume into the GC-MS system. Elution in forward direction with dichloromethane resulted in a broad fraction of more than 1 mL. Backflush elution, however, was possible in approximately 210 µL. Compared to the silica gel fraction (360 µL), backflush elution of the TCPIP column allowed a band compression of almost 42 %. This clearly showed the ability of the TCPIP column to retain PAHs from a rather large fraction volume from the first column dimension.

Transfer of the fraction into GC-MS occurred by the retention gap technique and fully concurrent solvent evaporation through the Y-interface [35]. It was observed that GC peaks of all PAHs began to tail after approximately 20 injections of standards. After change of the precolumn, peak shapes recovered. The reason for this observation was found in the usage of dichloromethane as the eluting solvent.

Over time, hydrochloric acid was probably formed in the HPLC solvent bottle. Despite being present in small amounts, every LC-GC transfer transported several nanograms into the precolumn, deteriorating the column's surface. Removal of hydrochloric acid is possible by addition of aluminum oxide to the solvent followed by filtration or re-distillation. Since these solutions are rather work- and time-intensive, another solution was sought. To conquer this problem, a polysiloxane-coated precolumn (0.1 µm film thickness) was used to shield the surface from hydrochloric acid. In fact, peak shapes remained virtually unaffected for more than 60 injections.

6.3.4 Polyunsaturates removal capacity

Twenty milligrams of extra virgin olive oil were injected into the LC-LC-GC-TOF-MS system. Chromatograms still showed a squalene peak. By comparison with a standard of known quantity, the peak corresponded to approximately 50 ng on column. To calculate the squalene removal capacity of the system, the same sample was cleaned up only by the first silica HPLC dimension. For this purpose, the sample was diluted by factor 6700:1. The silica fraction contained the whole squalene content of the sample. In this way, a squalene peak was obtained whose peak area could be multiplied by the dilution factor and could be compared to the LC-LC peak area. LC-LC generated a peak approximately 8000 times smaller than a single silica cleanup. Further removal of squalene was possible by increasing the amount of *n*-hexane used for flushing of the TCPIP column. An increase in the method's runtime and a slight loss of

smaller PAHs were the consequences of this approach. However, the residual squalene did not disturb the detection of PAHs in any way.

6.3.5 Generic cleanup capability for multiple food matrices

Alkyl, steryl, and wax esters of fatty acids are the first eluting compounds after the end of the PAH fraction [33]. Full spectra acquisition with adequate sensitivity is an inherent property of TOF-MS. Because of this, unwanted compounds could be easily traced in the PAH fraction. Special care was taken to examine all TOF-MS TIC chromatograms for these compounds. In fact, traces of water and other polar residues in high amounts were able to shift the PAH fraction to lower retention times on the silica column. This way, esters of fatty acids could be transferred to the TCPIP column. *n*-Hexane was not able to elute these compounds from the silica backbone of the TCPIP column. Thus, these compounds could be transferred into the GC-MS system during dichloromethane elution.

Only samples containing high amounts of emulsifiers, e.g., margarine or mayonnaise, showed traces (low picogram range) of C_{16} or C_{18} fatty acid ethyl esters. These compounds were partially formed from ethanol and fatty acids during the saponification step of the generic sample preparation. However, PAH detection and quantitation was undisturbed in any case. The sample preparation protocol was adapted as follows to minimize these undesired compounds.

Destruction of emulsifiers during the extraction process was realized by a short and mild saponification with an aqueous solution of KOH [42]. A high percentage of emulsifiers is composed of phospholipids (lecithins). Saponification allows for a rapid cleavage of the phosphoric acid ester bonds [43]. Afterward, removal of traces of water from the obtained *n*-hexanic phase was performed by dispersing a powder mixture of solid citric acid and sodium sulfate into it. Citric acid had two functions. On the one hand, it neutralized residues of KOH; on the other hand, it was able to chelate present metal ions, mainly Ca^{2+} and Mg^{2+} [43]. For example, emulsifiers based on Ca/Mg-phosphatidates are highly soluble in nonpolar solvents. Emulsions can evolve, and water residues can be trapped in the *n*-hexanic phase. By addition of a chelating agent, the solubility of phosphatidates is changed, and a removal from the *n*-hexanic phase becomes feasible. Thus, microemulsions can be destroyed, and the trapped water can be adsorbed by sodium sulfate. The best results in terms of residual fatty acid ethyl esters were obtained with a dispersion time of 2 min and consecutive centrifugation.

On the basis of this optimized sample preparation, several food matrices were screened for unwanted compounds inside the PAH fraction. In Fig. 6.3, the overlay of the corresponding TOF-MS TIC chromatograms is shown. As can be seen, few unwanted compounds are present in the chromatograms. This clearly demonstrates the cleanup potential of the designed system.

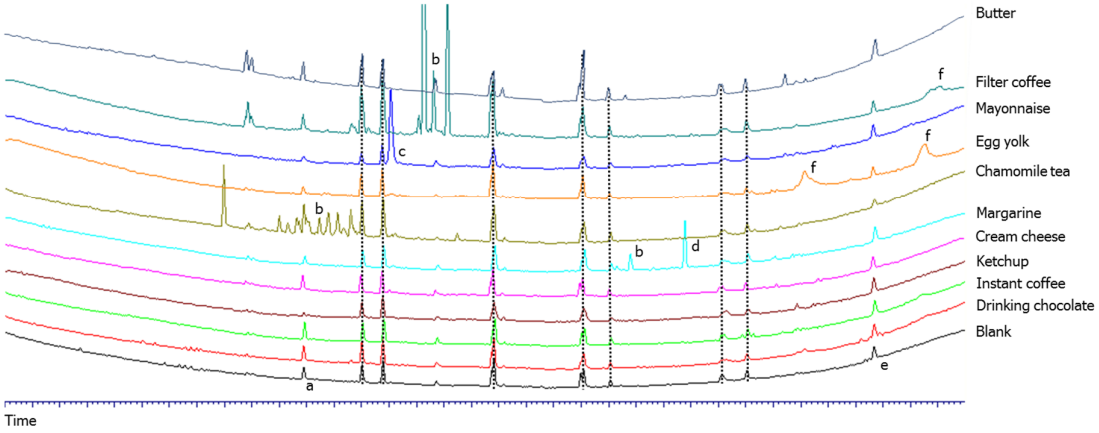

Fig. 6.3. Overlay of LC-LC-GC-TOF-MS TIC chromatograms (50 – 500 amu) of screened food matrix types (dotted lines represent PAH ISTDs; a: diphenyl sulfone from HPLC PEEK capillaries; b: nonregulated alkylated aromatic compounds; c: C_{18} fatty acid ethyl ester; d: stigmasta-3,5-diene; e: solvent impurity; f: polysiloxanes from vial septa). The baseline reflects dichloromethane solvent tailing at low retention times and column bleeding at higher GC oven temperatures.

6.3.6 Validation of the LC-LC-GC-MS method

The experimental setup was tested in terms of sensitivity, linearity, robustness, trueness, and precision. All measurements were performed with a DSQ II single quadrupole mass spectrometer. In SIM mode, it offered superior sensitivity compared to that of TOF-MS.

Ten-point calibration curves ranging equidistantly from 0.05 µg/kg to 0.5 µg/kg (1 – 10 pg) in *n*-hexane were constructed. This range was chosen because it was near the desired LODs and LOQs. The parameters are summarized in Table 6.4.

Table 6.4. Sensitivity and linearity parameters of the designed LC-LC-GC-MS method

Compound	Average response factor (n=10)[a]	LOD [µg/kg][b]	LOQ [µg/kg][c]
7H-Benzo[c]fluorene	0.37 (±1.29 %)	0.02	0.05
Benz[a]anthracene	0.37 (±1.89 %)	0.02	0.06
Cyclopenta[c,d]pyrene	0.37 (±2.02 %)	0.02	0.06
Chrysene	0.35 (±1.31 %)	0.02	0.05
5-Methylchrysene	0.23 (±1.55 %)	0.02	0.05
Benzo[b]fluoranthene	0.26 (±2.26 %)	0.02	0.06
Benzo[k]fluoranthene	0.22 (±3.03 %)	0.02	0.07
Benzo[j]fluoranthene	0.24 (±5.60 %)	0.02	0.07
Benzo[a]pyrene	0.22 (±3.24 %)	0.02	0.06
Indeno[1,2,3-cd]pyrene	0.25 (±3.78 %)	0.02	0.06
Dibenz[a,h]anthracene	0.30 (±2.55 %)	0.02	0.07
Benzo[g,h,i]perylene	0.32 (±4.31 %)	0.02	0.06
Dibenzo[a,l]pyrene	0.19 (±2.37 %)	0.01	0.04
Dibenzo[a,e]pyrene	0.18 (±1.47 %)	0.02	0.05
Dibenzo[a,i]pyrene	0.14 (±2.30 %)	0.02	0.05
Dibenzo[a,h]pyrene	0.07 (±4.44 %)	0.01	0.03

[a]: $Rf = \frac{Area(Analyte)}{Area(ISTD) * standard\ concentration}$

[b]: $3.3 * \frac{Residual\ standard\ deviation}{Slope}$; [c]: $10 * \frac{Residual\ standard\ deviation}{Slope}$

Linearity of the calibration curves for all PAHs could be assumed due to the obtained coefficients of determination ($R^2 > 0.998$) and average response factors with relative standard deviations below 6 %. Extension of the calibration range in routine measurements up to 5 µg/kg showed no indications of linearity issues. LODs and LOQs varied from 0.01 to 0.02 µg/kg and from 0.03 to 0.07 µg/kg, respectively, fully complying with the requirements of EU Regulation 836/2011. In fact, they were tenfold below the requirements and readily compatible with even tighter and future PAH regulations.

6.3.7 Recovery

Olive oils without blank values below the LOQ were not available. Instead, recoveries were determined by spiking a blank (sunflower oil) with PAHs at three different concentration levels (0.1, 0.5, 1.0 µg/kg). Furthermore, a margarine sample was spiked at 0.1 µg/kg. Since this sample was also not free of PAHs (> LOQ), blank values had to be subtracted from the quantified results. In all cases, recoveries for all EU priority PAHs ranged from 82 to 111 %. Due to the good sample cleanup properties verified by TOF-MS and the high recoveries for several matrices, the evaluation of matrix-matched calibrations was not pursued.

6.3.8 Precision and trueness

Precision was determined by multiple injections of extra virgin olive oil. Repeatability was calculated from six consecutive injections of six independently diluted olive oils. Reproducibility is based on the quantitation results of triplicate measurements on three successive days. Additionally, results measured on the Master TOF-MS were included. A FAPAS quality control olive oil was analyzed in triplicate to get an impression about the trueness of the LC-LC-GC-MS method.

Relative standard deviations of repeatability and reproducibility ranged from 1.9 to 8.1 % and 2.6 to 9.3 %, respectively. According to Thompson, the acceptable relative standard deviations below 120 µg/kg under repeatability and reproducibility conditions are fixed to 14.7 and 22 % [10]. All obtained results fully complied with these limits. Furthermore, regarding the QC sample, all PAHs were quantified within the indicated confidence intervals. According to the material data sheet, confidence intervals were calculated from the statistical evaluation of a proficiency trial. Intervals were based on a z-score range of ±2. All important validation data are summarized in Table 6.5.

Table 6.5. Validation data for the determination of EU priority PAHs[a]

Parameter	Criterion	Obtained value
Precision	RSD_r: < 29.3 %	RSD_r: 1.9 – 8.1 %
	RSD_R: < 44 %	RSD_R: 2.6 – 9.3 %
Trueness[b]	|z-score| ≤ 2	-1.60 < z < -0.64
Recovery	50 – 120 %	82 – 111 %
LOD	≤ 0.3 µg/kg for each PAH	0.01 – 0.03 µg/kg
LOQ	≤ 0.9 µg/kg for each PAH	0.03 – 0.07 µg/kg

[a]: Based on PAH4 criteria

[b]: Trueness calculations based on FAPAS QC oil sample [34]

6.3.9 Quantitation of PAHs in extra virgin olive oil

In Fig. 6.4, the SIM traces of all EU priority PAHs are shown. The sample was independently diluted six times and analyzed by LC-LC-GC-MS. The quantified concentrations of PAH4 (benz[a]anthracene, chrysene, benzo[b]fluoranthene and BaP) were 0.32, 0.67, 0.28, and 0.25 µg/kg, respectively. Standard deviations for all four compounds were below 0.02 µg/kg. Maximum concentration limits defined by EU regulation 835/2011 were not exceeded.

All peaks in the chromatogram can be attributed to PAHs. The 16 regulated ones have to be safely separated from the unregulated ones, which was possible by the chosen GC column.

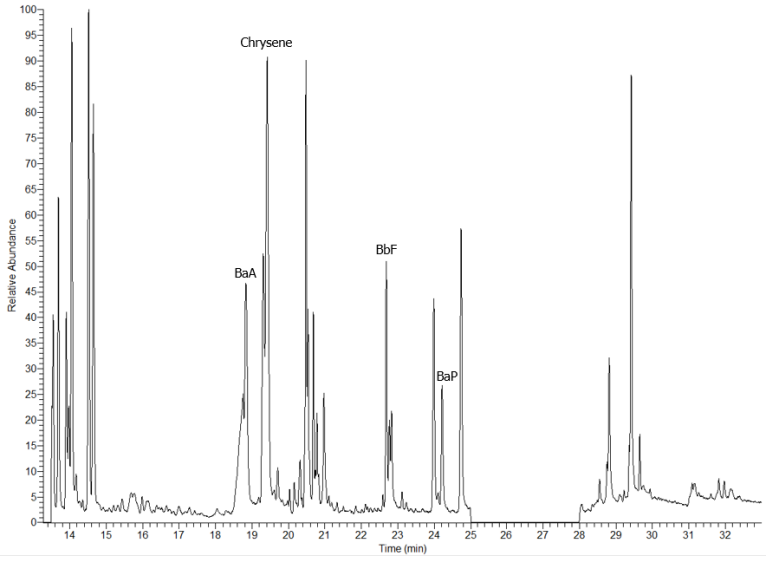

Fig. 6.4. Extracted ion chromatogram of EU priority PAHs of extra virgin olive oil (measured on DSQ II in SIM mode). Concentrations (PAH4) of benz[a]anthracene (BaA), chrysene, benzo[b]fluoranthene (BbF) and BaP are 0.32, 0.67, 0.28, and 0.25 µg/kg, respectively.

6.3.10 Sample throughput considerations

After homogenization, the generic sample preparation was possible in approximately 20 min. The total method runtime including GC cool-down time was 52 min. The HPLC part of the method was finished after 40 min. Interlacing of HPLC and GC parts allowed a subsequent sample to be injected immediately.

6.4 Conclusion

Two-dimensional heart-cut LC-LC-GC-MS for the detection of PAHs in foodstuff was employed to conquer the inadequacy of a silica-based LC-GC-MS method to deal with matrices containing high amounts of polyunsaturates such as olive oil. Coupling of silica and π-electron-acceptor-modified silica was found to be the best combination. Polyunsaturates could be removed to a high degree. Screening of several fatty and nonfatty foodstuffs with TOF-MS detection did not show significant amounts of residual matrix compounds down to the low picogram range. This is the first report of a fully automated LC-GC based heart-cut method able to handle various complex foodstuffs with minimal manual work.

The obtained validation data fully complied with EU Regulation 836/2011. Measurement of a QC sample underlined the trueness of the method. Therefore, the designed method can be used for detection of priority EU PAHs in foodstuff without known limitations. Combined with an easy and quick generic sample preparation, the method's benefits are the very high cleanup potential and sample throughput.

The suitability of the method for lighter PAHs will be subject of further investigations. EPA (Environmental Protection Agency) PAHs include two ring and three ring PAHs and are commonly analyzed in the environmental and consumer product sectors. Biomonitoring of complex organisms could be a second field of application that would profit from the advantages of the presented method.

6.5 References

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6.6 Supporting Information

Table S-6.1. Probed stationary phases for naphthalene and squalene separation including additional material information

Manufacturer	Phase	Column dimension [mm]	Particle size [µm]	Particle shape	Surface area [m²/g]	Pore size [Å]	Stationary phase notes
Agilent	OPT	50 x 2.1	30, 60				Polyamide-DVB
Agilent	Plexa	50 x 2.1	45	spherical	550	100	polar enhanced (Sty-OH)-DVB
Agilent	PPL	50 x 2.1	125	spherical	600	150	Sty-DVB
Agilent	Chromspher Pi	80 x 3.0	5	spherical		120	TCPIP modified silica gel
Benson	BP-OA-Ag	125 x 4.1	9				(Sty-SO_3Ag)-DVB
Biotage	Evolute ABN	50 x 2.1	30, 50			40	(Sty-OH)-DVB
Biotage	Isolute ENV+	50 x 2.1	90		1000–1100	800	(Sty-OH)-(DVB-OH)
Hamilton	PRP-1	150 x 2.1	5	spherical	415	100	Sty-DVB
Hamilton	PRP-X200	150 x 2.1	10	spherical	415	100	(Sty-SO_3H)-DVB
Jordi Labs	Fluorinated DVB	50 x 4.6	5–25	spherical		80	perfluorinated DVB
Macherey Nagel	Chromabond Easy	50 x 2.1	80		675	50	Sty-DVB with weak ion exchanger
Macherey Nagel	Chromabond HR-X	50 x 2.1	85	spherical	1000	55–60	Sty-DVB
Merck	LiChrolut EN	50 x 2.1	40–120		1200		Et-Sty-DVB
Phenomenex	SDB-L	50 x 2.1	100		500	260	Sty-DVB
Phenomenex	Strata-X	50 x 2.1	33		800	85	NVP-Sty-DVB
Supelco	ENVI-Chrom P	50 x 2.1	80–160	spherical	900	110–175	polar enhanced Sty-DVB
Supelco	SupelMIP PAH	50 x 2.1					Sty-DVB with chrysene cavities
Supelco	Supel-Select HLB	50 x 2.1	55–60		400–410	87	hydrophilic modified Sty-DVB
Thermo	HyperCarb	150 x 2.1	5	spherical	120	250	PGC
Waters	Oasis HLB	50 x 2.1	30		830	80	NVP-DVB

DVB: Divinylbenzene
NVP: N-Vinylpyrrolidone
PGC: Porous graphitic carbon
Sty: Styrene
TCPIP: Tetrachlorophthalimidopropyl

Table S-6.2. Peak performance parameters of naphthalene and squalene on the probed stationary phases

Phase	t_r(Sq) [min]	w_{05}(Sq)	A_s(Sq)	t_r(Na) [min]	w_{05}(Na)	A_s(Na)	t_m [min]	k(Sq)	k(Na)	α(Na/Sq)	R_s(Na/Sq)
OPT	0.74	0.43	0.70	1.90	0.86	1.12	0.37	1.00	4.14	4.14	1.06
Plexa	0.59	0.56	1.99	1.26	1.10	1.99	0.25	1.36	4.04	2.97	0.48
PPL	0.62	0.70	2.39	1.18	0.92	2.33	0.29	1.14	3.07	2.70	0.41
Chromspher Pi	1.96	0.52	2.47	13.32	0.92	12.61	1.32	0.48	9.09	18.75	9.31
BP-OA-Ag	3.60	0.97	8.85	4.83	1.10	5.39	1.90	0.89	1.54	1.72	0.70
Evolute ABN	0.54	0.50	1.75	1.07	1.02	2.42	0.26	1.08	3.12	2.89	0.41
Isolute ENV+	0.90	0.90	1.69	2.47	1.60	1.44	0.29	2.10	7.52	3.57	0.74
PRP-1	1.37	0.40	3.21	2.46	0.48	5.23	1.21	0.13	1.03	7.81	1.46
PRP-X200	1.43	0.31	2.40	2.88	0.50	6.24	1.21	0.18	1.38	7.59	2.11
Fluorinated DVB	2.77	0.59	1.08	6.61	0.87	4.89	1.62	0.71	3.08	4.34	3.10
Chromabond Easy	0.52	0.82	8.06	1.78	1.76	2.18	0.26	1.00	5.85	5.85	0.58
Chromabond HR-X	0.63	0.61	1.79	1.18	0.87	1.74	0.27	1.33	3.37	2.53	0.44
LiChrolut EN	0.63	1.10	8.71	2.27	1.07	1.60	0.32	0.97	6.09	6.29	0.89
SDB-L	0.59	0.58	1.90	0.98	0.79	2.28	0.27	1.19	2.63	2.22	0.34
Strata-X	0.73	0.45	0.62	1.65	0.86	0.77	0.31	1.35	4.32	3.19	0.83
ENVI-Chrom P	0.67	0.66	1.52	1.22	0.85	1.72	0.25	1.68	3.88	2.31	0.43
SupelMIP PAH	0.50	0.76	3.93	1.27	1.54	5.08	0.20	1.50	5.35	3.57	0.40
Supel-Select HLB	0.62	0.47	1.19	1.43	1.86	1.12	0.25	1.48	4.72	3.19	0.41
HyperCarb	2.94	0.27	2.80	4.89	0.57	10.03	1.21	1.43	3.04	2.13	2.74
Oasis HLB	0.69	0.38	1.09	1.72	0.77	1.50	0.35	0.97	3.91	4.03	1.06

A_s: Asymmetry factor calculated at 10 % peak height

w_{05}: Peak width at half maximum

k: Capacity factor

α: Selectivity factor

R_s: Resolution

Table S-6.3. Quantified PAH concentrations [µg/kg] for various food matrices shown in Fig. 6.3

Compound	Butter	Ketchup	Mayonnaise	Egg yolk	Chamomile tea	Margarine	Cream cheese	Filter coffee	Instant coffee	Drinking chocolate	Olive oil	Sunflower oil
7H-Benzo[c]fluorene						< LOQ		< TQL			0.11	
Benz[a]anthracene					< TQL	0.13		0.25			0.32	
Cyclopenta[c,d]pyrene						< LOQ		< TQL			0.13	
Chrysene					0.43	0.14		0.35			0.67	
5-Methylchrysene						< LOQ					0.08	
Benzo[b]fluoranthene						0.21					0.28	
Benzo[k]fluoranthene						0.11					0.15	
Benzo[j]fluoranthene	< TQL	< TQL	< TQL	< TQL	< TQL	0.11	< TQL	< TQL	< TQL	< TQL	0.16	< LOQ
Benzo[a]pyrene						0.19					0.25	
Indeno[1,2,3-cd]pyrene						< LOQ					0.10	
Dibenz[a,h]anthracene						0.17					0.15	
Benzo[g,h,i]perylene						0.20					0.32	
Dibenzo[a,l]pyrene						0.05					0.06	
Dibenzo[a,e]pyrene						0.03					0.07	
Dibenzo[a,i]pyrene						< LOQ					0.11	
Dibenzo[a,h]pyrene						< LOQ					0.05	

Each sample was measured in duplicate (n=2).

TOF-LOQ (TQL) was 0.75 µg/kg for Dibenzopyrenes and 0.25 µg/kg for all other PAHs. LOQ measured with quadrupole MS was below 0.1 µg/kg for all PAHs.

Table S-6.4. Data of FAPAS QC extra virgin olive oil in comparison to measured data

Compound	QC mean concentration [µg/kg]	Measured concentration (n=3) [µg/kg]	Calculated z-score
Benz[a]anthracene	1.49 ± 0.33	1.05 ± 0.01	-1.34
Chrysene	3.31 ± 0.73	2.62 ± 0.00	-0.95
Benzo[b]fluoranthene	2.09 ± 0.46	1.68 ± 0.01	-0.90
Benzo[k]fluoranthene	1.49 ± 0.33	1.13 ± 0.01	-1.09
Benzo[j]fluoranthene	1.48 ± 0.33	0.96 ± 0.01	-1.60
Benzo[a]pyrene	2.13 ± 0.47	1.83 ± 0.01	-0.64
Indeno[1,2,3-cd]pyrene	1.39 ± 0.31	1.04 ± 0.01	-1.15
Dibenz[a,h]anthracene	0.84 ± 0.18	0.63 ± 0.02	-1.15
Benzo[g,h,i]perylene	1.87 ± 0.41	1.41 ± 0.02	-1.12
Dibenzo[a,e]pyrene	1.43 ± 0.32	1.07 ± 0.02	-1.14
Dibenzo[a,i]pyrene	0.84 ± 0.19	0.63 ± 0.02	-1.12

Table S-6.5. List of measured PAHs and ISTDs with regulation types, retention times, and analyte ions

Compound	Type	t_r [min]	Quantifier ion [*m/z*]	Qualifier ion [*m/z*]
Naphthalene	EPA	---[a]	128	129
Acenaphthylene	EPA	6.2	152	153
Acenaphthene	EPA	6.3	153	154
Fluorene	EPA	6.9	166	165
Phenanthrene	EPA	8.7	178	179
Anthracene	EPA	8.8	178	179
Fluoranthene	EPA	11.9	202	203
Pyrene	EPA	12.7	202	203
7H-Benzo[c]fluorene	EU	14.0	216	215
Benz[a]anthracene	EPA/EU*	18.9	228	229
Cyclopenta[c,d]pyrene	EU	19.2	226	227
Chrysene	EPA/EU*	19.4	228	229
5-Methylchrysene	EU	20.8	242	241
Benzo[b]fluoranthene	EPA/EU*	22.7	252	253
Benzo[k]fluoranthene	EPA/EU	22.8	252	253
Benzo[j]fluoranthene	EU	22.9	252	253
Benzo[a]pyrene	EPA/EU*	24.2	252	253
Indeno[1,2,3-cd]pyrene	EPA/EU	28.8	276	277
Dibenz[a,h]anthracene	EPA/EU	28.8	278	279
Benzo[g,h,i]perylene	EPA/EU	29.4	276	277
Dibenzo[a,l]pyrene	EU	31.2	302	151
Dibenzo[a,e]pyrene	EU	31.8	302	151
Dibenzo[a,i]pyrene	EU	32.2	302	151
Dibenzo[a,h]pyrene	EU	32.4	302	151
Naphthalene-d_8		---[a]	136	
Acenaphthylene-d_8		6.1	160	
Acenaphthene-d_{10}		6.2	164	
Fluorene-d_{10}		6.8	176	
Phenanthrene-d_{10}		8.6	188	
Anthracene-d_{10}		8.7	188	
Fluoranthene-d_{10}		11.8	212	
Pyrene-d_{10}		12.6	212	
Benz[a]anthracene-d_{12}		18.7	240	
Chrysene-d_{12}		19.2	240	
Benzo[b]fluoranthene-d_{12}		22.6	264	
Benzo[k]fluoranthene-d_{12}		22.7	264	
Benzo[a]pyrene-d_{12}		24.1	264	
Indeno[1,2,3-cd]pyrene-d_{12}		28.7	288	
Dibenz[a,h]anthracene-d_{14}		28.7	292	
Benzo[g,h,i]perylene-d_{12}		29.3	288	

*: PAH4 representatives

[a]: Naphthalene and its ISTD were completely lost in the second HPLC dimension due to low retention.

6.6.1 Interaction of naphthalene and squalene with various stationary phases

In Table S-6.1, all available information of the probed stationary phase materials are summarized. Because of commercial interests and pending patents, only few reliable information were available.

Peak performance parameters for all materials are collected in Table S-6.2. As can be seen from the asymmetry factors A_s, most materials exhibit a significant tailing for naphthalene. This is a typical observation for a strong interaction between an analyte and the stationary phase. On few materials, e.g., LiChrolut EN, even squalene exhibits strong interactions with the column surface. As can be derived from the chromatographic resolution, these materials are therefore not suited for a baseline separation of both analytes ($R_s < 1.5$).

All experiments were performed with 10 µL injections of 1 µg/µL standard solutions and *n*-hexane as the mobile phase (300 µL/min). The amount on column was therefore 1 µg per analyte. These conditions do not reflect the situation in a heart-cut system. In the designed system, the injection volume from the first column was 360 µL with 35 % dichloromethane (126 µL). Thus, far more retention of naphthalene was needed.

Even the Chromspher Pi column was not able to retain naphthalene in a heart-cut configuration. Acenaphthylene trapping was only partially possible. Beginning with fluorene, trapping of heavier PAHs could be observed in HPLC-UV experiments. The enrichment of lighter PAHs would need a stationary phase exhibiting stronger π-electron acceptor properties. To our knowledge, no (commercial) phases are available at the current time. Synthesis and characterization of suited materials could be an interesting field of research for the future.

6.6.2 Elution behavior of PAHs and polyunsaturates on silica gel

Twenty milligrams of extra virgin olive oil in *n*-hexane were injected onto a 25 cm x 2.1 mm silica column. The PAH fraction was transferred into GC-MS. A peak massively overloading the whole system could be seen in the TIC of the TOF-MS chromatogram. The mass spectrum clearly identified (isomerized) squalene as compound. It rendered the evaluation of the chromatogram for PAHs virtually impossible. Because of column overloading effects, extensive peak broadening for most compounds was observed. During the elution of squalene,

the detector of the TOF-MS went into saturation prohibiting the detection of any other compound.

Olive oil contains approximately 5 – 7000 mg/kg of squalene [1]. An injection of 20 mg of olive oil would therefore transfer about 100 µg of squalene into the GC-MS system if it would not be removed by HPLC beforehand. To test this assumption, HPLC-UV measurements with pure squalene injections were performed (again approximately 100 µg on column). An overlay of the elution profile of squalene with a PAH standard containing all EU priority PAH (10 ng per PAH on column) is shown in Fig. S-6.1. Although HPLC-UV suggests that a separation of squalene and EU priority PAHs seems feasible, LC-GC-TOF-MS measurements revealed that squalene residues still could be found several minutes after the end of the PAH fraction. HPLC-UV clearly underestimated this effect. Because of missing chromophores, squalene could not be detected very sensitive by UV detection.

Extensive tailing of squalene was most probably related to the high amounts overloading the HPLC column. Mass overload results in severe peak distortion and pronounced peak tailing [2]. Grob et al. observed similar effects for triglycerides tailing from size-exclusion chromatography (SEC) columns [3]. Sticking of analytes to valve surfaces, e.g., rotors and stators, was found by them as additional reason.

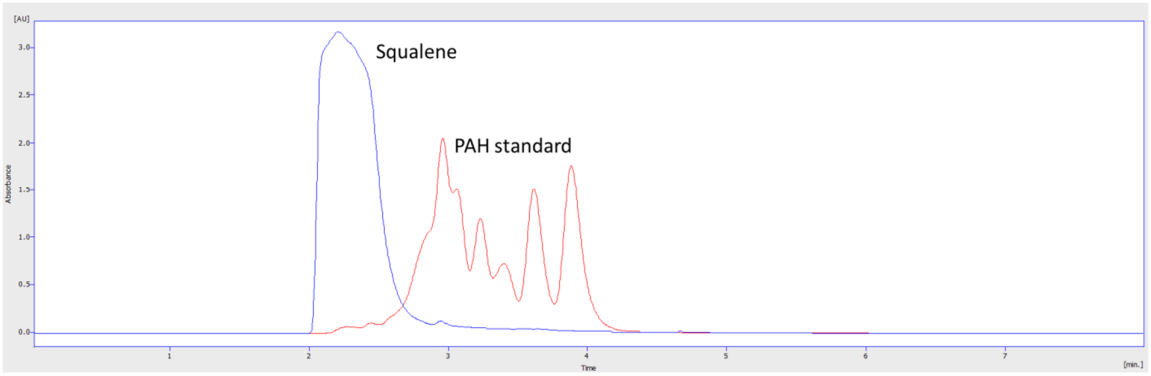

Fig. S-6.1. Overlay of HPLC-UV chromatograms of squalene and a PAH standard (Column: Restek Allure Si (250 mm x 2.1 mm, 5 µm, 60 Å), Eluent: *n*-hexane/MTBE (98:2, v/v), wave length: 205 nm, blue trace: 100 µg squalene on column, red trace: PAH standard with 10 ng per compound)

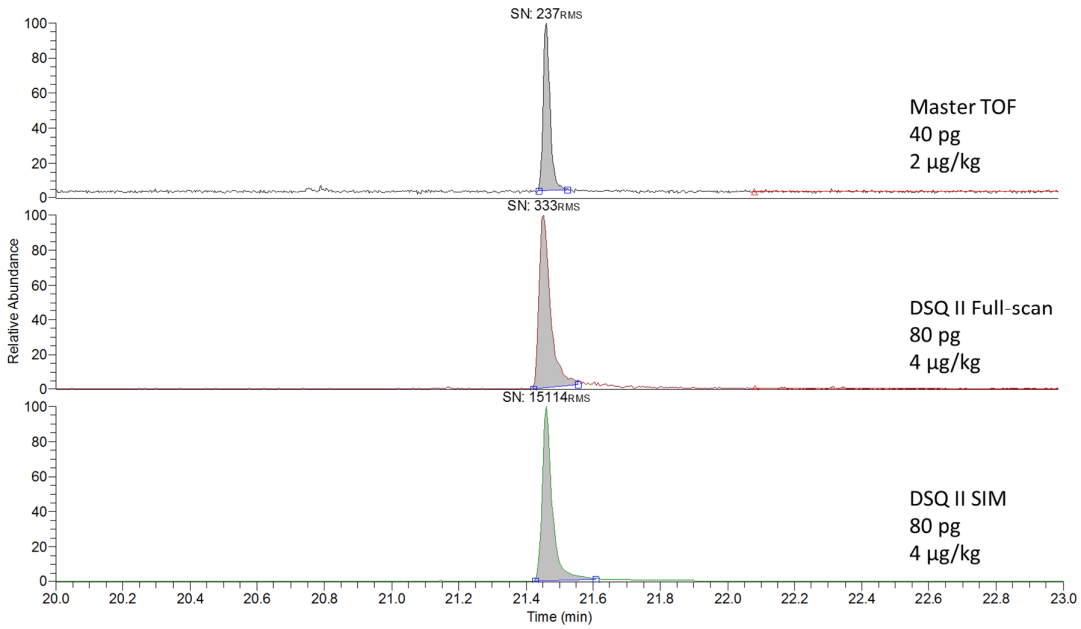

Fig. S-6.2. Sensitivity comparison of TOF-MS and quadrupole MS in full-scan and SIM mode for PAH detection (Extracted ion chromatogram (5-Methylchrysene, *m/z*: 242), 40 and 80 pg on-column, TOF-MS: 5 Hz (50–500 amu), quadrupole MS: 3 Hz (120–305 amu) for full-scan, 100 ms dwell-time for SIM)

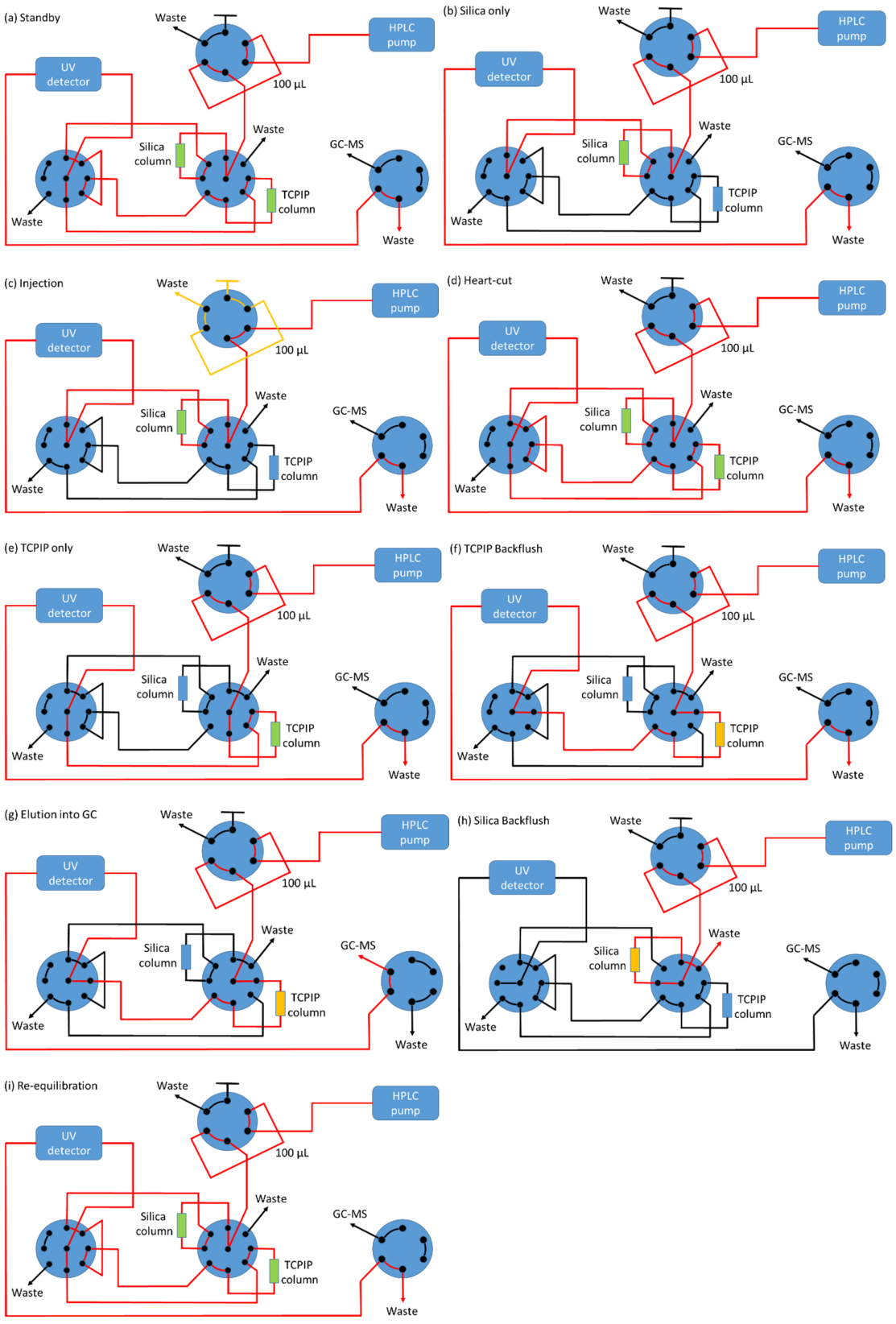

Fig. S-6.3. Detailed step-by-step valve schemes for LC-LC-GC-MS

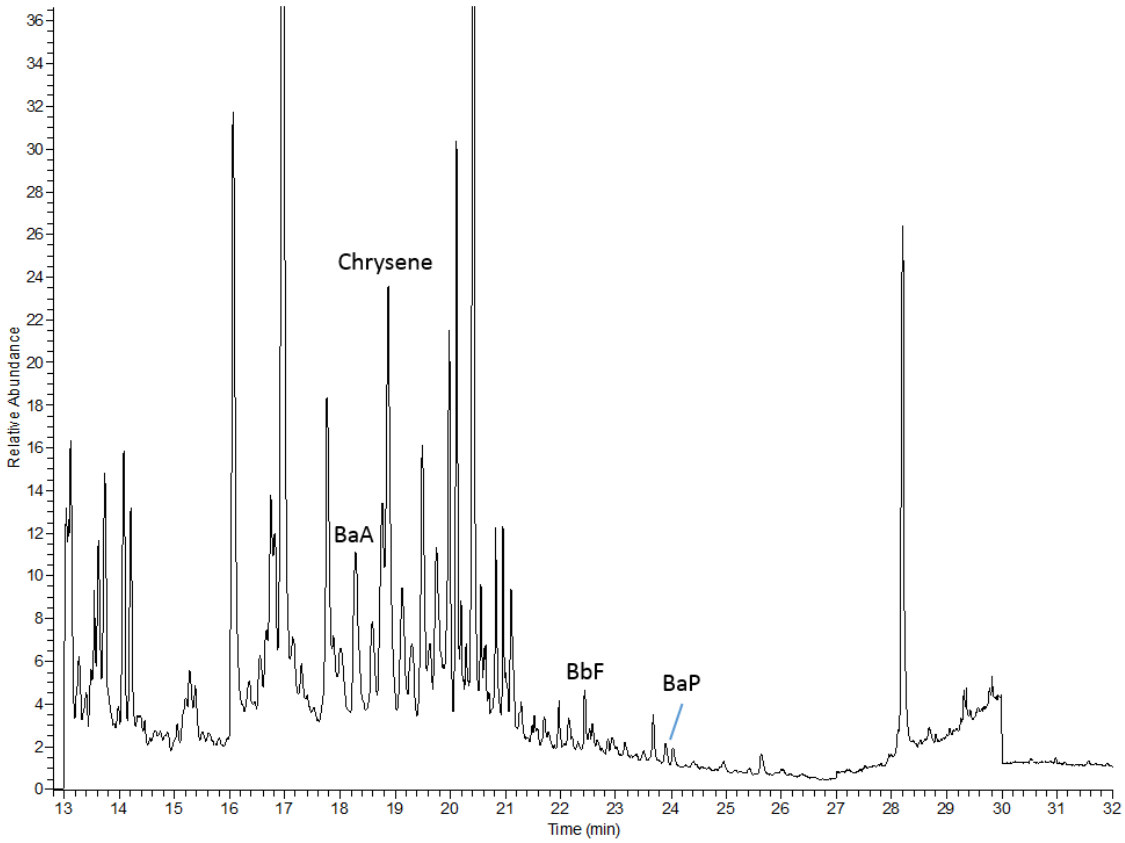

Fig. S-6.4. Extracted ion chromatogram of EU priority PAHs of olive pomace oil subjected to LC-LC-GC-MS. Concentrations (PAH4) of benz[a]anthracene (BaA), chrysene, benzo[b]fluoranthene (BbF) and BaP are 1.4, 3.3, 0.2, and 0.1 µg/kg, respectively.

6.6.3 References

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7. Determination of mineral oil aromatic hydrocarbons (MOAH) in edible oils and fats by online liquid chromatography–gas chromatography–flame ionization detection (LC-GC-FID) – Evaluation of automated removal strategies for biogenic olefins

Abstract

The existence of olefins in foodstuffs, such as edible oils and fats, can be problematic for the determination of mineral oil aromatic hydrocarbons (MOAH) by LC-GC-FID. Removal of these interfering substances by HPLC based on polarity differences is not possible. During gas chromatographic separation heavily overloaded peaks are observed rendering the detection of small mineral oil contaminations almost impossible. Therefore, removal of these olefins is necessary before subjection of the sample to LC-GC-FID. Derivatization of olefins to increase their polarity proved to be a valuable tool in the past. In the scope of this work, bromohydrin reaction, hydroboration, and epoxidation were examined for their potential for derivatization of unsaturated hydrocarbons. The efficiency of the bromohydrin reaction was highly dependent on the solvent composition while hydroboration did not show a removal of olefins under feasible reaction conditions. Epoxidation by *meta*-chloroperbenzoic acid (*m*CPBA) delivered the best removal of olefins. Precision and trueness of the results, however, were relying on the exact reaction conditions and timing. Hence, an automated epoxidation technique was developed as part of this work. Good precision (RSD_r < 1.5 %) and recovery (95 – 102 %) for MOAH were observed for sunflower and olive oils spiked with a lubricating mineral oil (24.5 mg/kg). The trueness of the method was verified by analyzing collaborative trial samples.

7.1 Introduction

Hydrocarbons of mineral oil origin account for a large proportion of the known contamination in foodstuffs [1]. According to an EFSA (European Food Safety Agency) statement from 2012, MOH (mineral oil hydrocarbons) contribute also to a high degree to contamination found in the human body [2]. They can be categorized into two main groups: Saturated (MOSH) and

aromatic hydrocarbons (MOAH) with alkyl chain lengths of 10 to approximately 50 carbon numbers [3]. While the first group consists of paraffinic and naphthenic saturated hydrocarbons, the second one is composed of alkylated (partially hydrogenated) aromatic hydrocarbons. The MOAH content of MOH can roughly range between 0 – 35 % depending on the nature of the mineral oil [4]. While crude oils exhibit higher MOAH contents, refined hydrogenated oils show little to no MOAH contribution.

The presence of MOH contamination in foodstuffs can be attributed to several sources. Packaging material made from recycled paperboard printed with mineral oil derived ink is one important origin. Additionally, lubricants during food processing, wax coatings directly applied to the food, environmental pollution, jute bags, etc. can be sources for contamination [5]. Found contaminations ranged from below 1 mg/kg up to several thousands of mg/kg [6]. In 2008, in Ukrainian sunflower oil more than 1000 mg/kg of mineral oil was found [7].

According to recent studies, acute toxicity upon oral intake of MOSH and MOAH is low [2]. Higher molecular MOSH are known to form microgranulomas in liver, spleen, lymph nodes, and other organs [1, 2]. Because of the structural similarities to polycyclic aromatic hydrocarbons (PAHs), some MOAH are suspected to have carcinogenic and mutagenic potential. It is known that alkylated PAHs, e.g., 1-methylpyrene, show increased carcinogenic potential compared to the parent compound (pyrene) [8]. I*n vitro* assays gave indication that MOAH from printing ink have genotoxic potential [9]. However, carcinogenicity data upon oral intake are neither available for MOSH nor MOAH to date.

Although no legislation is established till now for upper limits of MOSH and MOAH, minimization of both substance classes was advised by the EFSA and other national authorities such as the German Federal Institute for risk assessment (BfR) [10]. Upper limits of 0.6 and 0.15 mg/kg for MOSH and MOAH, respectively, were proposed in the past years derived from a temporary ADI (acceptable daily intake) of 0.01 mg/kg body weight (for a 60 kg person) and a suspected MOAH contribution of 25 % [11]. In 2012, however, this ADI was withdrawn by the JECFA (Joint FAO/WHO Expert Committee on Food Additives) due to insufficient scientific data. In 2014, upper limits of 2.0 and 0.5 mg/kg for MOSH and MOAH, respectively, found in foodstuffs packaged in recycled cardboard were proposed in the latest draft for the 22nd amendment of the German consumer goods regulation [12].

7.1.1 Analytics of MOSH and MOAH

The determination of MOSH and MOAH is routinely performed by online HPLC-GC-FID hyphenation. This method is based on a work by Biedermann et al. [13]. However, first publications regarding this topic and HPLC-GC hyphenation can be found already in the early 1990es [14].

Shortly, HPLC on bare silica gel is used for the separation of MOH components from the food matrix (lipids, sugars, etc.). Additionally, MOAH are separated from MOSH. The high capacity for retention of triglycerides allows the direct injection of edible oils upon dilution [15]. Detection limits of approximately 5 mg/kg were reported for selected edible oils. For low-fat containing matrices, such as rice or pasta, detection limits as low as 0.5 mg/kg were feasible [13].

After the HPLC separation step, the according fraction is transferred to GC by means of a large-volume on-column transfer technique. Typically, a retention gap method with partially concurrent solvent evaporation in combination with a solvent vapor exit (SVE) is employed for this purpose. It allows the quantitative recovery of volatile compounds, such as *n*-decane, for the necessary transfer volumes of several hundred microliters. GC-FID is used for further separation and detection. Because of the variety of MOH compositions, the FID is virtually the only detector capable for quantitation. Its quasi-unity response allows quantitation without specially adapted standards.

One remaining apparent problem with individual matrices is the co-elution of biogenic olefins during the HPLC separation. Some monoterpenes are partially eluted in the MOSH fraction, while polyunsaturates, such as carotenes, squalene, and sterenes, can be found in the MOAH fraction. Because of their natural abundance, these compounds form large peaks overloading the subsequent GC separation column pretending false-positive or overestimated quantitative results [13]. Because of the low content found in edible oils and fats, the co-elution of biogenic olefins in the MOSH fraction is mostly negligible and therefore out of the scope of this work.

Separation of MOAH from polyunsaturates by HPLC was found to be unsuited [13]. Alternatively, additional sample cleanup steps were developed [13, 16, 17]. Treatment of the sample with elemental bromine was used to derivatize the biogenic unsaturated hydrocarbons. Because of the toxicity of bromine and insufficient selectivity, epoxidation by *meta*-chloroperoxybenzoic acid (*m*CPBA) was proposed. Increased HPLC retention of the

polyunsaturates was the aim in both cases. Consequently, a removal of these compounds during the HPLC separation became feasible.

Typical reaction conditions for *m*CPBA epoxidation found in the literature include the use of dichloromethane as solvent and possible sub-ambient cooling for improved selectivity [18]. Quenching of the reaction is normally done by washing the sample with a reducing agent, such as sodium thiosulfate, afterward. Initial addition of sodium bicarbonate or a subsequent washing step was reported to improve the recovery of acid labile epoxides by removal of *meta*-chlorobenzoic acid formed during the reaction as sodium salt [19].

The proposed reaction route by Biedermann et al. for the determination of MOAH in edible oils and fats consisted of direct application of *m*CPBA in dichloromethane at sub-ambient temperatures, i.e., ice bath cooling [13]. Thirty milligrams of *m*CPBA were typically recommended for 300 mg of edible oils. Afterward, the sample was washed with a sodium carbonate solution (10 % aq.). After a second aqueous wash and workup, the solvent was carefully evaporated and the sample was reconstituted in *n*-hexane.

The authors realized that *m*CPBA also attacked certain MOAH constituents due to its oxidation potential. Roughly 20 % of MOAH were reported to be lost even at sub-ambient temperatures. Higher *m*CPBA amounts further increased the loss of MOAH. The presence of a food matrix containing high amounts of unsaturated fatty acids was found to be beneficial for the recovery of MOAH. Unsaturated fatty acids were reported to be attacked prior to MOAH compounds. In absence of a matrix, high losses for all polycyclic aromatic compounds, such as PAHs or thiophenes, were observed. Therefore, addition of uncontaminated edible oil as buffering agent was recommended for samples containing only small amounts of unsaturated fatty acids [13].

However, collaborative trials showed high variances in the obtained results possibly originating from varying reaction time, temperature, and reagent amount. Increase of method robustness is therefore appreciated.

Consequently, other solutions for this obstacle were already explored. Mondello et al. tried to remove the polyunsaturates by a second online LC-cleanup step [20]. After a first cleanup on silica, the MOAH fraction was separated from the polyunsaturates on Ag^+-treated silica gel. To that end, a commercial silica HPLC column was flushed with silver nitrate. Squalene from olive oil could be retained while MOAH with up to three aromatic rings were eluted in a transfer volume exceeding 2 mL. MOAH with larger ring systems were retained too strongly on the prepared column. Even further, no information were given by the authors regarding the elution

behavior of sterenes or carotenes present in vegetable oils. Moreover, stability of silver-ion impregnated HPLC columns is known to be limited [21].

For these reasons, aim of the current work was to explore possibilities for removal of polyunsaturates from the MOAH fraction offering increased robustness. HPLC separation techniques were not further pursued. During development of an LC-GC method for the determination of PAHs in a variety of foodstuffs, it was found that removal of polyunsaturates also removed large amounts of alkylated mono-aromatic hydrocarbons on donor-acceptor HPLC columns [22].

Alternatively, derivatization of the polyunsaturates was further explored. Treatment of samples with N-bromosuccinimide (NBS) as safe bromine supplier as well as hydroboration were examined for their suitability for removal of polyunsaturates. Optimization and automation of *m*CBPA epoxidation was also investigated as it would represent an important achievement to increase method robustness.

7.2 Experimental

7.2.1 Samples

Extra virgin olive oil and refined sunflower oil were obtained at the local supermarket and used for method development and validation. Additionally, edible oil samples from a collaborative trial performed in 2015 within the CEN/TC275/WG13 work program (European Committee for Standardization) organized by ITERG (Pessac, France) were available. They consisted of refined olive pomace oil, extra virgin olive oil, and palm oil.

7.2.2 Chemicals and solutions

Acetonitrile, dichloromethane, ethanol, and *n*-hexane were from LGC Promochem (Picograde quality, Wesel, Germany). The internal standard (ISTD) for MOH quantitation (Cat. No. 31070) and an EPA-PAH standard (Cat. No. 31011) were supplied from Restek (Bellefonte, PA, USA). A lubricating oil standard (K009) for spiking experiments was obtained from the Federal Institute for Materials Research and Testing (BAM, Berlin, Germany). 9-Borabicyclo(3.3.1)nonane (9-BBN, 0.4 M in hexanes), dibenzothiophene (DBT, 98 %) *meta*-chloroperoxybenzoic acid (*m*CPBA, ≤77 %), N-bromosuccinimide (NBS, ReagentPlus®, 99

%), and sodium thiosulfate (purum p.a., ≥98 % (RT)) were from Sigma Aldrich (Steinheim, Germany). Sodium formate and sodium sulfate were from Fluka (Buchs, Switzerland). Quantofix® Peroxide 25 test stripes were obtained from Macherey-Nagel (Düren, Germany). Water was supplied from a Milli-Q water purification system (Merck, Darmstadt, Germany).

7.2.3 Sample preparation

General

Three hundred milligrams of an edible oil or fat were weighed into a 10-mL autosampler vial. The vial was placed onto the autosampler, which added 50 µL of the ISTD solution (100 ng/µL in *n*-hexane) to the sample.

Bromohydrin reaction

The autosampler added 650 µL of *n*-hexane, 3 mL of ethanol/water (90:10, v/v) and 700 µL of an NBS solution (100 mg NBS in acetonitrile/water (75:25, v/v)). The vial was placed into an agitator and was shaken at a speed of 500 rpm (revolutions per minute) for 30 min at 40 °C. Afterward, 2.5 mL of an aqueous sodium formate solution (100 g/L) were added to destroy excess NBS and induce phase separation. The vial was shaken at 750 rpm for 30 s. Five hundred microliters of the *n*-hexanic upper phase were transferred into a 2-mL autosampler vial prefilled with a spatula tip of sodium sulfate. The dried organic phase was subjected to LC-GC-FID.

Hydroboration

The autosampler added 550 µL of *n*-hexane and 100 µL of a 9-BBN solution in hexane. The vial was placed into an agitator and was shaken at a speed of 500 rpm for 12 h at 60 °C. Afterward, the sample was directly injected into the LC-GC-FID system.

Epoxidation

The autosampler added 650 µL of *n*-hexane and 500 µL of an ethanolic *m*CBPA solution (200 mg/mL) to the sample. The vial was placed into an agitator and was shaken at a speed of 500 rpm for 15 min at room temperature. Afterward, 500 µL of ethanol and 2 mL of an aqueous

sodium thiosulfate solution (100 g/L) were added to destroy excess *m*CPBA and induce phase separation. The vial was shaken at 750 rpm for 30 s. Five hundred microliters of the *n*-hexanic upper phase were transferred into a 2-mL autosampler vial prefilled with a spatula tip of sodium sulfate. The dried organic phase was subjected to LC-GC-FID.

7.2.4 LC-GC-FID method

LC-GC-FID experiments were performed on a system from Axel Semrau (Sprockhövel, Germany). It consisted of a 1260 Infinity HPLC system (binary pump and variable wavelength detector by Agilent Technologies, Waldbronn, Germany), Master GC with flame ionization detector (DANI Instruments S.p.A., Cologno Monzese, Italy), and a DualPAL autosampler (CTC Analytics AG, Zwingen, Switzerland).

Three rotatory switching valves (VICI AG International, Schenkon, Switzerland) were used to guide the HPLC eluent from the HPLC into the GC. The GC was equipped with an on-column interface and a solvent vapor exit. The on-column interface, the carrier gas, and solvent vapor exit were controlled by CHRONECT LC-GC from Axel Semrau.

Typically, 50 µL (corresponding to 15 mg of edible oil or fat) of the prepared sample were injected onto an Allure Si HPLC column (250 mm x 2.1 mm, 5 µm, 60 Å, Restek, Bellefonte, PA, USA) without additional column temperature control. The mobile phase consisted of *n*-hexane and dichloromethane. Starting at 100 % *n*-hexane with 300 µL/min, the mobile phase was changed to 65 % *n*-hexane in 1.5 min after injection. It was held until 6.0 min. After elution of the MOAH fraction (4.5 – 6.0 min), the column was backflushed with dichloromethane at 500 µL/min for 9 min. Afterward, the column was reconditioned with *n*-hexane at 500 µL/min for 15 min.

LC-GC transfer occurred by the retention gap technique and partially concurrent solvent evaporation (PCSE) through the Y-interface [23]. An uncoated, deactivated precolumn (MXT Hydroguard, 10 m x 0.53 mm, Restek, Bellefonte) was followed by a steel T-piece union connecting to the solvent vapor exit and a separation column coated with a 100 % dimethyl polysiloxane film (MXT-1, 15 m x 0.25 mm x 0.10 µm, Restek, Bellefonte, PA, USA).

From HPLC, the MOAH fraction was transferred to the GC (resembling 450 µL) at a carrier gas inlet pressure of 65 kPa (hydrogen) in addition to an oven temperature of 60 °C. The elution window was verified by UV detection at 230 nm. The solvent vapor exit was opened 0.5 min prior to elution of the MOAH fraction and was closed 0.3 min after the fraction was transferred.

At this time, the carrier gas inlet pressure was set to 150 kPa and maintained throughout the whole analysis. The oven temperature was programmed at 30 °C/min from 60 °C (4 min) to 400 °C (4 min, total run time 18.00 min). The FID base temperature was set to 350 °C. The gas flows for air, hydrogen, and nitrogen were set to 280, 40, and 25 mL/min, respectively.

Data processing was performed with Clarity 6.2 (DataApex, Prague, Czech Republic). Quantitation was based on 2-methylnaphthalene (2MN) used as ISTD. The MOAH content was calculated following the equation

$$C = \frac{A_{MOAH} * m_{ISTD}}{A_{ISTD} * m_{Sample}}$$

with C: Content [mg/kg], A_{MOAH}: MOAH hump area without sharp peaks on top, A_{ISTD}: peak area of ISTD, m_{ISTD}: mass of ISTD [mg], m_{Sample}: mass of test sample [kg].

For compound identification, the FID was replaced by a Bruker EVOQ GC-TQ triple quadrupole mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The ion source and transfer line temperatures were set to 230 and 320 °C, respectively. Data acquisition was performed in full-scan mode (50 – 750 amu) at a rate of 3 spectra/s with EI ionization at 70 eV. Data processing was performed with Bruker MS Workstation 8.2.

7.3 Results and Discussion

7.3.1 Bromohydrin reaction

Opposed to elemental bromine, NBS supplies an electrophilic bromine atom in a safe manner. In general, NBS allows most of the reactions which are also observed with elemental bromine. Because of this, conversion of an olefin into the corresponding bromohydrin, i.e., addition of bromine and hydroxide onto an olefinic double bond, was studied as a possible derivatization reaction for removal of polyunsaturates.

In literature, formation of bromohydrins is most efficiently performed in acetonitrile, acetone, or tetrahydrofuran (THF) containing min. 5–20 % of water [24, 25]. Direct application of 100 mg of NBS was tried under these conditions on olive oil (300 mg) diluted by *n*-hexane (700 µL). Unexpectedly, the results were not too promising. High residual amounts of squalene could be identified in the LC-GC-FID chromatograms.

Interestingly, the use of ethanol as sole solvent showed significantly better results, even though removal of squalene was still not quantitative (as compared to epoxidation as reference). It is assumed that the corresponding ethoxybromide is formed under these conditions [26]. Nevertheless, addition of 10 % of water further increased the removal of squalene. Higher water contents did not improve the situation. Emerging phase separation could have been responsible for this observation despite vigorous shaking. Best results were found at a temperature of 40 °C and a reaction time of 30 min. In Fig. 7.1, the individual removal of squalene from the used olive oil for selected solvent systems is shown. A comprehensive compilation of the results can be found in the supporting information.

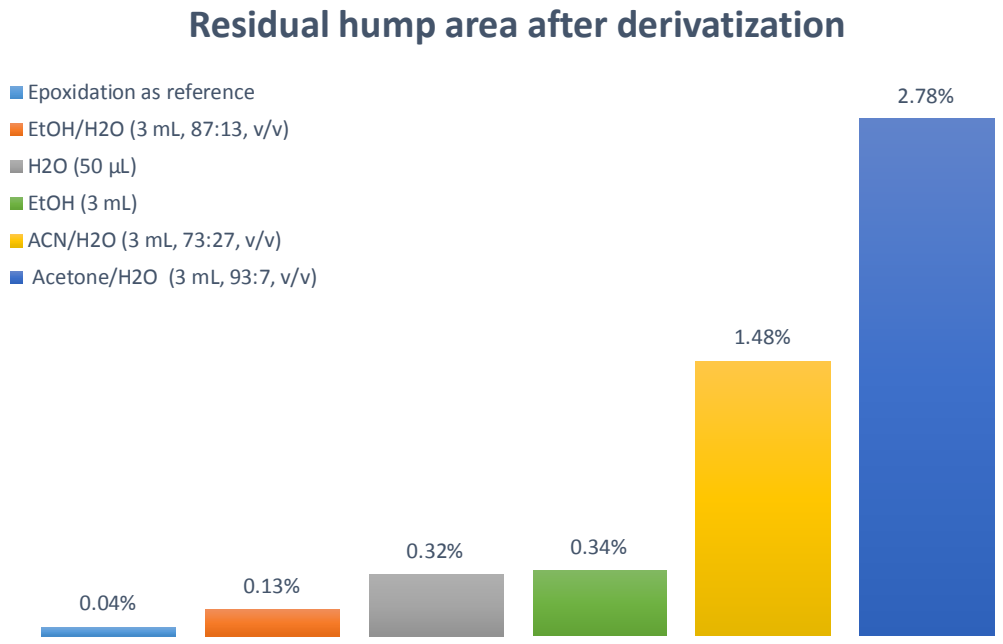

Fig. 7.1. Removal of squalene from olive oil by bromohydrin reaction under optimized reaction conditions for selected solvent systems (A hump area of 100 % corresponds to the squalene content prior to derivatization – Reaction conditions: 300 mg of olive oil, 50 µL of ISTD solution, 0.65 mL of *n*-hexane, specific solvent composition, 100 mg of NBS, 30 min, 40 °C).

The use of ethanol bore one problem. NBS oxidized ethanol in a few minutes even at room temperature, which could be observed by the emerging orange color of the solution [27]. Thus, stock solutions of NBS placed on the autosampler were prepared in a mixture of acetonitrile/water (75:25, v/v). This mixture allowed dissolution of the necessary NBS amount. The total water content was adapted accordingly. The influence on the results was negligible compared to addition of NBS as solid.

An EPA-PAH standard (3 mg/kg) was directly subjected to the bromohydrin reaction to get an estimation about its selectivity. Almost all PAHs were firstly brominated (as verified by mass spectrometry) before they were further oxidized, even in the presence of an edible oil as possible buffering agent. It is noteworthy that brominated PAHs were still eluted in the MOAH fraction on HPLC. Due to the quasi-unity response of the FID, quantitation was therefore not affected. Thus, as long as the substances were only brominated, but not further oxidized, recovery was quantitative. Furthermore, recovery for sunflower and olive oils spiked with a lubricating oil (MOAH content of 35 mg/kg) was virtually quantitative indicating that highly alkylated aromatic compounds could be retrieved.

Compared to epoxidation, however, quantitation of the residual hump after optimized bromohydrin reaction gave significantly higher amounts for the used olive oil (approximately the threefold, compare the two columns on the left in Fig. 7.1). This indicated non-quantitative removal of polyunsaturates rendering the reaction unsuitable as single cleanup step. Nevertheless, as add-on procedure for persistent polyunsaturates in individual foodstuffs, such as spices or algae, it could still be a valuable tool.

7.3.2 Hydroboration

Hydroboration is typically performed with a BH_3-THF complex in an inert atmosphere, thus, reaction conditions not suitable for automation by an autosampler. However, 9-BBN is known to be a longtime stable alternative to BH_3 allowing essentially the same reactions.

Hydroboration of olive oil with 9-BBN in *n*-hexane with subsequent injection into LC-GC-FID showed no removal of squalene even after 12 h at 60 °C. Literature recommended the use of THF as reaction solvent [28]. In most other solvents, significantly slower reaction kinetics were observed [29]. If the reaction occurred at all, retention of the formed organoboranes did not differ sufficiently from MOAH for a successful removal by HPLC.

Usual oxidation of organoboranes with H_2O_2/NaOH was avoided, since it was hardly automatable. Besides corrosiveness, the application of NaOH on an edible oil enabled saponification, which hindered a clear identification of the *n*-hexanic upper phase. Therefore, hydroboration was abandoned as removal step for polyunsaturates from edible oils and fats.

7.3.3 Optimization and automation of epoxidation

For the sake of automation, it was tried to omit the use of dichloromethane as reaction solvent. Solvent evaporation is a time consuming step always involving the risk of losing volatile compounds. Instead, *n*-hexane was tried as reaction solvent from the beginning. Solubility of *m*CPBA in *n*-hexane was too low for a quantitative removal of polyunsaturates in edible oils and fats. Solubility (>100 mg/100 µL) and stability of *m*CPBA in ethanol, however, proved to be well suited for an automated approach. One hundred milligrams dissolved in 500 µL of ethanol were added to the *n*-hexanic sample. Removal of the ethanol from *n*-hexane was easily possible by addition of water. Comparison with the traditional method for freshly spiked sunflower and olive oils (24.5 mg/kg of MOAH) showed virtually the same results.

One reason for insufficient robustness of traditional epoxidation reported in collaborative trials could be the missing quenching step with a reducing agent. A sodium carbonate wash does not remove excess *m*CPBA from the dichloromethanic phase and formed *meta*-chlorobenzoic acid would be removed in the subsequent HPLC step anyhow. Even during evaporation of dichloromethane, *m*CPBA could further react with the sample. Although solubility of *m*CPBA in *n*-hexane is low (1.4 mg/100 µL), continuing reaction would still be possible during sample storage on the LC-GC instrument.

Application of a peroxide test stripe after finished reaction of *m*CPBA with spiked sunflower and olive oils did not show traces of left peracid in the solution supporting the hypothesis that excess *m*CPBA is consumed by unsaturated fatty acids. In absence of a matrix, however, high residual amounts of peracid were detected. A carbonate wash did not reduce these amounts significantly. It is obvious that under these conditions epoxidation occurs mainly uncontrolled and why Biedermann et al. needed to add a buffering agent for samples containing few unsaturated fatty acids. That is precisely the reason why carbonate washing was replaced by sodium thiosulfate washing. Thereafter, a peroxide test did not show any traces of left peroxides.

The influence of the matrix on the recovery of MOAH was further examined. Therefore, an EPA-PAH standard (3 mg/kg) was derivatized with and without addition of an edible oil. The obtained results indicated that no PAHs were lost in both cases. Recoveries ranged from 92 to 104 %. Opposed to the literature, in which PAH losses were reported in absence of an edible oil, the results provided here show that PAH oxidation can be prevented by appropriate quenching and automated sample handling.

Derivatization of a DBT standard, however, exposed substantial losses of approximately 80 % without addition of a matrix. In presence of an edible oil, the losses were reduced to approximately 45 %. DBT is known to be oxidized unintentionally during epoxidation and is therefore well suited as indicator for worst-case MOAH losses [13, 30]. In literature, complete loss of DBT was observed in absence of a matrix [13]. Thus, slightly better results were obtained for automated epoxidation, even with significantly higher amounts of peracid (100 mg/300 mg matrix) compared to the traditional method (30 mg/300 mg matrix).

In Table 7.1, the obtained results for automated and traditional epoxidation are compared.

Table 7.1. Comparison of automated and traditional epoxidation for spiked sunflower oil

	Recovery [%]					
	MOAH[a]		**DBT[b]**		**EPA-PAHs[c]**	
	Autom.	**Trad.**	**Autom.**	**Trad.**	**Autom.**	**Trad.[d]**
No matrix	95	107	20	< 5	93–103	> 55
Sunflower oil	102	105	55	65	94–101	102
Virgin olive oil	101	99	56	65	92–104	95

[a]: BAM K009 lubricating oil MOAH spiking of 24.5 mg/kg
[b]: Dibenzothiophene (DBT) spiking of 30 mg/kg
[c]: EPA-PAH spiking of 3 mg/kg
[d]: Results obtained from [13]

MOAH recovery was quantitative for traditional and automated epoxidation indicating that the used mineral oil for spiking possibly did not contain many easily oxidable compounds, e.g., thiophenes. Recovery of DBT was slightly lower for spiked oil samples in the case of automated epoxidation. This clearly reflects the higher amount of used *m*CPBA opposed to traditional epoxidation. Higher amounts were chosen and validated, because for individual foodstuffs, such as palm fatty acid distillates, higher amounts of *m*CPBA were needed to obtain a sufficient cleanup.

7.3.4 Validation of the automated epoxidation approach

Repeatability and recovery were determined by six individual workups and injections of spiked olive and sunflower oils. According to Horwitz, the allowed relative standard deviation under repeatability conditions for a spiking of 24.5 mg/kg is fixed at 6.6 % [31]. The obtained repeatability was better than 1.5 % for both oils and complied therefore with the Horwitz requirements. The recovery ranged from 95 to 102 % including the internal standards.

Since no certified reference materials were available for the determination of MOAH in edible oils and fats, collaborative trial material was used instead. The three oil samples were analyzed in duplicate on three successive days to get an impression of the reproducibility and trueness of the automated epoxidation with subsequent LC-GC-FID analysis (see Table 7.2).

Table 7.2. Comparison of collaborative trial MOAH results and values obtained by automated epoxidation

	CT[a] MOAH Mean value [mg/kg]	Horwitz RSD_r [%]	CT RSD_r [%][b]	Automated epoxidation [mg/kg]	RSD_R [%][b,c]
Virgin olive oil	1.7	9.8	33	1.5	18.2
Olive pomace oil	44.7	6.0	6	70.6	2.7
Palm oil	11.4	7.4	9	13.6	5.5

[a]: Collaborative trial
[b]: To eliminate the influence of the individual laboratories regarding differing chromatogram integration etc., CT repeatability was assumed as intermediate precision and therefore compared to the reproducibility of own measurements.
[c]: Based on the quantitative results in duplicate on three successive days (n = 6)

In general, good agreement between quantitative collaborative trial mean values and automated epoxidation results was observed. However, for the extra virgin olive oil a reproducibility exceeding the predicted Horwitz limit was observed in own measurements. This was related to the low quantified amount, which was hardly distinguishable from the chromatogram baseline. As can be derived from the collaborative trial results, the participants struggled with the same problem.

For the refined olive pomace oil significantly higher amounts were quantified in own experiments. This was clearly related to the high-boiling mass distribution of the MOAH contamination in this case. Contaminations exceeding elution temperatures of 350 °C were

detected. The use of metal GC columns allowed GC oven temperatures of 400 °C needed for the elution of these high boiling compounds. It is doubtful if all participants of the collaborative trial were aware of this problem.

7.4 Conclusion

At the moment, epoxidation proved to be the method of choice for removal of polyunsaturates from edible oils and fats. However, precise control of reaction conditions and timing is of upmost importance for precise and valid results. Automation is therefore inevitable.

During optimization of the reaction conditions, the use of sub-ambient temperatures was not required. Solvent evaporation and sample reconstitution was no longer necessary after exchange of the reaction medium. Recovery of MOAH in spiked sunflower and olive oil samples was virtually quantitative for the used lubricating mineral oil. Even PAHs could be quantitatively recovered in absence of a matrix, which is most probably related to the use of an efficient quenching step. Quantitative results for collaborative trial samples verified the trueness of automated epoxidation. Precision complied with the Horwitz criteria for contaminations higher than approximately 3 mg/kg making the method amenable to the analysis of edible oils and fats in routine environments. Lowering the limit of quantitation is possible by sample enrichment [13].

Exploration of other derivatization reactions showed that bromohydrin formation could be an interesting tool for complex foodstuffs, for which epoxidation does not afford a reasonable cleanup, such as spices or algae. As single cleanup step, the bromohydrin reaction showed insufficient results and substantial losses of non-alkylated PAHs. Compared to literature, rather unconventional reaction conditions proved to be the most successful. Additional optimization of the reaction conditions could further improve the situation.

Another alternative, namely hydroboration, was not suited at all for removal of polyunsaturates in edible oils under feasible reaction conditions. Anyhow, exploration of new possibilities for removal of polyunsaturates will be pursued. The unlimited suitability of the developed methods also for the removal of olefins from the MOSH fraction, namely monoterpenes etc., will be another important aspect of future research.

7.5 Acknowledgements

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7.6 References

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7.7 Supporting Information

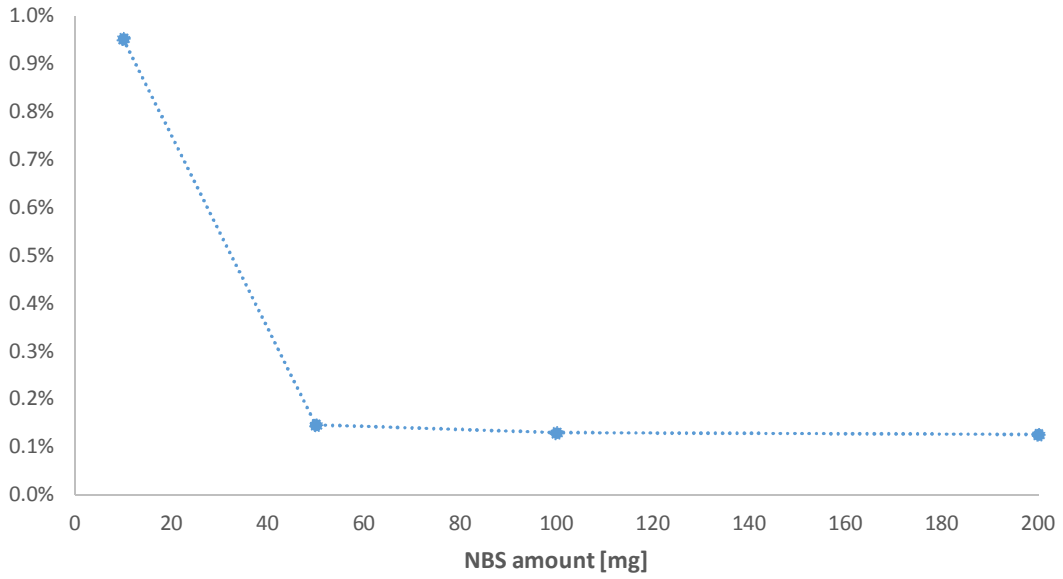

Fig. S-7.1. Residual hump area after reaction in dependence of the used NBS amount (Reaction conditions: 300 mg of olive oil, 50 µL of ISTD solution, 0.65 mL of *n*-hexane, 3 mL of EtOH/H_2O (87:13, v/v), 30 min, 40 °C)

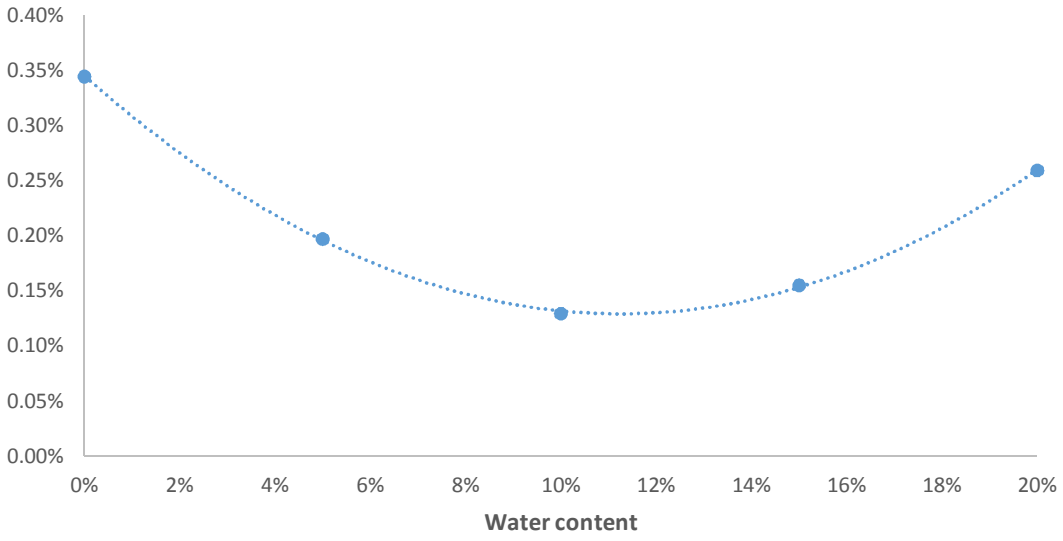

Fig. S-7.2. Residual hump area after reaction in dependence of the water content in the solvent (Reaction conditions: 300 mg of olive oil, 50 µL of ISTD solution, 0.65 mL of *n*-hexane, 3 mL of EtOH/H_2O (75-x:x, v/v), 100 mg of NBS, 30 min, 40 °C)

8. General Conclusions and Outlook

The here presented thesis dealt with the development and validation of analytical methods for the determination of various compound classes in a broad range of foodstuffs by use of LC-GC hyphenation. The connection of both chromatographic techniques combined into one analytical system offered apparent advantages for routine environments, in which robustness and high sample throughput are necessary prerequisites. HPLC opens very efficient cleanups of complex food matrices, such as edible oils, coffees, or teas, while capillary GC with its selective detectors (FID, MS) is an established cornerstone for robust and sensitive quantitation in routine laboratories.

The individual chapters of this work approached analytical methods in the context of "Unwanted contaminations in food (PAHs, MOHs)", "Quality assurance of edible oils and fats (sterols, stigmasta-3,5-diene)" and "Food nutrition analysis (vitamins D_2/D_3)". Hence, the chosen analyte spectrum ranged from nonpolar hydrocarbons up to slightly polar steroid derivatives. Determination of these compounds in a broad range of complex foodstuffs requires highly efficient cleanup routines, which are generally time-consuming and error-prone. LC-GC hyphenation is a logical step as it enables automated sample cleanup with minimal manual work and, consequently, an increase in sample throughput.

Although this type of technique is known since approximately 30 years, it has been hardly used beyond the scientific community. Technical difficulties were reported to affect the instrument robustness. Because of this, initial work in the context of this thesis involved the validation of a method for the detection of high-temperature refining of extra virgin olive oils. Opposed to the usual time-consuming column chromatography, LC-GC hyphenation allowed direct injection of a sample upon dilution. More important than this, validation showed that high precision, sensitivity, and robustness were achieved by use of a modern hard- and software solution.

In the following course, it was realized that a solid workflow involving LC-GC hyphenation frequently requires adaption to other sample preparation techniques. It is often the case that samples have to be prepared in one or the other way prior to injection. Automation of these steps by autosamplers and online coupling with LC-GC hyphenation was therefore evaluated.

The determination and quantitation of the sterol distribution of edible oils and fats was chosen as a showcase model. Although a manual ISO method from 1999 is existent, which was recently revised, collaborative trials regularly show insufficient precision among participating

laboratories [1]. For these reasons, the underlying ISO method was completely automated by connecting LC-GC hyphenation with the necessary sample preparation (addition of the internal standard, saponification, and extraction). The precision and trueness of the described automated approach, as compared to collaborative trial results, showed that standardization of workflows was an important aspect during method development.

The final chapters of the thesis were dedicated to the enlightening and overcoming of deficiencies of classical LC-GC hyphenation based on an HPLC cleanup strategy relying on polarity differences. In literature, multidimensional HPLC cleanups were rarely reported for improved sample purification. Moreover, the few publications investigating such approaches can merely be classified as proof of concept not yet evaluated for routine use [2, 3]. Thus, a simple heart-cut two-dimensional HPLC cleanup suited for high-throughput application was employed in the scope of this work. An HPLC cleanup based on polarity and aromaticity differences able to efficiently extract PAHs allowed their analysis in a wide range of foodstuffs.

For MOAH analytics, another approach was pursuit. Because chromatographic removal of matrix constituents proved to be hardly feasible, derivatization of the interfering matrix compounds was chosen. Sample derivatization was previously reported in literature [4]. However, precisely automated conditions for these reactions were found to be essential to allow high analyte recovery with appropriate removal of the chromatographic interference.

The key findings in this work confirmed the inherent relationship between LC-GC hyphenation and other analytical tools. The choice of the latter was vital for the success of the here developed analysis methods. The validation parts of all five methods in this work proved that extensive automation (sample preparation, sample cleanup, separation, and detection) was of upmost importance to provide high accuracy (precision and trueness). Moreover, high robustness and sample throughput were achievable by elimination of manual work. Accordingly, these methods are already partially in use in routine environments [5]. Just recently, a big German discounter requested the MOH analysis of all own-brand products by online HPLC-GC-FID [6].

Subsequent work in this field of research should involve the expansion of LC-GC hyphenation for new analyte groups previously not considered. Extension of the application pool and steady collection of validation data facilitates the chain of reasoning for necessary standardization work of national authorities and responsible working groups. Even though the analytical performance is evident, widespread distribution of such methods in routine environments is only possible by standardization.

From the analytical point of view, HPLC cleanup modes suited for LC-GC hyphenation should be further investigated. As was shown for PAH analysis, polarity differences between the analytes and matrix constituents are not always existent. Specialized separation modes, e.g., donor-acceptor chromatography, SEC, or molecular-shape recognition, could solve this obstacle [7, 8]. Alternatively, removal of matrix compounds by specialized derivatization as shown for MOAH contamination could be of analytical value.

The online coupling of multiple HPLC cleanup stages is surely another interesting research issue. As was also shown for PAH analysis, stacking of multiple cleanup stages drastically increased the sample purity before subjection to the gas chromatographic separation dimension. The two-dimensional heart-cut HPLC cleanup based on polarity and aromaticity differences was only possible because of the compatibility of the necessary LC mobile phases and transfer volumes. This is by no means a matter of fact.

Consequently, a robust online solvent exchange between multiple HPLC cleanup stages without loss of the analytes of interest is of vital importance. As was previously shown in literature, the hyphenation of SEC with normal-phase LC-GC (SEC-LC-GC) or normal-phase with reversed-phase HPLC could be of high value for comprehensive sample cleanups [9, 10]. Especially for applications in which the analytes of interest are not limited to a single substance class, multistage cleanup techniques could be of interest. For instance, the determination of pesticides in fatty foods requires multiple cleanup steps covering several chromatographic techniques [11].

Even though the use of aqueous reversed-phase HPLC with gas chromatographic methods was already explored in the past, its use could be interesting for specialized analysis questions [12]. Good recovery of volatile compounds and stable chromatographic conditions are only two questions which have to be conquered.

Apart from this, online solvent exchange of aqueous eluents for other fields of research, e.g., heart-cut LC-LC or comprehensive two-dimensional LCxLC, could be of value. While in the

past mainly trapping techniques, such as online solid phase extraction (SPE) or temperature-dependent trapping, were employed for incompatible eluent compositions in the second dimension, robust online solvent exchange could represent a generic key achievement [13–15]. Previously described vacuum solvent evaporation interfaces were either designed for (few microliters of) pure organic solvents or were exhibiting loss of volatile material [10, 16, 17].

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9. Appendix

9.1 List of Abbreviations

2MN	2-Methylnaphthalene
ADI	Acceptable daily intake
BfR	Bundesinstitut für Risikobewertung
BTEX	Benzene, toluene, ethylbenzene, xylenes
C_{18}	*n*-Octadecyl carbon chain
DVB	Divinylbenzene
ECD	Electron capture detector
EFSA	European Food Safety Agency
EPA	EPA US Environmental Protection Agency
FAME	Fatty acid methyl ester
FCSE	Fully concurrent solvent evaporation
FID	Flame ionization detector
GC	Gas chromatography
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatography
IOC	International Olive Council
ISTD	Internal standard
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
LVI	Large volume injection
*m*CPBA	*meta*-Chloroperbenzoic acid
MOH	Mineral oil hydrocarbons
MOSH	Mineral oil saturated hydrocarbons
MOAH	Mineral oil aromatic hydrocarbons
MS	Mass spectrometry
NARP	Non-aqueous reversed-phase
NBS	N-bromosuccinimide
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyl

PCSE	Partially concurrent solvent evaporation
PGC	Porous graphitic carbon
PS	Polystyrene
PTV	Programmable temperature vaporization
QC	Quality control
SEC	Size-exclusion chromatography
SNR	Signal-to-noise ratio
SSL	Split/Splitless
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SVE	Solvent vapor exit
TCPIP	Tetrachlorophthalimidopropyl
TIC	Total ion current
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TOF	Time of flight
VOC	Volatile organic compound

9.2 List of Figures

9.3 List of Tables

9.4 List of Publications

Book chapters

1. Nestola, M.; Becker, E. In *Der HPLC-Experte: Möglichkeiten und Grenzen der modernen HPLC*; Kromidas, S.; Wiley-VCH: Weinheim, **2014**; pp 61–100.

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1. Nestola, M.; Friedrich, R.; Bluhme, P.; Schmidt, T. C. *26. Doktorandenseminar*, Hohenroda, **2016**.
2. Nestola, M.; Friedrich, R.; Bluhme, P.; Schmidt, T. C. *44. Lebensmittelchemikertag*, Karlsruhe, **2015**.
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6. Nestola, M. *ANAKON*, Essen, **2013**.

9.5 Curriculum Vitae

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

9.6 Erklärung

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

„Multidimensional high-performance liquid chromatography–gas chromatography (HPLC-GC) hyphenation techniques for food analysis in routine environments"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im April 2016

Marco Nestola

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