GC-MS/MS and two-dimensional LC-MS/MS approaches for the determination of contaminants and residues in food

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Submitted by

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Summary:

This work presents a cost- and time-effective analysis for polycyclic aromatic hydrocarbons in various food matrices via gas chromatography tandem mass spectrometry (GC-MS/MS) and a method for the determination of pesticides, plant growth regulators, mycotoxins, tropane alkaloids and pyrrolizidine alkaloids in whole grains with a two-dimensional liquid chromatography tandem mass spectrometry system (2D-LC-MS/MS).

The development of a GC-MS/MS method for the determination of polycyclic aromatic hydrocarbons in eleven food matrices is described in publication I. State of the art and challenges for the analysis of these compounds concerning sample preparation and detection are outlined. Furthermore, the legal basis regarding the maximum levels and the analytical requirements set by regulations are described. In the first step of method development an appropriate column setting for the GC-MS/MS analysis for the optimal separation of possible interferences from the target analytes was investigated. The second part of this work was the development of a suitable sample preparation procedure conducted on the basis of the QuEChERS (quick, easy, cheap, effective, rugged and safe) method. Different extraction solvents and dispersive solid phase extraction sorbents and salts were investigated. Finally, the developed method was validated for the determination of the food relevant polycyclic aromatic hydrocarbons in eleven food matrices.

Publication II presents a two-dimensional LC-MS/MS method for the determination of 370 pesticides, mepiquat and chlormequat, atropine and scopolamine, 9 regulated mycotoxins and 30 pyrrolizidine alkaloids with a minimized sample preparation in oats and whole wheat grains. The 2D-LC-MS/MS system applied to food analysis is described. An optimized analyte extraction procedure, different matrix matched calibration standards, optimized parameters and interfering matrix effects are explained and described. The validation of the finalized method of each compound class is further discussed regarding other published analytical procedures for the target analytes, limit of detection, limit of quantification, matrix effects and the fulfillment of the analytical requirements according to the SANTE guide 2015.

Zusammenfassung

Diese Arbeit präsentiert eine kosten- und zeiteffiziente Analyse für die Bestimmung von polyzyklischen aromatischen Kohlenwasserstoffen in verschiedenen Lebensmitteln mittels Gaschromatographie Tandem-Massenspektrometrie (GC-MS/MS) und eine Methode für die Bestimmung von Pestiziden, Pflanzenwachstumsregulatoren, Mykotoxinen, Tropanalkaloiden und Pyrrolizidinalkaloiden in Weizen und Hafer mittels eines zwei-dimensionalen flüssigkeitschromatographischen Tandem-Massenspektrometrischen Systems (2D-LC-MS/MS).

In Publikation I ist die Methodenentwicklung für die Bestimmung von polyzyklischen aromatischen Kohlenwasserstoffen in elf Matrizen mittels GC-MS/MS beschrieben. Der derzeitige Stand der Technik und mögliche Schwierigkeiten in der Analyse dieser Kontaminanten bezüglich Probenvorbereitung, Detektion und Erfüllung der analytischen Anforderungen werden beschrieben. Im ersten Schritt der Probenvorbereitung wurde die optimale Säulenkonfiguration für das GC-MS/MS System für die unabdingbare Trennung möglicher chromatographischer Störungen von den Zielanalyten untersucht. Der zweite Teil dieser Arbeit beinhaltet die Entwicklung einer adäquaten Probenvorbereitung, beruhend auf den Prinzipien der QuEChERS (quick, easy, cheap, effective, rugged and safe) Methode. Verschiedene Extraktionslösungen und dispersive Festphasenextraktions Sorptionsmittel wurden untersucht. Nach der Optimierung all dieser Parameter wurde die vorliegende Methode erfolgreich für elf Matrizen validiert.

Publikation II präsentiert eine zwei-dimensionale LC-MS/MS Methode mit einer Minimierung der Probenvorbereitung für die Bestimmung von 370 Pestiziden, Mepiquat und Chlormequat, Atropin und Scopolamin, 9 rechtlich geregelten Mykotoxinen und 30 Pyrrolizidinalkaloiden in Weizen und Hafer. Beschrieben wird der Aufbau des 2D-LC-MS/MS Systems und die damit verbundenen Parameter, sowie die optimierten Extraktionsbedingungen, Matrixeffekte und verschiedene der Matrix angepassten Kalibrationen. Des Weiteren wird die validierte Methode mit den bestehenden in der Literatur angewandten Methoden in Bezug auf Nachweisgrenzen, Bestimmungsgrenzen, Matrixeffekten und die Erfüllung analytischer Vorgaben gemäß dem SANTE Dokument 2015 verglichen.

1 Polycyclic aromatic hydrocarbons

1.1 Human health and legislation

Polycyclic aromatic hydrocarbons (PAHs) consist of two or more fused aromatic rings increasing their toxicity and nonpolar properties proportionally to the number of rings [1]. PAHs are products of incomplete combustion or pyrolysis of organic matter and can be found in air, water, soil, fuels, foods and cigarette smoke exerting harmful effects on human health (Fig. 1). The source of the PAHs has to be further distinct from its origin: anthropogenic or natural origin. Anthropogenic sources like burning of fossil fuels and industrial processes predominate. Wood fires and volcanic eruptions belong to natural generation of PAHs. The source of contamination for certain foods are environmentally related and/or cooking and food manufacturing processes like smoking, grilling, frying, barbecuing, toasting and roasting [2-5] (Fig. 2). The potential of PAHs to be carcinogenic and genotoxic was evaluated by the scientific Committee on Food (SCF) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [6]. SCF and JECFA concluded 15 and 13 PAHs, respectively to be carcinogenic and genotoxic. Some selected PAHs and their chemical properties are shown in table 1. The best known representative of these environmental contaminants is benzo[a]pyrene (BaP) and is considered as a group 1 carcinogen [7]. Initially BaP was used as a marker for the occurrence and genotoxic effects of PAHs in certain foods. In February 2005 a call for all European member states was published recommending further investigations into the levels of PAHs in various foods [8]. In 50% of all samples BaP could be detected, but additionally in 30% other carcinogenic PAHs occurred also in the absence of BaP [6]. The most abundant PAH in the absence of BaP was chrysene (CHR). Data on the oral carcinogenic effect was available for eight PAHs, namely BaP, benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (Bkf), benzo[ghi]perylene (BghiP), chrysene (CHR), dibenz[a,h]anthracene (DahA) and indeno[1,2,3-cd]pyrene (IcdP). Therefore, these eight PAHs (PAH8) were investigated in certain foods with the addition of four PAHs (PAH4), namely BaP, BaA, CHR and BbF and two PAHs (PAH2), namely BaP and CHR (Fig.3). All PAH categories were evaluated regarding their occurrence above the LOD. The EFSA Panel on Contaminants in the Food Chain (CONTAM) concluded that PAH4 and PAH8 were better suited as indicators for the occurrence of PAHs in food [6]. This is still ongoing research since it has been shown that the PAH contamination profile of 115 olive oil samples, cooking oil and fats did not resemble the PAH8 for the occurrence of PAHs [9].

Fig 1: Human exposure to PAHs and their harmful effects regarding cancer [adapted from Rengarajan et al., 2015]

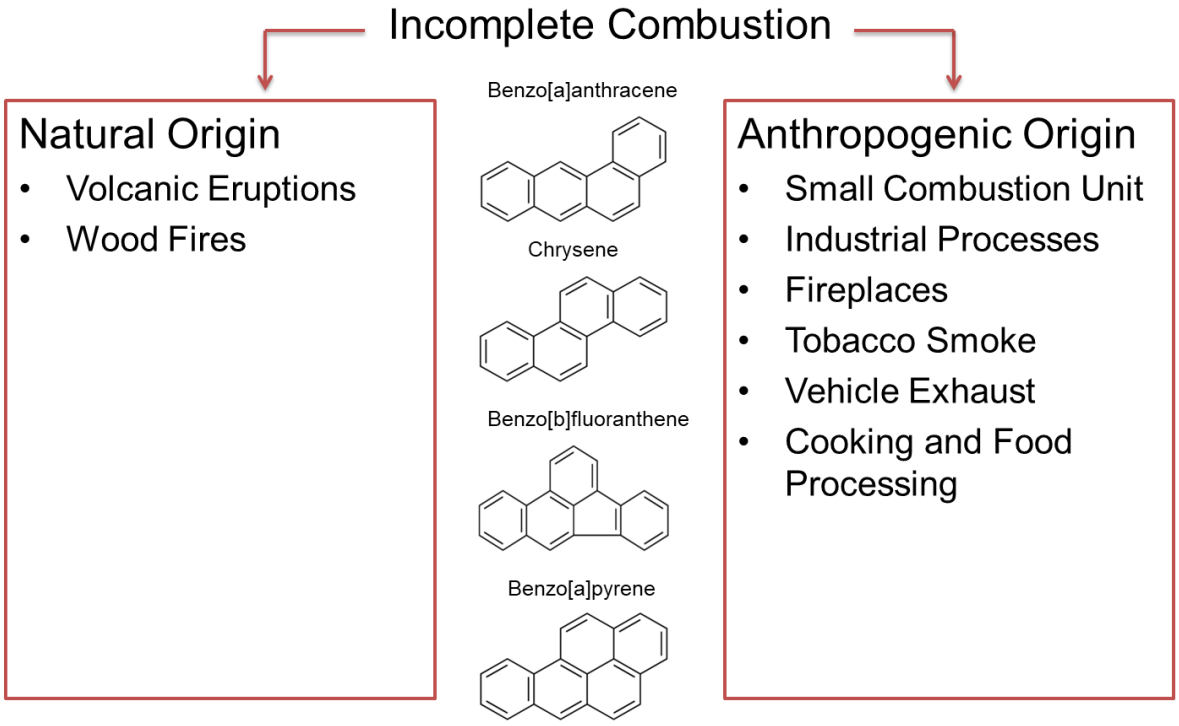


Fig 2: Chemical structures of the PAH4 and the formation of PAHs [adapted from 10]

Name (CAS No.)	Melting point (°C)	Boiling point (°C)	Molecular formular	Hazard designation
Benzo[a]anthracene	160	435	$C_{18}H_{12}$	May cause cancer, toxic to aquatic organisms
Benzo[a]pyrene	175	495	$C_{20}H_{12}$	May cause cancer, genetic defects, harm to unborn children, impair fertility, toxic to aquatic organisms
Benzo[b]fluoranthene	168	481	$C_{20}H_{12}$	May cause cancer, toxic to aquatic organisms
Benzo[k]fluoranthene	217	481	$C_{20}H_{12}$	May cause cancer, toxic to aquatic organisms
Benzo[j]fluoranthene	166	480	$C_{20}H_{12}$	May cause cancer, toxic to aquatic organisms
Chrysene	255	448	$C_{18}H_{12}$	May cause cancer, toxic to aquatic organisms

Table 1: Selected PAHs and their chemical properties with their impact on human health and the environment [adapted from 11]

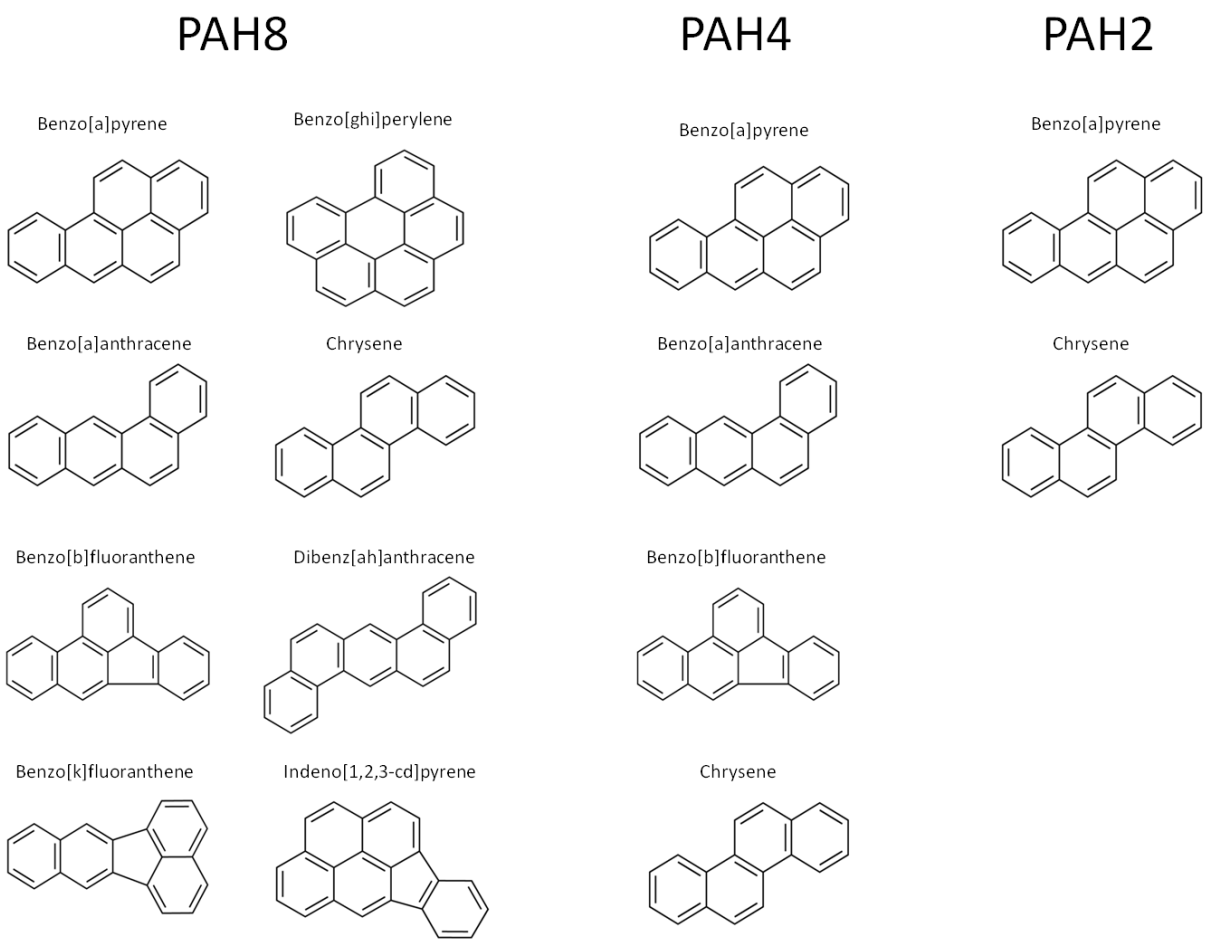


Fig 3: Chemical structure of priority PAHs investigated by the EFSA [6]

PAHs are generally non-reactive. In order to exert the genotoxic and carcinogenic effect PAHs need metabolic activation through mainly P450 enzymes in the liver [12]. In this process the formation of reactive intermediates may occur exerting the genotoxic effects. In addition, it is known that PAHs can absorb light energy and enter an excited state, which in return transfers energy to other molecules. This process results in the formation of free radicals increasing the oxidative stress in biological cells. Furthermore, PAHs in the excited state are able to react with other molecules in the cell, i.e. proteins, nucleic acids, amino acids, etc, which again results in the formation of free radicals damaging the cells, ultimately leading to cancer [12].

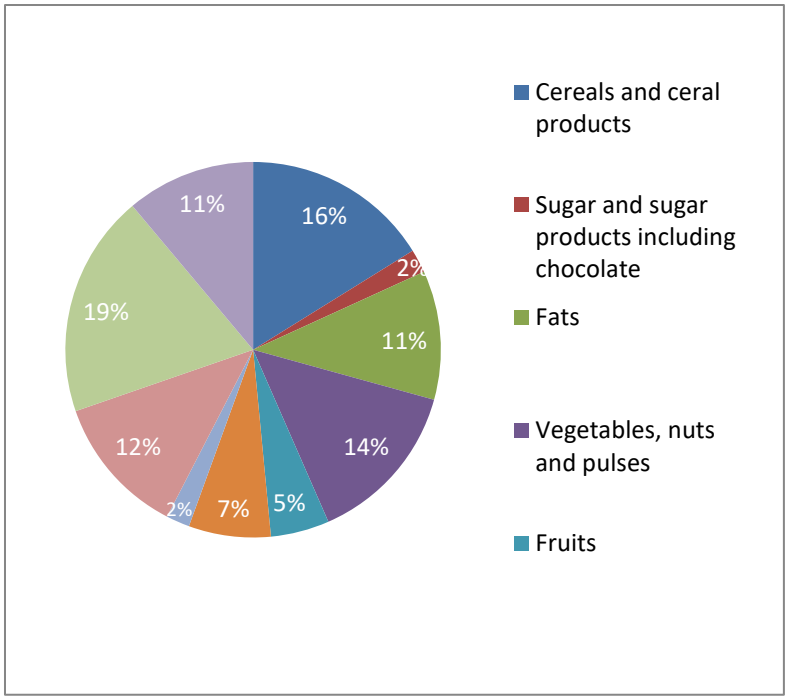
To calculate the consumer risk from exposure to PAHs the EFSA estimated the margin of exposure (MOE) with a bench mark dose lower limit set at 10% ($BMDL_{10}$) increase in tumor developing animals compared to a control group [6]. The MOEs of an average consumer for all PAH combinations were highly exceeding the 10000 mark indicating a low concern for consumer health for dietary exposure. However, high level consumers are closer or below 10000, which shows potential concerns for consumer health and the possibility of risk management steps. Regarding the occurrence of PAHs in certain foods the CONTAM panels conclusion was the use of the PAH4 or the PAH8 as indicator PAHs with the remark that the detection of PAH8 do not add much value compared to the PAH4 [6]. Different food commodities are therefore regulated with certain maximum level (ML) of BaP and the sum of the four PAHs (BaA, BaP, BbF and CHR) in the Commission Regulation 835/2011 amending the Regulation (EC) No 1881/2006, including oils and fats, cocoa beans and derived

products, coconut oil, smoked meat and products, smoked fish and products, smoked sprats, smoked crabs, smoked mollusks, cereal based foods for infants and children, infant formulae and dietary food for infants [13; 14]. The separate ML for BaP is still established for the comparison of previous and future data. In future regulations the ML of BaP will be reassessed regarding the need for a separate ML [14]. Since PAHs are considered to be carcinogenic, the decision for setting certain MLs is driven by the ALARA principle (**A**s **L**ow **A**s **R**easonably **A**chievable). The monitoring process also revealed lower background levels of PAHs. Additionally, technological advances in food preparations like smoking and roasting decreased PAHs contamination, thus the MLs for BaP and the sum of PAH4 were lowered in 2014 for the majority of the regulated food commodities [14]. In 2015 MLs for PAHs were established in different foods in the Regulation 1881/2006 amended by the Regulation 2015/1933 [15]. The added food commodities were cocoa fibres, banana chips (they are fried in coconut oil), certain food supplements derived from botanical ingredients and from propolis, royal jelly or spirulina, and dried herbs and spices [15].

2. PAH occurrence

2.1 PAHs in cereals

Cereals, cereal products and seafood were the highest contributor concerning the dietary exposure to the PAH4 (Fig. 4) [6, 16]. Although cereals and cereal products in general are not regulated with a respective ML, except for cereal based foods for infants, wheat flour was chosen to be validated in publication I, due to the high consumption of wheat and wheat related products in Europe [17]. The data for PAHs in vegetables and cereals is still limited, but low concentrations are expected. It has been evaluated that even these low levels highly contribute to the exposure of PAHs due to the high consumption of these products [14]. Kacmaz and colleagues investigated bread and breakfast cereals on the Turkish market for the occurrence of the PAH4. In all samples BaP and CHR was found and the concentrations were between 0.19 and 0.46 µg/kg for bread and 0.10 and 0.87 µg/kg for breakfast cereals, thus not exceeding the ML of 1 µg/kg [18]. In another study the contamination level of 19 PAHs in bread in the bakery chain was investigated by Ciecierska and Obiedzinski [19] in whole wheat grains, rye, three different flours from these grains, brans and their dough. The most abundant PAHs in whole grains of wheat and rye were light PAHs (PAHs comprising three to four rings). In wheat and rye bran, which is obtained after a grinding process of the grains, the amount of light PAHs decreased from an average of 78% to 69%. As for the heavy PAHs BaA and BkF formed the majority, while BaP, BghiP and IdcP remained undetected in the raw materials. The authors stated that the decrease of PAHs from whole grains to flour and brans is due to the environmental contamination, which is concentrated in the external parts of these foods. Similar PAH profiles were observed in bread with the exception that low levels of the PAH4 were detected in breads baked at higher temperatures. Additionally, BaP was only detected in the crust baked at the highest temperature. The remaining heavy PAHs including the dibenzopyrenes were not detected in bread [19]. The selection of the carcinogenic PAH4 as indicators for the occurrence of PAHs is therefore, according to this study a suitable choice. Higher baking temperatures lead to higher concentrations of the investigated 19 PAHs in all parts of the bread, including the loaf, crust and crumb. The authors concluded that the raw materials for the processed foods had a relatively low impact on the final PAH concentrations. Even though the concentrations of the PAHs were proportional to the baking temperature the levels were still low. Only when using unconventional high baking temperatures the ML for the sum of the PAH4 was exceeded [14; 19]. It has been reported that the calculated MOE derived from cereals and cereal products is eleven to twelve times higher than that reported from the EFSA opinion in 2008 [6; 19]. Nevertheless, a recent study conducted in Japan showed that the main dietary



lFig 4: Dietary exposure to PAH4 in Europe [adapted from lDuedahl-Olesen 2013; EFSA 2008]

exposure of the Beijing population concerning the heavy PAH (BaA, CHR, BbF, BkF, BaP, IcdP, BghiP and DahA) is derived from cereals (32.9%) and meat (37.0%) [12]. In contrast to the findings of Ciecierska and Obiedzinski [19] BaP was detected in all analyzed breakfast cereal products in the range from 0.2 to 16 µg/kg [20]. A study conducted in Latvia reported that 14% of all analyzed cereal samples exceeded the ML for the sum of the PAH4, although only 35 samples were taken [21]. The most contaminated breads were rye breads and the most abundant PAH found in cereals was CHR. The LODs for the PAH4 were between 0.002 and 0.006 µg/kg, therefore, this study applied a highly sensitive method for the detection of the PAH4, but the low number of samples analyzed does not allow any general assertions. The authors also compared their findings with other studies showing that, if their method would have been applied in other studies, the contamination profile would be more representative due to the lower LODs [21]. It is known that the available data for the occurrence of PAHs in cereal products is limited [14], but still most studies listed above analyzed a low number of samples ranging from 6 to 38 [18; 21-23]. One exception was the study conducted by Ciecierska and Obiedzinski [19] with 126 samples investigating PAHs in the bakery chain. In order to reach a conclusion for the establishment of a ML in cereals and cereal based foods future studies should focus on applying methods for low LODs, since low concentrations can be expected and a larger sample size.

2.2 PAHs in oil:

Several studies showed that also oils and fats of animal and plant origin highly contribute to the exposure to PAHs [6, 16, 24; 25;]. Oils are prone to contamination of PAHs due to their lipophilic character in two major ways: atmospheric contamination and during processing. Raw materials for oil production are dried with burning natural gas or even heating oil leading to contamination. Even though the refining process of oils reduces PAH concentrations by treating the oils with active carbon and deodorization [26] virgin olive oils, which are not refined, are highly valued in Europe [27]. Additionally, certain PAHs are affected in different ways in the refining process. Deodorization mostly affects light weight PAHs up to three rings, while the treatment with active carbon reduces the amount of heavy PAHs from four to six rings [61]. Additionally, the formation of PAHs during cooking with oil is influenced by various factors: oil temperature, volume of oil used, types of foods cooked and types of oils used [29]. Alomirah and co-workers [9] evaluated the concentration of BaP, PAH8, the sum

of BaP toxic equivalents and the sum of sixteen PAHs selected by the US Environmental Protection Agency (EPA) [30]. While the mean concentration of BaP, the PAH8 and the BaP equivalents were below the maximum limit of 2 µg/kg [14], higher concentrations occurred when analyzing the sixteen PAHs exceeding the ML of 25 µg/kg. Therefore, the sum of the sixteen PAHs cannot be compared to regulatory limits [9]. A study of Guillen and Sopelana [31] found a large number PAHs (33-44) in different seed oils with a slightly higher number in virgin olive oils (47-53). However, higher concentration were observed in seed oils compared to different olive oils, even with the higher number of PAHs found in virgin olive oils. Additionally, different batches of each oil brand were investigated revealing a significant difference in total PAH contents and heavy PAHs, which was the case in olive oils. The authors concluded that PAH patterns give insight into the source of contamination [31]. The investigation of 9 PAHs in different brands of olive oil showed that 97% of the virgin olive oil samples were below the ML of 2 µg/kg for BaP. This study was conducted before the regulation of the PAH4, therefore no sum of the PAH4 was reported [32]. Another study reported no olive oil and other oil samples exceeded the ML for BaP and the sum of the PAH4 [33]. The above cited articles in this chapter investigated edible oil contamination and almost no food product was exceeding the ML of 2 µg/kg. Nevertheless, the report of the EFSA [6] showed that edible oils and fats are the fourth highest contributor to dietary exposure concerning the PAH4.

2.3 Seafood, smoked seafood and meat products

According to Duedahl-Olesen [16] and the EFSA [6] seafood and seafood products are the highest contributor to dietary exposure to PAHs (Fig. 4). Seafood and seafood products are contaminated with PAHs in two major ways; environmentally or during food processing. Oil spills and leaks can contaminate locally harvested fish and seafood [34], while food processing techniques like roasting, grilling and smoking also contribute to the exposure of PAH. On the other hand, fish were caught at St. Helena in the South Atlantic near a historic wreck known to be leaking oil. It was reported that no fish sample contained four ring PAHs or heavier ones comprising no consumer health risk [35]. Nevertheless, oil spills are associated with unnatural high concentrations of PAHs in fish [36]. Seafood samples (shellfish, crab, shrimps) taken from 11 coastal cities in South China revealed detectable, but low concentrations of PAHs. The authors stated that the concentrations of the sum of 16 PAHs were slightly higher than those reported in Spain and lower than the area affected by the Deepwater Horizon oil spill in the Mississippi river [37]. Low concentrations were also observed when analyzing 280 samples in Korea [38]. In general PAH concentrations in fresh fish are low due to low contamination levels and the ability of the fish to metabolize PAHs avoiding accumulation in the muscle meat. Therefore, no ML has been established for fresh fish [14]. On the other hand bivalve mollusks accumulate PAHs more than vertebrate fish. Nevertheless, food processing including smoking along with adjustable smoking parameters increases the possibility of PAH exposure to the consumer.

Mohammadi and colleagues investigated 80 smoked fish samples with naphthalene being the most abundant PAH. The authors concluded that the fish's skin might protect the fish from high molecular PAHs like the PAH4, but not from "lighter" ones like naphthalene [39]. It is also important to note that the smoking procedure and the resulting PAH contamination depends on the wood type used. For example using alder for smoking resulted in higher contamination levels compared to using beech [40]. 25 PAHs were analyzed in 180 industrial

smoked fish products with the aim to evaluate critical processing parameters responsible for PAH contamination. In general indirect smoking resulted in lower levels of PAHs compared to direct smoking. In case of salmon, hot smoking lead to higher concentrations than cold smoking. The contamination with PAHs derived by smoking also showed different distributions in various body parts. The highest levels of PAHs were found in the skin, followed by the outer layer of the fish muscle and the inner part of the muscle [40, 60]. The PAH contamination in the skin was five times higher than in the underlying tissue, indicating that the skin acts as a barrier for PAHs. The authors concluded that the surface exposure to smoke and the fat content of the fish are two essential parameters for PAH contamination [40]. Essumang and colleagues [2] investigated PAH generation during smoking of fish with three types of wood and a smoking duration from 2 to 8 h in a traditional kiln. All fish samples (n = 108) smoke-cured with acacia and mangrove, so called hard woods, in the range from 2 to 8 h exceeded the ML of BaP set by the Commission [14]. The authors estimated a high consumer health risk with the consumption of fish smoke-cured with hard woods. The EFSA stated that the lowered ML in 2014 is achievable with the available knowledge and technology [14]. This applies in particular to the food industry with controlling the smoking procedure.

Despite the possible protective effect of PAH penetration of the fish skin, the above mentioned smoking procedures and the resulting PAH contamination also applies to meat products [41]. It has also been shown that sugar-smoking might be an alternative to traditional smoking with wood. The duration of the sugar-smoking process correlated with an increased PAH contamination, which also holds true for traditional smoking, despite that in the work of Chen and colleagues [41] no BaP was detected in all meat samples analyzed. An interesting factor concerning smoked meat products was reviewed by Simko [42]. The contamination with BaP in hot smoked sausages was lowered due to the cooking in boiling water. BaP was decreased from 4.8 µg/kg to 1.9 µg/kg after 20 min of cooking. Also the packaging of products affects the concentration of BaP. A smoked duck skin was analyzed prior to packaging in a low-density polyethylene material and was stored for 24 h. The initial concentration of BaP was decreased from 3.5 µg/kg to 0.9 µg/kg due to migration of BaP into the packaging material [43]. On the other hand PAHs are known to diffuse into the meat bulk after some time and even during the smoking process. Therefore, it would be beneficial to pack smoked meat as soon as possible to increase the chance of absorbing the PAHs into the packaging material, which should have a higher affinity to PAHs compared to the meat [42]. Another way to decrease PAH contamination is the utilization of liquid smoke flavoring (LSF). The time of exposure to LSF to reach the favored taste of the product is considerably reduced. It has been shown that LSF compared to traditional smoking reduces the BaP content up to 1666 times [42].

The scientific evidence shows that the contamination of PAHs in smoked seafood and meat products can be significantly reduced by either avoiding contamination by the use of the appropriate wood type, using liquid smoke flavoring and/or by decreasing the contaminated foods by cooking methods, the packaging material and the speed of the packaging process.

2.4 Home food preparation

Home food preparation has a higher impact on the exposure to PAHs than industrial food processing [5, 60]. Especially cooking oil fumes, generated at high temperatures by the interaction of edible oils and food components, are believed to increase PAH emission. The

effect on PAH formation was investigated in deep-frying and frying in different oils with chicken nuggets, potatoes, eggs and hairtails [29]. The formation and the composition of PAHs in deep-frying and frying depended on the oils used and to a certain degree on the cooked foods. The majority of PAHs detected had three to four rings (i.e. BaA and CHR) regardless of the cooking oil. Additionally, the gas-phase PAHs were more abundant (83%) than the particulate-phase PAHs. The majority of the five and six ring PAHs occurred in the particulate-phase. The authors concluded that deep-frying lead to a higher formation of BaP and total PAHs compared to frying due to the higher oil temperatures and volumes used. Regarding the investigated oils rapeseed oil showed the highest formation of PAHs, followed by olive oil, peanut oil and soybean oil [29]. Besides frying, grilling is a very popular way to prepare foods. Therefore, Saito and colleagues [44] investigated the emission of 19 PAHs when grilling corn, trout, beef, prawns, and pork. The particles of the oil mist were collected, which revealed higher concentrations of PAHs in fatty foods, being pork, trout and beef. The PAH profile consisted mainly of three and four ring PAHs with none of them being one of the PAH4. An average of 27% consisted of heavier PAHs like BbF, BaP and IcdP. Another contamination risk bears barbecuing fatty meat. The thermal decomposition of fatty droplets might lead to a higher PAH contamination of the products compared to barbecue practices avoiding fat pyrolysis [6]. Rose and colleagues investigated the formation of PAHs by different cooking procedures [5]. The formation of 27 PAHs was monitored after grilling, frying, barbecuing and roasting with different heating sources and distances from the heating source various kinds of meat and toasting white bread. In general, the PAH formation when grilling, roasting and frying showed little evidence. Beef burgers barbecued with charcoal showed the highest amount of the PAH8 when compared to the other meat varieties and sausages. The highest concentration could be observed when barbecued with charcoal and woodchips. An increase in cooking time revealed moderate increases of PAHs in some foods, but on the other hand the concentration in beef burgers seemed to decrease. Overall the authors stated that many barbecued foods would exceed the ML of PAHs [5; 13], making it a concern of public health.

Industrial smoking of fish and meat products under good manufacturing practice (GMP) conditions results in low levels of PAH contamination. Controlling the process of smoking is of utmost importance to ensure low contamination levels, since it has been shown that uncontrolled conditions, like home smoking and smoking in developing countries without the application of GMP principles, lead to high levels of PAHs [42]. Since home smoking is not as common as barbecuing there is still no reason to underestimate the risk of exposure. For example home smoking of fish resulted in BaP concentrations up to 11 µg/kg [60], which is five times the ML of today [14].

Various institutions have introduced guidelines to avoid or minimize the formation of PAHs while barbecuing, outlining the importance of the effects of PAHs on human health. [45].

2.5 Sample preparation and detection of PAHs

PAHs are light sensitive and can be decomposed by photoirradiation and oxidation, which should be avoided during the sample preparation. Low molecular PAHs (PAHs up to four rings) are more volatile than the heavier PAHs, which can result in possible losses during evaporation to dryness. For the determination of the PAH4 evaporation to dryness is not associated with high analyte loss, since BaA, BbF, CHR and BaP are all four to six ring PAHs.

The sample preparation of PAHs is usually laborious and time-consuming due to the lipophilic properties of these compounds, the fat content of the matrices and the required low detection limits [46]. In order to reach low LODs and reduce the maintenance of the chromatographic system the removal of fat is of utmost importance. The traditional sample preparation for the determination of PAHs consists of three steps including saponification, liquid-liquid extraction (LLE) and a clean-up usually a solid phase extraction (SPE) or a gel permeation chromatography (GPC). After diluting the sample a single SPE can also be performed for the extraction and clean-up for edible oils. A Soxhlet extraction or pressurized liquid extraction (PLE) is most commonly applied for the extraction of the fat content of the matrices [2; 16]. Further clean-up steps include SPE, GPC (or size exclusion chromatography (SEC)) and column chromatography [37, 47]. An overview of various applications is given in table 2.

Recent studies have introduced the QuEChERS (quick, easy, cheap, effective rugged and safe) sample preparation technique for the determination of PAHs. The QuEChERS extraction and clean-up procedure is quite popular due to the reduction of time and costs. Initially the QuEChERS method was developed for the analysis of pesticides in fruits and vegetables [48]. Since then, various modifications have been published concerning a variety of analytes and matrices, also including non-food matrices like biological fluids, soil and sediments [49].

The main analytical methods for the determination of PAHs are HPLC coupled to a fluorescence detector (FLD) and GC-MS [6]. Both methods have shown to deliver accurate results with good sensitivity as compared to HPLC coupled to an ultraviolet or a photo-diode array detector. The detection of PAHs with GC-MS was also encouraged with the possibility to use isotope labeled internal standards. Nevertheless, it is very important to note that the separation of existing isomers and chrysene from triphenylene must be accomplished chromatographically in order to avoid false positive results. Fig. 5 shows an example of a GC-MS/MS setup, which is able to separate the interfering PAHs concerning the PAH4.

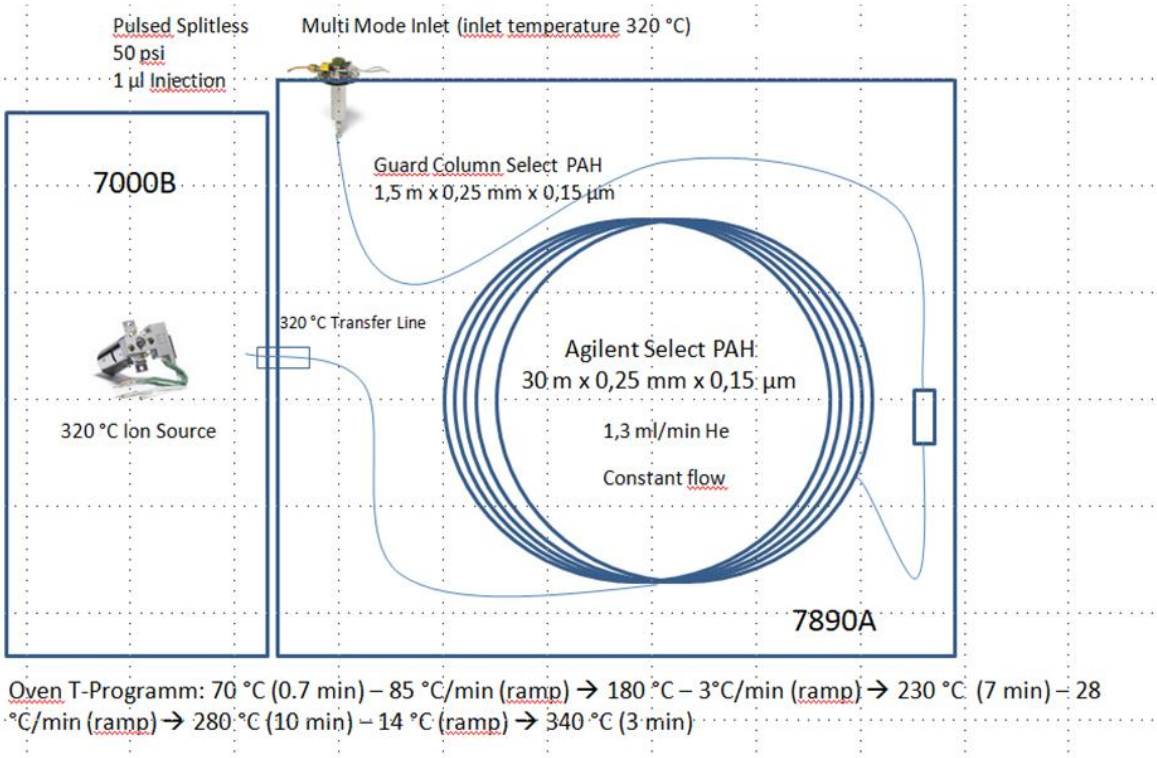


Fig 5: GC-MS/MS setup for the confident separation of interfering PAHs used in publication I

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References	Matrix	Sample preparation	Chromatographic system	Numer of PAHs	Limit of detection
Wang et al., 2016	Oil	LLE, GPC	LC-FLD	14	2.5 - 10 µg/kg
Guillen et al., 2004	Olive pomace oil	LLE, SPE	GC-MS	23	0.06 - 0.25 µg/kg
Moret and Conte 2002	Oil	Dilution with n-hexane and SPE	LC-FLD	15	-
Veyrand et al., 2007	Oil	ASE, SPE	GC-MS/MS	19	0.01 – 0.15 µg/kg
Simon et al., 2007	Oil	GPC, SPE	GC-HRMS	EU 15 + 1	-
Simon et al., 2007 (PT analysis)	Oil	Saponification, SPE	GC-MS	EU 15 + 1	-
Simon et al., 2007 (PT analysis)	Oil	LLE, SPE	LC-FLD	EU 15 + 1	-
Simon et al., 2007 (PT analysis)	Oil	Saponification, LLE, SPE	GC-HRMS	EU 15 + 1	-
Simon et al., 2007 (PT analysis)	Oil	DACC	LC-FLD	EU 15 + 1	-
Kishikawa et al., 2003	Milk	Saponification, LLE	LC-FLD	12	0.0013 – 0.076 µg/kg
Lee et al., 2015	Milk	Saponification, LLE, SPE	GC-MS	8	0.04 – 0.20 µg/kg
Kayali-Sayadi et al., 1998	Tea	SPE	LC-FLD	8	0.016 – 0.14 µg/kg
Chen and Lin., 1997	Meat	Soxhlet, LLE, SPE	GC-MS	16	5 - 50 pg
Chen et al., 2014	Meat	MAE, column chromatography	GC-MS	43	0.053 – 6.8 µg/kg
Kamankesh et al., 2014	Meat	MAE-DLLME	GC-MS	16	0.15 – 0.3 µg/kg
Ledesma et al., 2014	Meat	Sonication, SPE	GC-MS	1 (BaP)	0.05 µg/kg
Mottier et al., 2000	Meat and coconut oil	Saponification, LLE, SPE	GC-MS	16	0.06 µg/kg
Essumang et al., 2013	Fish	Soxhlet, column chromatography	GC-MS	16	1.00 – 2.00 µg/kg
Lee et al., 2015	Fish	Alkaline degradation, LLE, SPE	GC-MS	8	0.12 – 0.20 µg/kg
Mohammadi et al., 2013	Fish	MAE-DLLME	GC-MS	16	-
Ni and Guo 2013	Seafood	Soxhlet, GPC, column chromatography	GC-MS	16	0.125 µg/kg
Duedahl-Olesen et al., 2010	Fish	PLE, GPC, SPE	GC-MS	25	0.2 – 2.6 µg/kg
Hwang et al., 2012	Seafood	SPE	GC-MS	16	0.01 – 0.05 µg/kg
Ciecierska and Obiedzinski 2013	Cereals and cereal products	Sonication, GPC	LC-FLD, GC-MS	19	0.06 – 0.47 µg/kg
Kacmaz 2016	Cereals and cereal products	Sonication, SPE	GC-MS	4	0.01 – 0.03 µg/kg
Kacmaz et al., 2016	Cereals and cereal products	PLE, SPE	GC-MS	4	0.05 – 0.015 µg/kg
Rozentale et al., 2016	Cereals and cereal products	Sonication, GPC, SPE	GC-MS/MS, GC-HRMS	4	0.002 – 0.006 µg/kg

DACC…Donor acceptor column chromatography

MAE…microwave accelerated extraction

DLLME…dispersive liquid-liquid microextraction

GC-HRMS…gas chromatography high resolution mass spectrometry

GC-MS…gas chromatography mass spectrometry

GC-MS/MS…gas chromatography tandem mass spectrometry

LC-FLD…liquid chromatography fluorescence detection

Table 2: Determination of PAHs in various food commodities with the respective sample preparation and detection

2.6 QuEChERS applications

The QuEChERS method is a user friendly sample preparation and an alternative to LLE and SPE. The samples are homogenized and analytes are extracted with an organic solvent with a subsequent partitioning step using a mixture of salts like anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl). The supernatant is decanted for a further clean-up, namely a dispersive solid phase extraction (dSPE) using various salts and sorbents to remove co-extracted materials including lipids, sugars, organic acids, proteins, pigments and excessive water (Fig. 6).

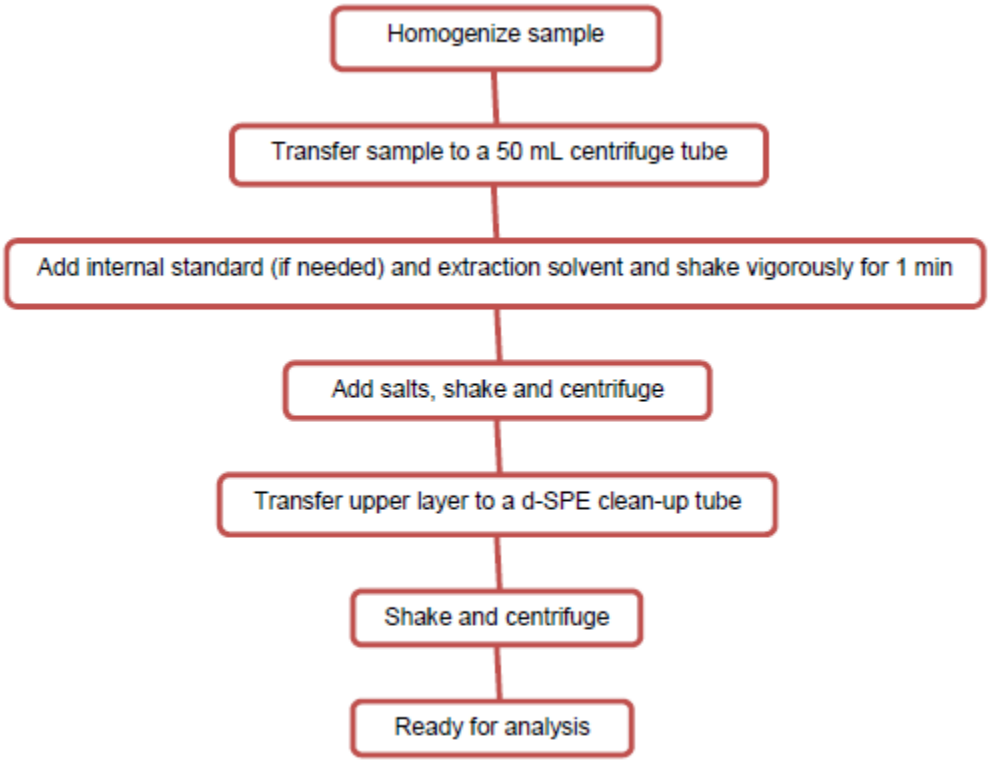


Fig 6: Flow chart of the QuEChERS sample preparation procedure

The most frequently used QuEChERS applications include the original unbuffered method [48], i.e. the European EN 15662 norm [50], the mini-multiresidue method [51] and the AOAC Official 2007.01 method [52]. The original unbuffered method is mostly used for the determination of non-ph sensitive pesticides, whereas the AOAC Official 2007.01 and the EN 15662 method are buffered and therefore used for ph sensitive analytes [53]. The d-SPE sample clean-up can be conducted with different salts and sorbents to meet the desired removal of co-extracted materials. Sugars, fatty acids, organic acids and some polar pigments are usually removed by using primary secondary amine (PSA). The sample clean-up with a mixture of anhydrous $MgSO_4$, PSA and C18 (octadecylsilan) is used for the removal of residual or co-extracted fat. In order to remove pigments of intensely colored samples graphitized carbon is utilized, while samples with high carotenoid or chlorophyll content are cleaned-up with a mixture of PSA and graphitized carbon. For all other samples a clean-up with a mixture of $MgSO_4$ and PSA is recommended [53]. Recently zirconia based clean-up sorbents (Z-Sep) have been

introduced as an alternative to the combination of PSA and C18 to remove pigments and lipids (Fig. 7). Z-Sep is a mixture of C18 and zirconium dioxide with a proportion ratio of 2:5. Z-Sep+ is zirconia and C18 dual bonded to silica. Despite the absorbent capabilities of Z-Sep concerning phospholipids, also electrostatic interactions have been observed regarding the removal of carboxylic acids. The absorbent mechanism is also ph dependent [54]. The clean-up with Z-Sep was investigated with avocados and almonds for pesticides, both comprising a high fat matrix [54]. Samples were extracted with ethyl acetate and Z-Sep was found to yield higher recoveries than Z-Sep+, which is used to remove larger amount of lipids and fats. A multi-class, multi-residue method for the determination of polychlorinated biphenyls (PCB), polybrominated diphenyl ethers, novel flame retardants and PAHs in fish was developed by Sapozhnikova and Lehotay [55]. The following QuEChERS clean-up sorbents regarding recoveries and relative standard deviations (RSD) were investigated: Z-Sep, Z-Sep+, C18 and PSA. Higher variability in recoveries and higher background noise were observed when samples were cleaned-up with C18 and PSA and Z-Sep+. Therefore, the method was validated with the Z-Sep clean-up [55].The latter study concludes that Z-Sep is superior to the other investigated sorbents. To the contrary concerning PAHs we did not find any differences when samples were cleaned-up with C18 or Z-Sep in publication I.

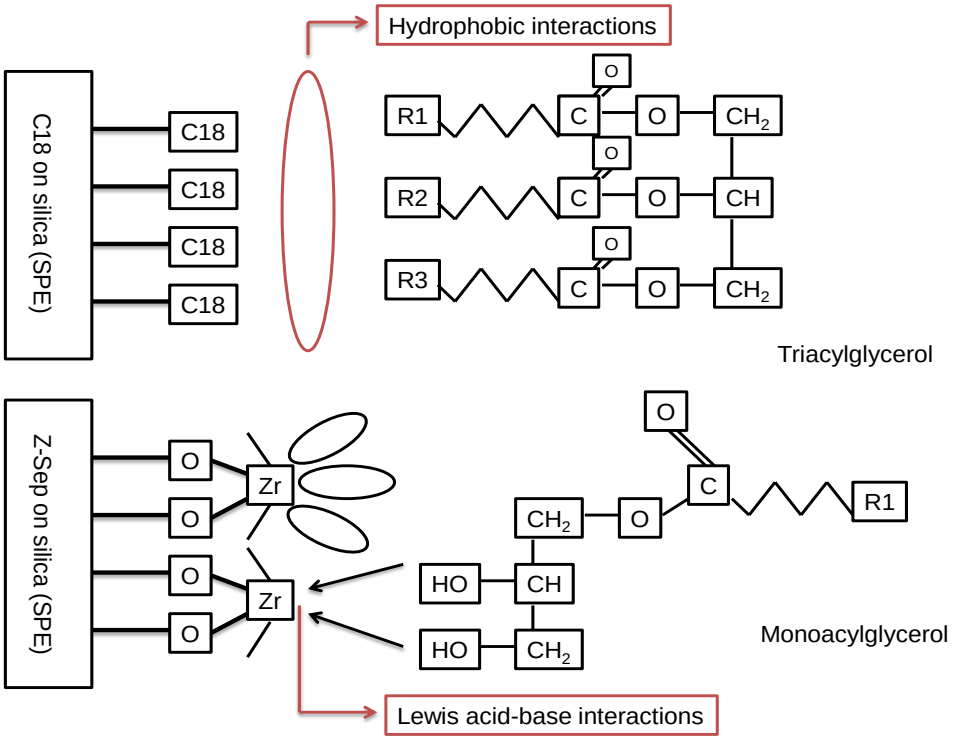


Fig 7: Retention mechanism of C18 and Z-Sep on silica [adapted from 56]

The latest application in the QuEChERS methodology is the enhanced matrix removal-lipid (EMR-lipid) sample preparation, which is advertised as a more effective clean-up for difficult matrices, especially fatty matrices [57]. For the determination of PAHs in salmon the d-SPE clean-up is applied prior to the removal of excess water, which is the subsequent step [57]. The removal of fats and lipids of the EMR-lipid material is due to its gel permeations chromatographic clean-up, separating molecules based on their size. Some authors have investigated the extraction efficiency of EMR-lipid on pesticides in fatty matrices [58; 59; 62]. Even though an official application has been published by

Agilent Technologies to our knowledge there are no publications concerning EMR-lipid and the determination of PAHs. Further experiments regarding the clean-up efficiency and recoveries comparing the EMR-lipid applications with other traditional QuEChERS clean-ups for the determination of PAHs are encouraged.

Initially acetonitrile was used for the extraction of pesticides from fruits and vegetables [48]. Modifications of this method range from adjusting vortexing and shaking duration [63] to different variations of clean-up sorbents [55; 64] and extraction solvents [65-67]. Applications of the QuEChERS method for various matrices concerning the determination of PAHs is given in table 2.

The QuEChERS method has also been modified and combined with other clean-up procedures. Pfannkoch and colleagues used the QuEChERS extraction with acetonitrile and the addition of salts followed by a stir bar sorptive extraction (SBSE) in seafood to concentrate the PAHs and remove coextracted materials [68]. A combination of the QuEChERS extraction with acetonitrile and a subsequent SPE was also applied [69; 70]. The QuEChERS sample preparation method is known for the high sample throughput. Not all of the cited studies in table 2 fulfilled the analytical criteria of the Regulation 836/2011, which includes LOD and LOQ of 0.3 µg/kg and 0.9 µg/kg, respectively. Nevertheless it has been shown that low detection limits can be achieved and all regulatory requirements can be met with the QuEChERS method. There is an obvious reduction in time and a higher sample throughput regarding the sample preparation of the QuEChERS method compared to the conventional sample treatment of PAHs. Cost effective and fast detection methods can further promote the monitoring process of these environmental and process contaminants. Apart from the detection instrument, the need for equipment is minimized for the sample preparation and therefore, the whole procedure for the determination of PAHs in food is more affordable for laboratories. Vice versa laboratories are able to promote the analysis of PAHs more cheaply, which possibly leads to higher food safety standards. The developed method in publication I achieved these goals, while all the analytical requirements were fulfilled [46].

3 Two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS)

The complexity of food products with interfering co-extracted materials from the sample preparation can make it difficult or even impossible to determine various compounds of interest in a monodimensional liquid chromatography (LC) system. The analysis of foods is usually conducted with one chromatographic dimension. Considering the complexity of various matrices, monodimensional chromatography might reach its limits resulting in unsatisfactory results. The aim of recent research to improve the resolving power of chromatographic systems and reduce sample preparation lead to the development of different 2D-LC systems. Multidimensional LC is generally distinct into “heart-cut” and “comprehensive LC” (LCxLC). The difference between these two techniques is the fractionation process. In heart-cutting some selected fractions are transferred from the first dimension (1D) to the second dimension (2D) containing the analytes of interest (Fig. 8). In LCxLC the whole sample is applied to both dimensions for separation (Fig. 9) [71].

An LCxLC system should (1) implement two separation dimensions for a sample mixture, (2) ensure the separation of the analytes in the 1D being consistent in the 2D and (3)

obtain the elution characteristics of both dimensions [72]. The term “orthogonality” describes a 2D-LC system with two independent separation mechanisms complementing their selectivities. The two chromatographic columns are i.e. connected via an interface with the function to cut and release fractions of the 1D to the 2D.

In general there are two types of LCxLC approaches; offline and online systems (Fig. 10). An offline collection of fractions with a concentration step of the fractions and the reintroduction of these fractions at a later time onto the 2D is the classical approach using an LCxLC system offline [73]. Offline methods are easier to apply than online approaches, but some downsides must be considered. These systems are difficult to automate, time-consuming, vulnerable to sample loss and yield low reproducibility. In online systems the fractions are collected in an interface with a sampling loop between the 1D and the 2D, while the effluent of the 1D is collected and directed to the 2D. In contrast to offline approaches, these systems are more reproducible and automated with faster analysis times. On the other hand, online methods are more difficult to implement [72]. Furthermore online LCxLC can be distinct in “continuous” and “stop-flow” mode. In the stop-flow mode the interface optionally consists of a valve without trapping or storage columns. The switching of the valve stops the flow for the 1D and enables the 2D to separate the eluted fraction. The flow on the 1D is restarted after one cycle and another fraction is collected for the subsequent separation on the 2D. This LCxLC system can be considered to be more time consuming, than systems with continuous flow rates. The application of a trapping column in a 2D-LC system in stop-flow mode has also been developed (Fig. 11) [74].

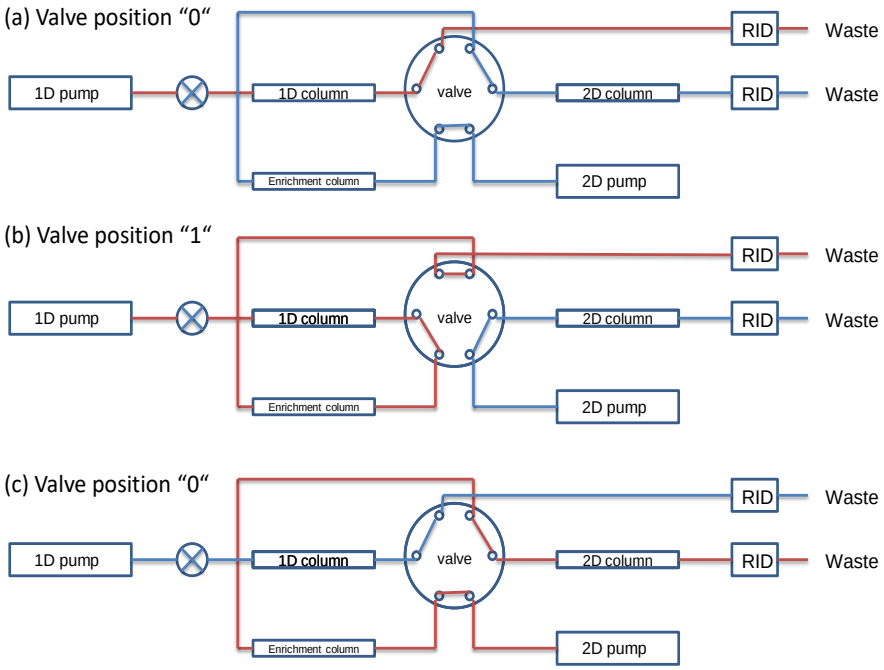


Fig 8: Schematic of heart-cutting 2D-LC approach. (a) Illustrates the heart-cutting procedure; (b) describes the storing of the analytes onto a enrichment column and (c) shows the separation on the 2D. (RID; refractive index detector) [adapted from 75]

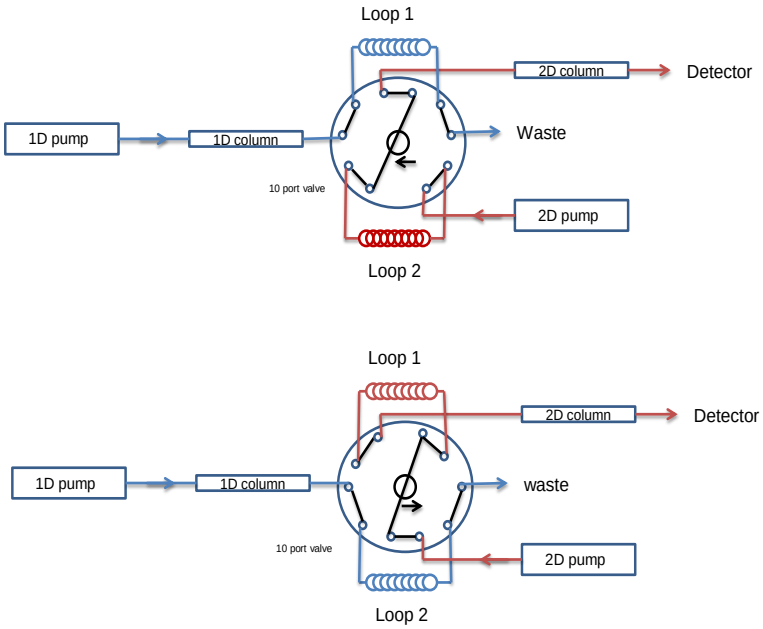


Fig 9: An example of a LCxLC interface with a 10-port valve and two sampling loops. The top of this figure describes how the effluent of the 1D is loaded to loop 1, while the fraction collected in loop 2 is backflushed to the 2D. The bottom describes the transfer of the fraction in loop 1 to the 2D, while the effluent of the 1D is loaded to loop 2 [adapted from 76]

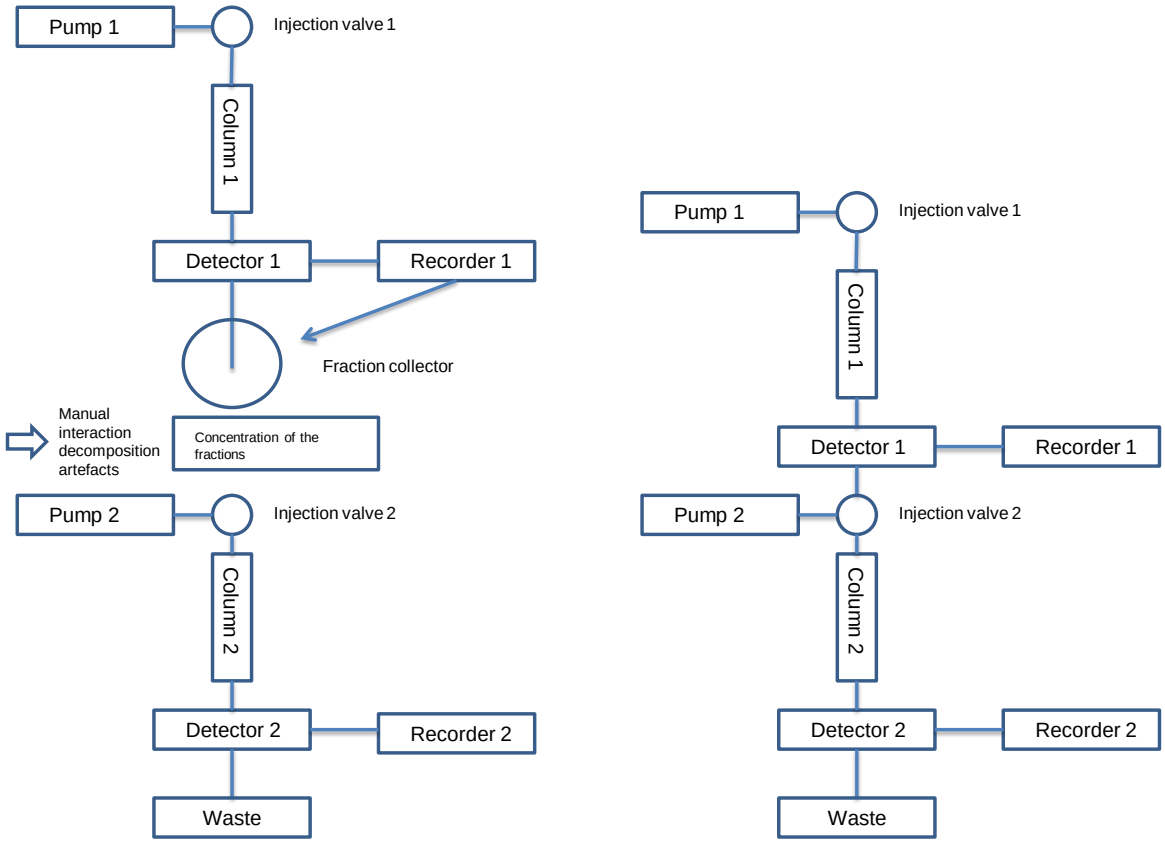


Fig 10: The left side of this graph illustrates an offline 2D-LC approach with a manual collection and concentration step. On the right an online 2D-LC is outlined with replacing the collection and

concentration step with a sampling loop. This system can also be operated in stop-flow mode [adapted from 73]

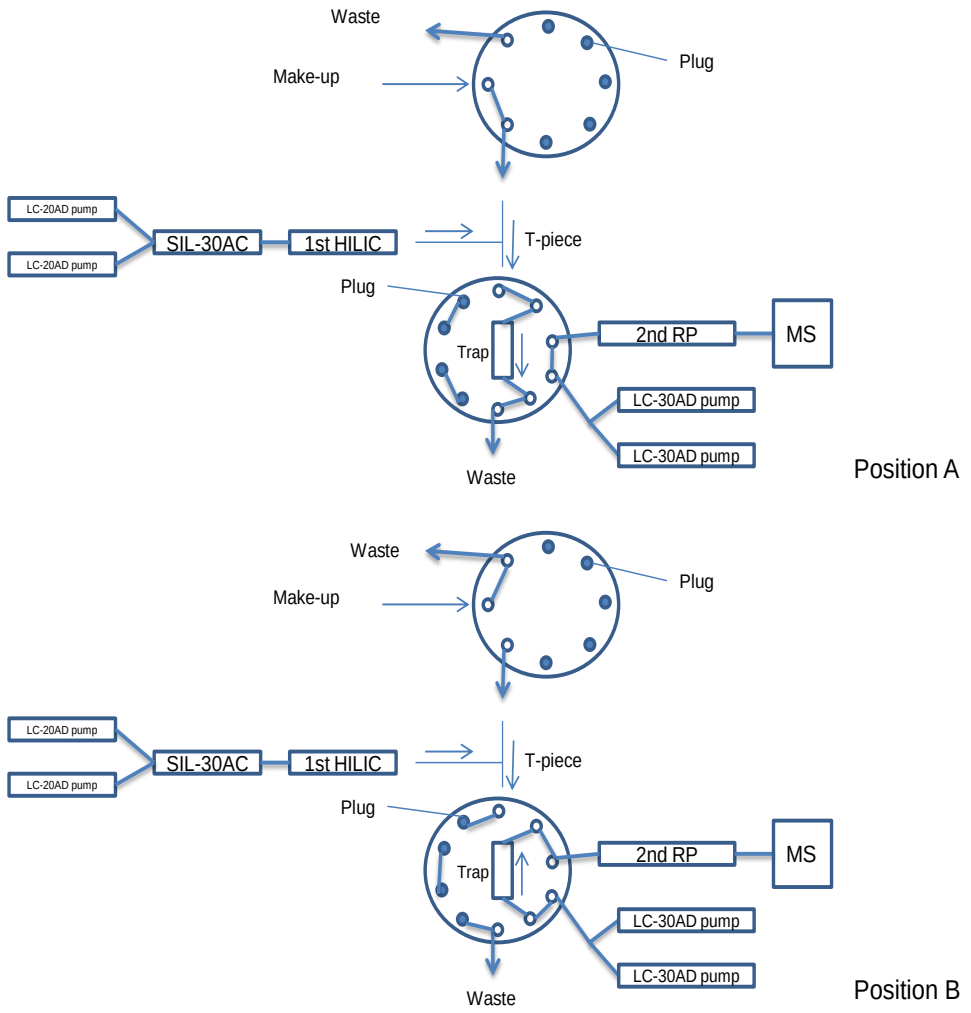


Fig 11: An example of an automated stop-flow 2D-LC system for the analysis of lipids. Position A describes the trapping of the analytes after the elution from 1D. Position B shows the elution from the trapping column and the separation in the 2D [adapted from 74]

Since the invention of the first online LCxLC system [73] further research has been dedicated to use the possibilities and advantages this technique has to offer. The first online 2D-LC approach consisted of a column filled with gel permeation chromatographic material in the 1D and the 2D being a reversed phase (RP) dimension. The columns were connected by an interface consisting of an eight-port loop valve [73]. LCxLC systems that have been developed mainly apply 8 and 10 port valves with two sample storage loops (Fig. 11), or no storage loop with one column being connected to two parallel columns (Fig. 12). The flow rate of the 1D in an LCxLC system with a storage loop must be lower than that of the 2D in order to perform properly. There is a wide variety of 2D-LC systems applied for the analysis of various compounds with a combination of different phases [72].

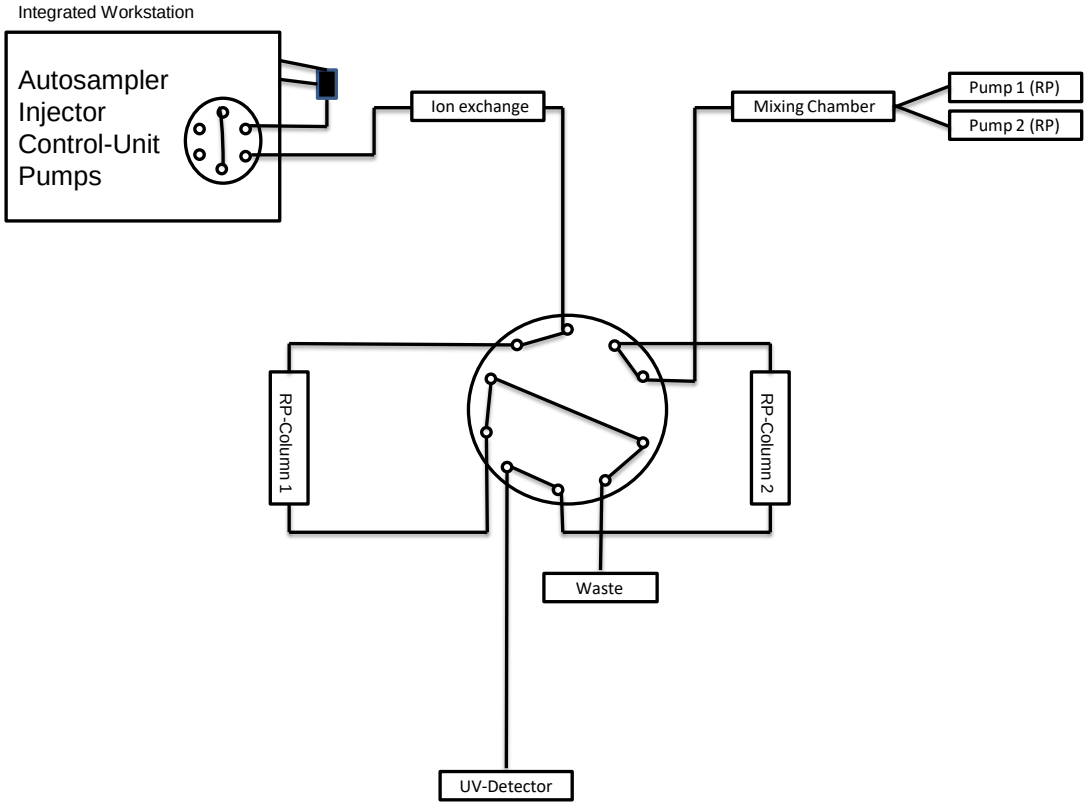


Fig 12**:** Example of a 2D-LC system using a parallel column assembly with no storage loops. The analytes eluting from the 1D are enriched alternatively on the head of each of the RP columns in a given time interval. When one RP column eluted all other analytes were directed to the other RP column [adapted from 77]

Various phases with different separation mechanisms can be applied bearing in mind the possibility of mobile phase immiscibility, precipitation of buffer salts and stationary column phase incompatibilities [72]. Incompatibilities in mobile phases arise when the elution strength of the effluent of the 1D is high for the 2D, which negatively affects peak capacity and resolution. Even when using a hydrophilic interaction chromatography (HILIC) column in 1D and a reversed phase column (RP) in the 2D these events may occur. Mobile phase incompatibilities can be resolved by increasing the flow rate in the 2D with the disadvantage of a higher dilution factor resulting in a decrease in sensitivity [74]. The length of the 2D column is usually shorter to avoid overlapping of the fractions of the 1D. This overlap would otherwise result in decreased separation ability of the 2D. Because of the continuous re-injection of the sample loop content onto the 2D, the sampling period must be the same as the time needed for analysis in the 2D. Another way to avoid mobile phase incompatibilities is the incorporation of a trapping column between the two dimensions. This enables the system to displace the effluent of the 1D while the analytes are focused on the trapping column and are eluted onto the 2D (Fig. 13) [74, 78]. These so called “packed loop interfaces” enable a re-concentration of the target compounds with narrow sample bands being transferred onto the 2D. To successfully focus the analytes on such an interface it is of utmost importance to choose a weak solvent in the 1D and a strong solvent in the 2D.

The development of a 2D-LC system not only requires the above mentioned necessities, but also the applicability with the detector. Usually a RP column is chosen for the 2D when analyzing with MS due to the amounts of organic solvents improving the ionization process. Mixed-bed columns, like strong cation exchange (SCX) and RP materials can be directly connected to MS with the gradient pulse increasing the solvent strength with a subsequent gradient for the analyte elution of the 2D. The applicability of a 2D-LC system with MS requires the following: (1) an interface to remove the mobile phase prior to the analytes entrance into the MS, (2) the chemical properties of the analytes includes low volatility and temperature instability and (3) a constant flow rate must be delivered by the LC pump [79].

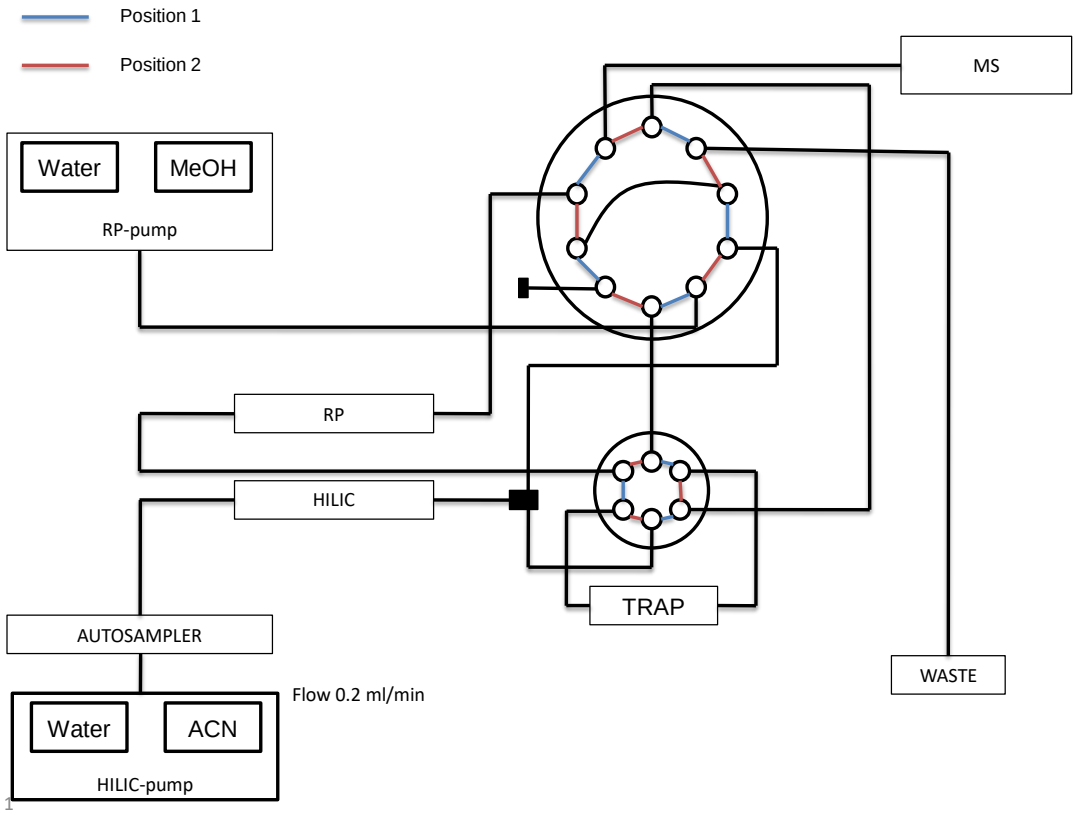


Fig 13: An example of using a packed loop interface in a 2D-LC setup. Analytes are saved on the trap column prior to the elution onto the RP column. Therefore, solvent immiscibilities are avoided and the separation on the RP column is maintained [adapted from 78]

The theoretical comparison of a monodimensional LC with a 2D-LC is well established. Furthermore, experiments have shown that the resolution of a 2D-LC system is superior at longer analysis times with more than 1 h, while monodimensional LC is better suited for short analytical runs with less than 1 min. While a monodimensional LC is suggested for the analysis of simple matrices, a 2D-LC should be considered when more complex mixtures are analyzed [28]. The superiorities of both systems are clear at extreme analysis run times. Nevertheless, it is still difficult to decide, which application yields the greater advantage for the determination of the target compounds.

3. 1 2D-LC method development

The method development of a 2D-LC system is a three step process including (1) the instrumental design, (2) the column selection and (3) optimization of the separation [80]. First, the selectivities of choice for both dimensions should be investigated with a monodimensional approach regarding the retention mechanism of the target analytes. Furthermore one must keep in mind the limitations of the arbitrariness of combining two LC columns, like i.e. solvent incompatibilities. An overview of the method development steps is given in Fig. 14.

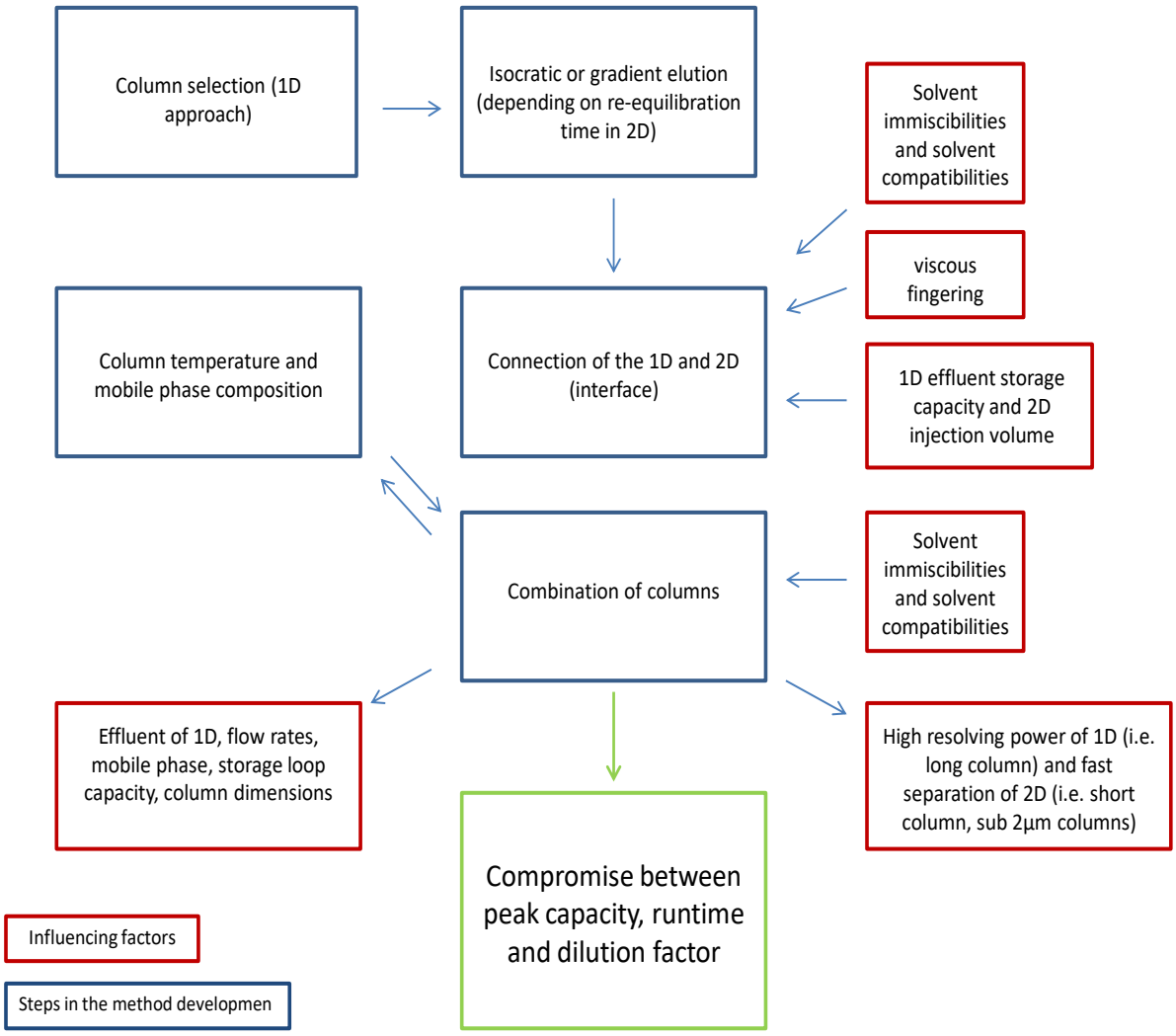


Fig 14: Main steps of the method development of a 2D-LC system with possible influencing factors and limitations.

Depending on the variety of compounds and their respective retention behavior an isocratic or a gradient elution is applied. A constant mobile phase might result in unsatisfactory separation of the target analytes. The gradient elution is therefore preferred in resolving complex mixtures due to the following advantages: an equal distribution of the analytes throughout the chromatogram, modified retention mechanisms and consistent peak widths. The modifiable parameters in a gradient solution vary for the retention mechanism of the column. In RPLC the amount of the organic solvent is modified, while in ion-exchange columns the ionic strength and the pH are adjusted. The time needed for equilibration in the 2D using a gradient elution must also be considered, thus reducing the

separation time. Columns with a long re-equilibration time are therefore restricted for the separation in the 2D like ion exchange columns [80]. The advantage of using a gradient elution in the 2D is the controllable elution strength for strongly retained analytes to ensure their detection within the given chromatographic analysis, thus avoiding carry-over into the subsequent run.

The next step of the method development concerns the connection of the 1D and 2D with an interface. Typically the interface consists of a two-position 10- or 8-port switching valve with two storage loops to enable fractionation of the 1D effluent. One sample loop is collecting the 1D effluent, while the stored fraction in the second loop is injected into the 2D.

One of the issues researches might face during the 2D-LC development is the so called “undersampling” or “remixing problem” in LCxLC. Undersampling is the loss of resolving power of the 1D [81]. Aiming for two to three fractions being transferred for every 1D peak is a good goal for avoiding the undersampling issue [82].

As mentioned above in an LCxLC system the entire sample must be subjected to both dimensions. Therefore, the separation of the 2D must be fast enough to comply with the amount of the 1D effluent, the separation of the 2D must not be impaired by the 1D flow rate and mobile phase and the storage capacity of the sample loops must suffice for the duration of the gradient. These principles restrict parameters, like flow rates, column dimensions and sampling storage loops capacity. For the selection of the column dimensions it is of utmost importance to utilize a column with a high resolving power for the 1D, while the separation of the 2D should be fast and applicable with the detector. The typical LCxLC column setup consists of a long 1D column (100-250 mm), ensuring resolution, while the 2D is a short column (≤ 50 mm). Especially the length of the 2D is important, when using a gradient elution due to the decrease in the re-equilibration time at the end of the analytical run. Furthermore, the chosen dimensions affect the dilution factor mostly concerning the 2D separation.

The theoretical peak capacity plotted against the analysis time yields pareto-optimal fronts with different column diameters in the 1D and 2D [83]. These results show that a diameter ratio (2D diameter/ 1D diameter) between 4 and 7 unravel the highest peak production (Fig. 15). A low flow rate in the 1D with a small diameter maintaining satisfactory separation leads to low volume fractions being collected by the interface and re-injected onto the 2D. The larger diameter of the 2D enables higher volume fractions to be injected without losing peak capacity and additionally, higher flow rates can be applied leading to a fast separation in the 2D. The downside of the high flow rates and the increased re-injection of small volumes onto the 2D is the higher dilution of the analytes. Developing LCxLC methods requires a compromise between peak capacity, the chromatographic runtime and the dilution factor [80].

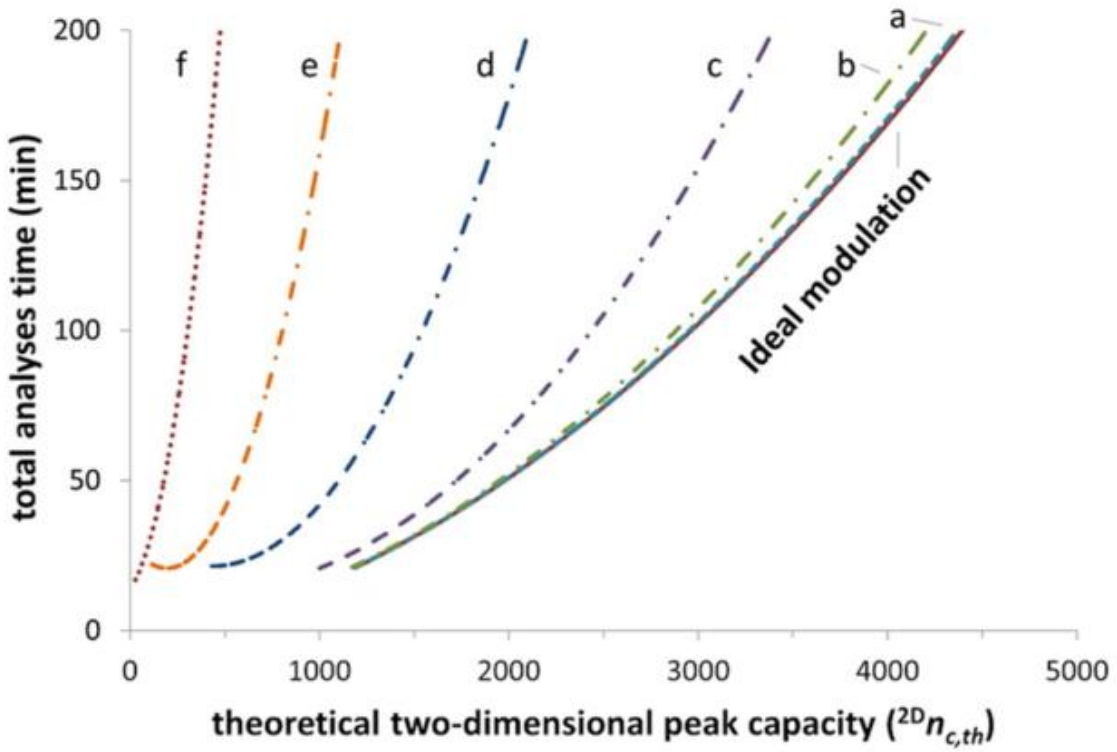


Fig 15: Pareto-optimal fronts are used to optimize theoretical peak capacity. The highest peak capacity ($^{2D}n_{c,th}$) is characterized by high $\frac{2D\ dimensions}{1D\ dimensions}$ ratios, which correlates to (a) being 7. Therefore, narrow 1D columns with intermediate particle size and wide 2D columns with small particle size yields high ratios [permission granted; 83]

There is a variety of columns to choose from when developing an LCxLC system. Most of the developed systems apply silica-based fully porous particle columns. Other possibilities might be the integration of monolithic, core-shell and polymeric beads based columns. The needed fast separation in the 2D might be accomplished with the use of sub-2 µm particle columns, which comes with the cost of high operating pressures. For the fast analysis in the 2D, core-shell and silica monolithic columns are more permeable, thus reducing pressure and furthermore their recent application in the LCxLC technology has increased [76]. 2.6 µm core-shell particle columns consist of solid silica cores coated with porous shells and possess the efficiency of a sub-2 µm particle column, but with the operating pressure of a 3 µm particle column. Monolithic columns consist of a material forming channels, which are connected by bigger pores, resulting in a better permeability than columns based on particles [76]. This morphology of these columns enables faster separations at higher flow rates, thus making them a promising tool for 2D separation. Furthermore there are two types of monolithic columns; silica-based and organic polymer monoliths. While silica-based monoliths are used for the separation of small molecules, organic polymer monoliths are superior in the separation of biopolymers [76]. However, the disadvantage of monolithic columns is the lower number of theoretical plates. Nevertheless, 2D columns need to be robust due to the high workload demanded for the fast separation.

Another essential part of the method development is the consideration of column temperatures and mobile phase compositions to improve selectivity and efficiency.

The implementation of the storage loop interface follows the evaluation of temperature and the mobile phase. There are two parameters, which need to be considered: 1D effluent storage capacity and 2D injection volume. To avoid increased peak width and lowered resolution it is recommended that the volume injected into the 2D should not exceed 15% of the dead volume of the 2D [84]. To avoid unnecessary analyte loss in the sample loop storage one must consider the parabolic flow profile. Friction on the walls of the channels of the LC system is inevitable and results in a flow profile with an increased velocity in the middle of the capillaries, while the flow near the walls is cut in half. When filling the storage loop all the way up analytes in the center might be lost due to their abundant concentration in the middle of the mobile phase, thus they are moving faster and 1D effluent is lost. Therefore, it is recommended that a fraction should not exceed half the volume of the storage loop (Fig. 16).

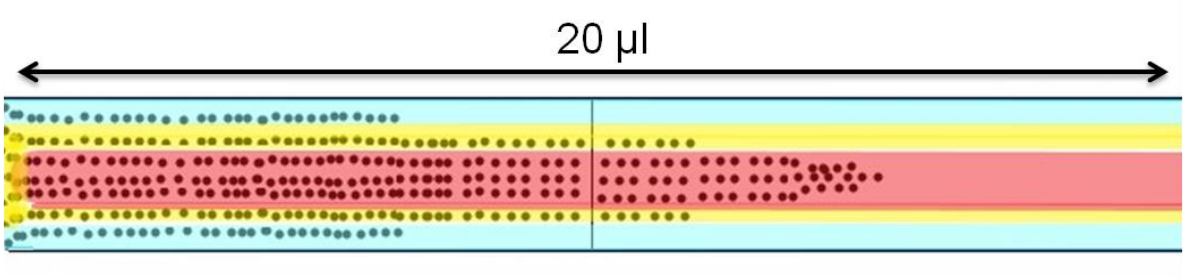


Fig 16: Analytes are shown as dots, while the colors represent the velocities with red describing the highest magnitude. The sample loop has been filled with 10 µl. When the sample volume equals the loop volume analytes pass the middle line would be lost [adapted from 80; Figure by Dr Suhas Nawada]

The next step consists of the selection of the appropriate columns. For analytes to be retained on a stationary phase it is important to identify the retention mechanism most suitable for the target analytes. Once these parameters have been evaluated, the mobile phase composition and gradient eluents (if applied) need to be considered. It is also possible that certain mobile phase compositions in combination with various selectivities might cancel the retention mechanism completely [80]. For example, C18 columns can be converted to GPC columns with the application of tetrahydrofuran as mobile phase.

The most critical part in the method development of an LCxLC system is the connection of the two dimensions and the corresponding compatibilities of the respective mobile phases. Therefore, the 1D effluent must be compatible with the 2D mobile phase to avoid detrimental effects on the 2D separation. The solvent strength of the 1D mobile phase must not exceed the 2D mobile phase due to the loss of retention on the 2D and the possible dispersion of the fractionated effluent in both directions in the 2D. Also differences in the mobile phase viscosity are important to consider. It can have detrimental effects on the chromatographic separation when the 1D effluent has a higher viscosity than the 2D mobile phase. This phenomenon is called viscous fingering, which are flow instabilities in the interface [85]. Solute bands are changing their form when entering the 2D jeopardizing the performance of the 2D (Fig. 17).

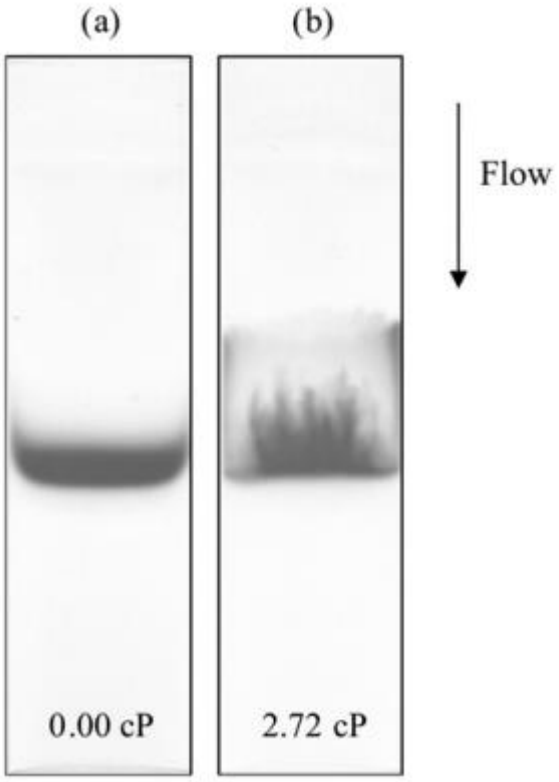


Fig 17: This graph illustrates the elution profile in (a) not experiencing viscous fingering and (b) experiencing viscous fingering [permission granted; 85]

A way to overcome the mismatches in solvent strength and viscosity is the application of a post-column dilution, thus diluting the 1D effluent to improve the separation on the 2D, despite the larger volume injected [86]. Switching sample storage loops with trap columns enables low volume injections onto the 2D. Trap columns can be applied in the following LCxLC setups: GPCxRP, SCXxRP (strong cation exchange columns), RPxRP and HILICxRP [80].

RP columns in the 2D are widely used due to their wide applicability and furthermore compatibility with the MS detector. Again, problems may arise with the combination of the 1D (normal phase columns (NP), GPC) with a RP column in the 2D due to solvent incompatibilities. The combination of two RP columns on both dimensions may at first result in low orthogonality, but on the other hand the selectivity of RP stationary phases can be tuned properly (Fig. 18). Choosing specific selectivities or adjusting pH in one of the dimensions yield higher orthogonality.

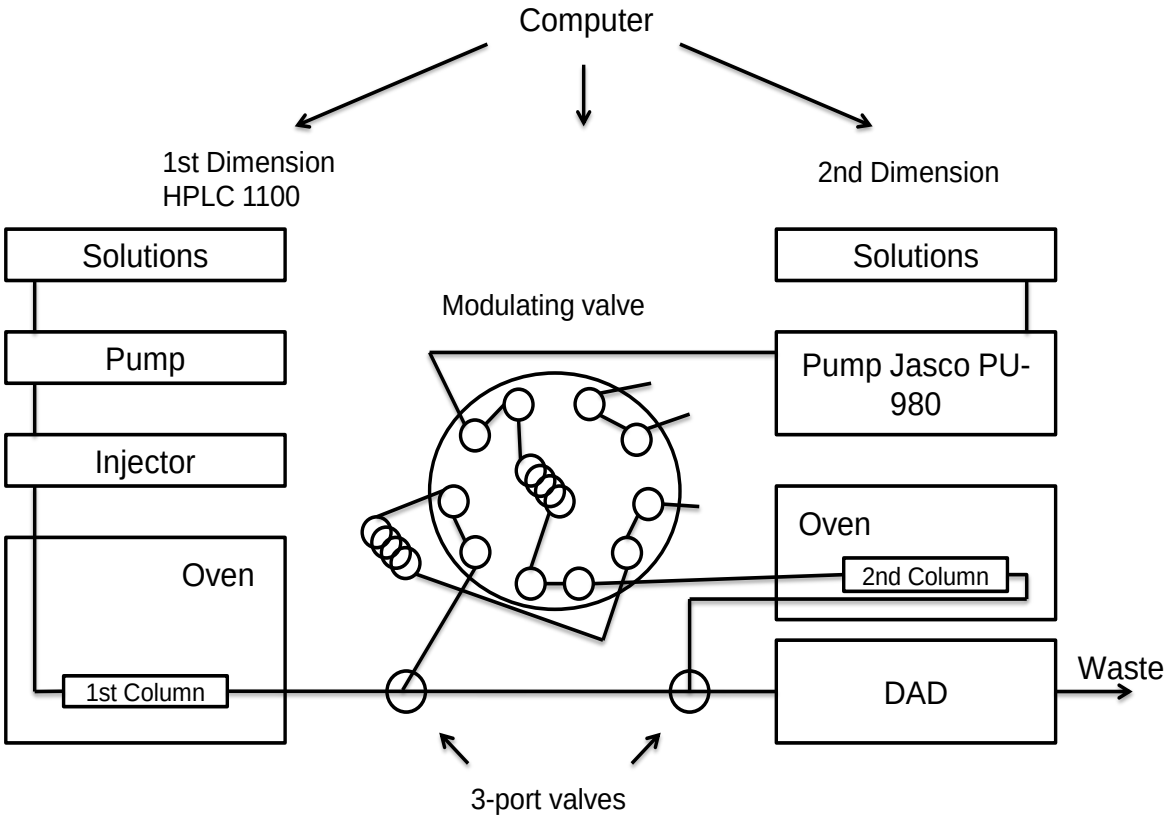


Fig 18: An example of a 2D-LC system using two RP columns for the separation of antioxidant phenolic compounds in wine and juice. Separated compounds from the 1D are directly measured by the DAD, while the remaining sample is directed to the 2D [adapted from 87]

The use of NP columns in LCxLC also struggles with mobile phase incompatibilities. The application of a RP in the 1D is accompanied with an aqueous phase, which in return jeopardizes proper retention on the NP in the 2D. One possible solution to this problem is to dilute the 1D fraction with an organic solvent to promote retention in the 2D. In addition NP columns have higher equilibration times, thus from this point of view not being a suitable choice for the 2D. Usually these columns are used in the 1D, decreasing the solvent incompatibilities. Two NP columns in both dimensions have been used for the determination of hydrophilic compounds (quillaja saponins) and furthermore the ability of a NP LCxLC system has been demonstrated to have a high orthogonality and good selectivity (Fig. 19) [88].

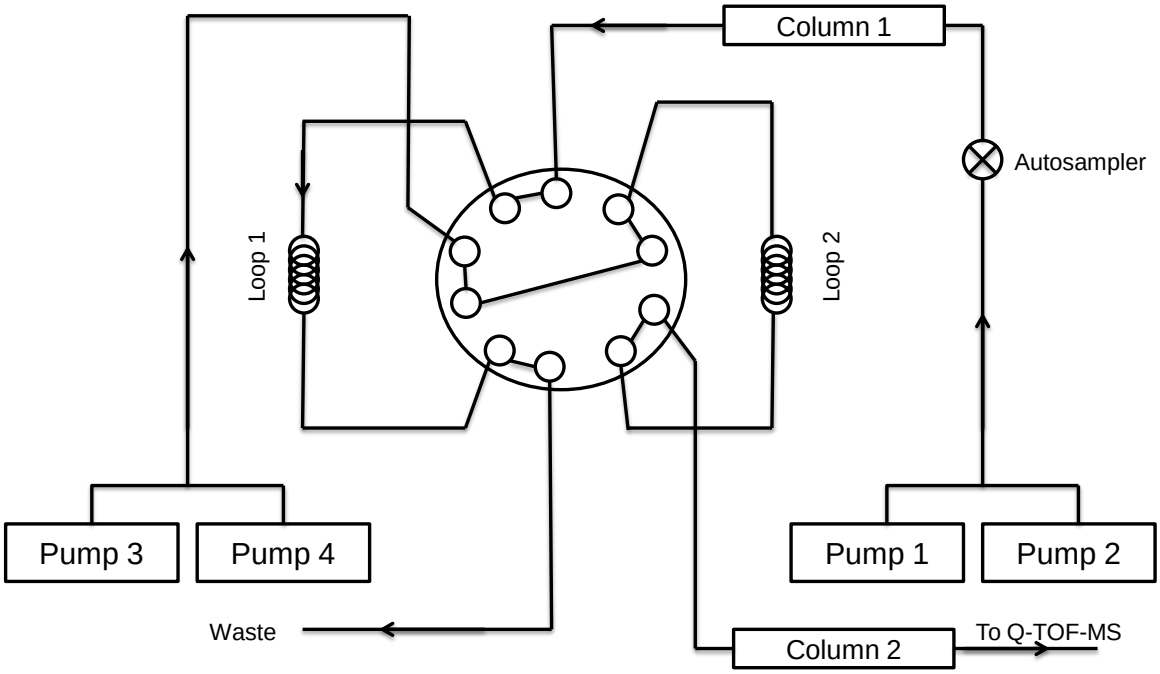


Fig 19: An example of a 2D-LC system using two HILIC columns for the determination of quillaja saponins with sufficient orthogonality. Each fraction of the 1D was stored at the 2 loops and were further injected onto the 2D [adapted from 88]

3.2 Applications of 2D-LC-MS/MS in food analysis

The resolving power of a 2D-LC system has been widely used in the determination of polyphenols in vegetable matrices [71]. The applications range from a combination of 1D RP and 2D RP to 1D HILIC and 2D RP columns. Applying two RP columns can be difficult due to the similarity of the separation mechanisms, thus leading to a low orthogonality of the system. This can in part be overcome by optimizing the mobile phase compositions and pH [71]. Another way to yield satisfactory selectivity is the selection of two columns with different polar interactions [76].

More promising results could be obtained with a combination of a HILIC in the 1D and a RP in the 2D, which enables higher orthogonality in comparison to two RP columns. These applications were coupled to a MS due to the typically high elution strength of the RP mobile phase [71]. For the analysis of furanocoumarins in celery, parsley and parsnips a so called selective LCxLC with UV detection was developed [28]. The main difference lies in the transfer of the 1D effluent to the 2D, which is, as the authors stated well timed. Fractions of the 1D are only collected and transferred to the 2D when additional separation is needed. This is accomplished by the use of sample loops to store fractions for the 2D until the 1D separation is completed. In addition the authors incorporated an interface, which enabled parallel sampling of the 1D effluent and the re-injection into the 2D [28]. Another 2D-LC system with the heart-cut approach was also applied for the

determination of furanocoumarins in oils [89]. The 1D was a bare silica column coupled to a chiral stationary phase column as the 2D via a 10 port valve connected to a photodiode-array detector.

The determination of anthocyanins is known to be difficult due to the conversion and interaction with other anthocyanins during a chromatographic run and their structural similarities yielding similar fragments when analyzed with tandem MS [90]. This process is supported by low optimal mobile phase velocity. To overcome this issue Willemse and colleagues developed an offline LCxLC method for the determination of anthocyanins in red grape skins, red cabbage, blueberries, black beans and red raddish with a HILIC in the 1D and a RP in the 2D. A highly acidic mobile phase with a low flow rate at high temperature (50°C) for the 1D was used to avoid the conversion of anthocyanins. The effluent of the 1D was collected with a fraction collector and only small volumes of the samples were re-injected onto the 2D to avoid band broadening and peak distortion [90].

Polyphenols were determined in wine and juices with an LCxLC system comprised of two RP columns coupled with a DAD detector. The complexity of both matrices could be overcome by the developed system due to the improvement of the separation efficiency to avoid co-elution of target analytes [87]. The sufficiently separated analytes of the 1D were directly sent to the detector, while other fractions for the need of additional separation were sent to the 2D (Fig. 18). Ma and colleagues used the heart-cutting technique for the determination of carbohydrates in milk powder. Using a RP column (C4) in the 1D and a specific NP column for carbohydrate analysis in the 2D enabled the researchers to skip the sample preparation process, which is known to be tedious and time consuming for the determination of glucose, galactose, fructose, saccharose and lactose with a refractive index detector [75]. A pre-separation or clean-up step was carried out by the 1D, while the final separation was completed by the 2D. In this study the 2D-LC method was compared to a 1D LC method, showing the superiority of the offline LCxLC method [75]. Ma and colleagues also used a heart-cut 2D-LC system with a packed loop interface for the determination of flavor enhancers in milk powder. The effluent of the 1D (C4 column) was trapped on a C8 material and further transferred to the 2D (C18 column) [75]. Furthermore the sample preparation could be reduced to a minimum due to the online clean-up of the 2D-LC system.

The analysis of carotenoids with LCxLC has been reviewed by Tranchida and co-workers [72]. In most of the described applications the 1D was a NP column with a RP column in the 2D. Saponification is usually applied in conventional carotenoid analysis resulting in artifact formation, which could be avoided by the outlined applications [72]. Also an UHPLC LCxLC system has been introduced with two RP columns enabling an improved fractionation of the 1D effluent.

The conventional sample preparation of PAHs is known to be time consuming and tedious. An offline LCxLC approach managed to skip the sample preparation process in smoked fish applying a silica column in the 1D and after the collection and concentration of the cleaned-up fraction, a RP column in the 2D [91]. The 1D column separated the PAHs from the interfering fat of the sample and the separation was carried out by the 2D (Fig. 20).

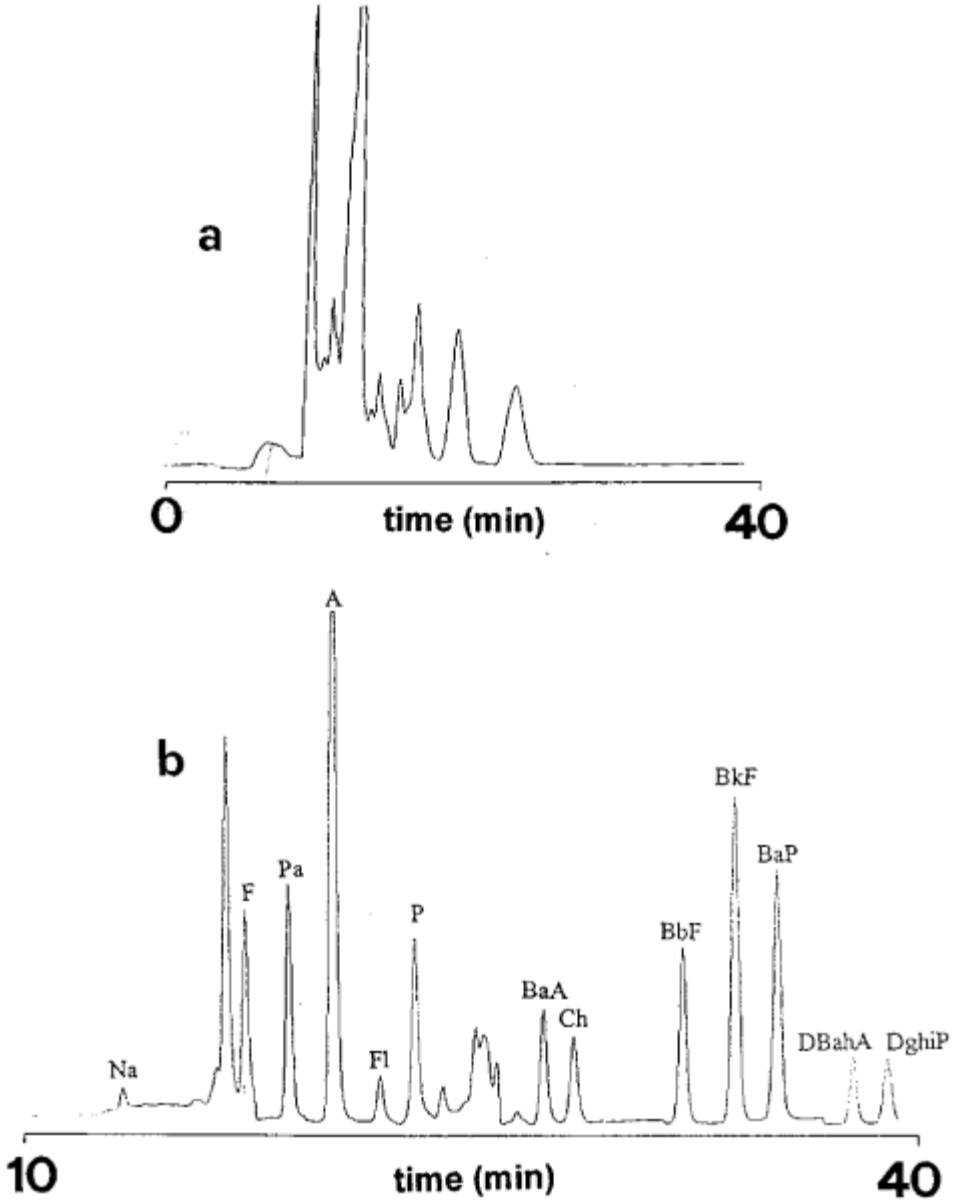


Fig 20: Determination of PAHs in a fortified trout fat sample with (a) a monodimensional analysis and (b) an offline 2D-LC analysis [permission granted, 91].

More food related LCxLC and heart cutting applications have been widely discussed by other authors [72; 92].

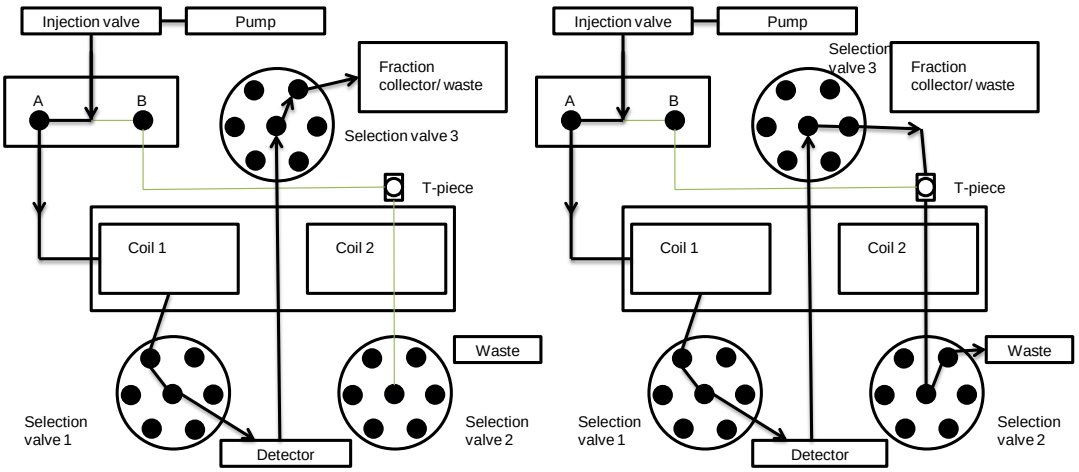
3.3 2D heart-cut LC in food analysis

In the case of pesticides, phenylurea herbicides in water were analyzed with two C18 columns with different dimension interfaced with a sample storage loop. The LCxLC system was compared with a 1D-LC method, showing an improved chromatographic performance with the LCxLC approach due to the implemented online clean-up and the reduced matrix effects [93].

Countercurrent chromatography (CCC) is described as a liquid-liquid partitioning process of the target analytes in two immiscible liquid phases. The main advantages of this chromatographic technique are stated by Englert and colleagues [94] including the possibility to handle raw materials with a high loading capacity, low solvent expenditure

and avoiding irreversible absorption. 2D-LC applications concerning the heart-cutting technique in countercurrent chromatography have also been proposed [94]. When operating 2D-CCC systems it is important to maintain the stationary phase in the column, which is accomplished by the use of miscible solvent systems without the disturbance of the inner band of the two phases. Usually, the setup of a 2D-CCC system is comprised by two CCC instruments, two pumps and detectors. However, recently a 2D-CCC heart cut system with only one CCC instrument, detector and pump has been proposed [94]. In this setup coil 1 and coil 2 were used as 1D and 2D, respectively with solvent selection valves transferring the coil 1 effluent to the 2D (Fig. 21).

a) b)



c)

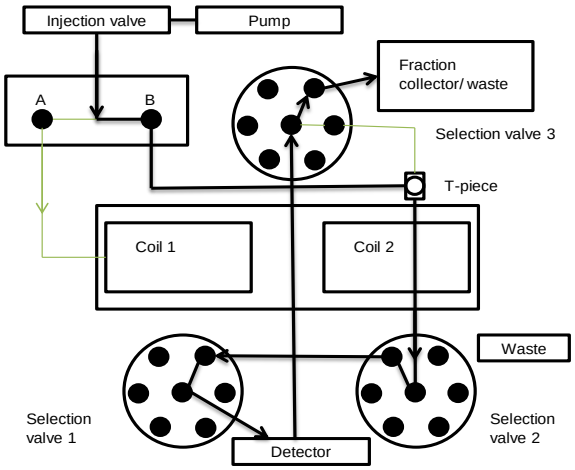


Fig 21: 2D-CCC setup with (a) being a one dimensional analysis, (b) showing the heart-cut procedure where the analytes are transferred from coil 1 to coil 2 and (c) showing the separation in coil 2 [94]

3.4 Pesticides

More than 1000 pesticides are available and widely used (Fig. 22) in modern agriculture to prevent respectively control weeds, pests and diseases of the harvesting crops. Pesticides are of major concern for human health and food safety. Excess use and misuse combined with long traveling distances may not only result in environmental contamination but also raises the exposure to humans. This is mostly the case in developing countries where also older pesticides are heavily used [95]. Pesticide residues are therefore incorporated into the food chain. Good agricultural practice (GAP) provides guidelines for the appropriate use of pesticides regarding the time they are allowed to be deployed and the time the crops are harvested [96]. Furthermore, policy development, pest management and pesticide risk reduction concerning public, occupational and animal health are supported by the GAP guidelines [96]. The aim of the “Integrated Pest Management” (IPM) is to keep the use of pesticides as low as possible to minimize human exposure and correlated health risks, protect the environment and to consider all alternative pest control techniques for implementation [96]. Applying these guidelines will reduce the possibility to find high levels of pesticide residues. It is of utmost importance to monitor pesticide residue levels to confirm the safety of the product for human consumption even though the processing of foods (e.g. peeling, dilution through homogenization) might lead to decreased pesticide concentrations [95]. Pesticides are regulated in various food commodities with a respective maximum residue level (MRL). So called residue trials are conducted for the establishment of a MRL, which should represent real life conditions like cultural practices, seasons, different climates and crops. When sufficient data is gathered, the Joint FAO/WHO Meeting on Pesticide Residues (JMPR)

sets a MRL for the given food matrix and pesticide. To estimate the dietary exposure several points have to be investigated, including pesticides in the soil and the contamination of crops, also regarding their metabolism, the contamination of animal tissues with pesticides from animal feed, processed foods and the residues after the production and short-term and long-term intake of pesticides [95].

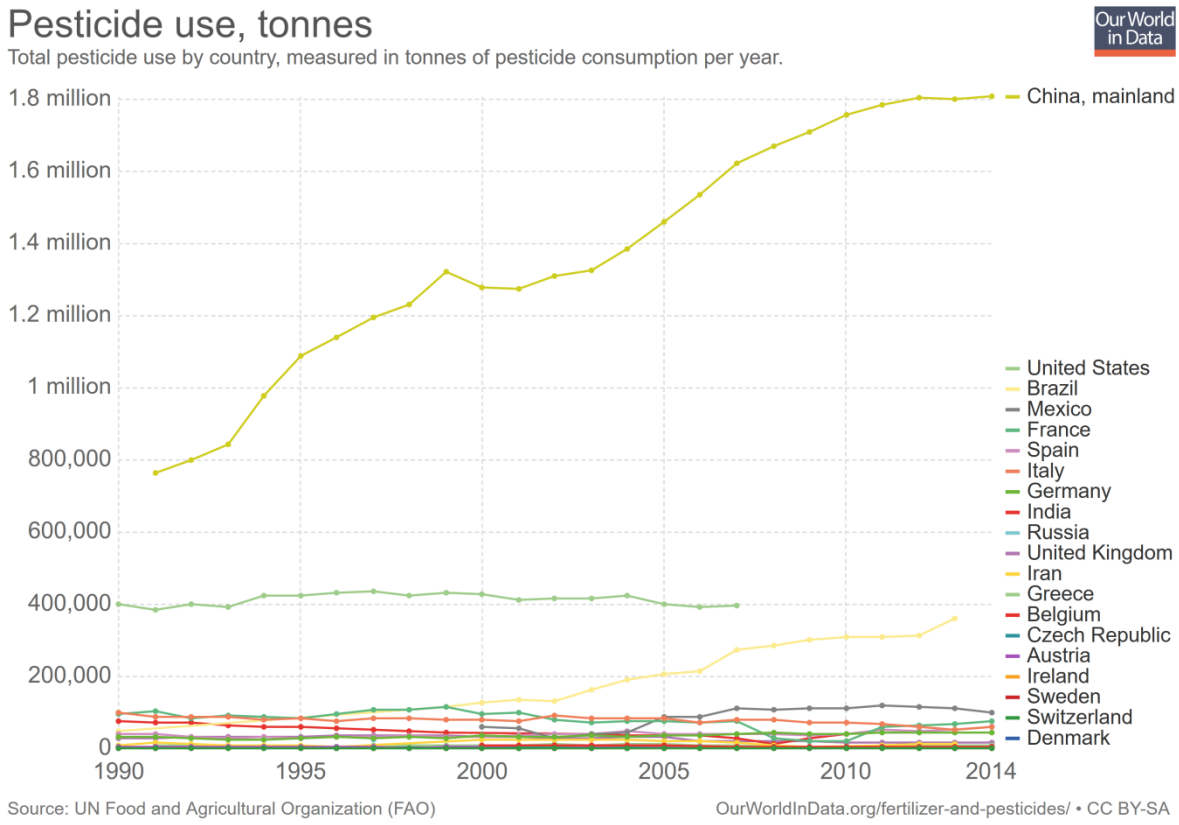


Fig 22: Pesticides used per tonnes in Europe, Asia, North and South America from a FAO questionnaire [97]

The importance of food processing on pesticide residues must not be underestimated. A meta-analysis has been conducted by Liang and colleagues [98] regarding fruits, their processing and the elimination or reduction of pesticide residues. Investigated were tap water washing, peeling, oven drying, sun drying, boiling and juicing. All but oven drying significantly resulted in a reduction of pesticide residues, suggesting several strategies to effectively lower pesticide concentrations. Peeling and juicing were the most effective food processing steps to reduce pesticides by 85%. On the other hand, oven drying was associated with an increase in pesticide contamination. Nevertheless, the authors stated that both reductions and increases in pesticides could be obtained by sun drying and oven drying due to properties of the pesticides and the matrix. These findings were consistent with the scientific literature [98].

Today, the determination of pesticide residues demands the simultaneous detection of various compounds with a wide range of polarities in a single analytical run. Usually, this is accomplished by the use of LC-MS/MS and GC-MS/MS. The SANTE document [11945/2015] provides a guideline for quality control and validation procedures for pesticide residue analysis in food and feed. The aim of this guide is to harmonize the quality management, make analytical results comparable, avoid false positive or false

negative results and implement analytical criteria like thresholds for relative standard deviation values [99].

Routine laboratories today demand a high sample throughput with cost effective methods, especially when it comes to the sample preparation process. Therefore, the sample preparation should be as little time consuming, tedious and expensive as possible, and at the same time possible matrix interferences like co-extracted materials should be removed. This commendable objective might not always be reached with the abundance of compounds analyzed in a single chromatographic run. Therefore, there are mostly two options to be considered: increasing the sample clean-up by expanding the sample preparation process and/or improve the chromatographic performance to achieve the analytical goals.

A widely used method for the determination of pesticide residues in various food matrices is the QuEChERS method (quick, easy, cheap, effective, rugged and safe) with a subsequent GC-MS/MS and/or LC-MS/MS detection. This sample preparation technique requires minimal laboratory setup, is easy to use and yields a high throughput of samples. Enjoying a great popularity since the publication of the original QuEChERS method by Anastassiades and Lehotay [48] this method has been modified in various ways to meet the requirements for other analytes, except pesticides as well. This sample preparation technique is more thoroughly discussed in publication II. Fig. 23 shows a schematic of the main steps in the QuEChERS sample preparations, which are most frequently used.

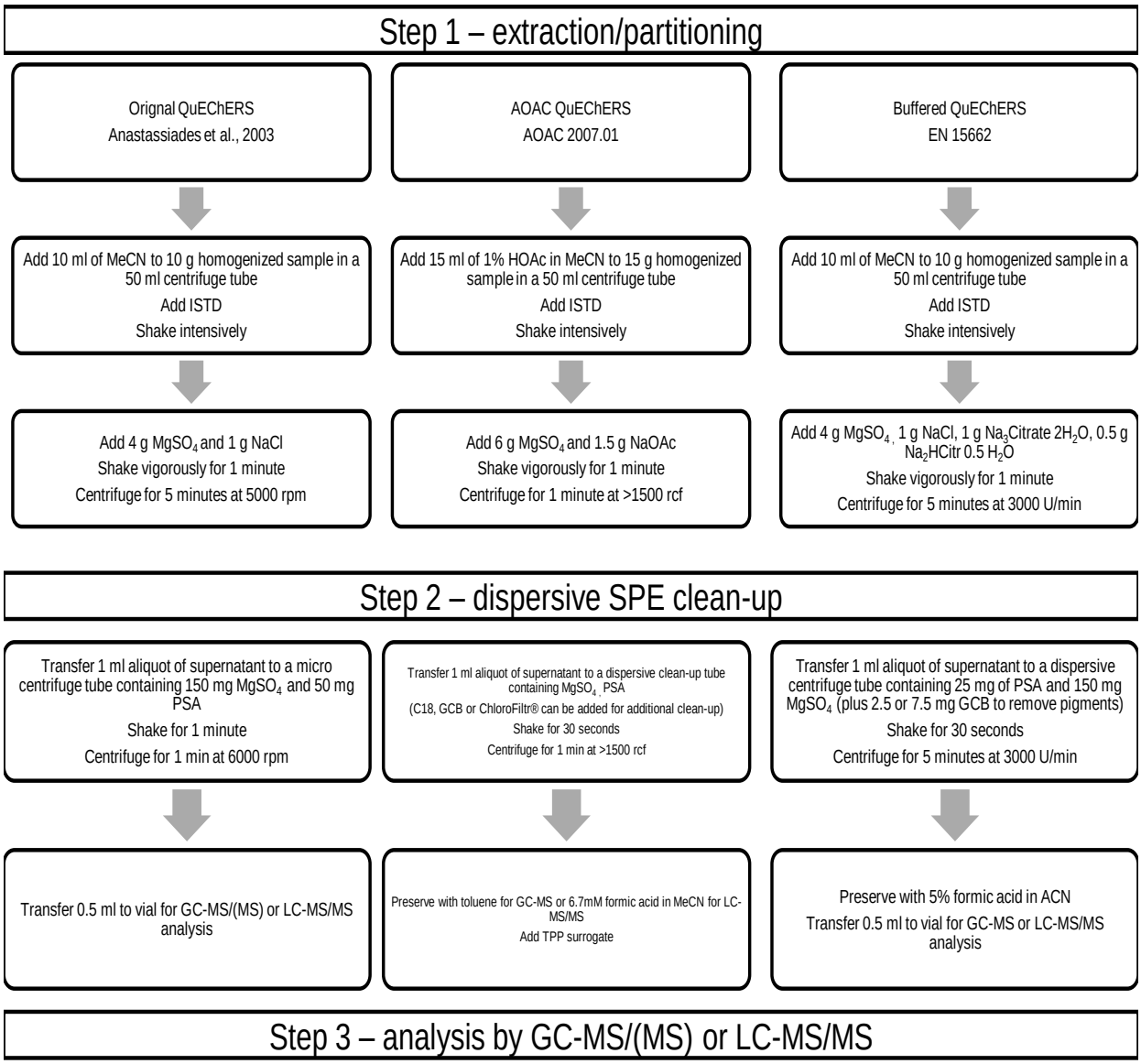


Fig 23: Illustration of the three priority QuEChERS methods. The original QuChERS method, the AOAC official method and the EN15662 European official method [adapted from 100]

Albeit the success story of the QuEChERS method, co-eluting matrix inferences still show up to be problematic for the confident determination of pesticide residues, especially in complex and difficult matrices. To avoid single compound methods various 2D-LC approaches have been proposed [78, 101]. For example, to demonstrate the ability of a 2D-LC system Robles-Molina and colleagues designed an automated parallel LC column system with a HILIC and a RP column for the determination various pesticides with high polarity differences [101]. The samples were prepared with two different sample preparation techniques (QuEChERS and QuPPe (quick polar pesticides method)). The injection of the prepared samples was conducted simultaneously onto both columns. The eluents were united and detected by the MS. To remove possible co-eluting matrix components Kittlaus and colleagues applied a 2D-LC heart-cut system with a packed loop interface with a HILIC in the 1D and a RP column in the 2D for the determination of more than 300 pesticides [78]. More 2D-LC systems are described in more detail in publication II.

3.5 Tropane alkaloids (TAs)

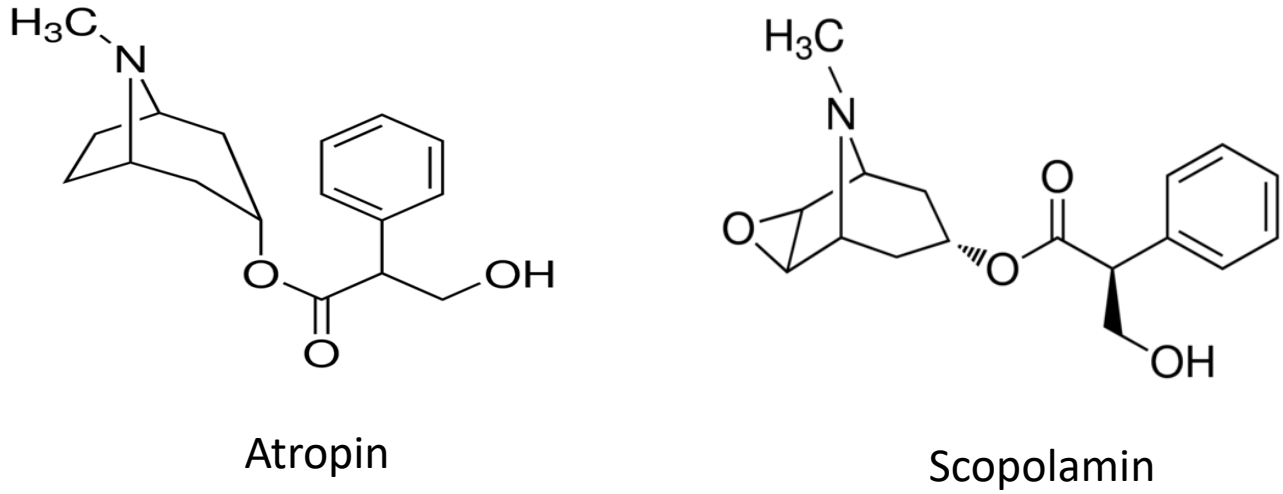


Fig 24: Chemical structure of atropine and scopolamine

TAs containing plants belong to the family species of *Brassicaceae*, *Solanaceae* and *Erythroxylaceae* [102]. These ornithine-derived compounds occur in these plants as mono-, di- and tri-esters, carboxylated and benzoylated with tropanes. More than 200 compounds of this substance class have been identified, but their occurrence in food and feed is still a matter of further investigations [102]. The (-)-enantiomeres are formed naturally with the most studied TAs being (-)-hyoscyamine and (-)-scopolamine. Atropine is the racemic mixture of (-)-hyoscyamine and (+)-hyoscyamine. Certain foods are contaminated with TAs due to co-harvesting TA containing plants (mostly seeds) with the edible crop or the plants themselves contain TAs. An Acute Reference Dose has been established for (-)-hyoscyamine and (-)-scopolamine, which would also protect from long-term exposure due to the non-genotoxic properties and the inability to exert chronic toxicity [102]. 124 food samples were investigated on the occurrence of atropine and (-)-scopolamine in the scientific opinion of the EFSA on TAs in food and feed [102]. 83% of the samples were below the LOD. Nevertheless, the most contaminated foods were for infants and young children, especially those based on cereals. But it has to be taken into account that samples taken for each food group were not well balanced, as can be seen in Fig. 25.

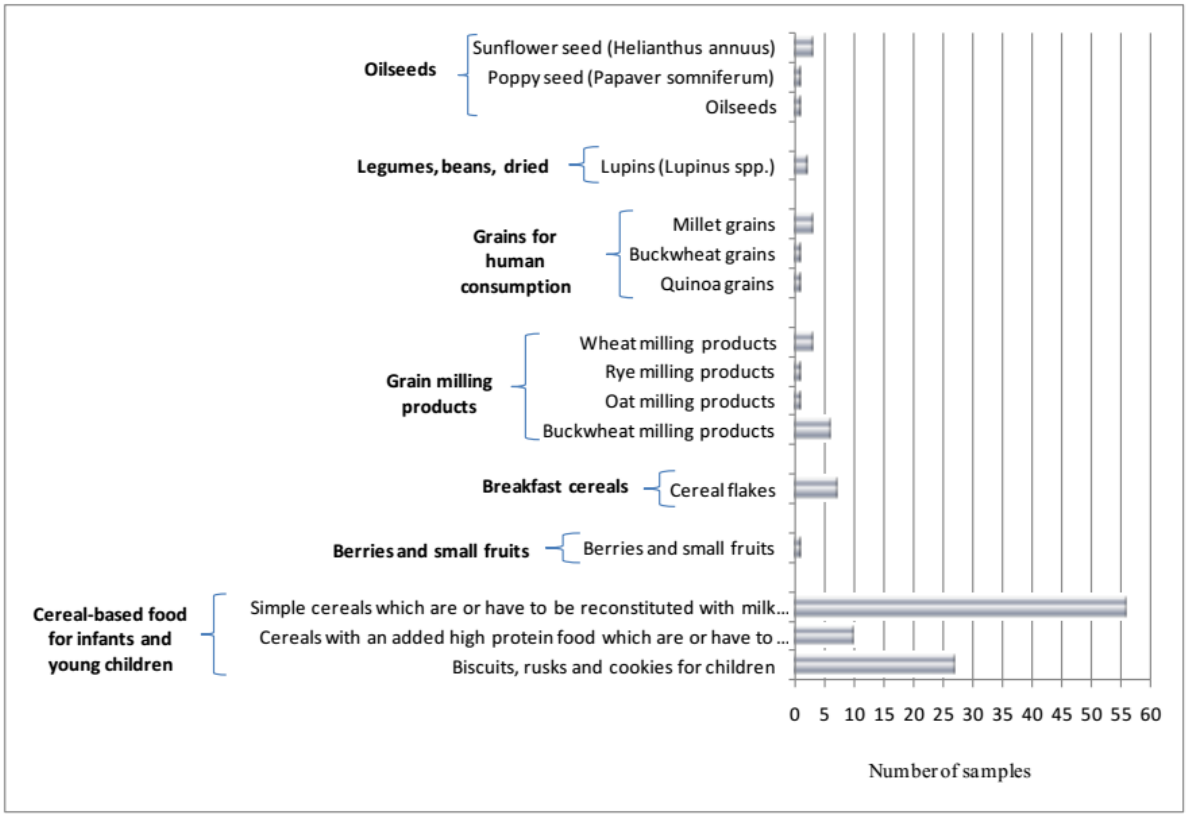


Fig 25: Distribution of investigated samples for TAs by the EFSA [102]

Therefore the EFSA encouraged the analysis of cereal-based infant foods [102]. Human health risk characterizations were only estimated for toddlers, since these were the only food groups where sufficient data was available. Only limited data on the occurrence of TAs in food products in general was available until 2013 (Fig. 26). More recently Mulder and colleagues [103] shed light on the occurrence of TAs in certain foods. 24 TAs in 1709 samples of plant derived products including cereals and cereal based infant and young children foods (206) were investigated. 20% of all cereal based foods for infants and young children were contaminated with TAs yielding the highest mean TA concentrations of all investigated food commodities (130.7 µg/kg). Atropine and scopolamine have been the most frequently detected TAs (Fig. 27) [103]. Another study by Mulder and colleagues [2015] showed that the contamination of cereal based infant foods was higher when the samples were collected in 2011 and 2012 compared to those collected in 2014. The authors stated that this might be due to the recommended measures for producer enforced by the EFSA [113].

The sample preparation for TAs is simple and straight forward, starting with the addition of an extraction solvent to the sample with a subsequent shaking, centrifugation and filtration step [105, 113]. The QuEChERS sample preparation has also been applied with a d-SPE cleanup [106].

It is important to promote monitoring of both TAs through the simplification of complex analytical methods with additional cost lowering applications. Today the method of choice for the determination of TAs in certain foodstuff is LC coupled with tandem MS [106; 107, 113].

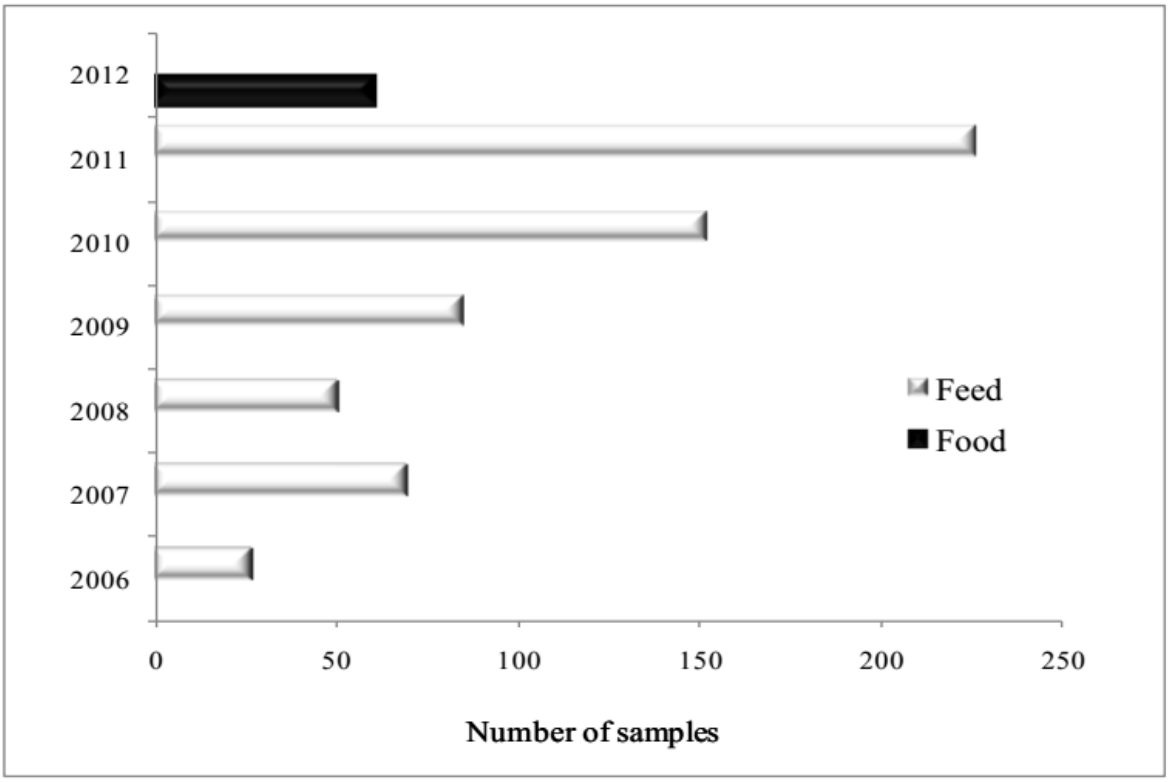


Fig 26: Number of samples investigated for the occurrence of TAs over the years [102]

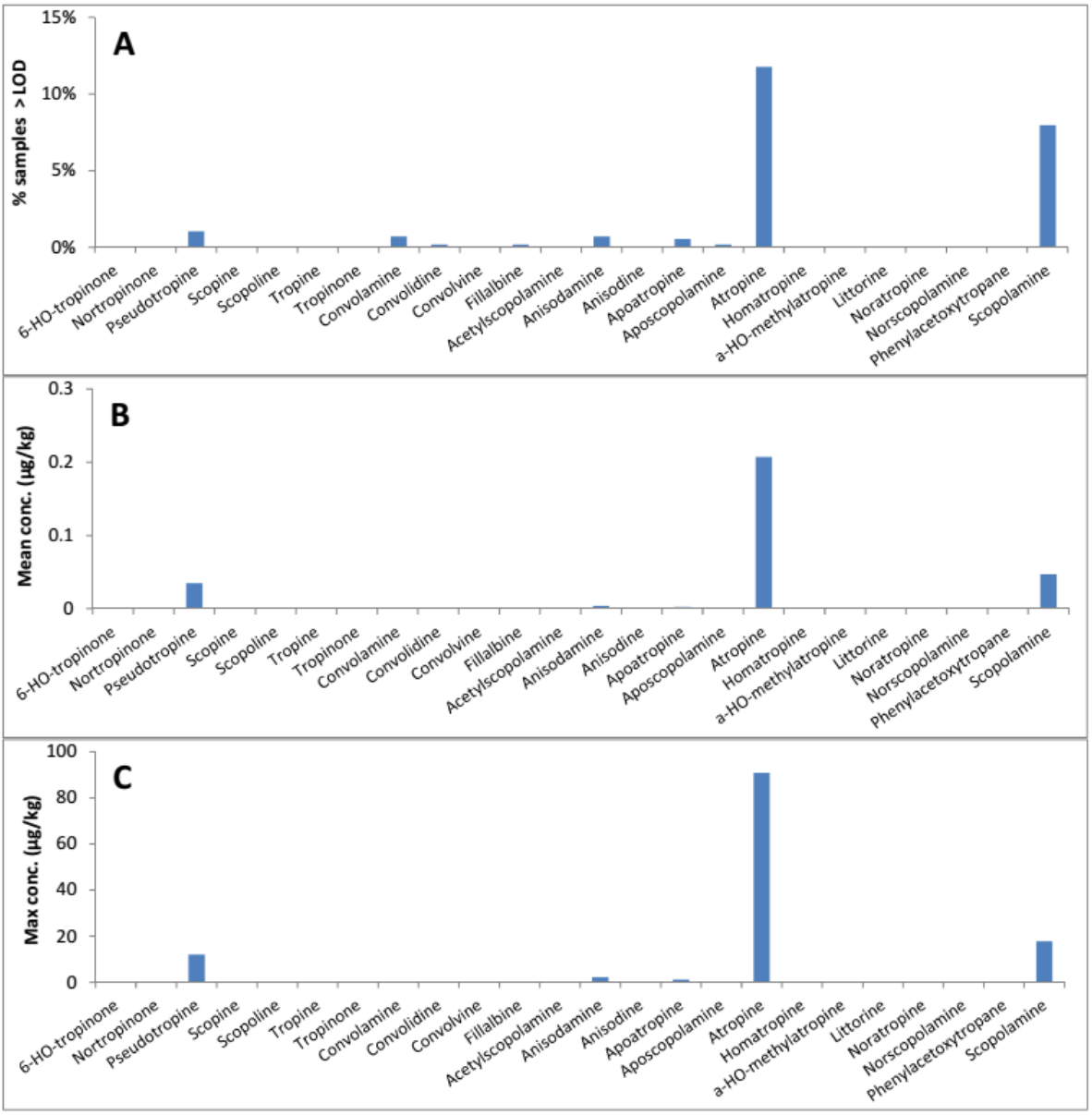


Fig 27: Illustration of 578 cereal based samples expressed in percentages with samples above the LOD, the mean concentration and the maximum concentration of the TAs [permission granted; 103]

3. 6 Pyrrolizidine alkaloids (PAs)

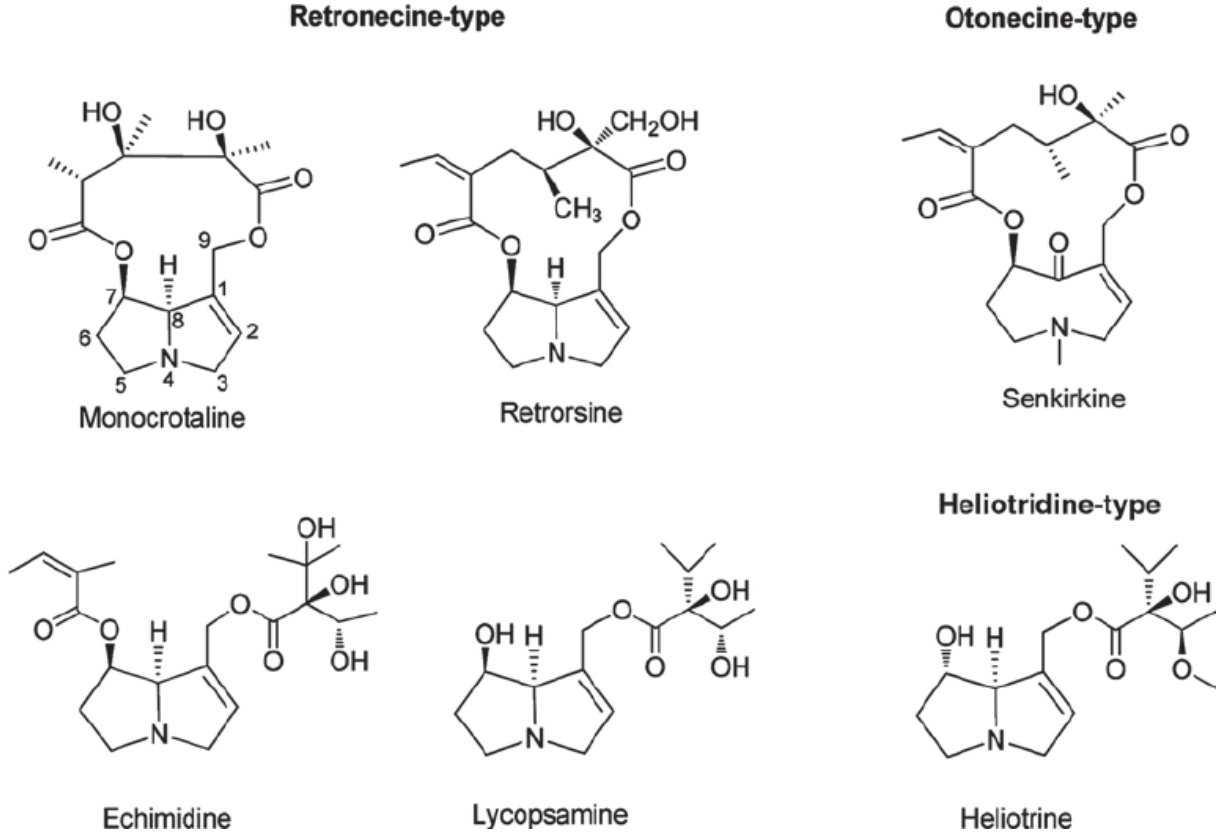


Fig 28: Chemical structure of certain PAs [108]

PA containing plants are found around the world including the following plant families: *Boraginaceae*, *Compositae*, *Leguminosae* and *Scrophulariaceae* [109]. More than 600 PAs are known, which all show the following structural similarities: a necine base, which is an amino alcohol, esterified with one or more necic acids [110]. PAs can occur in their tertiary base or their N-oxide form. Most of the characterized PAs are ester-PAs of hydroxylated 1-methylpyrrolizidine or otonecine necine bases (Fig 28) [110]. PAs occur in different food commodities ranging from cereal products, vegetables, honey, milk and eggs with the way of contamination being through harvesting the crops with the PA containing weeds and the animal consumption of such plants. Therefore carry-over is possible in contrast to TAs [110]. The main health impact of PAs on humans is the acute and chronic liver toxicity. These adverse health effects are well known due to documented outbreaks of PA poisoning in the USSR, India and Afghanistan [109]. Staple food crops were contaminated with PA containing plants, which were growing among the crops. Some conditions promote the occurrence of PA containing plants like drought and poor rainfall. More recently in 2008 an outbreak of liver disease of unknown origin in Ethiopia was reported where hundreds of people have died [111]. Evidence pointed in the direction of PA contaminated teff and maize consumed by the Ethiopian population. However, not all PAs are considered toxic. Only the 1,2-unsaturated PAs are considered to be genotoxic carcinogens including the acute toxic effects with no tolerable daily intake [110].

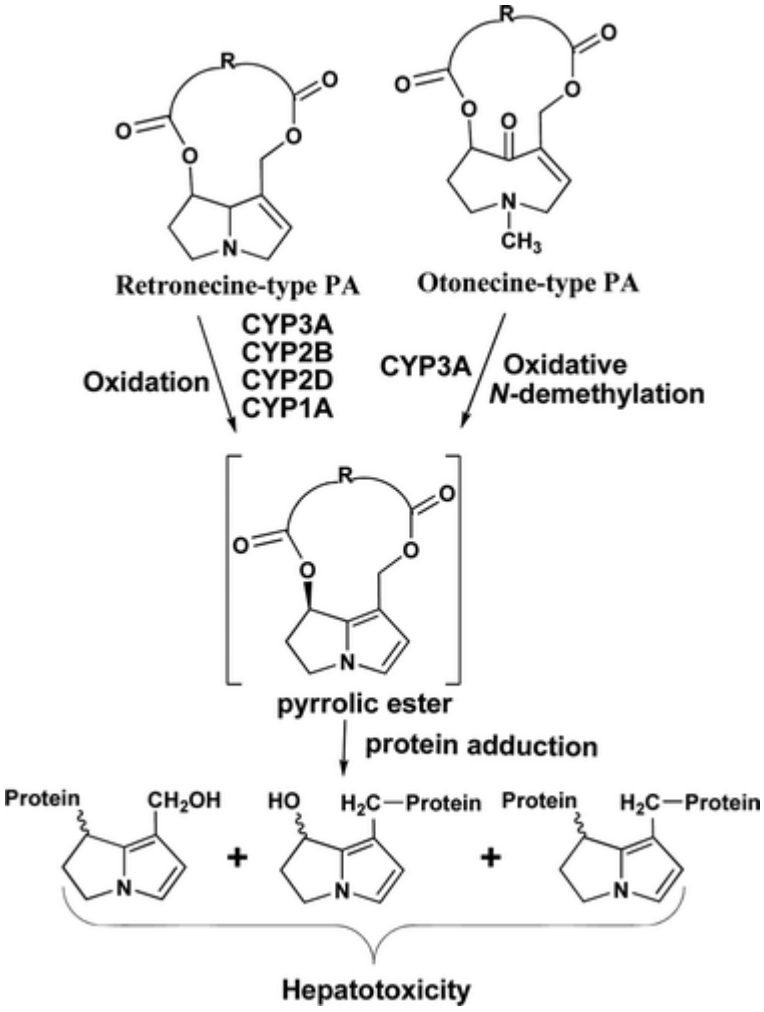


Fig 29: Metabolic activation of PAs by hepatic cytochromes P450s and the formation of hepatotoxic pyrrole-protein adducts [permission granted; 112]

These compounds exert their toxic effect through the metabolic activation by cytochromes P450s in the liver, which results in the formation of reactive intermediates (Fig. 29) [112].

To date, no respective maximum levels have been established for PAs in various food commodities. In the scientific opinion on PAs of the EFSA in 2011, only honey and feed were included into the final report due to the lack of data of other foodstuffs. In 2015 Mulder and colleagues [103] published a scientific report about the occurrence of PAs in food with 1105 food samples derived from animal and plant sources (excluding cereals) with 28 and 35 PAs, respectively investigated. The most contaminated food products were herbal teas, followed by food supplements, milk and eggs. Studies point out that data needs to be gathered to assess exposure risk for humans in case of PA contaminated grains [114].

Today, the methods of choice for the determination of PAs in certain foodstuff are LC-MS/MS [103; 115] or GC-MS/MS [110]. For the sample preparation of PAs a SPE [103, 115; 116] clean-up was introduced. A more straight-forward approach was published by Huybrechts and Callebaut [117]. The PAs were extracted with an extraction solvent with 37% hydrochloric acid to denature proteins to avoid any further clean-up in tea, honey and milk. Additionally, the authors stated that the weak elution strength of the solvent enabled a high on-column injection to skip any concentration steps [117]. That method was also compared with a modified QuEChERS approach and with an extraction with acidified methanol. Both, the QuEChERS method and the extraction with acidified methanol yielded high extraction efficiencies with the disadvantage of the analytes dissolved in a strong solvent limiting the injection volume and calling for a concentration step [117].

3.7 Mycotoxins

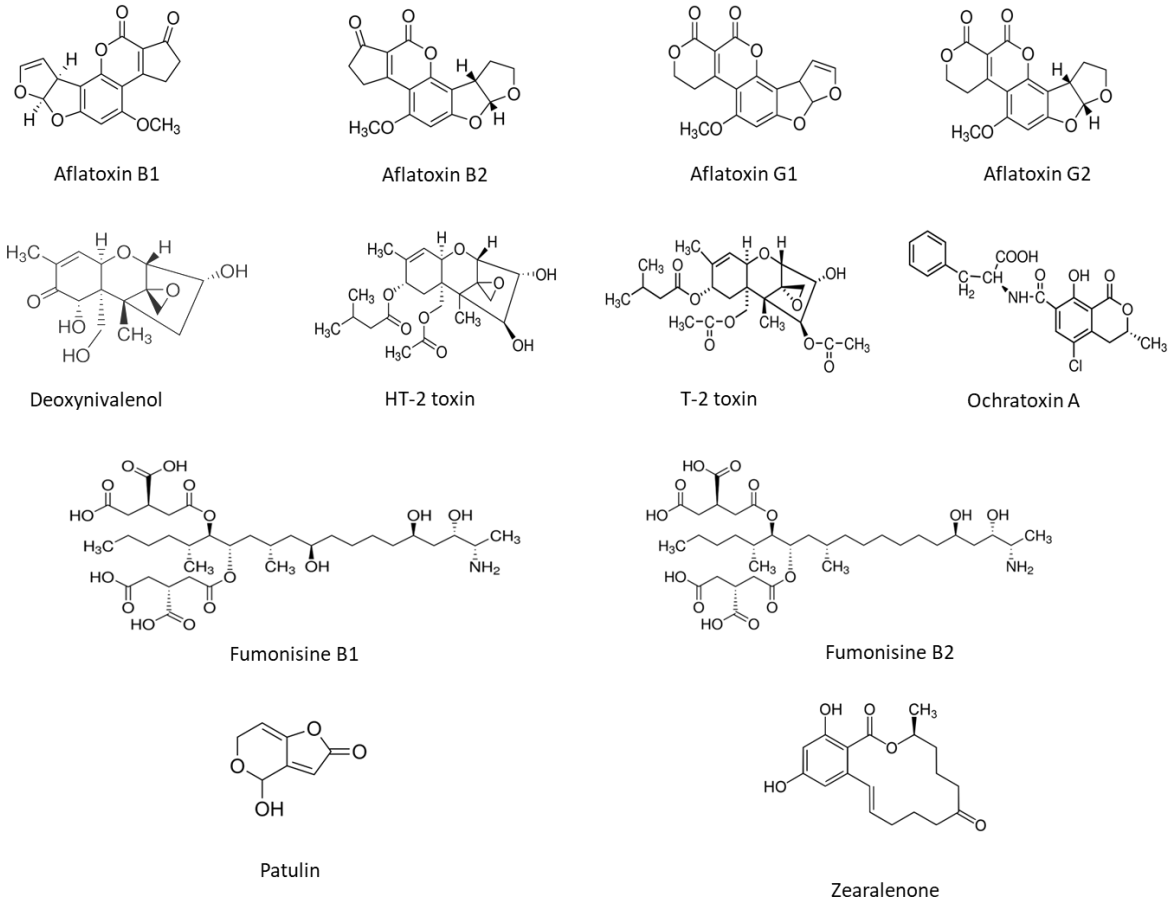


Fig 30: Chemical structure of mycotoxins regulated in Commission Regulation 1881/2006

Mycotoxins are secondary plant metabolites produced by a wide variety of fungi possibly contaminating foods from field to fork. One of the most common contaminations of mycotoxins regarding oats are *Fusarium* toxins. These toxins, like zearalenone (ZON) and trichothecenes (deoxynivalenol (DON), HT2 toxin and T2-toxin) are produced by moulds belonging to the *Fusarium* species [118].

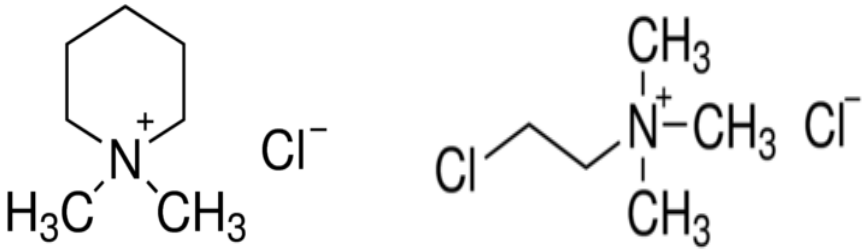
Even though MLs for mycotoxins have been established in a variety of food commodities the two *Fusarium* toxins HT2 and T-2 toxin are not regulated with respective MLs [13]. The recommendations for HT2 and T-2 toxin concerning cereals and cereal products including cereal based infant foods range from 15 µg/kg to 2000 µg/kg for the sum of both [119]. LOQ of HT2 and T-2 toxin should not exceed 5 µg/kg in processed cereal products, while the LOQ of unprocessed cereals should be below 10 µg/kg [119]. The other regulated mycotoxins are aflatoxin B_1, B_2, G_1, G_2, M_1, DON, zearalenone (ZON), ochratoxin A (OTA), patulin, fumonisine B_1 and B_2 [13]. The estimated maximum levels of mycotoxins are based on the tolerable daily intake (TDI) of a given population. The human exposure to mycotoxins in central Europe can be considered to be low, except for DON and its metabolite DON-3-O-glucuronide, which exceeded the TDI [120]. Results from the second French total diet study showed the highest mean concentration of mycotoxins in wheat and cereal products [121]. Concerning the single compounds and their recommendation regarding their lower bound assumptions only DON exceeded the TDI. These findings are in good accordance with Gerding and colleagues [120]. The concluding remarks of both studies indicate no existing consumer risk in central Europe

regarding aflatoxins, OTA, patulin, ZON and fumonisines [120, 121]. Fusarium toxins were investigated in 93 oat samples in Sweden showing that one of the most prevalent toxins was DON [118].

The method of choice for the determination of mycotoxins in certain foods is one dimensional LC-MS/MS [118, 122; 123]. Also a 2D-LC system was developed by Breibach and Ulberth [124] for the determination of aflatoxin B_1 in cereal based baby foods, neat maize material and maize based feed material. The authors reported decreased signal suppression with more satisfactory results. The 2D-LC system was comprised of a RP column in the 1D and a RP column in the 2D with alternate selectivities to reach a certain degree of orthogonality with a 100 µl storage loop to trap the 1D effluent [124].

The sample preparation for mycotoxins can be considered to be straightforward [122; 125]. The method of Sulyok and colleagues applies a mixture of acetonitrile, water and acetic acid (79/02/1; v/v/v) for the extraction of the mycotoxins with no further clean-up [122]. Tsiplakou and colleagues used a different extraction solvent (acidified methanol) with no additional clean-up [125]. The QuEChERS method has also been compared with an ultrasonic-assisted extraction with a mixture of acetonitrile, water and formic acid (80:19:1, v/v/v) [126]. Concerning animal based foods the QuEChERS method prevailed against the extraction with the mixture of acidified acetonitrile and water. On the other hand, the application of the mixture of acetonitrile and water yielded higher recovery rates compared to the QuEChERS method when mycotoxins were extracted from animal feed [126]. Similar ratios (80:20; acetonitrile:water) have been chosen for the extraction of various analytes in publication II, except for formic acid.

3.8 Plant growth regulators



Mepiquat chloride Clormequat chloride

Fig 31: Chemical structure of mepiquat chloride and chlormequat chloride

In publication II two quaternary ammonium plant growth regulators namely, mepiquat (MQ) and chlormequat (CCC) were analyzed. Both plant growth regulators are mainly used on cereals and oil seeds [127, 128] with a growth retardant effect to avoid unwanted longitudinal growth without compromising plant productivity by inhibiting the biosynthesis of gibberellins. Additionally, plant growth regulators promote flower formation and prevent premature fall of fruit and increase lodging resistance. The main factor for lodging is the culm length, which is increased proportionally with precipitation, fertilization with nitrogen and day length. The longer the culm the more likely lodging occurs, which in return can significantly decrease the crops yield [129]. Therefore, shortening the stem with plant growth regulators is one of the primary goals. Especially MQ and CCC are applied in early phases of stem elongation and CCC successfully exerted stem shortening effects to prevent lodging of wheat, barley and oats [129]. The question has been raised if plant

growth regulators still have an existence authorization in modern agriculture. On the one hand, there are different varieties of lodging resistant crops in which cases the application of plant growth regulators seems unnecessary. But with an increasing risk of lodging and possible harvest losses plant growth regulators applied in accordance with GAP seem reasonable.

MQ and CCC are regulated with respective MRLs with 0.02 mg/kg and 0.01-9 mg/kg in cereals including buckwheat and other pseudocereals, respectively [130; 131]. Both MRLs have been reviewed in 2015 and 2016, respectively [127, 128]. The investigation by the EFSA shows no risk for consumers for both compounds, although both reports stated that further investigations have to be undertaken, since their risk assessment is considered to be indicative. To the same conclusion came two Danish researchers [132], when observing levels of MQ and CCC in cereals on the Danish market. They estimated the daily exposure through the consumption of cereals would not exceed 1% of the acceptable daily intake.

MQ and CCC are usually determined with an LC-MS/MS approach [127, 128; 132-136;]. A SPE for the sample preparation of MQ and CCC is widely used and recognized [134-136]. Additionally, an automated online SPE was introduced by Riediker and colleagues [134]. For CCC alone a more straightforward sample preparation was developed with a “dilute and shoot” approach with water as an extraction solvent [133]. A similar extraction strategy was used in publication II.

4. Publications

4.1 Publication I

I Comparing d-SPE Sorbents of the QuEChERS Extraction Method and EMR-Lipid for the Determination of Polycyclic Aromatic Hydrocarbons (PAH4) in Food of Animal and Plant Origin, Urban, M., Lesueur, C. Food Anal Methods. 2017; 10(7); 2111-2124

Brief description:

This paper describes an easy and fast method for the determination of PAHs in various food matrices with GC-MS/MS. The sample preparation is based on the QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction method, which utilizes less laboratory equipment than the conventional sample preparation for the determination of PAHs. Furthermore, we were able to show that all regulatory requirements can be fulfilled with the proposed method.

Novelty statement:

We report the successful application of the QuEChERS method on 11 food matrices for the determination of the PAH4 fulfilling all regulatory requirements including the successful extraction of the PAH4 of naturally contaminated foods.

Food Anal. Methods
DOI 10.1007/s12161-016-0750-9

Comparing d-SPE Sorbents of the QuEChERS Extraction Method and EMR-Lipid for the Determination of Polycyclic Aromatic Hydrocarbons (PAH4) in Food of Animal and Plant Origin

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Abstract A Quick, Easy, Cheap, Rugged, Effective, and Safe (QuEChERS) method for the determination of benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[a]pyrene (PAH4 including the interfering PAHs triphenylene, cyclopenta[c,d]pyrene, benzo[k]fluoranthene, benzo[j]fluoranthene) was developed and validated with GC-MS/MS in foods of plant and animal origin. PAHs were extracted with acetonitrile, and different clean-ups with various compositions of sorbents, including zirconia-based sorbent (Z-Sep), primary-secondary amine (PSA), anhydrous magnesium sulfate ($MgSO_4$), octadecylsilane (C18 endcapped), and enhanced matrix removal (EMR)-lipid material, were tested. Another important focus of this study was the separation of critical pairs, which is essential for the qualification and quantification of PAH4. To investigate the developed methods, samples were spiked beneath their maximum levels (MLs) and recoveries and peak shapes were compared. The clean-up with 900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18 was chosen to be validated in salmon, mussels, shrimps, bacon, cutlets, wheat flour, curry spice powder, infant formula, infant follow-up formula, and infant foods. Recoveries for all analytes were between 75 and 108%, combined with standard deviation between 2 and 20%. Limits of detection (LODs) and limits of quantification (LOQs) were between 0.04 and 0.34 μg/kg and between 0.1 and 1 μg/kg, respectively.

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Keywords QuEChERS sample preparation · GC-MS/MS · Polycyclic aromatic hydrocarbons · Animal food · Plant-based food

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants consisting of two or more fused aromatic rings. They can be subdivided into "light PAHs" consisting of two to three rings and "heavy PAHs" from four to six rings, with toxicity increasing proportionally to the number of rings (Purcaro et al. 2013). The main sources of human exposure for non-smokers are certain foods (EFSA 2008). PAHs are generated through food processing, involving high temperatures, combustion gases, or flame contact, like roasting, grilling, drying, and smoking (Essumang et al. 2013; Ledesma et al. 2014; Varlet et al. 2007; Kamankesh et al. 2015). Therefore, household food preparation and industrial food production contribute to the exposure of PAHs. Benzo[a]pyrene, classified as a group 1 carcinogen (IARC Monograph On The Evaluation Of Carcinogenic Risks To Humans 2009), was considered to be an insufficient indicator for the occurrence of carcinogenic PAHs in food. The scientific panel of Contaminants in the Food Chain (CONTAM) concluded that the initial eight PAHs benzo[a]pyrene (BaP), benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF), chrysene (CHR), benzo[k]fluoranthene (BkF), benzo[g,h,i]perylene (BghiP), dibenzo[a,h]anthracene (DbahA), and indeno[1,2,3-c,d]pyrene (I123cdP), known as PAH8, finally reduced to the four specific PAHs BaP, BaA, BbF, and CHR, known as PAH4, are better suited for this task. The existing maximum levels (MLs) for food of animal origin, cocoa beans and products, vegetable oils, fats, and infant foods have been updated in the Regulation No. 835/2011

(Commission Regulation (EU) No 835/2011), amending Regulation No. 1881/2006 (Commission Regulation (EC) No 1881/2006).

The highest contributor to the dietary exposure of PAH4 is seafood and seafood products, followed by cereals and cereal products, vegetables, nuts and pulses, meat and meat products, fats and vegetable oils, fish and fishery products, fruits, and at last cheese (Duedahl-Olesen 2013).

The analysis of PAHs in fatty samples, as in food of animal origin, is commonly conducted by gas chromatography coupled to mass spectrometry (GC-MS) (Ledesma et al. 2014; Kamankesh et al. 2015; Sadowska-Rociek et al. 2015; Sapozhnikova and Lehotay 2015), GC with flame ionization detection (GC-FID) (Olatunji et al. 2014), high-performance liquid chromatography with fluorescence detection (HPLC-FLD) (Zhao et al. 2014), and UV detection (HPLC-UV) (Kumari et al. 2012). The conventional sample treatment and extraction is generally time-consuming, including ultrasonic extraction (Ledesma et al. 2014), liquid-liquid extraction (Schulz et al. 2014), saponification (Olatunji et al. 2014), microwave-assisted extraction (MAE) (Kamankesh et al. 2015), supercritical fluid extraction (SFE) (Yusty and Davina 2005), Soxhlet extraction (Ikechukwu et al. 2012), accelerated solvent extraction (ASE) (Martorell et al. 2010), or pressurized liquid extraction (PLE) (Hitzel et al. 2013), followed by a size exclusion chromatography (SEC) (Hitzel et al. 2013; Huertas-Perez et al. 2015). New approaches for the determination of PAHs in various matrices, including solid phase extraction (SPE) (Ledesma et al. 2014; Olatunji et al. 2014; Hitzel et al. 2013; Jung et al. 2013; Chukwujindu et al. 2014) and stir bar sorptive extraction (SBSE) (Pfannkoch et al. 2015), have been investigated and developed. A fast and simple sample preparation technique is the Quick, Easy, Cheap, Rugged, Effective, and Safe (QuEChERS) method. Several authors reported the successful extraction of PAHs from various food matrices with the QuEChERS method (Sapozhnikova and Lehotay 2013; Smith and Lynam 2012; Surma et al. 2014; Luzardo et al. 2013; Forsberg et al. 2011; Escarrone et al. 2014; Madureira et al. 2014), although the original method was developed for the determination of pesticides in fruits and vegetables (Anastassiades and Lehotay 2003).

The QuEChERS method includes an extraction step with the addition of an extraction solvent, usually acetonitrile, and liquid-liquid partitioning using extraction salts like $MgSO_4$ and NaCl. The sample clean-up consists of a dispersive solid phase extraction (d-SPE) using various sorbents to minimize co-extracted materials and improve chromatographic performance. In this study, we investigated different sorbents to examine the most effective clean-up procedure regarding the determination of PAHs. The following sorbents were used: primary-secondary amine (PSA) to remove fatty acids and sugars, C18 to remove non-polar materials, and zirconia-based sorbent (Z-Sep) to minimize interferences from fatty matrices and pigments and enhanced matrix removal lipid (EMR-lipid), which is a recently invented material applied at difficult matrices. Several authors investigated the efficiency of the EMR material concerning multi-residue pesticide analysis in fatty matrices (Lopez-Blanco et al. 2016; He et al. 2016; Dias et al. 2016; Vazquez et al. 2016)

There are several problems to address when analyzing PAHs in complex food matrices. PAHs can be bound strongly to the matrix (especially fatty matrices), which complicates their extraction. The separation of the critical pairs such as triphenylene (TPH) and CHR or BbF and its isomers BkF and benzo[j]fluoranthene (BjF) is essential for the qualification of PAH4 due to their high stability concerning fragmentation and the structural similarities shared with interfering PAHs.

The aim of this study was to develop a QuEChERS method for a broad range of foods from animal origin from moderate to high fat content, plant-derived products and infant foods for a high sample throughput and a cost-effective analysis of PAH4 and their possible interfering PAHs BaA, cyclopenta[c,d]pyrene (CPP), TPH, CHR, BbF, BkF, BjF, and BaP for more confident results with GC-MS/MS. Furthermore, monitoring PAHs would be encouraged due to lower costs and speed of analysis. A naturally contaminated reference material (smoked fish product FAPAS T0664QC) has been used to investigate the extraction efficiency and recovery of PAH4 with the QuEChERS method. The developed method should be implemented into a routine laboratory and fulfill the requirements set by the regulation (EU) No. 836/2011 (Commission Regulation (EU) No 836/2011).

Material and Methods

Reagents and Materials

All reagents and solvents were of analytical grade. Acetonitrile (VWR, Germany) and acetone (VWR, Germany) were obtained for sample preparation and GC-MS/MS analysis. Standards of BaA, CHR, BbF, and BkF were purchased from Supelco (USA), CPP and BjF from LGC Standards (Germany), and TPH and BaP from Sigma-Aldrich (Germany). The four deuterated PAHs benzo[a]pyrene-d12 (BaP-D12), benzo[a]anthracene-d12 (BaA-D12), chrysene-d12 (CHR-D12), and benzo[b]fluoranthene-d12 (BbF-D12) used as internal standards (ISs) were purchased from Sigma-Aldrich (Germany). De-ionized water was obtained from a Milli-Q system (Millipore USA). Anhydrous magnesium sulfate ($MgSO_4$), sodium chloride, di-sodium hydrogen citrate (DHS), tri-sodium citrate salts (TSCD), ready-to-use kits containing 900 mg $MgSO_4$, 150 mg primary-secondary amine (PSA), and 150 mg C18 (endcapped) were purchased from Bekolut

(Germany). Bondesil PSA was acquired from Varian (USA) and $MgSO_4$ from VWR (Germany). The EMR-lipid material was donated from Agilent Technologies (USA), Supel QuE Z-Sep was obtained from Supelco (USA), Plastipak 5-ml syringes from BD (USA), and polytetrafluoroethylene (PTFE) syringe filters with a pore size of 0.45 μm and a diameter of 13 mm were purchased from Whatman (UK). Fifteen-milliliter and 50-ml polypropylene centrifuge tubes were purchased from Falcon (USA).

Instrumentation

For homogenization, a Robot Coupe Blixer 3 (Robot Coupe, Austria) was used. Centrifugation was carried out with an Eppendorf 5430 centrifuge, and Collomix (Collomix, Germany) was used to vigorously shake the samples. For evaporation, a Biotage TurboVap LV (Biotage, Austria) was used.

A GC 7890A system (Agilent Technologies, USA) equipped with a 7690 autosampler (Agilent Technologies, USA) and connected with a 7000B triple quadrupole mass spectrometer (Agilent Technologies, USA) was used for the analysis of the PAHs. Chromatographic separation was achieved with a fused silica capillary column Select PAH (Agilent Technologies, USA) with a length of 30 m, 0.25 mm i.d., and 0.15 μm film thickness. Additionally, a Select PAH column (Agilent Technologies, USA) of 1.5 m length, 0.25 mm i.d., and 0.15 μm film thickness was installed as a guard column. An inert fused silica column with a length of 1.5 m and 0.25 mm i.d. was obtained from Agilent Technologies (USA). The guard column was connected from the inlet to the analytical column through a purged ultimate union (Agilent Technologies, USA) to enable backflush set at −0.5 ml/min and start at 40 min. Helium (99.999%) was used as carrier gas with a constant flow rate of 1.1 ml/min for the guard column and 1.3 ml/min for the analytical column. The filament multiplier delay time was set at 3.7 min. The oven temperature was programmed as follows: initial temperature of 70 °C held for 0.7 min, raised at 85 °C/min to 180 °C, then ramped at 3 °C/min to 230 °C, held for 7 min, ramped at 28 °C/min to 280 °C, held for 10 min, and at last ramped at 14 °C/min to 340 °C and maintained for 3 min. The total runtime was 45 min. The injection temperature was set at 320 °C with a pulsed splitless injection at a volume of 1 μl. The transfer line and both quadrupoles were set at 320 and 180 °C, respectively. Retention times, transitions, and collision energies are described in Table 1. The mass spectrometer was programmed as followed: electron impact ionization was set at 70 eV with an emission current of 35 μA. The electron multiplier voltage was operated through a gain factor of 9 at 2422 V. The analyses were carried out in multiple reaction monitoring (MRM) mode, after the determination of the retention times and collision energies. The acquired data was evaluated with the MassHunter Workstation software B.07.00/Build 7.0.457.0 (Agilent Technologies, USA).

Standards

Stock solutions were prepared in acetonitrile for each compound at a concentration of 1000 mg/kg and stored at −18 °C in the dark. A PAH mix standard solution was prepared by diluting 100 μl of each compound in 10 ml acetonitrile. The concentration of the PAH spiking solution ranged from 50 to 500 μg/kg.

Stock solutions for the individual ISs were prepared in acetonitrile at a concentration of 1000 mg/kg, and subsequently, 100 μl of each IS was diluted in 10 ml acetonitrile to create an IS mix solution. The concentration of the IS spiking solution ranged between 250 and 1000 μg/kg.

The calibration curve consisted of nine standards (0.2, 0.5, 1, 2, 5, 10, 20, 50, 75 μg/kg) including all the compounds and IS at a concentration of 10 μg/kg in all standard solutions.

Samples

The matrices salmon, shrimps, mussels, cutlet, bacon, curry spice powder, wheat flour, infant formula, infant follow-up formula, cereal-based baby foods with fruits, and baby foods with vegetables and meat were bought at local grocery stores. About 500 g to 1 kg of salmon, shrimps, mussels, cutlet, and bacon were homogenized in a Robot Coupe Blixer 3 with dry ice and stored at −18 °C. No homogenization was needed for the curry spice powder, infant formula, infant foods, and wheat flour. These samples were stored at room temperature.

The seafood and meat matrices have been chosen to cover a broad range of fat content ranging from lean matrices (shrimps, mussels) to high-fat matrices (salmon, bacon). Baby foods were chosen in a way to cover the infants' diet in its first year of development. Wheat is a widely consumed grain in Europe (http://ec.europa.eu/eurostat/statistics-explained/index.php/Agricultural_production_-_crops#Publications), due to the high consumption of bread and pasta and therefore was chosen as a representative matrix not regulated by Regulation 1881/2006. A curry spice powder was chosen, due to its composition as a mixture of different spices to cover the regulated dried spices (Commission Regulation (EU) 2015/1933).

Extraction Procedure

QuEChERS Method

The sample preparation consisted of a buffered QuEChERS method, extracting the analytes with acetonitrile. Two grams (for curry spice powder), 5 g, or 10 g of sample (for all matrices, except curry spice powder) was weighed in a 50-ml

Table 1 Retention time, qualifier, quantifier, and collision energies (CE) for the detection of PAHs

PAHs	$R_{t(min)}$	Transition for quantification ion (*m/z*)	Collision Energy (eV)	Transitions for qualification ion (*m/z*)	Collision energy (eV)
Benzo[a]anthracene	27.470	228 ➜ 226	30	228 ➜ 224,202 ➜ 200	40 30
Triphenylene	27.705	228 ➜ 226	40	228 ➜ 202,224 ➜ 222	30 40
Cyclopenta[c,d]pyrene	27.640	226 ➜ 224	50	226 ➜ 225,224 ➜ 222	30 40
Chrysene	27.765	228 ➜ 226	40	228 ➜ 224,228 ➜ 202	40 30
Benzo[b]fluoranthene	32.930	252 ➜ 250	30	253 ➜ 251,252 ➜ 248	30 60
Benzo[k]fluoranthene	33.080	252 ➜ 250	40	253 ➜ 251,252 ➜ 226	30 40
Benzo[j]fluoranthene	33.191	252 ➜ 250	50	250 ➜ 248,248 ➜ 246	50 50
Benzo[a]pyrene	35.742	252 ➜ 250	30	252 ➜ 226,250 ➜ 248	40 30
Benzo[a]anthracene-D_{12}	27.470	240 ➜ 236	40	240 ➜ 238,240 ➜ 212	20 30
Chrysene-D_{12}	27.765	240 ➜ 236	40	240 ➜ 238,236 ➜ 234	30 30
Benzo[b]fluoranthene-D_{12}	32.753	264 ➜ 260	40	264 ➜ 262,260 ➜ 256	30 50
Benzo[a]pyrene-D_{12}	35.526	264 ➜ 260	40	264 ➜ 262,260 ➜ 256	40 50

centrifuge tube and spiked with the PAH mix spiking solution and the IS mix standard solution. Ten grams was only used in the first experiment to investigate the influence of the amount of sample taken and compared with 5 g. Five milliliters of Millipore water was added, and the samples were shaken in the Collomix for 1 min. Ten milliliters of acetonitrile was added, and the samples were shaken for 2 min in the Collomix. Subsequently, a mix of salts containing 4 g anhydrous $MgSO_4$, 1 g of sodium chloride, 0.5 g of DHS, and 1 g of TSCD was added to the samples and the tubes were shaken for another 1 min in the Collomix. The samples were centrifuged at 6000 rpm for 5 min. Eight milliliters of the upper acetonitrile phase (to avoid losses of analytes) was transferred to 15-ml centrifuge tubes containing the investigated d-SPE clean-up sorbents, mixed in the Collomix for 30 s and centrifuged at 6000 rpm for 5 min. Three milliliters of the extract was transferred to a glass test tube and evaporated to dryness under a gentle stream of nitrogen (10.5 bar, 42 °C) to reach a certain concentration factor. The samples were reconstituted in 0.3 ml acetonitrile, vortexed, and filtered through a 0.45-μm PTFE filter into a GC vial.

EMR-Lipid Method

The procedure of the EMR-lipid differs from the application described above. After the analyte extraction with acetonitrile, the supernatant is directly transferred to the d-SPE tube for clean-up and the removal of residual water is conducted afterwards.

Usually, the EMR-lipid material is activated with additional water (Lucas and Zhao 2015), and therefore, the efficiency of the EMR-lipid was also tested with the addition of 5 ml Millipore water, with and without the extraction salts (4 g $MgSO_4$, 1 g NaCl, 0.5 g DHS, and 1 g TSCD). Therefore, we were able to directly compare the extraction method with the d-SPE clean-up with 900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18.

Three EMR-lipid applications have been investigated:

Application 1: 5 g of sample was weighed in a 50-ml centrifuge tube, 10 ml acetonitrile was added, and the samples were shaken for 2 min in the Collomix. Subsequently, the 50-ml tubes were centrifuged at 5000 rpm for 5 min. Ten milliliters of the supernatant was transferred to the EMR-lipid d-SPE tube, vortexed for 1 min, and centrifuged at 5000 rpm for another 5 min. Eight milliliters of the upper layer was transferred to the EMR polish tubes containing 2 g of salts (1:4, NaCl/MgSO4) to remove residual water, vortexed, and centrifuged at 5000 rpm for 5 min. Three milliliters of the extract was evaporated to dryness under a gentle stream of nitrogen (10.5 bar, 42 °C). The samples were reconstituted in 0.3 ml acetonitrile, vortexed, and filtered through a 0.45-μm PTFE filter into a GC vial.

Application 2: 5 g of sample was weighed in a 50-ml centrifuge tube, 5 ml Milli-Q water was added, and the samples were shaken for 1 min in the Collomix. Afterwards, 10 ml

acetonitrile was added and the samples were shaken for 2 min in the Collomix. A mix of salts containing 4 g $MgSO_4$, 1 g of sodium chloride, 0.5 g DHS, and 1 g of TSCD was added, and the samples were shaken for 1 min in the Collomix. The samples were centrifuged at 5000 rpm for 5 min. Subsequently, 10 ml of the upper phase was transferred to the EMR-lipid d-SPE tube, vortexed for 1 min, and centrifuged at 5000 rpm for 5 min. Eight milliliters of the extract was transferred to the EMR polish tube, vortexed, and centrifuged at 5000 rpm for 5 min. Three milliliters of this extract was evaporated to dryness under a gentle stream of nitrogen (10.5 bar, 42 °C). The samples were reconstituted in 0.3 ml acetonitrile, vortexed, and filtered through a 0.45-μm PTFE filter into a GC vial.

Application 3: Similar to application 2 without the addition of water.

Optimization of Chromatographic and Mass Spectrometric Conditions

Two guard column configurations have been investigated concerning the separation of the critical pairs. The Select PAH column (1.5 m) and the inert fused silica column (1.5 m), both identical in column dimensions, have been applied. To evaluate the most suitable injection technique, splitless and pulsed splitless injections were investigated with altering the injection volume from 1 to 3 μl and the injection temperature of 70 °C held for 0.1 min and ramped at 850 °C/min to 320 and 320 °C as a constant temperature. For the optimized MS/MS conditions, precursor ions were selected and exposed to collision energies reaching from 10 to 60 eV in intervals of 10 eV. Two time segments were applied in MRM mode starting at 12 min detecting BaA, TPH, CHR, CPP, BaA-D_{12}, and CHR-D_{12}, followed by switching the acquisition at 30 min to detect BbF, BkF, BjF, BaP, BbF-D_{12}, and BaP-D_{12}. The dwell time was set to 10 ms for the first time segment and 17 ms for the second segment to allow 4.4 Hz. The analytes were confirmed by one precursor ion and two product ions shown in Table 1. Standards, natural contaminated reference materials from FAPAS (T0664QC), and spiked samples were used to determine the performance parameters of the GC-MS/MS analysis.

Investigation of the Appropriate QuEChERS Method

To select the most suitable extraction method, salmon was selected due to the high fat content and the known complexity of the matrix (Forsberg et al. 2011). Blank salmon samples and fortified blank salmon samples were extracted and analyzed in duplicates.

QuEChERS

Fortification of blank salmon samples was carried out spiking 50 μl, and respectively 100 μl, of a PAH mix standard with a concentration of 100 μg/kg to 5 g, and respectively 10 g, of sample to reach a concentration of 1 μg/kg. The fortified samples were vortexed and stored at room temperature for 30 min in the dark to avoid UV light exposure before extraction.

$MgSO_4$ + PSA + C18, Z-Sep, and EMR-lipid were investigated regarding the recovery rates and the chromatographic separation of the PAHs.

To evaluate the effects on matrix interference of the sample weight, 5 and 10 g, respectively, of salmon were weighed and fortified at 1 μg/kg, and three different clean-ups were investigated: (1) 900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18, (2) 500 mg Z-Sep, and (3) 500 mg $MgSO_4$ + 500 mg Z-Sep.

Since no significant difference could be observed concerning recovery rates and peak shapes between 5 and 10 g of sample, further experiments were carried out with 5 g of sample. In order to investigate the influence of the amount of $MgSO_4$, 5 g of salmon was weighed and fortified at 1 μg/kg and six sets of d-SPE sorbents were evaluated: 150 mg PSA + 150 mg C18 or 500 mg Z-Sep + 150 mg PSA were combined with 500, 1500, and 2500 mg $MgSO_4$.

EMR-Lipid

Fortification of blank salmon samples was carried out spiking 100 μl of a PAH mix standard with a concentration of 100 μg/kg to 5 g sample to reach a concentration of 2 μg/kg (ML). The fortified samples were vortexed and stored at room temperature for 30 min in the dark to avoid UV light exposure before extraction.

Validation

The method used for validation was the QuEChERS method with 900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18 as d-SPE clean-up (Fig. 1). Validation took place with salmon, shrimps, mussels, cutlet, bacon, curry spice powder, wheat flour, infant formula, infant follow-up formula, cereal-based baby foods with fruits, and baby foods with vegetables and meat. Blank matrices—as far as available—were spiked at two concentrations and analyzed with seven replicates on each level and on different days. The spiked analyte concentrations of the various food matrices were as follows: salmon, shrimps, cutlet, bacon, wheat flour (0.5 and 2 μg/kg), mussels (0.5 and 5 μg/kg), curry spice powder (1 and 10 μg/kg), infant formula, infant follow-up formula, cereal-based baby food with fruits, and baby food with meat and vegetables (0.5 and 1 μg/kg). To fulfill the analytical performance criteria set by the Commission Regulation 836/2011, the recovery rates had to be between 50 and 120% and LODs and LOQs of ≤03 and

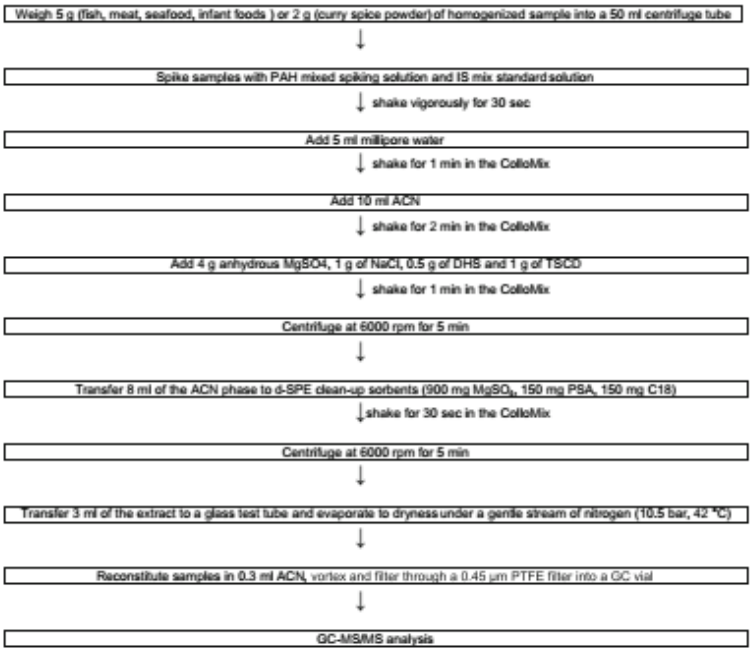

Fig. 1 Scheme of the final QuEChERS sample preparation procedure

≤0.9 µg/kg, respectively, for each of the PAH4 to be reached. The repeatability should be ≤22%. The certified reference material (CRM) smoked fish product from FAPAS (T0664QC) was used as quality control material for the validation experiments. The batch was considered valid, when the PAH concentrations of the CRM were within its given satisfactory range. LODs and LOQs were determined in the fortified blank samples with a signal-to-noise ratio of 3 and 10, respectively (Table 2a–c).

Results and Discussion

Optimized Chromatographic and Mass Spectrometric Conditions

The injection conditions, the column configuration, and the temperature program of the GC-MS/MS analysis have been altered various times, until the optimized separation shown in Fig. 2 could be achieved. An inlet temperature of 320 °C was chosen to assure the evaporation of the heavier PAHs and prevent their deposition in the liner. A pulsed splitless injection was necessary to enable the rapid transfer of the PAHs onto the column with 1 µl injection volume. Two guard column configurations have been investigated concerning the separation of the critical pairs. The Select PAH column (1.5 m) and the inert fused silica column (1.5 m), both identical in column dimensions, have been applied. Using the Select PAH column resulted in an improved separation of the critical pairs. Chromatographic separation of the critical pairs is crucial for the qualification and quantification of PAH4, due to the structural similarities including the critical pairs, the high stability of the PAHs, and the same transitions achieved by using high collision energies. The separation of TPH and CHR is of utmost importance, since there are only a few columns available achieving this goal. The runtime of 45 min can be considered as a long runtime for a routine laboratory, but in this case it is acceptable, due to the constant and confident separation of the critical pairs, which is in accordance with the findings of other authors (Oostdijk 2010). The calibration curves showed a linear regression of $R^2 \geq 0.99$.

The critical pair TPH and CHR could be separated with the Select PAH column under the applied chromatographic conditions, even though a baseline separation was not possible at this time. These results are in accordance to the literature (Oostdijk 2010). Four lots of mussels were analyzed according to the selected QuEChERS method with 900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18, and TPH and CHR could be found in each lot. Therefore, separating these two PAHs is essential to avoid false-positive results, since only CHR is

Table 2 Validation parameters ($n = 7$) of salmon, mussels, shrimps, bacon, and cutlet spiked at 0.5 and 2 μg/kg

Matrix	Compounds	Recoveries %	RSD %	Matrix	Compounds	Recoveries %	RSD %	LOD (μg/kg)	LOQ (μg/kg)
Salmon (0.5 μg/kg)	BaA	102	2	Salmon (2 μg/kg)	BaA	94	8	0.06	0.18
	TPH	96	8		TPH	93	9	0.11	0.33
	CPP	98	7		CPP	88	8	0.08	0.25
	CHR	101	7		CHR	98	11	0.13	0.4
	BbF	103	5		BbF	93	5	0.04	0.12
	BkF	107	6		BkF	98	8	0.05	0.15
	BjF	93	9		BjF	85	9	0.04	0.11
	BaP	94	8		BaP	89	4	0.08	0.25
Mussels (0.5 μg/kg)	BaA	94	5	Mussels (5 μg/kg)	BaA	99	3	0.12	0.35
	TPH	91	21		TPH	95	8	0.1	0.29
	CPP	100	5		CPP	91	10	0.06	0.19
	CHR	89	17		CHR	103	11	0.15	0.45
	BbF	102	16		BbF	93	5	0.07	0.2
	BkF	87	16		BkF	96	7	0.07	0.2
	BjF	93	15		BjF	104	7	0.09	0.27
	BaP	75	20		BaP	94	8	0.05	0.15
Shrimps (0.5 μg/kg)	BaA	100	3	Shrimps (2 μg/kg)	BaA	97	6	0.07	0.21
	TPH	102	5		TPH	93	7	0.07	0.22
	CPP	102	4		CPP	95	8	0.04	0.13
	CHR	104	5		CHR	103	8	0.09	0.27
	BbF	94	4		BbF	85	3	0.05	0.15
	BkF	95	5		BkF	90	10	0.05	0.14
	BjF	99	9		BjF	102	7	0.04	0.132
	BaP	81	5		BaP	88	3	0.12	0.36
Bacon (0.5 μg/kg)	BaA	99	4	Bacon (2 μg/kg)	BaA	99	7	0.14	0.43
	TPH	97	8		TPH	93	9	0.05	0.15
	CPP	100	7		CPP	90	7	0.08	0.24
	CHR	102	7		CHR	99	6	0.08	0.24
	BbF	77	5		BbF	82	5	0.06	1.9
	BkF	84	4		BkF	93	8	0.07	0.22
	BjF	79	8		BjF	87	5	0.04	0.12
	BaP	105	4		BaP	92	10	0.14	0.42
Cutlet (0.5 μg/kg)	BaA	96	6	Cutlet (2 μg/kg)	BaA	98	6	0.04	0.12
	TPH	96	6		TPH	95	8	0.07	0.21
	CPP	99	8		CPP	91	5	0.04	0.13
	CHR	101	8		CHR	97	8	0.11	0.32
	BbF	93	6		BbF	89	9	0.05	0.16
	BkF	93	10		BkF	94	6	0.03	0.1
	BjF	100	9		BjF	89	11	0.05	0.16
	BaP	88	8		BaP	90	4	0.06	0.19
Infant formula (0.5 μg/kg)	BaA	96	7	Infant formula (1 μg/kg)	BaA	87	8	0.16	0.49
	TPH	94	4		TPH	89	7	0.14	0.42
	CPP	93	9		CPP	90	7	0.06	0.19
	CHR	95	3		CHR	96	6	0.04	0.13
	BbF	94	7		BbF	86	3	0.11	0.34
	BkF	95	3		BkF	88	4	0.1	0.29
	BjF	93	5		BjF	91	3	0.11	0.32
	BaP	103	9		BaP	89	6	0.14	0.41

Table 2 (continued)

Matrix	Compounds	Recoveries %	RSD %	Matrix	Compounds	Recoveries %	RSD %	LOD (μg/kg)	LOQ (μg/kg)
Infant follow-up formula (0.5 μg/kg)	BaA	92	13	Infant follow-up formula (1 μg/kg)	BaA	93	11	0.16	0.47
	TPH	90	11		TPH	88	6	0.13	0.4
	CPP	90	5		CPP	83	4	0.12	0.35
	CHR	95	7		CHR	98	8	0.16	0.47
	BbF	91	5		BbF	84	8	0.1	0.31
	BkF	84	11		BkF	87	7	0.13	0.39
	BjF	95	9		BjF	92	9	0.1	0.31
	BaP	98	10		BaP	96	7	0.14	0.41
Infant cereal-based food (0.5 μg/kg)	BaA	90	7	Infant cereal-based food (1 μg/kg)	BaA	90	8	0.16	0.49
	TPH	85	4		TPH	84	8	0.12	0.37
	CPP	86	10		CPP	83	7	0.06	0.18
	CHR	97	10		CHR	98	10	0.13	0.39
	BbF	86	5		BbF	86	3	0.06	0.17
	BkF	88	5		BkF	90	3	0.06	0.18
	BjF	90	6		BjF	89	4	0.07	0.22
	BaP	99	4		BaP	94	7	0.09	0.26
Infant food with meat (0.5 μ/kg)	BaA	90	7	Infant food with meat (1 μ/kg)	BaA	89	6	0.11	0.32
	TPH	90	9		TPH	87	8	0.11	0.33
	CPP	90	7		CPP	85	6	0.04	0.13
	CHR	92	11		CHR	97	10	0.1	0.3
	BbF	83	6		BbF	83	9	0.05	0.14
	BkF	88	7		BkF	85	5	0.06	0.17
	BjF	87	5		BjF	88	9	0.06	0.19
	BaP	101	7		BaP	95	8	0.07	0.2
Wheat flour (0.5 μg/kg)	BaA	95	9	Wheat flour (2 μg/kg)	BaA	94	8	0.04	0.13
	TPH	92	6		TPH	89	5	0.06	0.19
	CPP	99	6		CPP	89	8	0.06	0.18
	CHR	102	9		CHR	101	9	0.08	0.25
	BbF	92	7		BbF	84	4	0.06	0.19
	BkF	94	7		BkF	91	5	0.08	0.23
	BjF	101	5		BjF	102	5	0.09	0.27
	BaP	96	9		BaP	92	4	0.07	0.2
Curry spice powder (1 μg/kg)	BaA	96	10	Curry spice powder (10 μg/kg)	BaA	91	5	0.27	0.8
	TPH	94	12		TPH	85	4	0.3	0.9
	CPP	108	7		CPP	86	6	0.15	0.46
	CHR	105	5		CHR	97	4	0.34	1
	BbF	98	4		BbF	89	4	0.17	0.5
	BkF	99	8		BkF	95	7	0.1	0.29
	BjF	104	12		BjF	85	15	0.12	0.36
	BaP	97	6		BaP	87	7	0.15	0.45

regulated in various foodstuffs (Commission Regulation No 835/2011). The separation of BbF from BkF and BjF could be achieved with all QuEChERS approaches, comprising an issue of minor concern.

QuEChERS Method Optimization

In the present study, the QuEChERS method was adapted for the determination of PAH4 in foods of plant and animal origin.

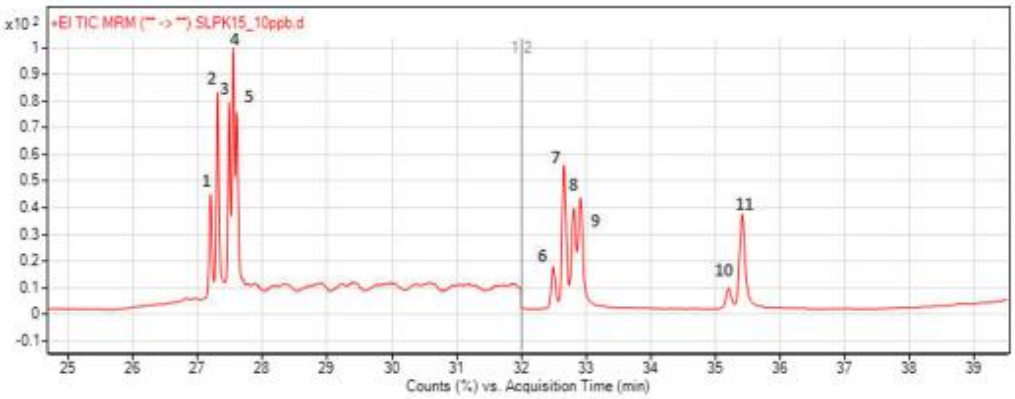

Fig. 2 Separation of the critical pairs in a 10 μg/kg standard with the described method. The analytes were dissolved in acetonitrile. *1* BaA-D12, *2* BaA, *3* CPP, *4* TPH, *5* CHR, *6* BbF-D12, *7* BbF, *8* BkF, *9* BjF, *10* BaP-D12, *11* BaP

Acetonitrile was chosen as the extraction solvent for the presented matrices, after the investigation of other solvents (mixture of acetone, ethyl acetate, and iso-octane (2:2:1; *v*/*v*/*v*)). The recoveries for the investigated solvent mixture ranged from 50 to 190%. Surma et al. (2014) used ethyl acetate as an extraction solvent and PSA with C18 as a d-SPE clean-up in ham. This experiment showed more promising results than the extraction with acetonitrile and the subsequent clean-up, resulting in low recovery rates (90% BaA, 55% CHR, 25% BkF and BbF, 39% BaP), contradicting our findings (recoveries with acetonitrile 91% BaA, 102% CHR, 89% BbF, 61% BkF, 89% BaP). To the same conclusion came another study (Forsberg et al. 2011) comparing the extraction of a mixture of acetone, ethyl acetate, and iso-octane (2:2:1; *v*/*v*/*v*) with the extraction of acetonitrile, showing low recovery rates using acetonitrile in salmon (24 to 88%). However, it has been demonstrated that ethyl acetate compared to acetonitrile increases the amount of co-extracted fat (Sapozhnikova et al. 2015).

A single sequence usually consists of a maximum of 30 samples, leaving its mark on the chromatographic

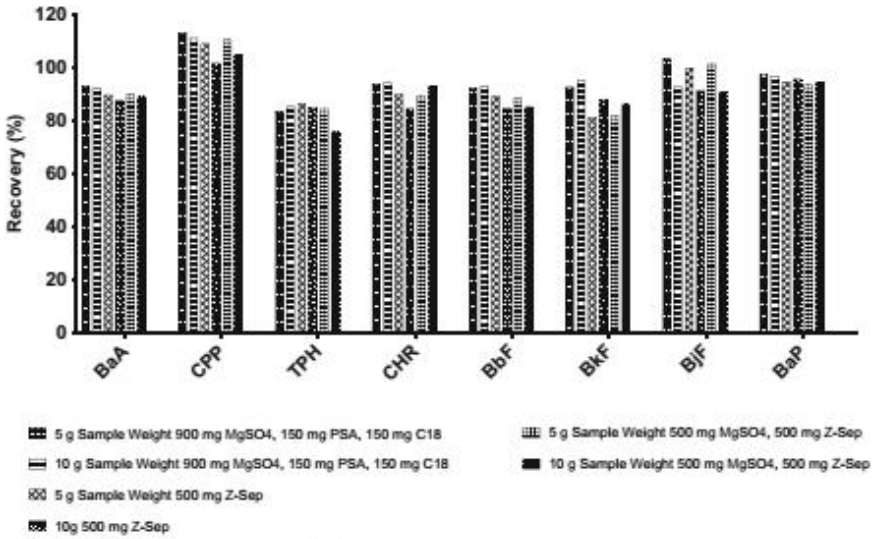

Fig. 3 Recoveries of two sample weights with three different clean-ups in salmon

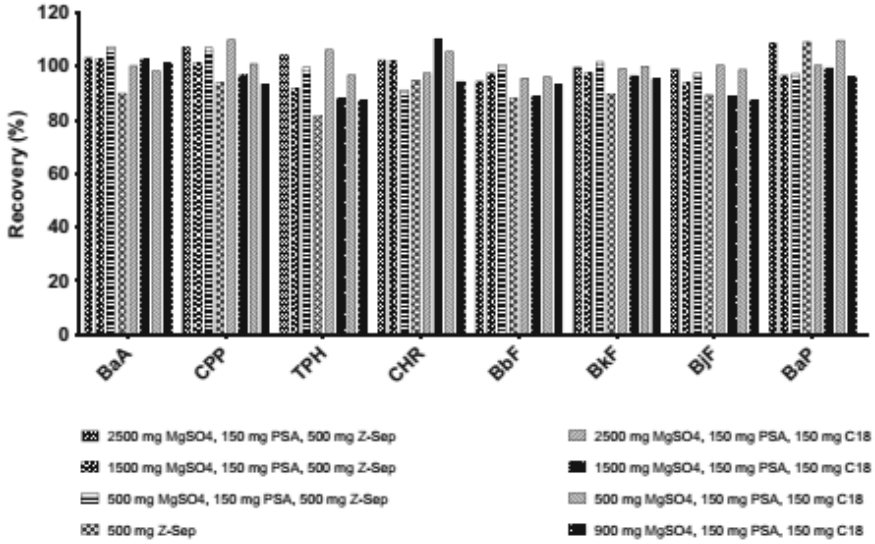

Fig. 4 Extraction efficiency of the clean-up steps with various amounts of MgSO4 with PSA combined with Z-Sep and PSA combined with C18 in salmon spiked with the nine contaminants (1 μg/kg)

performance. In this sample preparation technique and column configuration, it was necessary to cut the guard column after every sequence to maintain the proper separation of the critical pairs. The shift of the retention times is compensated by the use of the IS.

The separation of TPH and CHR and BbF, BkF, and BjF did not differ by weighing 5 or 10 g of sample and also not between the different clean-ups. The recovery rates shown in Fig. 3 ranged from 88 to 92% for BaA, 102 to 113% for CPP, 76 to 87% for TPH, 90 to 95% for CHR, 85 to 93% for BbF,

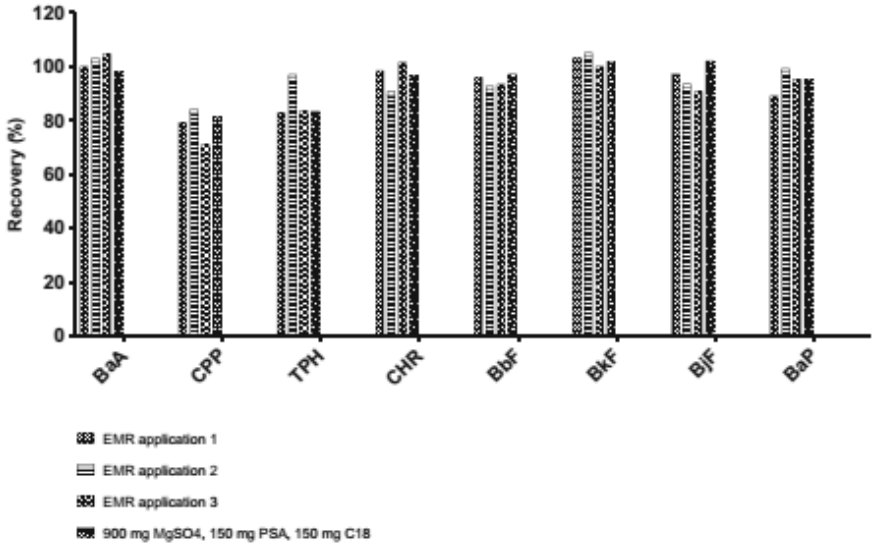

Fig. 5 Recoveries of the three described EMR-lipid applications compared with the C18 clean-up in salmon

81 to 95% for BkF, 90 to 103% for BjF, and 83 to 92% for BaP.

Since there were no differences analyzing 5 or 10 g of sample, a sample weight of 5 g was selected for further experiments. As shown in Fig. 4, no difference could be observed in the recovery rates and the separation of the critical pairs in using various amounts of $MgSO_4$ combined with the other sorbents (PSA + Z-Sep and PSA + C18). Recoveries ranged from 98 to 107% for BaA, 93 to 110% for CPP, 82 to 106% for TPH, 91 to 110% for CHR, 89 to 101% for BbF, 90 to 102% for BkF, 87 to 101% for BjF, and 96 to 110% for BaP. Sapozhnikova and Lehotay (2013) investigated three different clean-up sorbents (C18 + PSA, Z-Sep, and Z-Sep+) for the development of a multi-class, multi-residue method for the determination of PAHs, polychlorinated biphenyl congeners, and polybrominated diphenyl ether in catfish. The final method included a clean-up with the Z-Sep sorbent showing slightly lower recoveries concerning the PAHs investigated in our study (BaA 71 to 101%, CHR 70 to 108%, BbF + BkF 71 to 107%, BaP 80 to 107%). They also noted that a d-SPE clean-up with $MgSO_4$, PSA, and C18 showed a higher variability, when analyzed in duplicate concerning the recovery rates, which does not hold true for the analytes and matrices investigated in this study.

Concerning the experiments with the EMR-lipid material, as shown in Fig. 5, the recovery rates did not significantly differ between the three EMR approaches and the 900 mg $MgSO_4$ + 150 mg PSA + 150 mg C18 clean-up. Recoveries ranged from 103 to 105% for BaA, 71 to 84% for CPP, 83 to 97% for TPH, 91 to 102% for CHR, 93 to 96% for BbF, 100 to 105% for BkF, 91 to 97% for BjF, and 89 to 99% for BaP. Han et al. (2016) investigated the extraction efficiency (amount of EMR lipid vs extract volume) and recovery rates of 15 PAHs in 4 different matrices. The ratios of amount of EMR lipid material and extract volume are well comparable, as are the recovery rates (60 to 120%).

No difference concerning the separation of TPH and CHR could be observed between Z-Sep, mixture of $MgSO_4$ + PSA + C18, and EMR-lipid. The $MgSO_4$ + PSA + C18 clean-up was preferred due to the slightly higher response of the analytes, the availability of ready-to-use kits, and the price.

Validation

In order to test the trueness of the method, a natural contaminated CRM of fish (FAPAS T0664QC) was analyzed in all batches. The results of the CRM are presented in Table 3. As can be seen, the QuEChERS method used is not only accurate but also precise for the extraction and analysis of PAHs in samples with a high fat content. Therefore, it is

Table 3 Validation parameters ($n = 7$) for smoked fish product, used as QC, and values compared with the estimated results from FAPAS (T0664QC)

Compound		Assigned value (µg/kg)	Range for $\lvert z \rvert \leq 2$	Value N1 (µg/kg)	Value N2 (µg/kg)	Value N3 (µg/kg)	Value N4 (µg/kg)	Value N5 (µg/kg)	Value N6 (µg/kg)	Value N7 (µg/kg)	Average recovery (%)	RSD (%)
	ML (µg/kg)											
Benzo[a]anthracene	∑ of PAH4 12	25.8	14.45–37.15	26.25	27.90	27.03	28.72	26.71	28.80	28.87	108	4
Chrysene	∑ of PAH4 12	31.64	17.72–45.56	33.37	34.61	35.29	32.57	27.76	35.10	31.43	104	8
Benzo[b]fluoranthene	∑ of PAH4 12	12.97	7.26–18.68	14.26	15.04	14.76	14.47	15.43	12.91	12.88	110	7
Benzo[a]pyrene	2	10.29	5.76–14.82	11.94	11.90	12.02	11.48	11.32	12.09	11.04	114	3
∑ of PAH4	12	78.36	43.88–112.83	85.81	89.44	89.11	87.24	81.22	88.91	84.22	110	4

possible to extract PAH from naturally contaminated fatty food matrices. Concerning the validation of the 11 matrices, the only compound where the requirements for the LOD and LOQ (marked in red) according to the current regulation (Commission Regulation No 836/2011) could not been reached is CHR in the curry spice powder. This might be due to the natural contamination of the used matrix. The new Commission Regulation 2015/1933 did set the maximum levels for dried spices and herbs at 10 μg/kg for BaP and 50 μg/kg for the sum of PAH4. These levels can easily be reached with the validated method. The recovery rates ranged from 87 to 102% with RSD ≤13% for BaA, 83 to 108% with RSD ≤10% for CPP, 84 to 102% with RSD ≤21% for TPH, 89 to 105% with RSD ≤17% for CHR, 77 to 103% with RSD ≤16% for BbF, 84 to 107% with RSD ≤16% for BkF, 79 to 104% with RSD ≤15% for BjF, and 75 to 105% with RSD ≤20% for BaP (Table 2a–c).

The results of this study are in good accordance with the literature comparing the recovery rates with other developed QuEChERS methods (Sapozhnikova and Lehotay 2013, 2015; Surma et al. 2014; Forsberg et al. 2011; Escarrone et al. 2014). The recovery ranges of the applied method of Escarrone et al. (2014), who used $MgSO_4$ and PSA for the determination of PAHs in rice grain, were between 70 and 90% for PAH4. Although the recoveries were in the acceptable range, LOQs, stipulated by the European commission (Comission Regulation (EU) 836/2011), could not been reached.

We also participated in two proficiency tests (PTs). One was a spiked curry spice powder and the other one was a naturally contaminated smoked pepper from the European Reference Laboratory (EURL). Both PTs were passed with z scores of 0.8 for BaA, 0.6 for BaP, 0.5 for BbF, −0.1 for CHR, and 0.5 for the sum of PAH4 for the curry spice powder and 0.0 for BaA, −0.8 for BaP, −0.2 for BbF, −0.1 for CHR, and −0.4 for the sum of PAH4 for the smoked pepper PT. Therefore, the trueness of this method has been proven not only by the use of a CRM but also by interlaboratory comparisons including naturally contaminated samples.

Comparing the recoveries of this study with Soxhlet extraction, accelerated solvent extraction (ASE) and SPE (Ikechukwu et al. 2012; Londono et al. 2013) (recovery ratios between 55 and 82% for PAH4) comprise the equality of the developed QuEChERS method with the advantages of a higher sample throughput and cost reductions.

Conclusion

In this work, a QuEChERS-based extraction for the determination of PAH4 including their possible interfering PAHs was developed and validated by GC-MS/MS. Extracting the analytes with acetonitrile combined with the $MgSO_4$ + PSA + C18 d-SPE clean-up resulted in the reduction of co-extractive materials, good accuracy, and precision for all analytes in the given matrices. Aiming at incorporating a simple sample preparation technique into a routine laboratory to enable a high throughput of samples in a cost-effective way is feasible with the selected method since 20 samples can easily be extracted in 4 h.

Salmon, shrimps, mussels, cutlet, bacon, curry spice powder, wheat flour, infant formula, infant follow-up formula, cereal-based baby foods with fruits, and baby foods with vegetables and meat were validated, and all requirements of the commission regulation 836/2011 including recoveries, repeatability, LODs, and LOQs were fulfilled. The exception was CHR in the curry spice powder, where the LOD and LOQ could not been reached due to the natural contamination of the sample. Nevertheless, all MLs according to the commission regulation 1881/2006 could be successfully achieved in all matrices. The validated method was also tested in its trueness, using a naturally contaminated CRM as quality control material showing good recovery rates and repeatability of all compounds of interest. We also demonstrated the extraction of PAH4 from a naturally contaminated CRM (smoked fish product). This developed method is fit for purpose for the determination of PAH4 in a routine laboratory, avoiding false-positive results by chromatographically separating the contaminants of interest from their interfering PAHs.

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Compliance with Ethical Standards

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Conflict of Interest Michael Urban declares that he has no conflict of interest. Céline Lesueur declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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4.2 Publication II

Simultaneous determination of pesticides, mycotoxins, tropane alkaloids, growth regulators and pyrrolizidine alkaloids in oats and whole wheat grains after online clean-up via two-dimensional liquid chromatography tandem mass spectrometry, Urban, M., Hann, S., Rost H. J. Environ. Sci. Health. Part B. 2019, DOI: 10.1080/03601234.2018.1531662

Brief description:

This work proposes a method for the determination of various residues and contaminants with a minimized sample preparation procedure in oats and whole wheat grains. The two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) system in combination with the applied sample preparation, based on a “dilute and shoot” approach, showed satisfactory results for the abundance of target analytes in both matrices. Implementing this 2D-LC-MS/MS system into laboratory has the capability to effectively reduce time and costs for the analysis of the investigated residues and contaminants alike.

Novelty statement:

We report an extension of the range of analytes and an adjusted sample preparation for an existing 2D-LC-MS/MS system with integrated online clean-up [78] for up to 400 pesticides and contaminants fulfilling the regulatory requirements for the abundance of analytes in oats and whole wheat grains.

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Simultaneous determination of pesticides, mycotoxins, tropane alkaloids, growth regulators, and pyrrolizidine alkaloids in oats and whole wheat grains after online clean-up via two-dimensional liquid chromatography tandem mass spectrometry

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ABSTRACT

In this study, a two-dimensional liquid chromatography tandem mass spectrometry method was developed and validated for the determination of pesticide residues and contaminants in whole wheat grains and oats. The samples were extracted with a mixture of acetonitrile and water and were injected into the two-dimensional LC-MS/MS system without any further clean-up or sample preparation. Samples were analyzed with four different matrix matched calibrations. Matrix effects were evaluated by comparing analyte signals in the respective matrix matched standard with the neat solvent standards. The final method was validated according to the current Eurachem validation guide and SANTE document. The number of successfully validated analytes throughout all three validation levels in oats and wheat, respectively, were as follows: 330 and 316 out of 370 pesticides, 6 and 13 out of 18 pyrrolizidine alkaloids and 7 out of 9 regulated mycotoxins. Moreover, both plant growth regulators mepiquat and chlormequat as well as the tropane alkaloids atropine and scopolamine met the validation criteria. The majority of pesticides showed limits of detection below 1 µg kg^{-1}, pyrrolizidine alkaloids below 0.7 µg kg^{-1}, tropane alkaloids below 0.2 µg kg^{-1}, growth regulators below 0.7 µg kg^{-1} and mycotoxins below 8 µg kg^{-1} in both matrices.

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KEYWORDS
Mycotoxins; plant growth regulators; pyrrolizidine alkaloids; tropane alkaloids; two-dimensional liquid chromatography tandem mass spectrometry

Introduction

Nowadays the need for analytical methods addressing the simultaneous determination of multiple analyte classes in a single analytical run is increasing. In reversed-phase liquid chromatography tandem mass spectrometry the variation of polarities of the selected target compounds often makes their accurate determination difficult.[1] The use of comprehensive approaches with more than a single column separation was investigated with methods including: heart-cut two dimensional liquid chromatography (2D-LC)[2] and comprehensive 2D-LC (LCxLC).[3] In an LCxLC approach choosing two independent separation mechanisms in two chromatography dimensions can result in a higher resolving power and improved chromatographic efficiency.[4] In LCxLC it is of utmost importance to mind possible solvent incompatibilities and immiscibilities. A high degree of orthogonality or analysis of different food commodities can be achieved by the combination of a hydrophilic interaction chromatography column (HILIC) phase in the first dimension coupled to a reversed phase (RP) in the second dimension.[5] The most frequently 2D-LC setup in food analysis is using the first dimension to remove unwanted compounds and coextracted matrix components and the second column for the chromatographic separation of the analytes.[6] Therefore, the first dimension is used in terms of a clean-up step and the second one for the analytical separation. Nevertheless, there are some limitations, which must be considered when combining different separation mechanisms, that is mobile phase immiscibilities, precipitation of buffer salts and stationary phase incompatibilities, which may negatively influence the chromatographic performance.[7] If the elution strength of the effluent of the first dimension is too high for the second dimension this may negatively affect peak capacity and chromatographic efficiency. In part, this can be compensated by increasing the flow rate of the second dimension, but increasing the flow rate leads to higher dilution factors and lower sensitivity.[8] Another option to overcome this issue is to use a packed loop interface to enable the replacement of the mobile phase of the first dimension while target analytes remain saved on the trapping column.[2,8]

All investigated analyte classes are of great concern for human health. Pyrrolizidine alkaloids (PAs) are known to be carcinogenic and genotoxic when chronically exposed to, while acute toxicity mostly affects the lungs and the liver with the latter being associated with accidents.[9] In terms of carcinogenic contaminants the ALARA principle (as low as reasonably achievable) is used to limit human exposure to

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carcinogens. Today, PAs are not regulated in foods with a respective maximum level and neither analytical criteria nor target analytes have been established.[9]

Oats and cereals are known to be frequently contaminated with mycotoxins, therefore playing a major role in human exposure. Mycotoxins are known to be cytotoxic, immunosuppressive, and carcinogenic and are of major concern for food and feed safety.[10] The analytical performance criteria for the analysis of mycotoxins have been laid down in the Commission Regulation 519/2014[11] for all regulated mycotoxins regarding recovery rates, RSDs and the respective concentrations.

As for tropane alkaloids (TAs) it is known that low amounts of atropine in human nutrition might trigger severe poisoning symptoms, which is why the EU encourages the development of sensitive methods with LOQs ranging from 5 µg kg^{-1} to a maximum of 10 µg kg^{-1} for agricultural raw materials, lower than 2 µg kg^{-1} for manufactured products and 1 µg kg^{-1} for cereal-based infant foods.[12]

Plant growth regulators are widely applied for the improvement of the fruit size, color, and shape and to influence the growth of the crops or fruits. Both chlormequat (CCC) and mepiquat (MQ) are classified as moderately hazardous by the WHO.[13] MRLs have been established in cereals ranging from 20 to 3000 µg kg^{-1}.[14]

Pesticides are widely used in modern agriculture and are of major concern for food safety. The SANTE guideline[15] lays down all analytical requirements concerning the validation of pesticides in food and feed. A general default MRL of 0.01 mg kg^{-1} is applied to every pesticide, except mentioned otherwise. As for food analysis various 2D-LC approaches have been proposed. For the determination of pesticides, a parallel LC column assembly with a HILIC and a RP dimension has been applied for the separation and determination of 41 pesticides with a variety of polarities in a single run.[16] The samples were simultaneously injected and the eluents were combined prior to mass spectrometry (MS) detection. The separation of isomeric PAs and PA oxides in plant extracts was achieved with a multiple heart-cutting approach.[17] For mycotoxins a heart-cut 2D-LC-MS/MS was applied for the determination of aflatoxin B1 in cereal-based baby foods.[18] Other two-dimensional LC approaches in food analysis include the determination of polycyclic aromatic hydrocarbons in nonfat and fatty foods,[19] veterinary drugs in honey, milk and meat, heterocyclic amines in meat[6] and polyphenols in various food commodities.[20]

The QuEChERS method (quick, easy, cheap, effective, rugged, and safe) is the most frequently used sample preparation technique for the determination of pesticides in various food commodities[21] and has been extended and modified for the extraction of other analytes, like polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polybrominated diphenyl ethers, and flame retardants.[22] Discovered and developed by Anastassiades and Lehotay, this method successfully reduced costs and time for the analytical procedure. Even though samples are cleaned-up with a dispersive solid phase extraction (dSPE) matrix effects occur,[23] which leaves laboratories with the choice of tolerating signal suppression, enhancement or switching to a more time-consuming sample preparation.

The sample preparation of the target matrices for the determination of pesticides usually includes a clean-up step due to the complexity of cereals. A SPE is widely used in order to remove co-extracted compounds, but the number of pesticides extracted is limited due to lower selectivity of these cartridges.[24] Several studies investigated pesticides in cereals using a dSPE clean-up.[24–26] Additionally, the QuEChERS technique seems to be the right choice for the determination of Tas,[24,27] Pas,[24,28] plant growth regulators[29] and mycotoxins.[30–32] But the recent approach of Kittlaus and colleagues[2] with an online clean-up implemented into a two-dimensional LC-MS/MS system with the determination of more than 300 pesticides showed impressive results with a time and cost saving sample preparation approach. The integrated online clean-up resembles the first step of the QuEChERS method and possibly improves the determination of various compound classes in one analytical run. In this work the aim was to further investigate the capabilities of this two-dimensional LC-MS/MS setup by Kittlaus and colleagues[2] with expanding the list of substances analyzed in one chromatographic run to 370 pesticides, 2 TAs, 30 PAs, 2 plant growth regulators and 9 regulated mycotoxins.

A brief description of the method is as follows: To minimize the sample preparation procedure an online clean-up was implemented. The polar analytes were retained by the HILIC in the first dimension, while the nonpolar analytes were stored on a packed loop interface. Subsequently the compounds retained on the HILIC were detected. All nonpolar compounds on the trapping column were eluted and retained by an analytical RP column. The sample preparation procedure was adapted[2] and modified to meet the needs of the analytes for extraction. Another aim of this study was to implement this method into a routine laboratory to reduce costs, simplify the sample preparation and ease the data analysis for a higher sample throughput. Additionally, more information about the contamination and residues of the samples can be gathered through the wide scope of compounds analyzed in one run, thus improving the monitoring of various analytes regarding time and costs. To the best of our knowledge, a multi 2D-LC-MS/MS method for the variety of analytes has not yet been developed.

Material and methods

Chemicals and reagents

Pesticides were purchased from Sigma Aldrich (Germany), LGC (Germany) or HPC (Germany) and were certified standards at analytical grade. Stock solutions of the individual analytes were prepared in acetonitrile or methanol at a concentration of 1000 mg kg^{-1}. Nine standard mixtures were prepared in acetonitrile with a concentration of 10 mg kg^{-1} of each of the substances. Again, all mixtures were united in a final pesticide mix at a concentration of

1 mg kg^{-1} in acetonitrile. All solutions were stored at −18 °C. Deuterated internal standards (IS) for the pesticides (diazinon-d10, propamocarb-D7, and diuron-D6) were purchased from Sigma Aldrich (Germany) and were diluted and stored in the same manner as the native pesticides. PAs, growth regulators, including the deuterated IS (mepiquat-D3 and chlormequat-D4) and TAs atropine and scopolamine were purchased from Sigma Aldrich (Germany) in residue analytical grade. ^{13}C-labeled IS for each of the investigated mycotoxins (aflatoxin B1, B2, G1, G2, deoxynivalenol, HT2 toxin, T2 toxin, ochratoxin A and zearalenon) and native standards were purchased from Romer Labs (Austria).

All analyte class stock solutions were prepared in acetonitrile at a concentration of 1000 mg kg^{-1}. A mixture of all the PAs was prepared in acetonitrile with a concentration of 10 mg kg^{-1} and further diluted to 1 mg kg^{-1}. The same schematic was used for the preparation of the stock solutions and working mixtures of the growth regulators and their respective IS and TAs. Formic acid and ammonium formate were purchased from Sigma Aldrich (Germany) in analytical grade.

Sample preparation and selected matrices

The investigated food commodities were oats and wheat (whole grains). Oats were chosen due to the relatively high fat content and wheat due to the high consumption in Europe. Wheat samples were taken from our in-house samples and organic oats were bought at local grocery stores. The samples were homogenized in a Robot Coupe Blixer 3 (Robot-Coupe, Austria) and 5 g were weighed into 50 mL polypropylene centrifuge tubes. For the validation procedure low, medium and high levels were spiked seven times with 100 µL IS solution of CCC and MQ. All other IS were added simultaneously with the sample injection by the 2D-LC-MS/MS system. Samples were left at room temperature for 30 min prior to the extraction step. Ten mL of a mixture of acetonitrile and water (80:20; v/v) were added and the samples were shaken for 2 min in the shaker (Collomix, Germany). Subsequently, the samples were placed in an overhead shaker (Heidolph Reax 2, Germany) and the analytes were extracted for 30 min at the medium speed setting (60 rpm). The samples were centrifuged at 3000 rpm for 5 min. For analysis an aliquot of the supernatant was filtered through a 0.45 µm PTFE filter into a LC vial.

Validation experiments

For validation two concentration levels were added to the samples for each of the analyte classes. Pesticides and growth regulators were validated at 2.5 µg kg^{-1} and 10 µg kg^{-1} sample dry mass; PAs and TAs at 1 µg kg^{-1} and 10 µg kg^{-1} and mycotoxins at individual levels. Aflatoxin B1, B2 and G1 at 0.1 µg kg^{-1} and 0.2 µg kg^{-1}, G2 at 1 µg kg^{-1}; deoxynivalenol (DON) at 20 µg kg^{-1} and 40 µg kg^{-1}; HT2-toxin and T-2 toxin at 5 µg kg^{-1} and 10 µg kg^{-1}; ochratoxin (OTA) at 1 µg kg^{-1} and 2 µg kg^{-1}; zearalenone (ZON) at 10 µg kg^{-1} and 20 µg kg^{-1}. For the final method a third validation level was chosen, comprising higher concentrations of the analytes with 50 µg kg^{-1} for pesticides, PAs, growth regulators and TAs and individual levels of the mycotoxins regarding the maximum levels of cereals for direct human consumption[33] with aflatoxin B1, B2, G1, and G2 at 1 µg kg^{-1}, DON at 750 µg kg^{-1}, HT2 toxin, T-2 toxin and OTA at 50 µg kg^{-1} and ZON at 250 µg kg^{-1}. All validation experiments focused on fulfilling the analytical requirements of the Eurachem validation guide 2014 and the SANTE 2015 document.

Whole wheat grains were analyzed with the following matrix matched calibrations: oat, wheat and mixed matrix matched calibration. Oats were analyzed with all prepared matrix matched calibrations.

LODs and LOQs were evaluated according to the Eurachem guide 2014.[34] The standard deviation s_0 of the low spiking level in the validation is used to calculate $s_0' = (^{s_0}/_{\sqrt{n}})$. n is the number of replicate analyte determinations when reporting results and was set to 1.[34] The LOD is 3 × s_0' and LOQ 10 × s_0'. LOD and LOQ were evaluated with the lowest spiking level in both matrices (Supporting Information).

For each validation experiment two analyte concentration levels were spiked with the addition of the IS of CCC and MQ with seven replicates while the rest of the IS was added by the 2D-LC-MS/MS system. Within every validation experiment a blank sample of the matrix was prepared to ensure a residue-free matrix or to perform a blank subtraction. All samples were prepared with 10 mL of a mixture of acetonitrile and water (80:20; v/v).

2 D-LC-MS/MS calibration and internal standards

The seven-point calibration for pesticides and plant growth regulators ranged from 0.5 to 50 µg kg^{-1}, for the PAs and TAs from 0.2 to 50 µg kg^{-1}. Individual calibration levels for the mycotoxins were as follows: aflatoxin B1, B2, G1, and G2 ranging from 0.01 to 5 µg kg^{-1}, DON from 2 to 1000 µg kg^{-1}, HT2-toxin and T2 toxin from 0.5 to 50 µg kg^{-1}, OTA from 0.1 to 50 µg kg^{-1} and ZON from 1 to 500 µg kg^{-1}. Deuterated IS were used for selected pesticides (diuron-D6, propamocarb-D7 and diazinone-D10), plant growth regulators (MQ-D3 and CCC-D4) and ^{13}C labeled IS for each of the analyzed mycotoxins. The mixture of IS from the pesticides and mycotoxins was injected by the 2D-LC-MS/MS system with a volume of 0.4 µL, while the IS for the plant growth regulators were added prior to the extraction of the samples. Therefore, the selected pesticides and mycotoxins were matrix-matched, while MQ and CCC were procedure-matched.

2 D-LC-MS/MS system

The 1290 Infinity II HPLC system (Agilent, Germany) consisted of a degasser (G1379B), a binary pump (G7120A), a quaternary pump (G7104A), a multisampler (G7167B) and a four column oven (G7116B) coupled to a triple quadrupole mass spectrometer 6470 (Agilent, Germany). The RP column was operated by the quaternary and the HILIC column by

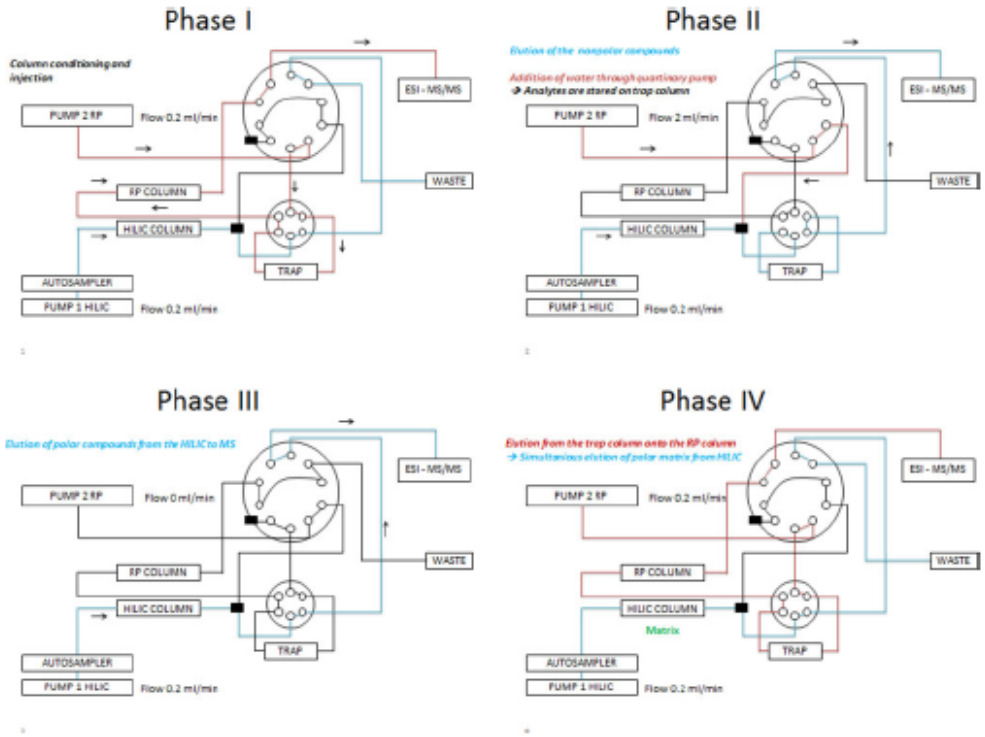

Figure 1. 2D-LC-MS/MS analytical run from phase HV.

the binary pump. For the connection of the two separation dimensions a 6-port valve and a 10-port valve (Valco Vici, USA) were used. The HILIC dimension consisted of a YMC-Pack Diol (2.1 mm ×100 mm i.d.; 5 µm particles; 120 Å) with a Diol-NP guard column (2.1 × 10 mm^2 i.d.; 5 µm particles; 12 nm) and the RP of a Phenomenex Synergi Fusion-RP (2 × 100 mm^2 i.d.; 2.5 µ particles; 100 Å) with C18 guard column from Phenomenex (Germany) (4 × 2.0 mm^2 i.d.). For storing of the analytes an Agilent ZORBAX SB-C8 (4.6 × 12.5 mm^2 i.d.; 5 µm particles; 80 Å) was used. The column temperatures were set to 30 °C for both columns and the injection volume was 5 µL.

The mobile phase of the HILIC dimension consisted of water (A) and a mixture of acetonitrile and water (90:10; v/v) (B), both containing 5 mM ammonium formate and 0.1% acetic acid. The flow rate was set to 0.2 mL min^{-1}. The gradient started with 100% B and was held until 2.5 min. Then the amount of B was reduced to 50% within 5 min and held for 8 min. Next, solvent B was increased to 100% within 3 min and held for 7.5 min.

The mobile phase of the RP dimension consisted of water (A) and methanol (B) with both containing 5 mM ammonium formate and 0.1% acetic acid. In phase I the RP column was conditioned until 1.10 min with 5% B with a flow rate of 0.2 mL min^{-1} (Fig. 1). B was reduced to 0% within 0.5 min and the flow rate was elevated to 2 mL min^{-1} in phase II (Fig. 1). The quaternary pump operating the RP dimension was connected by a T-piece with the trapping column to maximize the storage of the nonpolar analytes on the C8 material through the addition of water. The flow rate of 2 mL min^{-1} was held until 2.00 min and subsequently the flow rate was set to 0. This is where the direct measurement of the polar compounds began described as phase III in Figure 1. In phase IV started the separation of the analytes on the RP column at 4.5 min with 5% B at a flow rate of 0.2 mL min^{-1}. B was increased to 50% within 0.5 min and again elevated to 100% until 16.5 min. This was held for 3 min. B was again reduced to 5% within 0.5 min and held for 6 min. The runtime of this method was 26 min. Switching times of the six-port valve were 0.00 min position 1, 1.20 min position 2 and 2.00 min position 1 and of the 10 port valve were 0.00 min position 1, 1.10 min position 2 and 4.5 min position 1 (Fig. 1).

At the MS/MS system the nebulizer gas temperature of ion source was set to 200 °C with a gas flow of 10 L min^{-1} and a nebulizer gas pressure of 30 psi. The sheath gas temperature was 375 °C with a flow of 11 L min^{-1}. The capillary voltage was set at 4000 V positive and 3000 V negative. The nozzle voltage was set at 300 V positive and 500 V negative. The mass spectrometer was running in the triggered

multiple reaction monitoring mode detecting the most abundant transition exceeding a threshold of 1000 and if so monitoring a second up to a third transition.

2 D-LC-MS/MS chromatographic run

The sample preparation procedure was minimized by the use of a HILIC column in the first dimension. Therefore, polar matrix components were reduced through partitioning between a thin water layer on the stationary phase and the organic mobile phase. The addition of water stored all non-polar analytes, not retained by the HILIC column, on a trapping column. After the detection of the polar compounds, the stored analytes were eluted from the trapping column and separated by an analytical RP column. The majority of the pesticides and mycotoxins were not retained by the HILIC column and were fixed onto the trapping column. Most of the PAs, the plant growth regulators MQ and CCC and both TAs atropine and scopolamine eluted from the HILIC column.

Matrix effects (MEs) measurements

The MEs were evaluated by comparing the peak area of the analytes in the matrix matched standards with the area in the neat solvent standards (mixture of acetonitrile and water 80:20; v/v), expressed as matrix induced signal enhancement or suppression (SSE).[35] Therefore, the analytes were spiked at similar concentrations (10 µg kg^{-1} for pesticides, PAs, TAs and plant growth regulators, 1.25 µg kg^{-1} for aflatoxins, 251 µg kg^{-1} for DON, 10 µg kg^{-1} for HT2 and T-2 toxin and 12.5 µg kg^{-1} for OTA, 125 µg kg^{-1} for ZON) to each of the following solvent/matrices: mixture of acetonitrile and water (80:20; v/v) comprising the neat solvent standard, blank oat matrix, blank wheat matrix and the extracted mixture of oat and wheat matrix (50:50; v/v). MEs were calculated according to the following equation: $SSE = \frac{average\ area\ of\ matrix\ matched\ standard}{average\ area\ of\ neat\ solvent\ standard} \times 100$. A value below 100% describes ionization suppression, while a value over 100% ionization enhancement.

Results and discussion

Optimizing sample preparation

The first experiment was conducted with oats regarding the extraction volume of the sample preparation. A matrix matched calibration was used. About 10 mL and 20 mL were used for the extraction volume of the sample in triplets for each of the two validation levels. About 10 mL and 20 mL correspond to a dilution factor of two and four, respectively. Nearly 68% of the 401 analytes were between 70 and 120% recovery of the lower spike level and 84% of the medium spike level with a volume of 20 mL extraction solvent. When using 10 mL 82% of the analytes were between 70 and 120% for the low level spike and 84% for the medium spike level. Therefore, a volume of 10 mL extraction solvent was chosen and the method was validated in oats and wheat.

Matrix matched calibration

For the validation experiments four matrix matched calibrations were prepared: three matrices, that is oat matrix, wheat matrix and a mix of both with an equal fraction of 50% oat and 50% wheat matrix were subjected to the sample preparation procedure and, after the entire procedure, spiked with the analytes of interest. Moreover, a fourth, so-called procedure matched calibration consisting of an oat matrix which was spiked with the respective levels of the calibrants *before* sample processing, was prepared. The results of the validation for all analytes meeting the criteria with a recovery range between 70 and 120% and a relative standard deviation (RSD) lower than 20% are shown in Figure 2a and 2b. The highest recoveries and lowest RSDs were achieved with the mixed matrix matched calibration shown in Figure 2c comprising the final method for both matrices in the validation process. A pesticide standard mixture (Restek) was analyzed to ensure the trueness of the measured values and to exclude analyte interactions in the calibration standards. This mixture was analyzed with every matrix matched calibration used. To the best of our knowledge no prepared analyte mixture for mycotoxins, PAs, TAs and growth regulators is currently available.

The results in Figure 2c show that a mixture of oats and wheat as a matrix matched calibration improves compensating for signal enhancement and signal suppression in both matrices. Especially the recoveries in oats improved compared to the analysis with the wheat matrix matched standard (Fig. 2a). Using one matrix matched calibration to quantify compounds of interest in a different matrix has been investigated concerning matrix effects.[36] Pesticides were analyzed in different commodities and the slopes of the calibration curves were compared in fruits and vegetables. The authors stated that plant-families can be analyzed with a specific representative of the species.[36] To the contrary, our findings suggest that the calibration standard prepared in a mixture of matrices yields higher recoveries compared to using one matrix for the calibration.

Influence of ME on signal enhancement and suppression

According to the SANTE guide[15] MEs should be in the range of 80–120%. Therefore, we consider analytes having SSE values in this range not to be influenced by matrix effects. MEs in LC-MS depend on matrix components coeluting with each individual pesticide making it difficult to compensate for matrix effects. The majority of analytes show ionization suppression (Fig. 3). Severe MEs can be observed in almost all analytes (97% in oats, 95% in wheat and 97% in the mixed matrix matched calibration) eluting from the HILIC column. The online clean-up in phase II is designed to remove coextracted materials for phase IV when the analytes elute from the RP column, thus all analytes eluting in phase III remain unpurified (Fig. 1). MEs are significantly decreased, when analytes elute from the RP column (Fig. 3). About half of the analytes (59% in oats, 50% wheat and 48% mixed matrix matched calibration) are not affected by co-eluting matrix components (MEs in the range

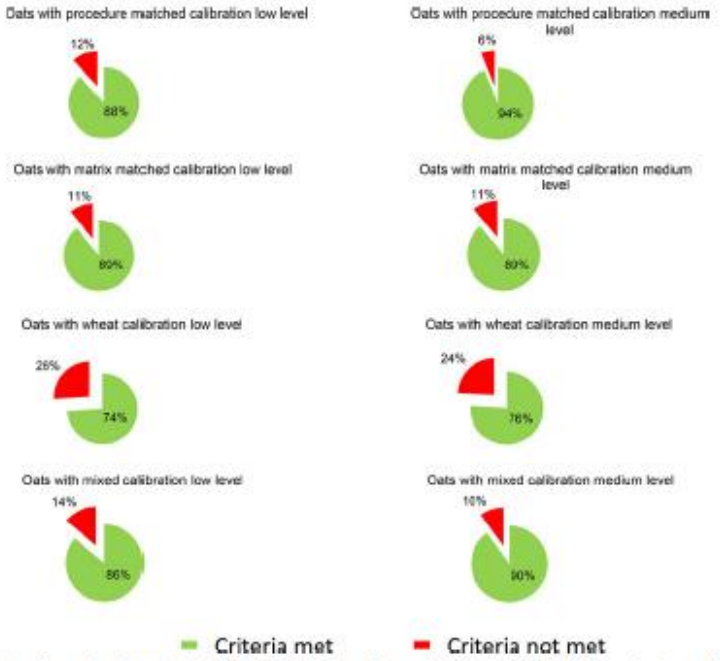

Figure 2. (a) Oats validation of low and medium spike level. (b) Wheat validation of low and medium spike level. (c) Oats and wheat validation with a mixed calibration of the low, medium and high spike level.

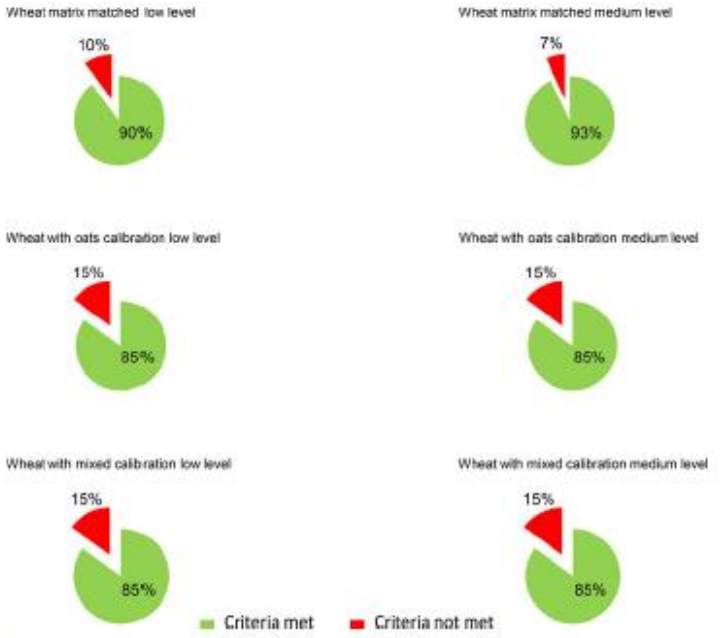

Figure 2. (continued)

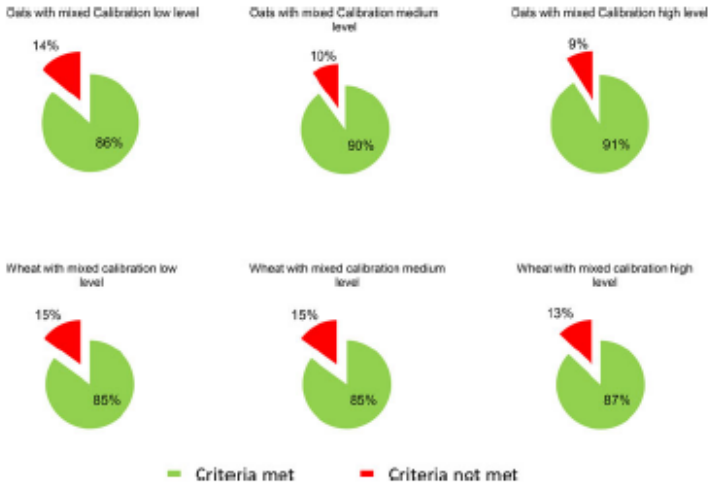

Figure 2. (continued)

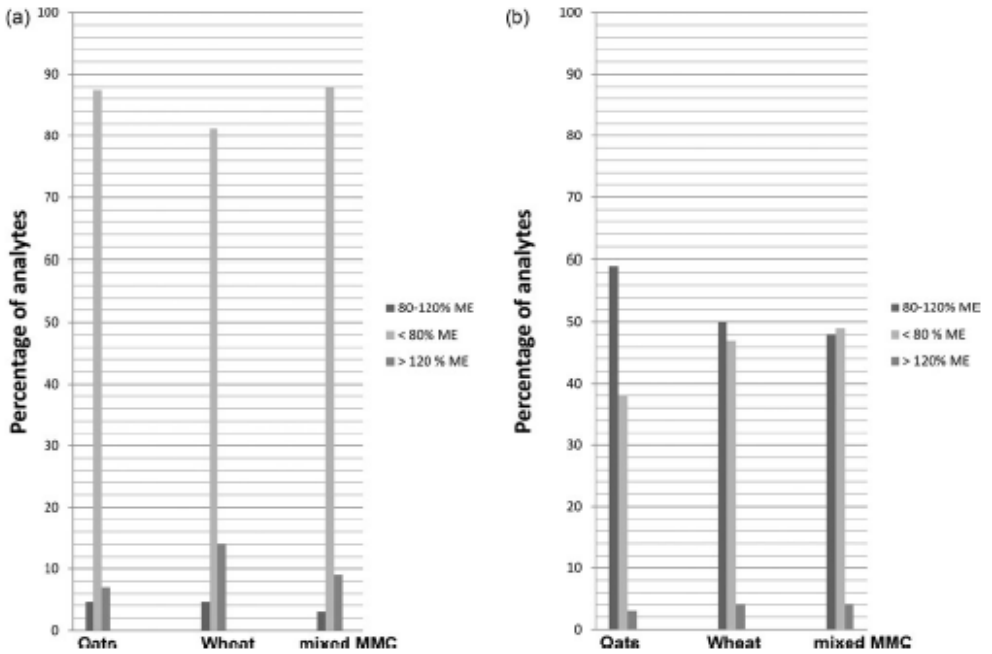

Figure 3. Matrix effects of all analytes in each of the investigated matrices including the mixed matrix matched calibration (MMC). (A) Analytes eluting from the HILIC in phase II and (B) eluting from the RP column in phase IV.

of 80–120%), while the other half shows SSE values below 80% (38% in oats, 47% in wheat and 49% in the mixed matrix matched calibration). Only a few compounds show signal enhancement. Malysheva et al.[37] investigated MEs expressed in SSE values of various types of grains, including oats and wheat. Ergot alkaloids were extracted with a liquid-liquid extraction procedure. Oats showed the highest signal suppression (10–50%), while wheat resulted in moderately lower SSE values (35–90%). The authors concluded that profound ME differences were observed between the grain

types, but not in grain varieties, which is in good accordance with our findings regarding the grain varieties. Mol and colleagues[38] investigated different extraction procedures ("dilute-and-shoot" methods) for the determination of 172 pesticides in various matrices including animal feed and maize. The extraction with acetonitrile showed the most promising results regarding MEs with more analytes falling into the range of 80–120%. The abundance of analytes in horse feed matrix showed signal suppression, which correlates with our findings due to the fact that traditional ingredients in horse feed are oats. The comparison of different matrices including maize, meat, milk, eggs and honey with animal feed exerting the highest MEs shows the high complexity of this matrix consisting in part of grains.

Therefore, a matrix matched calibration is highly advisable and required due to the complexity of the investigated matrices (Fig. 3). The ultimate goal was to evaluate one matrix matched calibration for both, wheat and oats. The final decision to use the mixed matrix matched calibration was driven due to the improved recovery rates in both matrices. Figure 3 shows slight differences in SSE values between the wheat matrix and the mixed matrix matched calibration, which in return might explain the improved recoveries of the target analytes in wheat and oats. All target analytes with their respective recoveries, LODs, LOQs, and RSDs evaluated with the final method (mixed matrix matched calibration) can be found in the supporting information.

Pyrrolizidine alkaloids

Initially 30 PAs were investigated with the 2D-LC-MS/MS method. Therefore, the solvent of the PAs was changed from acetonitrile to a mixture of acetonitrile and water (80:20; v/v), which corresponds to the extraction solvent used in the sample preparation. All PAs eluted directly from the HILIC in Phase III (Fig. 1). Unfortunately, separating indicine, intermedine, and lycopsamine, indicine-*N*-oxide, intermedine-*N*-oxide and lycopsamine-*N*-oxide, senecivernine and senecionine, senecivernine-*N*-oxide and senecionine-*N*-oxide and retrorsine-*N*-oxide from Jacobin-*N*-oxide was not achieved with the current method. The separation of isomeric PAs and PA oxides was achieved with a multiple heart-cutting 2D-LC-QToF-MS approach in plant extracts.[17] However, the main aim of our study was to incorporate as many analytes as possible into a single chromatographic run, although it meant to exclude 12 of the initially 30 PAs. Altering the gradient was no option due to the influence on other analytes investigated. Eighteen PAs were further investigated in the two matrices with the different matrix matched calibrations and dilution factors. To meet the validation criteria by the SANTE guidelines the recoveries and RSDs of the PAs needed to be between 70 and 120% and below 20%, respectively. The best results in oats were achieved with the procedure matched calibration, which indicates the loss of analytes during the sample preparation and matrix interferences. In the case of wheat more PAs met the analytical criteria being 12 for the low level and 13 for the medium spike level with the mixed matrix matched calibration. Considering the initial thirty PAs, the best results achieved were 12 PAs (40%) for the low level and 13 PAs (43%) for the medium level validation. Overall recoveries and RSDs of the investigated PAs shows that this sample preparation with the current method is not fit for purpose for quantification of all PAs in oats and wheat. The successfully validated PAs in the final method with the mixed calibration approach in both matrices are shown in Table 1.

In our study we could show that the major issue of the low recoveries of the PAs derived by analyte losses during the sample preparation, since the procedure matched calibration resulted in higher recoveries. We used a 4:1 mixture of acetonitrile and water for the extraction of the PAs, while other studies have been conducted with different extraction solvents and the addition of the QuEChERS sample preparation.

Martinello et al.[39] used the QuEChERS sample preparation technique for the extraction of PAs and TAs from honey. The PAs were extracted with sulfuric acid and a QuEChERS protocol was conducted with the addition of salts for partitioning and a subsequent clean-up with PSA and magnesium sulfate ($MgSO_4$). A further modified QuEChERS approach has been evaluated by Bolechova et al.[28] in animal feed. The extraction solvent (acetonitrile: water; 50:50; v/v) was added and a liquid-liquid partitioning was conducted with no further clean-up. In this study the authors analyzed five PAs and the method was validated with two levels being 5 and 100 µg kg^{-1}.[28] The recoveries of this study ranged from 72 to 98% for both levels analyzing senecionine, retrorsine, seneciphylline, monocrotaline, and senkirkine.[28] Comparing these results with our work shows that similar recoveries were achieved with a lower spike level of 1 µg kg^{-1} in three of the five PAs with recoveries ranging from 81% to 92% for seneciphylline, monocrotaline and senkirkine and with 291% recovery for retrorsine in wheat. Senecionine was excluded in our work since we were not able to sufficiently separate this compound from senecivernine. Regarding the medium spike level of 10 µg kg^{-1} recoveries ranged from 86 to 118% for all analytes comparable of those considered in the study of Bolechova and colleagues.[28] In our study acetonitrile and water in the ratio of 4:1 as an extraction solvent was used, while Bolechova and colleagues[28] used an acidified 1:1 ratio. Methanol in various amounts ranging from 60 to 100% has also been used as an extraction solvent by Mudge and colleagues.[40] The authors stated that using methanol in the range of 60 and 80% resulted in no significant differences in PA recovery.[40] As for our findings, this might not hold true for the extraction with acetonitrile and water (4:1) resulting in analyte loss.

Mycotoxins

The recoveries and RSDs for the mycotoxins of the final method with the three spike levels are shown in Tables 2–4. For the reduction of costs IS for all mycotoxins are automatically added by the LC system. The low-level spikes of aflatoxins B1, B2, and G1 with 0.01 µg kg^{-1} yield good

Table 1. Recoveries, RSD, LODs and LOQs for pyrrolizidine alkaloids in oats and wheat evaluated with a mixed calibration (50/50; v/v; oats and wheat) at the low, medium and high spike level.

Spike level	Compounds	Concentration (µg kg^{-1})	Recovery (%) in oats	RSD (%) in oats	Recovery (%) in wheat	RSD (%) in wheat	LOD (µg kg^{-1}) in oats	LOQ (µg kg^{-1}) in oats	LOD (µg kg^{-1}) in wheat	LOQ (µg kg^{-1}) in wheat
Low level	Erucifoline	1	117	10.2	103	8.1	0.18	0.60	0.12	0.42
	Heliotrine	1	70	5.0	–	–	0.05	0.18		
	Echimidine	1	–	–	95	6.5	–	–	0.09	0.31
	Europine	1	–	–	84	7.4	–	–	0.09	0.31
	Jacobine	1	81	16.8	100	9.5	0.20	0.68	0.14	0.48
	Retrorsine	1	77	9.0	–	–	0.10	0.35		
	Lasiocarpine-N-oxide	1	–	–	77	5.5	–	–	0.08	0.28
	Monocrotaline	1	–	–	81	12.1	–	–	0.15	0.49
	Seneciphylline	1	–	–	92	18.6	–	–	0.26	0.86
	Seneciphyllin-e-N-oxide	1	113	5.7	100	8.5	0.10	0.32	0.13	0.43
	Senkirkine	1	–	–	91	4.9	–	–	0.07	0.22
	Trichodesmine	1	–	–	78	16.4	–	–	0.19	0.64
Medium level	Erucifoline	10	82	3.5	101	4.8				
	Heliotrine	10	85	4.3	–	–				
	Echimidine	10	115	3.0						
	Europine	10	–	–	106	5.3				
	Jacobine	10	79	8.4	98	4.9				
	Retrorsine	10	70	4.2	–	–				
	Lasiocarpine-N-oxide	10	–	–	111	3.1				
	Monocrotaline	10	–	–	86	6.6				
	Seneciphylline	10	–	–	90	5.7				
	Seneciphyllin-e-N-oxide	10	82	3.5	102	4.8				
	Senkirkine	10	–	–	110	5.6				
	Trichodesmine	10	–	–	81	4.9				
High level	Erucifoline	50	85	3.1	106	1.1				
	Heliotrine	50	84	1.6	–	–				
	Echimidine	50	–	–	111	1.7				
	Europine	50	–	–	105	1.7				
	Jacobine	50	86	6.9	101	2.0				
	Retrorsine	50	71	4.6	–	–				
	Lasiocarpine-N-oxide	50	–	–	108	2.2				
	Monocrotaline	50	–	–	84	3.2				
	Seneciphylline	50	–	–	94	2.8				
	Seneciphyllin-e-N-oxide	50	85	3.1	107	2.1				
	Senkirkine	50	–	–	102	1.2				
	Trichodesmine	50	–	–	92	2.8				

All compounds listed meet the analytical criteria by SANTE.[45]

Table 2. Recoveries, RSD, LODs and LOQs for tropane alkaloids, growth regulators, and mycotoxins in oats and wheat evaluated with a mixed calibration (50/50; v/v; oats and wheat) at the low spike level.

Analyte class	Compounds	Concentration (µg kg^{-1})	Recovery (%) in oats	RSD (%) in oats	Recovery (%) in wheat	RSD (%) in wheat	LOD (µg kg^{-1}) in oats	LOQ (µg kg^{-1}) in oats	LOD (µg kg^{-1}) in wheat	LOQ (µg kg^{-1}) in wheat
Tropane alkaloids	Atropine	1	84	4.7	87	3.6	0.06	0.20	0.05	0.16
	Scopolamine	1	73	10.9	95	5.6	0.12	0.40	0.08	0.27
Growth regulators	Chlormequat	2.5	90	7.9	102	8.0	0.54	1.79	0.61	2.05
	Mepiquat	2.5	97	8.3	91	4.6	0.60	2.00	0.32	1.05
Mycotoxins	Aflatoxin B1	0.1	101	13.6	107	7.2	0.02	0.07	0.01	0.04
	Aflatoxin B2	0.1	111	17.2	105	19.0	0.03	0.10	0.03	0.10
	Aflatoxin G1	0.1	119	6.9	103	12.2	0.01	0.04	0.02	0.06
	Aflatoxin G2	0.1	261	23.0	169	31.0	0.12	0.41	0.10	0.35
	Deoxynivalenol	20	105	9.4	94	13.6	5.91	19.71	7.66	25.52
	HT2 toxin	5	40	12.8	19	13.7	0.38	1.28	0.19	0.64
	T2 toxin	5	29	9.8	24	7.4	0.21	0.70	0.13	0.44
	Ochratoxin	1	84	15.3	77	9.6	0.19	0.64	0.11	0.37
	Zearalenone	10	80	12.8	80	15.9	1.54	5.13	1.90	6.32

Table 3. Recoveries, RSD, LODs and LOQs for tropane alkaloids, growth regulators and mycotoxins in oats and wheat evaluated with a mixed calibration (50/50; v/v; oats and wheat) at the medium spike level.

Analyte class	Compounds	Concentration (µg kg^{-1})	Recovery (%) in oats	RSD (%) in oats	Recovery (%) in wheat	RSD (%) in wheat	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
Tropane alkaloids	Atropine	10	77	6.4	119	2.2		
	Scopolamine	10	79	6.3	108	5.8		
Growth regulators	Chlormequat	10	96	6.5	102	8.0		
	Mepiquat	10	97	3.3	97	4.6		
Mycotoxins	Aflatoxin B1	0.2	92	19.4	104	6.0		
	Aflatoxin B2	0.2	113	7.1	90	17.1		
	Aflatoxin G1	0.2	104	10.2	81	18.2		
	Aflatoxin G2	0.2	127	23.1	112	26.9		
	Deoxynivalenol	80	98	7.2	79	9.7		
	HT2 toxin	10	30	6.3	19	13.7		
	T2 toxin	10	28	9.1	25	7.3		
	Ochratoxin	2	82	7.9	75	9.3		
	Zearalenone	20	83	8.7	89	7.5		

Table 4. Recoveries, RSD, LODs and LOQs for tropane alkaloids, growth regulators and mycotoxins in oats and wheat evaluated with a mixed calibration (50/50; v/v; oats and wheat) at the high spike level.

Analyte class	Compounds	Concentration (µg kg^{-1})	Recovery (%) in oats	RSD (%) in oats	Recovery (%) in wheat	RSD (%) in wheat	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
Tropane alkaloids	Atropine	50	70	3.4	109	1.2		
	Scopolamine	50	81	3.8	108	2.5		
Growth regulators	Chlormequat	50	104	4.3	101	4.4		
	Mepiquat	50	93	3.1	93	1.7		
Mycotoxins	Aflatoxin B1	1	90	5.9	93	7.6		
	Aflatoxin B2	1	84	17.5	85	5.6		
	Aflatoxin G1	1	82	8.6	70	8.8		
	Aflatoxin G2	1	100	8.1	99	7.0		
	Deoxynivalenol	750	99	4.0	77	4.1		
	HT2 toxin	50	23	12.4	21	8.2		
	T2 toxin	50	27	4.3	28	3.7		
	Ochratoxin	50	89	4.9	82	3.3		
	Zearalenone	250	83	4.8	85	8.5		

recoveries and RSDs fulfilling the analytical criteria. Aflatoxin G2 was validated at a concentration of 1 µg kg^{-1}. This level of validation can be considered acceptable, since aflatoxins are regulated as a sum of all four with a maximum level (ML) of 4 µg kg^{-1} and aflatoxin B1 with 2 µg kg^{-1} for cereals and cereal products.[33] DON was validated at 20 µg kg^{-1} for the low, 40 µg kg^{-1} for the medium and 750 µg kg^{-1} for the high level with the latter comprising the lowest ML in cereals and cereal products.[33] The MLs for OTA in unprocessed cereals and cereals for direct human consumption are 5 µg kg^{-1} and 3 µg kg^{-1}, respectively. The validation levels of OTA at 1, 2 and 50 µg kg^{-1} yield satisfactory results regarding recoveries and RSD and are acceptable for the determination of OTA in cereals. The same holds true for the validation of ZON with 10, 20, and 125 µg kg^{-1}. The validation criteria for HT2 and T-2 toxin could not be fulfilled. The recoveries are low ranging from 19 to 24% in wheat, 28 to 40% in oats for both analytes and RSDs from 6.3 to 13.7% for HT2 and 7.3 to 9.8% for T-2 toxin in both matrices. Although HT2 and T-2 toxin are regulated for unprocessed cereals and products no ML has been established.[33] The LOD of HT2 and T-2 toxin is set at 15 µg kg^{-1}.[11] The peaks of the native analytes and IS are detected with a LOD of 0.015 µg kg^{-1} for HT2 toxin and 0.0007 µg kg^{-1} for T2-toxin at a signal to noise ratio of 3:1 in oats and wheat with acceptable peak shape, therefore suggesting that the developed method can be used as a screening for these two analytes. An in-house method for the determination of HT2 and T-2 toxin has been established and accredited.[48]

Sample preparation procedures for HT2 and T-2 toxin are described by Li and colleagues including liquid–liquid extraction, SPE and QuChERS in wheat flour.[31] An extraction procedure for HT2 and T-2 toxin in oats has been applied with a mixture of acetonitrile and water (84:16; v/v) leading to recoveries ranging from 91 to 110% described by Gottschalk et al.[10] Because this extraction solvent resembles the mixture used in this work indicates a suitable choice. The use of a matrix matched calibration should exclude drastic matrix effects but does not account for an explanation for the low recoveries. Analyzing HT2 and T-2 toxin in triplets with a procedure matched calibration in oats resulted in low recovery rates with 49 and 17%, respectively.

Tropane alkaloids

Both TAs elute in phase III directly from the HILIC column (Fig. 1). The requirements for recoveries and RSDs of the TAs were all fulfilled in all calibration strategies ranging from 75 to 119% and 2.1 to 6.4% for atropine and 72 to 116% and 3.4 to 10.9% for scopolamine, respectively. Recoveries and RSDs of the final method are shown in Tables 2–4.

The analytical requirements for the determination of TAs in oats and wheat are therefore fulfilled with LOQs going far below the recommendations (Tables 2–4). Although

signal suppression occurred in oats and on the other hand signal enhancement in wheat due to matrix interferences the recoveries were between 70 and 120%. Cirlini and colleagues used a simple sample extraction with slight differences regarding the extraction solvent (methanol and water in a volume ratio 3:2 and 0.2% acetonitrile in a volume ratio 3:2), shaking-time (90 min) and the dilution factor (1:50).[42] LODs and LOQs are well comparable with 0.03 µg kg^{-1} and 0.1 µg kg^{-1} for scopolamine and 0.09 µg kg^{-1} and 0.30 µg kg^{-1} for atropine in Cirlinis work, 0.08–0.12 µg kg^{-1} and 0.27–0.4 µg kg^{-1} for scopolamine and 0.05–0.06 µg kg^{-1} and 0.16–0.20 µg kg^{-1} in our method, respectively. The QuEChERS method has also been applied for the determination of the two TAs. LODs and LOQs reported by the authors for atropine and scopolamine were 0.04 and 0.2 µg kg^{-1} and 0.4 and 2 µg kg^{-1} with recoveries ranging from 76 to 92% for buckwheat, respectively. In addition to reach these analytical requirements the samples were diluted after the analyte extraction.[27] These results compare very well to those of our method which is, in addition, saving costs and time for the analysis of atropine and scopolamine.

Growth regulators CCC and MQ

MQ and CCC are known to be very polar and ionic compounds.[43] Both analytes can be successfully retained with the used HILIC column. Initial experiments were conducted with the addition of the IS of CCC and MQ directly by the multisampler of the 2D-LC-MS/MS system resulting in low recoveries (50–60%). The applied sample preparation results in analyte loss leading to recoveries below 70% for the matrix matched approach, suggesting the addition of the IS directly to the samples prior to extraction. Another method includes a higher dilution during sample preparation.[44] In this case it was no option, since we were able to show that a higher dilution factor affects overall recoveries. Recoveries and RSDs are shown in Table 2–4 reaching from 91 to 104% with the highest RSD being 11.7% for the procedure matched approach. Xue et al.[44] extracted various plant growth regulators with methanol and water (90:10; v/v) and used a clean-up with PSA to remove possible interfering matrix components. The extraction solvent to sample ratio was optimized from 2:1 to 4:1 increasing the recovery rates from 50–70% to 70–90%. Ion suppression was observed in case of CCC and MQ, which is in good accordance with our findings. LOQs in this study were between 1.79 and 2.05 µg kg^{-1} for CCC and 1.05–2.00 µg kg^{-1} for MQ. Xue and colleagues reported higher LOQs with an additional dispersive solid phase extraction clean-up, being 10 µg kg^{-1} for CCC and 5 µg kg^{-1} for MQ in buckwheat.[44] In another study CCC and MQ were extracted with the addition of an IS for CCC with a mixture of methanol and water and subsequently added to a SPE cartridge for a further clean-up in apples and beer.[45] The samples were spiked at 5 µg kg^{-1} for CCC and 10 µg kg^{-1} for MQ yielding recoveries higher than 78%. The reported LODs in apples for CCC and MQ reported by Esparza et al. were 1.0 µg kg^{-1} and 2.5 µg kg^{-1}, respectively. They also stated that a higher buffer concentration lead to more symmetric and narrower peaks, compromising with a concentration of 50 mM to avoid complications with the mass spectrometer. The mobile phase of the 2D-LC-MS/MS method had a concentration of 5 mM resulting in broader peak shapes, which is in good accordance with the other authors findings.[45] Again, in case of the sample preparation no additional clean-up is needed for the determination of CCC and MQ in our method, therefore saving time and costs, while still being able to reach low LODs and LOQs.

Pesticides

The results with the mixed matrix matched calibration of the validation for all pesticides in oats and wheat are shown in Figure 4. More detailed information about the single pesticides is shown in the supporting information. Slightly more compounds passed the analytical criteria in oats compared to wheat. 344 and 323 out of 370 pesticides in oats and wheat respectively show good results at the 50 µg kg^{-1} spike considering the quadratic calibration. The quadratic tendency of the pesticides might be due to the incomplete retention of the analytes at higher concentrations. In phase II more water is added by the quaternary pump with a flow rate of 2 mL min^{-1} to store the analytes onto the trapping column. But still the binary pump operating the HILIC flow rate adds a mixture of acetonitrile and water (90:10; v/v) with a flow rate at 0.2 mL min^{-1}. This step in phase II results in concentration dependent analyte loss reducing the response at higher pesticide concentrations. Pesticides measured directly from the HILIC column showed almost no trend towards a quadratic calibration. The other analyte classes (PA, TA, growth regulators) elute directly from the HILIC. Mycotoxins are retained by the trapping column and are analyzed with the addition of an IS partially compensating for analyte losses. IS are also used for some of the pesticides (proparmocarb, diazinon and diuron) resulting in a linear calibration curve. The adjustment of the HILIC gradient was not necessary due to satisfactory results with the mixed matrix matched calibration achieved (Fig. 2c). Additionally, the linearity of the calibration curve was improved by the use of the tMRM.

The maximum residue levels (MRL) of carbofuran, carbosulfan and carbofuran-3-hydroxy are lower in various food commodities like pome fruits, potatoes and animal based products with 1 µg kg^{-1} according to the Regulation (EU) 2015/399.[46] Therefore, carbofuran and its metabolites were validated at a concentration of 1 µg kg^{-1} in oats, even though the MRL for cereals is set at 10 µg kg^{-1}. The recoveries for carborfuran, carbosulfane and carborfuran-3-hydroxy are 97, 91, and 67% and RSDs 3.7, 2.5, and 4.5%, respectively. Carborfuran-3-hydroxy, not meeting the analytical criteria for recoveries, was validated at 2.5 µg kg^{-1} with 91% in oats and 101% recovery in wheat. Still all three pesticides meet the MRL.

The number of successfully validated pesticides in oats and wheat were 330 and 316 out of 370, respectively throughout all three validation levels with the minimized sample preparation and the online clean-up with the mixed

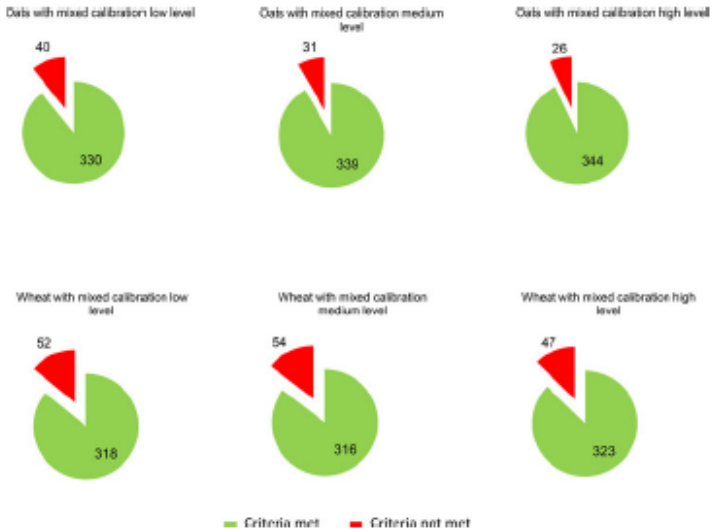

Figure 4. Oats and wheat validation of pesticides with a mixed calibration of the low, medium and high spike level.

matrix matched calibration approach. Matrix interferences have been investigated by Kittlaus et al.[47] with the post-column infusion approach. A higher retention of matrix components compared to the analytes was achieved especially for wheat flour. These interferences were successfully separated from the high polar compounds. The authors also stated that the diol column leads to a similar partitioning step resembling the QuEChERS method when the water is removed from the samples with the addition of the salt mixture.[2] Other coextracted compounds are removed by intelligent valve time switching. The most common sample preparation for pesticide residue analysis is the QuEChERS method.[21] The need for a d-SPE clean-up can be questionable looking at the results of another study.[48] Different QuEChERS clean-up sorbents including C18, PSA and graphitized carbon black (GCB) have been evaluated regarding pesticides in soil. In conclusion the authors stated that the matrix effects with the QuEChERS clean-up were more pronounced compared to the matrix effects without any additional clean-up. In another study 109 pesticides were investigated in vegetables, fruits and wheat regarding the matrix effects with and without the QuEChERS d-SPE clean-up analyzed with GC-MS/MS.[49] Recoveries and precision data revealed no differences between the clean-up step with C18 and PSA in wheat. The online clean-up of the 2D-LC-MS/MS system is considered to be sufficient, since it has been shown that matrix effects are reduced[2] and the data for accuracy and precision for the investigated pesticides in this study reveal satisfactory results.

Conclusion

The demand of fast and cost-effective analysis with a broad range of analytes is increasing. To meet these requirements and improve the monitoring process the 2D-LC-MS/MS system has been successfully applied for the determination of several analyte classes in oats and wheat. The 2D-LC-MS/MS system was initially developed for the determination of more than 300 pesticides with a cost effective and minimized sample preparation technique and an incorporated online clean-up. The adjustment of the extraction solvent from acetonitrile to a mixture of acetonitrile and water (80:20; v/v) lead to a satisfactory extraction of additional analyte classes. The recoveries and RSD of the majority of compounds are in the accepted range

Even though coextracted matrix components are minimized by the implemented online clean-up the use of a matrix matched calibration is still necessary and advisable. Therefore, matrix matched calibrations concerning their application to a wider variety of matrices should be further investigated. In our study we could show that a mixed matrix matched approach improves recovery rates compared to single matrix matched calibrations in oats and wheat. Additionally, different amounts of each matrix component could be further investigated.

To the best of our knowledge this is the first 2D-LC-MS/MS method for the determination of multiple analyte classes in one analytical run. The overall recoveries and RSDs of all analytes showed satisfactory results, therefore being of valuable use to a routine laboratory to effectively save time, costs and further improve the monitoring of contaminants and residues for safe foods.

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Disclosure statement

Michael Urban, Stephan Hann and Helmut Rost declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Not applicable.

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5. Conclusion

The main goal of this work was to investigate, evaluate and develop analytical procedures to reduce costs and safe time for the determination of the given analytes. In publication I we were able to show that the QuEChERS method can be successfully applied to various matrices for the determination of the PAH4. In this process we evaluated the most effective QuEChERS protocol to remove possible co-eluting matrix interfering compounds. As a result one QuEChERS method could be applied to eleven matrices. Additionally, we demonstrated that the extraction of the PAH4 of naturally incurred samples is possible yielding high recoveries and good precision. All analytical criteria set by the Regulation 836/2011 were fulfilled. The QuEChERS method for the determination of the PAH4 in various food commodities can be of great use for routine laboratories. The necessity for expensive instrumentation for the conventional sample preparation (e.g. GPC) and extended “know-how” of the laboratory personal is no longer needed and minimized, respectively, due to the QuEChERS application and its ease of use. However, this sample preparation technique is not fit for the determination of extremely low concentrations, which can be considered the main downside of this technique concerning the determination of PAHs. It is of utmost importance to be aware of the fact, that this QuEChERS protocol was developed for the current state of legislation and the respective maximum levels. Nevertheless, even if the maximum levels are lowered in the future this method can easily be applied for the routine screening of PAHs in the investigated food commodities.

In publication II we extended the scope of analytes of an existing fully automated heart-cut 2D-LC-MS/MS system with a broad range of polarities and compound classes in oats and wheat [78]. The sample preparation procedure was adopted and modified to meet the analytes need for extraction. The integrated online clean-up resembles the partitioning step of the QuEChERS method [78]. Despite the difficulty of implementing and maintaining a 2D-LC-MS/MS [72] with such an abundance of analytes, this system safe costs and time. As for the use in a routine laboratory one must consider the following: implementing and maintaining the proposed 2D-LC-MS/MS system cannot be considered trivial. Troubleshooting might as well increase the downtime due to the complexity of the 2D-LC system. On the other hand, the 2D-LC system enables a reduction in time and costs concerning the sample preparation due to the integrated online clean-up. Additionally, due to the use of a HILIC column in the first dimension, an abundance of analytes with a broad range of polarities can be analyzed in one chromatographic run with satisfactory recoveries and RSDs. Although only two matrices have been studied and further research is warranted, all analytes are of interest in the investigated food commodities. The successful long term routine use of the 2D-LC system still has to be proved to confirm the ability to withstand the pressure of the demanded fast analysis and high sample throughput.

Compared to the conventional sample preparation procedure we were able to minimize the sample preparation to a reasonable degree to obtain confident results and satisfactory detection limits in both publications. The methods proposed in this work are a promising tool to optimize the monitoring process of the investigated analytes and ensure higher food safety standards due to the reduction of costs and time.

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7. List of figures

Curriculum Vitae

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Environmental analysis

Instrumental chemistry

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PUBLICATIONS IN SCIENTIFIC JOURNALS

Urban M, Lesueur C. Comparing d-SPE Sorbents of the QuEChERS Extraction Method and EMR-Lipid for the Determination of Polycyclic Aromatic Hydrocarbons (PAH4) in Food of Animal and Plant Origin, Food Anal Methods. 2017; 10(7); 2111-2124

Urban, M., Hann, S., Rost H. Simultaneous determination of pesticides, mycotoxins, tropane alkaloids, growth regulators and pyrrolizidine alkaloids in oats and whole wheat grains after online clean-up via two-dimensional liquid chromatography tandem mass spectrometry, J. Envirom. Sci. Health. Part B. 2019, DOI: 10.1080/03601234.2018.1531662