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Validation of QuEChERS for screening of 4 marker polycyclic aromatic hydrocarbons in fish and malt.

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Abstract

Application of QuEChERS as a new quick and easy method for screening of polycyclic aromatic hydrocarbons (PAHs) was developed, validated and used for quantification (GC-QTOF-MS) of 4 PAHs, namely benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene and benzo[*a*]pyrene. A clean-up step using Zirconium based dispersive SPE was applied. Validation based on spiked samples (0.5, 1 and 4 µg/kg) revealed a limit of quantification (LOQ) for the 4 PAH compounds in the range of 0.14-0.24 µg/kg for fish and 0.12-0.24 µg/kg for raw barley. Overall recoveries ranged from 88 to 117% with repeatability and in-house reproducibility from 2.6 to 16%. The method was applied to 9 malt samples which were found to contain the sum of 4 PAH from <0.36 µg/kg for raw barley to 26 µg/kg for peat smoked barley. For 5 smoked fish samples the sum of PAH4 ranged from <0.34 µg/kg for cold smoked salmon to 2.2 µg/kg for hot smoked mackerel. In general it was found that the method could be used as a fast screening for evaluation of smoked fish according to EU Commission maximum concentrations of benzo[*a*]pyrene (2.0 µg/kg) and the sum of PAH4 (10 µg/kg).

Keywords: GC-Q-TOF-MS, PAH4, QuEChERS, benzo[*a*]pyrene, fish, malt

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), is a group of organic compounds that contains two or more aromatic rings. PAHs have a low solubility in water and are highly lipophilic (IPCS, 1998). PAHs are formed by incomplete combustion of organic materials, such as fossil fuels or wood, waste incineration and tobacco smoke. Human exposure to PAHs occurs as a result of exposure to environmental sources as well as food intake, where the PAHs are formed during processing of the food, such as drying, smoking or barbecuing.

Studies have shown that some PAHs are genotoxic and carcinogenic. In 2002, the Scientific Committee of Food (SCF) evaluated 33 PAHs resulting in 15 of these showing evidence of genotoxicity and 14 of the 15 showing carcinogenic effects in experimental animals (SCF, 2002). After this, the European commission established maximum limits for benzo[*a*]pyrene (BaP) for certain food (EC 2005b). At the same time the European commission established a recommendation on data collection of 15 PAHs in food namely benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*ghi*]perylene (BghiP), benzo[*a*]pyrene, BaP, chrysene (CHR), dibenz[*ah*]anthracene (DBahA), indeno[1,2,3-*cd*]pyrene (IND), benzo[*j*]fluoranthene (BjF), cyclopental[*cd*]pyrene (CP), dibenzo[*ae*]pyrene (DBaeP), dibenzo[*ah*]pyrene (DBahP), dibenzo[*ai*]pyrene (DBaiP), dibenzo[*al*]pyrene (DBalP) and 5-methyl chrysene (5MCHR)) (EC 2005a). At the same time JECFA re-evaluated the results of SCF and concluded that 13 out of the 15 PAHs SCF found were clearly genotoxic and carcinogenic (JECFA, 2005). They identified an additional compound, benzo[*c*]fluorine (BcF). In 2008, the EFSA Panel of Contaminants in the Food Chain (CONTAM Panel) reviewed data of occurrence and toxicity of the now 15+1 PAHs. EFSA concluded that the sum of 8 PAHs (PAH8: BaA, BaP, BbF, BkF, BghiP, CHR, DBahA and IND) and 4 PAHs (PAH4: BaA, BaP, CHR and BbF) should be used as a marker of carcinogenic effects and genotoxicity of PAHs in food, where PAH8 did not provide a significant amount of extra information compared to PAH4 (EFSA, 2008). Therefore, the legislation was changed to include a maximum level of BaP and the sum of PAH4 (EC 2006 with amendments).

PAH analysis of food generally consists of three steps, an extraction of PAH and fat, followed by one or more clean-up steps, and finally the detection. Analysis of PAHs in food can be tedious and time consuming because of interfering compounds such as fat, and the method often depends on the used matrix. An example is the analysis of PAH in olive oils with liquid-liquid extraction (LLE), adsorption chromatography, thin layer chromatography (TLC) followed by detection by gas chromatography (GC) with flame ionization detection (FID), GC-FID (Menichini, E. et al., 1991). Sample preparation techniques for PAH analysis have also included saponification (Rose et al., 2007, Akdogan et al., 2016) followed by purification by e.g. gel permeation chromatography (GPC) or solid phase extraction (SPE) or both. Also coffein complex formation has been used for clean-up (Welling and Kaandorp, 1986). Recent methods has included faster extraction methods such as microwave assisted extraction (MAE) or pressurized liquid extraction (PLE), however still combined with additional time-consuming clean-up steps such as GPC or SPE (Purcaro et al., 2009; Jira, W et al., 2008; Duedahl-Olesen et al., 2010). Detection principles vary and include e.g. liquid chromatography (LC) combined with fluorescence detection (FLD) (Purcaro et al. 2009; Akdogan

et al., 2016) and ultra violet (UV) detection (Dost and Ideli, 2012), GC combined with flame ionization detection (Menichini et al., 1991; Olatunji et al. 2014) or the more recent combination of both LC and GC with mass spectrometry (MS) detection (Hollosi and Wenzl, 2011; Rose et al., 2007; Jira et al., 2008; Duedahl-Olesen et al., 2010). Research in faster extraction methods and the producer driven need for fast screening techniques has led to the introduction of new methods. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method has shown to provide the same qualities of results as the traditional methods in food and feed analysis of e.g. pesticide analysis and mycotoxins (Payá, P *et al*, 2007 and Cunha, S. *et al* 2010). Acetonitrile is often used in the extraction step and an extraction salt including dessicant magnesium sulphate ($MgSO_4$) and sodium acetate (NaOAc) or sodium chlorid (NaCl) are applied. The clean-up step was simplified by replacing e.g. a slow GPC separation with simply adding dispersive-SPE material to the sample. Single attempts on application of QuEChERS for PAH analysis has been applied on few matrices e.g. meat (Surma et al., 2014), fish (Forsberg, N.D. *et al*., 2011) and tea (Sadowaska-Rociek, A. *et al*., 2013) all using GC-MS detection. The benefits of using the QuEChERS method instead of the other methods, is that the method is less time-consuming and more effective, while 10 to 12 samples can be processed within 35-40 minutes, instead of using 24 work hours (3 work days) with the traditional method. Thereby the capacity of sample preparation is increased tremendously. Furthermore, the method is more environmental friendly with its minimal use of solvents and non-special requirement of equipment in the clean-up step. In this study, we therefore present a validated fast QuEChERS method combined with GC-Q-TOF-MS detection (accurate mass) for screening analysis of 15 PAH focusing on 4 marker PAH in two distinct matrices namely malt and fish with high dry matter contents and various fat contents, respectively.

2. Material and methods

2.1. Chemicals

Acetone and toluene of glass distilled grade and acetonitrile of HPLC grade S was all purchased from Rathburn (Microlab, Århus, DK). n-hexane (HPLC grade) was purchased from Merck, Germany. Deionised water (18.2 M Ω cm^{-1}) produced by Mill-Q system from Millipore, (Molshein, France). An extraction salt, Supel™ QuE non-buffered (Product nr. 55294-U) and Supel™ QuE Z-sep+, 500 mg (Product nr. 55296-U) both obtained from Supelco, USA. FAPAS 0668 smoked fish, ISO certified reference material for quality control (truness) were obtained from FAPAS, Fera Science Ltd, National Agri-Food Innovation Campus (Sand Hutton, York, UK). The certified material contained BaA (32 ± 6.68 µg/kg), CHR (31.4 ± 6.90 µg/kg), BbF (14.8 ± 3.26 µg/kg), BaP (12.6 ± 2.77 µg/kg), BghiP (7.08 ± 1.56 µg/kg) and IND (6.63 ± 1.46 µg/kg).

Standard solution (STD), consisting of 15 PAHs: Acenaphthene (ACN), Acenaphthylene (ACY), Anthracene (ANT), BaA, BbF, BkF, BghiP, BaP, CHR, DBahA, Fluoranthene (FLA), Fluorene (FLU), IND, Phenanthrene (PHE), Pyrene (PYR) each at 1000 ng/mL. Internal standard solution (ISTD), 1000 ng/mL of each of 15 deuterium labelled PAHs: Acenaphthene D_{10} (ACN D_{10}), Acenaphthylene D_8 (ACY D_8), Anthracene D_{10}, (ANT D_{10}), Benz[*a*]anthracene D_{12},(BaA D_{12}),

Benzo[*b*]fluoranthene D_{12} (BbF D_{12}), Benzo[*k*]fluoranthene D_{12} (BkF D_{12}), Benzo[*ghi*]perylene D_{12} (BghiP D_{12}) Benzo[*a*]pyrene D_{12} (BaP D_{12}) Chrysene D_{12} (CHR D_{12}) Dibenz[*ah*]anthracene D_{14} (DBahA D_{14}) Fluoranthene D_{10} (FLA D_{10}) Fluorene D_{10} (FLU D_{10}), Indeno[1,2,3-*cd*]pyrene D_{12} (IND D_{12}) Phenanthrene D_{10} (PHE D_{10}), Pyrene D_{10} (PYR D_{10}) Both were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany) stored at -20°C.

2.2. Samples and sample preparation

For the validation, two types of fish, a raw cod (0.6 % fat) and a raw salmon (12 % fat) obtained from the local fishmonger, Mørkhøj, DK and raw barley with 91 % dry matter were used. Smoked fish from the local fish monger (Mørkhøj, Denmark) and malt samples (dry matter 91-96 %) from Maltbazaren (Maltbazaren ApS est 2004) were purchased for analysis. Bones and skin were removed from the fish before homogenization (Kenwood, FP220 series, Hampshire, UK). Barley and malt samples were homogenizied by a Coffee mill (Bosch KM13 coffee grinder). These matrices with variable fat content and high dry matter content were applied to evaluate QuEChERS method for PAH analysis of variable matrices.

2.3. Sample preparation with QuEChERS

Internal standard, ISTD (fixed concentration of 40 ng/mL) was added to 2.5 g homogenized fish or 5 g homogenized barley or malt in a 50 mL Centrifuge tube (Sarstedt AG&Co, Numbrecht, Germany) followed by 10 mL milliQ Water, 10 mL Acetonitril, and a ceramic stone. Samples were mixed 15 min. by a Genogrinder (SPEX sample prep, Ramcon, Denmark) at 750 rpm. Supel™ QuE non-buffered salt was added and mixed for 5 min. at 750 rpm. The sample was then centrifuged (Varifuge 3.0 R, Heraus, Denmark) at 4000 G for 10 min. The supernatant, approximately 9 mL, was extracted and frozen at -80°C. The next day, the sample was thawed and centrifuged for 5 min. at 4000 G and 5°C. Then 7 mL was transferred to a new tube and the Z-sep+ dispersive SPE material was added and mixed for 1 min at 750 rpm followed by centrifugation for 5 min (4000 G, 5°C). 5 mL supernatant was then transferred to a glass tube and evaporated to dryness with nitrogen. Lastly, the sample was dissolved in toluene (200µl), shaken, filtered and transferred to a GC-glass vial before analysis by GC-Q-TOF-MS. Spiked samples at 0.5, 1 and 4 µg/kg were made for validation by addition of standard STD and as described above ISTD. Blind samples without spike as well as reagent blanks (no sample matrix, only reagents) were included in every series to make sure that no PAHs was detected in the fish and malt used for fortification and that no background levels from solvents, salts or dispersive material were found.

Dry matter were determined by drying 0.5 g sample at 102°C for 3 hr followed by desiccating to constant weight.

Fat content was determined by gravimetric determination of acetone:hexane (1:3) extracts from pressurized liquid extraction (PLE) at 110°C (6 min heating), 2 cycles with a purge time at 60 sec and 100% flush volume at 1500 psi.

2.4. GC-Q-TOF-MS

Analysis was performed using an Agilent GC-Q-TOF-MS (Santa Clara, USA). The GC Model was 7890A and the MS was 7200 Accurate-Mass Q-TOF GC/MS. Separation was achieved by two coupled HP5MS ultra inert capillary columns (Agilent Technology, 15 m x 250 µm x 0.25 µm).

Helium was used as carrier gas at 1.2 ml/min. Programmed Temperature Vaporization (PTV) injection of 3 µL at 50 °C in solvent-vent mode , for 0.8 min. with a temperature raise to 290°C held for 2 min, followed by an increase to 330°C kept for 10 min. The column separation was obtained by a temperature gradient starting at 70°C for 3.3 min, increased to 180°C at the rate of 50°C/min, then increased to 230°C at a rate of 4°C/min, and then further increased to 280°C with 3 °C/min, and finally raised to 310°C with 14 °C/min and kept for 10 min. After each run a column backflush was used, in order to avoid column contamination. The mass spectrometer was operated in electron ionization mode, with an electron energy of 70 eV, and a mass range of m/z 50-550 and a scan range of 5 spectra/sec. Single compound concentration calculations were based on the relative response for each compound (compound response area/ISTD compound response area) and linear calibration using eight concentration levels (0, 10, 25, 50, 100, 200, 300 and 500 ng/mL). Identification of single PAH compounds was based upon 3 digit accuracy mass to charge values (m/z) (Table 1) and retention times and the PAH content was quantified by calculating the relative response from single compounds using calibration curves.

2.5. Method validation

For method validation two series of raw barley, one serie with salmon and one serie with trout, each serie with four replicates at each of three concentration levels (0.5, 1 and 4 µg/kg), were prepared and analysed for the content of 15 PAHs. Before calculation of validation parameters results were corrected with content in matrix blind samples (levels in Table 2 and supplementary material Tabel A.2). The validation parameters included linearity, the limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability (SD_r) and in-house reproducibility (SD_R) according to ISO 17025 (ISO/IEC 17025:2005). The LOD and LOQ were calculated according to ISO 5725-2 (1995) using three times and six times the relative standard deviation for the low concentration level (0.5 µg/kg). Recoveries were calculated for each matrix and spike level by dividing the detected single compound concentration with the theoretical spiked concentration and multiplied by 100. The relative repeatability standard deviation (RSD_r) and the relative in-house reproducibility standard deviation (RSD_R) was calculated for each concentration level (0.5, 1.0 and 4.0 µg/kg) from the series of fish and raw barley, respectively according to ISO 5725-2 (1995). The series were prepared on different days and calculations were made by using single sided varians analysis. A FAPAS proficiency test material 0668 based on smoked fish (FAPAS 2016) was used for evaluation of the trueness of the new method analysing 3 replicates (1g material each) for 6 PAH compounds (BaA, CHR, BbF, BaP, BghiP, IND).

3. Results and discussion

The modified QuEChERS method with a non-buffered extraction salt ($MgSO_4$ and NaCl) and zirconium based dispersive (Z-sep$^+$) clean-up was validated for 15 PAHs in raw fortified fish (0.6 and 12 % fat) and fortified raw barley (dry matter 91%). The values for 4 PAHs, namely BaA, CHR, BbF and BaP are reported. Additional results (other 11 PAH) can be found in the supplementary material with detection parameters named Table A.1, validation results for fish and raw barley (Supplementary material Table A.2), respectively and the additional 11 PAH in 5 Fish and 9 Malt samples as Table A.3.

3.1. Method validation

The linearity of the calibration curves were tested for all 15 single PAHs. All calibration curves had a determination coefficient (R^2) above 0.99. Linear graphic inspection as well as non-systematic residual plots confirmed the linear correlation (data not shown).

For validation of fish, salmon (12% fat) and trout (0.6% fat) were fortified and analysed using 4 replicates at each of 3 concentration levels. The average recovery of the 4 PAHs in the fortified fish samples was found to be 89-117 % for spike levels of 0.5, 1.0 and 4.0 µg/kg (Table 2). Recoveries between 50 and 120 % are generally accepted for the 4 PAHs in food with maximum limits (EC 2007). All our average recoveries for BaA, CHR, BbF and BaP was within the acceptance criterion. For the total 24 spiked fish samples, recovery values ranged between 83-108% for BaA, 100-123% for CHR, 86-111% for BbF and 93-111% for BaP (data not shown). These values indicated an acceptable recovery for all samples of the method. In comparison, our traditional method had slightly lower, however very similar recoveries between 73 and 106 % for the two lowest spike levels (Duedahl-Olesen et al., 2010). Previously, lower recoveries for the 4 PAHs on salmon and mackerel by QuEChERS of 56-96 % (Forsberg, N.D. *et al*., 2011) and of 87-99 % (Ramalhose, M.J. *et al*., 2009) have also been reported. For comparison, we generally do not accept recoveries below 70% in our laboratory. For other PAHs than PAH4, only ANT at spiked fish concentration 1.0 µg/kg had average recoveries large than 120%, namely 121% (Supplementary material Tabel A.2 Fish). These values indicated acceptable recoveries for all 15 PAHs in fish.

The relative repeatability standard deviation, RSD_r and the relative in-house reproducibility standard deviation, RSD_R, for the 4 PAHs in fortified fish were between 2.6 and 9.4 % (Table 2) and at acceptable levels well below 22% (EC 2007). Other 11 PAHs had RSD_r and RSD_R values between 2.2 and 73%, with unacceptable levels for ACY (RSD_r = 27% and 24% and RSD_R = 68% and 25%), ACN (RSD_R = 73% and 23%) and PHE (RSD_R = 30%) (Supplementary material, Table A.2 Fish).

The LOD for the 4 PAHs were below the maximum LOD of 0.3 µg/kg required by the EU regulation (EC 2007). Namely 0.07-0.12 µg/kg (Table 2) and LOQ`s were 0.14–0.24 µg/kg (Table 2) for fish. The results were slightly lower than LOD values for the 4 PAHs of 0.2 µg/kg obtained in the same laboratory with PLE followed by GPC and SPE clean-up for fish (Duedahl-Olesen *et al*., 2010). Our low LOD´s were similar to other studies using QuEChERS method for PAH analysis in e.g. horse mackerel with LOD´s of 0.04-0.09 µg/kg (Ramalhose, M.J *et al*., 2009). Five of 11 other PAHs had LOD´s above the required 0.3 µg/kg for PAH4 in the European Regulation (EC 2007). These higher levels were found for ACN (0.77 µg/kg), ACY (0.71 µg/kg), PHE (0.43 µg/kg) and BghiP (0.35 µg/kg) (Supplementary material Table A.2 Fish).

Background levels from fish generally resulted in higher concentrations for salmon compared to cod (data not shown). For PAH4 in fish background concentrations were all below LOD (Table 2). Background concentrations above LOD were found for PHE (1.1 µg/kg) followed by ACY (0.98 µg/kg), ANT (0.67 µg/kg), FLU (0.35 µg/kg) and FLA (0.15 µg/kg), whereas compounds such as

ACN (0.60 µg/kg) had high background concentration levels close to but below the calculated LOD (Supplementary material Table A.2 Raw barley). A large variation in concentration data due to high background concentrations results in unacceptable RSD_r and RSD_R levels (ACY, ACN and PHE) at low spike concentrations (0.5 and 1.0 µg/kg) as well as high LOD's (ACN, ACY, PHE, BghiP).

For evaluation of the trueness of the new method, triplicate analysis of a FAPAS proficiency test material based on smoked fish (FAPAS 2016) was analysed. The 6 PAH average recoveries (n = 3) were 46% for BaA (assigned value 31.2 µg/kg), 71 % for BaP (assigned value 12.6 µg/kg), 94% for CHR (assigned value 31.4 µg/kg), 104 % for B(ghi)P (assigned value 7.08 µg/kg), 111% for BbF (assigned value 14.8 µg/kg) and 120% for IND (assigned value 6.63 µg/kg). Only the trueness for BaA was unacceptable (below 70%). The accuracy (precision and trueness) of the new method in fish is therefore acceptable for CHR, BkF and BaP, whereas only precision where acceptable for BaA. Indications of BaA signal suppression will be discussed under discussion of figure 1.

For validation of raw barley, two series with 4 replicates at each of three concentration levels (0.5; 1.0; 4.0µg/kg) were used. The average recoveries for the 4 PAHs were 88-111 % (Table 2). Generally, recoveries for BaP, BbF and BaA ranged from 73-110%, 76-104% and 85-110%, respectively (data not shown) and therefore were acceptable for all replicates. For CHR the average recoveries were between 105 and 111% (Table 2), with recoveries ranging from 93 to 128% for the 24 replicates. Recoveries for cereals analysed by QuEChERS has been reported to be similar to our results, namely between 86-91 % for the 4 PAHs (Kacmaz *et al*., 2016). Our results therefore indicate good recoveries for the 4 PAHs, however for the other 11 PAH compounds, a large average recovery range was obtained. Especially ACY (121%), PHE (134%), ANT (132%) and PYR (127%) had recoveries above 120% at the lowest spike level (0.5 µg/kg). PHE (124%) and ANT (142%) also had recoveries above 120% for spiked concentration levels of 1 µg/kg and ANT (147%) also at high spiked level at 4.0 µg/kg (supplementary material A.2 Raw barley).

The relative repeatability standard deviation, RSD_r and the relative in-house reproducibility standard deviation on different days, $RSD_{R,}$ in fortified raw barley were between 2.7 and 16 % for the 4 PAHs (Table 2) indicating that the QuEChERS method is precise for cereals. For the other 11 PAHs RSD_r and RSD_R values were found to be between 2.1 and 49%, only with acceptable levels at all spiked concentrations for FLA, PYR, BkF, DBahA, and IND (Supplementary material A.2 Raw barley). Only BbF, BaA, CHR, BaP, BkF, FLA, and IND had acceptable recoveries as well as acceptable RSD_r and RSD_R levels when comparing to criterion for matrices with EU maximum limits (EC 2007). These results were similar to results reported by Kacmaz *et al.* with RSD_r and RSD_R values between 5.4-17.0% for cereals.

The LOD for the 4 PAHs in barley was 0.06-0.12 µg/kg and LOQ values were between 0.12 and 0.24 µg/kg (Table 2). These detection limits were more than twicethe LOD's previously reported for cereals (0.01-0.03 µg/kg) by Kacmaz *et al.* using n-hexane and cyclohexane for QuEChERS (Kacmaz *et al*, 2016). Their results indicated that lower detection limits might be obtained by exchange of solvents. However, preliminary experiments on spices using n-hexane resulted in high

concentrations of ethereal oils making the final extract impossible to apply for GC analysis (data not shown). Also, Cloutier *et al.* indicated that acetonitrile was inefficient for QuEChERS extraction of PAH in fatty samples (Cloutier, P-L. et al., 2017), which could be the reason for the obtained low trueness of BaA in the reference material (FAPAS). Our validation results however, indicated that not all PAH compounds were affected by the use of acetonitrile. Detection limits above 0.3 µg/kg and at higher levels than validation of fish were obtained for FLU (1.4 µg/kg), PHE (0.57 µg/kg), BghiP (0.39 µg/kg) and ANT (0.36 µg/kg).

Similar to fish validation, background levels from barley resulted in concentrations above LOD for ANT (3.8 µg/kg), ACY (1.8 µg/kg) and PHE (1.4 µg/kg) (Supplementary material Table A.2 raw barley). FLU (0.75 µg/kg), ACN (0.40 µg/kg), FLA (0.36 µg/kg), PYR (0.33 µg/kg), and BaP (0.10 µg/kg) were also found in high background concentrations (Table 2 and supplementary material Table A.2 raw barley). These high background concentrations resulted in a large data variation and especially unacceptable RSD_R levels (ACY, ACN, FLU, PHE, ANT and BghiP) at low spike concentrations (0.5 µg/kg) as well as high LOD's (Supplementary material Table A.2 raw barley).

Due to the challenges on single PAHs in both fish and raw barley the chromatograms were studied further. Figure 1a shows a chromatogram for standard 25 ng/ml for BaA and CHR with a fortified cod (1.0 µg/kg) in Figure 1b. In Figure 2a, the standard 25 ng/ml for BbF and BaP is shown and a spiked barley (1.0 µg/kg) in Figure 2b. Spiked levels at 1.0 µg/kg correspond to a level of 25 ng/ml. Both matrices seem to result in matrix effects. In Figure 1b an enhanced signal seems to be obtained when matrix is included, however no interferences or suppression for BaA seems to be visible (rt = 20.7 min). For the reference material (FAPAS) a lower signal than expected for BaA was visible without interfering peaks. However, for FAPAS, an increased baseline was identified when comparing the full scan chromatogram to that of fortified fish (data not shown). The low BaA recovery for the smoked fish in the FAPAS material indicate a non-identified signal depression for BaA in smoked material compared to non-smoked. The used reference material (FAPAS) also included concentration levels approximately ten times higher than the EU maximum limits and therefore levels not previously studied by fortification etc. To study the phenomenon further, a matrix-matched calibration curve based on smoked fish could be analysed. In Figure 2b for barley, be matrix suppression as well as several interfering peaks is visible.). The interferences was first expected to be due to the solid extraction material (salt and SPE), however a reagent blank (no matrix) revealed no carry over from the used material (solvents, salts and dispersive SPE) to the chromatograms of the detected single PAHs (data not shown). The use of a corresponding internal standard for each of the PAHs and use of relative responses minimize the influence of these matrix effects on our results. The zirconium clean-up step has previously been deemed relevant for challenged matrices (Al-Thaiban et al., 2018), however due to our results dried low fat material such as barley as well as smoked fish might be even more challenging. It was not possible to obtain cereal based reference material for evaluation of trueness for barley.

3.2 Concentration of PAH in smoked fish and malt

The developed QuEChERS method was used to determine PAH4 in processed food products similar to the validated matrices, namely fish and malt. Focus was on different smoking techniques

316 such as hot and cold smoked salmon, as well as the heat source (e.g. beech and alder) (Table 3).
317 After cold smoked salmon (PAH4 < 0.34 µg/kg), the alder wood hot smoked salmon (PAH4 = 0.69
318 µg/kg) contained the lowest concentrations of PAH4, whereas beech wood hot smoked salmon had
319 PAH4 concentrations of 1.1 µg/kg. Beech wood hot smoked mackerel had the highest PAH4
320 concentration (2.15 µg/kg), whereas beech wood hot smoked herring only contained 0.55 µg/kg.
321 Our results with higher PAH4 levels in hot smoked compared to cold smoked salmon are similar to
322 previously reported results for smoked fish products (Duedahl-Olesen *et al*., 2010). However, for
323 both BaP and PAH4 our results are well below the EU maximum limit of 2 µg/kg and 12 µg/kg,
324 respectively (EC 2006). ACY and ANT were found in highest concentrations and at levels up to 27
325 µg/kg in beech wood hot smoked fish compared to cold smoked fish (up to 1.4 µg/kg) and alder
326 wood smoked fish (up to 12 µg/kg) (Supplementary material Table A.3). These results could
327 indicate a mitigating action at fish mongers on PAH levels when applying hot smoking procedures.
328 ACY and ANT are not included in the 15+1 PAHs considered to affect the human health and might
329 be omitted for control of matrices complying with the EU regulation. Our results on different
330 smoking conditions are however not as clear as Duedahl-Olesen et al., due to the use of too few
331 samples. More samples should be included for verification of a trend towards lower PAH4 levels in
332 smoked.
333
334 Nine malt samples were analysed, four were smoked with beech or peat (Table 3). The sum of
335 PAH4 varied widely depending on the malt type. The amount of PAH4 in smoked malt was found
336 to be highest for peat smoked malt (15 and 26 µg/kg), followed by beech smoked malt (1.0 and 0.9
337 µg/kg) with nonsmoked malt PAH4 levels below LOD (Table 3). The latter results were similar or
338 even lower than the concentrations found by Kazmac *et al.* of 0.23 - 0.87 µg/kg in cereal based
339 breakfast products (Kazmac et al., 2016). Furthermore, a small amount of IND was detected in all
340 malt samples and not in the fish samples (supplementary material Table A.3). For malt samples the
341 dominating PAHs were ACY and FLU with concentration levels ranging from 0.6 µg/kg in raw
342 barley to 25 µg/kg in peat smoked barley and from <1.5 µg/kg in beech smoked barley to 91 µg/kg
343 in raw barley, respectively. In comparison malt and beech smoked barley had high concentration
344 levels of FLU whereas peat smoked barley also had high concentrations of BaA, BbF and BaP
345 (Table 3) as well as ACY, PHE, ANT, FLA and PYR (Supplementary material Table A.3).
346
347 Due to the slightly variable validation results we conclude that the QuEChERS method should only
348 be used for screening with further verification of results close to maximum EU limits. Screening for
349 EU maximum limits includes four PAHs, namely BaP, BaA, CHR and BbF. All these compounds,
350 were within acceptable ranges for the precision and roubustness of the method. The validation
351 described in this study illustrates that it is necessary to validate the method for new PAHs as well as
352 matrices before inclusion of these in the method. Method validation for fish revealed nonacceptable
353 precision for ACN, whereas for barley ACY, ANT, PHE and PYR precisions were nonacceptable.
354 More purifications steps could be included in the method to avoid matrix enhancement (fish) or
355 matrix suppression (barley and malt) resulting in more time-consuming sample preparation.. This
356 was however not the goal for our screening method and we suggestthatfurther method development

of QuEChERS could include exchange of salts, solvents, dispersive SPE material and application of matrix-matched calibration curves for improved BaA trueness in smoked fish.

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Legends:

Table 1. Compound names, abbreviations, formula and m/z values for PAH4 with internal standards (For additional 11 PAHs, see Supplementary material Table A.1).

Table 2. Background concentrations (blind) in non-spiked matrices (N=3), recovery, relative repeatability standard deviation (RSDr), relative in-house reproducibility standard deviation (RSD_R), limits of detection (LOD) and quantification (LOQ) values for single PAHs in spiked fish samples (N = 8) and spiked raw barley (N = 9). (For additional 11 PAHs, see Supplementary material Table A.2 Fish and A.2 raw barley, respectively)

Table 3. Dry matter (DM) and fat per cent, PAH4 single compound concentrations (µg/kg) for fish and malt samples with heat source used for smoking. S = smoked. (For additional 11 PAHs, see Supplementary material Table A.3)

Legends:

Figure 1 - EIC chromatogram for BaA and CHR. The left peak is BaA and the right peak is CHR. Illustration a) shows the EIC chromatogram for standard 25 ng/ml and b) a spiked cod at 1 µg/kg.

Figure 2- EIC chromatogram for first peak, BbF, the second peak, BkF and the last peak, BaP. Illustration a) shows the EIC chromatogram for standard 25 ng/ml and b) shows a spiked raw barley at 1 µg/kg.

Table1

Compound	**Abbreviation**	**Formula**	**m/z**
Benz[*a*]anthracene	BaA	$C_{18}H_{12}$	228.094
Benz[*a*]anthracene D_{12}	BaA D_{12}	$C_{18}D_{12}$	240.197
Chrysene	CHR	$C_{18}H_{12}$	228.094
Chrysene D_{12}	CHR D_{12}	$C_{18}D_{12}$	240.197
Benzo[*b*]fluoranthene	BbF	$C_{20}H_{12}$	252.094
Benzo[*b*]fluoranthene D_{12}	BbF D_{12}	$C_{20}D_{12}$	264.197
Benzo[*a*]pyrene	BaP	$C_{20}H_{12}$	252.094
Benzo[*a*]pyrene D_{12}	BaP D_{12}	$C_{20}D_{12}$	264.197

Table 2

Matrix	Parameter	Spike level (µg/kg)	BaA	CHR	BbF	BaP
Fish	**Blind (µg/kg)**		<0.12	<0.11	<0.08	<0.07
	Recovery (%)	**0.5**	97	114	99	103
		1.0	96	117	100*	105
		4.0	89	111	102	102
	RSD_r (%)	**0.5**	5.2	7.0	5.6	4.2
		1.0	8.0	3.3	7.2*	5.4
		4.0	4.9	2.6	7.4	4.3
	RSD_R (%)	**0.5**	9.4	7.0	5.6	4.2
		1.0	8.0	3.3	7.2*	5.4
		4.0	6.2	2.6	9.3	6.4
	LOD (µg/kg)		0.12	0.11	0.08	0.07
	LOQ (µg/kg)		0.24	0.22	0.17	0.14
Raw barley	**Blind (µg/kg)**		<0.09	<0.09	<0.12	0.10
	Recovery (%)	**0.5**	94	109	92	95
		1.0	96	105	88	95
		4.0	99	111	97	97
	RSD_r (%)	**0.5**	5.7	6.2	9.4	4.2
		1.0	2.7	5.0	6.0	6.3
		4.0	3.7	3.3	6.3	4.8
	RSD_R (%)	**0.5**	16	11	9.6	4.3
		1.0	3.7	5.6	6.0	6.3
		4.0	9.3	7.4	12	16
	LOD (µg/kg)		0.09	0.09	0.12	0.06
	LOQ (µg/kg)		0.18	0.18	0.24	0.12

*Outlier removed after Dixon outlier test (p=0.002)

Table 3

TYPE	Sample	Heat source	DM %	Fat %	BaA (µg/kg)	CHR (µg/kg)	BbF (µg/kg)	BaP (µg/kg)
FISH	**Herring, Hot S**	Beech	38	14	0.15	0.22	0.11	0.07
	Salmon, Cold S	Beech	41	15	0.10	<0.10	<0.08	<0.06
	Salmon, Hot S	Alder	48	18	0.27	0.20	0.10	0.12
	Salmon, Hot S	Beech	42	20	0.36	0.36	0.24	0.16
	Mackerel, Hot S	Beech	56	32	0.71	0.80	0.45	0.19
MALT	**Caramel**	Unknown	94	4.2	0.08	0.04	0.18	<0.05
	Barley1	Raw	96	5.1	<0.09	<0.08	<0.14	<0.05
	Barley2	Raw	91	2.3	<0.09	<0.08	<0.14	<0.05
	Barley, Pale	Raw	93	4.1	<0.09	<0.08	<0.14	<0.05
	Barley, Pale, S	Beech	93	3.9	0.34	0.23	0.31	0.12
	Barley, Wey Pale	Raw	96	4.2	<0.09	<0.08	<0.14	<0.05
	Barley, Wey S	Beech	96	3.9	0.24	0.23	0.33	0.08
	Barley, S	Intense Peat	96	4.7	8.08	2.28	12.25	3.65
	Barley, light, S	Intense Peat	95	3.6	4.39	1.24	7.19	2.31

Figure 1

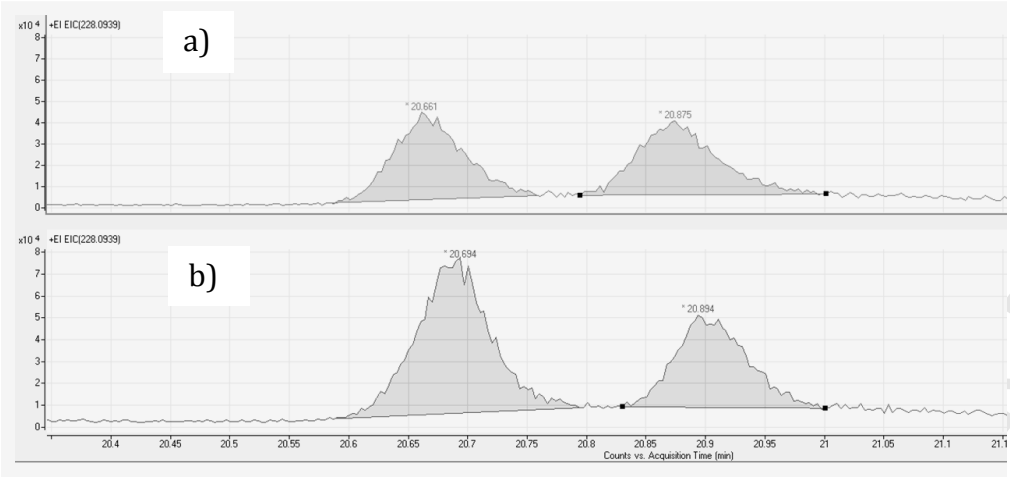
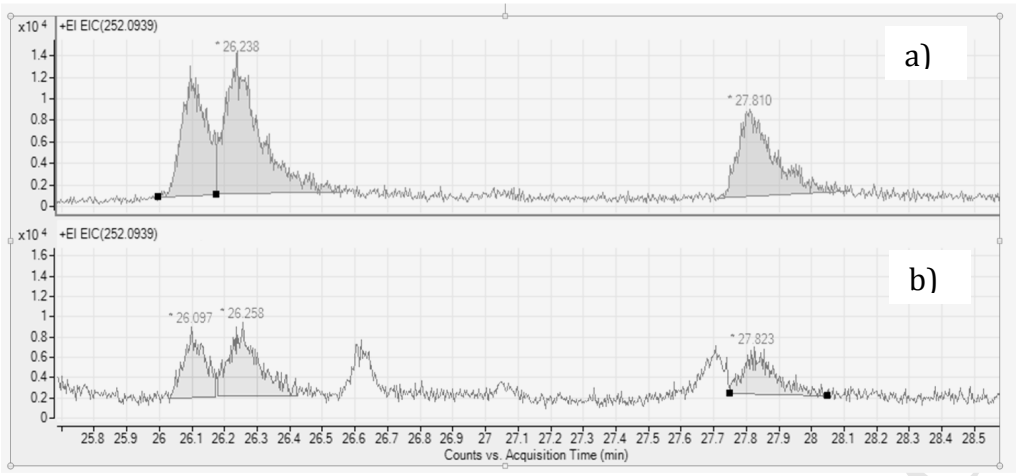


Figure 2



Highlights:

- Successful validation of QuEChERS screening method for PAH4 analysis.
- PAH4 average recoveries between 93 and 117 % with LOD's below 0.12 µg/kg.
- Peat smoked malt contained high PAH4 levels ranging from 15 to 36 µg/kg.