

Determination of PAH4 in edible oils with automated sample preparation

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Keywords: PAH, automated sample preparation, Vanquish Core, edible oils, online SPE, fluorescence detection, PAH4 (VO (EC) No 1881/2006)

Application benefits

- Substantially shorter analysis time (1 h) compared to 80 min in AN196 and 8–10 h with traditional methods
- Method modernization from Thermo Scientific™ UltiMate™ 3000 system to Thermo Scientific™ Vanquish™ Core system
- Simplified sample preparation compared to classical method



Goal

Determination of legally regulated PAH4 (benzo[a]pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene) in edible oils for batch release. The legal maximum quantities are 2 µg/kg for benzo[a]pyrene and 10 µg/kg for the sum of PAH4 (VO (EC) No 1881/2006).¹ Furthermore, referring to the customers' requirements, the sensitivity for benzo[a]pyrene should be at least a factor of 10 below the legal limit of 2 µg/kg. The same sensitivity should be achieved for the other PAHs, too. Determination of PAH4 in edible oils with automated sample enrichment and matrix elimination (online solid phase extraction - online SPE). Transfer of existing method from UltiMate 3000 to Vanquish Core series.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds, comprising more than 200 known structures, which arise from incomplete combustion processes and thus, present ubiquitous environmental pollutants in air, soil, and water. Per definition, the molecules are composed of two or more condensed aromatic rings without heteroatoms, resulting in very non-polar and uncharged structures. Many of them are considered carcinogenic and mutagenic, and due to their omnipresence, provide a public health concern as human exposure is unavoidable. However, human intake is particularly driven by the diet, with edible oils and fats being major contributors because of the lipophilic nature of PAHs. Thus, monitoring of PAH amounts in food, especially fats and oils is mandated.

Formerly, the European Commission considered benzo[a]pyrene a good indicator of PAH contamination, and it was used as the only marker for the occurrence of PAHs in food. As the scientific knowledge grew, the PAH4 system, a system of four compounds, namely benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, and chrysene (Figure 1) was introduced in 2011. Since then, the Regulation No. 835/2011 established limits of 2 µg/kg benzo[a]pyrene and 10 µg/kg sum of PAH4 for edible oils and fats.¹

The analysis of PAHs usually requires an extraction/clean-up procedure, which is followed by gas or liquid chromatographic separation and detection. While the extraction from water can be straightforward (e.g., by the enrichment on hydrophobic column material) the handling of oil and fat matrices is more complex. Applied techniques range from alkaline digestion over liquid-liquid extractions, evaporation and enrichment with packed columns or solid phase extraction (SPE) cartridges.^{2,3} Improved selectivity was found with the introduction of donor-acceptor complex chromatography (DACC) sorbents, which retain PAHs based on strong π - π interactions.⁴ Solvents without π -electrons are used to elute bulk oil components like fatty acids and triglycerides. Molecules with π -electron systems like PAHs can then be eluted by a proper solvent that neutralizes the π - π interactions. However, manual sample preparation is time and solvent consuming, and error

prone. Those challenges are solved by the implementation of an automated process, developed by van Stijn et al.⁵ It combines minimum preparation of the oil sample with online sample clean-up by a DACC column coupled to the analytical HPLC column with fluorescence detection (FLD). The concept was optimized and adopted into the DIN EN ISO 22959:2009 norm, describing a method for the analysis of up to 17 PAHs in animal and vegetable fats and oils.^{6,7} The current application note showcases the utilization of the online SPE clean-up coupled to HPLC-FLD for the analysis of PAH4 in a routine laboratory for oil testing implemented with the Thermo Scientific™ Vanquish™ Core HPLC system.

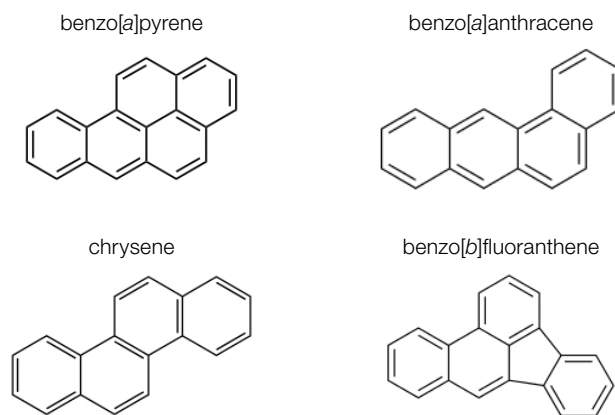


Figure 1. Analytes

Experimental details

Chemicals

- Water, UltraPure, 18.2 M Ω , Thermo Scientific™ Barnstead™ GenPure™ water purification system
- Acetonitrile, Optima™ LC-MS, Fisher Chemical™, Fisher Scientific (P/N A955-1)
- Isopropanol, Optima™ LC-MS, Fisher Chemical, Fisher Scientific (P/N A461-1)
- Benzo[a]anthracene, 100 µg/mL in ACN, Fisher Scientific (P/N P806330)
- Chrysene, 100 µg/mL in ACN, Fisher Scientific (P/N P813040)

- Benzo[b]fluoranthene, 100 µg/mL in ACN, Fisher Scientific (P/N P806460)
- Benzo[a]pyrene, 100 µg/mL in ACN, Fisher Scientific (P/N P806800)
- Benzo[b]chrysene, 10 µg/mL in ACN, LGC Dr. Ehrenstorfer (P/N L20550000AL)
- Benzo[e]pyrene, 10 µg/mL in ACN, LGC Dr. Ehrenstorfer (P/N L20645000AL)
- Perylene, 10 µg/mL in ACN, LGC Dr. Ehrenstorfer (P/N L20915000AL)
- Benzo[k]fluoranthene, 10 µg/mL in ACN, LGC Dr. Ehrenstorfer (P/N L20580000AL)
- Benzo[a]fluoranthene, 10 µg/mL in ACN, LGC Dr. Ehrenstorfer (P/N L20560000AL)

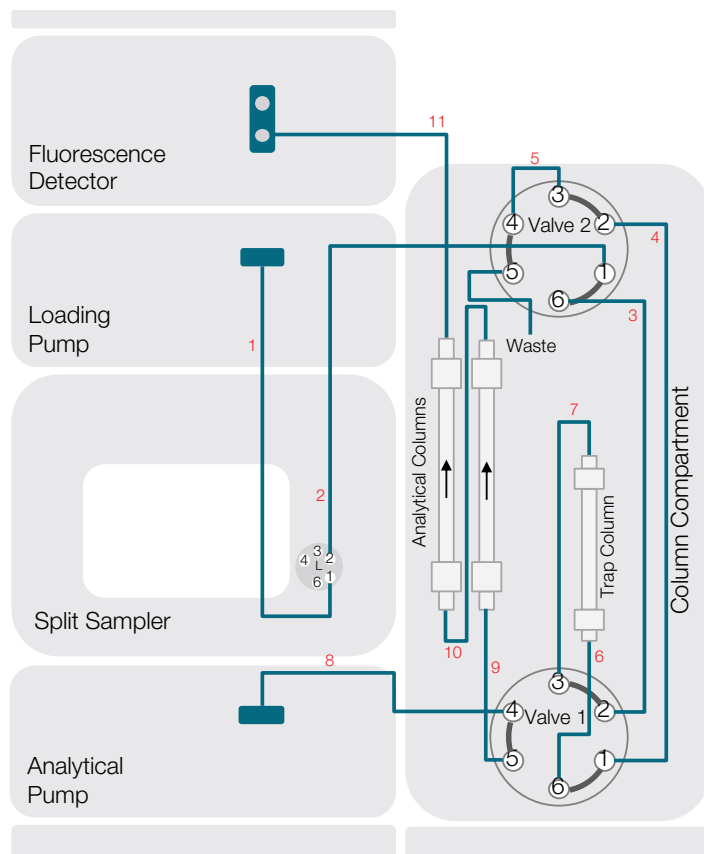
Sample handling

- Thermo Scientific™ Titan3™ PTFE syringe filters (0.45 µm, 17 mm) (P/N 44513-NPL)
- Syringe, 10 mL, B. Braun™ (P/N 4606051V)
- Thermo Scientific 18 mm Screw top headspace vials, 12 mL (P/N 10-HSV)
- Thermo Scientific 18 mm Magnetic screw caps (P/N 18-MS-C-ST201)

HPLC instrumentation

Vanquish Core HPLC system consisting of:

- Vanquish System Base C (P/N VC-S01-A)
- 2 x Vanquish Quaternary Pump C (loading pump, analytical pump) (P/N VC-P21-A)
- Vanquish Split Sampler CT (P/N VC-A12-A) (with 1000 µL sample loop, P/N 6851.1980)
- Vanquish Column Compartment C (P/N VC-C10-A) (with two 2-position/6-port valve 70 MPa, P/N 6230.1520)
- Vanquish Fluorescence Detector F, with Dual PMT (P/N VF-D51-A) (with 8 µL flow cell, P/N 6079.4230)



1	Viper Capillary, ID × L 0.18 × 550 mm, SST	6040.2355
2	Viper Capillary, ID × L 0.18 × 450 mm, SST	6040.2365
3	Viper Capillary, ID × L 0.18 × 650 mm, SST	6040.2395
4	Viper Capillary, ID × L 0.18 × 750 mm, SST	6040.2370
5	Viper Capillary, ID × L 0.18 × 150 mm, SST	6040.2360
6	Viper Capillary, ID × L 0.18 × 150 mm, SST	6040.2360
7	Viper Capillary, ID × L 0.18 × 250 mm, SST	6040.2385
8	Viper Capillary, ID × L 0.18 × 350 mm, SST	6040.2375 ¹
9	Viper Capillary, ID × L 0.18 × 250 mm, SST	6040.2385
10	Viper Capillary, ID × L 0.18 × 250 mm, SST	6040.2385
11	Viper Capillary, ID × L 0.18 × 450 mm, SST	6040.2365

¹ included in the shipkit of the System Base Vanquish Core (VC-S01-A-02)
SST = Stainless steel

Figure 2. Configuration of the system

Samples

Samples were provided by customers in the context of a customer application development.

- Coconut oil crude
- Sunflower oil crude
- Synthetic triglyceride
- Coconut oil native
- Olive oil extra virgin

Sample preparation

- Weigh 1 g of sample into a 5 mL volumetric flask.
- Fill up to the mark with isopropanol.
- Warm the sample to 35 °C in a water bath to prevent phase separation between the oil and isopropanol.
- Filtrate the sample by a syringe filter (PTFE, 0.45 microns) into a 10 mL vial.
- Place the capped vial into the autosampler.

Preparation of calibration samples

- Weigh approximately 1 g of rapeseed oil (analyte free matrix for calibration provided by customer) into a 5 mL volumetric flask.
- Add the according amount of standard solution (see Table 2) and fill up to the mark with isopropanol.

- Warm the sample in water bath to 35 °C to prevent phase separation between the oil and isopropanol.
- Filtrate the sample by a syringe filter (PTFE, 0.45 microns) into a 10 mL vial.
- Place the capped vial into the autosampler.

Preparation of stock solutions

- **S1 mix:** Combine 100 µL of the 100 µg/mL standard solutions of benzo[a]pyrene, benzo[b]fluoranthene, benzo[a]anthracene, and chrysene and dilute with acetonitrile to 10 mL (concentration: 1 µg/mL).
- **S2 mix:** Dilute 100 µL of S1 with isopropanol to 10 mL (concentration: 10 ng/mL).
- **Internal standard solution:** Dilute 100 µL of the 10 µg/mL solution of benzo[b]chrysene with acetonitrile to 10 mL (concentration: 100 ng/mL).

Table 1. Dilution of standards for external calibration in solvent

Level	Concentration	Volume standard solution S2 mix	Volume internal standard solution	Volume isopropanol
1	0.1 µg/kg	20 µL	20 µL	9960 µL
2	0.25 µg/kg	50 µL	20 µL	9930 µL
3	0.5 µg/kg	100 µL	20 µL	9880 µL
4	2.5 µg/kg	500 µL	20 µL	9480 µL
5	5.0 µg/kg	1000 µL	20 µL	8980 µL
6	10.0 µg/kg	2000 µL	20 µL	7980 µL

Table 2. Dilution of standards for matrix calibration

Level	Concentration	Volume standard solution S2 mix	Volume internal standard solution	Volume isopropanol
1	0.1 µg/kg	10 µL	10 µL	Fill up to 5 mL
2	0.25 µg/kg	25 µL	10 µL	Fill up to 5 mL
3	0.5 µg/kg	50 µL	10 µL	Fill up to 5 mL
4	2.5 µg/kg	250 µL	10 µL	Fill up to 5 mL
5	5.0 µg/kg	500 µL	10 µL	Fill up to 5 mL
6	10.0 µg/kg	1000 µL	10 µL	Fill up to 5 mL

Table 3. Dilution of standards for direct injection

Level	Concentration	Volume standard solution	Volume internal standard solution	Volume acetonitrile
1	2 ng/mL	S2 mix: 200 µL	200 µL	600 µL
2	5 ng/mL	S2 mix: 500 µL	200 µL	300 µL
3	10 ng/mL	S1 mix: 10 µL	200 µL	790 µL
4	50 ng/mL	S1 mix: 50 µL	200 µL	750 µL
5	100 ng/mL	S1 mix: 100 µL	200 µL	700 µL
6	200 ng/mL	S1 mix: 200 µL	200 µL	600 µL

Chromatographic conditions

Table 4. Chromatographic conditions (columns required by customer)

Parameter	Value
Columns	1x Nucleodur™ PAH, 125 x 4 mm, 3 µm 1x Nucleodur PAH, 100 x 4 mm, 3 µm
Trap column	DACC column - ChromSpher™ Pi, 3 × 80 mm, 5 µm
Mobile phase	A: Water B: Acetonitrile C: Isopropanol
Flow rate	See gradient (Table 5)
Column temperature	30 °C, forced air mode
Autosampler temperature	35 °C
Autosampler wash solvent	100 % Acetonitrile
Injection volume	500 µL (5 µL for direct injection)
Data collection rate	5 Hz
Response time	1.0 s
Detection	Fluorescence (settings see Table 6)

Chromatography Data System

The Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.3 was used for data acquisition and analysis.

Table 5. Gradient of analytical pump and loading pump and valve switching times

Time [min]	Loading pump				Analytical pump			Valve switching times	
	Flow rate [mL/min]	Solvent A [%]	Solvent B [%]	Solvent C [%]	Flow rate [mL/min]	Solvent A [%]	Solvent B [%]	Valve 2	Valve 1
0.0	0.35	0	0	100	0.80	30	70	1_2	6_1
10.0	0.35	0	0	100	---	---	---	---	---
10.1	0.55	30	70	0	---	---	---	---	---
12.1	---	---	---	---	---	---	---	6_1	---
14.0	---	---	---	---	0.80	30	70	---	---
14.1	---	---	---	---	0.40	30	70	---	---
14.8	---	---	---	---	---	---	---	---	1_2
14.9	---	---	---	---	0.40	30	70	---	---
16.5	---	---	---	---	0.80	30	70	---	---
18.0	---	---	---	---	0.80	30	70	---	6_1
20.9	0.55	30	70	0	---	---	---	---	---
21.0	0.50	0	100	0	---	---	---	---	---
46.0	0.50	0	100	0	0.80	0	100	---	---
46.1	0.50	0	0	100	---	---	---	---	---
50.0	---	---	---	---	---	---	---	1_2	---
51.9	---	---	---	---	0.80	0	100	---	---
52.0	---	---	---	---	1.20	100	0	---	---
55.8	0.50	0	0	100	1.20	100	0	---	---
55.9	0.35	0	0	100	0.80	30	70	---	---
56.0	0.35	0	0	100	0.80	30	70	---	---

Table 6. Settings for the fluorescence detector

Time [min]	Excitation wavelength [nm]	Emission wavelength [nm]	Sensitivity	Lamp mode
10.0	270	385	5	Long Life
30.5	270	385	5	High Power
35.3	290	430	7	High Power
39.0	287	410	6	High Power
45.0	285	398	6	High Power
56.0	Stop acquisition			

Results and discussion

Description of the method

The method used for this application was based on the previously published Application Note 196.⁶ Analog fluidic connections were set up with the Vanquish Core HPLC system, and only minor changes in the valve switching times, column dimensions and particle size, and flow rates were applied. To shorten the runtime, shorter columns with smaller particle size and dimensions were used. At the same time the flow rates of the analytical columns were reduced.

Figure 3 illustrates the different method stages. During the initial step, the loading pump is used to transfer the sample to the DACC column to remove the oil matrix and concentrate the analytes. At the same time, the analytical column is equilibrated (Figure 3A). In the second step, the upper valve is switched to remove the isopropanol from the DACC column with a mixture of water/ACN (30/70) in reversed flow (Figure 3B). When the solvent exchange is complete, the lower valve is switched to allow elution of the analytes onto the analytical column (Figure 3C). After completion of the analyte transfer, the DACC column is switched out of the analytical flow path, washed with pure ACN, and reconditioned with isopropanol. The analytical separation is carried out simultaneously (Figure 3A).

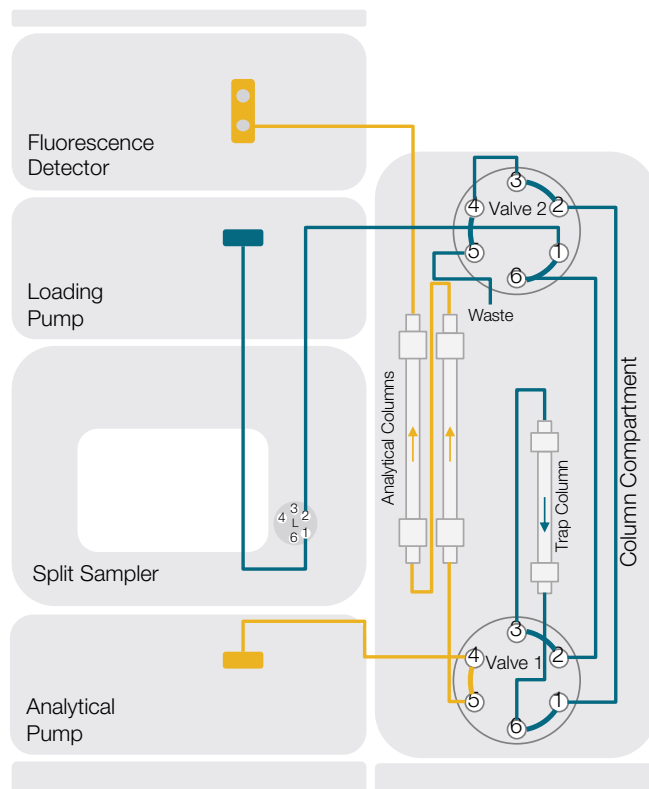


Figure 3B. Backflush the trap column with the loading pump to remove isopropanol; equilibration of the analytical column by the analytical pump

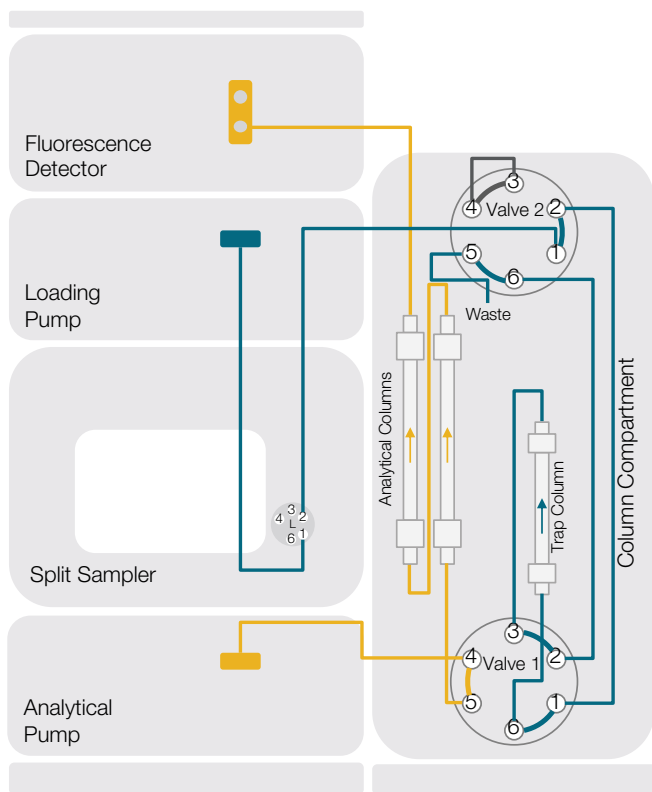


Figure 3A. Injection of the sample on the trap-column via the autosampler and loading pump; equilibration of the analytical column with the analytical pump

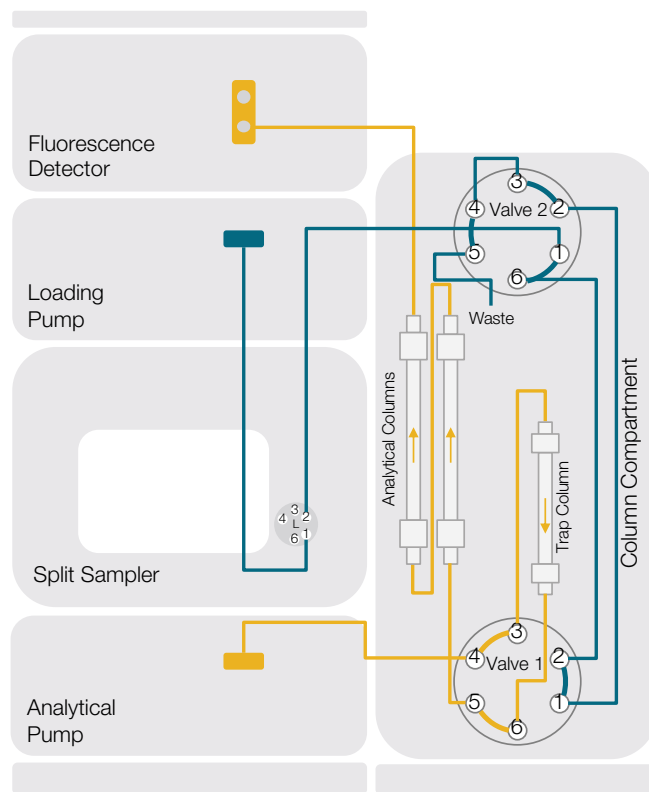


Figure 3C. The trap column is switched into the analytical flow path and the PAHs are eluted onto the analytical columns.

Determination/optimization of the FLD parameters

The settings for the fluorescence detector were optimized by performing emission and excitation scans as described in the Fluorescence Method Development Handbook.⁸ This is necessary because optimal emission and excitation wavelengths can vary between the different PAH. For this the capability of the fluorescence detector to perform online 3D scan experiments was used. In short, to determine the optimal emission wavelength, the excitation wavelength is kept constant and a defined emission wavelength range is scanned. The experimental determination was carried out by direct injections on the analytical columns (replumbing required). The resulting wavelength switching times were applied to the final method, which included the trapping step (Table 6).

Interference of adjacent peaks

Referring to the customers' requirements, the method was tested for potential interferences of the PAH4 with benzo[e]pyrene, perylene, benzo[k]fluoranthene, and benzo[a]fluoranthene.⁷ The black chromatogram in Figure 4 clearly shows that benzo[e]pyrene and benzo[k]fluoranthene are well separated. Only with perylene and benzo[b]fluoranthene co-elution can be seen (blue chromatogram). Since the response of perylene is only 3% of that of benzo[b]fluoranthene with the chosen detection parameters at the same concentration level, the interference is negligible. With the chosen detection parameters benzo[a]fluoranthene shows no response. Thus, it appears that no interference of the above listed analytes with the PAH4 was determined, proving the suitability of the chromatographic separation and detection parameters.

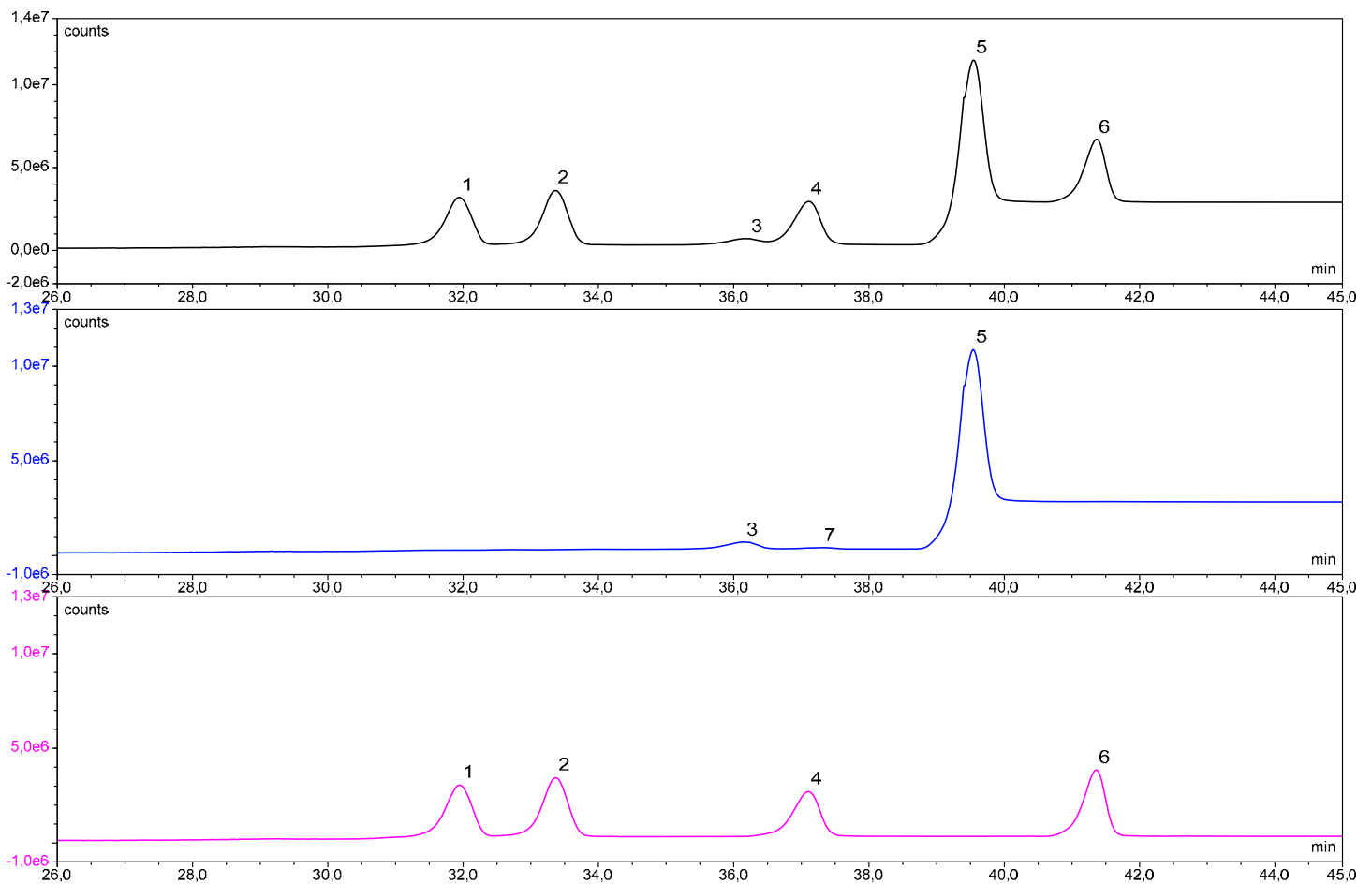


Figure 4. Stacked display of a mixture of all analytes (black chromatogram), a mixture containing the adjacent peaks (blue chromatogram), and a mixture containing the PAH4 (pink chromatogram). 1: benzo[a]anthracene. 2: chrysene: 3: benzo[e]pyrene. 4: benzo[b]fluoranthene. 5: benzo[k]fluoranthene. 6: benzo[a]pyrene. 7: perylene.

Trapping efficiency

The efficiency of the analyte trapping on the DACC column was investigated by a comparison of standard solutions injected to the trapping setup or directly to the analytical column. For the direct injection measurements, the system was replumbed accordingly. To avoid direct injection of large sample volumes, the injection volume was decreased from 500 μL to 5 μL while simultaneously the concentration was increased 100-fold (Table 3). The differences in peak areas between trapping and direct injection were small ($\pm 10\%$), demonstrating efficient trapping of the analytes.

Calibration in analyte free matrix

To correct for matrix influences, the calibration samples were generated with the addition of an analyte-free matrix provided by the customer (Figure 4). Such an analyte-free matrix can be obtained by treatment of an oil with charcoal.^{6,7} The suitability of benzo[*b*]chrysene as an internal standard was evaluated first. Our experiments showed good results for refined oils, since no interference with matrix components was observed. However, with crude oils, a significant increase in the peak area of benzo[*b*]chrysene compared to the calibration standards with matrix was observed. The large interfering peak at the same retention time as the internal standard was also seen in chromatograms of crude oils without added benzo[*b*]chrysene. (Figure 6). Therefore, the calibration for all samples was performed without using the internal standard (Table 7).

Table 7. External calibration data for investigated substances.

Compound	Calibration function	Coefficient of determination	Relative standard deviation
Benzo[<i>a</i>]anthracene	$Y = 352035x + 4907$	0.99923	3.51%
Chrysene	$Y = 416544x + 15938$	0.99905	3.87%
Benzo[<i>b</i>]fluoranthene	$Y = 275295x + 5172$	0.99898	4.04%
Benzo[<i>a</i>]pyrene	$Y = 306577x + 37041$	0.99695	6.76%

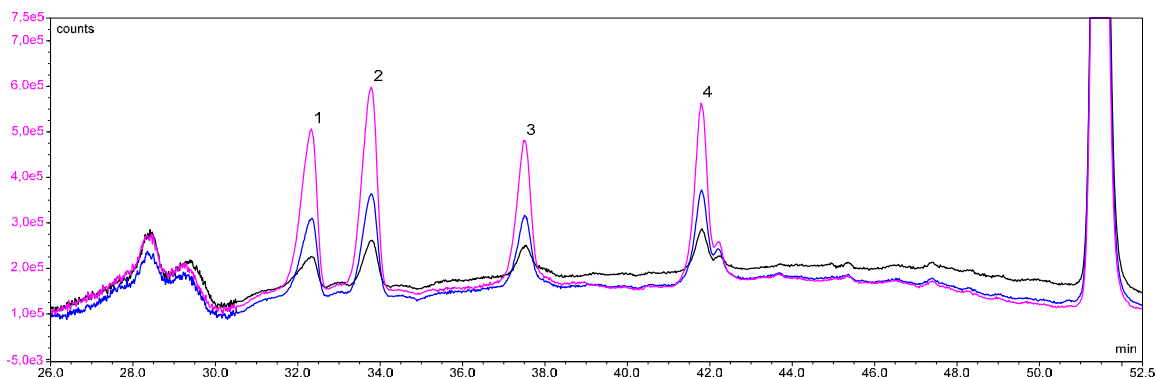


Figure 5. Overlay of the chromatograms of the three matrix calibration standards with the lowest concentrations. No interference with matrix components from the blank matrix can be observed. Black chromatogram: 0.1 $\mu\text{g}/\text{kg}$. Blue chromatogram: 0.25 $\mu\text{g}/\text{kg}$. Pink chromatogram: 0.5 $\mu\text{g}/\text{kg}$. 1: benzo[*a*]anthracene. 2: chrysene. 3: benzo[*b*]fluoranthene. 4: benzo[*a*]pyrene.

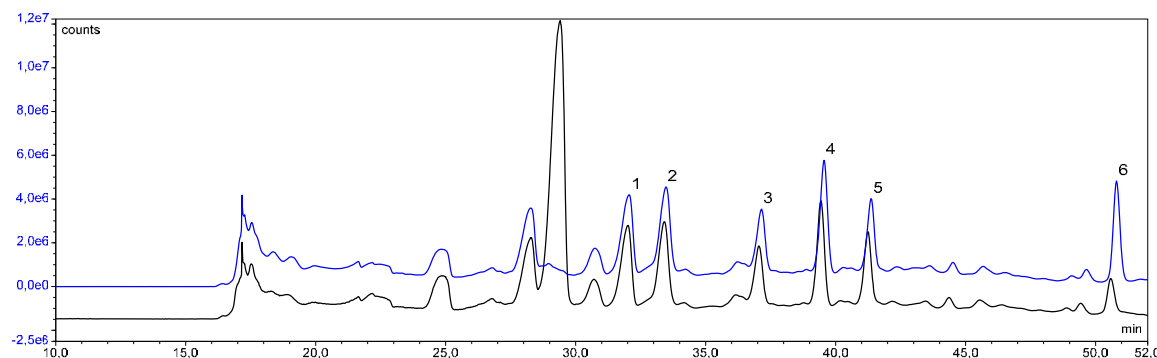


Figure 6. Overlay of the chromatograms of two samples of crude sunflower oil with (blue chromatogram) and without (black chromatogram) addition of internal standard (offset of 10%). 1: benzo[*a*]anthracene. 2: chrysene. 3: benzo[*b*]fluoranthene. 4: benzo[*k*]fluoranthene. 5: benzo[*a*]pyrene. 6: benzo[*b*]chrysene (IS).

Sensitivity

The limit of quantification was estimated by injecting matrix calibration standards with concentrations that correspond to an amount of 0.1 µg of analyte/kg sample (black chromatogram, Figure 5). The peaks of the analytes distinctly stand out from the noise, ensuring proper peak identification and integration. The signal-to-noise ratio (S/N) for all analytes is 19 or greater (Table 8), which is above the limit of quantitation typically defined at a signal-to-noise ratio of 10. The calculation was carried out by comparing the height of the baseline noise from the current blank run with the peak height. By applying higher sensitivity values in the FLD instrument method settings there is still room for improving sensitivity. With increasing the sensitivity setting by one step, the peak-height will increase roughly by a factor of two. Additional ways to further improve sensitivity could be to increase the sample amount (e.g., 2 g/5 mL) or to further increase the injection volume.

Table 8. S/N ratio of PAH standard mixture with analyte free matrix (0.1 µg/kg)

Compound	S/N	Sensitivity (1–8)
Benzo[a]anthracene	20	5
Chrysene	29	5
Benzo[b]fluoranthene	19	7
Benzo[a]pyrene	26	6

Sample analysis

The results of the sample analysis are shown in Table 9. All investigated PAHs could be detected in the crude oils. The same was true for the refined oils, whereas none of the PAH4 were detectable in the synthetic triglyceride (Table 9). In the case of the crude coconut oil, very high contents up to roughly 90 µg PAH/kg oil were determined and were out of the calibration range. The reproducibility was good for all analyzed samples with relative standard deviations below 5%, even in the crude coconut oil where very high contents of PAH were present (overlay of five consecutive injections, Figure 7). The recovery was determined by spiking the samples with an amount of 5 µg/kg for each analyte. The recoveries determined by this approach were between 91 and 131% (Table 10). The carryover performance was investigated by injecting a standard corresponding to a PAH content of 500 µg PAH/kg oil. In the following injection of isopropanol, no peaks were detected (Figure 8). Only for benzo[b]chrysene a small peak was visible. However, this peak equals to a carryover percentage of only 0.07%, demonstrating negligible carryover.

Table 9. Contents of the analyzed samples. Values in parentheses represent the relative standard deviation of the content (n=5).

Sample	Benzo[a]anthracene	Chrysene	Benzo[b]fluoranthene	Benzo[a]pyrene
Sunflower oil crude	4.73 µg/kg (2.4%)	4.32 µg/kg (1.8%)	3.70 µg/kg (1.4%)	3.22 µg/kg (4.4%)
Synthetic triglyceride	n.d.	n.d.	n.d.	n.d.
Coconut oil crude	88.94 µg/kg* (0.6%)	68.96 µg/kg* (0.8%)	21.46 µg/kg* (0.7%)	17.05 µg/kg* (2.8%)
Olive oil extra virgin	0.50 µg/kg (0.7%)	0.66 µg/kg (1.1%)	0.08 µg/kg* (1.9%)	n.d.
Coconut oil native	0.29 µg/kg (3.4%)	0.22 µg/kg (3.4%)	0.09 µg/kg* (2.9%)	0.07 µg/kg* (8.4%)

* content outside of calibration range

Table 10. Spike recovery of all samples

Sample	Benzo[a]anthracene	Chrysene	Benzo[b]fluoranthene	Benzo[a]pyrene
Sunflower oil crude	96.8%	101.8%	100.0%	91.1%
Synthetic triglyceride	96.8%	95.9%	95.3%	95.1%
Coconut oil crude	96.9%*	96.6%*	98.4%*	94.4%*
Olive oil extra virgin	120.0%	121.7%	116.6%	130.6%
Coconut oil native	108.7%	112.6%	106.2%	114.0%

* content outside of calibration range

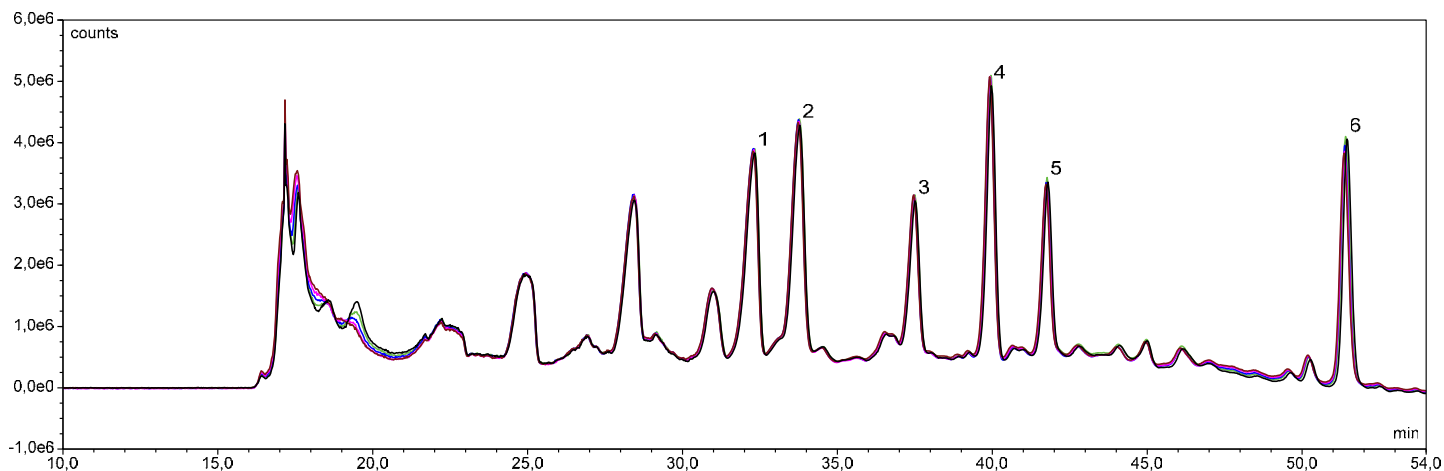


Figure 7. Overlay of chromatograms of five serial injections of the sample sunflower oil crude. 1: benzo[a]anthracene. 2: chrysene: 3: benzo[b]fluoranthene. 4: benzo[k]fluoranthene. 5: benzo[a]pyrene. 6: benzo[b]chrysene.

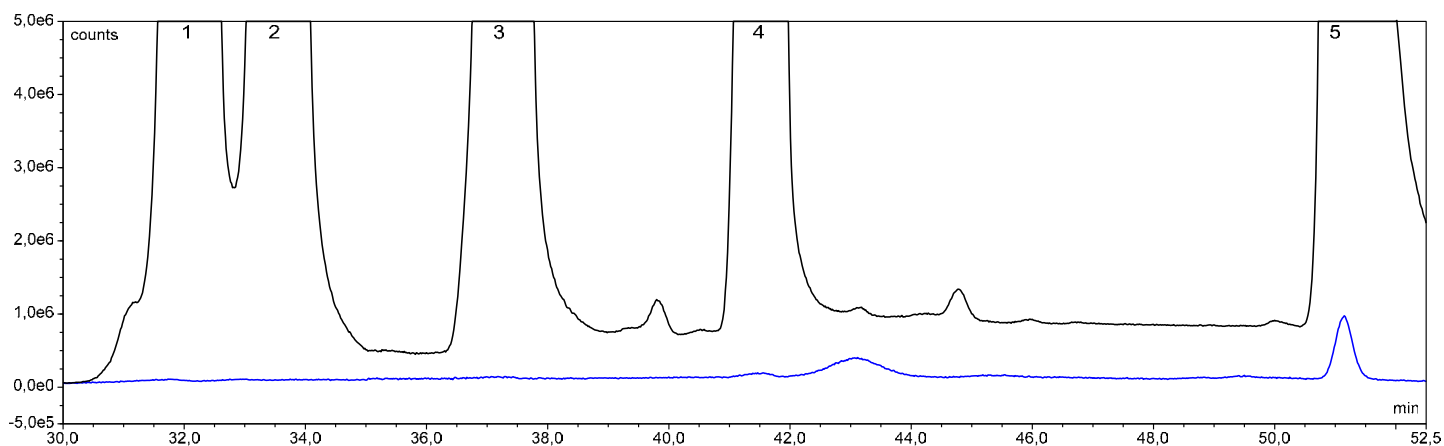


Figure 8. Injection of a standard solution with high concentration (500 µg/kg, black chromatogram), followed by an injection of solvent (blue chromatogram). 1: benzo[a]anthracene. 2: chrysene: 3: benzo[b]fluoranthene. 4: benzo[a]pyrene. 5: benzo[ab]chrysene.

Robustness of the method

In Application Note 196,⁶ a good robustness of the DACC column was observed. During this project, the tested column produced similar results initially and after several hundred injections.

Conclusion

- The presented analysis system enables an automated, reliable, and sensitive determination of the PAH4 in edible oils that are regulated by European Law.
- The use of an online solid phase extraction to concentrate the analytes and remove the matrix eliminates the need for complex sample preparation, which is required by many other methods.

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