Experimental design optimization of chromatographic separation for polycyclic aromatic hydrocarbons in vegetable oils

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HPLC with an electron acceptor stationary phase called tetrachlorophtalimidopropyl (TCP) was used to separate Polycyclic Aromatic Hydrocarbons (PAHs) in vegetable oils without any preparation of the samples being studied. Using an hexane/methylterbutylether mobile phase then an hexane/dichloromethane one, it was possible to separate triglycerids and tocopherols from PAH fraction. A fractional factorial design 2^{7-4} was used to perform the fractionating then the separation of the PAH fraction with the minimum of experiments. Seven factors were examined and the chromatography quality was evaluated through four answers. Finally, the predictions of the models and the characteristics of the separation were compared: the agreement was very good.

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are found everywhere (atmosphere, water, food...). Their dangerous impact on human health and environment has not to be demonstrated. It requires the implementation of reliable trace analysis methods in a wide variety of media. Strict regulations exist for food products. Control organisms such as DGCCRF (Direction Régionale de la Concurrence et de la Répression des Fraudes, France) have thus to test a wide variety of samples. In particular, vegetable oils and fats have been widely investigated. In these matrices, PAHs occurence may arise from atmospheric pollution, soils contamination, oilseeds drying procedure (old furnaces for example or gases) or contamination through extraction solvents.

The determination of PAHs in lipids is difficult because of the low concentration of individual PAHs ($\mu g.kg^{-1}$). Furthermore, complex extraction and clean-up procedures are required to isolate the PAHs from the oil or food components [1-7]. High performance liquid chromatography (HPLC) with fluorescence detection is an appropriate technique to determine the amount of PAHs as it enables good resolution of the compounds and trace evaluation.

The PAHs extraction procedure currently used has several drawbacks [8-9]:

– It requires large amounts of solvents (several liquid-liquid extractions), with formation of emulsions;

– some of those solvents are toxic;

– it is time consuming;

– several clean-up procedures are necessary;

– PAHs are only partly recovered due to the numerous experimental steps.

Finally, the chromatographic procedure uses an octadecyl stationary phase with hydroorganic eluents (mixture acetonitrile/water or methanol/water) which are poor solvents for PAHs.

We propose an alternative chromatographic method with an electron acceptor stationary phase of tetrachlorophtalimidopropyl (TCP) modified silica synthesized in the laboratory [10]. The mobile phases are mixtures of organic solvents: hexane/methylene chloride, methyltertiobutylether (MTBE), tetrahydrofuran (THF), etc., which are good solvents of PAHs. Such a system enables direct injection of oil on the chromatographic column avoiding the extraction procedure, losses during concentration steps and thus saves time and solvent. The composition of oil is relatively complex as it mainly contains triglycerids (98 %) and tocopherols, which are among the 2 % minor compounds. The chromatographic procedure implicates isolation of the PAHs from those major constituents of oil, followed by their separation.

The aim of this study is to optimize the experimental procedure of this complex chromatography while performing the minimum of experiments thanks to an experimental fractional factorial design.

Material and method

HPLC-grade hexane and methylene chloride were purchased from Scharlau (Barcelona, Spain). Methyltertiobutylether (MTBE) was purchased from Aldrich (Steinheim, Germany). PAHs standards were obtained from Supelco (Bellefonte,

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PA, USA). Vegetable oils come from ITERG (Pessac, France).

Chromatographic studies were performed with a modular HPLC apparatus consisting in a PU-980 Model gradient pump module (Jasco), and two detectors: a UV–975, UV-Visible detector (Jasco) first, and a RI 1530 refractometer (Jasco) second.

The stationary phase was synthetized in the laboratory according to a method previously described [10]. The column dimensions were: 250 × 4.6 mm. Silica was Kromasil 100 Å, 5 µm (Eka Nobel, Bohus Sweden).

Normal phase conditions using Hexane/CH_2Cl_2 and Hexane/MTBE mixtures were used.

The method requires a double detection:

– a refractometer for detection of triglycerides which do not absorb UV light;

– a UV detector for tocopherols and PAHs. Wavelenght detection was 290 nm for tocopherols and 254 nm for PAHs. The change in wavelength occurred after the tocopherol peak (at 10 min).

Oil was diluted in hexane in order to lower the injected sample viscosity. The maximum available quantity that can be injected without saturation of the analytical column was then determined and used later on. We injected 50 mg of soja oil using a 100 µL injection loop. Oil was doped with seven PAH among the most toxic ones as described in NF T90 115 Norm:

1 : Phenanthrene
2 : Benzo(a)anthracene
3 : Benzo(k)fluoranthene
4 : Dibenzo(a,h)anthracene
5 : Benzo(b)fluoranthene
6 : Benzo(a)pyrene
7 : Benzo(ghi)perylene

The chromatographic procedure has to start with preliminary elution of triglycerids and tocopherols before PAHs elution. Indeed, Hexane/MTBE mixture is a good candidate as MTBE has the same polarity as CH_2Cl_2. Furthermore, it hinders hydrogen bonds between the residual silanols of silica (stationary phase) and triglycerids as well as tocopherols. It makes possible the elution of these compounds earlier than with methylene chloride and their separation from the PAHs fraction. It was necessary to optimize this preliminary chromatography and to check the separation of triglycerid and tocopherols from the PAHs. In this aim we used phenanthrene which is not considered as one of the major toxic PAH but which is known to be eluted at first. Thus we used it as a marker towards tocopherols.

A hexane/CH_2Cl_2 mixture associated with a gradient elution method was set up next in order to separate the investigated PAHs. Trace analysis implies fluorimetric detector use. The optimisation of the experimental procedure described in this work is not compatible with fluorimetric detection which requires wavelength programmation that is not possible when elution times are unknown.

Optimization

The chromatographic separation quality has been evaluated through 4 answers:

– Y_1 : analytical duration measured by the elution time of benzo(ghi)perylene (to be minimized);

– Y_2 : elution time difference between tocopherol and phenanthrene peaks (to be maximized);

– Y_3 : global resolution depending on the average peaks half band width (to be minimized);

– Y_4 : time separation between benzo(b)fluoranthene and benzo(k)fluoranthene pics (to be maximized).

We investigated the influence of seven factors $X_{1\to7}$ characteristic of the experimental procedure described below (Fig. 1).

The oil injection is followed by elution with a mixture of Hexane/MTBE in order to eliminate tocopherols:

– 1st factor: X_1 is the MTBE concentration;

– 2nd factor: X_2 is the duration of this elution.

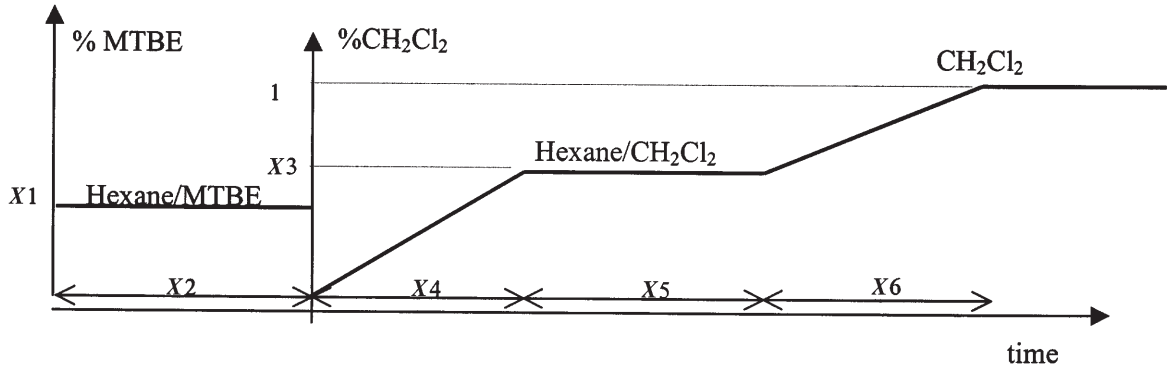

Figure 1. Experimental procedure diagram.

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Table I. Experimental domain of the seven factors.

Factors	*low level : –*	*high level : +*
MTBE % in Hexane : X_1	25 %	45 %
hexane/MTBE plateau duration (min) : X_2	17	25
CH_2Cl_2 v/v at the end of gradient : X_3	20 %	50 %
1st hexane/CH_2Cl_2 gradient duration (min) : X_4	5	15
hexane/ CH_2Cl_2 plateau duration (min) : X_5	0	10
2nd hexane/CH_2Cl_2 gradient duration (min) : X_6	15	25
Pump flow rate (mL.min^{-1}) : X_7	1	1.5

Table II. Fractional factorial 2^{7-4} design and experimental results: experiments 9 and 10 are replicates of 3 and 8; 10 and 12 are quasi-central experiments; the last line of the table is the calculated quasi-central result.

exp	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Y_1	Y_2	Y_3	Y_4
1	–	–	–	+	+	+	–	91.78	23.56	0.39	2.73
2	+	–	–	–	–	+	+	55.90	10.32	0.453	2.42
3	–	+	–	–	+	–	+	69.76	14.43	0.451	2.02
9	–	+	–	–	+	–	+	70.46	15.01	0.400	2.04
4	+	+	–	+	–	–	–	83.47	16.23	0.611	2.23
5	–	–	+	+	–	–	+	58.85	15.12	0.325	2.15
6	+	–	+	–	+	–	–	68.88	15.84	0.578	3.25
7	–	+	+	–	–	+	–	75.71	22.49	0.386	2.72
8	+	+	+	+	+	+	+	74.66	10.12	0.623	3.45
10	+	+	+	+	+	+	+	77.28	10.76	0.720	3.81
11	0	0	0	0	0	0	–	78.35	18.37	0.396	2.77
12	0	0	0	0	0	0	–	78.07	17.11	0.440	2.76
Calculation	*0*	*0*	*0*	*0*	*0*	*0*	–	*79.38*	*18.93*	*0.467*	*2.74*

The PAH elution phase starts with an increasing concentration gradient in CH_2Cl_2 described by:

– 3rd factor: X_3 is the CH_2Cl_2 concentration at the end of the gradient phase;

– 4th factor: X_4 is the gradient phase duration.

A plateau at concentration X_3 is then applied.

– 5th factor: X_5: plateau duration.

The plateau is followed by a second gradient, which drives to pure dichloromethane.

– 6th factor: X_6 gradient duration;

– 7th factor: X_7 is the pump flow rate.

The various factors have been fixed at two levels as indicated in table I.

The first aim was, due to the time constraint, to drastically reduce the number of experiments. Thus, we chose a fractional factorial design 2^{7-4} with 8 experiments. The four independent generators are: $I \equiv X_1X_2X_4 \equiv X_1X_3X_5 \equiv X_2X_3X_6 \equiv X_1X_2X_3X_7$,. Due to a modification of the experimental domain during the factorial design experimentation in order to restrain to the linear range for the answers, only poor statistical investigation with four degrees of freedom has been carried out: only one replicate of experiments 3 and 8 (exp 9 and 10) and two quasi-central experiments have been done (exp 11, 12). The experiments and their results are presented in table II. They have been performed in a random order.

Such a design only allows the estimation of the first order influences of the factors: $b(X_{1\to7})$. With NEMROD software [11], we obtained these effects on the four answers with their standard deviations and R^2 values as described in table III.

Interpretation

In order to determine the optimal experimental settings, we minimized mathematically the calculated answer: Y_1Y_3/Y_2Y_4. We obtained a minimum and thus an optimal

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Table III. First order effects of the seven factors on the four answers: Analytical duration: Y_1, Phenanthrene retention time: Y_2, Resolution: Y_3, Benzo(k), benzo(b) fluoranthene separation: Y_4. The significant terms are written in bold type.

	Effect									
Answer	*average*	*b(X_1)*	*b(X_2)*	*b(X_3)*	*b(X_4)*	*b(X_5)*	*b(X_6)*	*b(X_7)*	*R2*	*σ(b)*
Y_1	**72.3**	**–1.53**	**3.73**	**–2.73**	**4.93**	**4.101**	**2.26**	**–7.08**	0.99	0.46
Y_2	**15.79**	**–2.88**	–0.12	–0.12	0.25	0.05	0.61	**–3.14**	0.97	0.38
Y_3	**0.468**	**0.098**	0.043	0.01	0.019	0.036	–0.005	0.001	0.91	0.02
Y_4	**2.65**	**0.237**	0.007	**0.292**	**0.04**	**0.265**	**0.23**	**–0.093**	0.98	0.002

– % MTBE : X_1 = 25%
– hexane/MTBE plateau : $X2$ = 17 min
– hexane/CH_2Cl_2 gradient :
X_4 = 5 min, X_5 = 0, X_6 = 25 min
– pump flow rate : X_7 = 1.13 mL.min^{-1}

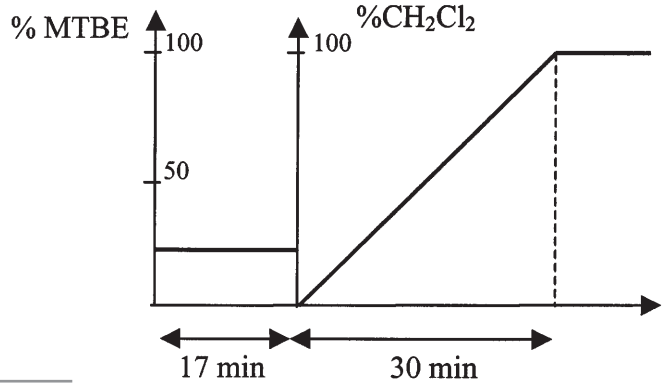

Figure 2. Optimal settings diagram.

chromatographic separation of the PAHs with the settings described in figure 2. Best results are obtained with the shortest preliminary Hexane/MTBE plateau at the lowest MTBE concentration. Only one gradient Hexane/CH_2Cl_2 is then applied during 30 min. This chromatogram is performed at low pum flow rate of 1.13 mL.min^{-1}

The validity of such results is supported by the large values of the R^2 coefficient of the regression which give good indication of the quality of the modelisation. The experimental variance has been estimated thanks to the two experiments at the centre of the experimental domain and the two replicates of experiments 3 and 8. The resulting standard deviation on the answers is indicated in table IV.

We checked the adequation between the experiments and the predicted values of the answers in two cases:

– In the central point, the differences between the calculated and experimental values as shown in the last line of table II are non-significant. This makes evident the linearity of the model.

– Furthermore, in order to check the previsional quality of the model and the quality of the separation we tried a validation experimental test using the optimized settings (Fig. 3 and Tab. IV). The model reproduces experiment because the gap between experimental results and calculated ones is non-significant.

Table IV. Calculated and experimental properties of the optimised oil chromatogram comparison.

	Calculated answer	*Experiment*	*σ*
analytical duration (min)	71.4	69.0	1.4
phenanthrene retention time (min)	21.2	19.8	1.17
peaks average half width (min)	0.25	0.25	0.06
benzo(b)/benzo(k)fluoranthene distance(min)	2.0	2.3	0.01

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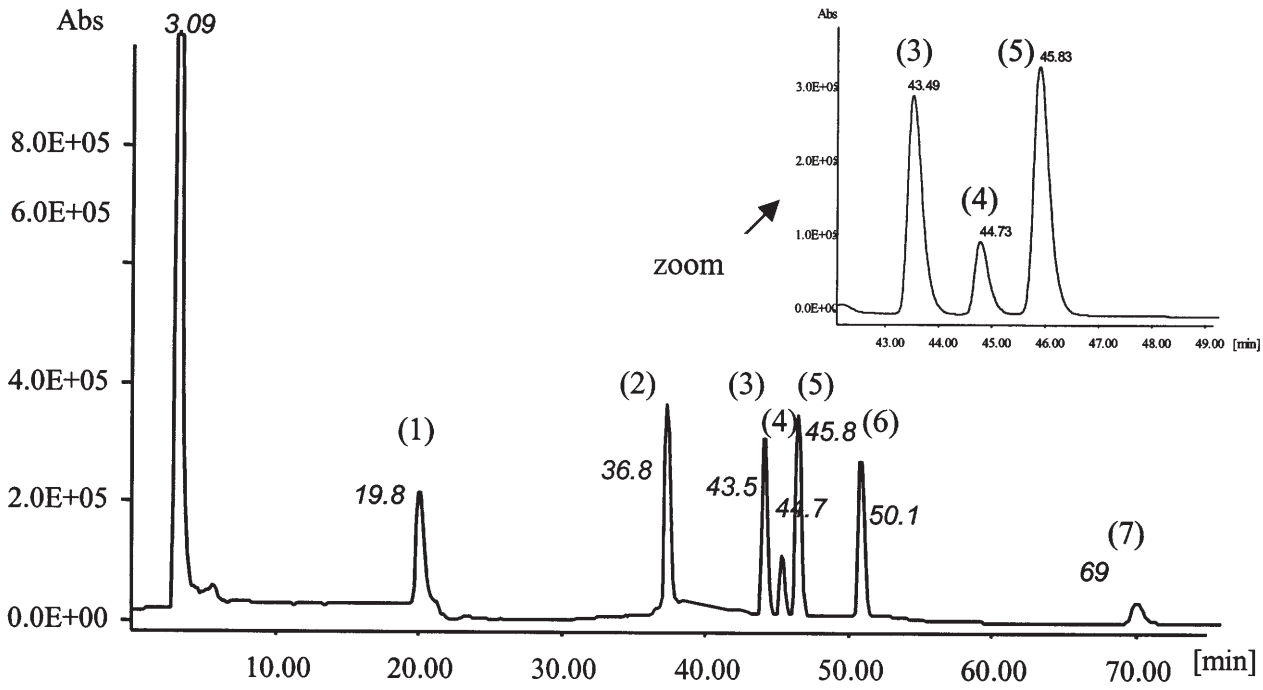

Figure 3. Oil chromatogram with optimized settings.

Conclusion

Thanks to a eight experiments fractional factorial design, we defined a satisfactory experimental procedure of a complex chromatography. These settings are the optimal ones, only in the investigated domain and are probably not the absolute optimum. We demonstrated the possibility of isolating, then separating six of the most toxic PAHs in a short analysis time (1 h 10 min), with direct oil injection on a silica-TCP column. The next step of this study is quantification, which requires a larger column, larger injection volume and fluorimetric detection due to the low concentration of the aromatic hydrocarbons.

References

1. Hopia, A.; Pyysal, H.; Wickström, K. *J. Am. Oil Chem. Soc.* **1986,** *63,* 889.
2. Larsson, B.K.; Eriksson, A.T.; Cervenka, M. *J. Amer. Oil Chem. Soc.* **1987,** *64,* 265.
3. Lawrence, J.F. *Int. J. Environ. Anal. Chem.* **1986,** *24,* 789.
4. Lawrence, J.F.; Weber, D.F. *J. Agric. Food Chem.* **1984**, *32*, 789.
5. Sagredos, A.N.; Shina Roy, D.; von Jan, E. *Fat Sci. Technol.* **1988**, *90*, 76.
6. Speer, K.; Montag, A. *Fat Sci. Technol.* **1988**, *90*, 163.
7. Welling, P.; Kaandorp, B. *Z. Lebennsm. Unters. Forsch* **1986**, *183*, 111.
8. Perrin, J.L.; Poirot, N.; Liska, P.; Thienpont, A.; Felix, G. *Fat Sciences Technology* **1993**, *95*, 46.
9. Thienpont, A.; Felix, G.; Perrin, J.L.; Liska, A. *Polycyclic Aromatic Compounds*, Synthesis, Properties, Analytical Measurements, Occurrence and Biological Effects **1993**, *3* (supp.), 337.
10. Felix, G.; Thienpont, A.; Dentraygues, P. *Chromatographia* **1992**, *34*, 177.
11. Mathieu, D.; Phan-Tan-Luu, R. NEMROD Software; Marseille: LPRAI.

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