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Jacqueline Claire Agnes van Der Wielen-Hustinx, John Jansen, Martijn J. Martena, Henk de Groot, Paul In T Veld

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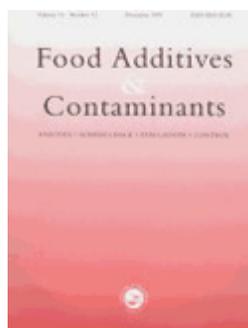
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## Determination of the level of Benzo[a]pyrene in fatty foods and food supplements

J.C.A. van der Wielen\*, J.T.A. Jansen, M.J. Martena, H.N. de Groot and P.H. in 't Veld

Food and Consumer Product Safety Authority (VWA) – Region South,  
V. Montgomerylaan 500 / PO Box 2168  
5600 CD Eindhoven, The Netherlands.

\* corresponding author - jacqueline.van.der.wielen@vwa.nl

A routine method was developed for the quantification of benzo[a]pyrene (BaP) in edible oils and food supplements. BaP is often taken as an indicator of the presence of polycyclic aromatic hydrocarbons. The method consists of on-line LC-clean up followed by injection to an HPLC-system with fluorescence detection. The method has good performance characteristics and gave good results in proficiency tests. From 2002 to 2004 about 1350 samples of oils and food supplements, were analysed using this method to test the level of benzo[a]pyrene. About 20% of the edible oils contained more than 1.2 µg/kg BaP (which is the limit applied by the Dutch Food and Consumer Product Safety Authority until 1 April 2005, and includes measurement uncertainty). In the case of food supplements more than 30% contained too high levels of BaP, ranging from 1.2 to 135 µg/kg.

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2  
3 **Key-Words:** BaP, routine LC-method, fluorescence detection, validation,  
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6 quantification, oils, food supplements.  
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## 10 **Introduction**

11  
12 Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental  
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14 contaminants, a number of which are considered to be human carcinogens. In  
15  
16 foodstuffs, PAHs can be formed directly as a result of some heat processes (charcoal  
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18 grilling, roasting, smoke drying) or by addition of smoke flavouring additives (Garcia-  
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20 Falcon et al. 2000; Simko 2002; Barranco et al. 2003). It has been estimated that  
21  
22 human intake of PAHs from food is considerably higher than that from air or drinking  
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24 water, edible oils and fats being the most significant sources because of their  
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26 lipophilic nature (Garcia-Falcon et al. 2000; Barranco et al. 2003). PAHs can be  
27  
28 removed from edible oils by treatment with activated charcoal, a practice that has  
29  
30 been widely used for a long time (Weisshaar 2002). More than 100 PAHs have been  
31  
32 found in nature; however, only 16 have been selected as priority pollutants by the  
33  
34 United States Environmental Protection Agency (EPA) on the basis of occurrence  
35  
36 and carcinogenicity (Barranco et al. 2003; Teixeira et al. 2003). In Commission  
37  
38 Recommendation 2005/108/EC, the Commission of the European Communities  
39  
40 (2005) also selected 15 priority PAHs, based on their carcinogenicity (some being  
41  
42 different from the EPA PAHs). At a special workshop organised jointly by the  
43  
44 European Commission (Directorate Joint Research Centre and Directorate Health  
45  
46 and Consumer Protection) and the European Food Safety Authority (EFSA), it was  
47  
48 decided to add one more PAH (benzo[c]fluorene) to this list, the later also  
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50 recommended by JECFA.  
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3 Benzo[a]pyrene is often used as an indicator of the presence of PAHs in water and  
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5 foodstuffs, even though it is well known that this compound is usually generated  
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7 together with many other PAHs which contaminate the food [Garcia-Falcon et al.  
8  
9 2000, Simko 2002, Bogusz et al. 2004). The Scientific Committee on Food (SCF  
10  
11 2002) concluded that BaP could be used as a marker for the occurrence and effect of  
12  
13 carcinogenic PAH in food. However further analysis of the relative proportions of  
14  
15 these 16 PAHs (15 EU + 1 JECFA) in foods is necessary, to review the suitability of  
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17 maintaining BaP as a marker (SCF 2002, Commission of the European Communities  
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19 2005).  
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27 The International Olive Oil Council recommended in 2001 a value of 2 µg/kg as a  
28  
29 maximum tolerable concentration of BaP as well as other PAHs in olive-pomace oil  
30  
31 (International Olive Oil Council 2001). It is generally recognised that total PAHs in a  
32  
33 refined oil or fat should not exceed 25 µg/kg, of which not more than 5 µg/kg should  
34  
35 be heavy PAHs (5- and 6-nuclear PAHs) (Fediol 2003). The SCF recommended, in  
36  
37 their opinion of 2002, that exposure to PAHs from food should be as low as  
38  
39 reasonably achievable (ALARA principle), due to the carcinogenicity and  
40  
41 genotoxicity, the most critical effects of PAHs. Prior to April 2005 the majority of  
42  
43 European countries set independently limits . The Dutch Food and Consumer  
44  
45 Product Safety Authority (VWA) had set a limit of 1 µg/kg for BaP in oils and food  
46  
47 supplements in 2002. Based on the ALARA principle a level of 1 µg/kg was used, as  
48  
49 the presence of BaP is avoidable, due to processing steps which can reduce the  
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51 content of PAHs. Based on a report by Kroese et al. (2001), which set the virtual safe  
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53 dose at 0.5 ng/kg bodyweight, an Acceptable Daily Intake (ADI) was calculated  
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55 based on an average of people weighing 60 kg. This ADI of 30 ng was taken as a  
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3 threshold above which additional measures were required in order to minimise the  
4 risk to public health. Due to the fact that several member states of the EU have set  
5 their own legal limits, the Commission of the European Communities (2005) issued a  
6 Regulation (No 208/2005) amending Regulation (EC) No 466/2001 with regard to  
7 polycyclic aromatic hydrocarbons. This regulation sets the maximum level for BaP at  
8 2 µg/kg for oils and fats for human consumption as well for those used as ingredients  
9 in foods and food supplements. In order to obtain more information about the  
10 patterns of PAH distributions (profiles) relative to BaP in various foods, the  
11 Commission recommended that monitoring studies be carried out on 16 PAHs (15  
12 EU and 1 JECFA) in various foods . This dataset can then be used to evaluate if  
13 maximum levels should be set for other PAHs and the accuracy of using BaP as a  
14 marker for all PAHs.  
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34 The principal problems associated with the determination of BaP in foods are the low  
35 detection levels (µg/kg) and the diversity of potential interferences present in food  
36 and food supplements (Garcia-Falcon et al. 2000). Although all sample preparation  
37 steps are important, chromatographic separation is considered as most significant for  
38 correct results (Simko 2002). There are several HPLC methods for the determination  
39 of PAH in edible oils, using on-line sample clean-up followed by injecting to an  
40 analytical C18-column and fluorescence detection (Garcia-Falcon et al. 2000, Moret  
41 and Conte 2002, Bogusz et al. 2004). Fluorescence detection is often used due to its  
42 selectivity and sensitivity (Barranco et al. 2003). GC-MS methods as well as HPLC  
43 methods are regarded as complementary in the analysis of PAHs (Bogusz et al  
44 2004).  
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3 The objectives of this study were (1) to develop a routine method for the  
4 determination of BaP and (2) to perform a monitoring study on edible oils and oil-  
5 based food supplements. The development of the method was based on the  
6 methodology of Stijn et al. (1996), in which PAHs were under investigation. In this  
7 method, a donor-acceptor complex chromatography (DACC) technique was been  
8 used for the on-line sample clean-up. PAHs are electron donors ( $\pi$ -electrons) and  
9 the strong interaction of the PAHs with an electron acceptor stationary phase results  
10 in retention of the PAHs and elution of (the bulk of) the other compounds in the oil.  
11 By coupling the DACC-column automated to the analytical column for separation of  
12 the PAHs (a LC-LC coupling), the method can be applied easily for routine analysis.  
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29 In 2002 a monitoring study was initiated to investigate the amounts of BaP in olive  
30 oils, of which several rapid alerts (a notification of a direct or indirect risk to human  
31 health deriving from Food or Feed, for which EU has set up a system) were reported.  
32 Soon it was decided to broaden the monitoring study to other kind of edible oils, for  
33 example corn oil and sunflower oil as well as Evening Primrose oil (*Oenothera*  
34 *biennes*). This latter oil is often used as an ingredient for food supplements, e.g. for  
35 fat burners. Because it was found that "special oils", which are being used as a raw  
36 material for food supplements quite often exceeded the limit of 1.2  $\mu\text{g}/\text{kg}$  BaP (the  
37 Dutch limit including measurement uncertainty) the research was broadened to  
38 further analyse food supplements. The food supplements of interest were oil  
39 capsules, e.g. fat burners, vitamin supplements.  
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## 58 **Method**

### 59 *Experimental Part*

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3 The method reported here is based on donor-acceptor complex chromatography  
4 (DACC) (Stijn et al. 1996). First, the samples were homogenized, then 10 ml  
5 isopropyl alcohol and 200 µl benzo[*b*]chrysene (10 ng/ml, Dr. Ehrenstorfer) were  
6 added to 500 mg of sample. The PAHs were extracted from the sample by shaking  
7 for 30 min. Benzo[*b*]chrysene was added for quality control purposes, to check the  
8 stability of retention times and recovery - it was not used as an internal standard for  
9 the correction of recovery. After filtration, the extract was injected on a DACC column  
10 (Chromospher-phi 7µm 80 mm). The DACC column was connected to an HPLC  
11 system (Agilent) with a switch system. The DACC column was first washed with  
12 isopropyl alcohol, then the PAHs are eluted from the column with 30% acetonitril/70%  
13 ethylacetate and finally the DACC column was cleaned with isopropyl alcohol. Only  
14 the eluted PAHs were injected onto the HPLC column by the switch system. The  
15 analytical column was a reversed phase column (Lichrocart 250-4 Lichrospher PAH  
16 RP-18 5 µm, 2\* in series), held at 30 °C. The HPLC conditions are listed in table I.  
17 Detection of the eluted PAHs was carried out by application of a variable wavelength  
18 fluorescence detector (Agilent). Excitation and emission wavelengths are given in  
19 Table II. A standard mix of PAHs was applied by Dr. Ehrenstorfer (PAH Mix 9, 10  
20 ng/µl of each PAH in acetonitril). This standard mixture contains the 16 EPA PAHs,  
21 however, not all 16 EPA PAHs can be detected with the method.  
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50 **[INSERT TABLE I ABOUT HERE]**

51 **[INSERT TABLE II ABOUT HERE]**

#### 52 *Sample collection*

53 All samples were collected by inspectors of the Dutch Food and Consumer Product  
54 Safety Authority (VWA). Several kinds of oils for human consumption (e.g. sunflower  
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3 oil, olive oil) were taken from shops (e.g. supermarkets) for analysis. When these  
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5 samples appeared to be above the limit, samples of the same charge and also of  
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7 other charges were collected at producers and/or importers before actions, like  
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9 discarding a whole charge from the market, were taken. All food supplements  
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11 collected were supplements on an oil base (oil capsules). For these samples the  
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13 same strategy was followed as for the oil samples; thus if a food supplement from a  
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15 shop appeared to be above the limit, same and other charges at producers or  
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17 importers side were collected and analysed.  
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## 24 **Results and Discussion**

### 25 *Method characteristics*

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27 The method of Stijn et al. (1996) was optimised giving special attention to  
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29 fluorescence detection. The following parameters were optimised: injection volume of  
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31 samples on DACC-column, back flush of the components of the DACC-column,  
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33 removal of isopropylalcohol from DACC-column before injection on the analytical  
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35 column, the level of concentration of acetonitrile necessary to strip off the PAHs from  
36  
37 the DACC column, column temperature of the analytical column, gradients of the  
38  
39 analytical column and wavelengths of the fluorescence detection. In addition,  
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41 benzo[*b*]chrysene was added to each sample for quality control purposes. If the  
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43 column switching system does not work correctly, retention times change and/or  
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45 there is no evidence of benzo[*b*]chrysene.  
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55 The method of Stijn et al. (1996) differs from the developed method in the injection  
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57 volume (250 µl compared to 100µl), column temperature of the HPLC-columns (20°C  
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59 compared to 30°C), the excitation and emission wavelengths of the fluorescence  
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3 detection of the PAHs and the back flush of the PAHs from the DACC column. The  
4 method of Stijn et al. (1996) uses both water/acetonitrile (15%/85%) and  
5 acetonitrile/ethyl acetate (30%/70%) to strip the PAHs off the DACC column, while in  
6 this method the PAHs were stripped off the DACC column by only 30% acetonitrile /  
7 70% ethyl acetate. The method of Stijn et al. (1996) shows peak identification of 17  
8 PAHs. Applying the method described in this study, 12 PAHs of the 16 EPA PAHs  
9 were identified,. 7 of these 12 identified PAHs are also mentioned in the list of the 15  
10 'priority PAHs' by the SCF. Figure 1 shows an example of a chromatogram of a  
11 standard containing 12 PAHs. Because BaP was considered a key component, the  
12 method has been especially validated for this component, however the other PAHs  
13 identified here, can also be quantified.  
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32 **[INSERT FIGURE 1 ABOUT HERE]**  
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36 For the in-house validation of the method, first the limit of detection and quantification  
37 (LOD and LOQ) were determined. The LOD was defined as the concentration which  
38 is three times higher than the standard deviation of the chromatographic background.  
39 The limit of detection determined in this way was 0.07 µg/kg. The limit of  
40 quantification has been defined as twice the limit of detection and was thus 0.14  
41 µg/kg. The calibration curve showed linearity with a correlation coefficient of at least  
42 0.9995 in the range of 0.14 up to 8.0 µg/kg. Therefore the range of application has  
43 been determined as 0.14 to 8.0 µg/kg. Samples containing higher values of BaP  
44 have to be diluted with isopropyl alcohol. The validity of the calibration was checked  
45 with each series of samples. The recovery of BaP from a spiked blank sunflower  
46 sample (determined from 6 replicate measurements under repeatability conditions)  
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3 was 99.9% with a relative standard deviation (RSD) of 2.8%. The reproducibility,  
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5 determined with different operators and different intervals of time and using a  
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7 standardised control material (SCM, sunflower oil) at a level of 1 µg/kg BaP was 0.18  
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9 µg/kg. The measurement uncertainty at the level of 1 µg/kg was established at 0.2  
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11 µg/kg. The recovery and the RSD were also determined under repeatability  
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13 conditions in a herbal food supplement (oil capsules), where they were 100.1% and  
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15 4.1% respectively. The reproducibility of using a food supplement at a level of 1.4  
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17 µg/kg was 0.40 µg/kg, slightly higher than for oil. The method performed well in  
18  
19 proficiency tests with oil samples (Central Science Laboratory 2003, FAPAS  
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21 Proficiency Testing 2004). The Z-scores obtained applying this method in these  
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23 proficiency tests were -1.3 respectively +0.8 for benzo[a]pyrene.  
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#### 34 *Samples analysed*

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36 For every series of samples the analytical protocol included several quality control  
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38 (QC) measurements, e.g. check of validity of calibration, and the analysis of QC  
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40 samples. A reference material (oil) was used as QC material for oils, whereas a  
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42 herbal food supplement in form of oil capsules was applied for the measurements of  
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44 food supplements. The mean values and the 95% confidence interval of these QC  
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46 materials are  $2.21 \pm 0.27$  and  $1.28 \pm 0.30$  respectively. The results of analysis of the  
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48 QC samples had to be within these limits. When all quality checks for a series of  
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50 samples are in line with the performance expected, samples can be evaluated on  
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52 their level of BaP.  
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58 Ten oil samples, taken from the market (shops), analysed with this DACC-  
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60 method were sent to a national research laboratory in the Netherlands, where they

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3 were analysed with another LC-method and a GCMS-method. These samples  
4 contained BaP levels ranging from 1 to 30 µg/kg. The results of each of the samples  
5 obtained by these three methods were similar, all results were within the  
6 measurement uncertainty of the level of BaP in the sample.  
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12 In the monitoring study about 1350 samples were analysed between 2002  
13 and 2004 for their BaP content, this being around 170 olive oils, 170 other oils for  
14 human consumption and 950 supplements. Additionally 60 further oils (raw  
15 materials), used as ingredients for food supplements, were also analysed. These  
16 latter oils were different kind of oils, collected at producers. The results are shown in  
17 figure 2.  
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29 **[INSERT FIGURE 2 ABOUT HERE]**  
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34 As can be seen in figure 2 about 80% of the olive oils and other oils for human  
35 consumption (eg. Sunflower oil) contained less than 1.2 µg/kg BaP, however there  
36 were some olive oils which contained up to 50 µg/kg BaP and one with 85 µg/kg BaP.  
37 The UK Food Standards Agency (2001) reported similar results with levels of BaP  
38 ranging from 9 to 43 µg/kg in some olive-pomace oil samples, originating from  
39 Greece, Spain and Italy. In the other oils for human consumption the amounts of BaP  
40 were much lower, the highest level rising to 9 µg/kg BaP. At inspections of producers  
41 and importers of oils for human consumption in the Netherlands by the VWA, it was  
42 noticed that producers and importers are aware of the possibility of contamination of  
43 PAHs and are taking action to reduce the amount of BaP in their oils. During the  
44 research over the last three years a slight decrease in the number of oil samples  
45 exceeding the limit of 1.2 µg/kg BaP was seen.  
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6 Nearly half of the raw materials (oils) which are used as a basis for food supplements  
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8 exceeded the limit of 1.2 µg/kg BaP, containing up to 80 µg/kg BaP, with 2  
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10 exceedances of 200 and 275 µg/kg BaP. During inspection of the producers of food  
11  
12 supplements it was found that some of them were not aware of the fact that BaP  
13  
14 could be present in their raw (oil) materials. These special oils were not controlled for  
15  
16 PAHs or other contaminants before dissolution of the supplements and encapsulation  
17  
18 of the products. Most of the food supplements under investigation were fat burners  
19  
20 (herbal extracts) and fat-soluble vitamin preparations. A lot of supplements with  
21  
22 tocopherols (vitamin E) were analysed. Tocopherols may interfere in the  
23  
24 determination of PAHs (Joint DG JRC/DG SANCO/EFSA workshop, 2005). However  
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26 with this DACC-method such problems were not found. Since the end products  
27  
28 contain additional substances apart from oils, which probably do not contribute to the  
29  
30 PAH contamination, the actual concentration of PAHs in those products, expressed  
31  
32 as a fraction of the sample weight, has to be lower than that of the oil itself. Of the  
33  
34 food supplements which were analysed 30% contained too much BaP with values of  
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36 140 and even 200 µg/kg. The percentage of food supplement samples that exceeded  
37  
38 the limit for BaP was the same in 2004 as in 2002. This suggests that companies  
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40 producing these type of products regularly ignore or are not aware of the presence of  
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42 PAHs in their raw materials (oils) and/or products. Thus, action on the part of the  
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44 producers as well as of the authorities is still needed to monitor this situation.  
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## 55 **Conclusions**

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57 The method developed for the determination of BaP in edible oils and oil based food  
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59 supplements is suitable to be applied as routine laboratory analysis and is relatively  
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3 fast (about 90 min per analysis). The validation characteristics of the method are in  
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5 line with international standards, as can be expected. The range of application is from  
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7 0.14 up to 8.0 µg/kg BaP, so even low levels of BaP can be determined. The  
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9 recovery in oil was 99.9% with an RSDr of 2.8%, in a food supplement the recovery  
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11 had a value of 100.1% with an RSDr of 4.1%. The performance of the method in  
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13 proficiency tests was good and samples analysed with other methods (LC and GC-  
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15 MS) showed similar results . With this method other PAHs can also be identified and  
16  
17 quantified. However, the method is not validated for these components at present.  
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19 For the time being, the method is single laboratory validated for BaP only. It is  
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21 intended to expand the validation to cover various PAHs, by a collaborative method  
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23 validation study to become an ISO standard (ISO/TC34/SC11 2005).  
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32 About 1350 samples were analysed for their BaP content, covering both oils for  
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34 human consumption and food supplements. In the oils about 20% exceeded the limit  
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36 of 1.2 µg/kg BaP applied by the VWA. In food supplements over 30% exceeded the  
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38 legal limit, which might be due to the raw materials (oil) which is being used as basis  
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40 for food supplements. Producers of food supplements need to become aware of the  
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42 presence of BaP in oils/oil extracts. More attention must be paid in the coming years  
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44 to the presence of BaP and other PAHs in food supplements. In addition, oils for  
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46 human consumption have to be checked, although to lesser extent than with food  
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48 supplements.  
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### 52 53 54 55 **Acknowledgement**

56  
57 This study resulted from the realisation that the levels of BaP were far in excess of  
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59 those which are acceptable. It was necessary to inform and inspect a very large  
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3 number of companies. We, the authors of this study, would like to thank the team  
4 who managed this project, being organisationally very complicated. A special thanks  
5 is due to the chemistry laboratory of VWA - region South, which performed all the  
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7 samples. The authors would also like to thank the National Institute of Public Health  
8 and the Environment (RIVM, The Netherlands), who carried out analysis using other  
9 methods, which enabled us to confirm our results.  
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Table I: HPLC conditions for the determination of PAHs

DACC column: Chromsper-pi 7 $\mu$ m, 80mm

Mobile phase: A: iso-propyl alcohol (cleaning), B: 30% acetonitril and 70% ethylacetate

flow rate: 0.35 ml/min

Analytical column: Lichrocart 250-4 Lichrosper PAH RP-18, 5 $\mu$ m (2x, connected in series)

Pre-column: Lichrocart 4-4 Lichrospher 100 RP-18, 5 $\mu$ m

Column temp: 30°C

Injection Volume: 100 $\mu$ l

Flow rate: 0.4 – 1.0 ml/min

Mobile phase:

Time (min)	Flow (ml/min)	Water (%)	Acetonitrile (%)	Ethyl acetate (%)
0	0.4	20	80	0
14.5	0.4	20	80	0
15.0	1.0	20	80	0
24.0	1.0	10	90	0
32.0	1.0	7	93	0
38.0	1.0	4	89	7
51.0	1.0	0	30	70
65.0	1.0	20	80	0
80.0	0.4	20	80	0
85.0	Stop time			

Table II: Wavelengths for fluorescence detection of PAHs after elution of LC system

Time (min)	Component	Excitation (nm)	Emission (nm)
25.80	Fluorene	275	350
26.84	Phenanthrene	260	390
28.00	Antracene	260	390
30.66	Pyrene	260	390
33.85	Benzo[ <i>a</i> ]anthracene*	260	390
35.08	Chrysene*	260	390
39.44	Benzo[ <i>b</i> ]fluoranthene*	290	430
41.79	Benzo[ <i>k</i> ]fluoranthene*	290	430
44.26	Benzo[ <i>a</i> ]pyrene*	290	430
46.67	Dibenzo[ <i>a,h</i> ]anthracene*	290	430
48.49	Benzo[ <i>g,h,i</i> ]perylene*	290	430
50.73	Benzo[ <i>b</i> ]chrysene (Internal Standard)	290	430
54.28	Coronene	300	450

\* PAHs regarded as 'priority PAHs' by the SCF

Figure 1: Chromatogram of a standard containing 12 PAHs

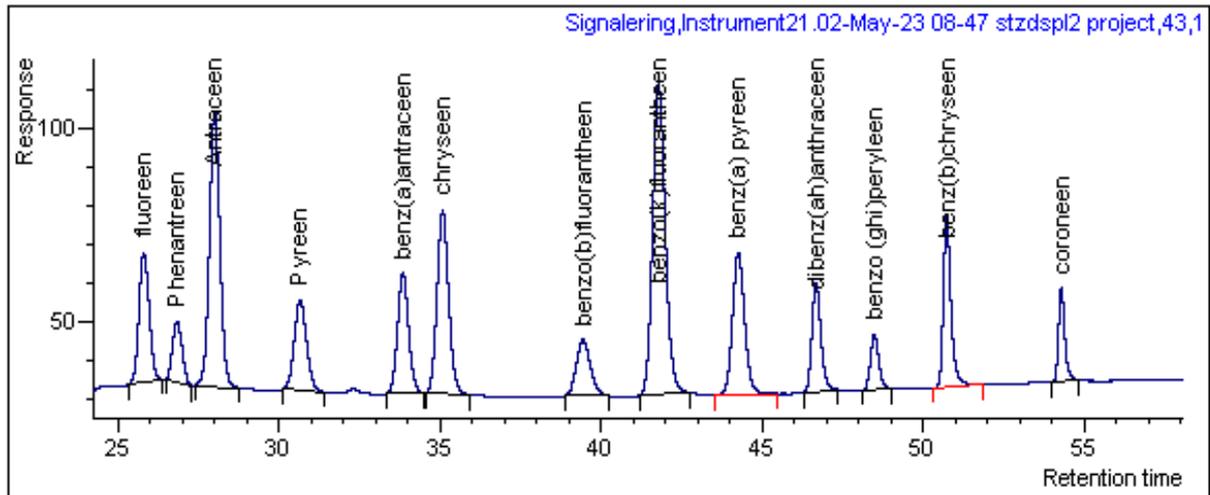
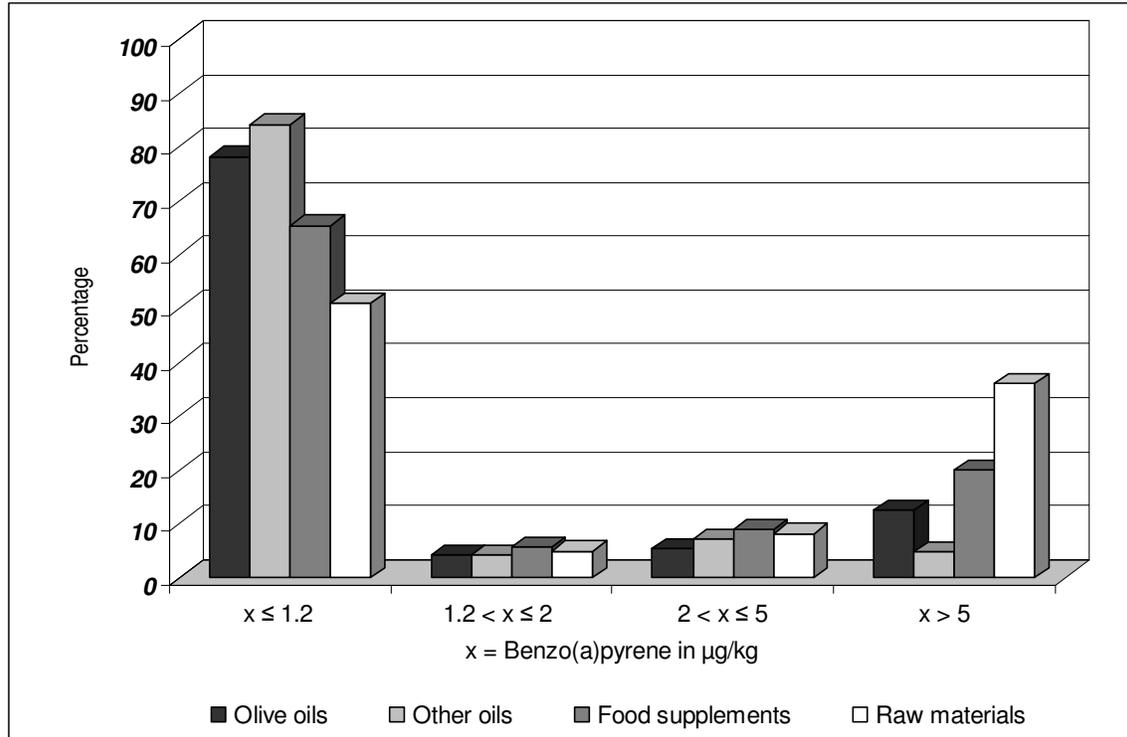


Figure 2: The levels of benzo[a]pyrene in several edible oils, foodsupplements and raw materials (oils) used for food supplements.



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