ОСНОВНІ ПУБЛІКАЦІЇ

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DETERMINATION OF BENZO[A]PYRENE IN SUNFLOWER SEEDS, EDIBLE OILS AND DERIVED PRODUCTS MANUFACTURED IN UKRAINE BY LIQUID CHROMATOGRAPHY USING FLUORESCENCE DETECTOR AND GAS CHROMATOGRAPHY USING MASS SELECTIVE DETECTOR

The method has been developed for qualitative and quantitative determination of benzo[a]pyrene in edible oils and oilseeds by HPLC using fluorescence detector. Sample preparation comprised alkaline hydrolysis of sample, liquid-liquid extraction of PAH and Florisil clean-up procedure. The benzo[b]chrysene was used as internal standard for quantification. Confirmation of positive results was carried out by GC-MS. The method described has been used for benzo[a]pyrene content determination in edible oils, oilseeds and derived products manufactured in Ukraine. The benzo[a]pyrene content in 24 samples of sunflower seeds and in 41 samples of crude sunflower oil from different regions of Ukraine ranged from 0.5 to 10.2 μg /kg of fat and from 0.8 to 21.0 μg /kg respectively.

Key words: *polycyclic aromatic hydrocarbons, benzo[a]pyrene, high performance liquid chromatography, gas chromatography-mass spectrometry, edible oils, oilseeds.*

Розроблено метод якісного та кількісного визначення бенз[а]пірену у насінні олійних культур та харчових оліях з використанням рідинної хроматографії з флуоресцентним детектором. Підготовка зразків включає лужний гідроліз зразка, екстракцію ПАВ в системі рідина-рідина та очистку на сорбенті Florisil. З метою кількісного визначення бенз[а]пірену в якості внутрішнього стандарту використовується бенз[b]хрізен. Позитивні результати підтверджуються методом газової хромато-мас-спектрометрії. З використанням розробленого методу було визначено вміст бенз[а]пірену у харчових оліях, олійному насінні та оліє жирових продуктах, вироблених на території України. Вміст бенз[а]пірену у 24 зразках насіння соняшнику та у 41 зразку сирої соняшникової олії, вироблених у різних регіонах України, коливався від 0,5 до 10,2 мкг/кг жиру та від 0,8 до 21,0 мкг/кг відповідно.

Ключові слова*: поліциклічні ароматичні вуглеводні, бенз[а]пірен, високоефективна рідинна хроматографія, газова хромато-мас-спектрометрія, харчові олії, олійне насіння.*

Разработан метод качественного и количественного определения бенз[а]пирена в семенах масличных культур и пищевых растительных маслах с использованием жидкостной хроматографии с флуоресцентным детектором. Пробоподготовка включает щелочной гидролиз, экстракцию ПАУ в системе жидкость-жидкость и очистку на сорбенте - Florisil. При количественном определении бенз[а]пирена используется внутренний стандарт – бенз[b]хризен. Положительные результаты подтверждаются методом газовой хромато-мас-спектрометрии. С помощью разработанного метода было определено содержание бенз[а]пирена в пищевых маслах, семенах масличных культур и масложировых продуктах, произведенных на территории Украины. Содержание бенз[а]пирена в 24 образцах семян подсолнечника и в 41 образце сырого подсолнечного масла, произведенных в разных регионах Украины, колебалось от 0,5 до 10,2 мкг/кг жира и от 0,8 до 21,0 мкг/кг соответственно.

***Ключевые слова**: полициклические ароматические углеводороды, бенз[а]пирен, высокоэффективная жидкостная хроматография, газовая хромато-масс-спектрометрия, пищевые масла, семена масличных культур.*

Introduction

Polycyclic aromatic hydrocarbons (PAH) are known as the widespread pollutants of edible oils and oil-containing alimentary products. Considering the fact that some PAH are potent carcinogens in human [5], it is necessary to establish the maximum allowable concentrations (MAC) thereof to protect public health and to monitor vendor compliance. Benzo[a]pyrene is used as a marker for carcinogenic PAH presence in food. As any PAH, it can be formed in foodstuffs when heated; it was found in furnace combustion gases, tobacco smoke, smoked meat and dairy products [1, 6]. The most probable oilseeds PAH pollution way is drying by flue gases containing smoldering products. PAH are also formed at waste, food and petrochemicals incineration [3, 4]. Benzo[a]pyrene MAC for oils and fats for human consumption or use as food ingredient is 0.002 μg/kg according to EC 208/2005 of 4 February 2005. However, an equivalent MAC norm is not still approved in Ukraine under excuse of lack of benzo[a]pyrene occurrence data for domestic edible oils and food products.

The most popular analytical methods for PAH determination are gas chromatography using flame ionization (FID) or electron capture detectors (ECD), GC-MS, and HPLC using diode-array detector (DAD), fluorescence detector or both connected in series [2, 7]. Determination of benzo[a]pyrene at 0.002 μg/kg level calls for high sensitivity and selectivity methods. Both fluorescence and mass-selective detector (MSD) do have high selectivity; however, the former is over 10 times more sensitive than MSD (see Table 1). High sensitivity means less sample consumption, larger volume after sample preparation, and, finally, significantly less matrix effect. As neither UV-detector, nor FID has such performance, those are not so much used for PAH determination.

Table 1

Limit of quantitation (LOQ) for benzo[a]pyrene in edible oils and fats

Analytical method	**LOQ, μg/kg**
HPLC with fluorescence detector	0,07
GC-MS	0,5
GC with ECD	5
HPLC with UV-detector	7

The objective of our work was to develop and to validate a method for benzo[a]pyrene determination in edible oils and oilseeds by HPLC with fluorescence detector. GC-MS has been used for positive results confirmation. The main purpose of the research was monitoring of benzo[a]pyrene content in sunflower seeds, oil and derived products manufactured in different regions of Ukraine.

Experimental

Chemicals and reagents

HPLC grade hexane (Pestanal®), acetonitrile, N,N-dimethylformamide, benzene, reagent grade sodium sulphate anhydrous and potassium hydroxide, activity grade II Florisil PR (60-100 mesh) have been used. All chemicals and solvents were made by Riedel-de-Haën.
Ultra pure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA).
Benzo[a]pyrene and benzo[b]chrysene were also Riedel-de-Haën made.

Standard solutions preparation

Benzo[a]pyrene and benzo[b]chrysene stock solutions (100 μg/ml) were prepared by commercial standards dissolving in acetonitrile. An intermediate standard solution of 10 μg/ml concentration was used for working solutions preparation. The working solutions of benzo[a]pyrene of 0.005 μg/ml to 1.0

μg/ml concentrations were prepared by further dissolving of intermediate solution.

Internal standard solution of benzo[b]chrysene 1.0 μg/ml concentration was prepared from stock solution.

Reagents purity check

50 ml of hexane was evaporated to dryness at 40 °C. The residue was dissolved in 1 ml of acetonitrile, left for 15 minutes and analyzed by HPLC. The benzene purity was checked in a similar way.

For the sake of purity check 50 ml of N,N-dimethylformamide was placed in a separating funnel and 100 ml water and 30 ml hexane was added subsequently. After 3-5 minutes the supernatant was taken and evaporated to dryness at 40 °C. Residue was dissolved in 1 ml of acetonitrile. Prepared solution was analyzed by HPLC.

The purity check was performed for every reagent batch. Solvents were considered suitable for the analysis if no benzo[a]pyrene peak was present on chromatogram.

Sample preparation

Seed samples were finely ground, and fat was extracted by hexane. Samples of oils and other products were homogenized and prepared as follows: 10 g of homogenized sample was weighed into a flask. 50 ml of potassium hydroxide solution and 50 μl of internal standard solution were added. The flask was fitted to the condenser and heated at 90 °C for 3 hours. Flask was cooled to ambient temperature, and 100 ml of water was added. Solution from flask was transferred into a separating funnel and extracted three times with 30-ml portions of hexane. The hexane layers were separated into one flask and washed by water until neutral pH. After adding of 50 ml of 9:1 v/v N,N-dimethylformamide:water the lower layer was separated. The extraction was repeated two times and followed by adding 100 ml of water. PAH were extracted from solution of N,N-dimethylformamide three times with 50-ml portions of hexane. Extracts were washed by water and filtered through the paper filter with sodium sulfate layer into the round-bottom flask. Extract was evaporated until 1-2 ml under reduced pressure at 40 °C and put to Florisil column. 40 ml of 1:1 v/v hexane:benzene was used as eluent. The eluate was collected into the round bottom flask and evaporated to dryness at 40 °C. Residue was dissolved in acetonitrile:water (8:2 v/v) mix.

Analytical system

The system consisted of HP 1100 liquid chromatograph (Hewlett Packard, USA), quaternary pump with vacuum degasser, autosampler, scanning fluorescence detector and column oven, and was controlled from PC with Chemstation software. The reversed-phase Hypersil MOS (200 x 2.1 mm, 5 μm particles) analytical column was used. The mobile phase consisted of acetonitrile and water in the gradient mode (from 20 to 100 % of water, v/v,). The flow rate was 0.3 ml/min. The excitation and emission wavelengths were set at 399 and 418 nm respectively.

The GC-MS system comprised gas chromatograph and HP 6890/5973 mass detector. The capillary column was a 30 m HP-5ms (5 % phenyl methyl polysiloxane) having 0.25 mm bore and 0.25 μm film. The carrier gas was helium at 1.0 ml/min flow. The injection port was kept at 250 °C, the oven temperature was programmed from 40 °C (1 min) to 320 °C. Ion source was kept at 320 °C and used 70eV electron impact ionization. Full scan data acquisition mode with 3.5 scan/sec scan rate was applied through the mass range from 35 to 450 u.

Results and Discussion

Benzo[a]pyrene Determination by HPLC

Retention time was used for benzo[a]pyrene identification. Benzo[b]chrysene, a substance rarely present in oils and fats, was used as the internal standard. Response of fluorescence detector for this compound is linear in 0.1 to 10 ng range and at 1.0 μg /ml is comparable with benzo[a]pyrene response.

Retention time of benzo[b]chrysene is close to retention time of benzo[a]pyrene (Fig.1).

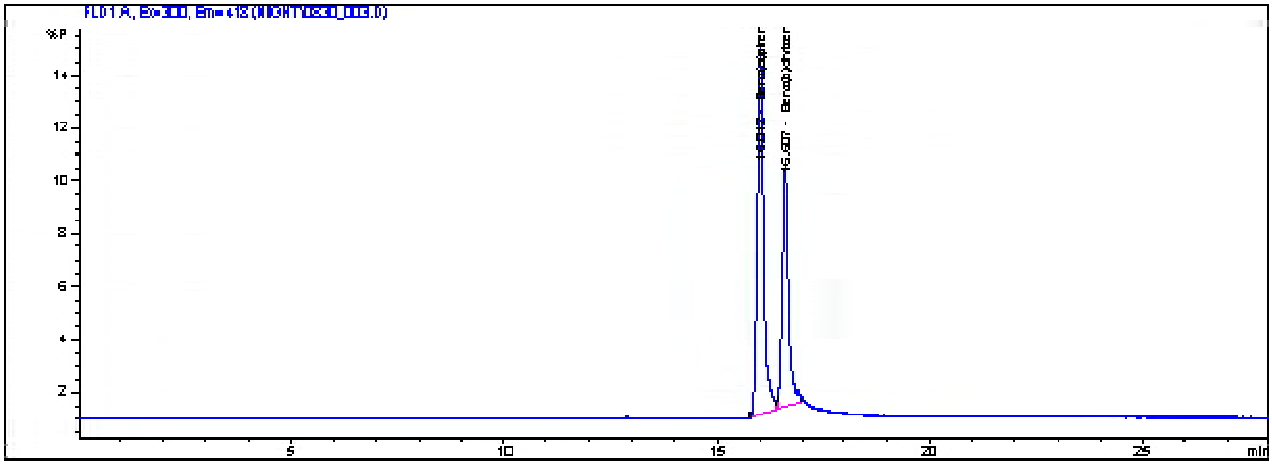

Fig.1 HPLC/fluorescence detector chromatogram of benzo[a]pyrene and benzo[b]chrysene (1.0 µg /ml) standard solution

The column used is also applicable for fat analysis; therefore it is not critical if some fat remains in the extract. The column is easily regenerated by tetrahydrofuran and acetone, its length is enough for separating all basic PAH.

The special attention should be given to results verification. It is also quite important to check the purity of all reagents. Some reagents available in the Ukrainian market are of poor quality and may contain residue of PAH. For example, some batches of commercially available N,N-dimethylformamide "for extraction" contained 5 µg/l benzo[a]pyrene as well as other PAH (Fig.2). Such reagents should not be used for PAH analysis.

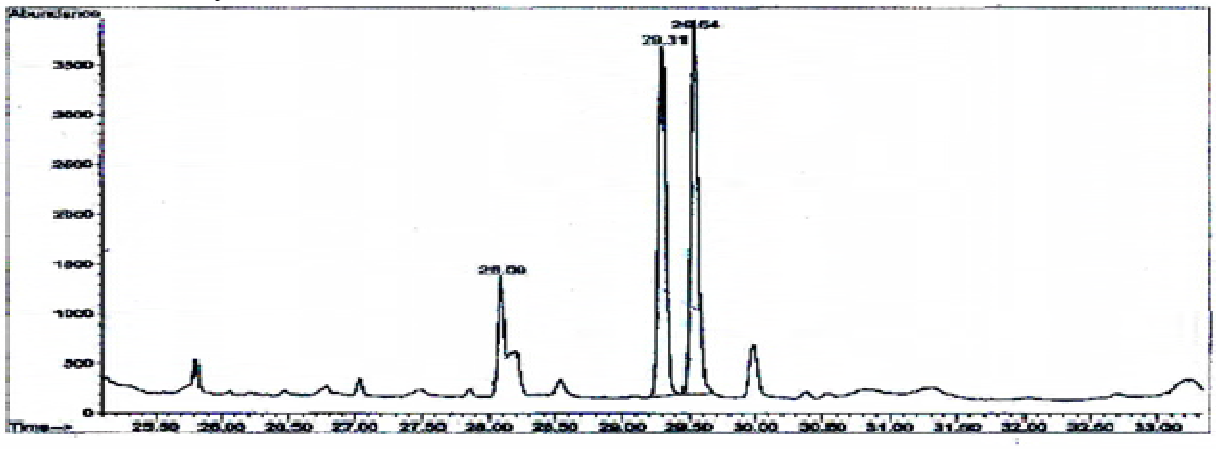

Fig.2 GC-MS chromatogram of N,N-dimethylformamide containing benzo[a]pyrene and other PAH

Results confirmation by GC-MS

Positive HPLC results require confirmation by GC-MS because the interfering substances co-eluting with PAH may result in overestimation or false positives.

The extracts prepared for HPLC were concentrated under nitrogen steam and dissolved in 50 ml of hexane for further GC-MS analysis. Identification of benzo[a]pyrene was done on the basis of retention time and mass to charge ratio (m/z) equivalence for sample extract and standard solution. The sample extract has to be cleared from fat before GC-MS analysis to prevent detector contamination.

Method performance

The method accuracy was checked up in two rounds of inter-laboratory testing. The results have confirmed efficiency of the developed method (Table 2). The relative error of parallel determinations did not exceed the reproducibility limit.

Table 2

Results of inter-laboratory testing of sunflower seed oils and sunflower seeds

Sample description	Benzo[a]pyrene weight content, μg /kg		Relative error, %*
	Mf_1	Mf_2	
Sunflower seeds	2,3	2,5	4,2
Sunflower seed oil (sample N1)	11	9,1	9,5
Sunflower seed oil (sample N2)	14	13	3,7
Sunflower seed oil (sample N3)	12	11	4,3
Sunflower seed oil, crude	4,5	4,0	5,9
Sunflower seed oilm refined	1,2	1,2	0,0

Mf_1 – benzo[a]pyrene content determined by other participant of the round;
Mf_2 – benzo[a]pyrene content determined by described method
* Relative error = (Mf2-Mean)/Mean x 100%

Results benzo[a]pyrene determination in seed oil

The described method was used for benzo[a]pyrene determination content in sunflower seeds, seed oil and derived products samples.24 samples of the sunflower seeds originating from different regions of Ukraine were analyzed. The occurrence of benzo[a]pyrene in the samples ranged from 0.5 to 10.2 μg /kg of fat. The benzo[a]pyrene content in 41 samples of crude sunflower oil was from 0.8 to 21.0 μg /kg (see Fig. 3-4). Maximum residual level of benzo[a]pyrene (2.0 μg /kg) was exceeded in 50 % samples of seeds and in 76% samples of sunflower oil.

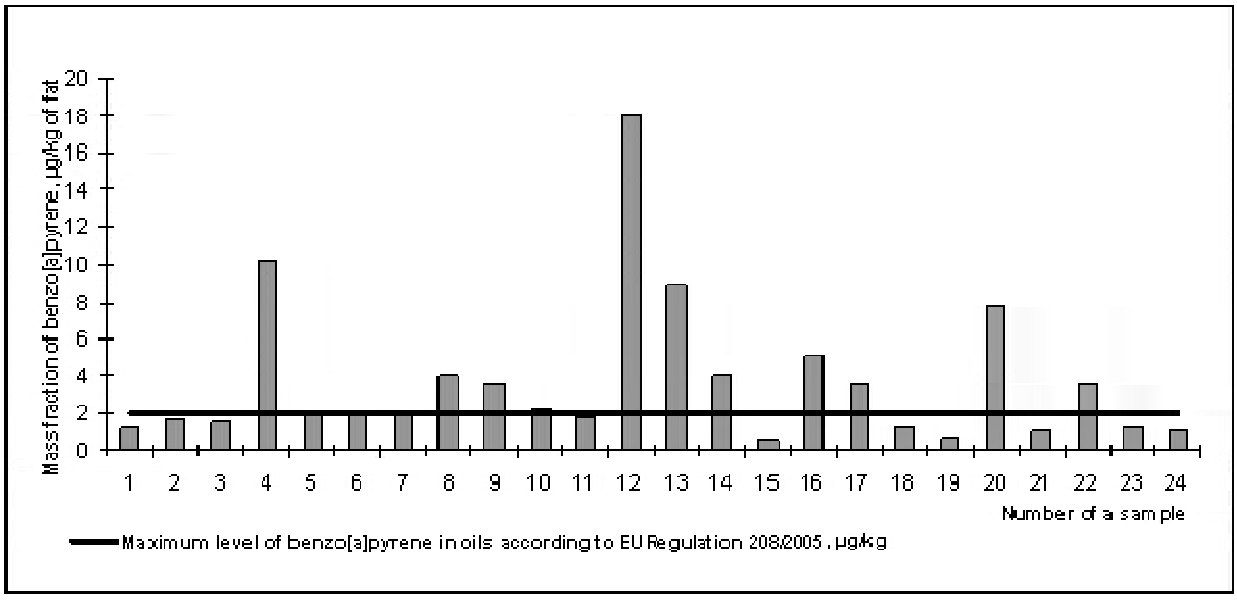

Fig.3 Occurrence of benzo[a]pyrene in sunflower seed samples

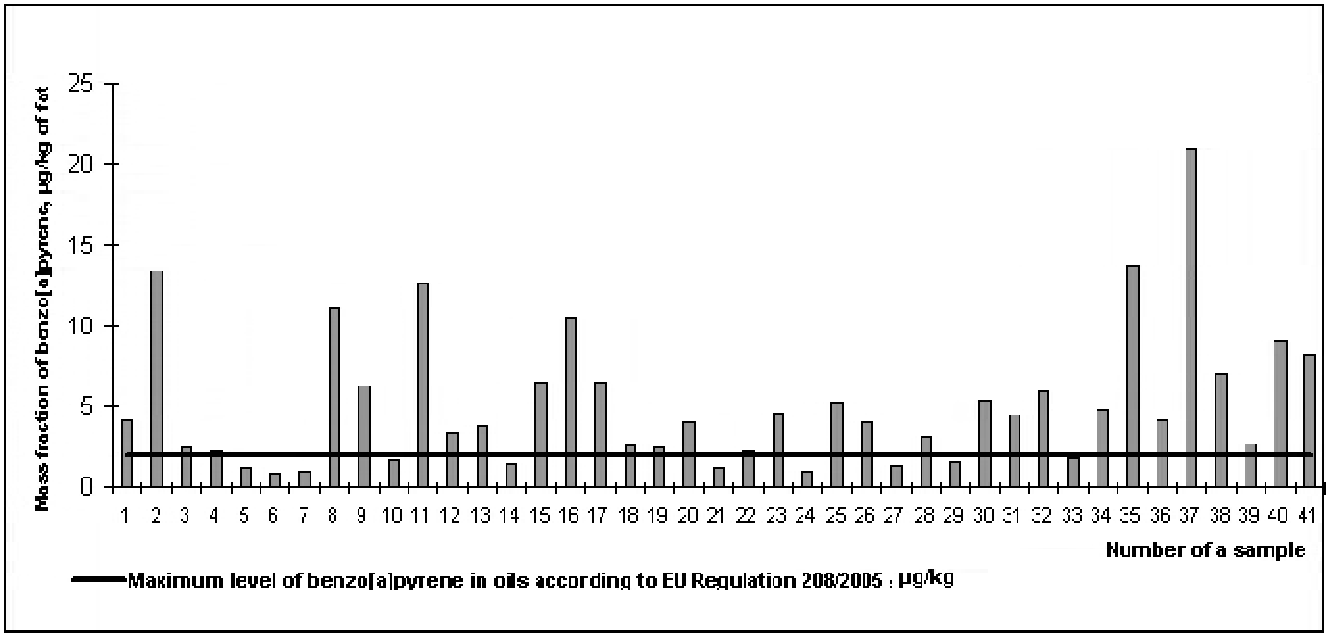

Fig.4 Result of determination of benzo[a]pyrene in the samples of sunflower seed oil

The benzo[a]pyrene was also present in margarines, mayonnaises, spreads etc. at 0.5 to 6.6 µg /kg, thus proving that the products manufactured from seed oil may have high PAH content. The extracts of these samples contained not only benzo[a]pyrene, but also other PAH (Fig.5).

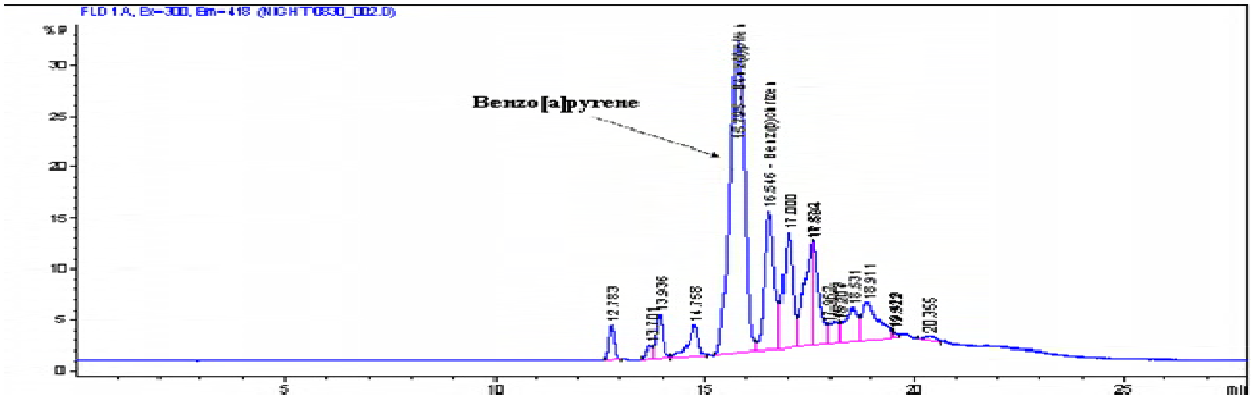

Fig.5 Chromatogram of mayonnaise extract (HPLC/fluorescence detector).

Conclusions

The results demonstrate the necessity to control benzo[a]pyrene content in edible oils and derived products. High-sensitivity HPLC using fluorescence detector has been shown to be an efficient method for determination of this compound. However, co-eluting interfering substances may result in overestimation or false positives. Therefore positive results require confirmation by GC-MS. All reagents and solvents used for analyses should be free from PAH residues.

The data so obtained will be informative in estimating the distribution of benzo[a]pyrene in sunflower seeds, edible oils and derived products manufactured in Ukraine. Our investigation confirms necessity of soonest possible legislative approval of MAC for benzo[a]pyrene in edible oils and others food products in Ukraine.

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