ISSN 2063-5346

DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHOD FOR ANALYSIS OF SOME POLYCYCLIC HYDROCARBONS IN SOYBEAN OIL

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Article History: Received: 01.02.2023 **Revised:** 07.03.2023 **Accepted:** 10.04.2023

Abstract

Some polycyclic hydrocarbons (PHs) present in almost all edible oils are carcinogenic, teratogenic, neurotoxic and mutagenic at certain levels. There are total 16 PHs which are present in various edible oils. Soybean oil is one of most widely and routinely used for cooking food by all over population. Hence the detection of PHs levels in various soybean oil brands available in market is necessary. Many analytical methods are available for the same but these methods are time consuming and secondly quantity of organic solvents required is also higher. For example HPLC can be used for analysis of PHs in edible oils but it takes more time for analysis. Hence a novel method is developed using supercritical fluid chromatography for analysis of some PHs in soybean oil which reduces time by 5 times as compared to normal HPLC method and also requires less amount of organic solvents for analysis because of the lower viscosity and higher diffusivity in the mobile phases of SFC. The method is validated using various validation parameters and statistically proved using ANOVA.

KEY WORDS- Polycyclic hydrocarbons, Soybean oil, HPLC, SFC, Cancer.

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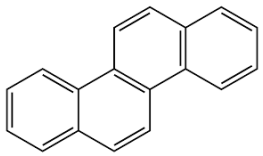
DOI: 10.31838/ecb/2023.12.s1.087

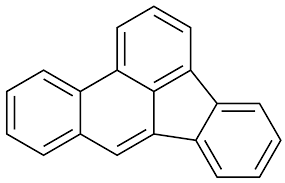
INTRODUCTION

Hydrocarbons are class of compounds comprised only of carbon and hydrogen and they are present by a wide margin in the predominant parts of edible oils, raw petroleum, handled petrol hydrocarbons (gas, diesel, lamp oil, fuel oil, and greasing up oil), coal tar, creosote, dyestuff, and pyrolysis side-effects[1]. Polycyclic hydrocarbons are a class of synthetic substances that occur normally in coal, raw petroleum, and fuel. They come out because of consuming coal, oil, gas, wood, trash, and tobacco. PHs can tie to or structure little particles in the air[2]. Polycyclic hydrocarbons are a large group of organic compounds with two or more fused aromatic rings. They are comprised of at least three benzene rings containing carbon and hydrogen. Contrasts in the setup of rings, might prompt contrasts in properties[3].

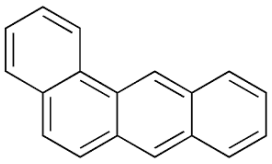
There are more than 100 polycyclic hydrocarbons present which have adverse effects on human health but fifteen of them are designated as major pollutant by the U.S. Environmental Protection Agency (USEPA) negatively affecting human health[4]. In 2002, Scientific Committee on Food (SCF) identified 15 polycyclic hydrocarbons that are potential enough to be genotoxicity and carcinogenicity[5]. In 2005, Joint Food and Agricultural Organization (FAO) and World Health Organization (WHO) Expert Committee on Food Additives (ECFA) added an additional polycyclic hydrocarbon to the list. This list is known as 15+1 EU Priority polycyclic hydrocarbon[6]. The list of PHs include benzo[a]anthracene (BaA), benzo[b]fluoranthene (BbF), indeno[1,2,3-cd]pyrene (IND), benzo[k]fluoranthene(BkF), benzo[a]pyrene (BaP), dibenzo-[a,h] anthracene (DBA), naphthalene (Nap), acenaphthene (Acp), acenaphthylene (AcPy), fluorene (Flu), phenanthrene (PA), anthracene (Ant), fluoranthene (FL), pyrene (Pyr), chrysene (CHR), and benzo(g,h,i)perylene (BghiP)[3]. Out of 16 PHs, following were taken into consideration for analysis



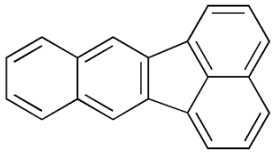
Chrysene (CHR)



Benzo[b]fluoranthene (BbF)



Benzo[a]anthracene (BaA)



Benzo[k]fluoranthene (BkF)

Figure 1- Important polycyclic hydrocarbons (PHs)

Polycyclic hydrocarbons are cancer-causing, teratogenic and mutagenic foreign substances that are poisonous to human wellbeing. They are exceptionally steady and are available in climate and food. So polycyclic hydrocarbons in food has drawn a lot of consideration over the most recent couple of years since it is straight forwardly connected with serious health issues[7].

It was accounted that food is one of the significant wellsprings of polycyclic hydrocarbons openness, including eatable oils because of their lipophilic nature and maximum usage[2]. PHs can undoubtedly enter the human body through the utilization of palatable oils because of their high lipophilicity[8]. Polycyclic hydrocarbons might be brought into eatable oils from the climate and drying process during creation. The presence of polycyclic hydrocarbons in vegetable oils might be credited to (i) climatic pollution of plant material, (ii) direct drying of the plant material with burning smoke, (iii) pollution through the dissolvable extraction (iv) take-up by the oil plants from debased soils[9].

Recently, European Union (Commission Regulation No. 208/ 2005) has set maximum levels of 2 ppb for benzo[a]pyrene in oils and fats for direct consumption or use as an

ingredient in foods[10]. Several countries like Spain, Italy, Portugal and Greece have established their own limits for the concentration of the following toxic and carcinogenic PHs such as benzo[a]anthracene, benzo[e]pyrene, benzo[b]fluoranthene, benzo[k] fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, benzo[ghi]perylene and indeno[1,2,3- cd]pyrene. Maximum limit for each individual single PH is 2 ppb and 5 ppb for the sum of the eight heavy PHs[11].

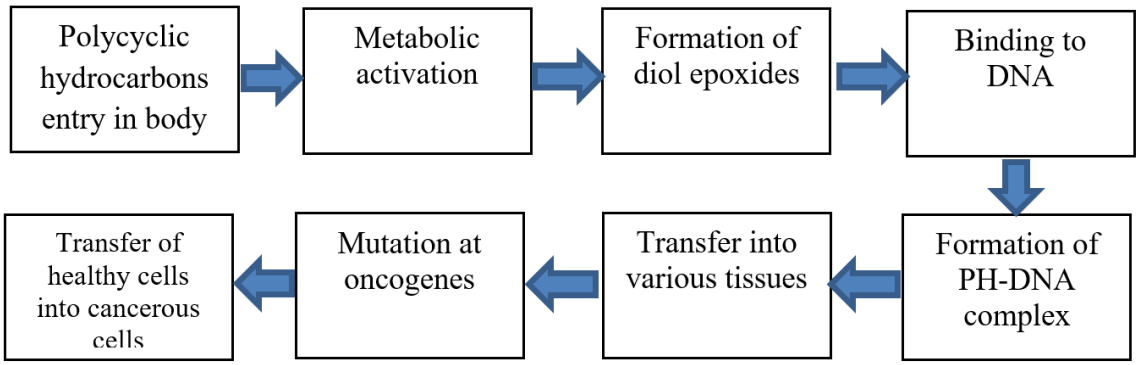
Polycyclic hydrocarbons have increased risk of skin and lung cancer[12]. Diet i.e. edible oil is the important source of polycyclic hydrocarbons for non-smokers; meat and meat products, cereals, and is also the major sources of polycyclic hydrocarbons[13]. A significant dietary source in oils and fats contaminate by polycyclic hydrocarbons are due to their lipophilic nature[14].

Soybean oil currently developed universally and is perhaps of the main vegetable oil as far as the amount delivered and used by the vast majority of populace as consumable oil. It is edible semi-drying oil. Its primary benefit is its non-yellowing property which is because of its low linolenic corrosive substance[15]. It is widely used all over country by lower and by middle level public sector. Hence it is an important research task to perform the evaluation of PHs in soybean oil similar to other oils. Numerous methods such as High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC), Ultra Violet (UV) Spectroscopy, Mass spectrometry (MS), Atomic absorption Spectroscopy (AAS) are available for the analysis of PHs from various edible oils including soybean oil but Supercritical Fluid Chromatography (SFC) is quite used. Secondly time required for above mentioned analytical methods is quite higher and the solvents required for are also has high volume[16]. Hence the aim of current research protocol includes (i) comparative analysis of soybean oil using HPLC and SFC, (ii) to observe the critical decrease in analysis time than required for ordinary HPLC examination, (iii) decrease in volume of organic solvent utilization than required for HPLC investigation.

Mechanism of action of polycyclic hydrocarbons[11]

Polycyclic hydrocarbons can easily reach the human being and show significant bioavailability mainly due to their lipophilic nature. They found in adipose tissue in higher amount however it can reach to almost all organs of the human body. Their main side effects include teratogenicity, neurotoxicity, genatotoxicity, cardiotoxicity, mutagenicity and carcinogenicity. The mechanism by which they act into the body is that they cause changes in the function of cell membrane and the enzymes which are involved in the functioning of cell membrane.

Molecular mechanism is depicted below



MATERIALS AND METHODS

Chemicals, solvents and reagents

Standard polycyclic hydrocarbons such as chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[a]anthracene (BaA), benzo[k]fluoranthene (BkF) were purchased from Merck laboratories. Hexane, methanol, N,N-dimethylformamide, acetonitrile (all HPLC grade) were procured from Bioera technologies. Purified water was used for the study. The SPE cartridges (500 mg, 3 ml) were procured from Agilent Technologies Inc.

Stock standard solutions were prepared by diluting the PHs standards in acetonitrile.

Oil sample preparation for HPLC[17]

Soybean oil commercially available pouch packing form of local brand was purchased at supermarkets in the region of Baramati in Maharashtra. Soybean oils (500 mg) was weighed in an Erlenmeyer flask using digital analytical balance and 7 ml of hexane was added. The mixture was taken into a 100 ml glass separating funnel and PHs were extracted twice with 8 ml of N,N-dimethylformamide–water (DMF-H2O) (6:1, v/v). The extract was combined and concentrated under a nitrogen flow until it reaches approximately 70 % of its starting volume. Then, 5 ml of water was added in the obtained solution and then solid-phase extraction (SPE) was performed. The SPE cartridges were washed with 7 ml of methanol and then by 7 ml of water using a Vacuum Manifold. Then the sample solution was applied and the column was washed with 15 ml of N,N-dimethylformamide–water (1:1, v/v), followed by 7 ml of water, all eluates were discarded. The cartridges were dried under vacuum for 30 min. Then PHs were eluted with 15 ml hexane at a flow rate of 3 ml/min. An eluate so obtained was subjected to dryness under a nitrogen stream. The residue so obtained was diluted in 1 ml acetonitrile, filtered through millipore filter into a HPLC vial and subjected to the HPLC analysis.

Oil sample preparation for SFC[18]

Pre-treatment method was used for oil sample preparation for SFC. In this method, 9 ml n-hexane is added to the 1 ml soybean oil. This mixture is vortexed for 5 min at ambient temperature and then centrifuged for 6 min. From this, 5 ml supernatant liquid is pipette out and filtered through PTFE having Millipore size of 0.24 µm size.

HPLC analytical method development

HPLC analytical method was developed on Thermo Scientific Vanquish Duo HPLC System having quaternary pumps on-line degasser, auto sampler and fluorescence detector. A C18 SUPELCOSIL 102TP column and a mobile phase having composition of acetonitrile and water, at a flow rate of 1 ml/min, were used to resolve the PHs. The gradient elution was started linearly from 65% to 70% acetonitrile in 25 min, followed 70% to 100% in 20 min acetonitrile and maintained 100% acetonitrile isocratic until 55 min, when finally returned to the initial conditions. The injection volume was set to 30 ml. The following excitation (ex) and emission (em) wavelength programme was used to determine the PHs: 4 min (268/398 nm) for chrysene, 6.5 min (312/507 nm) for benzo[b]fluoranthene, 7.2 min (290/430 nm) for benzo[a]anthracene, 11.75 min (300/500 nm) for benzo[k]fluoranthene. Data were acquired and processed with suitable software.

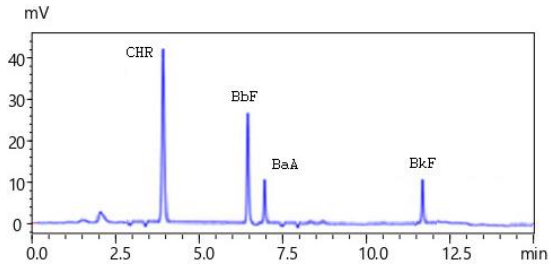


Figure 2- Chromatogram of PHs by HPLC

SFC analytical method development

SFC analytical method was developed Waters Xevo G3 QTof having StepWave XS detector. The wavelength used was 298 nm. The column, Shim pack UC-X (250 nm x 2.1 mm I.D., 5 µm) at temperature of 40ºC was used. Mobile phase having composition of carbon dioxide and methanol (1:2) at a flow rate of 4 ml/min was used. Time program was set at 0 to 2.5 min, 2.52 to 2.9 min and 2.91 to 3.0 min. BRP setting was done at 15 MPa at 50ºC. Glass vial having capacity of 1.5 ml was used.

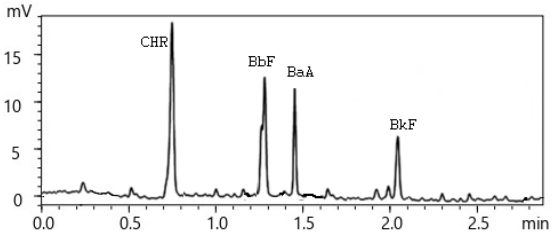


Figure 3- Chromatogram of PHs by SFC

Statistical analysis

The software Statistica was used to perform the analysis of variance (ANOVA). PHs contamination levels in different periods of

time were compared by Turkey test (95% confidence)[19].

Identification and quantification of PHs

The identification of isolated PHs was done by comparison of their retention times with those obtained by injecting standards in the same conditions. Confirmation of peak identity was done by impelling the extracts with pure standards. The quantification of compounds was done using the external standard plot method. A mixed standard stock solution with PHs was prepared in acetonitrile and stored at 4ºC for 2.5 months. From this solution, diluted solutions ranging from 0.5 to 250 ng/ml, were used to construct linear regression lines.

Validation study

Accuracy, precision (inter- and intraday repeatability), linearity, specificity, limit of detection (LOD) and limit of quantification (LOQ) are used as validation parameters. The accuracy and repeatability of the method were estimated by doing recovery tests. Recovery (accuracy) was determined by spiking a blank control sample of soybean oil with the PHs studied at 0.5, 1.0, 1.5 and 5.0 mg/kg and the values were reported as average % recovery. Interday and intraday repeatabilities, expressed as the % of relative standard deviation (% RSD), were checked by analysing the same sample control spiked with PHs standard sample during the same day. In addition, the precision of the chromatographic system was carried out by injecting the same oil sample extract, fortified with a working standard PHs solution (1.2 mg/kg). Linearity was tested by the square correlation coefficients (r^2) of the calibration curves. LOD and LOQ were determined using matrices. Specificity was confirmed by analysis of the blank oil sample control, which was produced with soybeans dried naturally in order to avoid any kind of contamination[20].

RESULTS AND DISCUSSION

In case of HPLC, the average recoveries obtained for the compounds (64–111%) were satisfactory for determinations at the mg/kg level and were in agreement with the criteria for methods of analysis. Intra- and interday repeatability of the extraction procedure, expressed as the percentage of relative standard deviation (% RSD), ranged from 64% to 98% and from 74% to 99%, respectively. For the chromatographic system, the RSD of the detector response was less than 1.5% (including intra- and interday repeatability) and the RSD of the retention times was lower than 0.09% for each compound. The PHs responses were linear over the concentration range studied, as showed in table 1. The LOD and LOQ for the target compounds varied from 0.12 to 0.65 mg/kg and from 0.06 to 0.35 mg/kg, respectively.

Table 1- Validation study of PHs by HPLC

PH	LOQ (mg/kg)	LOD (mg/kg)	Linear range (mg/kg)	Regression coefficient (r^2)	Intra-day precision RSD (%)	Inter-day precision RSD (%)	Mean recovery ±RSD (%)
CHR	0.06	0.27	0.5-50	0.9994	76±5.4	95±6.7	3.1
BbF	0.34	0.64	1-250	0.9998	88±3.7	85±4.5	1.1
BaA	0.23	0.12	0.5-50	0.9993	89±2.1	99±2.8	2.3
BkF	0.15	0.25	0.5-50	0.9984	64±6.8	74±3.6	4.1

In case of SFC, the calibration curves for standard PHs were created in range between 0.8 mg/L to 500 mg/L and for isolated PHs at range between 0.8 mg/L to 100 mg/L. The LOD, repeatability and linearity was mentioned in table 2.

Table 2- Validation study of PHs by SFC

PH	% RSD (mg/L)	Linearity	LOD (mg/L)
CHR	0.23	0.9999	0.6
BbF	0.21	0.9997	0.4
BaA	0.65	0.9998	0.6
BkF	0.38	0.9999	0.7

HPLC has required time of 12.5 min (figure 1) for analysis of PHs from soybean oil while same analysis was required within 2.5 min by using SFC (figure 2). This clearly reduced analysis time by 5 times as compared to HPLC. Second important thing is the total running cost owing to the use of less volume of organic solvent for SFC than HPLC. In research protocol, it has been 30 ml of n-hexane was required for HPLC while only 0.6 ml of methanol was required for SFC which uses liquid CO_2 (only 6.8 ml) which has very low cost.

CONCLUSION

Comparative HPLC and SFC chromatogram for four important PHs shows that HPLC has required more time for analysis of PHs while same analysis was required less time by using SFC. This clearly reduced analysis time by five times as compared to HPLC. In the research protocol it has been observed that less volume of organic solvents are required for SFC as compared to HPLC. Finally it can be concluded that, a novel SFC method was investigated for quantitative analysis of some important polycyclic hydrocarbons (PHs) in soybean oil and it can be used widely at in research and development level for multiple analysis and quality control of soybean oil in food industries.

AUTHOR CONTRIBUTION All authors contributed equally for investigation.

ABBREVIATIONS

HPLC- High Performance Liquid Chromatography, SFC-Super Critical Fluid Chromatography, UPLC- Ultra Performance Liquid Chromatography, UV- Ultra Violet, AAS- Atomic Absorption Spectroscopy, MS-Mass Spectrometry, PHs- Polycyclic Hydrocarbons, LOD- Limit of Detection, LOQ- Limit of Quantitation, RSD- Relative Standard Deviation, CHR- Chrysene, BbF-Benzo[b]fluoranthene, BaA-Benzo[a]anthracene, BkF-Benzo[k]fluoranthene, USEPA- United States Environmental Protection Agency, SCF-Scientific Committee on Food, WHO- World Health Organization, FAO- Food and Agricultural Organization, ECFA- Expert Committee on Food Additives, PTFE-polytetrafluoroethylene, ex- excitation, em-emission.

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