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MONITORING OF GAMMA IRRADIATION EFFECTS ON POLYCYCLIC AROMATIC HYDROCARBONS AND MICROBIAL LOAD IN PEA SEEDS USING GC-MS ANALYSIS

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A qualitative characterization of 16 polycyclic aromatic hydrocarbons (PAHs) in pea seeds before and post gamma irradiation has been carried out using gas chromatography-mass spectrometry (GC-MS). Seeds were exposed to gamma irradiation at 1, 5, 10 and 15 kGy doses. PAHs were extracted and their concentrations were correlated with the peak height, at each dose and compared to control samples. PAHs peaks decreased with the increase of gamma irradiation dose. Total bacterial and total fungal counts decreased from 2.43 and 1.66 $\log_{10}$ cfu g^{-1}, respectively (non-irradiated), to <1 $\log_{10}$ cfu g^{-1} after gamma irradiation. The results indicate the potential utilization of gamma irradiation for PAHs and microbial inactivation from pea seeds.

Keywords: polycyclic aromatic hydrocarbons (PAHs), GC-MS, gamma irradiation, decontamination, pea seeds

Introduction

Human foods are sometimes polluted by a variety of chemical compounds or microorganisms considered dangerous for human health. Polycyclic aromatic hydrocarbons (PAHs) are classified as the most important compounds that pollute human food and cause many health problems. PAHs have received great attention in the recent years. PAHs are ubiquitous environmental pollutants and formed from both natural and anthropogenic sources. Natural sources include forest fires, volcanic explosions and degradation of biological materials, which lead to the formation of these compounds (Blumer and Youngblood, 1995; Ilnitsky *et al.*, 1977; White and Lee, 1980). Major anthropogenic sources include the burning of coal, coke productions, automobiles, commercial incinerators, and wood-burning stoves (Lesage and Jackson, 1992). There are hundred compounds of PAHs, which occur in a complex mixture in the environment. The United States Environmental Protection Agency (US EPA) selected 16 PAHs, which are frequently found in the

environmental samples including the food chain. These 16 PAHs were considered as priority pollutants by US EPA priority pollutant list (Mumtaz *et al.*, 1996).

Polycyclic aromatic hydrocarbons enter the food chain by various routes such as atmospheric deposition, transfer from the soil, and deposition and transfer from water. Additionally, PAHs are formed in food directly by conventional processing such as cooking, drying, smoking, grilling, and frying (Phillips, 1999; Camargo and Toledo, 2002; Simko, 2002). Food consumption is the main source of PAHs intake. Therefore, the importance of research on PAHs in food has been emphasized, and the improvement of qualified approaches to reduce their contents in our food (SCF, 2002) is a matter of great concern nowadays.

There are several methods that can be employed for the removal of various classes of pollutants from contaminated environment, such as advanced oxidation processes (AOPs), which include UV photolysis, photo-catalysis, Fenton reagent and radiolysis of water (Burrows *et al.*, 2002). In addition, the radiation process is one of the most powerful AOPs, where irradiation with a beam of accelerated electrons or gamma radiation is employed for the decomposition of various pollutants like pesticide residues. The radiolytic degradation of pollutants was employed in recent years for the treatment of natural waters and wastes from organic pollution such as PAHs (Ribeiro *et al.*, 2015; Jiménez-Becerril *et al.*, 2016). Gamma irradiation becomes an important technology in the food industry, including the preservation of a variety of fruits and vegetables (Thayer, 2004). Additionally, the decontamination of food by ionizing radiation is a safe, efficient, environmentally clean and energy efficient process (Farkas, 1998). Many studies investigated the food irradiation, and monitored PAHs in food (Rey-Salgueiro *et al.*, 2009; Yebra-Pimentel *et al.*, 2012; Zelinkova and Wenzl, 2015; Li *et al.*, 2016). Nevertheless, limited studies focused on the effect of gamma irradiation on the removal of polycyclic aromatic hydrocarbons from food (Khalil and Al-Bachir, 2015; Khalil *et al.*, 2016).

In low-income developing countries, legumes such as peas are considered the main source of protein and energy because of the high price of meat, and the consumption of these legumes per capita has increased in recent years. The current study highlights the use of gas chromatography-mass spectrometry (GC-MS) in investigating the effect of gamma irradiation on the decontamination of pea seeds polluted with 16 PAHs. The peak heights were used to point out the changes in the studied PAHs. Furthermore, the irradiation effects on the microbial load of pea seeds have been investigated.

Materials and methods

Sampling and irradiation treatments

Pea (*Pisum sativum*) seeds (crop year 2015/2016) were collected, samples were dried and transferred (about 20 g of seeds) into plastic bags for irradiation treatment using different doses of gamma rays (1, 5, 10 and 15 kGy) in a ^{60}Co package irradiator (ROBO, Russia) (dose rate 730 Gy. h^{-1}). Middle doses (around 1 kGy) and high doses (more than 10 kGy) are recommended for both disinfestation

and decontamination of dried food respectively (Farkas and Mohácsi-Farkas, 2011). Irradiation was performed at room temperature (20°C) and the absorbed dose was determined by the measurement of chloride ions or hydrogen ions by means of Oscillotitrator (OK302/2, Radelkisz, Budapest, Hungary) using alcoholic chlorobenzene dosimeter. Ethanol chlorobenzene was prepared by mixing 24 ml chlorobenzene, 4 ml distilled water, 0.04 ml acetone and 0.04 ml benzene to 100 ml ethanol.

Chemicals and reagents

The mixed standard solution of 16 EPA PAHs (0.1 mg L^{-1}) was purchased from AccuStandard (New Haven, USA), and consists of 16 PAHs namely: Naphthalene (NAP), Acynaphtalene (ACY), Acynyphtalene (ACP), Fluorene (FLR), Phenanthrene (PHE), Anthrancene (ANT), Fluoranthene (FLT), Pyrene (PYR), BenzoAnthrancene (BaA), Chrysene (CHR), Bezo[b]Fluoranthene (BbF), Bezo[k]Fluoranthene (BkF), Benzo[a]Pyrene (BaP), Dibenzo[ah]Anthrancene (DhA), Benzo[ghi]Perylene (BgP) and Indeno[1,2,3-cd]Pyrene (ICP). The working standard solution was prepared in acetonitrile and stored at 4°C in the dark. Chromatography silica gel (70-230 mesh, 60 Å) was obtained from Sigma-Aldrich (St. Louis, USA). Dichloromethane (DCM), n-hexane and acetonitrile were of high performance liquid chromatography (HPLC) grade and were supplied by Sigma-Aldrich (St. Louis, USA). Ultrapure water was obtained from a high performance water purification system (SG-ultra-clear-distilled water, Siemens Water Technologies GmbH, Barsbüttel, Germany).

PAHs extraction

The extraction of PAHs from pea samples (bran and endosperm) was achieved using solid phase extraction (SPE) with silica cartridges according to the method reported by Moret and Conte (2002). After collection of the PAHs fraction, it was concentrated to near dryness under a nitrogen stream on a rotary evaporator. The residual solvent was allowed to evaporate spontaneously, at room temperature, in order to minimize volatile PAHs losses. Next, the residue was dissolved in 2 mL of acetonitrile and filtered on a 0.45µm filter (syringe) before the injection into GS-MS apparatus.

GS-MS analysis

An Agilent gas chromatography coupled with mass spectrometry (GC-MS model GC-6890) with an inert selective mass detector 5973 was used in PAHs analysis. The capillary column was DB-35 (30x0.2mm, film thickness 0.25 µm). The operating conditions were as follows: carrier gas, helium, with a flow rate of 1 ml /min; the volume injected was 1 µl of sample extract and the ionization mode was electron impact. The GC-MS system was operated under the following conditions: injection temperature 250ºC, source temperature 250°C, fragment energy of 70eV, mass spectra were acquired using an ionization voltage 70ev. The initial temperature of the column was 50°C (held for 2 min), then heated to 170°C at a rate of 2°C/min (held for 7 min), then heated to 250°C at a rate 4°C/min (held for 10 min). The same conditions of temperature programming were used for pea samples to calculate the retention index (RI). The identification of components in

pea seeds was based on RI. Individual components were identified by comparison of both mass spectra and their GC retention data; other PAHs identifications were made by comparison of mass spectra with those in the data system libraries and cited in the literature (Adams, 2007).

Microbiological analysis

The microbial load was determined using standard spread plate method (AOAC, 2010). The product of pea seeds (10 g) was homogenized with 90 ml of sterile physiological water (9 g NaCl L^{-1}). The homogenate was then serially diluted and appropriate dilutions were plated on agar plate counts (APCs) (Oxoid, CM 325, UK) for total bacteria counts (30 ºC, 48 h) and Dichloran Rose-Bengal Chloramphenicol Agar (DRBC) (Merck, 1.00466, Germany) for fungus (25 ºC, 5 days). After plating, the colony forming units (CFUs) were counted, and microbial counts were expressed by means of log CFU.

Statistical analysis

The normality of values distribution was first tested with the Shapiro-Wilk test. Then, one-way ANOVA statistical test using SUPERANOVA computer package (Abacus Concepts Inc, Berkeley, USA) software program (Snedecor and Cochran, 1988) was used to distinguish significance between groups, followed by multiple pairwise comparisons using a post-hoc test (Tukey test). The results were considered statistically significant at $P \leq 0.05$.

Results and discussion

The effect of irradiation on the microbial load of pea seeds

The effects of different doses (5, 10 and 15 kGy) of gamma irradiation on the microbial load contamination of pea seeds by bacteria and fungi are shown in Table 1. The total bacterial count and the total fungal count of non-irradiated (control) sample were 2.43 and 1.66 log cfu g^{-1}, respectively (cfu = colony forming unit). The exposure of green pea seeds to gamma irradiation showed significant ($p<0.05$) reduction of surface microorganisms, and microbial population was below detection limit (less than 1 $\log_{10}$ cfu g^{-1}) at 5, 10 and 15 kGy. Letters (a, b) within each microbial load at different irradiation dose denote significant difference ($P < 0.05$). Literature search revealed that gamma irradiation could effectively reduce the initial microbial load in a variety of commercially valued products (Al-Bachir, 2015; Al-Bachir, 2016; Al-Bachir and Al-Adawi, 2015). Gamma rays cause different degrees of cell damage. Biological damage is mostly indirect, and mediated by reactive oxygen species (ROS), such as hydroxyl radical (HO.), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), single oxygen, generated by water radiolysis (El-Beltagi *et al.*, 2011). The effect of irradiation may be beneficial or could cause damage, depending on the conditions of irradiation (power, intensity, distance and exposure times) and on the characteristics of the biological material to be subjected to the treatment (Paez *et al.*, 2011). Regarding fungi, Calado *et al.* (2011) reported that both yeast and *Aspergillus parasiticus* load on chestnuts did not survive at all when they have been irradiated with a dose of 10

kGy. Aziz *et al.* (2007) reported that irradiation of maize seeds at a dose of 5 kGy inhibited the toxigenic molds and mycotoxin formation in seeds. Reduction in fungi has been achieved in peanuts by using dose of irradiation ranged from 5 to 10 kGy (De Camargo *et al.*, 2012).

Table 1. The effect of gamma irradiation on microbial load of pea seeds

Parameters	Irradiation dose (kGy)				
	control	5	10	15	P-Value
Total bacterial count (log_{10} cfu. g^{-1})	2.43±0.08[a]	<1[b]	<1[b]	<1[b]	**
Total Mold (log_{10} cfu. g^{-1})	1.66±0.10[a]	<1[b]	<1[b]	<1[b]	**
Total Coliform (log_{10} cfu. g^{-1})	<1	<1	<1	<1	

ab Means values in the same column not sharing a superscript are significantly different.

*Significant at p<0.01.

The effect of gamma irradiation on 16 EPA PAHs in pea seeds

For the determination and evaluation of the degree of degradation of 16 EPA PAHs, a GC-MS analysis method of detection has been optimized.

In non-treated pea seed samples, all 16 PAHs were detected in the studied seeds. Their peaks are clearly indicated in the GC-MS graphs (Figure 1). These compounds are NAP, ACY, ACP, FLR, PHE, ANT, FLT, PYR, BaA, CHR, BbF, BkF, BaP, DhA, BgP, and ICP.

As demonstrated in Figure 2, 1kGy of gamma irradiation increased some PAHs of pea seed contents, namely FLR and PYR, while ANT, CHR, BaP were significantly decreased by the effect of the same dose, respectively as their curves indicated. Unexpectedly, the ACY curve could not be detected after the application of 1 kGy of gamma irradiation.

The increase of some PAHs concentration after gamma irradiation with 1kGy could be due to the fact that other PAHs with larger molecules were decomposed, combined and transformed in these PAHs for which higher concentrations were noticed. Previous research studies report that FLR is a main compound resulting from the radiolytic degradation of FLT (Butt and Qureshi, 2008; Popov and Getoff, 2005), which is in agreement with our result presented in Figure 1 that remarkably indicated an increase in FLR peak at 1kGy of gamma irradiation. Zhang *et al.* (2008) have also reported that the diuron (a stable herbicide) degradation decreased by the dose of 1kGy of gamma irradiation, with the increase of the initial concentration.

NAP removal from water by the effect of gamma irradiation was more efficient at a higher initial NAP concentration, and decreased with the increase of the applied dose (Cooper *et al.*, 2002). As shown in Figure 3, a dose of 5 kGy of gamma irradiation completely removed three compounds as their curves were not detectable by the GS system (NAP, ACP and FLR), and significantly decreased nine compounds of PAHs by their curve registration. These PAHs respectively are PHE, ANT, FLT, PYR, BaA, CHR, BbF, BkF and BaP.

A dose of 10 kGy was more effective, and removed three additionally PAHs curves (ACY, PHE and ANT) from the studied pea seeds, while this dose caused a decrease in seven PAHs curves (Figure 4) in irradiated pea seeds versus non-irradiated samples (Figure 1), respectively (FLT), (PYR), (BaA), (CHR), (BbF), (BkF) and (BaP). In this stage, it is important to note that DhA and BgP curves were not affected by both 5 and 10 kGy of gamma irradiation (Figure 3, 4).

In irradiated pea seeds compared to control samples, 15 kGy of gamma dose removed six new PAHs compounds from pea seeds (Figure 5), and reduced more compound curves of PAHs such as BaA, CHR, BkF and BaP, respectively. DhA and BgP peaks, which were not affected by 5 and 10 kGy of gamma dose, were completely removed by 15 kGy dose of gamma irradiation. Figure 5 clearly shows that only 4 PAHs are present in the studied pea seeds, out of 16 PAHs identified in the control samples (Figure 1).

The effect of gamma ray irradiation on PAHs load of pea seeds was varied from one PAHs congener to another. This observation explained that the effect of irradiation on a given compound depends on the composition of food matrix and the amount of energy required to the decomposition of these PAHs in pea samples. However, radiolytic degradation by gamma irradiation of PAHs depends efficiently on their structure, initial concentration and surrounded environment, which explained why different effects of gamma irradiation on PAHs were observed with all used doses (Butt and Qureshi, 2008; Khalil and Al-Bachir, 2015). However, the PAHs contamination rarely consists of a single compound, but rather of a mixture of compounds that can affect the environment (Popov and Getoff, 2005). The highest dose of gamma irradiation (15kGy) was the most effective dose and caused a significant decrease in all PAHs peaks with a percentage of removal ranging from 80 to 100% of GC analysis peaks. This result is in agreement with our finding in wheat kernels, where 15 kGy of gamma irradiation reduced all 16 EPA PAHs from 154 to 21 $\mu g.kg^{-1}$ (Khalil and Al-Bachir, 2015). Actually, this decrease in PAHs concentrations at such a low dose was important, since high doses of irradiation have been applied for decontamination and improving the hygienic quality of dried food. Pea seeds are one of several food groups approved for irradiation in Syria for microbial control, and similarly dried products are cleared up using a 30 kGy dose in different countries (Al-Bachir, 2007; IAEA, 2008).

In fact, the occurrence of PAHs in food has been extensively studied all over the world. Data from surveys indicate that the most abundant PAHs in foodstuffs are the low molecular weight PAHs from the EPA list (particularly those of two or three benzene rings). These PAHs are less relevant from the toxicological point of view and do not contribute to the genotoxic and carcinogenic potential of PAHs (Zelinkova and Wenzl, 2015). PAHs can degrade in different ways including photo oxidation, chemical oxidation, and bioremediation, since these methods are not reliable when applied to food. This monitoring study demonstrated that gamma irradiation appears to be an appropriate way to destroy these compounds without changing food properties.

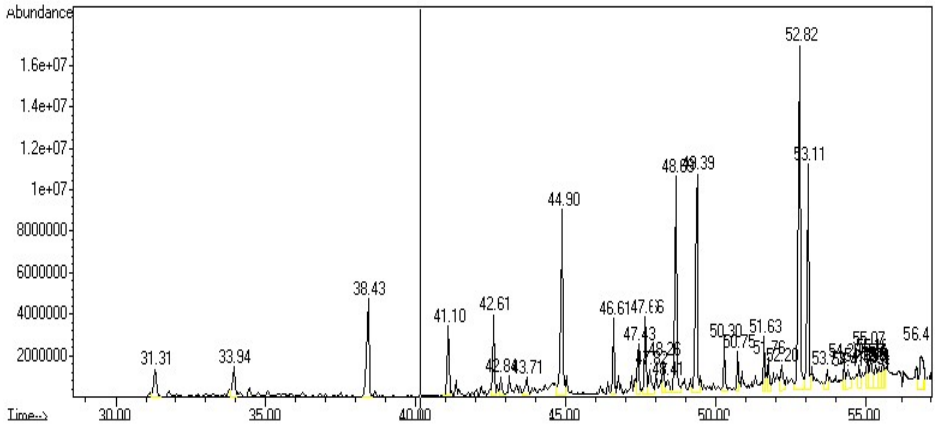

Figure 1. Control: not treated samples of pea seeds; the 16 EPAPAHs are identified according to their retention times (RT) as follow: 31.3 (NAP), 33.9 (ACY), 38.4 (ACP), 41.1 (FLR), 42.6 (PHE), 44.9 (ANT), 46.8 (FLT), 47.4 (PYR), 48.4 (BaA), 49.3 (CHR), 50.3 (BbF), 51.6 (BkF), 52.8 (BaP), 53.11 (DhA), 55.4 (BgP), 56.4 (ICP).

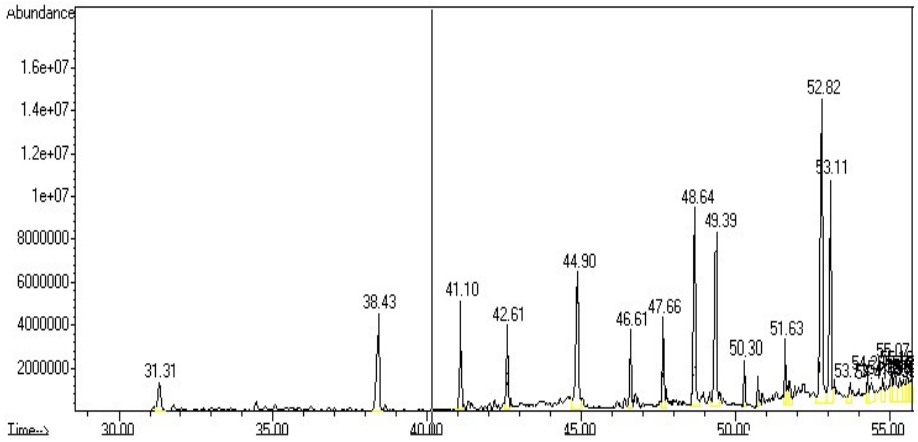

Figure 2. Effect of 1 kGy dose of gamma irradiation on 16 EPAPAHs in pea seeds, 15 EPAPAHs are seen according to their RT by the GC-MS graph analysis: 31.3 (NAP), 38.4 (ACP), 41.1 (FLR), 42.6 (PHE), 44.9 (ANT), 46.6 (FLT), 47.6 (PYR), 48.6 (BaA), 49.3 (CHR), 50.3 (BbF), 51.6 (BkF), 52.8 (BaP), 53.11 (DhA), and 55.4 (BgP).

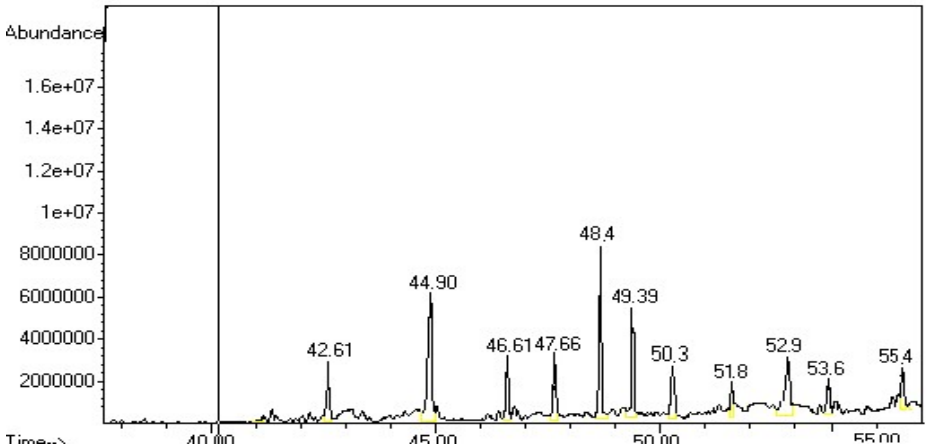

Figure 3. Effect of 5 kGy dose of gamma irradiation on 16 EPAPAHs in pea seeds, 11 EPAPAHs are seen according to their RT by the GC-MS graph analysis: 42.6 (PHE), 44.9 (ANT), 46.6 (FLT), 47.6 (PYR), 48.4 (BaA), 49.3 (CHR), 50.3 (BbF), 51.8 (BkF), 52.9 (BaP), 53.6 (DhA), and 55.4 (BgP).

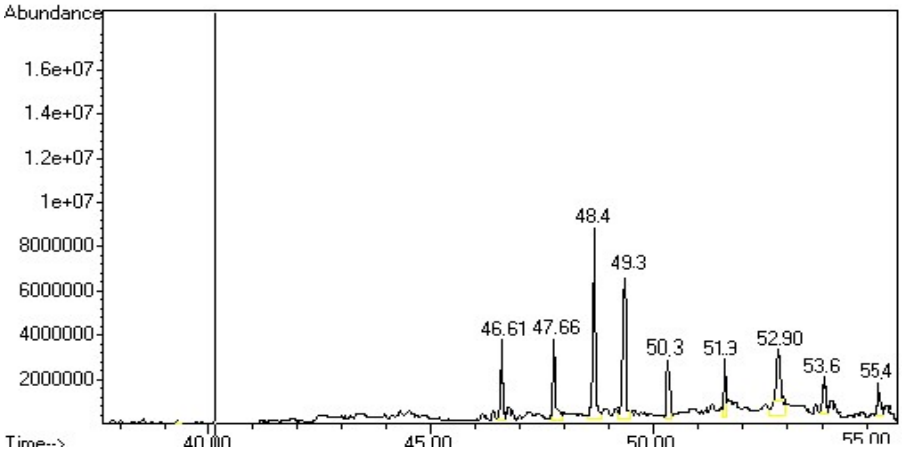

Figure 4. Effect of 10 kGy dose of gamma irradiation on 16 EPAPAHs in pea seeds, 9 EPAPAHs are seen according to their RT by the GC-MS graph analysis: 46.6 (FLT), 47.6 (PYR), 48.4 (BaA), 49.3 (CHR), 50.3 (BbF), 51.3 (BkF), 52.9 (BaP), 53.6 (DhA), 55.4 (BgP).

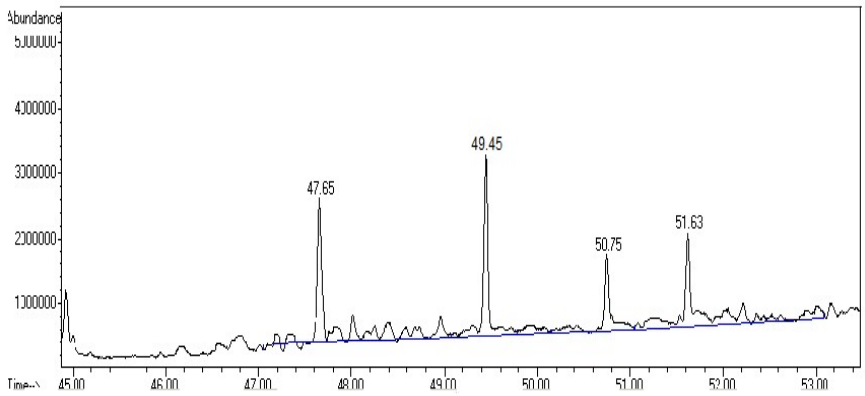

Figure 5. Effect of 15 kGy dose of gamma irradiation on 16 EPAPAHs in pea seeds, 4 EPA PAHs are seen according to their RT by the GC-MS graph analysis: 47.6 (BaA), 49.4 (CHR), 50.7 (BkF), and 51.6 (BaP).

Conclusions

The present study revealed that GC-MS analysis could be successfully employed to observe the effect of gamma irradiation on the decontamination of pea seeds from PAHs contaminates, by monitoring the peaks of the GC-MS chromatograms. The highest dose (15 kGy) has demonstrated effective treatment for PAHs pea seed load decontamination. Moreover, gamma rays can easily penetrate within legumes and food samples. The results of this work showed that gamma irradiation could be efficiently used for decontamination PAHs compounds, where their elimination seems difficult using other techniques because of their structure and their high immobility in foods. Further quantitative and qualitative studies are required on PAHs from different food sources, with wider irradiation dose ranges, to confirm the fate of each PAHs reduction due to gamma irradiation treatment.

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