Faculty of Biotechnology

Graduation Project

RS-401

QuEChERS Method Followed by dispersed Solid Phase Extraction Method for Gas Chromatographic-Mass Spectrometric Determination of Polycyclic Aromatic Hydrocarbons in Milk

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I. Abstract

Polycyclic aromatic hydrocarbons (PAHs) commonly refers to a large class of organic compounds containing two or more fused aromatic rings made up of carbon and hydrogen atoms. PAHs are formed and released during incomplete combustion or pyrolysis (burning) of organic matter such as waste or food, during industrial processes, fuel burning and other human activities. PAHs are also formed in natural processes, such as carbonization. This study aimed to determine the concentrations of polycyclic aromatic hydrocarbons (PAHs) in both commercial packed milk and raw milk from several cities all over Egypt. It was found that the concentrations of PAHs in the raw milk was higher than commercial packed milk. A number of PAHs have shown carcinogenic effects in experimental animals and it has been concluded that benzo[a]pyrene is carcinogenic to humans. The analysis was carried using a modified QuEChERS procedure followed by injection on gas chromatography coupled to tandem mass spectrometry. The results showed that 88% of packaged milk contained one or more compound of PAH, and in raw milk 93% of the samples contained one or more compound of PAH.

The specificity and sensitivity of GC/MS technique was highly significant of approximately 99%. The applicability of gas chromatography was clear, mostly allPAHs compounds were detected and analyzed properly.

Keywords:PAHs, Gas Chromatography, QuEChERS Method, and dispersed Solid Phase Extraction Method.

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List of Abbrevviations:

No.	Abbreviations	Full name
1	PAH	Polycyclic Aromatic Hydrocarbons
2	WHO	World Health Organization
3	SCF	Scientific Committee on Food
4	UV	Ultra violet
5	QCAP	The central laboratory of residue analysis of pesticides and heavy metals in food
6	HACCP	Hazard Aalysis Critical Control Points
7	IAEA	International Atomic Energy Agency
8	ATSDR	Agency for Toxic Substances and Diseases Registry
9	DNA	Deoxyribonucleic acid
10	GC/MS	Gas chromatography mass selective detector
11	APPI	Atmospheric Pressure Photoionization
12	IQ	Intelligence quotient
13	EU	European Union
14	SPE	Solid Phase Extraction
15	n.d.	Not detected
16	LOQ	Limit of quantification
17	LOD	Limit of detection
18	IARC	International Agency for Research on Cancer
19	AA-EQS	Annual Average Environmental Quality Standards
20	EPA	Environmental Protection Agency

III. Introduction

The term ‘polycyclic aromatic hydrocarbons’ (PAHs) commonly refers to a large class of organic compounds containing two or more fused aromatic rings made up of carbon and hydrogen atoms. PAHs are formed and released during incomplete combustion or pyrolysis (burning) of organic matter such as waste or food, during industrial processes, fuel burning and other human activities. PAHs are also formed in natural processes, such as carbonization. The general characteristics common to these chemicals are high melting and boiling points, low vapor pressure and very low water solubility which tend to decrease with increasing molecular mass. PAHs are soluble in many organic solvents and therefore, lipophilic (soluble in fat).

Furthermore, a variety of PAHs are naturally present in crude oil (0.2–7 %) and coal, with configurations ranging from two to six rings and arising from the chemical conversion of natural precursors for PAHs, such as steroids and terrenes (Harvey 1998; Albers 2002; Feng *et al*., 2009; Pampanin and Sydnes 2013). PAHs were one of the first atmospheric pollutants to be identified as being carcinogenic (ATSDR 1995; WHO 2006). Their presence in food implies a potential risk to human health according to the International Agency for Research on Cancer (IARC) (IARC 2012). Additionally, the Food and Agriculture Organization of the United Nations (FAO/WHO) (WHO 2006) and the Scientific Committee on Food (SCF) (EC 2002) consider PAHs to be genotoxic and carcinogenic and recommend monitoring the presence of PAHs in food.

The main techniques used for the detection and quantification of PAHs from food matrices include gas chromatography (GC) coupled to mass spectrometry (MS) and high performance liquid chromatography (HPLC) with fluorescence detection (FL). HPLC coupled to MS or GC tandem MS (MS/MS) and time of flight (TOF) are also used. Fat extraction and clean-up steps are often required for the determination of PAHs from complex matrices such as food. The applications of both conventional and recently developed extraction and clean-up techniques, such as pressurized liquid extraction (PLE) and QuEChERS (quick, easy, cheap, effective, rugged and

safe), are presented. For the clean-up step, solid-phase extraction (SPE), including conventional SPE, dispersive SPE, solid-phase micro-extraction (SPME) and molecularly imprinted polymer (MIP) SPE, as well as gel permeation chromatography (GPC) and donor–acceptor complex chromatography (DACC). Sixteen PAHs are considered particularly important regarding the environmental monitoring of organic pollutants (EPA 1986).

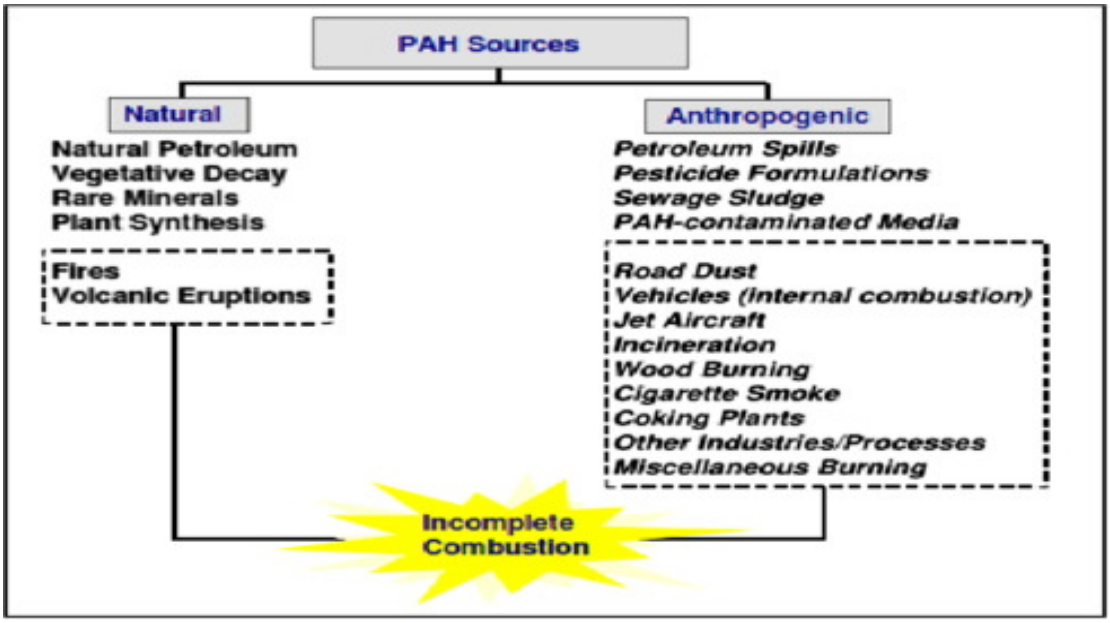


Figure 1: Sources of PAHs, (Abdel-Shafy and Mansour, 2016).

1. Physicochemical Properties of PAHs

Knowledge of the physico-chemical properties of the PAHs (Table 1) allows for an understanding of the distribution profile of these contaminants in food matrices, as well as their interactions and stability, which will guide the choice of strategies used for their extraction from food. In the environment, PAHs occur as compounds containing between two and seven conjugated rings and have molar masses ranging from 128 to 302 g/mol (Ming-Ho 2005). According to the United States Environmental Protection Agency, the physico-chemical characteristics of these PAHs, such as solubility and vapor pressure, are important factors that drive the distribution of these contaminants in soluble and particulate phases throughout atmospheric, aqueous and biotic media. In general, the solubility of PAHs in water decreases with increasing molecular weight and ranges from highly insoluble, such as for benzo(g.h.i)perylene

(solubility 0.0003 mg/L), to slightly soluble, such as for naphthalene (solubility 31 mg/L). PAHs can also be classified as moderately to highly soluble, with octanol-water partition coefficients (log KOW) ranging between 3.37 and 7.10 (IPCS 1998; Meire *et al*., 2007). Most PAHs are of low volatility and have a high tendency to adsorb onto organic particulate matter. In the atmosphere, PAHs containing five or more aromatic rings are found predominantly in association with particulates, usually on small (2.5 um) particles, such as fly ash and soot. PAHs with two or three rings are almost entirely found in the vapor phase, whereas those with four rings exhibit an intermediate behavior (EC 2002). In aqueous environments, PAHs are generally found adsorbed on particulates and humic matter or dissolved in any oily contaminant that may be present in the water, sediment or soil (EFSA 2008b). PAHs are chemically stable and resistant to degradation by hydrolysis. In the presence of light, they are susceptible to oxidation and photo-degradation. Depending on various parameters (such as the type of adsorption onto particles and molecular mass), the half-lives of PAHs in air range from a few hours to days. In soil, PAHs may also be degraded by microbial activity. The estimated half-lives of PAHS in soils vary from several months to several years (Harvey 1998; EC 2002).

Table 1: Nomenclature, CAS Reg. No., Synonyms and Structural formula of 16 PAHs.

Nomenclature	***CAS Reg.No.**	**Synonym (s)**	**Chemical Formula**
Acenaphthene	83-32-9	1,8-dihydroacenaphthene; 1,2-dihydroacenaphthylene	$C_{12}H_{10}$
Acenaphthylene	208-96-8	Cyclopenta[d,e]naphthalene	$C_{12}H_8$
Anthracene	120-12-7	Anthracin; para-naphthalene	$C_{14}H_{10}$
Benz[*a*]Anthracene	56-55-3	1,2-Benz[*a*]anthracene; benzanthracene	$C_{18}H_{12}$
Benzo[*a*]pyrene	50-32-8	BaP;benzo[*def*]chrysene; benz[*a*]pyrene	$C_{20}H_{12}$
Benzo[*b*]fluoranthene	205-99-2	3,4-Benz[*e*]acephenanthrylene	$C_{20}H_{12}$
Benzo[*ghi*]Perylene	191-24-2	1,12-Benzoperylene; 1,12-benzperylene	$C_{22}H_{12}$
Benzo[*k*]fluoranthene	207-08-9	8,9-Benzfluoranthene; 11,12-benzofluoranthene	$C_{20}H_{12}$
Chrysene	218-01-9	Benzo[*a*]phenanthrene; 1,2-benzophenanthrene	$C_{15}H_{12}$
Dibenz[*a,h*]Anthracene	53-70-3	dibenzo[*a,h*]anthracene, 1,2:5,6-Benzanthracene	$C_{22}H_{14}$
Fluoranthene	206-44-0	1,2-Benzacephthalene	$C_{16}H_{10}$
Fluorene	86-73-7	diphenylenemethane; 2,2′-methylenebiphenyl	$C_{13}H_{10}$
Indeno[1,2,3-*cd*]pyrene	193-39-5	1,10-(*ortho*-Phenylene)pyrene; 1,10-(1,2-phenylene)pyrene	$C_{22}H_{12}$
Naphthalene	91-20-3	Naphthalene	$C_{10}H_8$
Phenanthrene	85-01-8	Phenanthrin	$C_{14}H_{10}$
Pyrene	129-00-0	Benzo[*def*]phenanthrene;β-pyrene	$C_{16}H_{10}$

Table 2: Physico-chemical properties and classification by the International Agency for Research on Cancer (IARC) regarding the carcinogenicity of the sixteen polycyclic aromatic hydrocarbons

PAHs	No of rings	Molecular weight (g/mol)	Solubility in water at 25 °C (mg/L)	Vapor pressure 25 °C (Pa)	Octanol-water partition coefficient Log KOW (−)	IARC group
Naphthalene	2	128	3.1×10^{1}	1×10^{1}	3.37	2B
Acenaphthylene	3	152	3.9	9×10^{-1}	4.00	Not classified
Acenaphthene	3	154	3.8	3×10^{-1}	3.92	3
Fluorene	3	166	1.9	8×10^{-2}	4.18	3
Phenanthrene	3	178	1.1	2×10^{-2}	4.57	3
Anthracene	3	178	4.5×10^{-2}	8×10^{-4}	4.54	3
Fluoranthene	4	202	2.6×10^{-1}	1.2×10^{-3}	5.22	3
Pyrene	4	202	1.3×10^{-1}	6×10^{-4}	5.18	3
Benzo(a) anthracene	4	228	1.1×10^{-2}	2.8×10^{-5}	5.61	2B
Chrysene	4	228	2.0×10^{-3}	8.4×10^{-5}	5.91	2B
Benzo(b) fluoranthene	5	252	1.5×10^{-3}	1.3×10^{-7}	5.80	2B
Benzo(k) fluoranthene	5	252	7.6×10^{-4}	1.3×10^{-7}	6.84	2B
Benzo(a)pyrene	5	252	3.8×10^{-3}	7.3×10^{-7}	6.50	1
Indeno(1,2,3-c, d)pyrene	6	276	6.2×10^{-2}	1.3×10^{-8}	6.58	2B
Dibenzo(a,h) anthracene	5	278	6.0×10^{-4}	1.3×10^{-8}	6.50	2A
Benzo(g,h,i) perylene	6	276	2.6×10^{-4}	1.4×10^{-8}	7.10	2B

2. Toxicity of PAHs

The toxicological properties of PAHs highlight the importance of determining these compounds in food, even at very low levels. PAHs are the most extensively studied compounds with regard to their cancer-inducing properties in laboratory animals. Researchers have shown that certain PAHs can cause cancer when inhaled, ingested and even after skin contact. Immunosuppression, hepatic hypertrophy and changes in the growth of other tissues have also been reported in laboratory animals (ATSDR 1995). The evidence of carcinogenicity in humans comes from occupational studies in workers exposed to known sources of PAHs, such as oil refineries, charcoal ovens and chimneys (ATSDR 1995; WHO 2006).

Many PAHs are considered toxic even at low concentrations. Lower molecular weight compounds consisting of two or three rings (such as naphthalene, anthracene, and phenanthrene) have high acute toxicity but little or no carcinogenic potential. Conversely, compounds with higher molecular masses that consist of four, five or six rings (such as benzo(a)pyrene) have low acute toxicity but greater 70 R. Pissinatti and S.V.C. de Souza carcinogenic potential (Meire *et al*., 2007). Acute effects affect mainly the liver and kidneys, leading to cutaneous inflammation, ulcerations and hyperkeratosis. Changes in the lymph nodes and immunosuppressive induction have also been reported (WHO 2006).

In 2012, the IARC updated the classification of benzo(a)pyrene to Group 1-Carcinogenic to humans (IARC 2012). This group includes 116 agents (or mixtures) with sufficient evidence of carcinogenicity in humans. In Table 4, the classification of the sixteen PAHs prioritized by the EPA is presented. PAHs are considered to be pro-carcinogenic, i.e., they must be altered by metabolic activation to generate the active carcinogen capable of reacting with DNA and other molecules (Ming-Ho 2005). The carcinogenicity of PAHs is associated with the complexity of the molecule, i.e., the number of benzene rings present (please refer to table 4). The mechanism of toxicity involves the oxidation of the aromatic rings by the enzymes of the cytochrome P450

family, which generates epoxide intermediates that covalently bind to critical sites in the DNA, causing replication errors and allowing mutation to occur (Boström *et al.*, 2002; EC 2002). The main sites are the amino groups of adenine and guanine (WHO 2006). Some PAHs and their metabolites also bind the aryl hydrocarbon receptor, resulting in the up-regulation of several enzymes involved in the metabolism of PAHs and exhibiting complex and non-linear dose-response curves when mixtures of PAHs are present (WHO 2006).

This phenomenon hinders the establishment of safe limits for the intake of these contaminants. Thus, a minimum safe threshold for chronic exposure has not been established by international agencies and health authorities, such as the Agency for Toxic Substances and Disease Registry (ATSDR), the SCF and the EFSA. Instead, these agencies recommend that exposure to PAHs be as low as is reasonably achievable (Bulder *et al*., 2016). Other international organizations have calculated a so-called “virtually safe dose” based on extrapolating the data obtained from experiments with laboratory animals. The EPA associates an intake of 0.14 ng/kg body weight/day of benzo(a) pyrene with a cancer risk of 1 10−6 . The Dutch National Institute of Public Health and Environment (RIVM) calculated the virtually safe dose for the same cancer risk to be 0.50 ng/kg body weight/day (Bulder *et al*., 2006).

3. PAH in food

3.1 Contaminations and occurrence

There are several routes of food contamination by PAHs, and contamination can arise from both environmental pollution (fruits, vegetables and grains grown in industrial regions) and food processing (such as smoking and roasting) (Camargo and Toledo 2002; Tfouni *et al*., 2012). PAHs from environmental pollution have both natural sources (bitumens, coal, forest and prairie fires, oil seeps, plant debris) and anthropogenic sources (fossil fuels and combustion). Since the PAH compositions of the two sources overlap, the significance of anthropogenic PAH in the

environment have been evaluated against a dynamic background of natural PAH (Yunker *et al*., 2002). Some authors have proposed the estimation of isomeric ratios to predict the source of the contamination (Budzinski *et al*., 1997; Yunker *et al*., 2002; Mannino and Orecchio 2008; Orecchio 2010). The values of ratios between anthracene (An) and anthracene plus phenanthrene (An + Phe) when lower than 0.10 indicate low temperature sources (petroleum) while ratios bigger than 0.10 indicate a dominance of combustion (Yunker *et al*., 2002), because phenanthrene and anthracene are isomers and phenanthrene is more thermodynamically stable than anthracene (Budzinski *et al*., 1997). BaA/ (BaA + Chr) ratios lower than 0.20 involve petroleum, from 0.20 to 0.35 indicate either petroleum or combustion and upper than 0.35 combustion. Also, IP/ (IP + BghiP) ratios between 0.20 and 0.50 imply liquid fossil fuel combustion (Yunker *et al*., 2002). The ratio between fluoranthene (Fl) and fluoranthene plus pyrene (Fl + Py) have also been applied for PAHs source typing (Budzinski *et al*., 1997).

In vegetables and fruits, PAH contamination mainly occurs via the deposition of particles from air pollution on their surfaces. The concentrations depend on the location of cultivation and production, and samples cultivated in highly industrialized areas or near roads and express highways generally have higher levels of PAHs than those cultivated in rural areas. The association between PAHs with higher molecular masses and suspended particulate matter in the atmosphere is a major source of contamination (Nielsen *et al.*, 1999). The concentrations of PAHs are generally higher at the surfaces of plants, such as fruits shells and edible leaves, than in the internal tissues. The higher levels of PAHs found in plants grown in areas with polluted air are especially evident in plants with a large exposed surface area, for example, lettuce, cabbage and spinach (EC 2002).

During the process of smoking, PAHs may be deposited on the surface and migrate into the food that is being smoked. Numerous factors in the smoking process influence the composition of the smoke and the absorption of PAHs by smoked food, with the combustion temperature

during the generation of the smoke being a particularly critical parameter (Wretling *et al*., 2010). In general, due to the lipophilic properties of PAHs, oils and fats are more susceptible to concentrated contamination from environmental sources or during the process of seed drying prior to oil extraction. Smoked foods, such as meat and fish, can also have high levels of PAH contamination. However, the contribution of these foods to the total ingested PAHs is not significant because the levels of these products in the population's diet are usually low (Codex Alimentarius 2009). A survey published in 2008 by EFSA, which involved data from sixteen European countries, indicated that fish and seafood, vegetable oils, meat, coffee and tea, food supplements and spices as the main food groups susceptible to PAH contamination. Those types of food presented levels exceeding 10 µg/kg for the sum of the following eight PAHs: benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-c,d)pyrene, dibenzo (a,h)anthracene and benzo(g,h,i)perylene (PPAH8). These results suggest that attention should be paid to coffee and tea, food supplements and spices because there are no current regulations for the PAHs levels in these food matrices (EFSA 2008a, b).

Additionally, Studies conducted in Italy (Lodovici *et al*., 1995) highlighted the fact that exposure to PAHs from contaminated food intake is significantly higher than environmental exposure or exposure through inhalation or skin absorption. According to the EFSA Report, the diet is the largest non-occupational source of PAH exposure for non-smokers. In this European study, based on the dietary profiles of the 16 countries involved, an average dietary exposure for the whole population (including the non-consumers) would provide 235 ng/day of benzo(a) pyrene and 1729 ng/day of all eight PAHs, whereas a diet high in PAHs would provide 389 ng/day of benzo(a)pyrene and 3078 ng/day of all eight PAHs. The products with the largest contributions to PAH exposure, considering the median consumption estimated for consumers only, were seafood, cereals, vegetables, meat, oils and fats, fish and coffee in terms of both benzo(a)pyrene alone and

all eight PAHs. It must be emphasized that, in this study, the number of cereal and coffee samples was significantly lower than the other matrices studied (EFSA 2008a, b). In this context, the use of benzo(a)pyrene as a marker for the presence of PAHs in food, as proposed by the SCF (EC 2002), is controversial. The survey data collected by the EFSA (EFSA 2008a, b) also show that the concentrations of benzo (a)pyrene and other PAHs, such as pyrene and benzo(a)anthracene, which are also considered toxic, presented a low correlation in products such as fish, crustaceans, tea and coffee. Among 9714 samples comprising 95 groups of Codex Alimentarius food matrices, 33 % had values exceeding the detection limit for one or more priority PAH, and benzo(a)pyrene was not detected.

According to the report published by the Agence Française de Sécurité Sanitaire des Aliments (AFSSA 2004), in which PAHs occurrence data from more than eight thousand samples in 44 different food groups were compiled, the highest levels of benzo(a)pyrene were found in dried fruits (48.10 µg/kg wet weight), olive pomace oil (17.7 µg/kg), smoked fish (5.28 µg/kg), grape seed oil (4.2 µg/kg), smoked meat products (3.27 µg/kg), fresh mollusks (3.09 µg/kg), and spices/sauces and condiments (2.16 µg/kg). Several studies have reported evidence of increased PAH concentrations in seafood after oil spills compared with baseline levels. According to Law (2002), examined PAH levels based on datasets covering background levels and seafood after oil spills from 19 studies and found average total PAH concentrations between 20 and 1600 µg/kg in baseline monitoring studies and between n 104 and 27,400 µg/kg after different oil spills. Studies of oils spills suggest that several factors may play a role in determining the duration of PAH contamination, including the amount of sedimentation and the likelihood of subsequent resuspension of the oil, the composition of the oil, the rate of biodegradation (which tends to be higher in warmer climates), and the particular species of interest (Gohlke *et al*., 2011). Among fishery products, finfish species (i.e., tuna, mackerel and salmon) generally present lower contamination levels than shellfish (i.e., bivalve mollusks), even if they originated from polluted

areas. Indeed, unlike bivalves, fish oxidize and metabolize PAHs to water-soluble compounds, which are eventually excreted (EU 2011b; Gohlke *et al.*, 2011; Purcaro *et al.*, 2013).

3.2 Regulations

Until 2011, the maximum permitted level described in European legislation was applied only for benzo(a)pyrene. The report published by the EFSA (2008a, b) concluded that benzo(a)pyrene was not a suitable marker for the occurrence of PAHs in food and that a system of four specific substances would be appropriate to determine the carcinogenic potency of PAHs in food. Thus, the European regulation 1881/2006 was amended by the regulation 835/2011 (EU 2011b) with regard to the permitted levels for benzo(a)pyrene only, as well as for the sum of benzo(a)pyrene, chrysene, benz(a)anthracene, and benzo(b)fluoranthene (PPAH4).

The regulation EU/835/2011 (EU 2011b) also updated the matrices to be monitored based on new occurrence data. Therefore, it was concluded that maintaining a maximum level for PAHs in fresh fish (muscle) was no longer appropriate, as PAHs are quickly metabolized in fresh fish. Other limits were added, namely, for cocoa beans and derivatives and coconut oil (limits described in terms of fat). Other matrices affected by this legislation are as follows: oils and fats, smoked products, bivalve mollusks and baby food for infants and young children. For the latter, the maximum permitted levels were set at 1.0 µg/kg (benzo(a)pyrene and PPAH4) and ranged up to 6 µg/kg (benzo(a)pyrene) or 35 µg/kg (PPAH4) for bivalve mollusks.

3.3 PAHs in Milk

4. Determinations and quantification

Many authors have reported the use of HPLC with a UV/FL detector as the detection and quantification technique for the analysis of PAHs from food (Londoño *et al.*, 2013). Liquid chromatography coupled to MS has also been applied (Cai *et al.*, 2012). However, CG is the most widely used technique for these analytical scopes (Diletti *et al.*, 2015)

According to Poster (2006), for these compounds, GC has better selectivity, resolution and sensitivity compared to liquid chromatography. These authors also report the ease of coupling GC with MS, allowing for the confirmation and quantification of the compounds of interest and indicating that GC is preferable to LC. The thermal properties of PAHs are an important factor: PAHs are readily volatile but are not degraded by the higher temperatures used in GC. Typical chromatograms obtained for PAHs in food matrices by GC coupled to MS. A study of the mass spectra of PAHs was carried out by Veyrand *et al*., (2007), who aimed to develop a method for the analysis of contaminants in food. According to these authors, the minimal fragmentation observed when using electron ionization under conventional conditions is attributable to the stability of PAH molecules, which results in a highly intense molecular ion signal and the presence of ions with the loss of two hydrogens. The same observation was reported by (Poster *et al.*,2006), who concluded that this stability makes PAHs amenable to GC. However, this characteristic makes the use of tandem MS, in which the precursor ion is fragmented into specific product ions, problematic. As a result, very few works have described the use of MS/MS for PAH analysis (Ballesteros *et al.*, 2006; Veyrand *et al.*, 2007; Smoker *et al.*, 2010).

HPLC coupled to MS is more commonly used for environmental samples than food samples. The higher detection limits needed for environmental samples can be easily achieved using this technique. The most commonly used ion sources in LC– MS analysis are electro spray ionization (ESI) and atmospheric-pressure chemical ionization (APCI). However, such sources are inefficient for non-polar compound ionization. Post-run chemical derivatization was proposed by several authors to overcome this problem, but this technique may result in deposits in the instrument and create the need for more frequent maintenance. The atmospheric-pressure photoionization (APPI) ion source extends the range of ionizable compounds to many non-polar substances. Furthermore, this type of interface shows less ion suppression than APCI or ESI. Additionally, to further increase the ionization efficiency, a dopant (e.g., acetone or toluene) can

be used (Purcaro *et al*., 2013). Thus, the low sensitivity of HPLC–MS has only recently been overcome.

4.1 Gas Chromatography

Gas chromatography (GC) is a widely applied technique in many branches of science and technology. For over half a century, GC has played a fundamental role in determining how many components and in what proportion they exist in a mixture. However, the ability to establish the nature and chemical structure of these separated and quantified compounds is ambiguous and reduced, and requires a spectroscopic detection system. The most used, is the mass spectrometric detector (MSD), which allows obtaining the "fingerprint" of the molecule, i.e., its mass spectrum. Mass spectra provide information on the molecular weight, elemental composition, if a high resolution mass spectrometer is used, functional groups present, and, in some cases, the geometry and spatial isomerism of the molecule (Table 3 and 4).

In a gas chromatographic system, the sample to be analyzed may be a liquid solution or a collection of molecules adsorbed on a surface, e.g., the solid-phase microextraction (SPME) system. During the transfer into the GC, the sample is volatilized by rapid exposure to a zone kept at relatively high temperature (200-300°C) and mixed with a stream of carrier gas (Ar, He, N2 , or H). The resulting gaseous mixture enters the separation section, a chromatographic column, which in its current version is a fused-silica tubular capillary coated internally with a thin polymer film. Upon their displacement through the column, analyte molecules are partitioned between the gas carrier stream (mobile phase) and the polymer coating (stationary phase), to an extent which depends mainly on their chemical structure. At the end of the separation section, the molecules reach a detection system in which a specific physical property (thermal conductivity) or a physico-chemical process (ionization in a flame, electron capture) gives rise to an electric signal which is

proportional to the amount of molecules of the same, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. identity. A data system permits to process these data to produce a graph of the variation of this detector signal with time (chromatogram). Thus, four principal sections are distinguish- able in the chromatograph: introduction (injector), separation (chromatographic column), detection, and data handling units.

Table 3: Represents physical, chemical properties of 16 Polycyclic Aromatic Hydrocarbons (PAHs) under study.

Name	**Molecular Weight (g/mole)**	**Melting point (^{O}C)**	**Boiling Point (^{O}C)**	**Log K_{ow}**	**Vapor Pressure (mm Hg)**
Acenaphthene	154.2	95	96.2	3.98	$4.47x10^{-3}$
Acenaphthylene	152.2	92-93	275	4.07	0.029
Anthracene	178.2	218	340	4.45	$1.7x10^{-5}$
Benz[*a*]Anthracene	228.29	158-159	435	5.61	$2.2x10^{-8}$
Benzo[*a*]Pyrene	252.3	179	312	6.06	$5.6x10^{-9}$
Benzo[*b*]Fluoranthene	252.3	168	481	6.04	$5x10^{-7}$
Benzo[*g,h,i*]Perylene	276.34	273	550	6.5	$1.03x10^{-10}$
Benzo[*k*]Fluoranthene	252.3	215.7	480	6.06	$9.59x10^{-11}$
Chrysene	228.3	255-256	448	5.16	$6.3x10^{-7}$
Dibenz[*a,h*]Anthracene	278.35	262	524	6.84	$1x10^{-10}$
Fluoranthene	202.26	11	375	4.9	$5x10^{-6}$
Fluorene	166.2	116-117	295	4.18	$3.2x10^{-4}$
Indeno[1,2,3-*cd*]Pyrene	276.3	163.6	530	6.58	$1x10^{-11}$
Naphthalene	128.17	80.5	218	3.5	0.087
Phenanthrene	178.2	100	340	4.45	$6.8x10^{-4}$
Pyrene	202.3	156	400	4.88	$2.5x10^{-6}$

(IARC Monographs, volume 92, 2010)

Table 4: Selective ions monitoring (SIM) of 16 PAHs

Compound(s)	**Target compound monitored SIM ions (*m/z*)**		
	Quantifier	**Qualifier (1)**	**Qualifier (2)**
Acenaphthene	153	154	152
Acenaphthylene	152	151	150
Anthracene	178	176	179
Benz[*a*]anthracene	228	226	229
Benzo[*a*]pyrene	252	253	250
Benzo[*b*]fluoranthene	252	253	250
Benzo[*g,h,i*]perylene	276	277	274
Benzo[*k*]fluoranthene	252	253	250
Chrysene	228	226	229
Dibenz[*a,h*]anthracene	278	279	276
Fluoranthene	202	203	200
Fluorene	166	165	167
Indeno[1,2,3-*cd*]pyrene	276	277	274
Naphthalene	128	127	129
Phenanthrene	178	176	179
Pyrene	202	200	203
Pyrene-d_{10}"Surrogate Std."	212	211	208

(Norman D. et al., 2011)

Where qualifiers are used for confirmation of each compound detected and reduce false positive due to interferences.

4.2 Use of Internal Standards

The isotope dilution mass spectrometry (IDMS) technique was first developed during the 1950s for analyzing inorganic elements. In 1970, it was extended to the field of organic chemistry, with applications in trace analysis to determine persistent organic pollutants and in medical tests (Sargent *et al.*, 2002; Mechlinska *et al.*, 2010). ID consists of modifying the natural isotopic composition of a target measured present in the sample by adding a known amount of an isotopically labeled analog (internal standard). In MS, unlike spectrophotometric techniques, there is a fixed relationship between the quantity or concentration of a particular substance and the instrument response. The sensitivity for a given compound may vary over time or in accordance with the calibration of the equipment. These variations are added to variations caused by, for example, losses during extraction or the introduction of the analytical sample into the chromatographic system. Adding the internal standard at the beginning of the analytical procedure allows for compensation of losses and errors throughout the analytical process (Sargent *et al.*, 2002). The most important criterion for selecting a standard substance is that it mimics as closely as possible the physico-chemical properties of the target analyte. This is achieved by using similar molecules that isotopically labeled, especially with 13C, 37Cl or 2 H. Because the amount of internal standard added to the sample is known, the recovery percentage can be calculated and used as an indirect measurement of the target compound recovery (Mechlinska *et al.*, 2010). IDMS is often used for the analysis of PAHs to overcome systematic errors in analysis of PAH. In fact, almost all of the work published using MS have used this technique (Diletti *et al.*, 2005; Liguori *et al.*, 2006; Rose *et al.*, 2007; Veyrand *et al.*, 2007; Danyi *et al.*, 2009; Lund *et al.*, 2009; Orecchio *et al.*, 2009; Belo *et al.*, 2012 Drabova *et al.*, 2012; So-Young *et al.*, 2013; Taylor *et al.*, 2013, Pincemaille *et al.*, 2014; Pissinatti *et al.*, 2014).

Thus, because extraction methods are usually time consuming and laborious and because the volatility of the compounds can result in loss during the extraction procedure, the use of isotope dilution is almost a necessity for this type of analysis, allowing for acceptable accuracy, even at low concentrations (µg/kg). 13C-labeled PAHs are preferred in MS methods due to their relatively high stability compared to deaerated species (Pissinatti *et al*., 2014), but the high cost of these PAHS limits their use. Although a number of 13C-labeled PAH standards are commercially available, there is still a need for 13C-labeled analogues for various PAHs, such as benzo(j)fluoranthene, dibenzo(a,h)pyrene, dibenzo(a,l)pyrene cyclopenta(c,d)pyrene, 5-methylchrysene and benzo(c)fluorine (EFSA 2008a, b). The lack of 13C-labeled standards may partly explain the high variation in the results reported for some PAHs in foods (Rose *et al*., 2007).

5. Hazard Analysis Critical Control Points (HACCP) and other control measures for PAHs

The amount of PAHs formed during cooking or processing of food depends markedly on a number of factors such as food type and heating and cooking methods. The following are some preventative measures that may be applied:

• Avoid direct contact of oil seeds or cereals with combustion products during drying processes

• Select lean meat and fish

• Avoid contact of foods with flames when barbecuing

• Use less fat for grilling

• Cook at lower temperatures for a longer time

These measures result in a significant reduction in the PAH contamination of foods. Broiling, i.e. with the heat source above the food, can significantly reduce PAH levels. Fat should not drip down onto an open flame, sending up a column of smoke that coats the food with PAHs. The use of medium to low heat and placement of the meat further from the heat source can greatly

reduce formation of PAHs. The intensity of flavour is not necessarily associated with the depth of the brown colour of grilled foods. It is therefore, not necessary to overcook the food to get the flavour. Cooking however, must always remain effective as regards inactivation of any possible contaminating bacteria. The PAH contamination of smoked foods can be significantly reduced by replacing direct smoking (smoke developed in the smoking chamber used traditionally in smokehouses) with indirect smoking. The latter is obtained by an external smoke generator, which in modern industrialised kilns, is operated automatically under controlled conditions. Also the use of smoke flavourings is generally considered to be less of a health concern than the traditional smoking process as it may minimise PAH contamination. A smoke flavouring (also known as 'liquid smoke') is produced from condensed smoke, which is then fractionated and purified to remove most PAHs. The waxy surface of vegetables and fruits can concentrate low molecular mass PAHs, mainly through surface adsorption. The concentrations of PAHs are generally greater on the plant surface such as the peel or outer leaves than on internal tissue. Consequently, washing or peeling may remove a significant proportion of the total PAHs. Particle-bound high molecular mass PAHs which remain on the surface are easily washed off, whereas low molecular mass compounds which are in the vapour phase, can penetrate the waxy layer of fruits and vegetables and are less efficiently removed by washing.

IV. Aim of the Study

The aim of this study is to determine the concentrations of polycyclic aromatic hydrocarbons (PAHs) in both commercial packed milk and raw milk from several cities all over Egypt. PAH are a class of 28 complex chemicals that are formed and released during incomplete combustion or pyrolysis (burning) of organic matter such as waste or food, during industrial processes and other human activities. PAHs are also formed in natural processes such as carbonisation. PAH benzo[a]pyrene, have shown various toxicological effects, such as haematotoxicity), reproductive and developmental toxicity and immunotoxicity. A number of PAHs have shown carcinogenic effects in experimental animals and it has been concluded that benzo[a]pyrene is carcinogenic to humans. The analysis will be carried using a modified QuEChERS procedure followed by injection on gas chromatography coupled to tandem mass spectrometry.

V. Material and Methods

This project was a collaboration between MSA University, and Central Lab of Residue Analysis of Pesticides and Heavy Metal in Food (Qcap). This study aimed to use a novel and validated methodology to analyze and monitor the presence of polycyclic-aromatic hydrocarbons in both commercial and raw milk with a high performance device GC-MSD.

6. Materials:

6.1 Samples Collection:

6.1.1 Commercial Packed Milk Sample Locations (Table 5):

Table 5: Packaged milk samples location used

Company	Location	Type of Milk	Sample Code
Lactel Milk	October	Full Cream	1
Lamar	October	Full Cream	2
Lamar	October	Skimmed	3
Juhayna	October	Full Cream	4
Danone	October	Full Cream	5
Juhayna	Giza	Full Cream	6
Juhayna	Giza	Skimmed	7
Danone	Giza	Skimmed	8
Bekhairo	Giza	Full Cream	9
Juhayna (Kids)	Giza	Omega 3	10

Lamar	Giza	Full Cream	11
Lamar	Giza	Skimmed	12
Lamar	Giza	Full Cream	13
Lamar	Giza	Half Cream	14
Juhayna	Abo Rawash	Skimmed	15
Almarai	Abo Rawash	Full Cream	16
Juhayna	Abo Rawash	Full Cream	17
Lamar	Abo Rawash	Skimmed	18
Danone	Abo Rawash	Full Cream	19
Juhayna (Kids)	Abo Rawash	Omega3	20
Lamar	Cairo	Skimmed	21
Juhayna	Cairo	Full Cream	22
Lamar	Cairo	Full Cream	23
Danone	Cairo	Full Cream	24
Juhayna	Cairo	Full Cream	25
Almarai	Cairo	Skimmed	26
Juhayna	Cairo	Full Cream	27
Almarai	Cairo	Full Cream	28

6.1.2 Raw Milk Samples locations (Table 6):

Table 6: Raw milk samples location used

Company	Location	Sample Code
Local SuperMarket	October	1A
Local SuperMarket	October	1B
Local SuperMarket	October	1C
Local SuperMarket	Dokki	1D
Local SuperMarket	Dokki	1E
Local SuperMarket	Dokki	1F
Local SuperMarket	Cairo	1G
Local SuperMarket	Cairo	1H
Local SuperMarket	Giza	1I
Local SuperMarket	Giza	1J
Local SuperMarket	Abo-Rawash	1K
Local SuperMarket	Abo-Rawash	1L
Local SuperMarket	Abo-Rawash	1M
Local SuperMarket	Dokki	1N
Local SuperMarket	October	1O
Local SuperMarket	Giza	1P

6.2 Equipment:

Table 7: Equipment used

Equipment	Model	Company	Country
GC-MS	7010-GC/MS	Angilent Technologies	America
Lab Centrifuge	Sigma 4-16 KS	Sigma	France
Rotary Evaporator	Heidolph WB4000	Heidolph VV2000	America
Micro pipettes	10 µl to 100 µl, and 100 µl to 1000 µl.	Hirschman Labogerate	America
Balance	AG 204	Mettler Toledo	Egypt
Water bath	BS-21	BS-21	Egypt
Shaker	Geno	Spex	America
Ultra Sonic	Transonic 460	Elma	America

6.3 Reagents:

Table 8: Reagents used

Chemical	Company	Country
Acetone, 99.9%	Lab Scan	Egypt
Acetonitrile, 99.9%	Lab Scan	Egypt
n-Hexance, 97%	Lab Scan	Egypt
QuEChERs salts & buffer (Package contains 4g ($MgSO_4$), 1g	Lab Scan	Egypt

(NaCl), 1g sodium citrate, & 0.5g disodium citrate sesquihydrate)		
Magnesium Sulfate	Fluka	Egypt
C18	Sigma Aldrich	Egypt
PSA	Sigma Aldrich	Egypt
Toluene	Biosolve	France
Standard PAHs	Fluka & Dr. Ehernstrofer	Egypt

6.4 Apparatus:

Table 9: Apparatus used

Apparatus	Company	Country
Polypropylene Centrifuge Tubes with Screw Caps (50ml & 15ml)	Agilent	America
Pasteur Pipettes	Agilent	America
Pipette Tips	Agilent	America
Injection Vials	Agilent	America
Blank Syring (10ml)	Agilent	America
Graduated Glass Pipettes (5ml &10ml)	Agilent	America
50ml Glass Flasks	Agilent	America
Vials Caps	Agilent	America

7. Methodology:

7.1 Solutions preparations and standards that will be used in all protocols:

7.1.1 Polycyclic Aromatic Hydrocarbons (PAHs) active ingredients:

Sixteen PAHs reference standard active ingredients of Naphthalene, Fluorene, Fluoranthene, Benz(a)Anthracene, Chrysene, Pyrene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Acenaphthene, Phenanthrene, Anthracene, Acenaphthylene, and Pyrene-d10 (surrogate standard) obtained from Sigma-Aldrich with purity > 95%. Benzo(g,h,i)Perylene and Dibenz(a,h)Anthracene were obtained as readymade of 100 μg/mL in methylene chloride and Indeno[1,2,3-cd]pyrene 200 μg/mL in methanol.

7.1.2 Stock Solution:

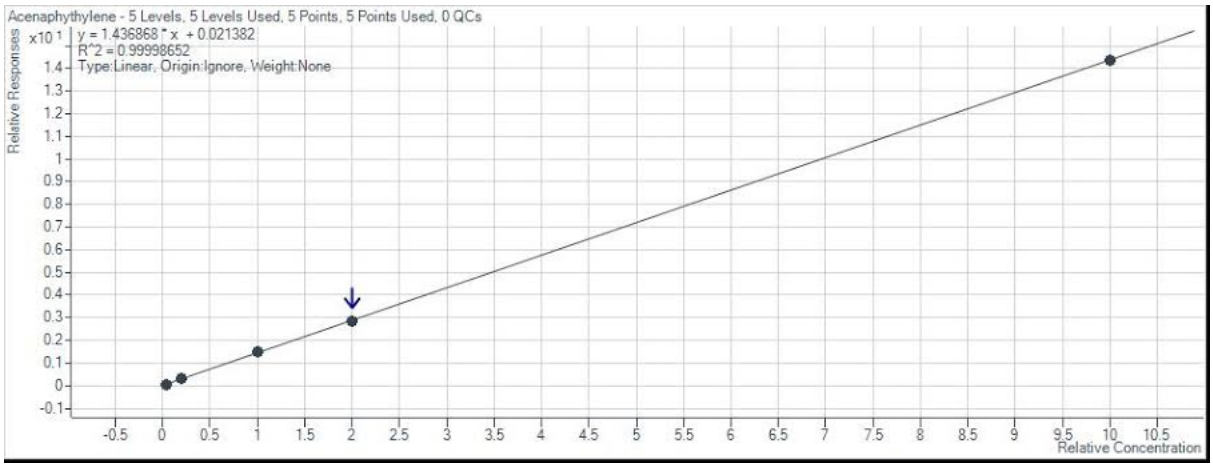
Reference standard solutions of concentration 1000 µg/ml were prepared and kept at -20 ± 2 ^{o}C. The solvent(s) used are appropriate to the analyte (solubility, stability) and method of analysis. Stock solution prepared in appropriate solvent(s) and should not negatively influence the stability of the PAHs employed (Toluene).

7.1.3 Spike Solution:

Mixture 1 µg/ml was prepared as spiking solution mixture stored in refrigerator at 4±2 ^{o}C.

7.1.4 Calibration mixture solution:

Five level calibration mixtures of concentration levels 2, 10, 50, 100, and 500ng/mL were prepared from serial dilution of the working solution in toluene where Pyrene-d10 maintained at level 50 ng/mL in all calibration levels and all stored in refrigerator at 4◦C. The preparation of multiple standards covering a broad concentration range will allow the construction of a linear calibration curve.



The below curves represents each PAH compound calibration curve

Figure 2: Represents Acenphythylenecalibration curve

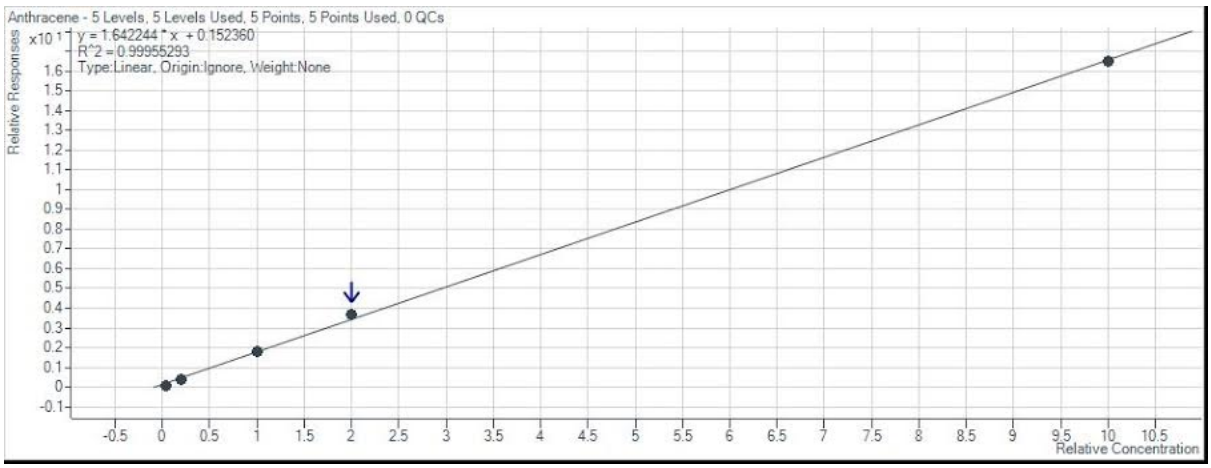


Figure 3: Represents Anthracene calibration curve

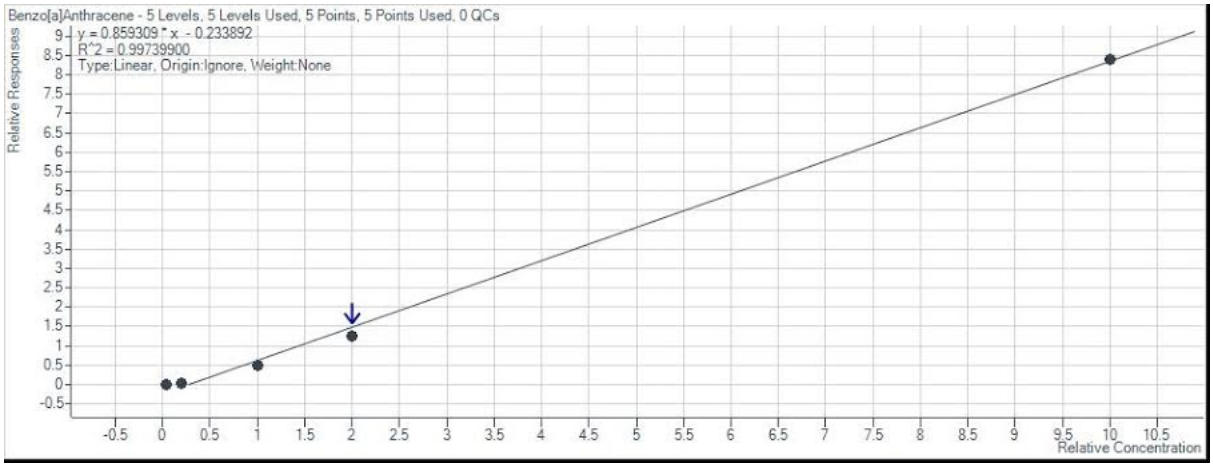


Figure 4: Represents Benzo[a]Anthracene calibration curve

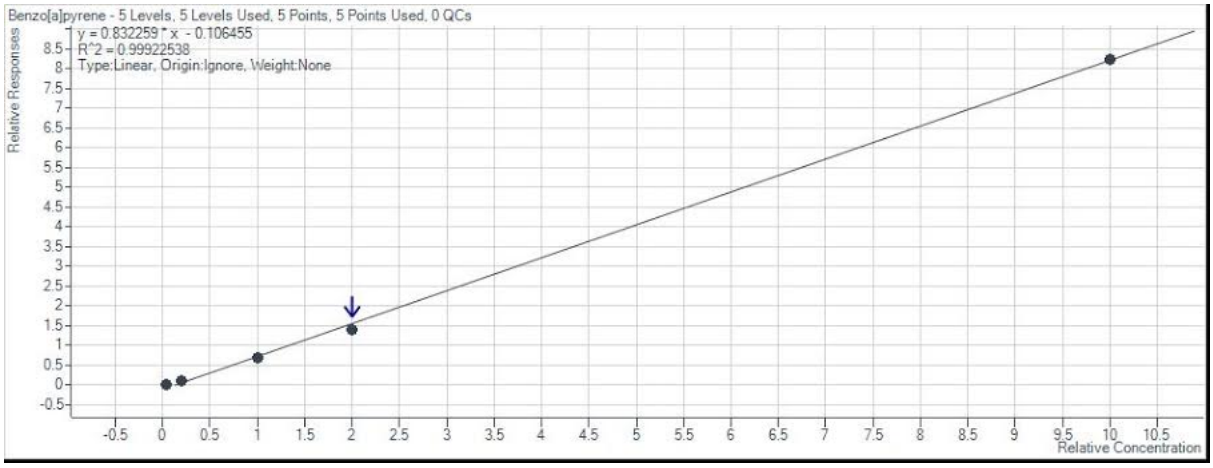


Figure 5: Represents Benzo[a]pyrene calibration curve

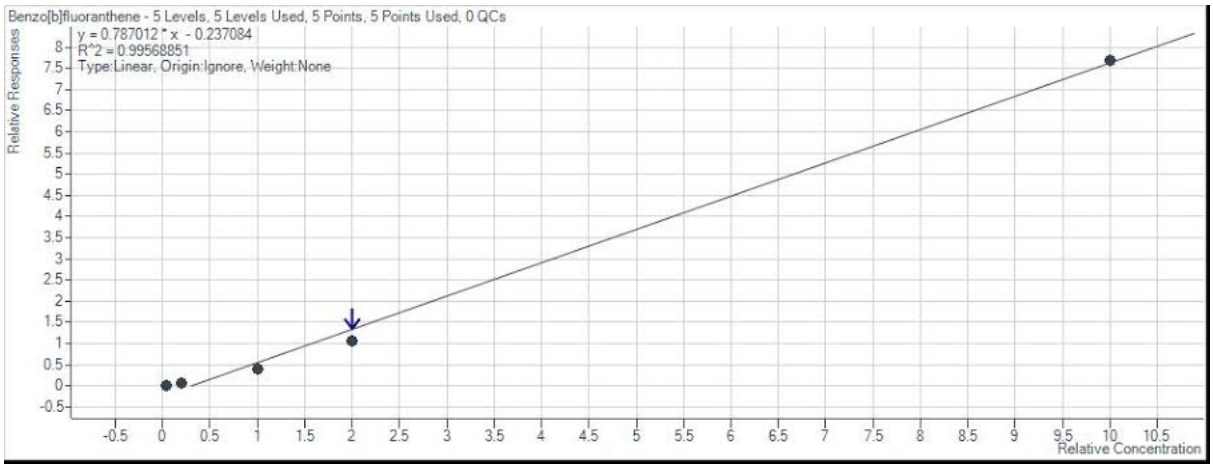


Figure 6: Represents Benzo[b]fluoranthene calibration curve

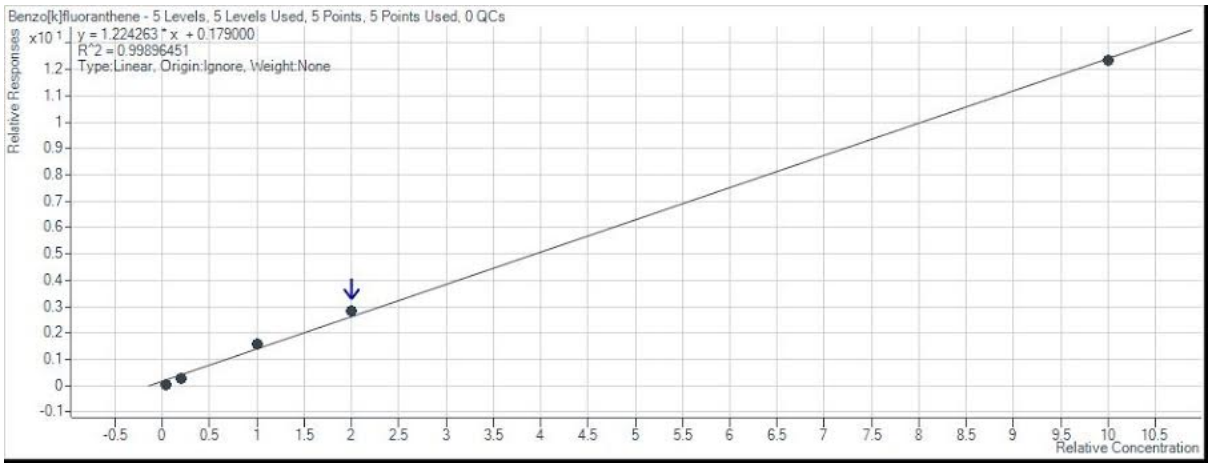


Figure 7: Represents Benzo[k[fluoranthene calibration curve

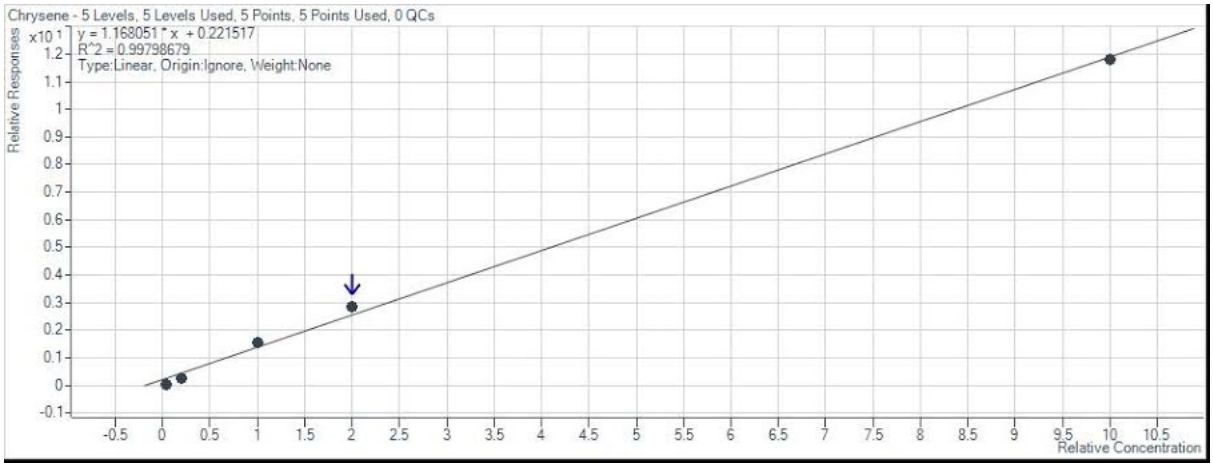


Figure 8: Represents Chrysene calibration curve

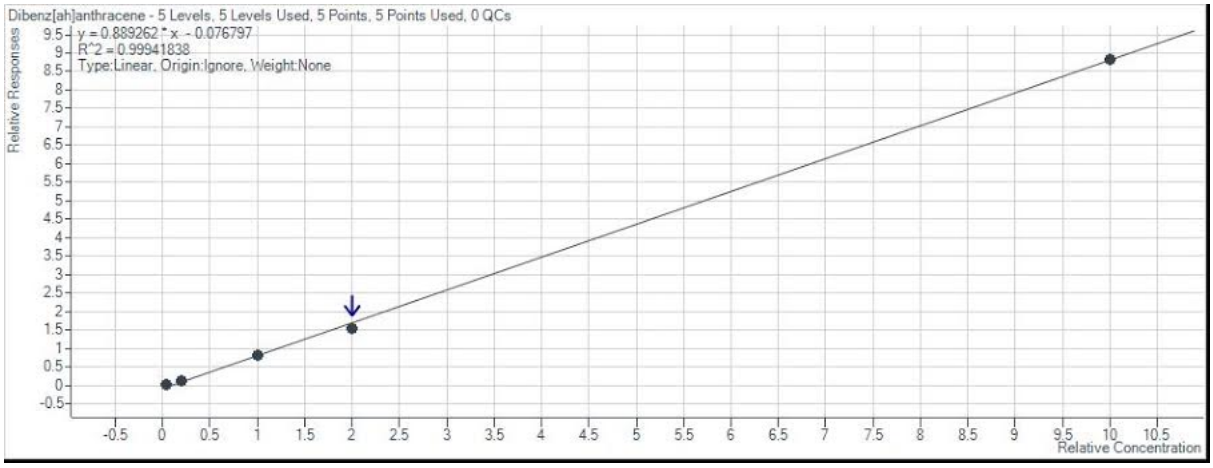


Figure 9: Represents Dibenz[ah]anthracene calibration curve

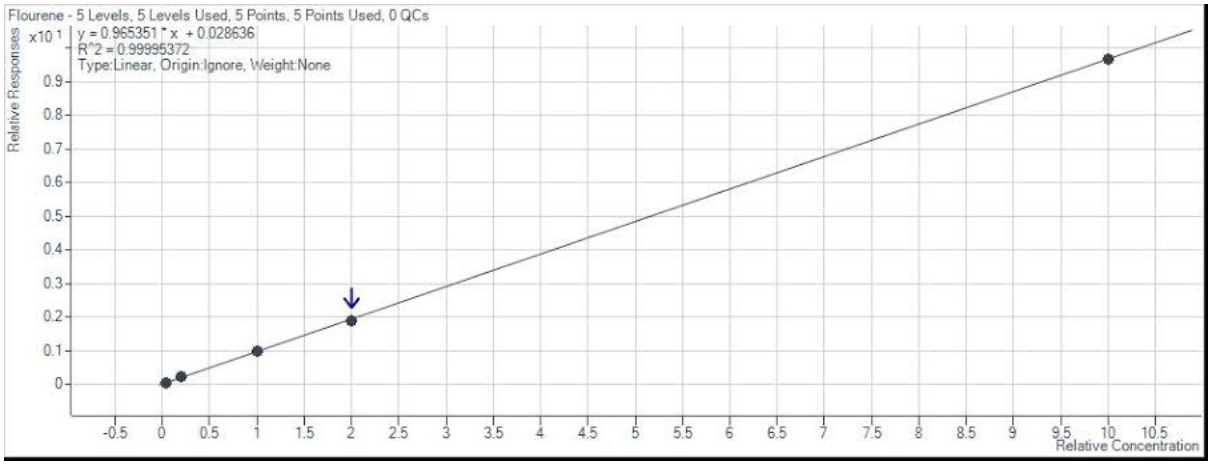


Figure 10: Represents Flourene calibration curve

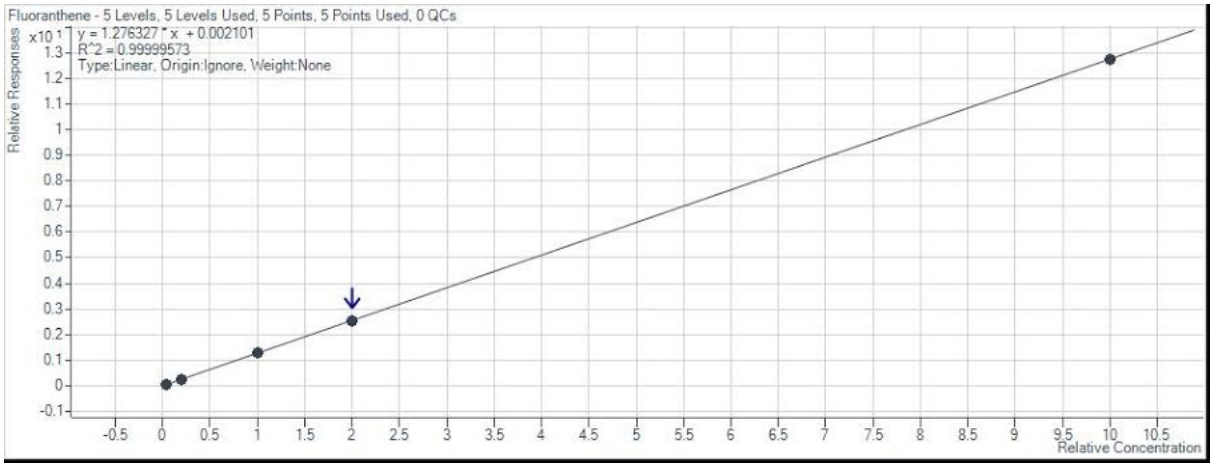


Figure 11: Represents Fluoranthene calibration curve

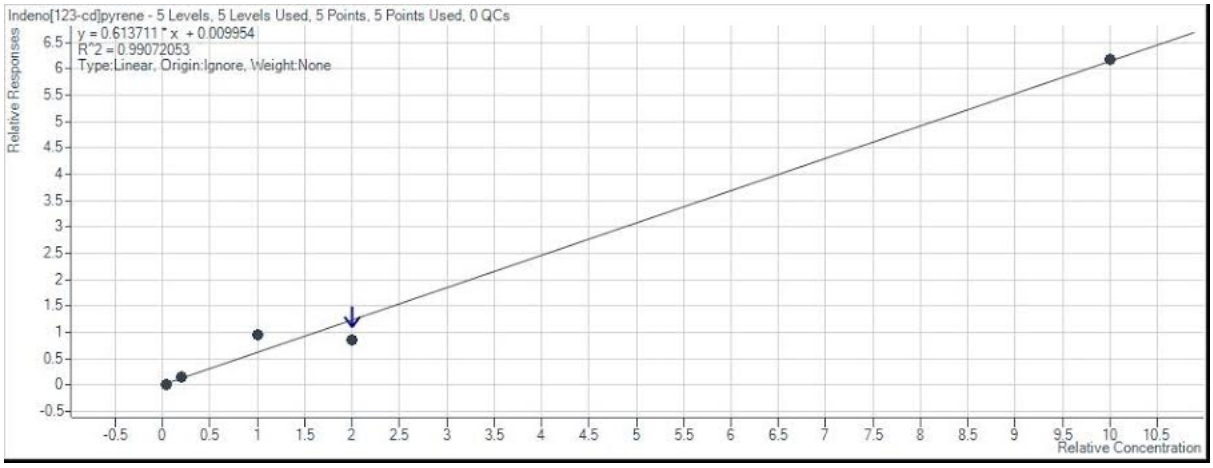


Figure 12: Represents Indeno[123-cd]pyrene calibration curve

Figure 13: Represents Napthalene calibration curve

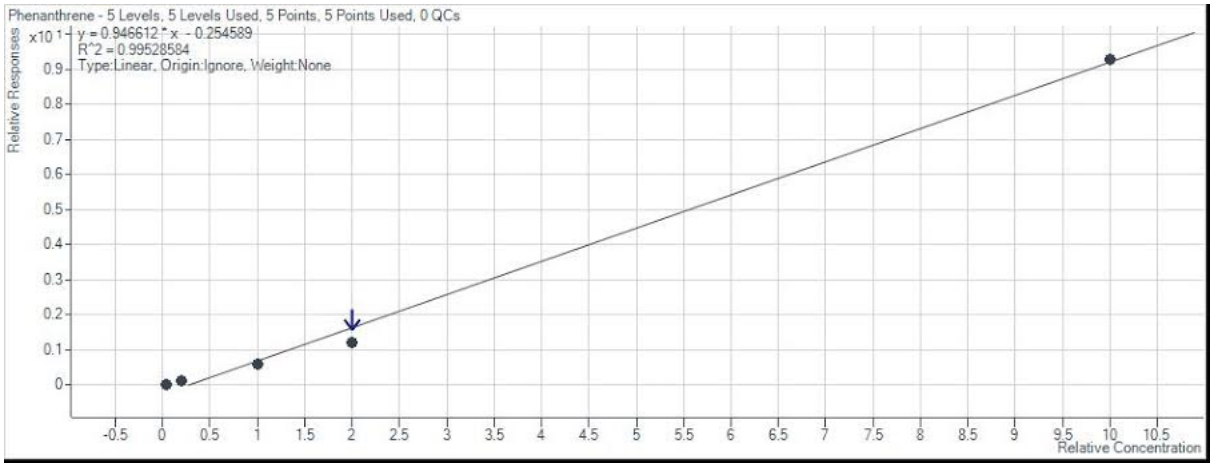


Figure 14: Represents Phenanthrene calibration curve

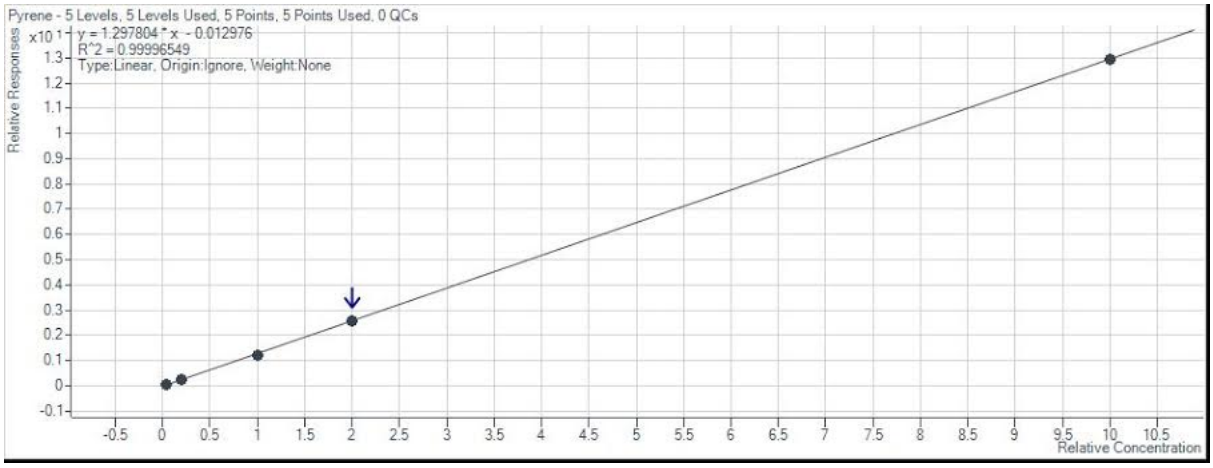


Figure 15: Represents Pyrene calibration curve

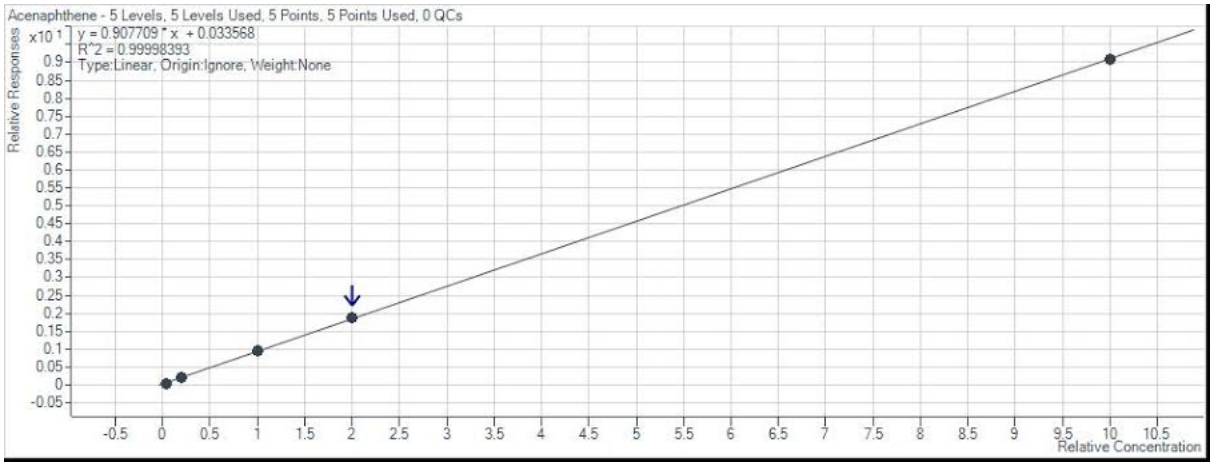


Figure 16: Represents Acenaphthene calibration curve

7.1.5 Standard Solution Pyrene-d10

Working standard of Pyrene-D_{10} with concentration 10µg/mL was prepared in Toluene used as surrogate standard in all samples.

7.2 GC/ MSD Determination:

7.2.1 Conditions:

Firstly, according to Hamzawy *et al*., (2016), the device column was calibrated. The PAHs standard compounds were also calibrated and adjusted. The conditions of the device were then adjusted as in (Table 10). The samples were then added in their places in the device for the injection process of the samples into the device start.

Table 10: GC/MSD conditions

Technique	GC-MSD
Column	0.25 mm, Film thickness: 0.52 µm, column length: 30 m.
Sample Concentration	Approx. 0.1-0.3 µg/mL
Injection Volume	1 µL
Temperature Temp: 260 ^{O}C Time: 36mins	90°C, hold 2 min, ramp 15°C/min to 180°C hold for 4 min ramp 10°C/min to 250°C, hold 2min ramp 10°C/min to 290°C, hold 10 min
Carrier Gas	Helium, constant flow 1.3 mL
Injector	300 °C, Splitless mode, 0.5 min at 100 mL/min

7.2.2 Chromatograms obtained of each PAH compound after real injection

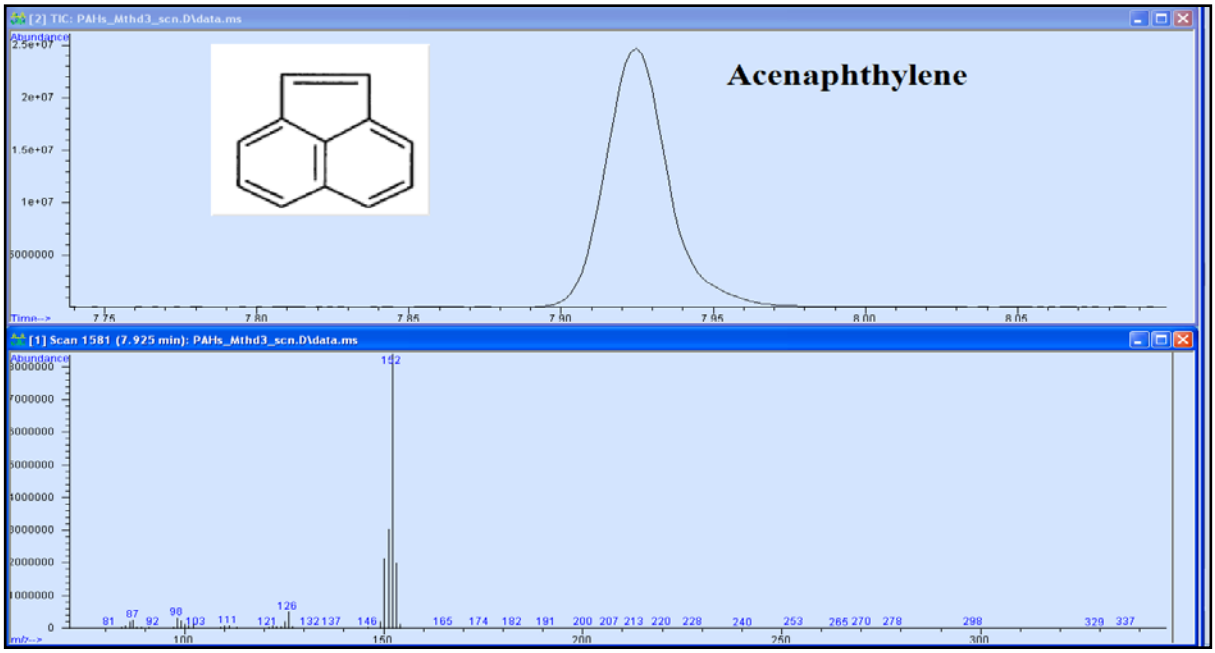


Figure 17: Represents Acenaphthylene chromatogram after injection on GC/MSD

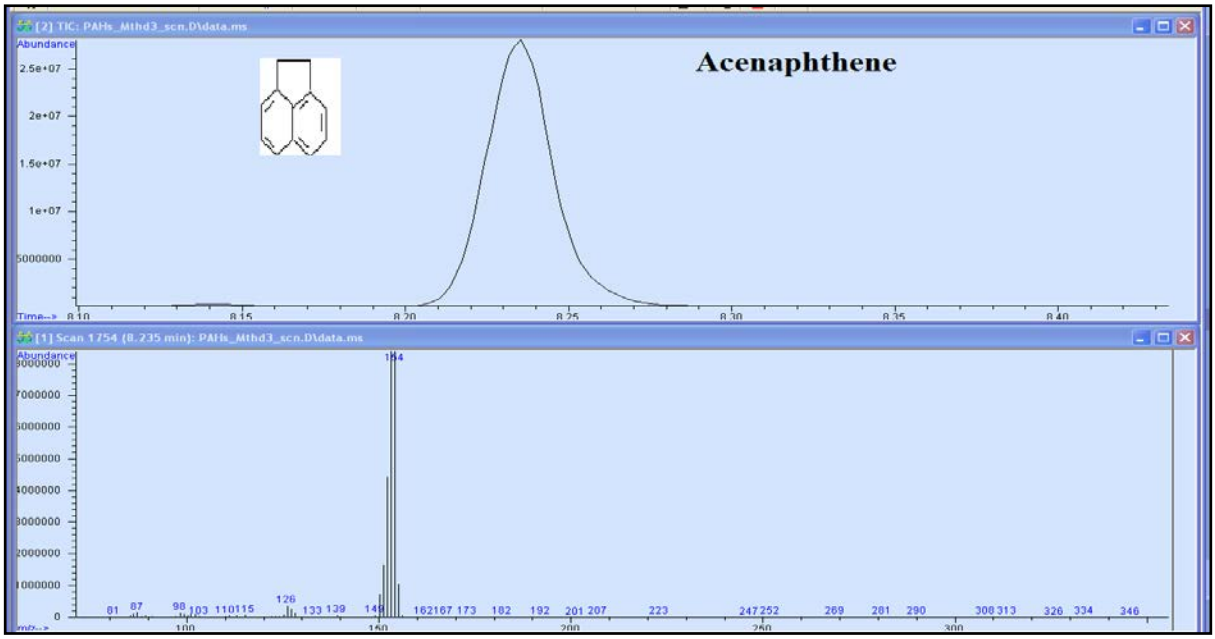


Figure 18: Represents Acenaphthene chromatogram after injection on GC/MSD

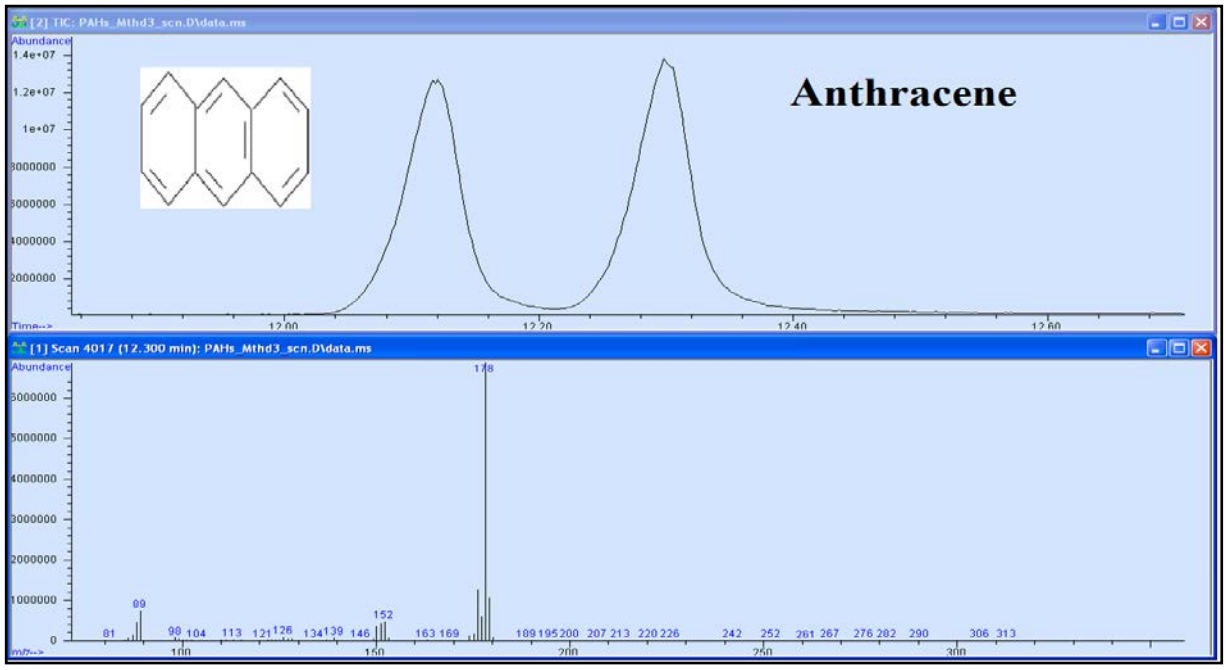


Figure 19: Represents Anthracene chromatogram after injection on GC/MSD

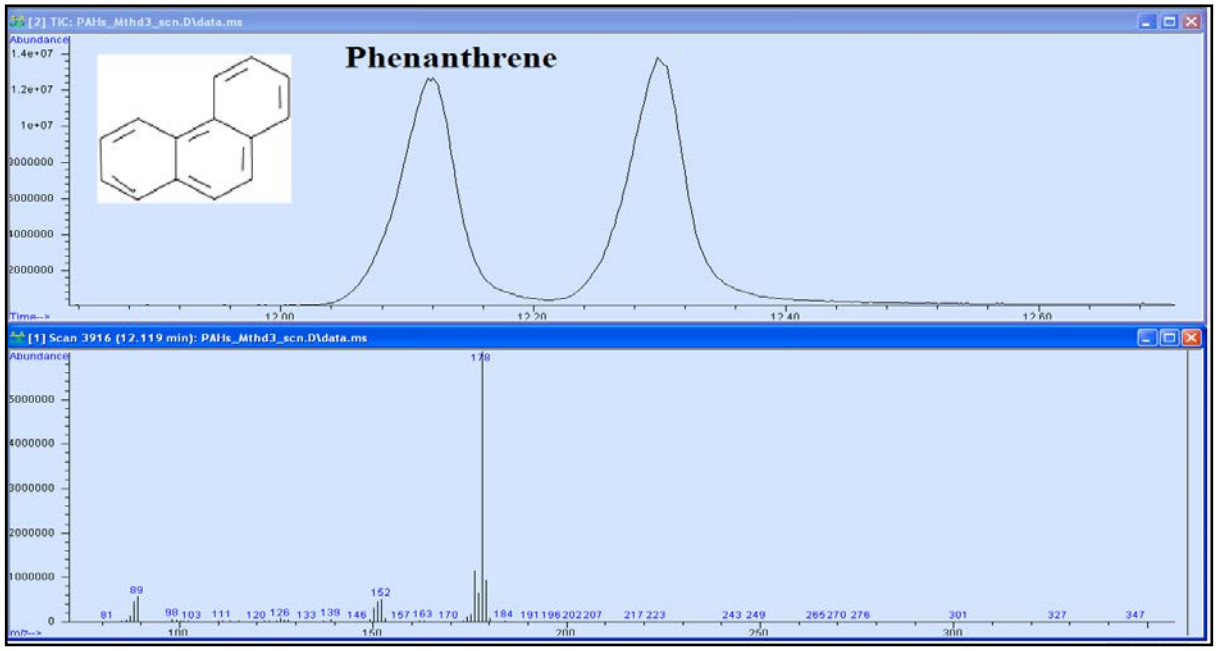


Figure 20: Represents Phenanthrene chromatogram after injection on GC/MSD

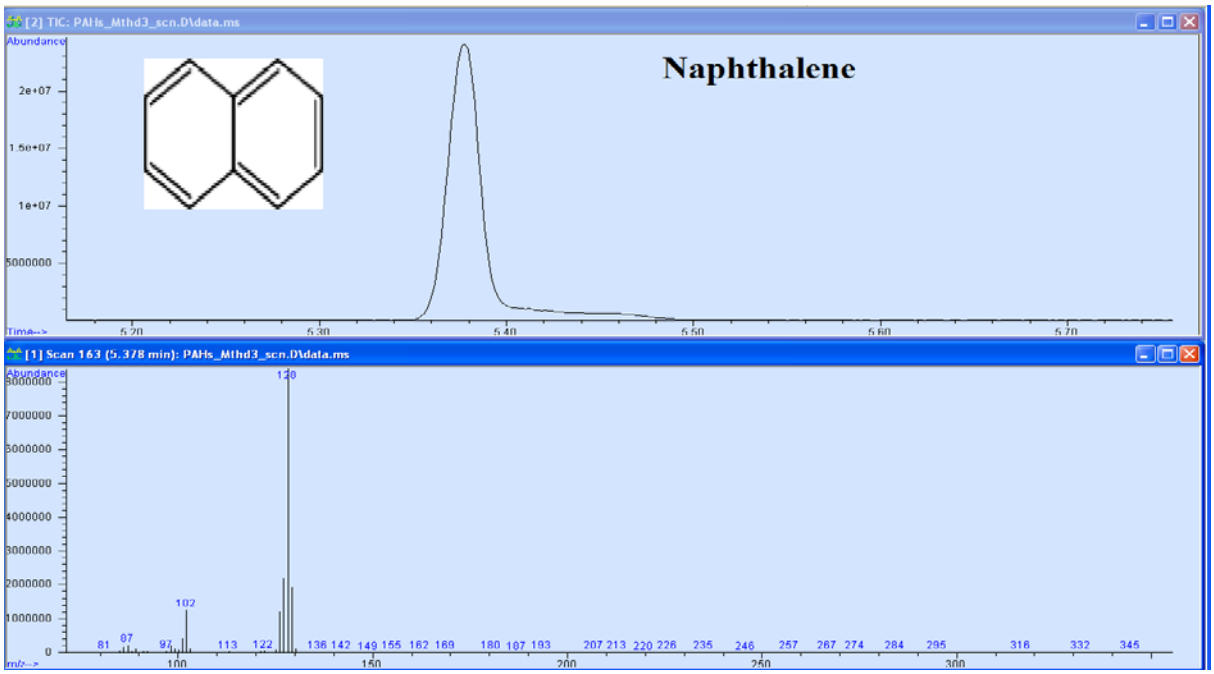


Figure 21: Represents Naphthalene chromatogram after injection on GC/MSD

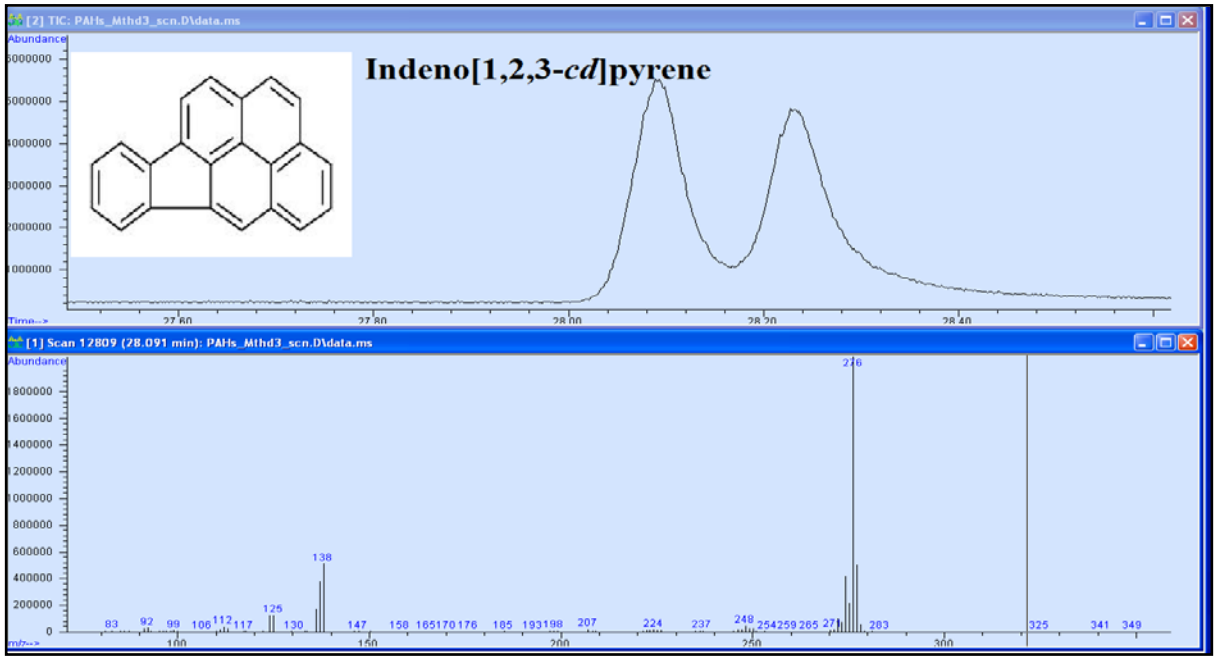


Figure 22: Represents Indeno[1,2,3-cd]pyrene chromatogram after injection on GC/MSD

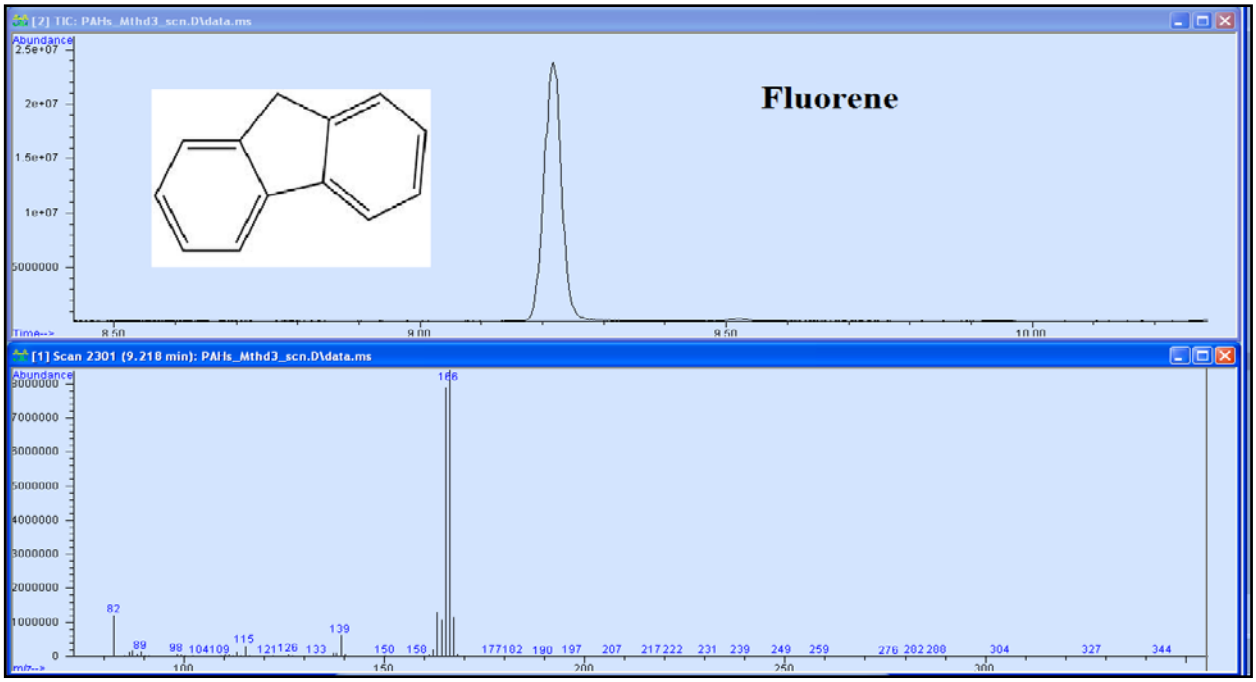


Figure 23: Represents Fluorene chromatogram after injection on GC/MSD

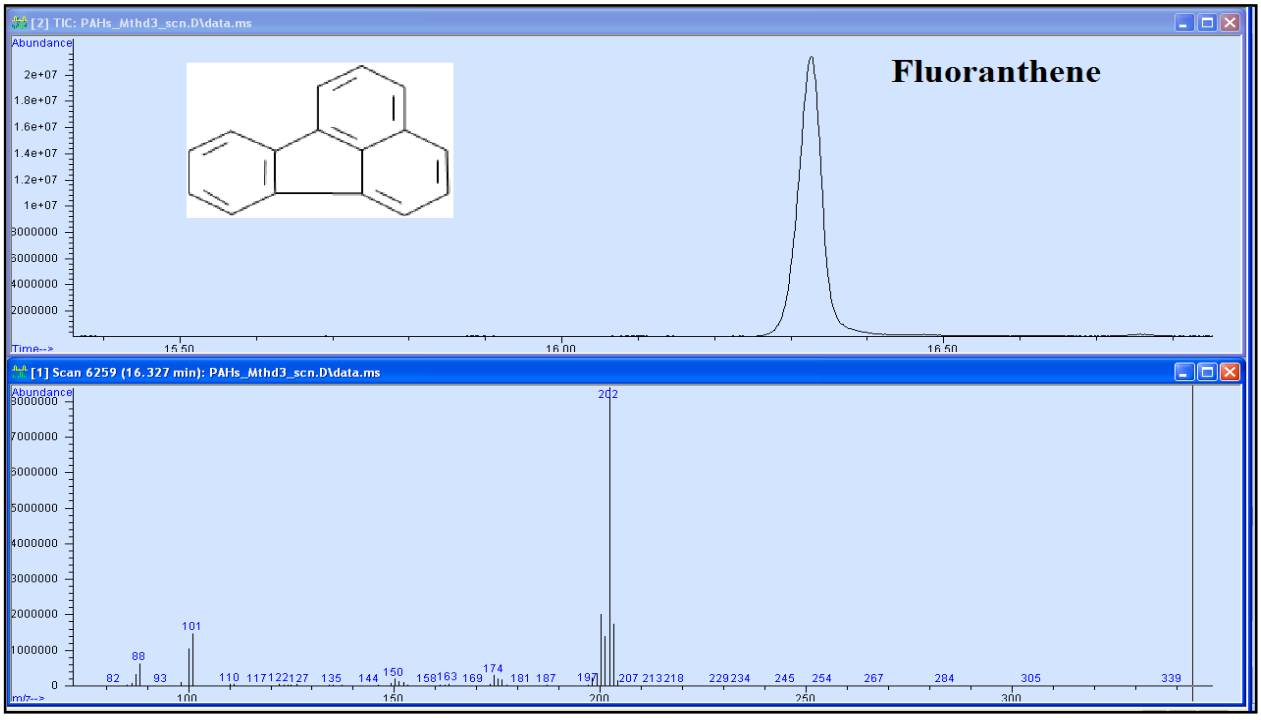


Figure 24: Represents Fluoranthene chromatogram after injection on GC/MSD

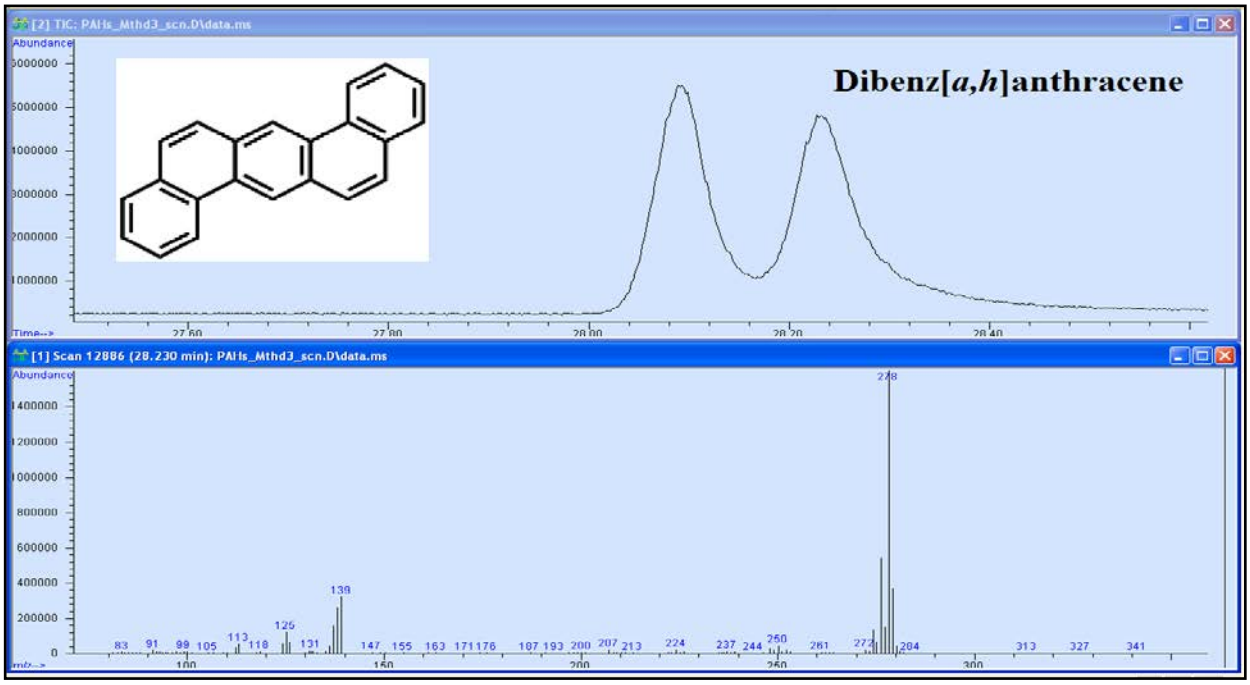


Figure 25: Represents Dibenz[a,h]anthracene chromatogram after injection on GC/MSD

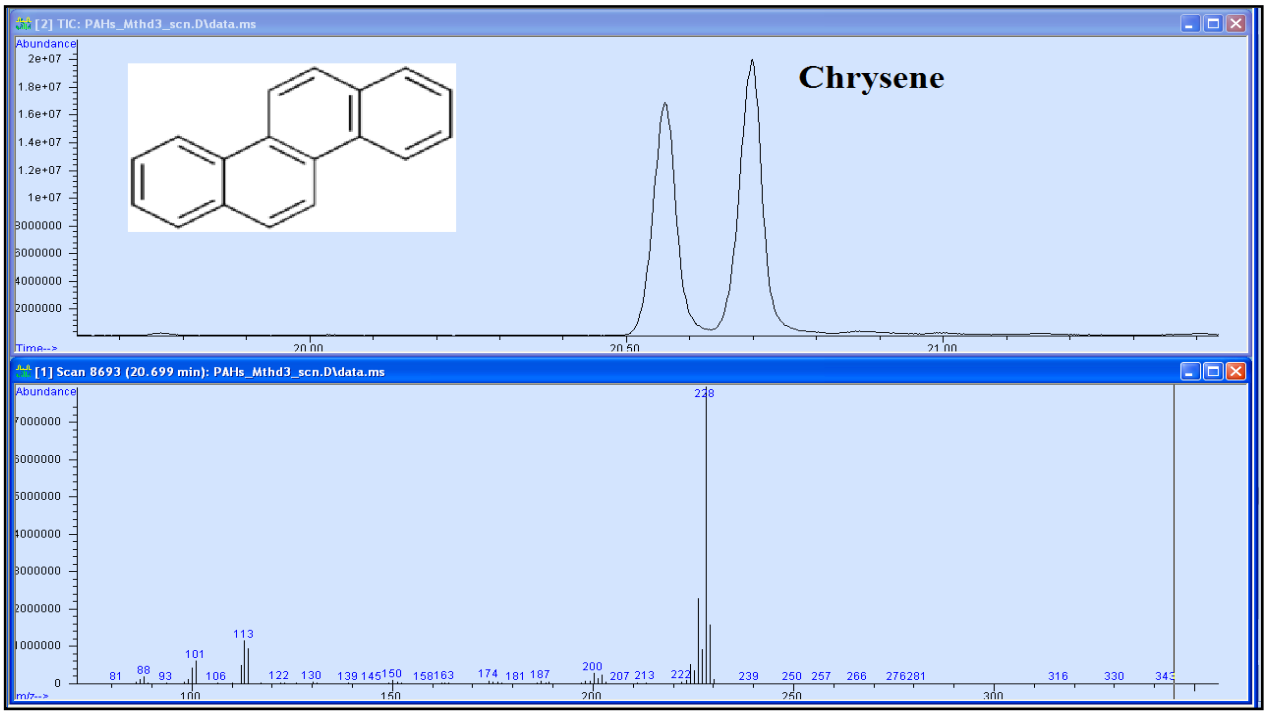


Figure 26: Represents Chrysene chromatogram after injection on GC/MSD

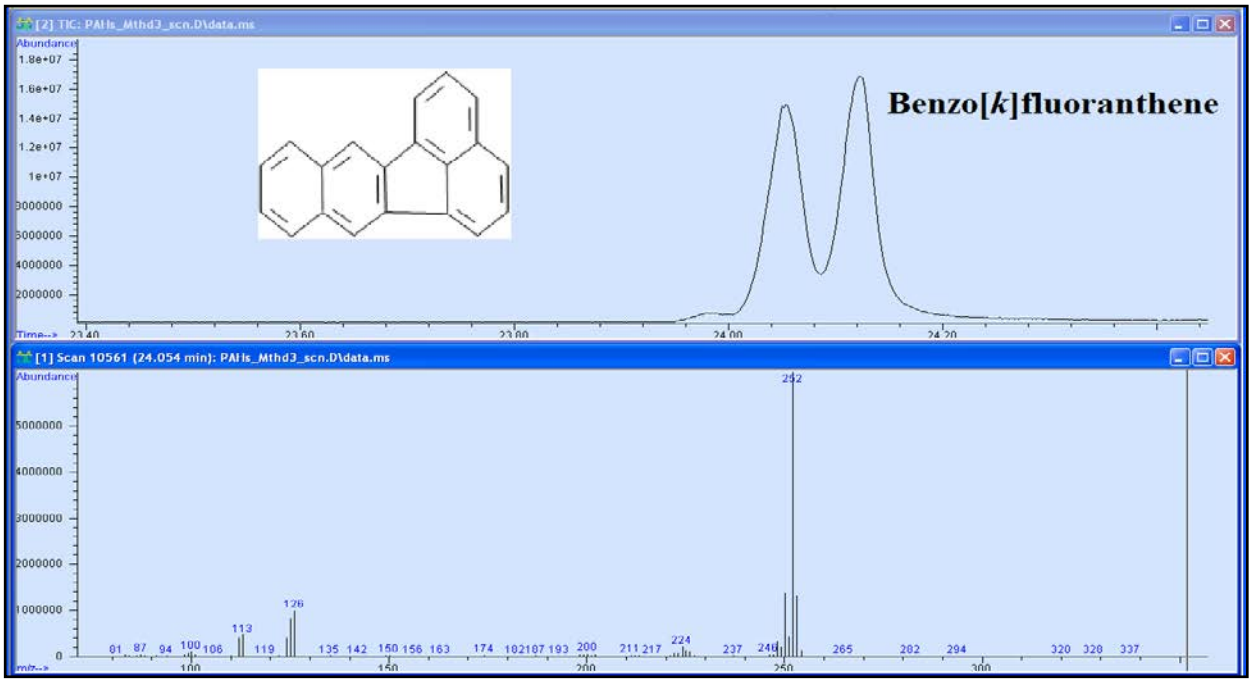


Figure 27: Represents Benzo[k]fluoranthene chromatogram after injection on GC/MSD

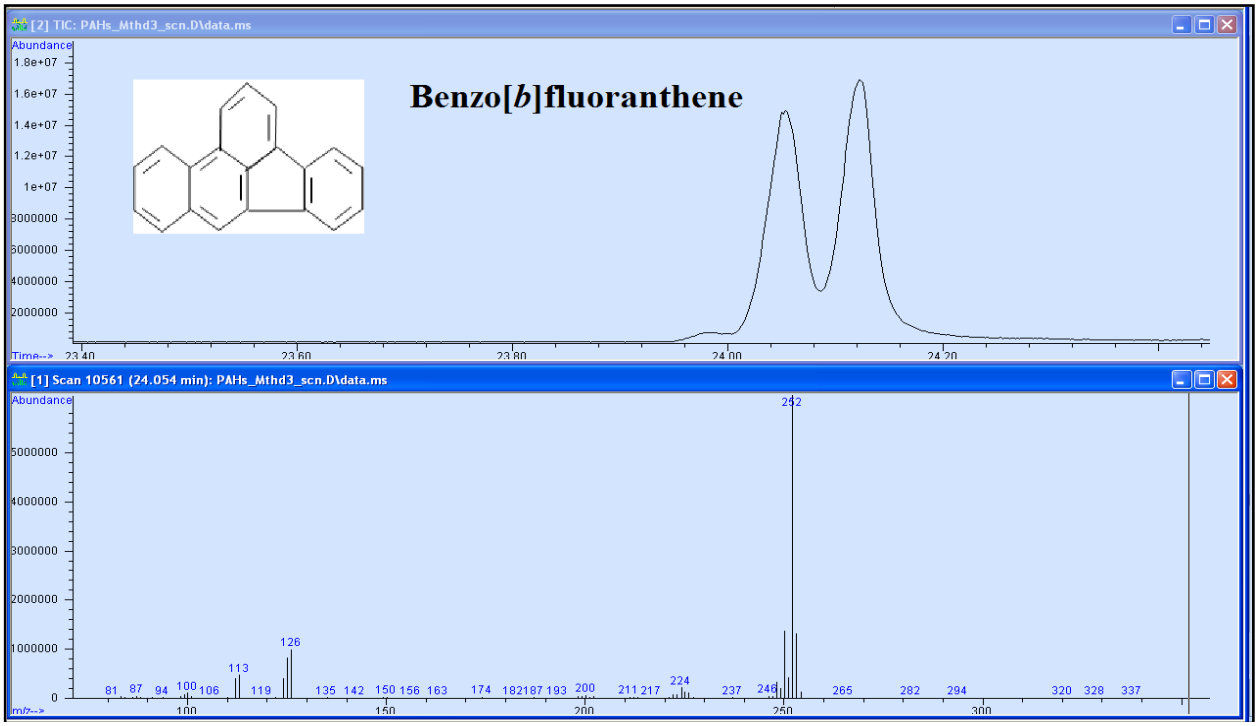


Figure 28: Represents Benzo[b]fluoranthene chromatogram after injection on GC/MSD

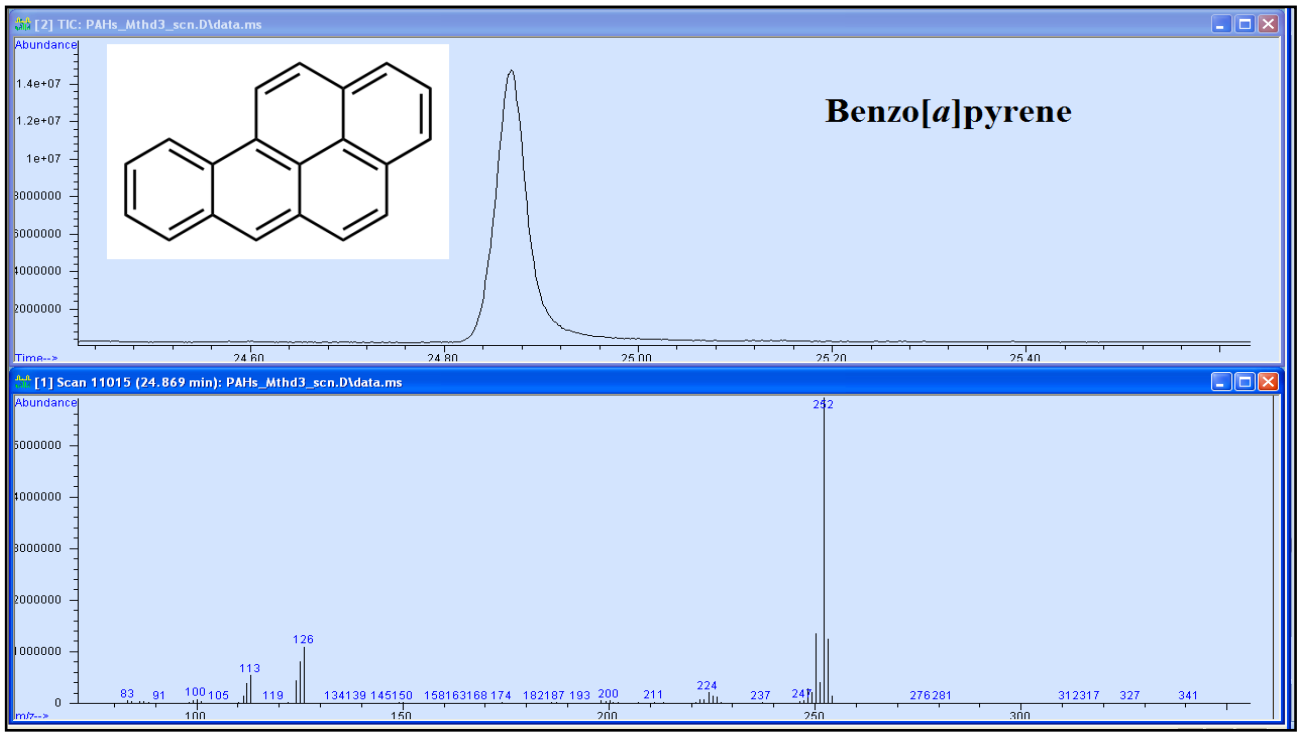


Figure 29: Represents Benzo[a]pyrene chromatogram after injection on GC/MSD

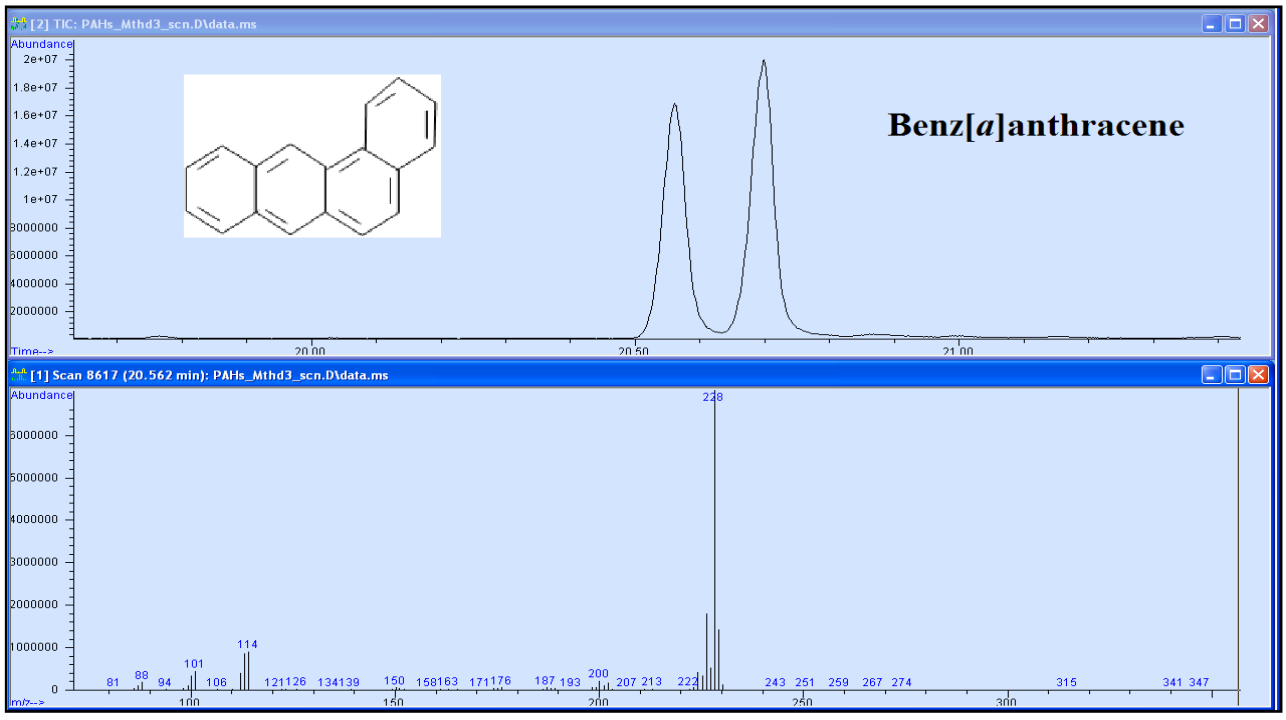


Figure 30: Represents Benz[a]anthracene chromatogram after injection on GC/MSD

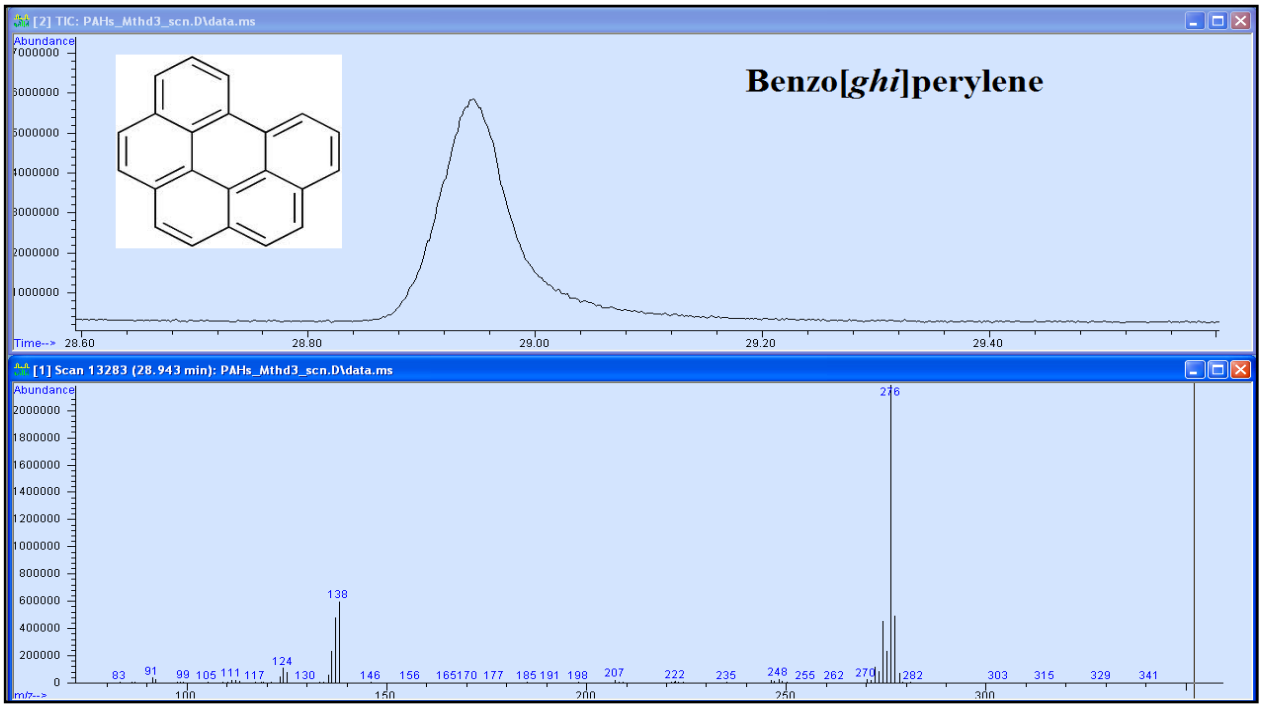


Figure 31: Represents Benzo[ghi]perylene chromatogram after injection on GC/MSD

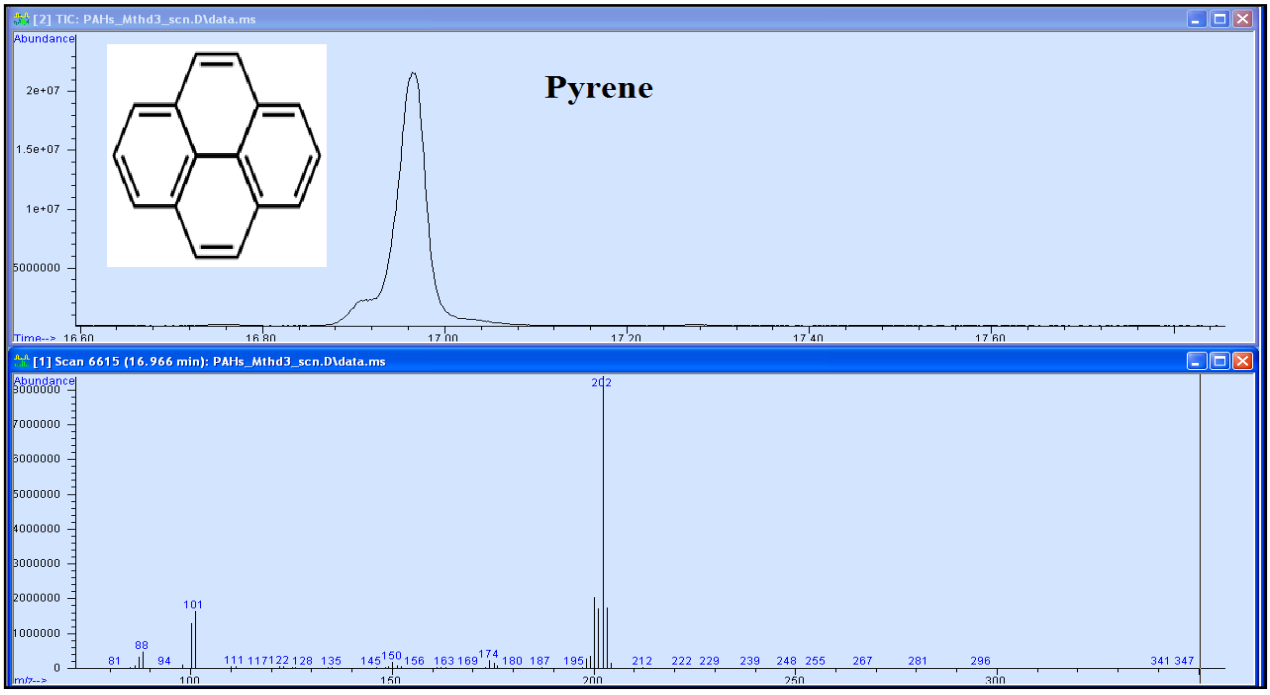


Figure 32: Represents Pyrene chromatogram after injection on GC/MSD

7.3 Analytical Procedures:

The extraction and GC determination part of the methodology was taken from Hamzawy *et al.,* (2016). But for the clean up part in the method it was modified because the referred method was applied on fish, meat, and chicken which are different commodity group referring SANTE/11813/2017 annex A defined that commodity groups and their representative were milk and milk products can be represented as milk. The Clean up method was chosen according to the method with best recovery and lowest matrix effect.

7.3.1 Method 1:

Clean up Evaluation

7.3.1.1 Samples Monitoring:

Total number of 9 samples were used in this protocol. These samples were the same. All samples were collected to determine the best clean up method that will be used with the least matrix effect.

7.3.1.2 Extraction Procedure:

7.3.1.2.1 Internal quality controls:

10g of milk was weighted in 50 ml Teflon centrifuge tube. The 9 samples were divided into 3 replicates and each replicate contains 3 samples. Firstly, 50ul/kg of Pyrene-d10 was added on all sample as a standard PAHs control (Please refer to annex 2.2 in the methodology for Pyrene-d10 preparation). Then 50ul/kg of spike solution was added on all samples in each replicate as internal quality control for the PAHs (Please refer to annex 2.2 in the methodology for spike preparation).

7.3.1.2.2 QuECHERs Method:

The procedure involves initial single-phase extraction of 10 g sample with acetonitrile in a 50 mL centrifuge tube, 10 mL acetonitrile were added and shaken vigorously by hand for 1

minute, then applied to complete extraction by addition of Agilent QuEChERs salts scathes containing:

4 g ± 0.2 g Magnesium sulphate anhydrous,

1 g ± 0.05 g Sodium chloride,

1 g ± 0.05 g Trisodium citrate dihydrate and

0.5 g ± 0.03g Disodium hydrogen citrate sesquihydrate

The tube closed and immediately shaken vigorously by hand for 1 minute and centrifuged for 5 minutes at 4000 rpm. The supernatant (ACN) was transferred into a single use 15mL centrifugation tube.

7.3.1.3 Clean up Process:

The Clean-up technique has three approaches optimized individually (Each approaches is made with 1 replicate (3 samples)): -

7.3.1.3.1 Clean up using Magnesium Sulfate.

This is done by addition of 0.9g of $MgSO_4$into 15mL centrifugation tube and shake with an aliquot for 30 sec and centrifuge for 2 minutes at 4000 rpm, then evaporate 2 ml of the upper layer and diluted with 2ml toluene in order to inject via GCMSD.

7.3.1.3.2 Clean up using C18 (Carbon 18).

This done by addition of 0.1 C18 into 15mL centrifugation tube and shakes with an aliquot for 30 sec and centrifuge for 2 minutes at 4000 rpm, then evaporate 2 ml of the upper layer and diluted with 2ml toluene in order to inject via GCMSD.

7.3.1.3.3 Clean up using Primary Secondary Amin (PSA) sorbent.

This done by addition of 25 mg PSA as dSPE into 15mL centrifugation tube and shakes with an aliquot for 30 sec and centrifuge for 2 minutes at 4000 rpm, then evaporate 2 ml of the upper layer and diluted with 2ml toluene in order to inject via GCMSD.

7.3.1.4 GCMSD Determination:

Please refer to 7.2 for GC/MSD conditions

7.3.2 Method 2:

After choosing the best clean up method and with a slight modification the final protocol is the following:

7.3.2.1 Samples Monitoring:

Total number of 54 samples were used in this protocol. These samples were 28 packed milk (Commercial milk) and 26 raw milk that was collected from different regions in Egypt (Please refer to annex 2.1 for samples collection). All samples were collected to determine and monitor the presence of PAHs.

7.3.2.2 Extraction Procedure:

7.3.2.2.1 Internal quality controls:

10g of milk was weighted in 50 ml Teflon centrifuge tube for all 54 sample. Firstly, 50ul/kg of Pyrene-d10 was added on all sample as a standard PAHs control (Please refer to annex 2.2 in the methodology for Pyrene-d10 preparation). Then 50ul/kg of spike solution was added on only 6 sample as internal quality control for the PAHs (Please refer to annex 2.2 in the methodology for spike preparation).

7.3.2.2.2 QuECHERs Method:

The procedure involves initial single-phase extraction of 10 g sample with acetonitrile in a 50 mL centrifuge tube, 10 mL acetonitrile were added and shaken vigorously by hand for 1 minute, then applied to complete extraction by addition of Agilent QuEChERs salts scathes containing:

4 g ± 0.2 g Magnesium sulphate anhydrous,

1 g ± 0.05 g Sodium chloride,

1 g ± 0.05 g Trisodium citrate dihydrate and

0.5 g ± 0.03g Disodium hydrogen citrate sesquihydrate

The tube closed and immediately shaken vigorously by hand for 1 minute and centrifuged for 5 minutes at 4000 rpm. The supernatant (ACN) was transferred into a single use 15mL centrifugation tube.

7.3.2.3 Clean up Process:

7.3.2.3.1 Clean up using Magnesium Sulfate and C18.

This is done by addition of 0.9g of $MgSO_4$and 0.1g of C18 into 15mL centrifugation tube and shake with an aliquot for 30 sec and centrifuge for 2 minutes at 4000 rpm, then evaporate 2 ml of the upper layer and diluted with 2ml toluene in order to inject via GCMSD.

7.3.2.4 GCMSD Determination:

Please refer to 7.2 for GC/MSD conditions

7.3.3 Method 3:

Effect of pasteurization on PAHs in Milk.

7.3.3.1 Samples Monitoring:

Total number of 6 samples were used in this protocol. These samples were raw milk samples. (Please refer to annex 2.1 for samples collection). All samples were used to determine and monitor the effect of pasteurization on PAHs.

7.3.3.2 Extraction Procedure:

7.3.3.2.1 Internal quality controls:

10g of milk was weighted in 50 ml Teflon centrifuge tube for all 6 sample. Firstly, 50ul/kg of Pyrene-d10 was added on all sample as a standard PAHs control (Please refer to annex 2.2 in the methodology for Pyrene-d10 preparation). Then 50ul/kg of spike solution was also added on all 6 sample as internal quality control for the PAHs (Please refer to annex 2.2 in the methodology for spike preparation).

In this step there areapproaches optimized individually:

7.3.3.2.1.1 Internal Quality Control:

2 samples were used as a control and no heat effect was done and it was followed by QuECHERs method till the end as mentioned below.

7.3.3.2.1.2 Effect of high temperature:

Firstly, after addition of internal quality controls 2 samples were placed in the water bath for 1 hour at 80^{o}C. Then it was followed by QuECHERs method till the end as mentioned below.

7.3.3.2.1.3 Effect of low temperature:

Firstly, after addition of internal quality controls 2 samples were placed in the water bath for 1 hour at 80^{o}C. Then cooled down in the freezer for 20 mins at -20^{o}C. Finally, it was followed by QuECHERs method till the end as mentioned below.

7.3.3.2.2 QuECHERs Method:

The procedure involves initial single-phase extraction of 10 g sample with acetonitrile in a 50 mL centrifuge tube, 10 mL acetonitrile were added and shaken vigorously by hand for 1 minute, then applied to complete extraction by addition of Agilent QuEChERs salts scathes containing:

4 g ± 0.2 g Magnesium sulphate anhydrous,

1 g ± 0.05 g Sodium chloride,

1 g ± 0.05 g Trisodium citrate dihydrate and

0.5 g ± 0.03g Disodium hydrogen citrate sesquihydrate

The tube closed and immediately shaken vigorously by hand for 1 minute and centrifuged for 5 minutes at 4000 rpm. The supernatant (ACN) was transferred into a single use 15mL centrifugation tube.

7.3.3.3 Clean up Process:

7.3.3.3.1 Clean up using Magnesium Sulfate and C18.

This is done by addition of 0.9g of $MgSO_4$ and 0.1g of C18 into 15mL centrifugation tube and shake with an aliquot for 30 sec and centrifuge for 2 minutes at 4000 rpm, then evaporate 2 ml of the upper layer and diluted with 2ml toluene in order to inject via GCMSD.

7.3.3.4 GCMSD Determination:

Please refer to 7.2 for GC/MSD conditions

VI. Results

This project was a collaboration between MSA University, and Central Lab of Residue Analysis of Pesticides and Heavy Metal in Food (Qcap). This study aimed to use a novel and validated methodology to analyze and monitor the presence of poly-aromatic hydrocarbons in both commercial and raw milk with a high performance device GC-MS. Total number of samples are 65 samples that was used in this study.

Method 1: Clean up Evaluation

Referring to the chromatograms and the below figures 33-40 and table 11 showed that the matrix effect of milk on PAH almost the same in all protocols except T2. Excluding Benzo[b]fluranthene, this compound matrix effect showed that T1 > T2 > T3. Matrix effect of Benzo[k]fluranthene showed that T1 > T2 <T3. Benzo[a]pyrene (target compound) matrix effect showed that T1 > T2 > T3.

Benzo[a]pyrene is considered the most important PAH compound and it is used as a monitor, therefore, T2 was chosen due it's moderate matrix effect on most of PAHs.

(The below results and graphs are obtained from the data found in results annexes, Annex 1)

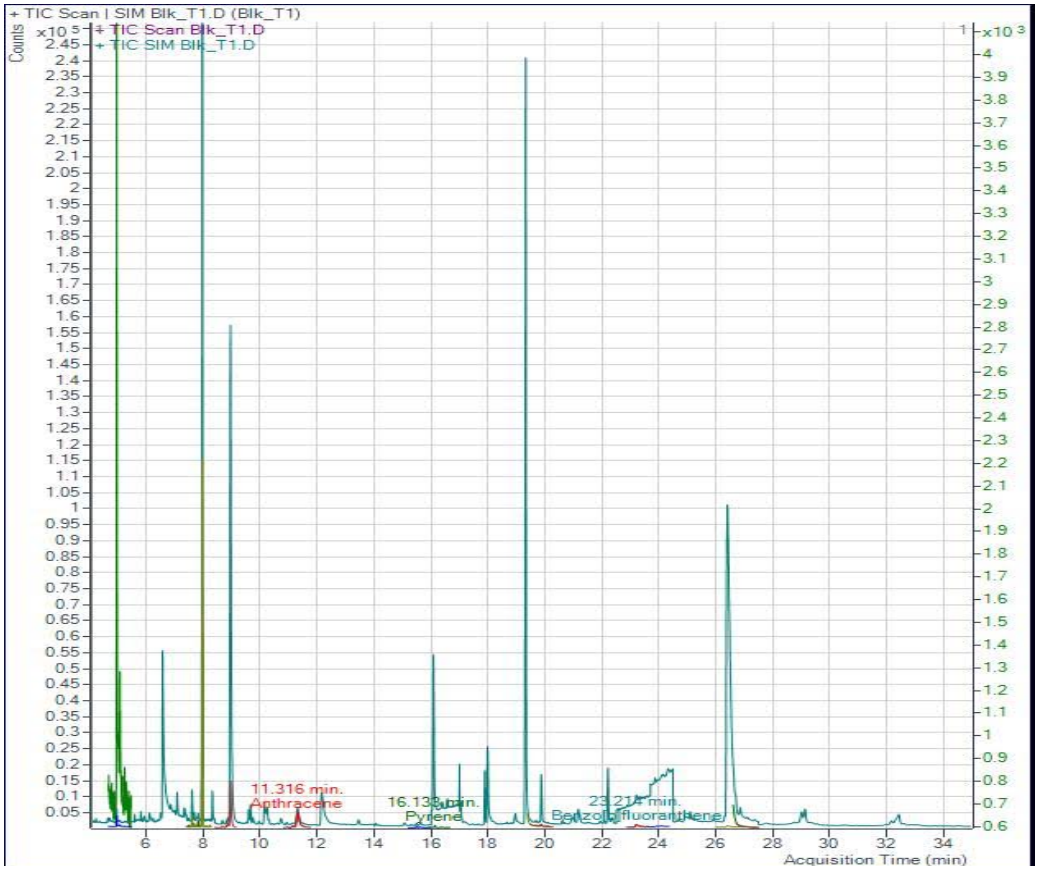


Figure 33: Represents the blank Std Matrx effect of T1

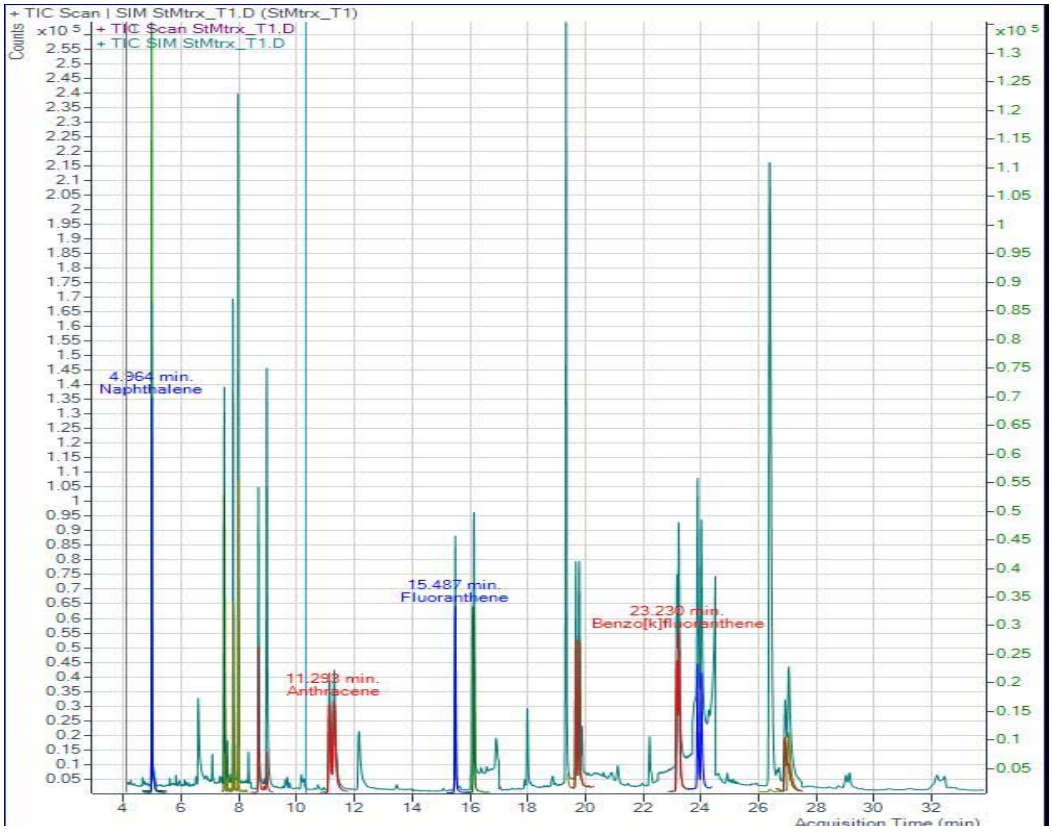


Figure 34: Represents the Std Matrx effect results of T1

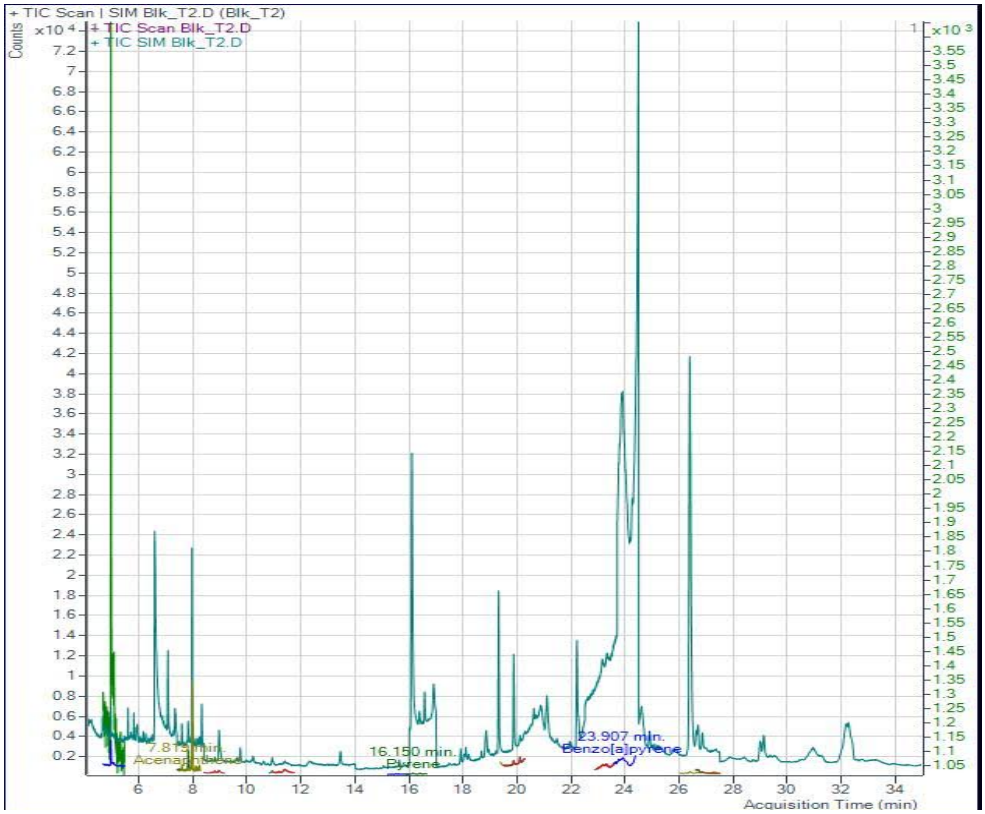


Figure 35: Represents the Std Matrx effect blank of T2

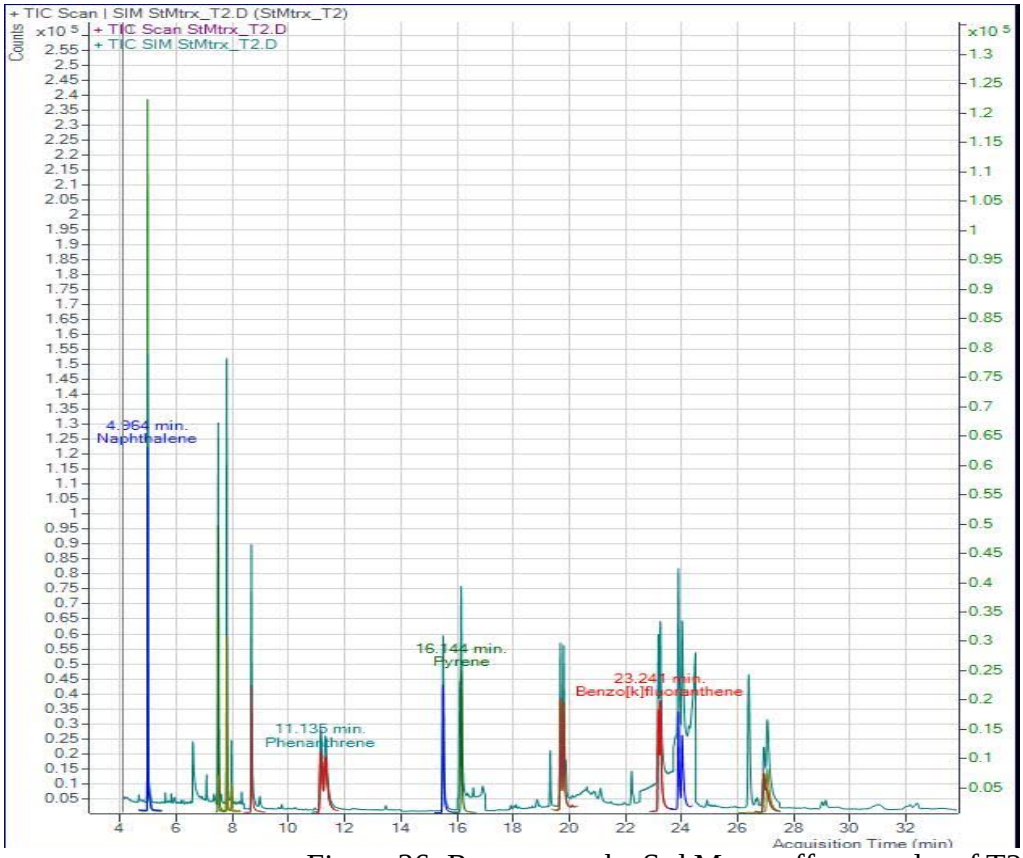


Figure 36: Represents the Std Matrx effect results of T2

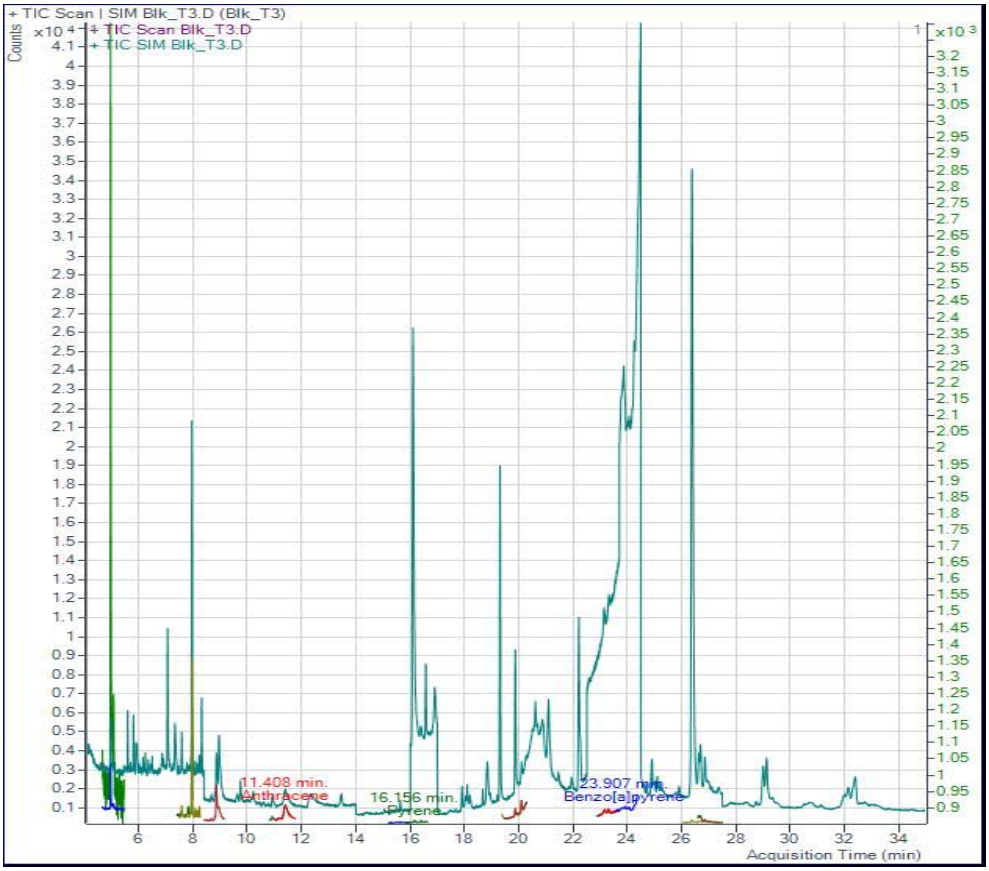


Figure 37: Represents the blank of Std Matrx effect of T3

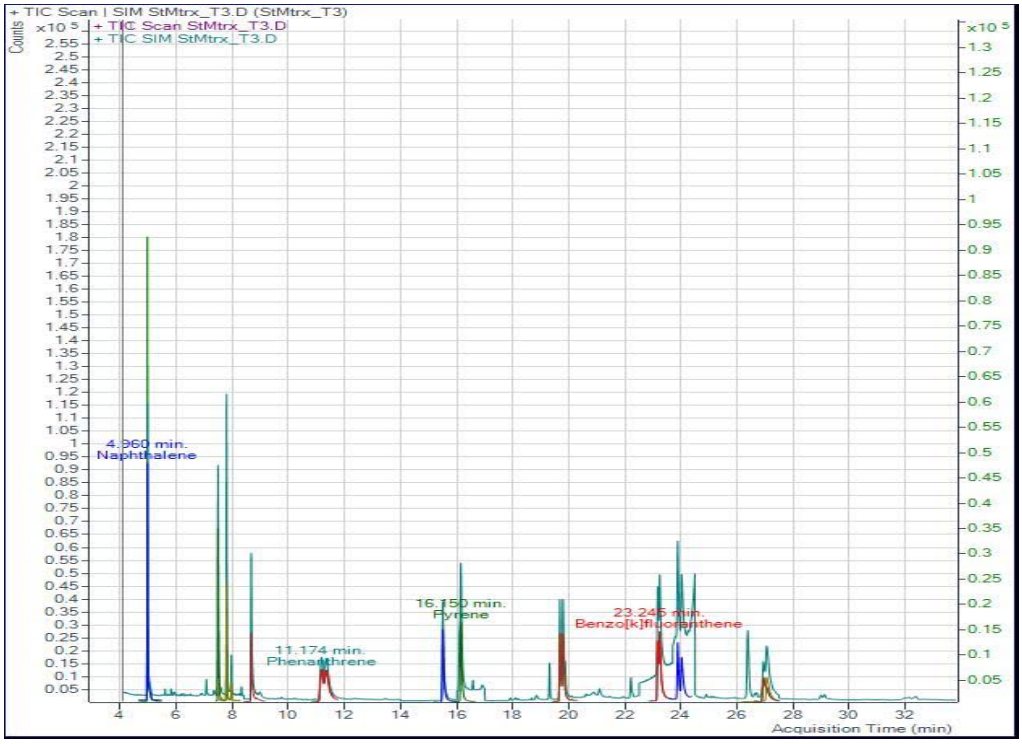


Figure 38: Represents the Std Matrx effect results of T3

Table 11: Represents the numerical data results of StdMtrx effect

Sample	StMtrx%_T1	Mean%_T1	SD_T1	CV_T1	StMtrx%_T2	Mean%_T2	SD_T2	CV_T2	StMtrx%_T3	Mean%_T3	SD_T3	CV_T3
Naphthalene	90%	31%	4.03	12.93	95%	27%	2.48	9.36	102%	13%	2.09	15.50
Acenaphythylene	102%	79%	2.90	3.68	114%	68%	1.94	2.86	121%	59%	2.74	4.63
Acenaphthene	101%	75%	2.62	3.47	111%	66%	1.38	2.09	119%	58%	2.99	5.15
Flourene	102%	80%	1.85	2.32	111%	72%	1.15	1.61	115%	67%	2.02	3.03
Phenanthrene	215%	78%	0.76	0.98	220%	73%	3.10	4.24	206%	74%	4.56	6.20
Anthracene	112%	74%	3.58	4.82	101%	77%	1.58	2.05	114%	73%	2.83	3.86
Fluoranthene	117%	78%	0.71	0.91	127%	72%	0.40	0.55	132%	69%	0.96	1.39
Pyrene	123%	78%	1.42	1.81	135%	71%	0.95	1.32	145%	67%	1.98	2.98
Benzo[a]Anthracene	208%	73%	0.85	1.17	206%	70%	4.02	5.75	199%	72%	1.77	2.46
Chrysene	93%	81%	1.69	2.09	102%	71%	2.03	2.84	112%	65%	2.69	4.16
Benzo[b]fluoranthene	215%	66%	2.41	3.66	197%	69%	3.92	5.71	195%	71%	4.78	6.71
Benzo[k]fluoranthene	144%	59%	7.07	11.99	142%	62%	2.45	3.97	155%	57%	2.13	3.76
Benzo[a]pyrene	172%	63%	1.87	2.99	108%	92%	2.12	2.31	88%	110%	3.19	2.91
Indeno[123-cd]pyrene	92%	47%	2.58	5.46	90%	47%	2.27	4.84	85%	46%	2.70	5.84
Benzo[ghi]perylene	93%	65%	0.96	1.49	109%	51%	1.42	2.77	114%	56%	3.05	5.50
Dibenz[ah]anthracene	152%	60%	2.80	4.68	148%	57%	4.45	7.84	140%	57%	3.40	5.93

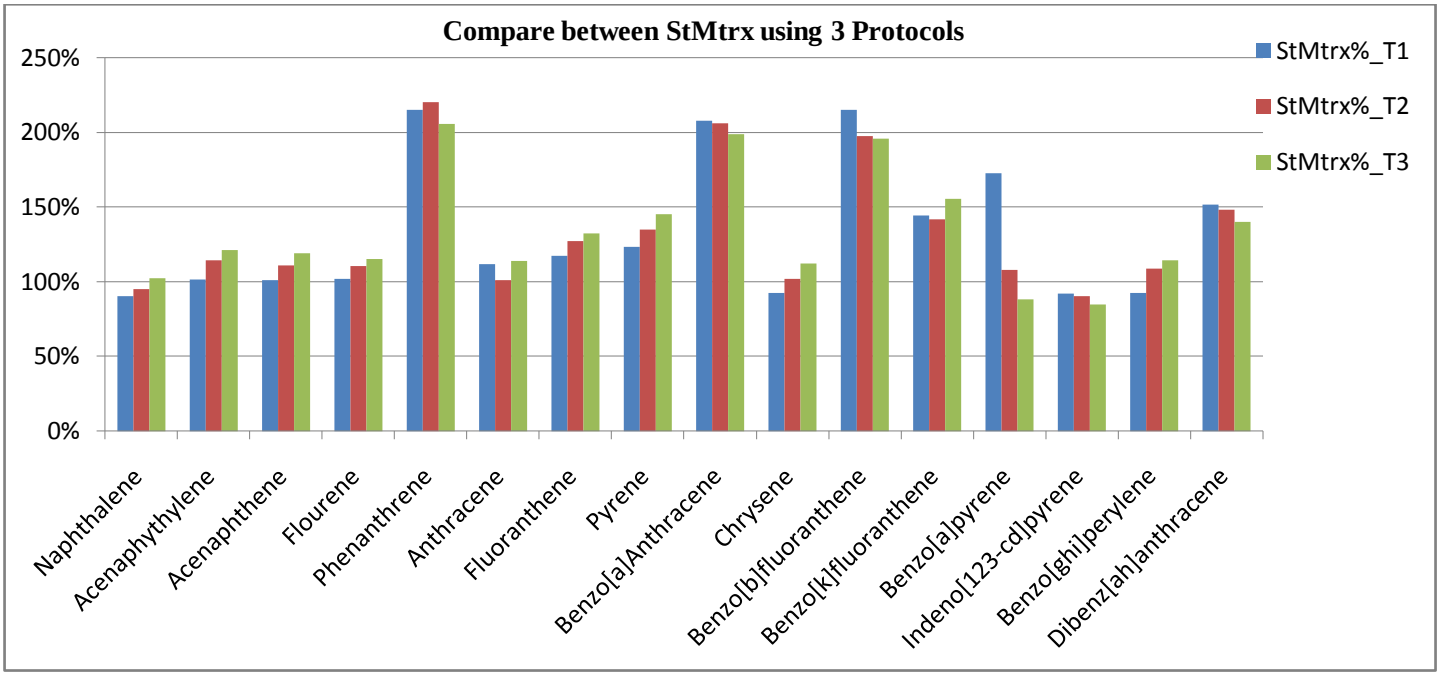


Figure 39: Comparison of StdMtrx effect on T1, T2& T3

The below figures 40 confirms the effect T2 on recovery. Benzo[a]pyrene shows moderate accepted recovery at 90% compared to T1 & T3 matrix effect

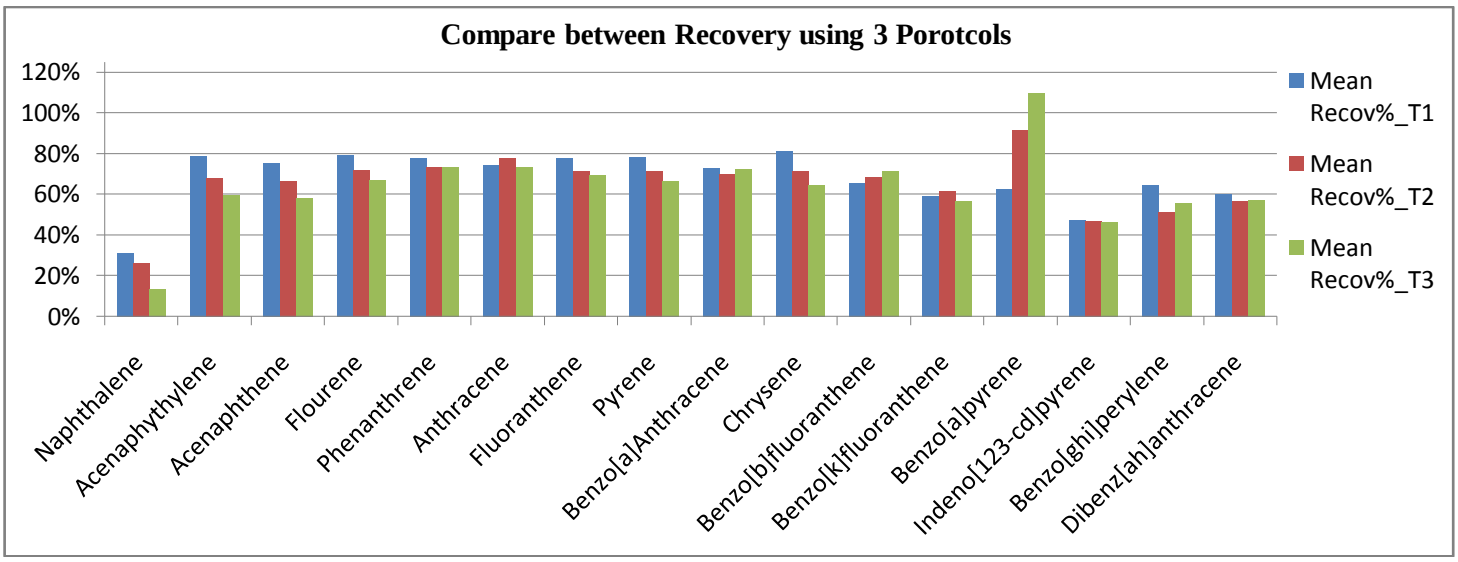


Figure 40: Comparison of the recovery between T1, T2 & T3

Method 2: Packed & Raw Milk Monitoring

1.1 Packed Milk Results

(The below results and graphs are obtained from the data found in results annexes, annex 2)

1.1.1 Samples with no detected PAHs compounds (Negative Results)

Figures 42 & 43 represent the results of the packaged milk code number 5 and 6 which show no detection of PAHs compounds.

Figure 39: Shows the results of package milk code 5

Figure 40: Shows the results of package milk code 6.

1.1.2 Samples with 1 detected compound

Figure 44 represents the result of package milk code 4, were benzo[b]fluranthene was detected with concentration 70 µg/g.

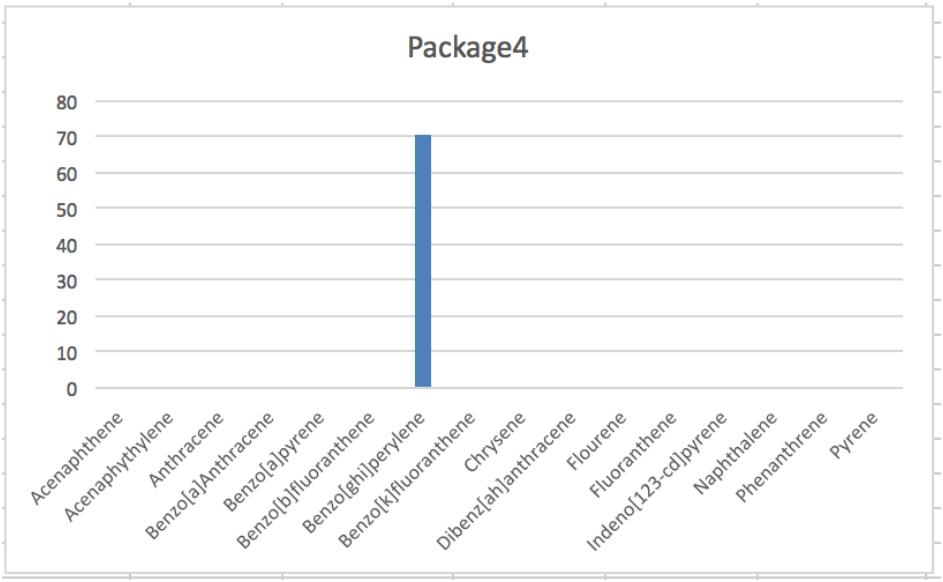


Figure 41: Shows the result of package milk code 4

Figure 45 represents the result of package milk code 2, were benzo[a]pyrene was detected with concentration 14 µg/g.

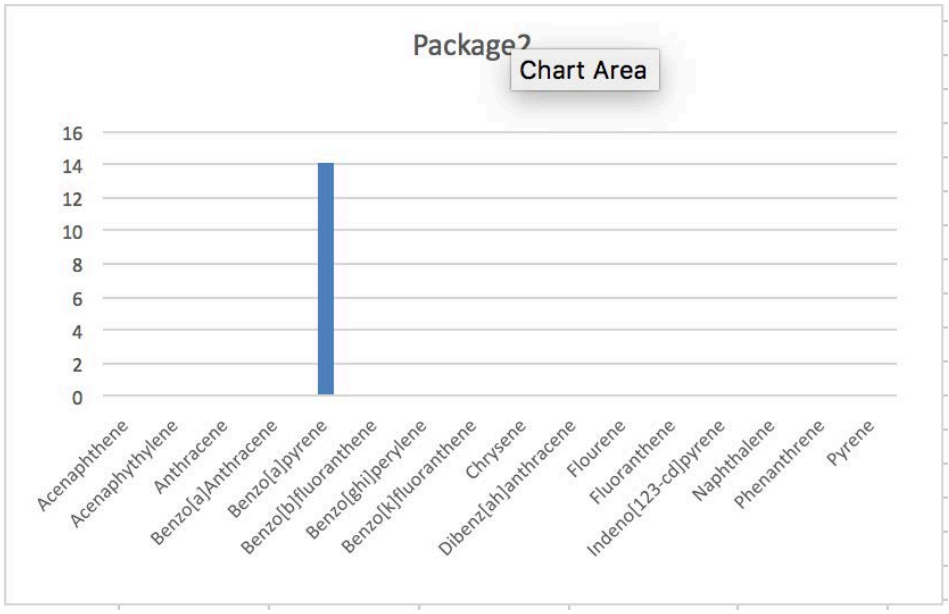


Figure 42: Shows the result of package milk code 2

Figure 46 represents the result of package milk code 7, were benzo[ghi]perylene was detected with concentration 55 µg/g

Figure 43: Shows the result of package milk code 7

1.1.3 Samples with 2 detected Compounds

Figure 47 represents the result of package milk code 3, were benzo[a]pyrene was detected with concentration 12 µg/g, and benzo[ghi]perylene was detected with concentration 62 µg/g

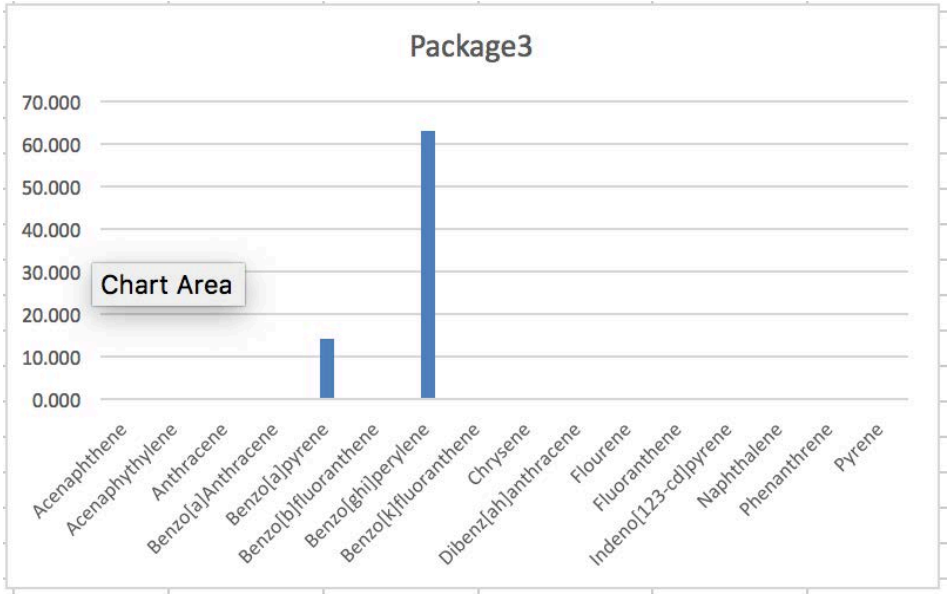


Figure 44: shows the result of package milk code 3

Figure 48 represents the result of package milk code 19, were Anthracene was detected with concentration 8.5 µg/g, and Fluoranthene was detected with concentration 2.3 µg/g

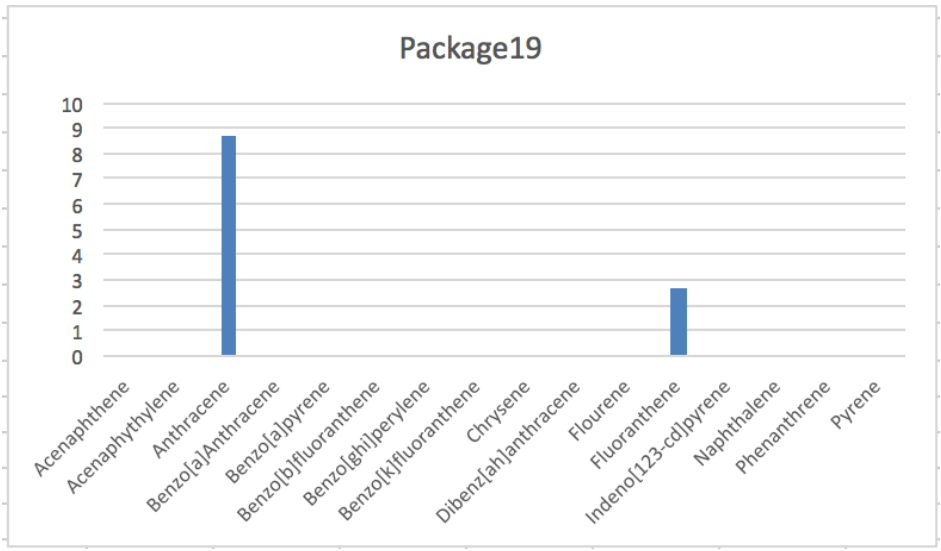


Figure 45: shows the result of package milk code 19

Figure 49 represents the result of package milk code 18, were Anthracene was detected with concentration 9.5 µg/g, and Fluoranthene was detected with concentration 2.7 µg/g.

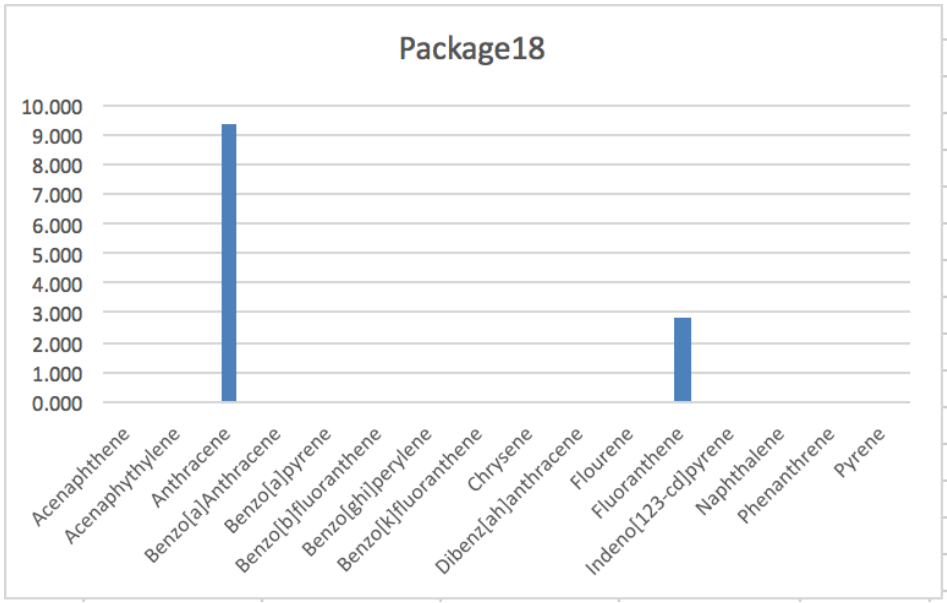


Figure 46: Shows the result of package milk code 18

Figure 50 represents the result of package milk code 21, were Anthracene was detected with concentration 8.1 µg/g, and Fluoranthene was detected with concentration 3 µg/g.

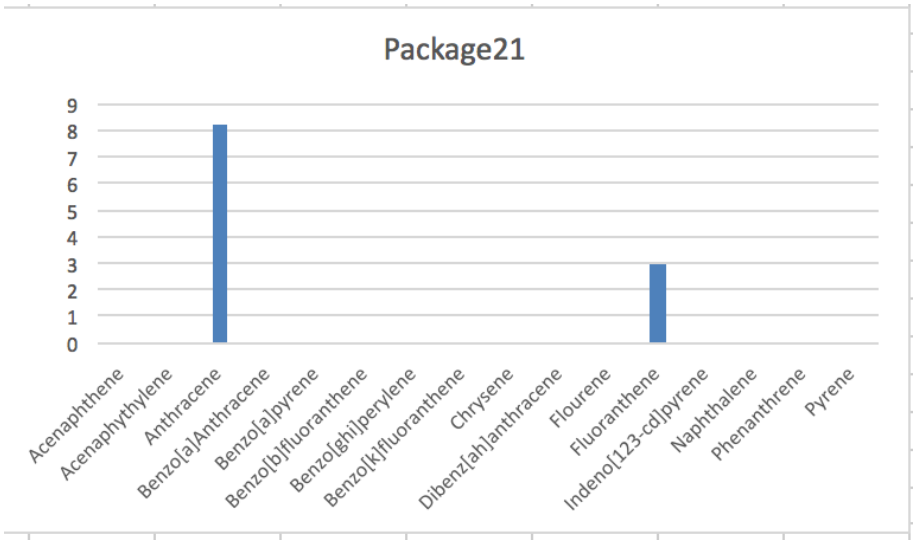


Figure 47: Shows the result of package milk code 21

Figure 51 represents the result of package milk code 22, were Anthracene was detected with concentration 8.5 µg/g, and Fluoranthene was detected with concentration 3 µg/g.

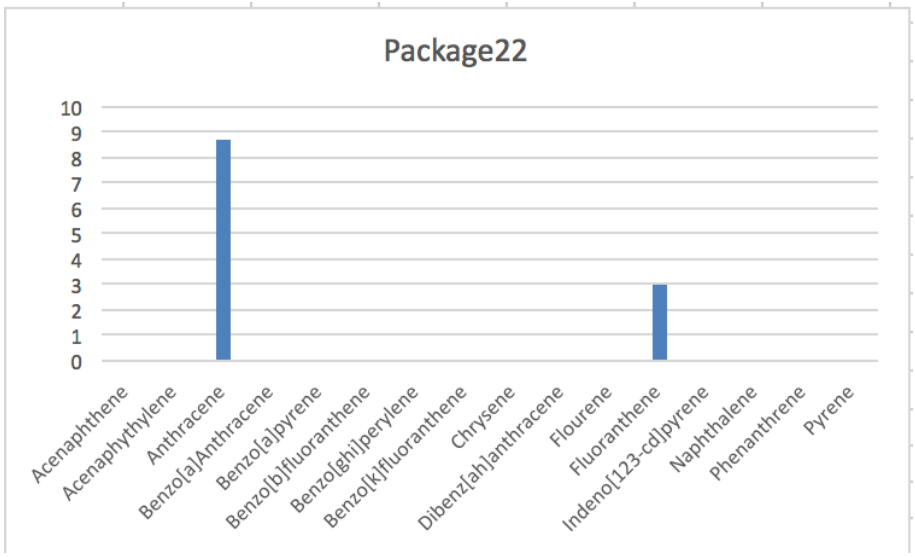


Figure 48: Shows the result of package milk code 22

Figure 52 represents the result of package milk code 23, were Anthracene was detected with concentration 8.9 µg/g, and Fluoranthene was detected with concentration 2.8 µg/g.

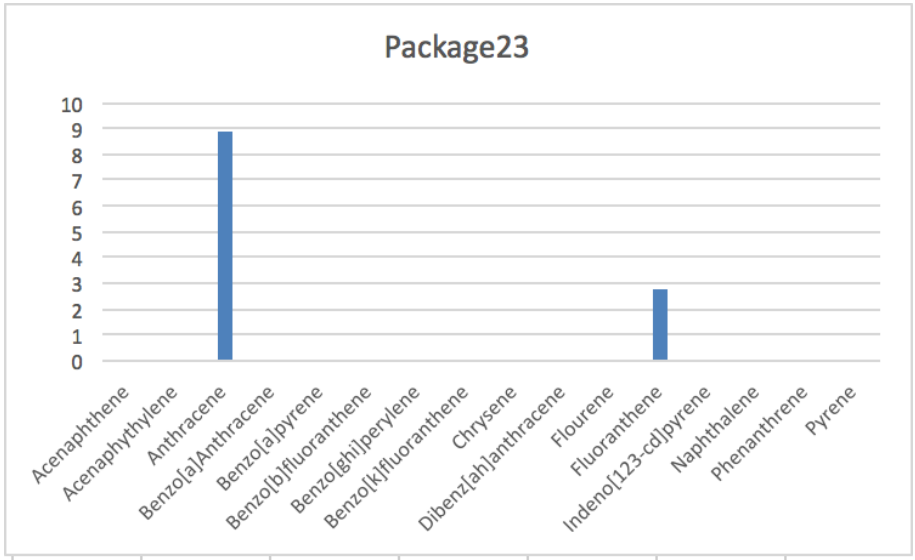


Figure 49: Shows the result of package milk code 23

1.1.4 Samples with 3 PAHs Compounds

Figure 53 represents the result of package milk code 16, were Anthracene was detected with concentration 8.5 µg/g, Fluoranthene was detected with concentration 2.7 µg/g, and Benozo[a]pyrene was detected with concentration 14.5 µg/g

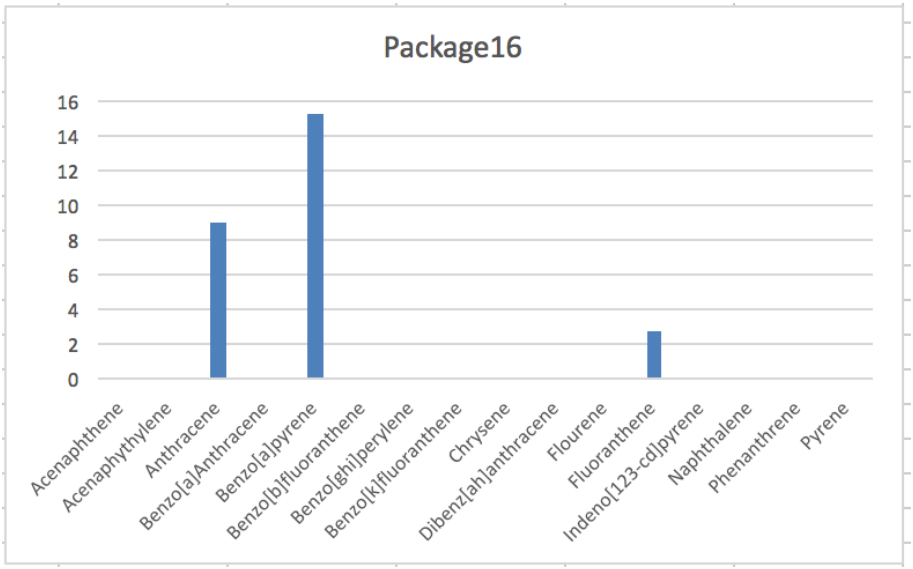


Figure 50: Shows the result of package milk code 16

Figure 54 represents the result of package milk code 20, were Anthracene was detected with concentration 8.4 µg/g, Fluoranthene was detected with concentration 3 µg/g, and Benozo[a]pyrene was detected with concentration 14.5 µg/g

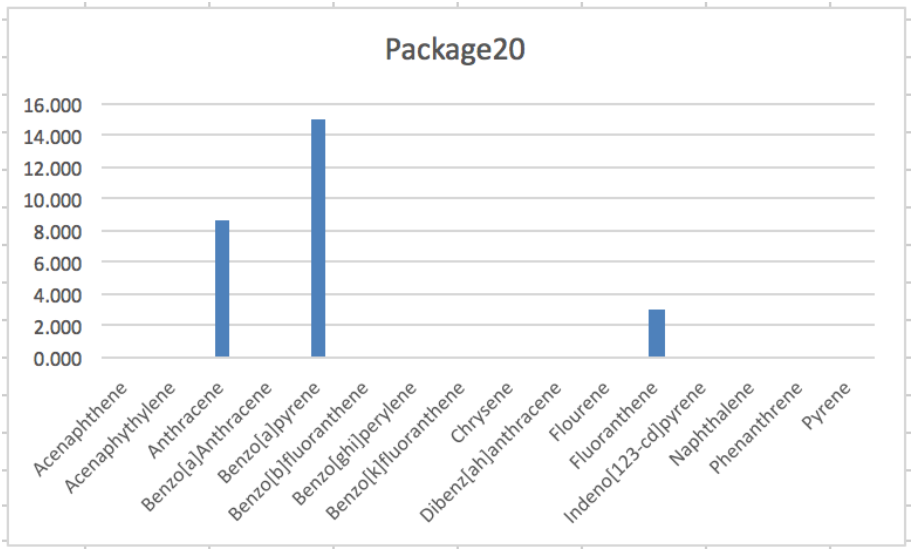


Figure 51: Shows the result of package milk code 20

Figure 55 represents the result of package milk code 17, were Anthracene was detected with concentration 9 µg/g, Fluoranthene was detected with concentration 3 µg/g, and Benozo[a]pyrene was detected with concentration 15 µg/g

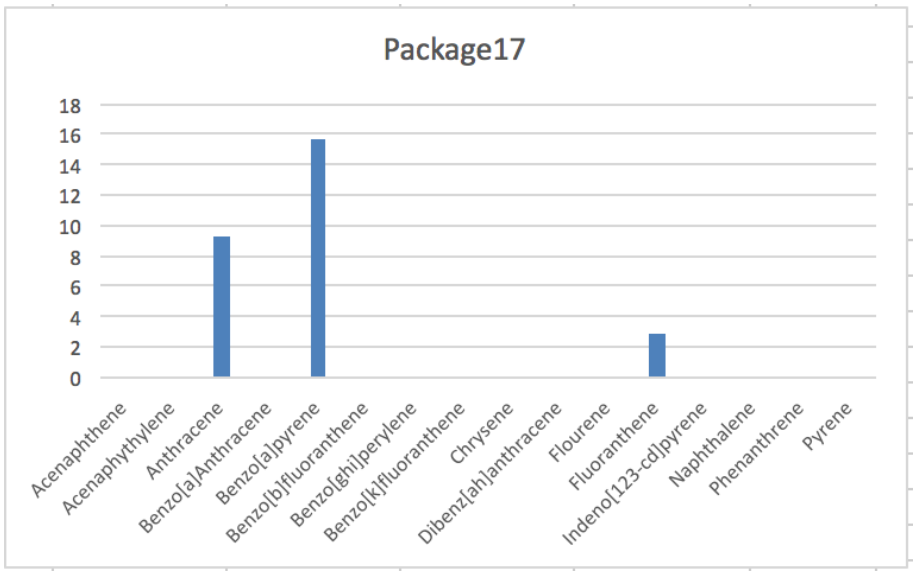


Figure 52: Shows the result of package milk code 17

Figure 56 represents the result of package milk code 24, were Anthracene was detected with concentration 9 µg/g, Fluoranthene was detected with concentration 3 µg/g, and Benozo[a]pyrene was detected with concentration 15 µg/g

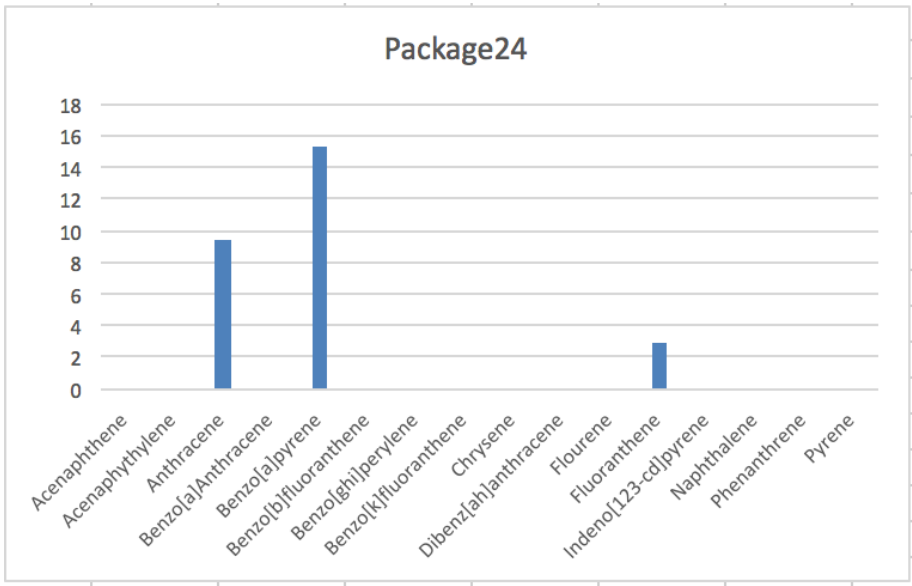


Figure 53: Shows the result of package milk code 24

1.1.5 Samples with 4 or more PAHs compounds

Figure 8 represents the result of package milk code 8, were Acenaphythylene was detected with concentration 3 µg/g, Anthracene was detected with concentration 11 µg/g, Benozo[a]pyrene was detected with concentration 15 µg/g, Flourene was detected with concentration 1.9 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4.2 µg/g, Phenanthrene was also detected with concentration 14.1 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

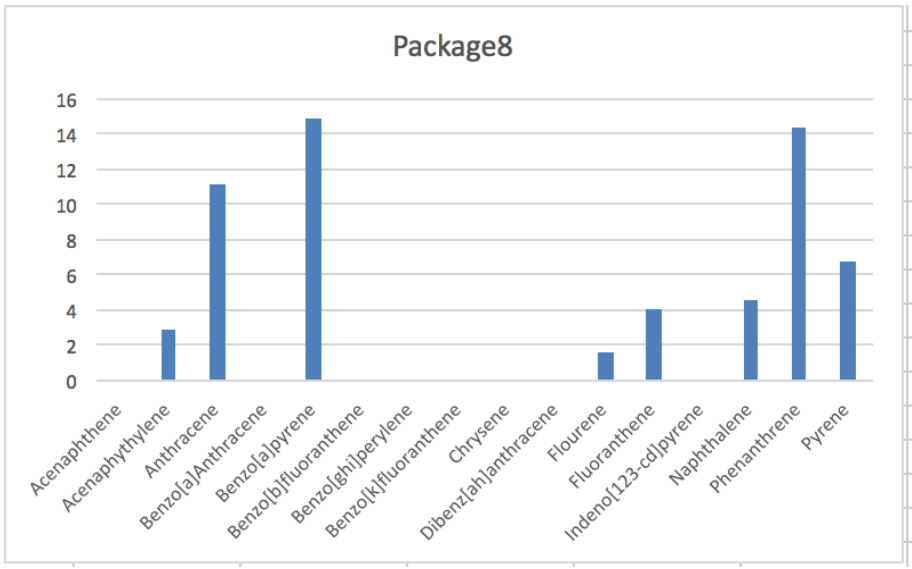


Figure 54: Shows the result of package milk code 8

Figure 58 represents the result of package milk code 9, were Anthracene was detected with concentration 11 µg/g, Benozo[b]fluoranthenewas detected with concentration 16 µg/g, Flourene was detected with concentration 1.9 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4.2 µg/g, Phenanthrene was also detected with concentration 15 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

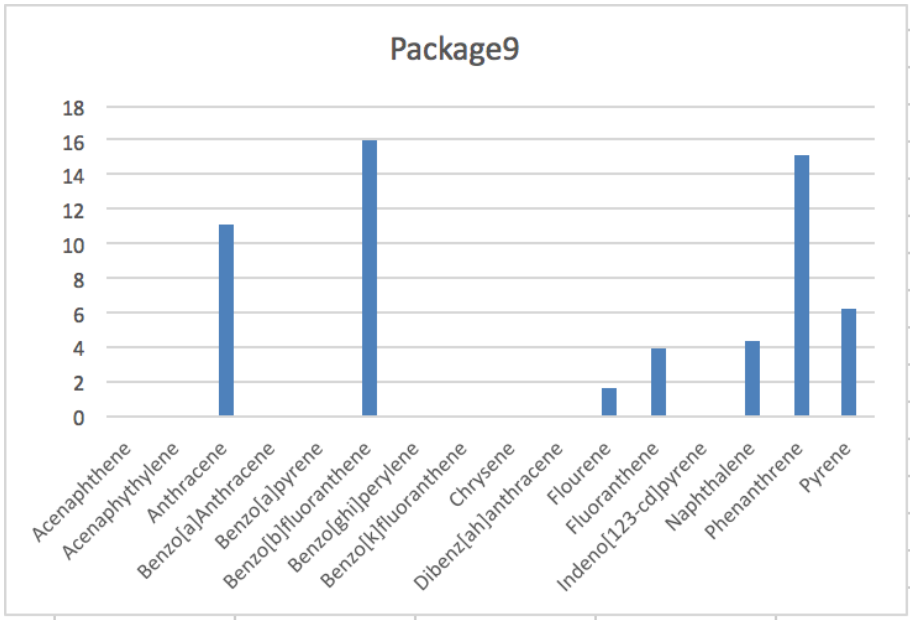


Figure 55: Shows the result of package milk code 9

Figure 59 represents the result of package milk code 11, were Anthracene was detected with concentration 11 µg/g, Benozo[b]fluoranthenewas detected with concentration 16 µg/g, Flourene was detected with concentration 1.9 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4.2 µg/g, Phenanthrene was also detected with concentration 15 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

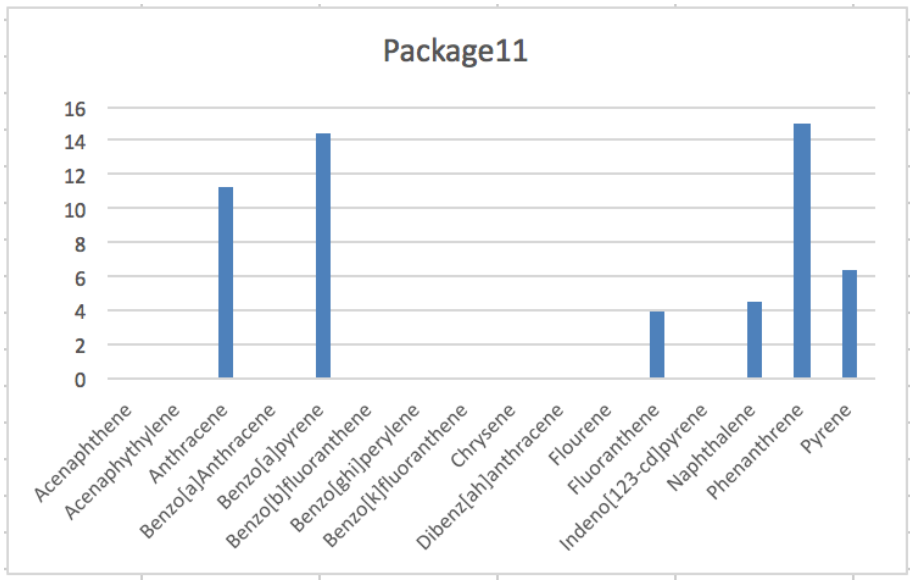


Figure 56: Shows the result of package milk code 11

Figure 60 represents the result of package milk code 10, were Anthracene was detected with concentration 11 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4 µg/g, Phenanthrene was also detected with concentration 15 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

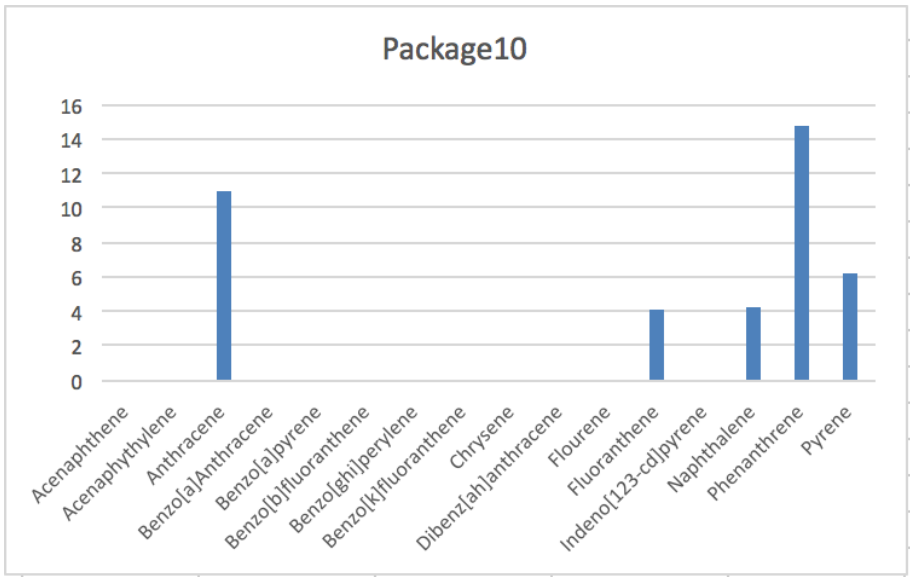


Figure 57: Shows the result of package milk code 10

Figure 61 represents the result of package milk code 13, were Anthracene was detected with concentration 11 µg/g, Benozo[a]pyrenewas detected with concentration 15 µg/g, Flourene was detected with concentration 3 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4.2 µg/g, Phenanthrene was also detected with concentration 13 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

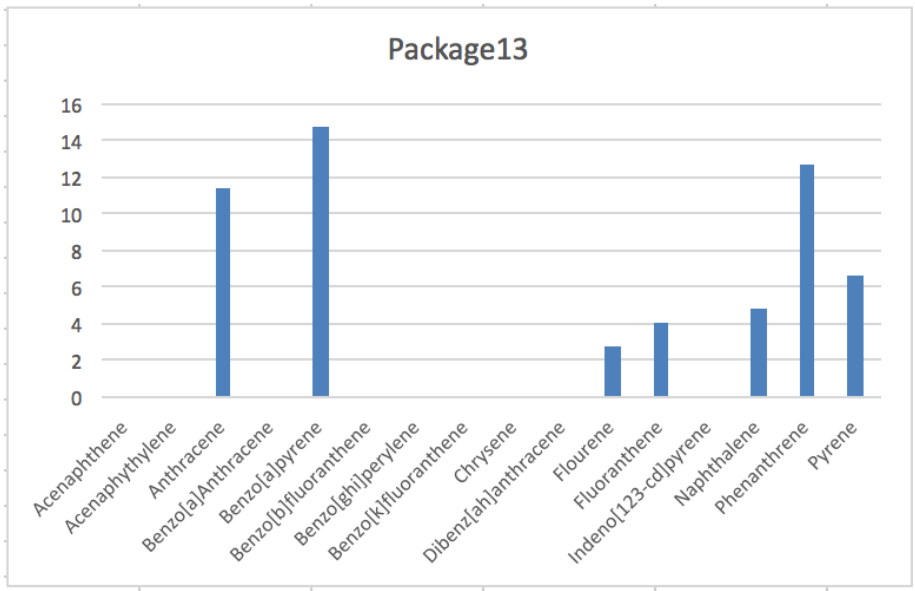


Figure 58: Shows the result of package milk code 13

Figure 62 represents the result of package milk code 12, were Acenaphthene was detected with concentration 2 µg/g, Anthracene was detected with concentration 11.5 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4.2 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

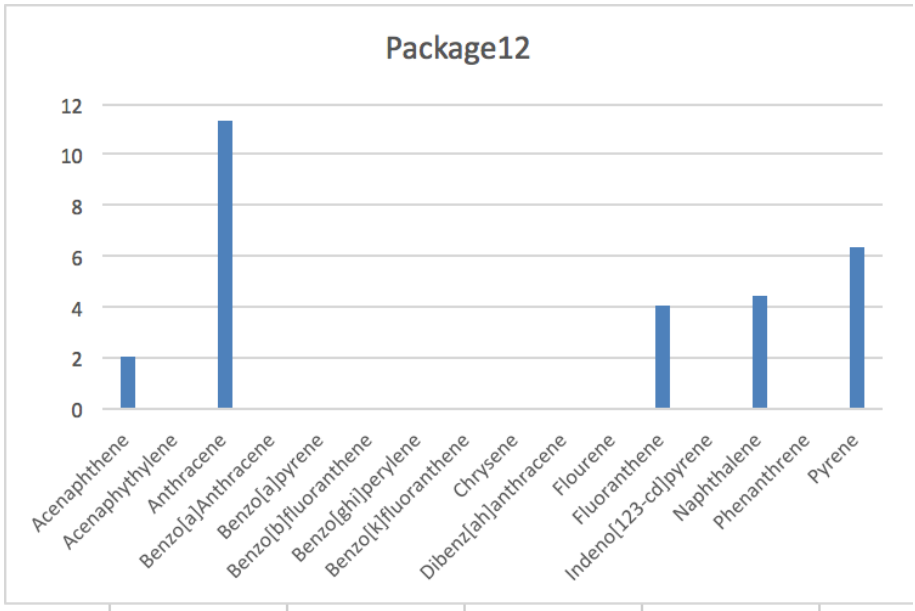


Figure 59: Shows the result of package milk code 12

Figure 63 represents the result of package milk code 14, were Acenaphythylene was detected with concentration 3 µg/g, Anthracene was detected with concentration 12 µg/g, Benozo[a]pyrenewas detected with concentration 14.5 µg/g, Benzo[k]fluroranthene was detected with concentration 3 µg/g, Flourene was detected with concentration 3 µg/g, Fluoranthene was detected with concentration 5 µg/g, Naphthalene was detected with concentration 9 µg/g, Phenanthrene was also detected with concentration 13 µg/g, and finally Pyrene was detected with concentration 8 µg/g.

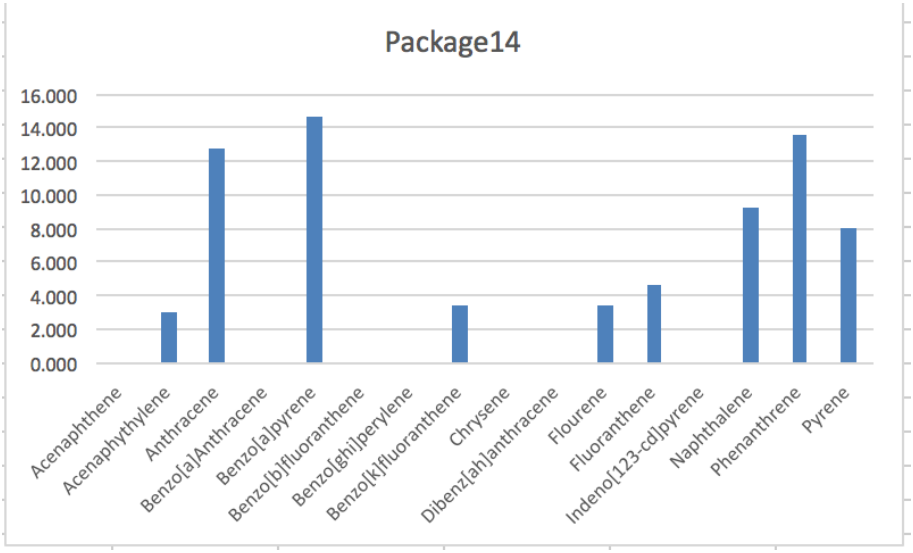


Figure 60: Shows the result of package milk code 14

Figure 64 represents the result of package milk code 15, were Anthracene was detected with concentration 11 µg/g, Benozo[a]pyrenewas detected with concentration 14.5 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 4.5 µg/g, Phenanthrene was also detected with concentration 11 µg/g, and finally Pyrene was detected with concentration 6.1 µg/g.

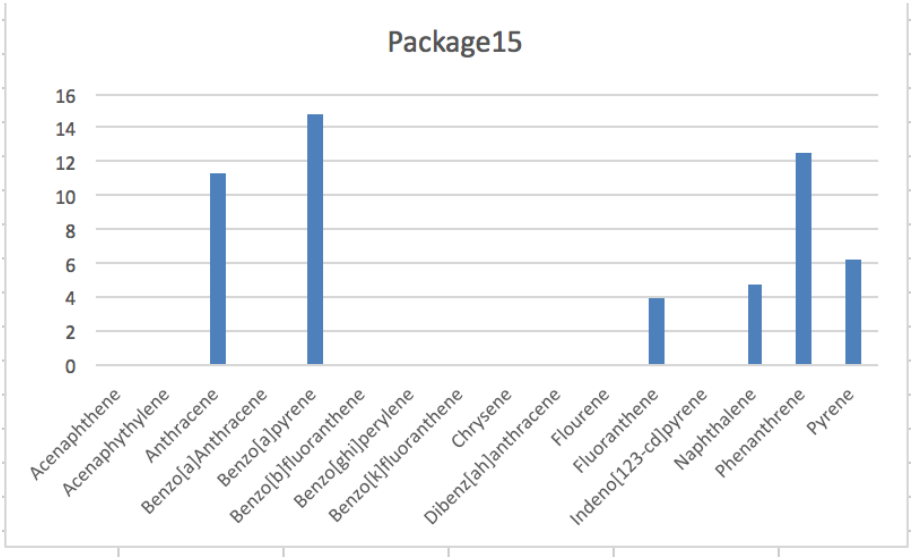


Figure 61: Shows the result of package milk code 15

1.1.6 Total Distribution of Packed Milk Results

The below figure(65) and table(11) represents the total distribution percentage comparison between all the detected compounds.

Table 12: Represents the percentage of detected positive compounds

Positive Compound (s)	Percentage
Not Detected	12%
One Compound	12%
Two Compounds	24%
Three Compounds	16%
Four Compounds or more	36%

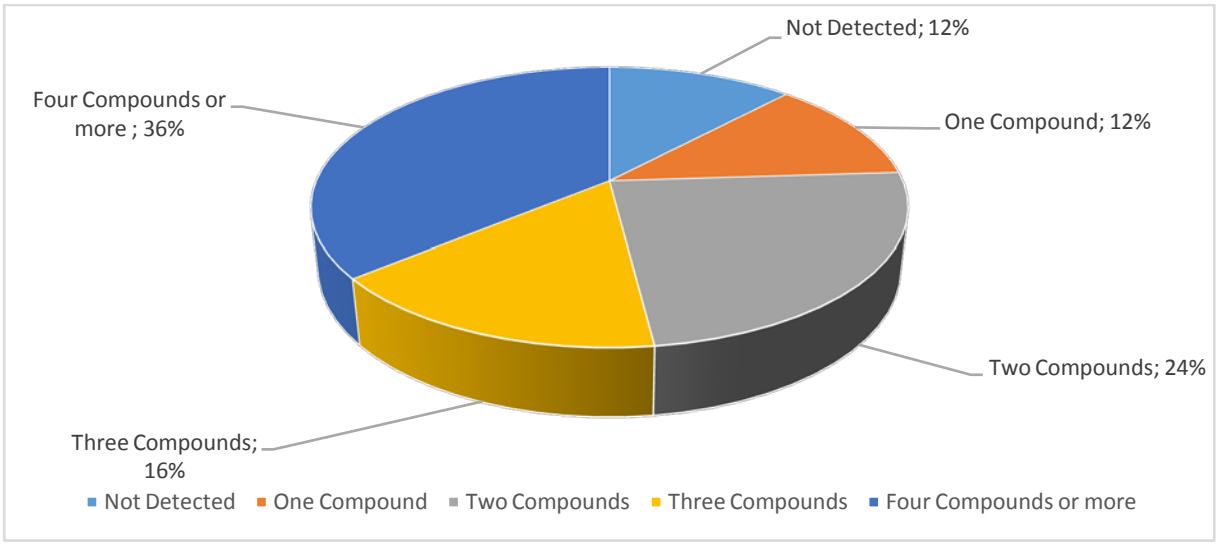


Figure 62: Represents the total distribution of packed milk results

7.4 Raw Milk Results:

(The below results and graphs are obtained from the data found in results annexes, annex 3)

3.1.1 Samples with no detected PAHs compounds (Negative Results)

Figure 66, 67, and 68 represents the result of the raw milk sample code 12, 19, and 26 which show no detection of PAHs compounds

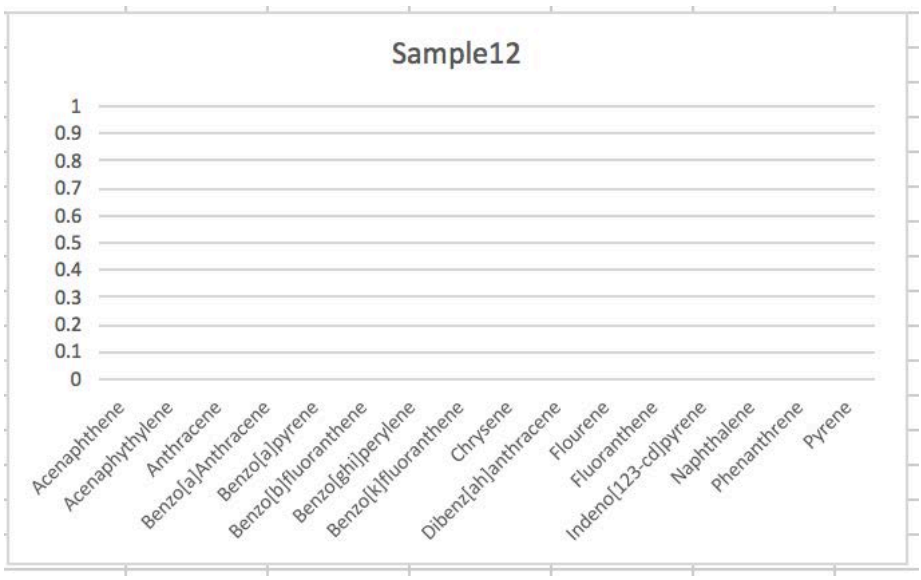


Figure 63: Represents the result of raw milk sample 12

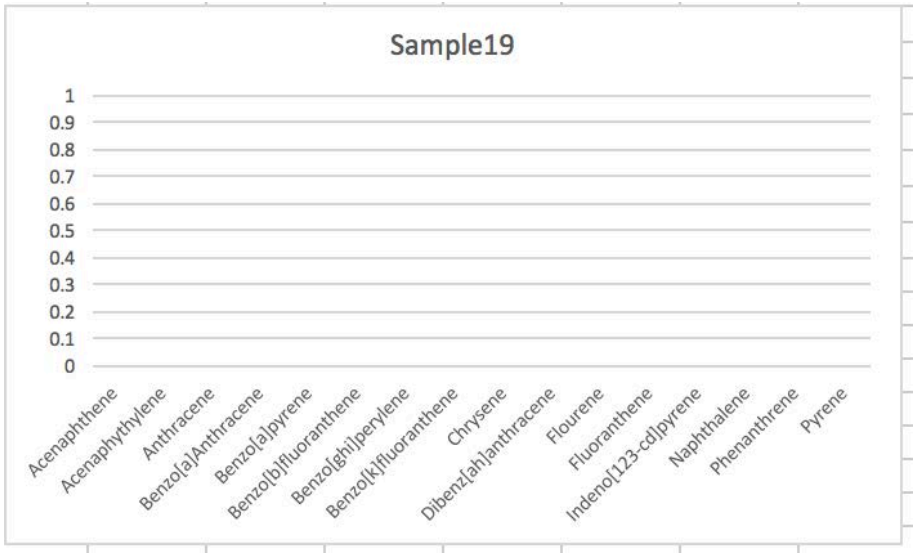


Figure 64: Represents the result of raw milk sample 19

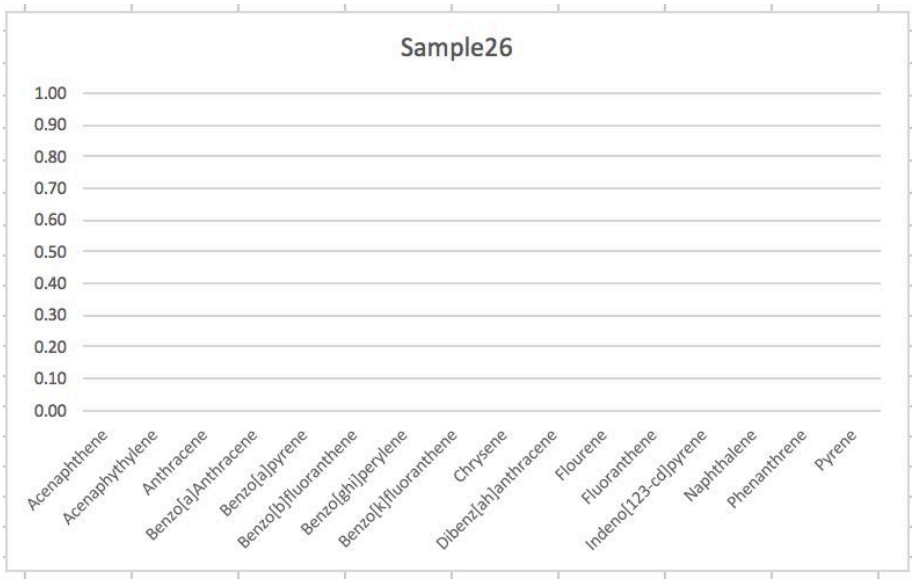


Figure 65: Represents the result of raw milk sample 26

3.1.2 Samples with 1 detected compound

Figure 69 represents the result of raw milk sample code 17, were Ideno[123-cd]pyrene was detected with concentration 14.5 µg/g

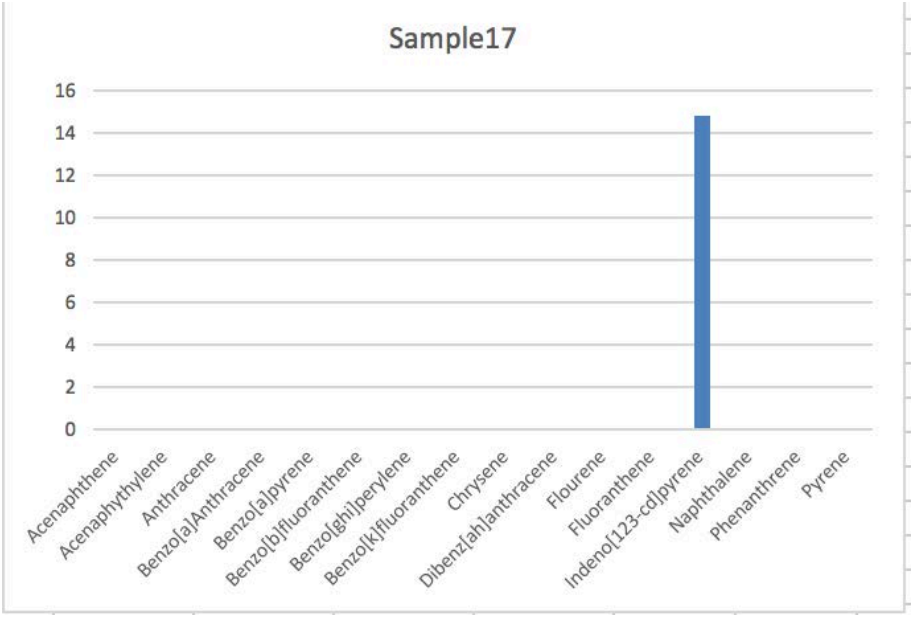


Figure 66: Represents the result of raw milk sample 17

Figure 70 represents the result of raw milk sample code 22, were Phenanthrene was detected with concentration 6.5 µg/g.

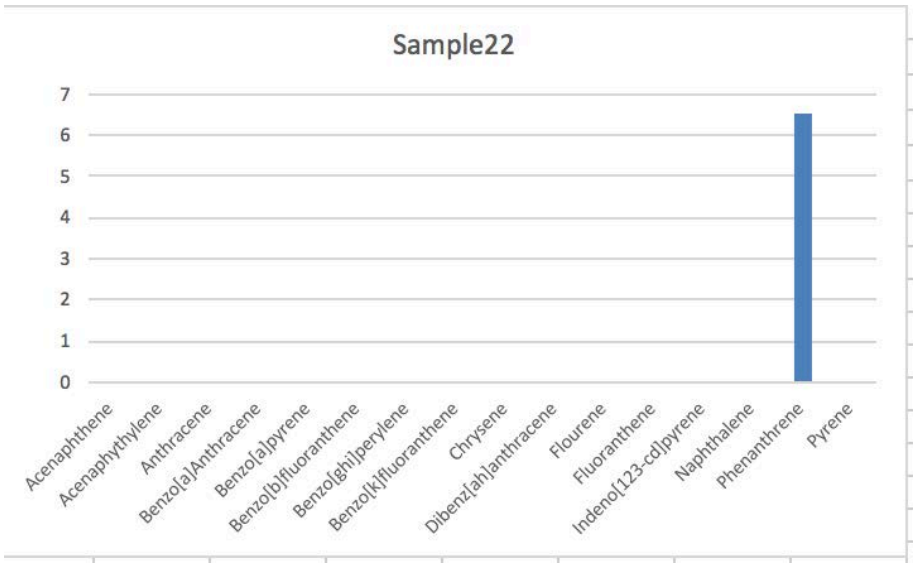


Figure 67: Represents the result of raw milk sample 22

3.1.3 Samples with 2 detected Compounds

Figure 71 represents the result of raw milk sample code 2, were Ideno[123-cd]pyrene was detected with concentration 70.5 µg/g, and Benzo[ghi]perylene was also detected with concentration 40.9 µg/g.

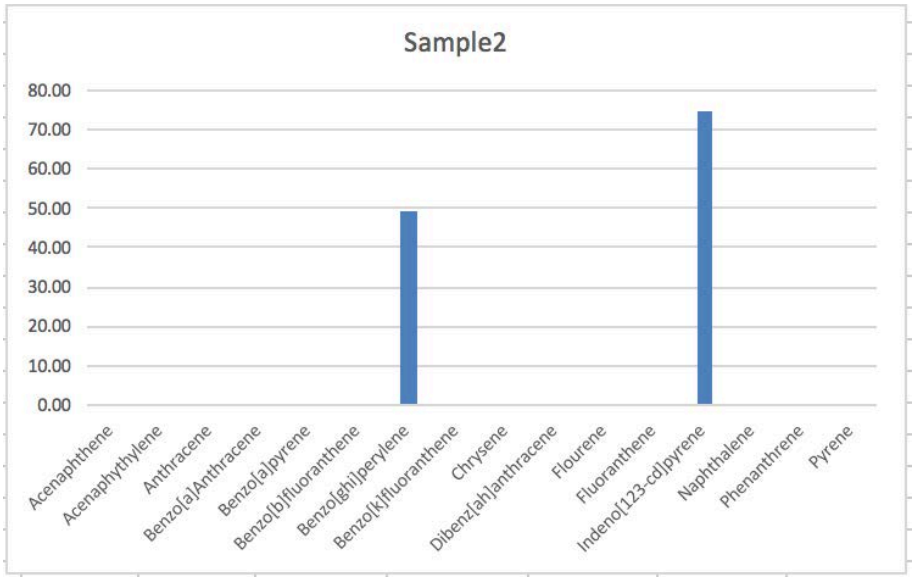


Figure 68: Represents the result of raw milk sample 2

Figure 72 represents the result of raw milk sample code 3, were Ideno[123-cd]pyrene was detected with concentration 17 µg/g, and Benzo[ghi]perylene was also detected with concentration 13 µg/g.

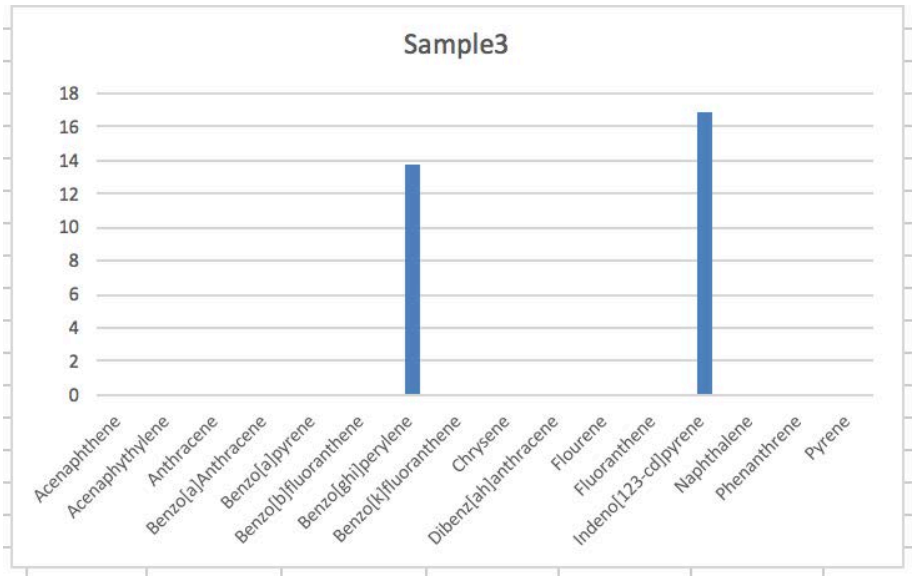


Figure 69: Represents the result of raw milk sample 3

Figure 73 represents the result of raw milk sample code 4, were Ideno[123-cd]pyrene was detected with concentration 41 µg/g, and Benzo[ghi]perylene was also detected with concentration 31 µg/g.

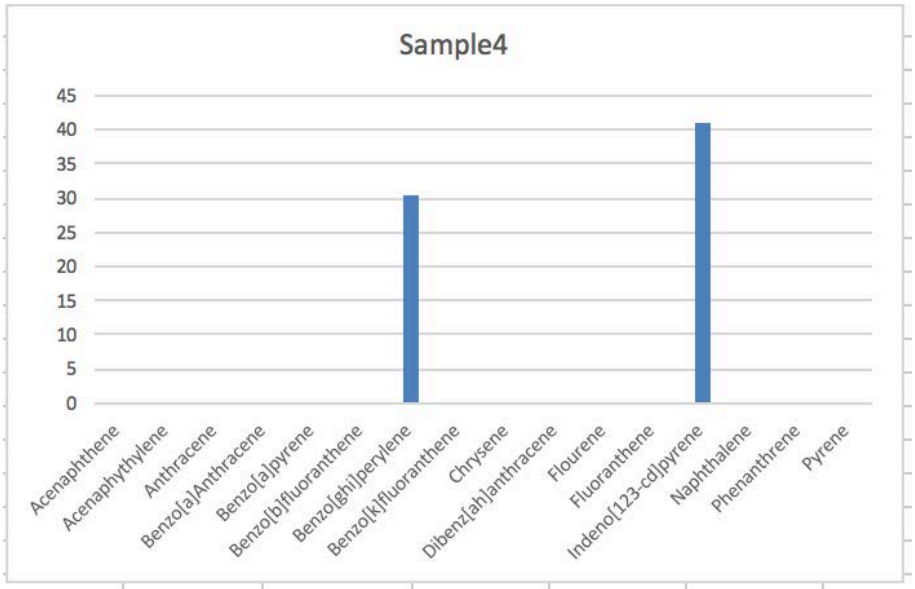


Figure 70: Represents the result of raw milk sample 4

Figure 74 represents the result of raw milk sample code 14, were Ideno[123-cd]pyrene was detected with concentration 5.9 µg/g, and Benzo[ghi]perylene was also detected with concentration 6.5 µg/g.

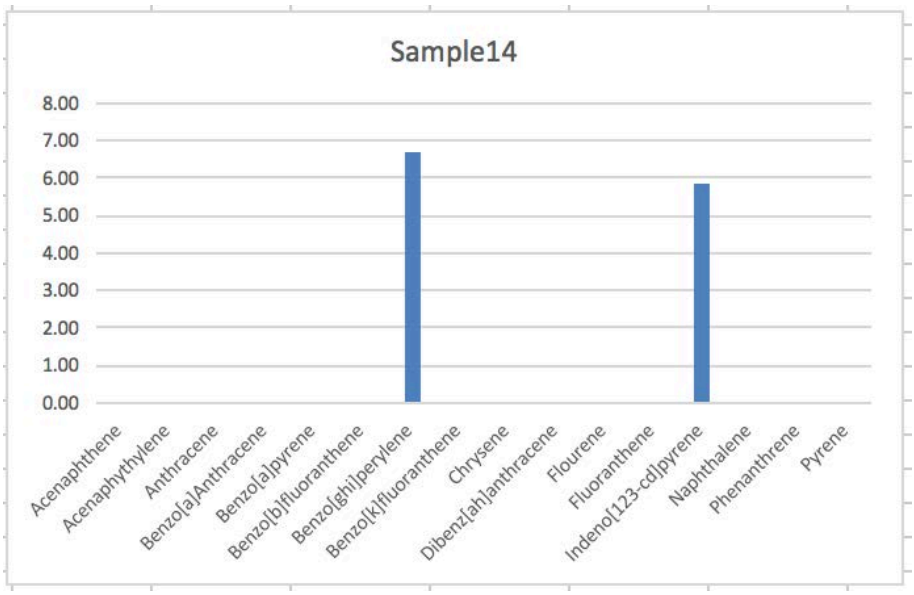


Figure 71: Represents the result of raw milk sample 14

Figure 75 represents the result of raw milk sample code 15, were Ideno[123-cd]pyrene was detected with concentration 36 µg/g, and Benzo[ghi]perylene was also detected with concentration 24 µg/g.

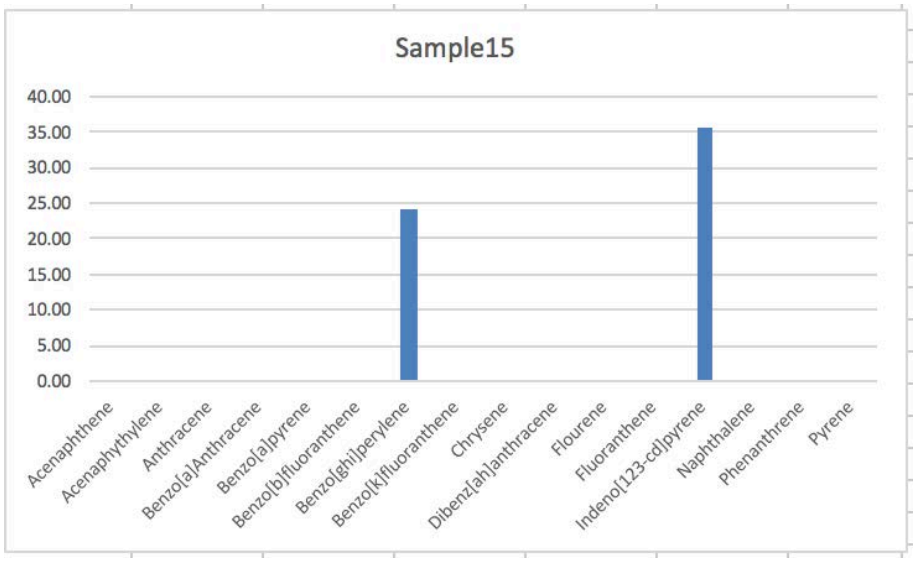


Figure 72: Represents the result of raw milk sample 15

Figure 76 represents the result of raw milk sample code 16, were Ideno[123-cd]pyrene was detected with concentration 29 µg/g, and Benzo[ghi]perylene was also detected with concentration 21 µg/g.

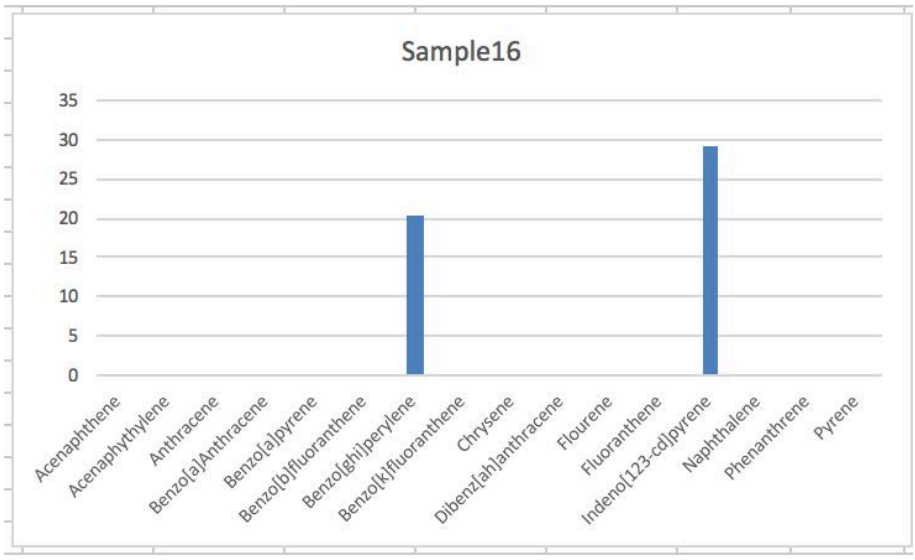


Figure 73: Represents the result of raw milk sample 16

Figure 77 represents the result of raw milk sample code 18, were Ideno[123-cd]pyrene was detected with concentration 44 µg/g, and Benzo[ghi]perylene was also detected with concentration 28 µg/g.

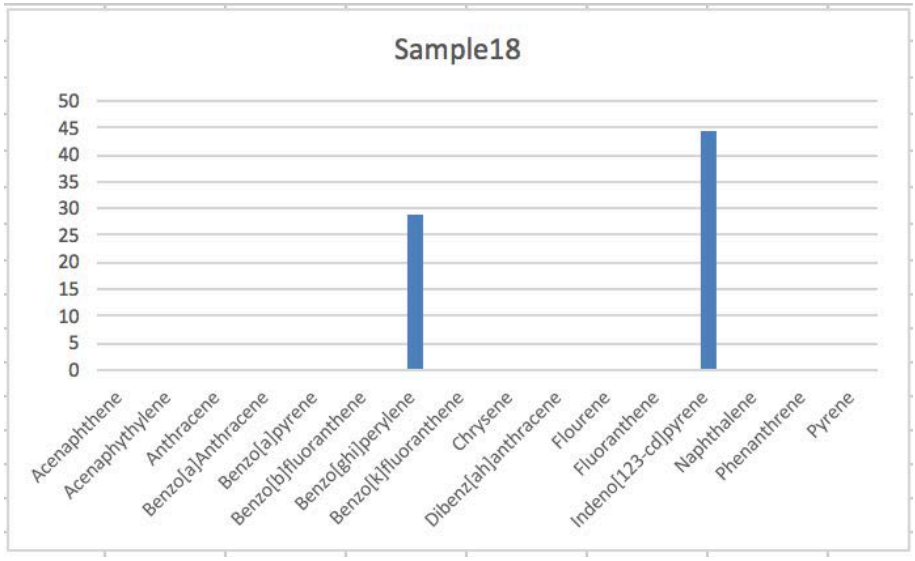


Figure 74: Represents the result of raw milk sample 18

Figure 78 represents the result of raw milk sample code 21, were Anthracene was detected with concentration 2.2 µg/g, and phenanthrene was also detected with concentration 7.5µg/g.

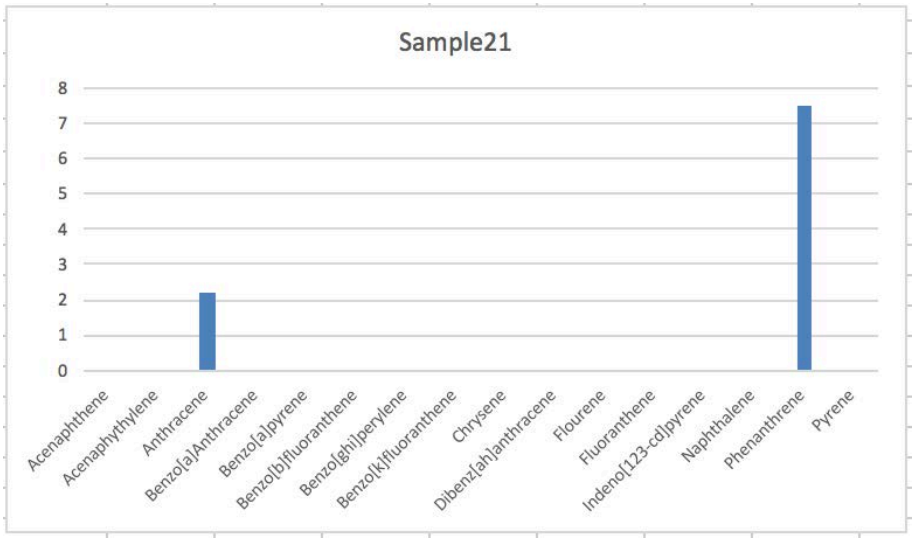


Figure 75: Represents the result of raw milk sample 21

Figure 79 represents the result of raw milk sample code 23, were Naphthalene was detected with concentration 1.5 µg/g, and phenanthrene was also detected with concentration 6.2 µg/g.

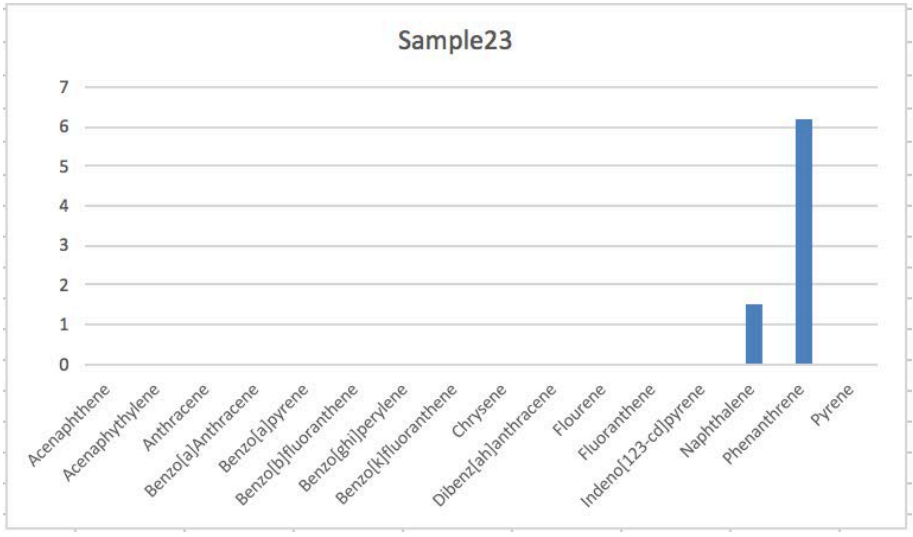


Figure 76: Represents the result of raw milk sample 23

Figure 80 represents the result of raw milk sample code 24, were Naphthalene was detected with concentration 2 µg/g, and phenanthrene was also detected with concentration 7.9 µg/g.

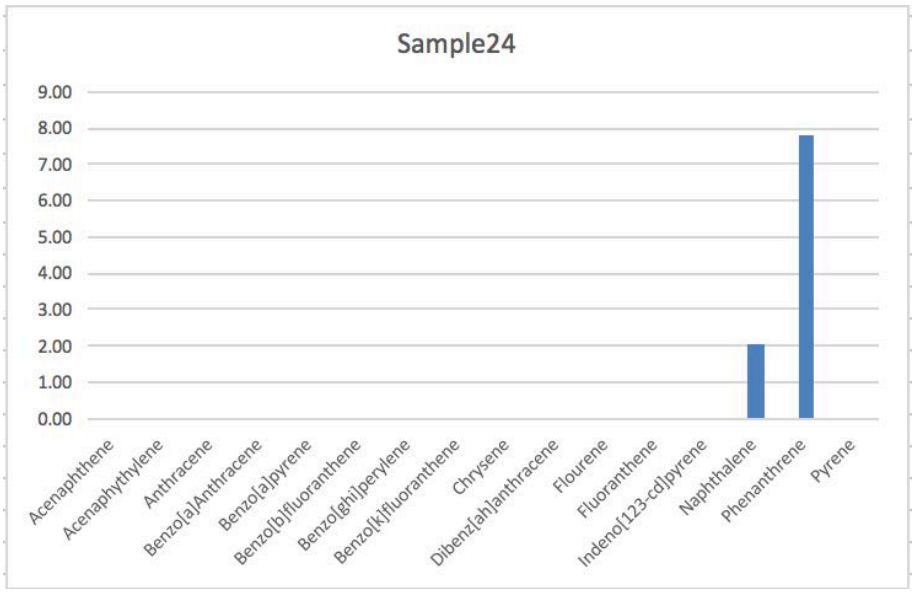


Figure 77: Represents the result of raw milk sample 24

3.1.4 Samples with 3 PAHs Compounds

Figure 81 represents the result of milksample code 6, were Indeno[123-cd]pyrene was detected with concentration 42 µg/g, Fluoranthene was detected with concentration 3 µg/g, and Benozo[ghi]perylene was detected with concentration 49 µg/g

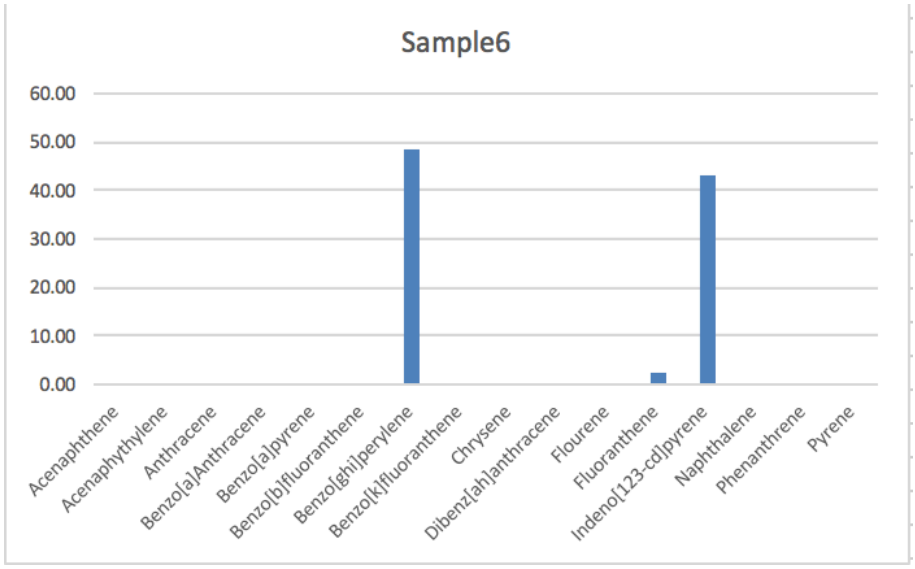


Figure 78: Represents the result of raw milk sample 6

Figure 82 represents the result of milk sample code 1, were Indeno[123-cd]pyrene was detected with concentration 90µg/g, Naphthalene was detected with concentration 3 µg/g, and Benozo[ghi]perylene was detected with concentration 69µg/g

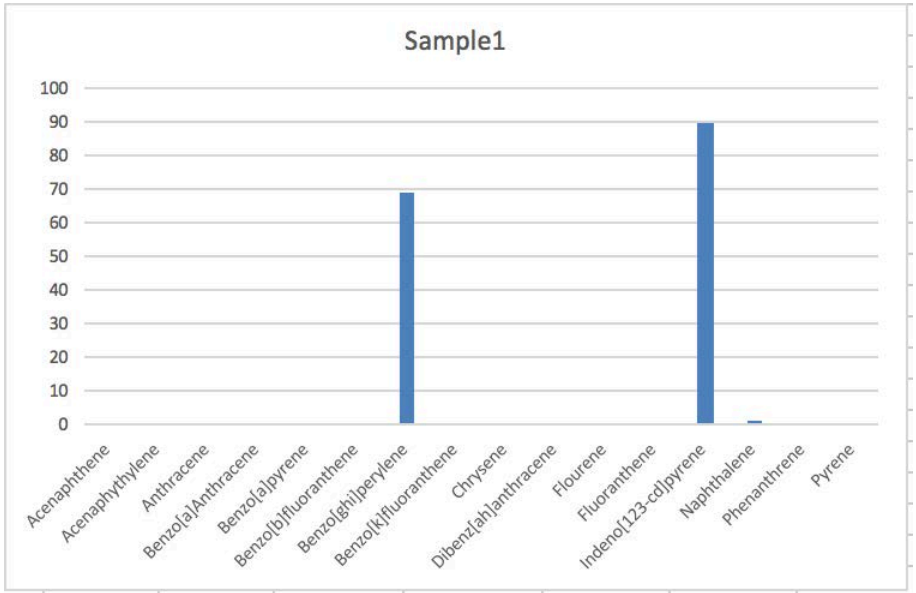


Figure 79: Represents the result of raw milk sample 1

Figure 83 represents the result of milk sample code 13, were Indeno[123-cd]pyrene was detected with concentration 78 µg/g, Dibenz[ah]anthracene was detected with concentration 8 µg/g, and Benozo[ghi]perylene was detected with concentration 58 µg/g

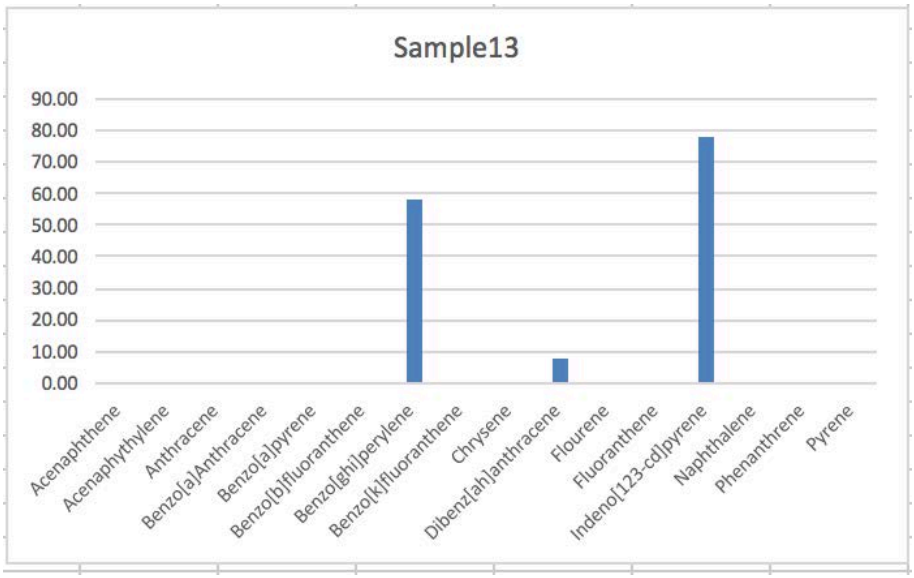


Figure 80: Represents the result of raw milk sample 13

Figure 84 represents the result of milk sample code 25, were Anthracene was detected with concentration 2.2µg/g, Naphthalene was detected with concentration 2.1 µg/g, and Phenanthrene was detected with concentration 9 µg/g

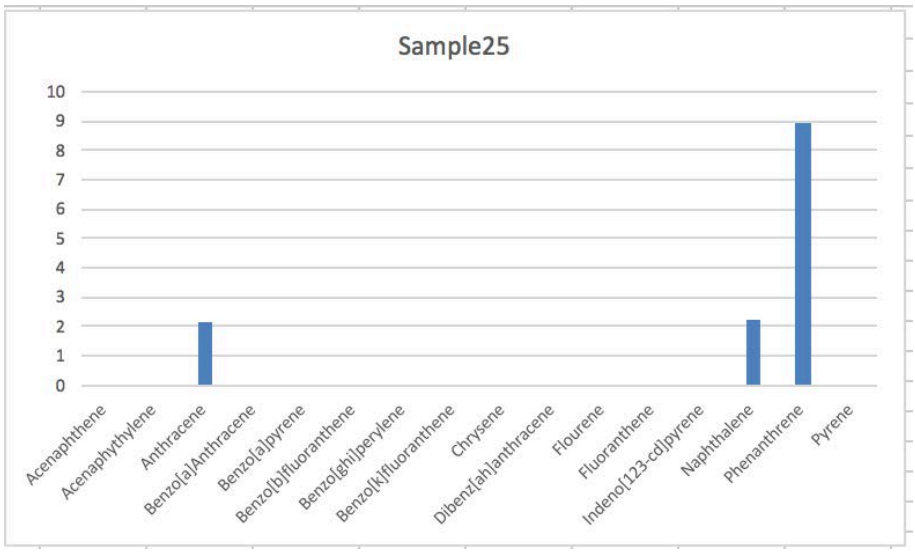


Figure 81: Represents the result of raw milk sample 25

3.1.5 Samples with 4 or more PAHs compounds

Figure 85 represents the result of milk sample code 5, were Anthracene was detected with concentration 18 µg/g, Acenaphthene was detected with concentration 9 µg/g, Acenaphythylene was detected with concentration 8, Benozo[ghi]perylenewas detected with concentration 20 µg/g,

Flourene was detected with concentration 10 µg/g, Fluoranthene was detected with concentration 4 µg/g, Ideno[123-cd]pyrene was detected with concentration 22 µg/g, Naphthalene was detected with concentration 95µg/g, Phenanthrene was also detected with concentration 35µg/g, and finally Pyrene was detected with concentration 3 µg/g.

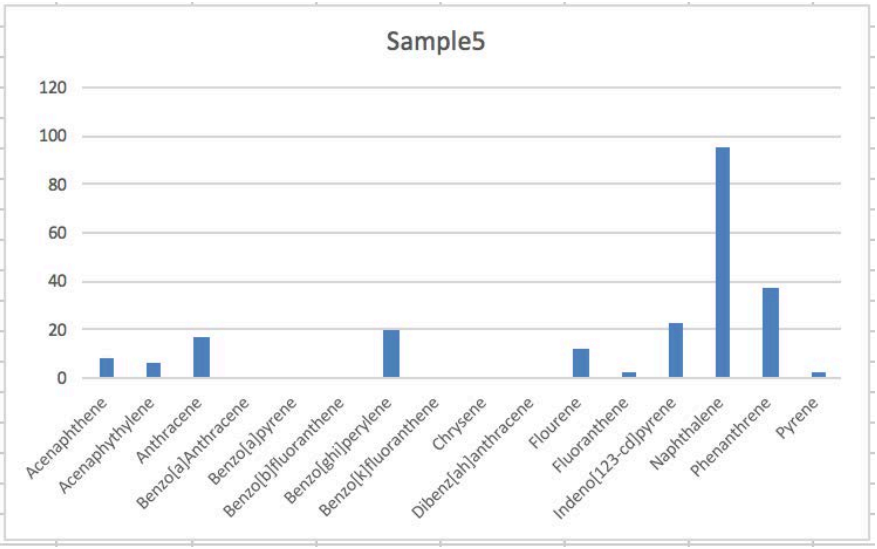


Figure 82: Represents the result of raw milk sample 5

Figure 86 represents the result of milk sample code 7, were Anthracene was detected with concentration 25 µg/g, Acenaphthene was detected with concentration 10 µg/g, Acenaphythylene was detected with concentration 9, Flourene was detected with concentration 14 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 124 µg/g, Phenanthrene was also detected with concentration 42 µg/g, and finally Pyrene was detected with concentration 3 µg/g.

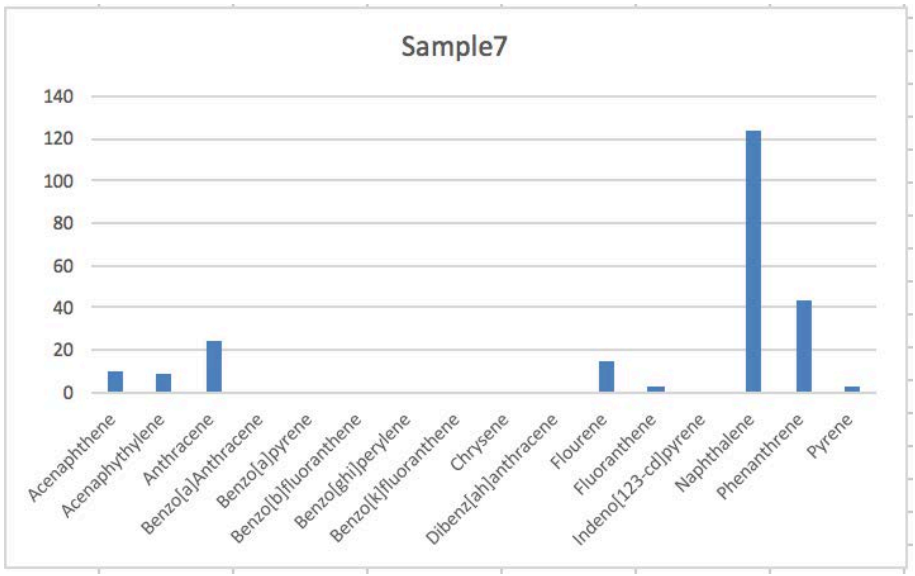


Figure 83: Represents the result of raw milk sample 7

Figure 87 represents the result of milk sample code 8, were Anthracene was detected with concentration 22 µg/g, Acenaphthene was detected with concentration 11 µg/g, Acenaphythylene was detected with concentration 7, Flourene was detected with concentration 17 µg/g, Fluoranthene was detected with concentration 4 µg/g, Naphthalene was detected with concentration 95µg/g, Phenanthrene was also detected with concentration 35 µg/g, and finally Pyrene was detected with concentration 3 µg/g.

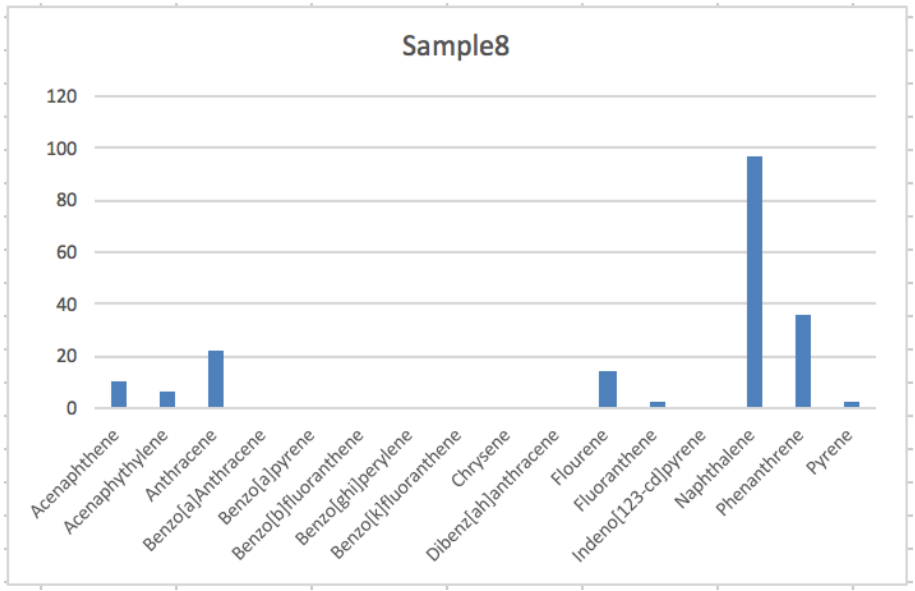


Figure 84: Represents the result of raw milk sample 8

Figure 88 represents the result of milk sample code 9, were Anthracene was detected with concentration 18 µg/g, Acenaphthene was detected with concentration 9 µg/g, Acenaphythylene was detected with concentration 8µg/g, Benozo[ghi]perylenewas detected with concentration 82 µg/g, Flourene was detected with concentration 10 µg/g, Chrysene was detected with concentration 4 µg/g, Ideno[123-cd]pyrene was detected with concentration 112 µg/g, Naphthalene was detected with concentration 74 µg/g, Phenanthrene was also detected with concentration 35 µg/g, and finally Pyrene was detected with concentration 3 µg/g.

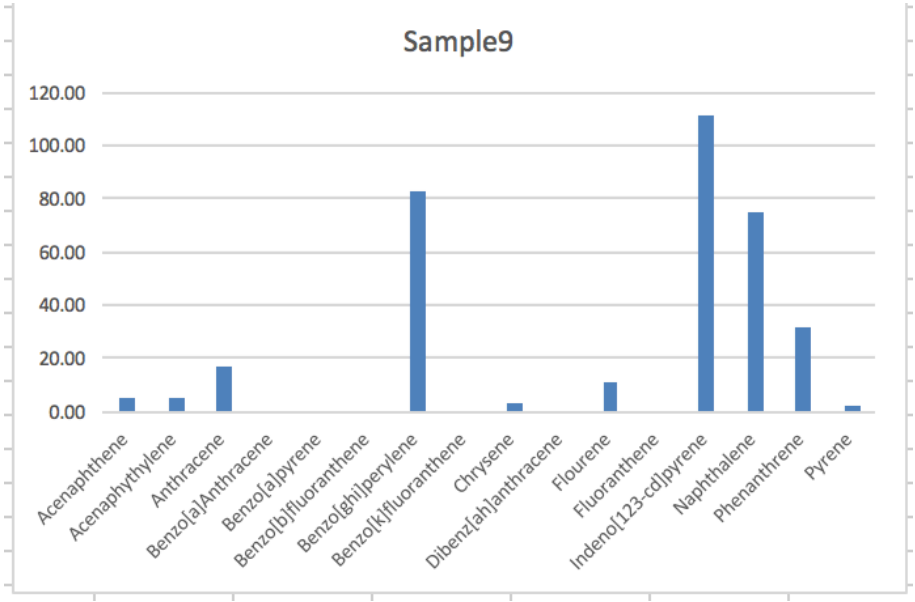


Figure 85: Represents the result of raw milk sample 9

Figure 89 represents the result of milk sample code 10, were Anthracene was detected with concentration 18 µg/g, Acenaphthene was detected with concentration 9 µg/g, Acenaphythylene was detected with concentration 10 µg/g, Benozo[ghi]perylenewas detected with concentration 63 µg/g, Flourene was detected with concentration 11 µg/g, Ideno[123-cd]pyrene was detected with concentration 85 µg/g, Naphthalene was detected with concentration 70 µg/g, and finally Phenanthrene was also detected with concentration 32 µg/g

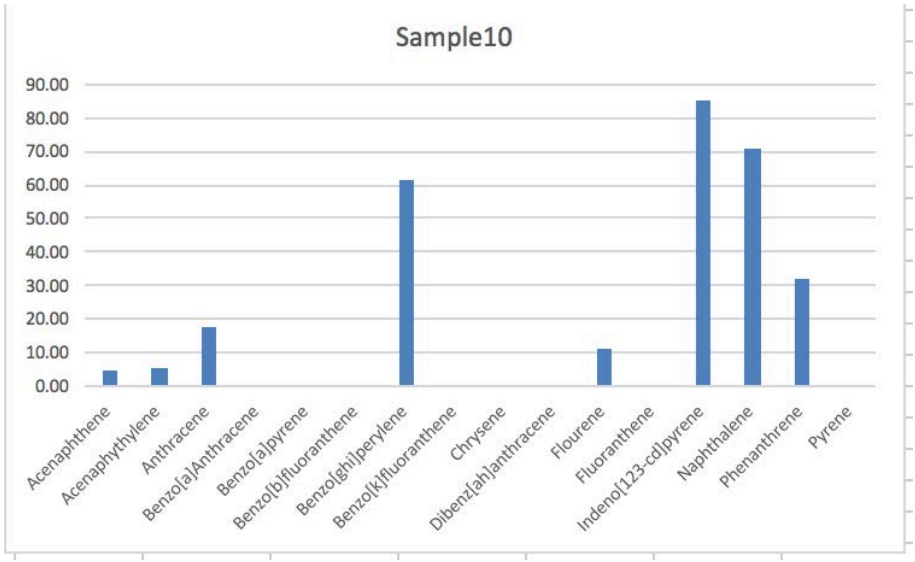


Figure 86: Represents the result of raw milk sample 10

Figure 90 represents the result of milk sample code 20, were Anthracene was detected with concentration 3 µg/g, Benzo[a]pyrene was detected with concentration 6 µg/g, Naphthalene was detected with concentration 3 µg/g, and finally Phenanthrene was also detected with concentration 13 µg/g

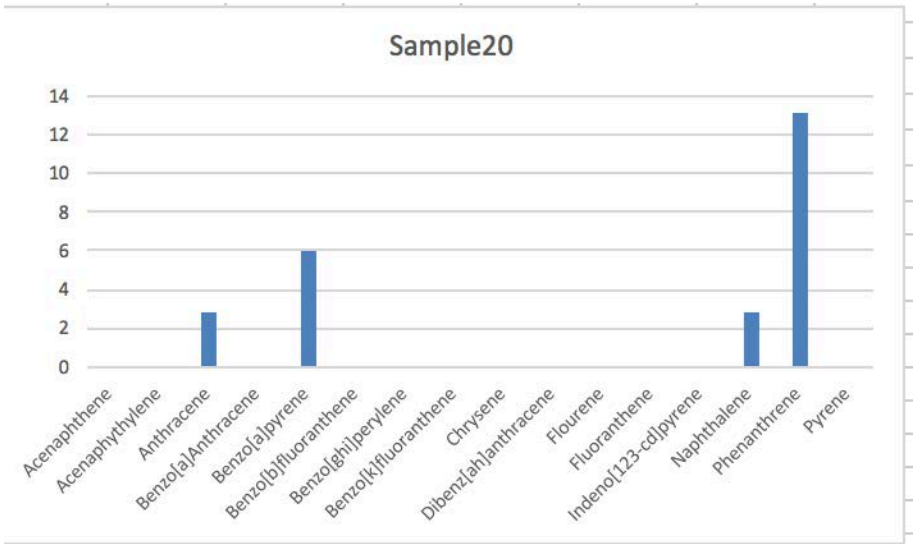


Figure 87: Represents the result of raw milk sample 20

3.1.6 Total Distribution of Raw Milk Results

The below figure (88) and table (13) represents the total distribution percentage comparison between all the detected compounds.

Table 13: Represents the percentage of detected positive compounds.

Positive Compound (s)	Percentage
Not Detected	7%
One Compound	7%
Two Compounds	41%
Three Compounds	15%
Four Compounds or more	30%

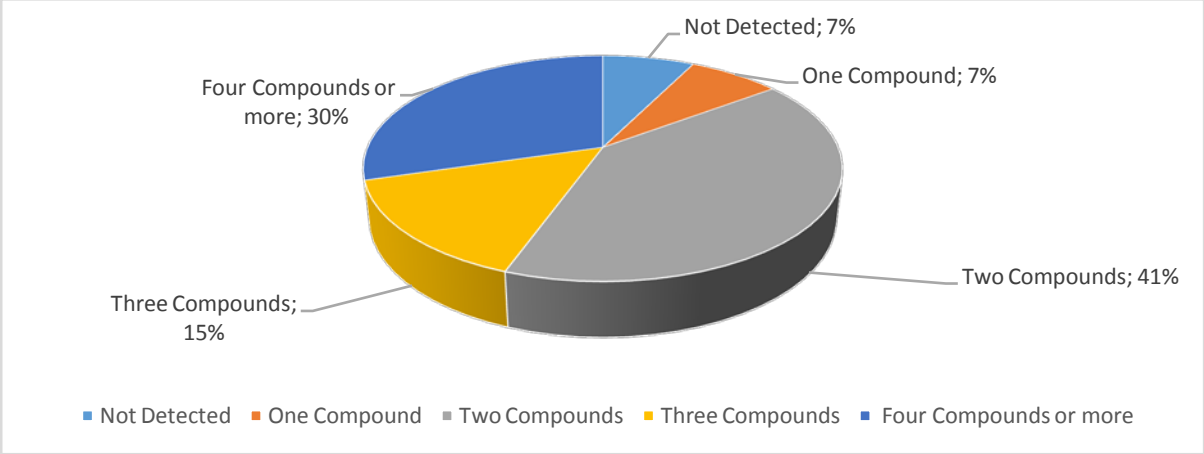


Figure 88: Represents the total distribution of raw milk results

7.5 Comparison between raw and packaged milk

(The below results and graphs are obtained from the data found in results annexes, annexes 2 & 3).

Figure 89 shows a total distribution comparison between raw milk packaged milk. It showed that raw milk has more detected compounds than packaged milk.

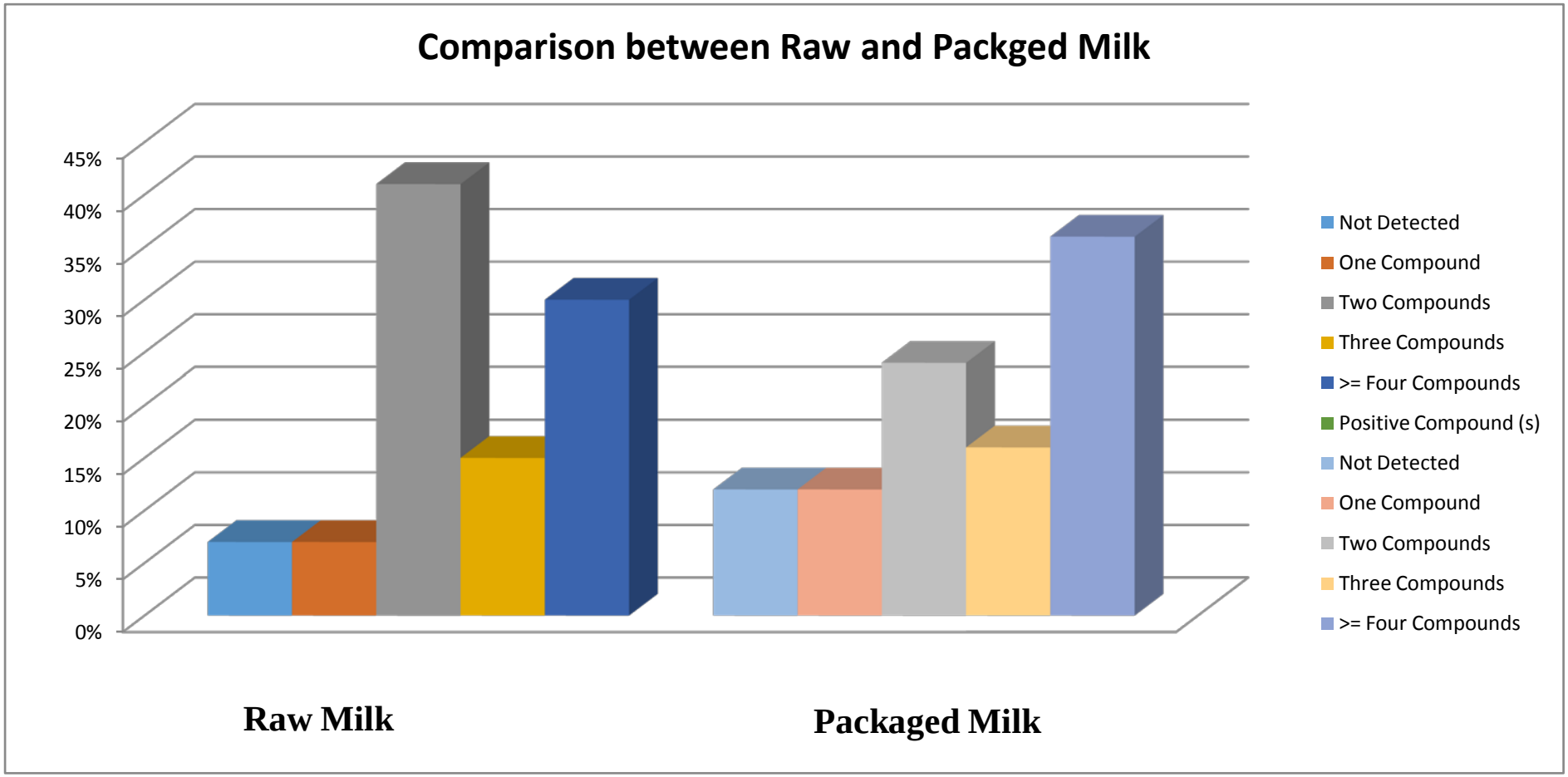
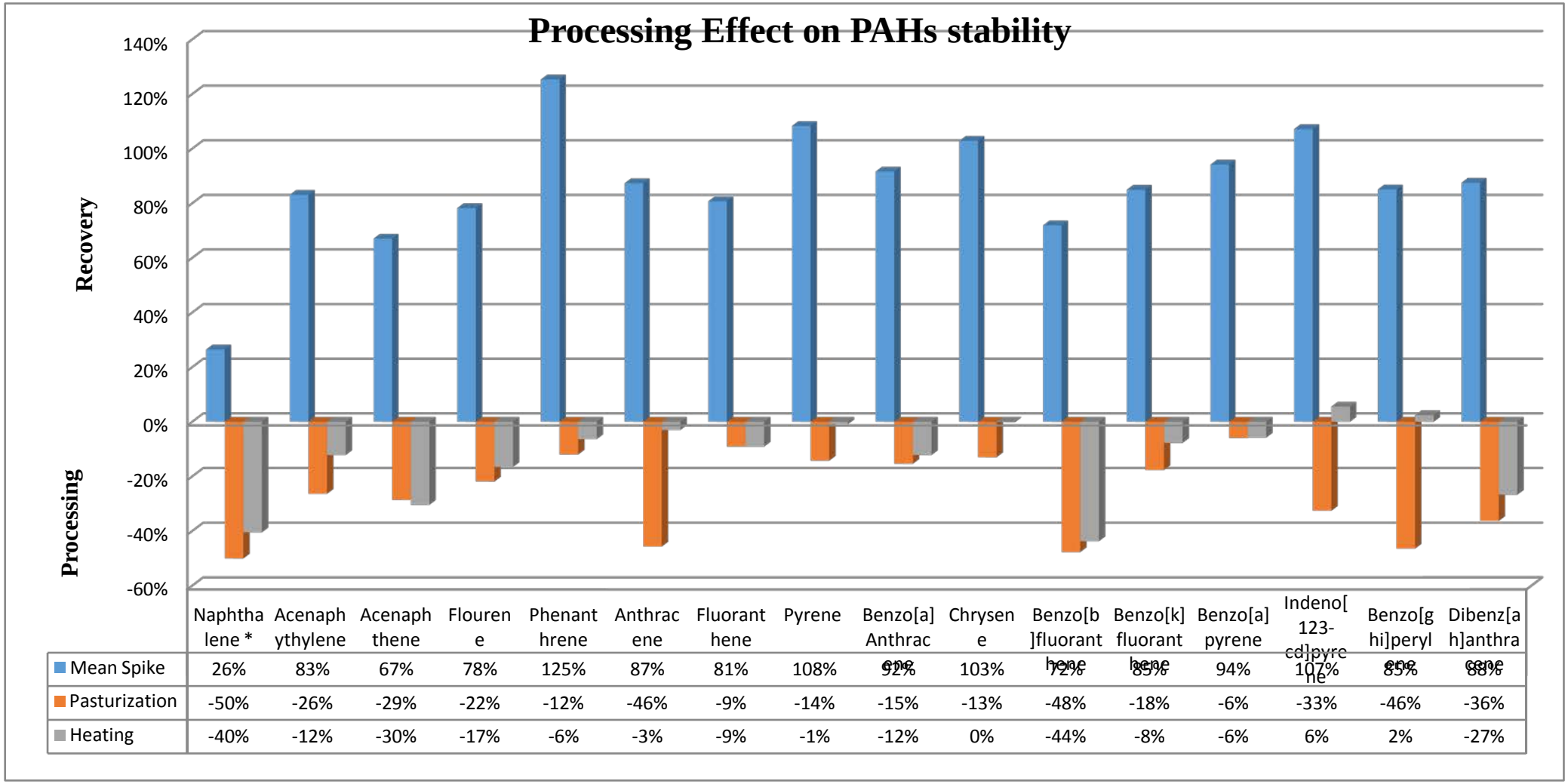


Figure 89: Shows a comparison between raw and packed milk.

Method 3: Effect of Pasteurization on PAHs in Milk.

(The below results and graphs are obtained from the data found in results annexes, annex 4)

Figure 90 represents the results for effect of heating, pasteurization and the stability of PAHs in milk. The result shows that there is a remarkable degradation of most of PAHs except for Benzo[a]pyrene, where Anthracene, and Chrysene the effect of pasteurization was more than heating. The concentration of Anthracene decreased by 46% while for heating 3%. For Chrysene there was no effect by heating, while pasteurization it decreased 13%. Overall, Pasteurization have more effect on the stability of PAHs rather than heating.



	Naphthalene *	Acenaphthylene	Acenaphthene	Flourene	Phenanthrene	Anthracene	Fluoranthene	Pyrene	Benzo[a] Anthracene	Chrysene	Benzo[b]fluoranthene	Benzo[k]fluoranthene	Benzo[a]pyrene	Indeno[123-cd]pyrene	Benzo[ghi]perylene	Dibenz[ah]anthracene
Mean Spike	26%	83%	67%	78%	125%	87%	81%	108%	92%	103%	72%	85%	94%	107%	85%	88%
Pasturization	-50%	-26%	-29%	-22%	-12%	-46%	-9%	-14%	-15%	-13%	-48%	-18%	-6%	-33%	-46%	-36%
Heating	-40%	-12%	-30%	-17%	-6%	-3%	-9%	-1%	-12%	0%	-44%	-8%	-6%	6%	2%	-27%

Figure 90: Shows the effect of pasteurization on PAHs in Milk

VII. Discussion

PAH are a class of 28 complex chemicals that are formed and released during incomplete combustion or pyrolysis (burning) of organic matter such as waste or food, during industrial processes and other human activities. PAHs are also formed in natural processes such as carbonization. PAH benzo[a]pyrene, have shown various toxicological effects, such as hepatotoxicity), reproductive and developmental toxicity and immunotoxicity. A number of PAHs have shown carcinogenic effects in experimental animals and it has been concluded that benzo[a]pyrene is carcinogenic to humans. The analysis was carried using a modified QuEChERS procedure followed by injection on gas chromatography coupled to tandem mass spectrometry.

The GC conditions in this study was conductedaccording to Hamzawy *et al*., (2016), the device column was calibrated. The PAHs standard compounds were also calibrated and adjusted. The conditions of the device were then adjusted as in (Table 10). The samples were then added in their places in the device for the injection process of the samples into the device start.on the other hand, (Alex Kim *et al.*, 2007) used a Varian 1200L GC-MS-MS. Mass range: full scan ion monitoring (m/z: 40-600), scan time was 0.5 second, with electron impact and quadruple analyzer. Split/split less injection (1:25 at 250°C) was onto a DB-1 column (60m length x 0.32 mm i.d. x 0.25 µm film thickness). Oven temperature Programme: - 60°C (hold for one min) to 200°C (at 5°C / min.) to 280°C (at 2.6°C / min.) to 320°C (at 20°C / min.) and hold at 320°C for 10 minutes. Helium used as a carrier gas at 1ml/min.

A few papers had been published on the optimization of programmable temperature vaporization injection mode on GCMS for the determination of 16 EPA PAHs. They reported an important increase in sensitivity by using PTV injection compared to splitless injection (Norlock F.M. *et al*., 2002, Yusà V. *et al*., 2006 and Fernández-González V. *et al*., 2008).

According to Smith *et al*., who used an Agilent 6890N GC system, Agilent 5975B series MSD and Agilent DB-EUPAH column (20 m × 0.18 mm × 0.14 μm) for chromatographic separation and detection of PAHs. The oven temperature program was at initial temperature 45°C for 0.8 min then ramped to 200°C with rate 45°C/min, ramped with rate 2.5 °C/min to 225 ^{o}C, ramped with rate 3°C/min to 266 °C, then 5 °C/min to 300 °C, 10 ^{o}C/min to 320 °C and hold for 4.5 min. The carrier was Helium at flow 1.0 mL/min for 0.2 min, 5 mL/min to 1.7 mL/min. In addition, Restek Rxi-17 column (20 m × 0.18 mm × 0.18 μm) was tested for separation comparison. The result shows lower bleed for the DB-EUPAH column with better signal to noise ratios for late eluting dibenzopyrene isomers (2010).

AlsoPrashant Rajput *et al.,* optimize temperature program for GC oven using capillary Agilent HP-5MS column (30 m × 0.25 mm × 0.25 μm) at constant flow rate of 1.3 mL/min of helium gas as follows, the initial oven temperature was 100°C, , ramp with rate 25°C/min to 150°C, ramp with rate 25°C/min to 200°C, rampwith rate 3°C/min to 230°C, ramp with rate 8 °C/min to 310 °C, hold for 3 min, the total run time of 30 min. Mass interface temperature was 280 °C, source 300°C and quadrapole180°C (2011).

Referring to figures from 33 & 34 which represents blank and standard matrix on blank results using protocol 1it shows that at retention time 16, 19.5, and 26 three main large peaks that’s interfered with our target PAHs compounds which make use false positive results using this protocol.

As stated in figures 35 & 36 which represents blank and standard matrix on blank results using protocol2 it shows that at retention time 16, 24, and 26 three main peaks with a lower response than found in protocol 1.

Referring to figure 37 & 38 it represents protocol 3 that shows at retention time 16, 24, and 26 three main peaks with high response which make use false positive results using this protocol.

Different extraction and sample clean-up techniques such as, soxlet extraction in spite of being the most popular method and overall recovery is good with smaller solvent and time consumption if batch (semi-automatic) system is used but it is time consuming, large solvent consumption, costly low selectivity, relatively small recovery for light PAHs (Method 3540C and US EPA, 1996)

Up to now, conventional methods of sample preparation used for PAHs determination in tea have included extraction using various solvents (acetone, ethyl acetate, dichloromethane, hexane) and clean-up solid-phase extraction (SPE) step, or gel permeation chromatography (Lin D. *et al.*, 2005 and Singh *et al.*, 2011).

Sanz-Landaluze J. *et al.*, used technique of cleanup from co-extracted interferences to assess most of PAHs in different matrices. These methods are expensive, time and chemicals consuming, use chlorinated solvent for extraction, and cause wrong results(2006).

Few previously published researches on the improvement of QuEChERs analytical method for determination of PAHs levels in animal origin tissue. The streamline of QuEChERs (quick, easy, cheap, effective, rugged, and safe) method for extraction of pesticides in tissues of high fat (>3.5%), inspires scientists to apply adjustments and improve this method for the extraction of veterinary drugs (Stubbings G. *et al.*, 2009).

As mentioned by Smith D. *et al.*, dispersed solid phase extraction and accelerated solvent extraction coupled for sample cleanup using gel permeation chromatography, which give a good PAHs recovery with short extraction time and small solvent consumption, but it had low selectivity (2010).

A simple and rapid method for determining polycyclic aromatic hydrocarbons (PAHs) in shrimp described where reverse phase chromatography using octadecyl silica (C_{18}) column and water/acetonitrile gradient elution used to clean-up analyte mixtures from co-extractive interferences. PAHs were quantitated using liquid chromatography-tandem mass spectrometry equipped with the atmospheric pressure photoionization (Photo Spray APPI) source operating in the positive-ion mode (Smoker M. *et al*, 2010).

In addition, Forsberg N. D. *et al*.,usedQuEChERs for extraction followed by dispersive SPE analysis and GCMS in SIM mode for PAHs quantification in fish (2011).

Mohd Marsin Sanagi, et. Al., developed a sensitive analytical method to provide a rapid, selective to determine polycyclic aromatic hydrocarbons (PAHs) in fresh milk by using a two-phase hollow fiber liquid-phase micro extraction (HF-LPME) method combined with gas chromatography–mass spectrometry (GC–MS). The standard addition method used to make calibration curves and to determine the residue levels for PAHs, Fluorene, Phenanthrene, fluoranthene, pyrene, and benzo[a]pyrene, thus removing sample pre-treatment steps such as pH adjustment. The HF-LPME method shows dynamic linearity from five to 500 mg/L for all target analyte with R2 ranging from 0.9978 to 0.9999. Under optimized conditions, the established detection limits range from 0.07 to 1.4 mg/L based on a signal-to-noise ratio of 3:1. Average relative recoveries for the determination of PAHs studied at 100 mg/L spiking levels are in the range of 85 to 110%. The relative recoveries are

somewhat higher than obtained by conventional solvent extraction, which requires saponification steps for Fluorene, and Phenanthrene, which are more volatile and heat sensitive. The HF-LPME method proves to be simple and rapid, and requires minimal amounts of organic solvent that supports green analysis (2011).

In 2013, a simple solid phase extraction (SPE) method followed by complete two-dimensional gas chromatography coupled to time-offlight (TOF) mass spectrometry has developed for analysis of (15 + 1) carcinogenic polycyclic aromatic hydrocarbons (PAHs). This method includes three critically assessed sample preparation approaches: (i) gel permeation chromatography (GPC), (ii) GPC followed by silica based SPE, and (iii) SPE employing PAHs-dedicated molecularly imprinted polymers (MIPs) (Drabova L. *et al*., 2013).

As stated by Yebra-Pimentel I. *et al.*,studied two of analytical techniques include extraction processes like ultrasound assisted solvent extraction (USAE) and ultrasound-assisted emulsification micro extraction (USAEME) for determination of 11 PAHs enhanced by the selected extraction methods. The recoveries ranging from 70% to 100% by USAE and from 70% to 108% by USAEME with LOQ range 0.020- 2.6µg/kg (2013).

As reported by *Dalena Surma et. al.*,the evaluation of an analytical technique for the determination of polycyclic aromatic hydrocarbons (PAHs) in food of animal origin using GCMSD. The results showed that the best recovery ratios 72.4–110.8 % with RSD% lower than 10 % for all PAHs tested by the method with ethyl acetate as an extraction solvent, primary–secondary amine and C_{18} sorbents and evaporation to dryness and dissolving the residues in the hexane. The LOQ ranged from 0.0003 to 0.0030 mg/kg for Pyrene and Benzo[a]Anthracene, respectively (2014).

Shih-Chun *et al.*,presented an ultra-high performance liquid chromatography-atmospheric pressure photoionization-tandem mass spectrometry (UHPLC-APPI-MS/MS) method for simultaneous analysis of 20 PAHs and 9 nitro-PAHs. The total run time is 15 minutes using positive mode and 11 minutes for negative mode, 50% time saving compared with GC/MS analysis time. Two pairs of precursor/product ions presented, which is critical for verification. This method separates and quantifies benzo[a]pyrene (the most toxic PAHs) and non-priority benzo[e]pyrene (isomers, little toxicity) to avoid miscalculation of PAHs levels, representing its significance for health-related researches. This fast, sensitive, and reliable method is the first UHPLC-APPI-MS/MS method capable of simultaneously analyzing 29 environmentally and toxicologically important PAHs and nitro-PAHs (2015).

According to Edward A. Pfannkoch *et., al.*,studied the using of a stir bar sorptive extraction (SBSE) technique to extract PAHs in water and (QuEChERS) extraction scheme extracts PAHs from food. This study uses SBSE to concentrate PAHs and remove matrix interference from QuEChERS extracts of seafood. This technique provides acceptable recovery (65–138%) with linear calibrations and LOD = 0.02 ppb, LOQ = 0.06 ppb (2015).

M Hernandez-Cordoba *et. al*., evaluated a procedure for the determination a total ten PAHs in various herbal using a solvent-free method based on headspace sorptive extraction (HSSE) coupled to gas chromatography-mass spectrometry (GC-MS). Different parameters affecting both the HSSE extraction and thermal desorption steps were enhanced using multivariate Plackett- Burman designs. D_{10}-phenanthrene was added to the samples not only to develop the repeatability of the method but also allowing the quantification of the mixture samples using calibration. The suggested technique reached to a detection limits ranging from 11 to 26 ng/L. The accuracy of the suggested method tested by

recovery test and by the analysis of a certified reference material. This solvent-free and simple method based on TD-GC-MS combination has proven to be a useful tool for the determination of ten volatile PAHs in herbal infusions, being helpful for their reliable control (2015).

Referring to table 11 & figure 39 and 40 which represents the numerical data results of matrix effect and recovery using the three protocols. Most of PAHs show lowest matrix effect with a moderate recovery using protocol two compared to protocol one and three. For Example, beno[a]pyrene which is known as the most carcinogenic compound of all PAHs compounds, in protocol one the matric effect was 172% with a recovery 63%, in protocol two the matrix effect was 108% and with a recovery 92%, and finally in protocol three the matrix effect was 88% with recovery 110%. Another example Anthracene which is one of the most detected PAHs compounds, in protocol one the matric effect was 112% with a recovery 74%, in protocol two the matrix effect was 101% and with a recovery 77%, and finally in protocol three the matrix effect was 114% with recovery 73%. This means that using protocol two will give us our main target which was removing co-elution that may give false positive results and get highest recovery among all PAHs compounds.

Total of 25 packaged milk where used to screen the presence of PAHs, Refer to Annex 2, & table 12 for the results and total distribution of the samples. It was found that 12% of samples no PAHs were detected, 12% of samples contained only one compound of total PAHs, 24% of samples contained two compounds of total PAHs, 16% of samples contained three compounds of total PAHs, and 36% of samples contained four or more compounds of total PAHs.

Total of 27 raw samples where used to screen the presence of PAHs, Refer to Annex 3, & table 13 for the results and total distribution of the samples. It was found that 7% of samples no PAHs were detected, 7% of samples contained only one compound of total PAHs, 41% of

samples contained two compounds of total PAHs, 15% of samples contained three compounds of total PAHs, and 30% of samples contained four or more compounds of total PAHs.

Refer to figure 89 that shows a total comparison distribution between packaged and raw milk samples, it showed that raw milk has more detected compounds than packaged milk. For example, for not detected compound and two compounds.

Referring to Annex 4 & Figure 90 which represents the results for effect of heating, pasteurization and the stability of PAHs in milk. The result shows that there is a remarkable degradation of most of PAHs except for Benzo[a]pyrene (based 3ala literature enu howa stability bt3to 3alia), where Anthracene, and Chrysene the effect of pasteurization was more than heating. The concentration of Anthracene decreased by 46% while for heating 3%. For Chrysene there was no effect by heating, while pasteurization it decreased 13%. Overall, Pasteurization have more effect on the stability of PAHs rather than heating.

Estimation of acceptable dietary intake (ADI)

As part of the current study, which aims to ensure the safety of food, consumed, distributed, produced, and sold on the Egyptian market. An estimation of Total Diet Study (TDS) is to measure the dietary exposure of the population of a country to particular chemicals that

may pose a risk to health if taken into the body in excessive amounts. In carrying out a TDS at national level, based on food consumption data, analyzed for particular chemical contaminants, food additives and nutrients present in the food. Dietary exposure to each chemical then estimated using the food consumption data and the level of the particular chemical present in each food.

The exposure estimates in the current study comparing with exposure standards established by international risk assessment bodies such as the European Food Safety Authority (EFSA), the Joint FAO/WHO, 2005 Expert Committee on Food Additives (JECFA) and the former EU Scientific Committee for Food (SCF). In order to reach a conclusion regarding the risk to Egyptian consumers from the presence of chemicals in the milk.

The estimated dietary intake of PAHs in a given milk obtained by multiplying the residue level in the food by the amount of that food consumed.

The Estimated Average Daily Intake (EADI) of PAHs calculated by the following equation

EADI, expressed in µg/kg body weight/day = Fi x Ri

Fi = Food consumption of the relevant commodity (kg/day),

All calculations for the determination of EDI were according to international guidelines (JECFA, 2006).

Food consumption rates selected based on the consumption data issued by WHO which is relatively within the same range of the Egyptian food balance sheet issued by economic affairs sector, ministry of agriculture (EFBS, 2011).

According to JECFA (WHO, GENEVA 2006), the estimated exposure limit for Benzo(a)Pyrene was 4.0 ng/kg body weight per day and the maximum limit was 10 ng/Kg body weight per day (estimates for Benzo(a)Pyrene as a marker for polycyclic aromatic hydrocarbons PAHs).

VIII. Conclusion

To conclude, this work aim to adapt, optimize and validate QuEChERs method (Anastassiades M. *et al*., 2003) for extraction of Polycyclic Aromatic Hydrocarbons (PAHs) followed by solid phase extraction for sample purification and gas chromatography mass spectrometer GCMS for determination of 16 PAHs in Milk at low LOQ level. Secondly, method two aimed to monitor the level of PAHs in both commercial packaged milk and raw milk samples and it was found that the packaged milk contains lower levels of PAHs compared to raw milk. In addition, measuring the stability of PAHs after the effect of pasteurization and heating.

From the pervious work, it is recommended to use commercial packaged milk over the raw milk. Even if the raw milk is contaminated with PAHs is heated using the normal still PAHs compounds will be present.

IX. Result's Annexes:

1.1 Annex I:

Table 14: Represents the numerical data results of StdMtrx effect

Sample	StMtrx%_T1	Mean%_T1	SD_T1	CV_T1	StMtrx%_T2	Mean%_T2	SD_T2	CV_T2	StMtrx%_T3	Mean%_T3	SD_T3	CV_T3
Naphthalene	90%	31%	4.03	12.93	95%	27%	2.48	9.36	102%	13%	2.09	15.50
Acenaphythylene	102%	79%	2.90	3.68	114%	68%	1.94	2.86	121%	59%	2.74	4.63
Acenaphthene	101%	75%	2.62	3.47	111%	66%	1.38	2.09	119%	58%	2.99	5.15
Flourene	102%	80%	1.85	2.32	111%	72%	1.15	1.61	115%	67%	2.02	3.03
Phenanthrene	215%	78%	0.76	0.98	220%	73%	3.10	4.24	206%	74%	4.56	6.20
Anthracene	112%	74%	3.58	4.82	101%	77%	1.58	2.05	114%	73%	2.83	3.86
Fluoranthene	117%	78%	0.71	0.91	127%	72%	0.40	0.55	132%	69%	0.96	1.39
Pyrene	123%	78%	1.42	1.81	135%	71%	0.95	1.32	145%	67%	1.98	2.98
Benzo[a]Anthracene	208%	73%	0.85	1.17	206%	70%	4.02	5.75	199%	72%	1.77	2.46
Chrysene	93%	81%	1.69	2.09	102%	71%	2.03	2.84	112%	65%	2.69	4.16
Benzo[b]fluoranthene	215%	66%	2.41	3.66	197%	69%	3.92	5.71	195%	71%	4.78	6.71
Benzo[k]fluoranthene	144%	59%	7.07	11.99	142%	62%	2.45	3.97	155%	57%	2.13	3.76
Benzo[a]pyrene	172%	63%	1.87	2.99	108%	92%	2.12	2.31	88%	110%	3.19	2.91
Indeno[123-cd]pyrene	92%	47%	2.58	5.46	90%	47%	2.27	4.84	85%	46%	2.70	5.84
Benzo[ghi]perylene	93%	65%	0.96	1.49	109%	51%	1.42	2.77	114%	56%	3.05	5.50
Dibenz[ah]anthracene	152%	60%	2.80	4.68	148%	57%	4.45	7.84	140%	57%	3.40	5.93

1.2 Annex II:

Table 15: Represents the numerical data results of Packaged milk

Compounds	SpPahs_1p	Package1	Package2	Package3	Package4	Package5	Package6	Package7	Package8	SpPahs_2p	Package8	Package9	Package10	Package11	Package12	Package13
Acenaphthene	108%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	107%	0.000	0.000	0.000	0.000	2.017	0.000
Acenaphythylene	108%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	114%	2.903	0.000	0.000	0.000	0.000	0.000
Anthracene	107%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	110%	11.168	11.099	10.900	11.232	11.294	11.369
Benzo[a]Anthracene	108%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	119%	0.000	0.000	0.000	0.000	0.000	0.000
Benzo[a]pyrene	102%	14.161	14.091	14.231	0.000	0.000	0.000	0.000	0.000	105%	14.809	0.000	0.000	14.472	0.000	14.785
Benzo[b]fluoranthene	77%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	76%	0.000	15.938	0.000	0.000	0.000	0.000
Benzo[ghi]perylene	91%	75.682	0.000	62.667	70.523	0.000	0.000	55.138	0.000	84%	0.000	0.000	0.000	0.000	0.000	0.000
Benzo[k]fluoranthene	116%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	109%	0.000	0.000	0.000	0.000	0.000	0.000
Chrysene	116%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	111%	0.000	0.000	0.000	0.000	0.000	0.000
Dibenz[ah]anthracene	108%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	102%	0.000	0.000	0.000	0.000	0.000	0.000
Flourene	121%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	118%	1.551	1.560	1.272	1.532	1.464	2.728
Fluoranthene	105%	3.179	0.000	0.000	0.000	0.000	0.000	0.000	0.000	110%	4.068	3.979	4.049	3.974	4.056	4.087
Indeno[123-cd]pyrene	94%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	120%	0.000	0.000	0.000	0.000	0.000	0.000
Naphthalene	100%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	100%	4.593	4.338	4.202	4.472	4.475	4.850
Phenanthrene	120%	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	140%	14.353	15.173	14.789	14.954	0.000	12.672
Pyrene	112%	3.864	0.000	0.000	0.000	0.000	0.000	0.000	0.000	109%	6.790	6.272	6.181	6.343	6.348	6.572

- Green color reflects to the positive PAHs compound found in a sample
- Orange color refers to LOQ
- Yellow: Spike solutions

Continuous data of table 15

Compounds	Package14	Package15	Package16	Package17	Package18	Package19	Package20	Package21	Package22	Package23	Package24	SpPahs_3p
Acenaphthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	99%
Acenaphythylene	3.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	105%
Anthracene	12.827	11.333	8.892	9.267	9.384	8.729	8.678	8.222	8.686	8.894	9.408	112%
Benzo[a]Anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	91%
Benzo[a]pyrene	14.596	14.838	15.210	15.604	0.000	0.000	15.041	0.000	0.000	0.000	15.249	61%
Benzo[b]fluoranthene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	60%
Benzo[ghi]perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	75%
Benzo[k]fluoranthene	3.384	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	85%
Chrysene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	110%
Dibenz[ah]anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	64%
Flourene	3.484	1.308	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	117%
Fluoranthene	4.624	3.965	2.744	2.876	2.789	2.698	3.002	2.917	2.949	2.775	2.885	106%
Indeno[123-cd]pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	80%
Naphthalene	9.278	4.674	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	88%
Phenanthrene	13.561	12.584	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	129%
Pyrene	8.042	6.220	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	106%

- Green color reflects to the positive PAHs compound found in a sample
- Orange color refers to LOQ
- Yellow: Spike solutions

1.3 Annex III:

Table 16: Represents the numerical data results of raw milk samples

Compounds	StMtrxPAHs	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	SpPAHs_1	Sample11	Sample12	Sample13
Acenaphthene	124%	0.00	0.00	0.00	0.00	8.05	0.00	10.22	10.38	5.40	4.89	86%	0.00	0.00	0.00
Acenaphythylene	123%	0.00	0.00	0.00	0.00	6.27	0.00	8.43	6.38	5.01	5.52	92%	0.00	0.00	0.00
Anthracene	120%	0.00	0.00	0.00	0.00	17.03	0.00	24.60	21.87	17.44	17.55	92%	0.00	0.00	0.00
Benzo[a]Anthracene	119%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	99%	0.00	0.00	0.00
Benzo[a]pyrene	119%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	98%	0.00	0.00	0.00
Benzo[b]fluoranthene	107%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	105%	0.00	0.00	0.00
Benzo[ghi]perylene	110%	68.58	49.07	13.74	30.33	19.46	48.27	0.00	0.00	82.72	61.50	94%	0.00	0.00	58.52
Benzo[k]fluoranthene	107%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.39	0.00	86%	1.40	0.00	0.00
Chrysene	103%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.26	0.00	105%	0.00	0.00	0.00
Dibenz[ah]anthracene	113%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	95%	0.00	0.00	7.97
Flourene	106%	0.00	0.00	0.00	0.00	11.66	0.00	14.31	13.73	10.78	11.46	108%	0.00	0.00	0.00
Fluoranthene	115%	0.00	0.00	0.00	0.00	2.33	2.11	2.97	2.42	1.95	1.86	99%	0.00	0.00	0.00
Indeno[123-cd]pyrene	117%	89.71	74.98	16.81	40.98	22.54	43.05	0.00	0.00	111.92	85.01	90%	0.00	0.00	78.01
Naphthalene	117%	0.09	0.00	0.00	0.00	95.64	0.00	123.70	97.13	75.39	71.23	87%	0.00	0.00	0.00
Phenanthrene	115%	0.00	0.00	0.00	0.00	37.65	0.00	43.82	35.35	31.46	31.96	97%	0.00	0.00	0.00
Pyrene	102%	0.00	0.00	0.00	0.00	2.75	0.00	2.88	2.45	2.46	1.86	109%	0.00	0.00	0.00

- Green color reflects to the positive PAHs compound found in a sample
- Orange color refers to LOQ
- Yellow: Spike solutions

Continuous data of table 16

Compounds	Sample14	Sample15	Sample16	Sample17	Sample18	Sample19	SpPAHs_2	Sample19	Sample20	Sample21	Sample22	Sample23	Sample24	Sample25	Sample26	SpPAHs_3
Acenaphthene	0.00	0.00	0.00	0.00	0.00	0.00	65%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	81%
Acenaphythylene	0.00	0.00	0.00	0.00	0.00	0.00	67%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	82%
Anthracene	0.00	0.00	0.00	0.00	0.00	0.00	85%	0.00	2.77	2.20	1.37	1.39	1.77	2.15	1.66	79%
Benzo[a]Anthracene	0.00	0.00	0.00	0.00	0.00	0.00	86%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	86%
Benzo[a]pyrene	0.00	0.00	0.00	0.00	0.00	0.00	77%	0.00	5.97	0.00	0.00	0.00	0.00	0.00	0.00	86%
Benzo[b]fluoranthene	0.00	0.00	0.00	0.00	0.00	0.00	105%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	108%
Benzo[ghi]perylene	6.68	24.03	20.35	0.00	28.72	18.69	101%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	106%
Benzo[k]fluoranthene	0.00	0.00	0.00	0.00	0.00	0.00	89%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	112%
Chrysene	0.00	0.00	0.00	0.00	0.00	0.00	96%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	123%
Dibenz[ah]anthracene	0.00	0.00	0.00	0.00	0.00	0.00	83%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	97%
Flourene	0.00	0.00	0.00	0.00	0.00	0.00	92%	0.00	1.32	0.00	0.00	0.00	1.00	1.14	0.77	101%
Fluoranthene	0.00	0.00	0.00	0.00	0.00	0.00	82%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	95%
Indeno[123-cd]pyrene	5.84	35.54	29.12	14.81	44.33	26.12	70%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	102%
Naphthalene	0.00	0.00	0.00	0.00	0.00	0.00	95%	0.00	2.79	1.71	1.71	1.52	2.05	2.26	0.00	93%
Phenanthrene	0.00	0.00	0.00	0.00	0.00	0.00	114%	0.00	13.17	7.50	6.50	6.17	7.84	8.94	0.00	112%
Pyrene	0.00	0.00	0.00	0.00	0.00	0.00	97%	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	113%

- Green color reflects to the positive PAHs compound found in a sample
- Orange color refers to LOQ
- Yellow: Spike solutions

1.4 Annex IV:

Table 17: Represents the numerical data results of Pasteurization and Heating

Sample	stmxB	R1_Pasturi	R2_Pasture	R1_Heat	R2_Heat	R1_Spike	R2_Spike	stmxB	R1_Pasturi	R2_Pasture	R1_Heat	R2_Heat	R1_Spike	R2_Spike	Mean_Pastur	Mean_Heat
Naphthalene *	58.68	31.30	27.33	42.10	27.77	18.77	12.24	117%	-47%	-53%	-28%	-53%	32%	21%	-50%	-40%
Acenaphythylene	51.41	44.09	42.31	46.25	44.03	44.03	41.39	103%	-14%	-18%	-9%	-13%	86%	81%	-16%	-11%
Acenaphthene	62.23	43.16	40.57	44.65	41.95	42.53	40.86	124%	-31%	-35%	-30%	-35%	68%	66%	-33%	-32%
Flourene	63.10	43.30	48.39	53.07	52.26	50.96	47.71	126%	-31%	-23%	-17%	-18%	81%	76%	-27%	-18%
Phenanthrene	57.75	50.78	52.50	56.00	52.15	77.64	67.09	115%	-12%	-9%	-3%	-10%	134%	116%	-11%	-6%
Anthracene	40.94	30.82	32.95	39.64	39.80	37.75	33.73	82%	-25%	-20%	-2%	-2%	92%	82%	-22%	-2%
Fluoranthene	67.49	55.38	51.31	52.41	53.24	55.73	53.21	135%	-18%	-24%	-26%	-24%	83%	79%	-21%	-25%
Pyrene	50.82	50.64	50.02	50.27	50.44	55.39	54.66	102%	0%	-2%	-1%	-1%	109%	108%	-1%	-1%
Benzo[a]Anthracene	59.68	53.66	45.67	51.90	52.94	52.63	56.70	119%	-10%	-23%	-13%	-11%	88%	95%	-17%	-12%
Chrysene	51.54	52.30	49.84	50.57	52.41	50.42	55.59	103%	1%	-3%	-2%	1%	98%	108%	-1%	0%
Benzo[b]fluoranthene	53.74	31.50	29.88	30.07	30.40	38.06	39.21	107%	-41%	-44%	-40%	-40%	71%	73%	-43%	-40%
Benzo[k]fluoranthene	53.54	49.49	47.11	50.92	47.88	42.45	48.48	107%	-8%	-12%	-4%	-10%	79%	91%	-10%	-7%
Benzo[a]pyrene	59.33	55.49	54.86	56.49	55.31	56.25	55.44	119%	-6%	-8%	-5%	-7%	95%	93%	-7%	-6%
Indeno[123-cd]pyrene	68.43	62.39	61.44	58.28	33.99	72.68	73.97	137%	-9%	-10%	-17%	-59%	106%	108%	-10%	-38%
Benzo[ghi]perylene	65.24	60.03	60.13	39.20	30.68	58.98	51.98	130%	-8%	-8%	-44%	-59%	90%	80%	-8%	-52%
Dibenz[ah]anthracene	52.59	37.98	36.78	40.43	36.68	47.06	44.99	105%	-28%	-30%	-21%	-27%	89%	86%	-29%	-24%

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