TÉCNICO LISBOA

Determination of PAHs in food matrices

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December 2019

A me stessa,
per la mia perseveranza.

A mia madre,
per i sacrifici, l'amore incondizionato e la fiducia in me.

A mio padre,
a te che sei la persona più importante della mia vita.

A mia sorella,
che questo percorso possa esserle di ispirazione.

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Resumo

Na atmosfera de um complexo urbano / industrial, quantidades consideráveis de óxidos de nitrogênio, dióxido de enxofre, pós suspensos, hidrocarbonetos aromáticos policíclicos e outros poluentes menos onipresentes, mas não menos nocivos, são introduzidos diariamente. No entanto, muitas pesquisas atribuem a uma classe específica de poluentes, os hidrocarbonetos aromáticos policíclicos (HAP), um papel de destaque entre as causas de risco para a saúde humana. Os HPA liberados no ar, essencialmente de fontes antropogênicas, como o tráfego veicular e os processos de combustão em geral, espalham-se rapidamente e podem permanecer na atmosfera por muito tempo, com conseqüentes problemas para o ser humano causados pelo contato com a pele e inalação prolongada e quase contínua ao longo do tempo. Mesmo com tempos variáveis, os HAPs se instalam mais cedo ou mais tarde nas superfícies de várias naturezas presentes na Terra. Deste modo, surge uma nova forma de contaminação que afeta alimentos, em particular os de origem vegetal. Diversas pesquisas e análises demonstraram de fato a presença de tais compostos nas drupas da azeitona e no óleo obtido a partir delas, bem como em muitas espécies de hortaliças (principalmente vegetais de folhas grandes e cereais). Para este processo de contaminação, outros são adicionados que derivam de tratamentos mal gerenciados (grelhar, fumar) ou de fenômenos de bioacumulação ao longo da cadeia alimentar. Portanto, é claro que é apropriado tentar reduzir a produção de HAPs e seu recrutamento através das rotas indicadas acima. Como prova disso, está em curso uma revisão da legislação em vigor à escala europeia, bem como nacional, para reconsiderar todas as implicações relacionadas com estes poluentes e definir diretrizes que permitam a identificação de padrões de qualidade para todas as matrizes que pode causar contaminação. A partir destas considerações, a ideia subjacente a esta tese toma forma, que visa avaliar analiticamente o conteúdo do HAP dentro de várias matrizes alimentares. O trabalho de tese foi realizado na ARPAM (Agência Regional de Proteção Ambiental das Marcas) no Departamento de Ascoli Piceno. Já há mais de 40 anos que a unidade de Ascoli Piceno realiza a determinação dos HAPs nas várias matrizes alimentares e ambientais, tanto para atender às exigências da lei, quanto para enfrentar a situação higiênico-ambiental criada na cidade de Ascoli Piceno. e seus arredores. Particularmente nos subúrbios orientais da cidade, no passado havia emissões industriais contendo HAP causadas pela presença de uma fábrica que produzia eletrodos de grafite e carbono amorfo, onde grandes quantidades de breu de carvão eram usadas entre as matérias-primas. material em que são encontradas altas concentrações de HAP. Graças à experiência adquirida, o Departamento ARPAM de Ascoli Piceno contribuiu, especialmente na década de 1990, para o desenvolvimento de métodos analíticos nos campos alimentar e ambiental através de estudos colaborativos organizados e geridos pelo Istituto Superiore di Sanità (ISS) e mais precisamente do Departamento de Química Toxicológica. Ao longo dos anos, vários procedimentos analíticos para a determinação de HAPs em matrizes ambientais e alimentares foram propostos. A partir das análises foi possível definir o estado da qualidade ambiental do território quanto à presença de substâncias consideradas perigosas e quanto ao seu retorno ao homem através da alimentação. Valores muito altos foram encontrados para HAPs leves em azeite extra-virgem. A partir disso, podemos deduzir que nosso território está poluído principalmente pelo tráfego de veículos e isso se reflete em uma quantidade significativa de HAPs leves no petróleo. Em todas as outras matrizes, não foi encontrado maior valor para o benzo[a]pireno e a soma de quatro HAPs em comparação com os limites impostos pela Comissão Europeia.

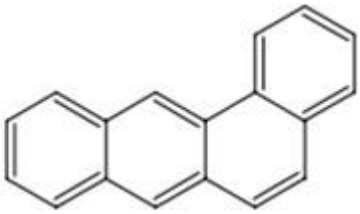
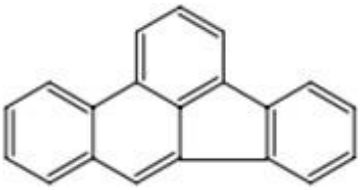
ABSTRACT:

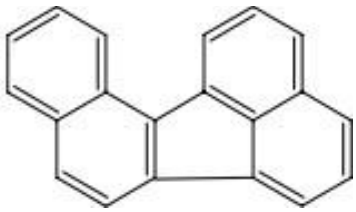
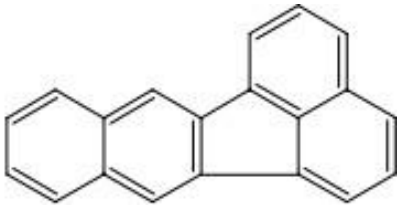
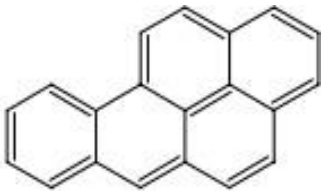
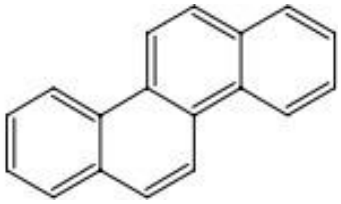
In the atmosphere of an urban/industrial complex, large quantities of nitrogen oxides, sulfur dioxide, suspended powders, polycyclic aromatic hydrocarbons and other less ubiquitous, but not less harmful, pollutants are introduced daily. However, many researchers attribute to a specific class of pollutants, the polycyclic aromatic hydrocarbons (PAH), a leading role among the causes of risk to human health. The PAHs released in the air, essentially from anthropic sources such as vehicular traffic and combustion processes in general, spread quickly and can remain in the atmosphere for quite long times, with consequent onset of problems for humans caused by skin contact and prolonged inhalation and almost continuous over time. Even if with variable times, PAHs sooner or later are deposited on the surfaces of various products present on the Earth. Due to this a further form of contamination arises and affects food, in particular those of plant origin. Various researches and analyzes have in fact shown the presence of such compounds in the drupes of the olives and in the oil obtained from them, as well as in many species of plant crops (mainly broad-leaved vegetables and cereals). To this form of contamination occurring in foods, others are added deriving from poorly managed treatments (grilling, smoking) or bioaccumulation along the food chain. Therefore, it is evident how much it is appropriate to try to minimize both the production of PAHs and their intake through the routes indicated above. As proof of this, a revision of the current legislation is in progress on a European scale, as well as a national one, in order to reconsider all the implications related to these pollutants and define guidelines that allow the identification of quality standards for all the matrices that can incur contamination. From these considerations the idea underlying the present thesis takes shape, with the aims to analytically evaluate the content of PAHs in various food matrices. The thesis work was carried out at the ARPAM (Regional Agency for Environmental Protection of the Marche) in the Department of Ascoli Piceno. It is now over 40 years that the Ascoli Piceno Structure performs the PAHs determination in the various food and environmental matrices and this, both to meet the requirements of the law, and to cope with the hygienic-environmental situation created in the town of Ascoli Piceno and its surroundings. In particular, in the eastern suburbs of the city, in the past there were industrial emissions containing PAHs caused by the presence of a factory where amorphous carbon and graphite electrodes were produced and where, among the raw materials, large quantities of pitch of fossil carbon were used, a material in which large concentrations of PAHs are found. Thanks to the experience acquired, the ARPAM Department of Ascoli Piceno contributed, especially in the 1990s, to the development of analytical methods both in the food and environmental fields through collaborative studies organized and managed by the Istituto Superiore di Sanità (ISS) and more precisely from the Department of Toxicological Chemistry. Therefore, over the years, several analytical procedures have been proposed for the determination of PAHs in environmental and food matrices. From the analysis it was possible to define the environmental quality status of the territory about the presence of substances considered dangerous and the extent of their return to humans through food. Very high values were found for light PAHs in extra virgin olive oil. From this we can deduce that our territory is polluted mainly by vehicular traffic and this is reflected in a significant amount of light PAHs in the oil. In all the other matrices in no case was found a higher value for benzo[*a*]pyrene and the sum of four PAHs than that imposed by the European Commission.

INTRODUCTION

1. Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of organic compounds that are composed of two or more fused aromatic rings with a pair of carbon atoms shared between rings in their molecules. PAHs containing up to six fused aromatic rings are often known as ‘‘small” PAHs, and those containing more than six aromatic rings are called ‘‘large” PAHs. The most extensively studied PAH is benzo(a)pyrene (BaP) and the most commonly analyzed PAHs are given in Table 1. The majority of research on PAHs has been conducted on small PAHs due to their availability. The simplest PAHs, as defined by the International Agency for Research on Cancer (IARC),[1] are phenanthrene and anthracene, which both contain three fused aromatic rings as we can see in Table **2.** Naphthalene, which consists of two coplanar six-membered rings sharing an edge, is another aromatic hydrocarbon. PAHs solely consist of carbon and hydrogen and do not contain heteroatoms. They are primarily formed by incomplete combustion or pyrolysis of organic matter and during various industrial processes. Consequently, the natural and anthropogenic sources in the environment are numerous. PAHs generally occur in complex mixtures which may consist of hundreds of compounds. The composition of these mixtures varies with the generating process.

Compound	Abbr.	MW	Structure
Benz[*a*]anthracene	BaA	228.3	
Benzo[*b*]fluoranthene	BbFA	252.3	

Benzo[*j*]fluoranthene	BjFA	252.3	
Benzo[*k*]fluoranthene	BkFA	252.3	
Benzo[*ghi*]perylene	BghiP	276.3	
Benzo[*a*]pyrene	BaP	252.3	
Chrysene	CHR	228.3	

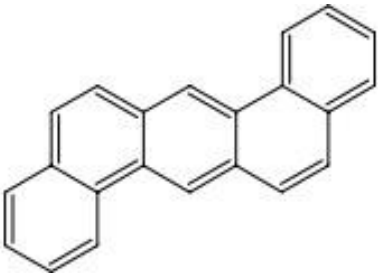
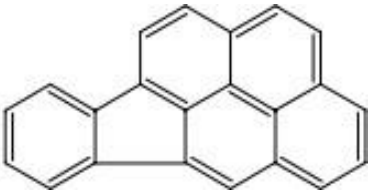
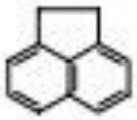
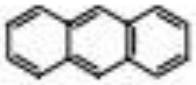
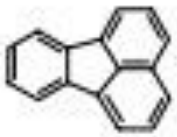
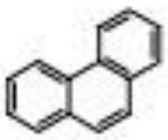
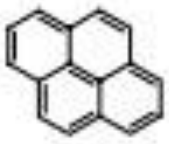
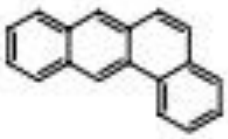
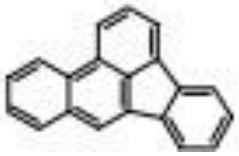
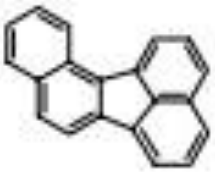
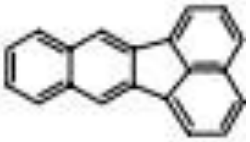
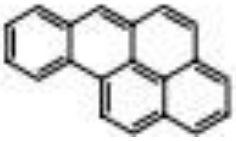
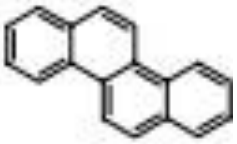
Dibenz[*a,h*]anthracene	DBahA	278.3	
Indeno[*1,2,3-cd*]pyrene	IP	276.3	

Table 1. Polycyclic aromatic hydrocarbons considered in the present thesis.

Abbr.: Abbreviation, MW: Molecular weight

For informational purposes, Table 2 lists all the PAHs that can be found in nature.

Name	Abbreviation	Structure
Acenaphthene	ACP	
Acenaphthylene	ACY	
Anthracene	ANT	
Fluoranthene	FLT	
Fluorene	FLR	
Naphthalene	NAP	
Phenanthrene	PHE	
Pyrene	PYR	
Benz[a]anthracene	BaA	
Benzo[*b*]fluoranthene	BbF	

Benzo[*j*]fluoranthene	BjF	
Benzo[*k*]fluoranthene	BkF	
Benzo[*ghi*]perylene	BgP	
Benzo[a]pyrene	BaP	
Chrysene	CHR	
Cyclopenta[*cd*]pyrene	CPP	
Dibenz[a,*h*]anthracene	DhA	

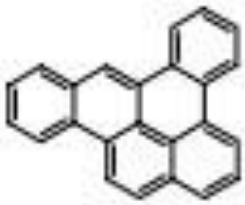
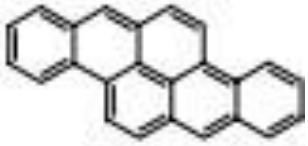
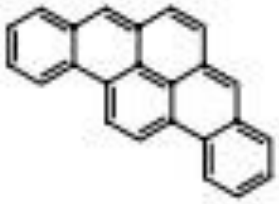
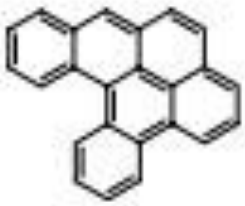
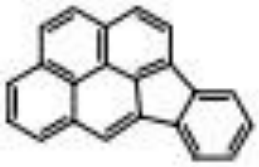
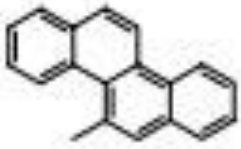
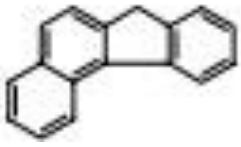
Dibenzo[*a*,*e*]pyrene	DeP	
Dibenzo[*a*,*h*]pyrene	DhP	
Dibenzo[*a*,*i*]pyrene	DiP	
Dibenzo[*a*,*l*]pyrene	DlP	
Indeno[1,2,3-*cd*]pyrene	IcP	
5-Methylchrysene	5MC	
Benzo[*c*]fluorene	BcL	

Table 2. Polycyclic aromatic hydrocarbons present in nature.

1.2 Sources of PAHs

PAHs derive from natural sources through three different processes: 1) pyrolysis of organic material at high-temperature (350°C – 1200°C), 2) creation of fossil fuel from organic material at low to medium temperature (100°C – 150°C) and 3) biosynthesis by microbes and plants. However, the environmental concern is on the anthropogenic sources of PAHs which can be domestic, agricultural, industrial or mobile (Figure 1).

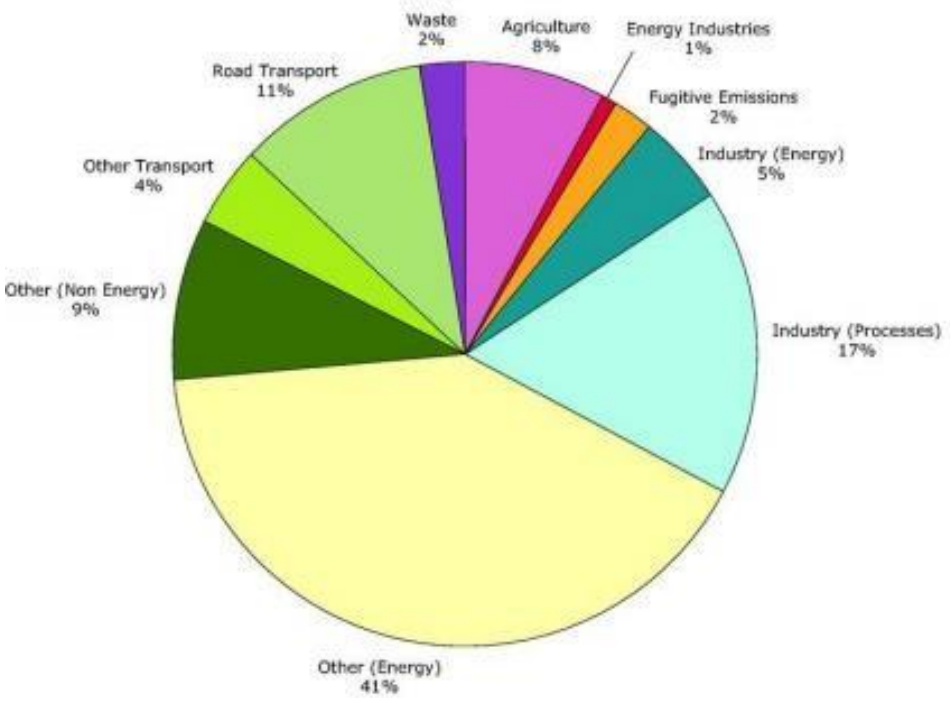


Figure 1. Sector share of PAH emissions.

The following three types: pyrogenic, petrogenic, and biological are the major PAH sources to the environment. In a process called pyrolysis, pyrogenic PAHs are formed whenever organic substances are exposed to high temperatures under low oxygen or no oxygen conditions. The destructive distillation of coal into coke and coal tar, or the thermal cracking of petroleum residuals into lighter hydrocarbons are pyrolytic processes that occur intentionally. Meanwhile, other unintentionally processes occur during the incomplete combustion of motor fuels in cars and trucks, the incomplete combustion of wood in forest fires and fireplaces, and the incomplete combustion of fuel oils in heating systems. The temperatures at which the pyrogenic processes occur are ranging from about 350 °C to more than 1200 °C. Pyrogenic PAHs are generally found in greater concentrations in urban areas and in locations close to major sources of PAHs. In addition, PAHs can also be formed at lower temperatures. It is worth mentioning that crude oils contain PAHs that formed over millions of years at temperatures as low as 100–150 °C. In this respect, PAHs formed during crude oil maturation and similar processes are called petrogenic. Such petrogenic PAHs are common due to the widespread

transportation, storage, and use of crude oil and crude oil products. Some of the major sources of petrogenic PAHs include oceanic and freshwater oil spills, underground and above ground storage tank leaks, and the accumulation of vast numbers of small releases of gasoline, motor oil, and related substances associated with transportation. It is well-known that PAHs can be formed during the incomplete combustion of organic substances. PAHs are also found in petroleum products. On the other hand, it is not well-known that PAHs can be produced biologically. For example, they can be synthesized by certain plants and bacteria or formed during the degradation of vegetative matter. The mode of PAHs formation can be either natural or anthropogenic. Examples of natural sources of PAHs formation include: forest and brush fires, volcanoes, bacterial and algal synthesis, petroleum seeps, erosion of sedimentary rocks containing petroleum hydrocarbons, and decomposition of vegetative liter fall. [2]

The mechanism of formation of PAHs is not fully clarified yet, it is thought to occur in two stages: pyrolysis and pyrosynthesis. At high temperatures, organic compounds are partially transformed into smaller and more unstable molecules (pyrolytic cracking: fragmentation into many parts of the fuel molecules in contact with the fire). These fragments, mainly radical, are recombined to form larger and more stable molecules such as PAHs (pyrosynthesis). The repolymerization reaction takes place above all in conditions of oxygen deficiency; in general, the rate of formation of PAHs increases as the oxygen / fuel ratio decreases; the fragments often lose some hydrogen atom, which forms water after being combined with oxygen during the various phases of the reaction: the carbon-rich fragments combine to form the polycyclic aromatic hydrocarbons, which represent the more stable molecules, with a high C / H ratio. After the processes of craking and partial combustion, in fact, there is a prevalence of the presence of radical fragments containing two carbon atoms that can react with an acetylene molecule to give a 4 carbon atoms radical (Figure 2).

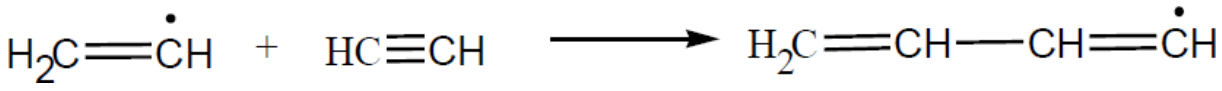


Figure 2. Formation of a chain of C_4 radicals.

The resulting radical can then be added to another acetylene molecule and cyclized to form a six component ring (Figure 3).

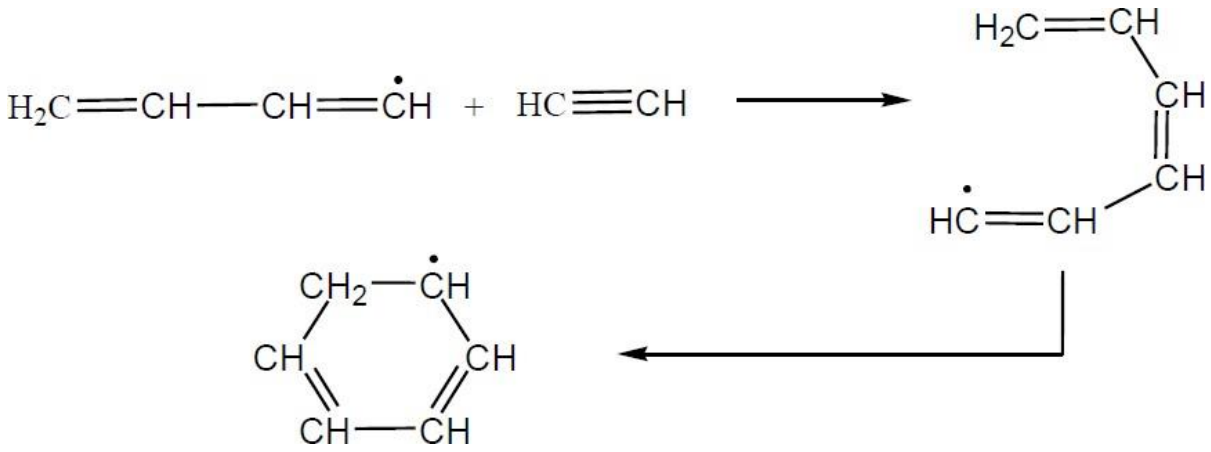


Figure 3. Formation of a 6 terms ring.

The radical can add further acetylene molecules giving rise to side chains which form condensed benzene rings (Figure 4).

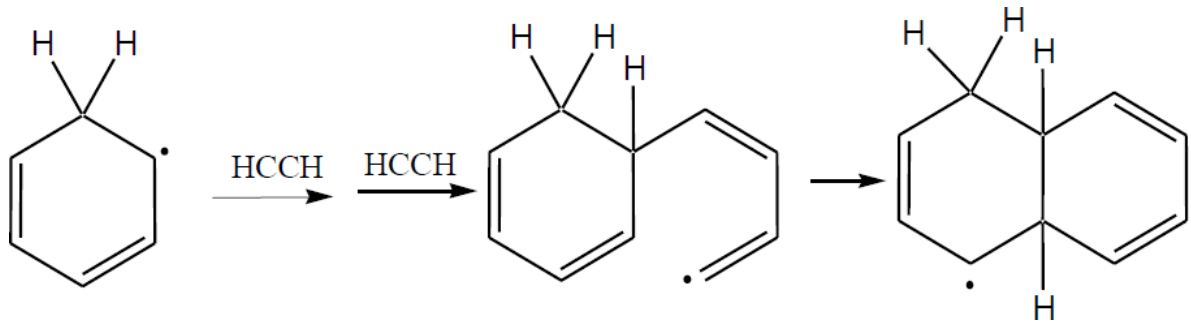


Figure 4. Formation of condensed benzene rings.

Moreover some types of food cooking can, for example, lead to the formation of PAHs, in particular heat treatments linked both to the preservation of the product (smoking), and to the preparation of the food (grilling, cooking, frying).

PAHs present in foods can be divided according to their origin in:

• PAHs of exogenous formation deriving from fuel combustion: the combustion fumes, consisting of PAHs in the solid phase, can come into contact with the food contaminating it on the surface. The main cause of the presence of PAHs in smoked foods is in fact due to the incomplete combustion of the fuel;

• PAHs of endogenous formation present on the surface of food due to severe heat treatments (high temperatures, long treatment times and proximity to heat sources). The high temperature causes pyrolysis of protides, lipids and carbohydrates. According to Larsson's studies (Larsson et al., 1983; 1987) [3], in the grilled meat products only the direct contact of the food with the flame gives significant production of PAH (up to 212 ppb of benzo(a)pyrene, B[a]P), while embers alone emit only small amounts (1-25 ppb of B[a]P). [4]

An additional source of contamination for food, in addition to the aforementioned, is environmental one. In fact, PAHs can also be found in broad-leaved vegetables such as lettuce and spinach for the deposition of these substances carried with the air on the leaves during growth. The cereals consumed in the raw state also contribute significantly. An Italian study (Lodovici et al., 1995) [5] shows that food is the most important source of exposure for humans to PAHs: from the data of this study it appears that in food products free from contamination, PAHs are detectable at concentrations lower than µg/kg (1-3) while in those exposed to anthropogenic sources of pollution such as vehicular traffic or domestic heating or, to a more incisive extent, to specific sources pollutants of industrial origin, they are detectable level of a few tens of µg/kg. These values are high when compared to the contribution that the substances themselves make through breathing, which is equal to 370 ng per day for total PAHs and 130 ng per day for carcinogenic PAHs. The presence of PAHs in food and in various environmental matrices can provide important information on the mechanism of contamination. In fact, from an analytical point of view, the chromatographic profile of the PAHs present in a sample and therefore the relative relationship between congeners is characteristic of the source of pollution: for example, in samples taken in areas with high vehicular traffic we will find a concentration of PAHs with a prevalence of low molecular weight molecules (more water-soluble) while in samples taken close to factories with particular emissions we will find a concentration of PAHs with the prevalence of molecules with high molecular weight (more liposoluble).

1.3 Characteristics of PAHs

The general characteristics of PAHs are high melting and boiling points (they are solid), low vapor pressure, and very low aqueous solubility. The latter two parameters tend to decrease with increasing molecular weight while resistance to oxidation and reduction increases. Aqueous solubility of PAHs decreases for each additional ring. Meanwhile, PAHs are very soluble in organic solvents because they are highly lipophilic. PAHs also manifest various functions such as light sensitivity, heat resistance, conductivity; corrosion resistance, and physiological activity. PAHs possess very characteristic UV absorbance spectra. Each ring structure has a unique UV spectrum, thus each isomer has a different UV absorbance spectrum. This is especially useful in the identification of PAHs. Most PAHs are also fluorescent, emitting characteristic wavelengths of light when they are excited (when the molecules absorb light). Specifically for this thesis work, in order to clarify the mechanism of contamination of crops and olive oil, it is interesting to report their water solubility as a function of the structure and molecular weight. Naphthalene is the most water-soluble PAH, its solubility in water is in the order of tens of mg/l (37.1 mg/l) and for the higher homologues it decreases exponentially depending on the number of condensed rings. Phenanthrene has three benzene rings and has a water solubility of about 1.3 mg / l, the pyrene of 0.13 mg / l, while in the five- and six-benzene components the solubility drops to the level of a few micrograms liter. Later, for simplicity of exposure, the most water-soluble components, from naphthalene to pyrene will be called "light", while those less water-soluble, from benzo[*a*]anthracene to benzo(*ghi*)perylene will be called "heavy".[6]

1.4 Biochemistry of PAHs

The ability of PAH-containing mixtures to induce cancer in humans has been known since 1775, when British surgeon Sir Percival Potter demonstrated the correlation between soot exposure and the incidence of scrotal cancer (Pott, 1963). [7] Research on the carcinogenesis induced by PAHs began with the isolation of benzo[*a*]pyrene from the fumes of coal in 1930, and the subsequent demonstration that benzo[*a*]pyrene induced tumors when, repeatedly, was brushed on the skin of the mouse [8] (Cook et al., 1933). Based on theoretical studies, it was suggested that the carcinogenic activity of PAHs was to be related to the presence of an electronic high density area called K-region. In particular, it was observed that PAHs presenting a K-region and a bay region are potent carcinogens and that those with a fjord regions or a sterically hindered bay region are even more potent carcinogens (Figure 5).

The correlation between the interactions of carcinogenic PAHs with DNA and their carcinogenic potency was later discovered by Brookes and Lawley in 1964 that demonstrated a positive correlation between the type of binding of a series of PAHs in the skin of the mouse and their carcinogenic potency (Brookes & Lawley, 1964). [9]

Based on these findings Baird and Brookes in 1973 tested the hypothesis that PAHs were activated in K-region in the epoxides that bind DNA [10] (Baird and Brookes, 1973). However, in 1977 Lehr and Jerina described the theoretical basis of the carcinogenic activity of PAH-diols and epoxides (Lehr and Jerina, 1977). [1]

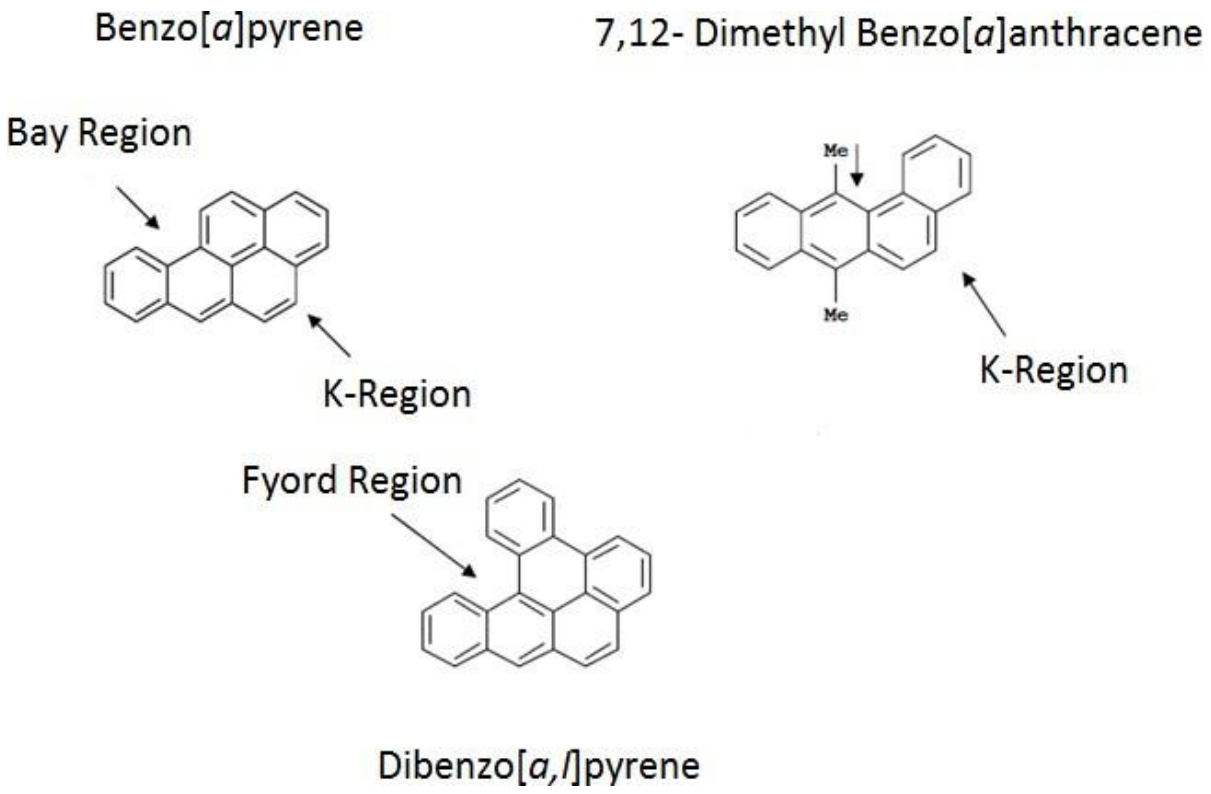
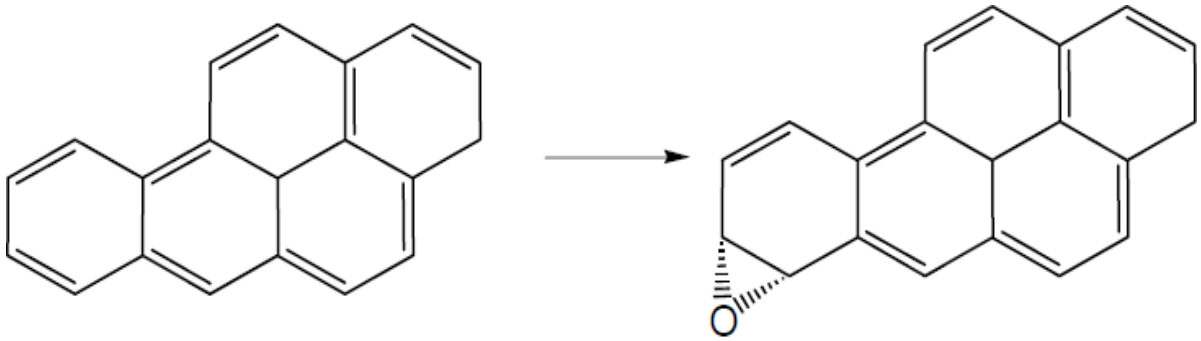


Figure 5. Structural formulas of some PAHs with high carcinogenic power.

The mutagenic and carcinogenic action of PAHs is a consequence of the transformations that these compounds undergo during the body's metabolic processes. The carcinogens are the intermediate products of the metabolism of PAHs, which the body produces to facilitate their elimination; the PAHs, in fact, are converted by the body into water-soluble rendering derivatives, therefore more easily eliminated (Baird et al., 2005).[2] Benzo[*a*]pyrene is the most known compound from the toxicological point of view and more frequently determined in the various matrices, both environmental and food. It has been used as an indicator of the class of PAHs, with regard to both levels of contamination and carcinogenic risk. This choice is due to the fact that the studies are epidemiological on the single component, because in nature the PAHs are always present in mixture and with precise concentration ratios, according to the polluting source. The observations on which the choice to use B[a]P is based as an indicator are: the substantial similarity, at least in terms of orders of magnitude, between the "profiles" of the PAHs with respect to the B[a]P (ie between the concentrations of PAHs, in particular carcinogens, and the concentration of B[a]P), observed in samples also of different origins; the carcinogenic potency of B[a]P relatively high compared to other PAHs; concentration levels of B[a]P similar or higher than those of other carcinogenic PAHs (IPCS, 1998). The first transformation to be followed by benzo[*a*]pyrene is epoxidation, catalyzed by cytochrome P450, in positions 7 and 8, the most reactive, which represent the so-called K region (Figure 6).

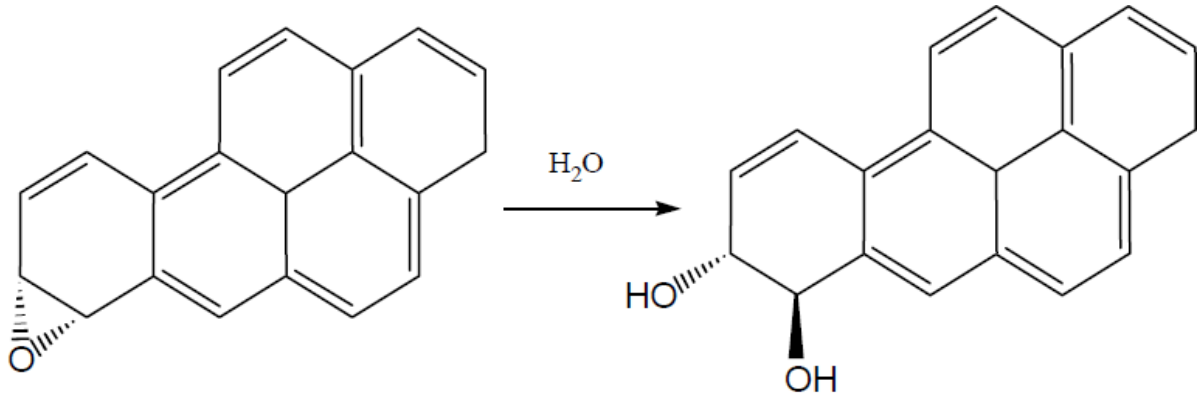


Benzo[*a*]pyrene

Benzo[*a*]Pyrene
7,8-epoxide

Figure 6. Epoxidation in positions 7 and 8.

The epoxide undergoes a nucleophilic attack by water, with the formation of a diol, which is more water-soluble and therefore more easily eliminated (Figure 7).

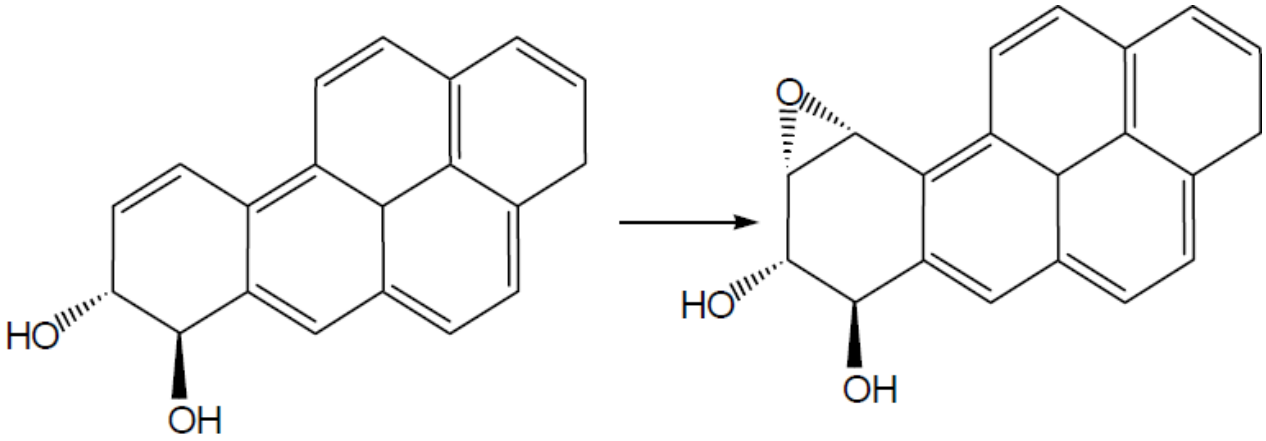


Benzo[*a*]pyrene
7,8-epoxide

Benzo[*a*]pyrene
7,8-diol

Figure 7. Formation of a diol.

This metabolite of PAHs can be further metabolized by cytochrome P450 to form an epoxide diol (Figure 8).



Benzo[*a*]pyrene
7,8-diol

Benzo[*a*]pyrene
7,8-diol - 9,10-epoxide

Figure 8. Metabolism of Benzo[*a*]pyrene.

It is believed that this compound is the actually carcinogenic species that binds to DNA through nucleophilic attack, for example by adenine (Figure 9). The covalent attack of the large hydrocarbon residue represents an evident damage to the DNA that causes mutations and a greater probability of carcinogenicity.

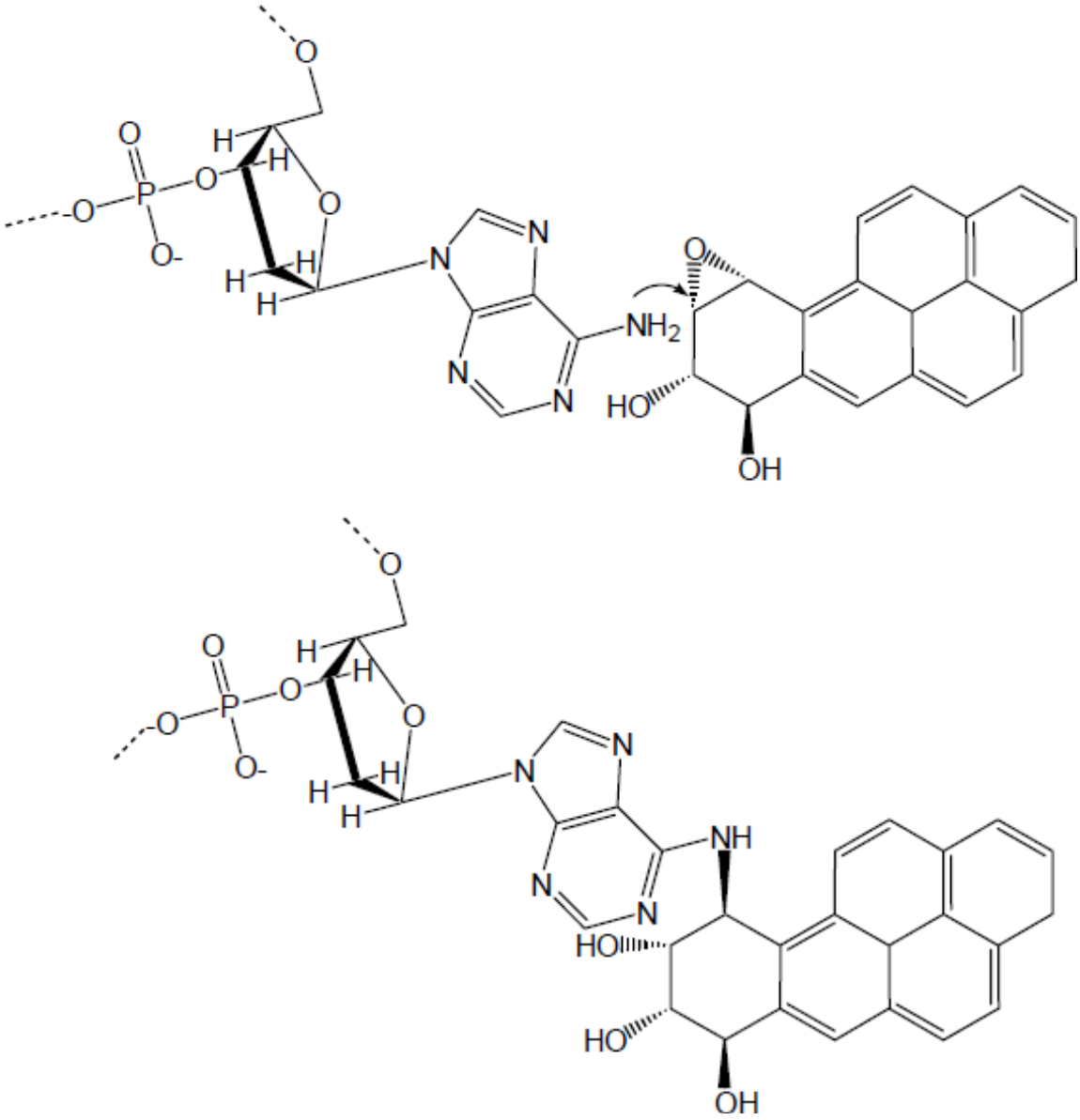


Figure 9. Reaction of Benzo[*a*]pyrene 7,8 diol-9,10 epoxide with DNA.

Information on the mechanism of carcinogenesis of PAHs, in which the induction of genetic alterations has a causal role, makes it possible to extend the observations on the animal from experiment to humans, being able to exclude indirect, species-specific mechanisms. On the other hand, there are no adequate studies to evaluate the carcinogenic effects of individual PAHs on humans. In real conditions, in fact, human exposure concerns complex mixtures of PAHs, in which other carcinogenic components are often present. For some of these mixtures (coal tar, fulgens) or types of exposure (coal distillation, coke production) there is in any case sufficient epidemiological evidence of carcinogenicity in humans (class IARC). In general, both for individual PAHs and for their complex mixtures, the evaluation of carcinogenicity overlaps with that of genotoxicity. This highlights the functional association between DNA damage/adduction formation[13], induction of mutations and long-term carcinogenic effects of PAHs. Always on the basis of experimental

toxicology, benzo[*a*]pyrene is defined as "initiator", i.e. substance which, following metabolic action, reacts with DNA causing irreversible effects also present in the stage of cell division and therefore transmitted to the offspring. For this reason, benzo[*a*]pyrene is also defined genotoxic and mutagenic. [14]

1.5 Metabolism of PAHs

Once PAHs enter the body they are metabolized in a number of organs (including liver, kidney, lungs), excreted in bile, urine or breast milk and stored to a limited degree in adipose tissue. The principal routes of exposure are: inhalation, ingestion, and dermal contact. The lipophilicity of PAHs enables them to readily penetrate cellular membranes. Subsequent metabolism renders them more water-soluble making them easier for the body to remove. However, PAHs can also be converted to more toxic or carcinogenic metabolites. After exposure, these molecules induce expression of phase I and II metabolizing enzymes including aldo-ketone reductases, cytochrome P-450s, catechol-O-methyltransferase, epoxide hydrolase, peroxidases, glutathione S-transferases, N-acetyltransferases, sulfotransferases, and other enzymes catalyzing conjugation reactions.

1.6 Health effects on humans

The presence of several aromatic rings gives PAHs low reactivity, high melting and boiling points and lipophilic character. Once ingested (or inhaled), PAHs are rapidly absorbed through the gastro-intestinal tract or the pulmonary epithelium, and distributed in various tissues (especially those rich in fat), including fetal ones. PAHs are extensively metabolised in various tissues and organs (lung, skin, esophagus, colon, liver, placenta, etc.). In general, the first step in the metabolism of PAHs, aiming to increase their hydrophilicity facilitating their excretion, is an oxidation. The metabolic pathway of the benzo[*a*]pyrene is the one studied in greater detail, and can be taken as an example (Figure 11).

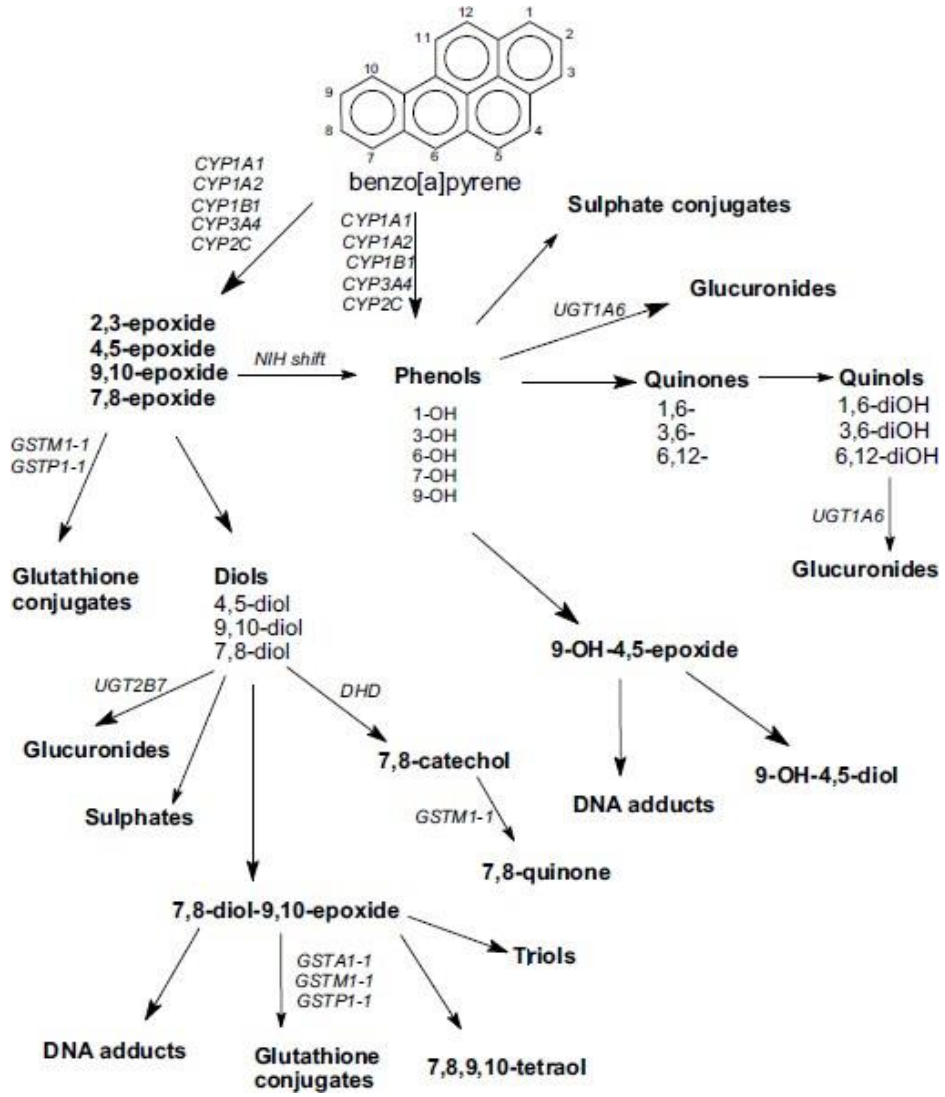


Figure 11. Metabolic pathway of benzo[*a*]pyrene. [15]

1.6.1 Sources of human exposure

There is a continuous concern regarding the exposure of the population to PAHs. This concern arises from the fact that PAHs are ubiquitous environmental pollutants that possess mutagenic, teratogenic and carcinogenic effects. This concern has driven the researchers to develop methods in order to assess the exposure of the general population to PAHs; one method that is highly used being the identification and quantification of urinary metabolites of PAHs. It should be noted that the general population is generally exposed to mixtures of PAHs rather than to a single PAH. The IARC has classified a number of individual PAHs compounds, based on animal experiments, as probable human carcinogens (Category 2A) or possibly human carcinogens (Category 2B) as we can see in Table 3. [16]

PAH	MW	No. aromatic rings	IARC Group
Naphthalene	128	2/3	2B
Fluorene	166	2/3	3
Phenanthrene	178	2/3	3
Anthracene	178	2/3	3
Fluoranthene	202	4	3
Pyrene	202	4	3
Benzo[a]fluorene	216	4	3
Benz[a]anthracene	228	4	2A
Chrysene	228	4	3
Benzo[b]fluoranthene	252	5	2B
Benzo[k]fluoranthene	252	5	2B
Benzo[j]fluoranthene	252	5	2B
Benzo[e]pyrene	252	5	3
Benzo[a]pyrene	252	5	1
Perylene	252	5	3
Benzo[ghi]perylene	276	6	3
Indeno[1,2,3-cd]pyrene	276	6	2B
Benzo[b]chrysene	278	6	3
Dibenzo[a,j]anthracene	278	6	
Dibenzo[a,h]anthracene	278	6	2A
Dibenzo[a,c]anthracene	278	6	

Table 3. The degree of evidence for carcinogenicity of PAHs in experimental animals, and overall evaluations of carcinogenicity to humans (evaluated by IARC and IPCS/WHO).

1.7 Legislation

In view of disparities caused by different maximum levels (ML) for PAHs in food in several Member States, the Commission set a harmonized maximum level for benzo[*a*]pyrene for the first time in 2005 [17] by Commission Regulation (EC) No 208/200510 amending Regulation (EC) No 466/200111 as regards PAHs. Benzo[*a*]pyrene was chosen because the Scientific Committee on Food (SCF) concluded in its opinion of 4 December 2002 that this compound can be used as a marker for the occurrence and effects of carcinogenic PAHs in food. Moreover, the data on occurrence and relative proportions of other carcinogenic PAHs than benzo[*a*]pyrene in food were considered insufficient by the Commission for setting further ML. The current MLs are laid down in the Annex, Section 6 of Commission Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs. [18] Maximum levels are especially set for foodstuffs containing fats and oils and foods where smoking and drying processes or environmental pollution might cause high levels of contamination. The lowest maximum levels are set for food for infants and young children. All maximum levels are expressed as µg/kg wet weight (Table 4) unless more specific footnote applies.

Foodstuff	ML (µg/kg wet weight)
Oils and fats (excluding cocoa butter) intended for direct human consumption or use as an ingredient in foods	2.0
Smoked meats and smoked meat products	5.0
Muscle meat of smoked fish and smoked fishery products, excluding bivalve molluscs. The maximum level applies to smoked crustaceans, excluding the brown meat of crab and excluding head and thorax meat of lobster and similar large crustaceans (*Nephropidae and Palinuridae*).	5.0
Muscle meat of fish, other than smoked fish	2.0
Crustaceans, cephalopods, other than smoked. The maximum level applies to crustaceans, excluding the brown meat of crab and excluding head and thorax meat of lobster and similar large crustaceans (*Nephropidae and Palinuridae*).	5.0
Bivalve molluscs	10.0
Processed cereal-based foods and baby foods for infants and young children	1.0
Infant formulae and follow-on formulae, including infant milk and follow-on milk	1.0
Dietary foods for special medical purposes intended specifically for infants	1.0

Table 4. Maximum levels (MLs) for benzo[*a*]pyrene as laid down in Regulation (EC) No. 1881/2006.

At a later stage, new data on occurrence of carcinogenic PAH in foodstuffs have been collected by the Member States in the framework of Commission Recommendation 2005/108/EC. The Commission asked the European Food Safety Authority (EFSA) to review the Scientific Committee on Food opinion taking into account the new occurrence data, other relevant new scientific information as well as the Margin of Exposure (MOE) approach. Within this review, EFSA was asked to re-assess the suitability of maintaining benzo(a)pyrene as a marker. The scientific group on contaminants in the food chain (CONTAM group) of EFSA adopted an opinion on polycyclic aromatic hydrocarbons in food on 9 June 2008. In this opinion, EFSA concluded that benzo (a) pyrene is not a marker suitable for the occurrence of polycyclic aromatic hydrocarbons in food and that a system of four specific substances (PAH4) would be the most suitable indicator of PAHs in food. For this reason, a new maximum levels for the sum of four substances (PAH4) (benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene) was introduced, whilst maintaining a separate maximum level for benzo(a)pyrene. The Annex to Regulation (EC) No 1881/2006 is amended as follows (Table 5):

Foodstuffs	Maximum levels (µg/kg)	
	Benzo(a)pyrene	Sum of benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene and chrysene (45)
Oils and fats (excluding cocoa butter and coconut oil) intended for direct human consumption or use as an ingredient in food	2,0	10,0
Cocoa beans and derived products	5,0 µg/kg fat as from 1.4.2013	35,0 µg/kg fat as from 1.4.2013 until 31.3.2015 30,0 µg/kg fat as from 1.4.2015
Coconut oil intended for direct human consumption or use as an ingredient in food	2,0	20,0
Smoked meat and smoked meat products	5,0 until 31.8.2014 2,0 as from 1.9.2014	30,0 as from 1.9.2012 until 31.8.2014 12,0 as from 1.9.2014

Muscle meat of smoked fish and smoked fishery products ([25])([36]), excluding fishery products listed in points 6.1.6 and 6.1.7. The maximum level for smoked crustaceans applies to muscle meat from appendages and abdomen ([44]). In case of smoked crabs and crab-like crustaceans (*Brachyura* and *Anomura*) it applies to muscle meat from appendages.	5,0 until 31.8.2014 2,0 as from 1.9.2014	30,0 as from 1.9.2012 until 31.8.2014 12,0 as from 1.9.2014
Smoked sprats and canned smoked sprats ([25]) ([47]) (*sprattus sprattus*); bivalve molluscs (fresh, chilled or frozen) ([26]); heat treated meat and heat treated meat products ([46]) sold to the final consumer	5,0	30,0
Bivalve molluscs ([36]) (smoked)	6,0	35,0
Processed cereal-based foods and baby foods for infants and young children ([3])([29])	1,0	1,0
Infant formulae and follow-on formulae, including infant milk and follow-on milk ([8])([29])	1,0	1,0

Table 5. New maximum levels for polycyclic aromatic hydrocarbons in foodstuffs.

This system ensures that PAH levels in food are kept at levels that do not cause health concerns and that the amount of PAH can also be controlled in those samples in which benzo(a)pyrene is not detectable, but where other PAHs are present.

2. Occurrence of PAHs in food

The waxy surface of vegetables and fruits is able to concentrate low molecular mass PAHs through surface adsorption and particle-bound high molecular mass PAHs can contaminate the surface due to atmospheric fallout. PAHs can also contaminate foods during industrial smoking, heating and drying processes that allow combustion products to come into direct contact with food. Contamination of cereals and vegetable oils (including seed oils and olive residue oils) with PAHs usually occurs during technological processes like direct fire drying, where combustion products may come into contact with the grain, oil seeds or the oil. PAHs are also formed as a result of certain home food preparation methods, such as grilling, roasting and smoking. High PAH concentrations have been reported in charcoal grilled/barbecued foods (such as fatty meat and meat products grilled under prolonged and severe conditions), in foods smoked by traditional techniques (fish in particular), and in mussels and other seafood from polluted waters. Smoked and grilled food may contribute significantly to the intake of PAHs if such foods are a large part of the usual diet. [19]

2.1 Extra Virgin Olive Oil

The Mediterranean is the historic home of the olive where it has been an important part of life for thousands of years. The olive, a symbol of peace, and the tree which produces olives (*Olea Europea*) are known to have been cultivated around the Mediterranean about 6000 years ago. The olive tree is one of the oldest plants in the world. Egyptians, Greeks and Romans tell stories and myths in their literature related to olive and its fruit.

Properties and Benefits:

If you squeeze an olive, you derive an oil that has been a symbol since ancient times, of tasteful and well-being. The olive is the only fruit which allows to obtain an oil with the single pressing, without any chemical intervention. It is digestible, rich in vitamins A, D, E, K, oleic acid, protects against the excesses of high bad cholesterol and increases, the “so-called” good cholesterol. Thanks to its content of antioxidants such as polyphenols, olive oil counteracts the processes that contribute to cellular aging. Oleic acid, the main component of extra virgin olive oil, stimulates the activity of the gall bladder and reduces the danger of the formation of gallstones. Particularly rich in oleocanthal, extra virgin olive oil plays an effective anti-inflammatory action. More oil is fresh (ie: more tingles in the throat), the higher the content of this substance. It also is rich in squalene that protects from ultraviolet rays of the sun.

Chemical composition of olive oil

The chemical composition of olive oil can be divided mainly in:

- *Saponifiable fraction*: represents about 98% of the total composition of oil. Mostly of this fraction is composed of triglycerides derived from esterification of glycerol with three molecules of fatty acids. Triglycerides can be simple triglycerides (esterified with only one type of fatty acid) or mixed triglycerides (esterified with different fatty acids). Both are presents in the oil in percentage of 55% simple and 45% mixed. The principal fatty acids esterified in olive oil triglycerides are: oleic acid (18:1 ω-9) in a range of 70-80%, linoleic acid (18:2 ω -6) at least 10%, palmitic acid (16:1 ω -7) in a range of 7-15% and stearic acid (18:0) less of 5%.

Monoglycerides and diglycerides are also present but in traces.

- *Unsaponifiable fraction*: includes lot of species like sterols, hydrocarbons, aliphatic and triterpenic alcohols, tocopherols, carotenes, hydrophilic phenols and some volatile compounds.

Types of olive oil:

There are numerous ways to differentiate olive oil on the basis of their nutritional content, flavor, processing method, harvesting method and packaging method.

Extra Virgin Oil – This is the highest grade of olive oil, which is known for its delicate flavor. This oil is unrefined and is obtained from the first pressing of the olives. International Olive Oil Council has established some standards, according to which an extra virgin can have only up to 0.8 percent of free acidity. The term virgin means that this oil has been derived from fruit of the olive tree only by using such methods which do not change or spoil the oil and its quality. It means that methods like chemical interaction, solvents mixing, radiation effect or microwave effect, etc. are not at all used during the processing of these oils. These are pressed by using cold pressed techniques which makes use of only cold pressure, and due to which a low acidity level is produced in these oils. It has a fruity delicate taste and its color varies from pale yellow to bright green.

Virgin Olive Oil - They come next to Extra virgin olive oils. They are known for their good taste. This oil is less expensive than the Extra virgin olive oil. They are also obtained from the first pressing like Extra virgin oils. It is made by olives which are little more ripened in comparison to those used for making extra virgin olive oil.

Refined olive oil – These oils are obtained from virgin olive oils. The refining is done by such methods which do not bring any change in their initial glyceride structure. This oil is actually tasteless. The odor of this oil is not pleasant and the flavor is also compromising in many terms. These are refined versions, and therefore they are not titled as virgin oils. Unlike virgin oils, they may undergo processing methods which include heat, chemical interact or filtration. They have a longer shelf life, and this advantage seems to be the only one favoring these oils.

Olive Oil –This oil contains both refined and virgin oils, and therefore is concerned to be a blend of both. The proportion of virgin olive oil and refined olive oil can be different depending up nth flavor desired by the manufacturer. As this oil contains refined olive oil, it is also place in the category of refined olive oil sometimes. It is transparent with hint of yellow color in appearance. It has a mild aroma and taste. It is also known as pure olive oil.

Pomace Olive Oil - This is made by that part of the olive which is left after all its water and oil content has been extracted by using various techniques like pressuring or centrifuging. Some solvents are used in order to collect the little residual oil. This oil lacks Olive oil's vitamins. It is usually blended with virgin olive oil so that people can use it as an option. It is however still considered good for high heat cooking. These oil products have been criticized sometimes for containing high levels of contaminants known as polycyclic aromatic hydrocarbons (PAHs), and due to which governments have set legal limits of PAHs in olive oil.

Mechanism of contamination:

Although virgin olive oil (VOO) should be naturally free of PAH, contamination can occur either directly during the processing in the mill or indirectly due to olive skin contamination by environmental sources. In this last situation, PAHs present in dust and particles from smoke and air pollution can contaminate olives via atmospheric fallout and this superficial contamination can be transferred to the final product. Aiming to identify and evaluate the sources of PAH contamination during the processing of VOO, the influence of factors such as the environmental pollution during olive growth, contamination during olive harvesting, contamination during extraction process and environmental pollution at the olive mill site has been studied. At that time, it was particularly motivating to clarify the mechanism of contamination of extra-virgin olive oil, that is the mechanism for the transfer of PAHs from industrial emissions to exposed olives, giving place to the phenomenon of accumulation (Figure 11).

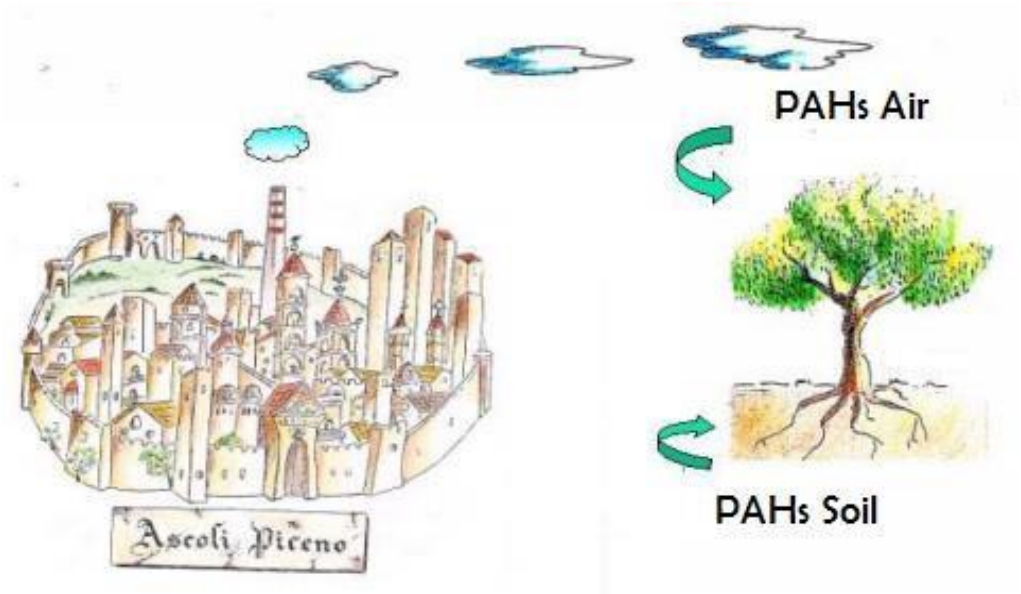
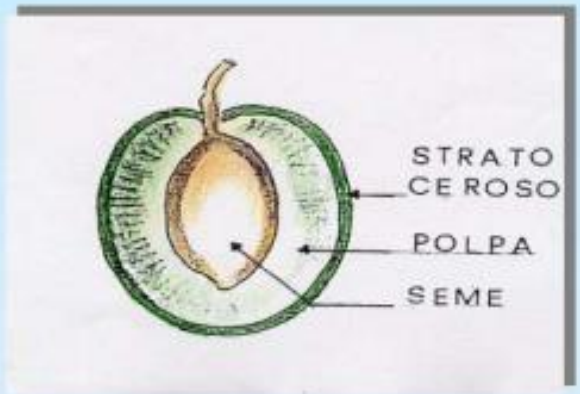


Figure 11. Atmospheric pollution by the PAHs in Ascoli Piceno.

Some authors claimed that the PAHs passed from the contaminated soil to the crops through the root system, someone else that the pollution happened due to the atmospheric deposition of the pollutants on the cultures, other authors supported the biosynthesis of PAHs in nature going to search, even in the crystalline ox eye. Subsequently it was verified that the crops exposed and not protected from atmospheric pollution such as salad, rosemary and grapes, showed a contamination from PAHs proportional to the surface of exposure. On the other hand, crops protected from atmospheric pollution, such as beans, peas and pine nuts, are not at all contaminated by PAHs. From the experimental evidence, the hypothesis that the PAHs found in the exposed crops derived from atmospheric depositions rather than radial absorption was advanced. This was confirmed by the data obtained by analyzing the various parts of the ripe olive drupe exposed for five months to the relapse of PAHs of industrial origin.



	IPA µg/Kg													
	DBT	FEN	ANT	FLU	PIR	BcFEN	BaA	Cr+Tr	B(b,j,k) FLU	BeP	BaP	PER	I.P.	B(g;h;i)P
STRATO CEROSO	7	58	3	32	20	3	3	9	4	2	2	--	3	4
POLPA	44	555	23	71	53	--	--	--	--	--	--	--	--	--
SEME	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Figure 12. Distribution of PAHs in the various parts of the olive drupe.

From the evaluation of the data in the Figure (Figure 12), the different composition of the PAHs present in the external part of the drupe, in relation to the one found in the pulp, is immediately evident. In the core are not detectable PAHs, confirming that the way of contamination of the crops is the aerial one and not through the radical absorption. Comparing the data on the PAHs found on the outside of the drupe with those of the pulp, it was also possible to clarify the process of accumulation of these substances in the plant structure. The percentage composition of PAHs in the pulp, compared to that on the surface, has shifted markedly towards the "lighter" components. This can be explained by the fact that the PAHs present on the external surface of the drupe, associated with condensation droplets or particle material, pass within the plant structure (Figure 13) by diffusion exploiting the concentration gradient existing between the most external parts with respect to the most internal parts.

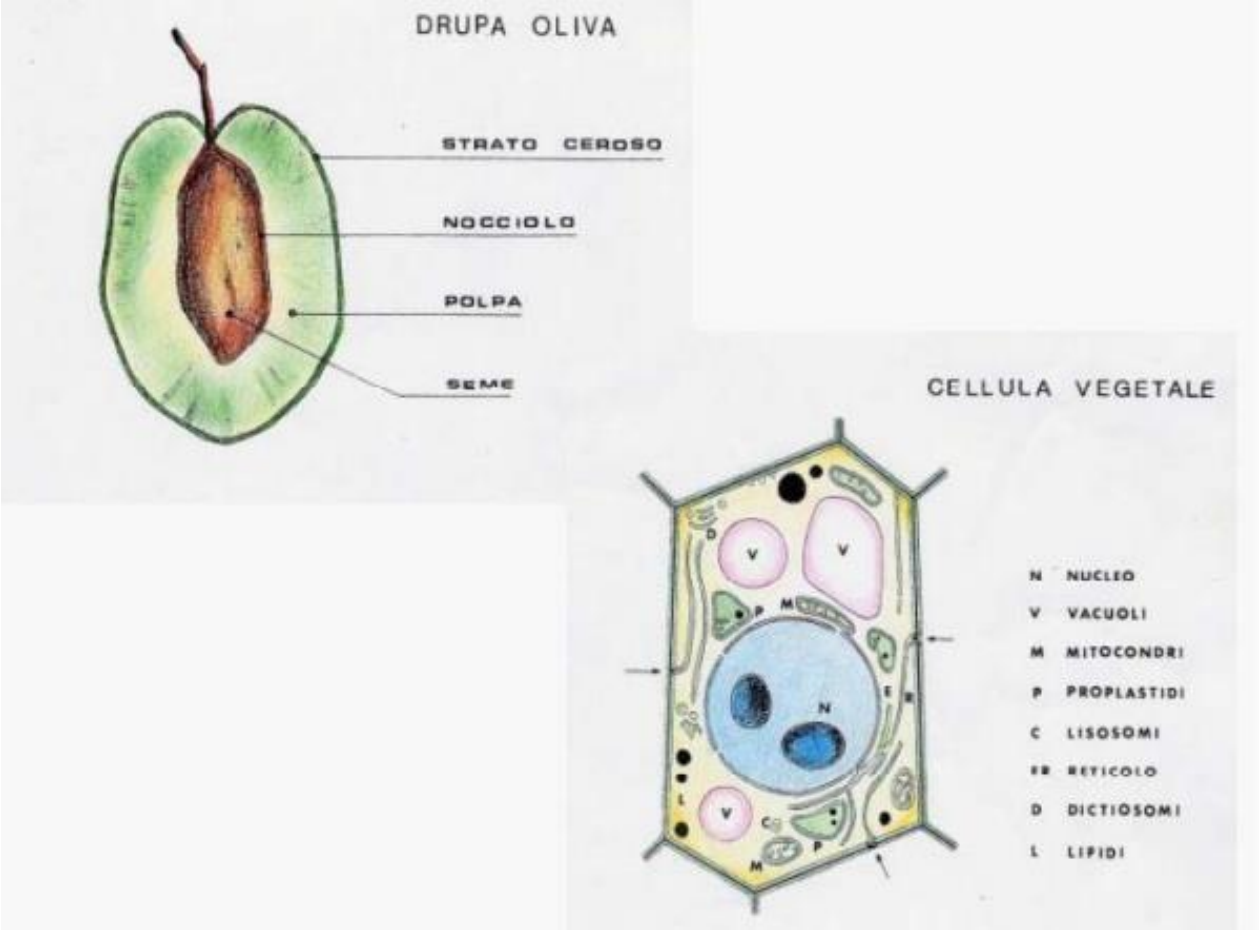


Figure 13. Plant structure of the olive drupe.

This step is not the same for all the components of PAHs, but it depends on their water solubility. In fact, the droplets of oil present in the drupe are suspended in the aqueous medium of the cell's cytoplasm and are reached by PAHs that have a certain water solubility, that is from the "lighter" ones. This hypothesis was confirmed by the following experiment (Figure 14):

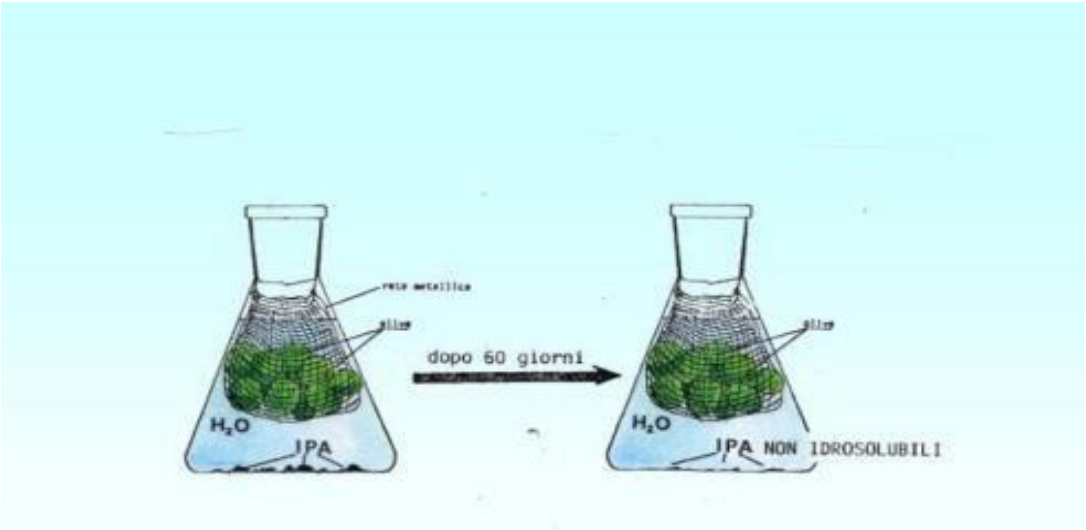


Figure 14. Simulation in the laboratory of PAHs contamination of olives.

In a flask containing distilled water, about 10 mg of each component of PAHs were introduced, both "light" and "heavy" ones, then 60 g of olives suspended from a metal net to avoid direct contact with PAHs on the bottom of the flask. After sixty days it was noticed that the PAHs at the bottom of the flask had visibly decreased and from the instrumental analysis of the olives it was possible to demonstrate that the "lighter" PAHs had passed inside the drupe, while the "heavier" ones had remained on the bottom of the flask. As the "light" PAHs leave the aqueous medium because they are accumulated in the oil droplets suspended in the cytoplasm, new solid phase PAH molecules pass into the aqueous medium to restore saturation equilibrium.

Sources of Contamination of Virgin Olive Oil

Aiming to identify and evaluate the sources of PAH contamination during the processing of VOO, it was studied the influence of factors such as the environmental pollution during olive growth, contamination during olive harvesting, contamination during extraction process and environmental pollution at the olive mill site. [20] Some authors identified nine PAHs (benz[*a*]anthracene (BaA), chrysene (CHR), benzo[*e*]pyrene (BeP), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), BaP, dibenz[*a*,*h*]anthracene (DhA), benzo[*ghi*]perylene (BgP), indeno[1,2,3-*cd*]pyrene (IcP)) and, comparing the total PAH concentration in olive oils with olive fruit surface extracted with hexane, they found identical values for both cases. This finding suggests that the contamination of olive oil is mainly from the olive skin. When establishing the influence of different levels of environmental pollution during olive growth, it was showed that total PAH content in olives and, consequently, in respective oil, is related to the level of air pollution in the vicinity of the olive grove. The same authors compared mechanical and handpicked harvesting, concluding that exposure to diesel exhaust fumes from the combine is the most important source of olive skin contamination, since the highest values of PAH were found in the olives harvested mechanically. The influence of the olive washing step, micronized talc (hydrated magnesium silicate) addition during oil extraction, and the environmental pollution at the mill site, were also assessed. The first two factors had no influence on PAH content of VOO, while the latter depends on several other issues, such as tank cleaning, installation of valves in the ventilation shafts to avoid intake of air pollution and the possibility of burning waste olive pomace in the facilities. According to the SCOOP task report of the European Commission (2004) [21] , from the 671 virgin and extra virgin olive oils (EVOO) analyzed, only 14 presented BaP levels above the maximum imposed by the EU (2 μg/kg), from which two samples presented levels between 5–20 μg/kg and one was above 20 μg/kg.

Sources of Contamination of Olive Pomace Oil

With the by-products obtained during olive oil production, other low-quality oils, such as olive pomace oil (OPO), can be produced. In olive pomace oil production, the dregs of crushed olives are dried and then extracted with organic solvents. For direct human consumption, this oil needs a refining process to remove unwanted minor components and undesirable organoleptic properties. Along olive pomace oil production, PAH contamination can occur during pomace drying and solvent extraction steps. [22] Sometimes, the olive pomace is dried by direct contact with combustion fumes. In such cases, the extent of PAH contamination is related to the type of fuel used and the exposure time necessary to eliminate water. [23] Thus, PAH content depends highly on the conditions used prior to the oil extraction process, and can be relatively high especially if harsh conditions are used. Nevertheless, PAH content is generally reduced during refining, either by the bleaching step, where the use of activated carbon and clay is recommended to remove the heaviest PAH, either by the deodorizing stage, where light PAH can be reduced together with other compounds, such as carotenoid pigments. The efficacy of the refining process can depend on the quality of the initial crude material, i.e., of the initial levels of PAH in the unrefined oil. A detailed study on the efficiency of the bleaching stage for the elimination of BaP in olive pomace oil was performed. These authors reported a slight reduction of BaP to values above the legislation limits (2 μg /kg) using earths in the bleaching stage, making necessary the use of active carbon in this step. Different procedures used along the refining process can possibly explain some differences in the values found in olive pomace oil. In fact, for a group of ten refined olive pomace oil samples, lower levels of PAH were reported in the five samples submitted to decoloring during refining. [24]

Occurrence of PAH in other vegetable oils

Due to their lipophilic nature, PAHs can easily contaminate oils and fats, which are a significant dietary source, either directly or indirectly by their incorporation into other foods such as cereal-based products.[25] Two main routes of PAH contamination in vegetable oils have been suggested, namely the contact of seeds with polluted surroundings and the drying process of oil seeds prior to oil extraction, by direct contact with combustion gases. Another reported form of contamination may arise from direct migration of PAH to the oil seeds from jute bags treated with mineral oils, which are used for raw material storing and transporting. [26]

Influence of Refining

PAH contamination in crude edible oils varies widely, but refined vegetable oils generally present lower levels than the crude oils, which can be attributed, at least in part, to the reduction observed through refining. [27] The influence of different steps during the refining process (neutralization, bleaching and deodorization) on raw soybean and sunflower oils PAH content was evaluated. The authors observed an evident decrease of PAH content, especially light PAH. After refining, total PAH decreased of 72% for sunflower oil and 87% for soybean oil. In both cases, the decrease of light PAH (71% and 88% for sunflower and soybean oils, respectively) was significantly higher than the decrease of heavy PAH (79% and 49% for sunflower and soybean oils, respectively) (Table 7). Regarding the different steps along refining, deodorization seems to have higher impact on decreasing total PAH levels, which agrees with other works. [28] Moreover, the deodorization process seems to have little effect on heavy PAH removing mainly light PAH, while higher condensed heavy PAH are mainly removed by activated charcoal treatment. The kind of treatment used during the bleaching step seems to be of major importance. In some works, an increase in light PAH content was observed after bleaching, as associated with the use of contaminated clay. On the other hand, a greater reduction in heavy PAH content can be achieved when using activated charcoal compared to activated earth or clay.

PAH content ($\mu g\,kg^{-1}$) during vegetable oil refining

	Sunflower oil				Soybean oil			
	Crude	Neutralized	Bleached	Deodorized	Crude	Neutralized	Bleached	Deodorized
Light PAH	15.59	11.06	7.50	4.53	63.59	42.56	43.45	7.79
Heavy PAH	1.77	0.73	0.46	0.37	1.74	1.60	1.25	0.89
Total PAH	17.36	11.80	7.96	4.90	65.33	44.16	44.71	8.67
Total % of reduction	–	32.0%	54.1%	71.8%	–	32.4%	31.5%	86.7%

Table 7. PAH content expressed in µg/kg during vegetable oil refining.

2.2 Cocoa

The manufacturing process of chocolate starts with the harvest of the ripe cocoa pods, culling of the cocoa beans from the pods and fermenting them. Afterwards the cocoa beans are dried, roasted, and winnowed. The collected cocoa nibs are alkalised and ground. Then the resulting cocoa liquor is blended with the other ingredients such as cocoa butter, sugar, milk, or emulsifiers and mechanically treated until the desired texture is reached. Conching, another mechanical treatment, and tempering follow next. Finally, the chocolate is moulded and packed. Within this manufacturing process are some critical steps during which cocoa and as a consequence chocolate may be contaminated with polycyclic aromatic hydrocarbons. The most critical step is drying of the cocoa seeds in their respective country of origin. Cocoa butter might contain higher levels of PAH than other oils and fats *. This is mainly due to inappropriate drying practices of the cocoa beans and the fact that cocoa butter cannot be refined as is done with other vegetable oils and fats. Cocoa butter is a main constituent of raw cocoa products (e.g. cocoa beans, cocoa mass, cocoa nibs, or cocoa liquor) and is present in chocolate and other cocoa products often consumed by children. Therefore maximum levels for PAH in cocoa beans and derived products were established on a fat basis since PAH concentrate in the fat fraction, the cocoa butter (Commission Regulation (EC) No 835/2011). It shall be mentioned that cocoa beans and derived products is the only food category for which maximum levels for polycyclic aromatic hydrocarbons (PAHs) are based on the fat fraction.

2.3 Early Childhood Products

Infants and young children are regarded as two particularly vulnerable groups in terms of food safety. These groups may be especially vulnerable to high exposures from contaminants at important stages of development. It is therefore essential that products intended for use by infants and young children must not contain contaminants in amounts that could lead to negative health effects. This is why it is important to monitor the level of PAHs in baby food. Most of the foods analyzed are cereal-based foods. The presence of polycyclic aromatic hydrocarbons in cereal-based foods is the result of the method of food preparation (e.g. roasting or drying) and the bioaccumulation in oil seeds. With regard to regulation, the lowest maximum levels are set for food for infants and young children. The carcinogenic and mutagenic activity of PAHs and the low legal limit of these compounds in baby food (controlled by EU Regulation 835/2011, setting a maximum of 1 ng/g for the total of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene) make selective the extraction and efficient development of the sample preparation method important and having higher priority in working with baby food, since PAHs can have a greater effect on children.

3. Materials and Methods

3.1 Preparation of the sample

In 1987, at the ARPAM (Regional Agency for Environmental Protection of the Marche) in the Department of Ascoli Piceno, by examining with Wood lamp a sample of locally produced extra virgin olive oil obtained from olives grown in areas exposed to PAHs of industrial origin, a light celestial coloring was noted (central sample in the Figure 15) instead of the characteristic brick red color. It was soon discovered that the abnormal coloring was due to the presence of significant amounts of PAH in the oil. [29]

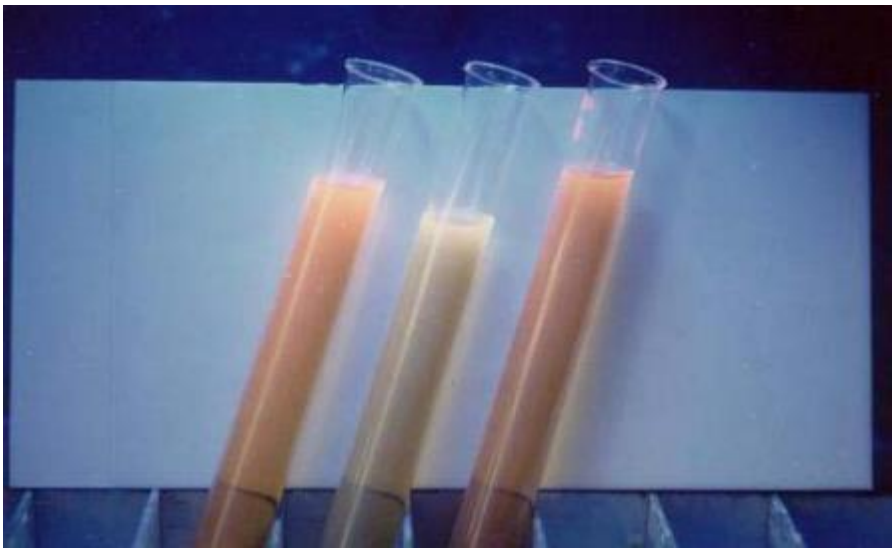


Figure 15. Three samples of extra virgin olive oil under Wood lamp.

Starting from the data obtained from the scientific literature, the following analytical procedure was developed over a period of a few months. This analytical procedure was validated by the "Istituto Superiore di Sanità" (ISS) through an external quality control ring test organized and managed by the ISS itself. The outcome of the test was more than satisfactory and the laboratory of Ascoli Piceno was enabled and encouraged to carry out the determination of PAHs in olive oil with this analytical procedure. The strong point is that a mixture of almost pure PAHs is obtained, so that, depending on the needs, it is possible to resort to different instrumental determinations, such as liquid-liquid chromatography (HPLC) with fluorophotometric detector as well as gas chromatography, to further lower the limit of determination of the analytical method.

3.1.1 Extraction

Ten grams of oil are weighed and dissolved in 25 ml of pentane. 5 µl of benzo[*a*]anthracene D12 solution at a concentration of 5 mg/l and 15 ml of DMSO are added to the pentane solution. Dimethylsulfoxide is a substance with electronic doublets and electrons π, so it is also very similar to PAHs. For this reason, DMSO is a specific solvent for PAHs and it allows the separation of aliphatic hydrocarbons from aromatic hydrocarbons and the latter from triglycerides found in vegetable oils and fats, resulting in a considerable advantage in subsequent instrumental determination. The extraction is performed by stirring in a 100 ml separating funnel for about 30 seconds. The clear DMSO solution, stratified in the lower part, drains into a 250 ml separating funnel with a ground necked flask. The extraction is repeated twice with 10 ml portions each of DMSO. To the portions of DMSO combined in a separating funnel, 75 ml of distilled water are added and three times extracted with 50 ml of cyclohexane each. The water complexes the DMSO and binds it more strongly than the PAHs. For this reason the solubility of PAHs in DMSO will decrease and will be moved to the cyclohexane phase. The cyclohexane solutions, which are also combined in a 250 ml separating funnel with a ground necked flask, are washed with 100 ml of distilled water and stirred to prevent the formation of emulsions. The aqueous solution below is eliminated, and the cyclohexane solution is filtered on anhydrous sodium sulfate placed in a funnel with glass wool and collected in a flask with a ground necked flask. Two washes of the anhydrous sodium sulfate were carried out with 10 ml each of cyclohexane and the cyclohexane solution obtained was evaporated through rotavapor at the temperature of 40°C. The solution is transferred to a vial, washing the sides of the flask twice and the solution obtained is evaporated at about 100 µl under the flow of nitrogen.

3.1.2 Purification

The sample is completely seeded on the starting line of a TLC plate. With a pencil we draw two parallel lines distant from the edge 1.5 cm (starting or sowing line of the sample) and 13.5 cm (solvent arrival line or end chromatographic runs). The vial washing liquids are deposited above the sample. The plate is placed in a chromatography tank containing toluene-hexane 50:50. The solvent is left to run until the distance of 12 cm is covered. After that the solvent is evaporated and the plate is quickly observed in the light of Wood to delimit the blue spot corresponding to the PAHs (Rf about 0.8). The silica portion corresponding to the PAHs is scraped with a flexible steel spatula on an aluminum sheet. The silica is transferred onto a tube with glass septum and eluted with 1 ml of dichloromethane portions until approximately 3 ml of eluate is obtained. It is evaporated to dryness under nitrogen flow, it is taken up with 100 µl of cyclohexane, 5 µl of perylene D12 solution at a concentration of 5 mg/l is added and the sample is ready for the gas chromatographic determination.

3.1.3 Instrumental analysis

The analysis is performed by a gas chromatograph (GC) Agilent Technologies 7890A. The gas chromatograph is combined with a Mass Selective Detector (MSD) that is 5975C (Agilent Technologies) as shown in Figure 16.

Figure 16. Agilent Technologies 7890A Gas Chromatograph coupled with a Mass Selective Detector (MSD), 5975C (Agilent Technologies).

- ***Inlet:*** PTV Inlet

	Rate (°C/min)	Value (°C)	Hold time (min)
Initial		45	0,43
Ramp1	720	320	5
Ramp2	10	200	0

- ***Mode:*** *Solvent split*
  Split on: 50 ml/min from 0 until 0.4 min
  Split off: From 0.4 min to 3 min
  Purge flow to split vent
  Split on: 100 ml/min at 3 min

- ***Flow gas saver:*** 20 ml/min after 10 min

- ***Column:*** RXi – 5ms, Varian CP8982VF – 17MS, 330°C, 60 m * 250 µm * 0.25 µm (Low-polarity phase; Crossbond diphenyl dimethyl polysiloxane)
  Flow: 2 ml/min

- ***Oven:***

	Rate (°C/min)	Value (°C)	Hold time (min)
Initial		45	4
Ramp1	20	180	5
Ramp2	8	320	20.75

- ***Total time of analysis:*** 54 min

- ***Detector:*** Mass Selective Detector MSD 5975C (Agilent Techonologies)
  Acquisition Mode: Scan
  Solvent delay: 10.0 min
  Scan parameters:
  Start time: 10.0
  Low Mass: 50.0
  High Mass: 550.0
  Temperatures MSD:
  Setpoint Quad: 150
  Setpoint Source: 230

3.1.3.1 Calibration of the instrument

The calibration protocol consists of the production of calibration solutions containing variable amounts of the standard analyte and the same amount of internal standard. The linear calibration model was obtained with the least squares method, and it is expressed by the equation Y = aX + b, where Y is the ratio of the areas of the analyte's chromatographic peaks and the internal standard, obtained from the analysis GC MS, and X is the ratio of the masses of the analyte and the internal standard in the solution. The gas chromatograph calibration is performed by constructing a calibration line. For this purpose, 5 solutions are prepared at the following concentrations:

25 µg/l – 50 µg/l – 100 µg/l – 250 µg/l – 500 µg/l

The solutions used for the construction of the calibration curve are obtained starting from certified stock solution (MIX PAHs) available on the market. PAHs mixture was purchased from UltraScientific. The stock solution was prepared as mentioned in the paragraph 3.2 at a concentration of 1 mg/l. To these solutions are added two internal standards at a concentration of 5 mg/l to obtain a final volume of 1 ml at a final concentration of a 0.25 mg/l. The surrogate standard used is deuterated benzo(a)anthracene and the internal standard used is deuterated perylene.
The calibration levels correspond respectively to concentrations in the actual oil samples:

25 µg/kg – 50 µg/kg – 100 µg/kg – 250 µg/kg – 500 µg/kg

For each concentration level, at least three replicates are injected into the gas chromatograph and for each analyte the calibration curve is constructed with the response factors (single PAH area / internal standard area) as a function of the ratio between the concentration of each analyte and that of the internal standard. The 5 solutions at different concentration levels have been prepared as follows:

- 25 µl MIX PAH + 50 µl Benzo(a)Anthracene D12 + 50 µl Perylene D12 + 875 µl cyclohexane
- 50 µl MIX PAH + 50 µl Benzo(a)Anthracene D12 + 50 µl Perylene D12 + 850 µl cyclohexane
- 100 µl MIX PAH + 50 µl Benzo(a)Anthracene D12 + 50 µl Perylene D12 + 800 µl cyclohexane
- 250 µl MIX PAH + 50 µl Benzo(a)Anthracene D12 + 50 µl Perylene D12 + 650 µl cyclohexane
- 500 µl MIX PAH + 50 µl Benzo(a)Anthracene D12 + 50 µl Perylene D12 + 400 µl cyclohexane

Calibration solutions are injected and the chromatogram is recorded. The chromatogram of a solution 250 µg/kg is shown below (Figure 17).

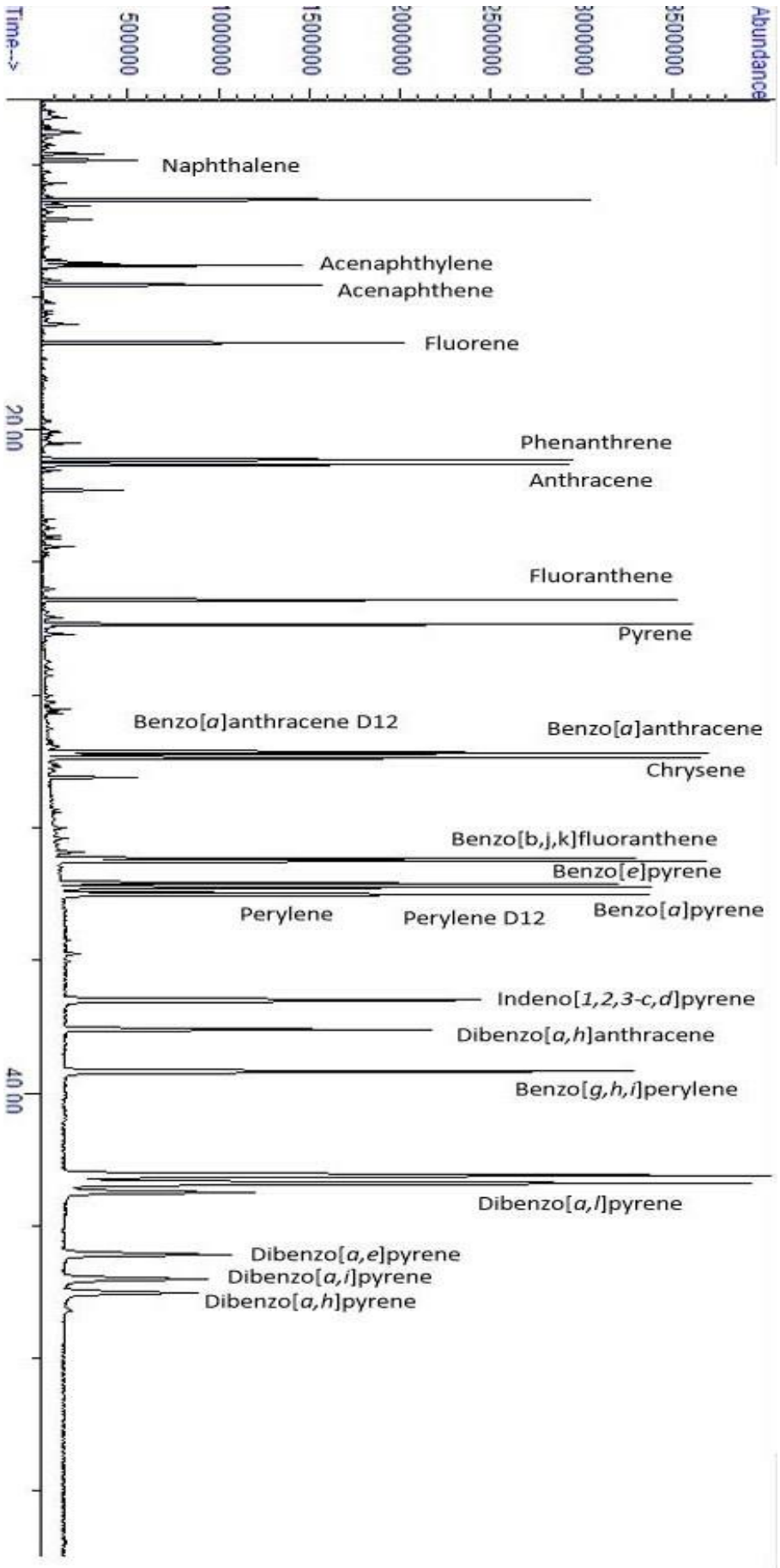


Figure 17. Chromatogram of a solution of PAHs at 250 µg/kg.

3.2 Extraction from cocoa

This method is used if the percentage of fat in cocoa is less than 50%. Five grams of cocoa powder are weighed and 5 µl of benzo[*a*]anthracene D12 solution at a concentration of 5 mg/l are added. Three extractions are carried out with dichloromethane with volumes of 50 ml, 40 ml and 40 ml, respectively. The dichloromethane solution obtained was evaporated through rotavapor at the temperature of 40°C to about 10 ml. At this point we can proceed with the DMSO procedure.

3.3 Extraction from childhood products

Five grams of sample are weighed and 5 µl of benzo[*a*]anthracene D12 solution at a concentration of 5 mg/l are added. Three extractions are carried out with dichloromethane with volumes of 10 ml. The final solution is collected in a 40 ml vial. The sample is sonicated for twenty minutes with a closed cap and then it is filtered on sintered septa containing anhydrous sodium sulfate. At last the sample is completely seeded on a TLC plate.

3.4 Samples collection

In this thesis work, a total of 59 samples were analyzed: 37 samples of oil, 7 samples of cocoa and 15 samples of products for children. The analyzed oil samples can be divided into:

- 15 sunflower oils
- 15 extra virgin olive oils
- 4 pomace oils
- 2 exhausted olive oil

The commercial samples were taken from the territorial ASUR inspectors of the region Marche and from the command for the protection of public health ("Comando per la Tutela della Salute Pubblica, N.A.S di Ancona"). The tables below summarize the data relating to the samples analyzed (Tables 7,8,9,10 and 11): sample number, sample type and territorial origin.

Sunflower Oil				
Sample Number	**Sample Type**	**Territorial Origin**	**Withdraw area**	**Manufacturer**
1	Commercial	Macerata	Supermercato Clagor Srl (MC)	Oleificio Zucchi Spa
2	Commercial	Ascoli Piceno	Stella Srl	OLITALIA Srl - Forlì
3	Commercial	San Benedetto del Tronto	M&T (Discount) - SBT	MIRA SUD Srl - Napoli
4	Commercial	Macerata	Supermercato Simply - Loro Piceno	OLITALIA Srl - Forlì
5	Commercial	Jesi	Supermercato EUROSPIN - Jesi	Eurospin Spa - Verona
6	Commercial	Macerata	Supermercato Camilletti	OLITALIA Srl - Forlì
7	Commercial	Fabriano	Supermercato 40	Bunge Italia Spa - Ravenna
8	Commercial	Ascoli Piceno	Supermercato Conad	Stab. Montesarchio (BN)
9	Commercial	Ancona	SMA Spa - Osimo	Oleificio Zucchi Spa
10	Commercial	Fano	ARCA Spa	Stab. Montesarchio (BN)
11	Commercial	San Benedetto del Tronto	Supermercato LIDL	LIDL Italia Srl
12	Commercial	Pesaro	Supermercato LIDL	LIDL Italia Srl
13	Commercial	Senigallia	SMA - Senigallia	Oleificio Zucchi Spa
14	Commercial	Urbino	Macelleria CAM	SECAM Spa Segrate (MI)
15	Commercial	Ascoli Piceno	Stella Srl	OLITALIA Srl - Forlì

Table 7. Samples of sunflower oil.

Extra Virgin Olive Oil				
Sample Number	**Sample Type**	**Territorial Origin**	**Withdraw area**	**Manufacturer**
1	Commercial	Macerata	Supermercato Camilletti	Frantonio La Rocca
2	Commercial	Fabriano	Supermercato 40	Carapelli Firenze
3	Commercial	San Benedetto del Tronto	Supermercato LIDL	LIDL Italia Srl
4	Commercial	Macerata	Supermercato Camilletti	Paradiso Coppini
5	Private	Ascoli Piceno	N.D.	N.D.
6	Commercial	Jesi	F.lli Marchigiani Srl	F.lli Marchigiani Srl
7	Commercial	Ascoli Pieno	Frantoio Agostini	Agostini Snc
8	Commercial	Ascoli Piceno	Oleificio Alessandrini	Oleificio Alessandrini
9	Commercial	Ascoli Piceno	Supermercato LIDL	Cavanna Oli Srl
10	Commercial	Ancona	Frantoio Fuselli e Graziani - Recanati	Frantonio Fuselli e Graziani Snc
11	Commercial	Ancona	Villa Igea	Coppini Angelo Spa
12	Commercial	Ancona	Frantoio Fuselli e Graziani - Recanati	Frantonio Fuselli e Graziani Snc
13	Commercial	Ancona	Frantoio Fuselli e Graziani - Recanati	Frantonio Fuselli e Graziani Snc
14	Commercial	Ancona	Frantoio Olive Contardi	Frantoio Olive Contardi
15	Commercial	San Benedetto del Tronto	Supermercato LIDL	LIDL Italia Srl

Table 8. Samples of extra virgin olive oil.

Pomace Olive Oil				
Sample Number	**Sample Type**	**Territorial Origin**	**Withdraw area**	**Manufacturer**
1	Commercial	Ancona	SMA Spa - Osimo	Oleificio Zucchi Spa
2	Commercial	Macerata	Supermercato Simply - Loro Piceno	OLITALIA Srl - Forlì
3	Commercial	Fano	Supermercato Conad	OT Srl - Forlì
4	Commercial	Urbino	Supermercato Conad	OIT Srl

Table 9. Samples of pomace olive oil.

Cocoa				
Sample Number	**Sample Type**	**Territorial Origin**	**Withdraw area**	**Manufacturer**
1	Commercial	Urbino	Supermercato RAC	Nestlè Italia Spa
2	Commercial	Ascoli Piceno	Demetra Srl - Colli del Tronto	Alce Nero Spa - Bologna
3	Commercial	San Benedetto del Tronto	Supermercato LIDL	LIDL Italia Srl
4	Commercial	Urbino	Supermercato Conad	Nestlè Italia Spa
5	Commercial	Ascoli Piceno	Coop "Città delle stelle"	Coop Italia
6	Commercial	Macerata	Supermercato Simply	SMA Spa
7	Commercial	Ancona	Leonardi Dolciumi	ELAH Dufour Spa

Table 10. Samples of cocoa.

Childhood products				
Sample Number	**Sample Type**	**Territorial Origin**	**Withdraw area**	**Manufacturer**
1	Commercial	Macerata	Supermercato Eurospin	Eurospin Italia Spa (VR)
2	Commercial	Grottammare	Angelici Srl	Humana - Milano
3	Commercial	San Benedetto del Tronto	Coccinella Snc	Prime pappe
4	Commercial	Senigallia	Supermercato LIDL Italia Srl	Plasmon - Latina
5	Commercial	Ascoli Piceno	Ophishop	Plasmon - Latina
6	Commercial	Urbino	Supermercato Conad	Stabilimento Carlo Erba
7	Commercial	Macerata	Supermercato Tigre	Mellin Spa - Milano
8	Commercial	San Benedetto del Tronto	GMF	Plasmon - Latina
9	Commercial	Fermo	Farmacia Lucia Pompei	Hipp e C
10	Commercial	Ancona	SMA Spa	Plasmon - Latina
11	Commercial	Senigallia	Punto vendita Ninna Nanna	Plasmon - Latina
12	Commercial	Ascoli Piceno	Supermercato Maxi Coal	Plasmon - Latina
13	Commercial	Fano	Supermercato Conad	Mellin Spa - Milano
14	Commercial	Pesaro	Eurospin Tirrenica Spa	GEOVITA Srl (AT)
15	Commercial	Urbino	Supermercato Conad	Mellin Spa - Milano

Table 11. Samples of childhood products.

3.5 Reagents and standards

The table below (Table 12) shows all the reagents used with the relative manufacturers and the percentage of purity.

Reagents and solvents	Manufacturers	Purity
Pentane	Carlo Erba	99%
Dimethylsulfoxide (DMSO)	Bisolve BV	99,90%
Distilled water	-	-
Cyclohexane	VWR International	99,90%
Sodium sulfate	Emsure	-
Glass wool	-	-
Acetone	VWR International	99,90%
Toluene	Sigma Aldrich	99,80%
Dichloromethane	VWR International	100%
Hexane	Panreac	98,50%
Glass plates	Analytical Chromatography	-

Table 12. Reagent used with the relative manufacturers and the percentage of purity.

Furthermore, the table below (Table 13) shows the composition of the PAH mixture used. PAHs mixture and internal standards (Benzo[*a*]anthracene D12 and Perylene D12) were purchased from UltraScientific. The true value and uncertainty value at the 95% confidence level for each analyte, determined gravimetrically, is listed below too.

PAH Mixture	
Analyte	**True Value**
Naphthalene	100,02 ± 0,5 µg/ml
Acenaphthylene	100,02 ± 0,5 µg/ml
Acenaphthene	100,03 ± 0,5 µg/ml
Fluorene	100,03 ± 0,5 µg/ml
Phenanthrene	100,02 ± 0,5 µg/ml
Anthracene	100,02 ± 0,5 µg/ml
Fluoranthene	100,02 ± 0,5 µg/ml
Pyrene	100,02 ± 0,5 µg/ml
Benzo[a]anthracene	100,03 ± 0,5 µg/ml
Chrysene	100,02 ± 0,5 µg/ml
Benzo[b]fluoranthene	100,02 ± 0,5 µg/ml
Benzo[k]fluoranthene	100,02 ± 0,5 µg/ml
Benzo[e]pyrene	100,02 ± 0,5 µg/ml
Benzo[a]pyrene	100,03 ± 0,5 µg/ml
Perylene	100,03 ± 0,5 µg/ml
Indeno[1,2,3-cd]pyrene	100,03 ± 0,5 µg/ml
Benzo[ghi]prylene	100,03 ± 0,5 µg/ml
Dibenzo[a,h]anthracene	100,03 ± 0,5 µg/ml
Dibenzo[a,l]pyrene	100,0 ± 0,5 µg/ml
Dibenzo[a,e]pyrene	100,0 ± 0,5 µg/ml
Dibenzo[a,i]pyrene	100,4 ± 0,5 µg/ml
Dibenzo[a,h]pyrene	100,4 ± 0,5 µg/ml
Benzo[j]fluoranthene	100,0 ± 0,5 µg/ml

Table 13. Composition of the PAH mixture used.

4. Results and Discussion

4.1 Method validation

This thesis work also concerned the validation of the test method for the research of PAHs in oil. The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. In this regard, the laboratory must use test methods and procedures defined by rules, techniques or official methods in force. The current legislation on foodstuffs requires control units to use accredited testing methods. The ARPAM laboratory in Ascoli Piceno is accredited by Accredia (Ente Italiano di Accreditamento) and in relation to the oils and fats matrix, the accredited methods are:

- Alkylesters

  (Reg CEE 2568/1991 11/07/1991 GU CEE n. L248 05/09/1991 All XX Reg CE 61/2011 24/01/2011 GU CE L23/1 27/01/2011)

- Free fatty acids

  (Reg CEE 256/1991 11/07/1991 GU CEE n. L28 05/09/1991 All II Reg CE 702/2007 21/06/2007 GU CE L 161 22/06/2007)

- Polycyclic aromatic hydrocarbons

  (MIP-AP-01 rev. 6 2015)

The method was validated by determining the following parameters:

- Selectivity
- Linearity
- Recovery
- Repeatibility
- Limits of quantification (LOQ)
- Uncertainty of measurement by Horwitz

4.1.1 Selectivity

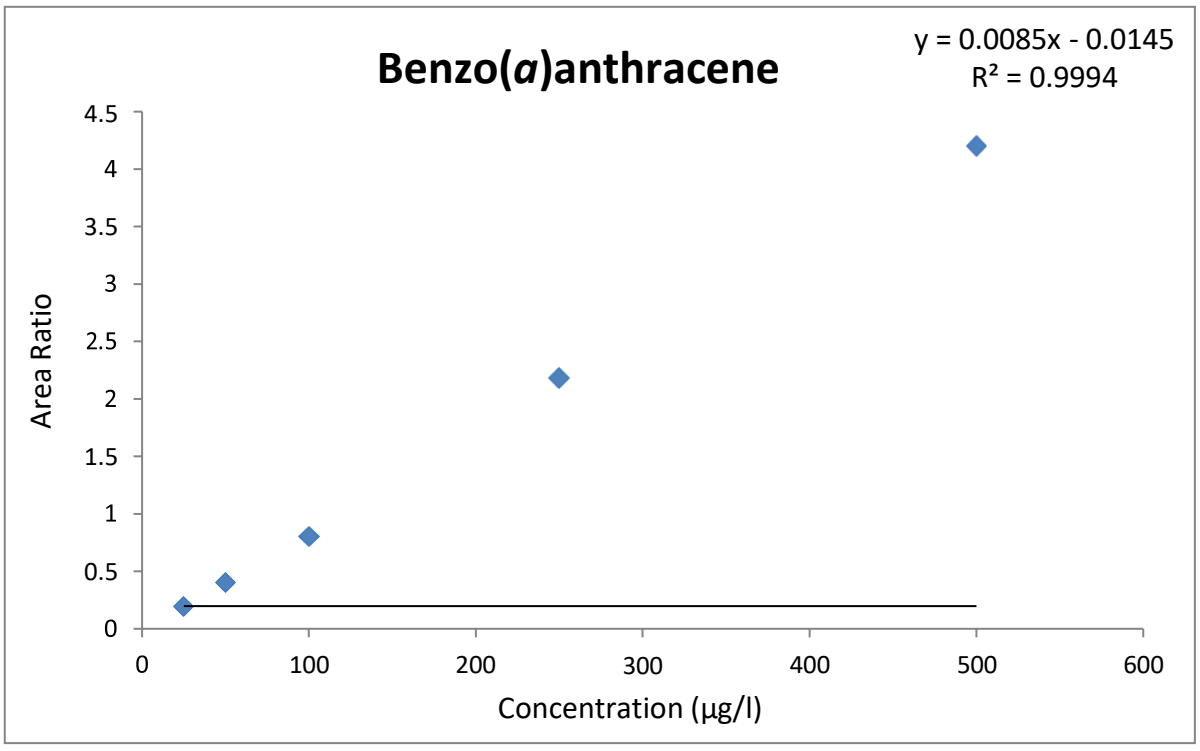
Selectivity is a parameter that can't be estimated numerically, but it is evaluated on the basis of the probability that the result of the test may be interfered by different origins; it must be established that the signal produced during the measurement phase can be uniquely ascribed to the analyte of interest. The analytical procedure used provides a purification phase in order to avoid false positives and false negatives and therefore to eliminate the interference. Moreover, the latter are attenuated with the use of the internal standard and deuterated surrogate standards. Finally, in chromatography to improve selectivity, the system can be coupled with a mass spectrometer. In fact, the MS detector allows us to extract the ions of interest.

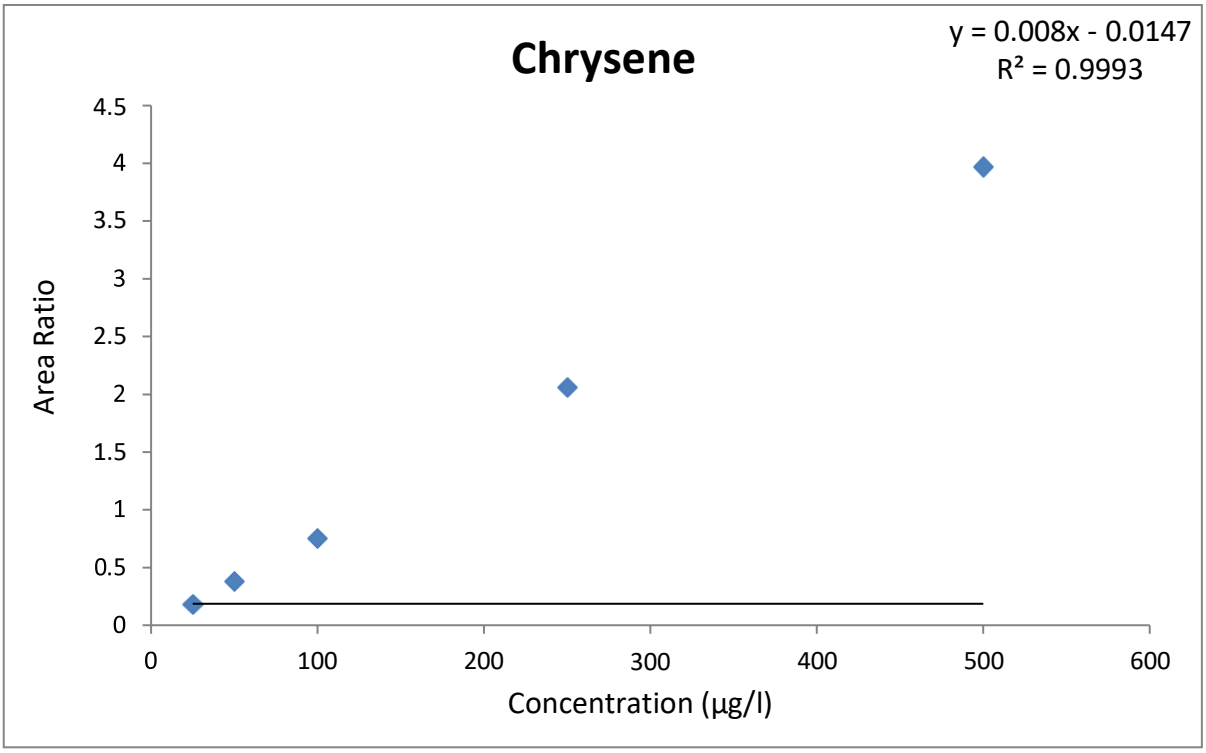
4.1.2 Linearity

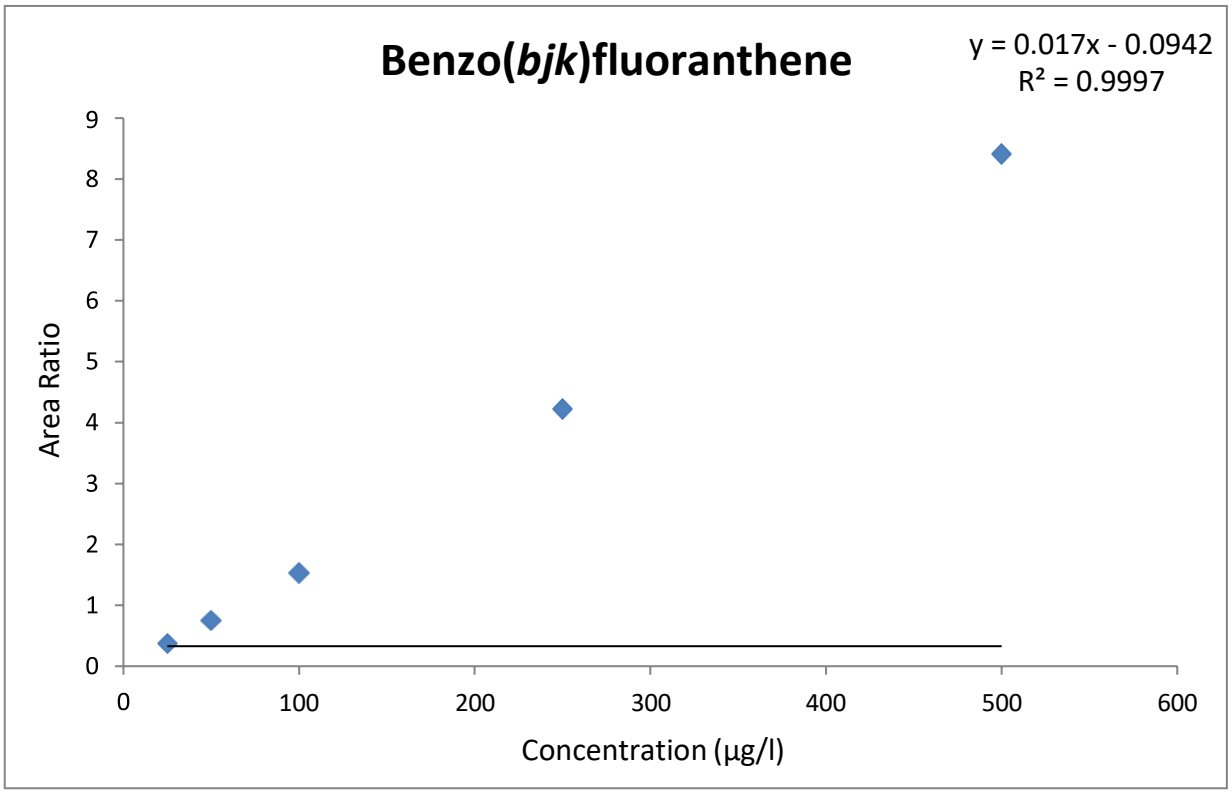
The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The width of the linearity interval was calculated in relation to the working interval (the measurement range of the laboratory-validated method goes from 25 µg/kg to 500 µg/kg) in 5 concentration levels. The preparation of the standards used for this purpose and the specifications of the calibration curve have already been described in the paragraph 3.1.3.1 (Calibration of the instrument).

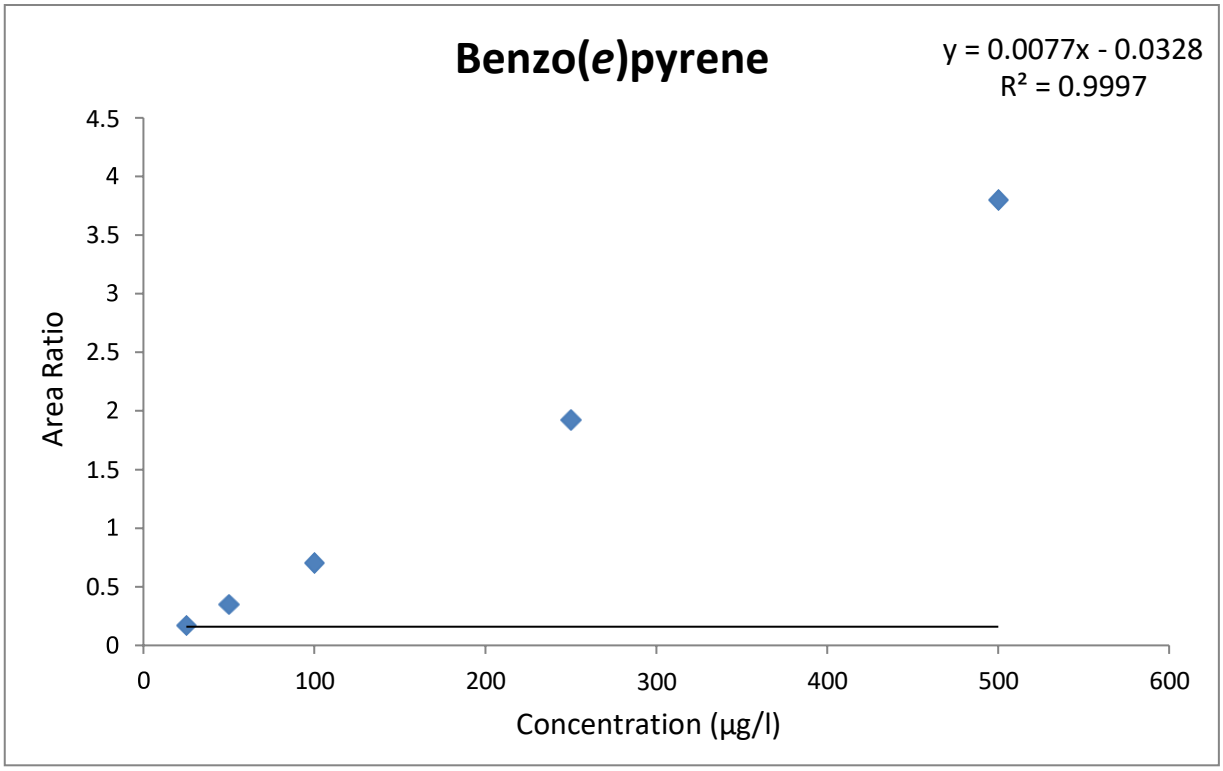
	STD 1	STD 2	STD 3	STD 4	STD 5
PAHs concentration in the standard solutions (µg/l)	25	50	100	250	500
Corresponding PAHs concentration in the sample (µg/kg)	0,25	0,5	1	2,5	5

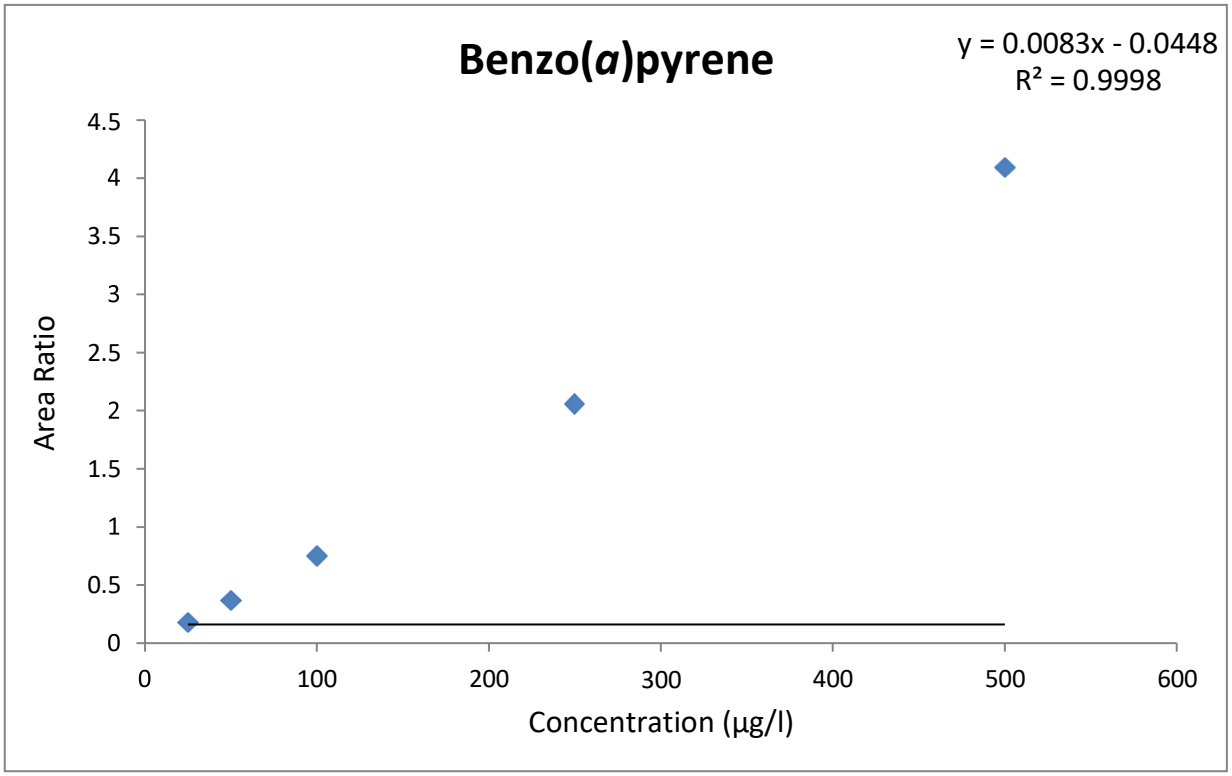
For each level, three replicates are injected and the line of response factors is constructed as a function of the concentrations for each analyte. The criteria of acceptability of the calibration curve have been established as the coefficient of determination $r^2 > 0.99$. The calibration lines are shown in Figure 18.

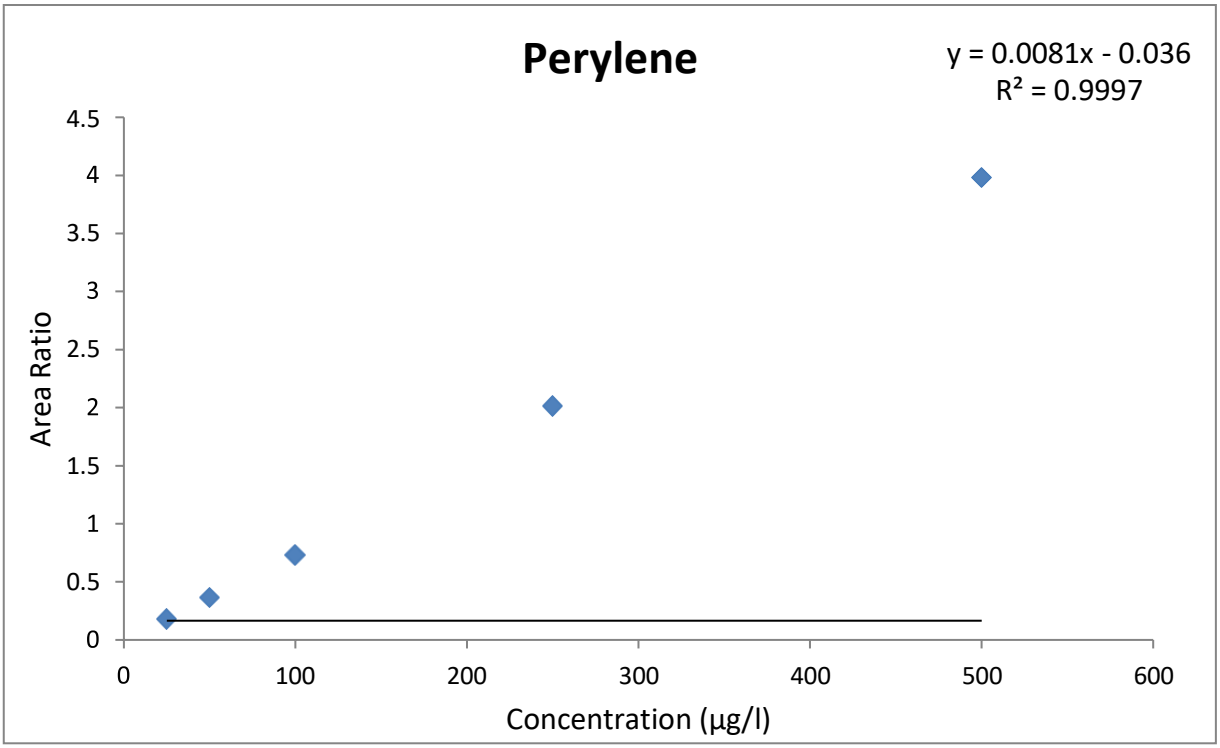


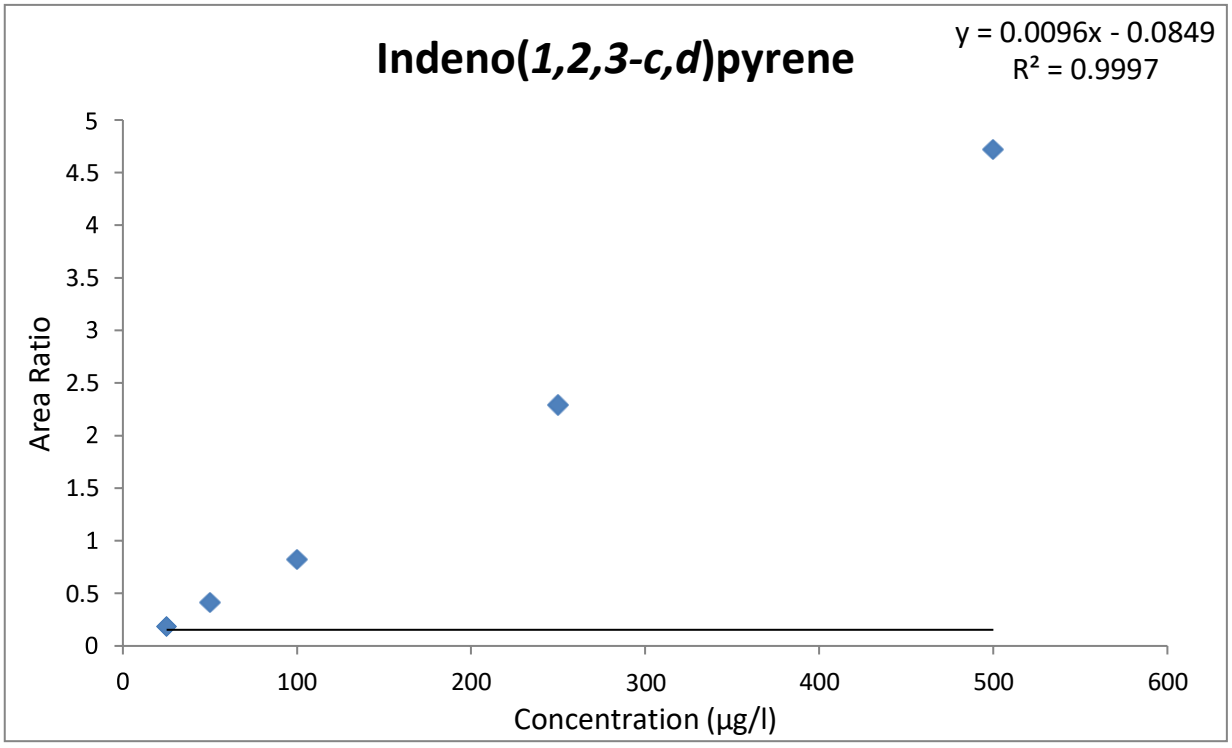

Chrysene
y = 0.008x - 0.0147
R² = 0.9993
Area Ratio
Concentration (µg/l)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
100
200
300
400
500
600

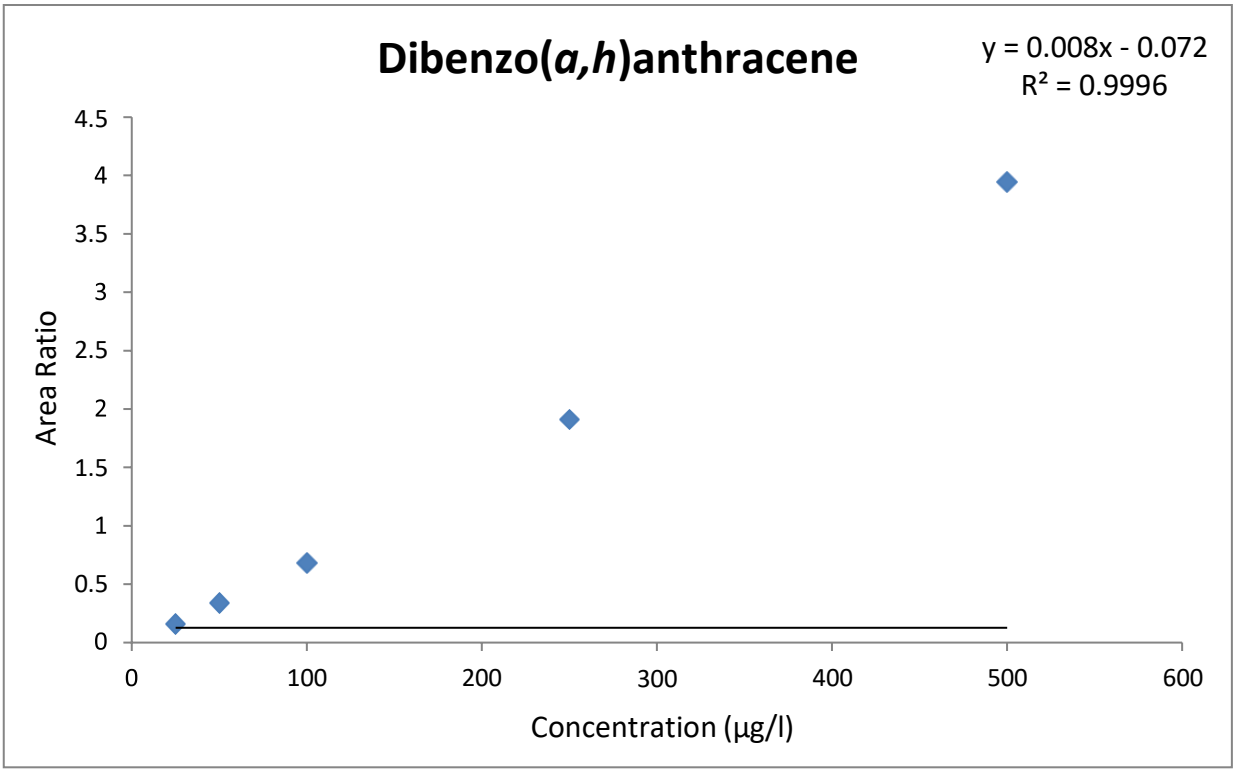

Benzo(*bjk*)fluoranthene
y = 0.017x - 0.0942
R² = 0.9997
Area Ratio
Concentration (µg/l)
0
1
2
3
4
5
6
7
8
9
0
100
200
300
400
500
600


Benzo(e)pyrene
y = 0.0077x - 0.0328
R² = 0.9997
Area Ratio
Concentration (µg/l)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
100
200
300
400
500
600


Benzo(a)pyrene
y = 0.0083x - 0.0448
R² = 0.9998
Area Ratio
Concentration (µg/l)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
100
200
300
400
500
600


Perylene
y = 0.0081x - 0.036
R² = 0.9997
Area Ratio
Concentration (µg/l)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
100
200
300
400
500
600


Indeno(1,2,3-c,d)pyrene
y = 0.0096x - 0.0849
R² = 0.9997
Area Ratio
Concentration (µg/l)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
100
200
300
400
500
600



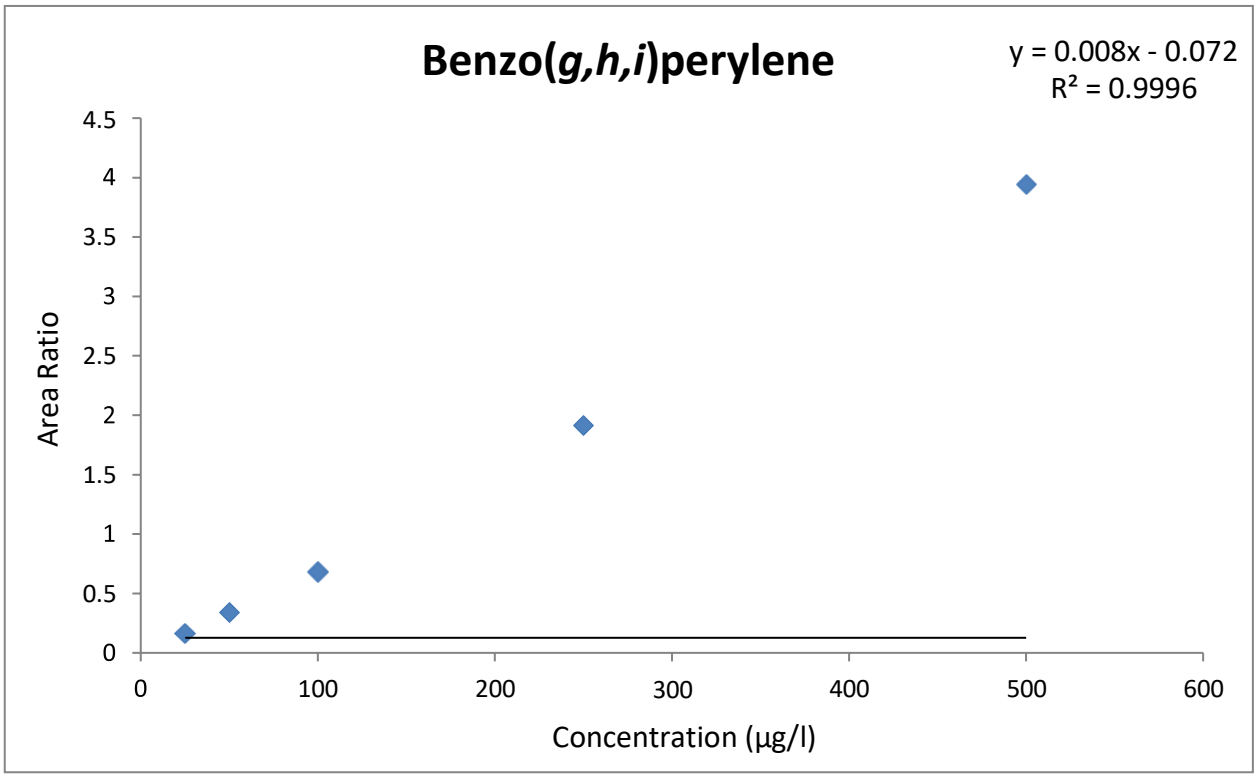


Figure 18. Calibration lines.

The linearity, evaluated by the linear regression coefficient (R^2), is very good for all the standard species in the studied ranges.

4.1.3 Recovery

The recovery test on all PAHs is performed during the validation of the method by adding to the real samples a known quantity of analytes and applying the normal chemical analysis procedure. For this scope, a sunflower oil poor in PAHs is analyzed. Nine replicas for each concentration level (LOQ 0.25 µg/kg, 1 µg/kg, 4 µg/kg) were made. The efficiency of the recovery of each PAH must respect the ranges indicated in the following table (Table 14):

Concentration	Range of recovery
< 1 µg/kg	50-120%
From 1 to 10 µg/kg	70-110%
> 10 µg/kg	80-110%

Table 14. Range of recovery for each PAH.

These criteria have been chosen keeping in mind the Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

For the first concentration level, it was added to the real sample 25 µl of MIX IPA and it was applied the normal chemical analysis procedure. For this reason we expected to find the same amount for each PAH at the end of the analytical procedure. The recovery should be between 50 and 120%. The results are shown in Table 15.

I Level (0,25 µg/kg)	B[*a*]A	Cr	B(*bjk*)F	B[*a*]P	IP	D(*a,h*)A	B(*g,h,i*)P
Replicate 1	0,27	0,29	0,51	0,28	0,24	0,21	0,29
Replicate 2	0,21	0,25	0,57	0,26	0,22	0,17	0,27
Replicate 3	0,29	0,31	0,5	0,25	0,21	0,18	0,29
Replicate 4	0,26	0,28	0,55	0,26	0,2	0,16	0,28
Replicate 5	0,26	0,29	0,49	0,25	0,18	0,18	0,3
Replicate 6	0,25	0,3	0,47	0,23	0,17	0,19	0,3
Replicate 7	0,26	0,3	0,49	0,23	0,18	0,2	0,3
Replicate 8	0,22	0,26	0,46	0,22	0,18	0,21	0,28
Replicate 9	0,27	0,29	0,57	0,28	0,24	0,2	0,3
Average	0,254	0,286	0,512	0,251	0,202	0,188	0,29
Standard Deviation	0,0251	0,0194	0,0415	0,0215	0,0266	0,0176	0,0112
CV%	9,8	6,8	8,1	8,6	13,3	9,34	3,9
Repeatibility limit	0,08171	0,06339	0,13523	0,07003	0,08747	0,10581	0,03646
Recovery %	102	114	102	100	81	76	116

Table 15. Results obtained for the first concentration level.

For the second concentration level, it was added to the real sample 100 µl of MIX IPA and it was applied the normal chemical analysis procedure. The recovery should be between 70 and 110%. The results are shown in Table 16.

II Level (1 µg/kg)	B[*a*]A	Cr	B(*bjk*)F	B[*a*]P	IP	D(*a,h*)A	B(*g,h,i*)P
Replicate 1	0,95	1,08	1,7	0,84	0,76	0,73	0,93
Replicate 2	0,93	1,08	1,65	0,82	0,74	0,72	0,91
Replicate 3	0,95	1,07	1,66	0,84	0,78	0,73	0,91
Replicate 4	0,92	1,09	1,64	0,77	0,73	0,71	0,87
Replicate 5	0,96	1,09	1,7	0,83	0,77	0,73	0,92
Replicate 6	0,95	1,09	1,64	0,83	0,75	0,72	0,9
Replicate 7	1,06	1,08	1,68	0,82	0,76	0,72	0,9
Replicate 8	0,83	1,08	1,7	0,85	0,77	0,75	0,96
Replicate 9	0,93	1,09	1,64	0,82	0,73	0,72	0,89
Average	0,942	1,083	1,668	0,824	0,754	0,726	0,91
Standard Deviation	0,0589	0,0071	0,0273	0,023	0,0181	0,0113	0,0255
CV%	6,3	0,7	1,6	2,8	2,4	1,6	2,8
Repeatibility limit	0,19209	0,02306	0,08898	0,07492	0,05904	0,03686	0,08314
Recovery %	94	108	83	82	75	73	91

Table 16. Results obtained for the second concentration level.

For the third concentration level, it was added to the real sample 400 µl of MIX IPA and it was applied the normal chemical analysis procedure. The recovery should be between 70 and 110%. The results are shown in Table 17.

III Level (4 µg/kg)	B[*a*]A	Cr	B(*bjk*)F	B[*a*]P	IP	D(*a,h*)A	B(*g,h,i*)P
Replicate 1	4,14	4,29	7,74	3,86	3,38	3,32	3,89
Replicate 2	4,28	4,15	7,99	3,99	3,6	3,59	4,03
Replicate 3	4,29	4,32	7,75	3,83	3,43	3,35	3,85
Replicate 4	4,2	4,38	7,71	3,85	3,47	3,41	3,88
Replicate 5	4,33	4,29	7,92	3,86	3,53	3,44	3,97
Replicate 6	4,04	4,31	7,75	3,83	3,45	3,37	3,94
Replicate 7	4,09	4,16	7,61	3,79	3,31	3,3	3,74
Replicate 8	4,24	4,08	7,98	3,97	3,61	3,6	4,04
Replicate 9	4,25	4,22	7,65	3,8	3,42	3,32	3,81
Average	4,207	4,244	7,789	3,864	3,467	3,411	3,906
Standard Deviation	0,0977	0,0976	0,1401	0,7	0,0989	0,1134	0,0996
CV%	2,3	2,3	1,8	1,8	2,9	3,3	2,6
Repeatibility limit	0,3187	0,31833	0,45699	0,22835	0,32243	0,36984	0,32494
Recovery %	105	106	97	97	87	85	98

Table 17. Results obtained for the third concentration level.

4.1.4 Repeatability

Repeatability was evaluated over the entire measurement range of the method and in particular nine replicates were performed on three concentration levels (LOQ 0.25 µg/kg, 1 µg/kg, 4 µg/kg). The acceptability criterion has been defined in relation to the variation coefficient that should be lower than 20% (CV < 20%) for the maximum acceptable repeatability. it was added to the real sample 25 µl of MIX PAHs and it was applied the normal chemical analysis procedure. This criterion has been chosen keeping in mind the Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Moreover, by means of the standard deviation of the replicas, the repeatability limit of the method was calculated at each level considered through the following formula:

$$= s_r * t * \sqrt{2}^{-}$$

Where:
r = repeatability limit
s_r = standard deviation
t = student variable for 95% confidence

The repeatability of the method is good with results. The latter, reported in terms of Standard Deviation percentage, are shown in Tables 15, 16 and 17 for each concentration level.

4.1.5 Limits of quantification (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The experimental determination of the LOQ was performed by means of repeatability tests at the lowest concentration level. In practice it has been verified that the maximum acceptable repeatability, defined as the coefficient of variation (CV < 20%), was also observed at the limit of quantification. Moreover the recovery must be greater than 70% (Recovery > 70%). Therefore it was added to the real sample 25 µl of MIX IPA and it was applied the normal chemical analysis procedure. Nine replicate were made. These criteria have been chosen keeping in mind the Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC. The results of this verification are shown in the following table (Table 18).

	Values obtained							Expected Value
I Level (0,25 µg/kg)	B[*a*]A	Cr	B(*bjk*)F	B[*a*]P	IP	D(*a,h*)A	B(*g,h,i*)P	Each PAHs
Replicate 1	0,27	0,29	0,51	0,28	0,24	0,21	0,29	0,25
Replicate 2	0,21	0,25	0,57	0,26	0,22	0,17	0,27	0,25
Replicate 3	0,29	0,31	0,5	0,25	0,21	0,18	0,29	0,25
Replicate 4	0,26	0,28	0,55	0,26	0,2	0,16	0,28	0,25
Replicate 5	0,26	0,29	0,49	0,25	0,18	0,18	0,3	0,25
Replicate 6	0,25	0,3	0,47	0,23	0,17	0,19	0,3	0,25
Replicate 7	0,26	0,3	0,49	0,23	0,18	0,2	0,3	0,25
Replicate 8	0,22	0,26	0,46	0,22	0,18	0,21	0,28	0,25
Replicate 9	0,27	0,29	0,57	0,28	0,24	0,2	0,3	0,25
Average	0,254	0,286	0,512	0,251	0,202	0,188	0,29	
CV%	9,8	6,8	8,1	8,6	13,3	9,34	3,9	
Recovery %	102	114	102	100	81	76	116	

Table 18. Limit of quantification values for the lowest concentration level.

As we can see, the sensitivity of the method was widely sufficient to quantify all the analytes present in the samples.

4.1.6 Uncertainty of measurement by Horwitz

This criterion is based on the assumption that random errors are inversely proportional to the concentration and given the prevalence of random errors in chemical tests. It is defined by an empirical mathematical expression that binds the concentration of the analyte (C) to the percent coefficient of variation (CVR%). The mathematical expression is taken from the observation of a large number of measurements, carried out at known concentrations, obtained from the various inter-comparison exercises (collaborative trials). Also in this case this method approaches the model based on the absolute independence of the obtained measurements, making the time factor vary in a consistent way. This relationship is of an empirical nature and has a field of application without limitations of the matrix and measuring range. The mathematical expression is as follows:

$$CV_R\,\% = 2^{(1-0.5logC)}$$

where $CV_R\%$ is the coefficient of percent variation of reproducibility and C is the concentration expressed as a mass fraction. To apply the Horwitz equation it is necessary to verify that the repeatability of the laboratory (S_r) is compatible with the reproducibility calculated with Horwitz (σ_R), according to the criteria previously exposed:

$$\sigma_R \frac{1}{2} \leq S_r \leq \frac{2}{3}\,\sigma_R$$

Where: $\sigma_R = 0.22 * C$

If the criteria are satisfied, then the standard deviation of reproducibility ("scarto tipo di riproducibilità") calculated according to Horwitz provides an estimate of the uncertainty ("incertezza composta") of the laboratory, while the extended uncertainty is equal to:

$$(y) = 2 * \sigma_R$$

where 2 is the coverage factor ("fattore di copertura") with a probability of 95%.
The following tables (Tables 19, 20 and 21) show the results obtained for each level concentration:

I Level (0,25 µg/kg)	Average (µg/kg)	Sr	σR=0,22C	Sr/σR	Lower limit	Upper limit
B[*a*]A	0,2544	0,0251	0,056	0,448	0,5	0,66
Cr	0,2856	0,0194	0,0628	0,309	0,5	0,66
B(*bjk*)F	0,5122	0,0415	0,1127	0,368	0,5	0,66
B[*a*]P	0,2511	0,0215	0,0552	0,389	0,5	0,66
IP	0,2022	0,0268	0,0445	0,603	0,5	0,66
D(*a,h*)A	0,1756	0,0324	0,0386	0,84	0,5	0,66
B(*g,h,i*)P	0,29	0,0112	0,0638	0,175	0,5	0,66

Table 19. Results obtained for the first concentration level.

II Level (1 µg/kg)	Average (µg/kg)	Sr	σR=0,22C	Sr/σR	Lower limit	Upper limit
B[*a*]A	0,9422	0,0589	0,2073	0,284	0,5	0,66
Cr	1,0833	0,0071	0,2383	0,03	0,5	0,66
B(*bjk*)F	1,6678	0,0273	0,3669	0,074	0,5	0,66
B[*a*]P	0,8244	0,023	0,1814	0,127	0,5	0,66
IP	0,7544	0,0181	0,166	0,109	0,5	0,66
D(*a,h*)A	0,7256	0,0113	0,1596	0,071	0,5	0,66
B(*g,h,i*)P	0,91	0,0255	0,2002	0,127	0,5	0,66

Table 20. Results obtained for the second concentration level.

III Level (4 µg/kg)	Average (µg/kg)	Sr	σR=0,22C	Sr/σR	Lower limit	Upper limit
B[*a*]A	4,207	0,098	0,925	0,106	0,5	0,66
Cr	4,244	0,098	0,934	0,105	0,5	0,66
B(*bjk*)F	7,789	0,14	1,714	0,082	0,5	0,66
B[*a*]P	3,864	0,07	0,85	0,082	0,5	0,66
IP	3,467	0,099	0,763	0,13	0,5	0,66
D(*a,h*)A	3,411	0,113	0,75	0,151	0,5	0,66
B(*g,h,i*)P	3,906	0,1	0,859	0,116	0,5	0,66

Table 21. Results obtained for the third concentration level.

As we can see from the tables above, the S_r/σ_R value is always smaller than the lower limit. These values are acceptable and this means that the values obtained are more precise than the official method. This finds an explanation in the fact that the criteria of acceptability of the Horwitz method were evaluated several years ago and there were no adequate tools. Now we have a more modern instrumentation and we are able to be more precise, so we obtain lower values than the values indicated by the Horwitz method. The strength of this method is certainly the simplicity of calculation. On the other hand, this method tends to overestimate the measurement uncertainty.

4.2 PAHs content in sunflower oil

In 15 samples of sunflower oil we quantified the amount of PAHs present. As we have previously said in the paragraph 1.7, Commission regulation (EC) 835/2011 has set a maximum level of 2 ppb for benzo[a]pyrene (BaP) in oils and fats intended for direct consumption or for use as an ingredient in foods and has set a maximum level of 10 ppb for the sum of 4 PAHs (benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene). In the graphs below the maximum level of 2 ppb is indicated with a red line that allows to see if in the analyzed samples there will be a surplus of benzo[*a*]pyrene. BaP is used as a marker of the occurrence and effect (toxic potential) of the whole class of genotoxic heavy PAHs. Below the graphs with the concentrations of PAHs found in each sunflower oil analyzed are shown. Moreover a chromatogram of a sample of sunflower oil is shown below.

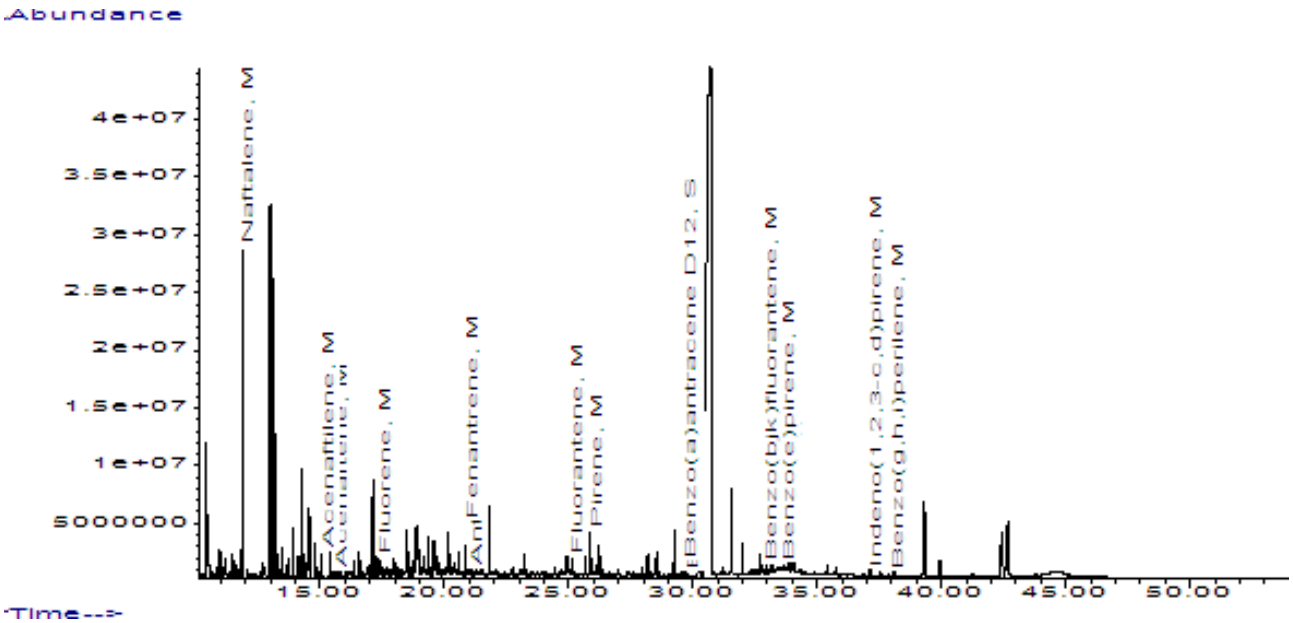


Figure 19. Chromatogram of a sample of sunflower oil.

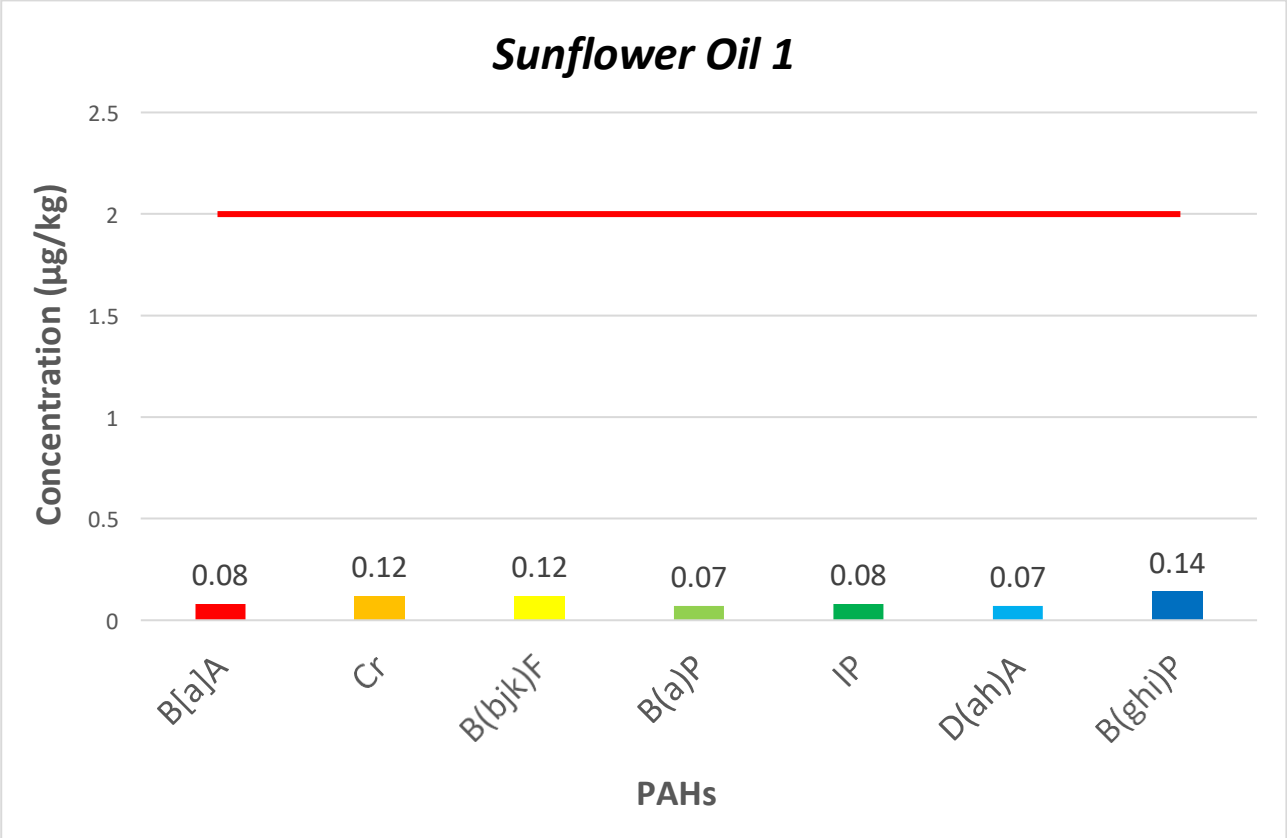


Figure 20. Sample of sunflower oil, Area vasta AV 3 (Macerata).

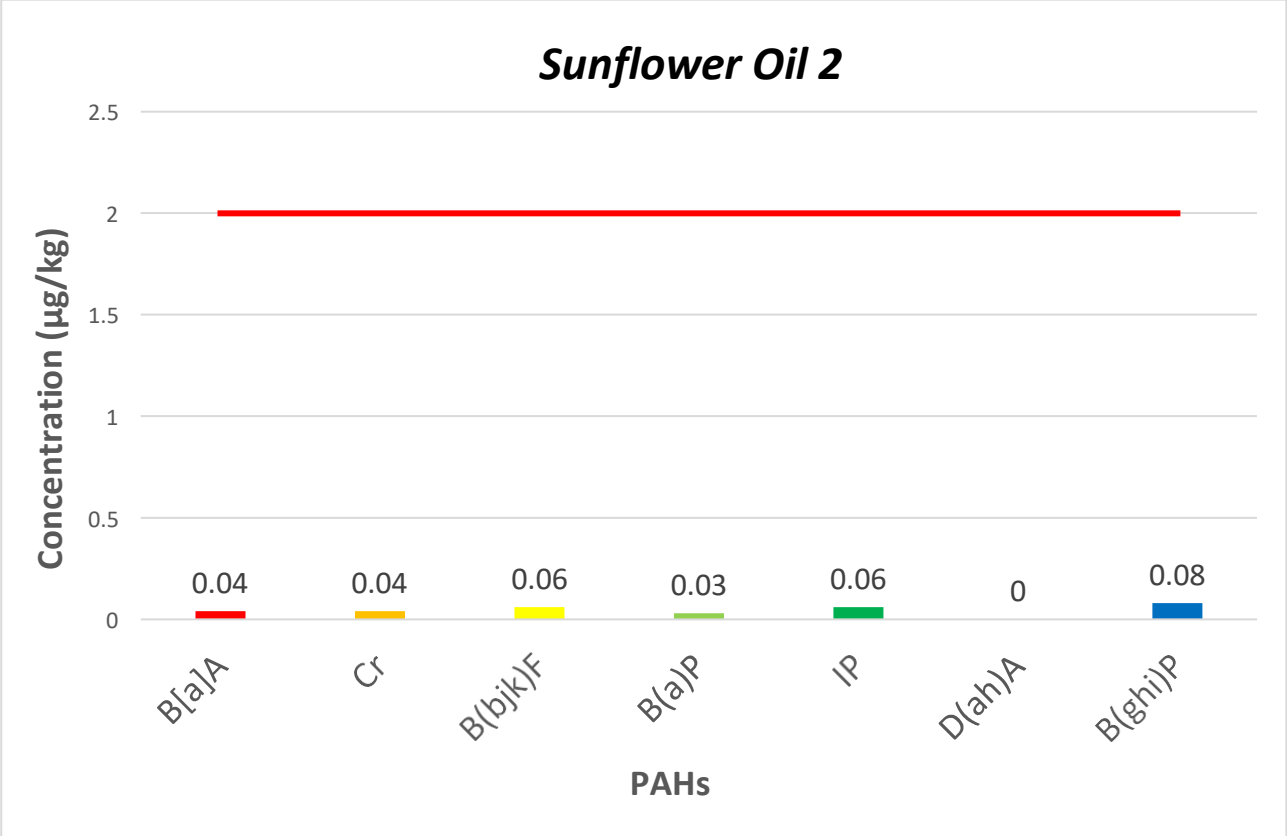


Figure 21. Sample of sunflower oil, Area vasta AV 5 (Ascoli Piceno).

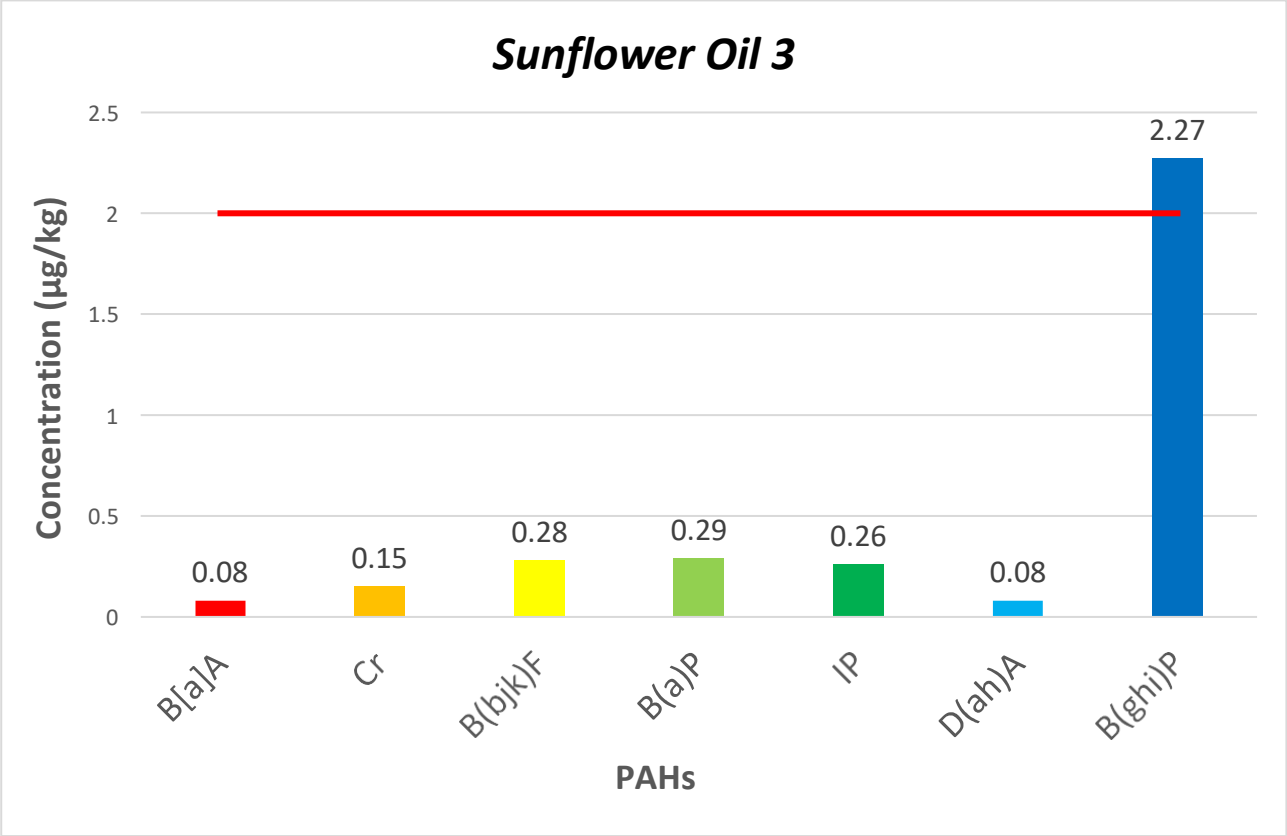


Figure 22. Sample of sunflower oil, Area vasta AV 5 (San Benedetto del Tronto).

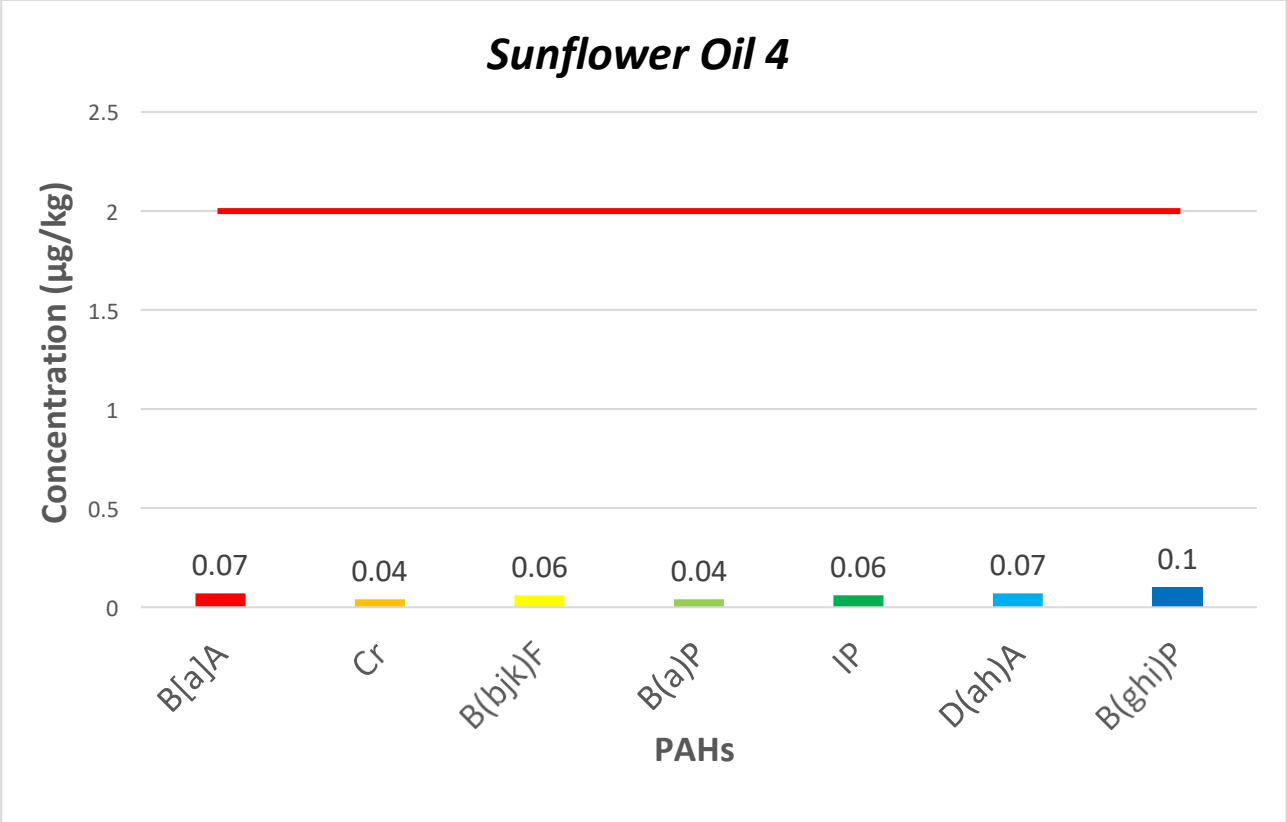


Figure 23. Sample of sunflower oil, Area vasta AV 3 (Macerata).

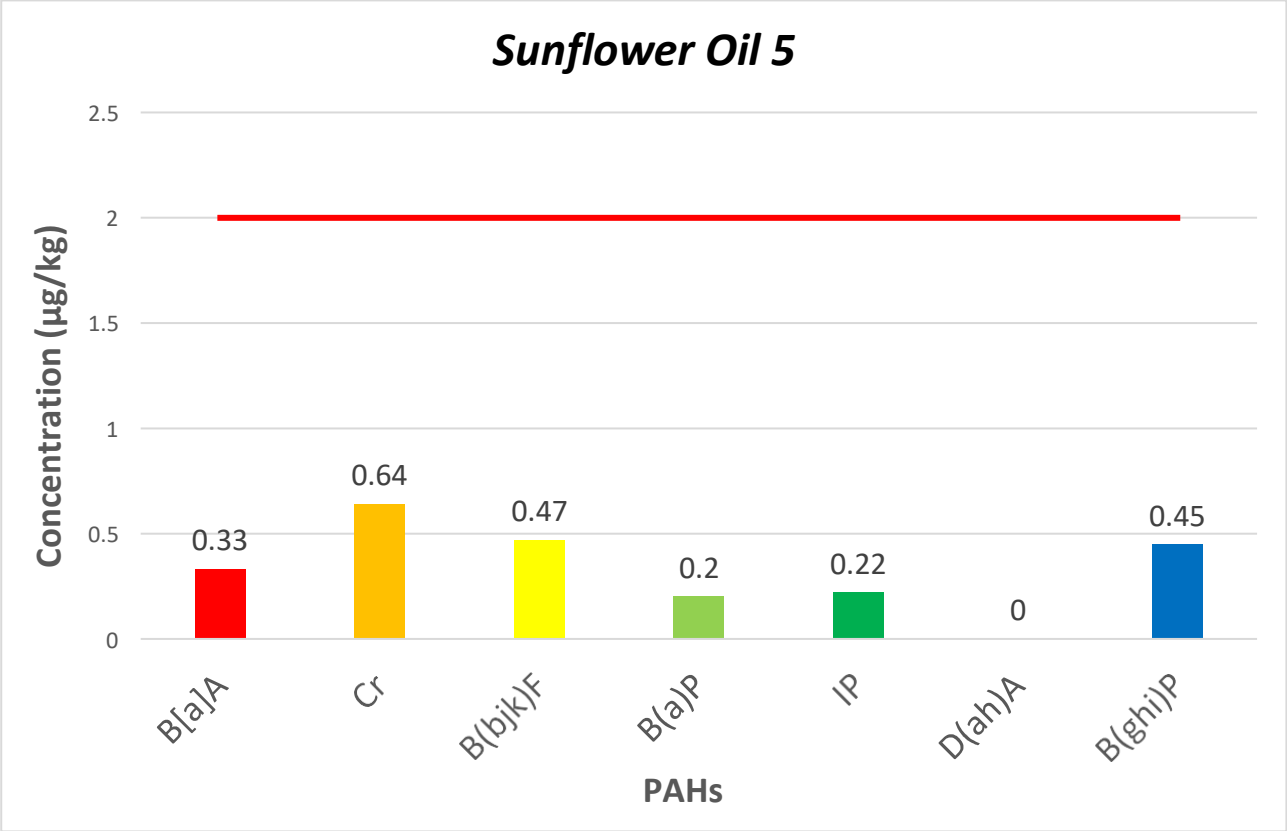


Figure 24. Sample of sunflower oil, Area vasta AV 2 (Jesi).

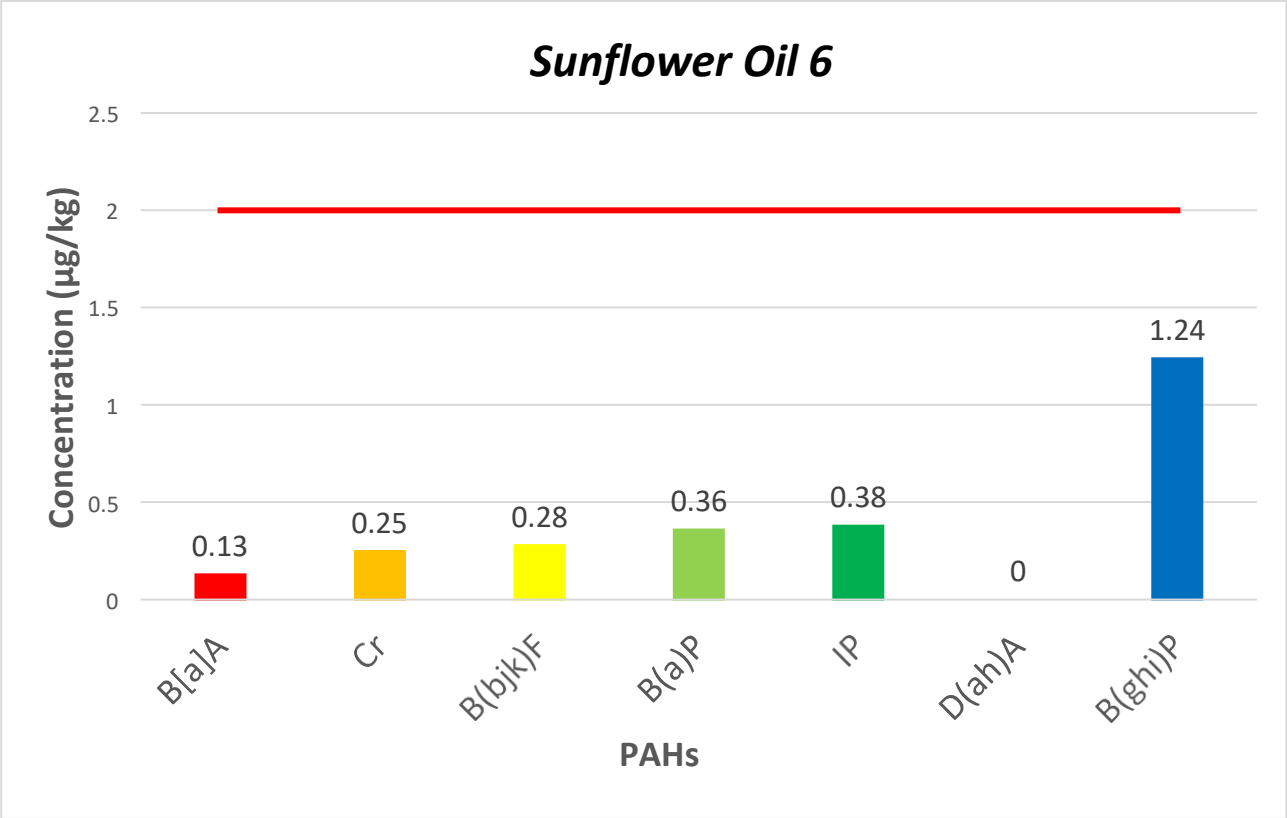


Figure 25. Sample of sunflower oil, Area vasta AV 3 (Macerata).

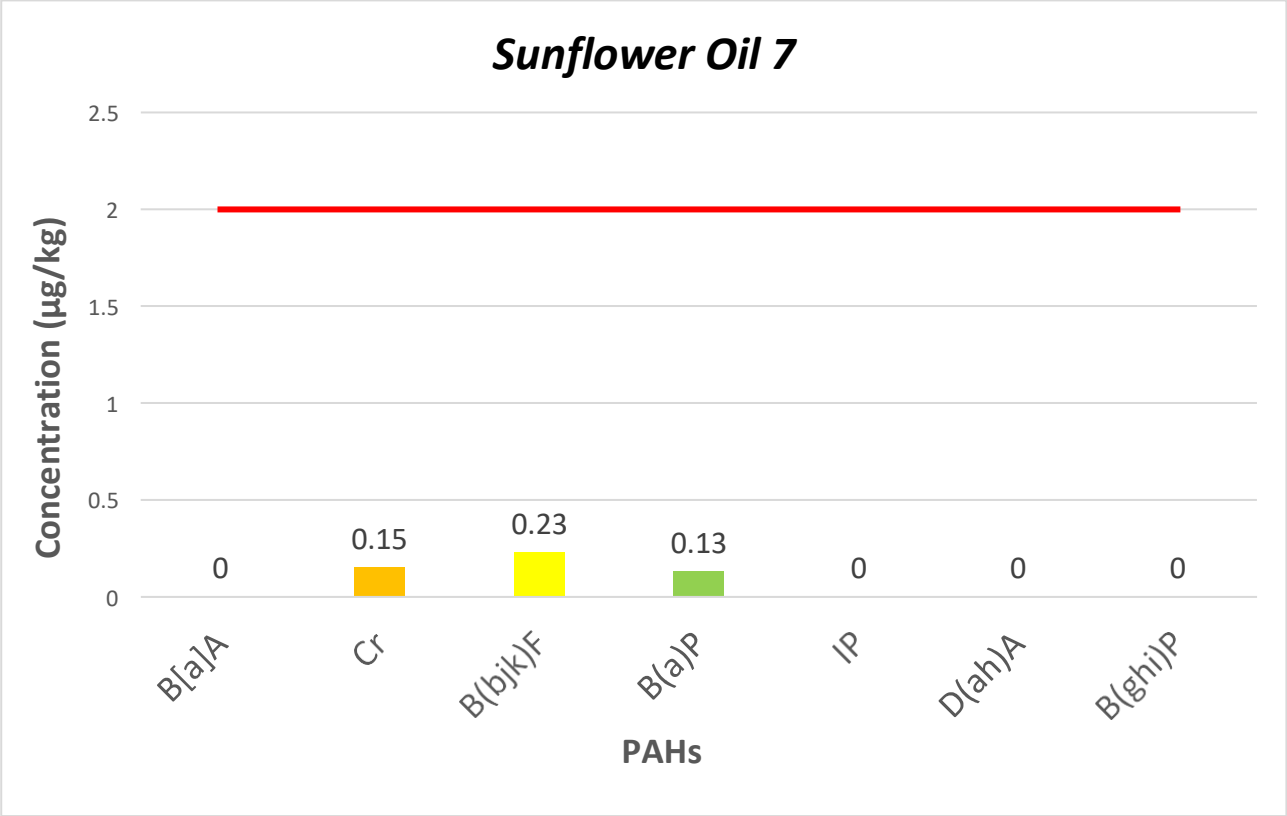


Figure 26. Sample of sunflower oil, Area vasta AV 2 (Fabriano).

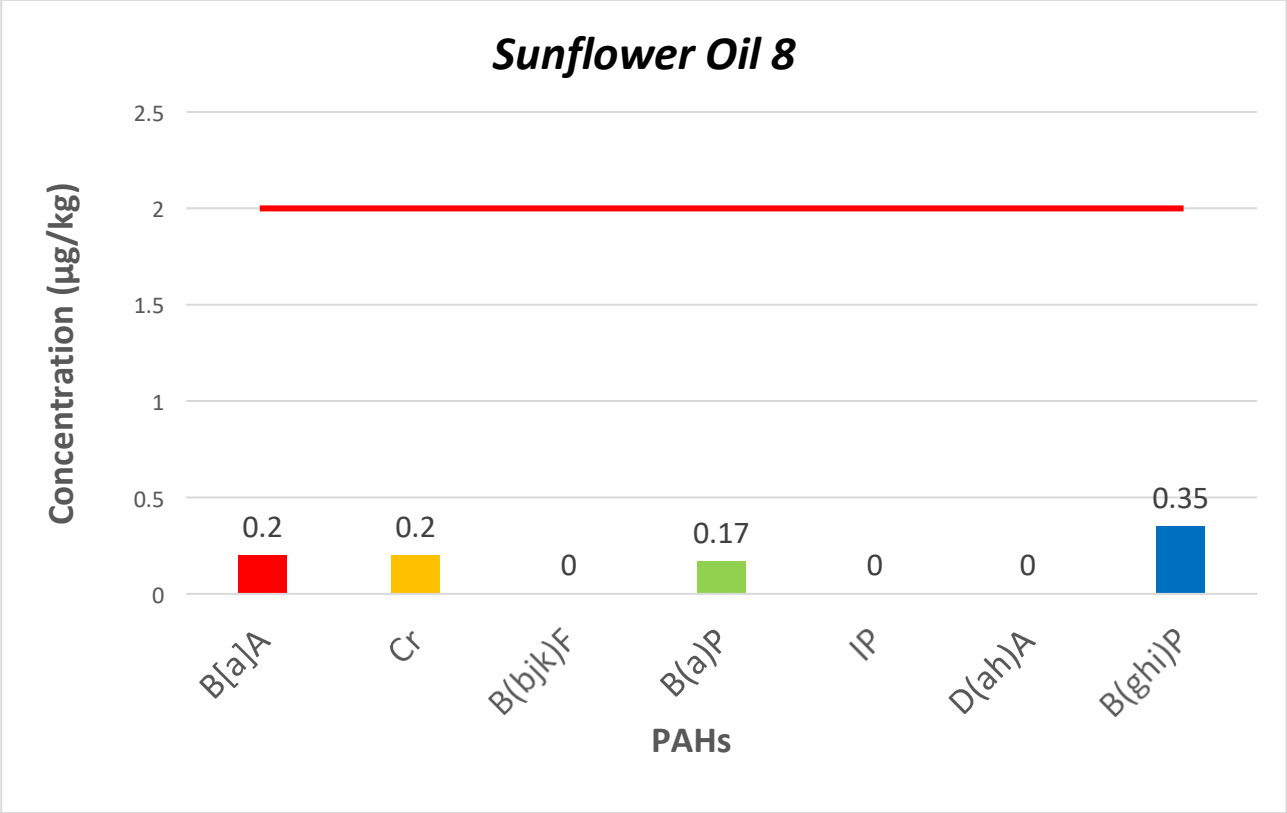


Figure 27. Sample of sunflower oil, Area vasta AV 5 (Ascoli Piceno).

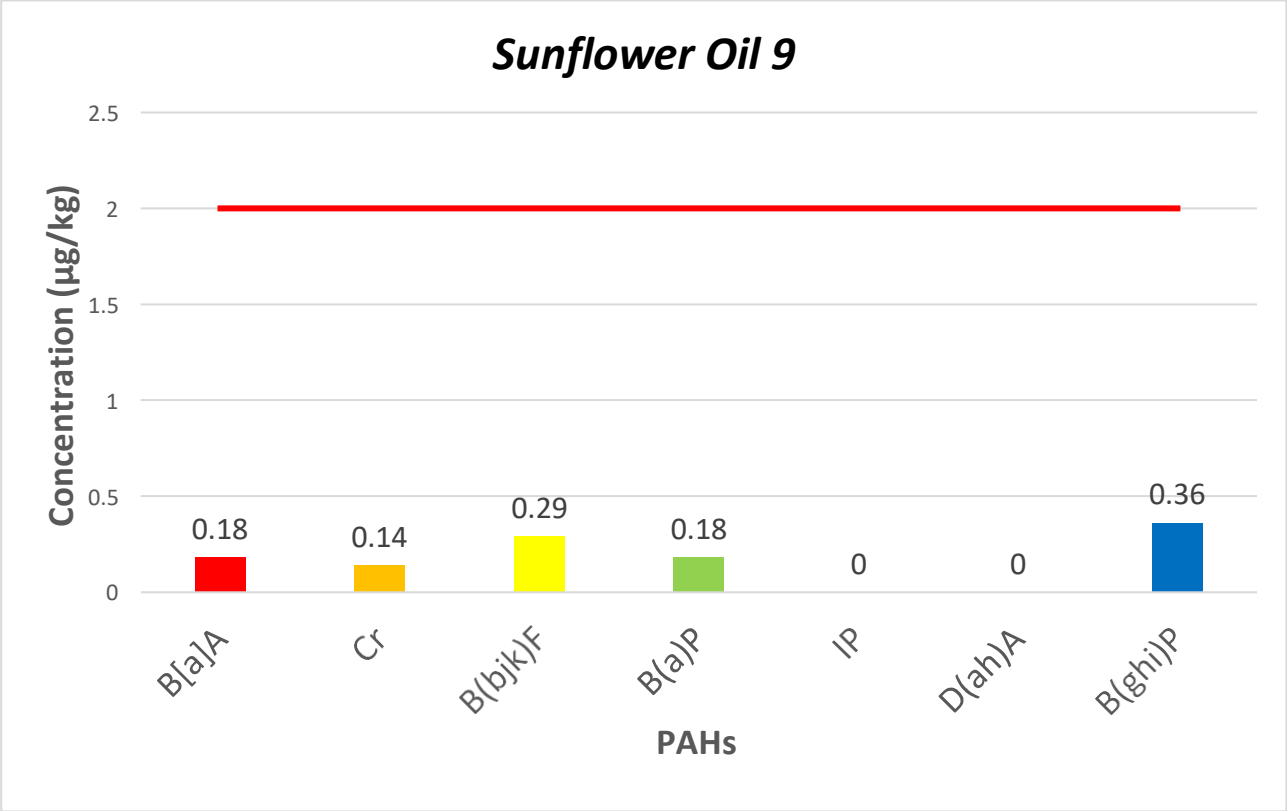


Figure 28. Sample of sunflower oil, Area vasta AV 2 (Ancona).

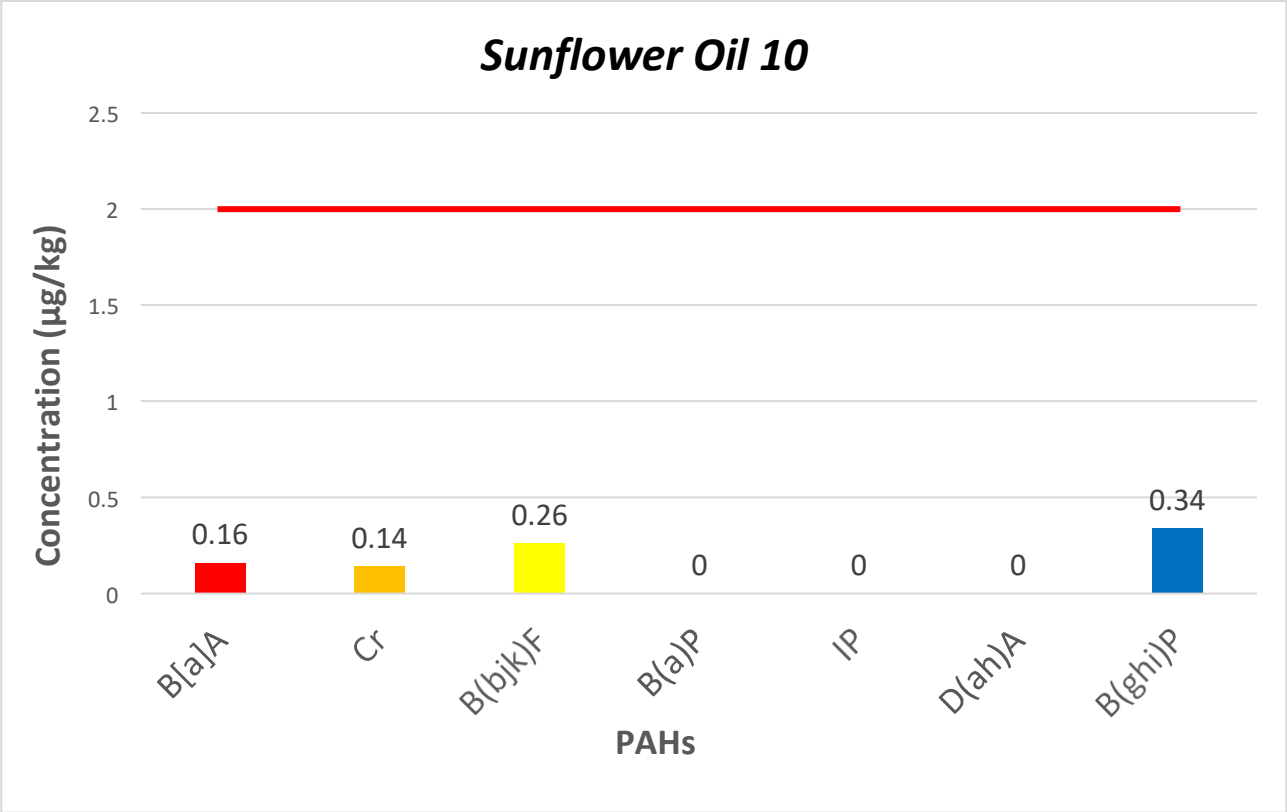


Figure 29. Sample of sunflower oil, Area vasta AV 1 (Fano).

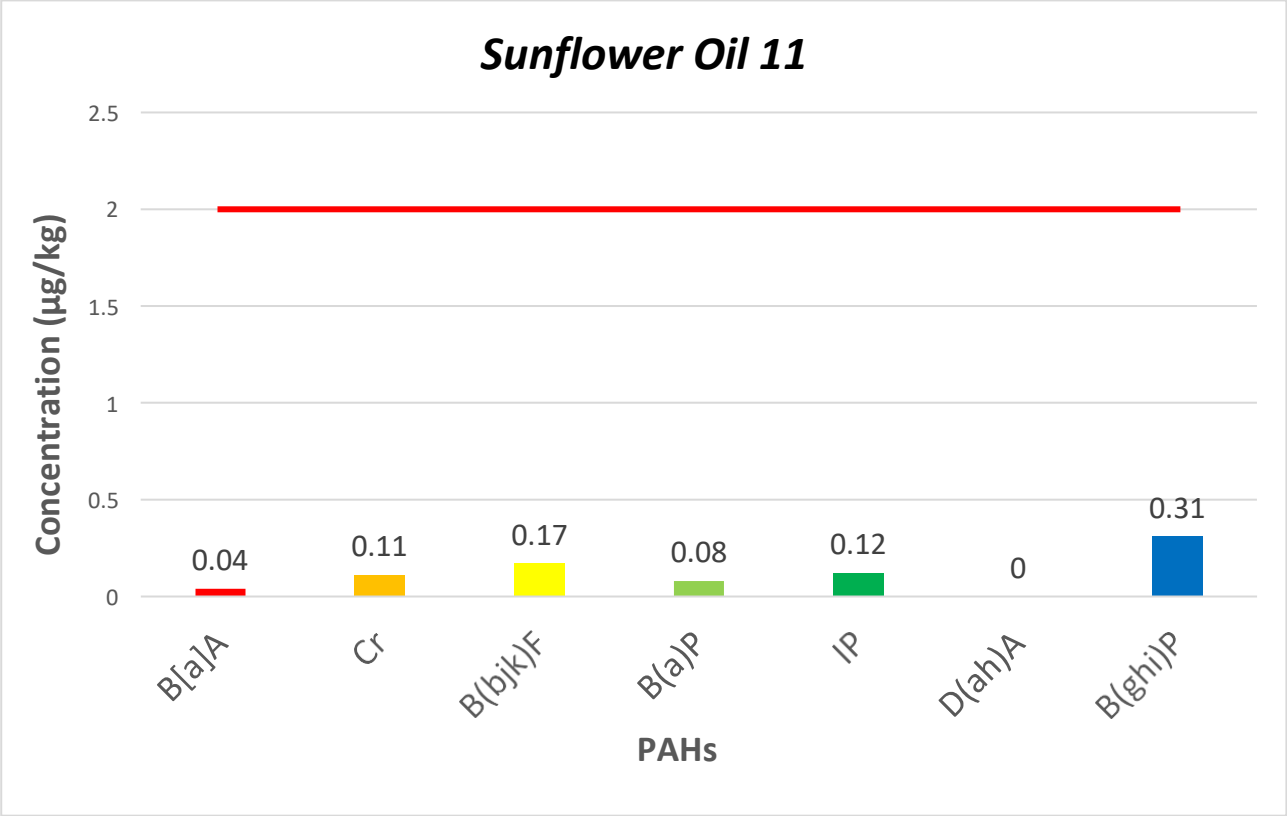


Figure 30. Sample of sunflower oil, Area vasta AV 5 (San Benedetto del Tronto).

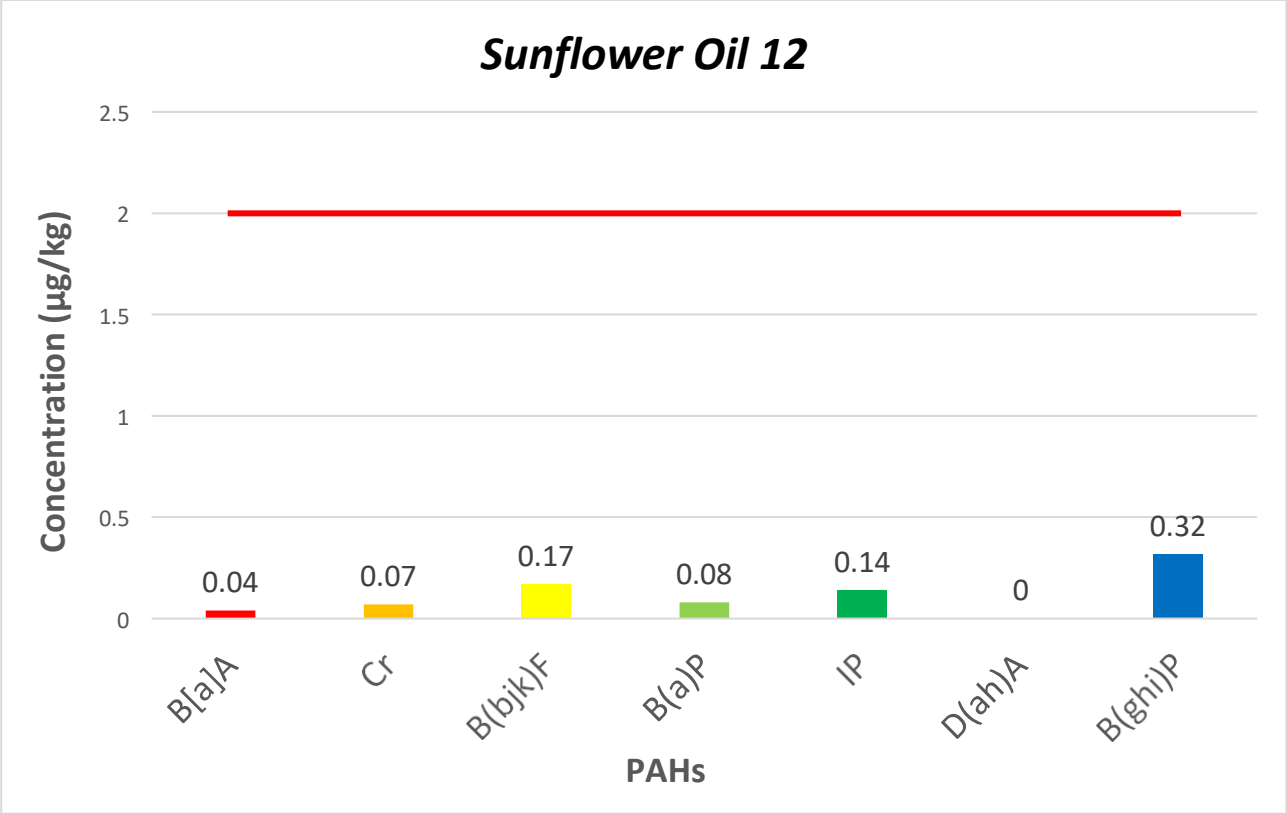


Figure 31. Sample of sunflower oil, Area vasta AV 1 (Pesaro).

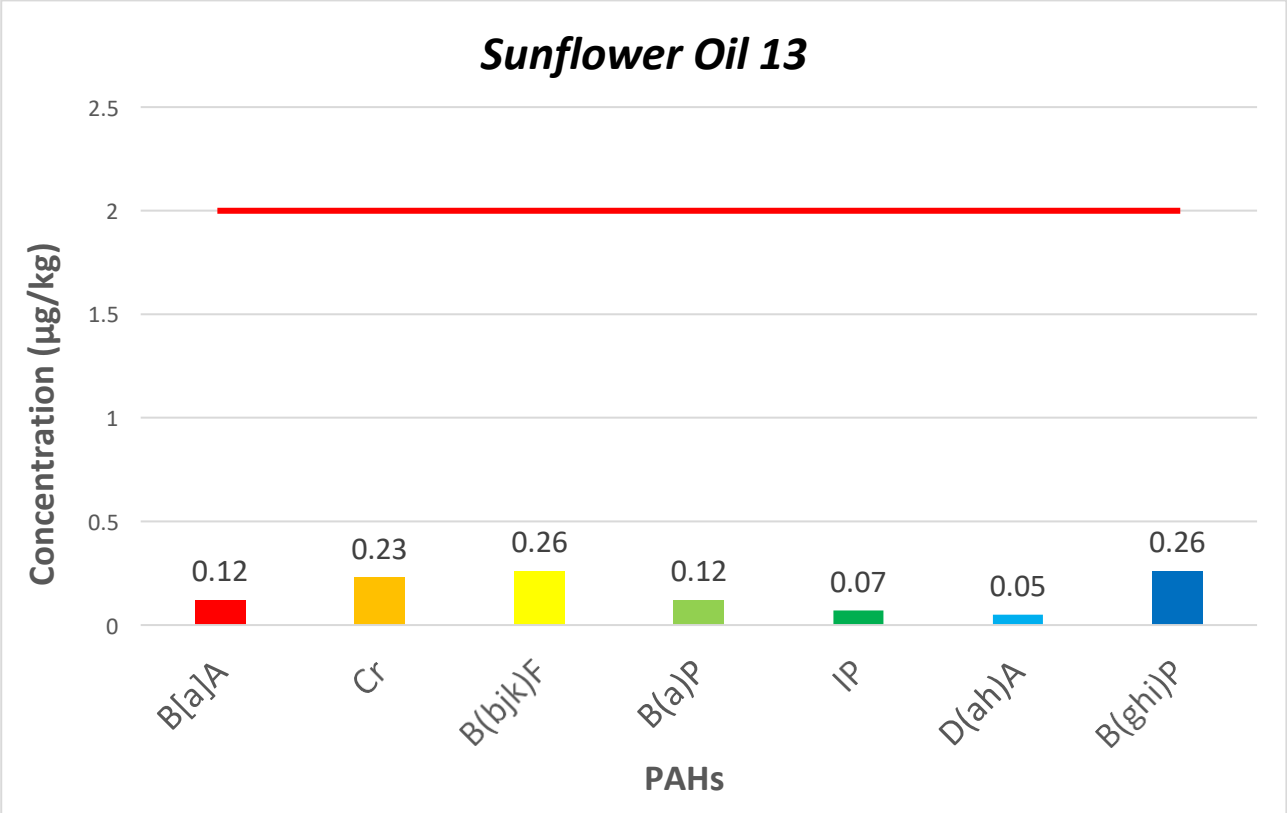


Figure 32. Sample of sunflower oil, Area vasta AV 2 (Senigallia).

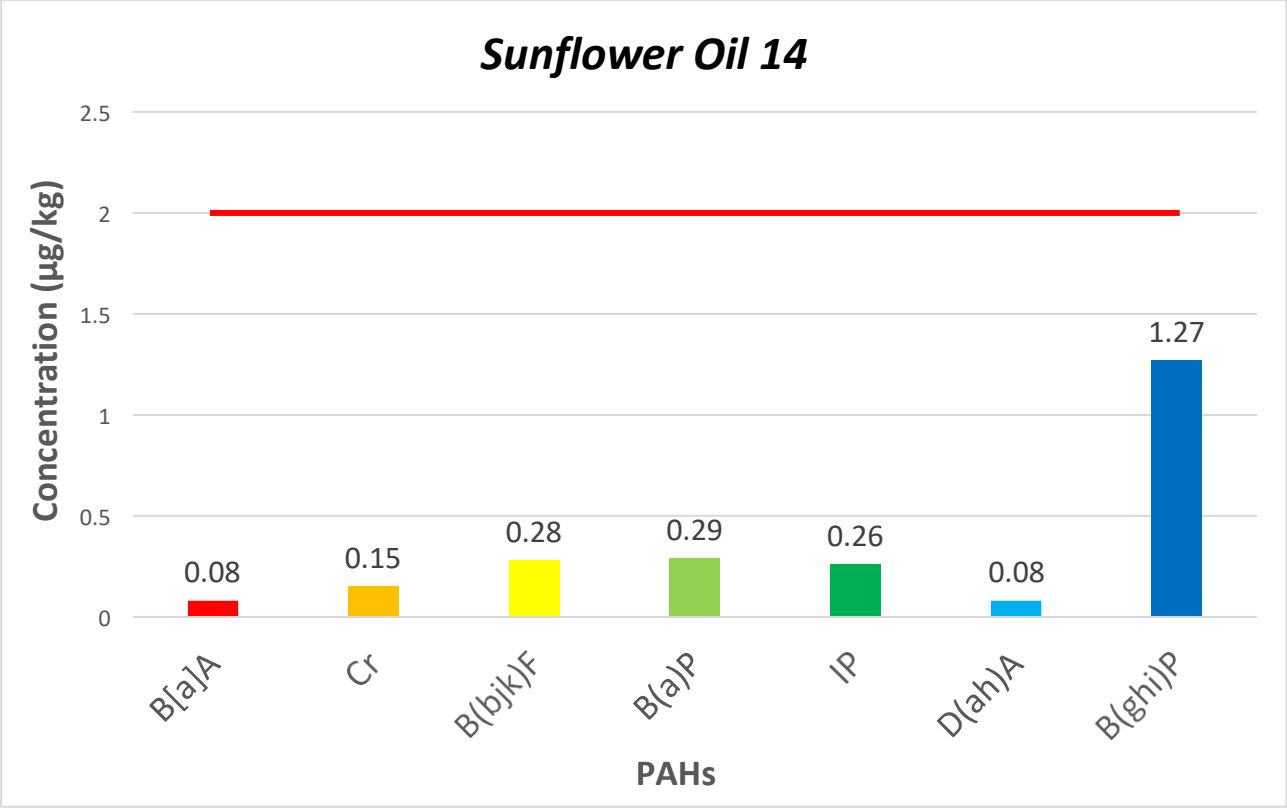


Figure 33. Sample of sunflower oil, Area vasta AV 1 (Urbino).

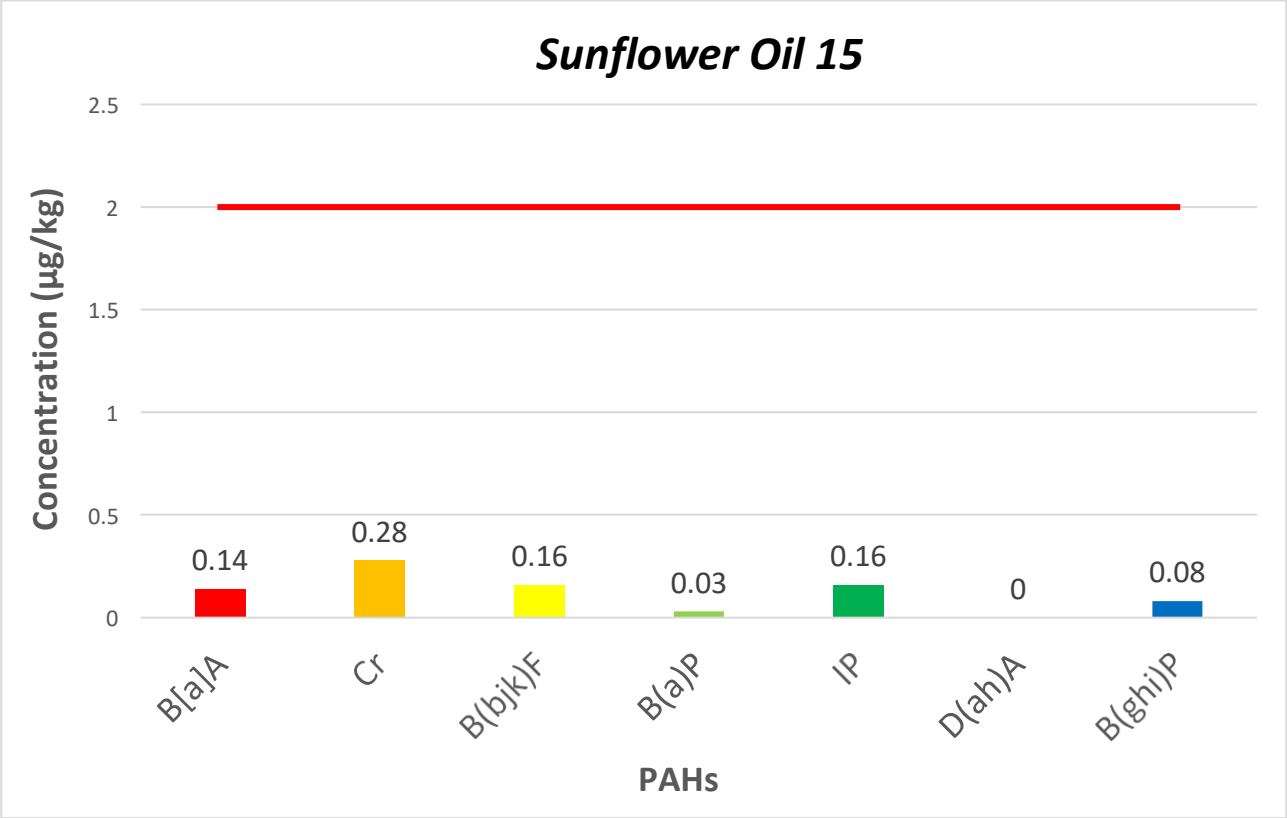


Figure 34. Sample of sunflower oil, Area vasta AV 5 (Ascoli Piceno).

In addition, the following table (Table 22) shows the results of the light PAHs in each oil analyzed.

	Light PAHs concentrations (µg/kg)						
Sunflower oil	**ACY**	**ACP**	**FLR**	**PHE**	**ANT**	**FLT**	**PYR**
Oil 1	0,13	0,14	0,92	2,09	0,17	0,79	1,61
Oil 2	0,11	0,11	0,82	1,74	0,16	0,57	1,04
Oil 3	0	0	0,13	2,52	0,17	5,14	16,55
Oil 4	0	0	0,24	1,18	0,13	0,55	1,18
Oil 5	0,2	0	0,73	4,18	0,51	1,91	3
Oil 6	0,21	0,12	1,23	4,37	0,49	1,45	2,18
Oil 7	0,24	0,06	0,95	4,74	0,5	1,95	3,48
Oil 8	0,35	0,56	3,63	8,13	0,53	2,75	5,72
Oil 9	0,38	1,13	2,39	7,14	1,01	4,02	6,79
Oil 10	0,2	0,32	1,74	6,93	0,58	4,43	14,42
Oil 11	0,6	1,19	3,34	6,26	0,41	1,58	3,92
Oil 12	0,44	0,86	2,61	4,65	0,27	1,24	3,35
Oil 13	0,26	0,48	1,59	4,41	0,28	1,35	2,88
Oil 14	0,24	0,12	1,72	4,18	0,16	1,95	1,18
Oil 15	0,21	0	1,24	4,65	0,51	0,55	2,18

Table 22. Concentration of light PAH in each sunflower oil analyzed.

In seed oils, low concentration values of PAH are generally found both because the seeds are protected from atmospheric pollution (the sunflower seed from the shell), and also because seed oils are subjected to grinding. But in this case they show an appreciable concentration of PAHs. This finds an explanation in the fact that some samples of oil analyzed have a high concentration of both "light" and "heavy" PAHs, most probably due to a phenomenon similar to the production of pomace oil, that is, the seeds are dried by direct contact with hot fumes, rich in PAHs, coming from the combustion of plant material and the subsequent processing and grinding phases were not effective for the complete removal of these substances. Nevertheless, under no circumstances is the threshold limit of 10 µg/kg exceeded for the sum of the four components. The limit of 2 µg/kg for benzo[*a*]pyrene was also not exceeded.

4.3 PAHs content in extra virgin olive oil

In 15 samples of extra virgin olive oil we quantified the amount of PAHs present. Also in this case, as well as for sunflower oils, the maximum level of benzo[a]pyrene (BaP) in oils and fats intended for direct consumption or for use as an ingredient in foods is 2 ppb while the maximum level for the sum of 4 PAHs (benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene) is 10 ppb. The level of 2 ppb is indicated with a red line in the graphs below. Below the graphs with the concentrations found in each oil analyzed are shown.

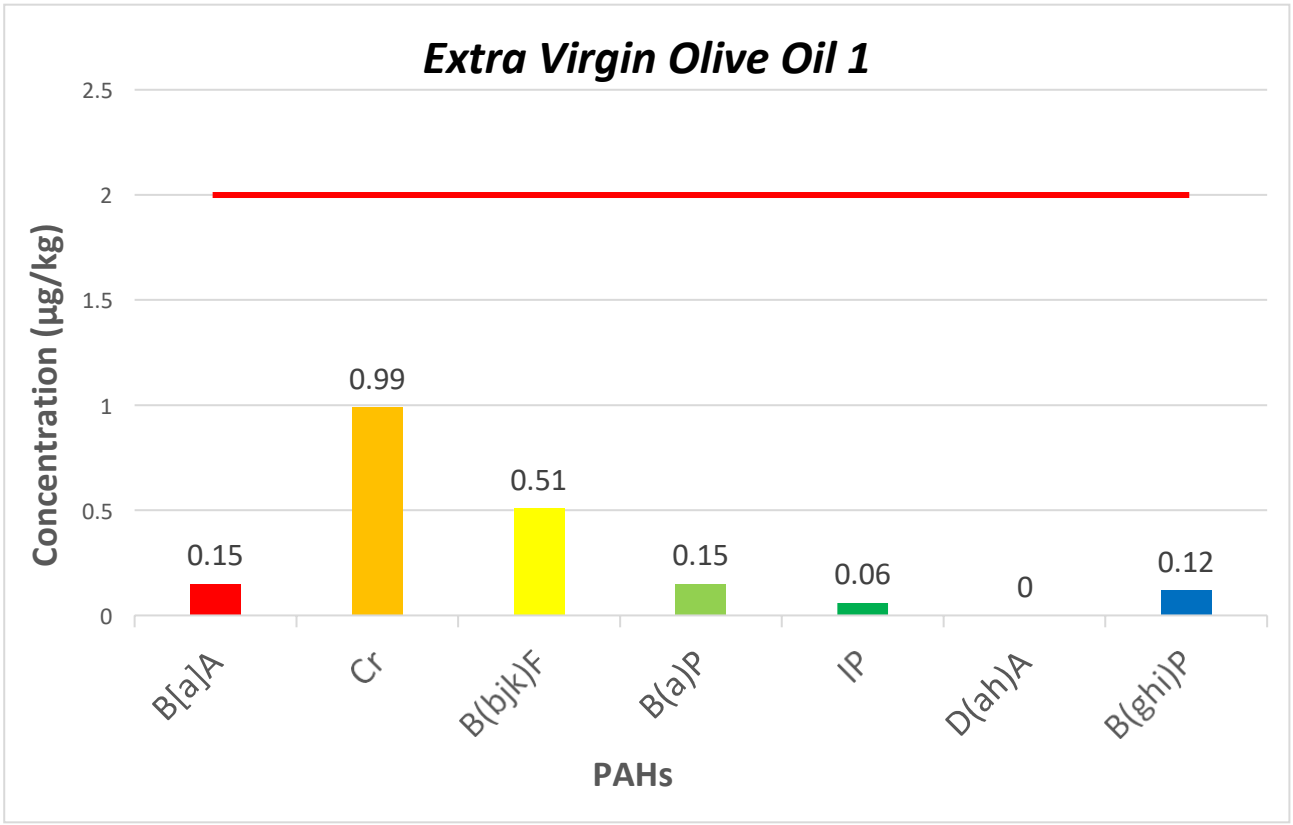


Figure 35. Sample of extra virgin olive oil, Area vasta AV 3 (Macerata).

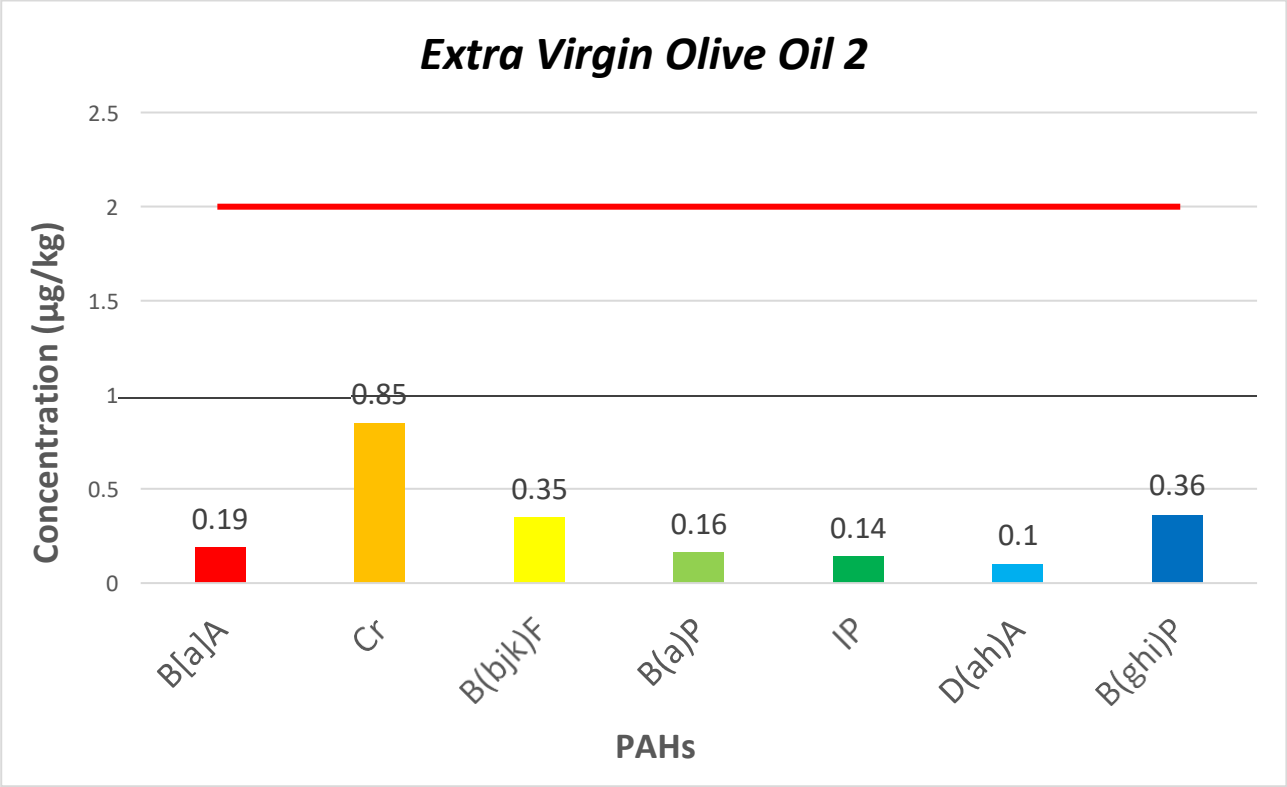


Figure 36. Sample of extra virgin olive oil, Area vasta AV 2 (Fabriano).

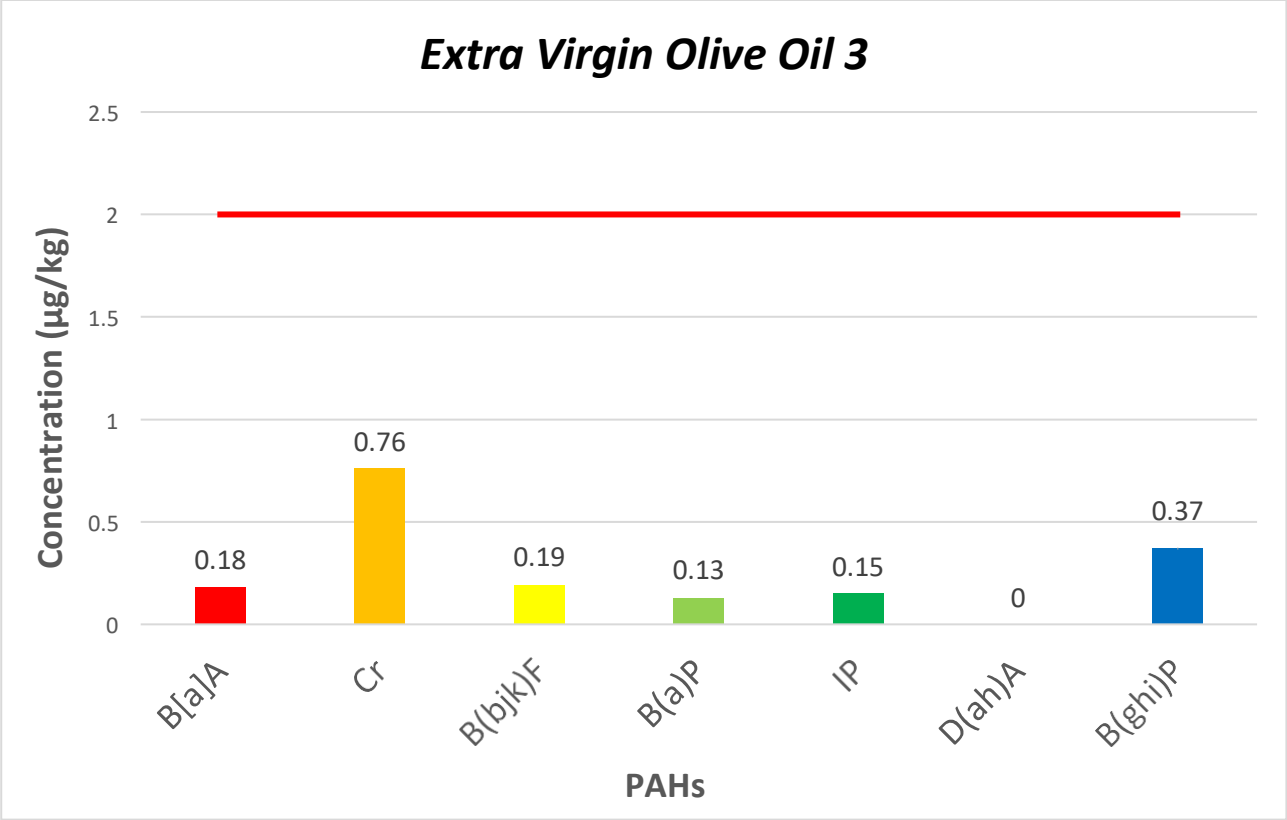


Figure 37. Sample of extra virgin olive oil, Area vasta AV 5 (San Benedetto del Tronto).

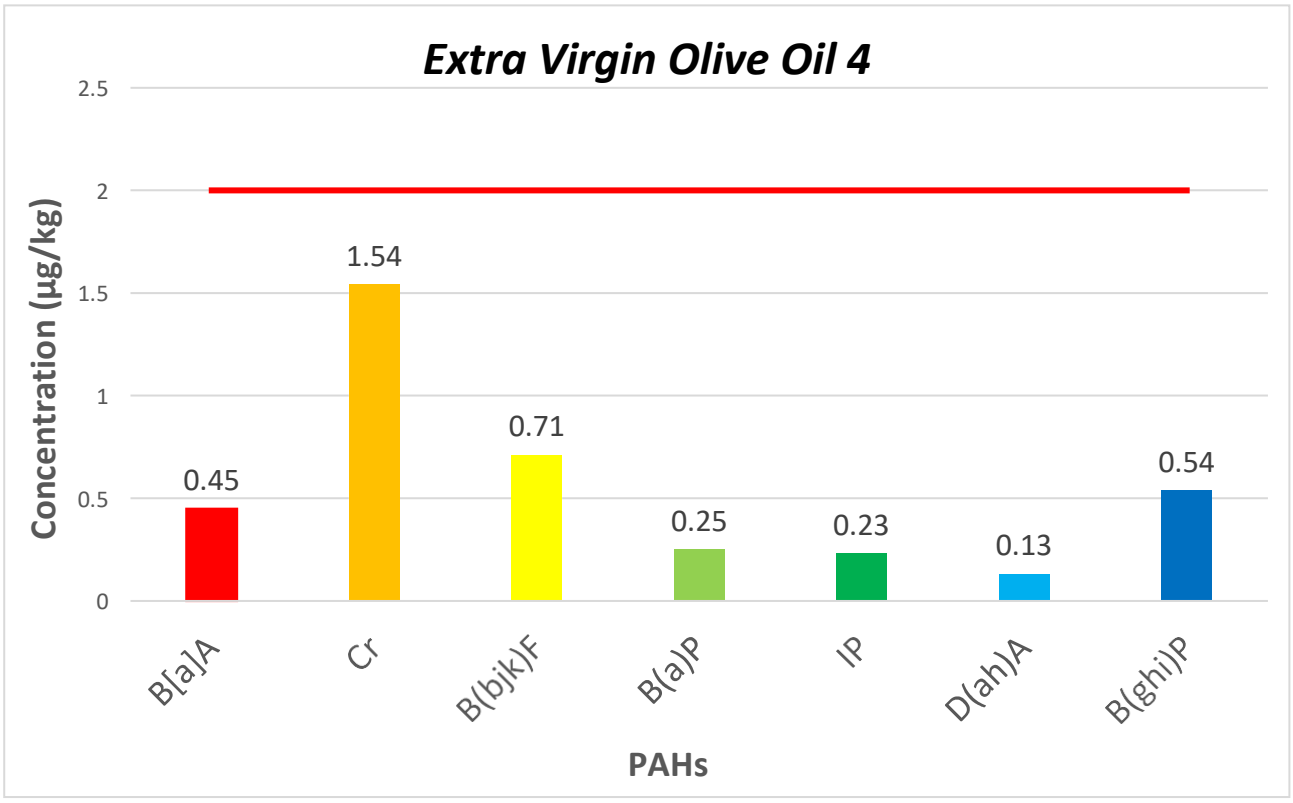


Figure 38. Sample of extra virgin olive oil, Area vasta AV 3 (Macerata).

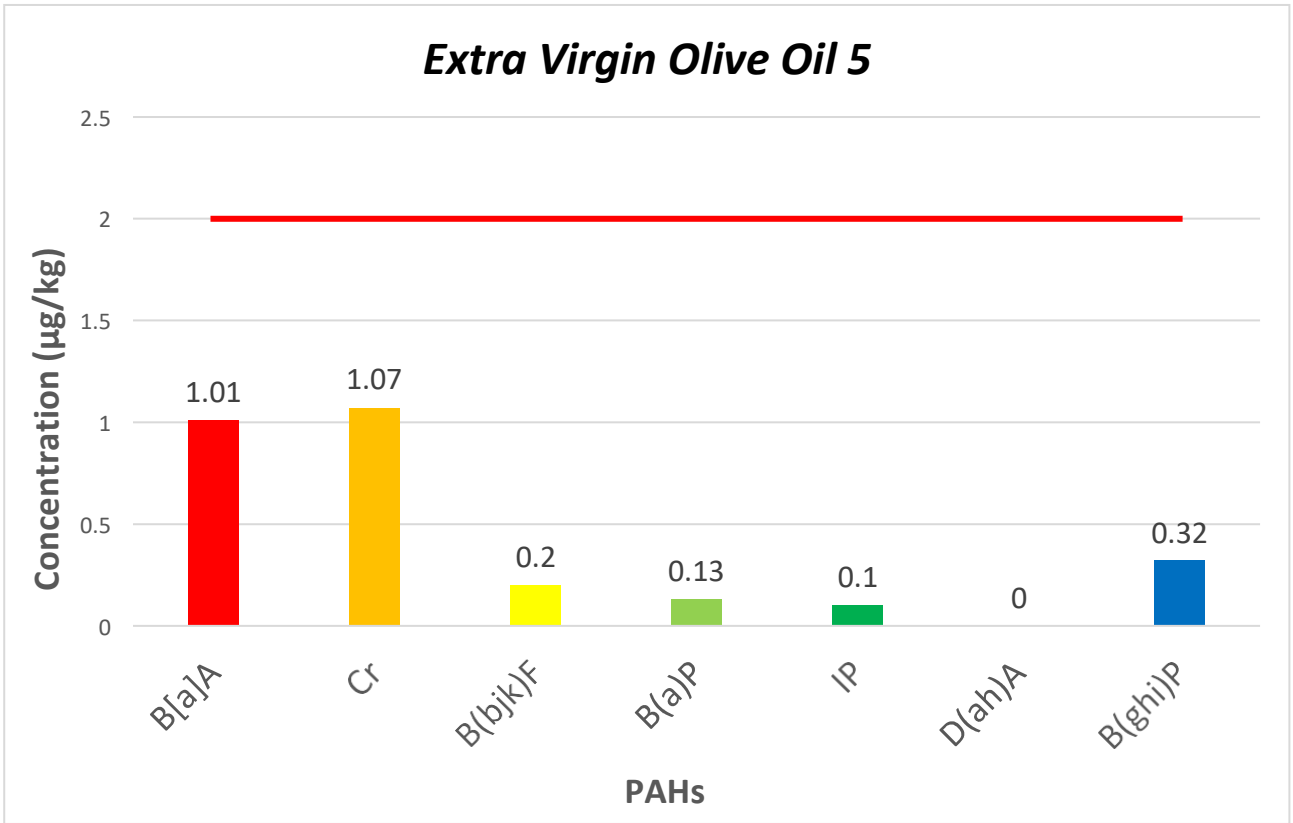


Figure 39. Sample of extra virgin olive oil, Area vasta AV 5 (Ascoli Piceno).

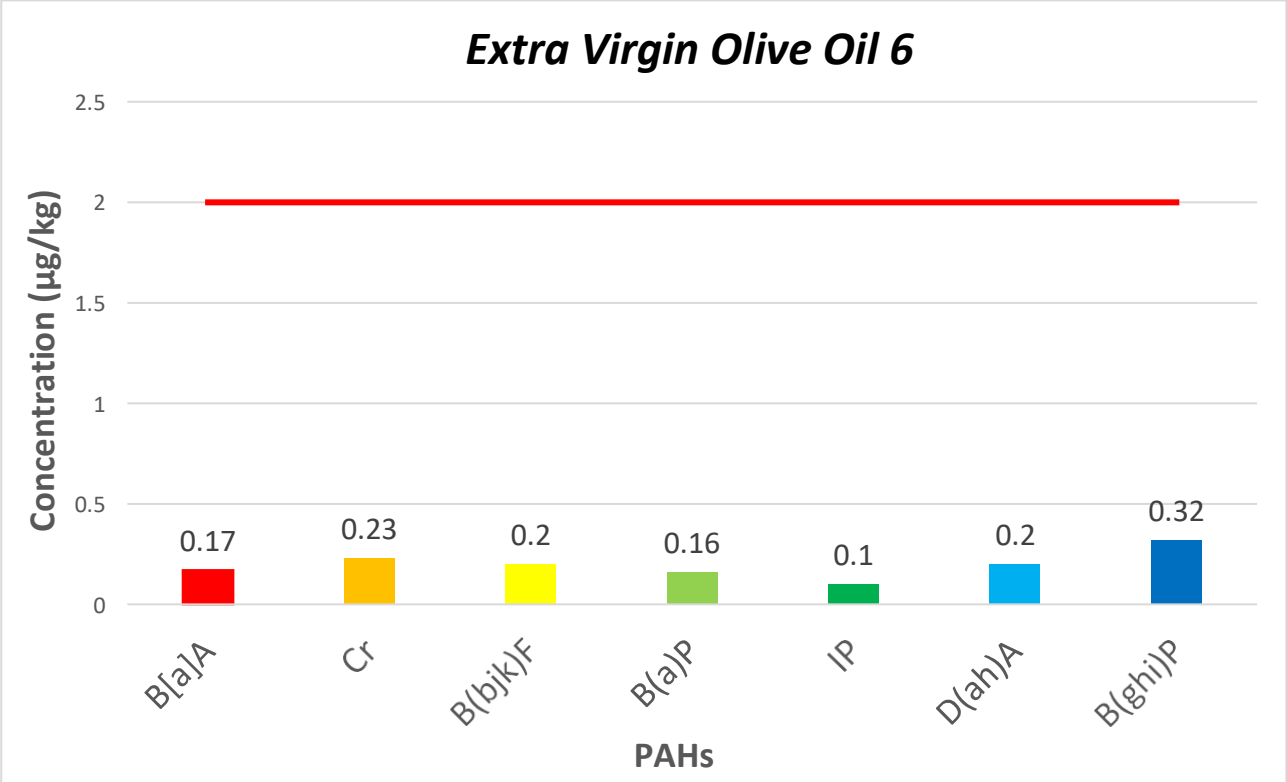


Figure 40. Sample of extra virgin olive oil, Area vasta AV 2 (Jesi).

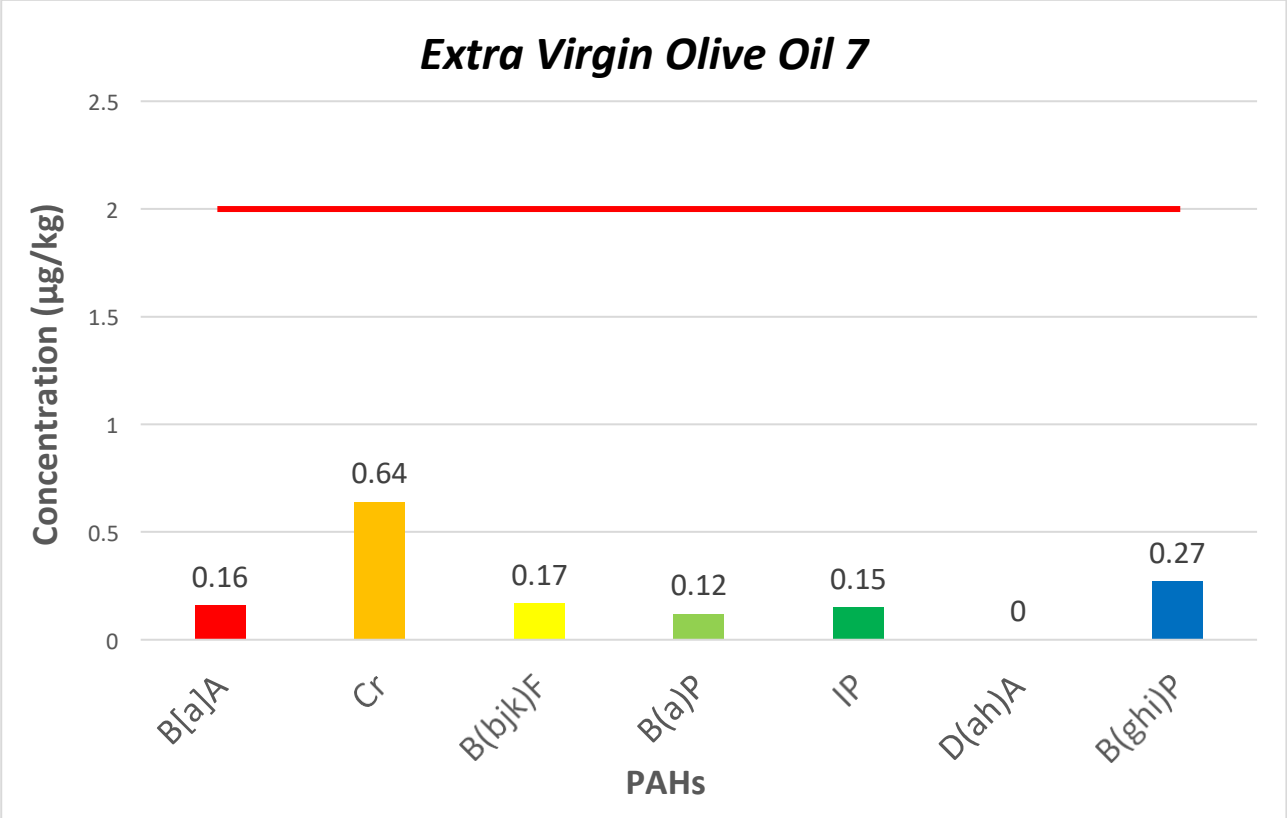


Figure 41. Sample of extra virgin olive oil, Area vasta AV 5 (Ascoli Piceno).

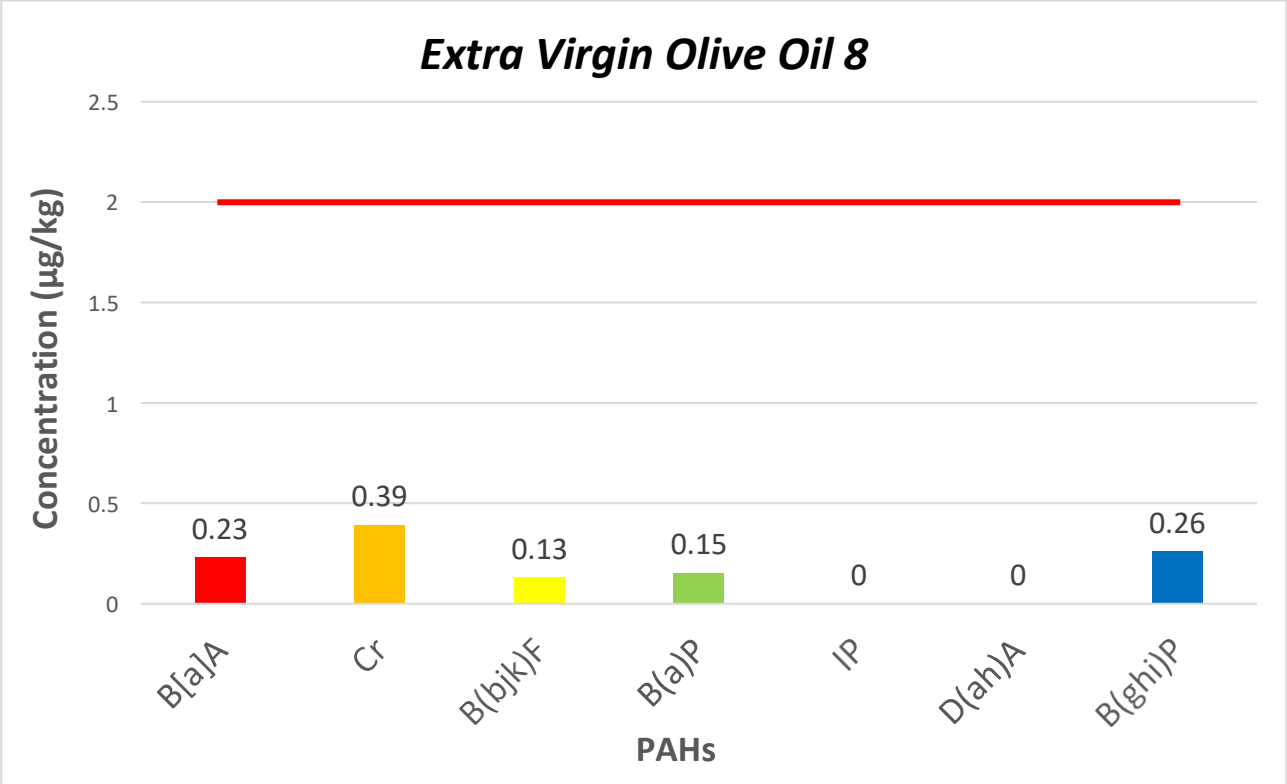


Figure 42. Sample of extra virgin olive oil, Area vasta AV 5 (Ascoli Piceno).

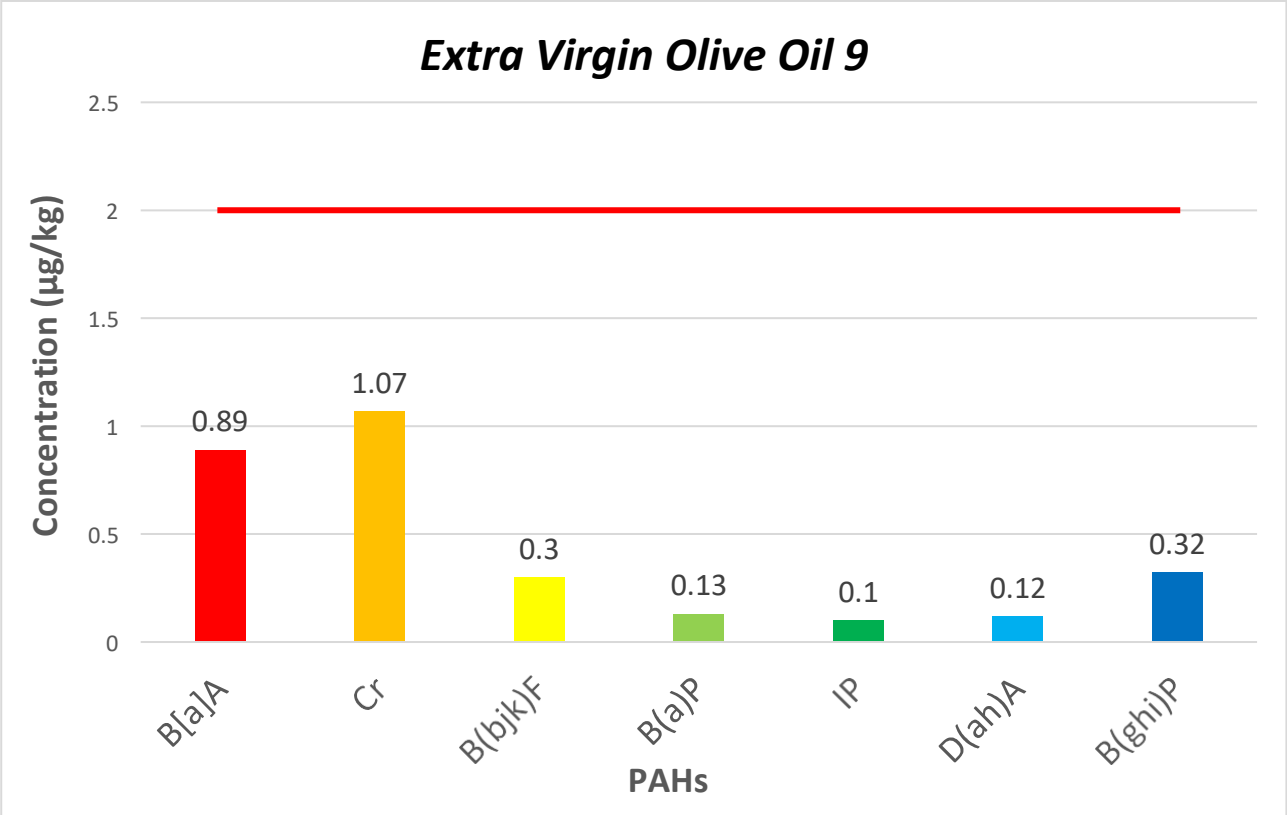


Figure 43. Sample of extra virgin olive oil, Area vasta AV 5 (Ascoli Piceno).

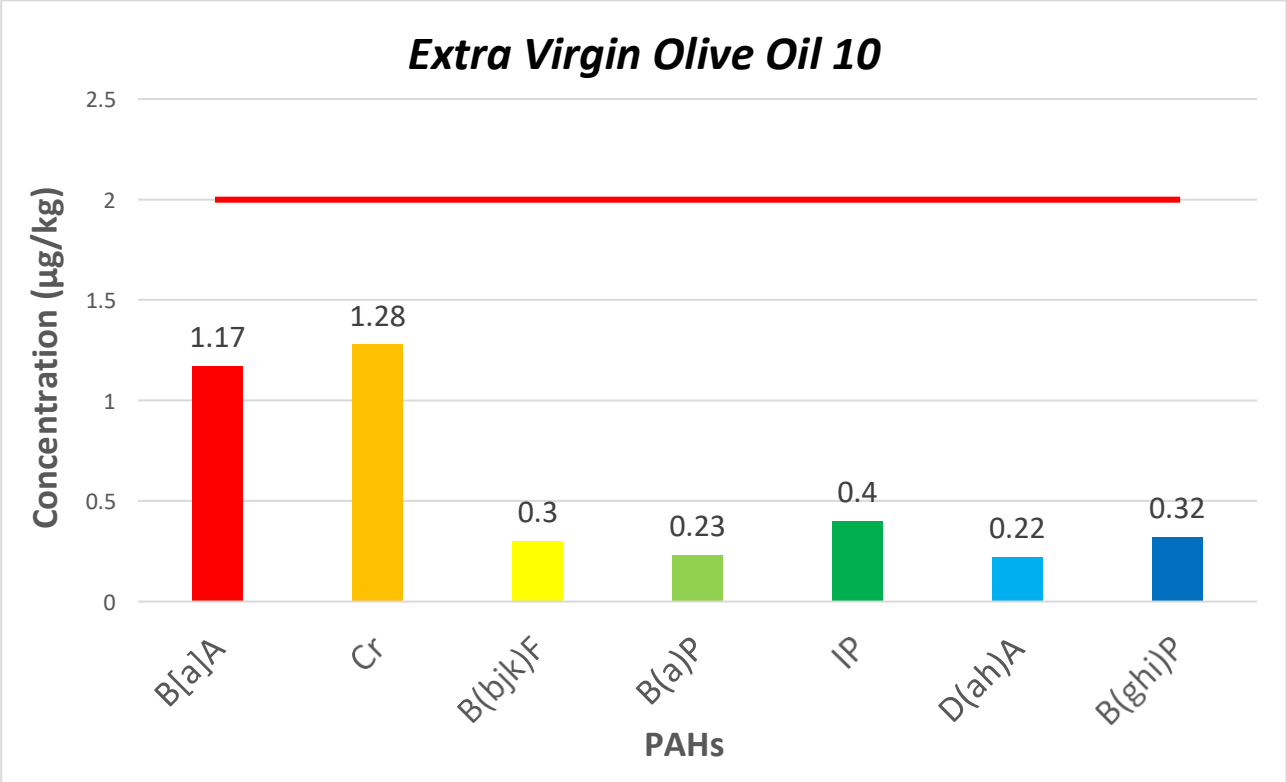


Figure 44. Sample of extra virgin olive oil, Area vasta AV 2 (Ancona).

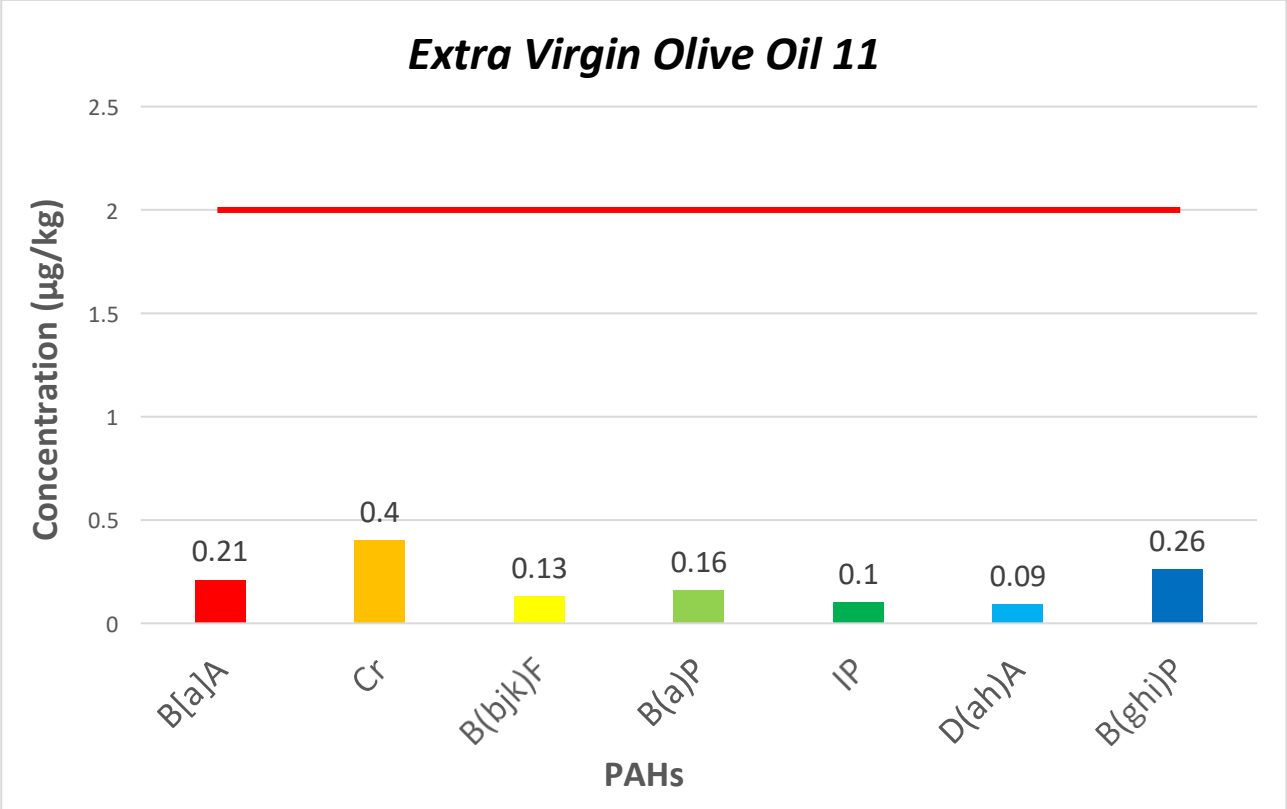


Figure 45. Sample of extra virgin olive oil, Area vasta AV 2 (Ancona).

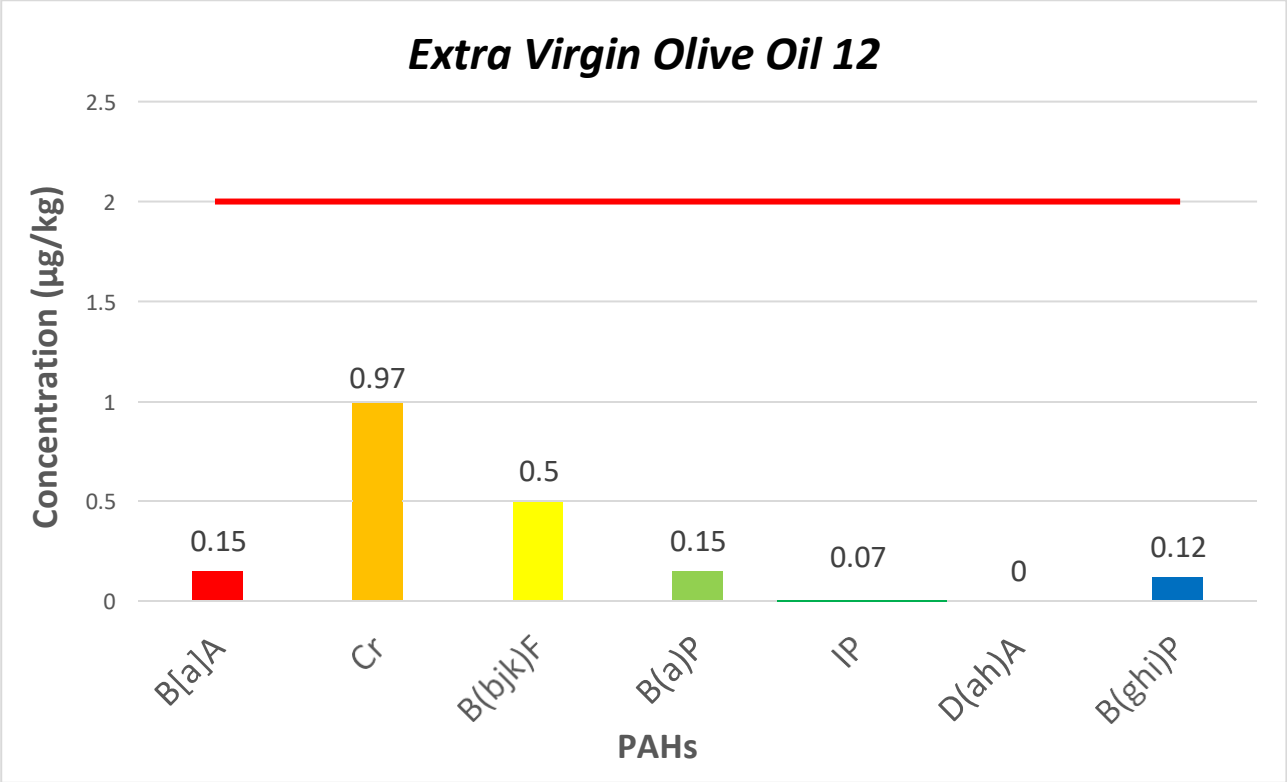


Figure 46. Sample of extra virgin olive oil, Area vasta AV 2 (Ancona).

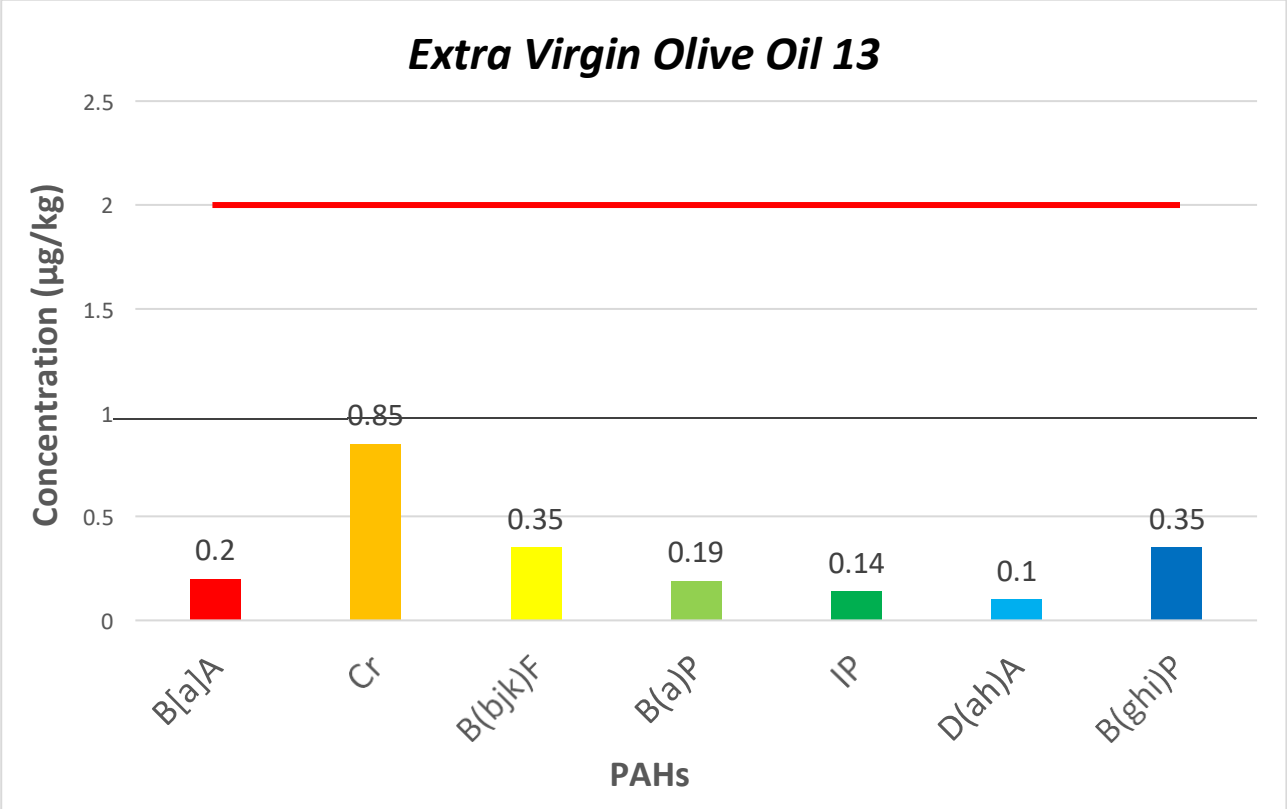


Figure 47. Sample of extra virgin olive oil, Area vasta AV 2 (Ancona).

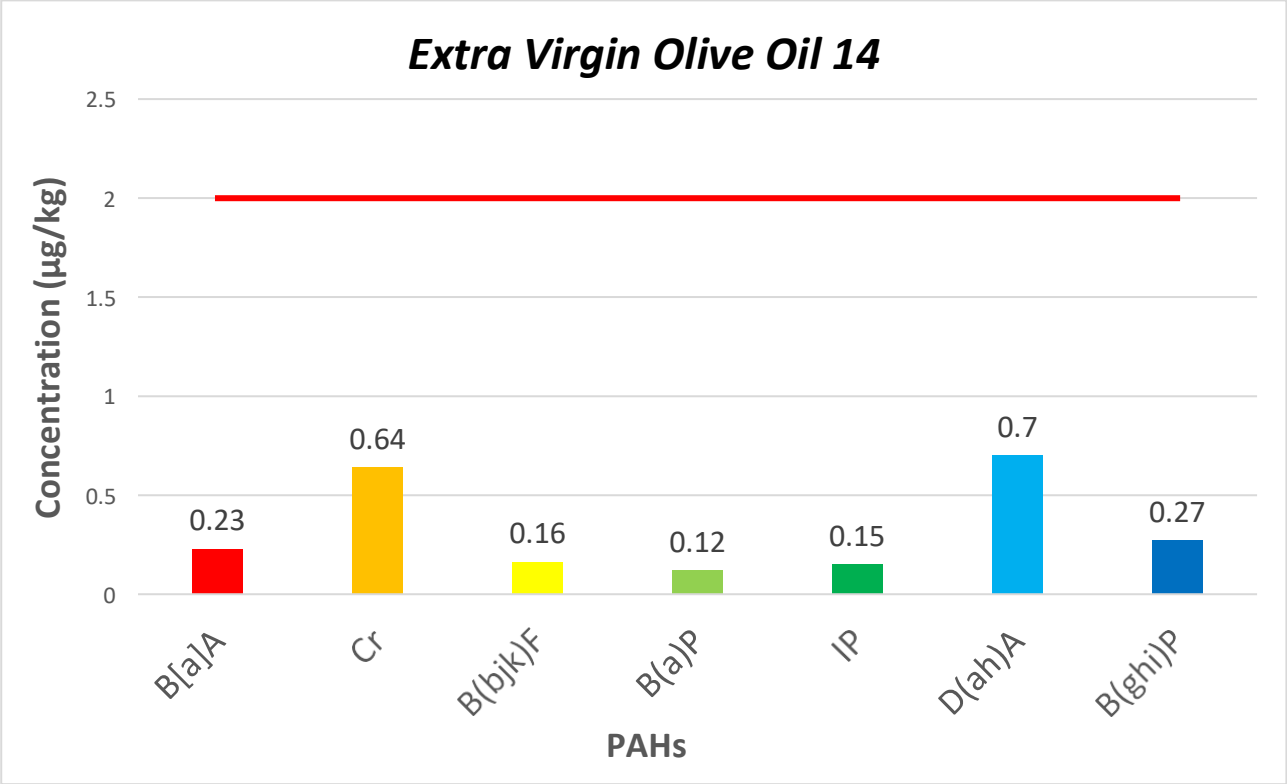


Figure 48. Sample of extra virgin olive oil, Area vasta AV 2 (Ancona).

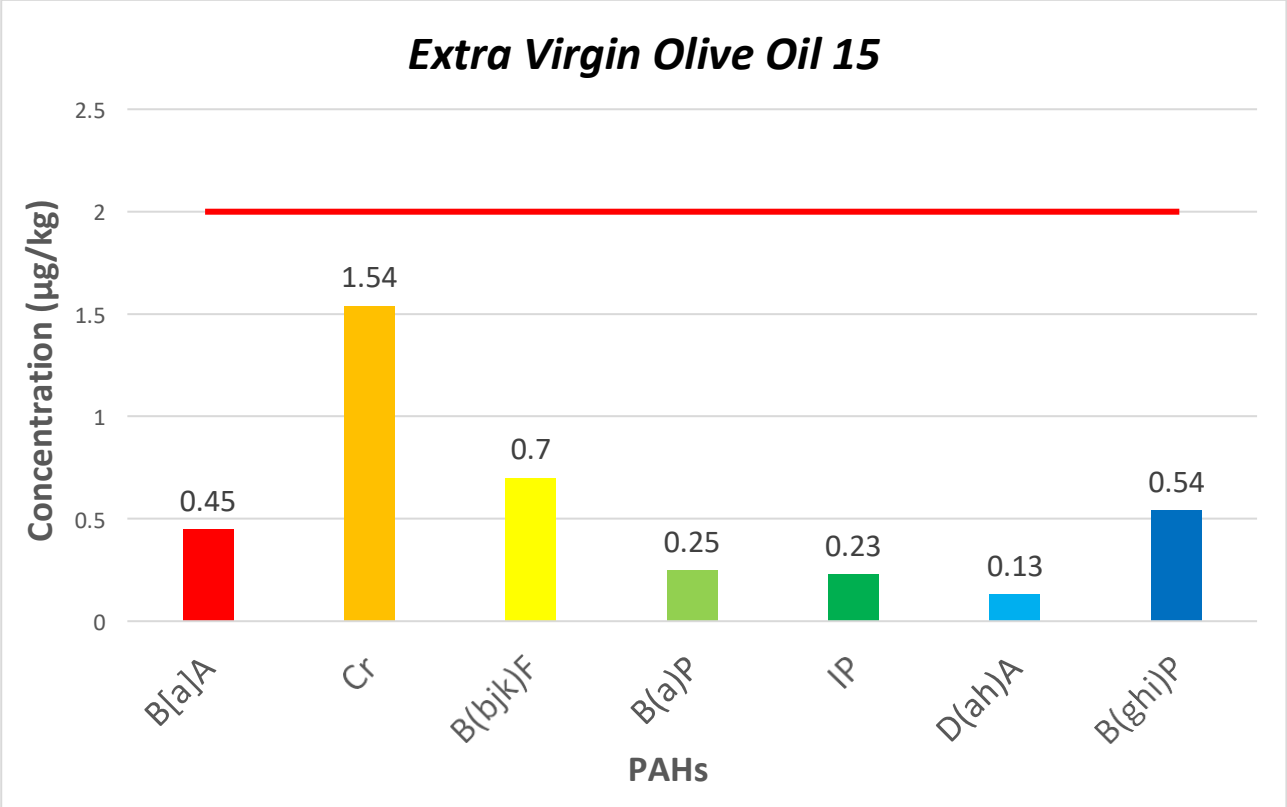


Figure 49. Sample of extra virgin olive oil, Area vasta AV 5 (San Benedetto del Tronto).

The following table (Table 23) shows the results of the light PAHs in each oil analyzed.

	Light PAHs concentrations (µg/kg)						
Extra virgin olive oil	**ACY**	**ACP**	**FLR**	**PHE**	**ANT**	**FLT**	**PYR**
Oil 1	1,99	1,74	3,67	16,38	1,08	4,9	5,46
Oil 2	0,91	0,78	2,29	9,22	0,58	2,57	4,07
Oil 3	0,6	0,58	2,1	7,54	0,5	2,43	4,1
Oil 4	0,95	1,8	2,37	9,43	0,64	2,82	4,43
Oil 5	0,82	0,61	2,45	10,66	0,64	3,31	4,84
Oil 6	0,38	0,2	1,74	7,14	0,35	5	3,92
Oil 7	0,2	0,3	3,35	6,93	1,59	2,6	3,36
Oil 8	0,67	0,31	2,61	6,26	0,55	2,7	2,8
Oil 9	0,45	0,06	1,59	4,65	0,32	3,41	1,1
Oil 10	0,26	0	1,73	4,41	0,21	4,9	2,15
Oil 11	0,23	0	1,25	4,18	0,5	2,62	4,72
Oil 12	0,21	0,01	2,64	4,65	1,3	3,3	5,07
Oil 13	1,17	0,18	2,99	8,13	1,43	4,8	4,42
Oil 14	1,16	0,15	3,06	4,37	0,3	5,01	3,65
Oil 15	0,99	1,3	1,68	4,74	0,76	4,36	5

Table 23. Concentration of light PAH in each extra virgin olive oil analyzed.

By transferring the data of the table (Table 23) in graph, we obtain the graph in figure (Figure 49) which indicates that the components with low molecular weight PAHs, in particular phenanthrene, fluoranthene and pyrene ("light" PAH), have a considerably higher concentration than those with higher molecular weight, such as benzo(a)anthracene, chrysene, benzo(bjk)fluorantenes and benzo(a)pyrene ("heavy" PAH). The cause of this has been clarified in previous investigations together with the mechanism of contamination and the mechanism of transfer of PAHs from the polluting sources to the olive drupe has been clarified in the paragraph 2.1 (Mechanism of contamination). In summary, the presence of PAHs in the oil is not due to the radical absorption of the tree, but to the atmospheric pollution to which the olives, from which the oil itself derives, have been exposed throughout their vegetative cycle, from July to November. It is useful to reiterate that the cause of the pollution of extra virgin olive oil is not to be found in the exposure of olives to sources of environmental pollution such as industrial emissions, but certainly to the polluting sources of a widespread type, such as vehicular traffic and the combustion of wood material, as responsible for the background pollution to which the olives are exposed during their vegetative cycle. Obviously the concentration of PAHs in the oil is directly proportional to the concentration of PAHs in the atmosphere of the olive grove.

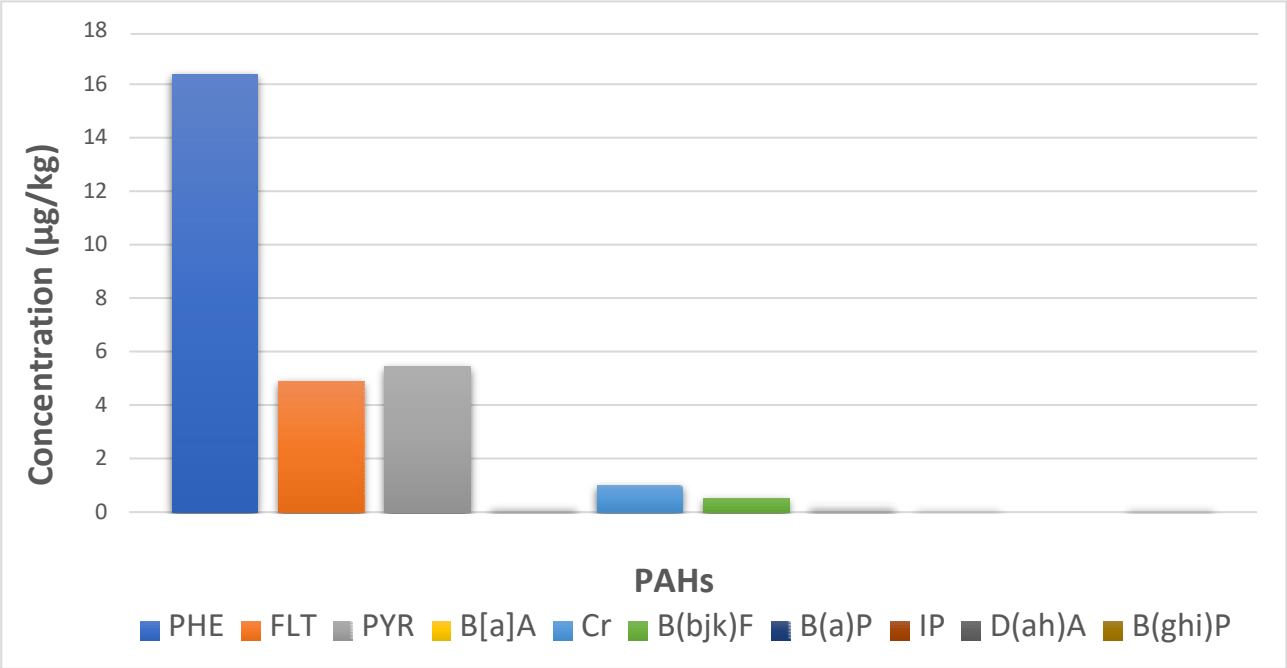


Figure 50. Total PAH (heavy and light PAH) in the oils analyzed.

Moreover, there is no significant correlation between the concentrations of light PAHs and those of heavy PAHs in extra virgin olive oils. This is due to the different diffusion in the atmosphere of the two classes of pollutants; the former travel in the form of vapors, being low boiling, and can travel considerable distances from the polluting source, while the latter travel mainly adsorbed on particulate material and therefore generally have a more limited range of influence. In light of the new legal limits imposed for PAHs in oils, it was checked whether the threshold of 10 µg/kg is exceeded, as a sum of the four components. In no case is the threshold limit exceeded. The highest value is 3 µg/kg and is also overestimated because it is obtained with the contribution of other components (benzo(*jk*)fluorantenes), as the chromatographic gas column used does not manage to separate it adequately from the corresponding isomers.

4.4 PAHs content in exhausted extra virgin olive oil

Frying is an important cooking practice that leads to the formation of substances that improve food taste and smell and of compounds that negatively affect the quality of foods. Frying is a cooking by convention and conduction in which the heat transmission medium is a fat (generally oil). The fatty substance must be resistant to high temperatures and with a high smoke point. In fact, frying is a process that puts a strain on the stability of the fat used. The reaction resulting from the set of temperatures and oxygen with the oil brought to the high temperatures required leads to the formation of harmful substances. The speed and extent of degradation (oxidation) depends on the temperature and the possible re-use of the oil for more frying, as well as the presence of previous frying residues. One of the many dangerous substances that can be formed are precisely the PAHs. The optimal temperatures for the formation of PAHs are between 660 to 740°C; however, several authors have studied the PAH concentration in cooking oil fumes (180-270°C). For this reason, some trials were carried out to investigate the fate of PAHs when oil was heated to frying temperatures. Aliquots of extra virgin olive oil previously analyzed (samples 1) and without PAHs were heated with a domestic electrical fryer at 180°C for various times. Below are shown the graphs with the concentrations of PAHs found in each oil analyzed. In particular Figure 51 shows PAH concentrations in the extra virgin olive oil after the first frying (heating time of 30 min), and Figure 52 shows PAH concentrations in the extra virgin olive oil after the fourth frying (heating time of 4 h).

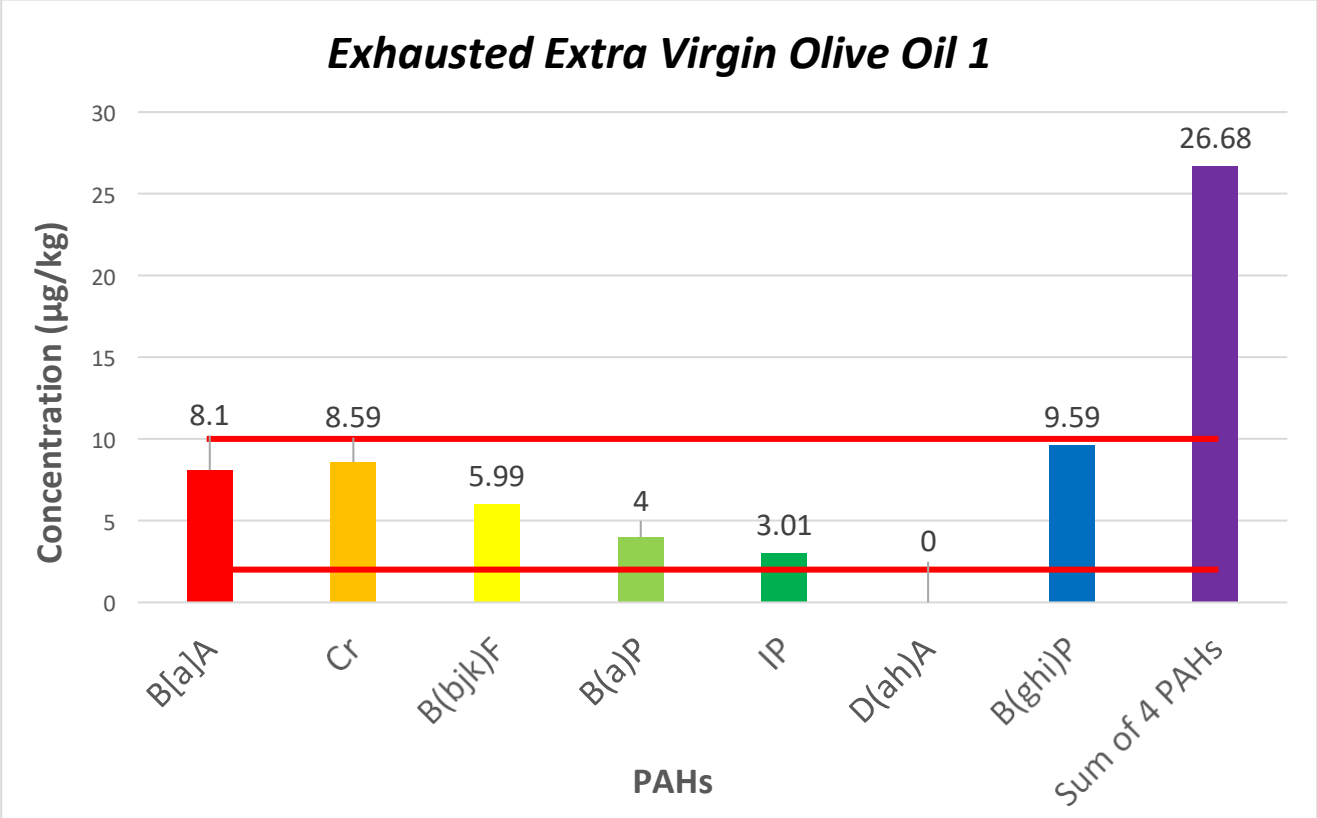


Figure 51. Sample of exhausted extra virgin olive oil.

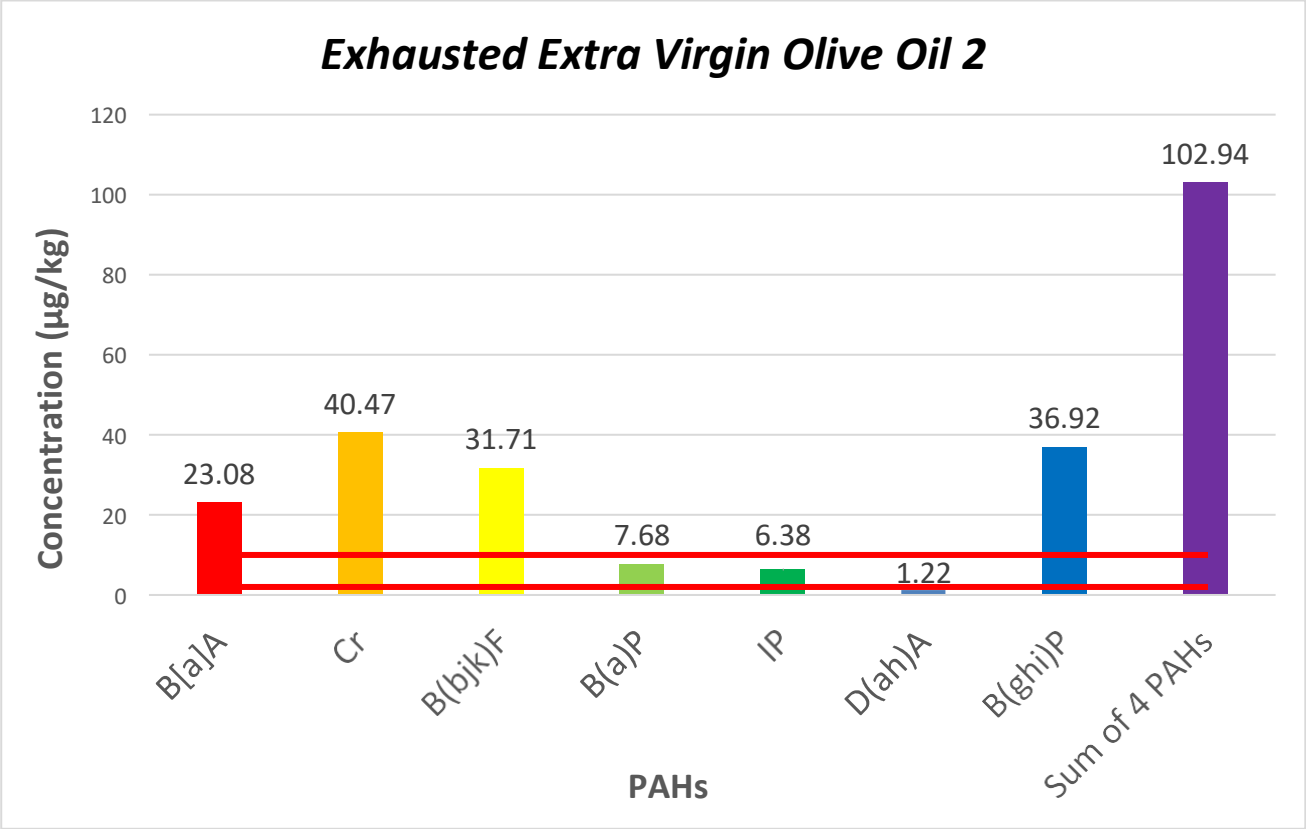


Figure 52. Sample of exhausted extra virgin olive oil.

Summing up all the data of the extra virgin olive oil before frying, after one frying and after four frying we obtain the graph below (Figure 53). The sample of extra virgin olive oil, which initially did not contain PAH or contained them in reduced quantities, has undergone a strong increase in these substances during the cooking process. In particular, it was possible to notice that there is a directly proportional relationship between frying time and the amount of IPA that are formed. PAHs concentration increased as heating time increased. As we can see from the graphs (Figure 50 and 51), the two fried oils exceeded the maximum permitted limit (10 µg/kg) set by the European Union for the sum of four PAH and they also exceeded the maximum permitted limit of 2 µg/kg for B[*a*]P. The results indicated that the cooking method significantly affected PAH emissions and frying increases the amounts of PAHs and B[a]P (4 µg/kg and 7.68 µg/kg respectively).

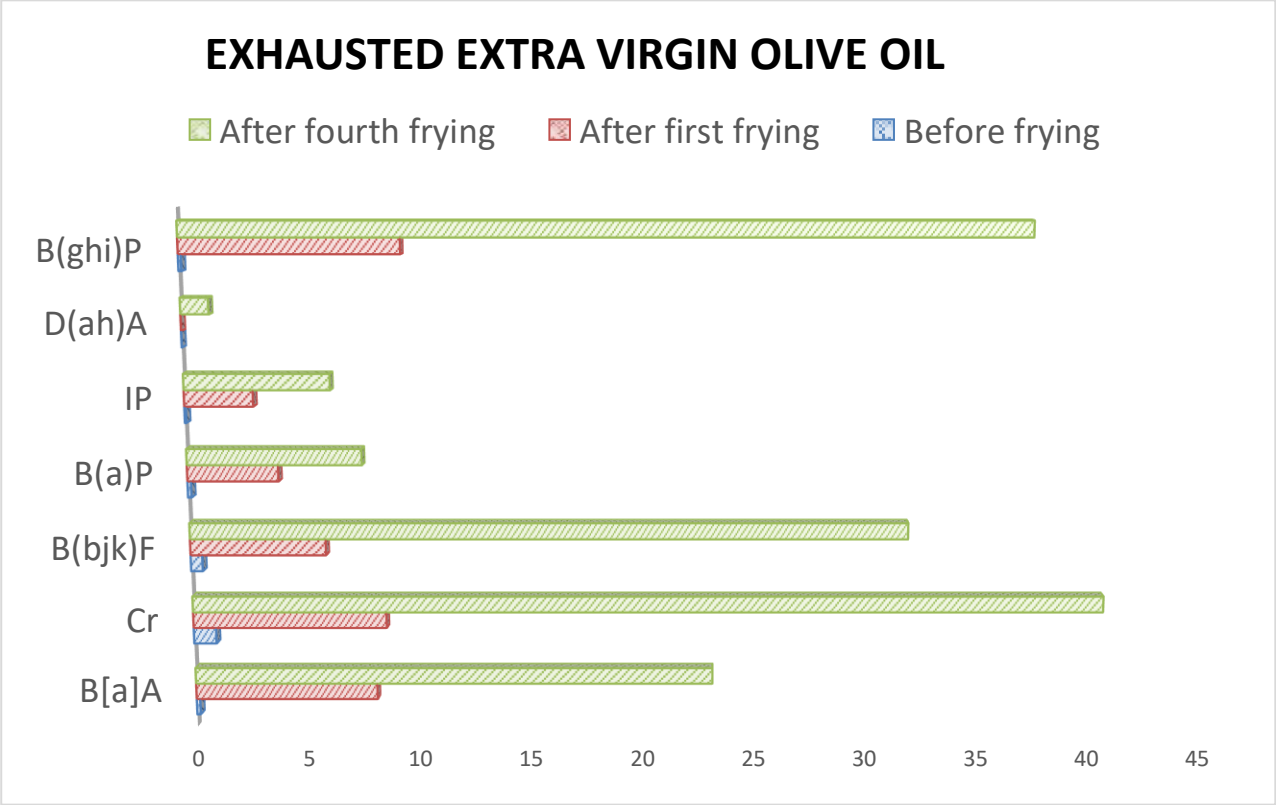


Figure 53. Sample of exhausted extra virgin olive oil before frying, after first frying and after fourth frying.

4.5 PAHs content in olive pomace oil

The pomace is the waste product of the production of oil from grinding and pressing of olives and consists of the solid parts of olive drupe remaining after oil extraction and water separation. The pomace oil extraction process is carried out with hexane on the previously dried pomace by direct contact with the hot fumes of the exhausted pomace combustion. In view of these considerations and the study of the production cycle, it is easy to identify the source and the mechanism of pollution from olive pomace oil; the smoke coming from the combustion of the exhausted pomace is in intimate contact with the raw material to which part of the combustion products, including PAHs, is released. For this reason, several samples of pomace oil taken at the regional level were analyzed. Below are shown the graphs with the concentrations of PAHs found in each olive pomace oil analyzed. Furthermore below is shown a chromatogram of a sample of olive pomace oil.

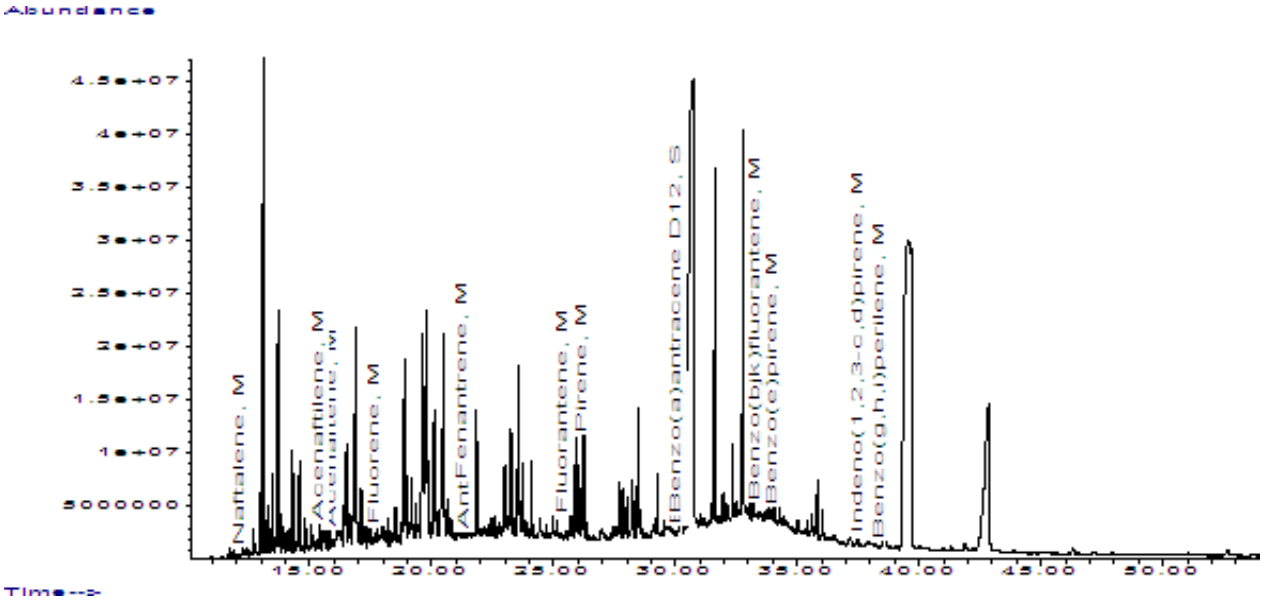


Figure 54. Chromatogram of a sample of olive pomace oil.

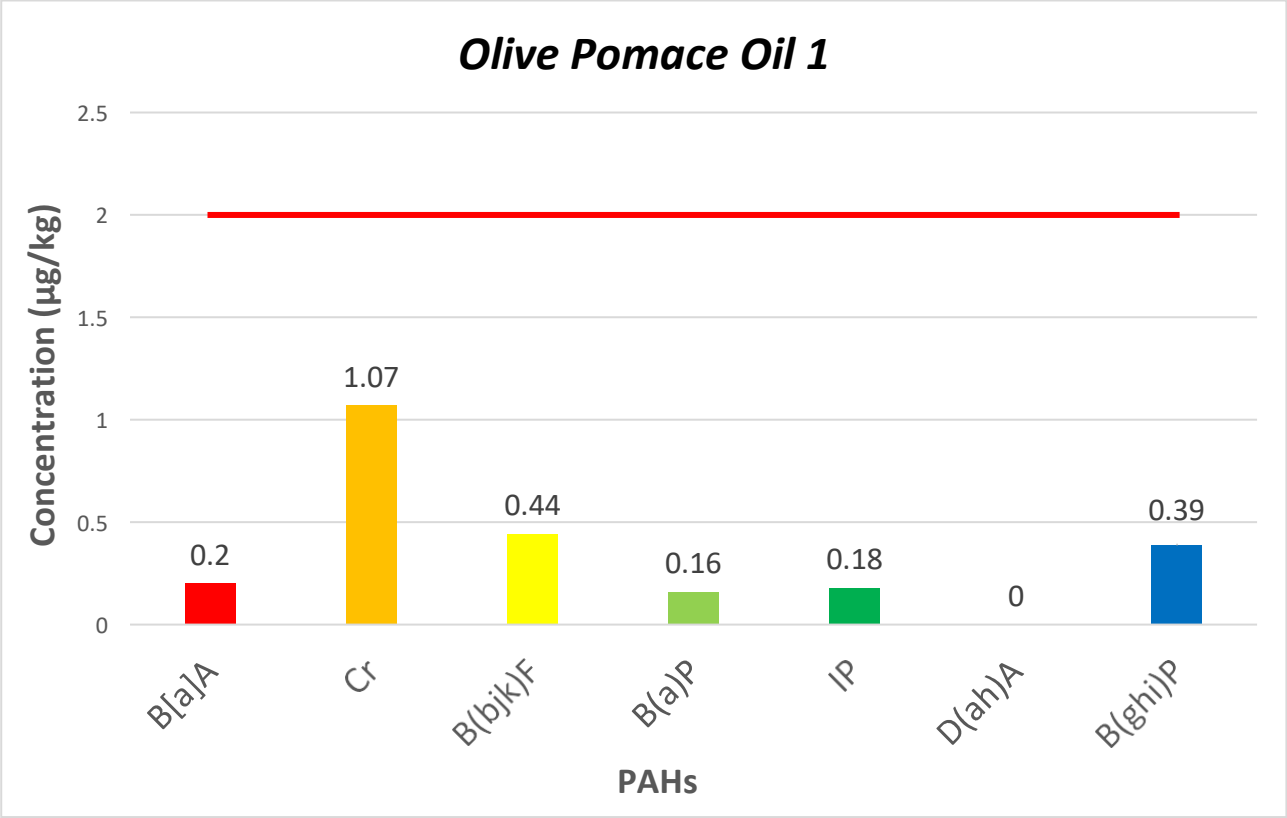


Figure 55. Sample of olive pomace oil, Area Vasta 2 (Ancona).

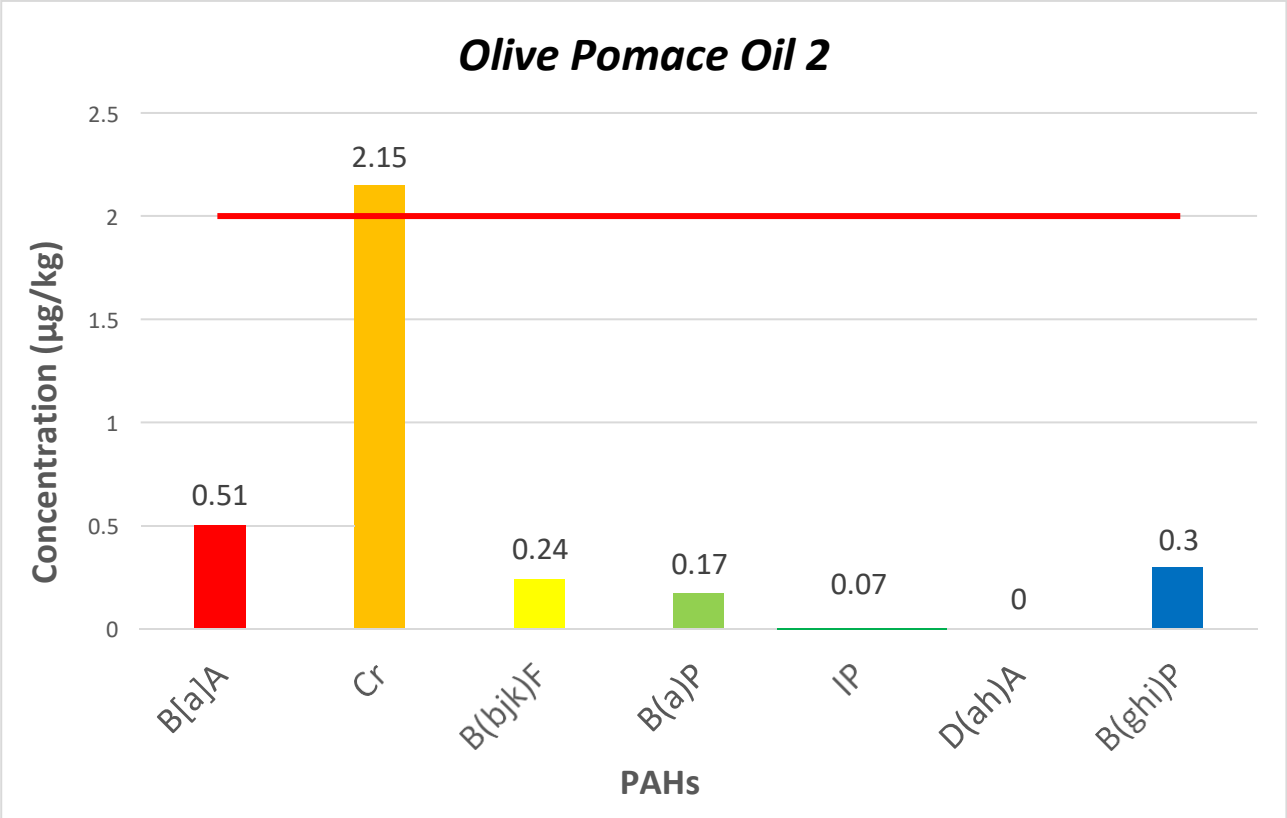


Figure 56. Sample of olive pomace oil, Area Vasta 3 (Macerata).

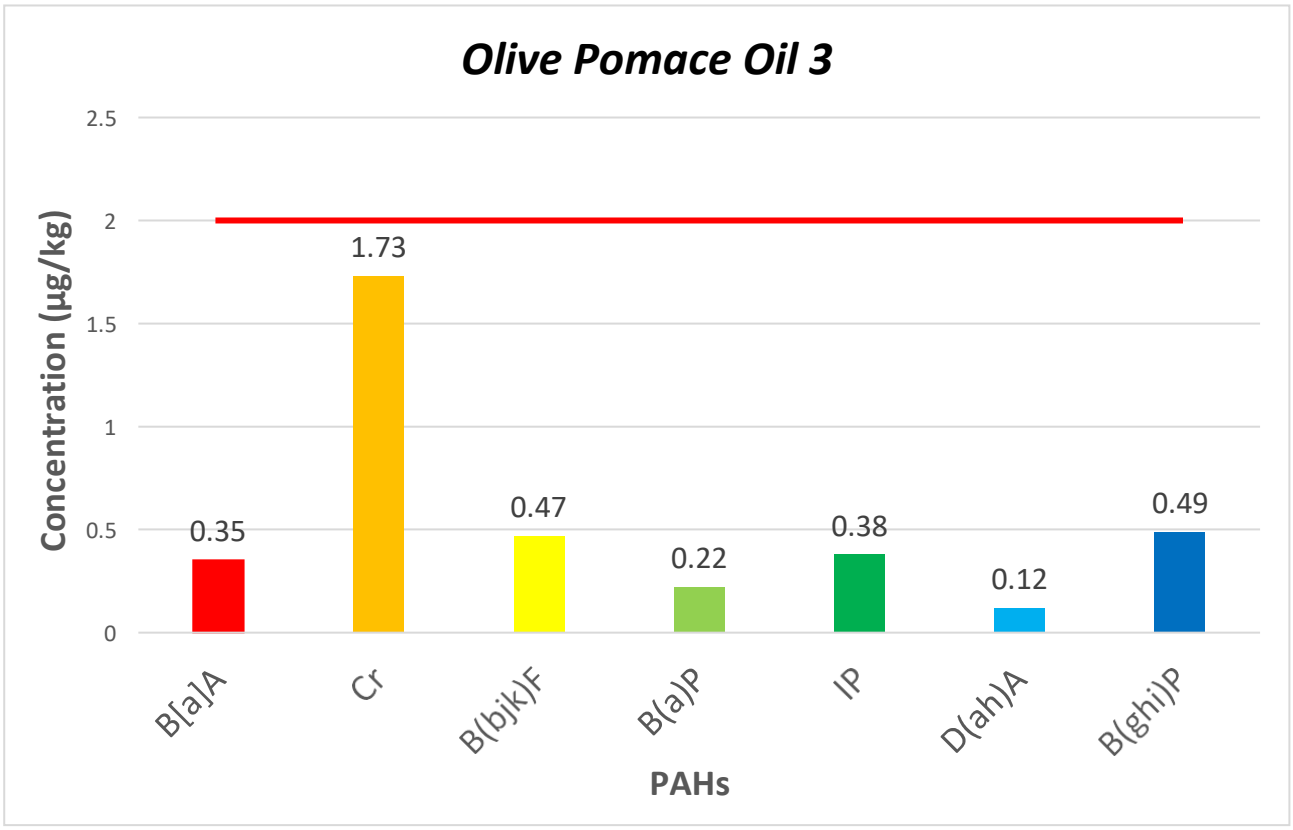


Figure 57. Sample of olive pomace oil, Area Vasta 1 (Fano).

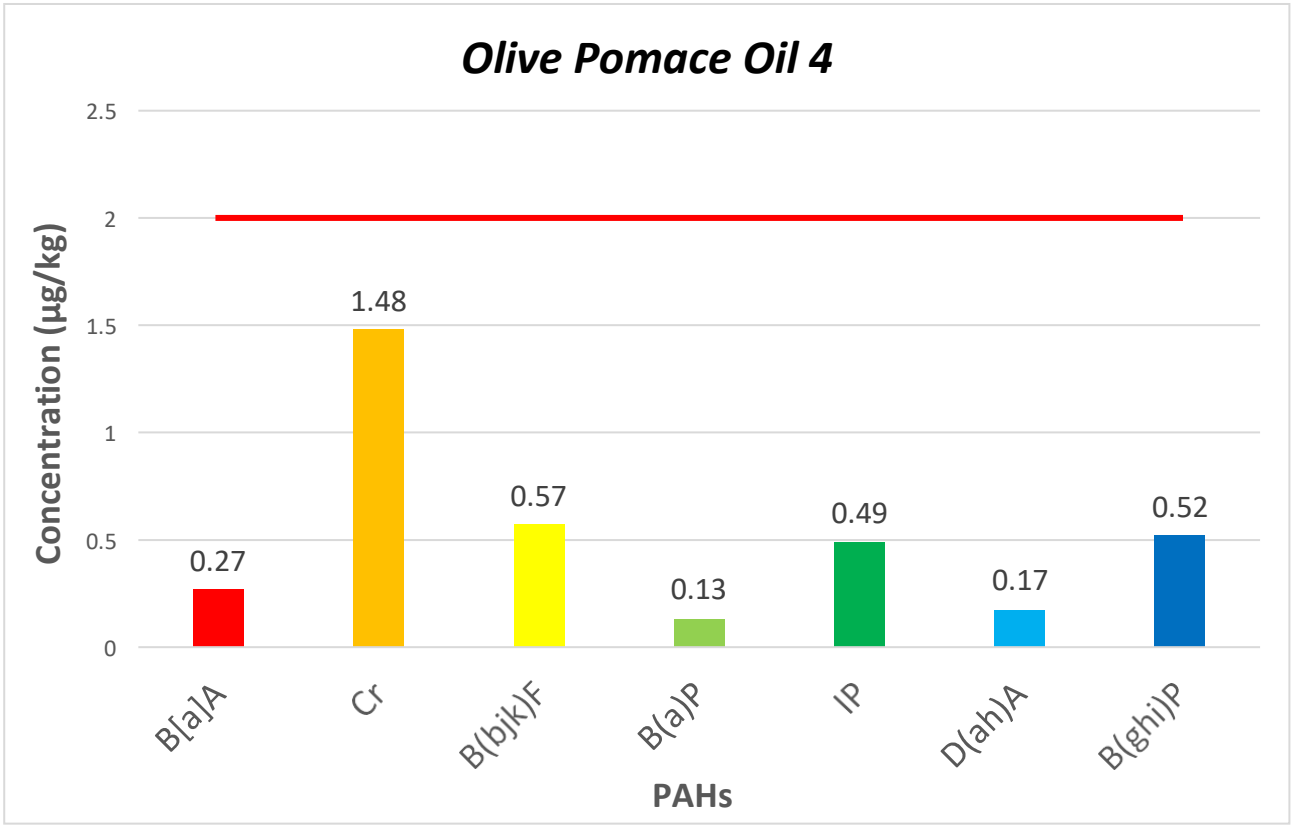


Figure 58. Sample of olive pomace oil, Area Vasta 1 (Urbino).

During olive oil production, other low-qualities oils, such as olive pomace oil, are sometimes produced. In olive pomace oil production, the dregs of crushed olives are dried and then extracted with organic solvents. For direct human consumptions, this oil needs a refining process to remove unwanted minor components and organoleptic properties. Along olive pomace oil production, PAH contamination can occur during pomace the drying and solvent extraction steps. Sometimes, the olive pomace is dried by direct contact with combustion fumes. PAH content depends highly on the conditions used prior to the oil extraction process, and can be relatively high especially if harsh conditions are used. Nevertheless, PAH content is generally reduced during refining, either by the bleaching step, where the use of activated carbon and clay is recommended to remove the heaviest PAH, either by the deodorizing stage, where light PAH can be reduced together with other compounds, such as carotenoid pigments. A broad range of concentrations has been found in the oils investigated and this can be attributed to the more or less thrust rectification to which they have been subjected. If the final rectification is not effective, PAHs remain in the final product (pomace oil) intended for human consumption. Anyway, under no circumstances is the threshold limit of 10 µg/kg exceeded for the sum of the four components and the limit of 2 µg/kg for benzo[*a*]pyrene was also not exceeded.

4.6 PAHs content in cocoa

The manufacturing process of chocolate starts with the harvest of the ripe cocoa pods, culling of the cocoa beans from the pods and fermenting them. Afterwards the cocoa beans are dried, roasted, and winnowed. The collected cocoa nibs are alkalised and ground. Then the resulting cocoa liquor is blended with the other ingredients such as cocoa butter, sugar, milk, or emulsifiers and mechanically treated until the desired texture is reached. Conching, another mechanical treatment, and tempering follow next. Finally, the chocolate is moulded and packed. Within this manufacturing process are some critical steps during which cocoa and as a consequence chocolate may be contaminated with polycyclic aromatic hydrocarbons (PAH). The most critical step is drying of the cocoa seeds. Cocoa butter contains higher levels of PAH than other oils and fats. This is mainly due to inappropriate drying practices of the cocoa. In seven samples of cocoa we quantified the amount of PAHs present and below are shown the graphs with the concentrations of PAHs found in each cocoa sample analyzed. It should be recalled that Commission regulation (EC) 835/2011 has set a maximum level of 5 µg/kg fat for benzo[a]pyrene (BaP) in cocoa beans and derived products and has set a maximum level of 30 µg/kg fat for the sum of 4 PAHs (benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene and chrysene). The limit of 5 ppb is marked with a red line in the graphs below. Again a chromatogram of a sample of cocoa is shown.

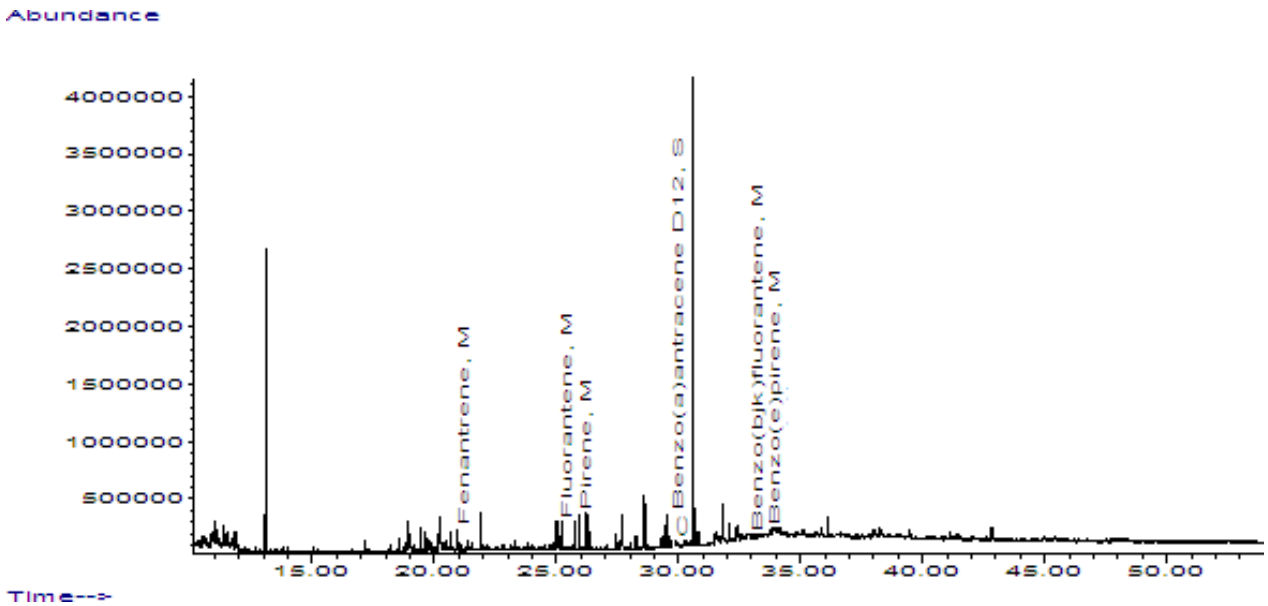


Figure 59. Chromatogram of a sample of cocoa.

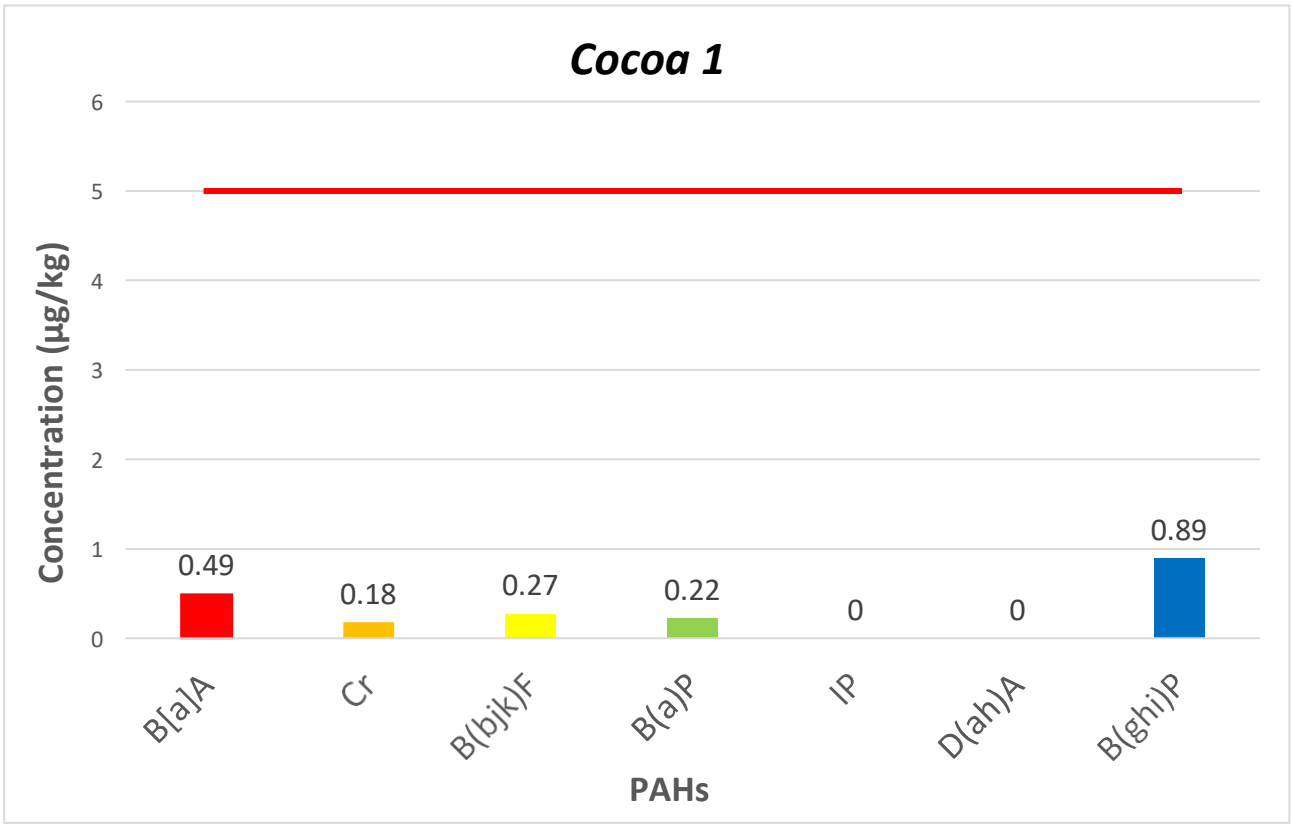


Figure 60. Sample of cocoa, Area Vasta 1 (Urbino).

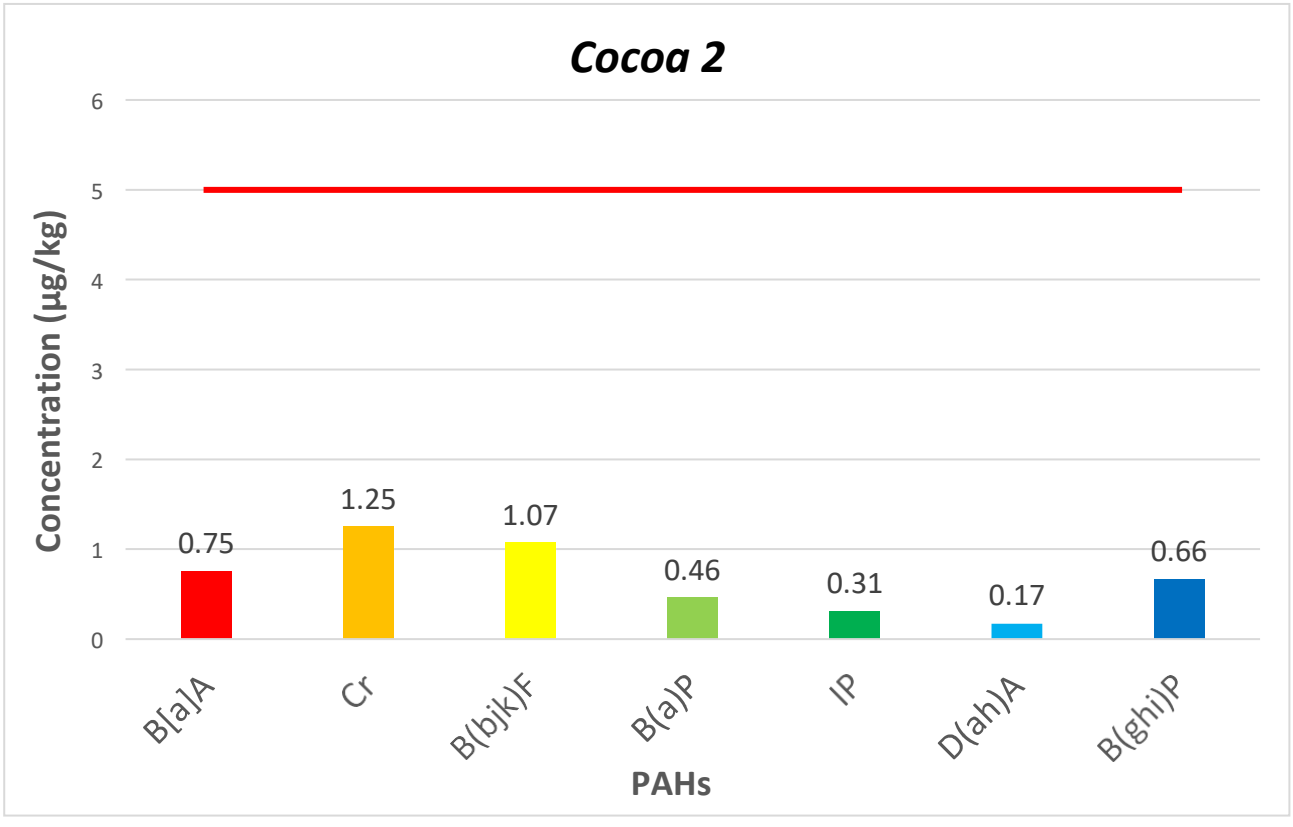


Figure 61. Sample of cocoa, Area Vasta 5 (Ascoli Piceno).

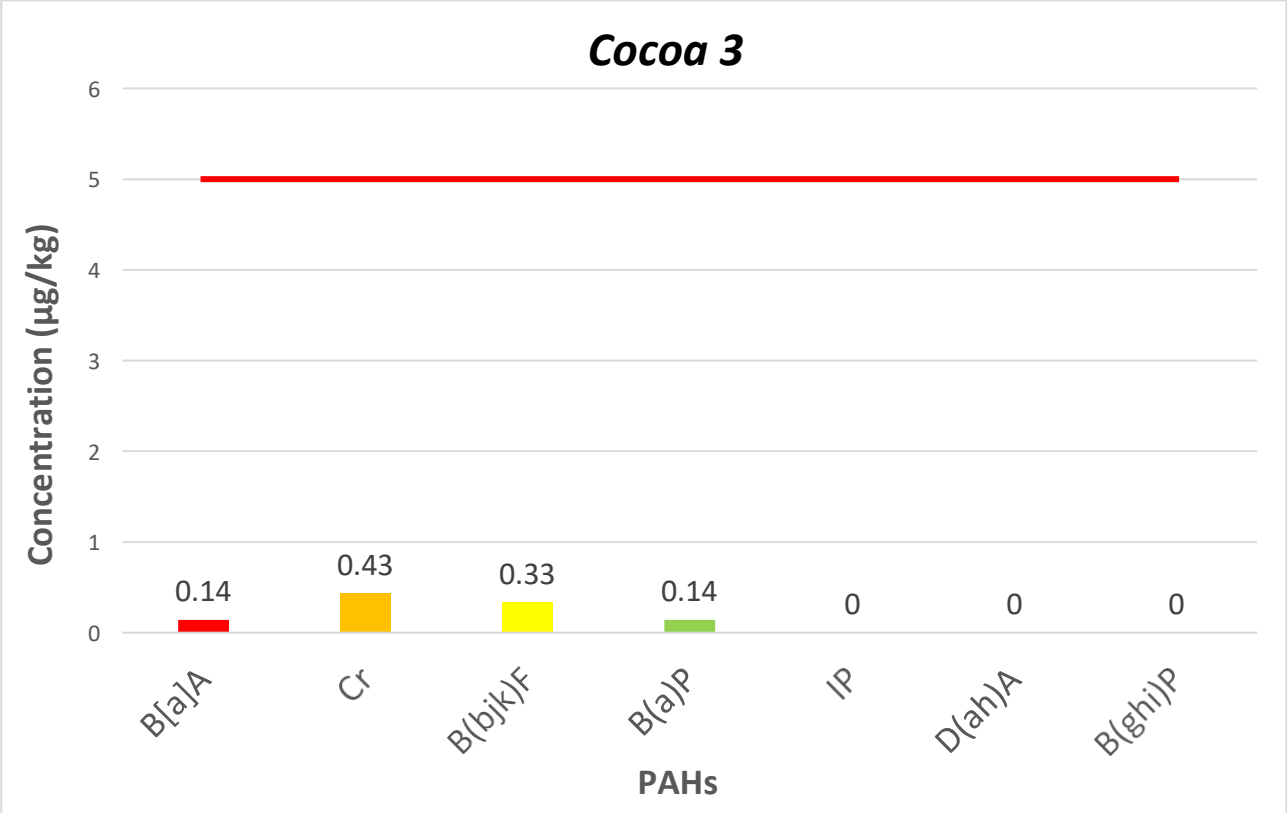


Figure 62. Sample of cocoa, Area Vasta 5 (San Benedetto del Tronto).

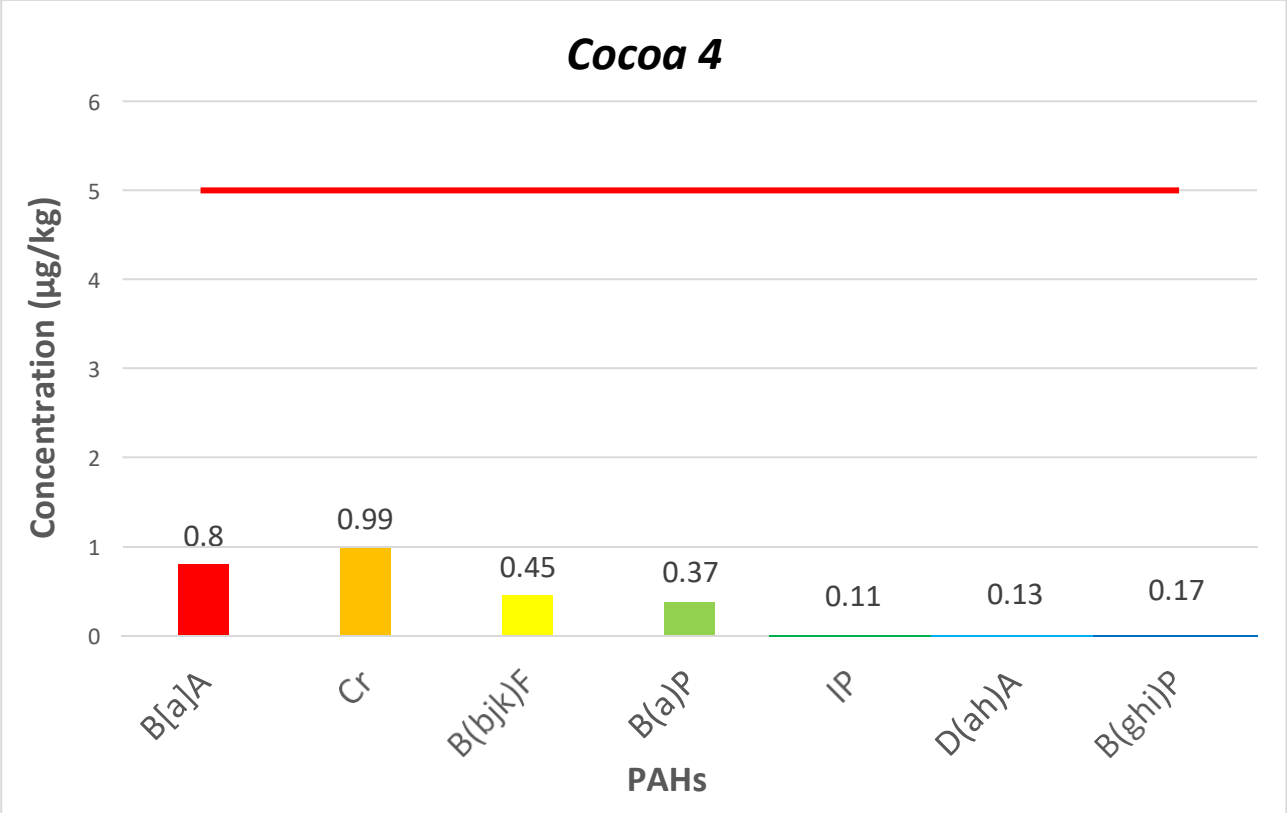


Figure 63. Sample of cocoa, Area Vasta 1 (Urbino).

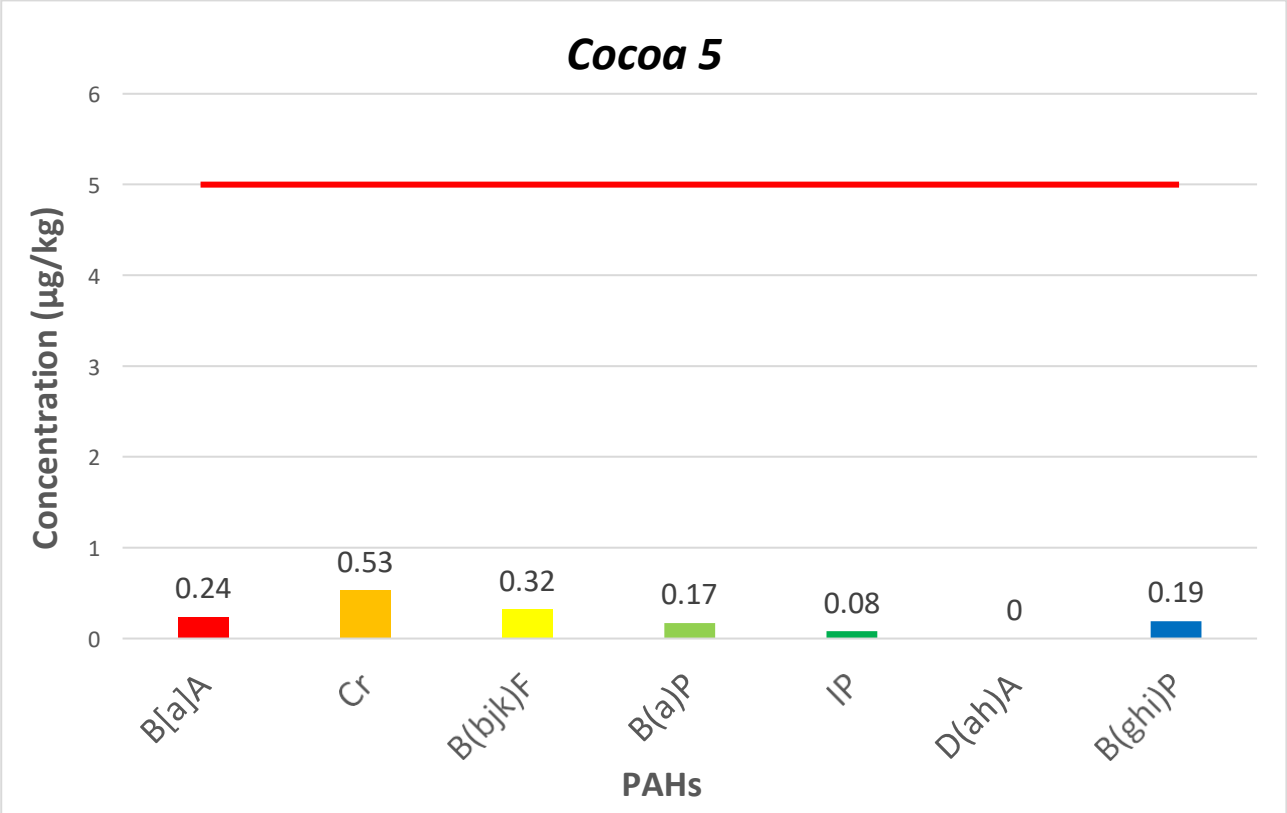


Figure 64. Sample of cocoa, Area Vasta 5 (Ascoli Piceno).

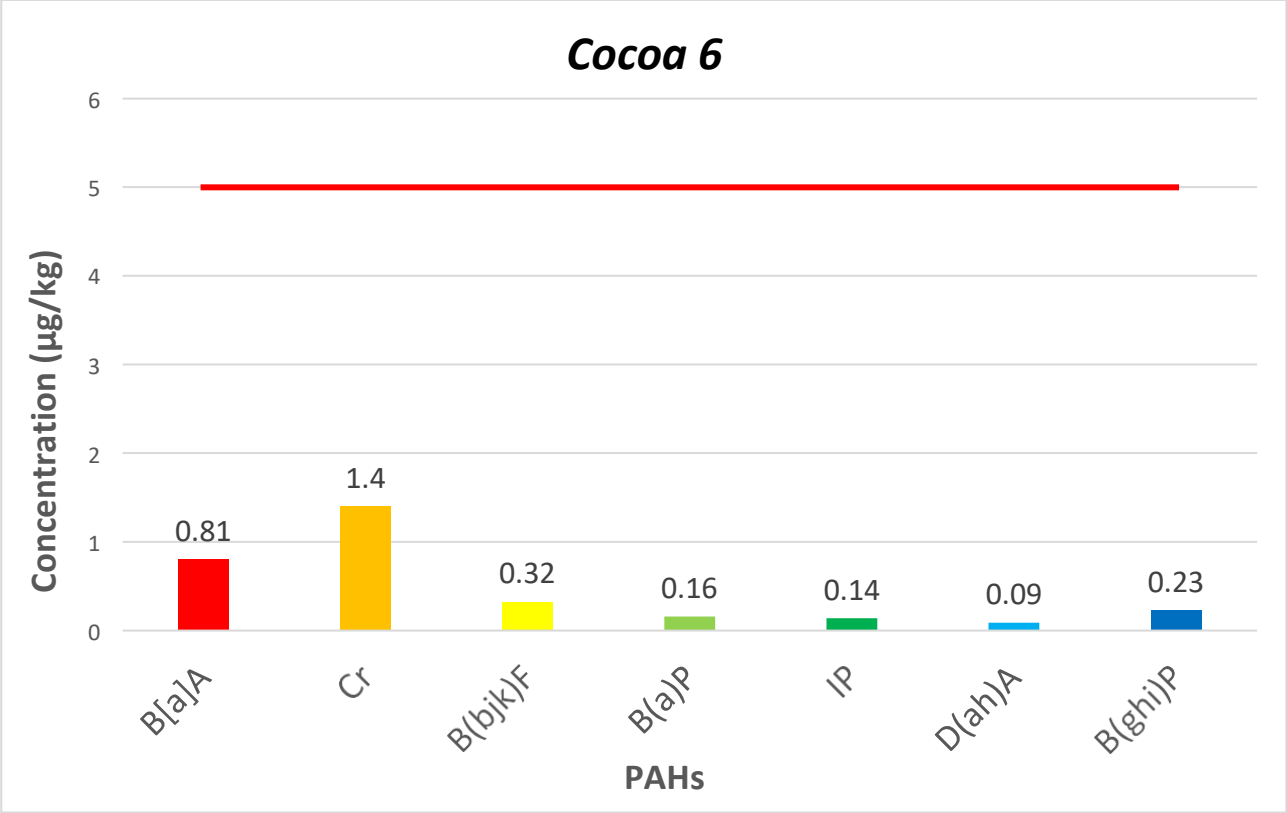


Figure 65. Sample of cocoa, Area Vasta 3 (Macerata).

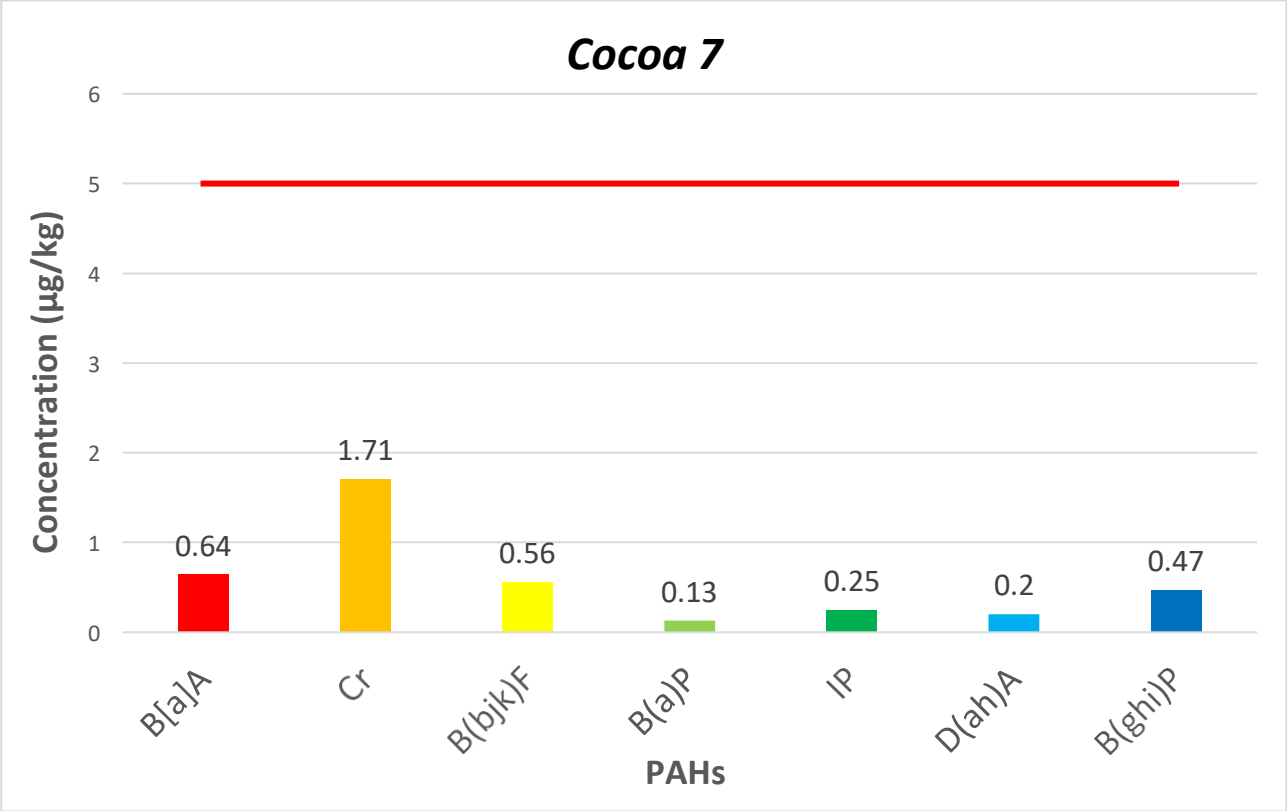


Figure 66. Sample of cocoa, Area Vasta 2 (Ancona).

Most PAHs content in cocoa bean originated from outside penetrate into cocoa cotyledon. The PAHs were supposed to come from smoke produced from wood and or/ fuel burning. Cocoa bean shell effectively absorbs the PAHs passing through and contacting with it. These evidences showed a risk for the PAH contamination in cocoa bean cotyledon from the contaminant in their shell. There is the possibility that the PAH compounds may be formed during cocoa beans drying and fermentation. From the results obtained it should be noted that the assigned analyte contents for B[*a*]P and the sum of four PAHs, expressed on fat basis did not exceed the above mentioned maximum levels. The B[a]P level detected in the analyzed cocoa beans was much lower than the 5 ppb maximum limit set by the European Union for cocoa infact the B[*a*]P contents in cocoa ranged between 0.13 and 0.46 µg/kg. The highest PAH contents were found for chrysene (0.18 to 1.71 µg/kg). In summary, it is noted that the current PAH contamination level of cocoa products can be deemed very slight overall.

4.7 PAHs content in early childhood products

For the protection of the health of infants and children, which is a vulnerable group, it is appropriate to define maximum levels at the lowest levels obtainable. For this reason Regulation 2005/108/EC specifies a maximum level of 1.0 µg/kg for BaP and also for the sum of 4 PAHs in processed cereal-based foods and baby foods for infants and young children, in infant formulae and follow-on formulae, including infant milk and follow-on milk, and in dietary foods for special medical purposes intended specifically for infants. Below are shown the graphs with the concentrations of PAHs found in each cereal-based product for children analyzed and a chromatogram of a sample of childhood product.

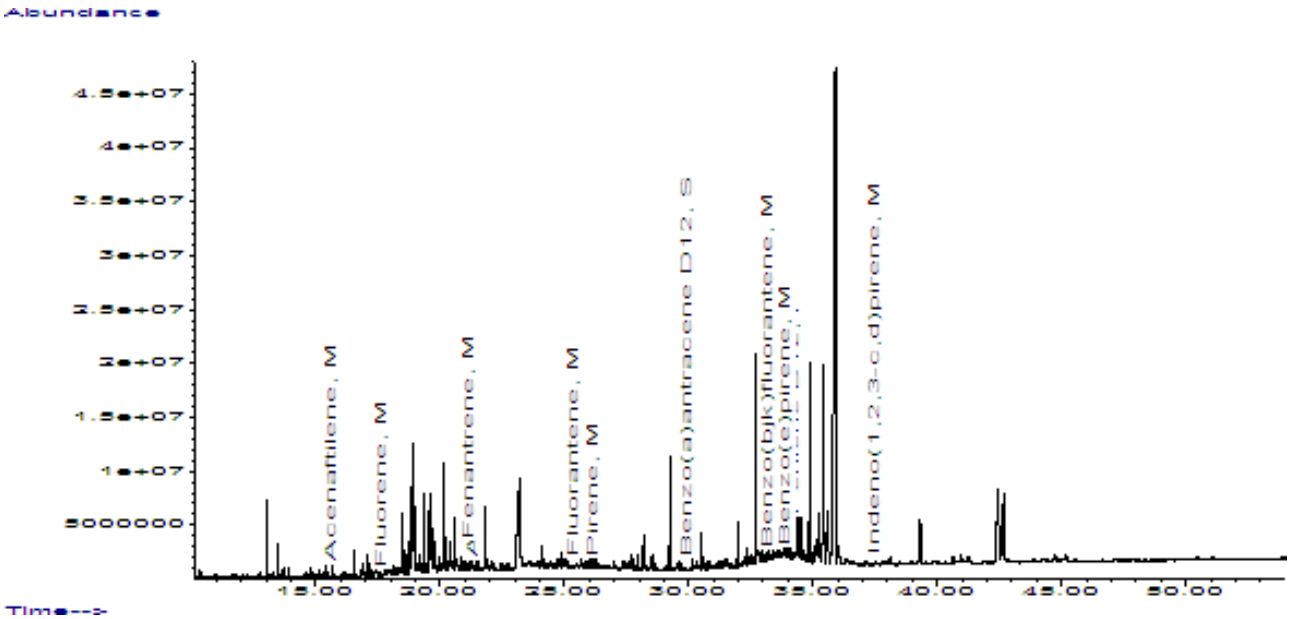


Figure 67. Chromatogram of a sample of cereal-based product for children.

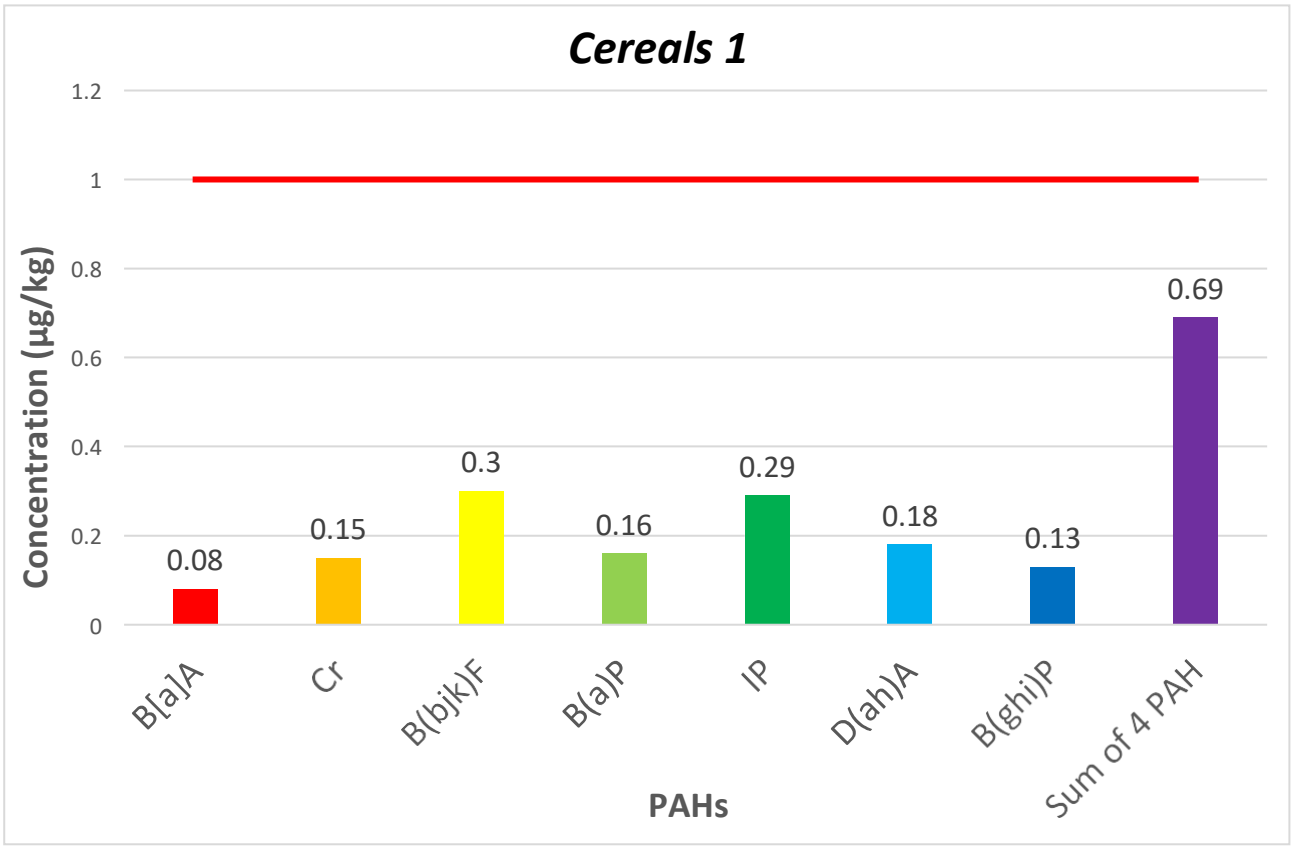


Figure 68. Sample of cereal-based childhood product, Area Vasta 3 (Macerata).

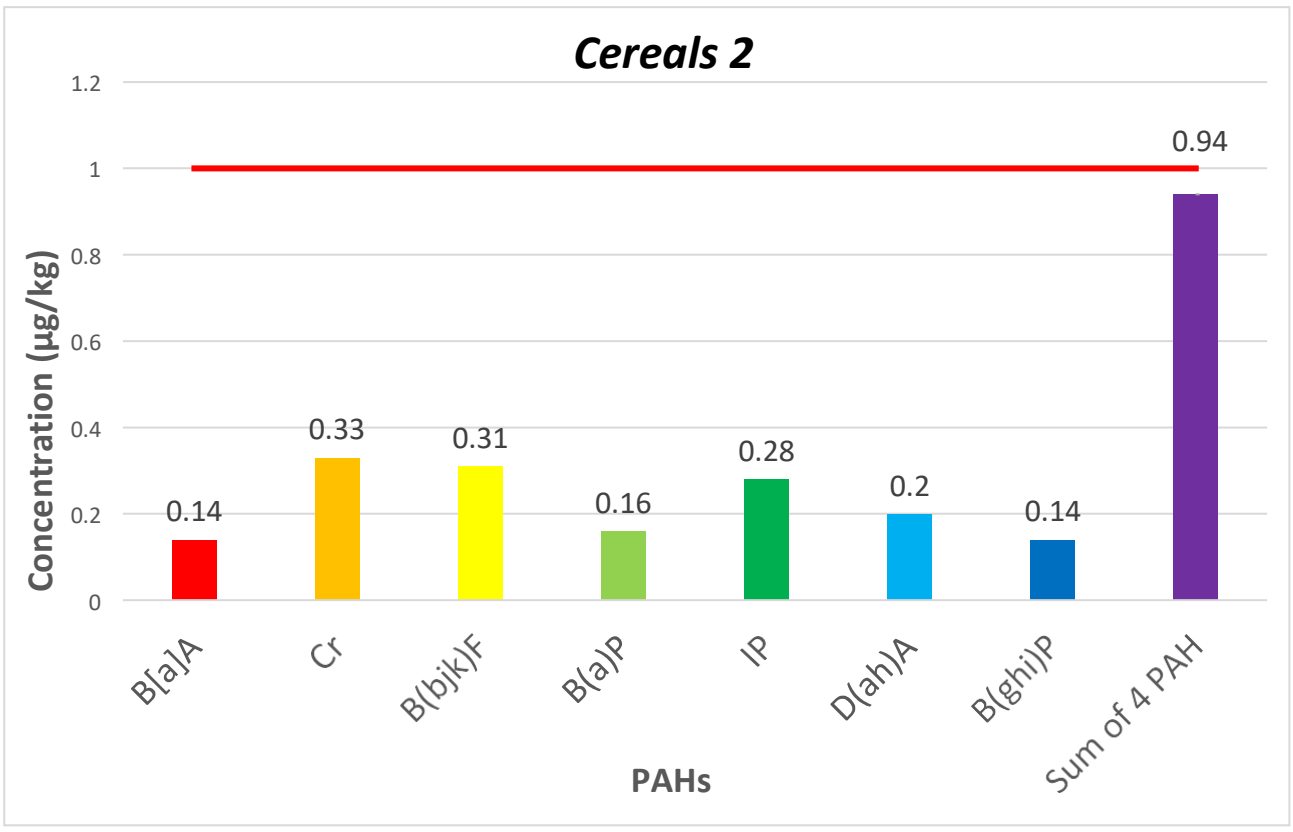


Figure 69. Sample of cereal-based childhood product, Area Vasta 5 (Grottammare).

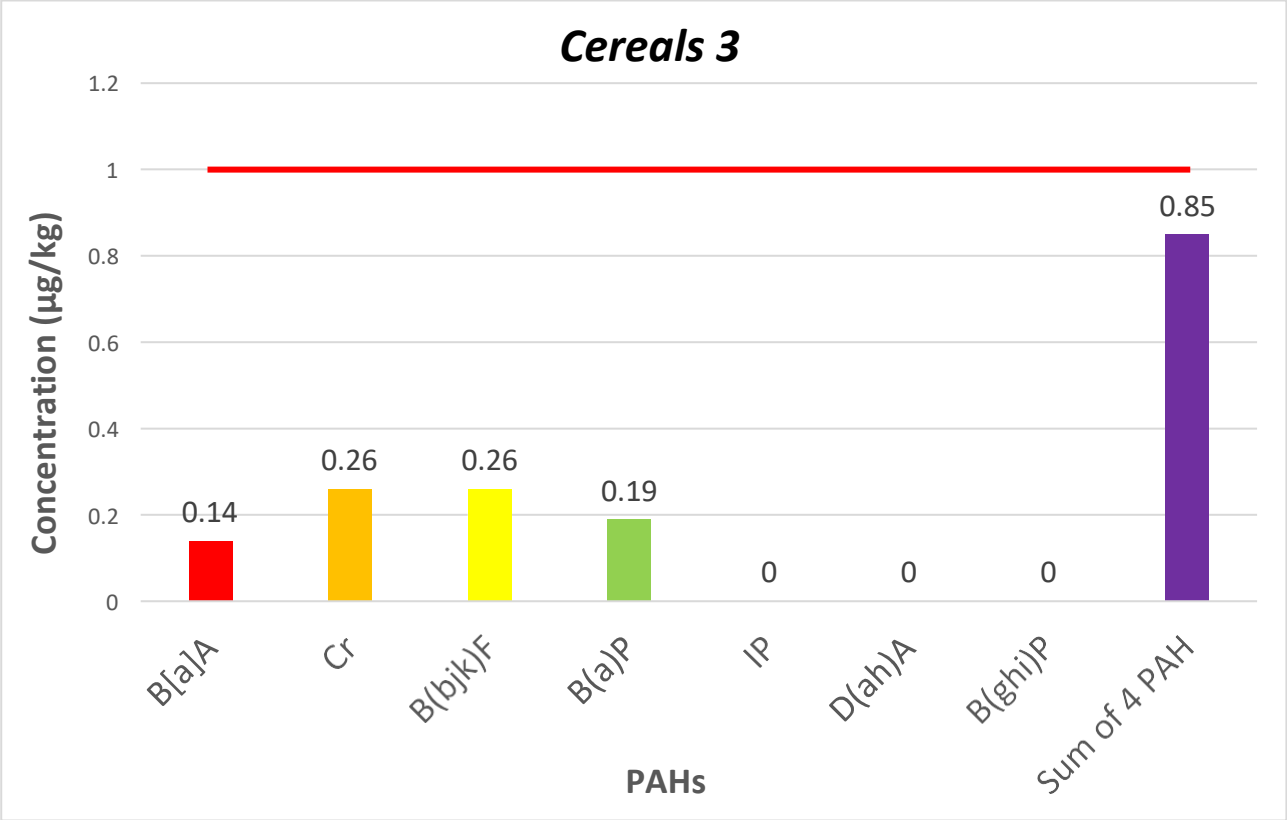


Figure 70. Sample of cereal-based childhood product, Area Vasta 5 (San Benedetto del Tronto).

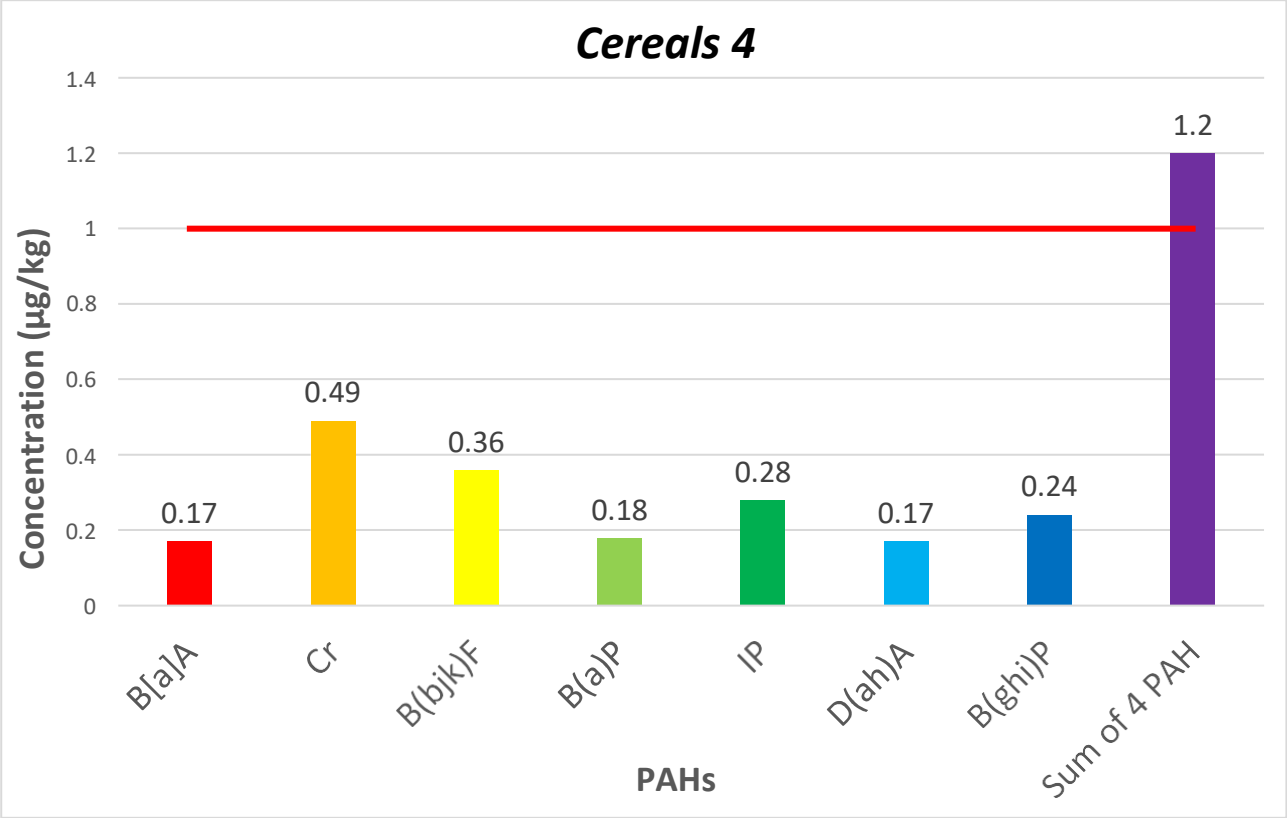


Figure 71. Sample of cereal-based childhood product, Area Vasta 2 (Senigallia).

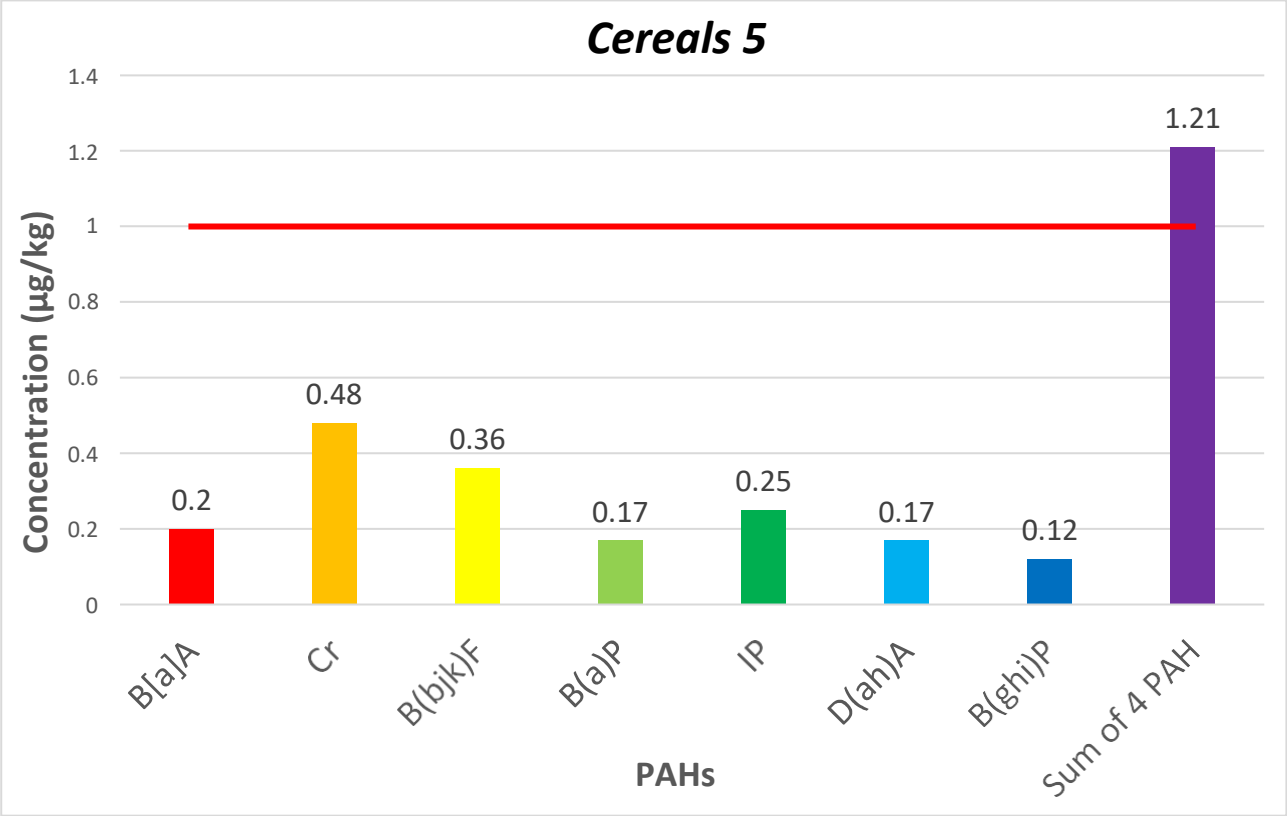


Figure 72. Sample of cereal-based childhood product, Area Vasta 5 (Ascoli Piceno).

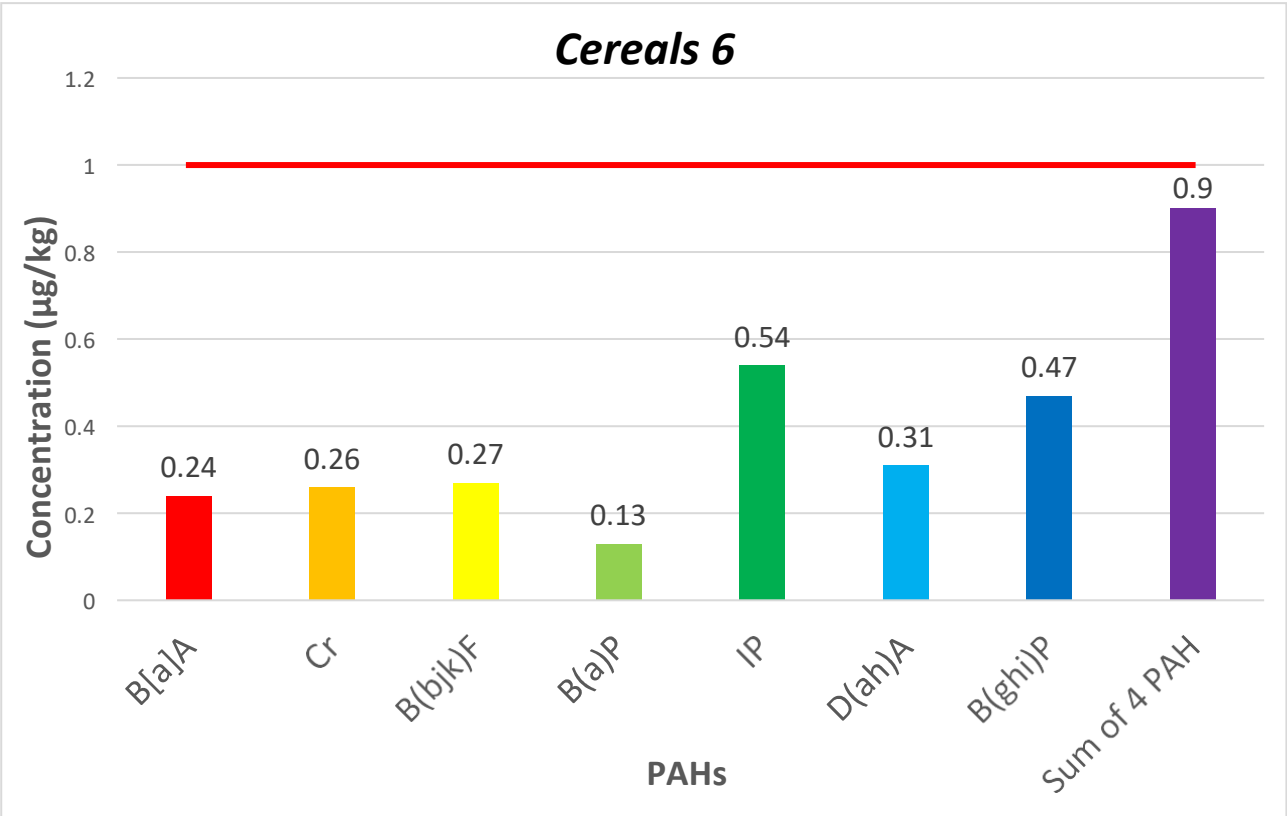


Figure 73. Sample of cereal-based childhood product, Area Vasta 1 (Urbino).

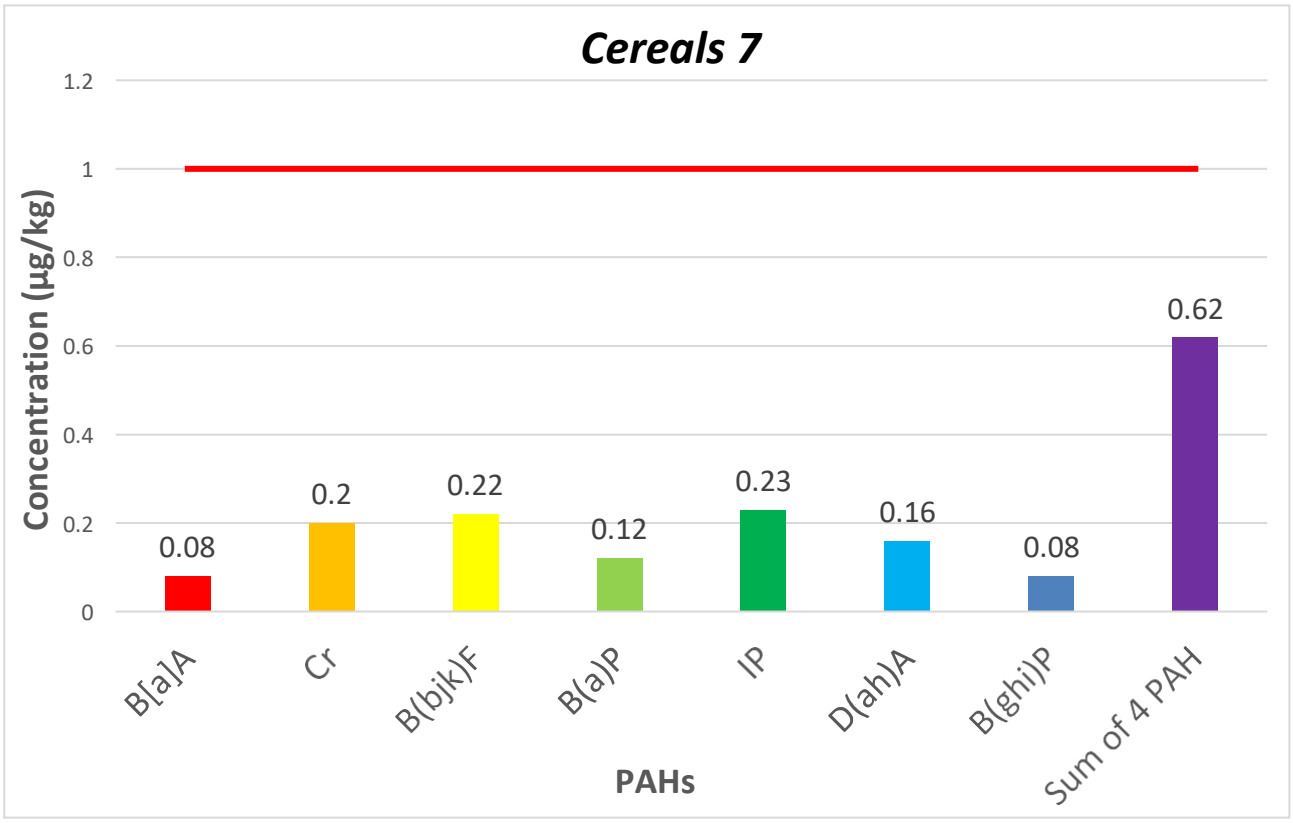


Figure 74. Sample of cereal-based childhood product, Area Vasta 3 (Macerata).

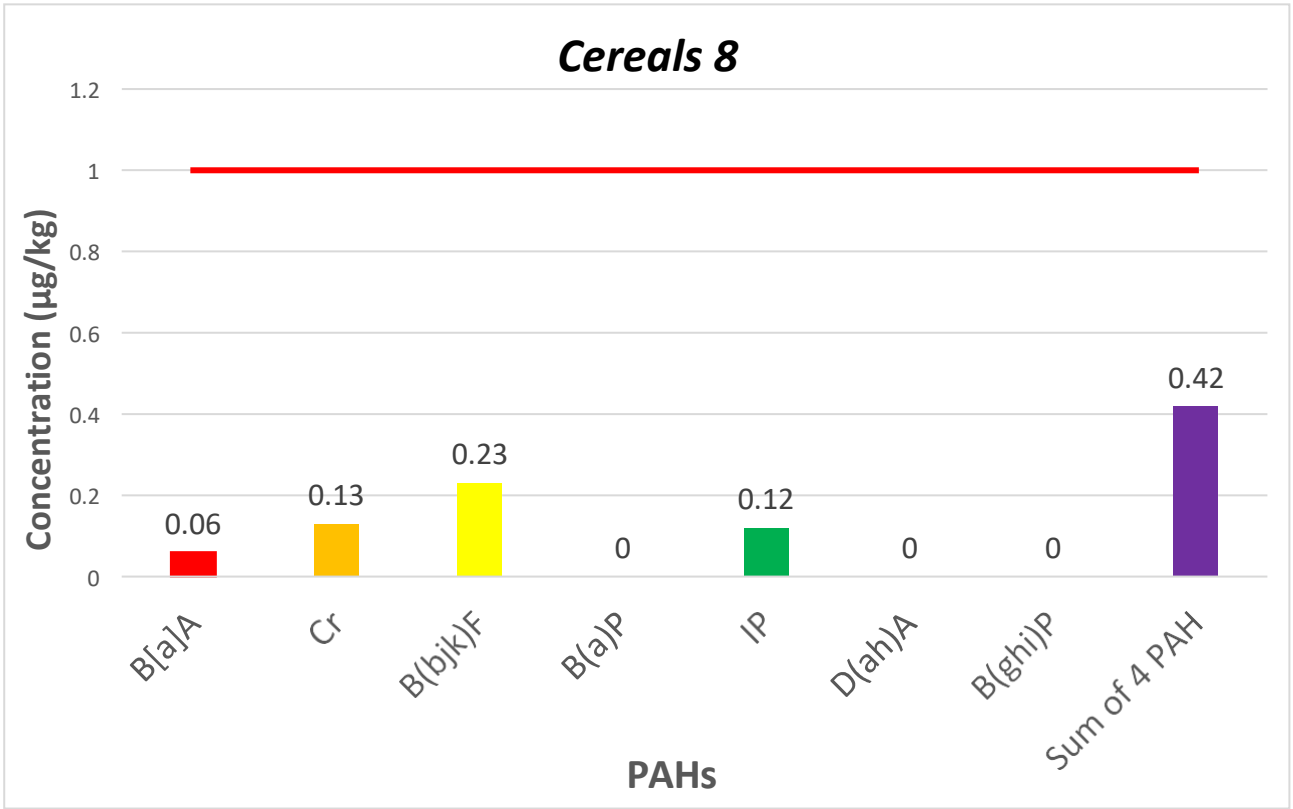


Figure 75. Sample of cereal-based childhood product, Area Vasta 5 (San Benedetto del Tronto).

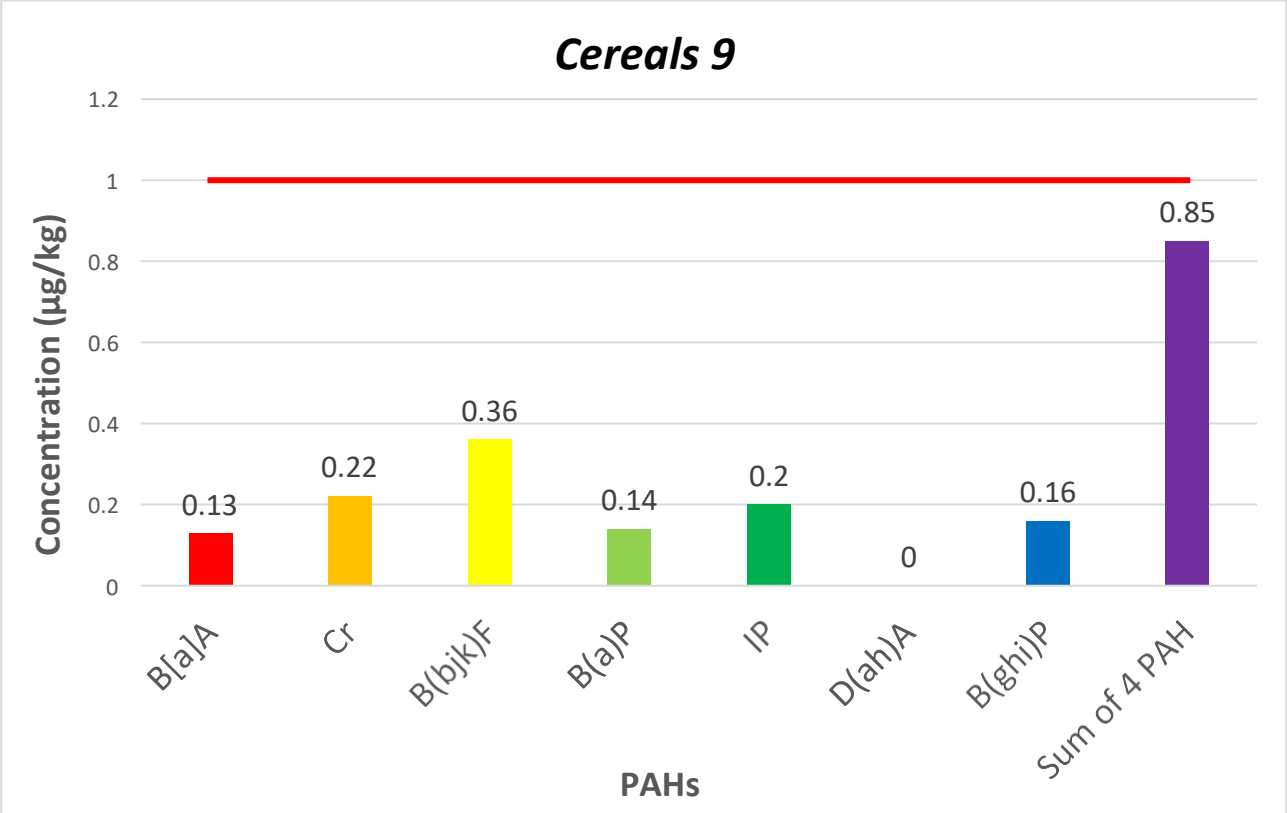


Figure 76. Sample of cereal-based childhood product, Area Vasta 4 (Fermo).

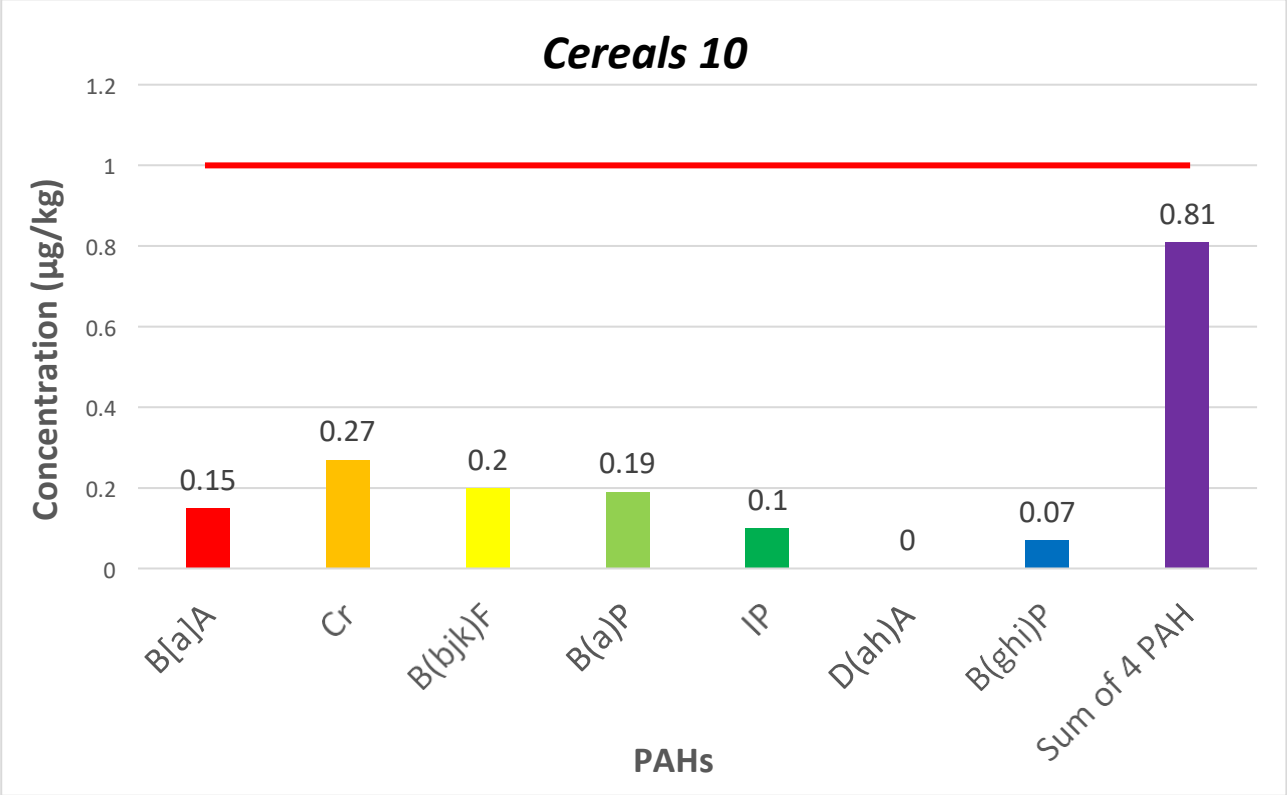


Figure 77. Sample of cereal-based childhood product, Area Vasta 2 (Ancona).

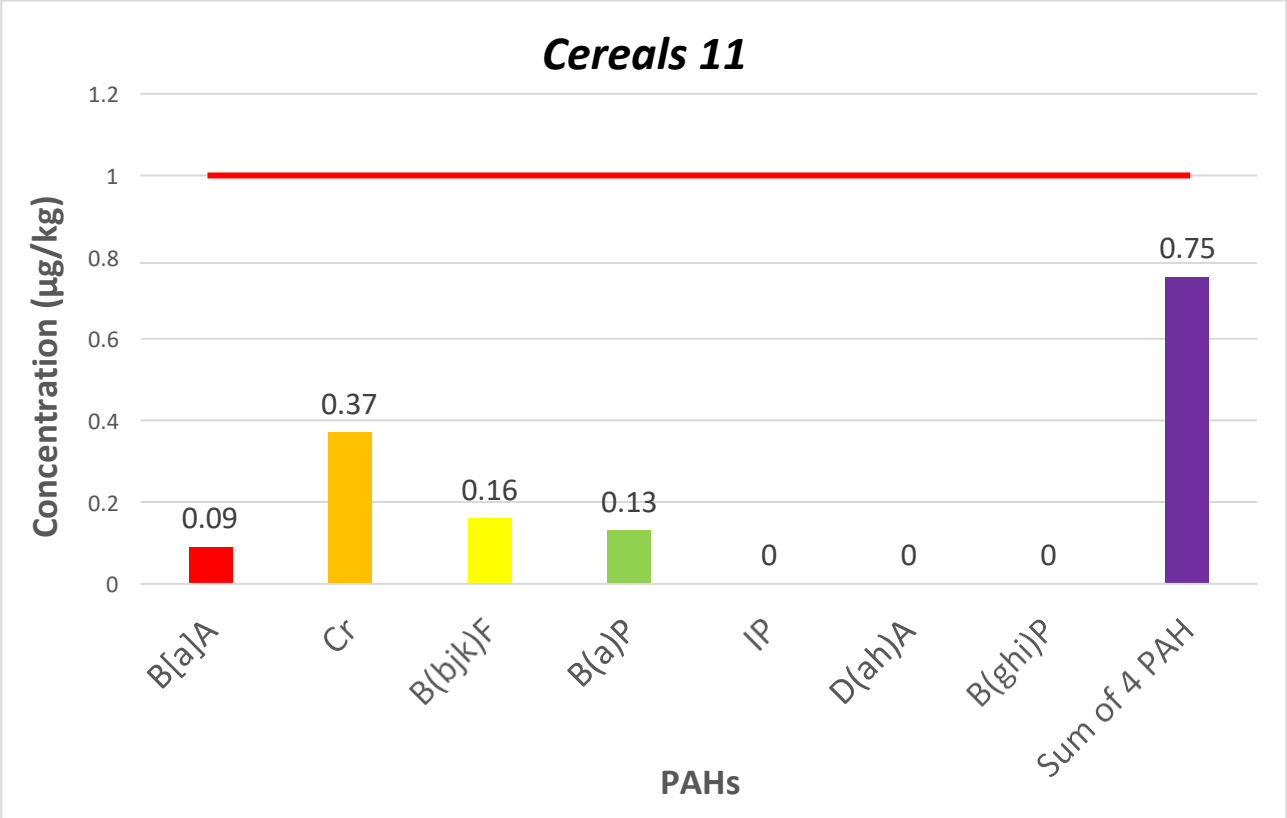


Figure 78. Sample of cereal-based childhood product, Area Vasta 2 (Senigallia).

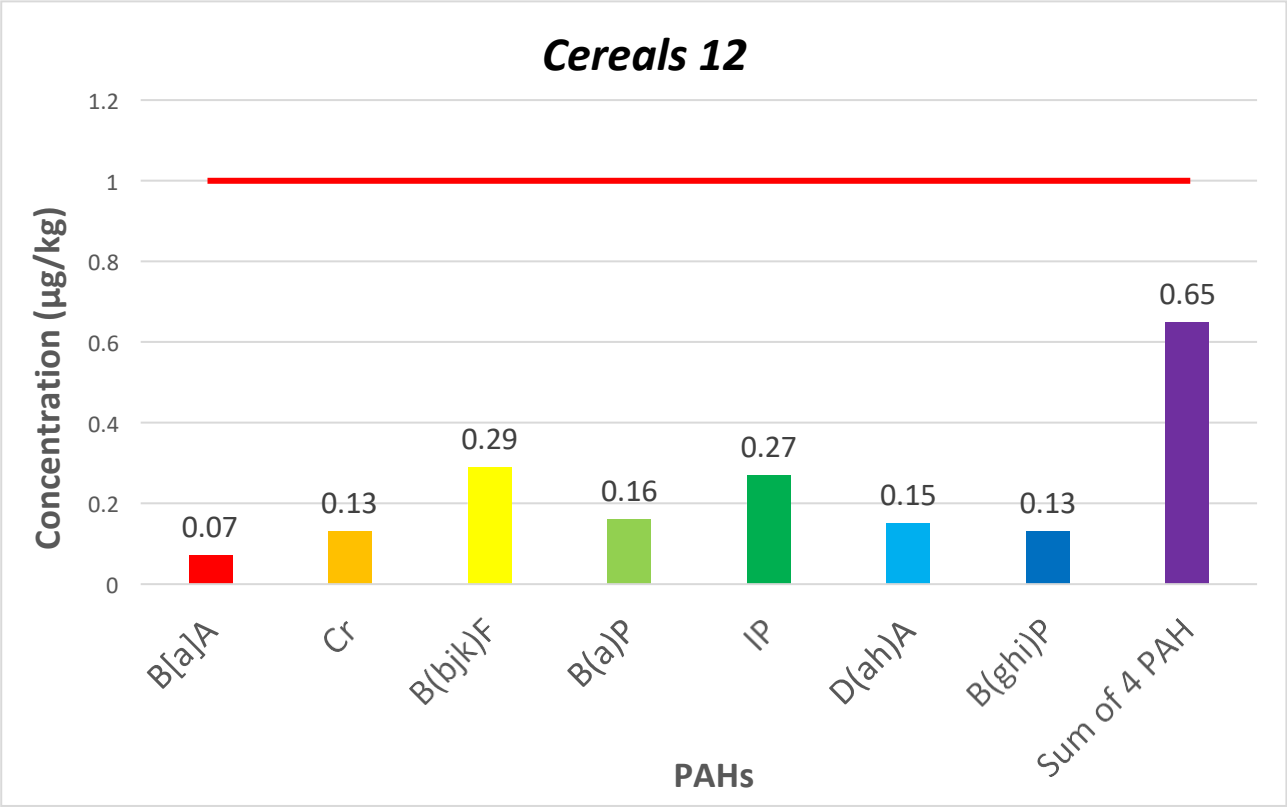


Figure 79. Sample of cereal-based childhood product, Area Vasta 5 (Ascoli Piceno).

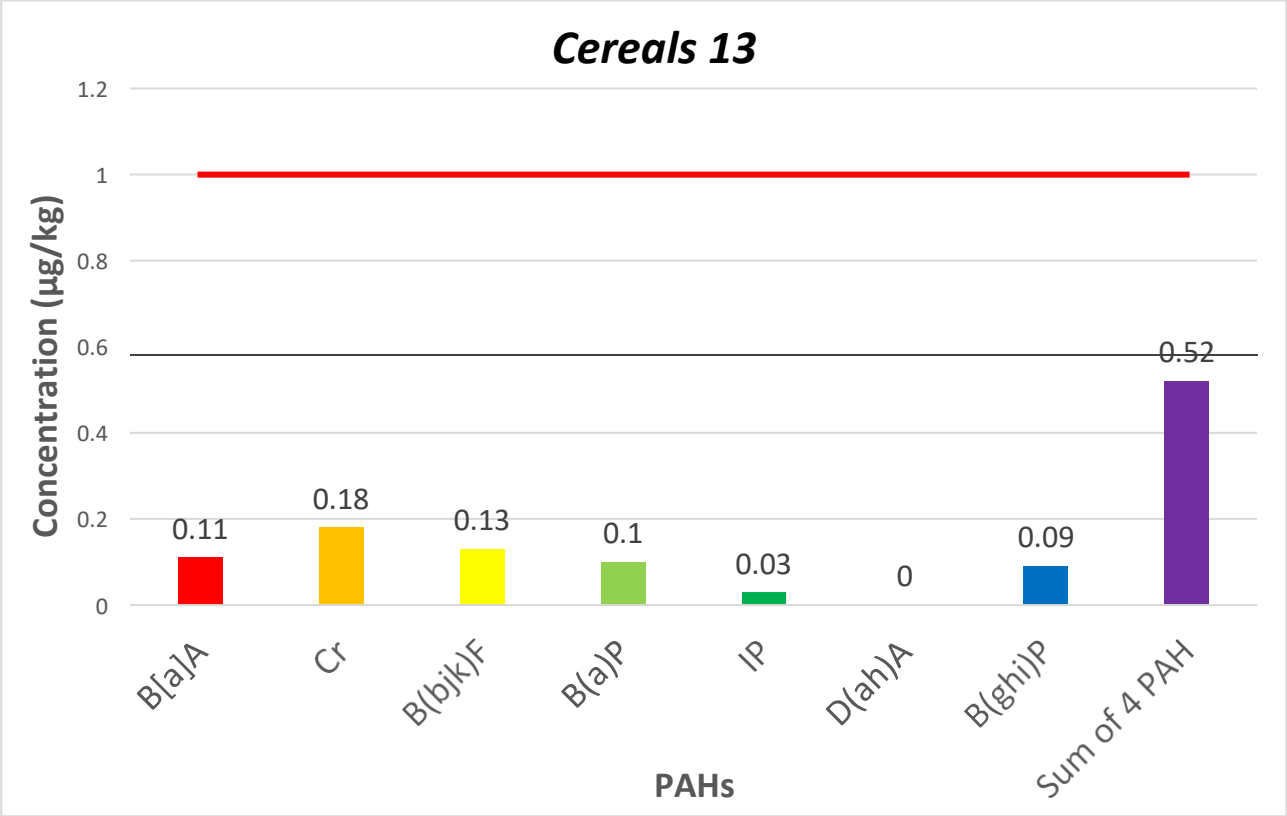


Figure 80. Sample of cereal-based childhood product, Area Vasta 1 (Fano).

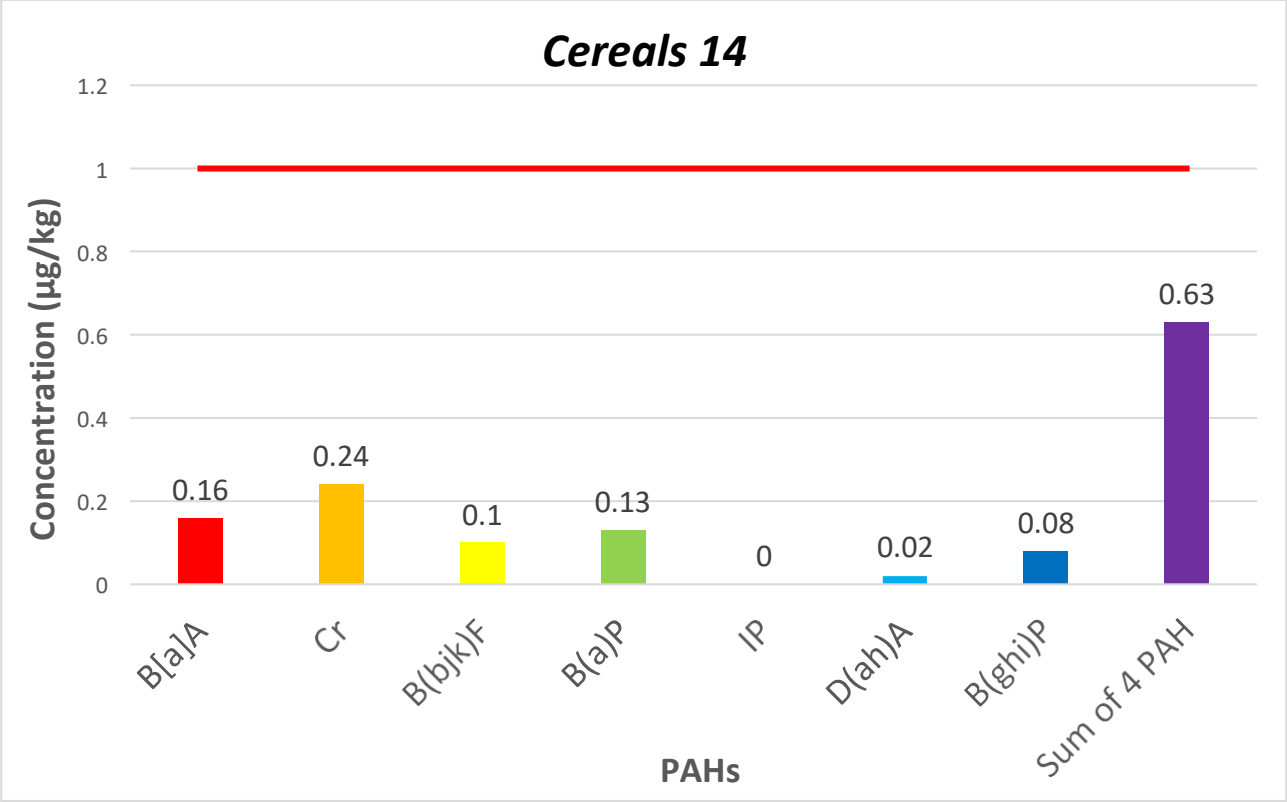


Figure 81. Sample of cereal-based childhood product, Area Vasta 1 (Pesaro).

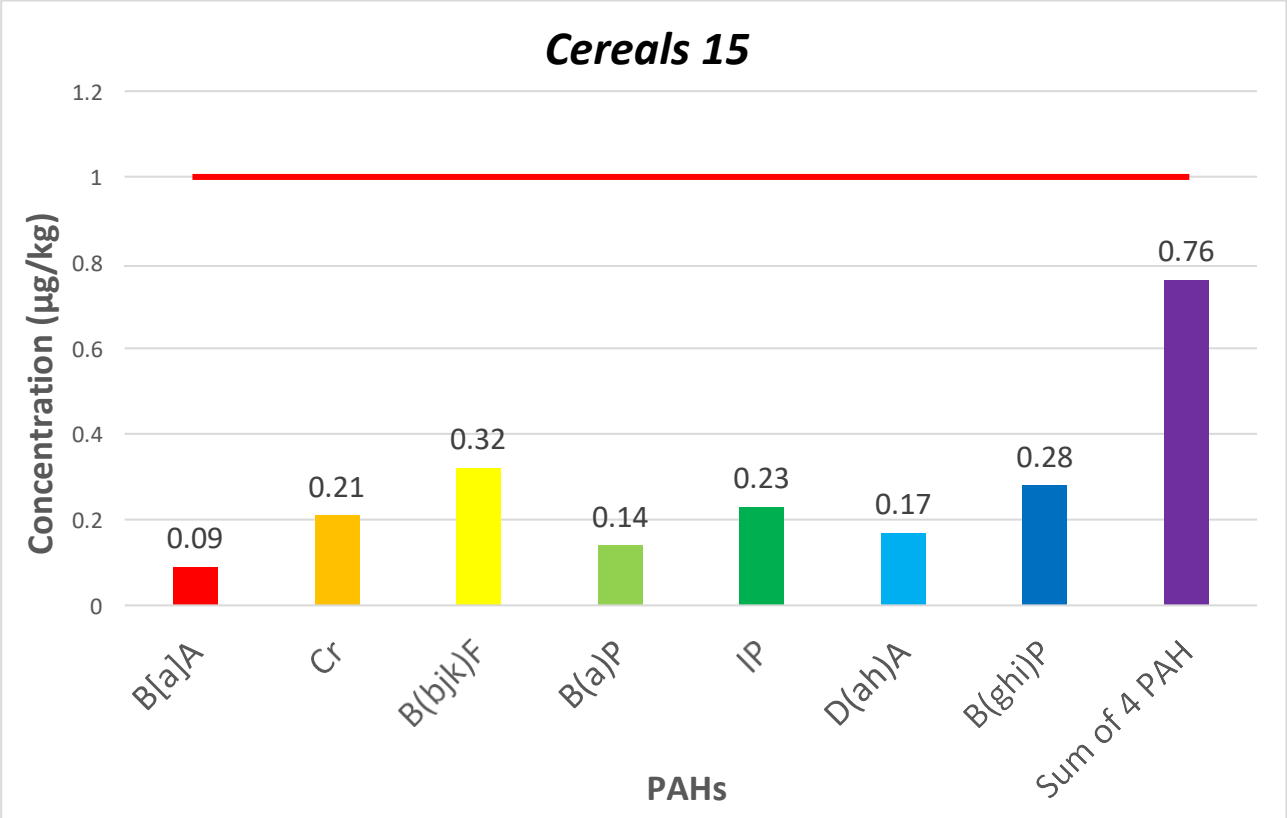


Figure 82. Sample of cereal-based childhood product, Area Vasta 1 (Urbino).

Cereals are important constituents of the human diet across the world both in terms of quantities consumed and nutritional value. Although the reported levels are overall rather low compared with those in seeds and edible oils the frequent occurrence of PAHs contamination and the large cereal consumption can also make them a significant source to human exposure. The occurrence of polycyclic aromatic hydrocarbons (PAHs) in cereal-based food is the result of different food preparation methods (e.g. roasting or drying) and the bioaccumulation in the oily seeds. Nevertheless, no sample result had a value above 1 µg/kg. Despite the low levels found, cereals and cereal products were identified as a major contributor to the intake of PAHs, owing to their high consumption. Therefore, PAH levels in these two product groups should be further monitored.

5. Conclusions

Since 1960 the prevailing activity in the territory of the province of Ascoli Piceno (Italy) has undergone a radical transformation from agricultural to industrial. In a very few years, habits and ways of working have changed. The diet has changed and the quality of life has certainly improved. On the other hand, dangerous substances dispersed in the environment have increased, both in terms of quality and quantity, and consequently the risk of intaking them with food has increased. In fact, food comes from the environment and, if the environment is contaminated, even food is contaminated. PAHs are present, in widely varying quantities, almost in all foods. This presence may be due to environmental contamination (mainly by deposition of atmospheric particulate material and by absorption from contaminated matrices, such as soil and river or sea water), or to formation during certain processing processes (especially drying through combustion fumes and smoking with traditional methods) and some heat treatments (in particular, grilling or frying). For this reason a study was carried out to assess the PAHs content in different food matrices such as oils, cocoa and products for children. The objective of this work was to determine the content of PAHs in food to verify if the limits imposed by the European Commission for benzo[*a*]pyrene and the sum of the four PAHs were respected. This has also allowed us to define the environmental quality status of the territory about the presence of substances considered dangerous and the extent of their return to humans through food. As for oils, three types of oil were analyzed: extra virgin olive oil, sunflower oil and pomace oil. When the olives are subjected to polluting sources containing PAHs, the resulting vegetable oil is contaminated by both light PAHs and heavy PAHs. The former are an expression of background pollution and concern extensive man-made areas (heavily trafficked areas). The latter are an expression of direct pollution by car-vehicle exhausts or discharges of equipment with an internal combustion engine used in the field for mechanized harvesting of olives (shaking or suction) and tree pruning, as well as mechanical handling of olives once collected. From the analysis of extra virgin olive oil, based on the results obtained, it is possible to have an indication about the location of the olive grove, whether it is in a pollution-free area or in an area affected by polluting sources. In the samples analyzed for heavy PAHs, in no case was found a higher value for benzo[*a*]pyrene and the sum of four PAHs than that imposed by the European Commission. On the other hand, very high values were found for light PAHs. From this we can deduce that our territory is polluted mainly by vehicular traffic and this is reflected in a significant amount of light PAHs in the oil. As far as sunflower oil is concerned, low concentrations of PAH are generally found both because the seeds are protected from atmospheric pollution (sunflower seeds from the shell) and because seed oils are subjected to grinding. In our case, however, the samples show an appreciable concentration of PAHs. This finds an explanation in the fact that sunflower oil is subjected to a similar treatment to that of pomace oil in which the seeds are dried by direct contact with hot fumes, rich in PAHs. However, in no case was the threshold limit of 10 µg/kg exceeded for the sum of the four components. The limit of 2 µg/kg for benzo[a]pyrene was also not exceeded. As regards pomace oils, higher PAH values were found than those found in extra virgin olive oils and sunflower seed oils. Knowing the production cycle of this oil it is easy to identify the source and the mechanism of pollution from olive pomace oil; the smoke coming from the combustion of the exhausted pomace is in close contact with the raw material to which part of the combustion products is released, including PAHs. However, in no case was the threshold limit of 10 µg/kg exceeded for the sum of

the four components and also the limit of 2 μg/kg for benzo[a]pyrene was not exceeded. On the other hand, it was interesting to evaluate the effect of cooking in a sample of extra virgin olive oil. The latter was analyzed as such and was subsequently analyzed after a single frying at 180°C for 30 minutes and after four frying at 180°C for 4 hours. The data obtained showed that the cooking process has significantly influenced PAH emissions and frying increases the amount of PAH and B[a]P. The sample of extra virgin olive oil, which initially did not contain PAH or contained in reduced quantities, has undergone a strong increase in these substances during the cooking process. In particular, it was possible to notice that there is a directly proportional relationship between frying time and the amount of IPA that are formed. In this case the two fried oils exceeded the maximum allowed limit (10 μg/kg) set by the European Union for the sum of four PAHs and also exceeded the maximum allowed limit of 2 μg / kg for B[a]P. With regard to cocoa, positive PAH concentration can be found due to inappropriate cocoa drying practices. The same goes for cereals used for baby products. In both cases the limits imposed by the law have been respected. In conclusion, keeping in mind the data obtained regarding the contamination of food which is an important indicator of the quality of life, no particularly critical situations have been identified that could compromise the natural process of sustainable development.

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