UNIVERSITY OF LATVIA

FACULTY OF CHEMISTRY

DEVELOPMENT AND APPLICATION OF SENSITIVE MASS SPECTROMETRIC METHODS FOR THE EFFECTIVE DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN FOOD

DOCTORAL THESIS

JUTĪGO MASSPEKTROMETRISKO METOŽU IZSTRĀDE UN PIELIETOJUMS POLICIKLISKO AROMĀTISKO OGĻŪDEŅRAŽU EFEKTĪVAI NOTEIKŠANAI PĀRTIKĀ

PROMOCIJAS DARBS

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The thesis is available at the Library of the University of Latvia, Raiņa Blvd. 19, Riga, Latvia.

CONTENTS

ABBREVIATIONS

AGC	Automatic gain control
APCI	Atmospheric-pressure chemical ionisation
APPI	Atmospheric-pressure photo ionisation
b.w.	Body weight
BaA	Benzo[a]anthracene
BaP	Benzo[a]pyrene
BAs	Biogenic amines
BbF	Benzo[b]fluoranthene
$BMDL_{10}$	Benchmark dose level of a 10%
Chr	Chrysene
CNT-1	Agglomerated MWCNT of trademark Baytubes®
CNT-2	Disperse MWCNTs of trademark Timestube™
CNT-OH	Hydroxyl derivate of disperse MWCNTs of trademark Timestube™
CNT-COOH	Carboxyl derivate of disperse MWCNTs of trademark Timestube™
DA	Dopant assisted
d-SPE	Dispersive solid-phase extraction
EFSA	European Food Safety Authority
EI	Electron impact
EPA	Environmental Protection Agency
ESI	Electrospray ionisation
EURL	European Union Reference Laboratory
FDA	Food and Drug Administration
FLD	Fluorescence detector
FS	Full scan
FWHM	Full width at half maximum
GC	Gas chromatography
GPC	Gel permeation chromatography
GRAS	Generally Recognised As Safe
HCD	High collision dissociation
HPLC	High performance liquid chromatography

HRMS	High resolution mass spectrometry
IARC	International Agency for Research on Cancer
ILC	Interlaboratory comparison
IQC	Internal quality control
IT	Maximum injection time
L. sakei	*Lactobacillus sakei*
LAB	Lactic acid bacteria
LC	Liquid chromatography
LLE	Liquid - liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
ML	Maximum level
MOE	Margin of exposure
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MWCNT	Multi-walled carbon nanotubes
NCE	Normalised collision energy
ND	Not detected
P. acidilactici	*Pediococcus acidilactici*
P. pentosaceus	*Pediococcus pentosaceus*
PAH	Polycyclic aromatic hydrocarbons
PAH4	Sum of BaP, Chr, BaA, BbF
RM	Reference material
RP	Resolving power
RSD	Relative standard deviation
RT	Retention time
SCF	Scientific Committee on Food
SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Selective reaction monitoring
TTA	Total titratable acidity
UV	Ultraviolet–visible detector
WHO	World Health Organization

ABSTRACT

Development and application of sensitive mass spectrometric methods for the effective determination of polycyclic aromatic hydrocarbons in food. Rozentāle I., supervisors Dr. Chem., Assoc. Prof. Bartkevičs V. and Dr. Chem., Prof. Vīksna A. Doctoral thesis in analytical chemistry, 130 pages, 20 figures, 26 tables, 161 literature references, 10 annexes. In English.

A new analytical methodology for the determination of polycyclic aromatic hydrocarbons (PAHs) by applying dispersive solid-phase extraction with multi-walled carbon nanotubes as sorbents for selective extraction of analytes has been elaborated. The method demonstrated its appropriate performance and underlined good reliability and practicability for the analysis of four EU-regulated PAHs.

For the first time, the analytical capabilities of Orbitrap mass spectrometry equipped with atmospheric pressure photoionisation ion source was applied and assessed for the quantitative determination of the PAHs in dark chocolate. This elaborated analytical method was demonstrated to be an accurate, precise, and a powerful tool for the determination of non-polar hydrophobic PAHs at trace levels, enabling its routine use even for difficult matrices.

Within the study two different gas chromatographic – mass spectrometric methods were developed, optimised and compared for the determination of PAHs at ultra-low contamination levels. Elaborated methods showed superior performance over single mass spectrometric detection and highlighted the need for proper control of cereal-based products with regards to low levels of contamination and high consumption.

A special attention in a study was drawn to risks associated with the consumption of smoked meat and smoked meat products in Latvia, with an emphasis to consumer's preferences and dietary habits. Furthermore, the present study reports data on the occurrence of PAHs in different foods and mitigation strategies for reducing the PAHs content were proposed.

POLYCYCLIC AROMATIC HYDROCARBONS, FOOD, OCCURRENCE OF PAHs, RISK ASSESSMENT, TANDEM MASS SPECTROMETRY, HIGH RESOLUTION MASS SPECTROMETRY, ORBITRAP

ANOTĀCIJA

Jutīgo masspektrometrisko metožu izstrāde un pielietojums policiklisko aromātisko ogļūdeņražu efektīvai noteikšanai pārtikā. Rozentāle I., zinātniskie vadītāji Dr. ķīm., asoc. prof. Bartkevičs V. un Dr. ķīm., prof. Vīksna A. Promocijas darbs, 130 lappuses, 20 attēli, 26 tabulas, 161 literatūras avoti, 10 pielikumi. Angļu valodā.

Promocijas darbā tika izstrādāta jauna un inovatīva paraugu sagatavošanas metode selektīvai policiklisko aromātisko ogļūdeņražu (PAH) noteikšanai, kas balstās uz dauzslāņu oglekļa nanocaurulīšu izmantošanu par dispersīvās cietfāzes ekstrakcijas sorbentu. Izstrādātā metode uzrādīja teicamas pielietošanas spējas, sniedzot ticamus un precīzus rezultātus Eiropas Savienības četru prioritāro PAH noteikšanā.

Pirmo reizi Orbitrap masspektrometra analītiskās spējas tika piemērotas un izvērtētas PAH kvantitatīvai noteikšanai tumšajā šokolādē, izmantojot atmosfēras spiediena fotojonizācijas jonu avotu. Izstrādātā metode uzrādīja selektīvu un precīzu PAH detektēšanu zemās piesārņojuma koncentrācijās, pavērot jaunas iespējas PAH rutīnās analīzēs sarežģītās matrices gadījumos.

Promocijas darbā tika izstrādātas, optimizētas un izvērtētas divas gāzu hromatogrāfijas – masspektrometrijas metodes PAH kvantitatīvai noteikšanai īpaši zemos piesārņojuma daudzumos. Abas metodes demonstrēja izcilas spējas un uzsvēra pienācīgās graudaugu produktu kontroles nepieciešamību, ņemot vērā produktu zemo piesārņojuma līmeni un augsto patēriņu uzturā.

Īpaša uzmanība promocijas darbā tika veltīta riskiem, kas saistīti ar kūpinātās gaļas produktu patēriņu Latvijā, ņemot vērā vietējo pātērētāju izvēli un paradumus. Darbā tika izpētīta PAH sastopamība lielā pārtikas produktu klāstā, kā arī tika piedāvātas un izvērtētas divas PAH piesārņojuma mazināšanas stratēģijas.

POLICIKLISKIE AROMĀTISKIE OGĻŪDEŅRAŽI, PĀRTIKA, PAO SASTOPAMĪBA, RISKA NOVĒRTĒJUMS, TANDĒMA MASSPEKTROMETRIJA, AUGSTAS IZŠKIRTSPĒJAS MASSPEKTROMETRIJA, ORBITRAP

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants, often with carcinogenic, teratogenic, and mutagenic effects [1-4]. Their physical and chemical properties, particularly high stability and lipophilicity, gives them ubiquity and capacity of accumulation in the living organisms and nature [5-6]. However, PAHs are not listed in a persistent organic pollutants (POPs) list by a Stockholm Convention, PAHs often goes in line with POPs and are classified as extremely toxic compounds for the environment and human health at a world scale [7-8].

Humans are exposed to PAHs through different pathways and it was proved in various studies that for the non-smokers the major route of exposure is consumption of food [1, 9-10]. Food is frequently contaminated with the PAHs, while the levels of PAHs found in food varies significantly. For example, the levels of PAHs found in unprocessed foods (fruits and vegatables, grains etc.) in rural areas reflect the background contamination (usually between 0.010 and 0.50 μg kg^{-1}) [11-13], whereas highly elevated concentrations of PAHs (up to 1 700 μg kg^{-1}) in smoked meat products can still be occasionally reported [14-18]. Hence, the importance of research on the PAHs occurrence in different foods is highlighted. A special attention should be drawn to a quantitatively minor foods or ingredients that can hold a significant potential to contaminate a wide range of products due to the wide spread use and large-scale distribution (for example, dried herbs and spices, cocoa).

Furthermore, to secure the public health, additional focus should be set to the risk assessment processes and mitigation strategies to reduce the chemical contamination of different food chain products with PAHs.

The practical relevance of the problem. Nowadays, food quality control institutions are established, food regulations and standards are developed, however, different methods and approaches may be used to assure food safety at appropriate level.

The low levels of the PAHs in food are often accompanied with the high consumption levels, and contrarily, high contamination levels often deals with low intake. Thus, due to the carcinogenic properties of the PAHs and its occurrence in food, the Scientific Committee on Food (SCF) recommended that the PAH contents in food should be “as low as reasonably achievable” in adherence with the so-called ALARA-principle [9]. Thus, methods for the PAHs determination should be sensitive enough to meet the aforementioned requirements and ensure the quality and safety of food [19-20].

The analysis of organic contaminants in food samples is hampered by interfering compounds present in the complex food matrices and the food matrix itself [5, 21-22].

Therefore, the most challenging task for analysts is to maximise recovery of analyte and minimise the accompanying interferences by proper extraction and clean-up procedures. In order to observe the better separation, extraction and clean-up, novel and selective methods are of a great importance.

Analytical approaches for the PAHs determination were historically based on both gas chromatography (mainly, gas chromatography with single quadrupole mass spectrometric detection (GC-MS)) and liquid chromatography (with both, fluorescence (FLD) and triple quadrupole (MS/MS) mass spectrometric detection) [1, 8, 10, 13]. In recent years, due to many advantages of high resolution mass spectrometry (HRMS) such as greater peak capacity and enhanced sensitivity, a general shift from single quadrupole mass spectrometric detection in case of GC, (GC-MS) and triple quadrupole detection, for LC systems, (LC-MS/MS) to HRMS has been observed [23-26]. Despite this fact, the usage of HRMS methods in PAHs analysis remains poorly investigated.

The aim of the work. Several aims were proposed during this thesis:

i. Elaboration of sensitive novel mass spectrometric methods for the simultaneous determination of the selected PAHs in different food samples;

ii. Estimating the occurrence of PAHs in different foods and risk assessment for these contaminants;

iii. The investigation of new strategies to reduce the contamination of PAHs in smoked food.

The approach used. The following objectives have been set in order to fulfil the aims of the thesis:

i. Development of an efficient sample extraction and clean-up procedure, together with the investigation of optimal conditions for the analysis of the PAHs using multi-walled carbon nanotubes as sorbents for dispersive solid-phase extraction (d-SPE);

ii. The elaboration of an efficient and reliable analytical method using liquid chromatography/high resolution Orbitrap mass spectrometry for the analysis of the PAHs in the samples of complex matrix (dark chocolate);

iii. The optimisation of GC-MS/MS and GC-HRMS instrumental methods for the determination of the PAHs at ultra low contaminaton levels and the comparative assessment of two investigated methodologies in terms of sensitivity, selectivity and robustness;

iv. Application of the developed analytical methods for the analysis of selected PAHs and the risk assessment associated with their presence in different foods;

v. Consideration of the ways for the PAHs reduction in smoked foods.

Scientific novelty.

i. The elaboration of a novel sample preparation method based on d-SPE with MWCNTs as sorbents for selective extraction of the PAHs;

ii. The development, optimisation and application of a HPLC-Orbitrap-MS analytical method with an APPI ionisation source for sensitive and selective determination of four EU marker PAHs;

iii. Comparative evaluation of GC-MS/MS based analytical method versus GC-HRMS based method in the analysis of PAHs;

iv. Assessment of Latvian population exposure to PAHs intake from smoked food;

v. Proposals for new mitigation strategies to prevent, eliminate or reduce PAHs contamination in smoked food.

Practical application of the work. The elaborated analytical methods could be applied for the extended monitoring of the occurrence of the selected PAHs in different foods, while the proposed strategies to reduce the PAHs contamination should be reassessed and can be suggested only in case of the lactic acid bacteria treatment.

Scientific publications.

1. **Rozentale, I.**; Zacs, D.; Bartkevics, V. Application of dopant-assisted atmospheric pressure photoionisation HPLC-MS method for the sensitive determination of polycyclic aromatic hydrocarbons (PAHs) in dark chocolate. *Journal of Chromatographic Science*[1], **2019**.
2. Zacs, D.; **Rozentale, I.**; Reinholds, I.; Bartkevics, V. Multi-Walled Carbon Nanotubes as Effective Sorbents for Rapid Analysis of Polycyclic Aromatic Hydrocarbons in Edible Oils Using Dispersive Solid-Phase Extraction (d-SPE) and Gas Chromatography—Tandem Mass Spectrometry (GC-MS/MS). *Food Analytical Methods*[2], **2018**, *11*, 2508-2517.
3. **Rozentale, I.**; Zacs, D.; Bartkiene, E.; Bartkevics, V. High levels of polycyclic aromatic hydrocarbons in traditionally smoked meat products from the Baltic states. *Food Additives and Contaminants, Part B*[3], **2018**, *11(2)*, 145-152.
4. **Rozentale, I.**; Yan Lun, A.; Zacs, D.; Bartkevics, V. The occurrence of polycyclic aromatic hydrocarbons in dried herbs and spices. *Food Control*[4], **2018**, *83*, 45-53.
5. **Rozentale, I.**; Zacs, D.; Perkons, I.; Bartkevics, V. A comparison of gas chromatography coupled to tandem quadrupole mass spectrometry and high-resolution sector mass spectrometry for sensitive determination of polycyclic aromatic hydrocarbons (PAHs) in cereal products. *Food Chemistry*[5], **2017**, *221*, 1291-1297.
6. Bartkiene, E.; Bartkevics, V.; Mozuriene, E.; Krungleviciute, V.; Novoslavskij, A.; Santini, A.; **Rozentale, I.**; Juodeikiene, G.; Cizeikiene, D. The impact of lactic acid bacteria with antimicrobial properties on biodegradation of polycyclic aromatic hydrocarbons and biogenic amines in cold smoked pork sausages. *Food Control*[4], **2017**, *71*, 285-292.
7. **Rozentale, I.**; Ancans, J.; Bartkiene, E.; Viksna, A.; Bartkevics, V. Efficacy of Ozonation Treatments of Smoked Fish for Reducing Its Benzo[a]pyrene Concentration and Toxicity. *Journal of Food Protection*[6], **2016**, *79(12)*, 2167-2173.
8. **Rozentale, I.**; Stumpe-Viksna, I.; Zacs, D.; Siksna, I.; Melngaile, A.; Bartkevics, V. Assessment of dietary exposure to polycyclic aromatic hydrocarbons from smoked meat products produced in Latvia. *Food Control*[4], **2015**, *54*, 16-22.

[1] *Peer reviewed journal, imprint of Oxford Academic (IF=1.037 (2017)), ISSN:0021-9665*
[2] *Peer reviewed journal, imprint of Springer (IF=2.245 (2017)), ISSN:1936-9751*
[3] *Peer reviewed journal, imprint of Taylor&Francis (IF=2.407 (2017)), ISSN:1939-3229*
[4] *Peer reviewed journal, imprint of Elsevier (IF=3.667 (2017)), ISSN:0956-7135*
[5] *Peer reviewed journal, imprint of Elsevier (IF=4.946 (2017)), ISSN:0308-8146*
[6] *Peer reviewed journal, imprint of IAFP (IF=1.510 (2017)), ISSN:1944-9097*

List of conferences.

1. 74th University of Latvia conference, Riga, Latvia, 2016. **Rozentāle, I.**; Bartkevičs, V.; Vīksna, A. Benzo[*a*]pirēna satura izmaiņas ozonēšanas ietekmē un ozonēšanas produktu toksicitāte (in book of abstracts/oral presentation);
2. 58th University of Daugavpils conference, Daugavpils, Latvia, 2016. Ciekure, E.; Siksna, I.; Bremmere, B.; **Rozentāle, I**. Consumption of grilled pork meat and potential exposure to polycyclic aromatic hydrocarbons (in book of abstracts/oral presentation).
3. 7th International Scientific Meeting, Struga, Republic of Macedonia, 2016. Bartkevics, V.; **Rozentāle, I.**; Bartkiene, E. Contamination of food products with polycyclic aromatic hydrocarbons: occurrence data, latest analytical developments (in book of abstracts/oral presentation).
4. 20th International Scientific Conference "EcoBalt 2016", Tartu, Estonia, 2016. Bartkevics, V.; **Rozentāle, I.** Polycyclic aromatic hydrocarbons in the environment and food products: occurrence data, risk assessment and analytical developments (in book of abstracts/oral presentation).

1. LITERATURE REVIEW

1.1. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds (comprising about 10,000 substances) containing two or more aromatic rings without any heteroatoms or substituents and are of special importance because of their widespread distribution throughout the environment and their potential toxic, carcinogenic, and mutagenic properties [2, 3, 6, 9, 27]. PAHs can be classified according to the number of condensed aromatic rings as light (2-3 rings) and heavy (4-6 rings) PAHs, the latter being more stable and toxic than the light ones [5].

PAHs are formed in all incomplete combustion processes with insufficient oxygen supply at >200°C temperatures and originate from natural (forest fires, volcanic activity) and anthropogenic sources (fossil fuel combustion, industrial and agricultural activities, power generation, municipal and medical waste incineration) [10, 28-29].

According to numerous studies, food is the main source of non-smokers exposure to PAHs [1, 30-31] and this fact highlights the importance of research about PAHs in food and the development of mitigation strategies to reduce such contamination [10]. PAHs occurring in foods are not present individually, but always exist as complex mixtures, so the occurrence of a whole range of PAHs in food and environment represents a cause for concern. Furthermore, PAHs that are not defined as carcinogens often may act as synergists of carcinogenesis [22].

PAHs that are found in foods may be formed during industrial food processing or domestic food preparation, such as smoking, drying, roasting, baking, frying, or grilling [21, 32-35]. Food can be also contaminated with PAHs present in the environment, i.e., PAHs can accumulate on the waxy surfaces of many vegetables and fruits. Indeed, the presence of PAHs in uncooked food, such as vegetables, seeds, and grains has also been demonstrated. For example, Fismes et al. (2002) have experimentally demonstrated the uptake of PAHs by fruit and vegetables grown in contaminated soils [11]. Another example of possible PAH contamination in foods is due to traffic exhaust, i.e., crops or livestock close to busy roads could be exposed to PAHs and nitro-PAHs (derivatives from PAHs), which often show higher mutagenic, carcinogenic, and toxic activity in comparison to their corresponding PAHs [36].

Benzo[*a*]pyrene (BaP) is the most studied compound among high molecular weight PAHs. It has been classified by the US Environmental Protection Agency (US EPA) as a priority pollutant: a compound selected on the basis of its known or suspected

carcinogenicity, teratogenicity or acute toxicity [37]. Since 2012, BaP has been ranked as a top example among human carcinogens in a report by the World Health Organization (WHO) International Agency for Research on Cancer [4]. Multiple animal studies in many species have demonstrated the carcinogenicity of BaP following administration by numerous routes [4, 33, 38-39]. In addition, BaP has been shown to cause genotoxic effects in a broad range of prokaryotic and mammalian cell assays, therefore its occurrence in the environment and food products is of great concern [40].

Despite the structural diversity of PAHs, only selected representatives are considered to be toxicologically significant, among which benzo[*a*]anthracene (BaA), chrysene (Chr), benzo[*b*]fluoranthene (BbF), and BaP are recognised in the EU as a subgroup of four priority PAHs (Fig. 1.1.) and are regulated in food products according to Commission Regulations (EU) No. 836/2011, 2015/1933 and 2015/1125 [1, 41-44].

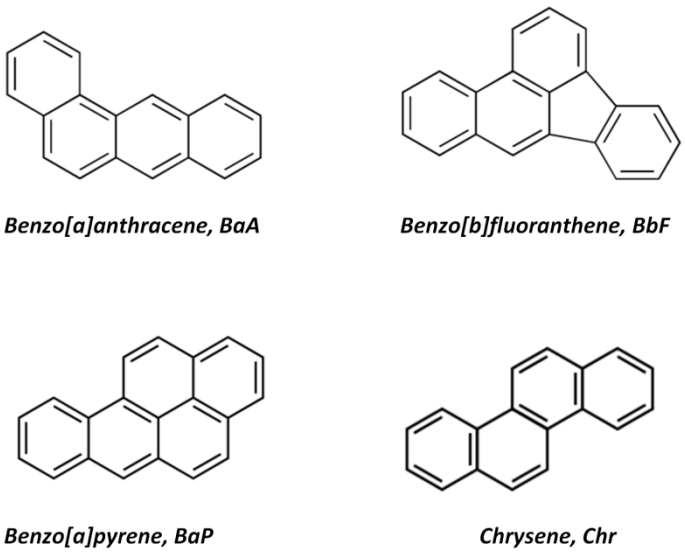

Fig. 1.1. **Four EU-regulated priority PAHs**

In October 2005 EFSA adopted the margin of exposure (MOE) approach for health risk assessment regarding PAHs in food [1]. The MOE is defined as a function of the potency of a given chemical assessed using animal carcinogenicity assays and human dietary exposure to the chemical in question and is calculated by dividing the lowest confidence limit of benchmark dose for a 10% increase in the number of tumour bearing animals compared to control animals ($BMDL_{10}$) by the mean and high level estimates of dietary exposure to BaP and PAH4. The reference $BMDL_{10}$ values among the accepted ones for the derivation of a MOE are 0.07 mg kg^{-1} b.w. per day for BaP and 0.34 mg kg^{-1} b.w. per day for the PAH4 [1]. In the Scientific Committee report EFSA also stated that a MOE of 10000 or higher is

considered of low concern from a public health point of view with respect to the carcinogenic effect and might be reasonably considered as a low priority for risk management actions [1].

1.2. Analytical methods for the determination of the PAHs

A large number of analytical methods for the determination of the PAHs in food have been proposed over the years, which relied on different purification, pre-concentration, and determination procedures [3, 45]. The main obstacle in the analysis of PAHs is that the major constituents of most matrices are high molecular weight compounds (e.g., triglycerides and fatty acids), thus laborious procedures need to be applied in order to separate analyte fractions from the matrix and to achieve the desired purity of the final extract. Hence, the search for alternative analytical protocols is still of great relevance.

Next important issue for the PAHs determination methods is associated with relatively low maximum permitted levels for some food products. For example, according to the latest European legislation concerning PAHs in food the content of BaP and PAH4 (sum of BaP, BaA, BbF, and Chr) in processed cereal-based foods and baby foods for infants and young children should not exceed 1.0 μg kg^{-1} [44]. Therefore, in case if all four regulated compounds had the same analytical performance the limit of quantification (LOQ) for individual PAH compounds should be below 0.25 μg kg^{-1} and limit of detection (LOD) – below 0.08 μg kg^{-1}.

Several GC-based studies on PAHs exposure in bread and/or cereal products with variable LOQs of PAH determination methods have already been published. For example, in a research from Poland dedicated to PAHs in the bakery chain [46], the LOQ of the reported method for PAH4 was 0.83 μg kg^{-1}, with the highest obtained value among PAH4 observed for Chr – 0.29 μg kg^{-1}. In a similar research from Spain regarding PAHs in toasted bread [47], the LOQs of the selected method for PAH4 were 9.8 μg kg^{-1} for the bread ash and 1.2 μg kg^{-1} for the bread samples, with highest LOQ obtained for Chr - 6.2 μg kg^{-1} and 0.75 μg kg^{-1} for the bread ash and the bread samples, respectively. Important to mention that by the time the last cited study was published, ML for processed cereals for PAHs was set at 1.0 μg kg^{-1} expressed by the content of BaP that was used as a marker for the occurrence of PAHs in foodstuffs, while MLs for other individual PAH4 have not been set yet [48]. Meanwhile, in the French total diet study [32], the specified LOQ values were 0.026 – 0.055 μg kg^{-1}. This underlines the fact that PAHs determination methods should be optimised and harmonised to provide the PAHs determination in different foodstuffs at the appropriate levels.

1.2.1. Sample preparation methods for the PAHs analysis

Due to its physical and chemical properties, PAHs tend to coextract with large amounts of matrix effects, thus the laborious purification procedure of extracts should often be applied. Gel permeation chromatography (GPC) as well as fat saponification have been recognised as most efficient clean-up methods in the analysis of foods with high fat content [3, 29]. However, these methods are time-consuming and additional clean-up steps (e.g., solid-phase extraction (SPE) or liquid - liquid extraction (LLE)) are typically needed.

Several novel analytical procedures based on the application of molecularly imprinted polymers [49, 50] and nanoparticles [45, 51-52] as SPE sorbents were recently published, indicating the new opportunities to design rapid analytical methods with promising isolation capability of desired analytes [53-54]. Considering the highly hydrophobic properties of multi-walled carbon nanotubes (MWCNTs), their unique tubular structure with the ability for π-stacking [55], these nanomaterials provide superior sorption potential towards aromatics, with a special emphasis on planar compounds. Thus, affirming the high sorption capability for planar aromatics, MWCNTs were found to be much effective in sorption of polychlorinated-dibenzo-*p*-dioxins in comparison to activated carbon [56]. The great potential of MWCNTs for selective sorption of planar aromatic contaminants causes increasing amount of scientific publications yearly [52, 54, 57-61]. However, despite the multiple studies in different fields of science, the application of MWCNTs in analytical food chemistry still remains poorly investigated and only a few published studies describe the application for the PAHs determination in complex food matrices [57, 60].

1.2.2. Instrumental methods for the sensitive determination of the PAHs

The analytical determination of PAHs can be carried out by both liquid chromatography (LC), including ultra-high performance LC, and gas chromatography (GC) with various detectors, or by comprehensive techniques (GCxGC and LCxLC) [5]. The LC determination of PAHs usually is carried out by liquid chromatography coupled to fluorescence (LC-FLD) or ultraviolet–visible detection (LC-UV) techniques, which are also described in some official methods according to the International Standardization Organization (ISO) and the United States Environmental Protection Agency (US EPA). However, it is well known that UV detection shows a number of disadvantages, such as selectivity problems and sensitivity limitations, and it cannot discriminate matrix interferences, especially in complex matrices. On the contrary, FLD is more selective and sensitive than UV detection, and it is currently the detection system of choice in LC analysis of PAHs. LC-FLD has been extensively applied for

the determination of PAHs in very different matrices, including foods and beverages, since it is simple and affordable compared to other detection systems [3].

Despite the advantages of FLD compared to UV detection, FLD can still show a lack of selectivity. Moreover, some authors describe certain selectivity problems due to the presence of alkylated PAHs, which are considered to be the main impurities of PAH fractions [21]. These compounds show fluorescence responses similar to the unsubstituted PAHs. Another disadvantage is the impossibility of using certain isotopically labelled compounds that cannot be distinguished by FLD from the native PAHs [3, 5].

Mass spectrometric detection methods in both LC and GC have become more and more popular because of the high selectivity that enables reliable confirmation of analyte identity and the possibility to use stable isotope labelled PAHs as internal standards [10]. So far, gas chromatography – single quadrupole mass spectrometry has been extensively used to isolate and quantify PAHs, however, application of tandem MS compared to single MS analysers provides a notable increase in sensitivity. For examples, in an analysis of fish matrix GC-MS/MS method showed an increase in sensitivity by five times [10]. Whereas, due to the improvements in robustness and sensitivity of high resolution mass spectrometry (HRMS) in recent years, there has been a growing interest in switching from using single or even triple quadrupole mass spectrometers to high resolution mass spectrometers [26].

The most commonly used ion sources in LC–MS analysis are electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation sources (APCI). However, such ion sources provide insufficient efficiency for the ionisation of non-polar PAHs. APPI appears to be a good alternative to ESI and APCI for the coupling of low flow rate separation techniques (<50 μL min^{-1}) and capillary LC, due to the lower concentration of the solvent vapour in the ion source. Furthermore, APPI is generally considered to be a compatible ionisation technique for non-polar compounds. In fact, APPI was found by various authors to be more complementary to ESI than APCI, due to its ability to ionise less polar molecules than those that can be ionised in APCI, and this type of interface also shows less ion suppression than APCI and ESI [5, 62].

Most of the studies based on the application of APPI in the analysis of PAHs were carried out using pure PAH standards [24, 63-68]. Only a few published studies describe the determination of PAHs in complex food matrices - LC-DA-APPI-MS/MS method for the determination of 15 + 1 EU priority PAHs in edible oil [69]; LC-DA-APPI-MS/MS method for the determination of 16 US EPA priority PAHs in shrimp samples [70], and LC-APPI-MS method for 16 US EPA priority PAHs analysis in oysters [71]. The LODs of the reported

methods were the following: 0.19 – 0.36 μg kg^{-1} for the oil, 0.02 – 0.51 μg kg^{-1} for the shrimp, and 0.013 – 0.13 μg kg^{-1} for the oysters.

1.3. Contamination of the processed foods with the PAHs

The occurrence of PAHs in processed foods is usually a consequence of numerous factors – nutrient contents in the foods (e.g., proteins, carbohydrates, and lipids), duration of cooking (drying and etc.), heat source type, distance from heat source, design of the cooking device, and the type of fuel used [33, 72]. Whereas according to Zelinkova and Wenzl (2015b), with respect to food groups, the highest levels of total PAHs were detected in meat and meat products, oils and fats and cereals [10].

1.3.1. Cocoa products

Cocoa is an important commercial crop – the raw material from which chocolate is manufactured. A number of manufacturing steps are necessary for the production of a homogenous chocolate of high quality with respect to flavour, consistency, and homogeneity. The manufacturing process includes some critical steps during which cocoa products including chocolate may be contaminated with PAHs. According to literature data [33], PAH contamination in chocolate mainly can be affected by drying, roasting, winnowing, blending, and fermenting of the cocoa beans, typically in their country of origin [33, 73].

Cocoa butter is a major constituent of raw cocoa products (e.g. cocoa beans, cocoa mass, cocoa nibs, or cocoa liquor) that is eventually present in chocolate and other processed cocoa products. It has been confirmed by many researchers that cocoa butter might contain even higher levels of PAH than other oils and fats and it cannot be refined by processes applied to other vegetable oils and fats [10, 34, 74]. Therefore, the maximum permissible levels for PAHs in cocoa beans and derived products were established on a fat basis since PAHs tend to concentrate in the fat fraction, which is the cocoa butter [75]. It should be mentioned that cocoa beans and derived products are the only food categories for which the maximum permissible levels of PAHs are based on the fat fraction. EU has established the maximum level for BaP and for the sum of the four marker PAHs (BaA, BbF, Chr, and BaP) as 5.0 μg kg^{-1} fat and 30.0 μg kg^{-1} fat, respectively. The second maximum level for the sum of four marker PAHs came into force on 1 April 2015 [43].

1.3.2. Processed cereal-based products

According to the studies on PAHs exposure, food is the main source of human exposure to PAHs, while cereals constitute one of the major contributing sources [30-31].

Bread is an essential food in human nutrition. It is a good source of energy, contains vitamins, proteins, lipids, and minerals, which are crucial for human diet. In many European countries (Latvia, Poland, Italy, Spain, France, etc.) bread is a major component of people's diet with the per capita consumption among the highest in the world [2, 31-32, 46]. Bread contamination by PAHs can be dependent on both the contamination of bakery raw materials, primarily flour, and the baking process. An important issue is also the temperature of the thermal treatment taking into account its impact on bread contamination level [46, 76]. It is considered that a maximum amount of PAHs is formed when materials are heated at temperatures in the range of 500–550°C, while the average bread baking temperature is 250°C [77-78].

According to the European Food Safety Authority (EFSA) report [1], prepared on the basis of the results of studies performed in 16 Member States dealing with PAHs in food, with special attention to BaP, the mean contents of this compound in bread, flour and grain were 0.22, 0.10 and 0.09 μg kg^{-1}, respectively. Moreover, the percentage of samples exceeding the limit of detection in the case of bread was only 8%, in flour 31%, and in grain 53%. Other research has revealed that, in samples of toasted bread originating from Kuwait, BaP was not detected in 10 of 18 samples whereas, in the rest of the samples, it varied from 2.8 μg kg^{-1} to even 16.5 μg kg^{-1} [79].

In general, processed cereal-based foods are usually characterised by low levels of PAHs, however, it should be noted that, due to their high volume of consumption, they can be a significant source of exposure to PAHs [30, 32, 46, 80].

1.3.3. Dried herbs and spices

During the last 15 years, PAHs have been an emerging issue in the herb and spice industry [81]. Spices and herbs have been used for flavour, colour, aroma, and preservation of foods and beverages for many hundreds of years. They are important ingredients in many processed foods, e.g., meat products, dairy products, and bakery products, and in the majority of culinary recipes. Aside from their efficacy, spices and herbs are classified as "all natural" or from "natural sources", therefore projecting high expectations regarding the quality of the product for consumers [81-83]. Moreover, with the ever-increasing use of herbs/spices and

the global expansion of the seasonings market, safety has become a major concern for both the health authorities and general public worldwide [84].

In general, the organoleptic and commercial quality of herbs and spices is affected by intrinsic quality parameters, as well as unintentional and intentional sources of contamination. Besides illegal colourants and other illegal food additives, the major groups of chemical hazards in spices and herbs comprise mycotoxins, pesticide residues, heavy metals, persistent organic pollutants, including PAHs, cross-contamination with allergens and toxic endogenous compounds [19, 83-87]. Thereby, even if used at low amounts, culinary herbs and spices can harbour potential health hazards.

Several studies have reported detectable levels of PAHs in various types of botanical food supplements. Significant levels of PAHs were detected mainly as a result of inappropriate drying processes [6, 83, 85-86]. While relatively high levels of PAHs were reported for food supplements that contained individual herbal ingredients such as ginkgo, ginseng, green tea, spirulina, liquorice root, rose flowers, and bee products such as propolis [6, 19, 88]. Data collection on the occurrence of PAHs in foodstuffs carried out within the framework of Council Directive 93/5/EEC and by the EFSA revealed that spices and herbs are often contaminated with PAHs, also at very high levels [1, 89]. Therefore, the maximum levels (MLs) for PAHs have recently been laid down [43], stating that dried culinary herbs and spices that are sold on the EU market from 1 April 2016 must not exceed the ML of 10.0 $\mu g\ kg^{-1}$ for BaP and the ML of 50.0 $\mu g\ kg^{-1}$ for the sum of BaP, BaA, BbF, and Chr. Cardamon and smoked fruits of Capsicum species are exempt from the MLs to enable these smoked products to remain on the market and because the consumption of these spices is low [43].

Despite the fact that the MLs for PAHs have recently been set, studies on the permissible limits and safety aspects of these contaminants are still insufficient, indicating an urgent need to focus more research on this issue.

1.3.4. Smoked meat

Smoking of meat and its products has been used for centuries, not only to achieve particular sensory profiles like taste, colour, and aroma, but also to ensure preservation based on the antimicrobial, antioxidant, and drying effects of this process [90-91]. The traditional smoking procedure includes the exposure of meat products to the smoke generated by controlled combustion of certain natural hardwoods, sometimes accompanied by aromatic herbs and spices. The formation of PAHs depends on the specific conditions of the smoking process [29, 92-93]. During smoking phenolic substances are generated, which have

considerable importance in organoleptic properties of smoked meat products. Besides that, phenolic compounds show antimicrobial and antioxidant properties. Nowadays, smoking technology uses mainly the specific effects of various sensory active compounds contained in smoke aromatisation of meat products with suitable organoleptic profile, widely demanded on the market [94]. As undesirable consequence of smoking, PAHs are generated during the incomplete combustion of wood [29, 95-97].

Smoked meat has been extensively monitored for PAHs and according to the EFSA, meat and meat products are one of the food categories contributing most to the dietary PAHs intake per day of European Union member state consumers [1]. This demonstrates an important role of PAHs studies for smoked food products.

Latvia has a long tradition of meat smoking. Smoked meat is produced not only by large meat processing facilities, but also at home and by small companies that produce products according to the traditional recipes. However, according to Zelinkova & Wenzl, (2015b) exactly traditional smoking, when the meat is put near the fireplace for several days, is characterised by higher observed levels of the PAHs contamination [10].

Since 2012, the maximum levels of PAHs in traditionally smoked meat and meat products in the European Union were set to 5.0 μg kg^{-1} for BaP and 30.0 μg kg^{-1} for PAH4. These maximum levels were lowered in September 2014 to 2.0 μg kg^{-1} for BaP and 12.0 μg kg^{-1} for the PAH4 [75]. However, in accordance to the Commission Regulation (EC) No. 1327/2014, a list of EU countries was specified that were allowed to continue using traditionally smoked meat and smoked meat products with levels of PAHs higher than those set out in Commission Regulation (EC) No. 835/2011 (5.0 μg kg^{-1} for BaP and 30.0 μg kg^{-1} for the PAH4) [75, 98]. Those EU Member States (Ireland, Spain, Croatia, Cyprus, Latvia, Poland, Portugal, Romania, Slovakia, Finland, Sweden, and the United Kingdom) should continue to monitor the presence of PAHs in traditionally smoked meat and smoked meat products and should establish programmes to implement good smoking practices where possible, within the limits of what is economically feasible and what is possible without losing typical organoleptic characteristics of those products [98].

1.4. Strategies to reduce the contamination of food with the PAHs

Due to PAHs adverse effects these compounds are being monitored in foods to ensure that public health is not endangered by violative contaminants concentrations, whereas strategies for the reduction of these contaminants are of major importance. Additional concern on the contamination of foods with PAHs arises from an increasing rate of PAHs exposure

world-wide [33, 99-100]. Therefore, technological procedures in food production to reduce the content of pollutants hazardous to public health should be desirably applied. Nowadays, in the food industry it is very common to use starter cultures or ozone to improve the characteristics of the foods, and the possibility that these microorganisms or procedures could lower the food contaminants content is of great relevance.

1.4.1. Ozone treatment

Ozone is one of the most powerful sanitisers. It was affirmed as Generally Recognised As Safe (GRAS) in the United States and approved by the Food and Drug Administration (FDA) as an antimicrobial agent that can be directly applied in the food industry [9, 101-102].

Ozone has been known as a disinfecting agent since 1893, when it was first industrially used for drinking water treatment [103]. Since that time, ozone has been widely applied in water processing, food processing and food storage as a powerful disinfectant and oxidising agent. However, ozonation was seldom reported to be used to destroy BaP in environmental samples [104-106]. Apart from the processes of BaP degradation, ozone is widely used to reduce the mycotoxin content in foods [101, 107-108]; it also can kill pests and has potential applications for the inactivation of microbes including bacteria, fungi, and viruses [109-111]. Ozone has a high penetration capacity and can be quickly decomposed to oxygen without producing any toxic residues, therefore it has numerous potential applications in food industry [112]. However, the identity of BaP derivatives that may form in foods during ozonation remains to be elucidated.

1.4.2. Lactic acid bacteria treatment

A great interest has been recently expressed in the biodegradation of chemical compounds using microorganisms [99, 113-116]. Lactic acid bacteria (LAB) are the dominant starter cultures employed in the production of fermented foods [117]. The use of bacteriocins or bacteriocins producing LAB with wide range of antimicrobial activity can improve the safety aspects of food by the control of the fermentation microflora and speed of maturation; can increase the shelf life of the products and inhibit the growth of certain pathogenic bacteria during the fermentation and ripening periods [118]. A few reports on the positive effect of lactic acid bacteria (LAB) against PAHs are also already available [12, 100, 119-120].

PAH degradation capabilities are associated with members of certain taxa such as *Pseudomonas*, *Sphingomonas*, and *Burkholderia*, independent of origin of the soil from which bacteria isolated [100, 119]. Moreover, genes responsible for PAH degradation are homologous and ordered. The results obtained by Abou-Baker et al., (2012) revealed that

PAHs was affected by *B. bifidium* strain during the incubation period [119]. For example, after 2 to 48 h of incubation, low weight PAHs as naphthalene, acenaphthylene, 2-bromonaphthalene, and acenaphthene were not detected in the various samples. However, 2-bromonaphthalene and acenaphthene that were appeared after 72 h of incubation were reduced by 75% and 88%, respectively.

2. EXPERIMENTAL PART

2.1. Chemicals and materials

Pesticide grade solvents (acetonitrile, cyclohexane, n-hexane, dichloromethane, toluene, m-xylene, acetone, and ethyl acetate), as well as Celite-545 were purchased from Sigma-Aldrich (Steinheim, Germany), while pesticide grade ethanol used for the study of BaP degradation was purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore-Q SP Reagent Water system (Millipore, Bedford, MA, USA) and pre-filtered through a 0.22 μm membrane filter. Anhydrous sodium sulphate and Strata SI-1 Silica solid-phase extraction (SPE) tubes (6 mL, 500 mg) were obtained from Supelco (Bellefonte, PA, USA) and Phenomenex (Torrance, CA, USA), respectively.

Four different types of MWCNTs were employed as potential sorbents for d-SPE. Agglomerated MWCNT of trademark Baytubes® C150P (abbreviated further as CNT-1) were obtained from Covestro AG (Leverkusen, Germany) with the purity greater than 95%, outer diameter (O.D.) of 13 nm, internal diameter (I.D.) of 4 nm, length >1 μm, and the density range of 1300–1500 kg/m^3 (according to data provided by manufacturer). Other three disperse MWCNTs – TNIM4 (abbreviated as CNT-2), TNIMH4 - hydroxyl derivate of TNIM4 (CNT-OH; hydroxy group content 2.5 wt.%), and TNIMC4 – carboxyl derivate of TNIM4 (CNT-COOH; carboxyl group content 1.6 wt.%) were purchased from Chengdu Organic Chemicals Company (Sichuan Sheng, China). The latter three MWCNTs were of >95% purity, with 5-10 nm I.D., 10–30 nm O.D., 10–30 μm length, and the average density of 2100 kg/m^3.

Four individual PAH standards (BaA, BbF, BaP, Chr) (50 mg L^{-1} each in acetonitrile) and isotopically labelled deuterated standards (BaP-d_{12}, BbF-d_{12}, Chr-d_{12}, BaA-d_{12}) (1 mg L^{-1} each in toluene) were purchased from LGC Standards (Bury, UK) and were stored at 4°C. The spiking solutions and calibration standards were prepared by serial dilution of stock standards in toluene and were stored in UV-protected glassware at 4°C.

Pure BaP standard (97% assay by HPLC) used for BaP ozonation and degradation study was purchased from Sigma-Aldrich (Steinheim, Germany). The BaP standard solution of 1 mg mL^{-1} concentration was prepared by weighing an aliquot of BaP standard in a 100 mL graduated flask and then dissolving in ethanol. The solution was stored at 4°C.

2.2. Microorganisms

Pediococcus acidilactici KTU05-7, *Pediococcus pentosaceus* KTU05-9 and *Lactobacillus sakei* KTU05-6 strains, previously isolated from spontaneous rye sourdough were obtained from the collection of Kaunas University of Technology. The lactic acid bacteria were grown in de Man Rogosa Sharpe (MRS) medium (Biolife, Italy). *P. acidilactici* KTU05-7, *P. pentosaceus* KTU05-9 and *L. Sakei* KTU05-6 strains were incubated at 32, 35 and 30°C, respectively, for 24 h and were used for further experiments.

2.3. Preparation of MWCNT-based sorbents for d-SPE and control of background contamination

Taking into account that relatively small amounts of MWCNTs are required for sufficient recovery of PAHs from the matrix aliquot, and in order to facilitate handling operations, MWCNTs were dispersed in Celite-545 at the ratio of 5/95 (w/w). Considering the affinity of MWCNTs towards sorption of planar aromatic compounds and the ubiquity of PAHs, there was a need to control the probable background contamination of SPE sorbents based on MWCNTs. In this respect the prepared MWCNT-based sorbent mixtures were washed with hot toluene under reflux conditions for 48 h, dried overnight at room temperature under aluminum foil and heated at 115°C for 4 h.

2.4. Sample preparation and clean-up

The presence of PAHs in 15 oil samples was determined using a novel sample preparation method based on d-SPE with MWCNTs, whereas rest 391 samples (bread and cereal products, dark chocolate, dried herbs and spices, smoked meats) were analysed using a sample preparation method based on extraction of PAHs with an organic solvent mixture, gel permeation chromatography and SPE. All the samples were minced, ground, cut or crushed and then thoroughly homogenised before the analysis. Smoked meat samles were homogenised (including skin and muscle) without bones. Dried herbs and spices samples were additionally sieved through a 1.5 mm sieve to ensure satisfactory homogeneity of the test samples.

2.4.1. Sample extraction and clean-up using MWCNT-based sorbents

A 1.0 gram aliquot of oil sample was placed in a 15 mL glass tube, spiked with 100 μL of isotopically labeled PAH surrogates in toluene (100 pg μL^{-1} of each surrogate) and n-hexane (10 mL) was added. The sample was vortexed and pre-cleaned d-SPE sorbent (1.0 g) consisting of 50 mg of MWCNTs and 950 mg of Celite-545 was added. After performing the d-SPE procedure by vigorous shaking of the glass tube for 25 min, the sample was centrifuged at 3000 rpm for 5 min and the upper n-hexane phase was eliminated. Subsequently, 10 mL of washing solvent was added and, after vigorous shaking for 5 min, the centrifugation was repeated and the washing solvent was removed. The resultant d-SPE sorbent was dispersed in 5 mL of toluene and the obtained slurry was quantitatively transferred into an extraction thimble for the semi-automatic Soxtec™ 2055 Extraction System (Hillerød, Denmark). PAHs were eluted from the d-SPE sorbent into a glass vessel with toluene (35 mL), according to the following program: immersion of the sample in refluxing solvent for 15 min with further rinsing of the thimble for 60 min, followed by careful elimination of the bulk of the solvent for 15 min. The residue was quantitatively transferred with n-hexane (5 mL) to a 10 mL glass tube and complete solvent evaporation was performed under a gentle stream of nitrogen. The resulting sample was reconstituted in toluene (50 μL) and analysed by the means of GC-MS/MS.

2.4.2. Sample extraction and clean-up using gel permeation chromatography and solid-phase extraction

A 2.75 g portion of each homogenised sample was mixed with 10-15 g of anhydrous sodium sulphate to absorb moisture. A 27.5 μL aliquot of toluene solution containing BaP-d_{12}, Chr-d_{12}, BbF-d_{12}, and BaA-d_{12} internal standards at 1.0 μg mL^{-1} concentration was added. The PAHs were extracted from test samples by adding 25 mL of dichlormethane/hexane (1:1, v/v) mixture and performing sonication for 20 min. After sonication, the supernatant was decanted and 15 mL of fresh solvent was added for another 20 min sonication cycle. To avoid the presence of solid particles, all the extracts were filtered. The combined extracts (~40 mL) were evaporated on a rotary evaporator (30°C, 500-100 mbar) to remove the solvents, and the residue was reconstituted in 5.5 mL of cyclohexane/ethyl acetate (1:1, v/v) mixture for further removal of high molecular mass compounds by the means of gel permeation chromatography (GPC). The extracts were centrifuged at 3000 rpm for 10 min and the solution was transferred into a glass GPC vial. The sample extracts were injected into an LC Tech Freestyle™ GPC system (Dorfen, Germany) consisting of an LC pump, autosampler, and a fraction collector.

High molecular mass substances were removed on a glass column (500 × 40 mm, 25 mm ID) filled with 50 g of Bio-Beads SX3 (Bio-Rad, Philadelphia, PA, USA) stationary phase with cyclohexane/ethyl acetate (1:1, v/v) mobile phase at a flow rate of 5 mL min^{-1}. The automated GPC program was as follows: dump time 0 – 21 min, collection time 21 – 45 min. The collected fraction was transferred to a round-bottom flask, evaporated (30°C, 130 mbar) to dryness on a rotary evaporator and the residue was redissolved in 3 mL of cyclohexane. Further clean-up was performed by using Strata SI-1 Silica SPE cartridges. The sorbent of the SPE cartridges was first conditioned with 5 mL of cyclohexane and then the extracts were loaded onto the cartridges. The analytes of interest were eluted from the column with cyclohexane (3 × 3 mL), the obtained fraction was evaporated under a nitrogen stream at 40°C, dissolved in appropriate organic solvent (50 µL of cyclohexane for GC-MS/MS or GC-HRMS analysis; 100 µL of acetonitrile for HPLC-HRMS analysis), and transferred into an autosampler vial for the further analysis of PAHs.

2.5. Instrumental analysis

2.5.1. Parameters of the HPLC-HRMS method

A Thermo Scientific Dionex Ultimate 3000 Series RS pump coupled to a Thermo Scientific Dionex Ultimate 3000 Series TCC-3000RS column compartments and a Thermo Fisher Scientific Ultimate 3000 Series WPS-3000RS autosampler controlled by Chromeleon 7.2 software (Thermo Fisher Scientific, Waltham, MA and Dionex Softron GmbH, Part of Thermo Fisher Scientific, Germany) were used for the analysis. A Pinnacle DB PAH 50 mm × 2.1 mm, ID 1.9 µm (Restek, Bellefonte, PA, USA) column was used for the chromatographic separation of target compounds at 30°C.

Mobile phase A consisted of 100% water, and mobile phase B consisted of 100% acetonitrile. The gradient was started from 50% B, then the phase B was increased to 90% from 0–1 min, after that the phase B was increased to 95% (1-2 min) and, finally, to 100% (2-5 min), and was held constant for 1 min. Finally, the mobile phase was brought back to the initial conditions and maintained for 1 min. The selected flow rate was 0.4 mL min^{-1} and the injection volume was 5 µL.

The determination of target analytes was performed using an HPLC-Orbitrap-MS system consisting of Thermo Scientific Dionex Ultimate 3000 HPLC instrument (Bremen, Germany) coupled to an Orbitrap Q-Exactive mass spectrometer (Bremen, Germany) equipped with a Thermo Scientific Ion Max APCI/APPI interface (Thermo Fisher Scientific).

The Ion Max source housing was equipped with a Syagen Photo-Mate vacuum UV light source (krypton discharge lamp, 10.0 eV) (Syagen Technology Inc., Tustin, CA, USA). The APCI probe was used as a nebulizer-desolvation device without applying corona discharge. The mass spectra were recorded in the positive ion mode. During the tuning procedure, the signals of target analytes were preliminarily optimised for the highest response of the corresponding ions. Introduction of the target compounds (native analytes and deuterated internal standards, 1 ng mL^{-1} in toluene) into the APPI interface of MS system was performed using a Chemyx Fusion 100T (Stafford, TX, USA) infusion pump at the flow rate of 50 µL min-1 through a T-piece connected to the capillary, which delivered the mobile phase to the ion source with a flow rate of 400 µL min^{-1}. Orbitrap-MS detection in tMS^2 mode was applied for the quantitative determination of selected compounds using the two most abundant ions of the respective molecular ion cluster for both the native components and the deuterated internal standards. The width of the ion-extraction window was 5×10^{-6} amu (5 ppm). External calibration of the Orbitrap-MS system was performed before each batch of samples over the m/z range of 50 – 2000 according to the guidelines provided by the instrument supplier. The details of the optimised instrumental parameters in tMS^2 mode are summarised in Table 2.1, while the average experimental mass of precursor ion as well as the ion masses used for the quantification and confirmation of all four marker PAHs are shown in Table 2.2.

Table 2.1

The optimised DA-APPI-Orbitrap-MS parameters for tMS^2 mode

APPI parameters	
Sheath gas flow	40 a.u.
Auxiliary gas flow	30 a.u.
Sweep gas flow	0 a.u.
Capillary temperature	300°C
Source heater temperature	350°C
S-lens radio frequency	35 a.u.
Dopant	Toluene
Dopant flow rate	50 µL min^{-1}
Orbitrap-MS parameters	
Detection mode	tMS^2
Maximum injection time	100 ms
Automatic gain control (AGC target)	2×10^5
MS resolving power	17,500 FWHM

a.u. - arbitrary unit

Table 2.2

Orbitrap-MS scan parameters of the detection of selected PAHs

PAH	Calculated exact mass $[M]^+$, *m/z*	Experimental mass $[M]^+$, *m/z*	Mass accuracy, ppm	NCE, %	Quantification ion[1] mass, *m/z*	Experimental quantification ion[1] mass, *m/z*	Mass accuracy, ppm	Confirmation ion[2] mass, *m/z*	Experimental confirmation ion[2] mass, *m/z*	Mass accuracy, ppm
BaA	228.0939	228.0943	1.6	100	226.0783	226.0792	4.0	202.0783	202.0792	4.5
Chr	228.0939	228.0945	2.7	100	226.0783	226.0792	4.0	202.0783	202.0792	4.5
BbF	252.0939	252.0936	-1.2	100	250.0783	250.0784	0.76	226.0783	226.0789	2.7
BaP	252.0939	252.0937	-0.67	100	250.0783	250.0785	0.84	226.0783	226.0788	2.6
BaA-d_{12}	240.1692	240.1697	2.1	110	236.1410	236.1419	3.8	212.1410	212.1417	3.3
Chr-d_{12}	240.1692	240.1703	4.6	100	236.1410	236.1419	3.8	212.1410	212.1417	3.3
BbF-d_{12}	264.1692	264.1683	-3.4	110	260.1410	260.1404	-2.3	236.1410	236.1409	-0.42
BaP-d_{12}	264.1692	264.1682	-3.8	120	260.1410	260.1405	-1.9	236.1410	236.1412	0.85

ppm – part per million

[1] $-[M-H_2]^+$ or $[M-D_2]^+$

[2] $-[M-C_2H_2]^+$ or $[M-C_2D_2]^+$

2.5.2. Parameters of the GC-HRMS method

The PAHs analysis was carried out on an Agilent Technologies 6890N gas chromatograph coupled with MicromassAutospec Premier high-resolution mass spectrometer. The GC-HRMS system was equipped with a ZB-50 capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) and operated in a splitless mode with helium as the carrier gas at a constant flow rate of 1.2 mL min^{-1}. The following GC-HRMS operating parameters were employed: injector temperature was set at 300°C; capillary line temperature 280°C; source temperature 280°C; electron energy 36 eV, and the ion trap current was 600 μA. The oven temperature was set initially at 90°C (1 min hold), increased to 215°C at 25°C min^{-1}. At 215°C, the temperature increased at a rate of 4°C min^{-1} to 235°C, at 235°C the temperature increased at a rate of 15°C min^{-1} and then to 320°C at a rate of 4°C min^{-1} (10.5 min hold). The total run time was 35 min. The injection volume was 1 μL.

The GC-HRMS system was operated in EI mode, at a resolving power > 10,000 (10% valley definition). The GC-HRMS system was controlled by MassLynx 4.1 software (Waters). The analysis was performed by operating the MS in the selected ion monitoring mode (SIM). The accurate mass of the monitored ions of each compound was individually optimised. Selected m/z values of fragments for the analysed compounds are given in Table 2.3.

Table 2.3

The compounds monitored by HRMS, with the respective analytical parameters

Analyte	**Retention time, min**	**Quantifier transition/ion, *m/z***	**Qualifier transition/ion, *m/z***
BaA	15.87	228.0939	240.1692
Chr	16.08	228.0939	240.1692
BbF	19.22	252.0939	264.1692
BaP	20.59	252.0939	264.1692
BaA-d_{12}	15.78	240.1692	–
Chr-d_{12}	15.97	240.1692	–
BbF-d_{12}	19.11	264.1692	–
BaP-d_{12}	20.47	264.1692	–

2.5.3. Parameters of the GC-MS/MS method

The analysis of PAHs was carried out on a Thermo Scientific Trace GC Ultra gas chromatograph coupled with Thermo Scientific TSQ Quantum XLS Ultra mass selective detector. This GC-MS/MS system was equipped with a Zebron ZB-50 (Phenomenex)

capillary column (30 m × 0.25 mm i.d. × 0.25 mm film thickness) and operated in a splitless mode. The injector temperature was kept at 260°C. The transfer line temperature was set at 320 °C. The operating conditions were as follows: helium was used as the carrier gas at a constant flow rate of 1.2 mL min^{-1}; inlet temperature 260°C; MS transfer line temperature 280°C. The oven temperature was set initially at 80°C (2 min hold), increased to 265°C at 15°C min^{-1}. At 265°C, the temperature was increased at a rate of 5°C min^{-1} to 290°C and then to 320°C at a rate of 20°C min^{-1} (12 min hold). The total run time was 38 min. The injection volume was 1 μL.

A tandem mass spectrometer with an electron impact (EI) interface was used, operating in the positive ion mode at electron energy of 70 eV; emission current of 50 μA and the source temperature of 250°C. The collision gas was argon, supplied at 1.0 mTorr pressure in the collision chamber. The system was controlled by Xcalibur software (Thermo Scientific). The data were acquired by operating the MS in selective reaction monitoring (SRM) mode. The transitions of each compound were individually optimised to produce suitable sensitivity and selectivity. Pseudo-SRM transitions were not selected in order to provide better selectivity and confirmatory capabilities of the analytical procedure. The obtained $[M]^+ \rightarrow [M–2H]^+$ transitions for quantification and $[M]^+ \rightarrow [M–2CH]^+$ transitions for the confirmation of selected PAHs are given in Table 2.4.

Table 2.4

The compounds monitored by GC-MS/MS, with the respective analytical parameters

Analyte	Retention time, min	Quantifier transition, *m/z*	Qualifier transition, *m/z*	Collision energy for both transitions, eV
BaA	17.34	228.1 → 226.1	228.1 → 202.1	25
Chr	17.54	228.1 → 226.1	228.1 → 202.1	25
BbF	20.76	252.1 → 250.1	252.1 → 226.1	30
BaP	22.44	252.1 → 250.1	252.1 → 226.1	30
BaA-d_{12}	17.28	240.1 → 238.0	–	30
Chr-d_{12}	17.47	240.1 → 238.0	–	30
BbF-d_{12}	20.66	264.1 → 260.1	–	30
BaP-d_{12}	22.32	264.1 → 260.1	–	30

2.6. Quality assurance/quality control

Identification criteria for the analytes of interest were based on the retention times of native PAHs and deuterated PAH surrogates, and the isotopic peak ratios of the SRM

transitions. The acceptable deviation of the isotopic peak ratio of two monitored ions or SRM transitions (target/confirmation) was within 15% of the value obtained for the medium calibration point. A minimum of five-point calibration curve was checked with relative response factors (RRFs) over the sample concentration range of 0.10–50.0 ng g^{-1} and was used for quantifying the analytes of interest in each sample run. The procedural blanks were taken through all steps of analytical procedure and were found to be uncontaminated with the analytes of interest. The quantification of analytes of interest was based on stable isotope dilution with the deuterated PAH surrogates and on internal standardisation.

2.7. Sampling and storage

In total, 406 different food samples were selected and analysed for the PAH content (see Table 2.5). These included 5 different food groups – bread and cereal products, dark chocolate, seasonings (dried herbs and spices), smoked meats and edible oils.

Bread and cereal products group included 35 samples from 15 different Latvian bakeries – 20 rye bread samples, 12 wheat bread samples and 3 cereals. All the samples were obtained from local markets and supermarkets and were immediately prepared upon arrival to the laboratory to avoid potential chemical composition changes. The final extracts were stored at -20°C before the instrumental analysis.

Dark chocolate samples included twenty six randomly selected samples originating from different countries and obtained from local supermarkets in Riga, Latvia. To avoid potential chemical composition changes, all samples were immediately prepared upon arrival to the laboratory, while the final extracts were stored at -20°C before performing the instrumental analysis.

Seasonings samples included: three types of commercial ground herbs: 25 basil samples from India, 25 oregano samples from Turkey, 24 thyme samples from Poland and one thyme sample from China, and three types of commercial ground spices: 25 blends of black pepper originating from Brazil and Vietnam, 25 nutmeg samples from Indonesia, and 25 sweet paprika / chilli blends originating from Brazil and China. In order to assess the possible influence of the year of production and pretreatment process on the PAH content, seasonings samples were grouped by the production year and pretreament type. All the samples were provided in 2014 by FUCHS Gewürze GmbH (Germany). To avoid potential chemical composition changes, all samples were immediately processed upon arrival to the laboratory and the final extracts were stored at -20°C before performing the instrumental analysis.

Smoked meat samples for the determination of PAHs were purchased in the time period from March to May 2014. For the study, 128 samples of various smoked meat products, produced by 48 different smoked meat manufacturers were obtained in local Latvian markets. Smoked meat samples included: smoked meat (pork, pork breast, chop, speck, smoked ham, smoked chicken), and smoked meat products (sausages, small sausages, semi-dry sausages, roulette and other). All samples were labelled, homogenised, and frozen at -20°C before the analysis.

Smoked meat samples for the determination of high levels of PAH were purchased in the time period from November 2016 until May 2017. The samples were selected with an emphasis to those with darker surface colour and origin from small-scale producers. A total of 52 smoked meat samples from 29 different producers in Latvia, 17 smoked meat samples from 10 Lithuanian companies and 8 samples from 7 Estonian producers were collected. All samples were labelled, homogenised, and frozen at -20°C before the analysis.

Edible oil samples (n=15) were collected during the period from February to May 2017 within the framework of a monitoring program for control of PAHs in Latvian food products. The samples were transported to the laboratory while protected from ambient UV radiation. To avoid potential chemical composition changes, the samples were immediately processed upon arrival and the final extracts were stored at -20°C prior to the instrumental analysis.

Table 2.5

Summary of the samples analysed for the PAHs content

Product group	Number of analysed samples
Bread and cereal products	35
Dark chocolate	26
Dried herbs	75
Edible oil	15
Spices	75
Smoked meats	180
Total	***406***

2.8. Smoked meat consumption data evaluation

The dietary survey of Latvian inhabitants was conducted in the year 2012 and almost two thousand participants from the age group of 19-64 were reached. Due to the fact that smoked meat consumption is a specific part of food consumption, focussed additional

research for smoked meat consumption in Latvia in the age group of 19-64 was carried out in 2014.

For dietary survey, the food frequency questionnaire and 24 h recall method were used. An additional questionnaire for smoked meat producers about processing technologies was used.

2.9. Risk characterisation

The actual contribution of smoked meat products to the overall exposure to BaP and PAH4 was assessed by estimating the MOEs, using the lower confidence limit of benchmark dose for a 10% increase in the number of tumour bearing animals compared to control animals ($BMDL_{10}$), as proposed by EFSA. Taking into account the findings of the EFSA study on PAHs in food, the $BMDL_{10}$ for BaP was 0.07 mg kg^{-1} b.w. per day, and the $BMDL_{10}$ for the PAH4 was 0.34 mg kg^{-1} b.w. per day. These values were used as a reference for the calculations of MOEs [1].

The MOE values were calculated by dividing the reference $BMDL_{10}$ values with the mean, median, 75 and 95 percentiles of the estimated dietary exposure to BaP and PAH4. In order to calculate the exposure of the whole population and specific population groups, data on the mean, median, 75 and 95 percentile consumption of smoked meat products were used. In accordance with the scientific opinion of EFSA on a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic, the MOEs of 10,000 or higher were assumed as to be of low concern from the viewpoint of public health and were considered as low priority for risk management actions [1, 121].

Comparison of the MOE indicators within different groups of consumers was carried out to conclude whether consumption of smoked meat products could present a risk to public health, taking into account the characteristic consumption patterns.

The one-way analysis of variance (ANOVA) and t-test with the Microsoft Excel Data Analysis Toolpack was used to test the differences between PAHs in different food samples. A criterion of $p < 0.05$ was considered to indicate statistical significance.

2.10. Ozone treatment to reduce PAHs contamination in smoked fish samples

2.10.1. Ozonation of BaP standard solution

Ozone gas was generated from purified extra-dry oxygen (purity ≥99.5%, AGA) using an ozone generator (OZ-3G, Kai Yuan, Guangzhou, China). The generated maximum ozone output was 3 g h^{-1}, meanwhile the maximum ozone concentration at the outlet was 10 mg L^{-1}. The oxygen flow rate was ~6 L min^{-1}, the current was 260 mA.

A standard solution of BaP was treated with 10 mg L^{-1} of ozone at the flow rate of 5 L min^{-1} for 1 min, 2 min, 5 min, 15 min, and 30 min. After the ozone treatment the samples were transferred into autosampler vials, labelled, and stored at -20°C until further analysis.

2.10.2. Ozonation and analysis of smoked fish samples

Ozonation experiments on PAHs content reduction were performed on a smoked fish sample with previously determined high PAH content. 300 g of smoked sprats were evenly hanged on metal rods and placed in a 60 L plastic box. The generated ozone gas at the maximum flow rate was introduced into the bottom of the box via plastic tube until the ozone concentration reached its maximum value of 20 mg m^{-3}.

The concentration of the introduced ozone was regulated by controlling the proportion of the ozone output from the generator and determined by a portable ozone sensor (A-22 Ozone Sensor, EcoSensors, California, USA). After the ozonation of sprats for 5 min the ozone gas flow was stopped, and the box was tightly closed. The first fifty gram portion of sprats after ozonation for 5 min was transferred to a plastic bag for further analysis of PAHs, while the remaining sprats were ozonated for additional 10 min (the ozone concentration in the box was again adjusted to 20 mg m^{-3}). After 10 min the second portion of fifty grams of sprats (ozonated for 15 min) was transferred to a plastic bag for further analysis of PAHs and the remaining sprats were ozonated for additional 15 min to obtain a sample with cumulative ozone exposure for a total of 30 min. The same procedure was repeated to obtain sprats with 45 and 60 min of ozone exposure. Ozone concentration was continuously monitored during all the experiments. All experiments were performed at room temperature and 65–75% relative humidity.

To avoid potential changes in chemical composition, all ozonated samples of sprats were immediately prepared for analysis and the final extracts were stored at -20°C before analysis.

The sample preparation procedure in the current study was based on GPC followed by SPE on silica gel and the determination of PAHs by the means of GC-MS/MS.

2.10.3. HepG2 and 3T3 cell culture and treatment

The HepG2 and 3T3 cell lines were suspended in standard cell cultivation medium DMEM/10%FBS (Biochrom, Germany) and seeded **i**n 96-well microplates (Sarstedt, Germany) at the density of 2500 cells per well (p/w) for HepG2 and 5000 cells p/w for 3T3. The cell lines were incubated at 37°C, 5% CO_2, and were left to adhere to the surface of the plate and allowed to grow for 48 hours. The cell cultivation medium was then drained and treated with 100 μL of fresh medium as control, 40% (v/v) solvent control solution (distilled H_2O), and BaP standard solutions at different concentrations corresponding to 1 – 8 μg mL^{-1} of BaP in the cell cultivation medium. Cells were imaged for up to 96 h by phase contrast imaging at 100× magnification, using an IncuCyte ZOOM microscope system (Essen Biosciences, USA). The kinetics of cell growth was monitored using the IncuCyte integrated confluence algorithm, where the confluence served as a surrogate for the number of cells.

2.11. Lactic acid bacteria treatment to reduce PAHs contamination in smoked meat samples

2.11.1. Antimicrobial activity determination

2% (w/w) of LAB cells were inoculated into a fresh MRS medium and propagated for 18 h. The cells were harvested by centrifugation (6000 g, 10 min, 4°C), and the supernatants then filtered through a 0.2 mm sterile Millipore filter to remove remaining cells. Supernatants were used for the determination of antimicrobial activities against various pathogenic and food spoilage bacteria. Agar well diffusion assay was performed for LAB antimicrobial activity evaluation. 0.5 McFarland Unit density suspension of each indicator bacteria strain were inoculated onto surface of cooled Mueller Hinton Agar (Oxoid, UK) using sterile cotton swabs. The wells (6 mm in diameter) were punched in agar and filled with 50 μL of LAB supernatants. The antimicrobial activities against tested bacteria were determined by measuring the diameter of inhibition zones (mm) after 48 h of cultivation at 37°C. Antimicrobial activity of LAB produced metabolites was tested against pathogens according to Cizeikiene et al. (2013) [115]. Antimicrobial activity tests were performed in three independent experiments and the average of inhibition zones was calculated.

2.11.2. Evaluation of potatoes juice as an alternative substrate for LAB propagation

Potatoes (var. Vinetta) tubers were obtained from a local farm (Mazeikiai, Lithuania) after 2014 harvest. The tubers were stored at 5°C in the dark. A juice of potatoes tubers was extracted by blending potatoes pieces without any additives. Potato mass was filtered through nylon mesh with pore size of approximately 150 μm to remove potato particles and obtained potato juice were sterilised at 121°C for 15 min and used for LAB propagation. 2% (w/w) of freshly prepared LAB cells suspension was inoculated in sterilised potato juice media and fermented at optimal temperatures: 32°C for *P. acidilactici* KTU05-7, 35°C for *P. Pentosaceus* KTU05-9 and 30°C for *L. sakei* KTU05-6 strain. The viable LAB cells in the potatoes juice was evaluated under standard serial dilution method on MRS agar medium at 30°C and expressed in $\log_{10}$ cfu mL^{-1}. The plates were incubated at 30°C temperature for 4 days under anaerobic conditions in a jar (Sigma–Aldrich, Broendby, Denmark) with anaerobic atmosphere generation bags (Sigma–Aldrich, Broendby, Denmark). The pH values were measured using a pH electrode (PP – 15, Sartorius, Goettingen, Germany). For total titratable acidity (TTA) determination, 10 mL of sample was homogenised with 90 mL of distilled water. After that the sample was neutralised with 0.1 M NaOH up to pH value 8.2. TTA was expressed as the amount (mL) of 0.1 M NaOH used to obtain the pH value 8.2. The analyses of fermented potatoes juice (count of viable LAB cells, pH, TTA and lactic acid content) was carried out after 24, 48 and 72 h of fermentation. LAB multiplied in an alternative substrate (potato juice) were used for surface treatment (before and after smoking) of cold smoked pork meat sausages.

2.11.3. Production of sausages for LAB experiments

The cold smoked pork sausages production was performed in the meat products production company “Nematekas” (Dovainonys, Lithuania). Sausages were made of 77% fresh pork, 20% frozen back fat, 2.4% salt (containing 0.4% sodium nitrite ($NaNO_2$)), 0.4% glucose, and 0.4% spice mix. Meat and fat was ground. After grinding, the batch was mixed 2 min in a mixer to distribute the added fat and spice mix. The mixture was vacuum-stuffed into natural casing (40 mm diameter, 240 mm length). Sausages treatment with LAB have been performed before (I) and after (II) smoking. (I) Formed fresh pork meat sausages, 400 g of each, were placed individually in a container with 1000 mL of fermented potatoes juice (containing on average 9.6 $\log_{10}$ cfu mL^{-1} of LAB) and were immersed for 60 min at 18°C. After immersion the sausages were drained and covered with plastic film. Samples were stored at room temperature (18-20°C) for 24 h. After 24 h sausages were ripened for 78 h in

24°C temperature under 93-86% humidity. After ripening fresh pork meat sausages were smoked at 16°C temperature for 130 min under 80-82% humidity. After smoking sausages were dried (8 days at 15°C under 75% humidity). The smoking and drying was carried out in the universal thermal camera (Bastramat 850 C-UP, Armsberg, Germany) with separate sawdust smoke generator. (II) The sausages after smoking and drying (400 g of each) were placed individually in a container with 1000 mL fermented potatoes juice (containing on average 9.6 $\log_{10}$ cfu g^{-1} of LAB) and covered with plastic film. Samples were stored at room temperature (18-20°C) for 24 h. The diagram of sausages preparation, additional biotreatment with LAB and sampling scheme is presented in Fig. 2.1. Control sample was prepared without treatment by LAB suspension. To evaluate the possible transition of PAHs into the liquid phase, control samples before and after smoking were treated with water. For cold smoking alder wood was used.

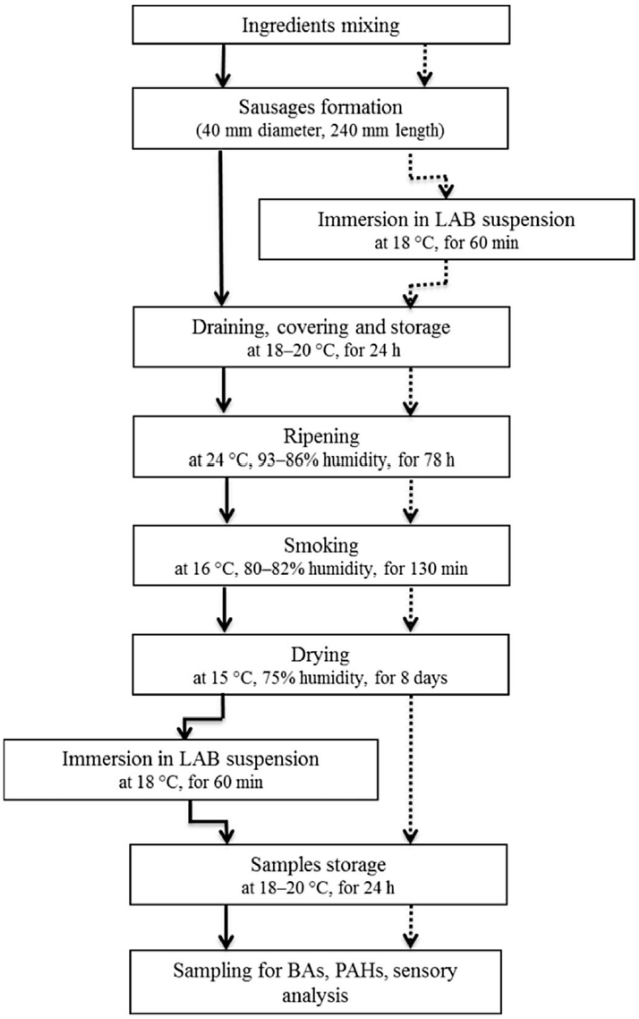

Fig. 2.1. **The diagram of sausages preparation, additional bio-treatment with LAB and sampling**

Sausages were collected for PAHs analysis. Geometry of the sausages and sampling locations for PAHs and BAs analysis are presented in Fig. 2.2.

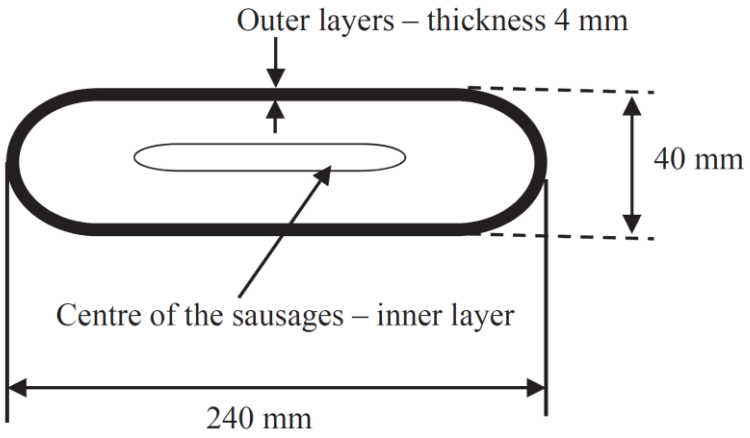

Fig. 2.2. **Geometry of the sausage and sampling locations for PAHs analysis**

2.11.4. Determination of polycyclic aromatic hydrocarbons (PAHs) in smoked pork meat sausages

The method for PAHs extraction from meat was carried out according to the procedures described in section 2.4.1. The sample preparation procedure included extraction of PAHs with an organic solvent mixture, GPC and SPE on silica gel. The determination of PAHs was performed by the means of GC-MS/MS.

3. RESULTS AND DISCUSSION

A set of experiments was performed following the different pathways to cover complex areas dedicated to issues of PAHs contamination in food. First, a novel analytical method for the determination of PAHs in edible oils by applying dispersive SPE (d-SPE) with MWCNTs as sorbents for selective extraction of analytes was elaborated. The whole procedure was optimised, including the type of MWCNT, as well as the extraction and elution conditions. Next, three different determination methods including HPLC-Orbitrap-MS, GC-HRMS, and GC-MS/MS were elaborated and optimised.

With the purpose to demonstrate applicability of elaborated methods the most challenging matrices were selected – edible oil as the most fatty matrix, to show the capability of sorption in fatty matrices, with regard to investigate the potential of the elaborated method for the application for other fatty matrices like canned smoked sprats and smoked meat; dark chocolate – as the only matrix that has the established MLs on a fat basis and thus requires ultra-low detection limits; bread – as a matrix that has the lowest established MLs.

The present study has been concentrated on the occurrence of 4 EU-regulated PAHs in different seasonings, while to monitor the PAHs in one of the food categories contributing most to the dietary PAHs intake, the smoked meat and smoked meats products from Latvia were analysed. With regards to latter the exposure of certain Latvian population groups to PAHs from smoked meat products was also assessed and risks associated with the uptake were evaluated. In addition, targeted smoked meat samples from the Baltic states were also analysed to assess the current situation in all three countries.

Concluding the ubiquitous PAHs contamination, two different strategies to reduce the PAHs contamination in processed foods were assessed - the effect of ozone treatment on BaP degradation in solutions and smoked products, and the influence of surface treatment with LAB in an alternative substrate for the reduction of PAHs contamination of the cold smoked pork meat sausages.

3.1. Innovative sample preparation method for the PAHs analysis – MWCNTs

The application of multi-walled carbon nanotubes (MWCNTs) in modern analytical methods has recently received increased attention due to its broad potential use as a sorbent for removing and immobilising various contaminants from different types of samples, including food. Hence, the study on an analytical method for the determination of PAHs in

edible oils by applying dispersive SPE (d-SPE) with MWCNTs as sorbents for selective extraction of analytes, followed by detection with GC-MS/MS was performed.

During a study it was observed that procedural blanks showed the background presence of significant quantities of PAHs in commercially available MWCNTs. For example, application of 50 mg of non-pretreated MWCNTs for the extraction of one gram of oil resulted in elevated BaA concentration by up to 0.6 μg kg^{-1} for CNT-1, 0.8 μg kg^{-1} for CNT-2, 1.7 μg kg^{-1} for CNT-COOH, and 2.5 μg kg^{-1} for CNT-OH, respectively. The source of this contamination is unknown; however, this fact should be considered during the application of MWCNTs as sorbents in PAH analysis. In order to avoid background contamination, all the prepared MWCNT-based sorbent mixtures were pre-treated until the sorbents did not show signals of the selected PAHs.

Experiment design for the optimisation of the extraction and clean-up procedure for the PAHs included the following steps: 1) selection of organic solvent for the dilution of oil samples to enhance the analyte sorption efficiency from the matrix; 2) selection of an appropriate solvent for analyte desorption and, 3) selection of the MWCNT sorbent type, considering a) sorption capacity of each MWCNT type towards PAHs in the presence of matrix; b) effectiveness of PAH desorption from the MWCNTs and, c) the affinity of MWCNTs towards matrix coextractives and selection of solvent for the washing step in order to reduce possible influence of matrix coextractives. All experiments devoted to the optimisation of extraction procedure were carried out using deuterated PAH surrogates as target compounds in order to eliminate the influence of the possible native PAH background in the laboratory environment and considering that isotopically labeled surrogates behaved analogously to the analytes in the presence of matrix components.

3.1.1. Selection of sample dilution and analyte elution solvents

One of the most convenient ways for providing better transfer of analytes from the oil matrix to d-SPE sorbent is decreasing the viscosity of the sample by dilution with an appropriate organic solvent (dilution solvent). Selection of such solvent should be based on the following aspects: a) miscibility of the sample matrix in the solvent; b) complete solubility of analytes in the solvent; c) the affinity of sorbent for the solvent molecules should be as low as possible in comparison to the affinity for analyte molecules, in order to ensure quantitative sorption of the analytes from the sample solution. Contrary to the case of sample dilution solvent, the affinity of sorbent for the elution solvent molecules should be at least comparable to the affinity for analyte molecules, providing for competitive desorption of the analyte. A number of organic solvents were tested as possible sample dilution and analyte elution media

and the results of sorption experiments expressed as analyte sorption efficiency are illustrated in Fig. 3.1. The best results among the tested aliphatic compounds as sample dilution solvents were obtained with n-hexane, from which the selected PAHs were almost completely adsorbed on the MWCNTs. Among the two tested aromatic elution solvents, toluene was found to be more efficient in comparison to m-xylene and provided better recovery of analytes from the MWCNT sorbents. The probable explanation of this effect is the stronger π-π interaction between the toluene molecules and MWCNT surface due to the smaller size of toluene molecules in comparison with m-xylene.

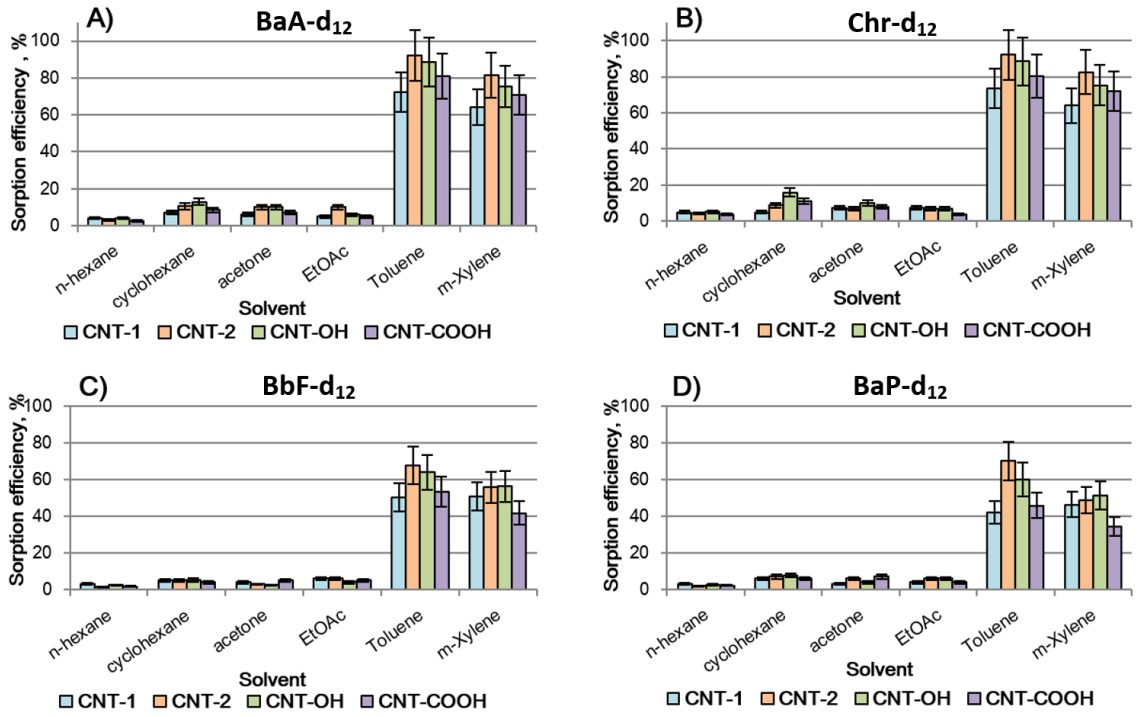

Fig. 3.1. **Sorption of the deuterated PAHs with different MWCNTs from standard solutions in selected organic solvents (CNT-1 – agglomerated MWCNT of trademark Baytubes® C150P; CNT-2 – disperse MWCNTs of trademark Timestube™; CNT-OH – hydroxyl derivate of CNT-2; CNT-COOH – carboxyl derivate of CNT-2)**

3.1.2. Selection of MWCNT sorbent

Absorption of PAHs on different MWCNTs

In order to assess the sorption potential of MWCNTs towards PAH analytes in the presence of matrix components, the capacity of each MWCNT type was evaluated in terms of recovery of PAHs-d_{12} from aliquots of oil treated (n=3 for each MWCNT type). As shown in Fig. 3.2, generally the lowest affinity among the tested sorbents towards the selected PAHs

was observed in the case of CNT-OH nanomaterial. The other three sorbents were found to provide comparable extraction efficiency under the fully optimised conditions, apart from BaP-d_{12}, for which CNT-1 was less effective than CNT-2 and CNT-COOH.

Recovery of analytes from MWCNT sorbents

Considering the planar structure of PAHs, the dominant sorption mechanism likely involved strong π-π electron interactions between the aromatic structure of analytes and the active surface of MWCNT sorbent [122].

Despite the known benefits of π-π electron interactions for quantitative and selective sorption of aromatics from the matrix, the strong affinity of MWCNT sorbents for PAH analytes resulted in poor absolute recoveries of selected PAHs-d_{12} during the initial desorption attempts. It was found that acceptable recoveries of the selected targets could be obtained by direct elution of the analytes with toluene, although the necessary volume of toluene was very high (up to 200 mL per sample) and was similar to the volume typically applied to elute the fraction of planar molecules from activated carbon in the analysis of polychlorinated dibenzo-p-dioxins and dibenzofurans [123-124]. In order to overcome this drawback and to reduce the necessary volume of elution solvent, washing with hot toluene under reflux conditions was applied for analyte elution from the MWCNT sorbent, and the total volume of toluene considered to be sufficient for the desorption procedure was 35 mL. It was found that the effectiveness of PAH elution from the MWCNTs increased with decreasing molecular weights of the analytes, showing absolute recoveries in the following order: BaA-d_{12} ~ Chr-d_{12} > BbF-d_{12} ~ BaP-d_{12}. The degree of analyte desorption was found to be highly dependent on the duration of refluxing, with 60 min extraction with toluene under reflux conditions selected as a reasonable compromise between the desorption efficiency of target PAHs and the labor and time consumption of the procedure. The best analyte recovery rates were in the range from 75 to 90%, observed for sorbents based on CNT-2 and CNT-COOH, (Fig. 3.2A and 3.2B), while sorbents prepared from CNT-1 and CNT-OH were less effective, providing absolute recoveries for BaP-d_{12} equal to only about 50% (Fig. 3.2D).

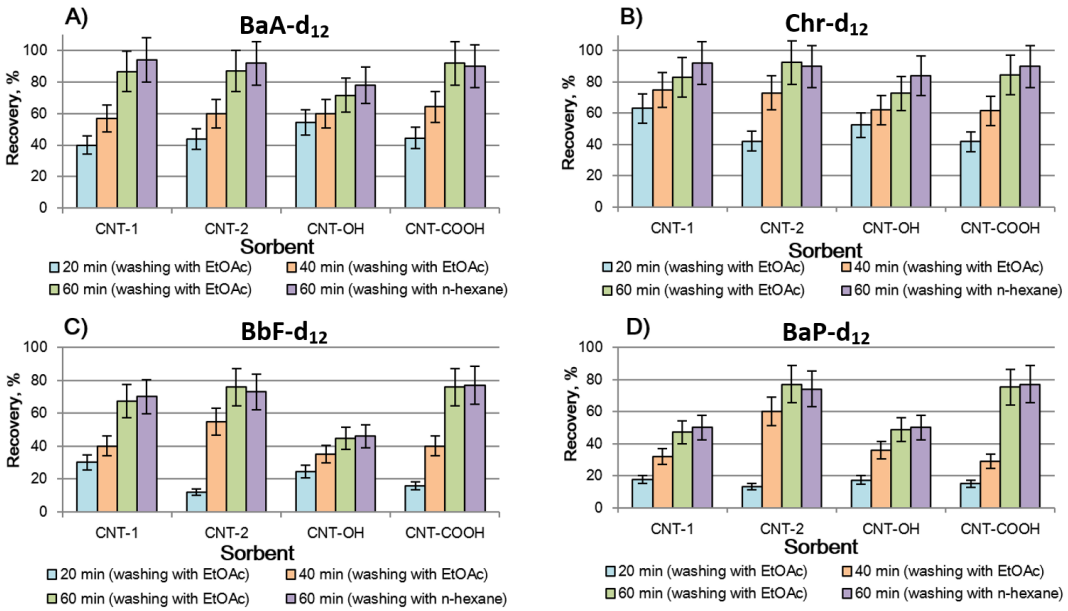

Fig. 3.2. **Recovery rates for deuterated PAHs observed with different MWCNTs, reflux times, and desorption solvents (CNT-1 – agglomerated MWCNT of trademark Baytubes® C150P; CNT-2 – disperse MWCNTs of trademark Timestube™; CNT-OH – hydroxyl derivate of CNT-2; CNT-COOH – carboxyl derivate of CNT-2)**

Evaluation of coextractive propensity of MWCNTs and the selection of washing solvent

Despite the selective sorbent properties of MWCNTs, the adsorption of some matrix components of aromatic nature (e.g., dyes) could be also expected. A greater role of sorption mechanisms other than π-π electron interaction could be expected in the case of CNT-OH and CNT-COOH sorbents due to the presence of hydroxy and carboxyl groups in their structure. The affinity of the tested MWCNTs towards matrix coextractives was evaluated by treating aliquots of oil diluted with *n*-hexane and further gravimetrical measurement of the residual matrix remaining after the evaporation of *n*-hexane. As it was expected, the chemically modified CNT-OH and CNT-COOH sorbents were found to be more susceptible to retaining the matrix in comparison to CNT-1 and CNT-2, pointing to different sorption mechanisms. The initial extraction experiments showed that post-extraction washing of the sorbent is clearly required. As shown in Fig. 3.3, *n*-hexane was not sufficiently effective as a post-extraction washing solvent, while the more polar EtOAc efficiently removed the remaining matrix components from the MWCNT sorbents without significant losses of analytes (Fig. 3.2).

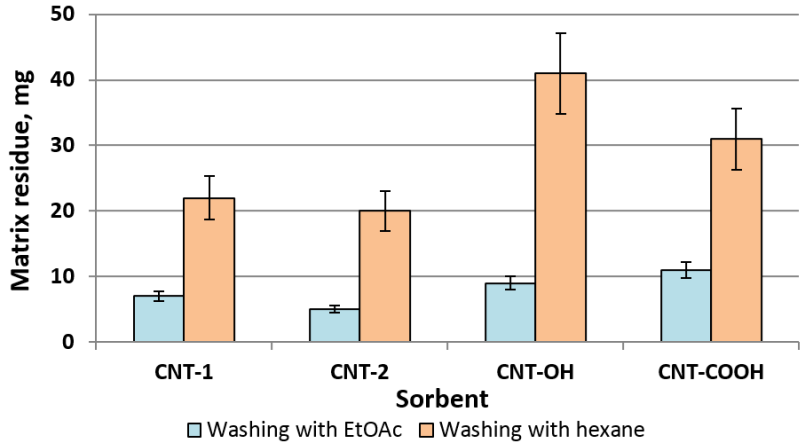

Fig. 3.3. **Matrix residue on the tested MWCNTs after post-extraction washing with different solvents, determined by extracting one gram of oil (CNT-1 – agglomerated MWCNT of trademark Baytubes® C150P; CNT-2 – disperse MWCNTs of trademark Timestube™; CNT-OH – hydroxyl derivate of CNT-2; CNT-COOH – carboxyl derivate of CNT-2)**

From the results observed it can be concluded that all the tested MWCNTs have a great potential for the sorption of PAHs from oil samples, providing good recoveries of analytes and acceptable purity of the final extracts. Based on the superior analyte recovery rates and the lower retention of matrix components, CNT-2 was used in the final method in combination with EtOAc as post-extraction washing solvent.

Method validation

The method was validated using spiked corn oil and the results of spiking experiments were corrected by taking into account the concentrations of pseudo-blank samples. The mean concentrations of PAHs in the pseudo-blank samples were: BaA – 0.19 μg kg^{-1}; Chr – 0.41 μg kg^{-1}; BbF – 0.24 μg kg^{-1}; BaP – 0.12 μg kg^{-1}. Essential parameters such as sensitivity, selectivity, linearity, accuracy, and precision were investigated and the overview of validation parameters for the elaborated method is given in Annex 1. Since isotope dilution and internal standardisation were used, solvent-matched calibration standards were chosen for quantitative purposes. Seven calibration levels covered the concentration range between 0.10 and 10.0 μg kg^{-1} and, in order to avoid heteroscedasticity, the calibration range was split into two parts. The lower part was applicable for LOD calculation and covered the range from 0.10 to 0.60 μg kg^{-1}, whereas the second calibration curve was set up for the whole calibration range between 0.1 and 10 μg kg^{-1}. The linearity of instrument responses was evaluated for each section based on visual inspection of the residuals of the linear regression curves. The

obtained correlation coefficients were greater than 0.998 with the residual values of less than 15% for all selected PAHs.

The calculation of method LOD was based on a single analysis on the test sample, ten independent analyses of the pseudo-blank, and equal probabilities (α=ß=0.05) for false positive and false negative detections. The pseudo-blank samples were processed by applying the whole analytical procedure. Homoscedasticity was assumed for the analyte concentration range between LOD and the spiking level, and the probabilities of type I and type II errors (α and ß errors) were set to 0.05. The method LOD and LOQ for each analyte were calculated based on the Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food [125]. The obtained method LODs and LOQs for single compounds were far below the levels set in Commission Regulation (EU) No. 836/2011 and 2015/1933 [42-43], with the actual values ranging from 0.06 to 0.21 μg kg^{-1} and from 0.19 to 0.71 μg kg^{-1} (Annex 1), respectively.

The results of spiking experiments show that the mean recovery values for target analytes ranged from 96 to 107% and all the obtained $HORRAT_r$ values were far below 2, thus completely meeting the criteria stated in the Commission Regulation No 836/2011 [42], while the intra-day and inter-day precision in terms of RSDs were in the range of 2–5% and 4–6%, respectively. The expanded uncertainties for individual PAH compounds and for the combined PAH4 group were calculated for the lowest validation level (1 μg kg^{-1}) with the refined level of confidence of 95% (k=2). The expanded uncertainty values for BaP and PAH4 were 10% and 11%, respectively.

The trueness of the method was demonstrated by analysis of a real sample that was inter-laboratory tested for the content of selected PAHs within the framework of the 14th inter-laboratory comparison organised by the European Union Reference Laboratory for Polycyclic Aromatic Hydrocarbons – “Four marker PAHs in food supplements”. Eq. (1) was applied for evaluation of the obtained results [126]. The measurement uncertainty was estimated as a first approximation from the determined intermediate precision. A statistically significant difference of the measurement result and certified value cannot be postulated at the 95% confidence level if Eq. (1) is fulfilled. Statistical evaluation of the measurement results did not indicate any bias.

$$2 \geq |zeta| = \frac{X_m - X_{ILC}}{\sqrt{u_m^2 + u_{ILC}^2}} \qquad (1)$$

X_m: the average measured value;

X_{ILC}: the values assigned for inter-laboratory comparison;

u_m: standard uncertainty of the measurement;

u_{ILC}: standard uncertainty of the assigned value.

The results derived from the analyses of this material represent a good agreement of the determined concentrations with the provided consensus values (Table 3.1). The calculated zeta scores of 0.71 for BaA, -0.22 for Chr, 1.0 for BbF, 0.71 for BaP, and 0.67 for the PAH4 indicated a good reliability of the elaborated method for the analysis of 4 EU-regulated PAHs in edible oils.

Table 3.1

Results obtained for the ILC 2014 – Four marker PAHs in food supplements (fish oil)

	X_{ILC}, µg kg^{-1} fat	u_{ILC}, µg kg^{-1} fat	X_m, µg kg^{-1} fat	u_m, µg kg^{-1} fat	zeta	Acceptance
BaA	3.3	0.3	3.6	0.3	0.71	Yes
Chr	3.6	0.4	3.5	0.2	-0.22	Yes
BbF	4.3	0.3	4.8	0.4	1.0	Yes
BaP	3.3	0.2	3.5	0.2	0.71	Yes
PAH4	14.5	0.6	15.4	1.2	0.67	Yes

Application to real samples and comparison with the GPC method

The applicability of the elaborated method was assessed through the analysis of selected PAHs in fifteen edible oil samples and the obtained analyte concentrations are outlined in Table 3.2. The concentrations of PAHs observed with the MWCNT sample preparation protocol varied from 0.19 to 7.0 µg kg^{-1} for BaP and from 1.6 to 31 µg kg^{-1} for PAH4, respectively. The concentrations of PAHs in five of the samples were above the MLs of 2.0 µg kg^{-1} for BaP and 10 µg kg^{-1} for PAH4 stated in Commission Regulation (EU) No 835/2011 [75], thus the efficiency of the method was evaluated over a broad range of analyte concentrations (from 0.1× to 3.5× of the ML for BaP and from 0.15× to 3× of the ML for the PAH4).

Table 3.2

The results of the analysis of selected PAHs in oil samples obtained with MWCNT and GPC sample preparation protocols (n=15)

No.	Oil type	BaA, µg kg^{-1}		Chr, µg kg^{-1}		BbF, µg kg^{-1}		BaP, µg kg^{-1}		PAH4, µg kg^{-1}	
		CNT protocol	GPC protocol	CNT protocol	GPC protocol	CNT protocol	GPC protocol	CNT protocol	GPC protocol	CNT protocol	GPC protocol
1	Unrefined linseed oil	3.3	3.6	4.0	4.3	2.6	2.4	3.2	2.9	13.2	13.1
2	Unrefined walnut oil	0.83	0.65	1.3	1.6	0.92	0.39	0.73	0.39	3.8	3.0
3	Unrefined linseed oil	6.2	6.9	7.5	8.4	5.5	5.3	6.6	6.0	25.8	26.6
4	Milk thistle oil	7.2	6.8	10.7	10.0	4.3	4.8	5.5	5.6	27.7	27.2
5	Unrefined milk thistle oil	0.92	0.66	1.4	1.1	0.56	0.43	0.51	0.41	3.4	2.6
6	Unrefined walnut oil	7.7	8.0	10.8	10.5	5.7	6.1	7.0	6.9	31.2	31.5
7	Unrefined almond oil	1.7	1.5	2.8	2.4	1.2	0.86	1.0	0.97	6.7	5.7
8	Unrefined rapeseed oil	0.90	0.74	1.7	1.5	0.56	0.44	0.47	0.32	3.6	3.0
9	Sea buckthorn oil	0.32	0.20	0.79	0.50	0.36	0.17	0.31	0.15	1.8	1.0
10	Unrefined olive oil	1.6	1.5	2.7	2.2	0.37	0.46	0.29	0.21	4.9	4.3
11	Unrefined olive oil	0.61	0.46	2.1	2.6	0.35	0.18	0.35	0.13	3.4	3.4
12	Unrefined olive oil	0.37	0.13	0.72	0.55	0.31	0.20	0.19	0.10	1.6	0.98
13	Linseed oil	0.97	0.81	1.6	1.1	1.0	0.72	0.43	0.67	4.0	3.3
14	Pumpkinseed oil	1.2	1.4	1.9	1.7	0.56	0.45	0.71	0.46	4.3	4.0
15	Olive extra virgin oil	2.9	2.5	4.1	4.6	3.5	3.8	5.3	5.0	15.8	15.8

A functional relationship between the data obtained by two different methods could serve as one of the approaches for the evaluation of comparability of the obtained results. In order to assess the efficiency of the elaborated d-SPE method, all tested oil samples prepared and analysed according to a novel MWCNT sorbent procedure, were additionally reanalysed using a well-established and validated GC-MS/MS method [127], in which a GPC based sample preparation procedure was applied. The functional relationship between d-SPE and GPC methods for selected PAHs is shown in Fig. 3.4. Two regression curves were plotted: the bisecting line and the functional relationship between the methods calculated by the regression method. As it has been shown, all selected PAHs practically merged with the bisecting line and the observed bias could be acknowledged to be in the range of method uncertainty, affirming the equivalency of the developed method in terms of providing reliable results. Taking into account all of the aforementioned considerations, it can be concluded that the developed method is convenient and rapid, while the whole proposed procedure using d-SPE for six parallel samples can be completed within less than 3 h.

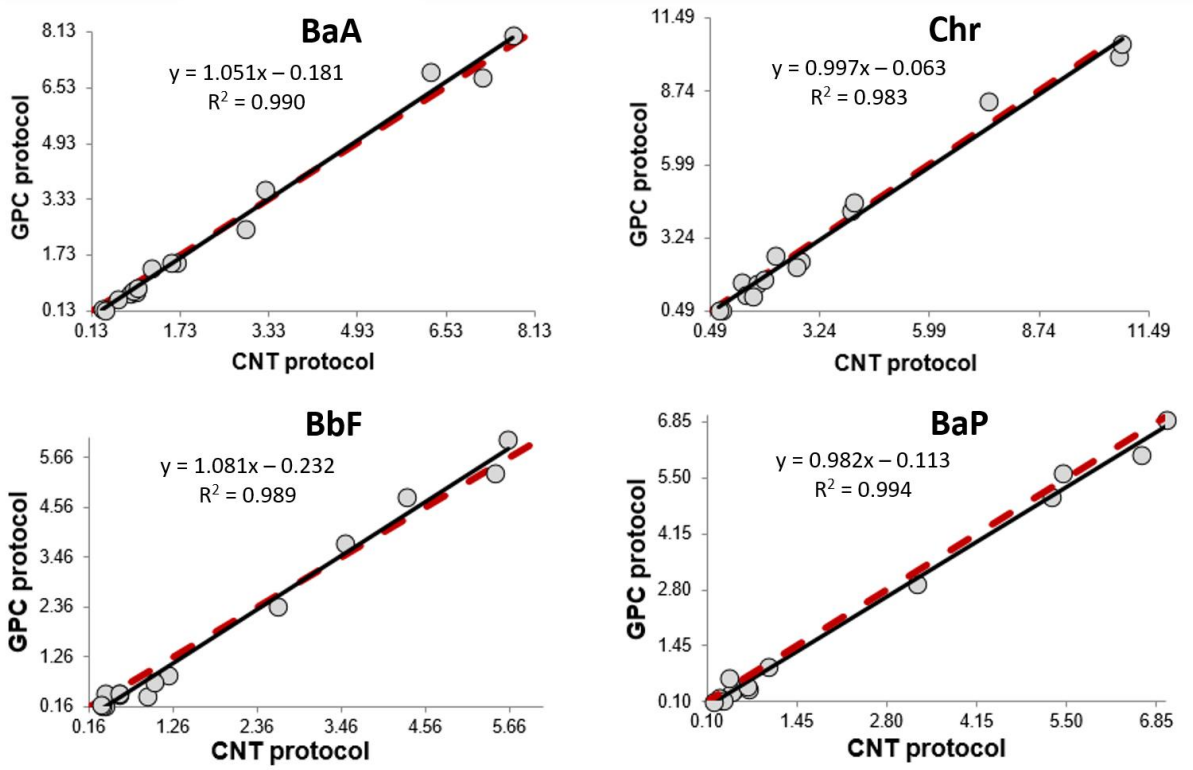

Fig. 3.4. **Representation of the functional relationships between results obtained by the elaborated d-SPE method and GPC method for selected PAHs**

3.2. Instrumental methods for the PAHs determination

Depending on the complexity of the food matrices and the sample preparation methodology to provide acceptable separation of the target analytes from the interfering food matrix coexrtactives, the instrumental methods published in the scientific literature for the determination of PAHs in food can be divided into two groups. The first group focuses on the determination of the PAHs by the means of HPLC coupled to FLD and MS detectors. The second group focuses on GC-MS determinations. Both groups were covered within the present study, thus representing recent trends in both possible instrumental analytical methods pathways. GC-MS/MS and GC-HRMS methods cover recent trends in GC-MS determination, however HPLC-HRMS opens new perspectives in HPLC-MS determinations of PAHs.

3.2.1. HPLC-Orbitrap-MS method for the determination of PAHs

The most common LC determination of PAHs is carried out by liquid chromatography coupled to fluorescence (LC-FLD) detection that is also proposed in some official methods according to the ISO and the US EPA. However, it is well known that FLD detection shows a number of disadvantages, such as selectivity problems and sensitivity limitations. To overcome these limitations MS or even HRMS detection can be used. Whereas, it would be not able to improve the sensitivity of determination without improving the ionisation efficientcy, the development and optimisation of a novel LC-APPI-Orbitrap-MS analytical method was performed.

Optimisation of the sample clean-up and HPLC conditions

Since the concentrations of PAHs in food are very low, generally in the range of parts per billion, the sample clean-up procedures usually consist of several extraction and purification steps [10]. The sample clean-up procedure for the present study was based on extraction of PAHs with dichlormethane/n-hexane mixture, gel permeation chromatography, and solid-phase extraction [see Section 2.4.2]. During the optimisation of the sample clean-up procedure, we attempted to analyse the chocolate samples without the final purification step (solid-phase extraction), and it was found that a sample clean-up apart from GPC was not strictly necessary for the APPI. However, for routine applications, the SPE clean-up step is very beneficial in order to avoid contamination of the ion source.

In order to achieve the highest possible instrumental selectivity for specific PAHs, the parameters of HPLC analysis were optimised. Based on previous studies regarding the analysis of PAHs using LC-MS [3, 8, 128], two different LC columns were tested -

LiChrospher PAH 250mm × 3 mm, ID 5 µm (Merck Millipore, Darmstadt, Germany) and Pinnacle DB PAH 50 mm × 2.1 mm, ID 1.9 µm (Restek, Bellefonte, PA, USA). The initial experiments were based on the manufacturer's recommendations and then optimised to achieve better separation of the analytes. Both columns demonstrated acceptable results even for the critical compounds – BbF and Chr. The main difference between the two tested columns was the total run time of the sample analysis. Sufficient separation of PAHs was achieved within a 6 min run on the Pinnacle DB PAH column, whereas LiChrospher PAH 250-3 showed acceptable results only after a 25 min run. Therefore, the selection of Pinnacle DB PAH analytical column was more reasonable. Figure 3.5 shows typical chromatograms for chocolate samples spiked with PAHs at 0.10 µg kg^{-1} concentration using these two analytical LC columns.

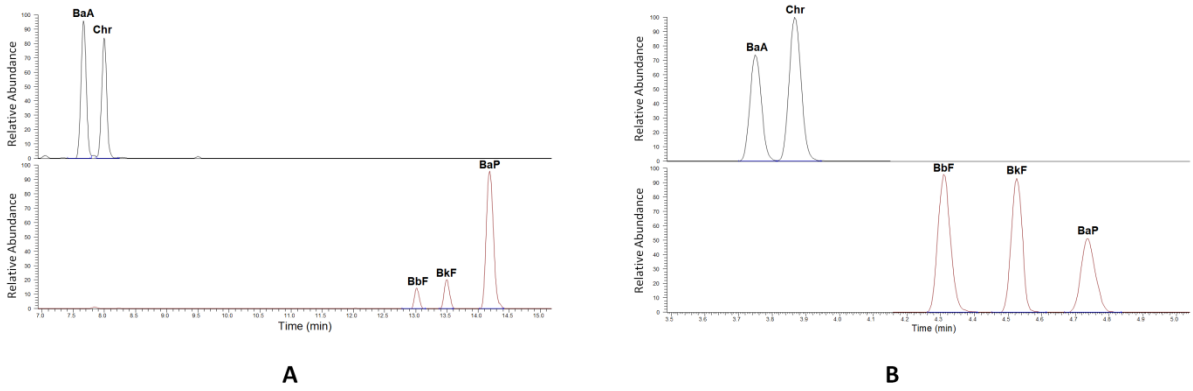

Fig. 3.5. **Chromatograms for chocolate samples spiked with PAHs at 0.1 µg kg^{-1} concentration using two different analytical LC columns: A – LiChrospher PAH 250-3; B – Pinnacle DB PAH**

The optimal column flow rate of 0.4 mL min^{-1} was used and was proved to be well suited for direct coupling with the APPI interface. Chromatographic separation of the analytes was achieved using water/acetonitrile as a binary mobile phase system and no column re-equilibration time between injections was needed.

Despite the fact that acetonitrile is not recommended as mobile phase solvent for APPI source due to its relatively low protonating efficiency, it was used successfully under our experimental conditions. The limitations for the use of acetonitrile are based on the low yield of acetonitrile ions responsible for the ionisation of PAHs, which is not sufficient for achieving sufficient ionisation of the analytes [63]. In our case, the ionising efficiency was greatly improved by adding a post-column doping agent to the mobile phase.

Several studies were performed during the last decade that compared the benefits of either single dopants or mixtures of dopants [64-66]. In our study, a screening aimed at the selection of several dopants and their combinations with the ionisation potential of less than

10 eV was performed, and these were (1) toluene, (2) anisole, (3) chlorobenzene, (4) toluene/acetone (50:50, v/v), (5) toluene/anisole (99.5:0.5, v/v), (6) toluene/chlorobenzene (99:1, v/v), and (7) ethanol/chlorobenzene/bromobenzene/anisole (98.975:0.1:0.9:0.025, v/v). Under our experimental conditions, pure toluene and pure anisole showed better and equivalent performance in terms of providing higher ion intensity for most of the selected PAHs. Similar results were obtained by Moriwaki et al. (2004) when toluene gave the highest peak areas for 12 EPA-PAHs and by Itoh et al. (2006) who reported that a mixture of toluene and anisole in the volume ratio of 99.5:0.5 provided the most suitable ionisation for the analysis of 16 PAHs [64, 129]. Due to the fact that most of our analytical standards were prepared in pure toluene, and to avoid the introduction of different solvents, toluene was finally selected in preference over other dopants as the most effective and convenient agent. The maximum sensitivity for the analytes was obtained by post-column introduction of toluene to the mobile phase at the flow rate of 50 $\mu L\ min^{-1}$.

Optimisation of the Orbitrap-MS method

The use of APPI ionisation mode in PAH analysis was previously described by several authors [64-65, 67-69, 130], however, limited selectivity, sensitivity, as well as strong interference might be occasionally encountered when using quadrupole MS instruments [71, 130]. The performance of Q-Exactive mass spectrometer functioning as a part of innovative hybrid high-resolution mass spectrometers (HRMS) combined the high resolving power (RP) performance of the Orbitrap with the high selectivity of the quadrupole. Hereby, in order to overcome the aforementioned limitations, we propose the use of HPLC-Orbitrap-MS as a promising technology for the routine analysis of PAHs.

In order to adjust the HRMS parameters and to optimise the method for determination of PAHs, systematic experiments were carried out for each of the Q-Exactive scan modes (FS, tSIM, and tMS^2). The effect of mass spectrometry (MS) parameters, such as automatic gain control (AGC target), maximum injection time (IT), resolving power (RP), normalised collision energy (NCE), and number of scans per chromatographic peak were examined.

During the optimisation of Q-Exactive scan modes, one of the essential criteria responsible for the sensitivity of method is finding the optimal values of two related parameters - AGC and IT. In the FS mode (m/z range of 100 – 500) with a complex matrix, the optimal AGC was configured to 1×10^6, while the IT was set to 200 ms. As a consequence, the detection of all species in the operating mass range was enhanced.

The AGC value for the tSIM mode was established at the same level as was found optimal for the FS mode, whereas the IT value was decreased. As a result, we observed a

slightly improved sensitivity and much higher intensities of the analyte signals in the spectra (Fig.3.6). The optimal AGC and IT values used for this detection mode were 1×10^6 and 120 ms, respectively.

In the case of the tMS^2 mode, the target ions were filtered from the total ion flux and a large portion of interfering ions were removed by applying a selective isolation window of narrow mass range (m/z 1) by the quadrupole. Thus, the ions were selectively accumulated in high collision dissociation (HCD) cell and subsequently fragmented. In order to avoid disrupting the analyte ions in the Orbitrap and to prevent distortions, the AGC was set to 2×10^5, to limit the amount of ions in the Orbitrap. At the same time, IT was set to 100 ms, still providing a sufficient number of scans per second. As a result, the proportion of target ions among the total ion population was greater, favouring lower detection limits compared to the FS and tSIM modes.

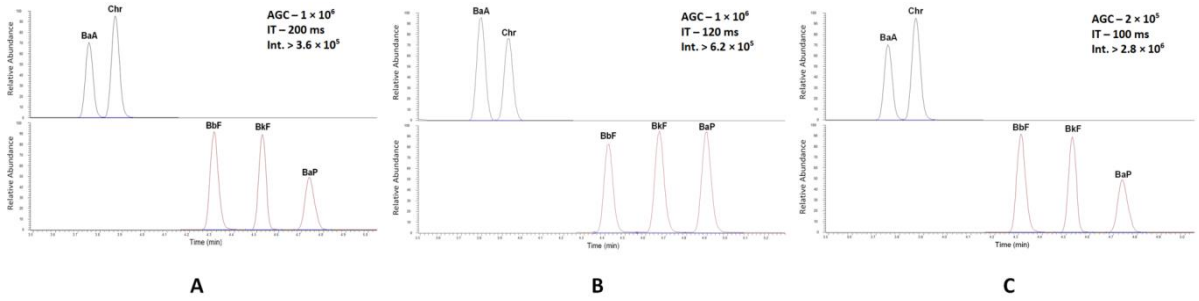

Fig. 3.6. **Chromatograms for the optimal AGC and IT values for the different Orbitrap-MS scan types: A – Full Scan mode; B – tSIM mode; C – tMS^2 mode**

Another important parameter to consider in relation to the selectivity of HRMS analysis is the resolving power (RP). Analysis at higher RP enables a better mass accuracy, thus a higher selectivity. Yet, high RP decreases the number of acquisitions during an analysis due to longer duty cycles [131]. For quantitative measurements with acceptable RSDs, it is generally agreed that a minimum of about 20 data points per peak is required [132]. The number of data points in relation to RP was studied in order to identify the best compromise between selectivity and quantitative parameters of the method. With these criteria, the selected RP for FS and tSIM was 35 000 FWHM, and 17 500 FWHM for tMS^2. Fig. 3.7 presents the optimal RP with the corresponding optimum acquisition points for tSIM and tMS^2 modes.

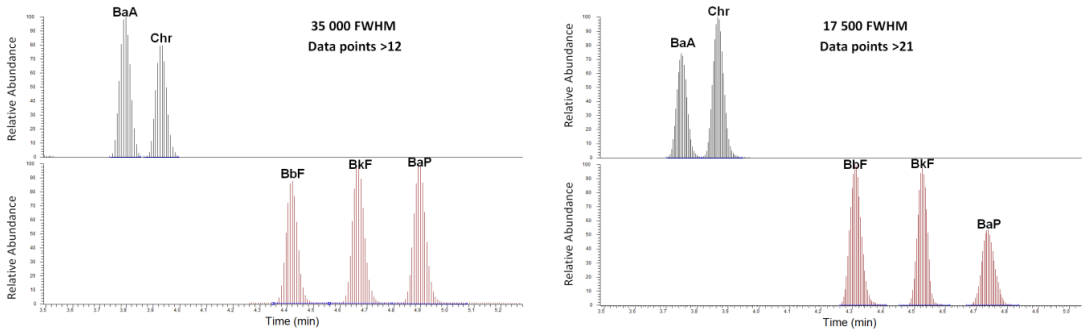

Fig. 3.7. **Optimal RP with corresponding optimum acquisition points for the different scan modes: A – tSIM mode; B – tMS^2 mode**

As described above, the efficiency of different scan modes was examined during the study. The FS and tSIM modes showed poorer performance in terms of selectivity compared to tMS^2, and many interfering peaks were observed for chocolate matrix when operating at low detection levels, especially near the BaA and BbF peaks (Fig. 3.8). Comparing the different scan modes in terms of sensitivity and amount of data points per peaks against the tMS^2 mode showed better performance (Fig. 3.7). Consequently, the tMS^2 mode resulted in a more accurate determination of PAHs and should be preferred when using complex matrices. Based on these observations, only the tMS^2 method was validated in the present study and used for the further analysis of chocolate samples.

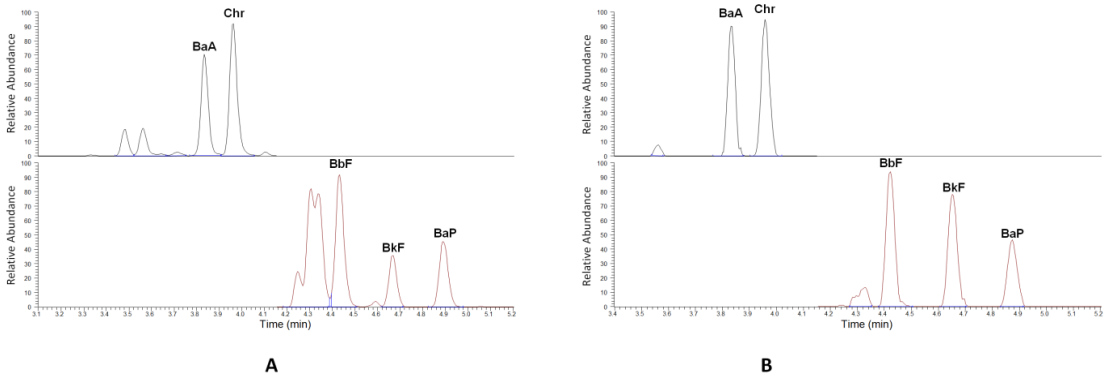

Fig. 3.8. **Chromatograms of the chocolate sample obtained by the different scan modes: A – tSIM mode; B – tMS^2 mode**

Validation of the method

The developed DA-APPI-HPLC-Orbitrap-MS method was validated according to the Commission Regulations (EU) No. 836/2011, 2015/1933, and 2016/582 [42-43, 133]. The validation of the method was performed using dark chocolate homogenate (pseudo-blank)

previously analysed for the PAHs content and found to contain quantifiable traces of selected substances. The mean concentrations of PAHs in the pseudo-blank sample were: BaA – 0.21 μg kg^{-1} fat; Chr – 0.31 μg kg^{-1} fat; BbF – 0.16 μg kg^{-1} fat; BaP – 0.071 μg kg^{-1} fat.

In order to evaluate the analytical performance of the developed method, essential parameters such as sensitivity, selectivity, linearity, accuracy, and precision were investigated. The overview of validation parameters such as linearity (r^2), linearity range, instrumental and method LODs and LOQs, as well as the recoveries and precision values for all the three different spiking levels during the intra-day and inter-day validation experiments are outlined in Annex 2.

As the analytical method had to be fit for the generation of reliable data at low concentration levels, an emphasis was given to the assessment of the lower limits at which analytes can be detected. Nine calibration levels covered the concentration range between 0.10 μg kg^{-1} and 5.0 μg kg^{-1} expressed on fat basis. To avoid heteroscedasticity, the calibration range was split into two parts. The lower part, which was applicable for LOD calculation, covered the range from 0.10 μg kg^{-1} to 0.30 μg kg^{-1} fat, whereas the second calibration curve was set up for the range between 0.20 μg kg^{-1} and 5.0 μg kg^{-1} fat and was used for the analysis of chocolate samples. The linearity of instrument responses was evaluated for each section based on visual inspection of the residuals of the linear regression curves. The obtained correlation coefficients were greater than 0.990 for all of the investigated compounds.

The calculation of method LOD (LOD) was performed according to Equation 1 under the conditions of performing a single analysis on the test sample, ten independent analyses of the pseudo-blank, and equal probabilities (α=ß=0.05) for false positive and false negative detections [125]. The pseudo-blank samples were processed by applying the whole analytical procedure. Homoscedasticity was assumed for the content range between method LOD and the spiking level, and the probabilities of type I and type II errors (α and ß errors) were set to 0.05. The method LOD and LOQ of each analyte were calculated based on the Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food [125], according to Eqs. (2) and (3), respectively. The factor 3.86 takes into account the number of experiments and the chosen error probabilities.

Equation (2) was used for the estimation of method LOD:

$$x_{LOD} = 3.86 \cdot \frac{S_{y,b}}{b} \qquad (2)$$

x_{LOD}: limit of detection for the method;

$S_{y,b}$: standard deviation of the pseudo-blank signals;

b: slope of the calibration curve close to LOD;

whereas Equation (3) was applied for the estimation of method LOQ:

$$x_{LOQ} = 3.3 \cdot x_{LOD} \qquad (3)$$

x_{LOQ}: limit of quantification for the method.

Due to the fact that in the case of Orbitrap-MS the extraction of exact masses within 5 × 10^{-6} amu (5 ppm) of the theoretical m/z from the full scan raw data for most of the target compounds provided for absence of background noise, the evaluation of the instrumental sensitivity via S/N ratio was not applicable and could result in overestimated results [134]. Thus, the instrumental LOD (*i*-LOD) and instrumental LOQ (*i*-LOQ) values were assessed from the analyses of pure standard solutions with analyte concentrations from 0.10 to 0.50 pg μL^{-1} (0.50 – 2.5 pg injected on-column). For the reliable detection taking into account the dynamic range of the detector, only signals with the intensities above 1 × 10^4 were used. The assessed *i*-LOD values for the analysed PAHs were: BaA – 1.2 pg injected on-column, Chr – 0.80 pg injected on-column; BbF – 1.0 pg injected on-column, and BaP – 1.0 pg injected on-column. The *i*-LOQs were BaA – 3.7 pg injected on-column, Chr – 2.4 pg injected on-column; BbF – 3.1 pg injected on-column, and BaP – 2.9 pg injected on-column, respectively.

The method LODs observed in the present study (0.016 to 0.024 μg kg^{-1} fat, see Annex 2) were approximately ten times lower than those obtained by Hollosi et al. (2011), and comparable to the lowest LODs reported by Smoker et al. (2010) and Cai et al. (2012) [69-71]. With regard to the instrumental LOD values, the most sensitive among the aforementioned methods was reported by Smoker et al. (2010), with the lowest established instrumental LOD at the level of 0.40 pg of BaP on-column [70]. However, the determination of LODs for the reported method was based on S/N ratio that is often associated with overestimated results. The instrumental LOD values reported by other authors were 6.3 pg of BaP on-column for edible oil matrix and 8.0 – 106 pg of individual PAHs for oysters.

According to the Commission Regulation No. 836/2011 amending the performance criteria for methods of analysis for PAHs, validated method recoveries for every single PAH4 compound should be in the range from 50 to 120%, and $HORRAT_r$ values should be less than 2 [42]. The mean recoveries for the elaborated method were within the recommended range – from 84% to 110%. All the obtained $HORRAT_r$ values were significantly lower than required (Annex 2).

The obtained mean RSD values for the inter-day validation ranged from 7% to 11%, while the mean RSD values for the intra-day validation were 10% for all the individual compounds.

The trueness of the method was estimated by analysis of an inter-laboratory comparison test material (EUPT-2015 – Four marker PAHs in cocoa products). Eq. (1) was applied for evaluation of the obtained results [126].

In general, the determined concentrations for the analytes included in the PT were in good agreement with the provided assigned values for both samples. The details are given in Table 3.3.

The overall quality assessment of validation data shows that our elaborated methodology provided acceptable performance for sensitive, selective, and accurate analysis of selected PAHs in chocolate samples.

Table 3.3

Results obtained for the ILC 2015 – Four marker PAHs in cocoa products

	X_{ILC}, µg kg^{-1} fat	u_{ILC}, µg kg^{-1} fat	X_m, µg kg^{-1} fat	u_m, µg kg^{-1} fat	zeta	Acceptance
Milk chocolate						
BaA	4.7	0.3	4.2	0.42	-0.99	Yes
Chr	5.8	0.3	5.3	0.53	-0.81	Yes
BbF	3.9	0.2	3.8	0.38	-0.20	Yes
BaP	3.9	0.2	3.5	0.35	-0.96	Yes
PAH4	18.2	0.5	16.7	3.4	-0.4	Yes
Cocoa powder						
BaA	4.4	0.32	4.7	0.47	0.46	Yes
Chr	6.2	0.39	5.9	0.59	-0.41	Yes
BbF	2.3	0.21	2.7	0.27	1.1	Yes
BaP	2.2	0.17	2.1	0.21	-0.49	Yes
PAH4	15.1	0.6	15.3	3.1	0.070	Yes

3.2.2. Comparison of GC-MS/MS and GC-HRMS methods for the determination of PAHs in cereal-based food

As it was mentioned above GC-MS/MS and GC-HRMS represent recent trends in instrumental determination of PAHs using gas chromatography. Both methods have appropriate sensitivity and selectivity for the analysis of these contaminants at trace levels. However, to evaluate the performance of aforementioned methods, it is relevant to compare the essential parameters of the determination methods such as sensitivity, selectivity, linearity, accuracy and precision. For this purpose, analysis of cereal-based foods with the PAHs at the ultra low contamination levels and the goal criteria for LOQs at the level of 0.10 µg kg^{-1} for the four individual PAHs was performed.

The analytical procedure for the analysis of PAHs used in this study was based on a sample preparation procedure described in a Section 2.4.2., however instrumental parameters

were thoroughly optimised to achieve the highest possible instrumental sensitivity for the selected PAH compounds.

As a result, both elaborated analytical methods demonstrated outstanding sensitivity and selectivity. Figure 3.9 shows typical chromatograms for bread samples spiked with PAHs at 0.10 µg kg^{-1} concentration. It is important to mention that PAHs concentrations in the pseudo-blank sample were: BaA – 0.089 µg kg^{-1}; Chr – 0.15 µg kg^{-1}; BbF – 0.097 µg kg^{-1}; BaP – 0.085 µg kg^{-1}.

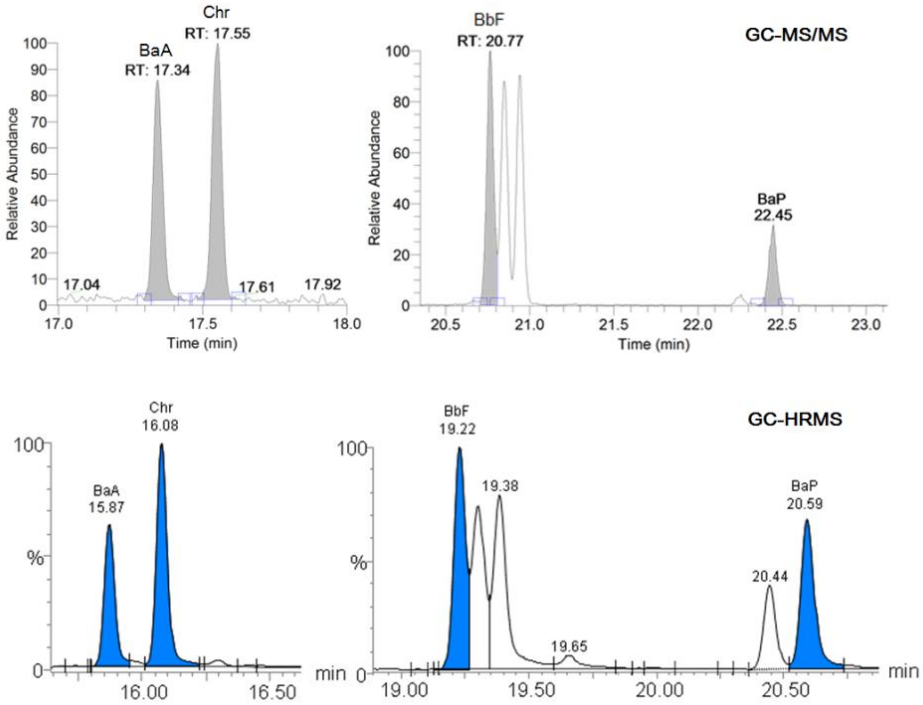

Fig. 3.9. **GC-MS/MS and GC-HRMS chromatograms of bread sample spiked with 0.10 µg kg^{-1} of analytes**

In order to perform a comparative assessment of two analytical methods for PAHs analysis in processed cereal-based foods, essential parameters such as sensitivity, selectivity, linearity, accuracy, and precision were compared. The main validation parameters such as linearity (r^2), LOQ, LOD, recoveries, RSD, and the $HORRAT_r$ values for elaborated tandem and high resolution mass spectrometric methods are given in Annex 3.

Assessment of validation data for both instrumental methods shows that the MS/MS technique produces a slightly lower r^2 for all 4 PAHs. The comparison of sensitivity between MS/MS and HRMS techniques indicates that the MS/MS technique is more sensitive in the case of BaP analysis and slightly inferior in the case of other 3 PAHs – BaA, Chr, and BbF.

According to the Commission Regulation No. 836/2011 amending the performance criteria for methods of analysis for PAHs, validated method recoveries for every single PAH4 compound should be in a range from 50 to 120%, but $HORRAT_r$ values should be less than 2 [42]. The recoveries for both of our studied methods were in the recommended range – from 86% to 119%, whereas HRMS produced slightly elevated recoveries for both 0.10 and 1.0 μg kg^{-1} spiked samples, while MS/MS indicated almost 100% recovery for all the investigated samples. All the obtained $HORRAT_r$ values were significantly lower than required.

The obtained RSD values ranged from 3% to 19% for both validation levels, while the mean RSD values ranged from 8% to 13% for the MS/MS method and 6% – 7% for the HRMS method.

The uncertainties for individual PAH compounds and for the combined PAH4 group were expressed as 2xRSD for the lowest validation level (0.10 μg kg^{-1}) and calculated according to equation: $u_{sum} = \sqrt{u_{BaA}^2 + u_{BaP}^2 + u_{BbF}^2 + u_{Chr}^2}$, where u – measurement uncertainty.

Uncertainty values for BaP were 13% in the case of HRMS and 21% in the case of MS/MS, while for the PAH4 the uncertainty values were 24% for HRMS and 35% for MS/MS, respectively.

In general, the obtained validation data indicated that the analytical characteristics of HRMS method are equivalent to the MS/MS method, and therefore both methods are perfectly appropriate for the application in PAHs analysis at low concentrations in processed cereal-based foods, such as bread.

In order to compare the results obtained by the GC-HRMS and GC-MS/MS methods, ten different bread samples were analysed with both analytical techniques. The observed differences of results were lower than 5% in the case of BaP and lower than 10% for PAH4, indicating a good agreement of the analytical performance of both elaborated methods. The obtained results are summarised in Table 3.4.

Table 3.4

The obtained HRMS and MS/MS results for 10 bread samples

Sample No.	Technique	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}	Difference BaP, %	Difference PAH4, %
1	MS/MS	0.20	0.32	0.33	0.17	1.0	-0.70	-7.4
	HRMS	0.23	0.34	0.35	0.18	1.1		
2	MS/MS	0.15	0.34	0.15	0.065	0.71	-0.10	-5.4
	HRMS	0.19	0.39	0.12	0.066	0.76		
3	MS/MS	0.069	0.16	0.15	0.094	0.48	-1.6	0.20
	HRMS	0.076	0.18	0.11	0.11	0.48		
4	MS/MS	0.068	0.18	0.12	0.039	0.41	-3.6	-6.8
	HRMS	0.10	0.20	0.097	0.075	0.48		
5	MS/MS	0.057	0.16	0.094	0.037	0.34	-2.6	-6.1
	HRMS	0.078	0.18	0.088	0.063	0.40		
6	MS/MS	0.045	0.15	0.078	0.031	0.31	-1.1	-2.3
	HRMS	0.061	0.16	0.064	0.042	0.33		
7	MS/MS	0.066	0.15	0.10	0.031	0.35	-2.4	0.40
	HRMS	0.078	0.14	0.067	0.055	0.34		
8	MS/MS	0.035	0.14	0.084	0.022	0.28	-0.60	0.80
	HRMS	0.047	0.15	0.052	0.028	0.28		
9	MS/MS	0.028	0.11	0.071	0.017	0.22	-1.6	-4.9
	HRMS	0.051	0.13	0.058	0.033	0.27		
10	MS/MS	0.051	0.15	0.085	0.029	0.31	-1.3	-4.4
	HRMS	0.064	0.18	0.074	0.042	0.36		

3.2.3. GC-MS/MS method for the determination of PAHs

In a present study the GC-MS/MS method for the occurrence data collection of the PAHs contamination was used. Utilised method was applied for the analysis of dried herbs and spices, and smoked meats. All the instrumental parameters were identical to those used for the GC-MS/MS and GC-HRMS comparative study, however to ensure that the performed method meets the requirements and specifications set in EU legislation as well as fulfills its intended purpose, the validation study for every desired food matrix was performed.

The performance of the GC-MS/MS method for the determination of PAHs in dried herbs and spices

In order to perform validation of the method for determination of four EU regulated PAHs in dried herbs and spices, essential parameters such as sensitivity, selectivity, linearity,

accuracy, and precision were assessed. According to the Commission Regulation No. 836/2011 amending the performance criteria for methods used in the analysis of PAHs, recoveries of the analytical method for every single PAH4 compound should be in a range from 50 to 120%, $HORRAT_r$ values should be less than 2, and specificity has been defined as method being "free from matrix or spectral interferences, verification of positive detection" [42]. Blank samples were analysed by the same procedure to determine any background contamination and no chromatographic peaks belonging to PAHs were detected.

The results show that analytical response of the target compounds exhibited good linearity over the range of 0.10 – 5.0 μg kg^{-1} with correlation coefficients varying from 0.9989 to 0.9999. The method LODs and LOQs were calculated as the analyte concentration giving a three and ten times signal-to-noise (S/N) ratio, respectively, for chromatographic peaks of PAHs in spiked samples. The method LOD values ranged from 0.04 μg kg^{-1} to 0.09 μg kg^{-1}, while mthod LOQ values were in the range of 0.13 – 0.31 μg kg^{-1}.

The obtained $HORRAT_r$ values were in a range of 0.41 – 0.73 and, accordingly, significantly lower than required by the relevant EU legislation. The obtained recoveries revealed in a study were in the recommended range – from 72% to 107%, with the lowest value for Chr – 72% at the spiking level of 1 μg kg^{-1} and the highest for BaP – 107% at the spiking level of 2 μg kg^{-1}. The obtained precision (RSD) values ranged from 2% to 15% for all spiking levels, while the mean RSD values ranged from 6% to 11%. The values of main validation parameters for the elaborated method of PAHs determination are summarised in Annex 4.

The trueness of the method was estimated by participation in the interlaboratory comparison testing program (EUPT-2016 – Four marker PAHs in smoked black pepper). The determined concentrations for the analytes included in the PT were in a good agreement with the provided assigned values for smoked black pepper sample, indicating the method's applicability for different types of seasonings. The performance of the participation was expressed by z-scores, that were graded with the absolute values between -0.7 – 0.9 for all the reported results (Table 3.5).

Table 3.5

Results obtained for the ILC 2016 – Four marker PAHs in smoked black pepper

PAH	X_{ILC}, μg kg^{-1}	u_{ILC}, μg kg^{-1}	X_m, μg kg^{-1}	u_m, μg kg^{-1}	z-score	Acceptance
BaA	34.2	2.1	29.7	3.0	-0.7	Yes
Chr	39.8	3.3	46.7	4.7	0.9	Yes
BbF	17.2	1.3	17.5	1.8	0.1	Yes
BaP	14.4	1.0	15.4	1.5	0.3	Yes
PAH4	106	4	109	22	0.3	Yes

In order to assess the validity of the obtained within the study results the internal quality control checks (IQC) for the different types of seasonings were also performed. Spiked samples were included in each analytical batch and the obtained results were plotted on a control charts. Mean recovery values were in a range of 90 – 94% for all four PAHs with the dynamic recovery ranges from 70 to 119%. Fig. 3.10. shows typical chromatogram for oregano sample spiked with PAHs at 2.0 μg kg^{-1} concentration.

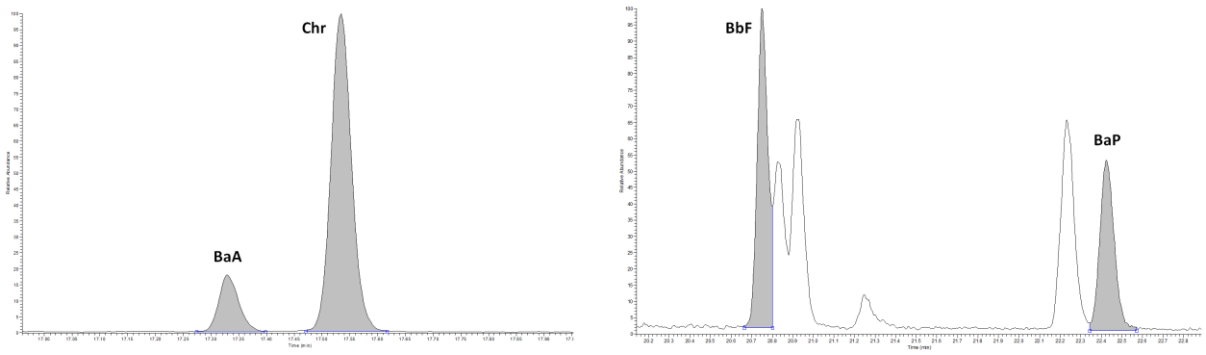

Fig. 3.10. **GC-MS/MS chromatogram of oregano sample spiked with 2 μg kg^{-1} of analytes**

In summary, the obtained results indicated that the proposed analytical method has a sufficient extraction efficacy, good linearity and reproducibility, satisfactory precision and accuracy, and reliable and valid results.

The performance of the GC-MS/MS method for the determination of PAHs in smoked meats

The method was validated using spiked smoked meat and the results of spiking experiments were corrected by taking into account the concentrations of pseudo-blank samples. The mean concentrations of PAHs in the pseudo-blank samples were: BaA – 0.07 μg kg^{-1}; Chr – 0.14 μg kg^{-1}; BbF – 0.09 μg kg^{-1}; BaP – 0.15 μg kg^{-1}. Essential parameters such as sensitivity, selectivity, linearity, accuracy, and precision were investigated and the overview of validation parameters for the utilised method is given in Annex 5. Since isotope dilution and internal standardisation were used, solvent-matched calibration standards were chosen for quantitative purposes. Seven calibration levels covered the concentration range between 0.10 and 10.0 μg kg^{-1} and, in order to avoid heteroscedasticity, the calibration range was split into two parts. The lower part was applicable for LOD calculation and covered the range from 0.10 to 0.60 μg kg^{-1}, whereas the second calibration curve was set up for the whole calibration range between 0.10 and 10 μg kg^{-1}. The linearity of instrument responses was evaluated for

each section based on visual inspection of the residuals of the linear regression curves. The obtained correlation coefficients were greater than 0.995 with the residual values of less than 15% for all selected PAHs.

The calculation of method LOD was based on a single analysis on the test sample, ten independent analyses of the pseudo-blank, and equal probabilities (α=ß=0.05) for false positive and false negative detections. The pseudo-blank samples were processed by applying the whole analytical procedure. Homoscedasticity was assumed for the analyte concentration range between LOD and the spiking level, and the probabilities of type I and type II errors (α and ß errors) were set to 0.05. The method LOD and LOQ for each analyte were calculated based on the Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food [124]. The obtained method LODs and LOQs for single compounds were far below the levels set in Commission Regulation (EU) No. 836/2011 and 2015/1125 [42, 44], with the actual values ranging from 0.02 to 0.03 μg kg^{-1} and from 0.06 to 0.09 μg kg^{-1} (Annex 5), respectively.

The results of spiking experiments show that the mean recovery values for target analytes ranged from 97 to 119% and all the obtained $HORRAT_r$ values were far below 2, thus completely meeting the criteria stated in the Commission Regulation No 836/2011 [42], while the intra-day and inter-day precision in terms of RSDs were in a range of 3–9%. The expanded uncertainties for individual PAH compounds and for the combined PAH4 group were calculated for the lowest validation level (0.50 μg kg^{-1}) with the refined level of confidence of 95% (k=2). The expanded uncertainty values for the individual PAHs were 20%.

The trueness of the method was estimated by participation in the interlaboratory comparison (ILC) testing program (EUPT-2014 – Four marker PAHs in smoked meat) [135]. The determined concentrations for the analytes included in the ILC were in a good agreement with the provided assigned values for smoked meat sample, indicating the method's applicability. The performance of the participation was expressed by z-scores, that were graded with the absolute values between -0.4 – 1.0 for all the reported results.

3.3. The occurrence of the PAHs in foods

Human beings are exposed to PAHs via air and drinking water, but mostly by intake of food, thus monitoring the safety of food products and proper management of the risks associated with PAHs are strictly needed. All the elaborated methods for the determination of

PAHs described above were applied for the analysis of real food samples, and the obtained occurrence data as well as main safety aspects will be discussed further.

3.3.1. PAHs in dark chocolate

The DA-APPI-HPLC-Orbitrap-MS method developed within the current study was applied for the analysis of dark chocolate samples. Dark chocolate is more often contaminated with PAHs because of the relatively high cocoa bean and cocoa butter content [73, 126]. The fat content in our analysed samples varied over the range of 30-66%.

Twenty six randomly selected dark chocolate samples were tested for the four EU marker PAHs. The total PAH content was found to be in the range from 1.1 to 16.1 μg kg^{-1} fat, with the mean content of 4.2 μg kg^{-1} fat. In all of the samples, the 4-ring PAHs (BaA, Chr) were detected at relatively higher concentrations than the 5-ring PAHs (BbF, BaP). Chr was especially prominent in the analysed samples, at the concentration range from 0.37 to 7.4 μg kg^{-1} fat. BaP, the most harmful of the indicator substances for the PAHs, had the mean concentration of 0.71 μg kg^{-1} fat, with 77% of the samples showing detectable concentrations of BaP. The determined concentrations of individual PAHs in the analysed chocolate samples are shown in Annex 6. The concentrations below the limit of detection were considered as not detected (ND), and the values below the LOD or LOQ were replaced by zero in order to describe the sample set by descriptive statistics methods. No samples exceeding the existing EU ML values were found.

The results obtained in the present study correlate well with the previously published survey results about the presence of PAHs in chocolate on the German market [136], where the highest PAH content was found for Chr+triphenylene (0.83 – 2.1 μg kg^{-1}), while the content of BaP ranged between 0.07 and 0.63 μg kg^{-1} (median: 0.22 μg kg^{-1}). Recalculating our results on a product mass basis as it was expressed in a study by Ziegenhals et al. (2009), the content of BaP ranged from ND (<0.02 μg kg^{-1}) to 0.98 μg kg^{-1} with the median value of 0.24 μg kg^{-1}, and the content of Chr was in the range of 0.06 – 2.1 μg kg^{-1} [136].

Another study concerning the content of PAHs in chocolate candies [137] indicated the median BaP content of 0.66 μg kg^{-1}, which was almost three times higher than our observed value of 0.24 μg kg^{-1}, whereas the mean BaP content of 1.5 μg kg^{-1} observed by Indian researchers was more than five times higher than our observed value of 0.27 μg kg^{-1}. This observation could be explained by the fact that some additional chocolate processing steps are included in the manufacturing of chocolate candies, which could act as additional sources for the formation and introduction of PAHs.

Commission Regulation (EU) No. 2015/1933 specifies a maximum level of 5.0 μg kg^{-1} fat for BaP in cocoa beans and derived products (excluding cocoa beans and derived products intended for use as an ingredient in food) [43]. All of the analysed samples conformed to the aforementioned maximum limit. BaP levels over the detection limit were found in 77% of the samples tested. The 95th percentile concentration was 0.29 μg kg^{-1} fat. An overview of the obtained results from this study is presented in Table 3.6.

The limits of 1.0 and 2.0 μg kg^{-1} fat for the individual PAHs concentrations were also applied for better characterisation of chocolate samples. Thus, 19% of the samples had BaP levels exceeding 1.0 μg kg^{-1} fat, and only 7.7% exceeded 2.0 μg kg^{-1} fat. As for PAH4, the specified maximum level of 30.0 μg kg^{-1} fat was set in Commission Regulation (EU) No. 2015/1933 [43], and no sample exceeding this level was found. The 95th percentile concentration for the PAH4 was established at the level of 1.6 μg kg^{-1} fat.

Table 3.6

The concentration of PAHs in chocolate and the percentage of samples exceeding the LOD, 1, 2, 5, or 30 μg kg^{-1} fat

PAH	Number of samples (%)					Concentration, μg kg^{-1} fat			
	>LOD	>1 μg kg^{-1} fat	>2 μg kg^{-1} fat	>5 μg kg^{-1} fat	>30 μg kg^{-1} fat	Median	Mean	P95	Max
BaA	100	27	12	0	0	0.49	0.91	0.38	3.6
Chr	100	35	15	7.7	0	0.87	1.4	0.65	7.4
BbF	100	27	19	0	0	0.74	1.2	0.51	4.8
BaP	77	19	7.7	0	0	0.59	0.71	0.29	2.9
PAH4	100	100	73	23	0	2.6	4.2	1.6	16.1

Judging from the results obtained in our study, chocolate seems to be consistently contaminated with a range of PAHs. In most of the cases this contamination is characterised by relatively low levels of PAHs, indicating that highly sensitive instrumentation with high trueness and efficiency level without any false positive/negative findings is preferable for reliable, precise, and accurate quantification.

3.3.2. PAHs in processed cereal-based foods

Applicability of the elaborated GC-MS/MS analytical method for ultra-sensitive determination of PAHs in processed cereal-based foods was checked by performing the analysis of 35 randomly collected Latvian bread and cereal samples.

The observed PAHs concentrations in processed cereal-based products available on the Latvian retail market are showed in Annex 7, while the summary with the mean, median, minimum, and maximum concentrations of single PAH compounds and the PAH4 compounds in Latvian cereal products and bread samples are shown in Table 3.7.

Table 3.7

The mean, median, and range of concentrations of single PAH compounds and PAH4 in Latvian cereal and bread samples

	BaA, μg kg^{-1}	Chr, μg kg^{-1}	BbF, μg kg^{-1}	BaP, μg kg^{-1}	PAH4, μg kg^{-1}
Cereals, n=3					
Mean	0.12	0.29	0.14	0.061	0.61
Median	0.12	0.32	0.15	0.062	0.67
Min-max	0.092-0.15	0.20-0.34	0.099-0.17	0.056-0.065	0.45-0.71
Rye bread, n=20					
Mean	0.18	0.28	0.17	0.084	0.71
Median	0.16	0.25	0.15	0.060	0.65
Min-max	0.045-0.41	0.15-0.61	0.078-0.37	0.010-0.24	0.31-1.6
Wheat bread, n=12					
Mean	0.094	0.20	0.13	0.064	0.49
Median	0.074	0.17	0.11	0.045	0.40
Min-max	0.028-0.25	0.11-0.42	0.065-0.33	0.017-0.17	0.22-1.1
Processed cereal-based foods, n=35					
Mean	0.14	0.25	0.15	0.075	0.63
Median	0.14	0.22	0.14	0.059	0.55
Min-max	0.028-0.41	0.11-0.61	0.065-0.37	0.010-0.24	0.22-1.6

Bread and cereal samples collected for this study were categorised into three groups: cereals (n=3), rye bread (n=20) and wheat bread (n=12). Rye bread samples were found to be the most contaminated with an arithmetic mean of the BaP content at the level of 0.084 μg kg^{-1} and an arithmetic mean of the combined PAH4 content – 0.71 μg kg^{-1} (see Table 3.7). The concentration of the PAH4 in cereals and wheat bread samples ranged from 0.45 μg kg^{-1} to 0.71 μg kg^{-1} for the cereals and 0.22 – 1.1 μg kg^{-1} for the bread samples, while mean of PAH4 were 0.61 μg kg^{-1} and 0.49 μg kg^{-1}, respectively. Measured BaP content in the analysed cereals and the wheat bread samples was rather similar, mean BaP concentration in the analysed cereals was established at the level of 0.061 μg kg^{-1} and at the level of 0.064 μg kg^{-1} for the wheat bread samples. Comparable results were obtained by Polish scientists, in the study of PAHs in the bakery chain [46]. The mean combined concentrations of 4 regulated PAHs reported in the study were in the range of 0.05 – 0.47 μg kg^{-1}, 0.23 – 0.45 μg kg^{-1}, and 0.21 – 1.3 μg kg^{-1} in the case of wheat-rye bread, rye bread, and whole rye bread, respectively, but the lowest levels of total PAHs contamination were found in wheat flour.

Among four individual PAHs chrysene was always the most abundant PAH with the amounts between 0.11 μg kg^{-1} and 0.61 μg kg^{-1}. The minimum Chr content at the level of 0.11 μg kg^{-1} was found among wheat bread samples, while maximum content (0.61 μg kg^{-1}) was observed among rye bread samples. The average Chr content was established at the level of 0.25 μg kg^{-1}.

Rye bread samples were also characterised by the most variable PAH content. For example, the BaA concentration range in rye bread samples was 0.045 – 0.41 μg kg^{-1}.

In general, the obtained results of the present study show that the BaP content in the analysed bread samples was notably lower than the EU maximum permitted level (ML) – 1.0 μg kg^{-1}, but in relation to the PAH4, 5 of 35 (14%) of the analysed bread samples had their values above the ML. The mean as well as the median PAH4 values for all the investigated bread samples were 0.63 μg kg^{-1} and 0.55 μg kg^{-1}, respectively (see Table 4), that also could be considered as a relatively high level, taking into account the ML value of 1.0 μg kg^{-1}.

The obtained results clearly indicate that the minimum LOD value (0.30 μg kg^{-1}) required by the legislation (Commission Regulation No. 836/2011) is not sufficient for appropriate analysis of the four priority PAH compounds in bread samples [42]. In case the analysis method with LOD of 0.30 μg kg^{-1} would be applied for this study, only one sample would exceed the MRL for the PAH4, and there would be no samples in which BaP would be detected at all, since all the BaP values found were lower than 0.30 μg kg^{-1}.

As it was mentioned above, the uncertainty of the developed methods for the PAH4 was 24% in the case of HRMS technique and 35% in the case of MS/MS method, therefore, by taking into account the uncertainty, the number of cases when the combined concentration of 4 PAHs exceeded the EU permitted level decreased from 5 to 1 or from 14% to only 3% (Table 3.8).

Table 3.8

The distribution of concentrations of PAH4 in bread samples

	<MRL-35% **<0.65 μg kg^{-1}**	**<MRL-24%** **<0.76 μg kg^{-1}**	**<MRL** **<1 μg kg^{-1}**	**>MRL** **>1 μg kg^{-1}**	**>MRL+24%** **>1.24 μg kg^{-1}**	**>MRL+35%** **>1.35 μg kg^{-1}**
Number of samples	22	28	30	5	2	1
Frequency, %	63	80	86	14	5.7	2.9

In a study performed by scientists from Spain [47] 24 bread samples were analysed and only 4 samples from 24 were found to contain PAHs (LOQ of the performed method ranged from 0.07 μg kg^{-1} to 0.75 μg kg^{-1}). The concentration of BaP was observed above the quantification limit (LOQ 0.07 μg kg^{-1}) only in three bread samples, with concentrations ranging from 0.13 to 0.23 μg kg^{-1}. If such LOQ would be applied to samples analysed in the

present study, only 11 samples from 35 (31% of the samples) would show detectable concentrations of BaP.

In another study concerning PAHs content in toasted bread [79], BaP was not detected in 10 of 18 samples (LOD 0.50 μg kg^{-1}; LOQ 1.5 μg kg^{-1}), whereas in the rest of the samples the BaP content varied from 2.8 μg kg^{-1} to 16.5 μg kg^{-1}. Furthermore, the total PAH concentration was in the range of 1.1 – 44.2 μg kg^{-1}. Remarkably, even the minimum calculated total PAH concentration would be non-compliant with the existing EU ML. This fact clearly shows that when such a low MLs are specified in legislation, sensitive and selective determination methods should be applied.

3.3.3. PAHs in dried herbs and spices

Samples of seasonings collected for the study were categorised into two groups: dried herbs and spices. The category of dried herbs included 25 oregano samples, 25 basil samples, and 25 thyme samples. The spices category combined 25 black pepper samples, 25 paprika samples, and 25 nutmeg samples. Detailed sample description is shown in Annex 8, while the summarised descriptions of every sample category are outlined in Annex 9. All the results were obtained by using the GC-MS/MS analytical technique.

In order to characterise the whole set of samples by descriptive statistics, all values below the LOD or LOQ were replaced by zero, and the confidence level was set at 95% to reflect a significance level of 0.05.

PAHs in oregano samples. For the determination of PAHs, 25 oregano samples produced from 2009 to 2014 were analysed. Oregano samples were found to be contaminated with the analysed PAHs at low levels (see Annex 9). BaP was present in 64% of the analysed oregano samples with the maximum concentration of 1.6 μg kg^{-1}. The observed concentration range of the PAH4 was 1.0 – 13.6 μg kg^{-1} with Chr as the most significant contributor at the mean concentration of 2.2 μg kg^{-1}. The confidence intervals at the confidence level of 95% were in the range of 0.21 – 0.54 μg kg^{-1} for BaP and 3.3 – 6.0 μg kg^{-1} for PAH4. In the oregano samples assessed by the production year there was a slight increase of PAHs content in years 2012 and 2013, with the average PAH4 content of 8.11 μg kg^{-1} in 2012 and 8.13 μg kg^{-1} in 2013.

The most contaminated oregano samples were produced in 2013, with the highest mean BaP content of 0.67 μg kg^{-1} and PAH4 reaching 8.1 μg kg^{-1}, respectively. Lower levels of PAHs were found in the oregano samples produced in 2010, with the average PAH4 content of 2.0 μg kg^{-1}. The highest concentrations among four individual PAHs were observed

for Chr, with the maximum content of 8.4 μg kg^{-1}. No oregano samples analysed in the current study exceeded the existing EU MLs.

PAHs in basil samples. Within the framework of this study, 25 basil samples produced in years 2010, 2011, 2013, and 2014 were analysed with regard to the PAHs content. Basil samples were found to be more contaminated with PAHs than oregano samples (see Annex 9). BaP was present at detectable levels in all analysed samples, with the average content of 1.5 μg kg^{-1}. The obtained confidence interval at the confidence level of 95% was established in the range of 1.2 – 1.7 μg kg^{-1}. As for PAH4, the obtained confidence interval was 10.2 – 13.1 μg kg^{-1}. Likewise for oregano samples, the most contaminated basil samples were produced in 2013, with the highest mean BaP content of 2.2 μg kg^{-1} and PAH4 content of 13.1 μg kg^{-1}. The lowest levels of PAHs were found in samples produced in 2011, with the mean PAH4 content of 9.9 μg kg^{-1} and BaP content of 1.1 μg kg^{-1}, respectively. Similarly to the oregano samples, in the case of basil the main contributor to the aggregated concentration of selected PAHs was Chr, with the highest mean content of 6.3 μg kg^{-1}. In order to assess the influence of the pretreatment process on the PAHs content in seasonings, the results from 21 cleaned and steam-treated basil samples were compared to 4 other basil samples that were only cleaned. The mean BaP and PAH4 concentrations in the four basil samples that were only cleaned turned out to be lower in comparison to those found in the cleaned and additionally steam treated samples (1.1 μg kg^{-1} and 8.0 μg kg^{-1} for basil that was only cleaned, but 1.6 μg kg^{-1} and 12.3 μg kg^{-1} for the cleaned and additionally steam treated basil, respectively). Although all investigated individual PAHs were present above the LOQ in all analysed basil samples, the existing EU MLs of 10.0 μg kg^{-1} for BaP and 50.0 μg kg^{-1} for PAH4 were not exceeded in any of the samples.

PAHs in thyme samples. Thyme samples included in this study were produced in 2010, 2011, 2014, and 2015, and were found to be the most contaminated samples in dried herbs category (see Annex 9). The mean BaP and PAH4 concentrations among the analysed samples were found to be 4.2 μg kg^{-1} and 25.8 μg kg^{-1}, respectively. The confidence interval for PAH4 concentration was 23.1 – 28.4 μg kg^{-1}. BaP was detected in all the analysed samples in the range of 1.3 – 5.7 μg kg^{-1}, with the confidence interval (confidence level of 95%) of 3.7 – 4.7 μg kg^{-1}. No significant correlation between the production year and PAHs content was observed. Similarly to the other dried herbs samples, Chr was the dominant congener, with the observed concentration range of 3.0 – 18.2 μg kg^{-1}, with the average amount of 9.2 μg kg^{-1}.

Among all the analysed thyme samples, PAHs content in organic thyme was the lowest, with PAH4 contamination at the level of 6.9 μg kg^{-1} and BaP content equal to 1.3 μg kg^{-1}. It is important to mention that organic thyme sample was produced in China, while the country of origin for the remaining thyme samples was Poland. However, this observation does not allow to conclude about the contamination risks regarding the country of origin, due to the insufficient number of analysed samples.

Similarly to basil samples, thyme samples were investigated for the possible influence of the pretreatment process on the PAH content and it was found that steam treatment might be associated with increased PAH contamination. Thus, the mean PAH4 concentration in the cleaned samples was significantly higher in comparison with the non-treated samples, at 18.5 and 6.9 μg kg^{-1}, respectively. Even though thyme samples were most contaminated in the dried herb category, no samples exceeded the acceptable MLs.

PAHs in black pepper samples. Twenty five black pepper samples produced in 2008 – 2010 were analysed for the PAHs content and a high variation of concentrations was observed. Thus, BaP content ranged from non-detectable levels (<0.05 μg kg^{-1}) to 6.6 μg kg^{-1} and PAH4 concentration was found to be in the range from 1.4 to 25.2 μg kg^{-1} (see Annex 9). The confidence intervals were established at the levels of 1.7 – 3.1 μg kg^{-1} for BaP and 10.3 – 16.0 μg kg^{-1} for the PAH4. In most of the cases, Chr was the dominant PAH at concentrations between 0.76 μg kg^{-1} and 10.9 μg kg^{-1}. Only one of the samples was produced in year 2008, and it was found to be the most contaminated with BaP (6.6 μg kg^{-1}) in the spices category. Samples produced in 2010 showed a slight decrease of PAHs content, with the average BaP and PAH4 concentrations of 2.2 μg kg^{-1} and 11.5 μg kg^{-1}, respectively. Based on the obtained results, it should be noted that contamination of black pepper samples with PAHs significantly decreased from 2008 to 2010. Thus, a decrease of PAHs content by a factor of two was observed from year 2008 (PAH4 of 22.5 μg kg^{-1}) to year 2010 (PAH4 of 11.5 μg kg^{-1}). A similar trend was observed for BaA, showing a decrease of concentration from 4.0 μg kg^{-1} in the samples from year 2008 to 1.5 μg kg^{-1} in the samples from year 2010. It should be noted that the concentrations of BaA, BbF, and Chr in black pepper samples also decreased, but to a lesser extent.

Two samples (8%) among the 25 analysed black pepper samples were found to exceed a half of the existing EU ML value for BaP (10 μg kg^{-1}). The relatively high contamination of black pepper might be explained by the additional processing step in black pepper production, where the still green, unripe drupes of the pepper plant are briefly boiled in hot water before

the drying process [138]. This additional step causes rupturing of cell walls, accelerating the browning during drying, and therefore could be an additional source of PAHs contamination.

PAHs in paprika samples. The analysed paprika samples were produced in 2010 and 2014. The concentration of PAH4 in paprika samples ranged from 2.9 μg kg^{-1} to 14.0 μg kg^{-1}, with the average concentration of 8.0 μg kg^{-1} (see Annex 9). The BaP concentration in analysed paprika samples was in the range of 0.33 – 2.2 μg kg^{-1}, with the mean concentration of 1.1 μg kg^{-1}. The confidence intervals were established at the levels of 0.92 – 1.3 μg kg^{-1} for BaP and 7.0 – 9.1 μg kg^{-1} for PAH4, respectively. Chr was observed as the dominant congener with the average content of 3.1 μg kg^{-1}.

The obtained results showed no significant differences between PAHs content and year of production. No significant differences in PAHs content were also observed between the hot and sweet paprika samples, however, the sweet paprika samples showed broader PAHs concentration ranges. Thus, the BaP concentration range in sweet paprika samples was 0.33 – 2.2 μg kg^{-1}, while the BaP concentration range in hot paprika samples was 0.70 – 1.1 μg kg^{-1}. All four EU regulated congeners were detected in 100% of the analysed samples, but none of the samples exceeded the ML values.

PAHs in nutmeg samples. A total of 25 nutmeg samples produced in 2009, 2010, 2011, and 2014 were analysed for the PAHs content, and this seasoning was found to be the least contaminated in the spice category (see Annex 9). In the majority of samples, the PAHs content was below the method LOQ. For example, BbF was present in 24% of the analysed nutmeg samples, while BaP was present in just 16%. The confidence interval of <LOQ - 0.22 μg kg^{-1} was calculated for BaP, and 1.9 – 3.8 μg kg^{-1} for PAH4. The measured levels of PAH4 varied within the range of 1.0 – 7.3 μg kg^{-1}, with the average value of 2.9 μg kg^{-1}. The most contaminated samples were produced in 2011, with the mean PAH4 content of 7.3 μg kg^{-1}. Lower levels of PAHs were found among the nutmeg samples produced in 2009, with the average PAH4 content of 1.3 μg kg^{-1}. Chr had the highest mean concentration of 1.4 μg kg^{-1} in all the analysed nutmeg samples. No nutmeg samples exceeding the existing EU ML values were found.

In summary, our results showed that both dried herbs and spices are contaminated with PAHs at low levels below the established EU MLs. Our elaborated analytical method for the analysis of PAHs indicated individual PAH congeners at detectable levels in 86% of the analysed samples, with the highest detection frequency for Chr, which was detected at levels above the established LOQ in 95% of the analysed samples. BaP, BbF, and BaA were

detected at levels above LOQ in 93%, 81% and 79%, respectively. Only basil and paprika samples showed all four individual EU regulated PAHs in 100% of analysed samples.

The aggregated PAHs content in the tested samples decreased in the order of thyme > black pepper > basil > paprika > oregano > nutmeg (Fig. 3.11). The same trend was observed for BaP and BbF, while the content of BaA and Chr decreased in the following orders: thyme > black pepper > paprika > oregano > nutmeg > basil and thyme > basil > black pepper > paprika > oregano > nutmeg, respectively.

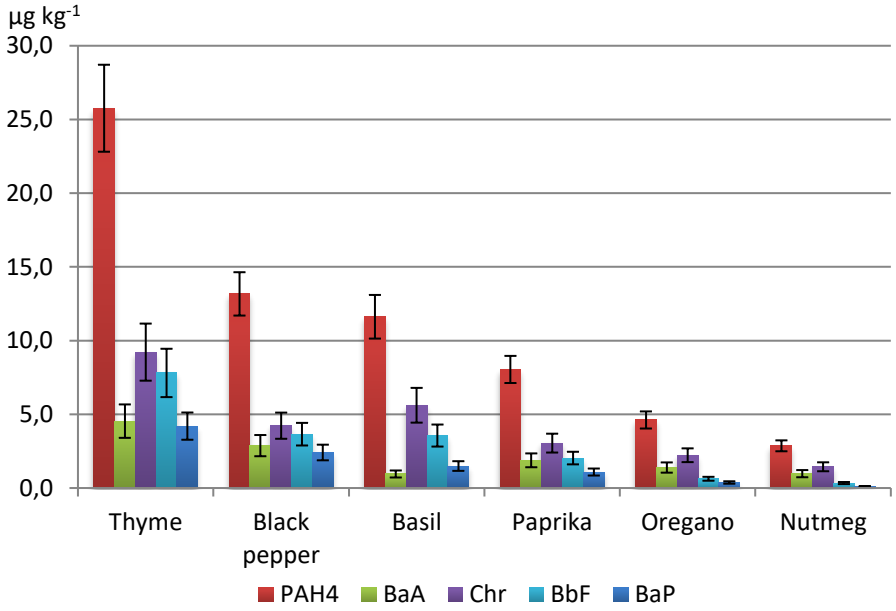

Fig. 3.11. **Mean levels of BaP (µg kg^{-1}) and the PAH4 (µg kg^{-1}) in seasonings**

Although some trends in the contamination with PAHs in various seasonings were discovered, the overall results showed no evidence of unusual contamination in specific types of products and, therefore, confirmed a prior observation [6] of non-systematic contamination connected with environmental pollution and processing techniques.

3.3.4. PAHs in smoked meat

The study on the occurrence of PAHs in smoked meats produced in Latvia was performed in two parts: 1) monitoring of the smoked meat samples and the assessment of dietary exposure to PAHs from smoked meat in Latvia; 2) occurrence of PAHs by applying a targeted approach, where smoked meat samples originating from small-scale producers and commercially available on the local market at farmer's markets, natural food stores, and farmer's fairs. All the results were obtained by the analytical method that included extraction

of PAHs with an organic solvent mixture, gel permeation chromatography, solid phase extraction, and followed by the GC-MS/MS determination.

PAHs in smoked meat and smoked meat products from Latvia

In a first study, 128 samples of smoked meat products from Latvia were analysed and the content of four PAHs was determined. Table 3.9 shows the mean, median, minimum, and maximum concentrations of single PAH compounds, as well as the total content of PAH4. In some samples the concentration of BaP and BbF was below the limit of quantification (LOQ).

Table 3.9

Mean, median, minimum, maximum of single PAH compounds and PAH4 in smoked meat products (n=128)

Compound	Mean, μg kg^{-1}	Median, μg kg^{-1}	Maximum, μg kg^{-1}	Minimum, μg kg^{-1}
BaA	2.4	0.76	14.2	0.052
Chr	2.4	0.82	14.5	0.10
BbF	0.82	0.32	4.6	<0.05
BaP	0.74	0.21	6.0	<0.05
PAH4	6.4	2.1	34.7	0.15

The median of BaP contents was 0.21 μg kg^{-1}, being significantly below the ML of 5.0 μg kg^{-1}. The higher content was observed for BaA and Chr with median values of 0.76 and 0.82 μg kg^{-1}, respectively.

Results showed (see Table 3.10) that all median values of individual PAH content and the mean levels of PAH4 were higher in smoked chicken samples (8.0 μg kg^{-1}), although no significant differences ($p > 0.05$) were observed. The highest level of PAH4 for individual samples was found in smoked pork speck (34.7 μg kg^{-1}) that could be due to the high concentration of Chr in some samples. An important factor for PAH contamination is the surface/mass ratio. General smoked chicken meat being of lesser size and thickness than smoked pork meat, showed a larger surface per unit of volume, which causes the elevated concentration of PAHs.

Table 3.10

Average and median concentration and range of individual PAHs and PAH4 of each meat type

	BaA, μg kg^{-1}	**Chr, μg kg^{-1}**	**BbF, μg kg^{-1}**	**BaP, μg kg^{-1}**	**PAH4, μg kg^{-1}**
Smoked pork, n=14					
Average	2.7	2.8	1.1	1.1	7.7
Median	1.1	1.3	0.37	0.29	3.1
Min-max	0.05-10.6	0.10-11.1	<0.05-4.6	<0.05-6.0	0.15-27.3
Samples >0	14	14	13	13	14
Smoked pork breast, n=18					
Average	2.3	2.3	0.81	0.60	5.9
Median	0.74	0.67	0.33	0.23	1.8
Min-max	0.06-9.6	0.10-11.2	<0.05-4.3	<0.05-3.0	0.16-28.1
Samples >0	18	18	16	16	18
Smoked chop, n=12					
Average	2.4	2.2	0.93	0.99	6.5
Median	1.1	0.88	0.44	0.24	3.0
Min-max	0.08-8.4	0.14-8.3	<0.05-3.0	<0.05-4.2	0.23-20.2
Samples >0	12	12	11	8	12
Smoked pork speck, n=10					
Average	2.7	3.1	1.1	0.91	7.8
Median	0.72	0.80	0.34	0.31	2.2
Min-max	0.13-12.2	0.25-14.5	0.11-4.3	<0.05-3.6	0.48-34.7
Samples >0	10	10	10	9	10
Smoked ham, n=4					
Average	1.9	2.17	0.81	0.65	5.5
Median	0.56	0.69	0.34	0.25	1.8
Min-max	0.12-6.3	0.29-7.0	0.11-2.5	<0.05-2.1	0.52-17.8
Samples >0	4	4	4	3	4
Smoked chicken, n=12					
Average	3.3	3.0	0.94	0.74	8.0
Median	2.3	2.0	0.62	0.47	5.4
Min-max	0.30-12.3	0.63-12.1	0.20-4.6	0.08-2.8	1.2-31.7
Samples >0	12	12	12	12	12

The PAH values observed in smoked chicken and smoked pork meat were higher than those reported for smoked meat products in Italy, Estonia, and Germany [128, 139-140]. However, data from previous studies show that during traditional smoking of meat high levels of BaP appear. Wretling et al. (2010) reported 9 (out of 38) samples with high BaP lavels ranging from 6.6 to 36.9 μg kg^{-1} in Swedish smoked meat samples exceeding the 5.0 μg kg^{-1} level [17]. High levels of BaP were detected in samples where traditional sauna smoking is used.

Also, our research shows that the higher concentrations of PAHs appear in samples from small producers, where traditional smoking methods could be used and the intensity of smoke deposition is uncontrolled, thus depends on the environmental conditions (temperature and relative humidity), and the type of wood used. In this case, the foodstuff is in direct

contact with all components of the generated smoke and it could be highly contaminated with PAHs. Contrary to traditional smoking methods, smoke production in industrial smoking ovens is closely controlled and the removal of undesirable compounds is facilitated by the smoke generators being separated from the smoking chamber [141].

All analysed sausages were distributed in three groups – smoked sausages, small sausages, and semi-dry sausages. The highest average concentrations of BaA (2.4 μg kg^{-1}), Chr (2.6 μg kg^{-1}), BbF (0.77 μg kg^{-1}), BaP (0.84 μg kg^{-1}), and PAH4 (6.6 μg kg^{-1}) were found in smoked sausage samples (see Table 3.11). Significant ($p < 0.05$) lower concentrations of BaP with an average value of 0.11 μg kg^{-1} were found in half dried sausage samples. Our results were higher than those obtained for Spanish [94, 142], Italian [5], and Swedish sausages, where the content of BaP was below the limit of detection [17].

Table 3.11

Average and median concentration and range of individual PAHs and PAH4 in smoked sausages and smoked products

	BaA, μg kg^{-1}	**Chr, μg kg^{-1}**	**BbF, μg kg^{-1}**	**BaP, μg kg^{-1}**	**PAH4, μg kg^{-1}**
Smoked sausage, n=21					
Average	2.4	2.6	0.77	0.84	6.6
Median	0.31	0.49	0.25	0.11	1.3
Min-max	0.08-14.2	0.13-11.2	<0.05-4.1	<0.05-4.2	0.22-33.7
Samples >0	21	21	18	14	21
Crackers or small sausages, n=10					
Average	1.9	1.9	0.68	0.67	5.2
Median	0.36	0.58	0.23	0.080	1.2
Min-max	0.19-13.1	0.26-12.8	0.13-4.0	0.05-4.6	0.63-34.6
Samples >0	10	10	10	10	10
Semi-dry sausage, n=8					
Average	0.43	0.54	0.22	0.11	1.3
Median	0.36	0.53	0.24	0.07	1.2
Min-max	0.13-0.88	0.21-1.0	0.10-0.33	<0.05-0.22	0.48-2.4
Samples >0	8	8	8	6	8
Roulette, n=6					
Average	0.35	0.45	0.25	0.15	1.2
Median	0.30	0.37	0.26	0.12	1.1
Min-max	0.12-0.78	0.21-0.88	0.12-0.39	<0.05-0.36	0.46-2.4
Samples >0	6	6	6	5	6

Low BaP content of 0.13 – 0.16 μg kg^{-1} was determined in Danish sausages [15] smoked by indirect smoking methods, followed by 0.24 – 0.33 μg kg^{-1} in smoked sausages from Serbia [143], 0.36 – 0.63 μg kg^{-1} in Portuguese traditional smoked meat and blood sausages [144], and 0.13 – 0.59 μg kg^{-1} [94] in Spanish traditional smoked sausage varieties

"Androlla" and "Botillo". In a study of Italian traditional smoked sausages "Pitina" the BaP content was found 0.8 μg kg^{-1} [5].

The higher concentrations of BaP and PAH4 were found in Portuguese traditional meat and blood sausages [18], where BaP levels in meat products were from 0.36 up to 4.8 μg kg^{-1} and in blood-derived products from 0.32 to 5.7 μg kg^{-1}. In these products very high BaA and Chr concentrations were found (up to 133 and 151 μg kg^{-1} respectively), hence the maximum PAH4 was found to be 295 μg kg^{-1}, which is several times higher than the 30.0 μg kg^{-1} ML for PAH4.

The proportion (%) of samples exceeding the maximum permitted level and planned maximum concentration of BaP from September 2014 are shown in Table 3.12, and for PAH4 in Table 3.13. The frequency of cases exceeding the EU specified limits for PAHs varied from 7% for smoked pork to 0% for other samples, although in the case if the 5.0 μg kg^{-1} ML for BaP of will be lowered to 2.0 μg kg^{-1} in September 2014, 14% of smoked meat products in Latvia will be non-compliant to the new permitted level of BaP. Regarding the PAH4, currently 3.9% of all smoked meat samples exceed the existing EU limit (30.0 μg kg^{-1}) and with the introduction of the new EU limits (12.0 μg kg^{-1}) more than 20% of samples will exceed that limit.

Table 3.12

The occurrence of BaP in smoked meat products

		<0.05 μg kg^{-1}	**≤2 μg kg^{-1}**	**>2 μg kg^{-1}**	**>5 μg kg^{-1}**
Smoked meat products, %	n=128	19	86	14	0.82
Smoked chicken, %	n=12	0	92	8.3	0
Smoked pork, %	n=12	7.1	79	21	7.1
Smoked pork breast, %	n=18	11	89	11	0
Smoked chop, %	n=12	33	83	17	0
Smoked pork speck, %	n=10	10	80	20	0
Smoked ham, %	n=4	25	75	25	0
Smoked sausage, %	n=21	33	81	19	0
Small sausages, %	n=10	0	90	10	0
Half-dried sausage, %	n=8	20	100	0	0
Roulette, %	n=6	17	100	0	0

The overall results indicate that the production of smoked meat products with BaP contamination levels below 2.0 μg kg^{-1} and PAH4 concentration below 12.0 μg kg^{-1} for manufacturers applying traditional smoking methods is problematic, and a greater effort of changing processing practices and regional consumption habits is needed.

Table 3.13

The occurrence of PAH4 sum concentrations in smoked meat products

		≤12 µg kg^{-1}	≤30 µg kg^{-1}	>30 µg kg^{-1}
Smoked meat products, %	n=128	79	96	3.9
Smoked chicken, %	n=12	75	92	8.3
Smoked pork, %	n=12	71	100	0
Smoked pork breast, %	n=18	78	100	0
Smoked chop, %	n=12	75	100	0
Smoked pork speck, %	n=10	80	90	10
Smoked ham, %	n=4	75	100	0
Smoked sausage, %	n=21	76	95	4.8
Small sausages, %	n=10	90	90	10
Half-dried sausage, %	n=8	100	100	0
Roulette, %	n=6	100	100	0

Consumption of smoked and grilled products in Latvia

Food consumption database is the significant information source for risk assessment, since it contains information on food consumption habits in Latvia. Due to the fact that smoked meat consumption is a specific part of food consumption, focussed research for smoked meat consumption in Latvia in the age group of 19-64 years was performed in 2014. The data for smoked meat and fish consumption is presented in Table 3.14.

Differences in meat consumption between men and women are statistically significant at exceptionally high levels ($p < 0.0001$ independent sample Student t-test, Mann-Whitney-Wilcoxon U-test). Women consume 171 g of meat per day, while men consume significantly more – 280 g per day.

Table 3.14

Consumption of meat and fish during a year

n = 1811	Mean	25th percentile	Median	75th percentile
Meat, kg	81.8	34.9	64.1	111
Fish, kg	13.0	2.6	6.7	15.4
Meat and fish, kg	94.8	42.1	73.7	125
Smoked meat and fish, kg	14.6	3.3	8.3	18.2
Grilled/barbequed meat and fish, kg	13.9	1.2	5.3	17.3
Smoked, grilled, barbequed meat and fish, kg	28.6	7.3	17.6	37.0
Smoked meat and fish, % from all meat and fish consumed	15.0	6.0	12.2	20.9
Grilled/barbequed meat and fish, % from all meat and fish consumed	14.0	2.1	8.2	21.6
Smoked, grilled, barbequed meat and fish, % from all meat and fish consumed	29.0	14.7	27.3	41.2

Assessment of consumer preferences in relation to smoked products

Respondents were asked to note products they use every day and those that are usually consumed on festive occasions. Semi-dry, dried sausages, and smoked chicken legs are consumed more on every day basis, but smoked whole chicken, pork chop, ham and chicken roulette are usually eaten on festive occasions. On average, 33% of consumers purchase smoked products in supermarkets, 23% buy at farmers markets, and 15% get them directly from farmers. Few families make smoked meat and fish products by themselves. Almost 15% do it on their own, while 10% mentioned that they have friends, relatives or neighbours who smoke meat for them.

It is possible to slightly reduce the consumed amounts of BaP and PAH4 if the skin or dark rind of meat is removed. Almost half of consumers (48%) replied that they remove skin of smoked chicken. These habits are more common among women (62%) than men (33%). The differences are statistically significant at high level (Pearson χ^2 test $p < 0.001$). The consumers who remove skin from smoked products also consume less smoked products than those who do not.

An average of 22% respondents have planned or have already reduced their smoked meat consumption to reduce intake of BaP and PAH4, but 23% mentioned that they would not change anything in their eating habits to reduce BaP and PAH and are worried that there will be changes in the taste of traditionally smoked products if levels of BaP and PAH4 have to be lowered. To detect what type and what colour of smoked meat products consumers prefer, they were offered photos of 4 groups of products and indicated their preferences. All the pictures shown in Fig. 3.12 contain products that are easily available on the market.

Most consumers prefer chicken that is prepared and looks like in Fig. 3.12 sample **a –** 31%, but 26% rate **d** as the favourite. Our concern is that the product in Fig. 3.12, sample d has such high level because this is one of most easily available products in all supermarkets and the only one available on market that is packed in convenient vacuum packaging.

Fig. 3.12. **Colour scale for assessment of surface colour of smoked chicken**

The same situation is with smoked pork fat, where 39% of consumers prefer products which look like sample **a** in Fig. 3.13, while only 7% prefer the one in Fig. 3.13 sample **c**.

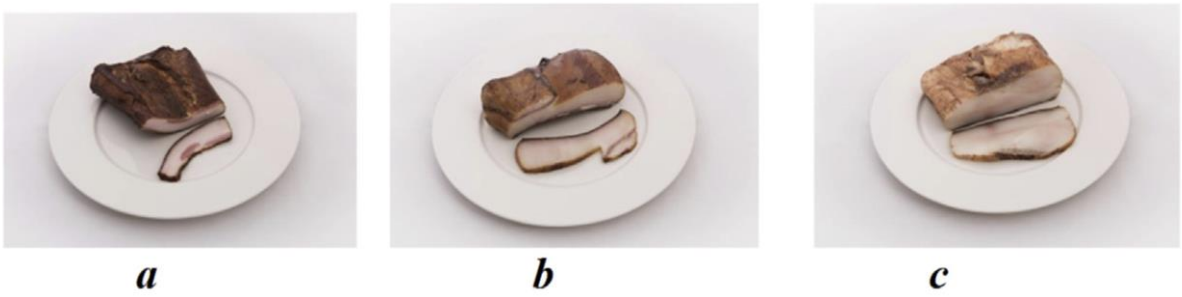

Fig. 3.13. **Colour scale for the assessment of surface colour of smoked pork fat**

In assessing the situation with home-made smoked sausages, we also concluded that most consumers (52%) prefer sausage with darker surface colour (sample **a** in Fig. 3.14).

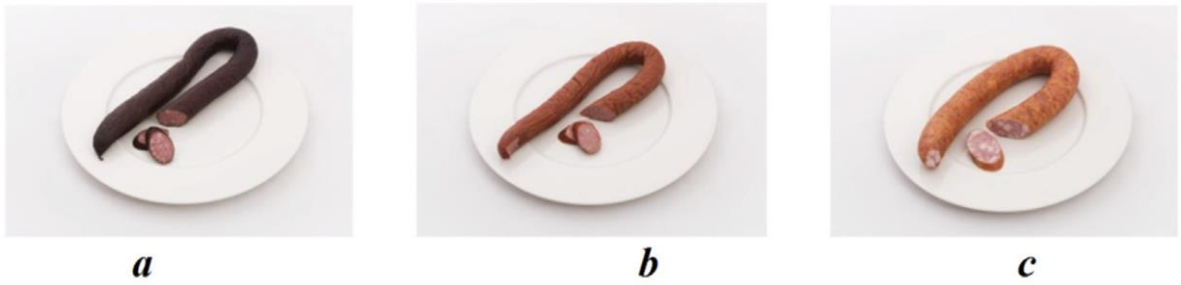

Fig. 3.14. **Colour scale for the assessment of home-made sausage colour**

This is a product typically eaten with bread and mainly chosen by seniors, as it may have a strong taste of garlic and other spices.

Targeted approach for the determination of the PAHs in smoked meat and meat products

Based on the aforementioned observations and according to the preferences of the consumers in Latvia, the research was extended and data on the occurrence of PAHs by applying a targeted approach, where smoked meat samples originating from small-scale producers, commercially available on the local market at farmer's markets, natural food stores, and farmer's fairs, and with an emphasis to those with darker surface colour were also collected and evaluated. In addition, targeted smoked meat samples from Lithuania and Estonia were also analysed for the content of PAHs in order to assess the situation in all three of the Baltic states.

An overview of the determined concentrations of individual PAHs in the analysed smoked meat samples from the three Baltic states is shown in Table 3.15. Meanwhile,

detailed descriptions of samples along with the respective contamination levels are presented in Annex 10.

Very large variations between the tested samples were observed with the concentration of BaP ranging from 0.05 to 116 μg kg^{-1} and the concentration of PAH4 ranging from 0.42 to 628 μg kg^{-1}. The smoked meat samples from Latvia were found to be the most contaminated – the PAH4 content was found to be in a range of 0.77 – 628 μg kg^{-1}, with the mean and median concentrations at 53.8 μg kg^{-1} and 13.3 μg kg^{-1}, respectively. Chr was found as the most prominent PAH in the analysed samples, at a concentration range in Latvian smoked meat samples from 0.27 to 215 μg kg^{-1}. BaA showed slightly lower concentrations than Chr, the detected concentrations in traditionally smoked meat samples produced in Latvia varied in the range of 0.25 – 171 μg kg^{-1}. Meanwhile, BaP, which is the most harmful of the PAHs, was detected in samples originating from Latvia at the levels of 0.11 – 116 μg kg^{-1}, with the mean concentration of 8.1 μg kg^{-1}. The content of BbF was at the same order of magnitude – from 0.12 to 126 μg kg^{-1}, with the mean concentration of 9.5 μg kg^{-1}.

Lithuanian and Estonian smoked meat samples were found to be contaminated with the PAHs to a lesser extent. The median BaP and PAH4 concentrations in smoked meats produced in Lithuania were 0.73 and 7.1 μg kg^{-1}, while smoked meats produced in Estonia contained BaP and PAH4 at the median concentrations of 0.18 and 1.8 μg kg^{-1}, respectively. Based on the results described above, Estonian smoked meat samples were found to be the least contaminated, however, only relatively few samples produced in Estonia were available for our study.

The 95^{th} percentile of PAH4 concentrations for the smoked meats from Latvia and Lithuania was established at the level of 29.7 μg kg^{-1} and 6.8 μg kg^{-1}, respectively. The results obtained from the analysis of smoked meat samples from Estonia were not included in this evaluation, because of insufficient sample number for reliable statistical evaluation. The 95^{th} percentile BaP concentrations were 5.0 μg kg^{-1} for the smoked meats from Latvia and 0.93 μg kg^{-1} for the samples from Lithuania. Hence, even the 95^{th} percentile concentrations for targeted Latvian smoked meat products are very close to the exceptional EU maximum permitted levels of 5.0 μg kg^{-1} for BaP and 30.0 μg kg^{-1} for PAH4 in traditional smoked meets in Latvia and exceeds the existing baseline EU MLs of 2.0 μg kg^{-1} (BaP) and 12.0 μg kg^{-1} (PAH4). At this point it is important to point out that all of the obtained results characterise only our special case, when the samples were collected with an emphasis to those with darker surface colour, smoked using traditional methods, and produced by small local producers.

Table 3.15

The mean and median concentrations, as well as the range of concentrations of individual PAHs and PAH4 in smoked meat samples

	BaA, μg kg^{-1}	Chr, μg kg^{-1}	BbF, μg kg^{-1}	BaP, μg kg^{-1}	PAH4, μg kg^{-1}
Smoked meat from Latvia, n=52					
Mean	16.4	19.8	9.5	8.1	53.8
Median	4.2	4.2	2.8	2.3	13.3
Minimum	0.25	0.27	0.12	0.11	0.77
Maximum	171	215	126	116	628
Confidence level (95%)	8.8	10.6	5.9	5.0	29.7
Smoked meat from Lithuania, n=17					
Mean	2.7	3.5	1.9	1.5	9.5
Median	1.1	2.5	0.99	0.73	7.1
Minimum	0.10	0.17	0.084	0.053	0.42
Maximum	19.5	21.1	8.5	7.1	56.2
Confidence level (95%)	2.3	2.5	1.2	0.93	6.8
Smoked meat from Estonia, n=8					
Mean	8.6	11.0	3.9	2.8	26.3
Median	0.61	0.80	0.24	0.18	1.8
Minimum	0.12	0.13	0.085	0.086	0.42
Maximum	59.1	74.7	24.6	16.8	175
Confidence level (95%)	17.1	21.6	7.1	4.9	50.7

Among the analysed meat samples from Latvia, 46% exceeded the existing EU maximum level for BaP and 48% - for PAH4. However, taking into account the amendment of 2014, specifying the list of countries that can apply MLs applicable before September 2014, i.e. 5.0 μg kg^{-1} for BaP and 30.0 μg kg^{-1} for PAH4, the fraction of meat samples from Latvia exceeding the MLs decreases to 31% for both BaP and PAH4. Nonetheless, the number of these cases is extremely high. Lower incidence of excessive contamination with PAHs was found among the samples produced in Lithuania and Estonia, which are not on the exception list of the EU 1327/2014 amendment [98]. However, from 24% to 29% of samples from Lithuania and Estonia still exceeded the EU norm for the concentrations of BaP and PAH. An overview of the results described above is presented in Table 3.16.

Table 3.16

The frequency of PAHs concentrations exceeding the ML in smoked meat products

	BaP	PAH4

	>2 μg kg^{-1}		>5 μg kg^{-1}		>12 μg kg^{-1}		>30 μg kg^{-1}	
	Number of samples	**Frequency, %**	**Number of samples**	**Frequency, %**	**Number of samples**	**Frequency, %**	**Number of samples**	**Frequency, %**
Latvian (*n=52*)	24	46	16	31	25	48	16	31
Lithuanian (*n=17*)	4	24	1	5.9	5	29	1	5.9
Estonian (*n=8*)	2	25	1	13	2	25	1	13

As to our knowledge, the levels of PAHs revealed in our study are rarely seen in recent studies. The majority of articles reporting high contamination with PAHs were published before 2000, when the extremely high levels of PAHs in smoked products caused an increasing interest from the EU authorities and resulted in establishing the maximum permitted levels for these contaminants. For example, Dyremark et al. (1994) and Rogge et al. (1991) reported individual PAH concentrations ranging from approximately 30 to 900 μg kg^{-1} for 3-, 4-, and 5-ringed PAH molecules, with the sum of PAHs reaching up to 1700 μg kg^{-1} [14, 145]. Despite the fact that extremely high concentrations of PAHs in smoked meat products are rarely observed in recent years, elevated concentrations of PAHs are still reported by the authors from several countries, for example, PAH4 concentrations up to 272 μg kg^{-1} were found in traditional blood sausages originating from Portugal [18]; up to 49 μg kg^{-1} of PAHs in smoked sausages originating from Denmark [15]; 31.2 μg kg^{-1} of BaP in smoked meat from Estonia [140]; 17.6 μg kg^{-1} of BaP in smoked belly of pork from Germany [16], and 36.9 μg kg^{-1} of BaP in Swedish ham produced by direct "sauna" smoking with birch logs [17].

Judging from the results obtained in our study, traditionally smoked meat in Latvia, Lithuania, and Estonia seems to be consistently contaminated with a range of PAHs. Although in most of the cases this contamination is characterised by relatively low levels of PAHs [127, 140], extremely high PAH contamination, as shown in a present study, can be still occasionally detected.

3.4. Risk characterisation in relation to meat consumption

The nutritional value of smoked meat is tarnished by its negative association with safety issues, like the presence of various toxic contaminants, including PAHs. Certainly, the actual toxicity of any compound depends on the dose and period of contact with the organism as well as on the individual characteristics of the human or animal host. In order to characterise the risks related to the presence of PAHs, the margins of exposure (MOEs), as adopted by the EFSA Scientific Committee for substances which are both genotoxic and

carcinogenic, were calculated. Calculations were performed for different groups of respondents according to the respective levels of consumption and two different scenarios were developed for evaluating the possible impact of smoked meat products on public health. For the 1st scenario, the data on average BaP concentration and average PAH4 for primary results were used. For the 2nd scenario, the results obtained applying targeted approach for the sampling of the smoked meat and meat products were used.

3.4.1. Risk assessment according to the mean PAHs contamination

Calculations of both the consumer exposure to BaP and PAH4, and the MOE indicators for all consumers of smoked meat products are demonstrated in Table 3.17

Table 3.17

The calculation of consumer exposure to BaP and PAH4

	Consumption of smoked meat products (kg day^{-1})			**Mean contamination (μg kg^{-1})**	**Consumer exposure (ng kg^{-1} b.w.day^{-1})**			**BMDL$_{10}$**
	Mean	**Median**	**95th percentile**		**Mean**	**Median**	**95th percentile**	
BaP	0.036	0.022	0.047	0.74	0.33	0.20	0.43	70000
PAH4	0.036	0.022	0.047	6.4	2.9	1.8	3.7	340000

The calculation of dietary exposures and MOEs suggests that MOE indicators for all consumer groups studied within this research far exceeds 10 000 – the MOE value derived on the base of precautionary approach and recommended by EFSA (Tables 3.18).

Table 3.18

MOE indicators within different groups of consumers for mean BaP and mean PAH4 concentrations

Group of consumers	**MOE (BaP)**			**MOE (PAH4)**		
	Mean	**Median**	**75th percentile**	**Mean**	**Median**	**75th percentile**
All consumers	209627	343026	160565	116868	191238	89516
19-35 years old	212702	350959	171199	118582	195660	95444
36-50 years old	198174	297260	148630	110482	165723	82862
51-64 years old	217697	383562	167808	121367	213836	93553
Men	172803	259204	127608	96338	144507	71142
Women	261486	424914	206019	180939	294025	142558

The comparison of MOE indicators for different consumer groups with regard to BaP exposure revealed that a relatively higher dietary exposure and thus a comparatively lower MOE is characteristic for men and middle-age consumers; nevertheless, the MOE values were significantly higher than 10 000. The comparison of MOE indicators for different consumer

groups with regard to the exposure to PAH4 indicated that a relatively higher dietary exposure and thus a comparatively lower MOE is characteristic for men, rather for women.

Thus, the MOE indicators calculated during the research demonstrate a low concern for consumer health at the mean, median, and 75th percentile exposures even in the case of worst possible scenarios.

3.4.2. Specific case with the elevated PAHs contamination

Considering the revealed concentrations of PAHs in smoked meat according to the preferences of consumers and the availability of the exposure data to smoked meat samples originating from the Baltic states, detailed risk assessment was made only for the smoked meats from Latvia. Meanwhile, in order to represent the central tendency of the PAHs distribution, MOEs were calculated just for the mean and 95th percentile values of the PAHs content.

Based on the previously determined consumption of smoked meat products (Table 3.17) and determined content of PAHs, the overall average dietary exposure for all consumers in Latvia is 324 ng day^{-1} (5.4 ng kg^{-1} b.w. per day assuming a body weight of 60 kg) for BaP (range: 229 – 426 ng day^{-1}) and 2153 ng day^{-1} (35.9 ng/kg b.w. per day assuming a body weight of 60 kg) for PAH4 (range: 1519 – 2832 ng day^{-1}). In view of these findings, the respective mean MOE for BaP among all consumer groups was 12952, with the minimum value of 9849 for men and maximum of 18350 for women. Slightly lower MOEs in a range of 7205 – 13430 were observed for the PAH4, with the mean value of 9475. Again, the lowest values were observed for men and the highest for women.

The characterisation of MOEs based on consumer age indicated the highest potential risk for middle-age (39–50 years) group of respondents – MOE of 8486 for PAH4 and 11602 for BaP. MOEs calculated for 95th percentile contamination were approximately 20% higher than those observed for the mean BaP and PAH4 content – between 12 517 and 24 434 for BaP and between 10 264 and 20 035 for PAH4. An overview of the obtained results is shown in Table 3.19.

Table 3.19

The calculated MOEs within the different groups of consumers in Latvia

Group of consumers	MOE (BaP)		MOE (PAH4)	
	Mean	95th percentile	Mean	95th percentile
All consumers	12952	16781	9475	13760
19-35 years old	14547	19331	10641	15850
36-50 years old	11602	15120	8486	12398
51-64 years old	12607	16509	9222	13537

Men	9849	12517	7205	10264
Women	18360	24434	13430	20035

The MOE approach within age/gender groups was already used to estimate the carcinogenic risk of different types of foods contaminated with PAHs. For example, Kang et al. (2014) reported MOEs for PAHs from edible oils in Korea at the levels of 4 000 000 for BaP and 137 000 for PAH4, and thus indicated a negligible risk to human health [146]. Similar results were obtained by Kim et al. (2014), who reported MOEs for PAH4 at the levels of 485 437 in the fish and shellfish group, 25 634 in the meat group, and 265 957 in the smoked products group [147]. Also, Veyrand et al. (2013), who assessed human dietary exposure to PAHs in a French total diet study, reported high MOE values – 150 000 and 230 000 for different age groups of consumers in France [32]. At the same time, Duedahl-Olesen et al. (2015) have recently published a detailed study on PAH contamination in Danish barbecued meat [148]. It revealed a health concern only in a worst case scenario – daily intake of highly contaminated barbecued meat, with the MOE values of 7 080 and 8 500 for commercially barbecued and home-grilled meat, respectively. Even though the study revealed levels less than 10 000, no consumer was believed to be exposed to these levels [148].

Nevertheless, taking into account the dietary habits and preferences of the Latvian consumers, as well as in accordance with the exposure assessment and risk characterisation, the comparison of MOE indicators revealed that the high levels of PAHs in traditionally smoked meat products from Latvia should raise concerns about the health of consumers and call for risk management actions.

3.5. Methods to reduce the PAHs content in food

As the PAHs are ubiquitous contaminants that could be found everywhere and, moreover, pose a potential threat to human health it is necessary not only to monitor the presence of these compounds but also elaborate methods of reducing of the PAHs content. Two different approaches were tested for this purpose – ozone treatment and lactic acid bacteria treatment.

3.5.1. Ozone treatment

The effect of ozonation on BaP standard solution

Initially, the effects of ozone treatment on a standard solution of BaP in ethanol were assessed. Ozone was applied to a solution containing high concentration of BaP (1.0 mg mL^{-1})

and immediate fading of the yellow colour within the first seconds indicated effective chemical transformation. It was established by GC-MS analysis that ozone treatment for 1 min reduced the BaP content in ethanol solution by 89% and after 5 minutes 99% of BaP was transformed. Additional ozone exposure further decreased the BaP concentration, although the overall changes after the first 5 min were insignificant (Fig. 3.15). Our results show that the degradation of BaP by ozone is consistent with pseudo-first order kinetics under the experimental conditions used in this study. Our data was fitted to the first order rate equation $[A]=[A]_0e^{-kt}$, where [A] is a BaP concentration (mg mL^{-1}) at the time t (min), $[A]_0$ is an initial BaP concentration (mg mL^{-1}) and k is the apparent first order rate constant (min^{-1}). This analysis yielded an apparent first order rate constant of 2.2 min^{-1} and an apparent half-life of 0.31 min for BaP in ethanol under these conditions. These results are consistent with the observations of Ottingen et al. (1999) who reported that BaP content was reduced by 99.7% after 1 min of ozonation and that BaP content was reduced to non-detectable levels after 10 min of ozonation [105].

The obtained results clearly indicate the high efficiency of ozonation treatment for the decomposition of BaP in solutions. Samples of ethanol solutions obtained in these experiments were injected into the GC-MS instrument operated in a full scan mode. Many low molecular mass products were detected in ozonated BaP solutions without any compound giving a major peak on the chromatogram. In general, the compounds identified by the mass spectral database represent the pathways of oxidative degradation of BaP described by other authors [149-152].

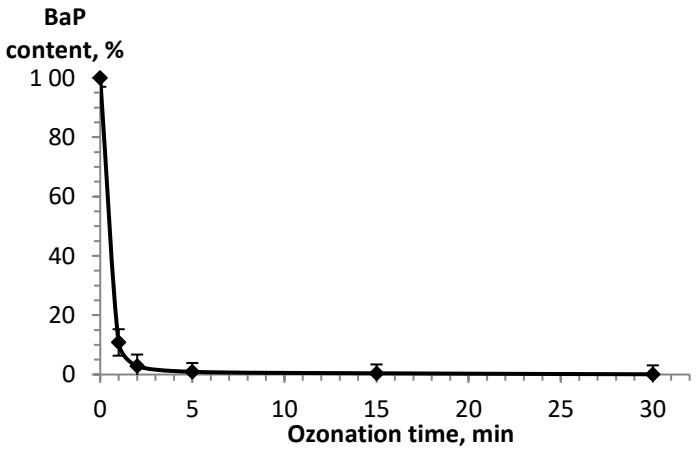

Fig. 3.15. **The effect of ozonation on a standard solution of BaP**

The effect of ozonation on smoked fish samples

Taking into account the pronounced effect of ozone on diminishing the concentration of BaP in ethanol solution, further experiments were performed in order to explore the possible application of similar processes for the removal of PAHs from smoked products. To study the effects of ozone, smoked sprats that were previously found to contain high concentrations of PAHs were treated under optimal ozonation conditions. The initial concentrations of PAHs and the changes after ozonation are summarised in Table 3.20.

The results clearly indicated the tendency for decrease of total PAHs concentration in smoked fish samples. The individual PAH concentrations in smoked fish decreased by 6 – 46% after 60 min, while the PAH4 concentration decreased by 22%. The concentration of Chr decreased by only 6% after ozonation for 60 min, moreover, positive and negative fluctuations in concentration were observed during the whole period of ozonation. These fluctuations did not exceed 10%, and were attributed to random errors, with the method uncertainty at around 20%. Similar results were obtained for BaA, even though fluctuations were less obvious in the case of BaA and the final decrease after 60 min of ozonation was 18%. The decrease in BbF and BaP concentration was more consistent, and the final degree of reduction was 34% and 46%, respectively. These observations indicate that PAH molecules containing an additional aromatic ring are more readily oxidised by ozone.

Table 3.20

The decrease of average PAH content in smoked fish samples during ozonation process (n=3)

Sample type	Time, min	BaA, μg kg^{-1}	BaA, %	Chr, μg kg^{-1}	Chr, %	BbF, μg kg^{-1}	BbF, %	BaP, μg kg^{-1}	BaP, %	PAH4, μg kg^{-1}	PAH4, %
Smoked sprats	0	4.5	100	5.2	100	2.4	100	2.8	100	14.9	100
	5	4.2	94	5.8	110	1.8	77	2.2	79	14.0	94
	15	3.7	81	4.9	94	1.8	75	2.0	72	12.4	83
	30	4.1	91	5.4	103	1.8	76	2.0	70	13.2	89
	45	3.6	79	5.0	97	1.8	77	1.9	67	12.3	83
	60	3.7	82	4.9	93	1.6	67	1.5	54	11.7	78

Previous studies on ozonation of food samples clearly showed that ozone has a strong potential to affect a wide range of quality traits in foods, such as colour, flavour, aroma, and the presence of vitamins. Both negative and positive effects are likely to occur [101, 109, 111, 153].

In vitro toxicity evaluation of products arising from the ozonation of BaP

A comprehensive assessment of results obtained in the present study shows that ozone has a significant effect on food quality. Unequivocally adverse effects of ozonation on food

safety were identified by *in vitro* studies of 3T3 and HepG2 cell culture viability and morphology after treatment with ozonated BaP solution.

In vitro toxicity evaluation of BaP ozonation products was based on four different BaP solutions obtained as 0.1%, 0.2%, 0.4% and 0.8% v/v dilutions of ozonated BaP solution, corresponding to 1.0, 2.0, 4.0, and 8.0 μg mL^{-1} of BaP and its degradation products in cell cultivation medium.

Non-ozonated BaP standard solution had no adverse effect on 3T3 cell division – control samples and samples with non-ozonated BaP reached the maximum confluence of 100% within 72 h.

The samples with BaP concentration of 8.0 μg mL^{-1} after ozonation for 1 and 2 min initially inhibited the cell growth rate in 3T3 cell cultures, although 100% cell confluence was achieved within 72 h (Fig. 3.16.). The samples with BaP concentration of 8.0 μg mL^{-1} after ozonation for 5 min had negative effect on cell proliferation, and only 30% of control sample confluence was achieved within 72 h, with many dead cells observed. BaP samples of lower concentration that were ozonated for 5 min showed no cytotoxic effects on 3T3 cells.

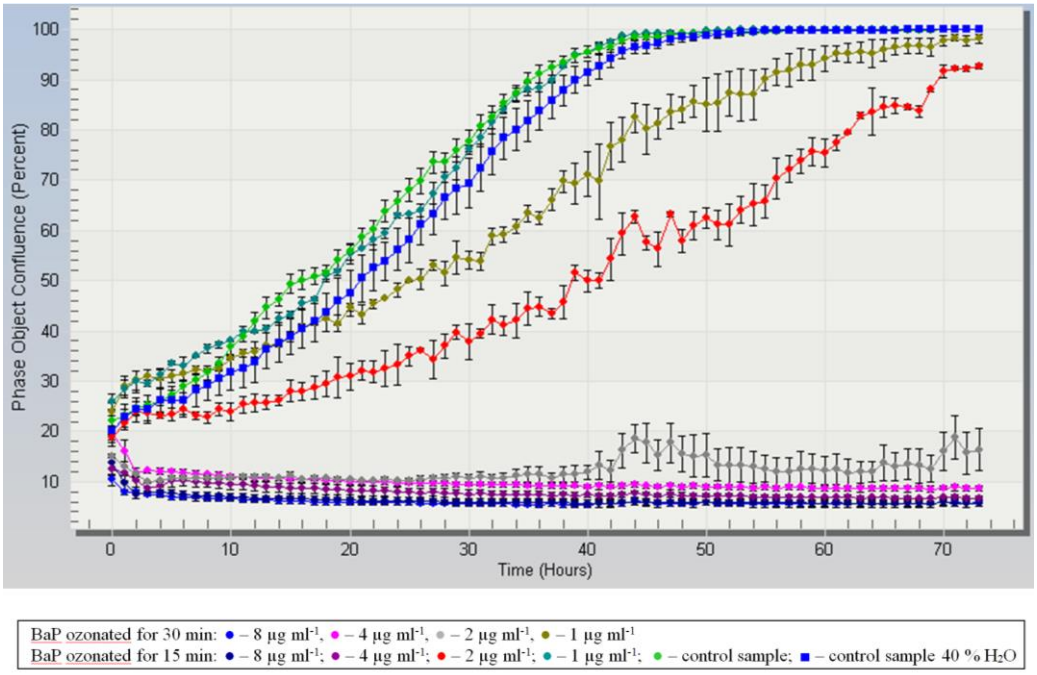

Fig. 3.16. **The changes in 3T3 cell confluence in the presence of ozonated BaP**

BaP samples that were ozonated for 15 and 30 min had cytotoxic effects that directly correlated with the initial concentration of BaP. The 2.0, 4.0, and 8.0 μg mL^{-1} solutions of BaP after ozonation for 30 min were highly toxic to 3T3 cells, with the number of living cells significantly reduced during the first incubation hours. Adding a 1.0 μg mL^{-1} BaP solution

after ozonation for 30 min decreased the rate of cell growth, but in the presence of this solution cells were able to reach confluence within 72 h, in contrast to the treatment with more concentrated ozonated BaP solutions. In the case of BaP solutions that were ozonated for 15 min, cytotoxic effects were observed at initial BaP concentrations of 4.0 and 8.0 μg mL^{-1}. Samples with the initial BaP concentration of 2.0 μg mL^{-1} slowed cell division in the growth medium, but 95% confluence was still achieved after 72 h cultivation.

Obvious changes in 3T3 cell morphology were observed after 48 h of exposure to ozonated BaP samples. Reduced cell growth rate and altered morphology were evident in cells treated with 8.0 μg mL^{-1} BaP that had been ozonated for 5 min, while cells exposed to BaP ozonated for 30 min showed complete cell death.

Similarly to 3T3 cell cultures, the viability of HepG2 cells was not significantly affected by treatment with non-ozonated BaP solutions, however, slightly lower proliferation rates were observed at the highest BaP concentrations (4.0 and 8.0 μg mL^{-1}).

Substantial cytotoxicity was observed in HepG2 cell cultures with BaP solutions that were ozonated for 5, 15, and 30 min. HepG2 cell viability in the presence of 2.0, 4.0, and 8.0 μg mL^{-1} of BaP that was ozonated for 15 and 30 min was negatively affected already during the first hours after the addition of ozonated BaP solutions. Cytotoxic effects were also observed with 8 μg mL^{-1} BaP solutions that were ozonated for 5 min. Cells remained viable at the lower BaP concentration of 4.0 μg mL^{-1}, however, the cell division rate was noticeably lower than in the control samples and cell morphology changes were observed.

BaP solutions that were ozonated for 30 min at the concentrations between 2.0 and 8.0 μg mL^{-1} were highly cytotoxic – these ozonated BaP solutions reduced cell viability and cell cultures did not reach even their initial seeding confluence throughout the 96 h period of cultivation. In contrast, BaP solutions at the lowest tested BaP concentration (1.0 μg mL^{-1}) that were ozonated for 30 minutes showed no cytotoxic effect and cell confluence was comparable to that of the control sample after 96 h of cultivation.

BaP samples ozonated for 15 min at the BaP concentration range of 4.0 – 8.0 μg mL^{-1} were cytotoxic to HepG2 cells. In contrast to these solutions, more dilute BaP samples that were ozonated for 15 min at concentrations between 1.0 and 2.0 μg mL^{-1} had no cytotoxic effects and cell confluence after cultivation for 96 h was similar to that of the control solution, see Fig. 3.17.

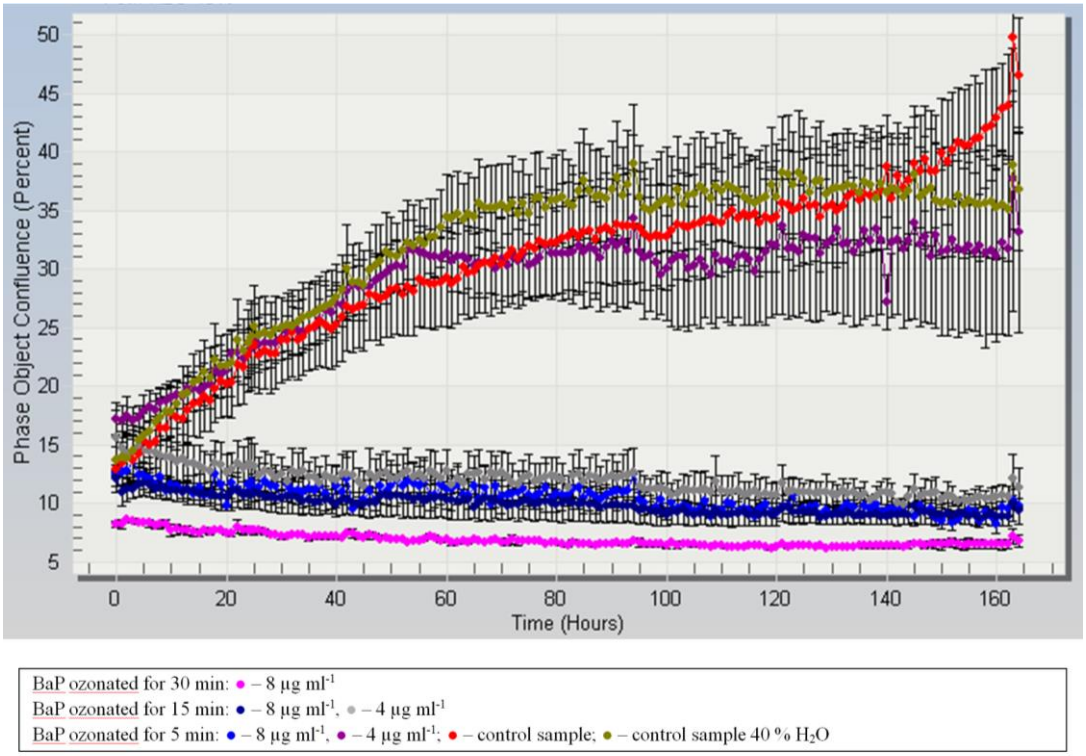

Fig. 3.17. **The changes in HepG2 cell confluence in the presence of ozonated BaP**

The BaP solutions that were ozonated for 5 min had no pronounced cytotoxic effect, but a significant reduction of cell proliferation rate was observed during the first 48 h of cultivation at the higher concentrations – 4.0 and 8.0 µg mL^{-1}. As cell cultivation continued, proliferation increased and reached the confluence of control samples. Presumably, the BaP ozonation products acted as a stress factor at the beginning that temporarily inhibited cell division, and after overcoming the stress, the cell division was even slightly stimulated.

Cytotoxic effect was not observed with BaP solutions that were ozonated for only 2.0 min. Although the initial cell division was inhibited at BaP concentration of 8.0 µg mL^{-1}, the cell growth rate recovered after a lag period. No adverse effects on cell growth were observed with 6.0 µg mL^{-1} BaP solution that was ozonated for 2 min.

In terms of food safety, when comparing the positive and negative effects of ozonation, the negative effects obviously had more influence on the safety of ozone treated fish products. It is also important to mention that smoked fish samples were ozonated for a twice longer time than BaP standard solution, for which the toxicity was evaluated. So it can be presumed that the toxicity of ozone treated smoked fish samples could be significantly higher.

In summary the current study indicates that ozone has a limited potential to degrade PAHs in smoked sprats. The most effective degradation by 60 min of ozonation was observed

for BaP and BbF, with contamination levels reduced by 46 and 34%, respectively. However, the content of BaA and Chr in analysed smoked fish samples remained at the same level.

According to the 3T3 and HepG2 cell's survival rate, morphology, and viability ozone-treated BaP standard solution inhibited the growth of 3T3 and HepG2 cells and long-term ozonated BaP standard solution (15 – 30 min) had strong adverse influence on cell viability and was highly toxic to 3T3 and HepG2 cells. At lower concentrations, cell proliferation was inhibited, and cell morphology was significantly changed indicating that even low concentrations can generate toxicity in primary cell types.

3.5.2. Lactic acid bacteria (LAB)

The PAHs content obtained in sausages even in not pre-treated with LAB suspension samples was below the maximum value currently allowed by European Union regulations. The results of PAHs formation in the outer layers and centre of the cold smoked sausages treated with LAB before and after smoking are presented in Table 3.21. The application of LAB for sausages treatment before and after smoking had significant influence on BaP and chrysene decreasing. In our experiment pH values of LAB bioproduct used for sausages treatment was in range 4.2 – 4.4. Zhao et al. (2013) reported that the maximum values of BaP binding rate of several LAB were obtained at pH 4.0 and 5.0 [154]. A significant effect of type of LAB applied for the fermentation on BaA ($F(6.8) ¼ 0.005$, $p ¼ 0.0001$), BbF ($F(12.0) ¼ 0.062$, $p ¼ 0.0001$), BaP ($F(5.6) ¼ 0.011$, $p ¼ 0.001$), and Chr ($F(26.7) ¼ 0.035$, $p ¼ 0.0001$) content in cold smoked sausages was found.

Significant changes were estimated in the different layers (outer or centre) of the sausages on BaA ($F(17.0) ¼ 0.005$, $p ¼ 0.0001$), BbF ($F(4.6) ¼ 0.062$, $p ¼ 0.035$), BaP ($F(29.1) ¼ 0.011$, $p ¼ 0.0001$), and Chr ($F(18.0) ¼ 0.035$, $p ¼ 0.0001$) content. Also, sausages treatment with LAB bioproduct before and after smoking had significant effect on BbF ($F(4.1) ¼ 0.062$, $p ¼ 0.049$), and BaP ($F(5.2) ¼ 0.011$, $p ¼ 0.027$) content. BaA and Chr formation in outer layers and centre of the cold smoked sausages were similar, and the differences were statistically insignificant. The interaction between all analysed factors (type of microorganisms, different part of sausages, treatment before and after smoking) on BaP ($F(3.6) ¼ 0.001$, $p ¼ 0.014$) and Chr ($F(3.9) ¼ 0.001$, $p ¼ 0.009$) content has been determined as statistically significant.

The results obtained in this study indicate that, during direct smoking process, the greatest amount of PAHs is formed in outer layers of sausages in comparison with inner layers of sausages. Similar results were reported by Ledesma et al. (2014, 2015) as they found that the greatest amount of BaP, PAHs content indicator, was deposited in the casing of the

meat product, not inside the product. These results are in agreement with other authors in smoked meat products [155-156]. Andrée et al. (2010) and Santos et al. (2011) reported that PAHs accumulate on the surface of the smoked meat product during smoking and then migrate into the products being smoked [144, 157].

Abou-Arab et al. (2010) reported that *Bifidobacterium bifidium*, *Streptococcus thermophilus* and *Lactobacillus bulgaricus* reduced PAHs content by 47, 88 and 92%, respectively [100]. Hongfei et al. (2013) reported that several LAB strains together might be beneficial for removing several toxic compounds [158]. Therefore, the mechanism of reducing toxic compounds is still unclear. Some researchers suggest that toxins are converted by specific enzymes produced by cells, therefore PAHs content decreasing after sausages treatment with LAB suspension could be achieved [159]. Other reports revealed that this process was due to the binding of the carcinogen to cell wall components, meanwhile, Tsuda et al. (2008) reported that exopolysaccharides played an important role in removing carcinogen [160-161].

To sum up, biopreservation using LAB and/or their antimicrobial metabolites represents an alternative for improving food safety. LAB strains used in a present research demonstrated good inhibition properties against all tested undesirable microorganisms, and could be used for cold smoked pork sausages surface treatment, in order to reduce biological/chemical contamination.

Table 3.21

PAHs content in outer layers and centre of cold smoked pork sausages

	L. sakei		***P. acidilactici***		***P. pentosaceus***		**Control**		
	BS	**AS**	**BS**	**AS**	**BS**	**AS**	**Non-treated**	**Treated with water BS**	**Treated with water AS**
Centre of sausage									
BaA, μg kg^{-1}	0.068 ± 0.005	0.054 ± 0.003	0.061 ± 0.004	0.063 ± 0.010	0.065 ± 0.007	0.062 ± 0.005	0.072 ± 0.006	0.069 ± 0.005	0.061 ± 0.003
Chr, μg kg^{-1}	0.15 ± 0.01	0.17 ± 0.01	0.20 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.10 ± 0.01	0.20 ± 0.01	0.17 ± 0.01	0.17 ± 0.01
BbF, μg kg^{-1}	0.021 ± 0.009	0.033 ± 0.005	0.030 ± 0.004	0.039 ± 0.002	0.031 ± 0.001	0.051 ± 0.004	0.033 ± 0.003	0.021 ± 0.002	0.024 ± 0.002
BaP, μg kg^{-1}	0.039 ± 0.004	0.030 ± 0.002	0.036 ± 0.007	0.034 ± 0.003	0.034 ± 0.005	0.042 ± 0.003	0.081 ± 0.007	0.079 ± 0.004	0.067 ± 0.005
PAH4, μg kg^{-1}	0.23	0.29	0.33	0.28	0.30	0.26	0.38	0.33	0.33
Outer layers of sausage									
BaA, μg kg^{-1}	0.072 ± 0.003	0.065 ± 0.004	0.069 ± 0.005	0.071 ± 0.011	0.069 ± 0.004	0.072 ± 0.003	0.086 ± 0.009	0.074 ± 0.003	0.068 ± 0.005
Chr, μg kg^{-1}	0.17 ± 0.01	0.18 ± 0.02	0.21 ± 0.01	0.16 ± 0.01	0.18 ± 0.01	0.12 ± 0.01	0.21 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
BbF, μg kg^{-1}	0.040 ± 0.007	0.051 ± 0.007	0.041 ± 0.005	0.062 ± 0.008	0.054 ± 0.007	0.073 ± 0.005	0.037 ± 0.005	0.048 ± 0.003	0.032 ± 0.005
BaP, μg kg^{-1}	0.062 ± 0.005	0.054 ± 0.006	0.051 ± 0.009	0.072 ± 0.007	0.068 ± 0.002	0.059 ± 0.004	0.106 ± 0.010	0.098 ± 0.007	0.081 ± 0.010
PAH4, μg kg^{-1}	0.34	0.35	0.37	0.37	0.38	0.33	0.44	0.40	0.36

BS – cold smoked pork sausages treated with LAB before smoking; AS – cold smoked pork sausages treated with LAB after smoking.

CONCLUSIONS

1. A novel nanomaterial-based d-SPE method for selective extraction of PAHs from edible oil was developed. The method showed good agreement between the results obtained with the elaborated method and a previously published GPC method, and thus, emphasized the great potential of nanomaterial-based sorbents in the analysis of the PAHs providing a respectable and less laborious alternative to commonly used sample preparation protocols.
2. The use of APPI interface in Orbitrap-MS system showed its superior performance in terms of method sensitivity and selectivity. The on-column instrumental LOD ranged from 0.8 pg to 1.2 pg for all four marker compounds, while the elaborated method LOD for chocolate samples varied from 0.016 to 0.024 μg kg^{-1} expressed on fat basis.
3. A comparative assessment of GC-MS/MS and GC-HRMS instrumental methods demonstrated that both methods have appropriate performance for the determination of the PAHs at ultra-low contamination levels. The obtained LODs for all individual PAHs were below 0.006 μg kg^{-1} and these LODs were lower than the values defined in the EU methods performance criteria more than 50 times.
4. The elaborated methods for the determination of the PAHs indicated a great variety of the observed PAHs in 406 different foods. The analysed foods were: bread and cereal products, dark chocolate, seasonings (dried herbs and spices), smoked meats and edible oils.
5. The MOE approach was utilised to assess the risks to Latvian consumers due to PAHs revealed in smoked meats. The obtained results indicated a potential concern for consumer health in Latvia. Moreover, the revealed PAHs contamination of bread and processed cereal-based products indicated a need to include these products into the risk characterisation study and to reassess possible human health risks.
6. Ozone treatment showed immediate effect for BaP solution, however, the impact of ozonation on smoked products was less pronounced even after prolonged ozonation. The *in vitro* toxicity evaluation showed that the cytotoxicity of BaP standard solution had significantly increased after the ozonation procedure, indicating a pronounced negative effect in terms of food safety.
7. The application of LAB for sausages treatment before and after smoking decreased both BaP and Chr content. The results also confirm that potatoes juice could be used as an alternative substrate for LAB cultivation, and the obtained fermented

bioproducts could be applied for surface treatment of cold smoked pork sausages to reduce PAHs content in final product.

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ANNEXES

Annex 1

The main validation parameters of the PAHs determination method using MWCNTs in edible oil

PAH	Calibration range, μg kg^{-1}	Linearity, r^2	LOD, μg kg^{-1}	LOQ, μg kg^{-1}	1st spiking level			2nd spiking level			HORRAT$_r$
					a	b	c	a	b	c	
BaA	0.10 – 10	0.998	0.09	0.30	108	5	6	107	2	4	0.34
Chr	0.10 – 10	0.999	0.21	0.71	104	3	5	105	2	4	0.31
BbF	0.10 – 10	0.998	0.08	0.27	107	3	5	101	4	6	0.38
BaP	0.10 – 10	0.999	0.06	0.19	98	2	4	96	4	5	0.31

1st spiking level – 1.0 μg kg^{-1}; 2nd spiking level – 2.0 μg kg^{-1}; a - Recovery (n=5), %; b - Intra-day precision, (n=5), %; c - Inter-day precision, (n=5), %

Annex 2

The main validation parameters for the HPLC-Orbitrap-MS determination method of the PAHs in dark chocolate

PAH	Calibration range, µg kg^{-1}	Linearity, r^2	LOD, µg kg^{-1}	LOQ, µg kg^{-1}	1st spiking level			2nd spiking level			3rd spiking level			HORRAT$_r$
					a	b	c	a	b	c	a	b	c	
BaA	0.10 – 5.0	0.995	0.024	0.081	86	14	12	88	7	7	84	11	10	0.71
Chr	0.10 – 5.0	0.993	0.016	0.054	94	10	17	85	3	4	86	9	10	0.49
BbF	0.10 – 5.0	0.990	0.021	0.069	94	12	16	92	9	8	91	7	6	0.63
BaP	0.10 – 5.0	0.997	0.019	0.064	110	16	14	102	8	10	95	8	7	0.72

1st spiking level – 0.10 µg kg^{-1} fat; 2nd spiking level – 1.0 µg kg^{-1} fat; 3rd spiking level – 5.0 µg kg^{-1} fat; a - Recovery (n=5), %; b - Intra-day precision, (n=5), %; c - Inter-day precision, (n=5), %

Annex 3

The main validation parameters of the GC-MS/MS and GC-HRMS methods for the PAHs determination in bread

PAH	Calibration range, µg kg^{-1}	r²		LOD, µg kg^{-1}		LOQ, µg kg^{-1}		Recovery, %						Precision, %						HORRAT$_r$	
		MS/MS	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS			HRMS			MS/MS			HRMS			MS/MS	HRMS
								A	b	Mean	a	b	Mean	a	b	Mean	a	b	Mean		
BaA	0.10 – 10	0.993	0.997	0.006	0.003	0.018	0.010	99	100	100	110	113	112	11	4	8	7	7	7	0.36	0.50
Chr	0.10 – 10	0.991	0.998	0.004	0.002	0.015	0.007	86	100	93	113	114	114	17	4	11	6	7	7	0.50	0.41
BbF	0.10 – 10	0.992	0.998	0.006	0.003	0.021	0.011	102	103	103	115	119	117	17	4	11	9	3	6	0.50	0.45
BaP	0.10 – 10	0.996	0.998	0.002	0.006	0.006	0.021	87	97	92	97	118	108	19	7	13	9	5	7	0.59	0.45

a – 0.10 µg kg^{-1}; b – 1.0 µg kg^{-1}

Annex 4

The main GC-MS/MS validation parameters of the PAHs determination method in dried herbs and spices

PAH	Calibration range, μg kg^{-1}	Linearity, r^2	LOD, μg kg^{-1}	LOQ, μg kg^{-1}	Recovery, %				Precision, %				HORRAT$_r$
					a	b	c	Mean	a	b	c	Mean	
BaA	0.10 – 5.0	0.9989	0.09	0.31	83	88	100	90	15	7	4	9	0.60
Chr	0.10 – 5.0	0.9999	0.04	0.14	79	72	81	77	5	9	4	6	0.41
BbF	0.10 – 5.0	0.9993	0.04	0.13	101	96	104	100	13	13	6	11	0.73
BaP	0.10 – 5.0	0.9992	0.05	0.18	100	100	107	102	15	3	2	7	0.45

a - 0.30 μg kg^{-1}; b – 1.0 μg kg^{-1}; c – 2.0 μg kg^{-1}

Annex 5

The main GC-MS/MS validation parameters of the PAHs determination method in smoked meat

PAH	Calibration range, µg kg^{-1}	Linearity, r^2	LOD, µg kg^{-1}	LOQ, µg kg^{-1}	1st spiking level			2nd spiking level			HORRAT$_r$
					a	b	c	a	b	c	
BaA	0.10 – 10	0.995	0.02	0.06	110	3	7	113	8	7	0.48
Chr	0.10 – 10	0.999	0.02	0.06	113	7	6	114	7	7	0.45
BbF	0.10 – 10	0.997	0.03	0.09	115	8	9	119	4	3	0.41
BaP	0.10 – 10	0.997	0.03	0.09	97	9	9	118	5	5	0.48

1st spiking level – 0.50 µg kg^{-1}; 2nd spiking level – 5.0 µg kg^{-1}; a - Recovery (n=5), %; b - Intra-day precision, (n=5), %; c - Inter-day precision, (n=5), %

Annex 6

The content of PAHs in the analysed dark chocolate samples, µg kg^{-1} fat (n=26)

No.	Country of production	Fat content, %	BaA, µg kg^{-1} fat	Chr, µg kg^{-1} fat	BbF, µg kg^{-1} fat	BaP, µg kg^{-1} fat	PAH4, µg kg^{-1} fat
1	Latvia	46	0.41	0.69	1.1	0.55	2.8
2	Spain	50	0.75	0.76	0.46	0.60	2.6
3	France	45	0.18	0.81	0.16	<0.06	1.1
4	France	51	0.73	1.1	0.54	<0.06	2.4
5	Germany	49	3.6	5.4	4.8	2.3	16.1
6	Switzerland	53	1.3	2.3	1.4	1.0	6.1
7	Lithuania	43	0.36	0.72	0.99	0.57	2.6
8	Estonia	57	0.72	0.74	0.32	<0.06	1.8
9	Germany	34	0.41	0.79	0.73	<0.06	1.9
10	Latvia	47	1.4	3.1	3.9	2.9	11.3
11	Germany	43	0.29	0.46	0.76	0.52	2.0
12	Belgium	59	3.5	7.4	3.2	0.87	15.0
13	Germany	45	1.5	1.7	2.8	0.69	6.6
14	Germany	56	0.38	0.71	0.36	0.21	1.7
15	Germany	67	0.41	0.83	0.30	0.56	2.1
16	Germany	62	0.47	0.91	0.48	0.75	2.6
17	Denmark	48	0.24	0.97	0.14	<0.06	1.4
18	France	35	0.23	0.61	0.96	0.55	2.4
19	Germany	58	0.49	0.37	0.18	0.08	1.1
20	Switzerland	45	2.7	0.94	3.0	0.89	7.5
21	Germany	55	0.49	1.0	0.53	0.76	2.8
22	Switzerland	41	0.84	0.94	0.97	0.88	3.6
23	Finland	58	0.58	1.0	0.76	1.9	4.3
24	Russia	31	0.34	0.60	0.34	<0.06	1.3
25	Russia	35	0.46	0.78	0.45	0.65	2.3
26	Russia	30	1.0	1.9	0.84	1.1	4.9

Annex 7

The mean, median, and range of concentrations of single PAHs and PAH4 in Latvian cereal and bread samples, µg kg^{-1} (n=35)

No.	BaA, µg kg^{1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
			Cereals, n=3		
1	0.092	0.20	0.099	0.056	0.45
2	0.15	0.34	0.15	0.065	0.71
3	0.12	0.32	0.17	0.062	0.67
			Rye bread, n=20		
1	0.15	0.23	0.12	0.14	0.64
2	0.16	0.24	0.16	0.11	0.67
3	0.20	0.32	0.24	0.20	0.95
4	0.33	0.41	0.24	0.093	1.1
5	0.32	0.50	0.29	0.24	1.3
6	0.41	0.61	0.37	0.23	1.6
7	0.14	0.22	0.15	0.090	0.60
8	0.14	0.20	0.19	0.052	0.58
9	0.22	0.36	0.17	0.059	0.82
10	0.18	0.29	0.16	0.040	0.67
11	0.15	0.21	0.092	0.032	0.48
12	0.15	0.21	0.099	0.010	0.46
13	0.20	0.29	0.21	0.052	0.75
14	0.22	0.31	0.13	0.060	0.71
15	0.15	0.20	0.15	0.041	0.54
16	0.18	0.26	0.14	0.076	0.65
17	0.11	0.25	0.11	0.065	0.53
18	0.068	0.18	0.12	0.039	0.41
19	0.057	0.16	0.094	0.037	0.34
20	0.045	0.15	0.078	0.031	0.31
			Wheat bread, n=12		
1	0.25	0.42	0.24	0.16	1.1
2	0.069	0.16	0.15	0.094	0.48
3	0.066	0.15	0.10	0.031	0.35
4	0.035	0.14	0.084	0.022	0.28
5	0.028	0.11	0.071	0.017	0.22
6	0.076	0.18	0.095	0.045	0.39
7	0.051	0.15	0.085	0.029	0.31
8	0.20	0.32	0.33	0.17	1.0
9	0.072	0.17	0.065	0.035	0.34
10	0.077	0.18	0.11	0.045	0.42
11	0.093	0.20	0.14	0.067	0.50
12	0.12	0.25	0.13	0.059	0.55

Annex 8

List of the analysed seasonings with detailed description (n=150)

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
Oregano, n=25									
1	rubbed oregano	Turkey	cleaning	2009	0.75	2.2	1.0	0.55	4.5
2	rubbed oregano	Turkey	cleaning	2009	0.78	0.58	<0.04	<0.05	1.4
3	rubbed oregano	Turkey	cleaning	2009	1.4	<0.04	0.96	0.54	2.9
4	rubbed oregano	Turkey	cleaning	2009	0.95	0.84	0.56	0.28	2.6
5	rubbed oregano	Turkey	cleaning	2009	<0.09	1.7	0.70	0.45	2.9
6	rubbed oregano	Turkey	cleaning	2009	2.5	0.36	<0.04	<0.05	2.8
7	rubbed oregano	Turkey	cleaning	2010	0.78	1.3	0.61	0.50	3.2
8	rubbed oregano	Turkey	cleaning	2010	0.76	<0.04	<0.04	0.25	1.0
9	rubbed oregano	Turkey	cleaning	2010	1.3	0.64	<0.04	<0.05	1.9
10	rubbed oregano	Turkey	cleaning	2010	0.96	<0.04	0.50	<0.05	1.5
11	rubbed oregano	Turkey	cleaning	2010	1.0	0.58	<0.04	0.45	2.1
12	rubbed oregano	Turkey	cleaning	2010	<0.09	2.4	<0.04	<0.05	2.4
13	rubbed oregano	Turkey	cleaning	2010	1.3	0.74	<0.04	<0.05	2.1
14	rubbed oregano	Turkey	cleaning	2012	1.5	1.2	0.73	0.28	3.7
15	rubbed oregano	Turkey	cleaning	2012	1.0	8.4	0.90	0.31	10.6
16	rubbed oregano	Turkey	cleaning	2012	1.3	7.5	0.80	0.42	10.1
17	rubbed oregano	Turkey	cleaning	2013	1.5	4.2	0.70	0.65	7.0
18	rubbed oregano	Turkey	cleaning	2013	2.4	4.2	1.1	0.90	8.6
19	rubbed oregano	Turkey	cleaning	2013	2.4	3.0	1.0	0.81	7.2
20	rubbed oregano	Turkey	cleaning	2013	2.7	0.87	1.2	<0.05	4.8
21	rubbed oregano	Turkey	cleaning	2013	2.4	8.1	1.5	1.64	13.6
22	rubbed oregano	Turkey	cleaning	2013	2.6	3.7	1.3	<0.05	7.6
23	rubbed oregano	Turkey	cleaning	2014	1.8	0.54	0.73	0.69	3.8
24	rubbed oregano	Turkey	cleaning	2014	1.0	1.7	0.93	0.68	4.4

Annex 8 continues

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
25	rubbed oregano	Turkey	cleaning	2014	1.7	0.91	0.49	<0.05	3.1
				Average	1.4	2.2	0.63	0.38	4.6
				Median	1.3	1.2	0.70	0.31	3.2
				Observed concentration range	<0.09 - 2.7	<0.04 - 8.4	<0.04 - 1.5	<0.05 - 1.6	1.0 - 13.6
				Confidence interval (95%)	1.1 – 1.7	1.19 – 3.3	0.44 – 0.82	0.21 – 0.54	3.3 – 6.0
Basil, n=25									
26	rubbed basil	Egypt	cleaned, steam treated	2010	0.72	5.7	2.2	1.1	9.8
27	rubbed basil	Egypt	cleaned, steam treated	2010	1.5	4.2	7.0	2.9	15.5
28	rubbed basil	Egypt	cleaned, steam treated	2010	<0.31	1.6	9.1	1.1	11.8
29	rubbed basil	Egypt	cleaned, steam treated	2010	0.32	5.5	2.7	0.94	9.4
30	rubbed basil	Egypt	cleaned, steam treated	2010	1.3	5.0	2.9	0.89	10.0
31	rubbed basil	Egypt	cleaned, steam treated	2010	1.0	8.2	5.8	2.4	17.4
32	rubbed basil	Egypt	cleaned, steam treated	2010	0.77	8.3	2.1	1.3	12.5
33	rubbed basil	Egypt	cleaned, steam treated	2010	<0.31	7.2	1.6	1.6	10.4
34	rubbed basil	Egypt	cleaned, steam treated	2010	<0.31	5.2	1.7	1.3	8.3
35	rubbed basil	Egypt	cleaned, steam treated	2010	2.6	9.7	3.8	1.5	17.6
36	rubbed basil	Egypt	cleaned, steam treated	2010	0.79	3.7	2.4	0.85	7.7
37	rubbed basil	Egypt	cleaned, steam treated	2010	0.90	5.2	3.4	0.97	10.5
38	rubbed basil	Egypt	cleaned, steam treated	2011	0.56	9.2	1.7	0.99	12.5
39	rubbed basil	Egypt	cleaned, steam treated	2011	0.08	3.4	2.6	1.3	7.4
40	rubbed basil	Egypt	cleaned, steam treated	2013	0.65	4.3	4.0	1.6	10.7
41	rubbed basil	Egypt	cleaned, steam treated	2013	<0.31	3.1	9.3	2.8	15.2
42	rubbed basil	Egypt	cleaned	2014	1.9	5.3	1.9	1.2	10.3
43	rubbed basil	Egypt	cleaned	2014	0.77	2.3	1.8	1.1	6.0
44	rubbed basil	Egypt	cleaned	2014	0.65	5.8	2.1	0.95	9.5

Annex 8 continues

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
45	rubbed basil	Egypt	cleaned	2014	0.55	2.0	2.4	1.2	6.1
46	rubbed basil	Egypt	cleaned, steam treated	2014	1.7	6.1	2.7	1.0	11.5
47	rubbed basil	Egypt	cleaned, steam treated	2014	1.2	8.5	3.4	2.0	15.1
48	rubbed basil	Egypt	cleaned, steam treated	2014	1.3	6.2	4.5	1.8	13.8
49	rubbed basil	Egypt	cleaned, steam treated	2014	0.55	5.3	4.1	2.5	12.5
50	rubbed basil	Egypt	cleaned, steam treated	2014	3.1	9.2	3.9	2.0	18.2
				Average	0.95	5.6	3.6	1.5	11.6
				Median	0.77	5.3	2.7	1.3	10.7
				Observed concentration range	<0.09 - 3.1	1.6 - 9.7	1.6 - 9.3	0.85 - 2.9	6.0 - 18.2
				Confidence interval (95%)	0.64 – 1.3	4.7 – 6.6	2.7 – 4.4	1.2 – 1.7	10.2 – 13.1
Thyme, n=25									
51	rubbed thyme	Poland	steam treated	2010	7.5	4.5	8.9	4.7	25.6
52	rubbed thyme	Poland	steam treated	2010	6.3	10.8	10.5	4.4	32.0
53	rubbed thyme	Poland	steam treated	2010	4.0	8.5	9.6	4.2	26.3
54	rubbed thyme	Poland	steam treated	2010	6.1	12.0	6.3	5.1	29.4
55	rubbed thyme	Poland	cleaned	2010	3.1	8.4	4.9	2.0	18.5
56	rubbed thyme	Poland	steam treated	2010	7.6	12.7	11.5	5.7	37.4
57	rubbed thyme	Poland	steam treated	2011	6.6	11.1	7.3	5.2	30.1
58	rubbed thyme	Poland	steam treated	2011	4.5	10.2	7.5	5.1	27.3
59	rubbed organic thyme	China	none	2011	0.81	3.0	1.8	1.3	6.9
60	rubbed thyme	Poland	steam treated	2011	4.9	8.0	7.9	4.2	25.0
61	rubbed thyme	Poland	steam treated	2011	5.5	10.4	10.3	4.7	30.9
62	rubbed thyme	Poland	steam treated	2014	5.9	9.3	8.5	4.5	28.2
63	rubbed thyme	Poland	steam treated	2014	5.4	8.8	10.0	4.8	29.1
64	rubbed thyme	Poland	steam treated	2014	5.1	9.0	8.4	4.5	27.0
65	rubbed thyme	Poland	steam treated	2014	5.5	10.6	9.5	4.7	30.3

Annex 8 continues

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
66	rubbed thyme	Poland	steam treated	2014	5.8	11.0	10.0	5.5	32.4
67	rubbed thyme	Poland	steam treated	2014	4.8	9.3	8.8	4.4	27.3
68	rubbed thyme	Poland	steam treated	2014	5.0	10.9	9.2	4.6	29.6
69	rubbed thyme	Poland	steam treated	2014	3.5	7.0	9.3	4.8	24.7
70	rubbed thyme	Poland	steam treated	2014	5.7	12.0	7.7	5.3	30.8
71	rubbed thyme	Poland	steam treated	2014	2.3	5.8	6.1	3.2	17.3
72	rubbed thyme	Poland	steam treated	2014	<0.09	18.2	2.4	1.8	22.4
73	rubbed thyme	Poland	steam treated	2014	2.3	6.2	5.9	3.6	18.0
74	rubbed thyme	Poland	steam treated	2014	2.5	6.3	6.0	3.4	18.1
75	rubbed thyme	Poland	steam treated	2015	2.9	6.6	6.8	3.4	19.8
				Average	4.5	9.2	7.8	4.2	25.8
				Median	5.0	9.3	8.4	4.5	27.3
				Observed concentration range	0.81 - 7.6	3.0 - 18.2	1.8 - 11.5	1.3 - 5.7	6.9 - 37.4
				Confidence interval (95%)	3.7 – 5.3	7.9 – 10.5	6.8 – 8.8	3.7 – 4.7	23.1 – 28.4
Black pepper, n=25									
76	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2008	4.0	6.1	5.8	6.6	22.5
77	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	2.3	2.2	1.3	0.60	6.5
78	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	7.2	3.5	2.6	1.4	14.7
79	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	2.9	3.2	3.5	1.8	11.4
80	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	5.5	3.6	4.6	2.0	15.6
81	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	4.3	2.8	5.5	1.9	14.4
82	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	5.4	9.6	4.4	3.6	23.1
83	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	2.3	2.6	4.1	1.6	10.5
84	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	3.4	2.2	3.6	2.3	11.5
85	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	5.2	4.2	5.9	6.5	21.7
86	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	6.4	10.9	4.4	3.5	25.2

Annex 8 continues

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
87	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	1.6	3.3	6.2	2.0	13.0
88	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	5.9	10.0	5.6	2.7	24.2
89	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	0.83	0.79	<0.04	<0.05	1.6
90	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	0.82	3.5	<0.04	4.0	8.3
91	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2009	<0.31	1.1	<0.04	<0.05	1.4
92	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	5.6	4.5	7.2	3.0	20.3
93	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	0.58	0.76	0.51	0.50	2.3
94	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	2.9	7.1	4.4	1.6	15.9
95	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	2.0	1.8	3.3	1.5	8.5
96	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	0.57	6.2	4.9	2.5	14.1
97	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	1.3	2.4	1.4	1.4	6.5
98	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	0.46	2.9	5.5	3.6	12.5
99	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	<0.31	2.4	4.6	2.9	10.1
100	ground black pepper	Blend of Brazil and Vietnam	steam treated, milled	2010	<0.09	8.2	2.4	3.0	13.6
				Average	2.9	4.2	3.7	2.4	13.2
				Median	2.3	3.3	4.4	2.0	13.0
				Observed concentration range	<0.09 - 7.3	0.76 - 10.9	0.51 - 7.2	<0.05 - 6.6	1.4 - 25.2
				Confidence interval (95%)	1.9 – 3.8	3.0 – 5.4	2.8 – 4.5	1.7 – 3.1	10.3 – 16.0
Paprika, n=25									
101	ground hot paprika	Blend of Brazil and China	steam treated	2010	2.7	3.8	2.2	1.1	9.7
102	ground hot paprika	Blend of Brazil and China	steam treated	2010	2.3	3.4	1.7	1.1	8.4
103	ground hot paprika	Blend of Brazil and China	steam treated	2010	2.2	3.6	2.1	1.0	8.9
104	ground sweet paprika	Blend of Brazil and China	steam treated	2010	2.3	3.9	2.2	1.3	9.6
105	ground sweet paprika	Blend of Brazil and China	steam treated	2010	4.1	2.8	3.1	2.0	12.0
106	ground sweet paprika	Blend of Brazil and China	steam treated	2010	2.2	3.6	2.9	1.0	9.6
107	ground sweet paprika	Blend of Brazil and China	steam treated	2010	3.4	5.0	3.4	2.2	14.0

Annex 8 continues

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
108	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.7	2.6	1.7	1.1	7.1
109	ground sweet paprika	Blend of Brazil and China	steam treated	2010	2.0	6.4	2.9	1.6	12.8
110	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.8	2.3	1.9	0.80	6.8
111	ground sweet paprika	Blend of Brazil and China	steam treated	2010	0.61	1.2	0.76	0.33	2.9
112	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.8	1.6	1.3	0.58	5.2
113	ground sweet paprika	Blend of Brazil and China	steam treated	2010	2.0	2.3	1.4	1.0	6.6
114	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.2	2.0	2.1	0.87	6.2
115	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.1	1.3	2.0	0.92	5.4
116	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.5	1.5	2.1	1.2	6.2
117	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.6	4.1	2.3	1.5	9.4
118	ground sweet paprika	Blend of Brazil and China	steam treated	2010	2.6	4.0	1.8	1.2	9.5
119	ground sweet paprika	Blend of Brazil and China	steam treated	2010	2.2	3.7	2.3	0.88	9.0
120	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.7	2.9	2.1	0.90	7.6
121	ground sweet paprika	Blend of Brazil and China	steam treated	2010	1.5	1.4	1.5	0.78	5.1
122	ground sweet paprika	Blend of Brazil and China	steam treated	2010	0.95	2.3	2.2	0.95	6.4
123	ground sweet paprika	Blend of Brazil and China	steam treated	2014	1.2	4.2	1.7	1.1	8.2
124	ground sweet paprika	Blend of Brazil and China	steam treated	2014	1.2	3.9	1.6	1.1	7.9
125	ground hot paprika	Blend of Brazil and China	steam treated	2014	1.4	2.6	1.8	0.70	6.4
				Average	1.9	3.1	2.0	1.1	8.0
				Median	1.8	2.9	2.1	1.0	7.9
				Observed concentration range	0.61 - 4.1	1.2 - 6.4	0.76 - 3.4	0.33 - 2.2	2.9 – 14.0
				Confidence interval (95%)	1.6 – 2.2	2.5 – 3.6	1.8 – 2.3	0.92 – 1.3	7.0 – 9.1
Nutmeg, n=25									
126	ground nutmeg	Indonesia	none	2009	1.2	1.7	0.77	0.64	4.4
127	ground nutmeg	Indonesia	none	2009	0.56	1.6	<0.04	<0.05	2.1
128	ground nutmeg	Indonesia	none	2009	0.35	1.4	<0.04	<0.05	1.8

Annex 8 continues

No.	Name/description	Origin	Pretreatments	Year of production	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
129	ground nutmeg	Indonesia	None	2009	0.53	0.87	<0.04	<0.05	1.4
130	ground nutmeg	Indonesia	None	2009	<0.09	<0.04	<0.04	<0.05	<0.22
131	ground nutmeg	Indonesia	None	2009	0.39	0.64	<0.04	<0.05	1.0
132	ground nutmeg	Indonesia	None	2009	<0.09	<0.04	<0.04	<0.05	<0.22
133	ground nutmeg	Indonesia	None	2009	<0.09	<0.04	<0.04	<0.05	<0.22
134	ground nutmeg	Indonesia	None	2009	<0.09	1.7	<0.04	<0.05	1.7
135	ground nutmeg	Indonesia	None	2009	0.88	2.0	<0.04	<0.05	2.9
136	ground nutmeg	Indonesia	None	2009	<0.09	<0.04	<0.04	<0.05	<0.22
137	ground nutmeg	Indonesia	None	2009	<0.09	<0.04	<0.04	<0.05	<0.22
138	ground nutmeg	Indonesia	None	2009	<0.09	1.8	<0.04	<0.05	1.8
139	ground nutmeg	Indonesia	None	2010	2.7	1.0	0.88	0.64	5.2
140	ground nutmeg	Indonesia	None	2010	0.65	1.9	0.91	0.61	4.1
141	ground nutmeg	Indonesia	None	2010	1.6	3.4	<0.04	<0.05	5.0
142	ground nutmeg	Indonesia	None	2010	1.7	0.80	<0.04	<0.05	2.5
143	ground nutmeg	Indonesia	None	2010	2.0	2.1	<0.04	<0.05	4.1
144	ground nutmeg	Indonesia	None	2010	0.91	2.3	<0.04	<0.05	3.2
145	ground nutmeg	Indonesia	None	2010	2.7	3.1	<0.04	<0.05	5.8
146	ground nutmeg	Indonesia	None	2011	2.6	4.6	<0.04	<0.05	7.3
147	ground nutmeg	Indonesia	None	2011	1.4	1.2	4.8	<0.05	7.3
148	ground nutmeg	Indonesia	None	2014	1.4	0.76	0.74	0.84	3.7
149	ground nutmeg	Indonesia	None	2014	0.48	0.64	0.40	<0.05	1.5
150	ground nutmeg	Indonesia	None	2014	2.4	2.5	<0.04	<0.05	4.9
				Average	0.97	1.4	0.34	0.11	2.9
				Median	0.65	1.4	<0.13	<0.18	2.5
				Observed concentration range	<0.09 - 2.7	<0.04 - 4.6	<0.04 - 4.8	<0.05 - 0.84	1.0 - 7.3
				Confidence interval (95%)	0.59 – 1.4	0.96 – 1.9	<0.04 – 0.74	<0.04 – 0.22	1.9 – 3.8

Annex 9

Average, median, observed concentration range and confidence interval at the confidence level of 95% of four individual EU regulated PAHs and PAH4 in dried herbs and spices (n=150)

Herb/spice	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
Oregano, n=25					
Average	1.4	2.2	0.63	0.38	4.6
Median	1.3	1.2	0.70	0.31	3.2
Observed concentration range	<0.09 - 2.7	<0.04 - 8.4	<0.04 - 1.5	<0.05 - 1.6	1.0 - 13.6
Confidence interval (95%)	1.1 – 1.7	1.2 – 3.3	0.44 – 0.82	0.21 – 0.54	3.3 – 6.0
2009, n=6					
Average	1.1	0.94	0.54	0.30	2.8
Median	0.86	0.71	0.63	0.37	2.8
Observed concentration range	<0.09 - 2.5	<0.04 - 2.2	<0.04 - 1.0	<0.05 - 0.55	1.4 - 4.5
2010, n=7					
Average	0.88	0.81	0.16	0.17	2.0
Median	0.96	0.64	<0.13	<0.18	2.1
Observed concentration range	<0.09 - 1.3	<0.04 - 2.4	<0.04 - 0.61	<0.05 - 0.50	1.0 - 3.2
2012, n=3					
Average	1.3	5.7	0.81	0.34	8.1
Median	1.3	7.5	0.80	0.31	10.1
Observed concentration range	1.0 - 1.5	1.2 - 8.4	0.73 - 0.90	0.28 - 0.42	3.7 - 10.6
2013, n=6					
Average	2.3	4.0	1.1	0.67	8.1
Median	2.4	3.9	1.2	0.73	7.4
Observed concentration range	1.5 - 2.7	0.87 - 8.1	0.70 - 1.5	<0.05 - 1.6	4.8 - 13.6
2014, n=3					
Average	1.5	1.1	0.72	0.46	3.7
Median	1.7	0.91	0.73	0.68	3.8
Observed concentration range	1.0 - 1.8	0.54 - 1.7	0.49 - 0.93	<0.05 - 0.69	3.1 - 4.4
Basil, n=25					
Average	0.95	5.6	3.6	1.5	11.6
Median	0.77	5.3	2.7	1.3	10.7
Observed concentration range	<0.09 - 3.1	1.6 - 9.7	1.6 - 9.3	0.85 - 2.9	6.0 - 18.2
Confidence interval (95%)	0.64 – 1.3	4.7 – 6.6	2.7 – 4.4	1.2 – 1.7	10.2 – 13.1
2010, n=12					
Average	0.88	5.8	3.7	1.4	11.8
Median	0.78	5.4	2.8	1.2	10.6
Observed concentration range	<0.31 - 2.6	1.6 - 9.7	1.6 - 9.1	0.85 - 2.9	6.0 -18.2
2011, n=2					
Average	0.32	6.3	2.2	1.1	9.9
Median	0.32	6.3	2.2	1.1	9.9
Observed concentration range	<0.09 - 0.56	3.4 - 9.2	1.7 - 2.6	0.99 - 1.3	7.4 - 12.5
2013, n=2					
Average	0.46	3.7	6.7	2.2	13.1
Median	0.46	3.7	6.7	2.2	13.1

Annex 9 continues

Herb/spice	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
Observed concentration range	<0.31 - 0.65	3.1 – 4.3	4.0 - 9.3	1.6 - 2.8	10.7 - 15.5
2014, n=9					
Average	1.3	5.6	3.0	1.5	11.4
Median	1.2	5.8	2.7	1.2	11.5
Observed concentration range	0.55 - 3.1	2.0 - 9.2	1.8 - 4.5	0.95 - 2.5	6.0 - 18.2
Thyme, n=25					
Average	4.5	9.2	7.8	4.2	25.8
Median	5.0	9.3	8.4	4.5	27.3
Observed concentration range	0.81 - 7.6	3.0 - 18.2	1.8 - 11.5	1.3 - 5.7	6.9 - 37.4
Confidence interval (95%)	3.7 – 5.3	7.9 – 10.5	6.8 – 8.8	3.7 – 4.7	23.1 – 28.4
2010, n=6					
Average	5.8	9.5	8.6	4.3	28.2
Median	6.2	9.7	9.3	4.5	27.9
Observed concentration range	3.1 - 7.6	4.5 - 12.7	4.9 - 11.5	2.0 - 5.7	18.5 - 37.4
2011, n=5					
Average	4.5	8.5	7.0	4.1	24.1
Median	4.9	10.2	7.5	4.7	27.3
Observed concentration range	0.81 - 6.6	3.0 - 11.1	1.8 - 10.3	1.3 - 5.2	6.9 - 30.9
2014, n=13					
Average	4.1	9.6	7.8	4.2	25.8
Median	5.0	9.3	8.5	4.5	27.3
Observed concentration range	<0.09 - 5.9	5.8 - 18.2	2.4 - 10.0	1.8 - 5.5	17.3 - 32.4
2015, n=1					
Average	2.9	6.6	6.8	3.4	19.8
Median	2.9	6.6	6.8	3.4	19.8
Observed concentration range	2.9	6.6	6.8	3.4	19.8
Black pepper, n=25					
Average	2.9	4.2	3.7	2.4	13.2
Median	2.3	3.3	4.4	2.0	13.0
Observed concentration range	<0.09 - 7.3	0.76 - 10.9	0.51 - 7.2	<0.05 - 6.6	1.4 - 25.2
Confidence interval (95%)	1.9 – 3.8	3.0 – 5.4	2.8 – 4.5	1.7 – 3.1	10.3 – 16.0
2008, n=1					
Average	4.0	6.1	5.8	6.6	22.5
Median	4.0	6.1	5.8	6.6	22.5
Observed concentration range	4.0	6.1	5.8	6.6	22.5
2009, n=15					
Average	3.6	4.2	3.4	2.3	13.5
Median	3.4	3.3	4.1	2.0	13.0
Observed concentration range	0.30 - 7.3	0.79 - 10.9	<0.04 - 6.2	<0.05 - 6.5	1.4 - 25.2
2010, n=9					
Average	1.5	4.0	3.8	2.2	11.5
Median	0.58	2.9	4.4	2.5	12.5
Observed concentration range	<0.09 - 5.6	0.76 - 8.2	0.51 - 7.2	0.50 - 3.6	2.3 - 20.3
Paprika, n=25					
Average	1.9	3.1	2.0	1.1	8.0

Annex 9 continues

Herb/spice	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}
Median	1.8	2.9	2.1	1.0	7.9
Observed concentration range	0.61 - 4.1	1.2 - 6.4	0.76 - 3.4	0.33 - 2.2	2.9 – 14.0
Confidence interval (95%)	1.6 – 2.2	2.5 – 3.6	1.8 – 2.3	0.92 – 1.3	7.0 – 9.1
2010, n=22					
Average	2.0	3.0	2.1	1.1	8.1
Median	1.9	2.9	2.1	1.0	8.0
Observed concentration range	0.61 - 4.1	1.2 - 6.4	0.76 - 3.4	0.33 - 2.2	2.9 – 14.0
2014, n=3					
Average	1.3	3.6	1.7	0.98	7.5
Median	1.2	3.9	1.7	1.1	7.9
Observed concentration range	1.2 - 1.4	2.6 - 4.2	1.6 - 1.8	0.70 - 1.1	6.4 - 8.2
Sweet, n=21					
Average	1.8	3.0	2.1	1.1	8.0
Median	1.7	2.8	2.1	1.0	7.6
Observed concentration range	0.61 - 4.1	1.2 - 6.4	0.76 - 3.4	0.33 - 2.2	2.9 – 14.0
Hot, n=4					
Average	2.1	3.3	1.9	0.98	8.4
Median	2.2	3.5	1.9	1.1	8.7
Observed concentration range	1.4 - 2.7	2.6 - 3.8	1.7 - 2.2	0.70 - 1.1	6.4 - 9.7
Nutmeg, n=25					
Average	0.97	1.4	0.34	0.11	2.9
Median	0.65	1.4	<0.13	<0.18	2.5
Observed concentration range	<0.09 - 2.7	<0.04 - 4.6	<0.04 - 4.8	<0.05 - 0.84	1.0 - 7.3
Confidence interval (95%)	0.59 – 1.4	0.96 – 1.9	0.00 – 0.74	0.00 – 0.22	1.9 – 3.8
2009, n=13					
Average	0.30	0.89	0.06	0.05	1.3
Median	<0.31	0.87	<0.13	<0.18	1.4
Observed concentration range	<0.09 - 1.2	0.64 - 2.0	<0.04 - 0.77	<0.05 - 0.64	1.0 - 4.4
2010, n=7					
Average	1.7	2.1	0.26	0.18	4.3
Median	1.7	2.1	<0.13	<0.18	4.1
Observed concentration range	0.65 - 2.7	0.80 - 3.4	<0.04 - 0.91	<0.05 - 0.64	2.5 - 5.8
2011, n=2					
Average	2.0	2.9	2.4	<0.05	7.3
Median	2.0	2.9	2.4	<0.05	7.3
Observed concentration range	1.4 - 2.6	1.2 - 4.6	<0.04 - 4.8	<0.05	7.3 - 7.3
2014, n=3					
Average	1.4	1.3	0.38	0.28	3.4
Median	1.4	0.76	0.40	<0.18	3.7
Observed concentration range	0.48 - 2.4	0.64 - 2.5	<0.04 - 0.74	<0.05 - 0.84	1.5 - 4.9

Annex 10

The obtained PAHs concentrations in smoked meat samples originating from Latvia, Lithuania and Estonia (n=77)

No.	Type	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}	MOE_{BaP}	MOE_{PAH4}
Latvian smoked meat								
1	Smoked pork lard	0.81	1.06	0.27	0.19	2.32	564516	219733
2	Smoked pork	0.47	0.56	0.19	0.21	1.44	497630	355153
3	Smoked pork	4.87	5.28	1.43	1.73	13.30	60764	38337
4	Smoked pork lard	0.52	0.61	0.18	0.13	1.43	840000	357895
5	Smoked pork lard	35.34	46.26	9.85	10.87	102.3	9663	4985
6	Smoked pork	0.42	0.43	0.16	0.25	1.26	415020	405728
7	Smoked pork belly	1.20	1.42	0.46	0.65	3.73	161538	136729
8	Smoked pork	0.79	1.24	0.30	0.41	2.74	256098	186472
9	Smoked ham	0.68	0.42	0.21	0.13	1.44	783582	353921
10	Smoked pork	4.93	6.50	3.00	5.21	19.64	20161	25967
11	Smoked ham	51.50	72.28	22.52	14.56	160.9	7211	3170
12	Smoked pork	0.25	0.33	0.16	0.14	0.88	736388	578955
13	Smoked pork	6.57	8.14	3.58	3.24	21.53	32394	23684
14	Smoked pork	7.63	7.27	4.68	4.70	24.27	22341	21011
15	Smoked pork	16.91	51.37	8.23	6.21	82.71	16918	6166
16	Smoked pork	123.77	126.28	43.39	32.77	326.2	3204	1563
17	Smoked pork	0.72	0.80	0.32	0.30	2.15	344424	237235
18	Smoked pork	5.40	7.58	2.39	1.87	17.24	56119	29579
19	Smoked pork roulette	0.98	1.14	0.38	0.36	2.85	294762	178882
20	Smoked pork belly	3.22	3.15	1.15	1.53	9.05	68486	56324
21	Smoked pork belly	26.62	26.35	10.07	12.61	75.66	8325	6741
22	Smoked ham	0.39	0.46	0.17	0.15	1.16	699633	438375
23	Smoked pork	0.26	0.27	0.12	0.11	0.77	940361	666324
24	Smoked pork lard	1.08	1.22	1.38	0.69	4.37	153002	116724
25	Smoked pork belly	0.47	0.51	0.56	0.23	1.77	448574	287576
26	Smoked pork	1.39	1.64	0.48	0.56	4.08	186199	125042
27	Smoked sausage	1.03	1.11	0.73	0.45	3.32	233971	153634
28	Smoked sausage	30.29	23.47	13.98	11.35	79.09	9251	6448
29	Smoked pork	3.43	2.85	3.12	2.12	11.53	49421	44233
30	Smoked ham	46.03	45.74	37.18	29.44	158.4	3566	3220
31	Smoked pork belly	7.79	10.05	7.74	3.80	29.38	27621	17359
32	Smoked ham	31.27	40.64	12.24	9.35	93.50	11227	5455
33	Smoked ham	28.21	34.28	13.50	11.13	87.13	9434	5854
34	Smoked pork belly	84.60	105.16	76.45	53.97	320.2	1945	1593
35	Smoked ham	170.66	214.78	125.98	116.12	627.5	904	813
36	Smoked pork	1.63	2.10	1.98	1.71	7.42	61442	68748
37	Smoked pork	22.06	26.92	11.20	9.88	70.05	10628	7280
38	Smoked ham	25.47	31.09	11.58	10.79	78.94	9730	6461
39	Smoked pork	8.00	9.79	4.27	3.54	25.60	29664	19920
40	Smoked pork	6.40	7.87	4.19	2.44	20.90	43044	24396
41	Smoked chicken	36.59	39.44	14.59	12.55	103.2	8369	4944
42	Smoked pork belly	1.61	1.39	1.83	1.39	6.23	75536	81890

Annex 10 continues

No.	Type	BaA, µg kg^{-1}	Chr, µg kg^{-1}	BbF, µg kg^{-1}	BaP, µg kg^{-1}	PAH4, µg kg^{-1}	MOE_{BaP}	MOE_{PAH4}
43	Smoked pork	0.76	0.83	0.29	0.42	2.29	251252	222283
44	Smoked pork cheeks	16.28	20.73	11.38	9.50	57.90	11057	8809
45	Smoked pork	1.54	1.70	3.12	6.87	13.23	15286	38557
46	Smoked ham	0.93	0.94	0.55	0.71	3.14	147220	162379
47	Smoked pork lard	1.94	2.42	0.55	0.78	5.69	135351	89623
48	Smoked pork	4.89	5.21	8.81	10.61	29.52	9895	17276
49	Smoked pork	14.19	16.07	6.92	5.92	43.10	17743	11833
50	Smoked pork belly	1.98	2.04	2.53	2.99	9.55	35096	53391
51	Smoked pork	0.75	0.95	0.72	0.91	3.33	115442	153195
52	Smoked pork belly	8.06	9.11	3.60	2.99	23.77	35124	21457
	Lithuanian smoked meat							
1	Cold smoked sausage	2.26	4.50	4.42	3.89	15.08	26960	33823
2	Smoked pork	3.77	4.80	4.03	2.51	15.11	41840	33761
3	Cold smoked sausage	1.08	2.54	2.14	1.33	7.08	79242	72014
4	Cold smoked sausage	1.03	2.82	2.31	1.69	7.84	62256	65035
5	Cold smoked sausage	3.65	5.72	4.09	2.29	15.74	45936	32392
6	Cold smoked sausage	1.89	2.45	0.62	0.71	5.66	148010	90081
7	Cold smoked sausage	0.11	0.17	0.08	0.05	0.42	1982549	1201862
8	Cold smoked sausage	0.29	0.28	0.14	0.11	0.81	970681	626252
9	Smoked ham	19.52	21.07	8.52	7.06	56.17	14876	9080
10	Smoked ham	0.75	0.85	0.36	0.34	2.29	312038	222304
11	Smoked pork	4.13	4.47	2.40	2.53	13.53	41495	37684
12	Cold smoked sausage	0.87	0.89	0.49	0.42	2.66	251476	191567
13	Cold smoked sausage	3.03	3.86	1.09	1.25	9.23	84055	55252
14	Cold smoked sausage	2.40	3.12	0.99	0.73	7.24	143920	70431
15	Cold smoked sausage	0.53	0.49	0.28	0.23	1.52	464593	334761
16	Cold smoked sausage	0.36	0.38	0.23	0.16	1.12	661830	453635
17	Smoked pork	0.10	0.17	0.13	0.09	0.49	1157733	1032434
	Estonian smoked meat							
1	Smoked ham	0.12	0.13	0.09	0.09	0.42	1216948	1206353
2	Smoked ham	0.23	0.38	0.13	0.11	0.86	988115	595406
3	Smoked pork	0.22	0.33	0.13	0.09	0.77	1201235	658971
4	Smoked pork	0.93	1.17	0.34	0.25	2.69	427883	189664
5	Smoked pork	0.29	0.42	0.14	0.11	0.96	976803	533597
6	Smoked pork	5.79	7.85	4.22	3.93	21.79	26745	23410
7	Smoked ham	2.11	3.06	1.35	1.07	7.59	97893	67214
8	Smoked ham	59.11	74.74	24.63	16.79	175.3	6255	2910

The research for doctoral thesis "**Development and application of sensitive mass spectrometric methods for the effective determination of polycyclic aromatic hydrocarbons in food**" was carried out at the Institute of Food Safety, Animal Health and Environment "BIOR" from 2015 to 2018.

I hereby confirm that I have written the doctoral thesis independently, that I have not used other sources or facilities that the ones mentioned and that the submitted electronic copy of the work is identical to printed version.

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