Integrated Masters in Bioengineering

Pine needles as biosamplers for emergent pollutants

Master's Thesis

of

Sara Fernandes Ramos

Developed within the discipline of Dissertation

conducted at

Laboratory for Process, Environmental and Energy Engineering,

Faculty of Engineering, University of Porto

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Universidade do Porto
Faculdade de Engenharia
FEUP

Department of Chemical Engineering

July 2013

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(Professor Lúcia Santos)

Beyond the door

There's peace

I'm sure

And I know there'll be no more

Tears in Heaven

To my grandmother, Alice Ramos

Thank you for everything

Acknowledgments

First I would like to thank LEPAE (Laboratory for Process, Environmental and Energy Engineering) and the Department of Chemical Engineering for providing the materials, equipment and facilities.

I take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this project.

 I would like to show my greatest appreciation to Professor Lúcia Santos. I can't say thank you enough for her tremendous support and help since the begging of this work.

I would like to express my gratitude to my supervisor Eng. José Avelino for the useful comments, remarks and engagement through the learning process of this master thesis. Without his encouragement and guidance this project would not have materialized.

Furthermore I would like to thank Vera Homem for introducing me to the topic, for the support and guidance on the way.

Besides my advisors, I would like to show my gratitude to Professor Arminda Alves, for the support and productive discussion.

I would like to thank Serafim Pereira and Maria de Fátima Ferreira for all the assistance with the rotary evaporator as well as for the patience and care demonstrated.

I wish to thank MIA 201 Lab Group: Mónica Santos, Raquel Sousa, Rúben Ribeiro, Leandro Figueiredo, Marzieh Moeenfard and Eduardo Silva for ensuring I was constantly engaged and entertained along the way, especially Mariana Mendes for allowing me to use some of her results.

The support outside the faculty was also essential so I would also like to thank my parents and two young sisters. They were always supporting and encouraging me with their best wishes. My friends were also essential during this semester, especially Inês Miranda for her constant support in the harder moments and Maria Mónica and Carlitos for their constant affirmation of positive energies, can't thank you enough. Also thank you to Francisco and Mané Peixoto for being constantly available to help me. To Professor Karl-Heinz van Pée and Dr. Eugenio Patallo thank you for the knowledge and laboratory skills I gained working with you.

Abstract

Pine needles have been widely used as passive biosamplers of emergent semivolatile pollutants due to their waxy outer cuticle, which favours the entrapment of lipophilic contaminants.

In line with previous research, this work intends to develop a multiresidue analytical method for POPs and emergent pollutants biomonitoring. Four different chemical classes were analysed: brominated flame retardants (BFRs), polychlorinated biphenyls (PCBs), polynuclear aromatic hydrocarbons (PAHs) and synthetic fragrances (musks). While the first three are better known chemicals, musks only recently have raised concern, due to their high consumption and release into the environment, their bioaccumulation and their endocrine disrupting potential. Musks have been detected in several environmental matrices (water, air), as well as biota (aquatic and terrestrial animals, humans), but no detection in vegetation has been attempted.

Two solid phase extraction cleanup methods approaches (glass columns or cartridges) eventually followed by gel permeation chromatography (GPC) were tested and compared. As sorbents florisil or neutral alumina were employed eluting with dichloromethane/hexane (DCM/Hex) eventually followed by DCM. GPC columns containing Biobeads S-X3 using dichloromethane/hexane 1:1 as eluent were tested as final cleanup step. Analysis was performed by GC-EI-MS using two different methods (one for BFRs+PCBs and the other for PAHs+Musks) on a 60 m CP-Sil 8 CB column.The final cleanup conditions used were SPE alumina glass columns eluted with 50 mL DCM/Hex (1:1) followed by GPC with 6 g of Biobeads eluted with 40 mL DCM/Hex (1:1). Final extract was diluted in 100 μL hexane and analysed in GC-MS.

LODs varied between 0.2 and 3.8 μg/L for PAHs with average recoveries of 65%, 0.4 and 4.5 μg/L for musks with average recoveries of 79%, 0.1 and 18.8 μg/L for PCBs with average recoveries of 48% and 0.01 and 12.5 for BFRs with average recoveries of 77%.

The concentrations of individual PCB congeners ranged between 0.04 and 2.11 ng/g with the higher concentration for PCB congener 77 (2.11 ng/g), 153 (0.75 ng/g) and 189 (0.56 ng/g). BFRs concentrations in pine needles are between 0.02 and 5.56 ng/g, where BDE 28 was the congener with the highest concentration (5.56 ng/g), followed by BDE 85, 99 and 100. The total concentrations of 16 PAHs ranged from 0.02 ng/g to 291.02 ng/g with higher concentrations for phenanthrene (291.02 ng/g), pyrene (158.27 ng/g), benzo[a]anthracene (74.42 ng/g) and fluorene (51.64 ng/g). Musks were found in concentrations ranging 0.03 and 12.15 ng/g with higher concentrations for cashmeran (12.15 ng/g), galaxolide (8.06 ng/g), phantolide (5.78 ng/g) and tonalide (1.26 ng/g).

Resumo

As agulhas de pinheiro têm sido amplamente utilizadas como biomarcadores passivos de poluentes emergentes, devido à sua cutícula cerosa exterior, o que favorece a retenção dos contaminantes lipofílicos.

De acordo com trabalhos anteriores, com este estudo pretendeu-se desenvolver um método analítico multiresíduo para monitorizar poluentes emergentes. Foram analisadas quatro classes químicas diferentes: retardadores de chamas bromados (BFRs), bifenilos policlorados (PCBs), hidrocarbonetos aromáticos policíclicos (PAHs) e almíscares (Musks). As três primeiras classes já foram bastante estudadas, contudo, só recentemente os musks começaram a gerar alguma preocupação na comunidade científica devido ao seu elevado consumo e consequente libertação no meio ambiente. Os musks foram detetados em várias matrizes ambientais (água e ar), bem como na biota (animais terrestres e aquáticos, seres humanos), contudo, não há publicações da sua deteção em vegetação.

Neste trabalho foram testados e comparados dois métodos de extração em fase sólida (SPE) (com coluna de vidro e cartuchos), seguido de uma cromatografia de exclusão de tamanho (GPC). Os sorbentes utilizados em SPE foram florisil e alumina neutra e a eluição dos compostos foi efetuada com diclorometano/hexano (DCM/Hex), sendo em alguns casos seguido por DCM. As colunas de GPC (Biobeads S-X3), testadas como passo final de *cleanup*, foram eluídas utilizando DCM/Hex (1:1). A análise foi feita por GC-EI-MS, utilizando dois métodos diferentes (um para BFR e PCB e o outro para os PHA e Musks) numa coluna CP-Sil 8 CB de 60 m. As condições finais de limpeza utilizadas foram colunas de vidro com alumina (SPE) e eluição com 50 mL de DCM/Hex (1:1), seguido por GPC, com 6 g de Biobeads eluídas com 40 mL de DCM/Hex (1:1). O extrato final foi diluído em 100 μl de hexano e analisado por GC-MS.

Os limites de deteção (LOD) variam entre 0,2 e 3,8 mg/L para os PAHs com recuperações médias de 65%, 0,4 e 4,5 mg/L para os musks com recuperações médias de 79%, 0,1 e 18.8 mg/L para os PCBs com recuperações médias de 48% e 0,01 e 12,5 mg/L para os BFRs com recuperações médias de 77%.

As concentrações de congéneres de PCBs variam entre 0,04 e 2,11 ng/g, com a maior concentração dos congéneres 77 (2,11 ng/g), 153 (0,75 ng/g) e 189 (0,56 ng/g). As concentrações dos BFR em agulhas de pinheiro são entre 0,02 e 5,56 ng/g em que BDE 28 tem a concentração mais elevada (5,56 ng/g), seguido pelos BDE 85, 99 e 100. As concentrações totais de 16 PAHs variam de 0,02 ng/g a 291,02 ng/g. Os musks foram encontrados em concentrações entre 0,03 e 12,15 ng/g, com concentrações mais elevadas para o Cashmeran (12,15 ng/g), Galaxolide (8,06 ng/g), Phantolide (5,78 ng/g) e Tonalide (1,26 ng/g).

Palavras-chave: Agulhas de pinheiro *Pinus pinaster*, PAHs, PCBs, BFRs, Musks, SPE, GPC, GC-MS.

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Notation and Glossary

ACN	Acetonitrile
Al_2O_3	Alumina
ASE	Accelerated solvent extraction
BC	Before Christ
BFRs	Brominated Flame Retardants
CLRTAP	Convention on Long-range Transboundary Air Pollution
CO_2	Carbon dioxide
DCM	Dichloromethane
d-PAHs	Deuterated PAHs
dw	Dry weight
EC	European Community
EU	European Union
GC-MS	Gas chromatography–mass spectrometry
GPC	Gel Permeation Chromatography
h	Hour
ha	Hectare
HBB	Hexabromobenzene
HBCD	Hexabromocyclododecane
Hex	Hexane
k_H	Henry's law constant
LEPAE	Laboratory for Process, Environmental and Energy Engineering
log K_{ow}	Octanol–water partition coefficient
log K_{oc}	Octanol–carbon partition coefficient
log K_{oa}	Octanol–air partition coefficient
MAE	Microwave assisted extraction
MarsX	Microwave accelerated reaction system for extraction
mbar	Milibar
Mg_2SiO_3	Florisil
min	Minute
NMCs	Nitro musk compounds
PAHs	Polycyclic Aromatic Hydrocarbons
PBBs	Polybrominated biphenyls
PBDEs	Polybrominated diphenyl ethers
PBEB	Pentabromoethylbenzene

PBT	Polybutylene terephthalate
PCBs	Polychlorinated Biphenyls
PeBDE	Pentabromodiphenyl ether
PLE	Pressurized liquid extraction
PMCs	Polycyclic musk compounds
POPs	Persistent Organic Pollutants
PVC	Polyvinyl chloride
REACH	Registration, Evaluation and Authorization of Chemicals
SC	Stockholm Convention
SFE	Supercritical fluid extraction
SiO_2	Silica
Musks	Synthetic Musks
SD	Standard Deviation
SPE	Solid Phase Extraction
S-PLE	Selective pressurized liquid extraction
TBBPA	Tetrabromobisphenol A
UNECE	United Nations Economic Commission for Europe
UNEP	United Nations Environmental Program
US	United States
US EPA	United States Environmental Protection Agency
USE	Ultrasonic Extraction
UV	Ultraviolet
ww	Wet weight

1 Introduction

1.1 Thesis Organization

This thesis is organized in several sections. In Section 1 an Introduction to the project is presented. Here are presented the studied compound classes, a general overview of the pine needles morphological structure and use and biosamplers as well as the basics of the most used cleanup methodologies in pine needles. Section 2 is the State of the Art, where all the advances in PAHs, PCBs and BFRs monitoring and extraction and quantitative analysis methods in pine needles are presented. Section 3 is the Technical Description of the work conducted, where the methods and material used are described. Results and Discussion are presented in Section 4, which is one of the most important sections of this thesis, as it is where the results obtained from the experiments conducted are presented and discussed. The main conclusions of this project are then presented in Section 5, followed by a general description of the limitations as well as some suggestions for future work to be performed under the scope of this project. In the appendix additional information can be found.

1.2 Background and Project Presentation

In the past few years there's been a growing concern about environmental pollutants since they can affect our everyday life. Semi volatile compounds, such as polycyclic aromatic hydrocarbons (PAHs), synthetic musks, polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs), due to their volatility can be transported throughout the world becoming a concern to countries that do not produce or even use such compounds. In order to monitor these pollutants, vegetation such as lichens, mosses, pine needles, among others, have been used as biomonitors.

The main purpose of this work is to develop and validate an extraction and cleanup method in order to analyse compounds of four different chemical classes (PAHs, PCBs, BFRs and Musks) using pine needles as matrix. Pine needles have been used to monitor several compound classes and multi residue methods have already been developed. However, synthetic musks have never been studied in pine needles, or any other type of vegetation and our focus is to be able to extract and analyse the main compounds from this class.

In this project, the studied compound classes will be presented showing their relevance for this study, their properties and what has been done so far in pine needles, in the Introduction and State of the Art section. The analytical methods used will be presented and the guide line used in the experimental execution of this work will be exposed in the Results as Discussion section. Further studies that weren't able to be performed will also be presented in the 'Future Work' section.

1.2.1   Emergent pollutants in the Environment

Since the Second World War scientists became aware that some emergent chemical compounds were able to persist in the environment for a long time, migrate through the entire world and accumulate to harmful levels both for human and wildlife (El-Shahawi et al., 2010).

Their physico-chemical properties allow them to occur either in vapour phase or adsorbed on atmospheric particles (dust) promoting their long-range migration through the atmosphere. Some of these properties are: very low water solubility, high lipid solubility, high toxicity, semi-volatility and long half-life in the environment. Chemicals with these properties are often called Persistent Organic Pollutants (POPs) (Rigét et al., 2010; Breivik et al., 2004; Lal et al., 2013). Because POPs are present either in vapour phase or dust they are easily transported to the ground either by deposition or by the rain. Hence, they may volatilize from soils, vegetation and water bodies into the atmosphere and because of their resistance to breakdown reactions in air they may travel long distances before being re-deposited. The cycle of volatilization and deposition may be repeated many times, allowing POPs to accumulate in areas far away from where they were used or emitted (Vallack et al., 1998; Wania and Mackay, 1999; Gon et al., 2007; Kallenborn, 2006; Tang, 2013). Although they have low water solubility, their high lipid solubility (solubility in oils, fats and liquid fuels) allows them to accumulate and biomagnify in fatty tissues of animals and humans, sewage sludge, soil matrix and other organic compounds present in water or on the ground. This implies that the more solid and liquid organic components are present in water, the higher is the probability of POP content (Wei et al., 2007; Polder et al., 2010; Passuello et al., 2010; Pozo et al., 2011; Nie et al., 2012).

There are many thousands of pollutants from different chemical classes. Among them are compounds such as organochlorine pesticides (OPs), industrial chemicals such as polychlorinated biphenyls (PCBs) (Pozo et al., 2011), phthalate esters, polybrominated and phosphate flame retardants (BFRs/PFRs), musks, biocides (Garcia-Jares et al., 2009; Muir, 2013), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) (Jones and Voogt, 1999; Wania and Mackay, 1999), hexachlorocyclohexane isomers (HCHs) and brominated diphenyl ethers (BDEs) (Zimmer et al., 2011).

The Industrial Revolution between the 18th and 19th century caused a transition from manual to machine production and new chemical manufacturing which allowed a new living style. This led to an enormous increase in the power generation, use of cars and aircrafts and agriculture industrialisation which has increased dramatically the number of pollutants in the environment. However, despite their deliberate manufacture, data on the total amount entering the environment and regional/global usage patterns is highly uncertain and often poorly known (Breulmann et al., 2002; Lohmann et al., 2007).

Unintentional POPs have been produced by numerous industrial activities throughout the years and issues associated with their formation, emission, transport, degradation and environmental risk have been widely investigated in the past decade (Nie et al., 2012). It is thought that more than 90% of

the human exposure to POPs is caused by consumption of contaminated food, being animal or fish derived products the main contributors to this exposure. It is therefore important to understand the different pathways of POPs on their way from "soil to table" (Porta et al., 2008; Polder et al., 2010). Also many indoor environments can act as concentrators of emissions from plastics, paints, and other building materials and inadequate ventilation coupled with the slow indoor degradation processes may increase indoor pollution levels. Hence, inhalation of indoor air is potentially another important exposure pathway to many pollutants (Garcia-Jares et al., 2009).

Oceans and large lakes represent major sinks for POPs and because of the previously mentioned volatilization cycle they can also represent a source of POP emissions since these compounds volatilize when air temperatures rise and therefore can be transported through the air to other places (Kallenborn, 2006; Rigét et al., 2010; Muir and Lohnmann, 2013; Koenig et al., 2013). Ocean currents can also be as important as atmospheric long range transport and responsible for POP deposition into coastal waters. Marginal seas are huge important regional issues. Examples of especially affected marine regions are Europe's marginal seas such as the Baltic, North, Mediterranean and Black Sea (Wania et al., 1998). Mountainous areas with high extensions of forests also play an important role in POPs concentration and transport in the environment because of the lipophilic nature of foliage (Belis et al., 2009).

Since 30 years ago there's evidence of long-range transport of these pollutants to remote regions where they have never been used or produced. Because of the consequent threats they pose to the global environment, the international community has, on several occasions, called for urgent global actions to reduce and eliminate releases of these chemicals. Several global and regional conventions have been signed with that purpose (Vallack et al., 1998; Lamon et al., 2009; Polder et al., 2010; Muir, 2013). The UNECE Convention on Long-range Transboundary Air Pollution (CLRTAP) has produced in 1998 a specific POP protocol, which entered into force on 23 October 2003 with the objective to control, reduce or eliminate discharges, emissions and losses of persistent organic pollutants (Zhang et al., 2005; Wei et al., 2007; Gon et al., 2007). The Registration, Evaluation and Authorization of Chemicals (REACH) system was created in the European Union (EU) in response to the ever-increasing concern about the production and use of many chemical substances lacking information on their environmental and health effects. REACH applies to all chemicals, not only those used in industrial processes but also those used in the day-to-day life, for example in cleaning products, paints, as well as in articles such as furniture, clothes or electrical appliances (Garcia-Jares et al., 2009). The Stockholm Convention (SC) on POPs coordinated through the United Nations Environmental Program (UNEP) is a global treaty intended to reduce or eliminate the use, discharge and emission of these compounds. It was adopted in May 2001 at a Conference of Plenipotentiaries in Stockholm, Sweden, and came into force three years later. In 2012, the SC targeted 22 POPs that are subject to elimination or restriction in production and use (Zhang et al.,

2005; Lohmann et al., 2007; Polder et al.; 2010; Pozo et al., 2011; Tang, 2013). Data regarding this subject is available online in the Stockholm Convention website (POPs convention, 2008).

Despite the fact that the use of these chemicals has either been phased-out or restricted, they are still found in the environment at levels that may cause negative effects to the health of individual animals and in some cases severe impacts on animal populations (Rigét et al., 2010). Exposure to POPs has been suggested to cause problems such as impaired neuronal, immune, and endocrine development and function, increased susceptibility to metabolic and cardiovascular diseases, cancer, birth defects and learning disabilities (Jones and Voogt, 1999; El-Shahawi, 2010; Zimmer et al., 2011).

In this work four classes of emergent pollutants were studied: polycyclic aromatic hydrocarbons (PAHs), synthetic musks, polychlorinated biphenyls (PCBs) and brominated flame retardants (BFRs). These classes were chosen based on their interest to the group were this thesis was developed, the Laboratory for Process, Environmental and Energy Engineering (LEPAE).

1.2.1.1   Polynuclear aromatic hydrocarbons (PAHs)

Polynuclear aromatic hydrocarbons (PAHs) are a complex class of organic compounds composed of two or more aromatic rings (Cecinato et al. 1999; Anyakora et al., 2005). Environmentally, the most significant PAHs range between naphthalene ($C_{10}H_8$) and coronene ($C_{24}H_{12}$). The differences between PAHs are in the number and position of aromatic rings, which leads to diverse physical and chemical properties among them (Chang-Chien, 1998; Manoli and Samara, 1999; Aue et al., 2000; ATSDR-PAHs, 1995).

They occur as colourless, white/pale yellow solids with low solubility in water, high melting and boiling points and low vapour pressure (Haritash and Kaushik, 2009). Table A.1 (Appendix A) presents molecular formulas, molecular mass, molecular structures and other properties for the 14 selected PAHs. It's possible to see that PAHs with higher molecular weight (4 or more aromatic rings) are less volatile, less water-soluble and more lipophilic than the lower molecular weight, with 2 or 3 aromatic rings. These differences are important in the PAHs distribution in the environmental media. (Anyakora et al., 2005)

PAHs are introduced in the environment by natural and anthropogenic processes. The main natural sources are volcanic eruptions, forest and prairie fires and biosynthesis (sediment diagenesis, tar pits and biological conversion of biogenic precursors). Anthropogenic sources include combustion of fossil fuels, waste incineration, coke and asphalt production, oil refining, aluminum production, combustion or pyrolysis of synthetic polymers and many other industrial activities. (Smith and Harrison, 1996; Tomaniová et al., 1998; Wilhelm et al., 2000; Anyakora et al., 2005)

Although PAHs' source strength in urban/industrial sites, they occur in rural and remote areas in relatively high concentrations due to their ability to be transported over long distances in gaseous and particulate phases and because they seem to be resistant to degradation on atmospheric particulates. Usually 2 to 4 ring PAHs occur in the gaseous phases, whereas PAHs with more rings appear almost exclusively in the particulate phase (Manoli and Samara, 1999; Hien et al., 2007).

PAHs are included in the European Community (EC) and United States Environmental Protection Agency (US EPA) priority list with the environmentally hazardous organic compounds classification because of its known or suspected carcinogenicity. Benzo(a)pyrene, a widely reported 5-ring PAH is an example of a compound known for its carcinogenic potency (Chang-Chien, 1998; Cecinato et al., 1999; Manoli and Samara, 1999; Anyakora et al., 2005).

PAHs' mutagenic and carcinogenic activity has been studied for a long time. Their biological activity mechanism is still unknown but is thought to involve oxidation and opening of the epoxide ring to carbocations that are related to the protonation of PAH structures (Ren et al., 1998; Cecinato et al., 1999; Aue et al., 2000; Anyakora et al., 2005). Because of that, they're studied in several environmental matrices including air, water, soil/sediments and plant tissue (Tomaniová et al., 1998).

Humans are exposed to PAHs via air and drinking water. However a major exposure route is by food. Food contamination with PAHs happens due to production practices like heat processing of meat and dairy products, such as charcoal grilling, roasting and smoking (Wenzl et al., 2006).

The 16 EPA priority PAHs are: Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene and Benzo[ghi]perylene (Manoli and Samara, 1999; Anyakora et al., 2005) and these are the ones that were studied in this project, except Benzo(a)anthracene and Indeno[1,2,3-cd]pyrene.

1.2.1.2   Synthetic Musks

Synthetic musks (Musks) are a new type of emerging contaminants. They are widely used as fragrances fixatives in a wide range of scented consumer goods like perfumes, lotions, sunscreens, deodorants, laundry detergents and air fresheners (Garcia-Jares et al., 2009; Hu et al., 2011).

Before the appearance of synthetic musks, natural musks have long been used as pharmaceutical ingredients and odorants, but also in religious ceremonies (Ravi et al., 2001). They were also employed as sedatives and stimulants to cure a variety of diseases in East Asian countries (Yang et al., 2003). Natural musks of animal origin were extracted from the exocrine odour glands of deer and consisted in macrocyclic ketones, lactones or alcohols and pyridine derivatives such as muscone,

civetone, dihydrocivetone and exaltone. Vegetal originated natural musks are exaltolide from angelica root and ambrettolide from ambrette seeds. The main problem with these natural musks is that they are found in very small quantities and as a complex mixture, therefore they are expensive to obtain (Ravi et al., 2001; Schmeiser et al., 2001).

Because of the perfume industry's need for large quantities of musks, but also due to ethical reasons, synthetic musks were developed offering the advantage of being cheaper and easier to obtain (Emig et al., 1996). Synthetic musks comprise four chemical groups, nitro, polycyclic, macrocyclic and alicyclic musks (Bester, 2009).

Nitro musk compounds (NMCs) were first synthesised at the end of the 19th century as substitutes for natural musks (Lee et al., 2010). They are synthetic di- and trinitro benzene derivatives with typical fragrance properties similar to those found in animals and plants, although structurally different from the natural ones (Schmeiser et al., 2001). They are widely used in the industrial production of numerous products because of the low production costs (Rimkus, 1999). The nitro musks ambrette, tibetene and moskene were banned in the European Union (Schmeiser et al., 2001) while musks xylene was restricted to 1% in perfumes, 0.4% in *eau de toilette* and 0.03% in other cosmetic products and musk ketone was restricted to 1.4% in perfumes, 0.56% in *eau de toilette* and 0.042% in other cosmetic products (Decree-Law Nº 189/2008).

Polycyclic musk compounds (PMCs) were developed in the 1950s and 60s. Since then, these synthetic musk compounds have been extensively used as fragrance ingredients in consumer products (Lee et al., 2010). The polycyclic musks are indane and tetraline derivatives, mainly substituted by methyl groups. These artificial musks do not occur in the nature, and there is no chemical or structural relationship with the natural musk compounds. Their industrial synthesis is relatively complex and, therefore, in comparison to nitro musks, are more expensive. Nevertheless, they are considered to be essential ingredients in fragrances for numerous consumer products (Rimkus, 1999; Bester, 2009). Tonalide and Galaxolide are the two largest volume products in this class, representing about 95% of the EU market and 90% of the US market for all polycyclic musks. Their use as an ingredient of cosmetics and detergents significantly decreased during the second half of the nineties in Europe due to negative publicity (Stevens, et al., 2003; HERA, 2004).

Macrocyclic musks are derived from natural odourants (Abramsson-Zetterberg and Slanina, 2002) and are large ringed (comprising often 10–15 carbons) ketones or lactones. The great cost of macrocyclic musks preparation currently limits their widescale usage. (Sumner et al., 2010)

Alicyclic musks were first introduced in 1975 with the trisubstituted cyclopentene derivative Cyclomusk. Structurally they are modified akyl esters, very diferent from the other musk classes (Eh, 2004).

In this project nitro and polycyclic musks were studied. Their properties, both physical and chemical, are similar to hydrophobic and semi volatile organic pollutants that are known to be persistent and biomagnify through the food chain (Garcia-Jares et al., 2009). Due to their high octanol–water partition coefficient (log K_{ow} values of 5.4–6.3) as can be seen in Table A.2 (Appendix A), musks are easily absorbed by particular organic matters and accumulate in the sediment. These pollutants can also be bio-accumulated in organisms and then threaten the aquatic ecosystems and food safety, due to their lipophilicity, persistence and biological effects (Guo et al., 2010; Hu et al., 2011).

So far it is known that polycyclic musks, have a potential as endocrine disrupters and can cause adverse chronic effects on wildlife due to their high bioaccumulation rates and persistency. However, further studies need to be performed (Paasivirta et al., 2002). Air quality can be affected by these compounds and evidence shows that they may play an important role in respiratory diseases. For these and many other reasons environmental issues have been raised (Garcia-Jares et al., 2009; Guo et al., 2010).

1.2.1.3   Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls are a group of organic chemicals that are persistent environmental contaminants (POPs). They can be odourless, mildly aromatic solids or oily liquids (US EPA- water, 2000; Gallagher et al., 2013). As can be seen in Figure 1, structurally they consist of two connected benzene rings carrying one to ten chlorine substitutes (Ross, 2004).
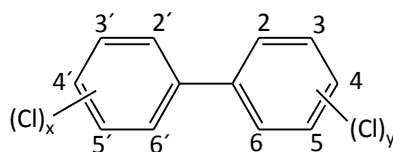
x + y = 1-10
Figure 1 - Basic chemical structure of PCBs

There are 209 so-called congeners that are structurally related and differ in number and/or position of the chlorines (Seeger et al., 1997). PCBs are thermally and chemically stable, non-flammable, with high boiling point, relatively long half-life, exceptional dielectric strength and high resistance to degradation at high temperatures. They're also recalcitrant to biodegradation (Clarke et al., 2010). Their highly lipophilicity and chemically stability allows them to partition into soil and sediment, bioconcentrate from water to aquatic animals and accumulate in the food chain (Patandin et al., 1999; Baba and Katayama, 2007; Hopf et al., 2009).

There are no natural sources of PCBs. They are released to the environment during their manufacture, use, and disposal as a mixture of congeners and impurities (US EPA-Polychlorinated Biphenyls, 2011). PCBs were manufactured in the United States from 1929 to 1977 primarily for

industrial use because of their remarkable electrical insulating properties and flame resistance and soon gained widespread use as insulators and coolants in transformers and other electrical equipment, until they were banned due to their persistence in the environment and living organisms (Everett et al., 2008; US EPA-PolychlorinatedBiphenyls, 2011). PCBs replaced combustible insulating fluids and thereby reduced the risk of fires in office buildings, hospitals, factories, and schools (US EPA- water, 2000; Furukawa and Fujihara, 2008; Hopf et al., 2009). They also were used in the manufacture of a wide variety of common products such as plastics, adhesives, paints and varnishes, carbonless copying paper, newsprint, way extenders, de-dusting agents, pesticide extenders, inks, lubricants, cutting oils, fluorescent light ballasts and caulking compounds (US EPA- water, 2000; Ross, 2004).

PCBs production are estimated to be between 1 to 1.2 million tons and it's thought that about one third of this quantity is still circulating in the environment (Furukawa and Fujihara, 2008; Clarke et al., 2010). Because of the amount of these compounds still circulating in the environment, PCBs have become serious global environmental contaminants. Their release into the environment is possible from hazardous waste sites, runoff from landfills, discharge of waste chemicals, illegal/improper disposal of industrial wastes and consumer products, leaks from old electrical transformers containing PCBs or burning of some wastes in incinerators (US EPA-Polychlorinated Biphenyls, 2011).

Once released into the aquatic environment, they can be adsorbed onto suspended particles or taken up and concentrated by aquatic organisms where they can bio-accumulate and biomagnify to about 200–70,000 times along the food chain and pose potential hazards to other organisms and human consumers (Furukawa and Fujihara, 2008; Dodoo et al., 2012). Some PCBs congeners and derivatives are as toxic as chlorinated dioxins and endocrine disrupters. However, they are converted from highly toxic PCBs to less toxic ones by anaerobic dechlorination activities of microorganisms (Baba and Katayama, 2007; Furukawa and Fujihara, 2008).

General population can be exposed to PCBs by contact with contaminated ground water, food stored in silos with PCB-coated interiors, fish consumption from contaminated waterways, caulking materials used in buildings built or refurbished prior to 1977, floor refinishing compounds, incineration of municipal waste and volatilization from landfills (Patandin et al., 1999; Hopf et al., 2009). PCBs effects on human health have been deeply studied. They enter the body via the lungs, the gastrointestinal tract or the skin. Eventually, they accumulate in fatty tissues or organs such as liver, kidneys, adrenal glands, brain, heart and skin where they can wreak havoc in diverse ways (Dodoo et al., 2012). Conditions like thyroid toxicity, effects on the immune system, hypertension, high blood pressure, endothelial dysfunction, reproductive impairment, chloroacne, skin discoloration, liver dysfunction, nervous and endocrine systems and cancer are the most common ones (US EPA- water, 2000; Ross, 2004; Everett et al., 2008).

In this study, out of the 209 PCB congeners, only 11 PCB were studied: -28, -77, -81, -105, -114, -138, -153, -156, -157, -189 and -209. Physico-chemical properties of these compounds are presented in Table A.3 (Appendix A). These congeners were selected due to their occurrence in the environment and presented in the State of the Art section. They have high octanol/water partitioning coefficients (log K_{ow}>4) and exhibit high affinity to adsorb onto lipophilic organic materials.

1.2.1.4   Brominated Flame Retardants (BFRs)

The idea of flame retardant materials dates back to about 450 BC, when the Egyptians used alum to reduce the flammability of wood. The Romans (about 200 BC) used a mixture of alum and vinegar to reduce the combustibility of wood. Nowadays, there are more than 175 chemicals classified as flame retardants (Alaee et al., 2003).

Technical flame retardant products contain brominated organic compounds including polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA) and polybrominated biphenyls (PBBs). In Figure 2 are shown these molecules structures (Wit, 2002; Garcia-Jones et al., 2009).
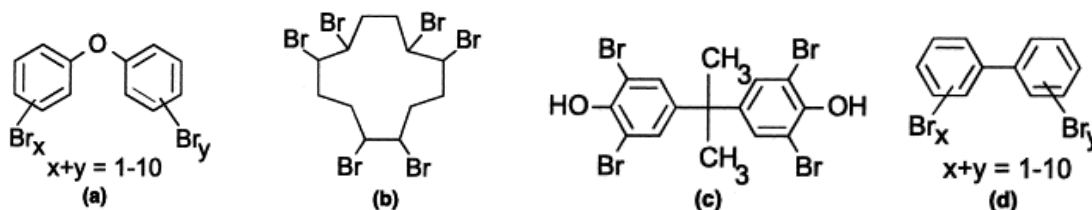

Figure 2 - The chemical structures of (a) PBDEs, (b) HBCD, (c) TBBPA, and (d) PBBs. (Wit, 2002)

There are a total number of 209 PBDE congeners. Technical PBDE products are produced by brominating diphenyl ether in the presence of a catalyst. The individual PBDE congeners are numbered according to the IUPAC system used for numbering PCBs based on the position of the halogen atoms on the rings (Rahman et al., 2001; Wit, 2002).

Brominated flame-retardants (BFRs) can be divided into three subgroups depending on the mode of incorporation of these compounds into the polymers: brominated monomers, reactive and additive flame retardants. A brominated monomer such as brominated styrene or brominated butadiene is used in the production of brominated polymers, which are then blended with nonhalogenated polymers or introduced into the feed mixture prior to polymerization, resulting in a polymer containing both brominated and non-brominated monomers. Reactive flame retardants, such as TBBPA, are incorporated into the polymeric materials by covalent bonding between the polymer and the flame retardant, whereas the additive types are dissolved in the polymer. Additive flame retardants, which include PBDEs and HBCDD are sometimes volatile and can tend to bleed, so their flame

retardancy may be gradually lost and they can enter the environment more easily (Rahman et al., 2001; Alaee et al., 2003).

Brominated flame retardants are the cheapest way of improving fire resistance. Alternatives are available, such as phosphorus and metal based compounds, but these are more expensive and can pose manufacturing problems. Their mechanism of flame retardancy is the same for all compounds in this group. With the application of heat they decompose before the matrix of the polymer, preventing the formation of flammable gases (Rahman et al., 2001). Flame retardants are used in resins and polymers. The major uses are in high impact polystyrene, flexible polyurethane foam, PVC, textile coatings (not clothing), furnishing, wire and cable insulation, electrical and electronic connectors, computer equipment and other interior parts (Rahman et al., 2001; Wit, 2002; Alaee et al., 2003; Garcia-Jares, 2009).

In 1992 the total world production of all brominated flame retardants was estimated at approximately 150,000 metric tons/year (Wit, 2002). Nowadays their production has increased greatly and in 2012 the worldwide consumption of these compounds amounts was around 2 million tons a year. Their use in plastics accounts for approximately 85% of all flame retardants, while textiles and rubber products account for the rest (Flame retardants online, 2013). Their increasing use has raised some concerns as they are a potential risk to health and to the environment. As they are lipophilic they tend to bioaccumulate and due to their high resistance to degradation processes, they may become persistent in the environment (Rahman et al., 2001).

Data on the toxicology of BFRs is limited and so are the consequences of their spread in the environment and in human health. Isolated cases of the occurrence of these compounds have been related to human illnesses including cancer and mass mortality of marine mammals (Garcia-Jares, 2009). Because of recent publications regarding the effects of BFRs in the environment and human health, industries are voluntarily replacing the lower brominated PBDEs with other flame retardants. As an example PeBDE technical products are currently in the process of being banned within the European Union (Wit, 2002). This year EPA will begin evaluating 20 flame retardant chemicals, conducting full risk assessments. EPA will use the information from these assessments to better understand the other chemicals in the group, which currently lack sufficient data for a full risk assessment (EPA, 2013).

Table A.4 (Appendix A) presents the physic-chemical properties of the BFRs analysed in this study. They vary from BDEs -28, -47, -99, -100, -138, -153, -154, -183 and -209, plus hexabromobenzene (HBB) and pentabromotolueno (PBT). BFRs have high log K_{OA} values (8-15) so they tend to partition onto atmospheric particles, they also have low vapor pressures, low water solubility and are very lipophilic, with log K_{ow} in the range 6–10.

1.2.2 Pine Needles

Considering that pine needles are an important part of this work, a brief description of their morphology and biosamplers use will be presented.

1.2.2.1 Morphology

Pine trees are the most predominant trees in the coniferous forests of the northern hemisphere (Stern, 1994a). Nowadays, the main pine trees present in Portugal are *Pinus pinaster* (885 trees/ 1000 ha) and *Pinus pinea* (130 trees/ 1000 ha) (CELPA, 2011).

In young pine trees, leaves are supported individually and twirl around the branches. Their leaves, also called needles are long and thin shaped. Mature trees produce needles in bunches and needle clusters are called fascicles. Needles evolved in order to allow the pine to survive and thrive in dry, cold climates. Leaves, stems and roots are all impregnated with resin which has the ability to dissuade insects and has also a lower freezing point than water (Krempels).

Figure 3 represents a section through a pine needle where are represented the main parts of the needle. The outer layer of the needle called the epidermis secretes a very thick cuticle which is commonly designated as the 'waxy layer'. The main function of this cuticle is to act as a barrier and reduce water evaporation. The stomata are pores found deeply in the epidermis that are used to control the gas exchange. Sunken stomata are one of many adaptations that help pine trees thrive in dry environments. Inside the epidermis there is a layer of mesophyll cells that are filled with chloroplasts. The mesophyll cells are surrounded by air spaces, which enables them to perform gas exchange. Air can move slowly in and out of these air spaces through the stomata. Also, in the middle of this pine needle there is a single vascular bundle. The bundle contains phloem and xylem. Phloem transports the sugars that are produced by photosynthesis from the leaves to the rest of the plant and xylem transports water and inorganic nutrients from the roots up to the rest of the plant. Resin ducts carry resin, which is a hydrocarbon-containing substance that may help to protect the leaves (McCauley, 2011; Gschmeissner, 2012; Esau, 1898; Stern, 1994b).
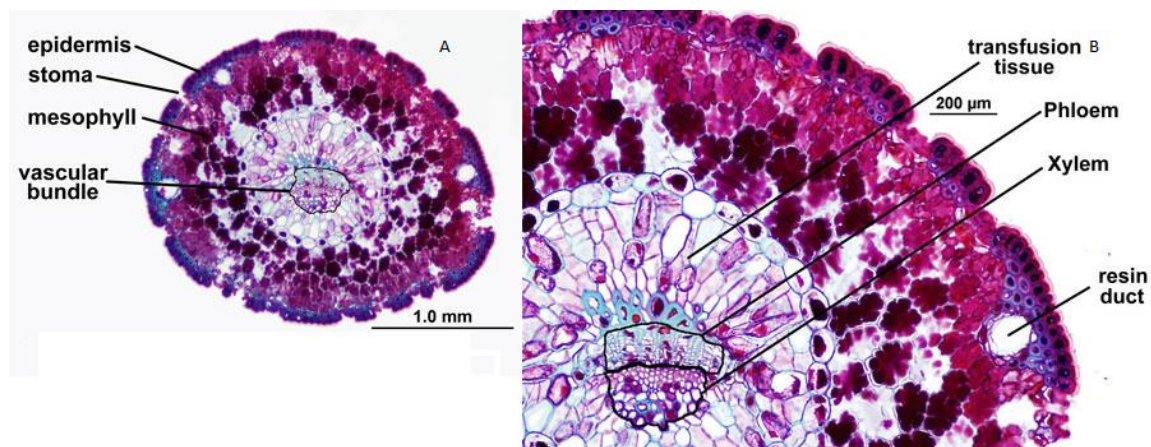
Figure 3 - Section through a pine needle; (A) pine needle cross section (epidermis, stoma, mesophyll and vascular bundle view) (B) pine needle cross section (transfusion and tissue, phloem, xylem and resin duct view) (McCauley, 2011)

The quantity, quality, and distribution of needle waxes vary between species, within species, and between needles on individual trees. They act as a defence mechanism protecting needle tissues against UV radiation and intense light in the visible range, minimize cuticular transpiration and shed water that might otherwise promote the growth of fungi (Woo et al., 2002; Tiwari et al., 2013).

1.2.2.2   Biosamplers use

Since the majority of plant surfaces that are exposed to air are covered with waxy or lipidic layers, plant foliage has been widely used as a non-destructive method for the detection of airborne pollutants since they can be sorbed and accumulated in these surfaces. This is considered a very cheap and convenient passive sampler (Tomaniová et al., 1998; Araújo et al., 2012). Pine trees, and more specifically pine needles, are a type of plant foliage that have been considered as biomonitor to assess the occurrence of a wide range of emergent pollutants in the environment (Ratola et al., 2009).

Because of their chemical properties such as low water solubility, high octanol-water partition coefficient and low vapour pressure, these type of pollutants tend to accumulate in lipidic tissues like pine needles waxy layer (Klánova et al., 2009). Depending on their properties these air contaminants can either be sorbed into the pine needle waxy layer or accumulate on its surface (Tomaniová et al., 1998). As evergreen plants, pine can accumulate atmospheric pollutants for several years being helpful to give mean concentration values, seasonal variations, long-term trends and relative comparisons (Klánova et al., 2009). The worldwide presence of different pine species also allows data comparison and bioaccumulation and transport patterns establishment between different parts of the world (Ratola et al., 2009). However, there's still a poor characterization of this sampler performance and it is known that different species have different uptake characteristics. Besides needles species, variations such as

needles age, structure and lipidic content also contributes to the end result of the contaminants characterization (Xu et al., 2004; Klánova et al., 2009).

1.2.3 Analytical methods for the determination of emergent pollutants (*in this project*)

Several analytical methodologies have been developed for the determination of emergent pollutants in pine needles with an exception for synthetic musks. Usually GC-MS analysis is the preferential method employed. However, before GC-MS analysis, samples must be extracted from their matrix and cleaned from naturally existing interferences and also allowing sample pre-concentration. Depending on the type of compounds of interest different types of extraction and cleanup can be employed.

1.2.3.1 Cleanup techniques

Cleanup techniques used during the development of the method of this work will be briefly presented.

Solid Phase Extraction (SPE)

Solid-phase extraction (SPE) is a sample preparation technique suitable for trace enrichment, matrix simplification and desalting (Żwir-Ferenc and Biziuk, 2006). The SPE principle involves a partitioning of solutes between two phases: liquid (sample matrix or solvent containing analytes) and solid (sorbent) phase. This sample cleanup technique enables the concentration and purification of analytes from solution by sorption on a solid sorbent and purification of the extract after extraction. The general procedure is to load a solution onto the SPE solid phase and either wash away the undesired components and elute after the desired analytes or elute at first the desired analytes with another solvent into a collection tube (Berrueta et al., 1995; Żwir-Ferenc and Biziuk, 2006).

SPE cleanup can be performed according to four general theory interactions, *reversed phase* which involves a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase and the analyte of interest is typically mid- to nonpolar, *normal phase* which involves a polar analyte, a mid-to nonpolar matrix and a polar stationary phase, *ion exchange* that is used for compounds in a solution where anionic (negatively charged) compounds can be isolated on an aliphatic quaternary amine group that is bonded to the silica surface and cationic (positively charged) compounds are isolated by using the silica with aliphatic sulfonic acid groups that are bonded to the surface, *polymer based* where sorbents are used to retain different type of compounds depending on which solid phase is used (Berrueta et al., 1995).

In order to obtain the analytes of interest in this work, a three step SPE methodology was chosen where the interferences are retained on the column while the analytes of interest pass through the sorbent bed and are collected with the elution solvent. Regarding this procedure, *first* the sorbent is conditioned with solvent to improve the reproducibility of analyte retention, reduce the carry through of sorbent impurities at the elution stage and activate the bonded phases to ensure consistent interaction between the analyte and the sorbent functional groups. The *second* step includes sample loading and slowly elution in order to cover all SPE's sorbent surface. Finally, at the *third* step the analytes of interest are eluted from the sorbent while interferences remain at the column (Poole et al., 2000).

The applicability of SPE is mainly determined by the sorbent used in the extraction column. Nowadays a large number of sorbents are available, and the most frequently used group of sorbents are chemically modified silica gel, polymer sorbents and graphitized or porous carbon. However, adsorptive inorganic oxides like silica, alumina and florisil (magnesium silicate) are widely used for normal phase (adsorption) SPE (Hennion, 1999; Augusto et al., 2013). Florisil (Mg_2SiO_3) is particularly suited to clean up of extracts from fatty compounds because it retains lipids preferentially. Alumina (Al_2O_3) can be substituted for Florisil for the cleanup of extracts of fatty compounds. Alumina is commonly used for purification of chemicals and for extraction of pollutants. Silica gel (SiO_2) is among the inorganic adsorbents that are most employed as support with a structure consisting of tetrahedral units of SiO_2 connected by siloxane bridges Si–O–Si. The silica gel is a material of high superficial area, resistant, porous and formed of irregular particles (Filho and Carmo, 2004; Żwir-Ferenc and Bizjuk, 2006; Buszewski and Szultka, 2012). Sorbents polarity plays a great deal in both interferences and analyte sorption. Table 1 resumes the adsorptive inorganic oxides' structure and polarity (Żwir-Ferenc and Bizjuk, 2006).

Table 1 - Sorbents for normal phase SPE (Adapted from Żwir-Ferenc and Bizjuk, 2006)

Sorbent	Structure	Polarity
Silica Gel	-SiOH	Slightly polar
Florisil	Mg_2SiO_3	Moderately polar
Alumina	Al_2O_3	Strongly polar

Adsorbent activity is usually controlled by water content, so in order to control adsorbent activity it is possible to intentionally add water or a drying agent (like anhydrous sodium sulfate) prior to sample loading (Poole, 2012).

Gel Permeation Chromatography (GPC)

Gel Permeation Chromatography (GPC) also known as gel filtration, gel chromatography or size exclusion chromatography is a column separation technique based on a non-ionic molecular effect separating molecules according to their size. A chromatographic column is packed with particles with a specific pore size, and the voids between the particles and the pores are filled with the chosen solvent. A small sample is introduced as a dilute solution onto the top of the column and the solvent is continually passed through (Williams, 1970; Macek et al., 2011).

In this type of chromatography, the chosen organic solvent is the mobile phase and the stationary phase consists of beads of porous polymeric material. GPC procedure is very similar to SPE consisting in column washing, sample loading and elution, however, at the end there's a column regeneration step where more solvent is added in order to remove the remaining interferences. Smaller compounds can enter the sorbents pores more easily and therefore spend more time in these pores increasing their retention time, on the other hand, larger compounds are eluted faster since they don´t fit in the pores.

1.2.3.2   Basic GC-MS principles

The GC-MS is an hyphenated analytical technique employing two major devices: the gas chromatograph and the mass spectrometer. The gas chromatograph uses a mobile phase (an inert gas) and a stationary phase (a column) whose properties will allow to separate chemical compounds from a mixture according to their chemical or physical properties as the sample travels through the length of the column. Molecules will elute at different retention times allowing the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio (Poole and Poole, 1991; Hubschmann, 2008). Figure 4 shows a diagram representation of a GC-MS system.
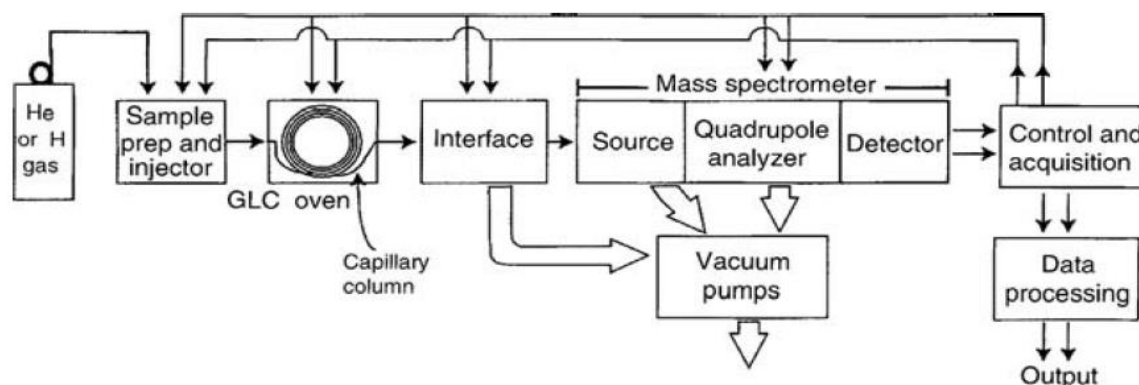

Figure 4 – GC/MS system diagram with a Quadrupole analyser (adapted from McMaster, 2008)

2 State of the Art

In this section are presented the methods of extraction and cleanup for the determination of the pollutants described before in pine needles.

Because of the extend literature concerning some classes of pollutants, this review only summarizes data after the year 2000. A compilation of review literature is given in Tables B.1, B.2 and B.3 (Appendix B).

2.1 Polynuclear Aromatic Hydrocarbons analysis

A general overview of PAH analysis in pine needles since 2000 was performed. Information regarding extraction and cleanup procedures, analytical method (including method validation parameters like limit of detection and recoveries) and detected concentrations is presented in Table B.1 (Appendix B).

Although several extraction methodologies have been used to determine PAH in pine needles, Soxhlet and ultrasonic extraction (USE) are the most used ones. Soxhlet has been widely applied to PAH extraction from pine needles because of its high extraction efficiency (60-100%) although it requires the use of large volume of organic solvents (100 – 1000 mL), it's time consuming (up to 48 h) and labor intensive. Among the high variety of solvents that can be used to extract the compounds, dichloromethane (DCM) (Hwang and Wade, 2008; Sun et al., 2010; Holoubek et al., 2000; Tian et al., 2008), hexane (Hex) (Hubert et al., 2003), acetonitrile (ACN) (Augusto et al., 2010) and their mixtures (Ratola et al., 2006) are the most employed ones. USE appeared as an alternative to Soxhlet extraction since extraction time is reduced because of the cavitation phenomena caused by the ultrasonds, even if it's less reproducible (Luque-García and Luque de Castro, 2003). With recoveries in the same range as Soxhlet extraction, USE employs similar solvents although with smaller volumes (15 - 100 mL) and reduced extraction times (10 min to 1 h) (Ratola et al., 2006, 2008, 2009, 2010, 2011; Amigo et al., 2011; Tomashuk et al., 2012; Capuano et al., 2005, Gorshkov, 2008; Hubert et al., 2003; Piccardo et al., 2005 and Wang et al., 2005). Even if they are less reported, methods like microwave assisted extraction (MAE) (Ratola et al., 2009), pressurized liquid extraction (PLE) (Ratola et al., 2006), accelerated solvent extraction (ASE) (Liu et al., 2006; Lehndorff and Schwark, 2009a, 2009b, 2004; Hubert et al., 2003) and supercritical fluid extraction (Lang et al., 2000) have been used to extract PAHs from pine needles with good overall results, similar solvents and reduced extraction times. However, special and sometimes expensive equipment are required.

In order to obtain a cleaner extract several cleanup methods can be employed. The most common one is solid-phase extraction (SPE) performed either with columns or cartridges. Sorbents like silica $(SiO_2)_x$, alumina (Al_2O_3) and florisil are the most used ones, with quantities ranging 0.5-20 g. The elution solvents used are acetonitrile (ACN), dichloromethane (DCM), hexane (Hex) and mixtures like DCM/Hex , pentane:DCM and pentane:chloroform with quantities from 8 to 200 mL. Some authors also use more than one sorbent per column like silica/alumina column (Sun et al., 2010; Liu et al., 2006) or florisil/alumina/silica columns (Tian et al., 2008). In order to improve the cleanup some authors performed a gel permeation chromatography after the SPE (Sun et al., 2010; Tian et al., 2008; Liu et al., 2006; Schröter-Kermani et al., 2006).

The first step towards the process of PAHs analysis is sampling where needles are collected and stored until analysis. Needles from different species have been studied so far like *Pinus nigra*, *Pinus strobus*, *Pinus pinea*, *Pinus pinaster*, *Pinus massoniana*, *Pinus sylvestris*, *Pinus taeda*, *Pinua thunbergii*, *Pinus densiflora* and *Pinus maximartinezii* (Table B.1 in Appendix). Different species of pine trees produce structural different pine needles so their entrapment ability toward some PAHs are different. Ratola et al. (2010 and 2011) showed that *P. pinaster* needles had the double average concentration of PAHs than *P. pinea* needles (748 vs. 399 ng/g-dw), Piccardo et al. (2005) showed that *P. pinaster* needles had higher concentrations than *P. nigra* needles (817 vs. 507 ng/g-dw), Tomashuk et al. (2012) obtained much higher concentrations for *P. nigra* when compared to *P. strobus* (4187 vs. 384 ng/g-dw) and Librando et al. (2002) obtained higher PAHs concentrations for *P. halepensis* needles than *P. pinea* (400-1000 vs. 300-700 ng/g respectively). Comparison between authors is not possible since needles are collected from different locations (with different pollution impact) and the number of analysed PAHs is also different.

Regarding the site of needle collecting, meaning rural, urban or industrial sites, there's a consensus that the increase of PAHs concentration follows the previous described order (Ratola et al., 2006, 2010; Lang et al., 2002; Piccardo et al., 2005; Tian et al., 2008; Lehndorff and Schwark, 2009b, 2004 and Hwang et al., 2003).

Amigo et al. (2011) found mean concentrations of 142 ng/g-dw for rural sites, 337 ng/g-dw for urban sites and 866 ng/g-dw for industrial sites, following the industrial>urban>rural trend. Lang et al. (2000) reported mean concentrations of 137 ng/g-dw for rural sites and 782 ng/g-dw for urban sites. Tian et al. (2008) found higher PAH incidence for urban and industrial sites than rural sites, however forests near pollution sources are more capable to adsorb PAHs from the atmosphere. Lehndorff and Schwark (2009b) only considering 3-ring PAHs also found an industrial>urban>rural trend. Hwang et al. (2003) obtained sum concentrations of 17 PAHs for rural/suburban sites (31-132 ng/g-dw), urban sites (102-192 ng/g-dw) and an industrial site (563 ng/g-dw).

Gorshkov (2008) found sum concentrations of 15 PAHs between 0.3 and 600 ng/g in Russia where phenanthrene is the most concentrated analyte with 241 ng/g and set a PAH concentration range of 20-1800 ng/g for heavily polluted sites (industrial sites) in a 2 to 3 years accumulation period. Also, Hwang and Wade (2008) obtained a sum concentration for 20 PAHs between 209 and 2226 ng/g-dw where phenanthrene was the predominant analyte and other 3- and 4-ring PAHs accounted for 79-97% in all samples. Hubert et al. (2003) don't report a range of values for their study (where they study 16 PAHs), however they show that phenantrene was the dominant compound in all polluted sites. Hwang et al. (2003) found that in rural/suburban sites 3-ring PAHs were predominant accounting for 63-73% of total analytes and phenanthrene was the major compound. Lehndorff and Schwark (2004) found concentrations of 18 summed 3–6-ring PAH range between 51 and 410 ng/g-dw in urban locations in Germany. Phenanthrene was the dominating PAH, followed by fluoranthrene and pyrene. Malawska et at (2002) found around 200 ng/g-dw in 17 summed 3 and 4 ring PAHs where phenantrene was the most common one. Liu et al. (2006) reported values for 15 PAHs ranging 280-3000 ng/g-dw from pollutes sites in China with major compounds such as phenanthrene, followed by fluorene, fluoranthene, pyrene and chrysene. In industrial sites 3-ring PAHs are the dominant group in pine needles. Augusto et al. (2010) found concentrations between 83 and 466.8 ng/g-dw when analyzed 16 EPA priority PAHs in Portugal. The most common analyte was phenanthrene (67.7 ng/g-dw), a 3-ring PAH, followed by pyrene, fluoranthene and naphthalene (18.6-204.0 ng/g-dw range). Based on this the PAH with the highest concentration in pine needles seems to be phenantrene.

On the other hand, Holoubek et al. (2000) found significantly high values both for rural and industrial sites (0.3-18,590.0 and 0.3-19,251.0 ng/g-dw respectively) in the Czech Republic where acenaphthylene was the most concentrated analyte in all locations. Also Amigo et al. (2011) reported that the most common PAHs in rural sites is naphthalene (2-ring PAH), this may be due to the fact that this is the most volatile PAH which facilitates the entrapment onto the needles.

The major uptake process for semivolatile organic contaminants from the air to the needle waxy layer is by gaseous sorption. In the case of PAHs, 3- and 4-ring PAHs exist mostly as the vapour phase and can easy be sorbed to the waxy layer. On the other hand, 5- and 6-ring PAHs directly accumulate on the pine needle surface in the form of particles (Hwang and Wade, 2008; Lehndorff and Schwark, 2009a). Because of that, during cold months PAH accumulation in pine needles is higher probably due to partitioning, contrasting to warmer months were PAH loss is due to increased volatilization (Lehndorff and Schwark, 2004; Piccardo et al., 2005). Also, in cold months there's a higher use of domestic heating and heavier road traffic which increases PAHs contamination sources (Amigo et al., 2011).

2.2 Synthetic Musks analysis

To the authors best knowledge no studies have been performed with musks and pine needles (or other type of vegetation).

2.3 Polychlorinated Biphenyls analysis

Like for PAHs, a general overview of PCBs analysis in pine needles since 2000 was performed. Information regarding extraction and cleanup procedures, analytical method (including method validation parameters like limit of detection and recoveries) and detected concentrations is presented in Table B.2 (Appendix B).

In order to extract PCBs from needles, most authors reported using the Soxhlet extraction. Different solvents, volumes and time of extraction were used, 325 mL of methylene chloride-hexane (3:1) for 16 h (Sajwan et al., 2009), toluene-methyl chloride for 7 h (Wyrzykowska et al., 2006 and 2007), 250 mL of cyclohexane-hexane (1:1) for 16 h (Xu et al., 2004), dichloromethane from 8 to 24 h (Klánová et al., 2009; Grimalt and Drooge, 2006 and Holoubek et al., 2000) and toluene for 24 h (Rappolder et al., 2009). Other extraction methods were also employed, accelerated solvent extraction (ASE) (Oberg and Peltola, 2009), microwave assisted extraction (MAE) (Kozul and Romanic, 2008), microwave accelerated reaction system for extraction (MarsX) (Romaníc and Klincic, 2012) as well as other described procedures. Recent technologies like selective pressurized liquid extraction (S-PLE) (Lavin and Hageman, 2012) and supercritical fluid extraction (SFE) (Zhu and Lee, 2002), allow simultaneous extraction and cleanup. With the exception of S-PLE and SFE, these methods alone are not enough to remove matrix-interfering compounds, like lipids and waxes, and further cleanup methods such as SPE and/or GPC are required. Regarding cleanup methods, common used sorbents or fat retainers include florisil (Oberg and Peltola, 2009; Xu et al., 2004 and Holoubek et al., 2000), sulfuric acid impregnated silica gel (Rappolder et al., 2007; Romanić and Krauthacker, 2007 and 2004; Wyrzykowska et al., 2007 and 2006; Chen et al., 2006 and Grimalt and Drooge, 2006), alumina and GPC as a final cleanup step. Table 2 presents a summary of extraction and cleanup methods and respective recoveries used in PCB studies.

Table 2 - PCB's extraction and cleanup methods

Extraction method	Cleanup	Recovery (%)	Reference
S-PLE		84	Lavin and Hageman, 2012
Soxhlet	SPE-Silica	100	Sajwan et al., 2009
ASE	SPE-Florisil	30-70	Oberg and Peltola, 2009
Unknown + saponification	SPE-Silica	34-57	Romanić and Krauthacker, 2007
Soxhlet	SPE-Silica + SPE-Alumina + HPLC	101	Wyrzykowska et al., 2007
Shaking + USE	SPE-Silica + SPE-Alumina + GPC	60-90	Chen et al., 2006
Soxhlet	SPE-Silica/Alumina	79-99	Grimalt and Drooge, 2006
Soxhlet	SPE-Silica mix + SPE-Alumina mix + HPLC	87-119	Wyrzykowska et al., 2006
Soxhlet	SPE-Florisil	84-92	Xu et al., 2004
Soxhlet	SPE-Florisil	80-98	Holoubek et al., 2000
SFE		89-97	Zhu and Lee, 2002

SPE- solid phase extraction, HPLC- high purification liquid chromatography, USE-ultrasonic extraction, ASE-accelerated solvent extraction

According to Table 2, S-PLE provides better recoveries (84%) than other combined methods. Lavin and Hageman (2012) concluded that this method reduces sample preparation time by 67% when compared with a conventional PLE method, since extraction and cleanup are combined in the same procedure. On the other hand, Zhu and Lee (2002) developed a method to extract PCBs from pine needles using supercritical fluid extraction technology. They determined optimum conditions such as extraction temperature (120 ^{o}C), CO_2 flow rate (2.5 mL/min) extraction time (50 min) and obtained recoveries of about 90%. Regarding extraction methods, Soxhlet is better than ASE, when the same cleanup method is used (SPE-Florisil) (Oberg and Peltola, 2009; Xu et al., 2004). Comparing cleanup methods, SPE-Silica mix + SPE-Alumina mix + HPLC showed better recoveries in both studies (Wyrzykowska et al., 2007 and 2006) as SPE-Florisil, Silica or Alumina alone.

Oberg and Peltola (2009) found a significant increase in deposition of PCBs under a high-voltage power line with concentrations ranging 240 and 1600 pg/g-dw. With 89 to 97% recovery, Capuano et al. (2005) obtained values ranging 0.7 and 30.1 ng/g-dw for 28 different PCBs near an incinerator power plant in Italy and Xu et al. (2004) found concentrations of 15 sum PCBs between 41.8 and 270.5 ng/g-dw from samples collected from 6 locations in Beijing city (China). Klánová et al. (2009) found high concentrations of PCB 28, 52, 153 and 101 in a urban area in Czech Republic, while validating pine needles as a passive air samplers for several pollutant compounds. Rappolder et al. (2007) also collected pine needle samples from urban areas in Germany and found concentrations between 0.3 and 1.1 ng/g-

dw where the PCB congener with the highest concentrations was PCB 153, followed by congeners 28, 52, 101 and 138. Both authors reported the same congeners to be dominant in these urban areas. Chen et al. (2006) found an average concentration of total 209 congeneres PCBs in pine neddles from Dalian (China) urban areas of 4.4 ng/g-dw, dominating groups were tri-, tetra- and penta-chlorinated biphenyls. Grimalt and Drooge (2006) found concentrations ranging 0.1 and 1.2 ng/g in the Pyrenean high mountains, of which most concentrated PCBs congeners were 101 (27%) and 138 (20%). In an industrial site, Wyrzykowska et al. (2007) reported total PCB concentrations ranging from 2.7 to 50 ng/g-ww, where the highest abundance of chlorinated groups are 6CB, 7CB and 8CB. Overall the most detected PCBs congeners are number 28, 52, 101, 138 and 153.

2.4   Brominated Flame Retardants analysis

A general overview of BFRs analysis in pine needles since 2000 was performed. Information regarding extraction and cleanup procedures, analytical method (including method validation parameters like limit of detection and recoveries) and detected concentrations is presented in Table B.3 (Appendix B). Regarding the extraction methods, both ultrasonic extraction (USE) and Soxhlet extraction are the main methodologies used in previous studies. The used solvents vary from DCM to DCM:Hex (1:1), methylene chloride:Hex (3:1) and Hex:acetone (1:1), with volumes ranging from 90 to 325 mL, although some authors don't report the complete methodology used. The preferential cleanup methods are in general SPE columns or cartridges. Ratola et al. (2011) reported in their study to have used SPE alumina cartridges with 5 g of sorbent followed by a florisil pipette column. On the other hand, Chen et al. (2009) used only a SPE alumina column and Sajwan et al. (2009) only a SPE silica column. Others, Tian et al. (2012) and Kannan et al. (2009), used a double sorbent SPE column with alumina and silica. Overall, recoveries reported are between 62.5 and 142% using the same extraction method (Soxhlet) and different SPE columns in the cleanup process. Tian et al. (2012) obtained recoveries between 62.5 and 142% and Sajwan et al. (2009) obtained a 100% recovery in general. Apparently the second author's method is more reliable since the recoveries are in narrower range than the first one. Some authors used an acid attack with sulfuric acid (H_2SO_4) either after or before the SPE cleanup procedure (Tian et al., 2012; Sajwan et al., 2009; Chen et al., 2009) in order to remove lipids from the sample. Kannan et al. (2009) used an HPLC purification methodology (size exclusion column) in order to obtain the same results.

Concerning pine needle species' ability to trap BFRs, it is impossible to compare results between authors, as they used different pine species (*Pinus halepensis, Pinus pinea, Pinus nigra, Pinus massoniana, Pinus taeda and Pinus korariensis*), collected sample at different sites and employed diverse extraction and cleanup methodologies. However, Ratola et al. (2011) studied three different

pine species, *Pinus halepensis, Pinus pinea and Pinus nigra*, and found that *P. halepensis* was the specie which accumulated the highest concentration of PBDEs, even though they were only detected in 18-72% of the samples analyzed. Also, BDE 209 was only found in this species. The species distribution in the territory may play also an important role on the analyte concentration since *P. halepensis* samples were collected mostly near urban and industrial sites, *P. nigra* were collected near remote areas and *P. pinea* was sampled in more coastal areas. Regarding urban areas, Kannan et al. (2009) found that PBDE-99 was the highest congener found in pine needles throughout the year with values reaching 1 ng/g-dw. Also, PBDE-47 showed relevant concentrations in that area reaching 0.3 ng/g-dw. In this study PBDE-209 was not analysed. Ratola et al. (2011) found in urban/industrial areas that PBDE-209 was the congener with the highest concentration reaching 13.04 ng/g-dw, other congeners, BDE-47, -99 and -183 also showed high concentrations, 0.937, 0.279 and 0.063 ng/g-dw, respectively. Sajwan et al. (2009) collected samples from an Aroclor-1268 (PCB mixture) disposal area in and around LCP Superfund Site and out of 11 analysed PBDEs, 6 were detected with total concentrations ranging 0.05 and 0.49 ng/g-dw where PBDE-99 was the most common one. Authors discuss that PBDE contamination in this site may not come from Aroclor source, but from local residential. The low concentrations found in this remote location are because of PBDEs low atmospheric transportability due to their high molecular weight. Tian et al. (2012) also collected samples from a contaminated rural area, an e-waste site, and analysed different BFRs including PBDEs, DBDPE, PTBPE, PBEB, PBT, HBB and PBBs. Needles' total concentrations were between 40.4 and 546 ng/g-dw, while the average concentration of BFRs in the leaf surface particles were around 5500 ng/g-dw. It is evident that leaf lipids are very important in the uptake and storage of these semivolatile compounds. However, large particles deposit on leaf surface easily because of their high deposition velocities. Overall, PBDE-209 accounted for 50% of the total PBDE burden, while others, -47, -99 and -183 were less significant. Overall, the most reported BDE congeners reported in pine needles are -47, -99, -183 and -209.

3 Technical Description

3.1 Chemicals and reagents

Dichloromethane and n-hexane, used for extraction and cleanup were supplied by VWR BDH Prolabo (Leuven, Belgium). Florisil (0.150 - 0.250 mm), neutral aluminium oxide 90 (0.063 – 0.200 mm), silica gel 60 (0.062 – 0.200 mm) and sodium sulphate used in the cleanup procedure were acquired from Merck (Darmstadt, Germany) and were activated overnight at 450 °C. Florisil, neutral aluminium oxide and silica cartridges containing 5 g of adsorbent with a 25 mL reservoir, also used for cleanup studies were bought from Isolute (Hengoed, United Kingdom). Bio-Beads S-X3 were acquired from Bio-rad (Amadora, Portugal). Individual PBDE standards (congener numbers 47, 85, 99, 100, 153, 154, 183) were bought as 50 µg/mL solution in isooctane by Sigma-Aldrich (St. Louis, MI, USA). A PAHs mix solution (containing Naph, Acy, Ace, Fluo, Phen, Ant, Flt, Pyr, BaA, Chry, BbF, BkF, BaP, IcdP, DahA and BghiP at 2000 µg/mL in DCM/benzene 1:1), a PCB mix (congener numbers 28, 138, 153, 209 as 10 µg/mL in isooctane) as well as musk xylene (100 µg/mL in acetonitrile) was obtained from the same source. Dr. Ehrenstorfer (Augsburg, Germany) supplied a mix of PCBs (congener numbers 77, 81, 105, 114, 156, 157, 167, 189 as 10 µg/mL in isooctane). Musk ketone and musk ambrette were bought as neat standards from the same supplier. HBB and PBT individual standards (each 50 µg/mL in toluene) and were acquired from Wellington laboratories (Guelph, ON, Canada). LGC Standards provided neat standards of Cashmeran, Celestolide, Traseolide, Phantolide, Tonalide, Galaxolide as well as standard solutions (10 µg/mL in cyclohexane) of musk moskene.

Helium with a purity of 99.9999% and nitrogen with a purity of 99.995% were supplied by Liquid Air (Maia, Portugal).

3.2 Standards preparation

Due to the volatility and light sensitivity of some compounds, standards were protected from light using amber glass vials or aluminium foil and stored at – 20 °C. As commercial standards were provided in a variety of solvents, special care had to be taken regarding its miscibility. Individual stock solutions containing 13.3 g/L of each neat musk standard (Cashmeran, Celestolide, Traseolide, Phantolide, Tonalide, Galaxolide) were prepared in cyclohexane as these standards were also used for other projects. Then a 4 mg/L musk stock solution in hexane was prepared by evaporating musk xylene, musks moskene and musk tibeten under a gentle stream of nitrogen and subsequently adding appropriate amounts of the former mentioned individual musks stock solutions and hexane as solvent. A stock solution of brominated flame retardants comprising PBDEs and HBB, PBT, all at a concentration level of 4 mg/L in hexane, was prepared by dilution of appropriate amounts of each commercial standard. A PCB

stock solution of 4 mg/L was prepared by mixing the commercial PCB mixes and dilution in hexane. Calibration standards in hexane were prepared for concentration levels ranging from 5 to 2000 µg/L.

3.3   Equipment

Ultrasonic assisted extraction was performed in a JP Selecta (Barcelona, Spain) ultrasonic bath with a nominal power of 420 Watt. SPE cartridge cleanup was performed using a Macherey-Nagel (Düren, Germany) Chromabond vacuum manifold and evaporations under a stream of nitrogen were made using the drying attachment of the same brand. Extracts were evaporated in a Buchi RE 111 rotavapor (Flawil, Switzerland).

Instrumental analysis of the samples was performed using a Varian 450-GC/240-MS system, of which details are given in the instrumental analysis chapter.

3.4   Samples

During all the experimental work *Pinus pinaster* needles from Santo Tirso were used. Samples were collected in May 2012. The needles as a whole were cut directly from the branch, wrapped in aluminum foil, transported to the lab in sealed plastic bags and kept in the freezer until analysis.

3.5   Extraction

Sample extraction method consists in withdrawing an approximate needed amount of needles from the freezer and keep in aluminium foil (previously decontaminated with acetone) at room temperature until defrosting is complete. Needles are cut in 1 cm length and weighed to 5 g into 250 mL Schott flasks. Then, samples are spiked with standards (100 µL of "Safe Pine Mix for spiking" with PAHs and Musks at 1.25 mg/L and BFRs + PCBs at 0.5 mg/L). After spiking, the flasks are shaken in order to impregnate the needles with the standards. To each flask 100 mL of DCM:Hex (1:1) is added and extracted in the ultrasound bath for 30 min. The closed flasks are removed from the ultrasound bath, protected from light and cooled down to room temperature. Extract are then transferred to pear shape flasks and reduced to 1 mL in a rotary evaporator (40 $^{\circ}$C bath, 5 $^{\circ}$C cooling system, 60 rotations/min and between -150 and -700 mbar vacuum pressure). If not used immediately the sample was kept in the freezer protected from light and sealed with Parafilm.

3.6   Cleanup process

For SPE cleanup glass columns (1 cm diameter) or cartridges packed with alumina, florisil or silica were tested. Alumina packed glass columns were chosen as the most suitable cleanup method. Conditioning was done using 50 mL of DCM:Hex (1:1) and after sample loading, analytes were eluted with 50 mL of the same solvent. The extract was evaporated in pear shaped flasks to approximately 1 mL on a rotary evaporator (40 ^{o}C bath, 5 ^{o}C cooling system, 60 rotations/min and between -150 and -700 mbar vacuum pressure). Afterwards, the extract was loaded onto GPC glass columns containing 6 g of Biobeads S-X3 and eluted with 40 mL of DCM:Hex (1:1). The first 15 mL were rejected and the remaining eluate (25 mL) collected. Volume reduction was subsequently done using rotary evaporation to about 1 mL, followed by evaporation to dryness under a gentle stream of nitrogen. Sample reconstitution was done with 100 µL of hexane before analysis by GC-MS.

3.7   Instrumental analysis

Instrumental analysis of the samples was performed on a Varian GC-MS system (Walnut Creek, CA, USA), equipped with a Varian 450-GC gas chromatograph, a CP-1177 split/splitless injector, a CP 8410 autosampler and a Varian 240-MS ion trap mass spectrometer. Ionization mode was by electron impact (EI) with ionization energy of 70 eV and filament emission current of 50 µA. System control, data acquisition and processing was done by Varian MS workstation v. 6.9.3 software. Chromatographic separation was carried out using an Agilent (Santa Clara, CA, USA) CP-Sil 8 CB capillary column (50 m x 0.25 mm, 0.2 µm film thickness) equipped with a fused silica deactivated retention gap (5 m × 0.530 mm). The employed carrier gas was helium at a constant flow rate of 1 mL/min. Injection volume was 2 µL in splitless mode (hold time 5 min) and injector temperature was set to 250 °C. Temperatures of the manifold, transfer line and ion trap were 50 °C, 250 °C and 250 °C, respectively. Two distinct oven temperature programs were employed. For PAHs and Musks, temperature ramping started at 60 °C (hold for 1 min), raised to 175 °C at 6 °C/min (hold for 11.11 min) and then to 300 °C at 5.5 °C/min (hold for 10 min). Total program time was 64 min. BFRs, PCBs and OCPs temperature was programmed from 110 °C (hold for 1.5 min) to 150 °C at 20 °C/min, to 220 °C at 5 °C/min (hold for 17.5 min) and finally to 300 °C at 5 °C/min (hold for 9 min). Total analysis time was 60 min.

In both cases detection was made employing the time-scheduled selected ion storage (SIS) capability of the ion trap. Identification of target compounds was done by comparing retention times and mass spectra to those of standards. Table 3 and Table 4 present the analysed compounds, retention times and used target ions for both employed GC-MS methods.

Table 3- PAHs and musks. Retention times, quantification and identification ions of the target

Retention time (min)	Compound	Target ions (m/z)
12.40	Naphthalene	127, **128**, 129
18.01	Acenaphthylene	151, **152**, 156
18.71	Acenaphthene	152, **153**
19.05	Cashmeran	**191**, 206
20.67	Fluorene	164, **165**, 166
23.92	Celestolide	173, **229**, 244
25.15	Phantolide	187, **229**
25.67	Phenanthrene	177, **178**
25.99	Anthracene	177, **178**
27.75	Musk ambrette	251, **253,** 254
28.42	Traseolide	**251,** 243
28.49	Galaxolide	215, **248**
28.86	Musk xylene	**282**, 283
29.01	Tonalide	**243**, 244
29.96	Musk moskene	**263**, 264
32.30	Musk tibeten	**251**, 252
34.23	Musk ketone	**279**, 280
36.49	Fluoranthene	**202**, 203
38.01	Pyrene	**202**, 203
45.52	Chrysene	226, **228**
50.33	Benzo[b]fluoranthene	251, **253**
51.47	Benzo[k]fluoranthene	251, **253**
51.64	Benzo[a]pyrene	252, **253**
55.76	Dibenz[*a,h*]anthracene	276, **278**
56.42	Benzo[*ghi*]perylene	**276**, 278

Table 4 - BFRs and PCBs. Retention times, quantification and identification ions of the target

Retention time (min)	Compound	Target ions (m/z)
15.03	PCB 28	186, **256**, 258
20.89	PCB 77	**292**, 294
21.37	PCB 81	**292**, 294
22.85	BDE 28	**248**
23.17	PBT	407, **488**
23.34	PCB 114	324, **326**, 328
24.07	PCB 153	**360**, 362
24.37	PCB 105	324, **326**, 328
26.03	PCB 138	358, **360**
29.99	HBB	**552**
30.79	PCB 156	358, **360**
31.23	PCB 157	358, **360**
32.78	BDE 47	**486**, 488
39.09	PCB 189	**394**, 396
41.69	BDE 100	**406**, 565
43.33	BDE 99	**406**, 565
45.77	PCB 209	498, **500**
46.04	BDE 85	**406**, 565
47.40	BDE 154	**484**, 644
49.05	BDE 153	**484**, 644
53.58	BDE 183	**564**, 724

Main quantification ions are shown in bold for both Tables.

3.8 Quality assurance and control

Because one of the analysed compound classes, synthetic musks, are present in most of the personal care products, some restriction in the use of this products had to be made. Scented cosmetics such as perfume, lotions and hand creams were avoided. Additionally, procedural blanks were extracted and analysed in order to identify and correct eventual contamination. Beyond several PAHs, some PCBs and musks, namely galaxolide and cashmeran were detected. Blank values were subtracted for all the concentrations reported. Also, all the glass material was rinsed with acetone and distilled water and the non-calibrated material was further subject to heating at 400 ^{o}C for at least 1 hour.

3.9 Waste treatment

The waste generated in this work consisted in organic solutions containing dichloromethane and hexane and analytes and mixtures of different sorbents (Na_2SO_4, silica, alumina, florisil) contaminated with target analytes. All these residues were collected in closed containers, properly labelled, and stored protected from light and from ignition sources for further treatment by the Environmental Management System of FEUP - EcoFEUP.

4 Results and Discussion

Vegetation samples, particularly pine needles, are very complex matrices and may be quite challenging in method development. The high number of target compounds of four different chemical classes with diverse physico-chemical properties posed additional difficulties in this undertaking. However, compromises had to be taken and were made based on the novelty of musks detection in vegetation. The obtained results will hereby presented and discussed and will serve as valuable inputs for further improvements within the *SAFEPINE project - monitoring pine contamination by emergent pollutants* objectives.

Experimental work comprised 3 phases. In the first phase, elution profiles were established which served as basis for tests with pine needle extracts of phase 2. During this one, the three commonly used SPE sorbents (alumina, florisil and silica) packed into two different devices (glass columns or cartridges) and eluting with two different solvent mixtures (DCM or DCM:Hex (1:1)) were tested and compared. Additionally, a final cleanup using GPC was tested to evaluate if further improvement was possible. Finally, in phase 3, pine needles samples were to be tested. Due to time and equipment restrictions, only one sample could be analysed. Even though, interesting conclusions could be taken about the suitability to use pine needles as biosamplers to evaluate atmospheric contaminations by musks.

4.1 Elution profiles

Cleanup method development started with the establishment of elution profiles. SPE glass columns containing 5 g of three sorbents, alumina, silica or florisil were tested. Elution was done employing two different solvents DCM or DCM:Hex (1:1). For the particular case of DCM:Hex (1:1) an additional elution with 20 mL DCM was done in order to check for still retained compounds. A graphic representation of obtained profile is given in Figure 5.
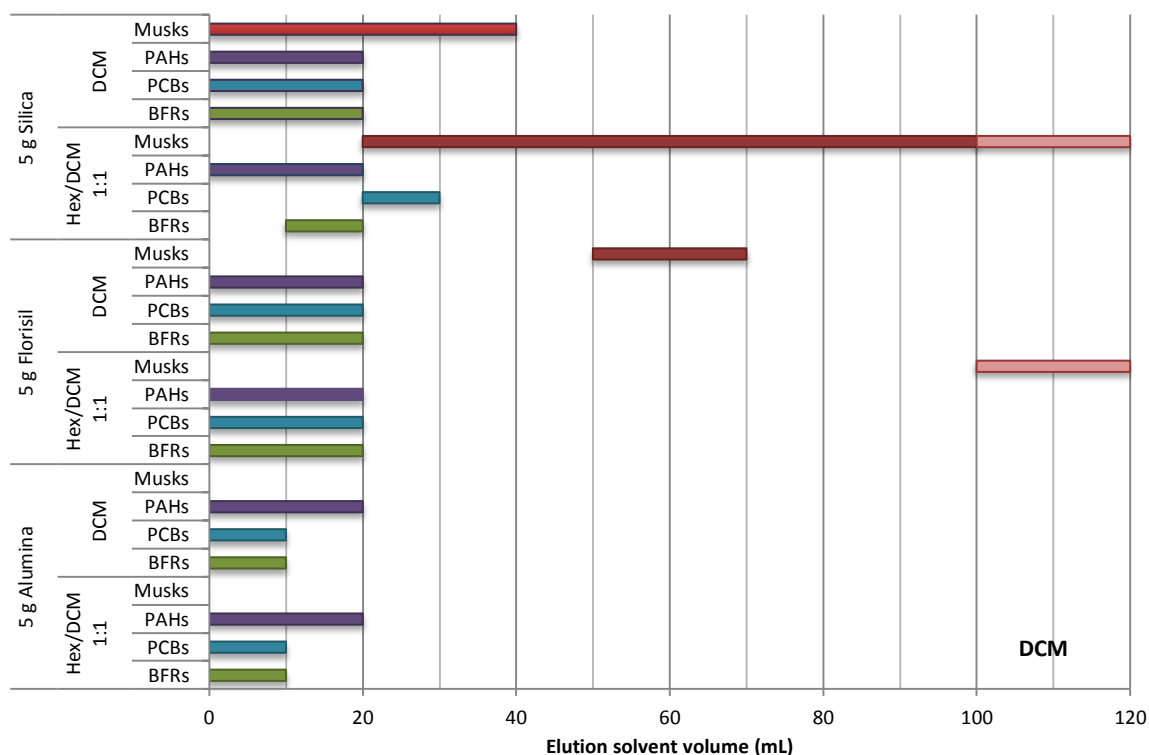
Figure 5 - Elution profiles of the SPE columns with different sorbents (silica, florisil and alumina) and solvents DCM and DCM:Hex (1:1).

Elution profiles result from a three-way equilibrium between the sample, the solvent and the sorbent. The solvent and the sample (in this case the analytes) compete for active sites on the sorbent where molecules are retained. This sorption may be due to different phenomena like polarity or hydrogen bonding, but depend also on structural properties of the molecules and the sorbent. The solvent on the other side displaces reversibly and continuously the retained analytes throughout the SPE column. Weakly retained analytes on the sorbent may be easily displaced by the solvent and therefore elute first, while strongly retained analytes take much more solvent to elute or may not elute at all. This displacement is mainly influenced by the analytes solubility in the solvent, but also due to polarities between sorbent and solvent. Usually, solvent's polarity should match the analytes polarity in order to allow an effective displacement.

In this case, four chemical classes, with differing properties between, but also within chemical classes were tested. A detailed discussion of the observed results and possible explanations regarding the occurring phenomena may be difficult, as beyond the above-described three-way equilibria, interactions and competitive sorption may occur. Therefore, a temptative interpretation of the results will be given.

Distinctive elution behaviour between used sorbents, but also regarding used solvents was observed. Analytes that show higher retention, require more solvent to be eluted and less retained analytes require lower amounts of elution solvent. The polarity order of the sorbents is

silica<florisil<alumina, from the least to the most polar. Regarding solvents, polarity increases as the amount of DCM increases, therefore pure DCM is more polar than the solvent mixture DCM/Hex (1:1).

Musks seem to be the most polar class of these compounds, and this may be the reason for which they are not eluted from alumina. As sorbent polarity is diminished and solvent polarity is increased musks are less retained and more easily eluted. PAHs are the most apolar class of compounds. However, no distinct elution behaviour was observed when sorbent or solvents were changed. As can be seen from Figure 5, these compounds are already poorly retained and therefore interactions are already weak. For this reason, no significant change in retention was observed. PCBs and BFRs also show similar behaviour for the three sorbents. They are slightly less retained on alumina and equally retained on florisil and silica. One exception is the combination of silica and DCM/Hex (1:1), both the least polar sorbent and solvent, respectively. In this specific case, small differences in polarity or structure of the compounds may be more relevant for the three-way equilibrium and therefore be responsible for this distinguished behaviour.

One important issue to be remembered is the fact that the fractions were manually collected and relevant parameters like flowrate or temperature could only be loosly controlled and may therefore influence significantly elution behaviour.

As noted before, musks were not recovered from alumina. However, according to this research group's expertise, alumina is very suitable for the retention of lipids and other vegetation related interferences (chlorophylls, waxes, phytosterols etc.) which led to the decision to test in the next phase this sorbent with pine needles extracts. Based on the diverse elution profiles, a compromise of elution solvents had to be found. For this, an elution scheme of 50 mL DCM/Hex (1:1) followed by additional 50 mL DCM was chosen, as this may elute all classes of compounds.

Within SafePine's research project, single GPC column cleanup of the extract was being tested during this thesis' research. Based on the obtained results, the possibility to use GPC as an additional cleanup step arose. Elution profiles for GPC columns containing 6 and 12 g of Biobead S-X3 (Figure 6) were kindly provided by the co-worker Mariana Mendes.
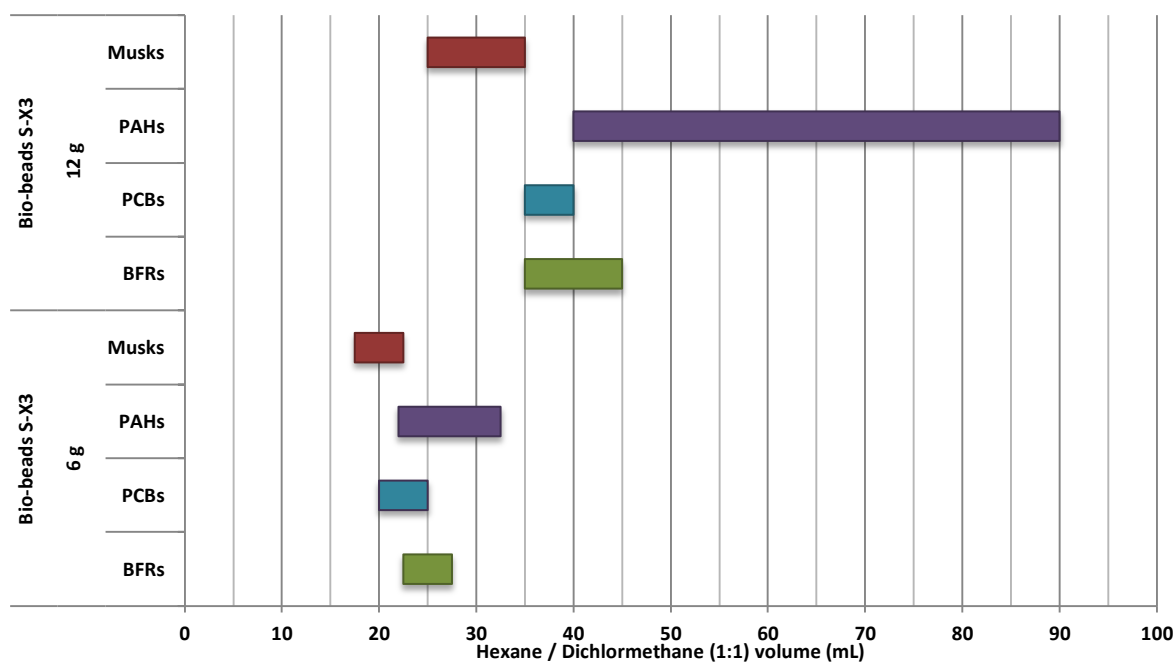
Figure 6 - Elution profiles of the GPC columns with 6 and 12 g of Bio-beads S-X3

Gel permeation chromatography is a separation technique based on molecular size. Smaller molecules tend to penetrate into the pores of the biobeads and elute later than molecules of bigger size, as these are unable to diffuse into the pores. Therefore, GPC separation involves usually two or more fractions. The first fraction containing interferences (macromolecules) is rejected and a second, containing analytes of interest, is collected. Due to diverse chemical properties of the analytes and to the fact that many interferences of pine needles are macromolecules (e.g. chlorophylls, lipids etc.), this technique seemed very suitable.

Elution profiles of GPC columns described in Figure 6, show that doubling the amount of Biobeads S-X3 from 6 g to 12 g, results in a proportional amount of the necessary solvent to start the elution of compounds (proportion factor 1.4-1.8). The volume necessary to completely elute each class of compounds increased slightly (except for PAHs for which the increase was more significant), but is not relevant in this case, as only interferents removal is intended.

Based on further results obtained by Mariana Mendes, using pine needle extracts (without prior SPE cleanup), 6 g and 12 g GPC columns showed similar results regarding cleanup capacity, namely for chlorophyll removal. Therefore, based on the fact that smaller amounts of solvent needed to be used, resulting in a shorter elution process, lower costs and reduced loss of analytes (due to the need to evaporate the solvent), the 6 g GPC column was chosen for additional cleanup. A total solvent elution volume of 40 mL of DCM/Hex (1:1) was chosen, of which a first fraction of 15 mL containing interferences was rejected.

4.2 Development of the cleanup methodology

The main cleanup parameters like the type of device, sorbent and type and volume of the elution solvent in the solid phase extraction (SPE) and influence of a GPC additional were optimized. *Pinus pinaster* needles were the samples chosen for this optimization process. This type of needles were chosen taken into account their complexity and was expected that the cleanup process method would work for needles of other species since they are considered less complex. A scheme of the cleanup process optimization is presented in Figure 7.

In every methodology performed, 5 g of pine needles (spiked with 10 ng/$g_{needles}$ of BFRs and PCBs standard mix and 25 ng/$g_{needles}$ of PAHs and Musks standard mix) were weighted and extracted in 50 mL of DCM/hex (1:1) for 30 minutes in an ultrasonic bath. These extraction parameters are the same as the ones optimized by Ratola et al. (2006). However, the cleanup method needed further optimization in order to be able to clean the extract as much as possible to analyse the four proposed classes of pollutants (PAHs, Musks, PCBs and BFRs).

Type of sorbent	
Conditions	Silica
	Alumina
	Florisil
Experimental conditions	5 g of sorbent
	SPE glass columns
	50 mL DCM/Hex (1:1) + 50 mL DCM

Extra cleanup steps	
Conditions	GPC additional step (40 mL DCM/Hex (1:1))
Experimental conditions	5 g of sorbent (Alumina, Silica and Florisil)
	SPE glass columns
	50 mL DCM/Hex (1:1) + 50 mL DCM

SPE stationary phase type	
Conditions	Glass columns
	Cartridges
Experimental conditions	5 g of sorbent (Alumina and Florisil)
	50 mL DCM/Hex (1:1) + 50 mL DCM
	GPC additional step (40 mL DCM/Hex (1:1))

Elution Solvent	
Conditions	50 mL DCM/Hex + 50 mL DCM
	50 mL DCM/Hex
Experimental conditions	5 g Alumina
	Glass columns
	GPC additional step

Optimized methodology	
Experimental conditions	5 g Alumina
	Glass columns
	50 mL DCM/Hex
	GPC additional step (40 mL DCM/Hex (1:1))

Figure 7 - Scheme of the cleanup optimization (variables need experimental conditions).

4.2.1 Comparison between SPE methodologies

Comparative studies between SPE devices (glass columns and cartridges) and sorbents (alumina, florisil and silica) were performed with and without an additional GPC cleanup step. Results are presented in Figure 8.
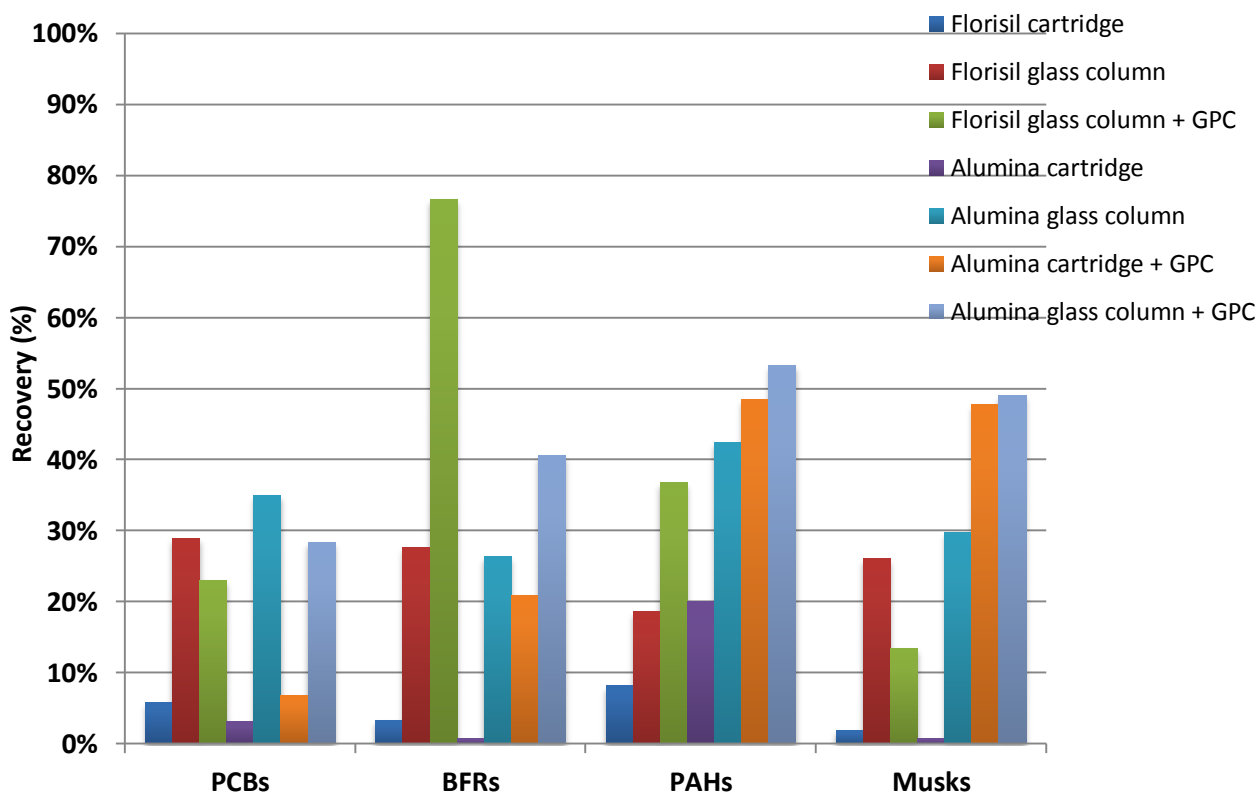
Figure 8 - Comparison of SPE cartridges and glass columns with Florisil and Alumina sorbents, with and without GPC additional step, spiked with 10 ng/g$_{needles}$ of BFRs and PCBs standard mix and 25 ng/g$_{needles}$ of PAHs and Musks standard mix and eluted with 50 mL DCM/Hex (1:1) plus 50 mL DCM

Recoveries of the analytes cleaned-up with SPE glass columns containing 5 g of silica, florisil or alumina and eluted with 50 mL DCM/Hex (1:1) + 50 mL DCM were compared. After solvent evaporation, the sample from the cleanup with silica column showed a green and very viscous appearance. Therefore, it was not considered to be amenable to GC-MS analysis and silica was considered to be not suitable to effectively cleanup this kind of extracts. For the other kind of sorbents and as can be seen in Figure 8, alumina showed slightly better recoveries for PCBs, BFRs and musks and significantly better recoveries for PAHs than florisil glass columns. It is noteworthy, that musks could not be eluted from alumina when elution profiles were made with standards, but are now recovered. This shows that the matrix plays an important role in the elution of compounds and that the lack of coextractives (lipids, chlorophylls, etc.) to occupy the active sites of the sorbents may be responsible for the fact that musk could not be eluted from the sorbent when pure standards were used.

Prepacked SPE cartridges are an alternative to SPE glass columns, as they require less handling (packing of the sorbent), are not breakable and may be stored (as they are usually packed in vacuum bags). However, they also offer some disadvantages as amounts and combinations of sorbents are reduced to the manufacturer's range of products, are more costly and require more equipment (vacuum pump, SPE manifold, needles etc.). Another important issue is the impossibility to bakeout and decontaminate the sorbent contained in the cartridges, as these devices may not be exposed to high temperatures. Ratola et al. (2006) proved that alumina cartridges are a good method to clean pine needles extracts in order to analyse PAHs. Since this class of compounds is one of the target chemical classes of this project, a decision was taken to test cartridges containing alumina or florisil. When cartridges and glass columns containing the same amount of the same sorbent (5 g of florisil or alumina) were compared, glass columns showed a clearly superior performance than the cartridges. One possible explanation for this may be due to the fact that although sorbent amount of the cartridges was the same as for glass columns, their diameter was different and consequently sorbent bed height of the cartridges was smaller. This means that contact time is lower and therefore cleanup may be negatively affected. Another reason is the necessity to use reduced pressure to elute solvent through SPE cartridges, which due to volatility of the analytes but also solvents posed and increased challenge in handling and may have affected recoveries.

As mentioned before, the possibility to use an additional cleanup step with GPC arose and was tested in order to improve recoveries. Two layouts were possible, one previous cleanup with GPC, removing firstly all bulk interferences followed by SPE, or first SPE followed by a final GPC cleanup procedure. Expertise acquired during Mariana Mendes work showed, that applying pine needle extracts to GPC columns reduced significantly the lifetime of the rather expensive Biobeads S-X3 as these are reusable. Additionally, fine particles may clogg the column, increasing the need of maintenance. SPE sorbents on the other side are disposable and therefore, the same problems don't arise, as sorbent is discarded after use. Therefore, first SPE was performed followed by GPC. Due to use-time restrictions of the GC-MS and based on the recoveries obtained, only florisil glass columns and alumina columns and cartridges followed by GPC cleanup were tested. For florisil columns the additional GPC step only increased the recoveries of BFRs and PAHs and even slightly decreased recoveries of PCBs and musks. A plausible reason for this behaviour could not be found, but may be due to the fact that these two classes of compunds are the first to elute, according to the elution profile established in Figure 6. Therefore, they may be partly coeluted with the interferents during GPC cleanup. For alumina as sorbent, the additional GPC cleanup on the other hand improved recoveries for all compounds. This improvement was more visible for the cartridges as they may contain more interferents and less for alumina glass columns, which already delivered a quite clean, extract.

Ratola et al. (2009) used alumina cartridges for PAHs cleanup, obtaining recoveries of 10-130%, while Gorshkov et al. (2008) used silica cartridges for the same purpose with a recovery of 85%. These values are not in the same range as the ones obtained in this study since with cartridges recovery values for florisil are less than 10% and for alumina less than 20%. With columns, most authors use a SPE followed by GPC methodology and recovery values are generally better. Sun et al. (2010) obtained between 60.5 and 96.8% recovery with silica/alumina (2:1), Liu et al. (2006) 80 to 110% with silica/alumina, Schroter-Kermani et al. (2006) obtained 70-120% with silica and Hubert et al. (2003) obtained 40-100% for florisil. In this study the SPE alumina columns recovery values are in the same range as the previous mentioned authors, however, a SPE column with silica and alumina proves to be a good combination to be performed in further tests.

Regarding BFRs cleanup in cartridges Ratola et al. (2011) reported recovery values between 99 and 139% with alumina cartridges followed by florisil columns. With SPE columns, Tian et al. (2012) obtained 62.5-142% recovery with alumina/silica, Sajwan et al. (2009) obtained 100% with silica and Chen et al. (2009) obtained 83.8-107.5% with alumina. In this study for BFRs the best results were obtained with florisil columns followed by GPC with recovery values near 80%. However, the alumina column followed by GPC seems to be the most suitable methodology to obtain all four classes, even if for this class it's only possible to obtain recoveries of 40%.

Regarding PCBs cleanup in cartridges Oberg and Peltola (2009) obtained 30-70% recovery with florisil cartridges followed by GPC. With SPE columns, Romanic and Krauthacker (2007) obtained 34-57% recovery with silica and Grimalt and Drooge (2006) obtained 79-99% recovery with silica/alumina. In this work, alumina columns followed by GPC, out of all tested methods, showed to deliver better recovery values, near 30%.

Resuming, alumina as sorbent delivered generally higher recoveries, especially when followed by an additional GPC cleanup. Glass columns performed better than cartridges of the same kind and therefore alumina glass columns with GPC cleanup will be chosen for further optimisation.

4.2.2   Elution solvent optimisation

Comparison of SPE methodologies allowed to chose the most suitable sorbent and device – alumina glass columns, as well as to decide in favour of an additional GPC cleanup step. As could be seen from the elution profile illustrated in Figure 6, alumina retained irreversibly musks. However, SPE assays displayed in Figure 8, showed that in fact alumina columns allowed the highest recoveries of this class of compounds. As explained before, matrix influenced significantly the elution and recovery of these compounds. Therefore, the need of the additional 50 mL DCM after the 50 mL DCM/Hex (1:1) elution solvent was put in question, as the coextractives contained in the matrix (lipids, waxes etc.) would be

eventually enough to completly elute musks. Additionally, during SPE assays, it was verified that only at the moment DCM was applied, visible intereferents (mainly chlorophylls) were leached. For this, elution only with 50 mL DCM/Hex (1:1) was tested against the until now used elution solvents combination (50 mL DCM/Hex (1:1) + 50 mL DCM).
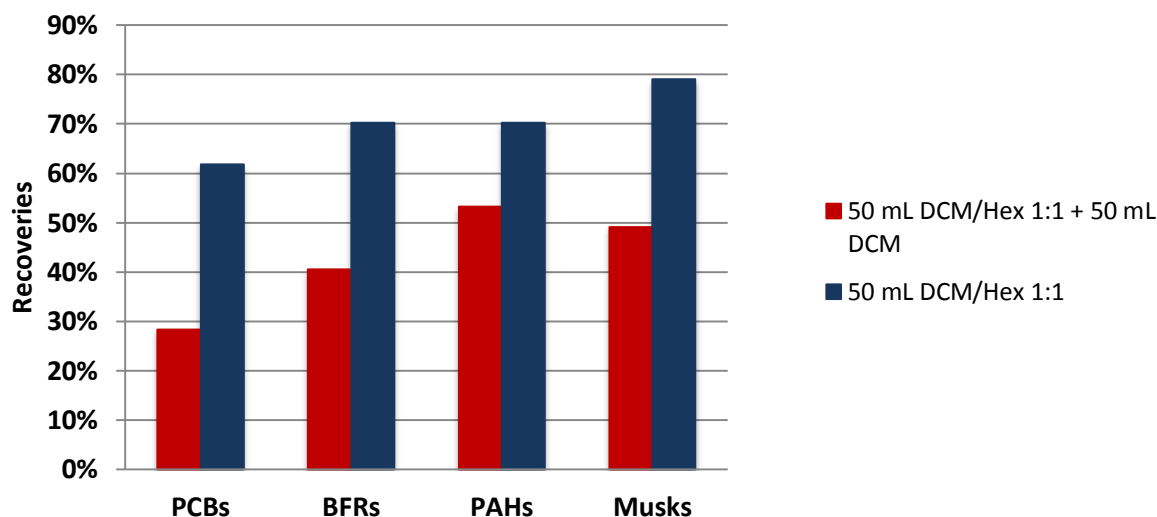
Figure 9 – Comparison of different elution solvents '50 mL DCM/Hex + 50 mL DCM' and '50 mL DCM/Hex' in SPE alumina glass column followed by GPC

As can be seen in Figure 9, elution with 50 mL DCM/Hex (1:1) is enough to elute all compounds and overall recoveries were higher. Comparing chromatograms for both methods, Figure 10 for BFRs and PCBs and Figure 11 for PAHs and musks, showed a possible reason for this. Chromatograms of DCM/Hex are 'cleaner' and peaks are better defined, which clearly improved detection and allows a better quantification. Highest average recoveries were obtained for musks, 79%, followed by 70% for the BFRs, 58% for the PAHs and 48% for the PCBs.

The stepwise optimisation, starting from elution profiles as point of departure, to SPE device and sorbent testing (with/without GPC) and final solvent optimisation, allowed obtaining a final analytical method. Samples of pine needles were extracted by an already defined extraction using 100 mL DCM/Hex (1:1) for 30 min in an ultrasonic bath. After solvent evaporation, cleanup will be performed using glass columns packed with 5 g of alumina and elution with 50 mL DCM/Hex (1:1). After another solvent evaporation, GPC columns containing 6 g of Biobeads S-X3 will be employed as additional cleanup. Finally, after solvent evaporation to dryness, the analyte will be taken-up in 100 μL of hexane and analysed using both GC-MS methods.
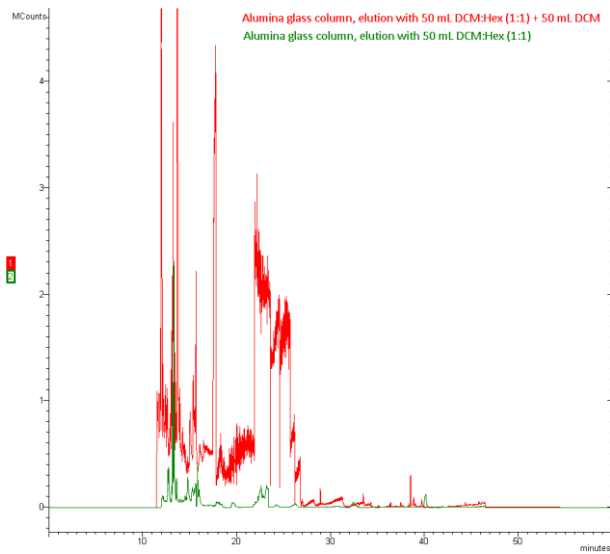
Figure 10 – Chromatogram obtained for alumina glass columns with DCM/Hex plus DCM in red and DCM/Hex in green for BFRs and PCB
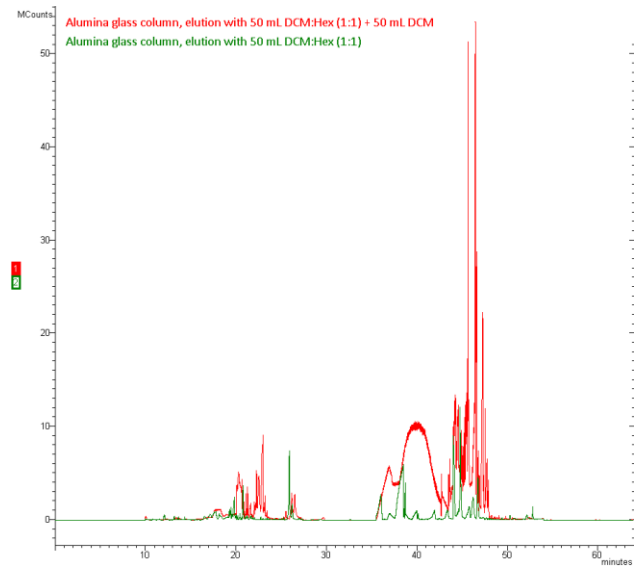
Figure 11 - Chromatogram obtained for alumina glass columns with DCM/Hex plus DCM in red and DCM/Hex in green for PAHs and Musks

4.3   Method validation

The identification of the 44 target compounds (13 PAHs, 11 musks, 11 PCBs and 9 BFRs) was based on their retention time and the identification ions displayed in Table 3 and Table 4. Detection was made using a time-scheduled selected ion storage (SIS) and coeluting compounds were separated based on their distinct target ions. As an example, Figure 12 and Figure 13 show the chromatograms of a standard obtained for BFRs and PCBs (Figure 12) and PAHs and Musks (Figure 13).
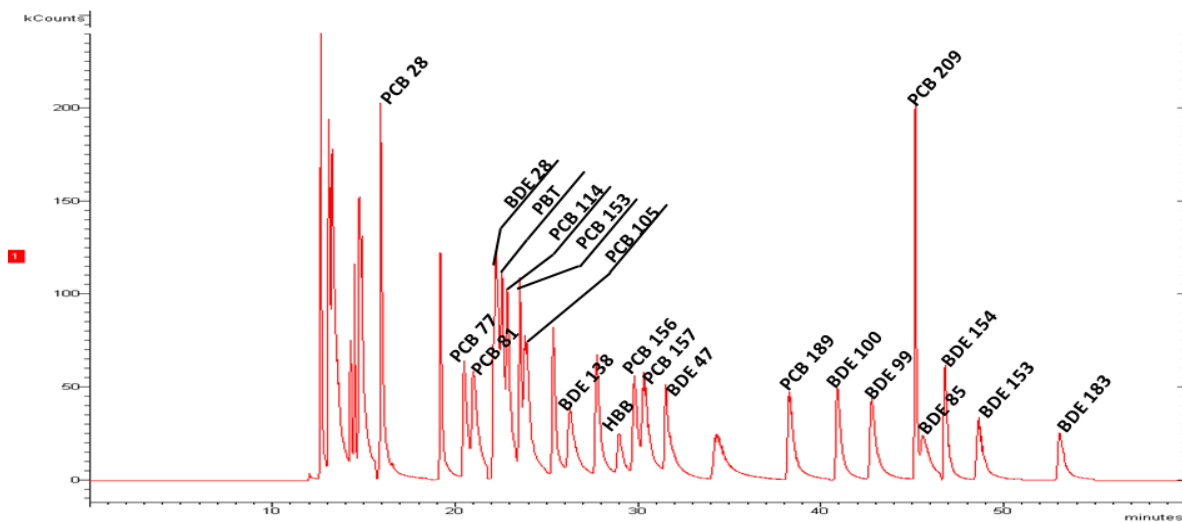
Figure 12 - SIS chromatogram of a standard solution of BFRs and PCBs (500 μg/L in hexane)
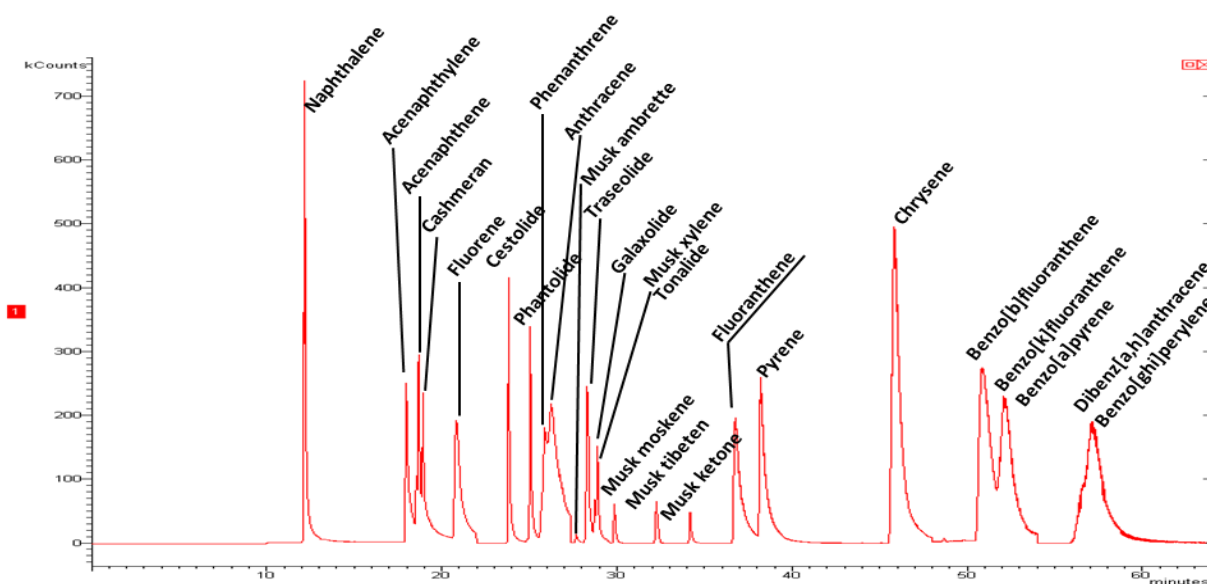
Figure 13 - SIS chromatogram of a standard solution of PAHs and Musks (1250 µg/L in hexane)

To verify that the developed cleanup method was suitable for the quantification of the selected compounds in pine needles, method quality parameters were evaluated for each compound.

4.3.1 Validation parameters

A calibration curve was obtained for each compound, by injections in duplicate of mixed standards at eight calibration levels. As can be seen further ahead, the analysed pine needles contained the studied compounds in very different concentration ranges within the same class. Limits of detection (LODs) and limits of quantification (LOQs) were calculated based on a signal-to-noise ratio of three (S/N = 3) and ten (S/N = 10), respectively. Recoveries were assumed to be 100% so that units could be converted from µg/L to ng/g.

For the PAHs class, calibration curves were obtained for each compound at the following calibration levels 25, 50, 100, 250, 500, 800, 1250 and 2000 µg/L. Results are presented in Table 5.

Table 5 – Validation parameters for the PAHs

Polynuclear aromatic hydrocarbons	Linearity range (μg/L)	R^2	LOD (ng/g)	LOQ (ng/g)	% Rec
Naphthalene	25-2000	0.999	0.004	0.015	22%
Acenaphthylene	25-2000	0.992	0.063	0.208	22%
Acenaphthene	25-2000	0.987	0.067	0.222	15%
Fluorene	25-2000	0.999	0.038	0.128	66%
Phenanthrene	25-2000	0.973	0.029	0.097	18%
Anthracene	25-2000	0.977	0.029	0.098	86%
Fluoranthene	25-2000	0.998	0.038	0.125	55%
Pyrene	25-2000	0.999	0.011	0.038	131%
Chrysene	25-2000	0.999	0.077	0.256	84%
Benzo[b+k]fluoranthene	250-2000	0.998	0.197	0.658	78%
Benzo[a]pyrene	500-2000	0.978	1.154	3.846	75%
Dibenz[a,h]anthracene	50-1250	0.969	0.048	0.159	109%
Benzo[ghi]perylene	50-1250	0.996	0.060	0.200	82%
				Average:	65%

For the PAHs class, the method exhibited a direct proportional relationship between the amount of each analyte and peak area with correlation coefficients $R^2 \geq 0.969$. LODs varied between 0.004 ng/g (for Naphthalene) and 1.154 ng/g (for Benzo[a]pyrene) while LOQs varied between 0.015 and 3.846 ng/g. The average recovery value for PAHs is 65% with the lower recovery for Acenaphthene (15%) and the highest for Pyrene (131%).

LODs obtained for this work are in the same range as reported by some some authors. Amigo et al. (2011) and Ratola et al. (2011) obtained LODs ranging between 0.08 ng/g (Fluoranthene) and 1.53 ng/g (Dibenz[a,h]anthracene). Librando et al. (2002) and Holoubek et al. (2000) reported LODs of 0.1 ng/g for every compound, Gorshkov (2008) obtained LOD values of 0.06 ng/g for the 16 EPA PAHs except for Fluorene (0.1 ng/g) and Piccardo et al. (2005) showed a LOD of 0.02 ng/g for each compound. However, Liu et al. (2006) reported higher LOD for the same compounds in this study, between 3.3 and 7.8 ng/g.

For Musks class calibration curves were obtained similarly as for PAHs at the following calibration levels 25, 50, 100, 250, 500, 800, 1250 and 2000 μg/L. Results are presented in Table 6.

Table 6 – Validation parameters for the Synthetic Musks

Musks	Linearity range (µg/L)	R²	LOD (ng/g)	LOQ (ng/g)	% Rec
Cashmeran	25-2000	0.986	0.068	0.227	42%
Celestolide	25-2000	0.999	0.013	0.042	85%
Galaxolide	25-2000	0.997	0.022	0.074	108%
Phantolide	25-2000	0.998	0.019	0.062	89%
Tonalide	25-2000	0.990	0.009	0.028	88%
Traseolide	25-2000	0.997	0.031	0.104	57%
Musk ambrette	50-2000	0.989	0.091	0.303	100%
Musk ketone	25-2000	0.992	0.042	0.139	70%
Musk moskene	25-2000	0.942	0.079	0.263	75%
Musk tibeten	25-2000	0.996	0.045	0.152	69%
Musk xylene	25-2000	0.986	0.024	0.079	87%
				Average:	79%

For the Musks class the method exhibited a direct proportional relationship between the amount of each analyte and the peak area with correlation coefficients $R^2 \geq 0.942$. LODs varied between 0.009 (for tonalide) and 0.091 ng/g (for musk ambrette) while LOQs varied between 0.028 and 0.303 ng/g. The average recovery value for Musks is 79% with the lower recovery for Cashmeran (42%) and the highest for Galaxolide (108%). Again, there are no studies with musk in pine needles, so it is not possible to compare the LOD values obtained.

Also for PCBs, calibration curves were made for each compound at the following concentration levels: 5, 10, 50, 100, 200, 350, 500 and 1000 µg/L. Results are presented in Table 7.

Table 7 – Validation parameters for the PCBs

Polychlorinated biphenyls	Linearity range (µg/L)	R²	LOD (ng/g)	LOQ (ng/g)	% Rec
PCB 28	50-350	0.966	0.071	0.238	102%
PCB 77	50-1000	0.988	0.006	0.019	59%
PCB 81	50-1000	0.995	0.006	0.019	59%
PCB 105	100-1000	0.968	0.667	2.222	20%
PCB 114	10-500	0.996	0.100	0.333	92%
PCB 138	50-350	0.931	0.019	0.062	137%
PCB 153	50-1000	0.931	0.003	0.009	60%
PCB 156	200-1000	0.980	0.364	1.212	33%
PCB 157	50-1000	0.993	0.031	0.104	60%
PCB 189	50-1000	0.986	0.055	0.182	134%
PCB 209	50-1000	0.999	0.001	0.002	108%
				Average:	78%

For the PCBs class the method exhibited a direct proportional relationship between the amount of each analyte and the peak area with correlation coefficients $R^2 \geq 0.931$. LODs varied between 0.001 (for PCB 209) and 0.667 ng/g (for PCB 105) while LOQs varied between 0.002 and 2.222 ng/g. The average recovery value for PCBs is 78%, being the lowest recovery for PCB 105 (20%) and the highest for PCB 138 (137%). Overall LODs obtained for this work are in the same range as some previous authors reported, although for some congeners they are a slightly higher. Grimalt and Drooge (2006) obtained LODs between 0.05 and 0.1 ng/g for 6 congeners, Xu et al. (2004) obtained LODs between 0.0004 and 0.09 ng/g for 15 congeners and Holoubek et al. (2000) obtained LODs of 0.1 ng/g for only 3 congeners.

For BFRs, calibration curves were obtained for each compound at concentration levels 5, 10, 50, 100, 200, 350, 500 and 1000 µg/L. Results are presented in Table 8.

Table 8 – Validation parameters for the BFRs

Brominated flame retardants	Linearity range (µg/L)	R^2	LOD (ng/g)	LOQ (ng/g)	%Rec
BDE 47	50-1000	0.997	0.001	0.004	100%
BDE 85	50-1000	0.994	0.010	0.035	82%
BDE 99	50-1000	0.996	0.125	0.417	103%
BDE 100	50-1000	0.994	0.250	0.833	106%
BDE 153	10-1000	0.994	0.046	0.154	102%
BDE 154	5-1000	0.998	0.0003	0.001	94%
BDE 183	5-1000	0.992	0.030	0.100	95%
PBT	5-1000	0.994	0.043	0.143	23%
HBB	5-1000	0.991	0.0002	0.001	63%
				Average	85%

For the BFRs class the method exhibited a direct proportional relationship between the amount of each analyte and the peak area with correlation coefficients $R^2 \geq 0.991$. LODs varied between 0.0002 ng/g (for HBB) and 0.250 ng/g (for BDE 100) while LOQs varied between 0.001 and 0.833 ng/g. Average recovery for BFRs is 85%, with lowest recovery for PBT (23%) and the highest for BDE 100 (106%).

LODs obtained for this work are in the same range as some authors previously reported. Ratola et al. (2011) related LOD between 0.011 and 0.070 ng/g for 7 congeneres, however, for BDE 209 the limit of detection is higher (0.232 ng/g). Chen et al. (2009) also reports a range of 0.003 and 0.014 ng/g for 7 congeneres and for BDE 209 a LOD of 0.222 ng/g. In this study, however, this congener was not studied.

4.4   Sample analysis

The aim of the development of analytical methods for BFRs, PCBs, PAHs and Musks was to analyse pine needle samples. These samples were mostly from *Pinus pinaster* and collected at two sampling campaings (spring and autumn) throughout Portugal in the ambit of Safe Pine's project tasks. However, given the reduced time available for the development of this thesis and the difficulties and limitations found during the method development, it was only possible to analyse one first sample of the screening campaign. Nevertheless, this analytical method will be used with other samples.

Figure 14 and Figure 15 show the chromatograms of the analysis of BFRs/PCBs and PAHs/Musks respectively in *Pinus pinaster* pine needles according to the developed analytical methodology.
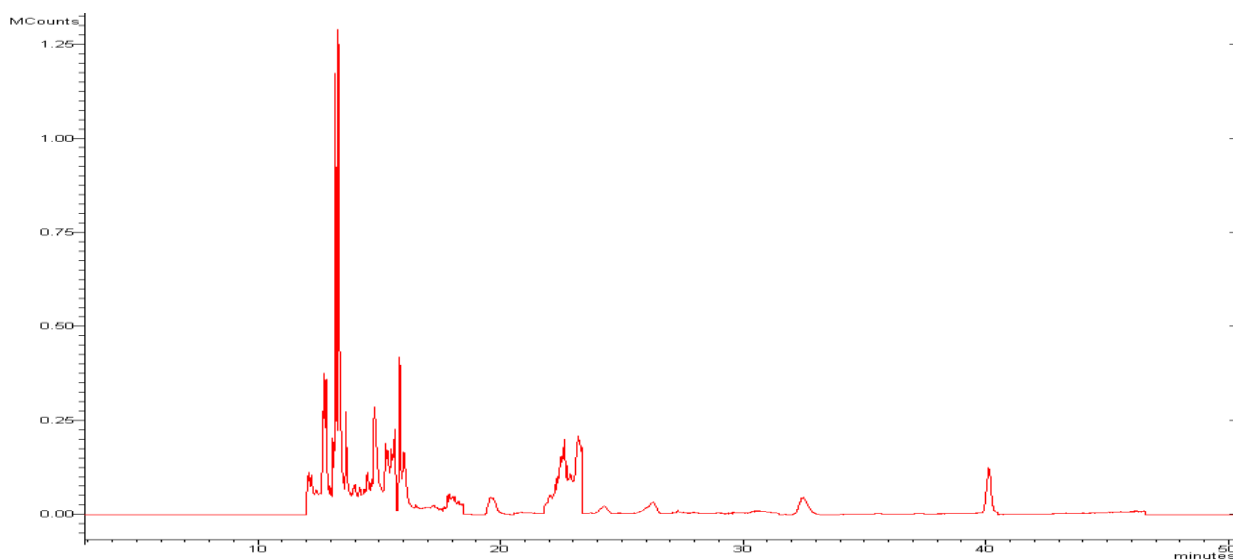
Figure 14 – Chromatogram of <u>Pinus pinaster</u> needles using the BFRs and PCBs method
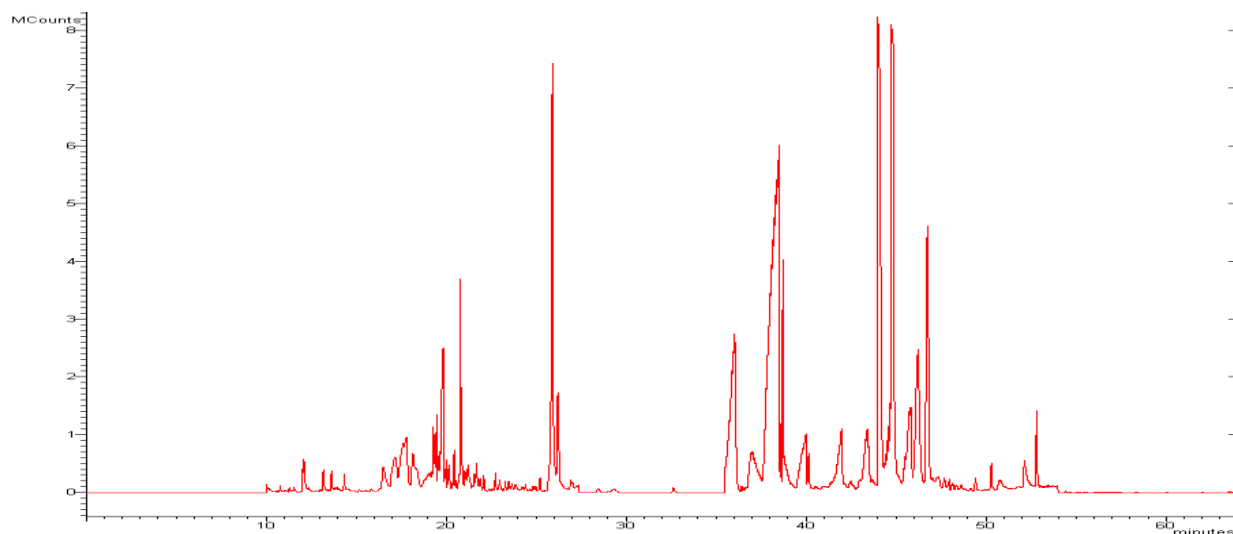
Figure 15 – Chromatogram of <u>Pinus pinaster</u> needles using the PAHs and Musks method

The concentrations (ng/g) obtained for Polychlorinated Biphenyls and Brominated Flame Retardants in *Pinus pinaster* pine needles are presented in Table 9 and Table 10, respectively.

Table 9 - Concentrations (ng/g) of Polychlorinated Biphenyls in Pinus pinaster needles

Compound	Concentration (ng/g)
PCB 28	0.23
PCB 77	2.11
PCB 81	0.04
PCB 105	<LOD (0.23)
PCB 114	0.24
PCB 138	0.27
PCB 153	0.75
PCB 156	<LOD (0.18)
PCB 157	0.08
PCB 189	0.56
PCB 209	n.d.
Total	4.28

(n.d.-not detected)

Table 10 - Concentrations (ng/g) of Brominated Flame Retardants in Pinus pinaster needles

Compound	Concentration (ng/g)
BDE 47	n.d.
BDE 85	0.56
BDE 99	0.34
BDE 100	<LOD (0.22)
BDE 153	0.05
BDE 154	0.02
BDE 183	<LOD (0.02)
PBT	<LOD (0.03)
HBB	n.d.
Total	0.97

(n.d.-not detected)

The concentrations of individual PCB congeners ranged between 0.04 and 2.11 ng/g with a total value of 4.28 ng/g for all 11 PCBs presented in Table 9. The higher concentration was found for PCB congener 77 with 2.11 ng/g, followed by congener 153 with 0.75 ng/g and congener 189 with 0.56 ng/g. PCBs congeners with higher incidence, reported in the literature, are congeners number 28, 52, 101, 138, 153 and 180. However, congeners 52, 101 and 180 were not studied in this project. Regarding the reported concentrations in literature, Öberg and Peltola (2009) in Sweden found concentrations ranging 0.24 to 1.6 ng/g-dw with a higher incidence for congeners 28, 52, 101, 118, 138, 153 and 180. With similar results, Klánová et al. (2009) in Czech Republic found high concentrations of PCB congener numbers 28, 52, 153 and 101. Rappolder et al. (2007) in Germany found concentrations between 0.3 and 1.1 ng/g-dw where the highest concentrations also belonged to PCB congener 153, followed by congeners 28, 52, 101 and 138. Grimalt and Drooge (2006) in Spain found concentrations ranging 0.1 and 1.2 ng/g where the main PCBs congeners were 101 (27%) and 138 (20%). PCBs concentrations found in this work are in the same range of magnitude with the highest level for PCB 77 which was not studied by the before mentioned authors.

Total concentration (ng/g) of 9 BFRs in pine needles is presented in Table 10. BDE 85 was the congener with the highest concentration (0.56 ng/g), followed by BDE 99 and 100. Among the BFRs, BDE 47 and HBB were not detected and BDEs 100, 183 and PBT were above LOD. In early studies regarding PBDEs in pine needles, Ratola et al. (2011) found concentration ranging between 0.027 and 13.04 ng/g-

dw in Spain where BDE 47 was the most frequently detected congener. Additionally, it was shown that the highest BFRs concentrations were found for BDE 209 > BDE 47 > BDE 99 > BDE 183. Kannan et al. (2009) reported a total concentration for 9 congeners reaching 1 ng/g-dw in Korea. For 15 BFRs, Tian et al. (2012) detected BDE 209, 47, 99 and 183 at concentrations between 40.4 and 546 ng/g-dw in pine needles from an e-waste site in China. In the USA, Sajwan et al. (2009) observed levels between 0.05 and 0.49 ng/g-dw for a total of 11 PBDE congeners near a LCP Superfund Site, where congener 99 was the most common one. Comparing this study, BDE 99's high concentration is in accordance with the published results. Also, Piccardo et al. (2005) mentioned that the morphological and physiological discrepancies between different conifer species could play an important role in the uptake of persistent organic pollutants, so the differences between BDEs were already expected since there are no BDE studies in *Pinus pinaster* needles.

In *Table 11* and Table 12 are presented the concentrations (ng/g) obtained for Polynuclear aromatic hydrocarbons (PAHs) and Synthetic musks respectively in *Pinus pinaster* needles.

Table 11 - Concentrations (ng/g) of Polynuclear aromatic hydrocarbons in <u>Pinus pinaster</u> needles

Compound	Concentration (ng/g)
Naphthalene	11.44
Acenaphthylene	21.38
Acenaphthene	1.86
Fluorene	51.64
Phenanthrene	291.02
Anthracene	19.66
Fluoranthene	46.53
Pyrene	158.27
Chrysene	28.20
Benzo[b+k]fluoranthene	2.82
Benzo[a]pyrene	1.66
Dibenz[a,h]anthracene	0.05
Benzo[ghi]perylene	<LOD (0.02)
Total	634.53

Table 12 - Concentrations (ng/g) of Synthetic musks in <u>Pinus pinaster</u> needles

Compound	Concentration (ng/g)
Cashmeran	12.15
Celestolide	n.d.
Galaxolide	8.06
Phantolide	5.78
Tonalide	1.26
Traseolide	0.68
Musk ambrette	n.d.
Musk ketone	<LOD (0.03)
Musk moskene	0.05
Musk tibeten	n.d.
Musk xylene	0.05
Total	28.03

(n.d. –not detected)

PAH concentrations in pine needles are presented in *Table 11*. The concentrations of 13 PAHs, classified as priority pollutants by US EPA, ranged from 0.05 ng/g to 291.02 ng/g with a total concentration of 634.53 ng/g. PAHs compounds with higher concentrations are phenanthrene (291.02 ng/g), pyrene (158.27 ng/g), fluorene (51.64 ng/g) and fluoranthene (46.53 ng/g). The values or ranges of contamination by PAHs reported in literature for pine needles are similar to the levels presented in

this study as well as the higher incidence compounds. Ratola et al. (2009) reported values between 213 and 1773 ng/g-dw where phenanthrene was the most abundant PAH, followed by fluoranthene, naphthalene and pyrene and in 2010 Ratola et al. showed that *Pinus pinaster* needles revealed higher mean entrapment levels than *Pinus pinea* (748 and 399 ng/g-dw respectively). Amigo et al. (2011) obtained mean values for the sum of 16 PAHs ranging from 96 ng/g-dw for remote sites to 866 ng/g-dw for industrial sites for *Pinus pinaster*. Piccardo et al. (2005) reported values between 10.41 and 817.41 ng/g-dw for the same pine species. Regarding other pine species, Tomashuk et al. (2012) found a range of 127-6111 ng/g-dw and an average of 4187 ng/g-dw in a metropolitan area in the USA, whereas Augusto et al. (2010) obtained values between 83.0 and 466.8 ng/g-dw with an average of 185.4 ng/g-dw for the 16 EPA PAHs in Portugal with higher incidence in phenanthrene (67.7 ng/g), fluoranthene (24.0 ng/g), pyrene (20.7 ng/g), naphthalene (18.6 ng/g) and fluorene (15.1 ng/g). Liu et al. (2005) reported a range between 280 and 3000 ng/g-dw of the 16 EPA PAHs minus naphthalene in Pinus *massoniana* from heavily populated regions of China, however, Tian et al. (2008) for the same needle species and number of PAHs found concentrations between 503.3 and 1172.6 ng/g-dw in the Pearl River Delta, China.

Regarding musks concentrations in pine needles, results are presented in Table 12. Higher concentrations were obtained for cashmeran (12.15 ng/g), galaxolide (8.06 ng/g), phantolide (5.78 ng/g) and tonalide (1.26 ng/g). To the author's best knowledge, there are no studies on the existence of musks in vegetation. Therefore it is not possible to compare the obtained results with published data. However, musks are compounds that are incorporated in products used in a daily basis as for example perfume, body lotions, detergents, air fresheners, etc. This means, they are introduced in the environment continuously. In personal care products Homem et al. (2013) found concentrations ranging from 2 ng/g in toothpastes to 882340 ng/g in perfumed body lotions where the most frequently detected musks were galaxolide (83%), cashmeran and exaltolide (75%). Taking into account that galaxolide and cashmeran were the musks found in higher concentrations, it can be supposed that these products can be a primary source of these compounds, either volatizing directly to the air or being disposed to the sewage system. Because of their bioaccumulative, lipophilic and difficult degradation nature, when they reach the wastewater treatment plants, they can't be totally removed. This means that, besides the air, wastewater is the first point where musks are spread into the environment. Lee et al. (2010) found musk ketone, musk xylene, galaxolide and tonalide in effluent wastewaters with concentrations ranging 960-2690 ng/L, whereas Yang and Metcalfe (2006) found concentrations ranging 4.8-390.2 ng/L for musk ambrette, musk xylene, musk moskene, musk tibetene, musk ketone, cashmeran, celestolide, phantolide, traseolide, galaxolide and tonalide in influent wastewaters. Overall, synthetic musks are found in high level in sewage waters and wastewaters proving that treatment processes are not efficient to remove such compounds and that from these sites, they're able to migrate

to other environmental systems. In these matrices, the higher concentrations found correspond to some of the musks found in pine needles in this study. In surface waters they're also detected with levels ranging 0.025-1141 ng/L (Villa et al., 2012) and in ground waters in levels of 270-573 ng/L (Arbulu et al., 2011) for the same musks found in sewage waters. Due to their high vapour pressure values, musks may volatilize from contaminated water basis contaminating air and being able to be transported to other places. Nevertheless, their presence in the air is also due to volatilization during use and production of personal care products. Musks compounds were found in a wastewater aeration basin with concentrations ranging 2 and 344306 ng/m^3 for galaxolide, musk xylene, musk ketone, tonalide and celestolide (Upadhyay et al., 2011), in indoor air with concentration ranging 2.6 – 1129 ng/m^3 for the same musks plus cashmeran, phantolide, traseolide and musk moskene (Regueiro et al., 2009) and in industrial air outside a cosmetics plant with concentrations of 14.89 ng/m^3 for cashmeran, celestolide, phantolide, traseolide, tonalide and galaxolide (Chen et al., 2007). Their presence in the air allows them to deposit in other water masses, sediments or plants. Chase et al. (2012) found concentrations of 8 musks compounds in sediments with concentrations between 10.24 and 24.12 ng/g and Hu et al. (2011) found concentrations of tonalide, galaxolide, celestolide, phantolide, traseolide, musk ketone and musk xylene in the same matrix with concentrations ranging 1.5 and 32.3 ng/g. The presence of musks in these different matrices shows that their volatilization cycle plays a major role in their transport through the environment and their persistence includes them in the persistent organic pollutants (POPs) group.

5 Conclusions

This study allowed us to obtain a multiresidue method for the simultaneous analysis of several semivolatile pollutant compounds of current concern (PAHs, PCBs, BFRs and Musks). Due to the different physico-chemical properties (polarity, solubility, boiling and melting point, vapour pressure, etc.) of the analysed pollutants and in order to obtain acceptable overall results in terms of performance and restrain on resources consumption, the need to find a compromise was necessary. As to the authors' best knowledge no studies concerning synthetic musks in vegetation were made, focus was given to the musk class.

Based on the overall results the alumina packed glass columns yielded the most satisfactory results for all classes. Regarding solvent, a single elution with 50 mL DCM/Hex is sufficient and yielded higher recoveries than a combined elution of 50 mL DCM/Hex + 50 mL DCM. The additional cleanup with GPC columns containing 6 g of Biobeads S-X3 delivered a cleaner extract as seen by the chromatograms and improved recoveries.

Total PCBs and BFRs amount was 4.28 and 0.97 ng/g w.w, respectively, where the highest levels were found for PCB congener numbers 77, 153 and 189 and BDE 85 and 99. Total PAHs and Musks amount was 634.53 and 28.03 ng/g w.w, respectively. PAHs showed the highest levels, of which Phenanthrene and Pyrene comprise more than 60%. Musks were found in the sample, namely Cashmeran (12.15 ng/g) and Galaxolide (8.06).

For the first time musks were detected in vegetation samples and this demonstrates the ability to use pine needles not only as biosampler for already known pollutants like PAHs, PCBs and BFRs, but also for this new class of emergent pollutants.

Results of this analytical method development and sample analysis allowed the presentation of a poster at the 14th EuCheMS International Conference on Chemistry and the Environment which took place from 25-28 of June, 2013 at Barcelona, Spain (poster presentend in Appendix C).

Pine needles are a complex type of matrix and their cleanup is always difficult, especially when target analytes of 4 different chemical classes were chosen. Therefore, the developed work during this thesis realisation was an ambitious and challenging undertaking in order to meet this research group's high standards. Yet, it was a rewarding learning process of new analytical techniques and the difficulties that have to be overcome to achieve results.

6 Limitations and Future Work

Throughout this work it was noticed that more tests and information would be of great value about the topic addressed in this dissertation. However, due to time, material and resources restraints no further studies could be performed and will be presented hereby as suggestion for future work.

The author is aware that this analytical method still has some potential for further improvements and these results, namely recoveries, may be improved if further tests will be done. One important issue is related with sorbent activation as until now only completely activated sorbents were used. Once alumina showed to be the most suitable sorbent, different deactivation levels should be tested and their effect on elution and cleanup of extracts.

SPE cartridges with the same diameter should also be tested in order to verify if this device is suitable for this kind of cleanup as it may offer some handling advantages over glass columns.

Due to the high cost and limited availability of mass-labelled standards (deuterium-labelled PAHs or musks or 13C-PCBs or 13C-PBDEs), no internal standards were used. However, the use of an internal standard is a good practice and may improve recoveries, as it will account for loss of analyte during handling (e.g. volatilization).

Finally and once the analytical method is completely optimised, tests with other kind of pine needles, namely *Pinus pinea* or *Pinus sylvestris* should be performed in order to verify this method's suitability to other kind of samples.

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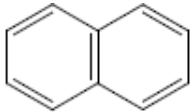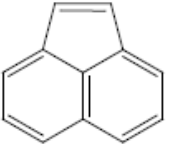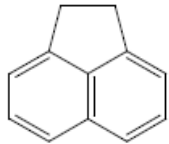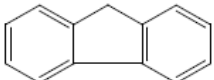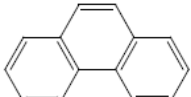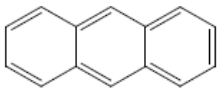
APPENDIX A – PROPERTIES

A1 PAHs PROPERTIES

Table A. 1 - Chemical structures and characteristics of the Polynuclear aromatic hydrocarbons (PAHs)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
Naphthalene 91-20-3 $C_{10}H_8$ 128.18		3.30	31.0000	217.9	1.133×10^1	4.40×10^{-4}	80.2	*2.960*	*5.190*
Acenaphthylene 20-89-68 $C_{12}H_8$ 152.20		3.94	16.1000	280.0	8.910×10^{-1}	1.14×10^{-4}	92.5	*3.750*	*6.272*
Acenaphthene 83-32-9 $C_{12}H_{10}$ 154.21		3.92	3.9000	279.0	2.860×10^{-1}	1.84×10^{-4}	93.4	*3.590*	*6.310*
Fluorene 86-73-7 $C_{13}H_{10}$ 166.22		4.18	1.6900	295.0	8.000×10^{-2}	9.62×10^{-5}	114.8	*3.700*	*6.790*
Phenanthrene 85-01-8 $C_{14}H_{10}$ 178.24		4.46	1.1500	340.0	1.600×10^{-2}	4.23×10^{-5}	99.2	*4.350*	*7.570*
Anthracene 120-12-7 $C_{14}H_{10}$ 178.24		4.45	0.0434	339.9	8.706×10^{-4}	5.56×10^{-5}	215.0	*4.310*	*7.550*

Table A. 1 - Chemical structures and characteristics of the Polynuclear aromatic hydrocarbons (PAHs) (Continuation)
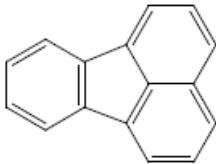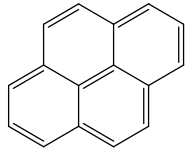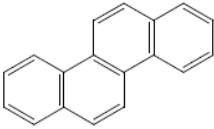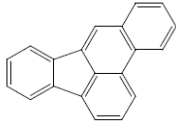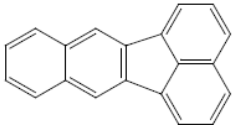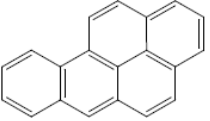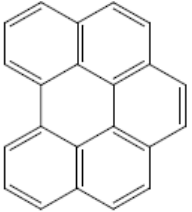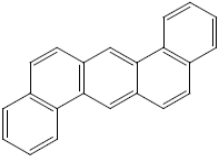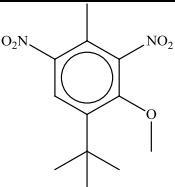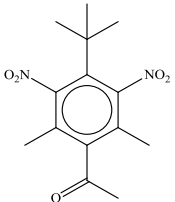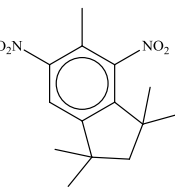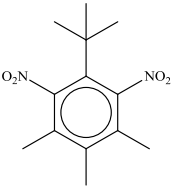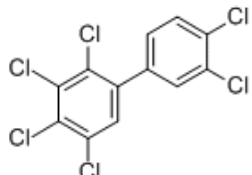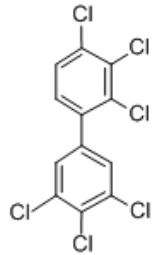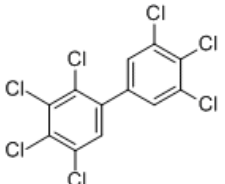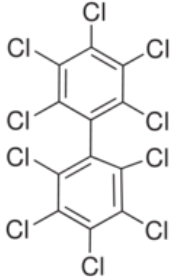
Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
Fluoranthene 206-44-0 $C_{16}H_{10}$ 202.26		5.16	0.2600	384.0	1.229×10^{-3}	8.86×10^{-6}	107.8	*4.800*	*8.880*
Pyrene 129-00-0 $C_{16}H_{10}$ 202.26		4.88	0.1350	404.0	5.999×10^{-4}	1.19×10^{-5}	151.2	*4.900*	*8.880*
Chrysene 218-01-9 $C_{18}H_{12}$ 228.30		5.81	0.0020	448.0	8.306×10^{-6}	5.23×10^{-6}	258.2	*5.042*	*9.480*
Benzo[b]fluoranthene 205-99-2 $C_{20}H_{12}$ 252.32		5.78	0.0015	*442.8*	6.666×10^{-5}	6.57×10^{-7}	168.0	*5.016*	*10.351*
Benzo[k]fluoranthene 207-08-9 $C_{20}H_{12}$ 252.32		6.11	0.0008	480.0	1.286×10^{-7}	5.36×10^{-11}	217.0	*5.302*	*10.732*

Table A. 1- Chemical structures and characteristics of the Polynuclear aromatic hydrocarbons (PAHs) (Continuation)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
Benzo[a]pyrene 50-32-8 $C_{20}H_{12}$ 252.32		6.13	0.0016	495.0	7.319×10^{-7}	4.57×10^{-7}	176.5	*5.320*	*10.859*
Benzo[ghi]perylene 191-24-2 $C_{22}H_{12}$ 276.34		6.63	0.0003	>500.0	1.333×10^{-8}	3.31×10^{-7}	278.0	*5.754*	*11.499*
Dibenzo[a,h]anthracene 53-70-3 $C_{22}H_{14}$ 278.36		6.75	0.0025	524.0	1.273×10^{-7}	1.41×10^{-7}	269.5	*5.676*	*11.779*

A2 – MUSKS PROPERTIES

Table A. 2 - Chemical structures and characteristics of Synthetic Musks

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour Pressure (Pa) (25 °C)	k_H (atm.m³.mol⁻¹) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
Musk ambrette (MA) 83-66-9 $C_{12}H_{16}N_2O_5$ 268.27		4.17	1.670	*367.93*	1.746×10^{-3}	1.41×10^{-8}	131.2	*3.726*	*10.409*
Musk ketone (MK) 81-14-1 $C_{14}H_{18}N_2O_5$ 294.31		4.60	1.900	*401.75*	5.333×10^{-3}	4.8×10^{-10}	135.5	*3.937*	*12.007*
Musk moskene (MM) 116-66-5 $C_{14}H_{18}N_2O_4$ 278.31		5.39	0.166	*377.04*	2.280×10^{-4}	2.05×10^{-7}	132.5	*4.345*	*10.467*
Musk tibetene (MT) 145-39-1 $C_{13}H_{18}N_2O_4$ 266.30		5.18	0.295	*367.48*	5.813×10^{-4}	2.91×10^{-7}	135.5	*4.228*	*10.105*

Data from EPI SuiteTM v4.11 and in Italic from syrres, Interactive PhysProp Database Demo

Table A. 2 - Chemical structures and characteristics of Synthetic Musks (Continuation)
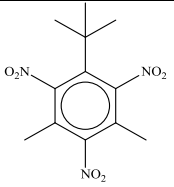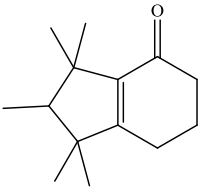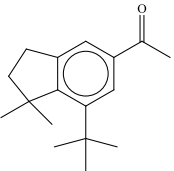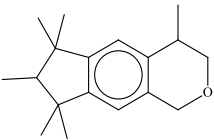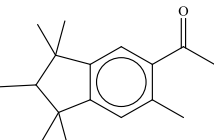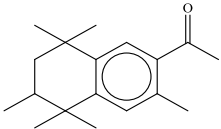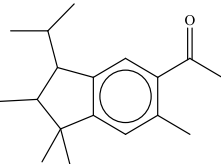
Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 ^{o}C)	Boiling point (^{o}C)	Vapour Pressure (Pa) (25 ^{o}C)	k_H (atm.m^3.mol^{-1}) (25 ^{o}C)	Melting point (^{o}C)	Log K_{oc}	Log K_{oa}
Musk xylene (MX) 81-15-2 $C_{12}H_{15}N_3O_6$ 297.27		4.45	0.472	411.56	8.466×10^{-5}	1.04×10^{-9}	110.00	3.825	11.821
Cashmeran (DPMI) 33704-61-9 $C_{14}H_{22}O$ 206.33		4.49	5.937	277.89	5.370×10^{-1}	1.42×10^{-4}	70.77	3.604	6.726
Celestolide (ADBI) 13171-00-1 $C_{17}H_{24}O$ 244.38		5.93	0.220	319.07	1.920×10^{-2}	3.18×10^{-5}	96.07	4.401	8.816
Galaxolide (HHCB) 1222-05-5 $C_{18}H_{26}O$ 258.41		5.90	1.750	325.00	7.266×10^{-2}	1.32×10^{-4}	102.64	4.098	8.168
Phantolide (AHMI) 15323-35-0 $C_{17}H_{24}O$ 244.38		5.85	0.255	317.61	1.950×10^{-2}	3.18×10^{-5}	98.68	4.357	8.736
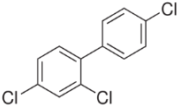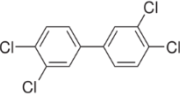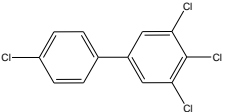
Table A. 2 - Chemical structures and characteristics of Synthetic Musks (Continuation)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 ^{o}C)	Boiling point (^{o}C)	Vapour Pressure (Pa) (25 ^{o}C)	k_H (atm.m^3.mol^{-1}) (25 ^{o}C)	Melting point (^{o}C)	Log K_{oc}	Log K_{oa}
Tonalide (AHTN) 1506-02-1 $C_{18}H_{26}O$ 258.41		5.70	1.250	331.88	2.520×10^{-2}	4.22×10^{-5}	106.87	4.274	7.945
Traseolide (ATII) 68140-48-7 $C_{18}H_{26}O$ 258.41		6.31	0.087	329.77	9.110×10^{-3}	1.94×10^{-5}	103.17	4.611	9.073

A3 – PCBs PROPERTIES

Table A. 3 - Chemical structures and characteristics of Polychlorinated biphenyls (PCBs)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 ^{o}C)	Boiling point (^{o}C)	Vapour Pressure (Pa) (25 ^{o}C)	k_H (atm.m^3.mol^{-1}) (25 ^{o}C)	Melting point (^{o}C)	Log K_{oc}	Log K_{oa}
PCB 28 7012-37-5 $C_{12}H_7Cl_3$ 257.54 2,4,4'-Trichlorobiphenyl		5.62	2.700×10^{-1}	*340.70*	2.600×10^{-2}	2.00×10^{-4}	*100.85*	*4.630*	*7.707*
PCB 77 32598-13-3 $C_{12}H_6Cl_4$ 291.99 3,3',4,4'-Tetrachlorobiphenyl		6.63	5.690×10^{-4}	*359.51*	2.186×10^{-3}	9.40×10^{-6}	*122.32*	*4.737*	*9.700*
PCB 81 70362-50-4 $C_{12}H_6Cl_4$ 291.98804 1,2,3-trichloro-5-(4-chlorophenyl)benzene		*6.34*	5.316×10^{-2}	359.51	1.506×10^{-1}	1.25×10^{-4}	122.32	4.576	8.632

Data from EPI SuiteTM v4.11 and in Italic from syrres, Interactive PhysProp Database Demo

Table A. 3 - Chemical structures and characteristics of Polychlorinated biphenyls (PCBs) (Continuation)
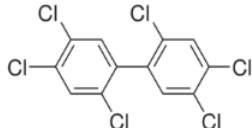
Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour Pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
PCB 153 35065-27-1 $C_{12}H_4Cl_6$ 360.88 2,2',4,4',5,5'-Hexachlorobiphenyl		7.75	9.500×10^{-4}	396.90	4.573×10^{-4}	2.30×10^{-5}	146.34	5.580	9.730
PCB 156 38380-08-4 $C_{12}H_4Cl_6$ 360.88 2,3,3',4,4',5-hexachlorobiphenyl		7.60	5.330×10^{-3}	396.90	2.146×10^{-4}	1.43×10^{-4}	146.34	5.273	9.833
PCB 157 69782-90-7 $C_{12}H_4Cl_6$ 360.88 2,3,3',4,4',5'-hexachlorobiphenyl		7.60	1.721×10^{-3}	396.90	1.032×10^{-2}	6.85×10^{-5}	146.34	5.273	10.153
PCB 189 39635-31-9 $C_{12}H_3Cl_7$ 395.32 2,3,3',4,4',5,5'-heptachlorobiphenyl		8.27	7.530×10^{-4}	415.60	1.733×10^{-5}	5.07×10^{-5}	163.50	5.644	10.953

Table A. 3 - Chemical structures and characteristics of Polychlorinated biphenyls (PCBs) (Continuation)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour Pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
PCB 209 2051-24-3 $C_{12}Cl_{10}$ 498.66 2,2',3,3',4,4',5,5',6,6'- PCB; Decachlorobiphenyl		8.27	7.430×10^{-6}	*471.68*	1.413×10^{-5}	9.40×10^{-3}	*199.37*	*5.644*	*8.685*

A4 - BFRs PROPERTIES

Table A. 4 - Chemical structures and characteristics of Flame Retardants (BFRs)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour Pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
BDE 47 5436-43-1 $C_{12}H_6Br_4O$ 485.80 2,2',4,4'-tetra-bromodiphenyl ether		6.77	1.460×10^{-3}	405.51	9.333×10^{-6}	2.97×10^{-6}	161.73	4.726	10.530
BDE 99 60348-60-9 $C_{12}H_5Br_5O$ 564,70 2,2',4,4',5-pentabromodiphenyl ether		7.66	7.860×10^{-5}	436.21	4.133×10^{-6}	1.18×10^{-6}	182.80	4.764	11.310
BDE 100 189084-64-8 564,7 2,2',4,4',6-pentabromodiphenyl ether		n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

(n.a. – not available)

Data from EPI SuiteTM v4.11 and in Italic from syrres, Interactive PhysProp Database Demo

Table A. 4 - Chemical structures and characteristics of Flame Retardants (BFRs) (Continuation)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 °C)	Boiling point (°C)	Vapour Pressure (Pa) (25 °C)	k_H (atm.m^3.mol^{-1}) (25 °C)	Melting point (°C)	Log K_{oc}	Log K_{oa}
BDE 138 182677-30-1 C$_{12}$H4Br$_6$O 643.59 2,2′,3,4,4′,5′- Hexabromodiphenyl ether		8.55	4.150x10^{-6}	466.91	3.733x10^{-7}	4.71x10^{-7}	197.14	5.710	13.265
BDE 153 68631-49-2 2,2',4,4',5,5'- hexabromodiphenyl ether 643,6		n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.	n.f.
BDE 154 207122-15-4 C$_{12}$H$_4$Br$_6$O 643,59 2,2′,4,4′,5,6′- hexabromodiphenyl ether		*8.55*	*4.148x10^{-6}*	*466.91*	*3.830x10^{-7}*	*4.71x10^{-7}*	*197.14*	*5.710*	*13.265*
BDE 183 207122-16-5 C$_{12}$H$_3$Br$_7$O 722,48 2,2′,3,4,5,5′- heptabromodiphenyl ether		*9.44*	*2.156x10^{-7}*	*497.61*	*4.390x10^{-8}*	*1.88x10^{-7}*	*211.48*	*6.203*	*14.554*

Table A. 4 - Chemical structures and characteristics of Flame Retardants (BFRs) (Continuation)

Common Name CAS n.º Molecular formula Molecular weight (g/mol)	Chemical structure	Log K_{ow}	Sol. in water (mg/L) (25 ^{o}C)	Boiling point (^{o}C)	Vapour Pressure (Pa) (25 ^{o}C)	k_H (atm.m^3.mol^{-1}) (25 ^{o}C)	Melting point (^{o}C)	Log K_{oc}	Log K_{oa}
BDE 209 1163-19-5 $C_{12}Br_{10}O$ 959.17 2,2',3,3',4,4',5,5',6,6'- decabromodiphenyl ether		12.11	1.000×10^{-4}	530.00	6.226×10^{-10}	1.19×10^{-8}	295.00	*7.679*	*18.423*
HBB 87-82-1 C_6Br_6 551.49 Hexabromobenzene		6.07	1.600×10^{-4}	*370.70*	2.173×10^{-6}	2.81×10^{-5}	*148.64*	*5.268*	*9.126*
PBT 87-83-2 $C_7H_3Br_5$ 486.62 Pentabromotoluene		*6.99*	*9.351×10^{-4}*	*351.60*	*1.950×10^{-5}*	*7.22×10^{-5}*	*130.01*	*6.066*	*9.602*

APPENDIX B - STATE OF THE ART OVERVIEW

Table B. 1 Overview on analytical methods for determination of PAHs in pine needles

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus nigra, Pinus strobus (10 g)	USA	16	USE: 60 mL DCM/Hex (1:1), 150 W, 10 min (2x) SPE - Florisil column: 5 g, 30 mL DCM/Hex (1:1) + 1.0 g of Na_2SO_4	GC-MS: HP-5MS column (30 m x 0.25 mm x 0.25 µm film thickness)	2.49-5,80 (µg/L)	-	127-6111	Tomashuk et al., 2012
Pinus pinea L., *Pinus pinaster* Alt. (5 g)	Portugal	16	USE: 30 mL DCM/Hex (1:1), 720 W, 10 min SPE - Alumina cartridges: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM	GC-MS: DB-5 column (30 m x 0.25 mm I.D. x 0.25 µm film thickness)	0.08-1.53	-	96-866	Amigo et al., 2011
Pinus pinea L., Pinus pinaster Alt. (5 g)	Portugal	16	USE: 30 mL, DCM/Hex (1:1), 720 W, 10 min SPE - Alumina cartridges: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM	GC-MS: DB-5 column (30 m x 0.25 mm I.D. x 0.25 µm film thickness)	0.08-1.53	-	251 - 2000	Ratola et al., 2011
Pinus pinea (2 g)	Portugal	16	Soxhlet: 200 mL ACN, 24h SPE – Florisil column: 30 mL ACN	HPLC: Agilent C18 and Phenomenex C18 columns, UV/visible detector (DAD/V–UV) and UV/fluodetector (FLD).	-	-	83.0-466.8	Augusto et al., 2010
Pinus pinea L., Pinus pinaster Alt. (5 g)	Portugal	16	USE: 30 mL, DCM/Hex (1:1), 720 W, 10 min (5 g needles) SPE - Alumina cartridges: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM	GC-MS: DB-5 column (30 m x 0.25 mm I.D. x 0.25 µm film thickness)	0.12-0.91	-	76-1944	Ratola et al., 2010

Table B. 1 Overview on analytical methods for determination of PAHs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus massoniana L. (5 g)	China	16	Soxhlet: (needles + Na$_2$SO$_4$) DCM, 48h SPE - Silica/Alumina (2:1) column: 60 mL DCM GPC: 80 mL DCM/Hex (1:1)	GC-MS (not specified)	-	60.5-96.8	1044.78-1927.20	Sun et al., 2010
Pinus nigra (10 g)	Germany	17	ASE: 120 ºC, 75 bar, Hex/DCM (99:1; v/v) Centrifugation: 3500 rpm MPLC: description not available	GC-MS: HP 1 column (25 m x 0.25mm I.D. x 0.25 µm film thickness)	-	-	3-2100 (ug/g-dw) 60-1500 (ng/g-dw)	Lehndorff and Schwark, 2009a and 2009b
Pinus pinea L., Pinus pinaster Alt. (5 g)	Portugal/ Spain	16	MAE: 90 mL DCM/Hex (1:1), 30min, 513W USE: 30 mL, DCM/Hex (1:1), 720 W, 10 min (5 g needles) SPE - Alumina cartridges: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM	GC-MS: DB-5 column (30 m x 0.25 mm I.D. x 0.25 µm film thickness)	MAE: 0.02-0.54 USE: 0.02-0.35	10-130	MAE: 221-1552 USE: 70-1773	Ratola et al., 2009
Pinus sylvestris L. (10 g)	Russia	15	USE: 100 mL Hex, 1h SPE – silica cartridge: 0.5 g, 5.5 mL Hex + 2 mL Hex:Benzene (9:1)	GC-MS: DB-5ms column (30 m × 0.25 mm) electron impact ionization	0.06-0.1	85	20-1800	Gorshkov, 2008
Pinus taeda (10 g)	USA	20	Soxhlet: 300 mL DCM 4h SPE - Alumina glass column: 10 g, 200 mL Pentane:DCM(1:1) SPE - Silica glass column: 20 g, 200 mL Pentane:DCM(1:1)	GC-MS: DB-5MS column (30 m x 0.25 mm x 0.25 µm film thickness) electron impact ionization	-	-	353-2226	Hwang and Wade, 2008

Table B. 1Overview on analytical methods for determination of PAHs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus pinea L. (5 g)	Greece	13	USE: 30 mL, DCM/Hex (1:1), 360 W, 10 min HF-LPME: Toluene, 20min, 1250 rpm	GC-MS: DB-5 column (30 m x 0.25 mm I.D. x 0.25 µm film thickness)	0.01-0.95	64-160	35 – 224	Ratola et al., 2008
Pinus massoniana (5 g)	China	15	Pre washed with deionized water Soxhlet: DCM, 48 h SPE – Florisil/Alumina/Silica column: 10 cm Si, 6 cm Al, 5 cm Flo, 2 cm Na_2SO_4, 70 mL DCM/Hex (1:1), GPC: 80 mL DCM/Hex (1:1)	GC-MS	-	30.3-112.4	503.3-1172.6	Tian et al., 2008
Pinus massoniana L. (5-8 g)	China	15	ASE: Acet:Hex (1:1), 1500 psi, 100 ºC, 20 min, 2x SPE - Silica/ Alumina Column: 5 g Al + 10 g Si + 2 g Na_2SO_4, 60 mL DCM GPC: 80 mL DCM/Hex (1:1)	GC-MS: HP5-MS column (30 m x 0.25mm x 0.25 µm film thickness)	3.3-7.8	80-110	280-3000	Liu et al., 2006

Table B. 1 Overview on analytical methods for determination of PAHs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus pinea L. (5 g)	Spain, Portugal	16	Soxhlet: 100 mL DCM/Hex (1:1) 24h USE: 30 mL DCM/Hex (1:1), 360 W, 10 min PLE: DCM/Hex (1:1), 10 min, 1500 psi, 150 ºC, 2 times SPE - Florisil cartridge/ column: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM SPE - Silica cartridge/ column: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM SPE - Alumina cartridge/ column: 5 g, 50 mL DCM/Hex (1:1) + 50 mL DCM	GC-MS: DB-5 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.22 – 0.71	Soxhlet: 65-102 USE: 72-100 PLE: 70-137	21.86 - 339.28	Ratola et al., 2006
Pinus sylvestris (5-10 g)	Germany	17	Undefined extraction SPE – Silica column GPC	GC-MS: HP5MS column (50 m x 0.2 mm x 0.33 µm film thickness)	0.04-0.35	70-120	8.2-121	Schroter-Kermani et al., 2006
-	Italy	13	USE: Hex SPE – silica	GC-MS: DB 5 column (60 m x 0.25 mm x 0.25 µm film thickness)	-	-	41.6-318.9	Capuano et al., 2005

Table B. 1 Overview on analytical methods for determination of PAHs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus nigra, Pinus pinaster (3 g)	Italy	9	USE: 15 mL DCM, 10 min, 2x SPE – Silica: 5 g, 8 mL DCM + 3 mL n-pentane:DCM (80:20)	HPLC: C18 reverse-phase column (5 mm, 4.6x150 mm) fluorescence detector	0.02	75-87	10.41-817.41	Piccardo et al., 2005
Pinua thunbergii (5 g)	China	16	USE: 30 mL Hex/Acet (2:1), 1 h SPE – Silica cartridge: 2 g, 8 mL Hex + 8mL DCM/Hex (1:1)	GC-MS: HP-5MS column (30 m x 250 mm x 0.25 0.25 µm film thickness)	Photolysis half-lives (t1/2, P): 12.9-65.4 h			Wang et al., 2005
Pinus nigra (10 g)	Germany	19	ASE: 120 ºC, 75 bar, Hex/DCM (99:1; v/v) Centrifugation: 3500 rpm MPLC: description not available	GC-MS: HP 1 column (25 m x 0.25mm I.D. x 0.25 µm film thickness)	-	-	51-355	Lehndorff and Schwark, 2004
Pinus sylvestris L. (5 g)	Germany	16	USE: 100 mL DCM, 10 min ASE: Hex, 40 and 120 °C, 15 Mpa, 3×10 min Soxhlet: 4 L Hex/100 g needles, 20h SPE-Florisil: 15 g, 160 mL DCM/Hex (1:1) + 3.5 g, 60 mL DCM/Hex (1:1) GPC	GC-MS: HP ultra 2 capillary column (25 m × 0.32 mm × 0.52 µm film thickness)	10-50 pg/g-dw	40-100	-	Hubert et al., 2003

Table B. 1 Overview on analytical methods for determination of PAHs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus densiflora, Pinus thunbergii, Pinus maximartinezii, Pinus taeda (10 g)	Korea, Mexico, USA	17	Soxhlet: 300 mL DCM 4h SPE - Alumina glass column: 10 g, 200 mL Pentane:CH2Cl2 (1:1) SPE - Silica glass column: 20 g, 200 mL Pentane:CH2Cl2 (1:1) HPLC purification: Phenogel, DCM (1.5 mL/min)	GC-MS: DB-5MS column (30 m x 0.25 mm x 0.25 μm film thickness) electron impact ionization	-	-	Korea:31-192; Mexico: 102-563; USA: 83-146 (ww) [31-563]	Hwang et al., 2003
Pinus halepensis, Pinus pinea (10 g)	Italy	15	Extraction not defined: Acetone, 30 min LLE: DMF, cyclohexane TLC: Silica gel plates, cyclohex:DCM (50:50)	GC-MS: HP5 column (30 m × 0.25 mm × 0.25 μm film thickness)	0.1	62	300-1000	Librando et al., 2002
Pinus sylvestris	Poland	17	Soxtec: DCM, 4h SPE – Florisil	GC-MS: HP-5 column (24 m x 0.2 mm x 0.33 μm film thickness)	-	81-96	194-1039	Malawska et al., 2002
Pinus sylvestris	Poland	17	Soxtec: DCM Desulfurization	GC-MS: HP-5MS column (25 m x 0.2 mm x 0.33 μm film thickness)	-	-	78-216 ppb	Migaszewski et al., 2002
Pinus sylvestris L. (10 g)	Czech Republic	16	Soxhlet: DCM, 8h SPE – Florisil: DCM/Hex	GC-MS: HP-5MS column (30 m x 0.25 mm x 0.25 μm film thickness)	0.1	80-98	0.3-19.0	Holoubek et al., 2000

Table B. 1 Overview on analytical methods for determination of PAHs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus strobus (15 g)	Russia	10	SFE: 5 mL Toluene, 15 min, 180 ºC, 350 atm USE: 500 mL DCM	GC-MS: HP5 column (30 m × 0.32 mm × 0.25 µm film thickness)	-	86-101	64-859	Lang et al., 2000

Table B. 2. - Overview on analytical methods for determination of PCBs in pine needles

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus strobus, Pinus nigra	Croatia	17	MARS: 20 mL DCM, 1200 W, 40 ºC, 15 min Alkaline and Acidic hydrolysis SPE-Silica column	GC-MS: SPB-5 column (60 m x 0.25 mm x 0.25 µm film thickness), SPB-1701 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.4 ng/mL	-	0-1.45 (1 year old needles) 0-2.84 (2 year old needles)	Romaníc and Klincic, 2012
Pinus radiata (10 g)	New Zealand	17	S-PLE: 35 g (Flo) + 10 g needles, 150 mL DCM:Hex (25:75), 100 ºC, 1500 psi, 1 cycle	GC-MS: DB-5MS column (60 m x 0.25 mm x 0.25 µm film thickness) Electron capture negative chemical ionisation	-	84	-	Lavin and Hageman, 2012
Pinus taeda (20 g)	USA	41	Soxhlet extraction: 325 mL Methylene Chloride:Hex (3:1), 16 h SPE-Silica column: 120 mL Hex + DCM:Hex (1:4) Acid attack: H_2SO_4	GC-MS	-	100	3.4-15 (ww)	Sajwan et al., 2009
Pinus sylvestris	Czech Republic	7	Soxhlet extraction: DCM SPE-Silica column GPC	GC-MS: DB-5MS	-	-	-	Klánova et al., 2009
Pinus sylvestris (1-2 g)	Sweden	18	ASE: Hex, 160 ºC, 12 Mpa SPE-Florisil cartridge: 5 mL DCM GPC: DCM	GC-MS: Rtx Dioxin 2 column (60 m x 0.25 mm)	-	30-70	0.28-1.60	Oberg and Peltola, 2009

Table B. 2. - Overview on analytical methods for determination of PCBs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus strobus, Pinus nigra (5 g)	Croatia	20	MAE: 20 mL DCM, 1200 W, 40 ºC, 15 min Alkaline and Acidic hydrolysis SPE-Silica column	GC-MS: SPB-5 column (60 m x 0.25 mm x 0.25 µm film thickness), SPB-1701 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.4 ng/mL	-	0.31-4.45 (1 year old needles); 0.27-7.20(2 year old needles)	Kozul and Romanic, 2008
Pinus sylvestris	Germany	18	Soxhlet: Toluene, 24 h SPE-Silica column: Si/44% H_2SO_4 + Si/33% 1 N NaOH SPE-Alumina column: 25 g	GC-MS: DB-XLB column (60 m)	-	-	0.266-1.046	Rappolder et al., 2007
Pinus strobus, Pinus nigra (15 g)	Croatia	7	Extraction: 50 mL DCM, 3 min mix and 24h rest (2x) Hot 7 mL Toluene:Hex (3:17) and cooling (0 ºC) (2x) Saponification: 5 mL KOH (1 mol/L in H2O): ethanol (1:1) SPE-Silica: 1 g (Si) + 2 g (33%-0.1 mol/L NaOH-Si) + 1 g (Si) + 4 g (44% H_2SO_4-Si) + 2 g (Si), 80 mL 4% diethyl-ether in Hex	GC-MS: SPB-5 column (60 m x 0.25 mm x 0.25 µm film thickness), SPB-1701 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.4 ng/mL	34-57	1.45-18.89 (1 year old needles); 3.2-30.07 (2 year old needles)	Romanić and Krauthacker, 2007

Table B. 2. - Overview on analytical methods for determination of PCBs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus sylvestris	Poland	9	Needle lyophilization and -20 ºC storage Homogenization with 300 mL toluene Soxhlet: Toluene and methyl chloride, 7h SPE-Silica column: Si gel (0.8 g) + 2% KOH–Si gel (3 g) + Si gel (0.8 g) + 44% H_2SO_4–Si gel (4 g) + 22% H_2SO_4–Si gel (4 g) + Si gel (0.8 g) + 10% $AgNO_3$–Si gel (8 g) + Na_2SO_4 (5 g), 200 mL Hex SPE-Alumina column: Al (10 g) + Na_2SO_4 (2 g), 20 mL Hex + 50 mL 0.5% methyl chloride in n-hex + 120 mL 50% methyl chloride in n-hexane 2D HPLC/PYE-HPLC: 20 mL 50% methyl chloride/Hex + 50 mL Toluene	GC-MS: DB-1 column (30 m x 0.25 mm x 0.25 µm film thickness)	-	101	2.7-50 (ww)	Wyrzykowska et al., 2007
Cedrus deodara	China	209	Shaking (1 h) or Shaking (1 h) + USE (10 min): chloroform/Toluene (2:1) SPE- Silica column: Silica + Acidic Silica + Na_2SO_4 SPE-Alumina column: Benzene + Hex/DCM (98:2) SPE-C18 cartridges: ACN	GC-MS: DB-XLB column (60 m)	-	60-90	4.389	Chen et al., 2006
Pinus uncinata (1-4 g)	Spain	6	Soxhlet: needles + Na_2SO_4 (5 g), 100 mL DCM, 24 h SPE-Silica/Alumina column: Si (8 g) + Al (8 g) + Na_2SO_4 (0.5 g), 20 mL Hex + 20 mL Hex:DCM (19:1)	GC-MS: HP-5 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.05-0.1	79-99	0.2-1.2	Grimalt and Drooge, 2006

Table B. 2. - Overview on analytical methods for determination of PCBs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus sylvestris L., Pinus thunberigii Parl. and Pinus densiflora Sieb and Zucc.	Poland, Japan		Needle lyophilization and -20 ºC storage Homogenization with 300 mL toluene Soxhlet: Toluene and methyl chloride in methyl alcohol, 7h SPE-Silica column: Si gel (0.8 g) + 2% KOH–Si gel (3 g) + Si gel (0.8 g) + 44% H_2SO_4–Si gel (4 g) + 22% H_2SO_4–Si gel (4 g) + Si gel (0.8 g) + 10% $AgNO_3$–Si gel (8 g) + Na_2SO_4 (5 g), 200 mL Hex SPE-Alumina column: Al (10 g) + Na_2SO_4 (2 g), 20 mL Hex + 50 mL 0.5% methyl chloride in n-hex + 120 mL 50% methyl chloride in n-hexane 2D HPLC/PYE-HPLC: 20 mL 50% methyl chloride/Hex + 50 mL Toluene	GC-MS: DB-1 column (30 m x 0.25 mm x 0.25 µm film thickness)	-	87.3-119	2.78-50.1 (ww-Poland); 3.77-72.5 (ww-Japan)	Wyrzykowska et al., 2006
-	Italy	28	USE: Hex SPE – silica	GC-MS: DB 5 column (60 m x 0.25 mm x 0.25 µm film thickness)	-	-	0.7-30.1	Capuano et al., 2005

Table B. 2. - Overview on analytical methods for determination of PCBs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus strobus, Pinus nigra (15 g)	Croatia	7	Extraction: 50 mL DCM, 3 min mix and 24h rest (2x) Hot 7 mL Toluene:Hex (3:17) and cooling (0 ºC) (2x) Saponification: 5 mL KOH : ethanol (1:1) SPE-Silica: 1 g (Si) + 2 g (33%-0.1 mol/L NaOH-Si) + 1 g (Si) + 4 g (44% H_2SO_4-Si) + 2 g (Si), 80 mL 4% diethyl-ether in Hex	GC-MS: SPB-5 column (60 m x 0.25 mm x 0.25 µm film thickness), SPB-1701 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.4 ng/mL	34-57	0.12-8.31	Romanić and Krauthacker, 2004
Pinus tabulaeformis (4-7 g)	China	15	Soxhlet: 250 mL CycloHex:Acet (1:1), 16 h Acid attack: H_2SO_4 Washing: 100 mL H2O (3x) Drying: Na_2SO_4 Acid attack: H_2SO_4 SPE-Florisil: 50 mL Hex	GC-MS: CP-Sil 8 CB column (50 m x 0.25 mm x 0.12 µm film thickness)	0.003-0.075	84-92	41.8-270.5	Xu et al., 2004
Pinus sylvestris (10 g)	Czech Republic	3	Soxhlet: DCM, 8 h Acid attack: H_2SO_4 SPE-Florisil column: Hex	GC-MS: NB-54 column (50 m x 0.2 mm x 0.1 µm film thickness)	0.1	80-98	<0.1	Holoubek et al., 2000
Pine needles (3 g)	Singapore	4	SFE: needles (3 g) + Na_2SO_4 (4 g), CO_2, 120 ºC, 200 atm, 2.5 ml/min, 50 min	GC-MS: DB-1 column (30 m x 0.25 mm x 0.25 µm film thickness)	0.5 (µg/L)	89-97	0.6-1.1	Zhu and Lee, 2002

Table B. 3 - Overview on analytical methods for determination of BFRs in pine needles

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus massoniana L. (10 g)	China	15	Pre washed purified water Soxhlet extraction Acid attack: 60 mL H_2SO_4 LLE: Hex SPE- Alumina/Silica column	GC-MS: DB-XLB column (30 m x 0.25 mm x 0.25 µm film thickness) and DB-5HT column (15 m x 0.25 mm x 0.10 µm film thickness)	0.04-0.4	62.5-142	40.4-546	Tian et al., 2012
Pinus halepensis, Pinus pinea, Pinus nigra (5 g)	Spain	8	USE: 30 mL DCM/Hex (1:1), 10 min, 360 W, 3x SPE-alumina cartridges: 5 g, 30 mL DCM/Hex (1:1) + 30 mL DCM SPE-Florisil pipette column: 3x3 mL Hex	GC-MS: HP-5MS column (15 m x 0.25 mm x 0.25 µm film thickness)	0.011-0.070 (0.232 BDE 209)	99-139	0.027-13.04	Ratola et al., 2011
Pinus taeda (20 g)	USA	11	Soxhlet extraction: 325 mL Methylene Chloride:Hex (3:1), 16 h SPE-Silica column: 120 mL Hex + DCM:Hex (1:4) Acid attack: H_2SO_4	GC-MS	-	100	0.05-3	Sajwan et al., 2009
-	China	8	USE: Hex:Acet (1:1) SPE-alumina Acid attack: H_2SO_4	GC-NCI-MS	3-15 pg/g-dw (222 pg/g-dw (209))	83.8-107.5	-	Chen et al., 2009

Table B. 3 - Overview on analytical methods for determination of BFRs in pine needles (Continuation)

Pine needles	Country	Analytes	Extraction and Cleanup	Analythical method	LOD (ng/g-dw)	% Recovery	Concentration ∑PAHs (ng/g-dw)	Reference
Pinus korariensis (10 g)	Korea	9	Maceration with Na_2SO_4 Soxhlet extraction: DCM, 16h SPE-Alumina/Silica column: 20 g Si + 10g Al, 100 mL DCM HPLC purification: size-exclusion column (250 x 22.5 mm)	GC-MS	-	-	0.08-1.17	Kannan et al., 2009

APPENDIX C – RESULTS

C.1. Elution Profiles

The elution profiles were performed in the begining of the experimental work in order to understand the compounds' behaviour towards the studied sorbents (alumina, florisil and silica) and assess the best elution solvent and its volume.

Prior to spiking, 10 mL were eluted to a clean vial, the blank sample. Columns were then spiked with standard *SPE Spike* (100 µL of "Safe Pine Mix for spiking" with PAHs and Musks at 1.25 mg/L and BFRs + PCBs at 0.5 mg/L). Elution was performed with the solvents used for conditioning and in 10 mL fractions in a total of 10 fractions. In the case of the solvent hexane-dichloromethane (1:1), in the end of the 10 fractions, was added 20 mL dichloromethane and eluted to a single vial. Samples were then evaporated at room temperature under a gentle stream of nitrogen until 500 µL, transferred to a smaller vial and evaporated again until dryness. Afterwards, samples were reconstituted in 50 µL hexane and analyzed in GC-MS.

Figure C.1. 1 represents the experimental setting were elution is performed in two columns with the same sorbent but with different solvents, DCM/Hex (1:1) in the first column and DCM in the second.

Figure C.1. 1 – Elution profiles experimental setting.

Elution profiles assays were also performed in the GPC columns. Columns were used with 6 g and 12 g of Biobeads and elution was performed with DCM/Hex (1:1) in 16 fractions of 2.5 mL for the 6 g column and 20 fractions of 5 mL for the 12 g column, after spiking with the same standards as the SPE columns. Samples were then evaporated at room temperature under a gentle stream of nitrogen until 500 µL, transferred to a smaller vial and evaporated again until dryness. Afterwards, samples were reconstituted in 50 µL hexane and analyzed in GC-MS.

C.2. SPE sorbent analysis and GPC influence

In order to verify the chromatographic differences obtained for SPE cleanup with florisil or alumina glass columns with or without GPC cleanup, chromatograms were compared. Figure C.2.1 and C.2.2 present the obtained chromatograms for BFRs+PCBs and PAH+musks, respectively, using a florisil column cleanup. In the same way, Figure C.2.3 and C.2.4 present the chromatograms for BFRs+PCBs and PAH+musks, respectively, using an alumina cleanup
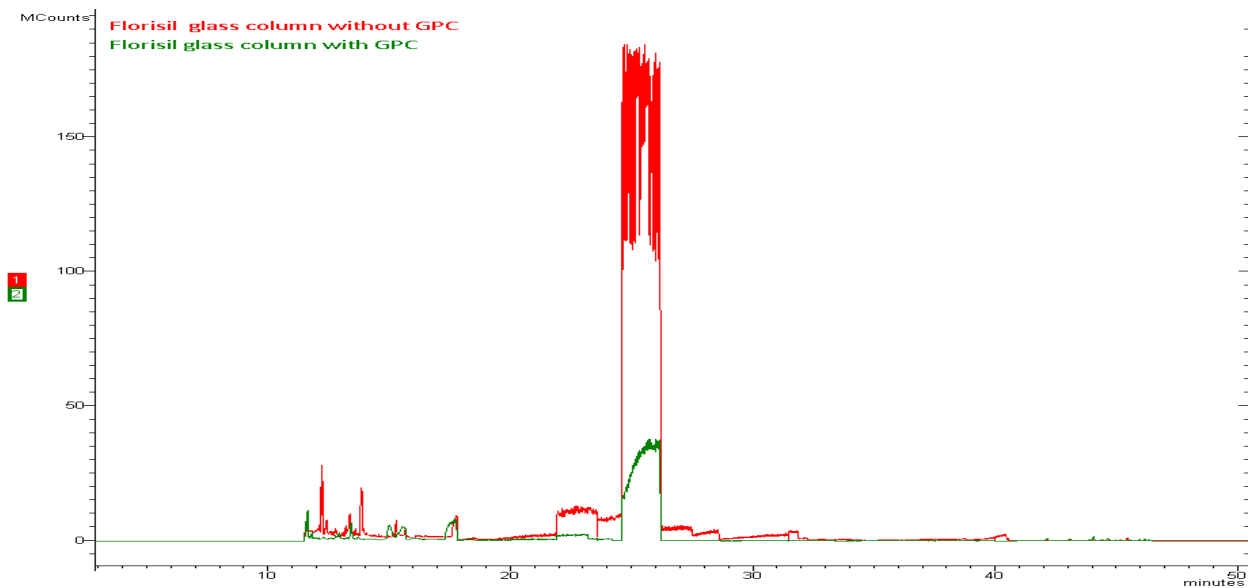
Figure C.2. 1 SIS Chromatograms for BFRs and PCBs in florisil SPE columns with and without GPC
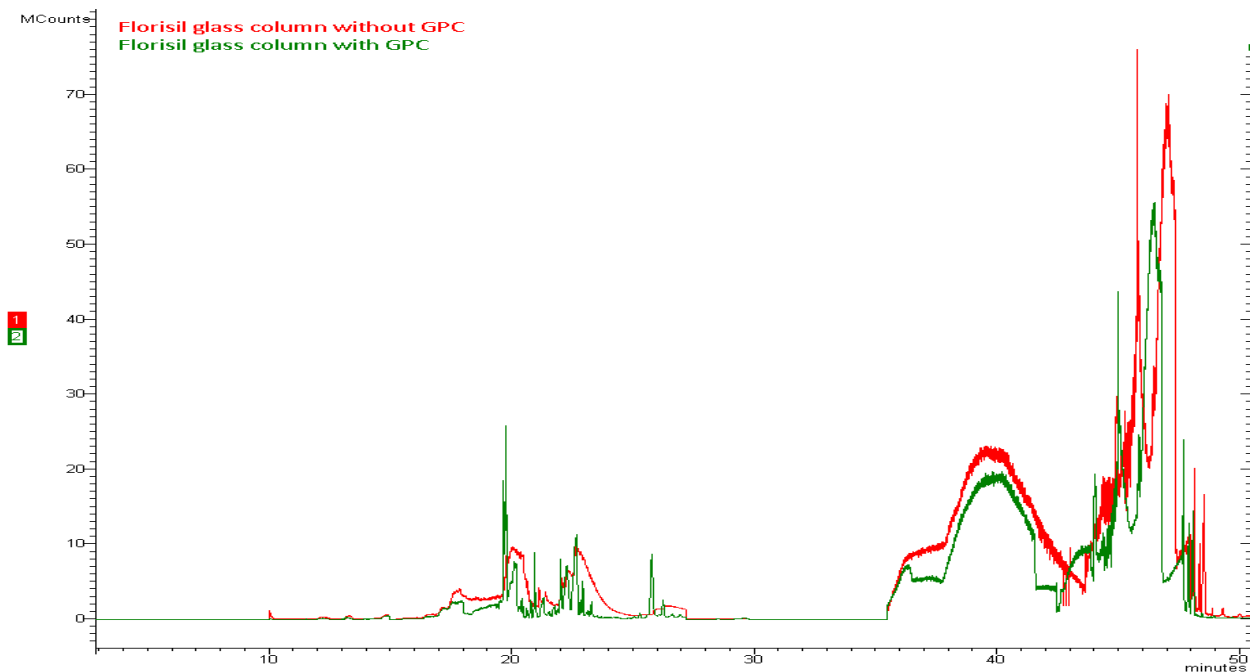
Figure C.2. 2 SIS Chromatograms for PAHs and Musks in florisil SPE columns with and without GPC
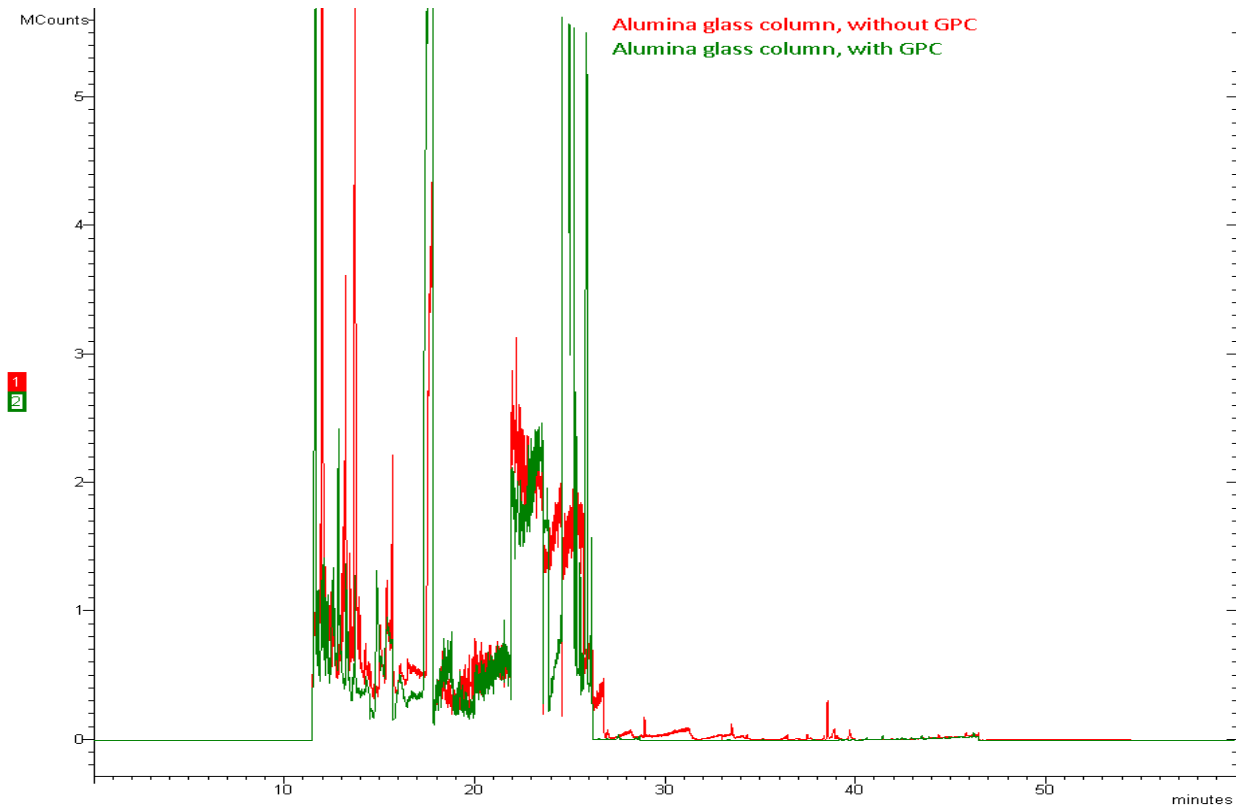
Figure C.2. 3 – SIS Chromatograms for BFRs and PCBs in alumina SPE columns with and without GPC
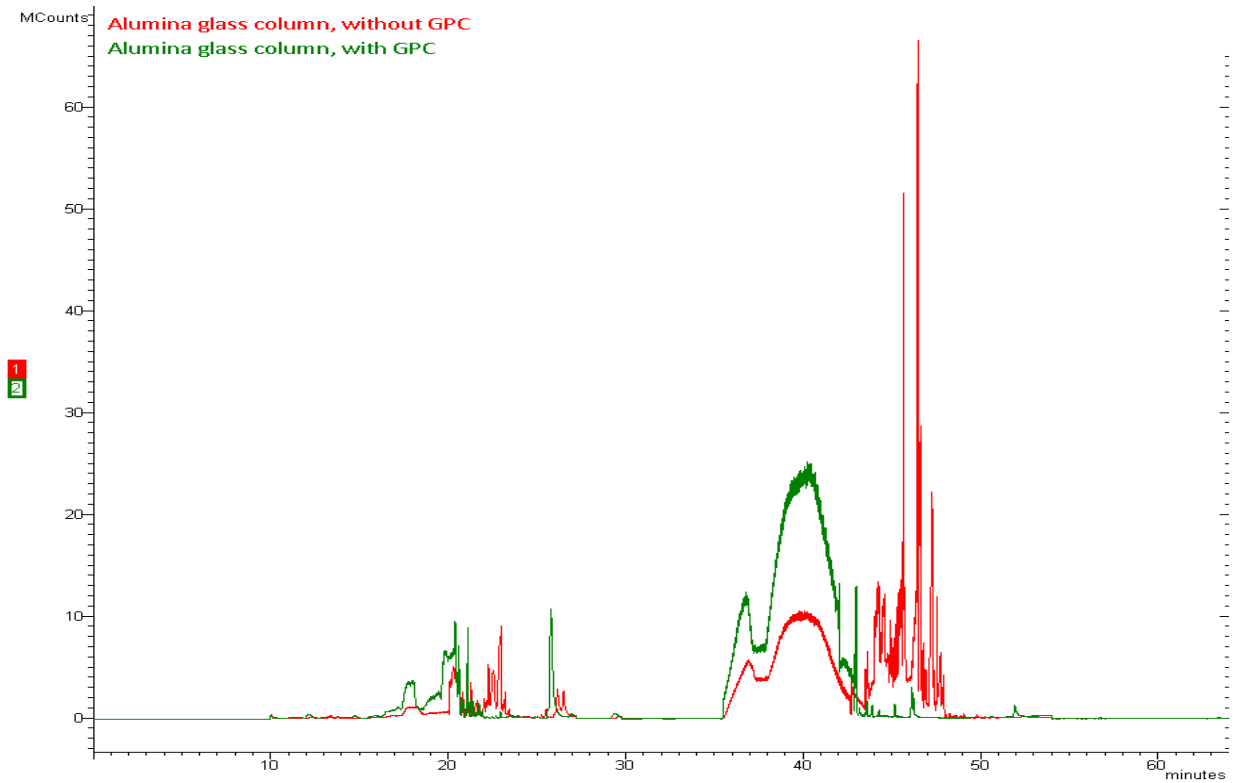
Figure C.2. 4 SIS Chromatograms for PAHs and Musks in alumina SPE columns with and without GPC

C.3. SPE stationary phase type evaluation

SPE glass columns were compared to SPE cartridges. Cartridges were conditioned prior to sample loading with 50mL hexane-dichloromethane (1:1). The extract were added to the cartridge and eluted with 50 mL hexane-dichloromethane (1:1), followed by 50 mL dichloromethane. Flow was mantained with the help of a vacuum pump, at a 1-2 drops/second, without ever allowing the sorbent to dry. The two fractions were collected into the same pear-shaped flask and pre-concentrated in a rotary evaporator to 1 mL and then transferred into amber glass vials. Extracts were evaporated at room temperature under nitrogen and reconstituted in 100 μL of hexane before GC-MS analysis.

Figure C.3. 1 represents the experimental setup of SPE cartridges. A vacuum pump is connected to the vaccum manifold and on top of the removable cover, the SPE cartridge is connected by means of small valves.

Figure C.3. 1 – SPE cartridges experimental setup

C.4. Real sample analysis

After the development of the cleanup method, a sample of *Pinus pinaster* needles was analysed. Figure C.5.1 shows a column after elution with 50 mL DCM/Hex (1:1). The main bulk of green color was retained in the begining of the column as can be seen in Figure C.5.1.

Figure C.5. 1 – Alumina column (SPE) after sample loading (Pinus pinaster needles) and elution with 50 mL DCM/Hex (1:1)

A GPC column during elution is represented in Figure C.4.2. This is the final clean up step and in this figure, interferences can be seen as a yellowish band that is rejected in the first fraction of the elution solvent.
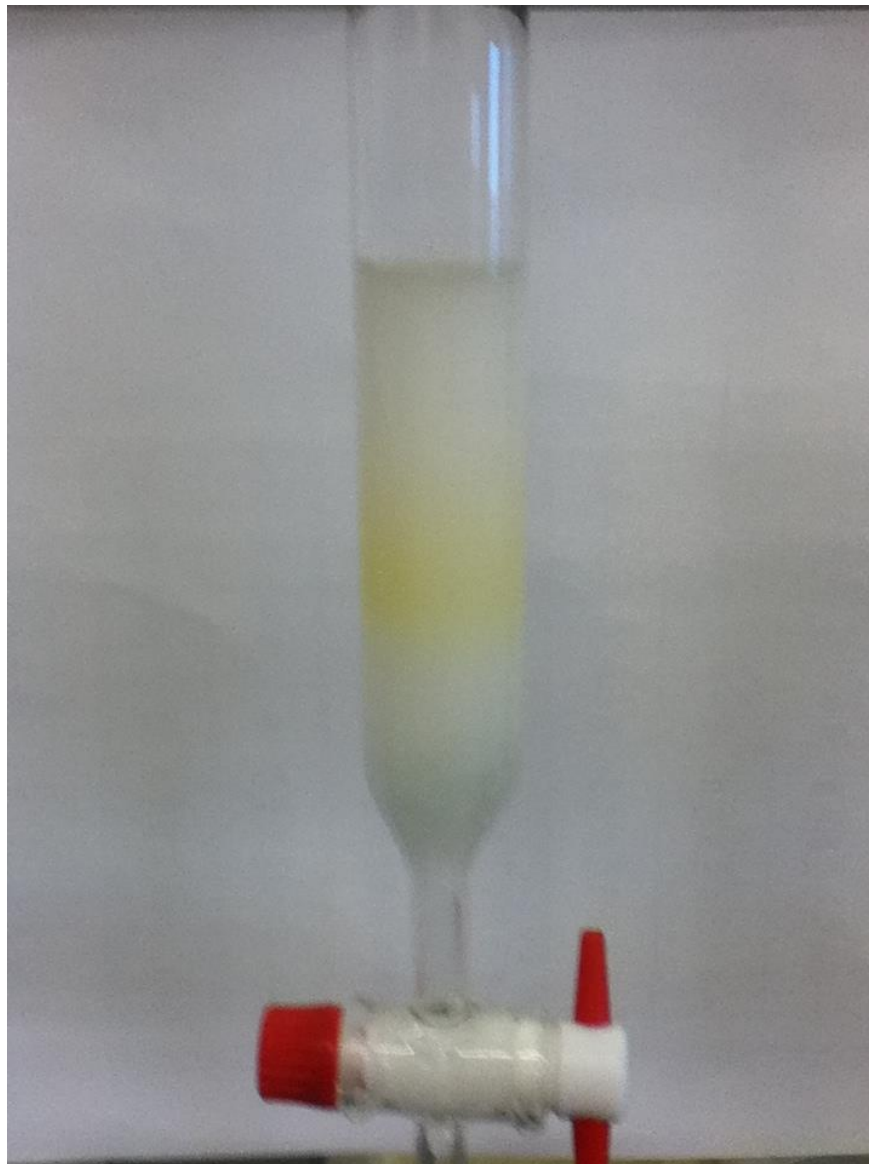
Figure C.4. 2 – GPC column with 6 g biobeads elution with 40 mL DCM/Hex (1:1)

C.5. Poster presentation

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