RISKS OF HUMAN EXPOSURE TO PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPs) - PARTICULAR CASE OF GALAXOLIDE

Patrícia Carla dos Santos Correia

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Supervision:

Arminda Alves (LEPABE/DEQ/FEUP), Full Professor

Lúcia Santos (LEPABE/DEQ/FEUP), Assistant Professor

Agostinho Cruz (CISA/ESTSP/IPP), Coordinator Professor

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À Carolina e ao Sérgio.

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Outline

This PhD thesis was developed at LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy), in the Chemical Engineering Department of the Faculty of Engineering of the University of Porto (DEQ-FEUP), throughout the period between September 2011 and September 2015, under the scholarship SFRH/BD/70945/2010 and the Project UID/EQU/00511/2013-LEPABE.

The main goal of this dissertation was to evaluate the direct and indirect human risk of exposure to galaxolide, a synthetic musk used in fragrances. In order to do that, the specific objectives of this work were: to determine the usage pattern of hygiene and personal care products by the North Region Portuguese population in general and, particularly in children, a more sensitive skin population; to validate and implement an analytical methodology for the extraction and detection of galaxolide in hygiene and personal care products and environmental matrices; to quantify galaxolide levels in the existing hygiene and personal care products more used by people (especially children); to correlate the levels of galaxolide in various hygiene and personal care products with the pattern of utilization of these products (application mode and frequency); to monitor galaxolide levels in various environmental matrices, in particular water and sludge from water treatment plants; to correlate galaxolide levels in environmental matrices with the seasonality and geographical location; to assess the risk of direct human exposure (dermal) and indirect (environmental) to galaxolide by the population from the North Region of Portugal.

The thesis structure is organized in three parts, with eight chapters, that include a general introduction (Part I), the risk of human exposure to galaxolide by dermal and environmental pathway (Part II), the conclusions, future work and list of references (Part III).

The first part corresponds to Chapter 1 that begins with the relevance and motivation for the theme of this thesis, followed by the outlined objectives. Finally, a description of the state of the art about the topics of interest regarding musks, particularly galaxolide, and a bibliographic review is updated, in order to introduce the main work described in the next chapters.

Part II is composed by Chapters 2 to 6 that correspond to papers already published or in preparation for submission to international peer-reviewed journals.

In Chapter 2, the usage pattern of personal care products is explored in order to achieve consumer habits that will be useful for risk assessment purposes. This was done using an online questionnaire, constructed by this team. The inclusion criteria were people resident in one of the municipalities of Portugal Northern Region. Data collected respect to the socio-economic characteristics of the sample and also to consumer habits of the respondents (414 individuals) like use/not use of some products' categories, products' marks choices and specific preferences of the products. This information was also complemented with data about the frequency and specific used amounts of each product category, by a diary of hygiene and personal care products use that some respondents accepted to fulfill.

Chapter 3 includes the development of the extraction method for galaxolide in personal care products, using a dispersive solid phase extraction, commonly named by QuEChERS. The analytical methodology for galaxolide quantification was performed by High Performance Liquid Chromatography with fluorescence detection (HPLC-FL), and galaxolide was determined in seven product types. Using these concentrations and published usage patterns, the risk of dermal exposure to galaxolide for a typical adult was estimated.

In Chapter 4, the risk of dermal exposure to galaxolide was examined for children from Oporto district. Data about the usage patterns of this specific population was collected through a questionnaire elaborated and conducted by the authors. The questionnaire was applied to 250 caregivers of children aged till 5 years old and resident in the Oporto district. The estimation of the risk of dermal exposure to galaxolide in children of this region of Portugal was achieved using the analytical method described in chapter 3 to quantify galaxolide in the mostly used personal care products.

Chapter 5 includes a comparison between the consumer patterns of adults and children's population, as well as a risk assessment using the dermal exposure risk of both populations.

Chapter 6 includes the adaptation and validation of the previously referred analytical method for the quantification of galaxolide in wastewater treatment plants matrices (residual waters and dehydrated sludge). The method was applied to the monitoring of galaxolide in residual waters (influent and effluent) and dehydrated sludge of an urban water treatment plant of Oporto, during three seasons, depending on the month that samples were collected: Autumn/Winter (November), Spring (May) and Summer (August). Apart from a seasonal

variation, an analysis of the daily and week/weekend variations of galaxolide concentrations in those environmental matrices was also performed. The estimations of the daily galaxolide *per capita* discharge was also explored and compared to the expected in terms of consumer habits.

Finally the last part of this thesis (Part III) includes the conclusions (Chapter 7), future work (Chapter 8) and references list (Chapter 9). A single references list was included to avoid the repetition of references due to a vast number of references common to all chapters.

CONTENTS

ABSTRACT

Synthetic fragrances are compounds widely used for several personal care and household perfumed products. One of the most known fragrances are musks that, due to their proprieties, are used as fixatives in a perfume composition. The polycyclic musk galaxolide is the most worldwide used synthetic fragrance in the last years. For this reason, galaxolide is widely studied and has been frequently detected in the environment, especially in water courses and respective fauna, at concentrations of the order of $ng.L^{-1}$ to $\mu g.L^{-1}$. However, there are few international studies about musks levels in personal care products and galaxolide risk of human dermal exposure and risk assessment. In Portugal, few data exists about galaxolide presence in environmental matrices. Additionally, in spite of its wide use, few studies have been conducted on the consumption patterns of personal care products. The present work was performed in the North Region of Portugal and includes galaxolide detection not only in environmental matrices, but also in personal care products, focusing two aspects: the incidence of human exposure, in adults and children, and its environmental impact. Therefore, the usage patterns of personal care products were necessary, and two population groups of Portugal were considered: children (0-5 years old) from Oporto district and adults from the North Region of Portugal. The collected data includes preference brands, mode and frequency of application of those products, besides some social and personal characterization of the inquired. A reliable analytical methodology, by High Pressure Liquid Chromatography with fluorescence detection, was validated for galaxolide, with an extraction method adapted to different consumer goods and environmental matrices (water and sludge from a wastewater treatment plant). The method presented low uncertainty and limits of detection of 0.001 $mg.kg^{-1}_{sample}$, for personal care products, 0.4 $\mu g.L^{-1}_{water}$, for wastewater and 0.004 $mg{\cdot}kg^{-1}_{dry\ sludge}$, for sludge samples. First, using the referred analytical method, galaxolide levels were determined in several personal care products with a large spectrum of utilization, in a range of 0.04 $mg.kg^{-1}_{sample}$ to 280.78 $mg.kg^{-1}_{sample}$, in adults' products, and 0.001 $mg{\cdot}kg^{-1}_{sample}$ to 300.480 $mg{\cdot}kg^{-1}_{sample}$, in children's products. Subsequently, the risk of dermal exposure to galaxolide was assessed, considering the collected consumer habits, corresponding to an estimated dermal exposure of 692 $\mu g.day^{-1}$ on the population of Portugal north region and 277 $\mu g{\cdot}day^{-1}$ on the children's population of Oporto district. With these exposure data, combined with the risk characterization data found in the literature, it was possible to conclude a risk assessment for

those children and adults' population. A maximum systemic exposure dose of 9.4 μg.kg_{bw}^{-1} for adults and 36.6 μg.kg_{bw}^{-1} for children, respectively corresponding to minimum margins of safety of 3191 and 820, leads to the conclusion that galaxolide is a safe ingredient considering the normal use of personal care products in both analyzed groups. Finally, an environmental exposure risk was accessed using wastewater and sludge samples from a wastewater treatment plant of a large urban center located in the region under study. Galaxolide was detected in all sludge samples, from 17.798 to 24.531 mg·$kg^{-1}_{dry\ sludge}$, and not all wastewater samples, from bellow 1.3 to 24.5 μg.L^{-1}, due to the non-polar character of galaxolide. The seasonal variation of galaxolide levels was evaluated in the course of a year, considering three seasons, and also the daily variation (nigh/day) and weekly variation (week/weekend). There was a significant difference between dry (May and August) and wet months (November) with smaller influent galaxolide concentrations, probably due to the dilution effect of the rain. Galaxolide mean concentrations were higher in influent night samples during the two hot seasons, but no significant difference was noticed in week/weekend galaxolide levels. Considering the wastewater treatment plant hydraulic parameters, the mean daily emissions of galaxolide from the served population was estimated in 1506 μg *per capita*. For the authors' best knowledge, this work represents the first one of this kind performed in Portugal and the first world galaxolide risk assessment considering children's population in all the revised literature.

Keywords: Galaxolide; Personal care products; Consumer patterns; Dermal and environmental exposure risk; Risk assessment.

RESUMO

As fragrâncias sintéticas são compostos amplamente utilizados para aromatizar diversos produtos de cuidado pessoal e de limpeza. Um dos grupos de fragrâncias mais conhecido são os *musks* que, devido às suas propriedades, são usados como fixadores na composição de perfumes. O *musk* policíclico *galaxolide* é a fragrância sintética mundialmente mais utilizada desde há vários anos. Por esse motivo, o *galaxolide* é o *musk* mais amplamente estudado, e tem sido detetado no ambiente, especialmente em cursos de água e respetiva fauna, em concentrações da ordem dos $ng.L^{-1}$ a $\mu g.L^{-1}$. No entanto, existem poucos estudos acerca dos níveis de *musks* em produtos de cuidado pessoal e respetivo risco de exposição ao *galaxolide* e avaliação de risco. Em Portugal, existem poucos dados acerca da sua presença em matrizes ambientais. Além disso, apesar da ampla utilização de produtos de higiene e cuidado pessoal, poucos estudos foram realizados sobre os seus padrões de consumo. O presente trabalho realizou-se na Região Norte de Portugal e inclui a análise de *galaxolide*, não só em matrizes ambientais mas também em produtos de cuidado pessoal, abordando-se duas vertentes: a incidência de exposição humana, em adultos e crianças, e o seu impacto ambiental em Portugal. Assim, primeiramente eram necessários os padrões de utilização dos produtos de cuidado pessoal e dois grupos da população Portuguesa foram considerados: crianças (0-5 anos) do distrito do Porto e adultos da região norte. Os dados recolhidos incluem as marcas preferenciais, modo e frequência de aplicação dos produtos, além da caracterização social e pessoal dos inquiridos. Foi validada uma metodologia analítica por cromatografia líquida de elevada pressão com deteção por fluorescência, capaz de detetar *galaxolide*, após extração por um método adaptado aos diferentes bens de consumo e às matrizes ambientais (águas e lamas de estações de tratamento de águas residuais). O método apresentou baixas incertezas e limites de deteção de 0,001 $mg.kg^{-1}_{produto}$, para os produtos de higiene pessoal, 0,4 $\mu g.L^{-1}_{água}$, para as águas residuais e 0,004 $mg{\cdot}kg^{-1}_{lama\ seca}$, para as amostras de lama. Primeiramente, usando o método analítico referido, determinaram-se os níveis de *galaxolide* existentes nos produtos de cuidado pessoal com maior espectro de utilização, numa gama de 0,04 $mg.kg^{-1}_{produto}$ a 280,78 $mg.kg^{-1}_{produto}$, em produtos de adulto, e 0,001 $mg{\cdot}kg^{-1}_{produto}$ a 300,480 $mg{\cdot}kg^{-1}_{produto}$, em produtos de criança. Posteriormente, foi avaliado o risco de exposição dérmica ao *galaxolide*, considerando os hábitos de utilização referidos, que correspondeu a uma exposição dérmica de 692 $\mu g.dia^{-1}$ na população adulta e de 277 $\mu g{\cdot}dia^{-1}$ nas crianças. Com

estes dados e com base em dados de caracterização do risco encontrados na literatura, foi possível concluir uma avaliação de risco para estas populações específicas. Para uma dose máxima de exposição sistémica ao *galaxolide* de 9,4 $\mu g.kg^{-1}_{peso\ corporal}$ para adultos e de 36,6 $\mu g.kg^{-1}_{peso\ corporal}$ para crianças, corresponderam margens mínimas de segurança de 3191 para adultos e de 820 para crianças. Desta forma, e considerando a utilização normal dos produtos higiene e cuidado pessoal, conclui-se que o *galaxolide* é considerado seguro. Finalmente, foi ainda avaliado o risco de exposição ambiental usando amostras do afluente, efluente e lamas de uma estação de tratamento de águas residuais de um grande centro urbano da região em estudo. O *galaxolide* foi detetado em todas as amostras de lamas, de 17,798 a 24,531 $mg{\cdot}kg^{-1}_{lama\ seca}$, e na maioria das amostras de águas, desde menos de 1,3 até 24,5 $\mu g.L^{-1}_{água}$, o que se deve às características apolares do *galaxolide*. Foi avaliada a variação sazonal dos níveis de *galaxolide* ao longo de um ano, considerando três épocas, assim como a variação diária (noite/dia) e a variação semanal (semana/fim-de-semana). Foi encontrada uma diferença significativa entre os meses mais húmidos (Novembro), como menor concentração no afluente, e os mais secos (Maio e Agosto), provavelmente devido ao efeito de diluição das chuvas. As concentrações médias de *galaxolide* foram superiores nas amostras de afluente durante as duas estações mais quentes, mas não se registou uma diferença estatisticamente significativa na variação entre as amostras recolhidas durante a semana e o fim-de-semana. Considerando os parâmetros hidráulicos da estação de tratamento de águas residuais, a emissão diária de *galaxolide* pela população abrangida foi estimada em 2,9 mg *per capita*. Não é do conhecimento dos autores a existência de mais nenhum estudo do género em Portugal, tratando-se ainda do primeiro estudo a nível mundial sobre a avaliação de risco do *galaxolide* na população infantil.

Palavras-chave: Galaxolide; Produtos de cuidado pessoal; Padrões de consumo; Risco de exposição dérmica e ambiental; Avaliação de risco.

Figure captions

Table captions

Nomenclature

ADBI - cestolide

AHDI – phantolide

AETT - versalide

AHTN - tonalide

ATII – traseolide

bw - body weight

C18 – octadecylsilane

CISA - Research Centre for Health and Environment (Centro de Investigação em Saúde e Ambiente)

CSTEE - Scientific Committee on Toxicity Ecotoxicity and the Environment

CV - coefficient of variation

DEP – diethyl phthalate

DEQ - Chemical Engineering Department

DPMI – cashmeran

DSPE - Dispersive Solid Phase Extraction

EcoFEUP - Environmental Management System of Faculty of Engineering of the University of Porto

ESTSP-IPP - School of Allied Health Technologies of the Polytechnic Institute of Porto

EU - European Union

USA – United States of America

FCT - Foundation for Science and Technology (Portugal)

FEDER - European Regional Development Fund

FEUP - Faculty of Engineering of the University of Porto

FL - fluorescence

GCB - graphitized carbon black

GC-MS – Gas Chromatography with Mass Spectrometry

HERA - Human and Environmental Risk Assessment

HHCB – galaxolide

HPLC – High Pressure Liquid Chromatography

kg_{dw} – kilogram of dry weight

K_{ow} - Octanol-water sharing coefficient

LEPABE - Laboratory for Process Engineering, Environment, Biotechnology and Energy

LLE - Liquid-Liquid Extraction

LOD - limit of detection

LOQ - limit of quantification

MA - musk ambrette

$MgSO_4$ - magnesium sulfate

MK - musk ketone

MM - musk moskene

MSPD - matrix solid phase extraction

MT - musk tibetene

MX - musk xylene

$NaCH_3COO$ - sodium acetate

NM - Nitro Musk

NO_3^- - nitrate anion

O_3 - ozone

OH• - Hydroxyl radical

OSPAR - Oslo and Paris Commissions for the Protection of the Marine Environment of the North-East Atlantic

PEC - Predicted Environmental Concentration

PCP - Personal Care Product

PPCP - Pharmaceuticals and Personal Care Product

PNEC - Predicted No-Effect Concentration

PM - Polycyclic Musk

PP - polypropylene

PSA - Primary and Secondary amine exchange polymer material

QuEChERS - Quick, Easy, Cheap, Effective, Rugged and Safe extraction method (or DSPE)

SCCNFP - Scientific Committee on Cosmetic Products and Non-food Products

SPE – Solid Phase Extraction

SPME - Solid Phase Micro-Extraction

SWECO - Swedish Environmental Protection Agency

USEPA - United States Environmental Protection Agency

UV – Ultraviolet

WWTP - wastewater treatment plant

λ- Wavelength

PART I – GENERAL INTRODUCTION

1 Introduction

1.1 Relevance and motivation

Every day people use various products and materials pleasantly perfumed, namely personal care and hygiene products, air fresheners, cleaners, or even objects that invade our senses with the synthetic fragrances that compose them. Thus, it is expected that direct contact with these products is the main cause to human exposure to fragrances.

Some compounds present in these products can contribute to human dermal exposure as they are skin absorbed, thus body penetrating. Inhalation due to products applied by spraying or residues in air or dust can also be a reality, such as ingestion through products applied in the oral mucosa (lipsticks and toothpaste) or even through the ingestion of water or food. As for indirect exposure via environment, it is expected that the waste from human use of personal care products, is the largest source of contamination, in particular through urban sewerage systems. The following issues are raised by the widespread and growing use of perfumed products: Does the continued and increasingly intense contact with synthetic fragrances have any direct influence (by the use of products) or indirect (via environment) in our body and in the ecosystem? Does it affects any particular population in a more severe extent, especially the ones with sensitive skin?

Given the concerns about some allergic reactions that these compounds may trigger in more sensitive skins, several fragrance-free proposals have emerged on the market for hygiene and personal care products. However, the European legislation does not oblige discrimination of the fragrances composition used in these products, since it is considered to be a "brand-secret”. Therefore, some doubts have been raised about the effective absence of some fragrances compounds. Thus, the development of reliable and expeditious quantification methods for these compounds is the only way of checking the human exposure risk to fragrances. The analytical method more frequently described in the literature is liquid-liquid extraction with gas-chromatography and mass spectrometry detection, which is time-consuming and expensive. Therefore, the development of a new methodology will be an added value for the overview of research in this area. An analytical method development, adapted to several types of matrices as different products of hygiene and personal care (emulsions, solutions,

suspensions, gels, etc.) or environmental matrices (soils, sediments, wastewater and sludge) is a real challenge.

And which fragrances to analyze? It is known that the more currently used and most studied musks are galaxolide, tonalide, musk ketone and musk xylene. Galaxolide is the most often detected *musk* and the one whose concentrations have higher levels both in hygiene and personal care products and environmental matrices. As such, its extraction methodology in hygiene and personal care products followed by analysis by high efficiency liquid chromatography with fluorescence detection was optimized in the first place. Later, it was tried to extend the study to the analysis of other musks. However, the attempt to adapt the analytical method for the detection of other musks was not achievable. In this way, and given the availability of analytical equipment existent in the laboratory where this project was carried out, galaxolide was the only chosen fragrance. Given these data, and knowing that galaxolide remains the most worldwide used musk, it was considered to be important to assess dermal human exposure (via hygiene and personal care products), as well as the environmental exposure (via wastewater and sludge from wastewater treatment plant).

1.2 Objectives of this thesis

As already mentioned, it was decided to direct this work only for galaxolide, the most predominant musk, trying to answer the following questions:

- What are the usage patterns of personal care and hygiene products in some specific Portuguese population?
- What are the common galaxolide concentrations found in those consumer goods in Portugal?
- What is the human exposure incidence to galaxolide according to those products usage patterns?
- What is the environmental distribution of galaxolide and especially in wastewater treatment plants?

Therefore, the specific objectives of this project are:

(i) To determine the pattern of personal care and hygiene products use in the general Portuguese population, and particularly in children, a more specific and sensitive population;

(ii) To validate and implement an analytical methodology, with High Performance Liquid Chromatography with fluorescence detection, including extracting procedures adapted to different products and environmental matrices;

(iii) To quantify the galaxolide levels in the most used consumer goods (especially by children) and correlate them with the usage patterns of those products (mode and frequency of application);

(iv) To monitor galaxolide levels in environmental matrices (water and sludges from wastewater treatment plants) and correlate them with the geographical location (proximity to possible direct sources such as urban centers and places of discharge of wastewater treatment plants), and with weather data (rainfall, temperature and atmospheric humidity);

(v) To assess direct (dermal) and indirect (environmental) human exposure risk to galaxolide by Portuguese population.

1.3 State of the art

Emerging Pollutants are all chemical substances that are not currently covered by the regulations, and whose (eco)toxicity data and occurrence in various matrices do not exist or are not exhaustive. These compounds pose a potential danger for the environmental ecosystems as well to the safety and human health, and include pharmaceuticals and personal care products (PPCPs) and other constantly new developed substances (Gómez, Herrera et al. 2011). PPCPs are synthetic organic chemicals used in a wide variety of products which include, among others, pharmaceuticals, sunscreens and fragrances (Lishman, Smyth et al. 2006).

Synthetic fragrances are currently one of the groups of PPCPs more widely used. They are compounds or mixtures of compounds that print a characteristic aroma in products, objects and, usually, in the human body, making them more pleasant and appealing. There is a list of over 2000 fragrances referenced in the EU, and musks represent one of the most important classes of these compounds (Bridges 2002, SWECO 2008). Musks are widely used, especially in personal care products (PCPs) as perfumes, deodorants, creams, lotions, makeup, bath and hair products, as well as in cleaning products, cigarettes, air fresheners and scented candles. Given the use of these compounds in a wide variety of products with current and daily use by the population, its impact, both on the environment and on human health, is an area of emerging and growing interest, since the long-term effects of these compounds are still unknown (Homem, Alves et al. 2016).

1.3.1 Natural musks and synthetic musks

Musks are natural or synthetic chemicals with perfume properties that operate as base notes in the mixture that composes the whole fragrance, because they have the main function to fix this mixture into products.

Natural *musks* are compounds extracted from exocrine glands of animals, such as some mammals (e.g. *musk deer*), reptiles, birds, insects, but also from roots, seeds and flowers of plants (OSPAR Comission 2004). However, due to ethical issues (defense and preservation of the species) and to the high cost and scarcity, the extraction was prohibited and these fragrances have been replaced, several decades ago, by synthetic compounds with similar perfume characteristics. Thus, today, most of the musks used in cosmetics and perfumed

products are of synthetic origin, which led to the exponential growth of the worldwide industry of musks (Rimkus 1999). Chemically, synthetic musks are divided into four major groups (Figure 1.1): the nitro musks (NMs), the polycyclic musks (PMs), the macrocyclic musks and alicyclic musks (Eh 2004).

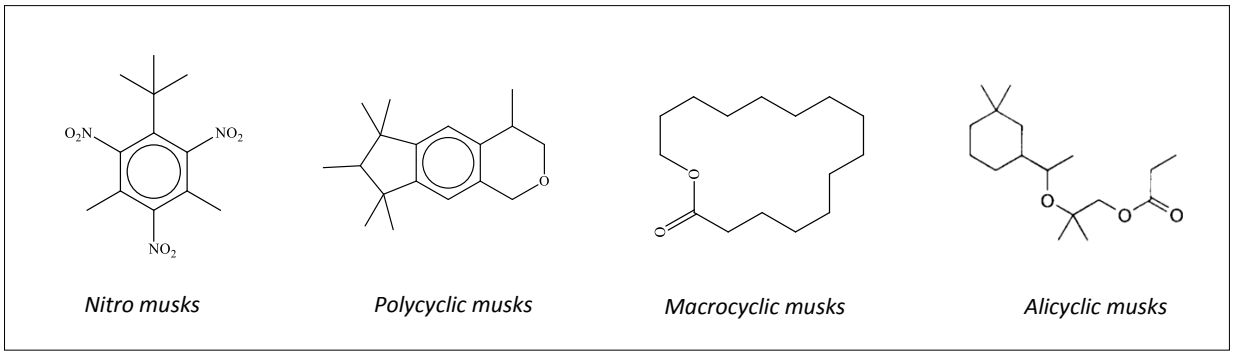


Figure 1.1 - Examples of chemical structure of synthetic musks: nitro musks (musk xylene), polycyclic musks (galaxolide), macrocyclic musks (exaltolide) and alicyclic musks (helvetolide).

The first nitro musk was musk moskene (MM) that arose accidentally as a derivative of the dinitrobenzene and trinitrobenzene during studies with explosives at the end of the 19th century (Rimkus, 1999). Consequently, other NMs emerged forming the first class of synthetic musks used at the beginning of the 20th century: musk ambrette (MA), musk ketone (MK), musk moskene (MM), musk tibetene (MT) and musk xylene (MX). These compounds had very similar characteristics to those of natural musks and were widely used in the industry due to its low cost of production. Nowadays, they require a lot of attention and concern once that they pose risks to the environment and to human health, and also due to its high persistence in the environment, bioaccumulation and some serious toxic effects.

In the 1950s, another important group of synthetic musks was introduced on the market, the polycyclic musks. The use of this new class of musks supplanted the previous one, NMs, due to its lower toxicity and higher molecular stability, derived from chemical structure with more than one ring (Figure 1.1). Consequently, the PMs have greater resistance to chemical degradation, especially in alkaline conditions (Roosens, Covaci et al. 2007). Some of the more relevant compounds of this category are galaxolide (HHBC), tonalide (AHTN), cestolide (ADBI), phantolide (AHDI), cashmeran (DPMI), traseolide (ATII) and versalide (AETT). The first two compounds are the more dominant on the market and the latter one was withdrawn from the market because it was considered to be neurotoxic (Bester 2009). PMs' industrial synthesis is

relatively complex and therefore more expensive, in comparison with the NMs. In spite of this, PMs are essential ingredients in the production of fragrances due to their typical olfactory properties and the ability to fix easily to materials (Homem, Silva et al. 2015a). Due to its wide application and the ability to spread and persist in the environment, HHCB and AHTN, have been subject of great attention by the scientific community (Bester 2009), being part of the List of compounds for Priority Action in EU (HERA 2004a).

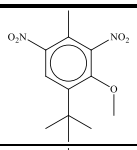
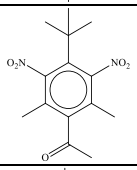
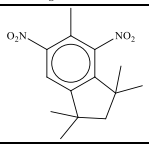
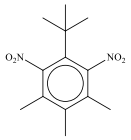
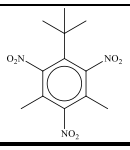
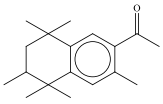
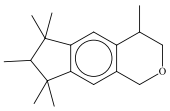
By the 1990s, macrocyclic musks arise to the market and, although more expensive, they offer an alternative to NMs and PMs since they have excellent fixative properties and are less recalcitrant (Gebauer and Bouter 1997, Abramsson-Zetterberg and Slanina 2002). In addition, they also have very similar characteristics to those of natural musks but have a more intense odor than PMs, which allows the use of smaller quantities to have the same results (Vallecillos, Borrull et al. 2013, Vallecillos, Pocurull et al. 2013). Macrocyclic musks are mainly used in perfumes and the more relevant ones are: ambrettolide, civetone, ethylene brassylate, exaltolide and musk muscone (Abramsson-Zetterberg and Slanina 2002).

The last class of musks, alicyclic musks, appeared with the compound cyclomusk discovered in 1975, but only helvetolide (Figure 1.1) was the first compound actually introduced in the market in 1990s. Recently, romandolide appeared, but the use of these musks is still very scarce (Eh 2004).

1.3.2 Physical-chemical properties of synthetic musks

Due to their low volatility, compared to other substances that compose the fragrance of a product, musks are used as fixatives (OSPAR Comission 2004). Analyzing the values of vapor pressure and the Henry's constant (Table 1.1), PMs (HHCB and AHTN) are more volatile than NMs, although MX has lower boiling point and is the most volatile of its class. In general, water solubility is low for all musks that, therefore, present non-polar characteristics, but there are some musks more soluble in water as MK, HHCB, MA and AHTN (Table 1.1). The octanol-water sharing coefficient (K_{ow}) is relatively high, which indicates a high lipophilicity. For all the identified properties, musks have a high potential for adsorption to organic matter and to bioaccumulation. Particularly, HHCB, a viscous and transparent colorless liquid, has more lipid affinity. For that, HHCB is insoluble in water, but soluble in alcohol and oil.

Table 1.1 - Physical-chemical properties of some nitro musks and polycyclic musks (Tas, Balk et al. 1997, Balk and Ford 1999)

Musk (abbreviation) Chemical name	**Chemical structure**	**Molecular weight**	**log K_{ow}**	**Solubility in water, 25 °C ($mg.L^{-1}$)**	**Vapour pressure (mPa)**	**Boiling point (°C)**	**Henry's Constant ($Pa.m^3.mol^{-1}$)**
Musk ambrette *(MA)* 1-*Tert*-butyl-2-methoxy-4-methyl-3,5-dinitrobenzene		268	3.7	1.67	3.3	369	nd
Musk ketone (MK) 1-(4-*Tert*-butyl-2,6-dimethyl-3,5-dinitrophenyl)ethanone		294	4.3	1.90	4.0×10^{-3}	369	6.1×10^{-3}
Musk moskene (MM) 1,1,3,3,5-Pentamethyl-4,6-dinitro-2H-indene		278	5.3	0.17	1.1	351	nd
Musk tibetene *(MT)* 1-*Tert*-butyl-3,4,5-trimethyl-2,6-dinitrobenzene		266	4.7	0.29	7.6	391	nd
Musk xylene *(MX)* 1-*Tert*-butyl-3,5-dimethyl-2,4,6-trinitrobenzene		297	4.9	0.49	3.0×10^{-3}	292	18×10^{-3}
Tonalide *(AHTN)* 1-(3,5,5,6,8,8-Hexamethyl-6,7-dihydronaphthalen-2-yl) ethanone		258	5.7	1.25	68	357	12.5
Galaxolide *(HHCB)* 4,6,6,7,8,8-Hexamethyl-1,3,4,7-tetrahydrocyclopenta[g] isochromene		258	5.9	1.75	73	362	11.3

nd – no data

All musks are molecules with at least one polar group and dimensions in the order of 9 to 12 Å (Beets 1982). The NMs and PMs have almost always an aromatic ring and, in addition to the polar group, two quaternary carbons at positions *ortho* or *meta*. The NMs have alkyl substituents, keto or methoxy, while in PMs the substituents may be methyl ether and carbonyl groups. PMs are compounds with 17 or 18 carbons derived from tetralin and indene, and have at least two rings, and the quaternary carbons can form a third ring, as in the case of HHCB (Table 1.1) (OSPAR Comission 2004).

Due to its fixing properties, NMs and PMs allow to obtain better balanced and more durable fragrances than other musks, since they are less volatile and can retard the release of other

more volatile compounds. However, the NMs are quite unstable in alkaline medium (OSPAR Comission 2004).

Macrocyclic musks have chemical structures similar to animal and vegetable natural musks, consisting of ketones and cyclic lactones from 10 to 15 carbons. These compounds are very lipophilic, but ethylene brassylate is the most soluble in water and the least volatile of this class (OSPAR Comission 2004).

Alicyclic musks, also called linear musks (Figure 1.1), consist of cycle-alkyl esters or linear chains with no similar sensory characteristics to any of the other groups. This compounds process a *fruity musk odor*, but they are still not very used (Eh 2004).

1.3.3 Synthetic musks degradation

Musks are semi-volatile organic compounds, and it is expected that a part can volatilize to the atmosphere and undergo chemical degradation with hydroxyl radical (OH•), nitrate anion (NO_3^-) as well as ozone (O_3). This hypothesis has been studied in the case of HHCB and AHTN, and the short half-life time in the atmosphere, 3.4 h for HHCB and 7.3 h for AHTN, suggests that these compounds don't go under atmospheric long range transport (Aschmann, Arey et al. 2001, HERA 2004b) and suffer medium range atmospheric transport of 1 to 1000 km (Villa, Vighi et al. 2014).

Reports also exist about the photodegradation of the AHTN at 20 °C using a high pressure mercury lamp, with a half-life time of 1.25 minutes in the presence of oxygen and 20 minutes in anoxic conditions, with any detected stable degradation product (HERA 2004b).

As regards biodegradation, some studies indicated that PMs are hardly biodegraded due to their high chemical stability. However, some species of fungi can degrade them in activated sludge and soils, causing more polar metabolites like diketone, peroxide, and O-methylated derivatives (Martin, Moeder et al. 2007). In HHCB case, HHCB hidroxycarboxilic acid and HHCB-lactone (Galaxolidone®) are formed (Figure 1.2), either by biological processes or by abiotic ones (Franke, Meyer et al. 1999). This last degradation product has already been detected in several matrices: sludge and residual water (Kupper, Berset et al. 2004, Reiner, Berset et al.

2007), superficial water (Franke, Meyer et al. 1999, Andresen, Muir et al. 2007), human milk (Reiner, Berset et al. 2007) and PCPs (Reiner and Kannan 2006, Lu, Yuan et al. 2011a).

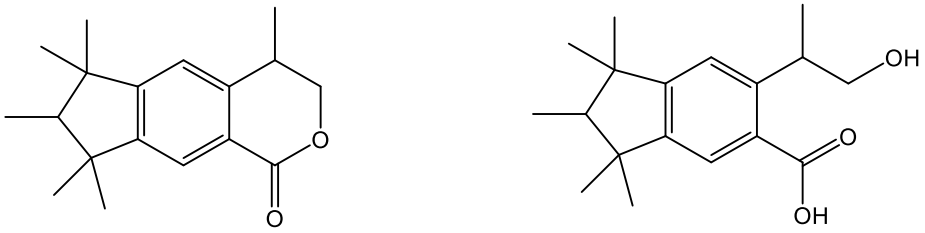


Figure 1.2 – Examples of HHCB degradation products: HHCB-lactone (left) and HHCB hidroxycarboxilic acid (right)

AHTN, which seems to suffer a more complex biological transformation (Franke, Meyer et al. 1999), can probably form (3,5,5,6,8,8-hexametil-5,6,7,8-tetrahidronaftalene-2-il) methanol (Faust, Nauroozi et al. 2011). Bacterial biodegradation of HHCB and AHTN can then proceed, but total mineralization is virtually non-existent (Balk and Ford 1999, HERA 2004b), which makes them recalcitrant compounds and may indicate a tendency for its environmental persistence.

It is known that NMs have a high photochemical reactivity and, therefore, MK, MM, MT and MX can suffer photodegradation (Sanchez-Prado, Lores et al. 2004). In addition, the nitro group (NO_2) reduction to amine (NH_2) may occur (Figure 1.3), forming 4-acetyl-1-tert-butyl-3,5-dimethyl-2-nitro-6-aminobenzene, from MK, 1,1,3,3,5-pentamethyl-4-nitro-6-aminoindene, from MM, and even 1-tert-butyl-3,5-dimethyl-4-amino-2,6-dinitrobenzene and 1-tert-butyl-3,5-dimethyl-2-amino-4,6-dinitrobenzene, from MX (Berset, Bigler et al. 2000, CSTEE 2004). For these reasons, all NMs can be degraded in several matrices and degradation products were already detected in blood (Riedel, Birner et al. 1999, Riedel and Dekant 1999), human urine (Riedel and Dekant 1999), aquatic fauna (Käfferlein and Goen 1998), sludge and residual water (Rimkus, Gatermann et al. 1999, Berset, Bigler et al. 2000, Herren and Berset 2000, Gatermann, Biselli et al. 2002) and superficial water (Rimkus, Gatermann et al. 1999).

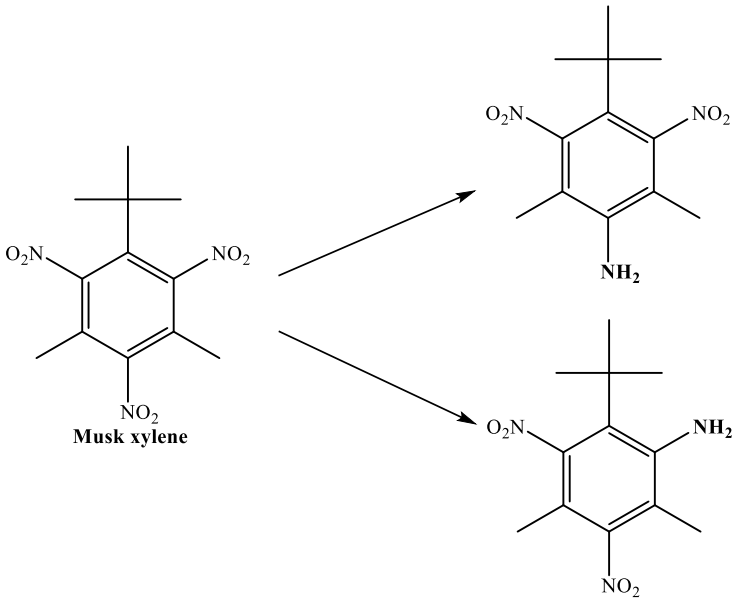


Figure 1.3 - Musk xylene degradation products: 1-terc-butil-3,5-dimetil-4-amine-2,6-dinitrobenzene (top) and 1-terc-butil-3,5-dimetil-2- amine-4,6-dinitrobenzene (bottom).

Macrocyclic musks and alicyclic musks are still little studied, but it is reported they can both undergo biodegradation (Eh 2004).

1.3.4 Toxicity of synthetic musks

Musks toxicity is greatly influenced by its highly lipophilic nature and by their increasingly intense use. In addition, musks degradation products may enhance the toxic effects of original compounds. All fragrances are associated with the possibility of allergic reactions, mutations and neurological changes. For instance, a study showed the possibility of the use of fragrances develop autism in children, linking it to the increasingly intense use of perfumed products in pregnancy (Bagasra, Golkar et al. 2013).

Several studies have reported the toxic effects of PMs and NMs in aquatic organisms (OSPAR Comission 2004) and endocrine activity has been target of some studies in fishes and mice (Seinen, Lemmen et al. 1999, Yamauchi, Ishibashi et al. 2008).

NMs are compounds with high toxicity: MA is neurotoxic and can cause photosensitivity reactions and phototoxicity (Cronin 1984); MT has estrogenic activity and, like all others, is potentially carcinogenic (Kevekordes, Zaulig et al. 1997, Liebl, Mayer et al. 2002). Other studies have demonstrated that the use of MX and MK can increase the risk of abortion, which led to the banning of their production in Japan (Wombacher and Hornbuckle 2009). In addition, all

NMs and MX in particular, can be degraded in products even more toxic than the original (Figure 1.3) during the water treatment process (Bester 2009).

PMs affect the liver, causing discoloration and an increase in its volume and/or weight, and galaxolide and tonalide are toxic to brain and lung cells (Ayuk-Takem, Amissah et al. 2014). In addition, it is known that PMs may cause some irritation and skin sensitization in humans. HHCB and AHTN are not considered mutagenic, carcinogenic or genotoxic, inducing the idea that the PMs are less toxic than the NMs (Roosens, Covaci et al. 2007). But, the use of AETT is forbidden because it was considered neurotoxic since it is degraded in γ-ketone (Bester 2009). Although HHCB was considered estrogenically inactive (Gebauer and Bouter 1997), recent studies state that both HHCB and AHTN are inhibitors of estrogenic activity by acting as antagonists when binding to androgen and progesterone receptors (Schreurs, Sonneveld et al. 2005) and also inhibiting the production of cortisol (Simmons, Marlatt et al. 2010).

Macrocyclic musks have also no estrogenic activity, with the exception of musk muscone, which presented moderate activity (Gebauer and Bouter 1997). There is still little information about environmental and human health risks, but the fact that they have a chemical structure similar to natural musks, indicates lower risks (OSPAR Comission 2004).

No information regarding the potential toxicity of the alicyclic musks was found in the literature.

1.3.5 Legal aspects of synthetic musks

Due to the extensive use of fragrances in various consumer goods, and given that natural fragrances are very expensive, NMs and PMs were increasingly being produced and used worldwide (OSPAR Comission 2004). However, in recent decades, concerns about the potential toxicity of some musks and its degradation products led to restrictions/prohibitions on the use of these fragrances in EU. Thus, according to the Regulation of the Council of the European Parliament relating to cosmetic products (European Parliment Council 2009), the following fragrances were forbidden in cosmetic products: MA since 1995 (European Communities Commission 1995); MM and MT since 1998 (European Communities Commission 1998); AETT since 2000 (European Communities Commission 2000). In addition, restrictions were

established to the use of MK, which can no longer be used in cosmetic products for oral application; maximum mass concentrations allowed for this musk in cosmetic products were also defined (European Communities Commission 2004): 1.4% in fine fragrances, 0.56% in toilette and 0.042% in other products. AHTN use was also restricted, with limits of 0.2 % in rinse-off products and 0.1 % in the leave-on ones. In the last ones, there is also an AHTN limit of 1 % for hydro-alcoholic products, and 2.5 % for fine fragrances (European Communities Commission 2008). The same European policy restricted AHDI with a maximum concentration of 2% in leave-on PCPs (European Communities Commission 2008). More recently, since 21 July 2014, the use of MX was completely banned in cosmetics and PCPs in EU (European Communities Commission 2011). Regarding macrocyclic musks and alicyclic musks, legislation is still scarce.

In general, the use of synthetic fragrances in consumer goods, while respecting the already referred legal limits, seems to pose no risk to human health. However, although not required, most fragrance suppliers deliver to PCPs producers a certificate indicating the safe use of the fragrance, as long as respecting a range of concentrations per PCP type, but with no discrimination of the fragrance composition (SCCS 2012). Additionally, despite the fact that the EU required a list of all ingredients used in the formulation of a PCP (in descending order of weight), it is not mandatory the discrimination of the fragrance composition, since this must be considered a protected trade secret of the mark. Thus, the composition of PCPs only refers "Parfum" or "Fragrance" instead of a detailed list of compounds. Recent research from the Environmental Working Group (EWG) found an average of 14 chemicals in 17 brands of fragranced products, none of them listed on the label, but it is possible to get up to 100, some of them in traceable quantities. So, given that there is only an auto regulatory mechanism in the industry itself, each brand can choose any ingredients to incorporate in the composition of a PCP, in particular the fragrance, without even having tested its safety for humans (EWG 2015). In addition, there are more than 2000 fragrances referenced that do not correspond to the entire compounds used by the industry for this purpose. There are even products where small quantities of fragrances are used to mask the odor of other substances without any reference in the formulation. This can happen even in products designated as *fragrance-free* (Bridges 2002). Furthermore, there are several *fragrance-free* PCPs where fragrances are frequently found (EWG 2015).

1.3.6 Synthetic musks consumption data

As a result of the restrictions described above, the consumption of NMs have decreased, which resulted in PMs' production and usage increase. Thus, in the 1990s, the PMs held approximately 70 % of the world market, with an annual production of around 4300 ton/year in 1987, increasing to 5600 ton/year in 1996 (Gebauer and Bouter 1997, Rimkus 1999). Later, the use of fragrances in such a vast number of consumer goods, and the fear of a consequent bioaccumulation in human tissues and pollution in various environmental matrices, have led to the decrease in consumption of PMs and NMs in Europe (Figure 1.4). This trend is most notorious in the northern Europe countries, probably due to cultural and advertising differences in relation to the other European countries (HERA 2004b). In fact, it is described that the use of musks is higher in the southern Europe countries than in the Nordic countries (OSPAR Comission 2004, HERA 2004b), which makes it especially important to study these aspects in countries such as Portugal. It is expected that this trend has been accentuated, although no recent data were found in literature.

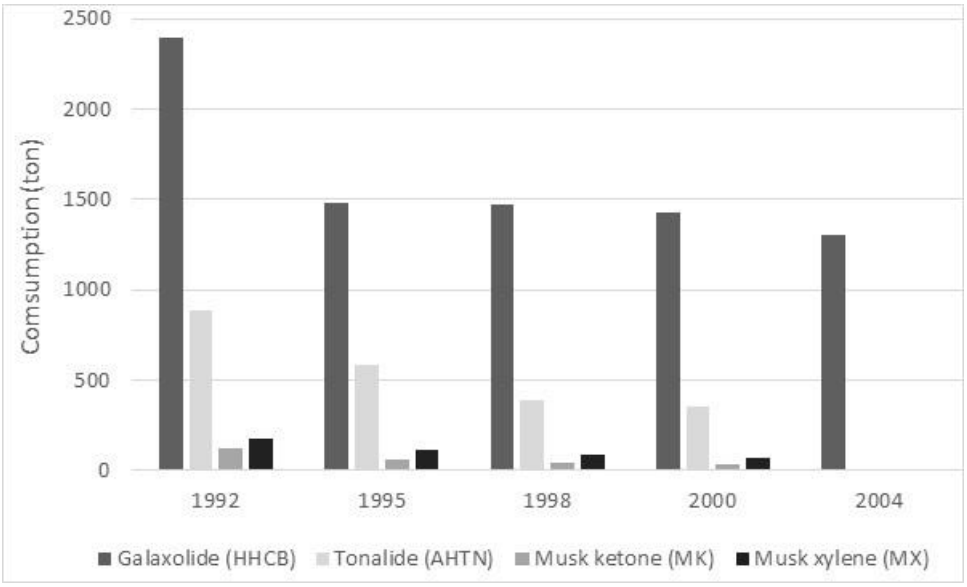


Figure 1.4 - Consumption data of the polycyclic and nitro musks more consumed in Europe between 1992 and 2004 (OSPAR Comission 2004, HERA 2004b, Clara, Gans et al. 2011). No available data for tonalide, musk ketone and musk xylene in 2004.

Nevertheless, the most globally used PMs are still HHCB and AHTN, and, for that, they are part of the List of compounds for Priority Action in the EU (HERA 2004a) due to its high production and use and also integrate the United States Environmental Protection Agency (USEPA) Toxic Substances Control Inventory (USEPA 2003). In fact, in the year 2004, HHCB and AHTN already

accounted for about 90% of the PMs market in the United States of America (USA), and about 95% of the same market in the EU (HERA 2004b). In the class of NMs the most used ones were MX, prohibited very recently, and MK, no longer practiced in some countries.

The low decrease in the use of PMs and NMs may be due to the fact that the more recent alternatives are not already very viable. In fact, the macrocyclic musks are very expensive and represent only a small proportion (3-4%) of the synthetic musks market (Abramsson-Zetterberg and Slanina 2002). In spite of being more economical, the alicyclic musks do not have great expression on the market because there are only two alternatives, romandolide and helvetolide (Eh 2004). Consequently, the PMs continue to be the more used class, with HHCB and AHTN representing around 95% of the European market (Clara, Gans et al. 2011), and HHCB remains the more used one.

1.4 The particular case of Galaxolide

As mentioned before, HHCB is the mostly worldwide consumed musk, being incorporated in daily used PCPs. The major route of exposure to HHCB is the direct utilization of those products, but the following path into the environment and the food chain, resumed in Figure 1.5, may be an alternative route of exposure.

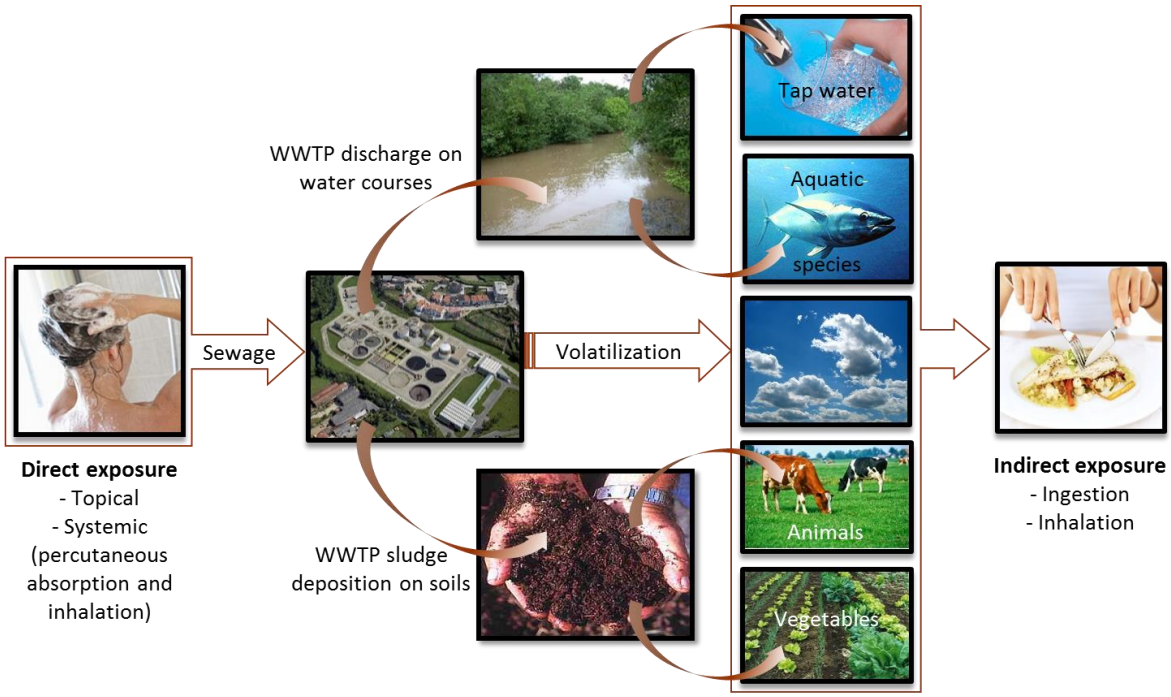


Figure 1.5 – Main pathway of galaxolide environmental and Human exposure

1.4.1 Galaxolide detection in environmental matrices

HHCB is the most used musk in the industry of PCPs and, for that, it has been found near industrial areas in high concentrations, with a maximum reported level of 595.48 $\mu g.L^{-1}$ (Chen, Zeng et al. 2007). Due to the extensive use of HHCB in formulations of PCPs with high consumption, a large percentage of this musk is launched on the network of wastewater sanitation, through which is introduced into the environment. It is expected that around 77% of the musks enter the sanitation system after use (HERA 2004b), mainly in large urban areas densely populated. Several studies carried out over the past few years, compiled by some authors, indicate the presence of HHCB in tributaries of wastewater treatment plants (WWTPs) in concentration between 0.00101 and 45.4 $\mu g.L^{-1}$ (OSPAR Comission 2004). The main way of removing these musks in WWTPs is adsorption to sludge and partial degradation, with

efficiencies of removal between 50 and 75% (OSPAR Comission 2004) and HHCB concentrations in sludge ranging from 1.4 to 703681 $\mu g.kg^{-1}_{dw}$ (Fooken 2004, Sang, Zhang et al. 2012, Chen, Ying et al. 2014, Vallecillos, Pedrouzo et al. 2014, Homem, Silva et al. 2015b). As a result of this removal process in the WWTPs, effluent should have on average lower concentrations of HHCB then the influent ones. Found concentrations ranged from less than 0.00014 to 33.54 $\mu g.L^{-1}$ (Balk and Ford 1999).

Due to the entry of these effluents into the aquatic environment, HHCB was found in groundwater and surface water in concentrations from less than 0.00005 to 12.5 $\mu g.L^{-1}$ (Eschke 2004). HHCB may persist even after water treatment procedures to obtain consumer waters with concentrations ranging from 0.00009 and 0.0281 $\mu g.L^{-1}$ (Vallecillos, Pocurull et al. 2012, Wang, McDonald et al. 2013, Wang and Khan 2014). Moreover, because of its environmental mobility, HHCB was found in rainwaters with a maximum level of 0.025 $\mu g.L^{-1}$ (Peters, Beeltje et al. 2008) and in snow in concentrations between 0.0028 and 0.0751 $\mu g.L^{-1}$ (Villa, Vighi et al. 2014).

Although HHCB enters in the environment through the aquatic sources, it is predictable a major distribution in sediments and suspended matter compared to water, given the physical-chemical characteristics of HHCB, namely high k_{ow} and low water solubility (Table 1.1). In fact, applying a model to HHCB transport and distribution in the environment, using parameters like water solubility, k_{ow}, vapor pressure, molecular formula and weight, it is expected a HHCB distribution mainly to sediment (55.6%), soil (38.6%), water (5.6%) and air (0.2%) (International Flavors & Fragrances 2003). Therefore, HHCB levels from 1.43 to 17993 $\mu g.kg^{-1}_{dw}$ (dry weight) were detected in suspended material and superficial water sediments (Fooken 2004). As for the entry of HHCB in soils, it can be done through the waters (near watercourses, rainfall and irrigation) or by deposition of bio solids resulting from digestion and dewatering of activated sludge from WWTPs. Due to the high K_{ow}, HHCB persistence in soil is long and increases with the organic carbon content of the soil (Yang and Metcalfe 2006). HHCB have already been detected in soils after 119 days (Macherius, Eggen et al. 2012), up to six months (Yang and Metcalfe 2006) or even 14 years after the deposition of activated sludge in soils (Dreher 2004). It were even reported HHCB concentrations from 0.35 to 29 $\mu g.kg^{-1}_{dw}$ in soils (Yang and Metcalfe 2006).

As mentioned before, air is the environmental compartment where a lower HHCB distribution is expected. However, given the moderate values of vapor pressure and the constant of Henry (Table 1.1), HHCB is considered a semi-volatile compound and it is very likely to have a small volatile fraction (Aschmann, Arey et al. 2001). HHCB was detected in indoor air, varying from 0.0652 to 1.256 $ng.L^{-1}$, and in outdoor air, from 0.0046 to 344.306 $ng.L^{-1}$ (Fromme, Lahrz et al. 2004, Peck and Hornbuckle 2004, Regueiro, Garcia-Jares et al. 2009, Ramírez, Marcé et al. 2010, Sofuoglu, Kiymet et al. 2010, Upadhyay, Sun et al. 2011). Given its log k_{ow} (Table 1.1), once in air, HHCB can be dust and particle adsorbed. Several studies have referred HHCB presence in dust with values that vary between 11.4 and 839000 $ng.g^{-1}$ (Fromme, Lahrz et al. 2004, Peck, Kucklick et al. 2007, Regueiro, Llompart et al. 2007, Lu, Yuan et al. 2011b), and in particles (0.00005 to 0.99 $ng.L^{-1}$) (Sofuoglu, Kiymet et al. 2010, Upadhyay, Sun et al. 2011, Wang and Khan 2014).

In Portugal, some studies regarding musks presence in environmental matrices have been performed (Silva and Nogueira 2010, Machado, Gonçalves et al. 2011, Salgado, Marques et al. 2011), with the following maximum concentrations of HHCB: 4670 $ng.L^{-1}$, in influents of WWTPs, 1270 $ng.L^{-1}$ in effluents of WWTPs, and 287 $ng.L^{-1}$, in superficial water.

1.4.2 Galaxolide detection in biological matrices

As previously mentioned, sludge deposition as fertilizer can cause contamination in soils and, consequently, may cause contaminants incorporation in crops used for human and other animal consumption and their introduction into the food chain. A study have already mentioned HHCB detection in carrots (0.22 – 4.35 $\mu.g^{-1}_{dry\ weight}$), barley (0.81 $\mu.g^{-1}_{dry\ weight}$) and meadow fescue plants (1.77 $\mu.g^{-1}_{dry\ weight}$) (Macherius, Eggen et al. 2012). Given the high log K_{ow} values (Table 1.1), some fragrances may enter food chain and bio accumulate. Furthermore, since the main entrance of HHCB in environment is made through watercourses, higher concentrations in water biological matrices are likely to occur. Thus, HHCB was found in various aquatic animals, and in larger quantities in freshwater fish, with maximum concentrations of 11.1 $mg.kg^{-1}_{lipid\ weight}$ (Franke, Meyer et al. 1999), as well as in mussels, in concentrations up to 0.850 $mg.kg^{-1}_{lipid\ weight}$ (Subedi, Yun et al. 2014). HHCB has even been found in animals of environments further away, such as the Arctic, which indicates a high environmental and trophic mobility (Mogensen, Pritzl et al. 2004). HHCB bioaccumulation is related to the fat

content of the organisms and is the result of a momentary exposure and not a chronicle one (HERA 2004b, Wan, Wei et al. 2007). However, and despite high log K_{ow} levels, Nakata and co-workers suggest that HHCB does not have a high tendency to bio accumulate (Nakata, Sasaki et al. 2007).

HHCB was detected in several human fluids and tissues: from 30 to 3600 $ng.L^{-1}$ in maternal milk, from 6.1 to 189000 $ng.kg^{-1}_{lipids}$ in fat tissue, from 0.301 to 6.9 $\mu g.L^{-1}$ in adult's blood and until 314.7 $ng.g^{-1}_{lipids}$ in umbilical cord blood (HERA 2004a, Hutter, Wallner et al. 2005, Kannan, Reiner et al. 2005, Kuklenyik, Bryant et al. 2007, Hutter, Wallner et al. 2009, Hutter, Wallner et al. 2010). A study carried out in Austria about the presence of musks in blood, have related the number of samples containing HHCB with gender, age, use of PCPs (body lotions and perfumes) and also the consumption of fish. This study concluded that young women (19-25 years) presented the highest levels of HHCB in blood, probably due to the higher use of PCPs compared to men and older women (26-43 years). In addition, it has been made a positive correlation between the presence of HHCB in blood and the frequency of PCP's use or the consumption of fish (Hutter, Wallner et al. 2005). Later, the same authors have confirmed this trend, obtaining blood maximum concentrations of 4100 $ng.L^{-1}$ for HHCB (Hutter, Wallner et al. 2009). Higher HHCB blood concentrations (6900 $ng.L^{-1}$) in older women (> 50 years) are described in another recent study. Concentration of HHCB may increase with age, due to the fact that older women have a drier skin and use more often hand, face and body creams compared to younger ones. In addition, skin physiological characteristics vary with age and, in more aged skins, penetration increases since the integrity is compromised (Hutter, Wallner et al. 2010), which is similar to the situation in younger and immature skins.

Thus, HHCB detection in biological matrices, namely human, is related with the PCPs used and with the habits of each population in hygiene and personal care. In individuals with sensitive skin, such as children, hygiene habits are necessarily different from those prevailing in the rest of the population, as well as the chosen related products are distinct and specific.

1.4.3 Galaxolide detection in hygiene and personal care products

Due to the nowadays intense use of hygiene and fragranced personal care products, it is expected that the largest HHCB exposure source is dermal contact, especially with leave-on

products used regularly (Cadby, Troy et al. 2002, Reiner and Kannan 2006, Kuklenyik, Bryant et al. 2007, Reiner, Berset et al. 2007). But, in spite of several studies made about HHCB distribution in environmental compartments and in living organisms, little is known about their distribution and use in PCPs.

The first description about the analysis of HHCB in PCPs dates back to 1998, where an after shave sample was directly analyzed by high-performance liquid chromatography (HPLC) with fluorescence (FL) and ultraviolet (UV) detection. The method presented a limit of detection (LOD) of 5 $\mu g.L^{-1}$ (HPLC-FL) and 1500 $\mu g.L^{-1}$ (HPLC-UV) (Schüssler and Nitschke 1998), and the described analytical method for HPLC-FL was adapted for the analysis of HHCB in this project, with some changes in the constitution and elution of mobile phase.

After this first study, only in 2006 HHCB was analyzed in 60 PCPs (body lotions, deodorants and perfumes), after liquid-liquid extraction (LLE) with hexane and Gas Chromatography with Mass Spectrometry (GC-MS) analysis. HHCB was detected in 72% of the analyzed samples in maximum concentrations close to 5000 $mg.kg^{-1}$ (in perfumes). The average recoveries were greater than 100% and the LOD of HHCB was 0.005 $mg.kg^{-1}_{sample}$ (Reiner and Kannan 2006).

Another study also held a LLE with hexane, but used water as a co-solvent and ended with a cleanup process in a silica column and elution with hexane and dichloromethane. This final process allowed to remove possible interferences, leading to high recovery rates (98-110 %) and low limits of quantification (LOQ) (0.017 $mg.kg^{-1}_{sample}$). The GC-MS analysis showed HHCB in 55% of the samples from 82 PHCP analyzed, being this the musk detected in higher concentration (22000 mg. kg^{-1}). These authors also observed higher concentrations of HHCB in perfumes, deodorants and body lotions (Roosens, Covaci et al. 2007).

Zhang and co-workers (2008) also used LLE with hexane followed by cleanup with a column of silica/alumina. This method has led to little lower recoveries than the methods mentioned above (78 %) with LOQ of the same order of magnitude (0.006 $mg.kg^{-1}_{sample}$). HHCB was detected in 61% of the 31 samples analyzed, having been obtained maximum concentrations of HHCB 1000 mg. kg^{-1} in perfumes and bath products (Zhang, Yao et al. 2008).

Martínez-Girón et al. (2010) used an extracting method similar to Roosens et al. (2007), but opted to use capillary electrophoresis to separate and quantify polycyclic chiral musks (HHCB

and AHTN) in samples of perfumes. This analytical method is not suitable for the analysis of products containing HHCB at low levels of concentration, given the high LOD (49 $mg.kg^{-1}_{sample}$) and LOQ (147 $mg.kg^{-1}_{sample}$) obtained. These authors detected HHCB average concentrations of 14.500 $mg.kg^{-1}$ with recoveries of 90-116% (Martínez-Girón, Crego et al. 2010).

Lu, Yuan and contributors (2011a) used an extracting method similar to Reiner and Kannan (2006) for musks determination in different PCPs. The LLE was made with hexane/ethyl acetate (1:1 v/v) and the aid of ultrasounds (US), followed by a cleanup in cartridges of SPE (solid phase extraction) with silica gel. Extraction with US allows lower consumption of solvent, requires less sample processing and is faster than conventional methods. Thus, this method has led to high recovery percentages (82 - 92 %) and low LOQ values (0.00301 $mg.kg^{-1}_{sample}$). In this study, HHCB presented the highest concentration in 52% of the samples and was the more frequently detected *musk* (73 %). The higher concentrations were detected in hair products (1010 $mg.kg^{-1}_{sample}$), lotions and creams (732 $mg.kg^{-1}_{sample}$) (Lu, Yuan et al. 2011a).

Llompart et al. (2013) developed a new methodology for synthetic musks analysis in various PCPs (creams, lotions, lotions, deodorants, bath products and hair) through matrix solid phase extraction (MSPD). The extraction used sodium sulphate as desiccant and Florisil as dispersing agent. This mixture was placed in a small column and the analytes were eluted with ethyl acetate or a mixture of hexane/acetone. This methodology has, as main advantages, high efficiency and low cost of extraction. The extracts obtained with high recoveries of HHCB (83 - 101 %), were analyzed by GC-MS with LOD of 0.0052 and LOQ of 0.011 $mg.kg^{-1}_{sample}$. The authors also observed that HHCB was present in all analyzed samples and was the musk present in greater abundance. On the other hand, the concentrations were lower in rinse-off products (0.0358-0.134 $mg.kg^{-1}_{sample}$), such as shampoos, conditioners, gels, bath, in relation to leave-on (0.044-3640 $mg.kg^{-1}_{sample}$), as the creams/lotions, lotions and deodorants (Llompart, Celeiro et al. 2013).

Homem and collaborators (2013) conducted a study by adapting the extraction methodology used in this project, the dispersive solid phase extraction (DSPE or QuEChERS), followed by GC-MS analysis, for the detection of 12 musks in various PCPs (body hair washes, toilet soap, skin moisturizers, roll-on deodorant and toothpaste). HHCB LOD (0.00001 $mg.kg^{-1}_{sample}$) was lower than the ones previously referred, with recoveries varying from 42 to 100%. HHCB was detected

in 83% of samples with concentrations from 0.002 to 882.340 $mg.kg^{-1}_{sample}$. The lowest HCCB concentration was found in toothpaste, while moisturizers and bath and hair products showed the highest concentrations. However, HHCB was not detectable in two body lotions, one of which a baby body lotion. In addition to this baby's PCPs, HCCB was also detected in a shampoo (0.025 $mg.kg^{-1}_{sample}$) and in a shower gel (0.015 $mg.kg^{-1}_{sample}$) (Homem, Silva et al. 2013).

Other recent study of this working team, have also analyzed children PCPs (bath gels, shampoos, soaps, toothpastes, body lotions and creams) using the same analytical method. Some concentrations were less than the LOQ, but, when detectable, HHCB varied between 0.0004 $mg{\cdot}kg^{-1}_{sample}$ on a shower gel and a toothpaste, and 20.83 $mg{\cdot}kg^{-1}_{sample}$ on a shampoo. This study also report HHCB concentrations in adults PCPs (bath gels, shampoos, hair conditioners, soaps, shaving products, toothpastes, deodorants, moisturizers and perfumes) varying between less than the LOD and 31124 $mg{\cdot}kg^{-1}_{sample}$ on a perfume (Homem, Silva et al. 2015a).

Finally, Nakata and co-workers (2015) analyzed several adults PCPs (perfumes, shampoos, body lotions, soaps and deodorants) by GC-MS, extracted by hexane LLE with ultrasounds and a silica column clean-up. This study have reported detectable HHCB concentrations until a maximum of 15000 $mg{\cdot}kg^{-1}_{sample}$ in perfumes have (Nakata, Hinosaka et al. 2015).

Thus, based on this literature review, the PCPs with larger quantities of HHCB are perfumes, deodorants, cosmetics and lotions/creams, all of them leave-on PCPs. Additionally, there are still few HHCB detection studies in PCPs, especially in children, and the compounds present in PCPs can be absorbed in a particular way in this population group, given the different skin physiology compared to a healthy adults. These are some of the topics that justify the interest of completion of this project, since only by determining the quantities of each fragrance in PCPs, it is possible to understand the risk of direct human exposure to these compounds, as long as the patterns of use are known and provided.

1.4.4 Galaxolide human exposure

As already mentioned, the dermal absorption seems to be the main form of direct human exposure to HHCB, although other less relevant sources of exposure, as inhalation and ingestion, may be present. Exposure to HHCB can occur due to oral contact with several

materials, such as the case of toys for babies (Masuck, Hutzler et al. 2011), or the ingestion of water and food. This ingestion is predictable due to the detection of HHCB in drinking water (Bruchet, Hochereau et al. 2005), several crops (Macherius, Eggen et al. 2012) and mainly due to the presence of HHCB in various aquatic species (Mogensen, Pritzl et al. 2004). The real risk of exposure by fish ingestion was already calculated (124 $\mu g.day^{-1}$), having always been considered inferior to direct exposure by dermal absorption (Roosens, Covaci et al. 2007). Maternal milk ingestion may also result in HHCB exposure risks for babies between 2.526 and 11.829 $\mu g.day^{-1}$ (Zhang, Liang et al. 2011, Yin, Wang et al. 2012). Inhalation can also occur during the application of PCPs in spray or aerosol, becoming more relevant in closed environments where this type of product is extensively used, as in hairdressers/barbers and perfumeries. Some studies seem, however, to indicate that inhalation is a less relevant exposure route to musks compared to dermal absorption, even when the inhalation involves powder ingestion in professional environments compared with home environments (Liu, Shi et al. 2013). Literature data shows HHCB exposure values, by powder ingestion, between 0.00022 and 0.115 $\mu g.day^{-1}$ (Lu, Yuan et al. 2011b, Nakata, Hinosaka et al. 2015).

Contaminants present in the air and dust may not only be inhaled or ingested but can also be absorbed through the skin, as demonstrated a study performed by Weschler and Nazaroff (2012). In fact, this may be an exposure route even more relevant than the inhalation or ingestion because, when dermal absorbed, the compounds don´t find the same enzymes and chemical barriers present in the digestive tract before its systemic absorption. In this study, a HHCB dermal flow of 280 $ng.h^{-1}.m^{-2}_{skin}$ from the air to the bloodstream was estimated. The reported HHCB concentration in air was 0.1 $ng.L^{-1}$ (Weschler and Nazaroff 2012), which is in the range of values found in literature mentioned before.

But the main route of exposure to HHCB is by dermal application of PCPs, even considering evaporation and rinsing of the product from the skin, and even when exaggerated scenarios are used for other routes of exposure (Cadby, Troy et al. 2002). The risk of exposure varies greatly with the type of products included in the analysis and with the range of concentrations found in each product group (Reiner and Kannan 2006). The first study found in the literature regarding dermal exposure risk combined HHCB concentrations in common use PCPs (perfume, shampoo, body wash and body lotion) with the patterns of use of these products, and reported a risk of exposure to 25100 $\mu g.day^{-1}$ (Ford 1998). Later, in a similar study, the risk of exposure

to HHCB was calculated considering several scenarios and ranged between 17 and 23700 $\mu g.day^{-1}$. In this study, it was concluded that HHCB is one of the most contributing musks to dermal exposure risk (Roosens, Covaci et al. 2007). A more recent study that used a broader range of products (shampoo, hair conditioner, body wash, facial cleanser, toilet soap, body lotion, face cream and liquid foundation), reported a HHCB exposure risk of 3060 $\mu g.day^{-1}$ (Lu, Yuan et al. 2011a). In Portugal, references to HHCB human exposure risks were only found in studies carried out by this research group. In addition to the studies carried out within the framework of this project, (Homem, Silva et al. 2015a) reported an average daily dermal exposure to HHCB of 1362 $\mu g.day^{-1}$ for adults. Other recently published study refer to the human dermal exposure risk to musks, estimating a HHCB daily dermal exposure of 395 $\mu g.day^{-1}$ (Nakata 2005). Some of the differences found between the exposure risks referred in the studies, can be justified not only by the detected HHCB concentrations in PCPs, dependent on the legislation in force in each country, as well as by different consumer habits that vary with the culture of the studied population. For this reason, it is of extreme importance studies about the patterns of use of PCPs in different regions.

PART II – HUMAN EXPOSURE RISKS TO GALAXOLIDE

2 Consumer patterns of personal care products in the Northern Region of Portugal[1]

Abstract

Personal care products are part of everyone´s daily routine, and that continuous contact with several compounds may have some negative effect in Humans. The safety evaluation of those products is based on each ingredient toxicity, concentration and also on the mean frequency and amount of product applied. Although there are published data in some countries, the usage of personal care products may vary along time and with the population. Therefore, it is essential to obtain the specific consumer patterns of the studied population. In order to do that, an online questionnaire was constructed, validated and applied to a sample of 414 individuals resident in 41% of the municipalities that compose the Northern Region of Portugal. The majority of the respondents were females (62.3%), from the age groups of 15-24 years old (54.1%) and 25-64 years old (43.4%), with normal to combination facial skin (57%), dry body skin (48%) and normal hair (32%) or hair with dandruff (21%). The analyzed types of products were facial and body moisturizers, deodorants, body wash products, shampoos, toothpastes, rinse-off and leave-on hair care products, facial and body sunscreens and other products. Each person used an average of seven products simultaneously and the most used ones were toothpaste (99.8%), shampoo (98.0%), body washer (97.8%) and deodorant (94.5%). The frequencies of use was of 1 event.day^{-1}, except for toothpaste (2 event.day^{-1}) and after shave (0.5 event.day^{-1}). The obtained daily used amounts were similar to other reported values for deodorant (0.9 g_{sample}.day^{-1}), shower gel (9.6 g_{sample}.day^{-1}) and shaving foam (2.0 g_{sample}.day^{-1}), but for other products, the daily amount values are lower than the obtained in other studies. This results indicated that, contrary to frequency of application, used amounts varied with the population under study. Body lotion was the most contributive product for systemic exposure, presenting the highest value of exposure per unit of body weight (69.9 mg_{sample}.day^{-1}.kg_{bw}), while deodorants present a higher exposure per unit area of skin (66.0 μg_{sample}.day^{-1}.cm^{-2}), being the most concerning for local effects (skin toxicity). These results may be useful for exposure assessment to any ingredient that composes a personal care product used in Portugal.

Keywords: Personal Care Products, toiletries, usage patterns, frequency of use, used amounts

[1] "Consumer patterns of personal care products in the Northern Region of Portugal"; P Correia, A Gerós, A Soares, A Cruz, L Santos, A Alves; submitted 2016

2.1 Introduction

Every day, several Personal Care Products (PCPs) are used, containing various substances to which people is exposed at very small amounts that may result in possible negative health effects (Cadby, Troy et al. 2002, Biesterbos, Dudzina et al. 2013). The safety evaluation of a PCP ingredient is not only based in its toxicological properties but also in PCPs consumer patterns, namely the frequencies and amounts per use (McNamara, Rohan et al. 2007). Consequently, for substances present in PCPs, exposure doses can only be obtained on a case-by-case basis, taking directly in consideration at least the following factors (SCCS 2012): the method of application of the PCP (directly or diluted; rinse-off or leave-on); the quantity of PCP used in each application; the frequency of application; the site and total area of skin contact; the duration of contact. All of these factors are related to consumer habits that can vary with the target population characteristics (age, gender, monthly income, hair and skin characteristics), marketing variables (fashion, trends, product innovation) and the region or country (e.g. local habits, seasonal variations) (SCCS 2012). Additionally, to evaluate the exposure to a substance present in PCPs, personal preferences must be accounted for, and also the co-use and the non-use of the products (Biesterbos, Dudzina et al. 2013). So, it is essential to achieve specific consumer preferences and habits of PCPs in each studied population. There are several studies respecting to PCPs use all over the world, but mostly in Europe (Table 2.1 and Table 2.2), where the most reported PCPs are body lotions, deodorants and shampoos, and the less referred ones are shaving products and sunscreens.

But in order to perform the exposure risk to a substance present in PCPs, the percentage of product retained on the skin after application (retention factor) should be taking into account. There are several studies referring to the retention factor (Table 2.3), but the main differences are only noticed among the following rinse-off PCPs: toothpaste, shampoo, hair conditioner and hair styling products. All leave-on PCPs are considered to have total retention on skin after application, except for spray deodorants which 15% may be lost at the time of application (Bremmer 2006).

Table 2.1 - Estimated mean application frequency (event. day^{-1}) of PCPs in several studies

PCPs	**Mean application frequency (event.day^{-1})**										
	Europe							**USA**			**Japan**
	(Cadby, Troy et al. 2002)	(SCCNFP 2002)	(Bremmer 2006)	(Nohynek, Antignac et al. 2010)	(SCCS 2012)	(Biesterbos, Dudzina et al. 2013)[a]	**Average values**	(CTFA 2004, CTFA 2005, Wu, Bennett et al. 2010)	(Loretz, Api et al. 2005, Loretz, Api et al. 2006, Loretz, Api et al. 2008)	**Average values**	(Nakata, Hinosaka et al. 2015)
Body lotion/cream	0.7	0.5	0.7	-	2.3	1.0	1.0	0.7	0.8	0.8	0.7
Facial creams	2.0	2.0	2.0	-	2.1	1.0	1.8	1.6	1.8	1.7	-
Toothpaste	-	2.0	1.5	-	-	2.0	1.8	-	-	-	-
Deodorant	1.0	1.0	1.0	-	2.0	1.0	1.2	1.0	1.3	1.2	1.0
Shower gel	1.1	2.0	1.5	-	1.4	1.0	1.4	0.7	1.4	1.1	1.1
Soap bar (hands)	6.0	6.0	5.0	-	-	-	5.7	-	-	-	-
Shampoo	1.0	1.0	0.7	-	1.0	0.4	0.8	0.7	1.1	0.9	1
Hair conditioner	-	1.0	0.3	-	0.3	0.4	0.5	0.6	1.1	0.9	-
Hair Styling	2.0	-	1.0	-	1.1	1.0	1.3	0.6	1.5	1.1	-
Sunscreen	-	-	2.5[b]	2.0	2.0	-	2.2	0.4[c]	-	0.4	-
Hand cream	-	-	2.0	-	2.0	2.5	2.2	1.3	2.1	1.7	-
Shaving products	-	-	1.0	-	1.0	1.0	1.0	-	-	-	-
Mouthwash	-	3.0	4.0	-	-	-	3.5	-	-	-	-

[a] Median amounts; [b] Assuming a sun exposure day, 15 days/year; [c] cool season

The analysis of the studies presented in Table 2.1 to Table 2.3, highlights the need to know the PCPs consumption patterns in each region, where the most relevant parameter seems to be the amount of PCP used in each application. The composition of PCPs and the specific concentrations of each ingredient can vary depending on the PCP type, manufacturing practices, and on the regional legislation (Lu, Yuan et al. 2011a).

Table 2.2 - Estimated mean daily application amount ($g_{\cdot sample}. day^{-1}$) of PCPs in several studies.

PCPs	Mean daily application amount ($g_{\cdot sample}. day^{-1}$)													
	Europe										USA			Japan
	(Cadby, Troy et al. 2002)	(SCCNFP 2002)	(HERA 2003)	(Colipa 2005)	(Bremmer 2006)	(Hall, Tozer et al. 2007, Hall, Steiling et al. 2011)	(Nohynek, Antignac et al. 2010)	(SCCS 2012)	(Biesterbos, Dudzina et al. 2013)	**Average values**	(CTFA 2004, CTFA 2005)	(Loretz, Api et al. 2005, Loretz, Api et al. 2006, Loretz, Api et al. 2008)	**Average values**	(Nakata, Hinosaka et al. 2015)
Body lotion/cream	5.7	4.0	-	7.8	5.7	4.5	-	7.8	3.6	5.6	14.4	8.7	11.6	5.7
Facial creams	1.6	1.6	-	1.5	1.2	0.9	-	1.5	0.4[a]	1.2	3.5	2.1	2.8	-
Toothpaste	-	2.8	-	2.8	2.1	2.1	-	2.8	2.2	2.5	-	-	-	-
Deodorant	0.5	0.5[b]	-	3.8[c]	2.9[d]	3.1[e]	-	1.2[e]	0.4[f]	1.8	1.7[g]	0.8[g]	1.3	0.5
Shower gel	5.4	10.0	-	18.7	7.5	11.3	-	18.7	1.2	10.6	25.5	14.5	20.0	-
Soap bar (hands)	4.8	4.8	-	0.04	4.0	5.4	-	20.0[h]	-	6.5	-	-	-	
Shampoo	8.0	8.0	-	10.5	14.3	6.0	-	10.5	2.4	8.5	23.6	12.8	18.2	8.0
Hair conditioner	-	14.0	-	-	4.0	-	-	3.9	2.1	6.0	28.2	13.8	21.0	-
Hair Styling	10.0	7.7	-	4.0[i]	2.1[j]	1.9	-	4.0	1.0[k]	4.4	10.0	4.4[l]	7.2	-
Sunscreen	-	-	-	-	30.0	-	18.0	18.0	0.4	16.6	-	-	-	-
Hand cream	-	2.4	-	2.2	3.4	1.1	-	2.2	0.4	2.0	-	-	-	-
Shaving products	-	2.0	-	-	2.0	-	-		1.3	1.8	-	-	-	-
Mouthwash	-	30.0	21.7	21.6	40.0	12.6	-	21.6	-	24.6	-	-	-	-

[a] Mean amount for day and night cream; [b] Deodorant type not specified; [c] Non-spray deodorant; [d] Mean amount for roller, stick and spray deodorant; [e] Mean amount for non-spray and spray deodorant; [f] Mean amount for cream, roller, stick and spray deodorant; [g] Solid deodorant; [h] liquid hand soap; [i] Mean amount for hairspray, hair foam and gel; [j] Mean amount for hair foam, spray and gel; [k] Mean amount for hair foam, gel, lotion and wax; [l] Mean amount for aerosol and pump hairspray.

It is important to know, not only the consumption patterns, but also the population preferences (brands, type of PCP for each use, among other). However, reaching this data and achieving the exposure patterns of a specific population is difficult due to the need of a large and representative sample size accepting to collaborate in the study, and also to the variability between individual habits of consumers (Hall, Tozer et al. 2007).

Table 2.3 – Reported estimated retention factors of product on skin after application of PCPs

PCPs	**Mean retention factor**			
	Europe			**Japan**
	(Cadby, Troy et al. 2002)	(Bremmer 2006)	(SCCS 2012)	(Nakata, Hinosaka et al. 2015)
Body lotion/cream	1.00	1.00	1.00	1.00
Facial cream	1.00	1.00	1.00	-
Toothpaste	-	0.03[a]	0.05	-
Deodorant	1.00	0.85[b]	1.00	1.00
Shower gel	0.01	0.01	0.01	0.01
Soap bar (hands)	-	-	0.01	0.01
Shampoo	0.01	0.10	0.01	0.01
Hair conditioner	-	0.10	0.01	-
Hair Styling	0.01	0.10	0.10	-
Sunscreen	-	1.00	1.00	-
Hand cream	-	1.00	1.00	-
Shaving products	-	-	0.01	-
Mouthwash	-	0.10	0.10	-

[a] For children it can vary between 0.14 and 0.35; [b] For spray deodorant (it is assumed 1.00 for other deodorant types).

Although there are several European studies (Table 2.1 and Table 2.2), to the authors´ best knowledge, there are no available data about the use of PCPs in Portugal. These data are essential to perform an exposure assessment to any substance present in the PCPs used in this region. Therefore, the objective of the present study is to provide data on the preferences of the PCP consumers in the Northern Region of Portugal, the daily frequencies and used amounts.

2.2 Experimental Section

2.2.1 Study population

In order to access consumer habits of PCPs in the Northern Region of Portugal, a online questionnaire (Annex A) was developed with a previous validation. The inclusion criterion was adults resident in one of the Northern Region municipalities of Portugal. Based on these inclusion criteria and on the final results of the Portugal Census 2011 (INE 2011), the population real size was estimated to be about 370,000 people with a population distribution mostly concentrated in the littoral region (Figure 2.1). Thus, with a margin of error of 5% and a confidence level of 90%, a sampling size of 271 persons was estimated to guarantee representativeness of the study.

2.2.2 Data collection

First, a 50 people sample answer to a paper questionnaire in order to validate and verify the questionnaire, permitting some corrections to the final online questionnaire. Then, the link to the questionnaire was divulged by e-mail to all students and workers of the Engineering Faculty - University of Porto, and also through several authors' personal contacts from January to February 2015 (two months). The questionnaire had an initial brief description of the purpose of the study and the possibility of respondents to provide their consent before answering (Annex A). The questionnaire intended to collect social-demographic characteristics, personal characteristics and consumer habits. The analyzed PCPs were body moisturizer, facial moisturizer (day and night), toothpaste, deodorant, bath gel/soap, shampoo, hair conditioner, hair care leave on products, sunscreen (face and body) and other products. The last part of the questionnaire was not mandatory, corresponding to a diary of registration about hygiene and PCPs usage during one week. In this diary, besides the body weight information, respondents had to indicate, for each used PCP, the initial weight of the package, the daily frequency of use over seven days and the final weigh. Respondents submitted the fulfilled questionnaires with no personal identification, and a number was attributed to each one, ensuring anonymity.

2.2.3 Data analysis

Statistical analysis was performed using Microsoft Excel 2007®. Data were analyzed in terms of social-demographic characteristics (residence municipality, gender, age, labor activity, academic qualification and monthly income), personal characteristics (type of face skin, type of body skin, type of hair) and consumer habits (PCPs category, percentage of use and co-use and preferred PCPs brands and types). With the data collected in the last part of the questionnaire (diary of utilization), namely the utilization frequency per week and the initial and final weight of the package ($W_{initial\ package}$ and $W_{final\ package}$, respectively), the daily frequencies and used amounts for each type of PCP were obtained, using the following equations (1) to (3):

$$Daily\ frequency\ (event.day^{-1}) = \frac{Frequency\ per\ week}{7} \quad (1)$$

$$Application\ ammount\ (g.event^{-1}) = \frac{(W_{initial\ package} - W_{final\ package})}{Frequency\ per\ week} \quad (2)$$

$$Daily\ amount\ (g.day^{-1}) = Daily\ frequency\ \times Application\ amount \quad (3)$$

2.3 Results and Discussion

The aim of this study was to collect information about the consumer patterns of PCPs in the Northern Region of Portugal. In order to create this data pool, an online questionnaire was fulfilled by individuals resident in one of the Northern Region municipalities of Portugal (inclusion criterion). Although 458 individuals have responded, due to the inclusion criteria, the final sample size was set at 414. This sample size is higher than the initially defined (271), corresponding to a representative sample population for a 4.1% margin of error and a 90% confidence level.

2.3.1 Socio-Demographic Characterization

Considering the inclusion criteria, the residence distribution of the 414 respondents includes 35 of the 86 municipalities included in the Northern Region of Portugal (Figure 2.1).

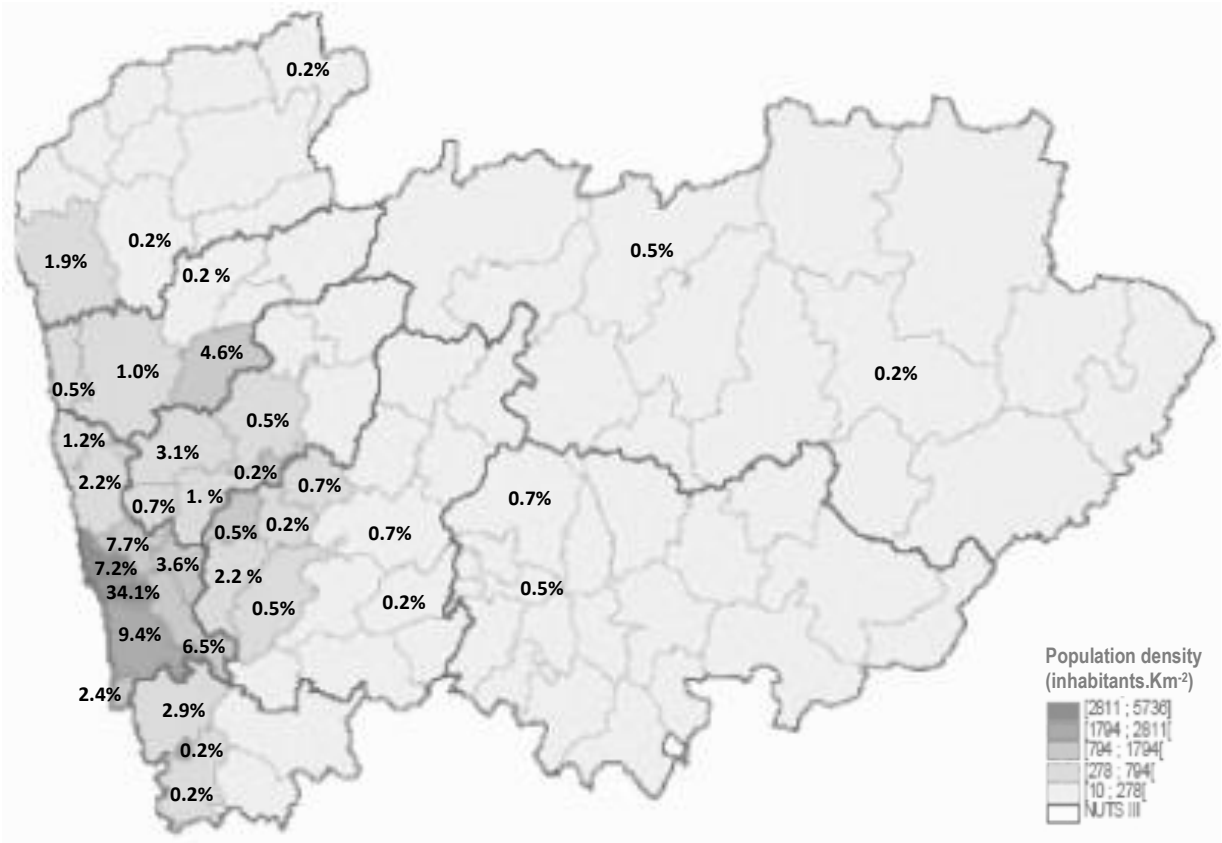


Figure 2.1 – Population density (INE 2011) and sample distribution of the respondents (N=414) in the municipalities of the Northern region of Portugal.

The sample residence distribution covers the most populated municipalities of the Northern Region of Portugal: Porto (34.1%), Gaia (9.4%), Trofa (7.7%), Matosinhos (7.2%), Gondomar (6.5%), Maia (3.6%) and Braga (4.6%). There are 51 municipalities not represented in the sample but all of them have a small population density (Figure 2.1). Therefore, it is assumed a good representability of the population under study by this sample.

When analyzing PCPs' usage patterns, another important parameter is gender because it is expected that females use more often and are more likely to use PCPs than males, as reported before (Wu, Bennett et al. 2010, Biesterbos, Dudzina et al. 2013, Den Hond, Paulussen et al. 2013). In the present study the majority of the respondents were females and the sample distribution is similar (62.3% female and 37.7% male) to the population distribution (52.1% female and 47.9% male).

All individuals who met the inclusion criteria were divided into four age categories corresponding to previous reported ranges (INE 2011). The distribution of the four age groups is shown in Figure 2.2, although two of the respondents did not answer this question. Age is an important parameter of distinction on consumer patterns especially when minors are included.

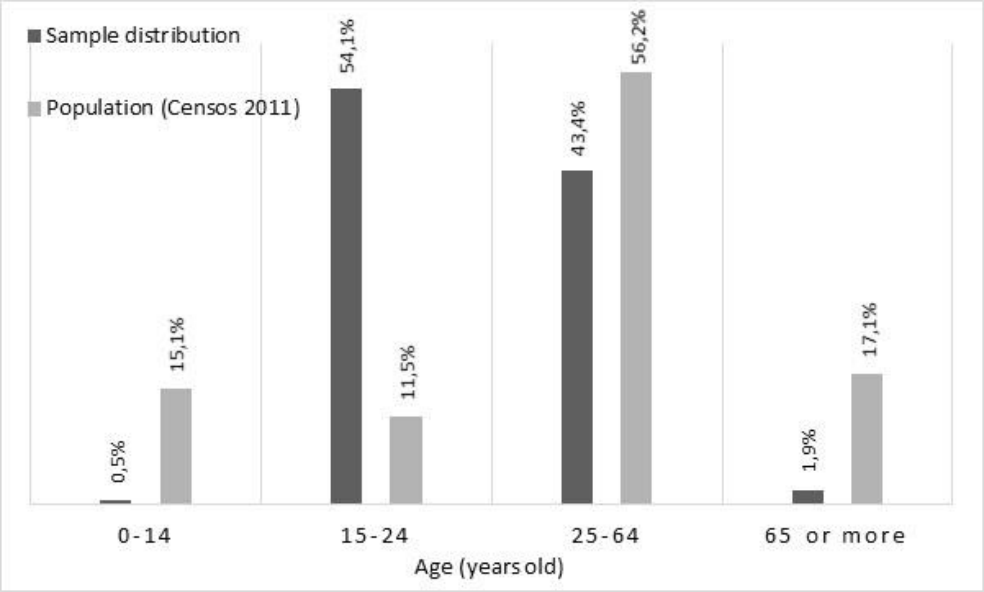


Figure 2.2- Distribution of inquired individuals by age (n=412).

This distribution is not according to Portugal Census 2011 because most of the Northern Region Portuguese population is from 25-64 years old category (56.2%), while the most relevant sample category is 15-24 years old (54.1%). These differences can be due to the fact that the

online questionnaire was mainly divulged to students and workers of a higher education institution, where most available individuals are students (younger ages). This is confirmed by the labor activity sample's distribution (Figure 2.3) with more students but also workers from the tertiary activity sector (includes the educational sector).

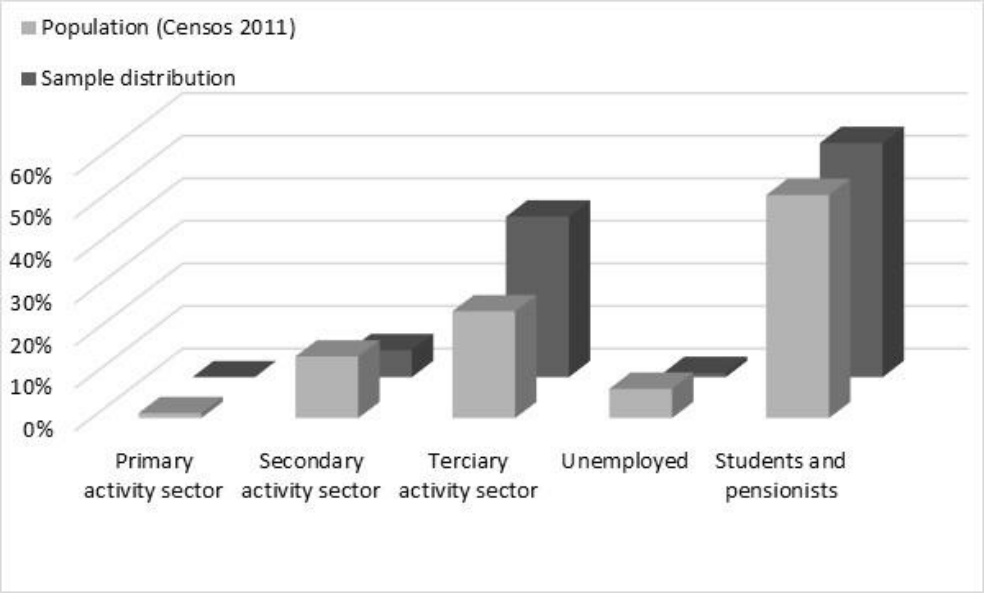


Figure 2.3 - Distribution of inquired individuals by labor activity (n=411).

The previous results are also corroborated by the distribution of the inquired individuals by academic qualification (Figure 2.4) where a higher educational level was found because individuals from the institution involved were, as expected, more graduated.

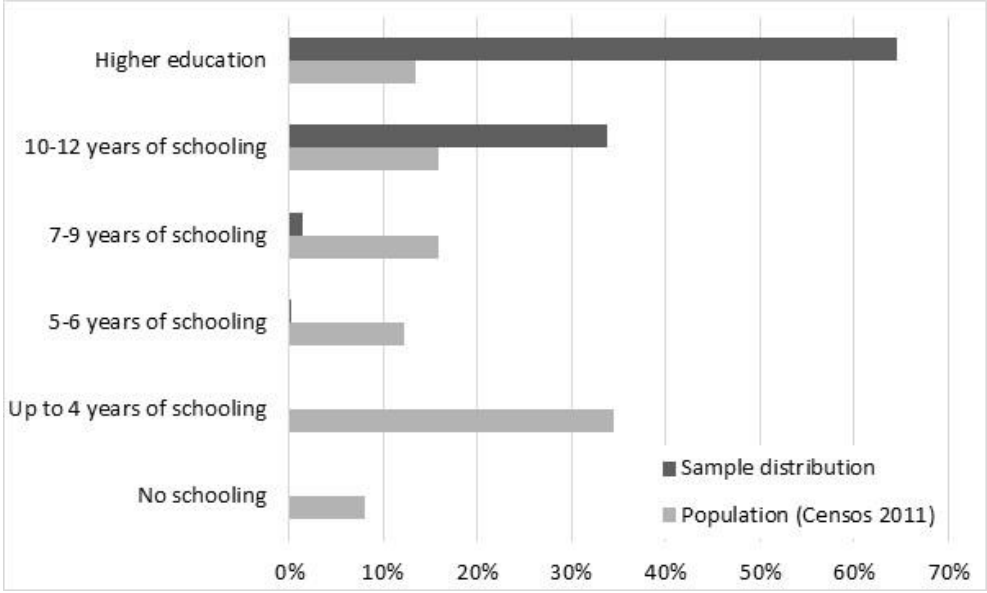


Figure 2.4 - Distribution of inquired individuals by academic qualification (N=414).

The monthly income (per capita) was obtained for 364 respondents, and the majority (60.4%) have one minimal wage or less, while only 4.9% have more than two minimal wages. With this mainly low monthly income, it is expectable that the economic purchasing power is reduced. This data are confirmed by the indication of the local of PCPs' purchase (Figure 2.5), where most respondents buy their PCPs in supermarkets and hypermarkets (72.7%), instead of pharmacies (5.1%), generally with higher PCPs' prices.

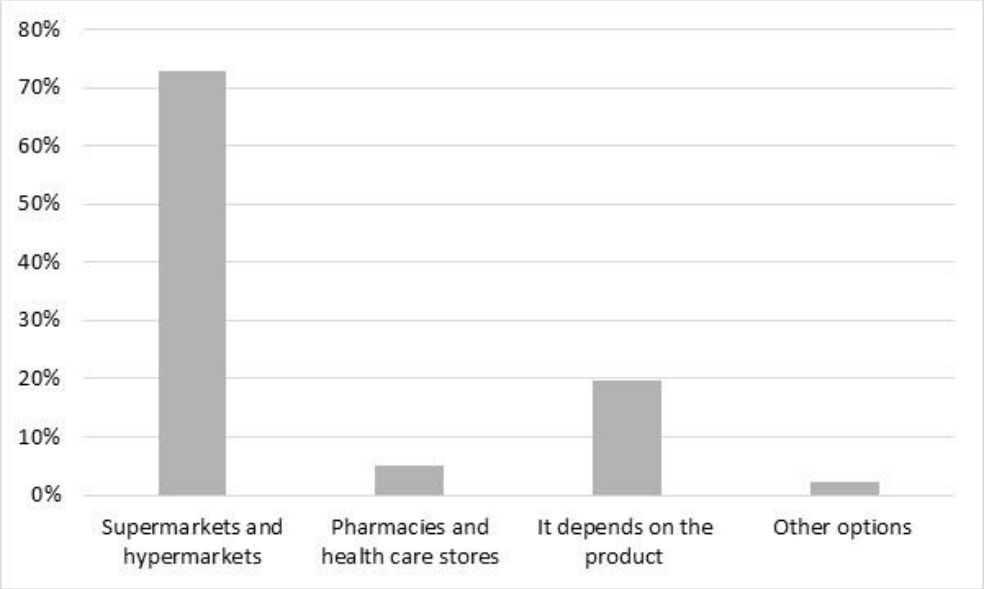


Figure 2.5 - Local of PCPs' purchase (N=414).

2.3.2 Personal characteristics

The questionnaire (Annex A) intended to obtain some skin and hair characteristics that could be useful to understand the consumer patterns. For instance, it is expected that people with oily hair or with dandruff increase the frequency of use of shampoo. It is also expected that individuals with dry or sensitive skin would apply more body lotion than someone with normal/combination/oily skin (Biesterbos, Dudzina et al. 2013). Therefore, questions about the skin type (face and body) and hair type were posed, but the number of respondents answering those questions were very low, varying from 51% (body skin type) to 84% (hair type) of the respondents. These are the respondents' perception about their skin and hair type, and not a professional evaluation. The facial skin type (Figure 2.6) was mainly normal to combination skin (57%), while body skin was considered to be dry for the majority of the respondents (48%).

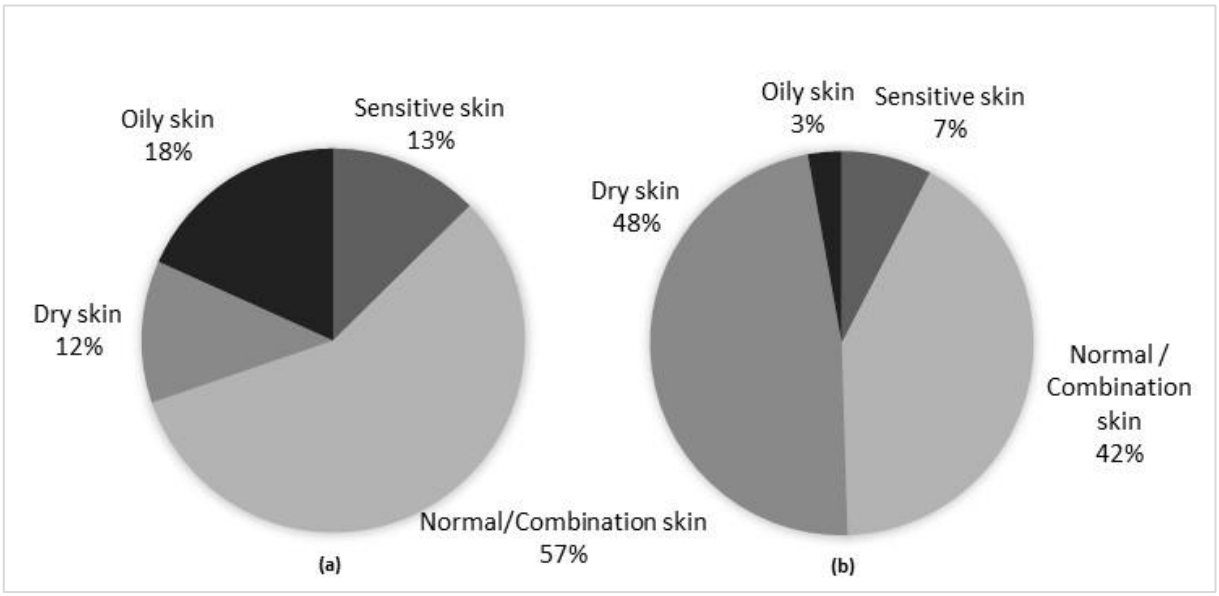


Figure 2.6 - Skin types of the inquired individuals: (a) face (n=267) and (b) body (n=210).

Another analyzed characteristic was the type of hair (Figure 2.7), and the plurality of the respondents classify it as normal hair (32%) or hair with dandruff (21%).

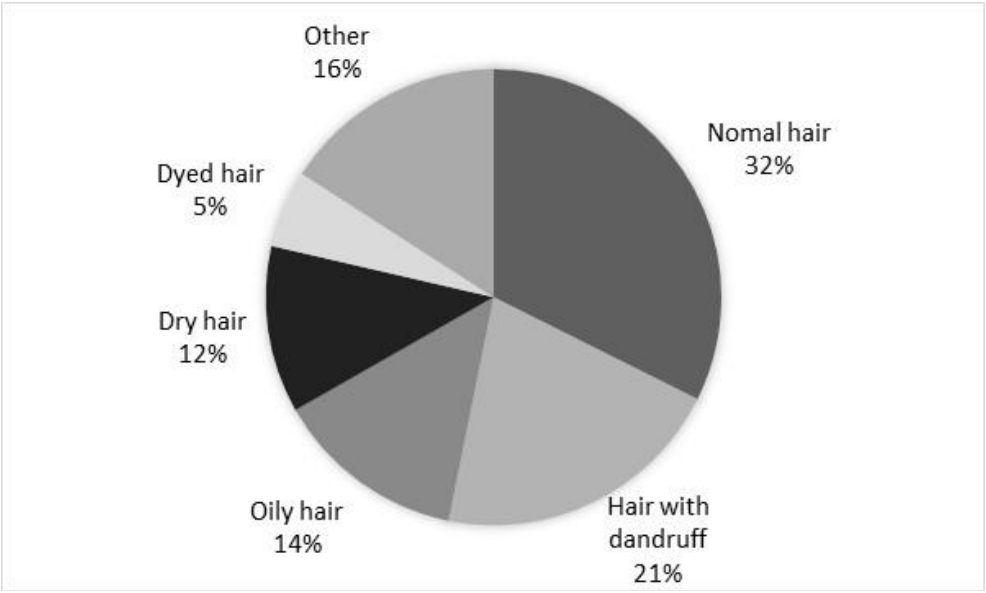


Figure 2.7 - Types of hair of the inquired individuals (n=346).

2.3.3 Consumer Habits

Using the responses to the online questionnaire, data were analyzed in terms of PCPs' use (percentage of use and co-use), PCPs' preferences (characteristics and brands) and PCPs' mean used amounts and frequencies. The analyzed PCPs were: body moisturizers, day facial moisturizer, night facial moisturizer, body sunscreen, facial sunscreen, toothpaste, deodorant,

body washers (bath gel/solid soap), shampoo, hair care rinse off products, hair care leave on products, and other products. This last category includes products that respondents could freely add, and the indicated ones were hand cream, shaving foam, mouthwash, aftershave, lip balm and face cleansing.

2.3.3.1 Personal Care Products' use

There are several socio-demographic parameters that may influence the PCPs' use, like gender, age, residence area and academic qualifications. For instance, two American studies reported similar responses for women resident across the four different regions of USA (according to USA Census), because no significant differences were found in usage patterns (Loretz, Api et al. 2005, Loretz, Api et al. 2008). However, other study from the same authors found significant differences across the country regions (Loretz, Api et al. 2006). Educational level can influence PCPs' preferences and use due to higher health awareness, as reflect the greater use of sunscreen in a study with 64% of the sample having higher education (Wu, Bennett et al. 2010). But, a higher educational level may increase or decrease the PCPs' usage patterns, as reported before for adolescents and adults, respectively (Den Hond, Paulussen et al. 2013). Nevertheless, only gender and age were considered in the present analysis for being the most relevant parameters influencing the usage patterns.

Personal Care Products' single use

The percentage of respondents using each type of PCP is presented in Figure 2.8 and Figure 2.9. Analyzing Figure 2.8.*a*, the most used PCPs are, as expected, toothpaste (99.8%), shampoo (98.0%), body washer (97.8%) and deodorant (94.5%), for being basic hygiene products. Leave on hair care products (22.6%), night facial moisturizer (24.4%) and other products (45.7%) are the less used PCPs. Shampoo and deodorant have already been reported as the most used PCPs among American adults, while the less prevalent were facial night cream and bath gel (Wu, Bennett et al. 2010). The "Other products" category (Figure 2.8.*b*) includes six types of PCPs, freely reported by respondents, where the most used ones are hand cream, shaving cream and mouthwash.

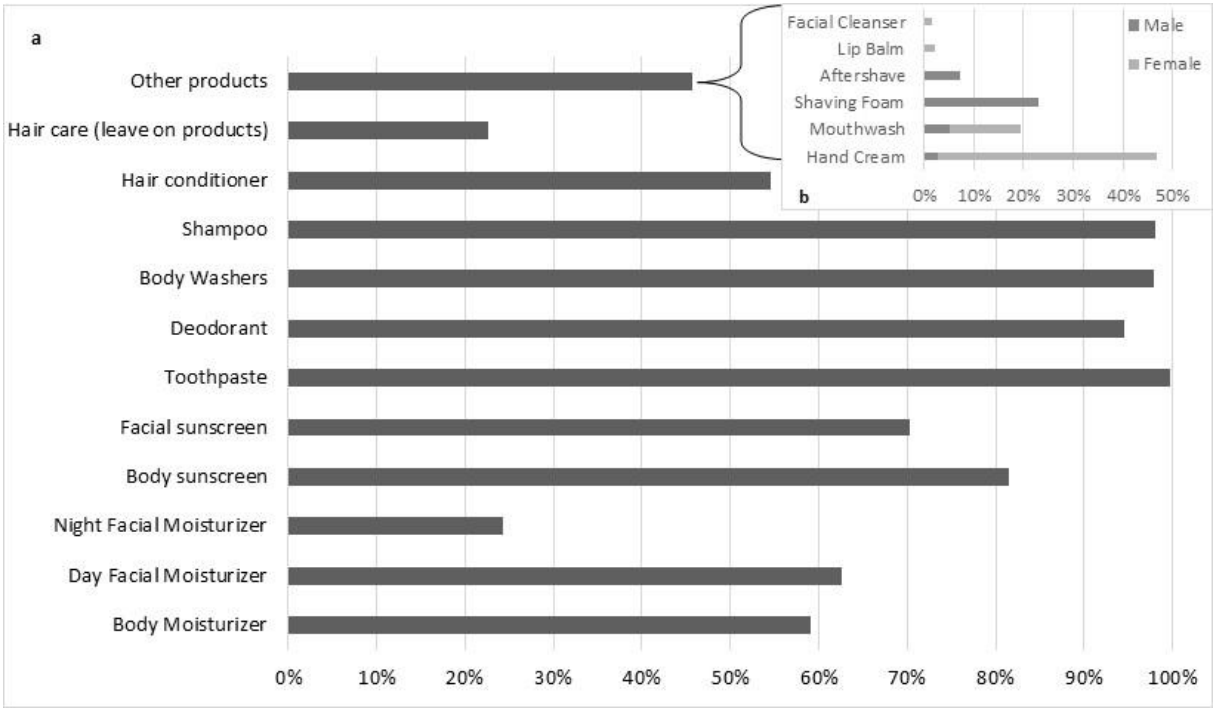


Figure 2.8 - PCPs percentage of users (N=414): (**a**) all products; (**b**) other products by gender.

The presented values in Figure 2.8.*a*, are consistent with the reported in Figure 2.9 because, for all population groups of gender or age, the most used PCPs are the same.

The differences between the percentage of usage by gender is reported in Figure 2.8.*b* and Figure 2.9.*a* where, as expected and reported before (Wu, Bennett et al. 2010, Biesterbos, Dudzina et al. 2013, Den Hond, Paulussen et al. 2013), women generally use more often PCPs than men. There are also more women using moisturizers and sunscreens than men, while for the other types of PCPs there are similar uses for both sexes. But, although the percentage of use for "Other products" is similar in both sexes (Figure 2.9), the PCPs mostly used in this category vary with gender (Figure 2.8.*b*): shaving products (shaving cream and aftershave) are, as expected, exclusively used by men, while lip balm and facial cleanser are used only by women. The present results are consistent with other values reported before for women (Loretz, Api et al. 2005, Loretz, Api et al. 2006, Loretz, Api et al. 2008): 76.0-99.7% for body lotions, 79.0-99.0% for day facial cream, 78.5-91.5% for shampoo, 92.9-97.1% for body washes, 97.1-99.4% for deodorant and 45.0-85.5% for hair conditioner.

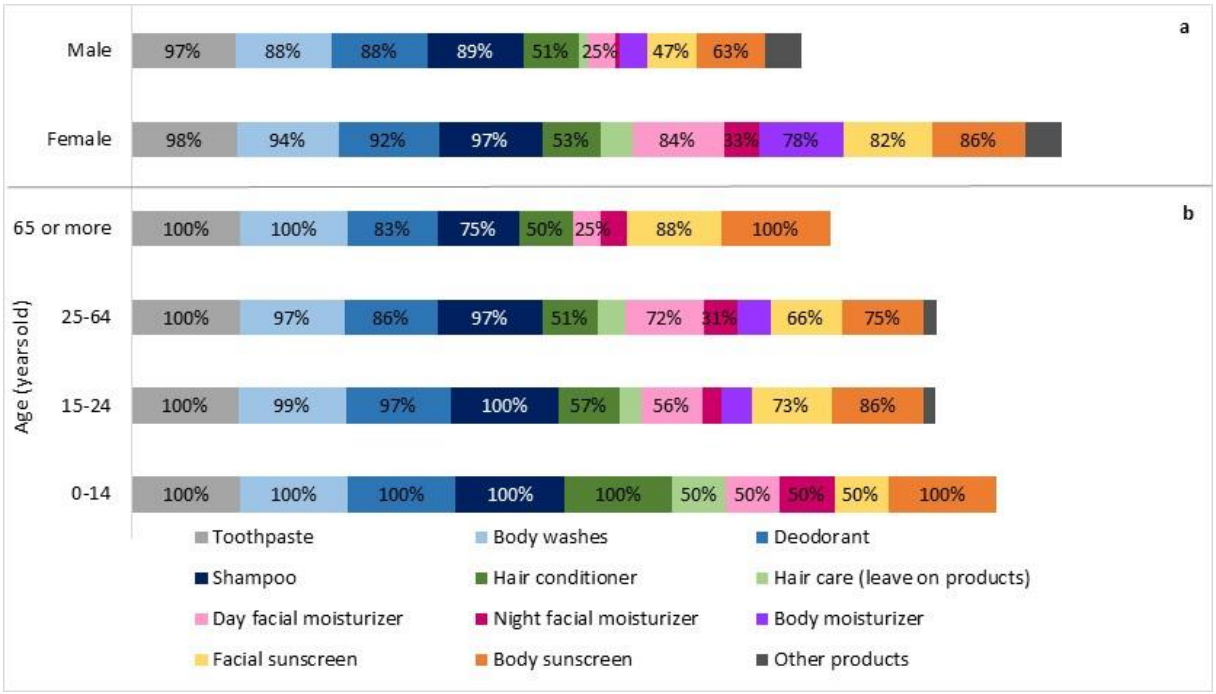


Figure 2.9 - PCPs percentage of users (N=414): by gender (**a**); by age (**b**).

The percentage of use is lower in the oldest individuals and higher in the younger ones. However, the lower number of respondents in the younger group may have introduced errors on the obtained results. Only the two middle age groups refer to use "Body moisturizer" or "Other products", and leave on hair care products are not used by any of the oldest respondents. The use of hair conditioner seems to decrease with age, and an American study (Loretz, Api et al. 2006) reported consistent values: 34.7% on the oldest group (60-65 years old) to 60.1% on the youngest respondents (60-65 years old). A study conducted in Belgium (Den Hond, Paulussen et al. 2013), reported differences on the PCPs' usage percentages of use when comparing adolescents (14-15 years old) and adults (20-40 years old). However, no significant differences were found among adolescents (14-15 years old) and among adults (20-40 years old). Actually, if only adults are considered, it is expected a small variation on the results, as stated in a study with the women´s USA population (Loretz, Api et al. 2006). Nevertheless, other American studies reported some differences on PCPs' use among adults' age groups (Loretz, Api et al. 2008, Wu, Bennett et al. 2010).

Personal Care Products' co-use

Besides the use or non-use of each kind of PCPs, it is important to know the population habits about the use of several PCPs, in order to consider an aggregate exposure approach. The first

thing to know is the number of PCPs simultaneously used in terms of age and gender (Figure 2.10).

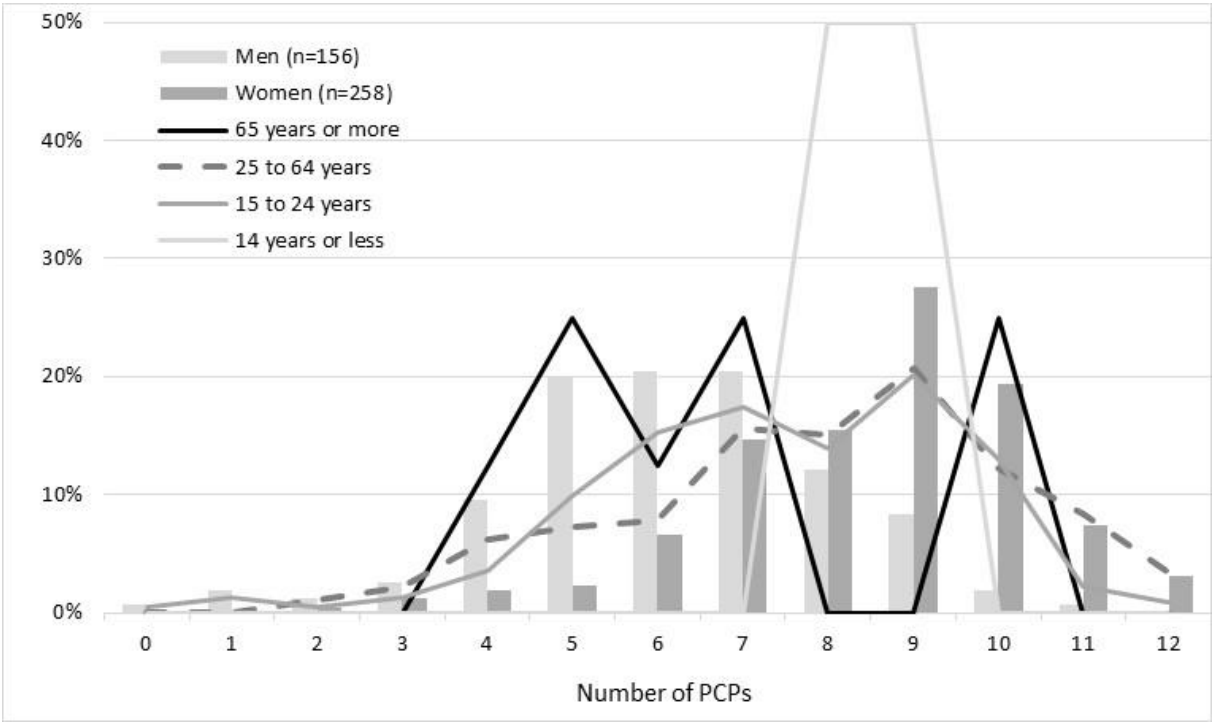


Figure 2.10 - Number of PCPs used simultaneously by age and gender.

It seems like the older and younger groups have more variation in the number of PCPs daily used, and that the middle groups are very similar. The mean number of PCPs used by each age group don't vary a lot: nine (0-14 years old), seven (15-24 years old), eight (25-64 years old) and seven (65 years old or more). But it should notice that the two extreme age groups have less respondents than the middle ones, which causes more dispersion. Analyzing Figure 2.10 it is clear that, as expected, women use more PCPs than men, with mean values of 8.5 and 6.2 products, respectively.

The previous results indicate that the co-use of PCPs is more influenced by the gender of the consumer than its age. So, an analysis was made taking into account the gender influence in the co-use of some PCPs. The simultaneous use of PCPs containing the same kind of substances contributes to aggregate exposure, which is important for a risk assessment (Wu, Bennett et al. 2010). Considering the usual hygiene and personal care moments, the most commonly associated PCPs are considered in this study (Figure 2.11): facial day moisturizer and facial night moisturizer; facial day moisturizer and body lotion; facial night moisturizer and body lotion; facial sunscreen and body sunscreen; toothpaste and mouthwash; shampoo and body wash;

shampoo and hair conditioner; shampoo and leave on hair care product. Almost all of this associations were already reported, being more or less correlated in terms of frequency of use (Wu, Bennett et al. 2010).

As expected, due to the higher number of PCPs' used simultaneously, women usually make more PCPs' use combinations. The most common combination is shampoo and body wash for both sexes, while the less used combination is toothpaste with mouthwash for women and facial day moisturizer with facial night moisturizer for men. About 52% of the respondents from both genders affirm to simultaneously use shampoo and hair conditioner. A smaller co-usage percentage of those PCPs was already reported (35%) (Biesterbos, Dudzina et al. 2013).

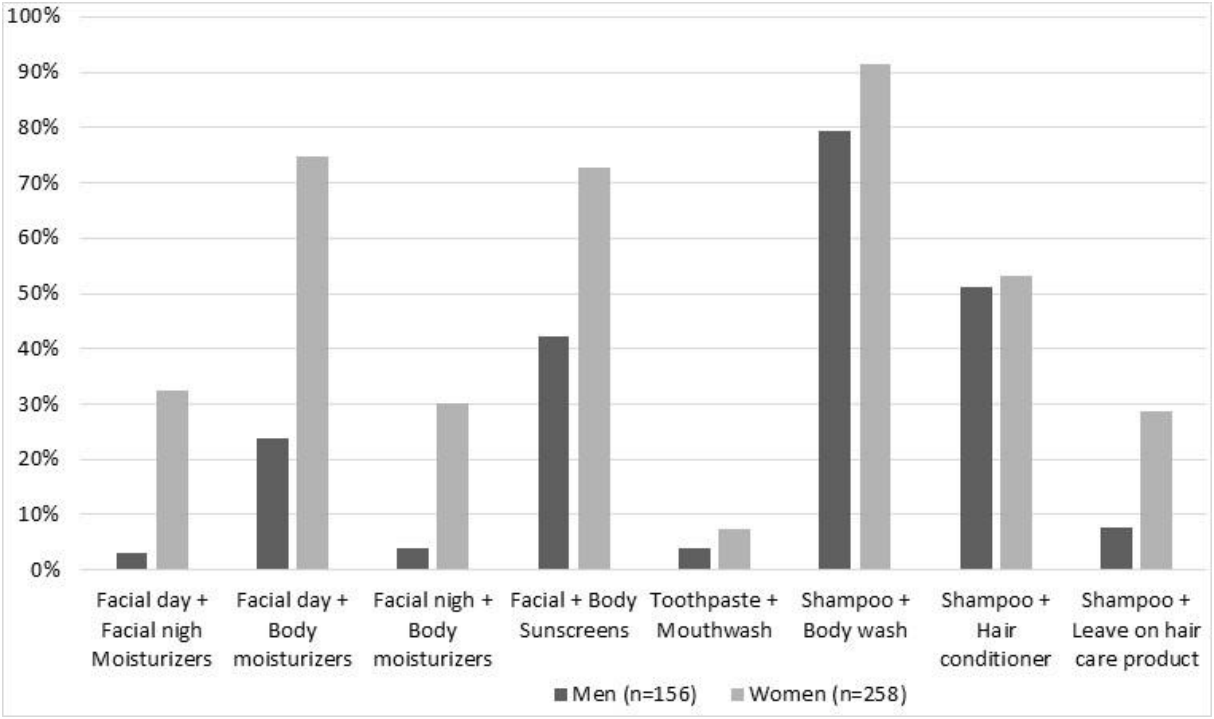


Figure 2.11 - Types of PCPs used simultaneously by each gender.

2.3.3.2 PCPs' preferences

PCPs characteristics

Each considered PCP has several types based on its characteristics and purposes. So, considering the different offers available in the market, each PCP was analyzed in terms of preference on types/characteristics.

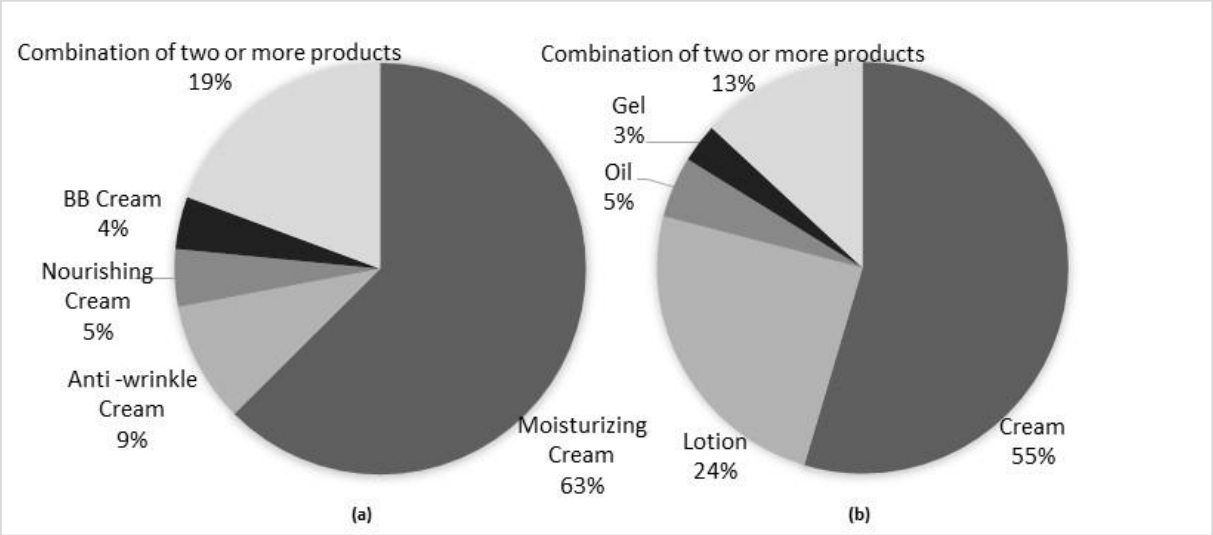


Figure 2.12 - Moisturizers types preferred by the inquired individuals: (a) Facial (n=243) (b) Body (n=229)

The majority of the respondents prefer facial cream moisturizers instead of anti-wrinkle, nourishing or BB creams. The results of Figure 2.12 are consistent with the percentage of use of facial creams (Figure 2.8), because moisturizing cream is mostly used as facial day cream and anti-wrinkle or nourishing creams are mainly used as facial night creams. These data are also explained due to the face skin type (Figure 2.6) because most people has normal to combination skin, and to age distribution of the sample because older people, that are more likely to use anti-wrinkle or nourishing creams, are one of the less representative age groups.

As for body moisturizers, the most commonly used types are creams and lotions. The results of Figure 2.12 are consistent with the type of body skin of the respondents (Figure 2.6) because, it is expectable that people with dry skin would prefer creams for the hydration of the skin.

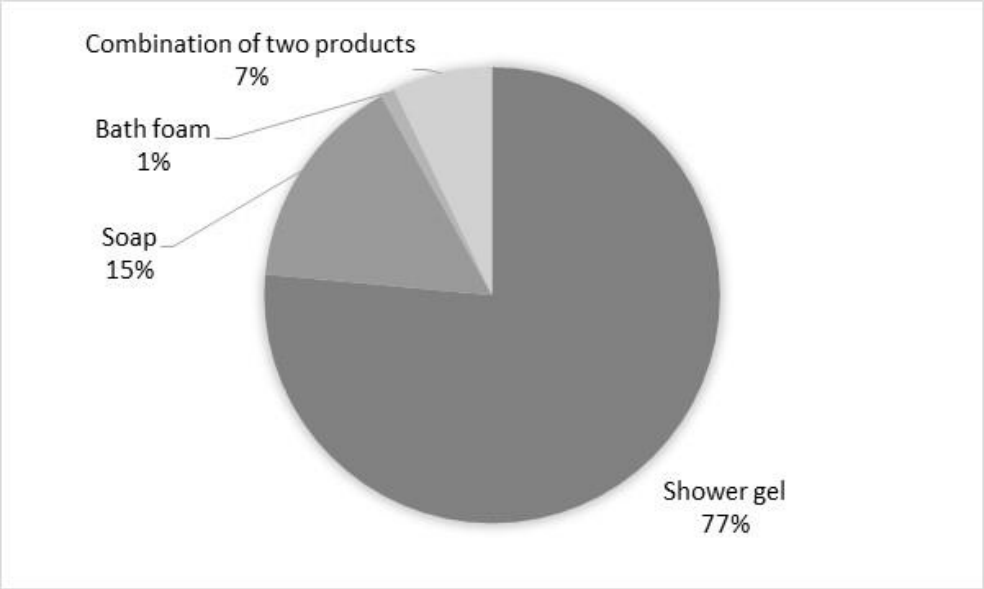


Figure 2.13 - Body washers types used by the inquired individuals (n=395).

The most commonly used type of body washer is, as expected, shower gel (Figure 2.13). This is consistent with other study that reported 77% for shower gel (Biesterbos, Dudzina et al. 2013), but is higher than an American study where only 34 to 41% of the respondents prefer to use shower gel (Wu, Bennett et al. 2010). Shampoo is used by 91% of the respondents (n = 375), while the other use a 2 in 1 product. For the rinse off hair care products (n = 211), 62% use hair conditioner, 17% a hair mask while 21% use both products. The most frequently used leave on hair care products are foam and serum, mainly used for several hair styling purposes (Figure 2.14). The purpose of the hair styling/care products are mainly the curl effect and the fixation.

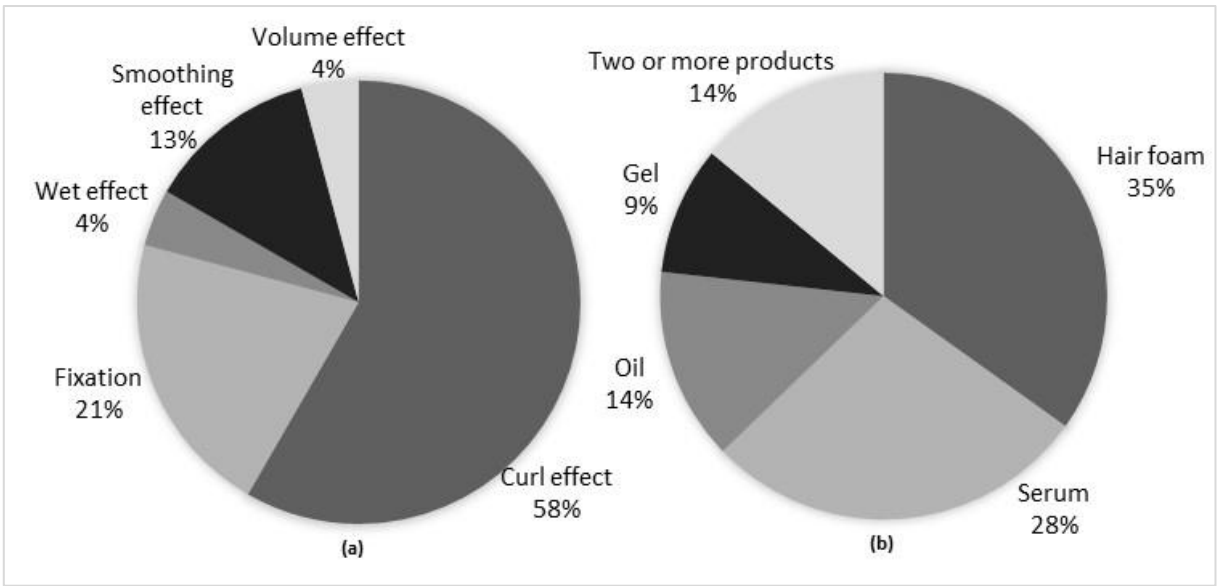


Figure 2.14 – Leave-on hair care/styling product: (a) effect preferred by the inquired individuals (n=48) (b) types preferred by the inquired individuals (n=86)

The preferred type of deodorant is roll-on (Figure 2.15), and respondents buy a variety of deodorant correspondent to the gender distribution of the sample, considering that women also use unisex deodorant: deodorant for women (53%), deodorant for men (38%) and deodorant unisex (9%).

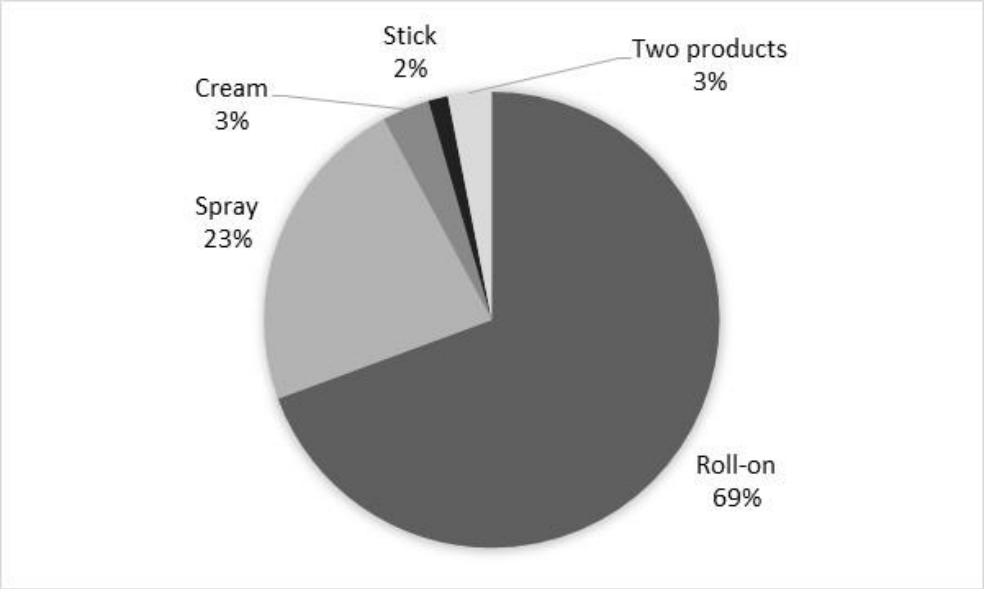


Figure 2.15 - Deodorant type preferred by the inquired individuals (n=356).

The majority of the sample individuals use toothpaste for oral hygiene (93%), while the others use a 2 in 1 product (toothpaste and mouthwash).

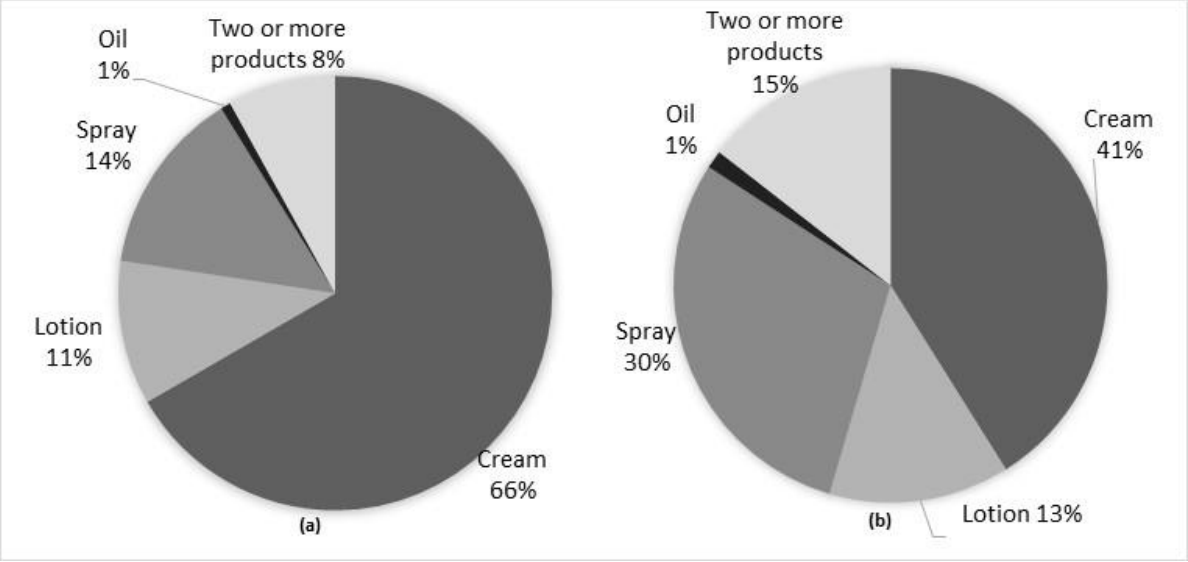


Figure 2.16 - Sunscreen type preferred: (a) Facial sunscreen (n=261); (b) Body sunscreen (n=297).

The most used sunscreen type is cream, especially for facial (Figure 2.16.*a*), although for body sunscreen, cream and spray preferences are similar (Figure 2.16.*b*). The preferred sunscreen protection factor (SPF) were SPF 30 and 50+ for both facial and body use (Figure 2.17).

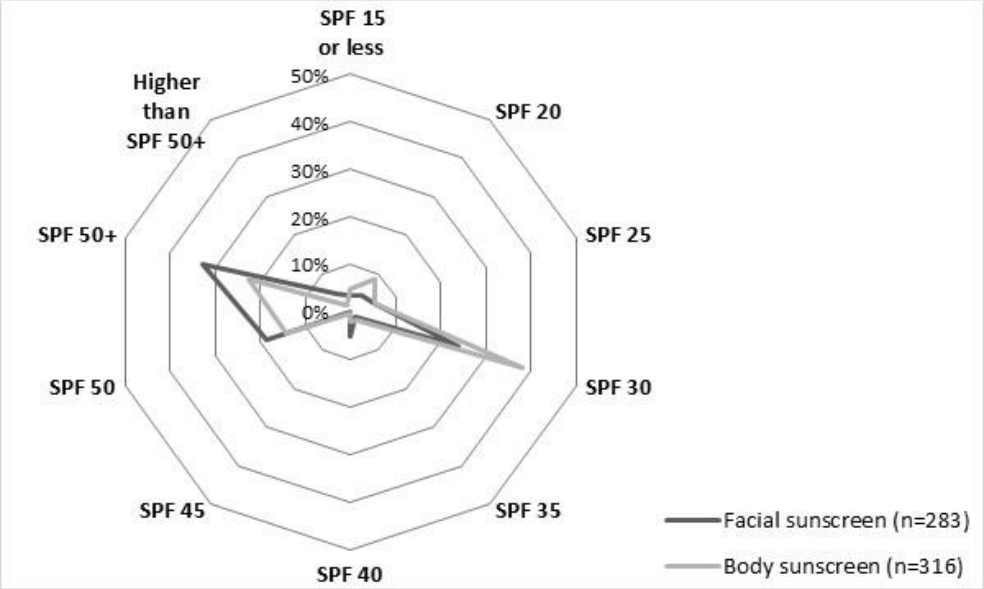


Figure 2.17 - Sunscreen Protection Factor (SPF) preferred by the inquired individuals (% respondents).

Personal Care Products' brands

In order to perform an exposure assessment with PCPs, the preferences of the consumers about the brands and specific products of each brand were also collected (Table 2.4), but the confidentiality of the brand names were ensured by a codification for each product.

The most referred brand by the respondents is M26, that is the preferred of the consumers for moisturizers (body and facial), deodorants and sunscreens (body and facial), being also the third one as body washer. For hair care PCPs, M19 and M34 are always referred and the last one is preferred. The three major brands of sunscreens are the same for facial and body application (M26, M27 and M19), although the order of preference vary between the last two. The majority of the referred brands of PCPs are normally purchased in supermarkets and hypermarkets, which is consistent to the consumer habits on the local of purchase (Figure 2.5). This is an important information for exposure assessment, because these should be the selected PCPs for being analyzed in terms of its composition substances of interest. The PCPs presentation are also useful for the selection of the extraction method.

Table 2.4 - PCPs brands preferences and most referred specific product of each brand.

	Brands			Specific product of each brand				
	Code	**Number of users**	**Utilization (%)**	**Code**	**Presentation**	**Number of users**	**Utilization by the brand users (%)**	**Utilization by the sample users (%)**
BODY MOISTURIZERS	M26	67	16.2	M26CC11	Cream	5	7.5	2.1
	M18	23	5.6	M18CC10	Cream	2	8.7	0.9
				M18LC11	Lotion	2	8.7	0.9
	M31	13	3.1	M31LC10	Lotion	4	30.8	1.7
	Other brands	132	31.9	-	-	-	-	-
	No answer; Don't Know/use	179	43.2	-	-	-	-	-
FACIAL DAY MOISTURIZER	M26	82	19.8	M26GR1	Gel Cream	10	12.2	4.0
	M28	21	5.1	M28CR5	Cream	7	33.3	2.8
	M27	20	4.8	M27CR4	Cream	5	25.0	2.0
	Other brands	127	30.7	-	-	-	-	-
	No answer; Don't Know/use	164	39.6	-	-	-	-	-
FACIAL NIGHT MOISTURIZER	M26	18	4.3	M26CR6	Cream	3	16.7	2.9
	M29	6	1.4	(a)	-	-	-	-
	M30	6	1.4	M30CR7	Cream	3	50.0	2.9
	Other brands	73	17.6	-	-	-	-	-
	No answer; Don't Know/use	311	75.1	-	-	-	-	-
TOOTHPASTES	M11	240	58.0	M11PD4	Paste	17	7.1	4.2
	M35	41	9.9	M35PD5	Paste	4	9.8	1.0
	M14	31	7.5	M14PD3	Paste	5	16.1	1.2
	Other brands	91	22.0	-	-	-	-	-
	No answer; Don't Know/use	11	2.7	-	-	-	-	-
DEODORANTS	M26	105	25.4	M26D2	Roll-on	7	6.7	1.9
	M18	71	17.1	M18D1	Roll-on	9	12.7	2.4
	M32	44	10.6	M32D3	Roll-on	4	9.1	1.1
	Other brands	155	37.4	-	-	-	-	-
	No answer; Don't Know/use	39	9.4	-	-	-	-	-
BODY WASHERS	M18	95	22.9	M18GB14	Gel	6	6.3	1.6
	M1	46	11.1	M18GB15	Gel	6	13.0	1.6
	M26	38	9.2	M18GB16	Gel	4	10.5	1.0
	Other brands	206	49.8	-	-	-	-	-
	No answer; Don't Know/use	29	7.0	-	-	-	-	-
SHAMPOOS	M34	69	16.7	M34CH12	Gel	3	4.3	0.8
				M34CH13	Gel	3	4.3	0.8
	M33	40	9.7	M33CH11	Gel	6	15.0	1.5
	M19	34	8.2	M19CH10	Gel	4	11.8	1.0
	Other brands	245	59.2	-	-	-	-	-
	No answer; Don't Know/use	26	6.3	-	-	-	-	-

Table 2.4 (*cont.*)

HAIR CONDITIONERS	M34	53	12.8	M34AC4	Cream	6	11.3	2.8
	M36	22	5.3	M36MC1	Cream	4	18.2	1.8
	M19	20	4.8	M19AC3	Cream	4	20.0	1.8
	Other brands	123	29.7	-	-	-	-	-
	No answer; Don't Know/use	196	47.3	-	-	-	-	-
HAIR STYLING PRODUCTS	M37	13	3.1	M37OC2	Oil	3	23.1	3.4
	M34	13	3.1	M34EC1	Foam	2	15.4	2.3
	M19	7	1.7	M19OC1	Oil	2	28.6	2.3
	Other brands	55	13.3	-	-	-	-	-
	No answer; Don't Know/use	326	78.7	-	-	-	-	-
FACIAL SUNSCREEN	M26	73	17.6	M26PL8	Lotion	7	9.6	2.5
	M27	57	13.8	M27PL6	Lotion	4	7.0	1.5
	M19	30	7.2	M19PL7	Lotion	5	16.7	1.8
	Other brands	115	27.8	-	-	-	-	-
	No answer; Don't Know/use	139	33.6	-	-	-	-	-
BODY SUNSCREEN	M26	110	26.6	M26PL8	Lotion	7	6.4	2.2
	M19	53	12.8	M19PL7	Lotion	5	9.4	1.6
	M27	42	10.1	M27PL6	Lotion	4	9.5	1.3
	Other brands	108	26.1	-	-	-	-	-
	No answer; Don't Know/use	101	24.4	-	-	-	-	-
OTHER PRODUCTS	M38 (Hand cream)	5	1.2	M38CM1	Cream	5	100.0	3.8
	M39 (Shaving foam)	10	2.4	M39EB1	Foam	4	40.0	3.0
	M40 (Mouthwash)	9	2.2	M40SO1	Solution	3	33.3	2.3
	Other brands	108	26.1	-	-	-	-	-
	No answer; Don't Know/use	101	24.4	-	-	-	-	-

2.3.3.3 PCP´s usage mean frequencies and amounts

Daily frequency of use and used amount

The daily frequencies and amounts of use were mainly obtained in the last part of the questionnaire (Annex A), the "Diary of hygiene and Personal Care Products use by adults", fulfilled by some respondents during one week. This kind of paper diaries, where consumers register the PCP weigh before and after use, and also the frequency of use, is a more accurate method to access the precise amounts and frequencies of use than an online questionnaire. Nevertheless, due to the difficulty to collect this data when dealing with large samples (Biesterbos, Dudzina et al. 2013), the obtained responses (Table 2.4) correspond to the PCPs'

usage by twenty six persons (6.3% of the total respondents). The respondents were aged between 13 and 68 years old (mean 35 years old), where most of them were females (69%) resident in Porto municipality (38%) and aged between 15 and 64 years old (89%). Besides the reduced number of respondents, it is believed that the results are representative of the population under study because the socio-demographic characteristics are similar to the obtained for the whole sample (see section 2.3.1). Using the collected data and equations (1) to (3), the daily frequency, application amount and daily amount were calculated (Table 2.5).

The most used PCPs by the diary respondents were shampoo (100%), toothpaste (92%) and shower gel (88%), while the less used ones were mouthwash (12%), shaving foam (12%) and facial sunscreen (15%). PCPs were generally used mostly by women, with facial night cream, body lotion, hand cream and facial sunscreen used exclusively by women, while shaving foam was used only by men. This results are consistent with other published data (Biesterbos, Dudzina et al. 2013) and with the main results obtained from all questionnaire respondents (Figure 2.8). The major differences between usage percentage of the diary respondents (Table 2.5) and the total respondents (Figure 2.8) were found for body lotion, hand cream, facial sunscreen and deodorant. The type of used deodorant was also reported in the diary responses and similar values were obtained for the preferences of all respondents (Figure 2.15): 56% use roll-on, 33% use cream and 11% use spray. So, besides some differences, we believe in the representativeness of these diary respondents compared to the total respondent's sample.

The frequencies of use in the present study (Table 2.5) have a small variation between PCPs, being all approximately of 1 event.day^{-1}, except for toothpaste (2 event.day^{-1}) and after-shave (0.5 event.day^{-1}). There is a higher variation in frequency when analyzing the mean values of frequencies in other published studies (Table 2.1), but comparing the averages of European, American and Japanese studies, the frequencies of PCPs' use seem to be independent from the location/population under study. In fact, the most different average value was found for sunscreen´s frequency of use, with 2.2 times a day in Europe and 0.4 times a day in USA, but they respect to sun exposure days and cool season, respectively. The frequencies of application in the present study (Table 2.5) are generally similar to the other studies (Table 2.1) for body lotion, facial creams (considering day and night cream co-use), toothpaste, deodorant, shower gel, shampoo, hair conditioner and shaving foam. The use frequency of mouthwashes, hair

styling products, hand cream and sunscreen presented here (Table 2.5) are lower than the obtained in other studies (Table 2.1). But it should noticed that the present study was performed in winter season, so the only value of sunscreen frequency comparable to this one is similar (CTFA 2004, CTFA 2005). So, frequency of application of PCPs seems to be a parameter with less variation, since the majority of the studies found in the literature don't differ a lot from the present one. For mouthwash, hand cream, and shaving foam, there was also a question on the main questionnaire about the frequency of these particular PCPs. The frequencies obtained from the questionnaire responses, confirm the results of Table 2.5: mouthwash (0.8 event.day^{-1}), hand cream (0.9 event.day^{-1}) and shaving foam (0.5 event.day^{-1}). This results also reinforce the representativeness of these small sample compared to the total sample obtained in the online survey.

Table 2.5 – Percentage of users, mean frequencies and application amounts of the analyzed PCPs.

PCP type	**% of users** (Diary respondents; n=26)	**Daily frequency** (event.day^{-1})	**Application amount** (g_{sample}.event^{-1})	**Daily amount** (g_{sample}.day^{-1})
Body lotion	38	0.8 ± 0.3	6.8 ± 6.7	4.1 ± 2.7
Day facial cream	73	0.9 ± 0.1	0.6 ± 0.5	0.6 ± 0.4
Night facial	35	0.9 ± 0.2	0.6 ± 0.3	0.5 ± 0.3
Toothpaste	92	1.9 ± 0.5	0.8 ± 0.4	1.5 ± 0.8
Deodorant[a]	69	1.1 ± 0.4	0.8 ± 0.6	0.9 ± 0.8
Shower gel/Soap[b]	88	1.0 ± 0.5	10.6 ± 6.8	9.6 ± 6.0
Shampoo	100	0.7 ± 0.4	7.6 ± 5.9	4.8 ± 3.8
Hair conditioner	54	0.6 ± 0.4	5.2 ± 3.2	3.0 ± 2.2
Hair styling[c]	15	0.6 ± 0.3	2.9 ± 3.8	2.4 ± 3.9
Facial sunscreen	15	0.6 ± 0.3	0.6 ± 0.6	0.5 ± 0.7
Hand cream	27	0.9 ± 0.5	0.9 ± 0.5	1.0 ± 0.8
Shaving foam	12	0.6 ± 0.2	3.2 ± 1.8	2.0 ± 1.7
Mouthwash	12	0.8 ± 0.4	12.1 ± 5.6	10.6 ± 8.0
Aftershave	-	0.5	-	-
Lipbalm	-	1.0	-	-
Facial cleanser	-	0.6	-	-

[a] Mean amount for roll-on, cream and spray deodorant; [b] Only one soap bar user; [c] Mean amount for hair gel, lotion and foam

The obtained daily amount values (Table 2.5) present a greater variability than the frequency of use of the different types of PCPs. This variability was also reported in other studies (Table 2.2) published between 2002 and 2015 and different countries. Therefore, usage patterns must be influenced by the culture of the population and that during such a long period, market shifts

may have played a role on those habits. But, when performing an average amount value for each region (Table 2.2), the American ones report a highest average daily amount for all PCPs, except for deodorant. The Japanese study shows mean amount of PCPs similar to the average value for European studies, also excluding deodorant values. Nevertheless, it should be noticed that there is a great variation on the type of deodorant included on each study and there is a small variation between the daily used amounts of shaving products. For sunscreens, the highest values found in literature refers to sunscreen applied in the whole body (face included), and were obtained assuming a sun exposure day with applications three times a day (Bremmer 2006) and two times a day (Nohynek, Antignac et al. 2010, SCCS 2012), which may justify the differences between them. The smallest daily amount of sunscreen (Biesterbos, Dudzina et al. 2013) seems to be related only to face application due to the huge difference to the other published values and to the resemblance to the amount of facial cream reported in the same study. The data obtained in the present study (Table 2.5) and the ones found in literature (Table 2.2) are very different. But, considering that Portuguese population should have comparable consumer patterns as the European population, only facial creams and shower gel present similar amounts of use, respectively 1.2 and 10.6 g.event^{-1}. The remaining PCPs reported in this work (Table 2.5) presented daily amount values lower than the average values obtained for European studies (Table 2.2), except for body lotion and shaving products with higher amounts of use. This results indicate that, contrary to frequency of application, used amounts vary a lot between studies and populations under evaluation, and that this parameter is an important one for exposure assessment.

Sunscreen use seasonal variation

Sunscreen is a specific PCP that is mainly used in hot seasons, like spring and summer, leading to a seasonal variation along the year. In order to evaluate this hypothesis, two questions were posed in the questionnaire, for facial and body sunscreen, relating to the frequency of use of those PCPs all year and in summer (Annex A). The collected and combined data are presented in Figure 2.18.

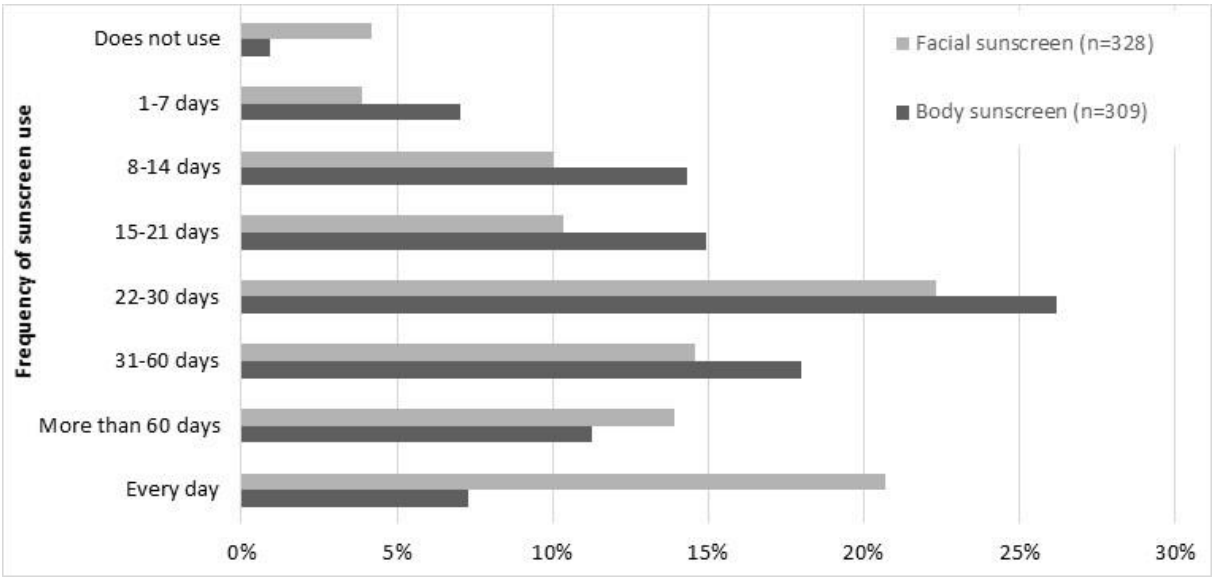


Figure 2.18 - Sunscreen frequency of use by the inquired individuals (% respondents).

The majority of the respondents use facial sunscreen from 22 to 30 days (22%) or on a daily basis (21%), while for body sunscreen most people use it from 22 to 30 days (26%) or 31 to 60 days (18%). Considering a maximum of 60 days for sun exposure in summer (2 months), 61.2% and 80.5% of the respondents use, respectively, facial and body sunscreen only in summer. Only the remaining consumers, use sunscreen regularly for more than 60 days or daily. A study conducted in USA, reported similar frequencies of facial sunscreens' use in cool and hot season for women, and that only men have a lower utilization in cool season (Wu, Bennett et al. 2010).

Exposure per body weight and per area of skin

Exposure is defined as the average amount of product used per day (daily exposure), the amount per body weight (McNamara, Rohan et al. 2007) or the amount per unit area of skin. The exposure per body weight is more relevant for the evaluation of systemic toxicity, while for skin irritation or phototoxicity the exposure per unit area of skin should be achieved (SCCS 2012). So, combining the daily amount (Table 2.5) with the PCP retention factor (adopted from SCCS 2012), a daily exposure ($g_{sample}.day^{-1}$) was calculated (Table 2.6). This exposure can be divided by a body weight or by the area of skin in which each PCP is applied. The respondents' body weight vary between 46 and 82 kg, with a mean of 60 kg, that is near the published data for Portuguese adult average weight of 57.5 kg (ECETOC 2001). Using each individual body weight, an exposure per unit of body weight ($mg_{sample}.day^{-1}.kg_{bw}$) was estimated (Table 2.6). The exposure per unit area of skin was achieved using the mean values of exposed skin surface for

each PCP application (SCCS 2012), although no data are available for the oral cavity and lips surface area (toothpaste and mouthwash). Analyzing the results of Table 2.6, the most relevant PCP for systemic exposure is body lotion for having the highest value of exposure per unit of body weight (69.9 $mg_{sample}.day^{-1}.kg_{bw}$). On the other hand, deodorants seem to be the most preoccupant PCPs in terms of skin toxicity due to its higher exposure per unit area of skin (66.0 $\mu g_{sample}.day^{-1}.cm^{-2}$). Additionally, both PCPs are leave-on products, resulting in greater opportunity for absorption by the skin or for skin sensitization. The obtained results are consistent with other European studies (Hall, Tozer et al. 2007) (Hall, Steiling et al. 2011), if we also consider the retention factor, for all PCPs considered in those studies (all except hair conditioner, shaving foam and sunscreen). But another older study (Colipa 2005) reported slightly different values for shampoo, toothpaste, shower gel and hair styling products, but very different exposures for body lotion (123.2 $mg_{sample}.day^{-1}.kg_{bw}$), facial moisturizer (24.1 $mg_{sample}.day^{-1}.kg_{bw}$), deodorant (55.0 $mg_{sample}. day^{-1}. kg_{bw}$), mouthwash (32.5 $mg_{sample}. day^{-1}. kg_{bw}$) and hand cream (32.7 $mg_{sample}. day^{-1}. kg_{bw}$). This results reveal that exposure varies with time and that actual data are essential for exposure assessment purposes.

Table 2.6 - Daily exposure based on the retention factor of PCPs on skin, exposure per body weight ($mg_{sample}.day^{-1}.kg_{bw}$) and exposure per unit area of skin ($\mu g_{sample}.day^{-1}.cm^{-2}$).

PCP	**Retention factor** (SCCS 2012)	**Daily amount of exposure** ($g_{sample}.day^{-1}$)	**Exposure** ($mg_{sample}.day^{-1}.kg_{bw}$)	**Exposure** ($\mu g_{sample}.day^{-1}.cm^{-2}$)
Body lotion	1.0	4.10 ± 2.70	69.9 ± 46.5	4.0
Day facial cream	1.0	0.60 ± 0.40	9.7 ± 5.8	17.0
Night facial cream	1.0	0.50 ± 0.30	8.5 ± 4.9	15.0
Toothpaste	0.05	0.08 ± 0.04	1.3 ± 0.7	-
Mouthwash	0.10	1.10 ± 0.80	14.3 ± 9.2	-
Deodorant[a]	1.0 [d]	0.80 ± 0.70	13.2 ± 10.2	66.0
Shower gel/Soap[b]	0.01	0.10 ± 0.10	1.6 ± 1.0	0.1
Shampoo	0.01	0.05 ± 0.04	0.8 ± 0.6	0.6
Hair conditioner	0.01	0.03 ± 0.02	0.5 ± 0.4	0.4
Hair styling[c]	0.10	0.20 ± 0.40	3.3 ± 5.1	3.0
Hand cream	1.00	1.00 ± 0.80	15.3 ± 15.1	18.0
Shaving foam	0.01	0.20 ± 0.20	0.3 ± 0.2	0.8
Facial sunscreen	1.00	0.50 ± 0.70	8.0 ± 10.6	14.0

bw - body weight; [a] Mean amount for roller, cream and spray deodorant; [b] only a soap bar; [c] Mean amount for hair gel, lotion and foam; [d] The retention factor of spray deodorant was 0.85 (Bremmer 2006)

2.4 Conclusions

The most used PCPs in the Northern Region of Portugal are toothpaste, shampoo, body washer and deodorant. The percentage of users for each kind of PCP is higher in the younger individuals and among women. This gender use also a higher number of PCPs than men, with mean values of nine and six products used simultaneously, respectively. The co-use of PCPs is more influenced by the gender of the consumer than its age, because no differences were registered on the co-use results found between age groups (mean value of seven). The most common combination is the use of shampoo simultaneously with body wash. Whenever different presentations of PCPs are available in the market, the preferred ones were: cream (60%) for moisturizers and sunscreens, toothpaste (90%) for oral care, roll on (70%) for deodorant, bath gel (80%) for body washers, shampoo (90%) for hair wash, hair conditioner (60%) for rinse-off hair care products, and hair foam (35%) for leave-on hair styling products. The most used Sunscreen Protection Factor is 30 and 50+ and the majority of the respondents use sunscreen only in summer (20-30%). The most referred brands of PCPs are purchased in supermarkets and hypermarkets, confirming the 73% of the respondents that affirm to buy their PCPs in those commercial stores. For the other PCPs, application the frequencies of application are mainly once a day, while daily amounts shows a great variability. The most contributive consumer good to systemic exposure to any ingredient of PCPs was found to be body lotion (70 $mg_{sample}.day^{-1}.kg_{bw}$), while deodorants raise concerns on skin toxicity due to their high exposure per unit area of skin (66 $\mu g_{sample}.day^{-1}.cm^{-2}$). These results may be used for the exposure assessment to any ingredient used in North region of Portugal Personal Care Products.

3 Human dermal exposure to galaxolide from personal care products[2]

Abstract

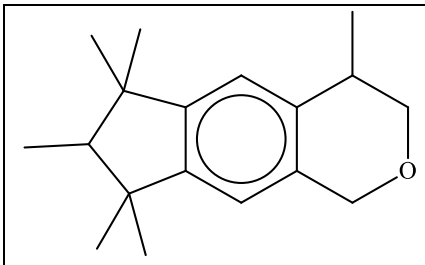
Musks are synthetic fragrances applied on personal care and household products as fixatives, by retarding the release of other fragrances with higher volatility. Galaxolide is the most used polycyclic musk since the 90th decade, and it has been detected in several environmental and biological matrices, particularly in human tissues and fluids. For exposure assessment purposes, large monitoring data need to be obtained and rapid but reliable analytical techniques are requested. The main objective of this study is to develop and validate a new and fast analytical methodology to quantify galaxolide in personal care products, and to apply this method to real matrices like skin care products (creams and lotions), shower products (soap bar), hair care products (shampoo and hair conditioner) and oral care products (toothpaste), in order to evaluate the human dermal exposure risk. A dispersive solid phase extraction is proposed, using QuEChERS methodology, followed by HPLC with fluorescence detection. Some extraction parameters were studied, like the ratio of sample/solvent amounts, the homogenization time, the salt addition effect and the used sorbents. The validation parameters of the developed method were: a linearity range of 0.005-1.002 mg.kg^{-1}sample, a limit of detection of 0.001 mg.kg$^{-1}_{sample}$, repeatability between 0.7 and 11.3% (variation coefficient of six standard injections), an intermediate precision of 2.5% (variation coefficient of six independent analysis of the same sample), mean recoveries ranging from 65% (soap bar) to 95% (body cream), and 3% of global uncertainty in most of the working range. The time of analysis, including the extraction steps, is 60 minutes, allowing a throughput of 4 samples.h^{-1}. Galaxolide was detected in all of the seven analyzed products in concentrations ranging from 0.04 ± 0.01 mg.kg^{-1}sample (toothpaste) to 280.78 ± 8.19 mg.kg^{-1}sample (perfumed body cream), which may correspond to a significant estimated daily human dermal exposure of 904 µg.day^{-1}.

Keywords: Human dermal exposure risk, Galaxolide, Personal Care Products, QuEChERS, HPLC-Fluorescence detection

[2] "Human Dermal Exposure to Galaxolide from Personal Care Products"; P Correia, A Cruz, L Santos, A Alves; International Journal of Cosmetic Science; 2013, 1-11, doi: 10.1111/ics.12043.

3.1 Introduction

Musks are synthetic chemicals used in household and personal care products (PCP) to improve its galenic and impart pleasant odoriferous characteristics. Because of their low volatility, they are applied as fixatives, retarding the release of the fragrances from the products, and helping to maintain the desired scent of the products. According to their physical-chemical properties, musks are organized in four main groups: nitro musks (NMs), polycyclic musks (PMs), macrocyclic musks (MMs) and alicyclic musks (AMs) (Arbulu, Sampedro et al. 2011). Initially, NMs were the most worldwide used musks, but, in the last decades, some concerns about their toxicity lead to restrictions on their use in Europe (OSPAR Comission 2004). As a consequence, PMs became more popular, particularly galaxolide (HHCB; 1,3,4,6,7-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran; Figure 3.1).



$C_{18}H_{26}O$

1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexa-methylcyclopenta-γ-2-benzopyran

Molecular weight (g.mol^{-1})	258.44
log K_{ow}	5.9
Water solubility, 25 °C (mg.L^{-1})	1.75
Vapor pressure, 25 °C (Pa)	0.0727
Henry's constant (Pa.m^3.mol^{-1})	11.3

Figure 3.1 - Galaxolide (HHCB) chemical structure, molecular formula, IUPAC chemical name and physical-chemical proprieties (Balk and Ford 1999): molecular weight, octanol-water partition coefficient (K_{ow}), water solubility, vapour pressure and Henry's constant

Data only available from 1992 to 2004 (OSPAR Comission 2004, HERA 2004b, Clara, Gans et al. 2011) may lead to the conclusion that HHCB mean consumption in Europe is slowing down: 2400 tons in 1992, 1482 tons in 1995, 1473 tons in 1998, 1427 tons in 2000 and 1307 tons in 2004. This trend probably appears due to some concerns attributed to PMs (HERA 2004b), and is more significant in the European northern region, whereas in southern countries a slight increase is verified, which can be explained by cultural and marketing differences (OSPAR Comission 2004).

The highest environmental contamination by HHCB occurs near cosmetics plants, where it has been found in influent wastewater (max. 595.48 µg.L^{-1}), effluent wastewater (mean 33.54 µg.L^{-}

[1]), sludge (max. 601270 μg.kg^{-1} dry weight) and air (max. 4.5 μg.L^{-1}) (Chen, Zeng et al. 2007). But due to its inclusion in almost all PCP and to its massive use, allied to frequent water rinsing of these products after application, a great part of HHCB enters the environment through residual water, and it is expected that 77% of the musks are discharged into the sewer system after used (Hu, Shi et al. 2011). In fact, HHCB has been found in wastewater treatment plants influents (from 0.029 to 45.4 μg.L^{-1}) and effluents (from bellow 0.0005 to 13.3 μg.L^{-1}), and in surface and ground-water (0.00009 to 12.47 μg.L^{-1}) in several countries of Europe, America and Asia (Eschke 2004, OSPAR Comission 2004, HERA 2004b, Yang and Metcalfe 2006, Moldovan, Chira et al. 2009, Silva and Nogueira 2010, Clara, Gans et al. 2011, Hu, Shi et al. 2011, Hu, Shi et al. 2011, Ramírez, Borrull et al. 2012, Yang and Ding 2012), as well as in drinking water from concentrations bellow 0.03 to few μg.L^{-1} (Eschke 2004, Bruchet, Hochereau et al. 2005). HHCB has also been found in sewage sludge of wastewater treatment plants all over the world, from 1.4 to 63000 μg.kg^{-1} dry weight (dw) (Rimkus 1999, Kupper, Berset et al. 2004, OSPAR Comission 2004, HERA 2004b, Peck, Kucklick et al. 2007, Wu and Ding 2010, Clara, Gans et al. 2011, Hu, Shi et al. 2011), and in sediments and suspended matter, from 0.2 to 13722 μg.kg^{-1}dw (Balk and Ford 1999, Rimkus 1999, OSPAR Comission 2004, HERA 2004b, Wu and Ding 2010). This bound of HHCB to organic matter is mainly explained by the high octanol-water partition coefficient, log K_{ow}, of about 6, and its relatively low water solubility of 1.75 mg.L^{-1} (Figure 3.1) (Balk and Ford 1999). Therefore soil adsorption of HHCB is also expected, from the deposition of biological sludge as a land application, and real measured concentrations have already been found above 1 μg.kg^{-1}dw, and it is also known that HHCB persists in soils at least for six months after application (Yang and Metcalfe 2006). Analysing other physical-chemical properties (Figure 3.1) like vapour pressure, 0.0727 Pa, and Henry's constant, 11.3 Pa.m^3.mol^{-1} (Balk and Ford 1999), it is expected that HHCB is easily volatilized (Aschmann, Arey et al. 2001) and consequently it has been found in indoor air, from 47.1 to 1256 ng.L^{-1} (Fromme, Lahrz et al. 2004, Regueiro, Garcia-Jares et al. 2009, Ramírez, Marcé et al. 2010, Sofuoglu, Kiymet et al. 2010), outdoor air, from 1.1 to 344306 ng.L^{-1} (Peck and Hornbuckle 2004, Ramírez, Marcé et al. 2010, Upadhyay, Sun et al. 2011), and dust, from 0.4 to 11400 μg.kg^{-1} (Fromme, Lahrz et al. 2004, Peck, Kucklick et al. 2007, Lu, Yuan et al. 2011b). Either from water, air or soils, HHCB reaches the biological food chain, and has been found in several biological matrices, from 0.00052 to 190 mg.kg^{-1} (Balk and Ford 1999, Rimkus 1999, Kannan, Reiner et al. 2005, Peck, Kucklick et al. 2007, Mottaleb, Usenko et al. 2009, Ramirez, Brain et al. 2009, Hu, Shi et al. 2011,

Subedi, Mottaleb et al. 2011), mostly of them aquatic fauna samples. Finally, HHCB has also been found in human matrices like blood, from below 0.003 to 6900 $ng.L^{-1}$ (HERA 2004a, Hutter, Wallner et al. 2005, Kuklenyik, Bryant et al. 2007, Hutter, Wallner et al. 2009, Hutter, Wallner et al. 2010), breast milk, from bellow 30 to 3600 $ng.L^{-1}$ (Kuklenyik, Bryant et al. 2007) or below 5 to 108000 $ng.g^{-1}$fat (Liebl, Mayer et al. 2002, HERA 2004a, Reiner, Wong et al. 2007, Schlumpf, Kypke et al. 2010), and human fat, from 6.1 to 189000 $ng.kg^{-1}$fat (HERA 2004a, Kannan, Reiner et al. 2005).

The major source of human exposure to musks is expected to be the dermal application, especially from intentional use of cosmetics and if these products are used on a regular basis and are not rinsed off after application (leave-on products) (Cadby, Troy et al. 2002, Reiner and Kannan 2006, Kuklenyik, Bryant et al. 2007, Reiner, Wong et al. 2007). There is limited information on dermal absorption rates for HHCB, but an *in vitro* study with human skin showed low absorption (about 0.4%) of the applied dose (HERA 2004a). This was confirmed by *in vivo* studies with human and rat skin that showed some percutaneous absorption (less than 2%), although low dermal permeation and distribution (Ford, Hawkins et al. 1999) or negligible skin permeability was verified (SCCNFP 2002). Nevertheless, other *in vivo* studies showed a HHCB absorption of about 40% by human skin (HERA 2004a) and 14% by rat skin (Api and Ford 1999). Additionally, it is expected that 22% of the applied dose of HHCB will evaporate from the skin (Ford, Hawkins et al. 1999). Inhalation exposure can also occur (Rogers, Isola et al. 2005, Kuklenyik, Bryant et al. 2007) but it appears to represent a minor route of exposure (Cadby, Troy et al. 2002, HERA 2004a).

Although lots of studies have been conducted about the presence of HHCB on different environmental and biological compartments, there are few reports that refer the detection of this musk in PCPs (Schüssler and Nitschke 1998, Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011a). On these cases, there are some differences between the results obtained in Asia, America or Europe (Table 3.1). For instance, in China, the highest HHCB mean concentration was found in perfumes (Zhang, Yao et al. 2008) and in hair care products (Lu, Yuan et al. 2011a). This last study detected trace levels of HHCB in a toothpaste (Table 3.1), suggesting that the use of this product is a minor source of exposure to HHCB (Lu, Yuan et al. 2011a). Nevertheless, a work performed in USA concluded that the exposure to HHCB varies a lot due to the wide range of

concentrations found in each group of products, and the highest mean concentrations of HHCB were found in perfumes, body lotions and antiperspirants (Table 3.1). All of those PCPs are leave-on products, which enhances the risk of human dermal exposure (Reiner and Kannan 2006). In Europe, a Belgium study found higher HHCB concentrations also on perfumes and deodorants (Roosens, Covaci et al. 2007). Additionally, as mentioned above, the use of HHCB is greater in south Europe than in north regions (OSPAR Comission 2004, HERA 2004b). So, it is crucial to study these aspects in southern Europe, even at a regional scale, measuring data that provides actual information, in order to evaluate the human exposure risk in this region, namely in Portugal. The studies referred above can contribute to a human risk exposure assessment to HHCB, but the social realities differ from one location and culture to another, leading to the existence of distinct PCPs and usage patterns. There are also few reports about the use habits of PCPs like creams/lotions, cosmetics, deodorants, bath and hair care products (Cadby, Troy et al. 2002, Loretz, Api et al. 2005, Loretz, Api et al. 2006). Additionally, the PCPs producers are not legally obliged to discriminate the composition of fragrances mixtures used on their formulations and these mixtures need only to be mentioned as “parfum” or “fragrance” (European Parliment Council 2009). Therefore, for exposure assessment purposes, large monitoring data need to be obtained, namely HHCB concentrations in PCPs.

Personal care products usually have a pleasant fragrance that is imparted by perfumed oils on its composition. These perfume oil is a mixture of several synthetic fragrances that are used to simulate natural desirable odours. The perfume oil quantities used in each product are extremely variable and depend on the kind of application of the product (skin, hair, mouth) and the target population (adults/children, men/women). For instance, toothpaste has a small amount of flower and fruit fragrances, combined with flavourings, like peppermint and others, that are responsible for its characteristic scent (Umbach 1991). Most of PCPs are solutions, suspensions and emulsions, applied in the skin, hair and mucosa. The most complex ones are emulsions that are stable systems of two insoluble liquids, one dispersed in the form of fine droplets (dispersed or inner phase) within the other (closer or outer phase). When the inner phase is composed by water and soluble compounds it forms a *water in oil* (W/O) emulsion, while when the inner phase is composed by oil and soluble compounds it forms an *oil in water* (O/W) emulsion. Lotions and creams are emulsions O/W or W/O, and the maximum percentage of disperse phase in a stable emulsion is considered to be 72.5%. In order to enable emulsion

stability, an emulsifier is needed, a compound with higher solubility in the outer phase than in the inner phase, that forms a layer between the two phases (Umbach 1991). Such distinctive characteristics of the PCPs that incorporate musks in their formulation pose a complex analytical challenge, in order to develop analytical methods able to quantify HHCB in a wide range of products.

Some analytical methods have been published for the extraction and the detection of HHCB in PCPs (Schüssler and Nitschke 1998, Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011a), but almost all are based in liquid-liquid extraction (LLE) with hexane, sometimes followed by clean-up procedures (e.g. silica columns), and gas chromatography with mass spectrometer detection (GC-MS), as shown in Table 3.1. These methods are applied to several types of products, like ethanolic and other solutions (perfumes, shampoo, shower gel), emulsions (lotions, creams, conditioners, toothpaste) and solid surfactants (bar soap), but they are time dispending methods, with high solvent consumption and sample manipulation, which leads to higher global uncertainties associated to final results. Most of the referred studies do not perform an uncertainty study, which is particularly important if the detected concentrations are in the frontiers of the detection limits of the methods. In the current study, HHCB is extracted applying a new extraction method, named QuEChERS, which reduces some of the problems associated to the other referred methods, and enables HHCB screening in a great number and variety of samples. These method, originally applied to pesticides extraction from fruits and vegetables (Anastassiades and Lehotay 2003), dues its name to the association of the terms Quick, Easy, Cheap, Effective, Rugged and Safe method. It is based in three simple steps, each one performed in a polypropylene conical tube, combined as an extraction, drying/partitioning and dispersive solid phase extraction (dispersive-SPE) method. The first step is the extraction using an adequate solvent, usually acetonitrile, followed by a drying/partitioning step, with salts as magnesium sulphate, $MgSO_4$, and sodium acetate or sodium chloride. A last dispersive-SPE cleanup step uses sorbents like primary and secondary amine exchange polymer material (PSA), and octadecylsilane (C18) or graphitized carbon black (GCB). The choice of salts and sorbents used is based on the analytes and also on matrices characteristics.

Table 3.1 - Determination of galaxolide (HHCB), tonalide (AHTN), musk ketone (MK) and musk xylene (MX) in different personal care products (PCPs): extraction and analytical methods, limits of detection (LOD) and quantification (LOQ), recovery percentages and concentrations of HHCB (Schüssler and Nitschke 1998, Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011a)

Musk	**PCPs**	**Extraction method**	**Analytical method**	**HHCB LOD / LOQ**	**HHCB recovery** (%)	**HHCB concentration** ($mg.kg^{-1}$sample)
HHCB **AHTN** (Reiner and Kannan 2006)	Perfumes Lotions/creams Antiperspirants Shaving cream Hair styling Soap bar Shower gel Shampoo Hair conditioner	-Extraction (hexane)	GC - MS Run time 51 min (HHCB Rt n.a.)	LOD 0.005 $mg.kg^{-1}$ sample	n.a.	< LOD - 4990 < LOD - 3740 0.801- 2250 < LOD - 1230 12.9 - 855 0.171 - 456 < LOD - 104 < LOD - 122 < LOD - 97
HHCB **AHTN** **MK** **MX** (Zhang, Yao et al. 2008)	Perfumes Shower products Hair care Lotions/creams Toothpastes	-Extraction (hexane) -SPE cleanup (silica/alumina column)	GC - MS Run time 35 min (HHCB Rt n.a.)	LOQ 0.006 $mg.kg^{-1}$ sample	78%	500 - 1000 0.1 1000 10 - 500 0.1 - 10 0.001 - 0.1
HHCB **AHTN** **MK** **MX** (Lu, Yuan et al. 2011a)	Hair care Lotions/creams Makeup Shower gel Soap bar Toothpastes	-Extraction (hexane) - LLE (ethyl acetate/hexane) -SPE cleanup (silica columns/Na_2SO_4)	GC - MS Run time 60 min (HHCB Rt n.a.)	LOQ 0.00301 $mg.kg^{-1}$ sample	82% (0.1 µg spike) 92% (1 µg spike)	< LOQ - 1010 < LOQ - 732 < LOQ - 72.8 < LOQ - 63.3 0.08 - 38.7 < LOQ - 0.02
HHCB **AHTN** **MK** **MX** (Roosens, Covaci et al. 2007)	Perfumes Deodorants Lotions/creams Shower products Hair care	-Extraction (hexane/water) -SPE cleanup (silica columns and Na_2SO_4)	GC - MS Run time 42 min (HHCB Rt 18 min)	LOQ 0.017 $mg.kg^{-1}$ sample	98% (500 $ng.g^{-1}$ sample spike) 110% (6 $ng.g^{-1}$ sample spike)	30 - 22000 5 - 1000 0.020 - 600 0.020 - 400 0.05 - 100
HHCB (Schüssler and Nitschke 1998)	After shave	-LLE (ethanol) -Dilution (water)	HPLC - FL Run time 35 min (HHCB Rt 33 min)	LOD 5 $\mu g.L^{-1}$	n.a.	n.a.
HHCB **AHTN** (Martínez-Girón, Crego et al. 2010)	Perfume	-LLE (hexane/water) -SPE cleanup (silica column) -Dilution (methanol)	Capillary electrophoresis Run time 45 min (HHCB Rt 28 min)	LOD 49 $mg.L^{-1}$ LOQ 147 $mg.L^{-1}$	90-116%	14500

Rt – Retention time; n.a.- not available

As far as the authors know, the QuEChERS methodology has never been applied to the analysis of musks, particularly HHCB, in personal care products. Besides the new method proposal, this work intends to display a complete set of validation parameters, including the global uncertainty associated to the results. This analytical method may be used in the future for exposure assessment purposes applied to a large number and type of personal care and household products.

3.2 Methods

3.2.1 Chemicals and samples

Seven personal care products were analysed, including two body creams, a body lotion, a shampoo, a soap bar, a hair conditioner and a toothpaste.

Galaxolide (HHCB) was obtained at 50% diluted in diethyl phthalate (DEP), from SAFC (St. Louis, USA). A working standard solution at 60 $mg.L^{-1}$ was prepared in absolute ethanol (*pro-analysis* grade, from Riedel-de Haën, Honeywell Specialty Chemicals Seelze GmbH, Hanover, Germany), as well as a stock solution at 600 $mg.L^{-1}$ in acetonitrile (HPLC isocratic grade, from VWR International, Pennsylvania, USA). These solutions were used for calibration purposes and for extraction spikes (recovery assays), respectively, and both were stored in the dark at -4 °C.

The HPLC mobile phase was prepared with deionised water and acetonitrile (the same as previously referred), acidified with glacial acetic acid (100%), *pro-analysis* grade, from Pronalab (Tlalnepantla, Mexico).

The extraction solvent was acetonitrile (the same as previously referred). Other tested co-solvents for extraction were methanol (HPLC isocratic grade, from VWR International), acetic acid (the same as previously referred) and deionised water.

The extraction salts and sorbents (named QuEChERS), ECMSSA50CT (6000 mg $MgSO_4$, 1500 mg sodium acetate) and ECMPSC1815C (900 mg $MgSO_4$, 300 mg PSA, 150 mg C18), were purchased from UCT (Bristol, UK).

3.2.2 Extraction method (QuEChERS)

The extraction method was adapted from a previous study (Anastassiades and Lehotay 2003), developed for pesticides. For the method development, four parameters were investigated: (1) type of solvent and necessity of co-solvent, (2) ratio sample/solvent amounts, (3) homogenization time and (4) salts and sorbents for sample drying/partitioning and clean-up steps.

The extraction method is described below. For solid matrices, as soap bar, preliminary trituration in a mortar was necessary. Pre-treated solid samples and liquid/semi-liquid samples

were rigorously weighed (2 g) directly into a polypropylene (PP) tube with conical bottom (Falcon, 50 mL), and5 mL of water were added as co-solvent. The mixture was shacked for 3 minutes, with a vortex mixer (IKA Vortex Genius 3) at maximum speed, and acetonitrile (15 mL) was added as extraction solvent. Extraction proceeded with a similar shaking step of 3 minutes and ultrasounds (P-Selecta) for 10 minutes. The drying/partitioning step was performed with the QuEChERS salts (ECMSSA50CT), which were added and immediately mixed, to avoid conglomerates formation, for 3 minutes in the vortex. To enable total phase separation, centrifugation was performed at 3700 rpm for 10 minutes (Hettich Zentrifugen Rotofix 32 A). The upper layer was transferred to a 50 mL PP tube, and the addition of the QuEChERS sorbents (ECMPSC1815C) enabled the dispersive-SPE clean-up, by vortexing for 3 minutes, followed by centrifugation (3700 rpm, 10 minutes). The upper layer was collected in a 50 mL PP tube, and, if the extract analysis was not performed immediately, extracts were stored in a freezer (-18 °C) for HPLC-FL analysis. All samples were, at least, four times extracted for quantification purposes and two times for recovery tests.

3.2.3 Blank issues/Quality assurance

HHCB can be found in many consumer products, as personal care and household products. Therefore, care was taken by the analyst not to wear personal products that contained fragrances. Glassware materials were washed using only organic solvents and water. To avoid cross contamination when handling samples, gloves were used by the analyst and changed for each product. All the materials and reagents used in the analysis were proved to be free of interferences, by performing two extraction blanks, for no HHCB was detected (below LOD).

Microsoft Excel 2007® software program was used for all statistical work.

3.2.4 Chromatographic analysis

HHCB was analysed by high pressure liquid chromatography with fluorescence detection, HPLC-FL (Schüssler and Nitschke 1998). An eight-point calibration curve was constructed by diluting the HHCB stock solution (60 $mg.L^{-1}$ in ethanol), in mobile phase, at concentrations ranging from 1.00 to 200.40 $\mu g.L^{-1}$ (1.00, 5.01, 10.02, 40.08, 80.20, 120.24, 160.32 and 200.40 $\mu g.L^{-1}$) equivalent to 0.005 to 1.002 $mg.kg^{-1}$sample.

Extracts were previously filtered by a syringe filter (PTFE membrane, 0.2 µm pore, 13 mm diameter, VWR International) and diluted with mobile phase, whenever the analyte concentration exceeded the higher concentration of the calibration curve.

Standard solutions and extracts were analysed by HPLC (Merck-Hitachi L6200A Intelligent Pump) with a Merck column LiChroCART® 250-4- LiChrospher ® 100 RP- 18 (5 mm). A manual injection volume of 120 µl was used (SGE Analytical Science, 250 µl, 250 F-LC) and the mobile phase, acetonitrile:water (acidified with acetic acid 17 mM) at the ratio 80:20, respectively, was kept in isocratic mode, at 1 $mL.min^{-1}$. Detection was performed at $\lambda_{excitation}$ = 280 nm and $\lambda_{emission}$ = 310 nm (Merck-Hitachi F-1080 Fluorescence Detector). Chromatograms were analysed with a Merck-Hitachi mode D-7000 Chromatography Data Station Software.

Total run time was 15 minutes, and the HHCB peak showed a retention time of 12 minutes.

3.3 Results and discussion

Few publications exist concerning the quantification of HHCB in PCPs (Schüssler and Nitschke 1998, Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011b). On the current study, six types of PCPs were analysed (body creams, body lotion, shampoo, soap bar, toothpaste and hair conditioner) and the main purpose was to obtain a fast, but reliable analytical method, that will enable the HCCB screening in a large number of samples in several types of personal care products, for further human exposure assessment.

3.3.1 Extraction method development

Due to the complex composition of PCPs, extraction and clean-up procedures have to be adopted before the chromatographic determination, either by GC or HPLC methods. The extraction of HHCB in personal care products has been mostly performed with hexane, by vortex shaking or sonication and centrifugation steps (Table 3.1). This procedure is, at least, twice repeated, and the extracts are then combined (Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011b). Further clean-up implies additional steps of solvent evaporation, SPE with silica columns, another solvent evaporation and recovery with an appropriate solvent (Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011b). These procedures result in time dispending methods, high solvent consumption, increased uncertainty of the results due to sample manipulation and additional costs of the analysis.

In this study, the basis for the method development was the previous application of QuEChERS to pesticides (Anastassiades and Lehotay 2003), where the extraction time is reduced comparatively to LLE extraction procedures, the solvent consumption is diminished, the solvent is environmentally compatible and the costs are accessible to allow future HHCB screening in a large number of samples. The original method, described by Anastassiades *et al.* (2003), was developed for pesticide extractions from food samples, where most of the analytes are polar substances, while HHCB is much less polar. Nevertheless, some less polar pesticides have also been successfully extracted with this method, and therefore it was thought that an improvement of the QuEChERS methodology could be performed, in order to allow the determination of HHCB in PCPs. The PCPs formulation presents a challenge, whenever such

different matrices, that include ethanolic solutions, emulsions and solid surfactants, are to be analysed by the same method. As a result of the samples composition, some parameters were adapted from the original method (Anastassiades and Lehotay 2003), essentially sample/solvent amount, shaking time, as also salts and sorbents used on sample drying/partitioning and clean-up steps.

Selection of the extraction solvent

Because almost all of the fragrance compounds have an oily nature (Umbach 1991), it was expected that, similarly, musks would be more easily extracted by non-polar solvents. Acetonitrile was found adequate, because of its low viscosity and intermediate polarity, and also its effectiveness as mobile phase in reversed-phase liquid chromatography. Additionally, acetonitrile has a low volatility that allows manipulation without great volume changes (Anastassiades and Lehotay 2003).

The first group of products tested were lotions and creams, and, in this case, the use of acetonitrile resulted in product homogenisation. This solvent was also adequate to the shampoo and the soap bar. However, the samples of hair conditioner and toothpaste were not efficiently homogenized by acetonitrile and therefore a co-solvent had to be used. Pursuing that objective, three solvents were tested: acetic acid, methanol and water. Only with this last solvent, the referred products were homogenised, and so, 5 mL of water was added prior to acetonitrile. Water enables the destruction of O/W emulsions (in this case, hair conditioner or toothpaste), and a consequent efficient extraction. In order to uniform the extraction method, water addition was also applied to the other products, a body cream, a shampoo and a soap bar, without loss of efficiency, as it is shown in Figure 3.2.

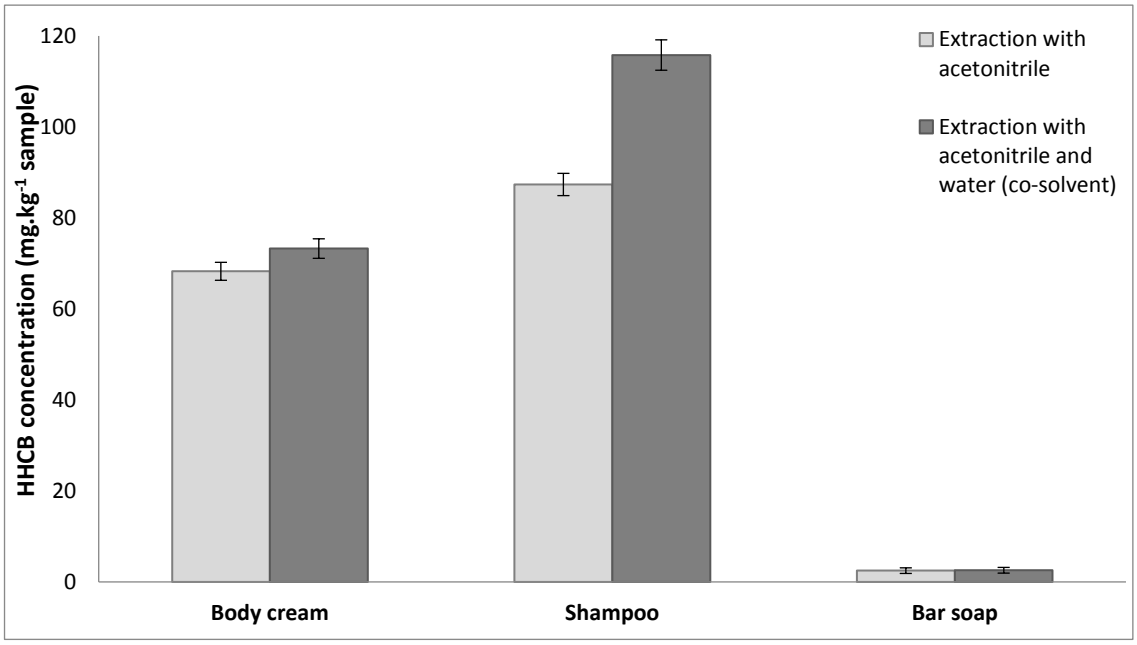


Figure 3.2 - Galaxolide (HHCB) concentration (mg.kg^{-1}sample) of three different personal care products (body cream, shampoo and bar soap): extraction using only acetonitrile ▁ compared to the extraction with co-solvent (water) addition ▁

Effect of the sample and solvent amount

In order to maximize the surface area and ensure better extraction efficiencies during shaking, some samples needed pre-treatment, as the soap bar which was triturated in a mortar. Due to its consistency (semi-solid or liquid), the other products tested in this study were used directly.

The tested sample amounts were 2 g and 10 g, but the latter resulted difficult to fit inside the 50 mL PP tube, due to the low density of almost all PCPs. But the lower sample amount could compromise the desirable low limit of detection of the method. Reproducibility and accuracy were improved when the sample weight was 2 g and, therefore, it was chosen. The low samples density also justified the change to 15 mL of solvent, instead of the 10 mL originally used.

Therefore, combining the sample amount of 2 g and the solvent volume of 15 mL, the concentration was changed from the original 1 g sample.mL^{-1} (Anastassiades and Lehotay 2003) to 0.13 g sample.mL^{-1}. This combination lead to an efficient method, using minimal size sample to provide statistically reliable results, while taking into account the degree of sample homogeneity (Anastassiades and Lehotay 2003). In this study HHCB was detected above LOD in all samples analysed. Other studies with the same kind of products, used similar concentrations: 0.06-0.15 g sample/mL (Roosens, Covaci et al. 2007), 0.02-0.1 g sample/mL

(Reiner and Kannan 2006), 0.012-0.02 g sample/mL (Lu, Yuan et al. 2011b) and 0.003 g sample/mL (Martínez-Girón, Crego et al. 2010).

Effect of the homogenization time

The homogenization time by vortex, after the solvent addition, is a crucial step to guarantee quantitative extraction yields. Three times were tested: 30 seconds, 1 and 3 minutes. The longer time proved to enhance the extraction, and that should be attributed to the complexity of the matrices, that needed more time to homogenise with acetonitrile. Other extraction methods of HHCB in personal care products refer a shaking time of 3 minutes (Roosens, Covaci et al. 2007, Martínez-Girón, Crego et al. 2010) or even 15 minutes (Reiner and Kannan 2006). An additional step of sonication for 10 minutes was added after the first vortex shaking to enhance extraction. The use of sonication (20 minutes) has been also described by other authors (Lu, Yuan et al. 2011b).

Effect of the salts addition

After acetonitrile addition, vortexing and sonication, salts were added in the partitioning/drying step, namely magnesium sulphate ($MgSO_4$) and sodium acetate. The first is a drying agent, which confers less polarity to the extract, originating the precipitation of certain polar compounds. Anastassiades et al. (Anastassiades and Lehotay 2003) proposed the use of 4 g of $MgSO_4$ to dry a 10 g sample of fruits and vegetables with an water content between 80-95%. Considering some formulations of personal care products (Table 3.2), water content ranges from 1 to 92%, which, for a 2 g sample, corresponds to 0.02 to 1.84 g of water. To ensure a better water removal, 6 g $MgSO_4$ was added. Larger quantities of $MgSO_4$ could difficult vortexing due to the formation of conglomerates and could also increase temperature to 40-45 °C, compromising the extraction efficiencies (Anastassiades and Lehotay 2003). Assuming that each $MgSO_4$ molecule joins to seven water molecules, because magnesium sulphate heptahydrate is the most commonly found, 6 g of this drying agent on the partitioning/drying step and the additional 900 mg on the clean-up step, as described above, enables a 7.2 g water removal. Even when water is used as co-solvent (5 mL or 5 g), this drying process results in a total water removal during the extraction of a wide range of products, including the PCPs analysed on the current study. The other partitioning step salt, sodium acetate, was used in this

study instead of sodium chloride, because it increases the aqueous phase polarity, decreasing even more water solubility of the less polar compounds, which enhances the extraction of HHCB.

Table 3.2 - Content ranges (%) of some group of compounds and pH ranges found in the composition of typical personal care products (PCPs) (Umbach 1991, Williams 1996)

Content / **PCPs**	Water (%)	Sugars (%)	Fatty acids (%)	Organic acids and alcohols (%)	Lipids and waxes (%)	Pigments (%)	Perfumed oils (%)	Flavour oils (%)	Others (%)	pH
Body Lotion	64-82	-	0-1.5	3.8-4.0	3-30	-	q.s.	-	0.7-1.8	5-6
Body Cream	29-70	-	0-4	2-30	2-47	-	q.s.	-	3-64	5-6
Shampoo	39-70	-	-	1-10	0.4-3	q.s.	q.s.	-	30-61	5.5-8.5
Bar soap	1-15	-	90-98 (salt)	-	-	-	0.5-2	-	0.6-1.35	8-10
Hair Conditioner	89-92	-	0-0.5	4-5	0-0.5	q.s.	0.4	-	2-3	3.5-5
Toothpaste	26-43	0-0.4	0-0.5	15-75	-	0-0.01	-	1.0-1.1	19-52	4-8

q.s.- *quantum satis, quantum sufficit*; a Latin phrase used in prescription writing that means “a sufficient quantity”

Effect of the sorbents

The basic formulation of some PCPs include a large number of compounds (Table 3.2) that are susceptible to be present in the extract and interfere in the chromatographic analysis. So, a dispersive-SPE clean-up step was performed with 300 mg of PSA and 150 mg of C18 sorbents. The PSA sorbent is used to remove sugars, fatty acids, organic acids and some pigments, while the C18 sorbent is used to remove lipids and non-polar interferences (Anastassiades and Lehotay 2003). Sugars only found in toothpastes, at very low mass percentages, and so it will be easily removed. Fatty acids are commonly used in soaps production, appearing as alkali salts (result of saponification), and in some emulsions (lotion, cream, hair conditioner, toothpaste) as emulsifiers. While no difficulties are expected in removing fatty acids from these PCPs, the high percentage of those compounds in soaps could be a problem in the clean-up step of the resulting extracts. The most used fatty acids for soap bar production are lauric acid, palmitic acid and oleic acid. Organic acids and alcohols are also extensively found in PCPs, but only the toothpaste could be difficult to clean due to the highest percentage (15-75%) of those

compounds. The main organic alcohols found on the toothpaste composition are sorbitol and glycerine, while acids, like benzoic acid and tartaric acid, are less common. Lipids and waxes are mainly found in lotions and creams, and the small quantities found (22% max.) are expected to be easily removed with C18 sorbent. Pigmentation of PCPs is normally very discreet or inexistent, exception made for make-up formulations, some hair care products and toothpaste (Umbach 1991, Williams 1996). The analysed hair care product included in this study presented almost colourless extracts. The most coloured product was the toothpaste, but the final dispersive-SPE clean-up used in this study was sufficient to remove all the pigments from the resulting extract, validating therefore the use of the sorbents and the amounts previewed. Graphitized carbon black (GCB) is the sorbent proposed for intensively coloured extracts (Anastassiades and Lehotay 2003), but it was found not necessary because clean extracts were obtained using PSA and C18 sorbents.

The resulting chromatograms for the extraction method proposed in this study proved an excellent resolution for HCCB peak, with no interferents in the vicinity (Fig. 3.3). The final extraction conditions were therefore:

- 2 g of sample (with pre-treatment in a mortar for solid samples);
- 5 mL of water as co-solvent, followed by vortexing during 3 minutes;
- 15 mL of acetonitrile as extraction solvent, followed by vortexing during 3 minutes and sonication for 10 minutes;
- drying/partitioning step with 6.0 g $MgSO_4$ and 1.5 g sodium acetate, centrifuged for 10 minutes at 3700 rpm;
- dispersive-SPE clean-up of the resulting upper layer with 900 mg $MgSO_4$, 300 mg PSA and 150 mg C18, centrifuged for 10 minutes at 3700 rpm
- extracts (upper layer) were preserved in PP tubes stored in a freezer (-18 °C).

Stability assays were performed with a standard solution (1.002 $mg.kg^{-1}$sample) stored in several conditions, and 8-months stability was confirmed, whether the standard solution is in glass or polypropylene tubes, each one stored in refrigerator (4 °C) and freezer (-18 °C).

3.3.2 Chromatographic analysis and method validation

The fluorescence chromatograms of an HHCB standard (0.601 mg.kg^{-1} sample) and the extract of a commercially available body cream are shown in Figure 3.3. The HHCB peak is well identified when comparing these two chromatograms, with a retention time of about 12 minutes. This main peak is followed, in all analysed chromatograms, by a small and non-symmetrical peak (12.5 minutes retention time), that has been described as a by-product of the technical synthesis of HHCB. The proposed analytical method is able to rapidly quantify HHCB when compared with other ones with larger retention times and more complex extraction procedure for HHCB (Table 3.1).

A calibration curve was constructed by plotting the peak areas of eight standard solutions against the respective galaxolide concentrations. The linearity range was from 0.005 to 1.002 mg.kg^{-1}sample, with an R^2 of 0.999.

The limit of detection, LOD, obtained considering a three times signal-to-noise ratio, was 0.001 mg.kg^{-1}sample (0.22 µg.L^{-1}), while the limit of quantification, LOQ, obtained considering a ten times signal-to-noise ratio was 0.004 mg.kg^{-1}sample (0.76 µg.L^{-1}). These limits are below the 5 µg.L^{-1} LOD of the original LLE with HPLC-FL detection method (Schüssler and Nitschke 1998), as well the 49 mg. L^{-1} LOD and a 147 mg. L^{-1} LOQ of a LLE with detection based on enantiomeric separation by a capillary electrophoresis method (Martínez-Girón, Crego et al. 2010). Only methods using LLE with GC-MS detection could achieve such low limits (Table 3.1).

The repeatability was evaluated by the coefficient of variation (CV) of the peak area of a standard solution injected six times on the same day at three levels of concentration: 11.3% (0.005 mg.kg^{-1} sample), 0.9% (0.401 mg.kg^{-1} sample) and 0.7% (1.002 mg.kg^{-1} sample). These results are comparable to those presented by a capillary electrophoresis method (Martínez-Girón, Crego et al. 2010).

An intermediate precision of 2.5% was obtained by the CV for six independent extractions of the same sample.

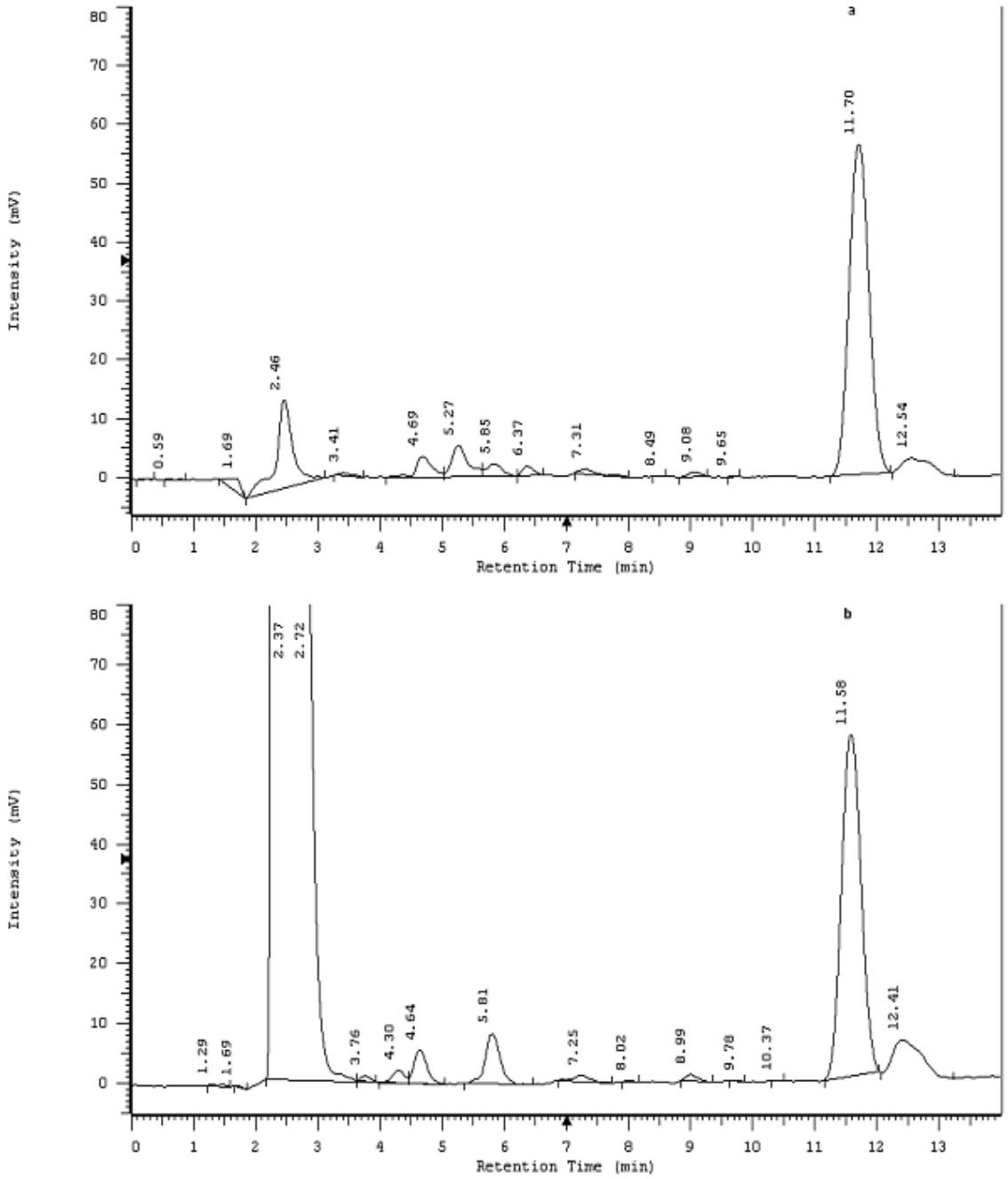


Figure 3.3 - Fluorescence chromatograms of (a) a galaxolide standard (0.601 mg.kg^{-1} sample) prepared in mobile phase and (b) an extract of a body cream (detected concentration of 68.27 mg.kg^{-1} sample, dilution factor of 1:100 in mobile phase)

Accuracy, evaluated by the recovery percentage after spiking additions at different concentrations to the different samples, was within the range of 65% (soap bar) to 95% (body cream) as may be seen in Table 3.3. These recovery results are consistent with other studies (Table 3.1) (Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011b). The lower recovery found for the soap bar may be attributed to the high alkalinity of soaps, with pH values between 8 and 10. In fact, the pH values of the other

products are lower (Table 3.2), and the recoveries found for those products were higher, which may indicate that extraction or detection of HHCB is better at acidic or neutral conditions. Additionally, the degradation of HHCB to its transformation products, HHCB-lactone or the respective acid, is pH-dependent (Bester 2009). So, in order to evaluate the influence of pH on HHCB detection, three standards were prepared at different pH values: pH 4.0, pH 7.0 and pH 10.0. The CV between HHCB concentration found for each standard and a normal standard (prepared in mobile phase) was 3% maximum, revealing that pH has no influence on HHCB detection. Recovery tests performed for a body cream, a shampoo and a bar soap, extracted with water (co-solvent) and acetonitrile, achieved mean recoveries of 98%, 81% and 53%, respectively, when an HHCB spike of 29.4 $mg.kg^{-1}$sample and a 100 times dilution of final extracts were used. These results seem to indicate that the use of water as a co-solvent doesn't change the method efficiency for these products, once similar results were obtained when the extraction was performed only with acetonitrile.

Dilution of extracts was performed whenever galaxolide concentration exceeds the calibration ranging. The influence of this dilution on the precision and accuracy was tested with a body cream extract (68.27 $mg.kg^{-1}$) diluted 1:100, 1:200 and 1:400. The resulting concentrations had a CV of 3.4% between them, which proves that dilution has no effect on the results.

The effect of matrix interferences was proved negligible for a body cream, with different spiked HHCB concentrations, because the obtained areas of the spiked samples lied within the linear fit limits of the calibration curve. The recovery percentages obtained were 95, 90 and 83% for HHCB spike levels of 0.06, 0.15 and 0.29 $mg.kg^{-1}$sample, respectively.

The global uncertainty was calculated using the *bottom-up* approach, adopted by the International Organization for Standardisation (ISO) and also adapted by the EURACHEM/CITAC Guide (Ellison, Rosslein et al. 2000). According to this approach, there are four main individual sources of uncertainty that must be taken into account, namely the standard preparation uncertainty, the calibration curve uncertainty, the precision uncertainty, and the accuracy uncertainty, as already described by Ratola et al. (Ratola, Martins et al. 2004). This method presents a global uncertainty ranging from 25% to 3% for concentrations from 0.0251 to 1.002 $mg.kg^{-1}$sample, and only the lowest concentration (0.005 $mg.kg^{-1}$sample) presents a high uncertainty of 123% (Figure 3.4).

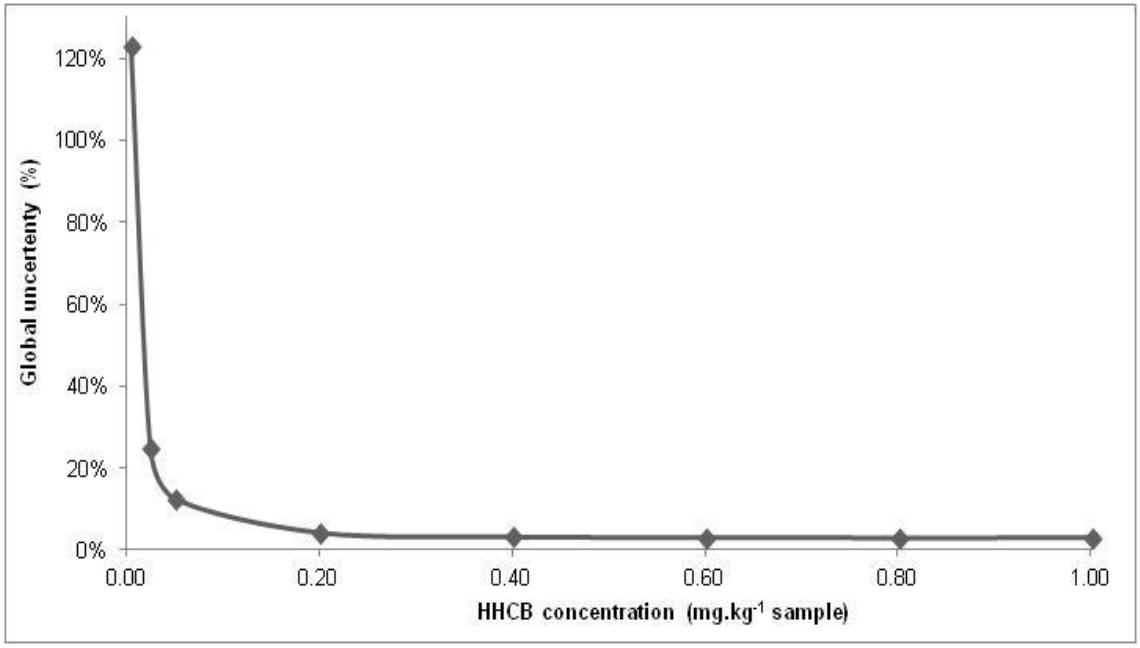


Figure 3.4 - Global uncertainty (%) of the current method, associated to galaxolide (HHCB) concentration (mg.kg^{-1} sample) on personal care products, estimated accordingly to EURACHEM/CITAC Guide (Ellison, Rosslein et al. 2000)

3.3.3 Galaxolide in personal care products and daily exposure estimates

The developed method was applied to the determination of HHCB in seven PCPs. HHCB was detected in all products analysed, within a wide range of concentrations (Table 3.3) consistent with other studies (Table 3.1). The lowest value was found on the toothpaste, 0.04 ± 0.01 mg.kg^{-1}sample, and the highest one in the perfumed body cream, 280.78 ± 8.19 mg.kg^{-1}sample (Table 3.3). All the concentrations presented in Table 3.3 are corrected with the recovery percentage. For all of the three body creams and lotions, a mean recovery of 89% was used, a value that resulted from the three recovery percentages obtained for the body cream.

Table 3.3 - Galaxolide (HHCB) concentration (mg.kg^{-1}sample) found for each personal care product (PCP) analysed on the current study, and recoveries (%) found for each used HHCB spike level (mg.kg$^{-1}_{sample}$)

PCPs	**HHCB concentration** (mg.kg $^{-1}$ sample)	**HHCB spike level** (mg.kg $^{-1}$ sample) [a]	**Recovery** (%)
Body lotion	7.31 ± 0.85	-	-
Body cream	68.27 ± 1.97	5.8 14.7 29.4	95 90 83
Perfumed body cream	280.78 ± 8.19	-	-
Shampoo	87.35 ± 2.45	29.4	80
Hair conditioner [b]	28.90 ± 1.10	29.4	90
Soap bar	2.48 ± 0.61	29.4	65
Toothpaste [b]	0.04 ± 0.01	14.7 29.4	73 80

[a] Extract 100 times diluted before analysis [b] Extraction made with water as co-solvent

Considering a typical adult consume profile, estimation of a daily exposure to HHCB was performed, exclusively using the analysed PCPs as sources. The formulated hypothesis, as the application site, mean daily application amount and percentage of skin retention are found in Table 3.4. The retention factors were based on a previous study (Cadby, Troy et al. 2002) that as similar products: 100% for leave on products (body lotions and creams) and 10% for rinse off products (shampoo, soap bar and toothpaste). For the hair conditioner, it was assumed a retention factor lower than the other rinse off PCPs, of only 1%, because a correct application is only made on hair tips and not on scalp. The final daily dermal exposure for each PCP was calculated multiplying the concentration of HHCB on the product for the daily application amount and the retention factor. A total daily dermal exposure to HHCB of 904 µg.day^{-1} was found, which was bellow other published results: 3060 µg.day^{-1} (Lu, Yuan et al. 2011a), 25100 µg.day^{-1} (Ford 1998) and 23700 µg.day^{-1} (Roosens, Covaci et al. 2007). Additionally, assuming a total evaporation of 22% of HHCB (Ford, Hawkins et al. 1999), only 705 µg.day^{-1} is effectively retained on the skin surface and is able to be systemically absorbed. The human skin absorption amount of HHCB has been reported to be about 0.1% (Ford, Hawkins et al. 1999). Assuming that this is the absorption average rate for a normal adult skin, the estimated global systemic human exposure to HHCB may be considered very low, although other studies are required specially when dealing with sensitive and more permeable skins, like children's or senior's.

Table 3.4 - Estimated daily dermal exposure to HHCB (μg.day^{-1}) using the analysed personal care products.

Personal care products	**Application site**	**Exposure route**	**HHCB concentration** (μg.kg^{-1} sample)	**Mean daily application** (g sample.day^{-1}) [a]	**Estimated daily HHCB retention** (μg.day^{-1}) [b]
Body lotions and creams	Whole body (includes face and hands)	Dermal	105.72 [c]	8.0	845.8
Shampoo	Scalp, neck and hands	Dermal	69.88	8.0	55.90
Hair conditioner	Hair tips and hands	Dermal	26.01	4.0	1.040
Soap bar	Whole body (includes face and hands)	Dermal	2.48	5.0	1.240
Toothpaste	Perioral region and mouth mucous membranes	Dermal and oral	0.03	2.0	0.0060
Estimated daily dermal exposure to HHCB (μg.day^{-1})					**904.0**

[a] Estimated values based on previous studies (Cadby, Troy et al. 2002, Loretz, Api et al. 2005, Loretz, Api et al. 2006, Hall, Tozer et al. 2007)

[b] Retention factors of 100% for body lotions and creams, 1% for hair conditioner and 10% for the other PCPs (Cadby, Troy et al. 2002)

[c] Mean HHCB concentration of the three body lotions/creams analysed on this study

3.4 Conclusions

As a result of this study, a quick and easy method was developed for the analysis of galaxolide in PCPs. This method showed an excellent global uncertainty of 3% when concentrations of the products studied were above 0.2 $mg.kg^{-1}$sample. All analysed products contained HHCB at concentrations ranging from 0.04 ± 0.01 $mg.kg^{-1}$ sample, on the toothpaste, to 280.78 ± 8.19 $mg.kg^{-1}$sample, on the perfumed body cream. A daily human exposure to HHCB of 904 $\mu g.day^{-1}$ was estimated considering dermal application of these PCPs as the only source. This validated analytical method will enable the future characterization of the presence of galaxolide in a huge variety of PCPs, in order to evaluate the trends of consumption and human exposure to this chemical.

4 Risk of children's dermal exposure to galaxolide through personal care products[3]

Abstract

Galaxolide is the most used fragrance since the early 1990s, and it has been largely detected in environmental and biological matrices. This polycyclic musk is present in almost all of our daily products, so the risk of human exposure is substantial, as it had been proved by its detection in human tissues and fluids. Due to the lack of information about the concentrations found in consumer products, monitoring data is needed for exposure assessment purposes. Dermal contact, mostly by personal care products, seems to be the major route of human exposure to galaxolide, and, due to the immaturity of young children's skin, exposure consequences can be worse in this population. The main objective of this study was to evaluate galaxolide levels in personal care products used by children of Oporto (Portugal), aged 0–5 years, and relate it with consumer habits. Consumer patterns were obtained through 250 questionnaires to caregivers of Oporto children. The 79 most used products were extracted by a dispersive solid phase extraction methodology known as QuEChERS and galaxolide was determined by High Performance Liquid Chromatography with fluorescence detection. The concentrations ranged between 0.001 ± 0.001 $mg \cdot kg^{-1}_{sample}$, on a baby wipe, and 300.480 ± 8.819 $mg \cdot kg^{-1}_{sample}$, on glycerin soap, which may correspond to an estimated daily dermal exposure of 277.10 ± 0.02 $\mu g \cdot day^{-1}$ on the population of Oporto children. This value is in the range of the results observed for adults, although no information of toxicological risk for children is available.

Keywords: galaxolide; dermal exposure risk; children; personal care products; QuEChERS; HPLC-fluorescence

[3] "Risk of Children's dermal exposure to galaxolide through personal care products"; P Correia, A Cruz, L Santos, A Alves, Cosmetics 2 (2); 2015; 93-109; doi:103390/cosmetics2020093

4.1 Introduction

Musks are synthetic fragrances widely used in innumerous daily products, like personal care products (PCP), in order to maintain the desired scent. They are applied as fixatives, because of their low volatility against other fragrances, retarding their release. There are four main groups of musks, according to their physical–chemical properties: nitro musks (NM), polycyclic musks (PM), macrocyclic musks (MM), and alicyclic musks (AM) (Arbulu, Sampedro et al. 2011).

The PM galaxolide (HHCB; 1,3,4,6,7-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran; Figure 4.1) is the most used musk in the world since the early 1990s (HERA 2004b).

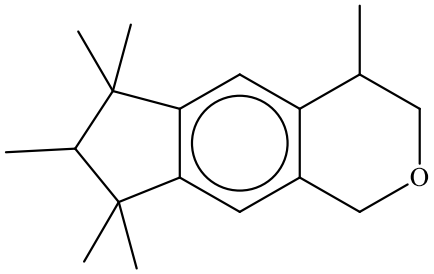


Figure 4.1 - Galaxolide (HHCB) chemical structure.

Due to the massive use of fragranced PCPs that are frequently water rinsed after application, it is expected that 77% of musks are discharged into the sewer system (Reiner and Kannan 2006). As HHCB has a high octanol-water partition coefficient (log K_{ow} = 5.9) and relatively low water solubility (1.75 mg·L^{-1}), this compound has been found in wastewater treatment plants (influents, effluents, and in sewage sludge) and even in environmental matrices (surface water, sediments, and suspended matter) (HERA 2004b). HHCB also reaches the biological food chain, and has been detected in several biological matrices, most of them aquatic fauna (HERA 2004b, Reiner and Kannan 2006). Therefore, the risk of exposure to HHCB by humans is significant, not only directly by PCPs and other products (dermal, oral, and inhalation exposure), but also by food and the environment. In fact, HHCB has already been found in human tissues and fluids like blood, human fat, and breast milk (HERA 2004b, Reiner and Kannan 2006).

The main source of human exposure to musks is expected to be by the dermal application of PCPs, especially by leave-on products (Reiner and Kannan 2006). Musks have been described as potentially endocrine disrupters, although low estrogenic effects have been reported for HHCB in humans at current levels of exposure. The same study has also reported that perfumes

that contain several fragrances, like HHCB, may play an important role in inducing genetic mutations that can lead to autism spectrum disorders, and the incidence of this disorder has increased in children since the early 1990s (Bagasra, Golkar et al. 2013). Nevertheless, the providing the details of the entire fragrance composition is not legally mandatory in European Union, forcing companies to only to refer to “Parfum”, “Fragrance” or “Aroma” in the product composition (European Parliment Council 2009). There are more than 2000 known fragrances used in PCPs like perfumes, moisturizers, bath or hair care products, or even cosmetic products (Bridges 2002). But there are also some PCPs with such a residual quantity of fragrances that are not referenced in the product’s composition, and can even be labelled as “fragrance-free”, namely some children or baby PCPs.

Children’s skin is different from adults’ skin, because after birth, and during the first months or years of life, the cutaneous structure and functions are in adaptation and maturation (Fluhr, Darlenski et al. 2010, Blume-Peytavi, Hauser et al. 2012). At this age, the skin barrier, that prevents the loss of water and the penetration of irritants or allergens, is less developed than in adults (Lavender, Bedwell et al. 2011), mainly due to the lack of some lipids, higher levels of water and pH (Stamatas, Nikolovski et al. 2011). Additionally, the cutaneous permeability can be altered, for example, by the use of soaps and detergents that can raise skin pH, especially in newborn babies (Lavender, Bedwell et al. 2011). So, the use of PCPs in children can damage the skin barrier, allowing allergens to enter through the skin (Lavender, Bedwell et al. 2011, Stamatas, Nikolovski et al. 2011). Consequently, depending on some predisposing factors and also on the presence of certain PCP ingredients, such as fragrances, children’s skin is more vulnerable to adverse reactions like atopic eczema, chemical dermal allergies and hypersensitivity (Fluhr, Darlenski et al. 2010, Lavender, Bedwell et al. 2011, Blume-Peytavi, Hauser et al. 2012), or even absorption into the bloodstream. A study in the elderly population, which also have the epidermal barrier altered, showed higher systemic absorption of musks than in healthy adults (Hutter, Wallner et al. 2010), but no studies were found in children. So, children’s dermal exposure risk to HHCB may be higher than in adults and no data is available regarding the use of PCPs by this population. The maturation process of skin may be beyond the first year of life (Stamatas, Nikolovski et al. 2011), and this should be taken into account when choosing the population under scrutiny.

Few reports refer to the detection of HHCB in PCPs themselves, even in the ones targeting adults. The reported HHCB concentrations ranged from below 0.003 to 22.000 $mg \cdot kg^{-1}$ (Schüssler and Nitschke 1998, Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011a), and only two of the seven studies are European (Roosens, Covaci et al. 2007). Patterns of fragrance consumption may differ around the world, and it is mentioned that North European countries consume less fragranced products than the southern ones (HERA 2004b). Only a previous study of this work team was performed in this latter region, namely in Portugal (Correia, Cruz et al. 2013), and there is not available data about the PCP consumption patterns, mostly only the risk of dermal exposure to musks by European children.

The main purpose of this work was to estimate the risk of children's dermal exposure to galaxolide in Oporto (Portugal). Because of the referred lack of information on the PCP consumer habits and PCPs' fragrance compositions, this particular study intends: (i) to find the most used PCPs among Oporto children, concerning moisturizers, toothpastes, bath products, diaper change products, and sunscreens; (ii) to quantify the HHCB concentration in each of those PCPs; (iii) to estimate the mean daily frequency, quantities, and site of application of each type of PCP in Oporto children, and (iv) to evaluate the risk of dermal exposure to HHCB in children.

4.2 Experimental Section

4.2.1 Questionnaire

A questionnaire regarding consumer habits (Annex B), previously validated with a 50-individual sample, was applied to child caregivers (mothers and fathers) that filled out a questionnaire for each child. The inclusion criteria were children aged between 0 and 5 years, and resident in the district of Oporto. Based on these inclusion criteria and on the final results of the Portugal Census 2011 (INE 2011), the population size was estimated to be 85,019, which corresponds to 30% of the population of up to 14 years of age living in the district of Oporto. Thus, with a margin of error of 5% and a confidence level of 90%, a sampling size of 270 children was estimated to guarantee representativeness of the study. Over 300 questionnaires were delivered on paper from January to March 2013 (three months). The questionnaire had a cover page with a brief description of the purpose of the study and the possibility of respondents to provide their consent before answering (Annex B). Respondents returned the completed questionnaires with no personal identification, and a number was attributed to each one, ensuring anonymity. The questionnaire was analyzed in terms of social-demographic characteristics and consumer habits, namely most used PCPs (brands and types), as well frequency and quantity of application. The PCPs were selected from the questionnaires results and complemented with two hypermarkets statistics of sales.

Besides this information, six of the 250 caregivers agreed to respond to a diary of hygiene and personal care products use by children (Annex B) where the used amounts were precisely quantified. These responses correspond to the utilization of PCPs by six children aged between 9 and 59 months, where half of them were from each gender. For each used PCP, the initial weight of the package was recorded as well as the daily frequency of use over seven days, after which the package was weighed again. Thus, it was possible to obtain used average quantities for each type of PCP (body moisturizer, face moisturizer, toothpaste, bath gel, soap, shampoo, hair conditioner, baby wipes, diaper change cream, and sunscreen). Statistical analysis was performed using Microsoft Excel 2007®.

4.2.2 Chemicals and Samples

Based on consumer habits, 79 PCPs were selected as the most used by this population (62 PCPs based on the questionnaire results and 17 PCPs from market data obtained from two of the greatest hypermarkets chains in the region). These selected PCPs were purchased in pharmacies and hypermarkets and the final sample was composed of: twenty one moisturizers, seven toothpastes, nine shampoos, thirteen "2 in 1" bath products, two soaps, one cleansing solution, four hair conditioners, six baby wipes, eight barrier creams, and eight sunscreens.

A commercial HHCB standard with a 50% purity in diethyl phthalate (DEP) was purchased from SAFC (St. Louis, MO, USA). A working standard solution of HHCB at 60 $mg \cdot L^{-1}$ was prepared in absolute ethanol (pro-analysis grade, from Riedel-de Haën, Honeywell Specialty Chemicals Seelze GmbH, Hanover, Germany) for calibration purposes. Stock solutions of HHCB at concentrations varying from 455 to 600 $mg \cdot L^{-1}$ in acetonitrile (HPLC isocratic grade, from VWR International, Radnor, PA, USA) and stored in the dark at −4 °C, were used for the spiking of samples for recovery studies.

The HPLC mobile phase was prepared with deionized water and acetonitrile, acidified with glacial acetic acid (100%), and the extraction solvent and sorbents were also the same as described in previous work by this team (Correia, Cruz et al. 2013).

4.2.3 Extraction and Analytical Method

The referenced PCPs were extracted by dispersive solid phase extraction, commonly named as QuEChERS, and HHCB concentration was determined by High Performance Liquid Chromatography with fluorescence detection (HPLC-FL), as described before (Correia, Cruz et al. 2013). At least duplicates of all samples were extracted and analyzed. Briefly, 2 g of samples were rigorously weighed and vigorously shaken for 3 min with 5 mL of water (co-solvent). Then, 15 mL of acetonitrile (solvent) was added, shaken for another 3 min, and homogenized in an ultrasonic bath for 10 min. The two sequential steps of extraction and cleanup were performed subsequently adding the corresponding QuEChERS sorbents and mixing, as described elsewhere (Correia, Cruz et al. 2013). To enable total phase separation, centrifugation was performed at 3700 rpm for 10 min at the end of each of these two steps. The supernatant was collected in a 50 mL PP tube and stored in a freezer (−18 °C) until HPLC-FL analysis. Validation parameters of this method were already reported in the previously mentioned study (Correia,

Cruz et al. 2013) and include: a linearity range of 0.005–1.002 $mg \cdot kg^{-1}$, a limit of detection (LOD) of 0.001 $mg \cdot kg^{-1}$, a repeatability below 11.3%, an intermediate precision of 2.5% and a global uncertainty below 4% for concentrations under 0.2 $mg \cdot kg^{-1}$. Accuracy was specifically evaluated by the recovery percentage for each type of PCP fortified with HHCB spikes (see section 4.3.2).

4.2.4 Blank Issues/Quality Assurance

The analyst avoided wearing scented personal products whenever assays were performed. Additionally, for each analyzed product, a fresh pair of disposable gloves were used to prevent HHCB cross contamination. Glassware was washed using non fragranced detergent and all the materials and reagents were tested to be free of interferences, by performing extraction blank assays, as no HHCB was detected (below LOD) as described before (Correia, Cruz et al. 2013).

4.3 Results and Discussion

4.3.1 Questionnaire

The aim of this study was to collect information to assess the risk of dermal exposure of children to the synthetic fragrance galaxolide contained in toiletries and personal care products. In order to create this data pool, a questionnaire for each child was given to caregivers of children aged between 0 and 5 years, all residents in the Oporto district (inclusion criteria). With these inclusion criteria, the final sample size was set at 250 children, corresponding to a representative sample population for a 5.2% margin of error and a 90% confidence level.

4.3.1.1 Social-Demographic Characterization of Children and Families

Children´s Characterization

Children's ages were selected assuming a previous reported range (Lavender, Bedwell et al. 2011). The children were divided into six age categories corresponding to each year of life, except for the first year, where the range was two half years. This was done because, during the first year, children experience exponential growth and major modifications on their skin structure, mainly during the first half year (Fluhr, Darlenski et al. 2010, Lavender, Bedwell et al. 2011). They were aged 0–5 months (8.3%), 6–11 months (9.1%), 12–23 months (19.0%), 24–35 months (21.3%), 36–47 months (26.1%), and 48–59 months (16.2%). The distribution of the six age groups by gender is shown in Figure 4.2, where 51% belongs to the female and 47% to the male gender. This distribution is according to Portugal Census 2011 (INE 2011) for the Oporto district: 52% female and 48% male.

The residence area of the inquired children included all 18 municipalities of the Oporto district with a distribution that mainly comprises the main city of Porto (26.8%) and also other municipalities like Gaia (16.8%), Amarante (15.2%), Matosinhos (10.4%), and Maia (8.8%). This distribution is slightly different from the real distribution based on the final results for the northern region of the Portugal Census 2011 (INE 2011), especially for the municipalities of Amarante and Gondomar.

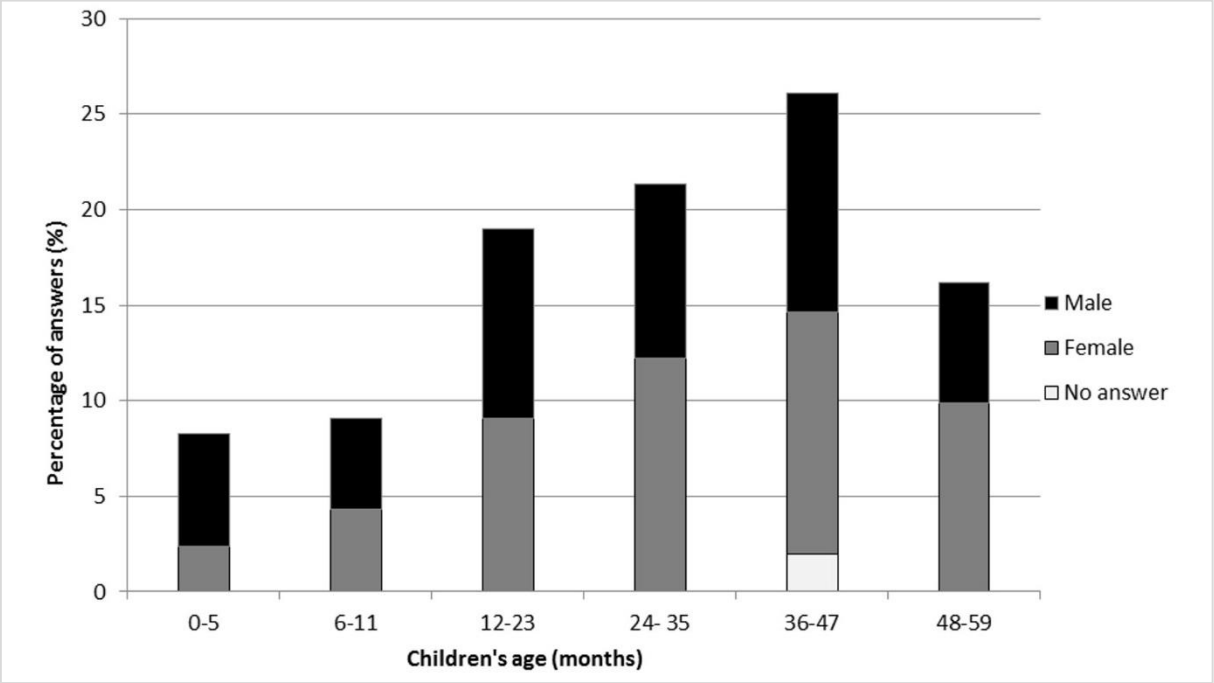


Figure 4.2 - Distribution of inquired children by age and gender (total 253 responses).

Caregivers and Families' Characterizations

Most questionnaires (89.2%) were answered by the children's mothers, 8.8% by the fathers, and 2.0% did not answer to this question. The caregivers, with an average of 34.7 ± 4.3 years, are mostly in charge with only one child under five years old (79.2%) and have an undergraduate degree (38.0%) or a Master's degree (18.8%), and few (4.0%) have less than six years of schooling (Figure 4.3).

In terms of professional occupation, 20.4% of caregivers work both in the area of "Education" as in "Health and social work", 16.4% in "Other service activities", and 8.8% in the "Industry", but 5.2% are "Unemployed" (Figure 4.4). Families are mainly comprised of three (45.0%) or four members (44.0%) and most of them (53.6%) reported a monthly income equal or superior to three minimum wages, while for 6.4%, monthly income was equal to the minimum wage (about 466 euros/month at the time of the study). Nevertheless, 9.2% of the respondents have chosen not to answer to this question.

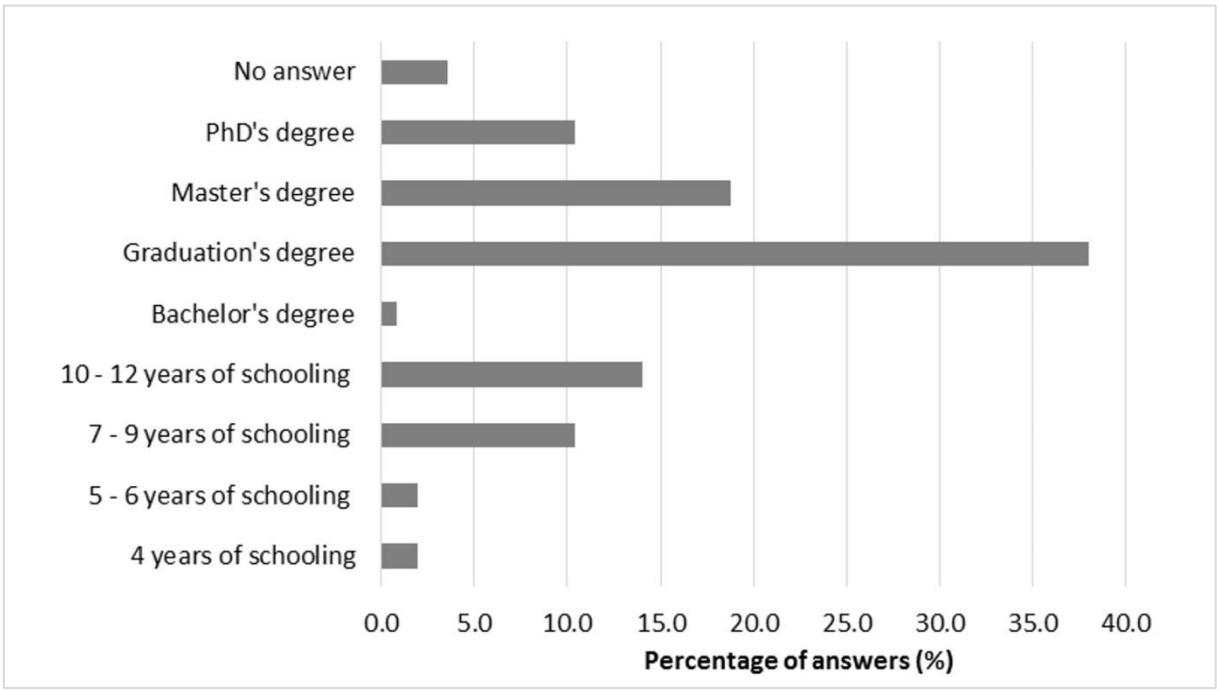


Figure 4.3 - Qualifications of caregivers.

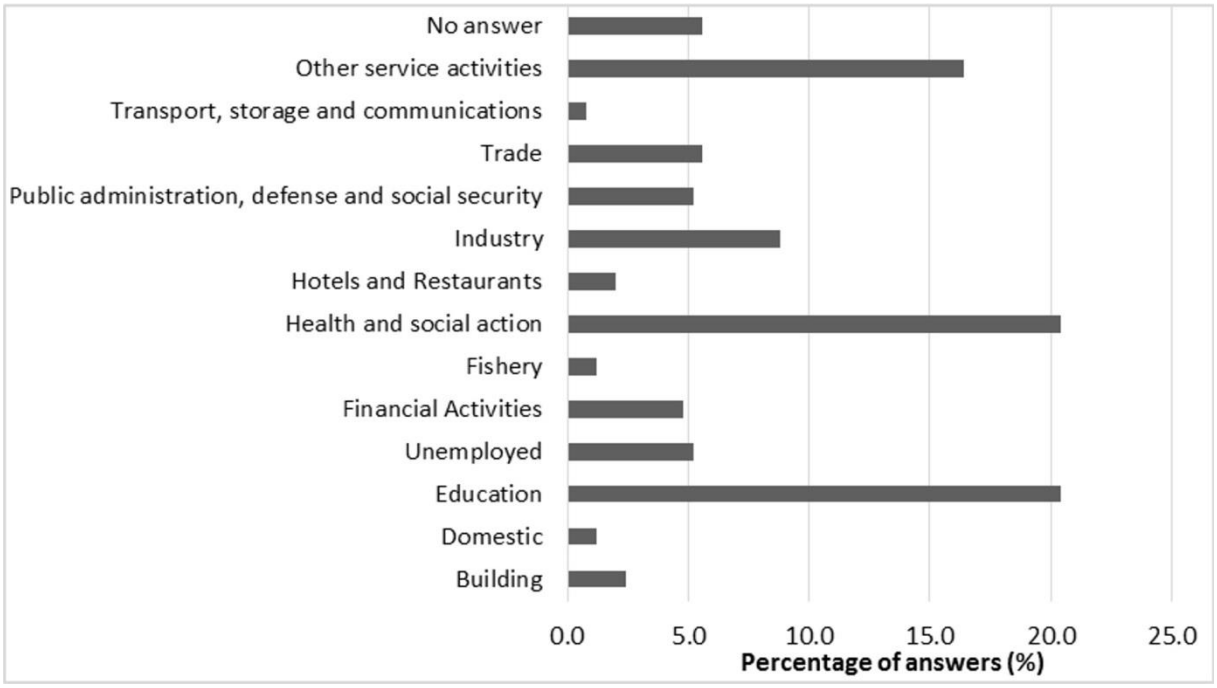


Figure 4.4 - Caregivers' professional occupations.

This section of the questionnaire, corresponding to the characterization of the caregivers and the families, was not mandatory, but only a minor fraction of respondents (2.4%) did not answer all of these questions.

4.3.1.2 Consumer Habits

The frequency and the mode of application (applied amount and site of application) of the PCPs used in children are described in Table 4.1. Notice that, regarding the mode of application, qualitative answers such as “a walnut of product” were inserted in order to facilitate the answers.

Moisturizing products are applied mostly once (52.8%) or twice (20.8%) a day on the face (92.8%), trunk (82.8%) and members (82.4%) as a thick layer (80.8%). Another study about PCP use in children in the United States of America (USA), made between 2010 and 2011, reported a similar frequency of 1.3 times a day for hydration (Gao and Simpson 2014).

Oral hygiene is performed one (24.0%) or two (46.4%) times a day, applying toothpaste on teeth and tongue (52.8%). The used amount of this kind of product corresponds mainly to the nail size of the little finger of the child (60.0%). Children who do not use toothpaste (16.4%) are mostly children under 12 months (15.6%), which is understandable, as most children at this age do not yet have dentition.

Table 4.1 - Frequency of utilization, applied amount and site of application of personal care products (PCPs) by the inquired children (percentage of answers below).

Parameters	Moisturizer	Toothpaste	Bath Gel/Soap	Shampoo	Hair Conditioner	Diaper Change Hygiene	Diaper Change Cream	Sunscreen
Frequency of utilization	Once a day (53%)	Two times a day (46%)	Once a day (68%)	Once a day (41%)	Once a day (10%)	Five times a day (mean)	Five times a day (mean)	Three times a day * (mean)
Applied amount	Thick layer (81%)	Nail of the little finger (60%)	A walnut of product (74%)	A walnut of product (94%)	A walnut of product (18%)	More than a wet wipe (43%)	Thick layer (39%)	Thick layer (51%)
Site of application	Face (93%)	Teeth and tongue (53%)	Wet skin (81%)	Wet scalp (92%)	Hair tips (22%)	Genital area (43%)	Irritated skin (40%)	All over the body (66%)

* On sun exposure days.

Children usually take a daily bath (68.4%) with application of a walnut of a bath gel (74.4%) directly onto wet skin (81.2%). Shampoo is also used on a daily basis by 40.8% of the children, while nearly a quarter (24.0%) only uses it three times a week. Typically, a walnut-sized amount of the product (93.6%) directly on the scalp (91.6%). This frequency of utilization is consistent with a report of 0.7 times a day for bath products and shampoo (Gao and Simpson 2014). Hair

conditioner is not used by the majority of the population (69.0%). When used, it is done once a day (10.0%) applying a walnut of the product (17.6%) at the hair tips (21.6%).

The diaper change is made from one (20.4%) to six (15.2%) times a day, with an average of five times per day, which is higher than the reported three times a day in the already referenced study [20]. For the hygiene of the genital area, most parents use one (23.2%) or more (42.8%) baby wipes, while others use a cleansing product directly on wet skin (31.6%). The diaper change barrier cream is applied as a thin (28.8%) or thick layer (38.8%), all over the genital area (29.6%) or only on irritated skin patches (40.0%).

Only a minority (8.0%) of the analyzed children do not use sunscreen, but all of them are children under 12 months of age, which is in fact an age group where any sun exposure should be avoided. The vast majority of the parents said they do not apply sunscreen every day of the year (83.6%), applying only in days of sun exposure, two (22.0%), three (26.4%) or four (20.4%) times a day, with an average of 3 times per day. The product is usually applied all over the body (65.6%) in a thin (32.8%) or thick layer (50.8%).

Based on the questionnaire results, 62 PCPs were selected as the most used by this population, and 17 other were added based on the sales statistics from two local hypermarkets (Table 4.2). These final 79 analyzed products were: twenty one moisturizers, seven toothpastes, thirteen bath gels, two soaps, one cleansing solution, nine shampoos, four hair conditioners, six baby wipes, eight diaper change creams, and eight sunscreens.

Table 4.2 - Selected personal care products (PCP) based on the level of use (%) obtained in the questionnaires or from hypermarket statistics; respective galaxolide (HHCB) concentrations (highest concentrations in bold) and PCP presentation.

PCP Type	PCP Code	PCP Utilization (%)	HHCB Concentration ($mg \cdot kg^{-1}_{sample}$)	PCP Presentation
Moisturizers (*N* = 70)	M1LC1	0.3	0.098 ± 0.007	Lotion
	M2CC1	0.5	0.022 ± 0.009	Cream
	M3LC2	**0.8**	**414.855 ± 0.016**	**Lotion**
	M4CC2	0.8	0.220 ± 0.009	Cream
	M5CR1	1.8	0.024 ± 0.007	Cream
	M2CC3	1.8	0.169 ± 0.010	Cream
	M6CC4	2.1	0.362 ± 0.012	Cream
	M2CC5	2.6	0.018 ± 0.011	Cream
	M7LC3	3.2	0.032 ± 0.009	Lotion
	M2CR2	3.9	0.034 ± 0.007	Cream
	M7LC4	4.7	0.406 ± 0.010	Lotion
	M2CC6	4.7	0.058 ± 0.007	Cream
	M8CC7	5.0	nd	Cream
	M5CC8	6.1	0.020 ± 0.011	Cream
	M9CC9	7.4	0.013 ± 0.011	Cream
	M4CR3	**7.4**	**105.397 ± 0.030**	**Cream**
	M4LC5	**11.3**	**184.174 ± 0.034**	**Lotion**
	M2LC6	15.0	nd	Lotion
	M5LC7	a	0.031 ± 0.010	Lotion
	M5LC8	a	0.023 ± 0.011	Lotion
	M7LC9	a	0.114 ± 0.008	Lotion
	Other PCPs (*n* = 49)	20.5	–	–
	No answer; Don't Know/use	1.1	–	–
Toothpastes (*N* = 24)	M10GD1	2.6	nd	Gel
	M10GD2	2.6	0.056 ± 0.001	Gel
	M11PD1	9.8	0.009 ± 0.008	Paste
	M12GD3	10.9	nd	Gel
	M13GD4	18.9	0.006 ± 0.006	Gel
	M14PD2	22.3	0.014 ± 0.010	Paste
	M15GD5	a	nd	Gel
	Other PCPs (*n* = 17)	12.8	–	–
	No answer; Don't Know/use	20.1	–	–

Table 4.2 - *Cont.*

PCP Type	PCP Code	PCP Utilization (%)	HHCB Concentration ($mg \cdot kg^{-1}_{sample}$)	PCP Presentation
Body Bath Products (*N* = 58)	**M16SB1**	**0.7**	**300.480 ± 0.017**	**Soap**
	M17GB1	1.0	1.385 ± 0.033	Gel
	M18SB2	1.0	0.712 ± 0.012	Soap
	M7GB2	3.5	0.033 ± 0.006	Gel
	M4GB3	**4.5**	**79.718 ± 0.019**	**Gel**
	M9GB4	5.6	0.035 ± 0.007	Gel
	M2SL1	8.0	0.075 ± 0.007	Solution
	M4GB5	8.4	0.063 ± 0.007	Gel
	M5GB6	9.1	0.025 ± 0.009	Gel
	M7GB7	10.5	0.100 ± 0.009	Gel
	M2GB8	24.0	0.444 ± 0.012	Gel
	M1GB9	[a]	0.010 ± 0.009	Gel
	M1GB10	[a]	0.068 ± 0.002	Gel
	M5GB11	[a]	0.012 ± 0.010	Gel
	M7GB12	[a]	0.226 ± 0.009	Gel
	M3GB13	**[a]**	**217.795 ± 0.012**	**Gel**
	Other PCPs (*n* = 42)	23.3	–	–
	No answer; Don't Know/use	0.3	–	–
Shampoos (*N* = 25)	M19CH1	1.7	0.038 ± 0.007	Gel
	M5CH2	10.4	0.193 ± 0.010	Gel
	M4CH3	**10.4**	**127.517 ± 0.030**	**Gel**
	M7CH4	52.2	0.005 ± 0.005	Gel
	M1CH5	[a]	0.035 ± 0.006	Gel
	M1CH6	[a]	0.796 ± 0.019	Gel
	M7CH7	[a]	0.089 ± 0.008	Gel
	M7CH8	[a]	0.346 ± 0.010	Gel
	M7CH9	[a]	0.209 ± 0.010	Gel
	Other PCPs (*n* = 16)	0.9	–	–
	No answer; Don't Know/use	24.3	–	–
Hair Conditioners (*N* = 8)	**M20AS1**	**5.9**	**251.796 ± 0.031**	**Solution**
	M7AC1	64.7	0.037 ± 0.006	Cream
	M7AC2	[a]	0.017 ± 0.011	Cream
	M7AS2	[a]	0.006 ± 0.006	Solution
	Other PCPs (*n* = 4)	23.5	–	–
	No answer; Don't Know/use	5.9	–	–
Baby Wipes (*N* = 26)	M17TL1	0.7	2.675 ± 0.009	Wet wipe
	M7TL2	3.0	[nd]	Wet wipe
	M4TL3	3.3	[nd]	Wet wipe
	M8TL4	4.6	0.001 ± 0.001	Wet wipe
	M21TL5	6.3	0.022 ± 0.011	Wet wipe
	M22TL6	43.4	0.154 ± 0.010	Wet wipe
	Other PCPs (*n* = 20)	17.4	–	–
	No answer; Don't Know/use	21.4	–	–

Table 4.2 - *Cont.*

PCP Type	PCP Code	PCP Utilization (%)	HHCB Concentration ($mg \cdot kg^{-1}_{sample}$)	PCP Presentation
	M2CF1	1.1	nd	Cream
	M23CF2	1.4	0.012 ± 0.011	Cream
	M5CF3	3.2	0.207 ± 0.008	Cream
Diaper	M24PO1	4.9	1.234 ± 0.025	Ointment
Change	M2PA1	5.6	0.016 ± 0.011	Paste
Products	**M4CF4**	**6.0**	**71.513 ± 0.010**	**Cream**
(*N* = 28)	M6PA2	15.1	0.016 ± 0.011	Paste
	M23PO2	21.1	nd	Ointment
	Other PCPs (*n* = 20)	18.3	–	–
	No answer; Don't Know/use	23.2	–	–
	M25PL1	5.2	0.074 ± 0.006	Lotion
	M4CM1	6.6	1.005 ± 0.023	Paste
	M26PL2	9.0	12.312 ± 0.010	Lotion
	M2PL3	9.4	0.051 ± 0.007	Lotion
Sunscreens	M2CM1	11.5	0.344 ± 0.010	Paste
(*N* = 31)	M27PL4	12.8	0.229 ± 0.010	Lotion
	M27CM2	15.3	0.394 ± 0.011	Paste
	M1PL5	[a]	**134.715 ± 0.025**	**Lotion**
	Other PCPs (*n* = 23)	21.9	–	–
	No answer; Don't Know/use	8.3	–	–

[a] Indicated by hypermarkets statistics; nd not detectable.

4.3.2 HHCB Concentrations

The final results for the analyzed PCPs are presented in Table 4.2. Product brands and designations were encoded as a PCP code representing the number of the mark (M#) followed by a product number according to the presentation and use of that product. For example, brand 1 has a body lotion (M1LC1), two bath gels (M1GB9, M1GB10), two shampoos (M1CH5, M1CH6) and a sunscreen lotion (M1PL5).

HHCB was detected in 70 of the 79 analyzed PCPs, within a wide range of concentrations that were consistent with other studies, although all of them respective PCPs for adults [3, 13–17]. The HHCB concentrations ranged from 0.001 ± 0.001 $mg \cdot kg^{-1}$, on a baby wipe (M8TL4) to 300.480 ± 8.819 $mg \cdot kg^{-1}$, on glycerin soap (M16SB1). The highest HHCB concentrations were found in ten products (bold PCP codes in Table 4.2), and five of them are from brand M4 (with nine analyzed products). The nine products with concentrations of HHCB below the limit of detection (LOD = 0.001 $mg \cdot kg^{-1}$ sample) are indicated as not detectable (nd) in Table 4.2 and two of them are from brand M2 (with twelve analyzed products). In this study, 29 of the analyzed

PCPs were labeled as "fragrance-free", but only six of them have actually a non-detectable amount of HHCB. Among these "fragrance-free" products, the detectable HHCB concentrations varied between 0.001 ± 0.001 and 71.513 ± 0.010 mg·kg^{-1}, and therefore, most of them cannot be considered as residual quantities. On the other hand, three of the 50 PCPs that include "parfum" or "fragrance" in their compositions, were found to have no detectable HHCB, indicating that those products may contain other fragrance ingredients, rather than HHCB.

All presented concentrations were corrected by their respective recovery (Table 4.3), determined as described before (Correia, Cruz et al. 2013), and ranging from 65% in lotion moisturizers and sunscreens (with a 320.0 mg·kg^{-1} sample spike) and 98% in soaps (with a 227.5 mg·kg^{-1} sample spike) and creams (with a 300.0 mg·kg^{-1} sample spike).

Table 4.3 - Selected personal care products (PCP) for the recovery assays of each PCP category, respective galaxolide (HHCB) concentrations, HHCB spikes, and recoveries (%).

PCP Category	PCP Presentation	PCP Code	HHCB Concentration (mg·kg^{-1}$_{sample}$)	HHCB Concentration Spike (mg·kg^{-1}$_{sample}$)	HHCB Recovery (%)
Moisturizers	Lotion	M7LC3	0.032 ± 0.009	320.0	65
	Cream	M4CR3	105.397 ± 0.030	300.0	98
Toothpastes	Paste	M14PD2	0.014 ± 0.010	300.0	80
	Gel	M15GD5	nd	227.5	80
Body Bath Products	Soap	M16SB1	300.480 ± 0.017	227.5	98
	Solution	M2SL1	0.075 ± 0.007	230.0	85
	Gel	M1GB9	0.010 ± 0.009	230.0	84
Shampoos	Gel	M4CH3	127.517 ± 0.030	300.0	81
Hair Conditioners	Cream	M7AC2	0.017 ± 0.011	227.5	75
Baby Wipes	Wet wipe	M17TL1	2.675 ± 0.009	227.5	68
Diaper Change Products	Paste	M27CM2	0.394 ± 0.011	230.0	79
	Ointment	M24PO1	1.234 ± 0.025	320.0	70
Sunscreens	Lotion	M7LC3	0.032 ± 0.009	320.0	65
	Paste	M27CM2	0.394 ± 0.011	230.0	79

nd not detectable

4.3.3 HHCB Risk of Dermal Exposure

Considering children's consumer habits previously obtained by the referenced questionnaire, an estimation of the total daily dermal exposure to HHCB of this population was performed (Table 4.4). The mean daily application amount was estimated based on the responses obtained from answers to the questionnaire "Diary of hygiene and personal care products use by children" (Annex B). Average retention factors on skin for each type of PCP were based on values reported in scientific literature (Cadby, Troy et al. 2002) for similar products: 100% for

leave-on products (body lotions, creams, wet wipes, and sunscreens) and 10% for rinse-off products (toothpastes, bath gels, soap bars, shampoos, and hair conditioners). The mean daily HHCB retention for each type of PCP was calculated as the product of mean HHCB concentrations on each group (considering measured concentrations in Table 4.2), mean daily applied amount, and the respective retention factor. Total daily dermal exposure risk was obtained by the sum of all HHCB retained from daily use of PCPs. The lowest mean HHCB concentrations (Table 4.4) were found in toothpastes and baby wipes, while the highest mean concentration corresponded to soap bars. Soap bars can irritate the skin due to its alkaline pH and due to the presence of some additives, like fragrances (Imai and Kuwabara 1992), which can raise the risk of exposure to HHCB. Nevertheless, these kind of PCPs were not the most relevant ones to the estimated total daily dermal exposure risk, as soap bars were only considered to be used for washing hands, and, therefore, used on a reduced exposure area. In cases where they are used for body wash, their influence to dermal exposure may be higher. The highest contribution for the estimated dermal exposure risk, then, was found to be from leave-on-products applied onto the whole body (Table 4.4): sunscreens, assuming a sun exposure day, and body lotions/creams, on the other days. Additionally, it is expected that penetration is greater for longer contact times and surface areas (Wester e Maibach 2002a). Consequently, leave-on-products applied onto the whole body, like moisturizers and sunscreens, should be the most absorbed ones. It is also reported that the penetration varies with the application site of the PCP, of which the most critical ones are the genitals, head and trunk (Wester e Maibach 2002b), so only this last site could more greatly affect the dermal exposure risk from moisturizers and sunscreens. Toothpastes were the PCPs that contributed less to the total dermal exposure risk, which is consistent with the small area of the oral cavity, small applied quantities, short contact time, and low detected HHCB concentrations found in these kind of products (some not detectable). Nevertheless, it should be noted that there is still some risk of oral exposure from toothpastes, although this is not a major route.

Table 4.4 - Estimated mean daily dermal exposure to HHCB (μg·day^{-1}) using the analyzed PCPs.

Personal Care Products (PCPs)	**Application Site**	**Exposure Route**	**Number of Analyzed PCPs**	**Mean HHCB Concentration[c]** (mg.kg$^{-1}_{sample}$)	**Mean daily Application Amount** (g.$_{sample}$.day^{-1})	**Retention Factor** (Cadby, Troy et al. 2002)	**Mean Daily HHCB Retention** (μg.day^{-1})
Body lotions / creams	Whole body	Dermal	18	37.539	1.4	100.0%	52.5541
Facial creams	Face	Dermal	3	35.152	0.5	100.0%	17.5759
Toothpastes	Perioral region and mouth mucous membranes	Dermal and oral	7	0.021	0.2	10.0%	0.0004
Bath gels	Whole body	Dermal	14	21.428	2.1	10.0%	4.4998
Soap bars	Hands	Dermal	2	150.431	1.1	10.0%	16.5474
Shampoos	Scalp, neck and hands	Dermal	9	14.359	0.9	10.0%	1.2923
Hair conditioners	Hair tips and hands	Dermal	4	62.964	0.6	10.0%	3.7779
Baby wipes[a]	Genital area	Dermal	6	0.713	21.7	100.0%	9.9853
Diaper change creams	Genital area	Dermal	8	12.167	0.7	100.0%	8.5166
Sunscreens[b]	Whole body	Dermal	8	18.672	8.4	100.0%	156.8443
Total daily dermal exposure to HHCB (μg·day^{-1})							**277.10±0.02**

[a] mean daily application calculated based on the questionnaire "Diary of hygiene and personal care products use by children" answers, that indicate an average of 7 baby wipes a day and about 3.1 g each wipe; [b] mean daily application calculated assuming a sun exposure day (3 times a day in thick layer, Table 4.1); because no answers were obtained for the referred diary to the questionnaire related to sunscreens, the application of a thick layer was assumed to be twice the amount of body lotions/creams (thin layer); [c] Mean HHCB concentrations for all the analysed PCPs in each category of products (*Table 4.2*).

The estimated total daily dermal exposure to HHCB was 277.10 ± 0.02 μg·day^{-1} (Table 4.4) considering the mean application amount for each kind of PCP used by all the children's age groups. Observing data in detail and the differences between the youngest and the oldest children consumer habits, we can notice that the total daily dermal exposure risk is about 132.38 ± 0.01 μg·day^{-1} for children less than 1 year old and 169.16 ± 0.02 μg·day^{-1} for children older than 4 years. The lower levels for these two age groups, at the lower and upper end of the studied population, are justified by the fact that the youngest ones do not yet use sunscreens (46.5%) or toothpastes (81.4%), while the oldest ones use no more diaper change products (53.7%). The middle-aged groups have a higher mean daily dermal exposure risk to HHCB (297.98 ± 0.02 μg·day^{-1}) because they already use sunscreens and still use considerable

amounts of diaper change products. All of the estimated daily dermal exposure risks are below other published results for adults 904 μg·day^{-1} (Correia, Cruz et al. 2013), 3060 μg·day^{-1} (Lu, Yuan et al. 2011a), 25100 μg·day^{-1} (Reiner and Kannan 2006) and 23700 μg·day^{-1} (Roosens, Covaci et al. 2007)), which is consistent with the fact that children's PCPs are expected to be less fragranced than adult's ones and the used amounts are also smaller in children. Although rather small amounts are predicted to be retained by children's skin, exposure risk can be higher than in adults, especially when the epidermal barrier is compromised due to some common children's skin disorders. To the authors' best knowledge, no studies were found about children's dermal exposure to HHCB, but a study in the elderly population, with an altered epidermal barrier, showed a higher systemic absorption of musks than in healthy adults (Hutter, Wallner et al. 2010). A common children's skin disorder that may alter epidermal barrier is atopic skin, but it should be mentioned that the six analysed body creams for this kind of skin have a mean HHCB concentration value of 0.134 mg·kg^{-1}sample, which is a low contribution for dermal exposure to HHCB in children.

Considering the estimated total daily dermal exposure risk to HHCB and a total evaporation factor of 22% (Ford, Hawkins et al. 1999), only 216 μg·day^{-1} remains on the skin surface and may be absorbed. There is limited information about HHCB dermal absorption, but the human skin absorption has been reported as 19.5%, of which only 0.1% eventually enters the systemic circulation (Ford, Hawkins et al. 1999). However, different rates are expected when dealing with children or baby skin (Fluhr, Darlenski et al. 2010, Lavender, Bedwell et al. 2011, Blume-Peytavi, Hauser et al. 2012). Considering the same level of absorption for all PCPs for children and adults, and assuming an average body weight (BW) of 17.2 kg for a 4-year-old child (Masuck, Hutzler et al. 2011), dermal exposure will be about 0.02 μg·kg$_{bw}^{-1}$·day^{-1}. To the author's best knowledge, no other values of dermal exposure of children to HHCB due to PCP use was reported. Only one study was found where dermal exposure of children to toys scented with several fragrances was evaluated and reported values were significantly higher, ranging from 8.6 to 605.0 μg·kg$_{bw}^{-1}$·day^{-1} (Masuck, Hutzler et al. 2011).

4.4 Conclusions

PCP consumption habits of children population from the Oporto district was established by a questionnaire applied to 250 children. The frequency and mode of application of the PCPs, estimated by the results of this survey, permitted to establish the common applied quantities. Additionally, HHCB was detected in 70 of the 79 most used PCPs in concentrations ranging from 0.001 ± 0.001 mg·kg^{-1}, found on a baby wipe, and 300.480 ± 8.819 mg·kg^{-1}, detected in glycerin soap. The frequency and mode of application of the PCPs, estimated by the results of this survey, permit researchers to establish the common applied quantities. With these results, it was possible to determine the children's dermal exposure to HHCB from personal care products in Oporto as 277.10 ± 0.02 µg·day^{-1}.

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4.6 Author Contributions

Arminda Alves, Lúcia Santos and Agostinho Cruz conceived and designed the experiments. Patrícia Correia performed the experiments and analyzed the data. All authors wrote the paper.

4.7 Conflicts of Interest

The authors declare no conflict of interest.

5 Estimation of galaxolide dermal risk assessment in adults and children[4]

Abstract

Synthetic fragrances are present in all kind of consumer goods, from detergents and household products to toiletries used in our routine. So everyone is daily exposed to fragrances, particularly through hygiene and Personal Care Products. Musks is one of the most important class of fragrances, since they have a function of fixing the odor to materials and also to skin. Therefore, this class of cosmetic substances represent a high exposure potential to human organism, particularly those with lipophilic characteristics, such as galaxolide, the most worldwide used polycyclic musk. For that, when conducting risk assessment to galaxolide human´s exposure, not only general population of a region but also sensitive groups, like children, should be analyzed. In this study, a 414 individuals' sample of the Northern Region Portuguese population, and a specific part of that population (250 children from 0-5 years old from Oporto district) were scrutinized about their consumer patterns of toiletries. It was concluded that the main differences were verified on the used amounts of each product type, rather than in the frequencies of application. Additionally, the specificity of the chosen children products led to differences on exposure risks for each population group: 277 $\mu g.day^{-1}$ for children and 692 $\mu g \cdot day^{-1}$ for the remaining population. The exposure data were combined with the risk characterization data found in the literature and the population mean body weight, conducting to a maximum systemic exposure dose of 36.6 $\mu g.kg_{bw}^{-1}$ for children and 9.4 $\mu g.kg_{bw}^{-1}$ for adults was achieved. Therefore, the minimum margins of safety, respectively 820 and 3191, permits to conclude that the use of galaxolide is safe considering the normal use of toiletries. Nevertheless, the simultaneous utilization of products with high concentrations of galaxolide, or the incorrect use of Personal Care Products for example in damaged skin, could result in loss of safety, especially for children, considering that a 100 minimum margin of safety is acceptable.

Keywords: Galaxolide, toiletries, dermal risk assessment, systemic exposure dose, margin of safety

[4] "Estimation of galaxolide risk assessment in adults and children's population"; P Correia, A Cruz, L Santos, A Alves; submitted 2016

5.1 Introduction

Fragranced products are part of nowadays human routine, including Personal Care Products (PCPs), household products and detergents, and even fresheners, decoration objects or children's toys. The human exposure to fragrances, namely synthetic musks, includes a vast number of different substances that should be extensively evaluated for their safety prior to their marketing. Some of these substances may have a negative effect in the health of the PCPs' consumers (Biesterbos, Dudzina et al. 2013) and the safety evaluation of a PCP ingredient is not only based on its toxicological properties, but also in the way that PCPs are used. Four phases are considered in risk assessment of hazardous substances (Gomez-Berrada, Gautier et al. 2013): hazard identification, hazard quantification, exposure risk and risk characterization.

The first phase of a risk assessment, the hazard identification, involves the identification of the inherent ability for a substance to cause adverse health effects. Even when people is exposed to very small amounts, acute exposure, to a specific skin site or to sensitive skin, may occur from a single PCP's use (short-term exposure), but also a continued and cumulative exposure (long-term exposure) through bath products, moisturizers, deodorants and various other personal care and hygiene products (Cadby, Troy et al. 2002). For instance, a short-term or acute exposure to fragrances may result in skin irritancy, allergenicity and phototoxicity (Cadby, Troy et al. 2002). But recent studies reported also the potential risk of fragrances, like polycyclic musks (PMs), to cause estrogenic or anti-estrogenic effects, probably resulting from a long-term exposure with systemic absorption (Yamauchi, Ishibashi et al. 2008, Hu, Shi et al. 2011).

The hazard quantification is the risk assessment phase where the No Observed (Adverse) Effect Level, NO(A)EL, for the identified hazard (in the first phase), is calculated for experimental species, establishing the toxicity of the compound at a certain level (Gomez-Berrada, Gautier et al. 2013). NO(A)EL is the highest dose level where no (adverse) health effects are observed. The HHCB NO(A)EL values found in literature vary between 50 and 150 $mg.kg^{-1}_{body\ weight}$ for rats (Api and Ford 1999, SCCNFP 2002, TOXNET 2012). It is also possible to calculate the Lowest Observed (Adverse) Effect Level (LO(A)EL) and the values found in literature vary between 10 $mg.kg^{-1}_{body\ weight}$ for red worm (Chen, Xue et al. 2011) and 100 $mg.kg^{-1}_{body\ weight}$ for rats (TOXNET 2012).

The exposure risk (or exposure assessment) is performed by the combination of the substance concentrations on the analyzed products and the respective exposure patterns. Exposure is defined as the average amount of product used per day or, even, per kilogram bodyweight (or skin area). In this phase, it is necessary to know the usage patterns, namely the frequency and used amounts and the substance concentration in each used PCP (McNamara, Rohan et al. 2007). But part of the substance applied on the skin may not be absorbed, since evaporation can occur. The evaporation of HHCB is dependent of its physical-chemical properties as well as its interactions with the other components of the product (Saiyasombati and Kasting 2003). Ford et al. (1999) estimated that about 22% of the HHCB volatilizes after skin application. The remaining HHCB may result in acute exposure and can cause allergic reactions in sensitive skin such as irritation, allergy and phototoxicity (Cadby, Troy et al. 2002). Although skin provides a protective barrier against several threats, some substances may penetrate the skin and became systemically available. However, a compound deposited in skin can only enter systemic circulation if it adheres to skin lipids, passes through several epidermal cells layers and reaches the dermis, where blood capillaries are present (Weschler and Nazaroff 2012). Two studies have reported HHCB dermal absorption to the bloodstream. In the first study it was estimated that 0.1% of HHCB passes into the bloodstream (Ford, Hawkins et al. 1999), and a second study (Weschler and Nazaroff 2012), refers the systemic absorption of compounds through the skin dependent on several factors. Among these factors are: the lipophilicity of the compounds, the surface area available for absorption, the thickness and composition of the epidermis (that varies with the body site), the duration of exposure, the amount of topically applied product, the concentration of the compound, the existence of occlusion, and others. In order to reach the dermis, a compound must pass through a layer of lipids and the superficial epidermis, composed by *Stratum Corneum*, SC, with lipophilic characteristics, and the *Viable Epidermis*, VE, with hydrophilic characteristics. However, these authors propose a way of calculating the flow, *J*, of any compound through the skin, considering the epidermis permeability coefficient (k_{pw}), the compound concentration trapped in skin surface lipids (C_l), the amount of PCP applied (A_a), the surface of the skin where the PCP is applied (S_p) and the skin surface lipids thickness (e_l):

$$J = \frac{k_{pw} C_l A_a}{S_p e_l} \quad (1)$$

HHCB is a lipophilic molecule that easily passes through SC after deposition on skin, but will find some resistance to migration through the VE, the innermost hydrophilic layer of the skin. However, disregarding this resistance and the possibility of some degradation by skin enzymes, equation (1) may be applied to HHCB absorption.

The first study found in the literature regarding dermal exposure risk due to PCPs use reported an exposure risk of 25100 μg.day^{-1} (Ford 1998). Afterwards, other dermal exposure risks to HHCB in adults were emerging: from 17 to 23700 μg.day^{-1} (Roosens, Covaci et al. 2007); 3060 μg.day^{-1} (Lu, Yuan et al. 2011a); 904 μg.day^{-1} (Correia, Cruz et al. 2013); 1362 μg.day^{-1} (Homem, Silva et al. 2015a) and 395 μg.day^{-1} (Nakata 2005). For the author's best knowledge, there is only a reported value, by this working team, of 277 μg.day^{-1} for dermal exposure risk to HHCB in children (Correia, Cruz et al. 2015).

The last phase of the risk assessment, the risk characterization, combines the hazard quantification and the exposure risk, by comparing nontoxic doses and exposure doses, to determine the probability that an adverse effect will occur. For PCPs, this probability includes an uncertainty factor, the Margin of Safety (MoS). The MoS is used because the NO(A)EL values are derived from repeated dose animal toxicity studies that need to be extrapolated to human population. A minimum value of 100 for MoS is used to consider that a substance is safe for use. That minimum MoS value results from the application of two subsequent factors of 10 times, one for the extrapolation from the animals' tested group to an average human, and another to consider the human variability (SCCS 2012). Human variability includes age, gender, ethnicity, sensitive subpopulations, differences in barrier function and genetic differences. When analyzing exposure to HHCB, the variability also include matrix effects because the consumer can be exposed in many different PCP's formulations (e.g. creams, shower gels, hair spray) that may be complex and contain ingredients that are irritant or penetration enhancers (Bremmer 2006).

The MoS is calculated using the equation (2) using the NO(A)EL and the SED, the systemic exposure dose (SCCS 2012):

$$MoS = \frac{NO(A)EL}{SED} \qquad (2)$$

The SED permits to evaluate the systemic availability of a cosmetic substance, and is determined by equation (3) where DA_s is the dermal absorption of the substance and BW is the bodyweight (SCCS 2012):

$$SED = \frac{DA_S}{BW} \quad (3)$$

The *DAs* is determined in the exposure risk phase, and corresponds to the substance daily systemic exposure.

For the author's best knowledge, there is only a published MoS for dermal exposure to HHCB of 58523 due to PCPs' use, respecting to European population (SCCNFP 2002). Another reported value of 350000 was related to the use of household cleaning products in the United States of America (USA) (HERA 2004a). But, the consumer patterns and PCPs market is surely different, not only because more than a decade have passed since the only risk assessment about HHCB in PCPs, but also because this study was performed at a large regional scale (Europe). So, it is essential to make recent risk assessment applied to new regions.

The objectives of the present study are to compare consumer habits of PCPs and HHCB concentrations found in those PCPs, and to perform an exposure assessment for two age groups of the Portuguese population: adults resident in the Northern Region of Portugal and children (0-5 years old) resident in the Oporto district.

5.2 Experimental section

5.2.1 Consumer patterns

Consumer habits of PCPs on people resident in the Northern Region of Portugal was obtained with a sample of 414 persons, by an online questionnaire (Annex A) from January to February 2015. The included PCP types were body moisturizer, facial moisturizer (day and night), toothpaste, deodorant, bath gel/soap, shampoo, hair conditioner, hair care leave on products, sunscreen (face and body) and other products. The consumer patterns include: PCP percentage of use, preferred brands and types, daily frequency of use and application amount per event. Additionally, some social-demographic characteristics (residence municipality, gender, age, labor activity, academic qualification and monthly income) and extra personal information (e. g. body weight, type of face skin, type of body skin, and type of hair) were also obtained. Statistical analysis was performed using Microsoft Excel 2007®.[5]

The consumer patterns of 250 children aged between 0 and 5 years, and resident in the district of Oporto were obtained from child parents' responses to a paper questionnaire for each child consumer habits (Annex B), from January to March 2013. The responses were analyzed in terms of social-demographic characteristics and consumer habits, namely most used PCPs (brands and types), as well frequency and quantity of application. Statistical analysis was performed using Microsoft Excel 2007®. More detailed information was already reported (Correia, Cruz et al. 2015).

5.2.2 HHCB concentrations in PCPs

An analytical method with QuEChERS extraction and High performance liquid chromatography with fluorescence detection (HPLC-FL) was developed and validated (limit of detection, LOD, of 0.001 $mg.kg^{-1}_{sample}$) for the quantification of HHCB in PCPs. For adults' population seven PCPs were analyzed, chosen randomly to represent the main types of PCPs (Correia, Cruz et al. 2013). Additionally, 79 children´s PCPs were selected (Correia, Cruz et al. 2015) including: 21 moisturizers, 7 toothpastes, 9 shampoos, 13 body washes, 2 soaps, 1 cleansing solution, 4 hair conditioners, 6 baby wipes, 8 barrier creams and 8 sunscreens.

[5] More detailed information is referred in Chapter 2.

5.3 Results and discussion

As referred before, PMs may have some negative effects on human health when daily used products are applied on skin. Special care must be taken with the most used ones, like galaxolide (HHCB), and sensitive skin individuals, like babies/children, that are the most vulnerable ones. In fact, some studies indicate that the immature skin of infants, may compromise the skin protection barrier (Lavender, Bedwell et al. 2011, Stamatas, Nikolovski et al. 2011). Additionally, adult's PCPs are not frequently used by children on most families routine, because there is a special concern with children's skin (by the caregivers and by the market choices), and there are some specificity like diaper change products. For that, a comparison was made between consumer patterns and HHCB concentrations found in each PCPs' age group, and then dermal exposure risk and subsequent risk assessment in those two stages of life, have been separately analyzed.

5.3.1 Consumer patterns

Data about the mean daily used amounts of PCPs are vital for the determination of the dermal exposure risk to HHCB. For adults' population, the consumer patterns have already been extensively collected, as referred before (Correia, Cruz et al. 2013). A first approach was made (Correia, Cruz et al. 2013) using European consumer patterns (Hall, Tozer et al. 2007) and assuming that they were similar to the Portuguese ones. Children's population consumer pattern, that was not accessible on the open literature, was also obtained for Oporto district (children from 0-5 years old) and published (Correia, Cruz et al. 2015). Apart from this study, there are few and recent comprehensive exposure data available about PCPs use in children population and all of them are from European (Nohynek, Antignac et al. 2010, Gomez-Berrada, Gautier et al. 2013, Gosens, Delmaar et al. 2014) or American (Wu, Bennett et al. 2010) studies (Table 5.1).

The studies found in the literature focus mainly on body lotions/creams and sunscreens, and none of them refers specifically to face cream used amounts and frequency, or to the soap bars frequency. There is a lot of variation between mean application amounts found in the literature, except for soap bars (for washing hands) and sunscreens (hot season with sun exposure). Correia et al. (2015) generally reported mean application amounts smaller than the

other studies, except for soap bars, baby wipes and sunscreens. The mean daily frequencies of the studied PCPs presents smaller variations than the used amounts. These results confirm the need of specific exposure assessments related to differences in consumer patterns among populations of different regions.

Table 5.1 - Application amount (g_{sample}.event^{-1}) and daily frequency (event.day^{-1}) of children's PCP use.

Studies / PCPs	**Portugal** Present study (Correia, Cruz et al. 2015)		**Europe** (Gosens, Delmaar et al. 2014)		**Europe** (Gomez-Berrada, Gautier et al. 2013)		**Europe** (Nohynek, Antignac et al. 2010)		**USA** (Wu, Bennett et al. 2010)	
	g_{sample}. event^{-1}	**event. day^{-1}**	**g_{sample}. event^{-1}**	**event. day^{-1}**	**g_{sample}. event^{-1}**	**event. day^{-1}**	**g_{sample}. event^{-1}**	**event. day^{-1}**	**g_{sample}. event^{-1}**	**event. day^{-1}**
Body lotions/creams	1.4	1.0	2.2	-	5.2[b]	1.5	-	-	-	0.6
Facial creams	0.5	1.0	-	-	-	-	-	-	-	-
Toothpastes	0.1	1.5	0.9	1.5	-	-	-	-	-	-
Shower gels	5.3	0.4	-	-	9.1	1.2	-	-	-	0.4
Soap bars (hands)	0.3	3.9	0.2	-	-	-	-	-	-	-
Shampoos	3.1	0.3	13.2	-	7.3	0.6	-	-	-	-
Hair conditioners	2.0	0.3	9.2	-	-	-	-	-	-	0.3
Baby wipes	6.2	3.5	5.0	5.0	-	-	-	-	-	-
Diaper change creams	0.4	1.8	-	-	3.7	2.2	-	-	-	-
Sunscreens (hot season)	2.8[a]	3.0	2.7	2.5	-	-	3.0[b]	2.0	-	0.6

[a] assuming a sun exposure day (3 times a day in thick layer that is twice the amount of a thin layer of body lotions/creams
[b] applied in body and face

On the other hand, the way that PCPs' are used may vary, not only between different regions, but also between different groups in the same region or country, as reported by Wu et al. (2010) and Biesterbos et al. (2013). So, the consumer patterns have also been collected for the Northern Region Portuguese adults' population and compared to the previously obtained for children (Figure 5.1). The PCPs' types used in such a different age groups, are obviously distinct, but the comparison have been made only between the PCPs' types used by both age groups.

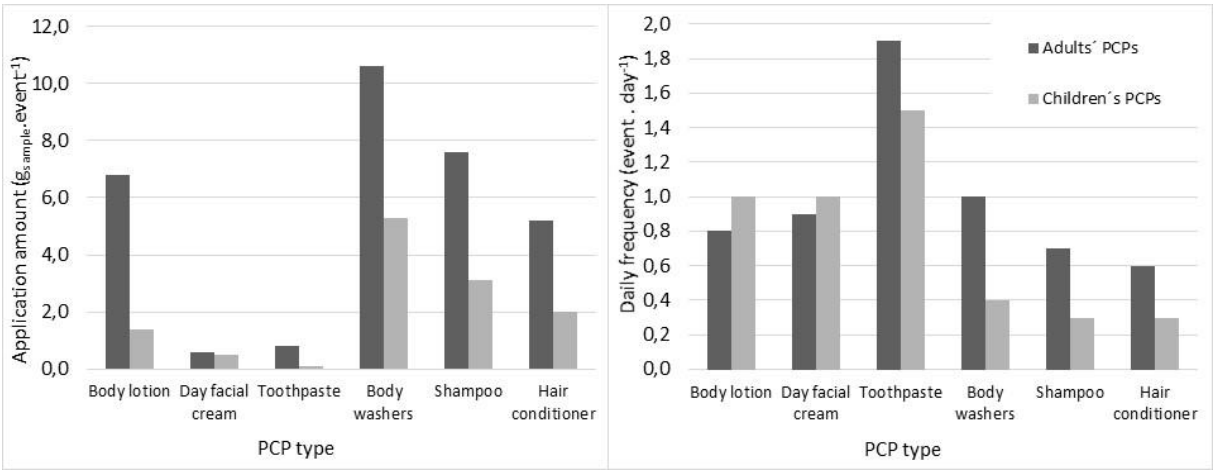

Figure 5.1- Mean used application amounts (left) and daily frequencies (right) of the similar analyzed PCPs for adults and children.

The mean application amounts of product for each utilization is generally higher for adults, except for facial cream. These results are expected because the skin area differences among the two age groups are evident. As for the daily frequencies of PCPs' use, there are some differences among bath products and adults seem to use them more often. In fact, adults usually take a daily bath while caregivers try to avoid children's skin damage for excessive bathing, doing that less often. Nevertheless, both age groups use body washers more often than hair care products (shampoo and hair conditioner), indicating that hair isn´t washed in every individuals' bath. A study about PCPs usage patterns in California households, reported that PCPs use of children and their parents may be correlated (Wu, Bennett et al. 2010) but in the present study, adults and children's population was not necessarily from the same family.

5.3.2 HHCB concentrations

The previously developed analytical method (Correia, Cruz et al. 2013) was used to determine HHCB concentrations in PCPs used in Portugal for adults and children. The adults population PCPs presented HHCB in all analyzed samples in concentrations ranging from 0.04 ± 0.01 mg.kg$^{-1}_{sample}$ (toothpaste) to 280.78 ± 8.19 mg.kg$^{-1}_{sample}$ (perfumed body cream). These levels of HHCB are in the range of all concentrations found in the revised similar literature (Reiner and Kannan 2006, Roosens, Covaci et al. 2007, Zhang, Yao et al. 2008, Martínez-Girón, Crego et al. 2010, Lu, Yuan et al. 2011a, Homem, Silva et al. 2013, Llompart, Celeiro et al. 2013, Nakata, Hinosaka et al. 2015, Homem, Silva et al. 2015a). For the authors best knowledge, there are only two studies referring the presence of HHCB and other musks in children's PCPs, both of them from different

authors of the same Portuguese investigation team as the current study (Homem, Silva et al. 2013, Homem, Silva et al. 2015a). In 2012, the Scientific Committee on Consumer Safety has already noticed that there wasn't enough and comprehensive exposure data for infants available in the open literature (SCCS 2012). So, the recently published report (Correia, Cruz et al. 2015) is an important contribution, for being the first analysis of HHCB that includes a vast number and type of children's PCPs, namely some types never approached before like hair conditioner, sunscreen or baby wipes. The HHCB concentrations found on the 79 selected PCPs used by children (0-5 years old) of Oporto, ranged between 0.001 ± 0.001 $mg \cdot kg^{-1}_{sample}$, on a baby wipe, and 300.480 ± 8.819 $mg \cdot kg^{-1}_{sample}$, on a glycerin soap. Besides this maximum value for children's PCPs be higher than the maximum value found on the analyzed adult´s PCPs, it should be noticed that this concentration respect to a glycerin soap, a non-specific children's PCP. A comparison between similar PCPs analyzed for each age group was made (Figure 5.2), although there are a vast number of PCPs' types that were only analyzed for children and, therefore, are not included here.

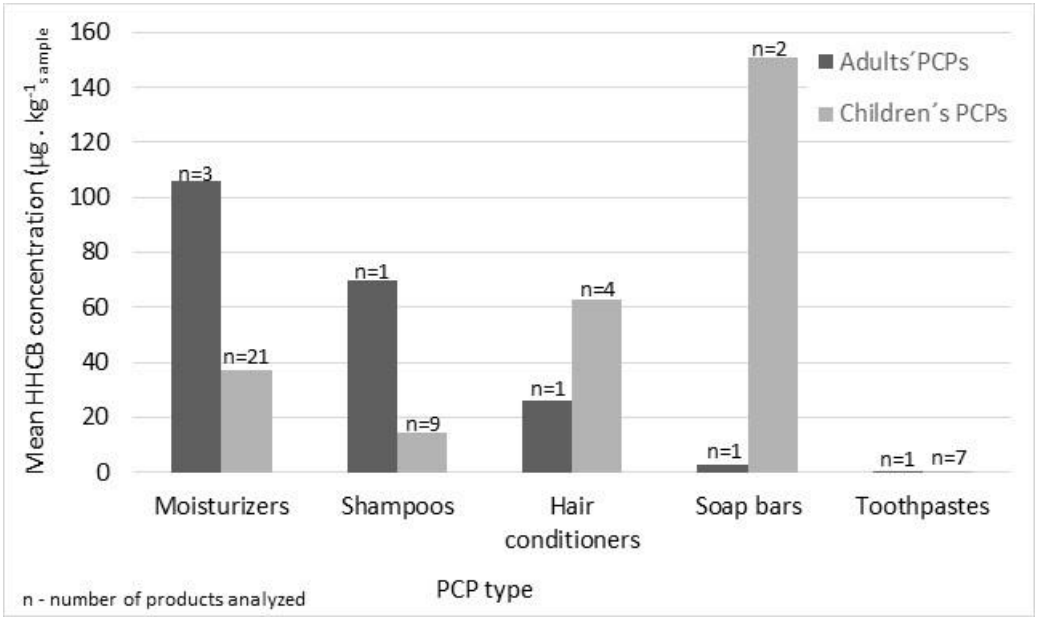

Figure 5.2 - Mean HHCB concentrations of similar analyzed PCPs for adults and children.

Although a scarce number of products were included in adults' analysis, as expected, almost all PCPs types presented higher mean HHCB concentrations for children´s PCPs, except for hair conditioners and soap bars. But the analyzed soap bars are not specific for children and one of the hair conditioner used for this age group has a very high level of HHCB (251.80 $mg \cdot kg^{-1}_{sample}$). Another study where adult's and baby's PCPs where analyzed, reported higher HHCB

concentration in an adult's body lotion, but baby's bath gel presented a contrary trend while adult's and baby's shampoos had similar HHCB concentrations (Homem, Silva et al. 2013).

But, the PCPs used in infants, especially in babies, are products where the fragrances should be present in minimum quantities, due to possible allergic reactions. In fact, nowadays, "fragrance-free" indication is a commercial advantage that should be well monitored, but, as previously reported by this team (Correia, Cruz et al. 2015), 79% of the analyzed PCPs that were labeled as "fragrance-free", have actually considerable quantities of HHCB that could reach 1.234 ± 0.025 $mg \cdot kg^{-1}$. So, fast and reliable analytical methods with low LOD, such as the one that was previously developed (Correia, Cruz et al. 2013, Correia, Cruz et al. 2015), present an extreme importance. The possible health dangers of fragrances has alerted the general community and pressed some brands to withdraw these compounds of their PCPs. But the majority of the analyzed brands are still applying in their children's PCPs formulations, HHCB, the most word wide used musk fragrance that may be seen as an indicator on fragrances use (Table 5.2). A brand of PCPs analyzed in this study (M7 in Table 5.2), have announced in May 2013 the beginning of the withdrawal of all nitro musks and polycyclic musks of their PCPs (EWG 2015). Up to the time of purchase of the products included in the study (between March and July 2013), that brand still have 13 of the 14 analyzed PCPs with detectable, although low, levels of HHCB (Table 5.2). But the highest mean levels of HHCB were found in brands M3 (316.325 $mg.kg^{-1}_{sample}$), M4 (67.305 $mg.kg^{-1}_{sample}$), M16 (300.480 $mg.kg^{-1}_{sample}$) and M20 (251.796 $mg.kg^{-1}_{sample}$). From those, the most concerning one is M4 because, from a broad of nine analyzed products, only one have no detectable levels of HHCB, and one of the PCPs containing HHCB has been labeled as "fragrance-free". The types of PCPs that presented lower levels of HHCB was toothpaste and diaper change products. In fact, it makes sense that toothpastes, being a PCP with high risk of ingestion, have less fragrances than other products, especially when respecting to children. As for diaper change products (wipes, creams, pastes and ointments), a specific PCP type of this this age group, HHCB absence is very important because it respects to a very sensitive skin, the genital area. This specific skin area changes during childhood due to the use of diapers that causes loss of capacity to maintain pH and the integrity of the skin barrier. This problem becomes more significant in the age group of 6-12 months in which, typically, occur the nappy dermatitis, an inflammation that increases susceptibility to infection and the change of absorption of chemical compounds (SCCS 2012).

Table 5.2 - Selected children's Personal Care Products (PCPs): analysed brands, PCP type and presentation, galaxolide (HHCB) mean concentrations (PCPs containing HHCB) and PCPs with no HHCB.

PCP Brand	PCP type	PCPs with HHCB	HHCB mean concentration ($mg.kg^{-1}_{sample}$)	PCPs with no detectable HHCB (LOD 0.001 $mg.kg^{-1}_{sample}$)
M1	Moisturizers	1 lotion	0.098 ± 0.007	-
	Body wash products	2 gels	0.039 ± 0.006	-
	Shampoos	2 gels	0.416 ± 0.013	-
	Sunscreens	1 lotion	**134.715 ± 0.025**	-
M2	Moisturizers	5 creams	0.120 ± 0.009	1 lotion
	Body wash products	1 gel + 1 solution	0.260 ± 0.010	-
	Diaper change	1 cream	0.016 ± 0.011	1 cream
	Sunscreens	1 lotion + 1 paste	0.198 ± 0.009	-
M3	Moisturizers	1 lotion	**414.855 ± 0.016**	-
	Body wash products	1 gel	**217.795 ± 0.012**	-
M4	Moisturizers	2 creams + 1 lotion	**96.597 ± 0.024**	-
	Body wash products	2 gels	**39.891 ± 0.013**	-
	Shampoos	1 gel	**127.517 ± 0.030**	-
	Diaper change	1 cream	**71.513 ± 0.010**	1 baby wipe
	Sunscreens	1 paste	1.005 ± 0.023	
M5	Moisturizers	2 creams + 2 lotions	0.024 ± 0.010	-
	Body wash products	2 gels	0.019 ± 0.010	-
	Shampoos	1 gel	0.193 ± 0.010	-
	Diaper change	1 cream	0.207 ± 0.008	-
M6	Moisturizers	1 cream	0.362 ± 0.012	-
	Diaper change	1 paste	0.016 ± 0.011	-
M7	Moisturizers	3 lotions	0.184 ± 0.009	-
	Body wash products	3 gels	0.120 ± 0.008	-
	Shampoos	4 gels	0.162 ± 0.008	-
	Hair conditioners	2 creams + 1 solution	0.020 ± 0.008	-
	Diaper change	-	< LOD	1 baby wipe
M8	Moisturizers	-	< LOD	1 cream
	Diaper change	1 baby wipe	0.001 ± 0.001	-
M9	Moisturizers	1 cream	0.013 ± 0.011	-
	Body wash products	1 gel	0.035 ± 0.007	-
M10	Toothpastes	1 gel	0.056 ± 0.001	1 gel
M11	Toothpastes	1 paste	0.009 ± 0.008	-
M12	Toothpastes	-	< LOD	1 gel
M13	Toothpastes	1 gel	0.006 ± 0.006	–
M14	Toothpastes	1 paste	0.014 ± 0.010	–
M15	Toothpastes	-	< LOD	1 gel
M16	Body wash products	1 soap bar	**300.480 ± 0.017**	–
M17	Body wash products	1 gel	1.385 ± 0.033	–
	Diaper change	1 baby wipe	2.675 ± 0.009	-
M18	Body wash products	1 soap bar	0.712 ± 0.012	–
M19	Shampoos	1 gel	0.038 ± 0.007	–
M20	Hair conditioners	1 solution	**251.796 ± 0.031**	–
M21	Diaper change	1 baby wipe	0.022 ± 0.011	–
M22	Diaper change	1 baby wipe	0.154 ± 0.010	–
M23	Diaper change	1 cream	0.012 ± 0.011	–
	Diaper change	-	< LOD	1 ointment
M24	Diaper change	1 ointment	1.234 ± 0.025	–
M25	Sunscreens	1 lotion	0.074 ± 0.006	–
M26	Sunscreens	1 lotion	12.312 ± 0.010	–
M27	Sunscreens	1 lotion	0.229 ± 0.010	–
	Sunscreens	1 paste	0.394 ± 0.011	–

5.3.3 Dermal exposure risk

The first dermal exposure risk to HHCB, by the use of PCPs in Portugal, was already published (Correia, Cruz et al. 2013) and, in order to perform that, consumer patterns of PCPs and HHCB concentrations in those PCPs were essential. The adults' consumer patterns, adapted from data of other European countries (Hall, Tozer et al. 2007), was used along with the referred HHCB concentrations and previously reported retention factors of HHCB on skin surface (Cadby, Troy et al. 2002), and a dermal exposure risk of 904 $\mu g.day^{-1}$ was obtained (Correia, Cruz et al. 2013). But, as referred before, the exposure risk may be influenced by the consumer pattern, which varies with the population under study (SCCS 2012). In fact, there is a lot of variation between mean daily application amounts found in the literature (Table 5.3), especially between European and American studies, except for toothpastes. Although small differences are noticed, the current study generally reported mean application amounts lower than the other European studies, except for soap bars. So, there was a need to recalculate the exposure risk using real consumer patterns of the Northern Region Portuguese population latter obtained, with the same PCPs' HHCB concentrations as used before. Using an estimation procedure already reported (Homem, Silva et al. 2015a), a new dermal exposure risk of 692 $\mu g.day^{-1}$ was obtained and compared with other estimations resumed in Table 5.3. Apart from the current team estimations for dermal exposure risk, including the current study and the former published one (Correia, Cruz et al. 2013), several consumer patterns' scenario where considered: European studies (Cadby, Troy et al. 2002, SCCNFP 2002, HERA 2003, Colipa 2005, Bremmer 2006, Hall, Tozer et al. 2007, Nohynek, Antignac et al. 2010, Hall, Steiling et al. 2011, SCCS 2012, Biesterbos, Dudzina et al. 2013) and American studies (CTFA 2004, CTFA 2005, Loretz, Api et al. 2005, Loretz, Api et al. 2006, Loretz, Api et al. 2008).

Table 5.3 - Estimations of daily dermal exposure risk to galaxolide (HHCB) using the reported HHCB concentrations and retention factors (Correia, Cruz et al. 2013) and several consumer patterns.

Personal care products (PCPs)	**HHCB concentration** ($mg.kg^{-1}_{sample}$)	**Portugal** (Current study)		**Portugal** (Correia, Cruz et al. 2013)[a]		**Europe**[b]		**USA**[c]	
		Mean daily application ($g_{sample}.day^{-1}$)	**HHCB retention** ($\mu g.day^{-1}$)	**Mean daily application** ($g_{sample}.day^{-1}$)	**HHCB retention** ($\mu g.day^{-1}$)	**Mean daily application** ($g_{sample}.day^{-1}$)	**HHCB retention** ($\mu g.day^{-1}$)	**Mean daily application** ($g_{sample}.day^{-1}$)	**HHCB retention** ($\mu g.day^{-1}$)
Moisturizers	105.72	6.2	655.464	8.0	845.760	8.9	940.908	14.4	1522.368
Shampoos	69.88	4.8	33.542	8.0	55.904	8.5	59.398	18.2	127.182
Conditioners	26.01	3.0	0.780	4.0	1.040	6.0	1.5606	21.0	5.462
Soap bars	2.48	9.6	2.381	5.0	1.240	7.8	1.9344	20.0	4.960
Toothpastes	0.03	1.5	0.005	2.0	0.006	2.5	0.008	2.5	0.008
Estimated daily dermal exposure to HHCB ($ug.day^{-1}$)		**-**	**692.2**	**-**	**904.0**	**-**	**1003.8**	**-**	**1660.0**
Differences to the current study		-	-	-	31%	-	45%	-	140%

a Mean daily application amounts obtained for the European population (Hall, Tozer et al. 2007)
b Mean daily application amounts found in all published European studies (Cadby, Troy et al. 2002, SCCNFP 2002, HERA 2003, Colipa 2005, Bremmer 2006, Hall, Tozer et al. 2007, Nohynek, Antignac et al. 2010, Hall, Steiling et al. 2011, SCCS 2012, Biesterbos, Dudzina et al. 2013)
c Mean daily application amounts found in American studies (CTFA 2004, CTFA 2005, Loretz, Api et al. 2005, Loretz, Api et al. 2006, Loretz, Api et al. 2008)

As expected, there are some differences between the estimations of daily dermal exposure to HHCB (Table 5.3), although the major difference is noticed between the Portuguese population and the American one (140%). Cultural and regional differences may be the cause of those disparities, and this is consistent with the fact that estimations using European consumer habits are closer to the Portuguese one, with maximum differences of 45%. It seems like, using the consumer patterns of cultural similar countries will not affect in a very large extent the exposure risk estimations.

The dermal exposure risk to HHCB here obtained (692 $\mu g.day^{-1}$) is, as expected, higher than the previously obtained for children (277 $\mu g.day^{-1}$) (Correia, Cruz et al. 2015), given the referred differences on consumer patterns and HHCB concentrations in PCPs used for each age group. For the authors' best knowledge, there are no reports of exposure risk to HHCB in children. The major contributor for this exposure was from body lotions in both age groups, excluding the sunscreen products since they were not considered on adult's estimation.

The previously reported exposure risks come from the direct application of the applied PCPs. But as referred before, some HHCB applied on the skin will evaporate and Ford et. al (1999) estimated that only 78% of the HHCB is absorbed. So, taking into account this evaporation rate, and disregarding the amount of HHCB withdrawal by clothing, the acute exposure to HHCB will be 540 $\mu g.day^{-1}$ (adult population) and 216 $\mu g.day^{-1}$ (children). Only this exposure to HHCB may

be available for systemic absorption and, as referred before, Ford et al. (1999) estimates that 0.1% of HHCB is absorbed, which would result, in the recent study, in 0.540 μg.day^{-1} and 0.216 μg.day^{-1} to adult people and to children, respectively. But Wescheler and Nazaroff (2012) considers the systemic exposure of compounds dependent of the absorption flow to the bloodstream. Therefore, applying the previously referred equation (1), the absorption flow of HHCB, J_{HHCB}, can be determined, using data from the same study (Weschler and Nazaroff 2012), as HHCB k_{pw} value ($3.0*10^{-4}$ m.h^{-1}), e_l for adults ($1.3*10^{-6}$ m^3.m$^{-2}_{skin}$) and children ($8.8*10^{-7}$ m^3.m$^{-2}_{skin}$). Thus, daily absorbed HHCB has been determined for both cases, taking into account the different S_p where each PCP type is applied (EPA 2011). Since bath is usually daily taken by most individuals, HHCB remains trapped in the skin for 24 hours (Bremmer 2006) during which HHCB dermal absorption occurs at a flow that able the absorption of more than 540 μg.day^{-1}, in adults, and 216 μg.day^{-1}, in children. Thus, although the first study referred an absorption of 0.1% (Ford, Hawkins et al. 1999), considering the worst case scenario, with an absorption rate of 100% inferred from the last study (Weschler and Nazaroff 2012), the maximum systemic exposure dose to HHCB is 540 μg.day^{-1} for adults and 216 μg.day^{-1} for children. These levels are higher than the ones found for powder inhalation, reported between 0.00022 and 0.115 μg.day^{-1} (Lu, Yuan et al. 2011b, Nakata, Hinosaka et al. 2015), which confirms that inhalation exposure to HHCB is less representative than the dermal route, regardless the absorption rate of HHCB by skin. Considering also the maximum levels, dermal exposure is more relevant than the ingestion of fish in adults, 124 μg.day^{-1} (Roosens, Covaci et al. 2007), and the ingestion of breast milk in children, 2.526 and 11.829 μg.day^{-1} (Zhang, Liang et al. 2011, Yin, Wang et al. 2012).

5.3.4 Systemic exposure doses to HHCB

The present obtained values respect to the effective dermal exposure risk of HHCB, after application of PCPs, evaporation and absorption of HHCB. But, as referred before, the safety of a cosmetic substance is evaluated by the SED value, calculated bellow for adults and children.

Adult's systemic exposure dose

Using equation (3) and considering a Portuguese adult average body weight (bw) of 57.5 kg (ECETOC 2001), 22% HHCB evaporation and HHCB total absorption, the maximum HHCB SED, would be 9.4 $\mu g.kg_{bw}^{-1}$. Currently there are no official data about HHCB maximum acceptable systemic levels in humans, but Slanina (2004) estimated a Provisional Tolerable Daily Intake (PTDI) for HHCB of 500 $\mu g.kg_{bw}^{-1}$. Therefore, the obtained value is well below the proposed PTDI, although this value is for ingestion of HHCB. Nakata Hinosaka et al. (2015) estimated a HHCB daily SED of 7.9 $\mu g.kg_{bw}^{-1}$ while Homem , Silva et al. (2015a) reported an average daily SED to HHCB of 22.7 $\mu g.kg_{bw}^{-1}$ for adults. The first SED value is from the same order of magnitude as the present study values, but the smallest level may be due, not only to differences in consumer patterns of Japanese population, but also to different regulations about musks' concentrations on PCPs used in this country. Homem, Silva et al. (2015b) analyzed 110 PCPs used by the Portuguese population but the maximum HHCB concentration (31124 $mg.kg^{-1}_{sample}$) was much higher than the reported in the present study, although lowest values were used for the absorption rate (10%), amount and frequency of application.

Children's systemic exposure dose

Due to the constant variation of weight during childhood, it is advisable to use average weights for each age and to estimate exposure risk variation in children (ECETOC 2001). Thus, taking into account the considered ages in the present study (less than 5 years) and the various exposure dermal risks described previously (Correia, Cruz et al. 2015), after 22% HHCB evaporation and skin total absorption, the effective exposure dermal risks conduct to the following HHCB daily SED for children (Table 5.4) calculated using equation (3). All values of SED for children (7.4 to 36.6 $\mu g.kg_{bw}^{-1}$), and the value found for adults (9.4 $\mu g.kg_{bw}^{-1}$), are quite below the proposed PTDI by Slanina (2004). However, it is important to note that this value is proposed for adults and not for children, where the susceptibility can be higher and a lower dose may be enough to cause harmful effects. It should also be noted that the value obtained for the adult population is very similar to the obtained value for the older children in the average estimation scenario (12.1 $\mu g.kg_{bw}^{-1}$).

Table 5.4 - HHCB systemic daily exposure dose for children.

Effective exposure dermal risk (µg.day^{-1})	HHCB systemic daily exposure dose (µg.kg$_{bw}^{-1}$)					
	< 6 months (5.9 kg) [a]	6-11 months (9.2 kg) [a]	12-23 months (11.4 kg) [a]	25-35 months (13.9 kg) [a,b,c]	36-47 months (16.2 kg) [b]	48-59 months (17.8 kg) [b,d]
103.3 (< 1 year old)	17.5	11.2	-	-	-	-
232.4 (1 to 4 years old)	-	-	20.4	16.7	14.3	-
132.0 (> 4 years old)	-	-	-	-	-	7.4
216.1 (average age estimation)	36.6	23.5	19.0	15.5	13.3	12.1

[a] Data obtained in the Child Specific Exposure Factors Handbook (EPA 2008)
[b] Data obtained by ECETOC (2001)
[c] Data reported by (Nohynek, Antignac et al. 2010); [d] Data reported by (Masuck, Hutzler et al. 2011)

5.3.5 Galaxolide dermal risk assessment

To conclude the risk assessment, the risk characterization of HHCB must be performed, calculating the margin of safety, Mos, by equation (2). It is also possible to calculate MoS substituting the NO(A)EL for the Lowest Observed (Adverse) Effect Level (LO(A)EL), but an additional factor of 3 is added to the calculation (SCCS 2012). The minimum reported NO(A)EL and LO(A)EL values for HHCB were 50 mg.kg$_{bw}^{-1}$ (SCCNPF 2002) and 10 mg.kg$_{bw}^{-1}$ (Chen, Xue et al. 2011). The most secure scenario should be achieved using the minimum NO(A)EL value and the maximum SED.

Adult's risk assessment

The SED values for HHCB obtained on the present study were 9.4 µg.kg$_{bw}^{-1}$. The minimum previously reported NO(A)EL value was 50000 µg kg$_{bw}^{-1}$, resulting in a minimum MoS value of 5319 or 3191. Only a MoS value from the dermal exposure to PCPs were found in the open literature, of 58523 (SCCNFP 2002). The obtained MoS in this study are lower than the reported one, indicating that the European population, in 2002, could be using HHCB in PCPs more safely than nowadays. But using the reported exposure risk values, a similar estimation of the MoS could be done. The exposure risk varied between 17 and 25100 µg.day^{-1} (Ford 1998, Roosens, Covaci et al. 2007, Lu, Yuan et al. 2011a, Nakata, Hinosaka et al. 2015, Homem, Silva et al. 2015a). So, assuming 22% of evaporation and total absorption, the MoS varies between 115 and 217391, and the lowest value could indicate a non-safe use of HHCB. An higher MoS was obtained for dermal exposure, but it respects to household products, 350000 (HERA 2004a).

Nevertheless, the minimum estimated MoS in this study, of 3191, is higher than 100 and, for that, HHCB should be considered safe for normal use in adults' Portuguese population. But accidental or exaggerated use of PCPs could result in loss of safety.

Children's risk assessment

The SED maximum value obtained for children was 36.6 $\mu g.kg_{bw}^{-1}$, which results in a minimum MoS of 1366 and 820 for children using the NO(A)EL or the LO(A)EL values, respectively. The estimated MoS are higher than 100 but due to the specific characteristics of children's skin, specially babies, it should be analyzed if the minimum MoS value of 100 is sufficient for children's risk assessment. In fact, this minimum MoS includes a factor of 10 for the species differences, because NO(A)EL is not determined in Humans, and another factor of 10 that respects to the individuals' variability (SCCS 2012). Former reports state that, if the skin is intact, dermal absorption in babies is similar to what is observed in adults and the major concern in safety evaluation between adults and children is the surface area/body weight ratio. But it has been reported that this parameter is already included in the individuals' variability factor (Nohynek, Antignac et al. 2010). Only very young babies may be considered to have potential risk factors, especially on toxicokinetic parameters and some special skin regions, like the nappy area. The differences in metabolization between children and adults, can result in reduced clearance or longer half-life in the organism: the lower activities of some hepatic enzymes seems to lead to less bioactivation of chemicals in children, decreasing the potential risk; on the other hand, skin metabolization is weaker in children and substances may be absorbed in higher doses. This two toxicokinetic parameters compensate one another, and there is also no need for an extra safety factor. Regarding to differences in skin body locations, nappy area needs a special attention. Although at birth this skin region is equal to one another, after some days of confining clothes and nappies, associated with urine and feces, several changes occur. The occlusion of the skin results in increasing hydration of the *SC*, pH, temperature, microbial presence and dermal irritation. Particularly, the increase in hydration has been associated with increased dermal penetration, although the relative effect of occlusion is dependent on the lipophilicity of the chemical. Due to this changes, skin barrier is compromised, and this area is subjected to inflammation and infection. Between 6 and 12 months of age, the typical nappy dermatitis is one of the most common deregulations of this skin region. Technology have improved nappies and this skin problem seem to be lightened, but it cannot be completely

avoided, which might enhance dermal absorption of substances in this region. So, in general cases there is no need of an additional safety factor when estimating MoS for children (SCCS 2012). But if skin is altered, like in the nappy zone, or even is not intact, like in atopic skin, it should be given special attention. Previously analyzed nappy area products (Correia, Cruz et al. 2015), include six baby wipes and eight diaper change products (creams, ointments and pastes). Despite two baby wipes, one cream and one ointment with no detectable HHCB, concentrations varied between 0.001 and 2.675 $mg.kg^{-1}_{sample}$ in baby wipes and 0.012 and 71.513 $mg.kg^{-1}_{sample}$ in diaper change products. Respecting to atopic skin, six specific moisturizers were analyzed and HHCB concentrations varied between 0.018 and 0.362 $mg.kg^{-1}_{sample}$. Assuming a total absorption of HHCB and that children with less than 1 year old change nappy more frequently than older ones and are more susceptible to HHCB dermal absorption, systemic exposure dose can be estimated. Using the mean estimation for the HHCB daily dermal exposure by children under 1 year old, the SED will be of 4.1 $mg.kg_{bw}^{-1}$ for nappy area products and 0.2 $mg.kg^{-1}_{bw}$ for atopic skin moisturizers. With these results, and using the previously referred NO(A)EL values, the minimum MoS will be of 2500 for nappy area products and 52631 for atopic skin moisturizers. Even when considering an extra safety factor for these cases, were skin barrier is compromised and dermal absorption can be greater, those high MoS values can guarantee a safe use of these PCPs regarding to HHCB exposure in children's population.

5.4 Conclusions

The consumer patterns and the range of HHCB concentrations found in PCPs used by the two analyzed groups of the Northern Region Portuguese population present some differences. The mean application amounts of PCP is higher in each adults' utilization, except for facial cream where children and adults use similar quantities (0.5 $g_{sample}.event^{-1}$). The daily frequencies are also similar for almost all kind of PCPs except for bath products (body washer, shampoo, hair conditioner), with higher usage in adults (4-7 $event.week^{-1}$) than children (2-3 $event.week^{-1}$). Nevertheless, the average weights of each population groups differ a lot, conducting to major differences in systemic exposure doses: 9.4 $\mu g.kg_{bw}^{-1}$ in adults and 36.6 $\mu g.kg_{bw}^{-1}$ for children. The risk assessment for HHCB dermal exposure was analyzed separately and the minimum margins of safety obtained (820 for children and 3191 for adults), indicate that HHCB use in PCPs is safe in both analyzed groups. But other scenarios, like accidental or exaggerated use of PCPs, or even the utilization of highly concentrated PCPs in much damaged skin, could result in loss of safety.

6 Risk of environmental exposure to galaxolide through residual waters and sludge[6]

Abstract

Musks is one of the most important and often used group of fragrances in perfumery. The growing demand for musks has created a worldwide synthetic musk industry, and today they are intensively applied in several consumer products, like Personal Care Products and household detergents, that are constantly rinsed off and enter the sewerage system. Wastewater treatment plants may not totally remove these substances, contaminating the receiving waters and the aquatic environment. Additionally, due to its low water soluble and lipophilic characteristics, musks may adsorb to the resulting sludge and contaminate soils when used as fertilizers, and even reach the food chain. This situation poses an environmental and human health risk, especially in over populated locations, where the implementation of new technologies and control measures is required. Galaxolide is the most world used musk, being considered an emerging pollutant that needs to be monitored in the environment. In this work a dispersive solid-phase extraction followed by High Performance Liquid Chromatography with fluorescence detection has been optimized to determine the levels of galaxolide in influent, effluent and sludge of wastewater treatment plants. The limits of detection and quantification were, respectively, 0.4 $\mu g.L^{-1}_{water}$ or 0.004 $mg.kg^{-1}_{dry\ sludge}$, and 1.3 $\mu g.L^{-1}_{water}$ or 0.013 $mg.kg^{-1}_{dry\ sludge}$. The recovery was 62.3% and 74.2% for a spike of 9.1 $\mu g.L^{-1}$, respectively, in an influent sample (4.0 ± 0.9 $\mu g.L^{-1}$) and in an effluent sample (1.3 ± 0.5 $\mu g.L^{-1}$), and 92.6% for a spike of 9.978 $mg.kg^{-1}_{dry\ sludge}$ in a sludge sample (19.210 ± 1.783 $mg.kg^{-1}_{dry\ sludge}$). The analytical method was validated with low uncertainty for sludge (<10% for concentrations above 0.100 $mg{\cdot}kg^{-1}_{dry\ sludge}$) and wastewater (<15% for concentrations above 12.1 $\mu g{\cdot}L^{-1}_{water}$). Samples collected along three seasons on an urban wastewater treatment plant in Portugal, presented a mean reduction rate on final effluent of 49-65%, while in sludge samples galaxolide was always detected. The mean daily *per capita* emission was estimated in 1506 $\mu g.day^{-1}$ and there was a significant seasonal variation between wet/dry seasons and between day/night collections.

Keywords: Galaxolide; QuEChERS; HPLC-fluorescence; environmental exposure risk; wastewater; sludge

[6] "Risk of environmental exposure to galaxolide through residual waters and sludge"; P Correia, M Mendes, V Homem, A Cruz, L Santos, A Alves; submitted, 2016

6.1 Introduction

Synthetic fragrances are present in various daily used products, such as perfumes, toothpastes, shampoos, body lotions, soaps or even household cleaning products (Roosens, Covaci et al. 2007). They include musks, whose main function is to fix fragrances in these materials. Due to their intensive daily use, musks are continuously introduced in the sewerage system and most of the traditional wastewater treatment plants (WWTP) are not able to effectively remove this type of compounds. The main purposes of a WWTP are the separation of the existing solids in the water to be treated and the reduction of the organic load. The treatment process may vary, but usually it includes a preliminary treatment (influent solids separation), primary treatment and secondary/biological treatment and, eventually, tertiary treatment. During this, the collected sludge is directed to the thickening step, anaerobic stabilization and dehydration (Sperling 2007). Musks are poorly soluble in water and have a lipophilic character (Ritter, Safety et al. 1995), and therefore it has been estimated that about 50 to 60% of dry solids of sludge is organic matter (LeBlanc, Matthews et al. 2006). So, it is expected that their removal way in WWTPs should be sludge adsorption, although chemical degradation may also occur during some tertiary treatments (Yang and Metcalfe 2006)(Ren, Wei et al. 2013). In fact, some studies (Yang and Metcalfe 2006, Salgado, Marques et al. 2011b) demonstrate that the level of adsorption by the sludge is high, and removal by biodegradation of musks is low. Therefore, effluents from WWTP, that enter the water natural courses, but, specially, the resulting WWTPs sludge, eventually used as soils fertilizers, are the primary vehicle for environmental contamination by musks and entrance in the food chain (Wilson 1996, Zeng, Sheng et al. 2005). Additionally, musks are bioaccumulative, persistent and only partially biodegradable (Ritter, Safety et al. 1995). Therefore the negative impact caused by musks on the environment and human health has been of concern among the scientific community and, there have been more and more studies in this area. Most studies have confirmed that the continued use of these compounds is directly reflected in aquatic environmental matrices like surface waters (Zeng, Sheng et al. 2005, Lv, Yuan et al. 2009, Posada-Ureta, Olivares et al. 2012). A recent revision work by (Homem, Silva et al. 2015b) reported that musks are present in WWTPs influent, effluent and sludge, with galaxolide (HHCB) being the most prevalent one. The reported HHCB concentrations on influents of urban WWTPs with domestic and industrial sewage, ranged between 0.00101 and 2.325 $\mu g.L^{-1}_{water}$. This variability may be explained, not only by differences on consumer habits, but also by sewage composition (domestic and/or industrial). On the other

hand, concentrations of galaxolide in the effluents and sludge are mostly influenced, not only by influent concentrations, but also by wastewater treatment type (secondary/tertiary) and sludge treatment. In fact, the removal of those compounds depends on the population type (urban/rural), industry types (fragrances related or not), origin of the sewage (domestic/industrial), size and type of WWTP.

This study was performed with wastewater and sludge samples from Parada WWTP that covers 75% of Maia municipality sewerage system and treats an average daily volume of 18000 m^3. The sewage composition of that urban WWTP, is from domestic (70%) to industrial (30%) origin, and the process includes a preliminary treatment (screening and grit removal), a primary treatment (decantation) and a biological treatment (activated sludge). The resulting sludge from all WWTP is treated by a thickening process, followed by anaerobic digestion (30 °C), secondary digestion and mechanical dehydration processes (Figure 6.1), with a total residence time of about 20 days (CMM 2005).

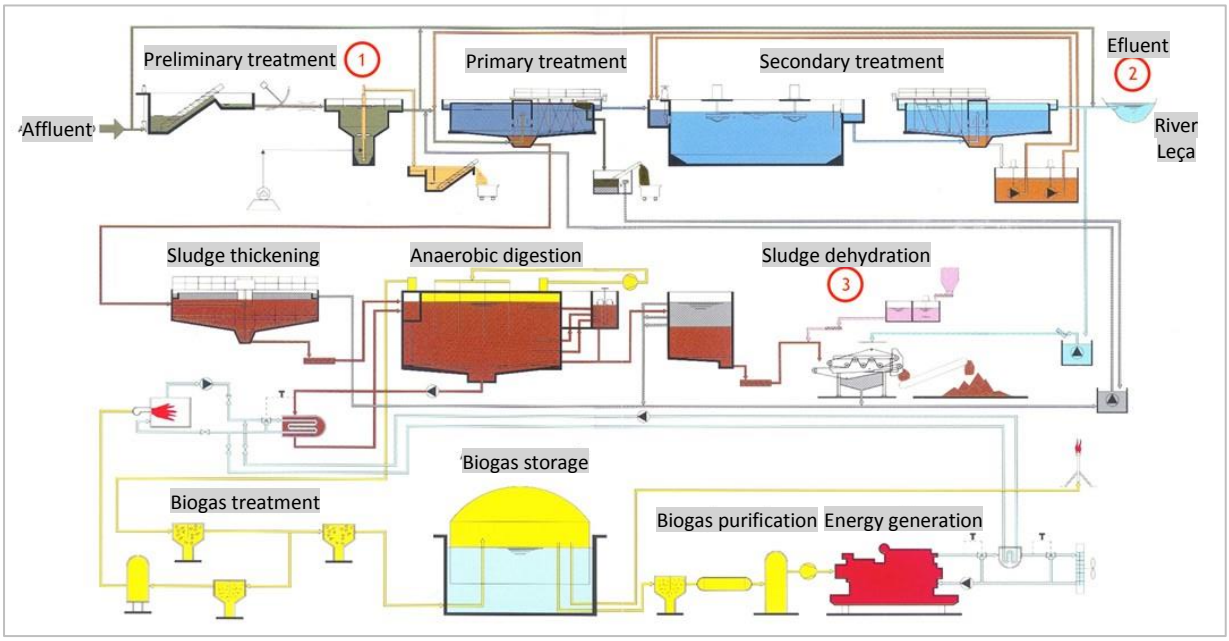

Figure 6.1 – Parada WWTP flowsheet (CMM 2002) and collection points: affluent (1), efluent (2) and sludge (3).

Data about HHCB detection in WWTPs (influents, effluents and sludge) with similar characteristics were collected (Table 6.1), in order to compare the present results with similar ones found in the literature. Despite several studies referring that musks are mainly removed from wastewater by sorption to sludge, there are few about HHCB presence in WWTPs sludge,

when compared to the detection in liquid WWTPs samples (Homem, Silva et al. 2015b). In Portugal, there are some studies about HHCB detection in urban WWTPs influents (domestic) and effluents, and the concentrations ranged, respectively, between 0.05 to 4.67 $\mu g.L^{-1}_{water}$ on influents and 1.270 $\mu g.L^{-1}_{water}$ in effluents (Silva and Nogueira 2010, Salgado, Marques et al. 2011, Salgado, Marques et al. 2011b). Nevertheless, this studies were not included in Table 6.1 because the analyzed sewage do not include a mixture of domestic and industrial sewage.

Table 6.1 - Galaxolide (HHCB) concentration in influent, effluent and sludge from WWTPs with a mix sewage (domestic and industrial), with no tertiary treatment and anaerobic sludge digestion.

Country	**Reference**	**Influent HHCB concentration** ($\mu g.L^{-1}_{water}$)	**Effluent HHCB concentration** ($\mu g.L^{-1}_{water}$)	**Dewatered sludge HHCB concentration** ($mg.kg^{-1}_{dry\ sludge}$)
China	(Zeng, Sheng et al. 2005)	-	-	5.656 - 21.214
	(Lv, Yuan et al. 2009)	1.2904	0.2126	-
	(Lv, Yuan et al. 2010)	1.4783 - 2.2143	0.1811 - 0.2422	3.2813 - 3.5602
	(Zeng, Cao et al. 2012)	-	-	1.0378 - 3.2991
	(Sang, Zhang et al. 2012)	0.3564 - 1.1066	0.3508 - 1.3148	0.2682 - 1.2692
	(Ren, Wei et al. 2013)	0.125 - 0.200	0.040 - 0.100	3.2813 - 3.5602
	(He, Chen et al. 2013)	0.306	0.186	-
	(Liu, Shi et al. 2014)	-	-	< 0.0022 - 4.1400
Spain	(Gómez, Gómez-Ramos et al. 2009)	-	1.259 - 8.697	-
	(Rosal, Rodríguez et al. 2010)	< 0.056 - 24.971	< 0.056 - 2.766	-
	(Vallecillos, Pedrouzo et al. 2014)	0.818 - 45.091	0.0015 – 0.900	-
	(Vallecillos, Borrull et al. 2015)	0.020 – 1.160	0.010 – 0.550	-
Germany	(Bester 2004)	1.409 - 2.325	0.652 - 0.795	2.709 - 3.342
	(Müller and Böhmer 2006)	1.44 - 1.79	-	6.020 - 23.000
Canada	(Yang and Metcalfe 2006)	0.2467 - 0.5675	0.2143 - 0.4721	5.7727 - 7.8967
	(Lishman, Smyth et al. 2006)	2.031	0.751	-
Austria	(Clara, Gans et al. 2011)	< 1.4 – 13.0	< 0.8 – 1.1	-
USA	(Reiner, Berset et al. 2007)	4.76 - 12.70	2.81 - 3.73	63.4 - 117.0

The objectives of the present study are to collect data from HHCB concentrations in three WWTP matrices (influent, effluent and dewatered digested sludge), in order to obtain the removal efficiency, and to evaluate the daily, weekly and seasonal variation. For the authors best knowledge, this is the first report about HHCB (and other musks) detection in sludge samples in Portugal, and the first one reporting the concentrations variability on sludge and wastewater.

6.2 Experimental section

6.2.1 Chemicals, solutions and standards

HHCB was purchased from SAFC (St. Louis, MO, USA) with 50% purity in diethyl phthalate (DEP). A HHCB stock solution was prepared at 42 $mg \cdot L^{-1}$ in absolute ethanol (pro-analysis grade, from Riedel-de-Haën, Honeywell Specialty Chemicals Seelze GmbH, Hanover, Germany) for calibration purposes. Using this stock solution, the highest HHCB standard (201.6 $\mu g \cdot L^{-1}$) was prepared in acetonitrile (HPLC isocratic grade, from VWR International, Radnor, PA, USA) and, subsequently, eight standards were prepared from the first one, resulting in nine standards (0.5 - 201.6 $\mu g \cdot L^{-1}$). Stock solutions of HHCB in acetonitrile were also prepared at concentrations of 4.55 and 455 $mg \cdot L^{-1}$, for the recovery studies of water and sludge samples, respectively. The highest HHCB standard was stored for quality control purposes and all solutions were also stored in glass recipients, refrigerated at 4°C and protected from light.

Mobile phase for chromatographic analysis was acetonitrile, either purchased (HPLC isocratic grade, from VWR International, Radnor, USA) or reused after recuperation (see section 6.2.6.), and deionized water acidified with glacial acetic acid. The extraction solvent (acetonitrile) and sorbents were the same as described in a previous work by this team (Correia, Cruz et al. 2013).

The extraction solvent was acetonitrile and the extraction salts and sorbents, ECMSSA50CT (6000 mg magnesium sulfate, 1500 mg sodium acetate) and ECMPSC1815C (900 mg magnesium sulfate, 300 mg PSA, 150 mg C18), were purchased from UCT (Bristol, UK).

6.2.2 Samples and sampling plan

This study was developed in collaboration with the Parada WWTP, who kindly provided the wastewater and sludge samples. Influent water samples were collected after screening and grit removal while effluent water samples were collected after the secondary treatment, right before the delivery to the receiving natural water course (river Leça). Sludge samples were collected after the mechanical dehydration process (centrifugation) of the collected sludge from the anaerobic digester (Figure 6.1) (CMM 2005). The initial sampling plan included four seasons: Autumn (November), Winter (February), Spring (May) and Summer (August). Due to the recurrent flooding of the river Leça during Winter season, affecting the WWTP facilities, it

was not possible to perform the February sampling and Autumn collection was considered to be representative of both seasons (Autumn/Winter). The final sampling plan is described in Table 6.2. Duplicate water samples were collected in plastic bottles during 5 consecutive days, from Sunday to Thursday, at three distinct times of the day: morning (9:00 am), afternoon (4:00 pm) and night (11:00 pm). Dehydrated sludge samples were collected whenever the centrifugation process was performed, because it was not a regular procedure. Spring and Summer sampling included also a sludge sample after about 20 days of the first sludge sample, in order to assess the effect of the sludge residence treatment time in the anaerobic digester. Samples were transported to the laboratory in an isothermal box and, when necessary, stored at 4 °C (without preservative) until extraction.

Table 6.2 - Sampling plan for wastewater (influent and effluent) and dehydrated sludge.

	Year	2013											2014												
	Season	Autumn / Winter											Spring							Summer					
	Month	November											May						June	August					September
Sample type	Hour\Day	17	18	19	20	21	22	25	26	27	28	29	25	26	27	28	29	30	12	24	25	26	27	28	29	18
Influent and Effluent	9:00 am	√	√	√	√	√	×	×	×	×	×	×	√	√	√	√	√	×	×	√	√	√	√	√	×	×
	4:00 pm	√	√	√	√	√	×	×	×	×	×	×	√	√	√	√	√	×	×	√	√	√	√	√	×	×
	11:00 pm	√	√	√	√	√	×	×	×	×	×	×	√	√	√	√	√	×	×	√	√	√	√	√	×	×
Sludge	9:00 am	×	×	×	×	√	√	√	√	√	√	√	×	√	×	×	√	√	√	×	×	√	√	√	√	√

√-sample collection; ×-no sample collection

6.2.3 Quality Assurance

Musks are present in many daily used products like PCPs and household commodities. So, to avoid samples contamination, the analyst tried to use as little as possible scented PCPs during the samples handling and extraction period, and a fresh pair of disposable gloves was used for each different sample. Additionally, the detergents used for cleaning tools and glassware were also fragrance-free, and all materials were rinsed with acetone and distillated water after washing. Additionally, the glass material was subjected to a high temperature (400 °C baked) in order to eliminate possible interferences.

Calibration was daily monitored injecting the highest standard (201.6 $\mu g \cdot L^{-1}$). The validation parameters include a limit of detection (LOD) and a limit of quantification (LOQ), respectively obtained considering a three and a ten times signal-to-noise ratio. Accuracy was also evaluated, using the recovery percentage for double extracts of each type of sample (WWTP influent, effluent and sludge) fortified with HHCB spikes. Those spikes were chosen based on the

common range of HHCB concentrations found on literature for this kind of samples: 9.1, 45.5 and 91.0 $\mu g \cdot L^{-1}_{water}$ for wastewater samples, and 9.978, 49.890, 99.781 and 199.561 $mg \cdot kg^{-1}_{sludge}$ for sludge samples. The global uncertainty was estimated using the bottom-up approach wherein each source of uncertainty is examined individually (Ellison, Rosslein et al. 2000).

Stability tests of extracts and standard solutions were also performed, analyzing HHCB concentration in an extract, stored in a polypropylene (PP) tube at -18 °C, and a HHCB standard solution (201.6 $\mu g \cdot L^{-1}$), stored in a glass recipient at 4 °C. HHCB concentration was monitored over time and a 10% error was established as being an acceptable range for consider HHCB concentration stable.

6.2.4 Analytical Method

Once arrived to the laboratory facilities, a part of the moist sludge sample was rapidly extracted (humid extraction) and the refrigerated wastewater samples were extracted as soon as possible, within a maximum period of 3 days and no pre-treatment. Autumn sludge samples were also dried in order to test a dry extraction procedure. The sludge drying was performed in a tray placed inside the hood, at room temperature for several days, until constant weight was achieved. Sludge pretreatment was concluded with the pulverization of dried sludge in an electric mill (Briel®, CG model, Portugal), followed by pulverization in a porcelain mortar and powder particle homogenization ($d \leq 500$ µm) with a sieve (Retsch AS 200 digit). The extraction procedure of those samples was performed as described before (Correia, Cruz et al. 2013), using a dispersive solid phase extraction (QuEChERS). At least duplicates of all samples were extracted and analyzed, after being rigorously measured (2 g of sludge samples and 5 ml of wastewater samples). Then, all samples were vigorously shaken for 3 min with 15 mL of acetonitrile and homogenized in an ultrasonic bath for 10 min. The two sequential steps of extraction and cleanup were performed adding and mixing the corresponding QuEChERS sorbents, as described elsewhere (Correia, Cruz et al. 2013). To enable total phase separation, centrifugation was performed at 3700 rpm for 10 min at the end of each of these two steps. The supernatant was collected in a 50 mL polypropylene (PP) tube and stored in a freezer (−18 °C) until analysis, as described before (Correia, Cruz et al. 2013). Afterwards, extracts were analysed by High Performance Liquid Chromatography with fluorescence detection (HPLC-FL) with 1

mL.min^{-1} mobile phase of acetonitrile:water (acidified with acetic acid 17 mM) at the ratio 80:20, respectively. The stationary phase was a Merck column LiChroCART® 250-4- LiChrospher ® 100 RP- 18 (5 mm) and the detection was performed at $\lambda_{excitation}$=280 nm and $\lambda_{emission}$=310 nm.

6.2.5 HHCB concentration corrections

Besides the quality assurance procedures, each batch of three extractions was always carried out with a blank assay, in order to verify cross contamination. Whenever the HHCB concentration found in blanks was higher than LOD, a correction was applied to the HHCB concentrations detected in wastewater or sludge samples extracted in the same batch.

Whenever sludge samples were extracted without dryness pretreatment (wet extraction) the final HHCB concentration was corrected for water content ($mg \cdot kg^{-1}_{dry\ sludge}$). In order to obtain the water content for sludge samples, a loss on drying test was performed for all Autumn samples. Therefore, wet sludge samples were weighted and then submitted to the previously described sludge drying procedure. The sludge weight was monitored along time until it stabilizes, and the weight loss corresponded to the sample water content.

6.2.6 Packaging, destination and treatment of waste

Resulting from the extraction process previously described, a solid residue was accumulated at the end of the centrifugation processes, consisting of a mixture of the adsorbed sample (water or sludge) and different sorbents, as magnesium sulfate ($MgSO_4$), sodium acetate ($NaCH_3COO$), primary and secondary amine exchange polymer material (PSA) and octadecylsilica (C18). This mixture was washed with a minimum water quantity and the resulting suspension was stored in a labeled and sealed container for subsequent routing by EcoFEUP (Environmental Management System of Faculty of Engineering of University of Porto) for treatment. The generated liquid waste from the chromatographic analysis and from the rejected extracts (after analysis), consisted generally in organic solutions containing acetonitrile, water and traces of the extracted compounds (including musks). For ecological and economic reasons, the mobile phase was recovered by fractionated distillation. The mixture forms an azeotrope which boils at 76.1 °C and which is composed of 86% acetonitrile and 14% water. To avoid the passage of

HHCB to the recovered acetonitrile and compromise its reutilization, the distillate was collected just until about a third of the initial mixture was left as a residue. Nevertheless, all obtained distillates were analyzed by HPLC- FL to ensure that they had no interferents on HHCB analysis. When some peak was detected in the proximity of HHCB retention time, the correspondent distillate was discarded and the whole distillation system was washed to restart a new recovery. The composition of the reusable distillates was expected to be of about 84% of acetonitrile, but all distillates were confirmed by refractometry (Abbe digital refractometer, Optic Ivy Men System®). The calibration was performed using the refraction indices of six aqueous solutions with 0, 20, 40, 60, 80 and 100% of acetonitrile. All distillates were then adjusted with water (acidified wit acetic acid) to the mobile phase composition (80% acetonitrile) in order to be reused.

6.3 Results and discussion

6.3.1 Method validation

Using the described analytical method, HHCB was detected at a retention time of 12.23 ± 1.40 minutes. Temperature can influence the adsorption ability of the HPLC column, because at low temperatures the stationary phase tends to retain more analytes and the retention times are longer. This analysis was performed at room temperature and, therefore, to evaluate the performance of the analytical method and to determine if the process is under control, the highest standard (201.6 µg·L^{-1}) was daily injected and a Shewhart control chart was built using the area of the HHCB peak along time (Figure 6.2). An acceptable control range for daily standards was established around the mean area ($\bar{x}$) , limited by an upper control limit, ($\bar{x} + 3s$), and a lower control limit, ($\bar{x} - 3s$). The basic criterion of rejection is that there is at least one point outside the control limits. Additional criteria may be used to increase the sensitivity of the control chart to small changes in the process, using lines $\bar{x} \pm 2s$ and $\bar{x} \pm 1s$. The resulting eight criteria, referred in normative ISO 7870-2 (International Organization for Standardization 2013), are fully acquired by the present work.

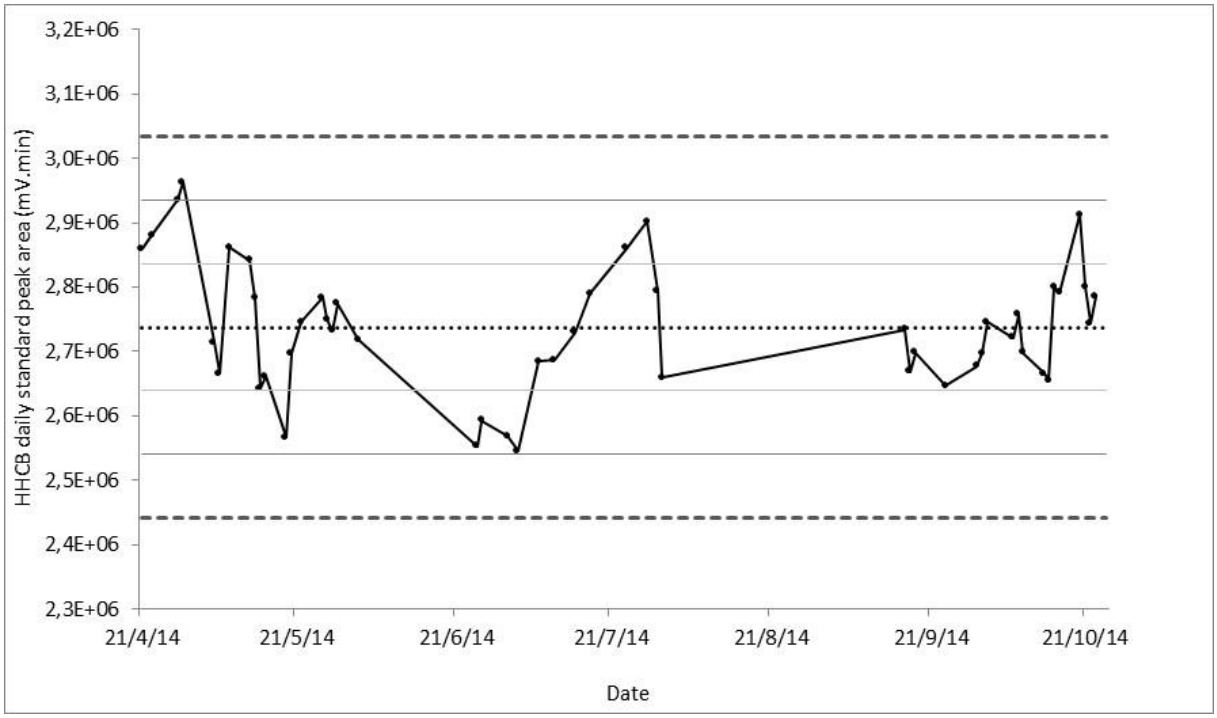

Figure 6.2 - Shewhart control chart for galaxolide daily injected standard (201.6 µg.L^{-1}).

Calibration was performed using nine standards (0.5 - 201.6 µg·L^{-1}) but, due to the HHCB concentration samples range, the final calibration curve was established with the most diluted

six standards. The calibration curve, with a linearity range of 0.5 – 80.6 $\mu g.L^{-1}$ (corresponding to 1.2 - 195.7 $\mu g.L^{-1}_{water}$ or 0.010 - 1.613 $mg.kg^{-1}_{dry\ sludge}$) was considered suitable for the analysis of HHCB, with good correlation coefficient (0.9999) and relative standard deviation of the slope (0.4%). The validation parameters of this method also included a LOD of 0.2 $\mu g.L^{-1}$ (0.4 $\mu g.L^{-1}_{water}$ or 0.004 $mg.kg^{-1}_{dry\ sludge}$) and a LOQ of 0.5 $\mu g.L^{-1}$ (1.3 $\mu g.L^{-1}_{water}$ or 0.013 $mg.kg^{-1}_{dry\ sludge}$). These limits are similar to the reported LOD for wastewater by Bester (2004) and to sludge LOD or LOQ reported by Liu, Shi et al. (2014), Clara et al. (2011) and Sang et al. (2012). Some studies refer smaller limits for wastewater (Lishman, Smyth et al. 2006, Yang and Metcalfe 2006, Lv, Yuan et al. 2009, He, Chen et al. 2013, Vallecillos, Pedrouzo et al. 2014) and sludge (Bester 2004). However, several other Gas Chromatography with Mass Spectrometry detection (GC-MS) analysis of HHCB reported higher values for wastewater (Zeng, Sheng et al. 2005, Clara, Gans et al. 2011, Zeng, Cao et al. 2012) and sludge samples (Clara, Gans et al. 2011, Sang, Zhang et al. 2012, Vallecillos, Pedrouzo et al. 2014).

Other parameters of this method were calculated based on the coefficient of variation (CV), and include a repeatability below 11.3% and a maximum intermediate precision of 23.2% for sludge samples and 19.6% for wastewater samples.

Accuracy was evaluated by the recovery percentage for each type of sample fortified with HHCB spikes (Table 6.3), using Autumn samples. Considering the HHCB concentration range of the analyzed samples, the mean recovery was found to be of 62.3% for influent samples, 74.2% for effluent samples, 92.6% for wet sludge and 66.4% for dry sludge samples (Table 6.3 bold values). The recovery values are similar to the ones found in the literature (Bester 2004, Zeng, Sheng et al. 2005, Yang and Metcalfe 2006, Lv, Yuan et al. 2009, Sang, Zhang et al. 2012, Zeng, Cao et al. 2012, Liu, Shi et al. 2014). The recovery values obtained in the wet sludge samples are better than the obtained for the dried sludge (Table 6.3). Because drying the samples would not improve the method validation, this extraction process was skipped, making it easier and quicker. Sludge samples were directly analyzed without drying.

The global uncertainty was found to be below 15% for concentrations above 12.1 $\mu g \cdot L^{-1}_{water}$ and also below 10% for concentrations above 0.100 $mg \cdot kg^{-1}_{dry\ sludge}$.

Table 6.3 - Mean recovery of HHCB in WWTP wastewater and sludge samples

SAMPLE		HHCB concentration[a, b]	HHCB spike[a]	Recovery[b] (%)
Wastewater	Influent	4.0 ± 0.9	9.1	**62.3**
			45.5	77.2
			91.0	82.5
	Effluent	1.3 ± 0.5	9.1	**74.2**
			45.5	84.7
			91.0	82.8
Dehydrated sludge	Wet sludge	19.210 ± 1.783	9.978	**92.6**
		18.614 ± 1.728	49.890	72.1
		22.303 ± 2.045	99.781	64.1
		20.203 ± 1.854	199.561	70.7
	Dry sludge	19.210 ± 1.783	9.978	**66.4**
		18.614 ± 1.728	49.890	74.7
		22.303 ± 2.045	99.781	64.9
		20.203 ± 1.854	199.561	51.8

[a] units of $\mu g \cdot L^{-1}_{water}$ for water samples and $mg \cdot kg^{-1}_{dry\ sludge}$ for sludge samples
[b] HHCB concentration without recovery correction and with a global associated uncertainty

Stability was also tested in stored extracts and standard solutions, and a total period of 32 weeks was achieved for constant HHCB concentration within a defined specification (± 10%). Therefore, the acetonitrile extracts and standard solutions were considered to be stable during the referred period and under the chosen stored conditions (extracts in PP tube at -18 °C and standard solutions in a glass recipient at 4 °C).

6.3.2 HHCB concentrations in wastewater and sludge samples

Parada WWTP samples were collected as planned in three seasons, with Autumn samples also representing Winter season, which was considered to be reasonable, because the collection dates (end of November) were proximal to the beginning of the coldest and rainy season. This assumptions were partially confirmed by the low temperatures found at the second half of November 2013 in Porto district (2.1 to 13.0 °C) (IPMA 2013), similar to the ones found in February 2014 (2.3 to 14.2 °C) (IPMA 2014). Nevertheless, precipitation has a more relevant influence on influent concentrations (due to infiltration of rainwater) than environmental temperature, and total precipitation in November 2013 (50.6 mm) (IPMA 2013) was much lower compared to February 2014 (362.2 mm) (IPMA 2014). Therefore, the concentrations found in November could not, actually, be similar to the ones that should be found in February,

but February campaign was impossible to perform due to the referred high precipitation and WWTP flood.

The wastewater and sludge samples were extracted and analyzed as described before and the obtained HHCB concentrations were corrected with the previously referred recovery percentage and, when necessary, with the cutback of the blank assay HHCB concentration. For sludge samples, an additional correction was made to the final HHCB concentration due to water content ($mg \cdot kg^{-1}_{dry\ sludge}$). A loss on drying test was performed for 13 days, and the mean sample water content was found to be 77.2 ± 2.0%. Therefore, all HHCB concentration values detected on sludge samples were corrected, assuming that all HHCB remains on the dried matrix. This assumption is based on HHCB physical-chemical proprieties (Balk and Ford 1999), like the high octanol-water partition coefficient (K_{ow} = 5.9) and its relatively high boiling point (330 °C at 760 mm Hg), which indicates a high lipid solubility and adsorption to organic matter, with low volatilization at room temperature. In fact, the main referred process of HHCB removal on WWTPs is sorption to sludge (Salgado, Marques et al. 2011b). The final HHCB concentrations found in wastewater and sludge samples are presented in Figure 6.3.

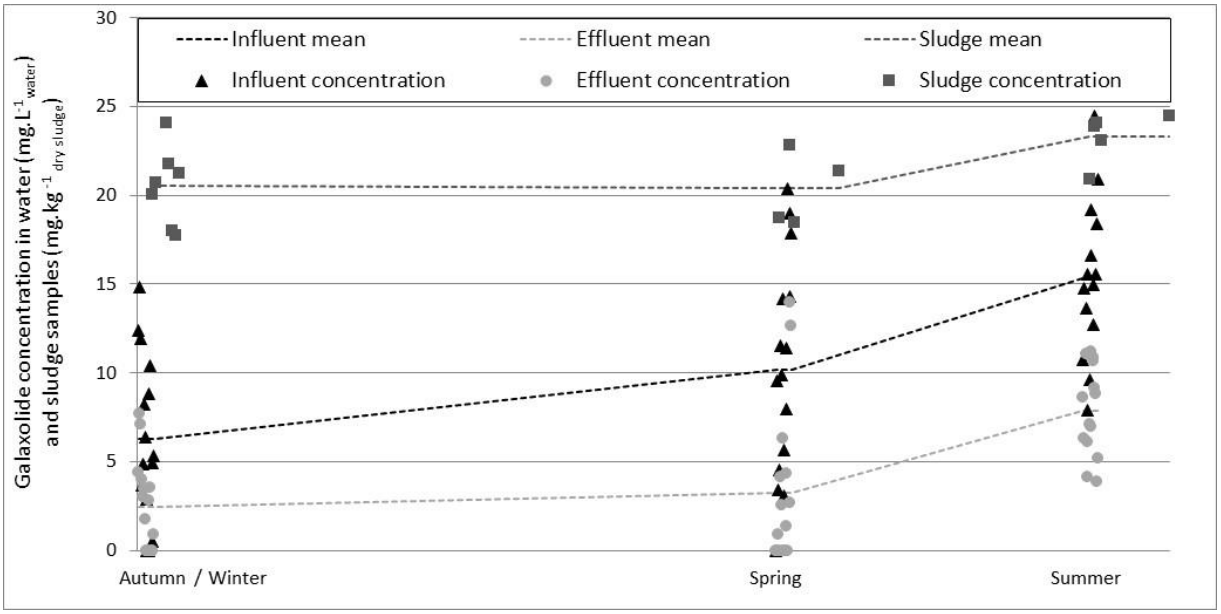

Figure 6.3 - Seasonal variation of HHCB concentration in wastewater and sludge samples.

There is a great dispersion of HHCB concentrations on both water and sludge matrix, which is reflected on the overlap of the concentration ranges (Figure 6.3). HHCB concentrations in dewatered sludge samples varied between 17.798 and 24.531 $mg \cdot kg^{-1}_{dry\ sludge}$, as expected, the

mean value of HHCB concentrations are always higher for influent (from < 1.3 to 24.5 $\mu g.L^{-1}$) than effluent samples to (from < 1.3 to 14.0 $\mu g.L^{-1}$). HHCB concentration range values are consistent with other studies in similar wastewater, as previously reported on Table 6.1. Nevertheless, these results present maximum values of the same order of two Spanish studies (Rosal, Rodríguez et al. 2010, Vallecillos, Pedrouzo et al. 2014) which can be hypothetically explained by the similar consumer patterns of the Iberian population, although the removal efficiencies have to consider all the influencing factors. In the present study, there was always a reduction in HHCB mean concentration between influent and effluent for each season: 61% in Autumn, 65% in Spring and 49% in Summer (Table 6.4). Similar removal rates have been reported (55 - 92%) (Lishman, Smyth et al. 2006, Reiner, Berset et al. 2007, Clara, Gans et al. 2011, Sang, Zhang et al. 2012, He, Chen et al. 2013).

Parada WWTP is located in Maia and treats sewage mainly from this municipality (75%) (SMM 2014). The influent is composed by 70% from domestic sources and the remaining of industrial origin (CMM 2005) and it is expected a high level of fragrances in this urban and mostly domestic influent. Especially because the domestic discharge may vary, not only seasonally but also between week/weekend or morning/afternoon/night, and considering an industrial discontinuous activity on those periods, the influent composition might present some variations. Analyzing seasonal variation on Figure 6.3, the mean concentration values seem similar between Autumn and Spring, for effluent and sludge samples, but there is some difference between the correspondent influent HHCB concentrations. Additionally, there is a remarkable difference for all matrices between Summer (dry season) and the other seasons. This perception is confirmed by the results reported in Table 6.4 for wastewater samples, where influent HHCB mean concentrations are significantly different between the three seasons, and effluent samples only differ between Spring and Summer. However, there were no significant differences between each season on sludge HHCB mean concentrations (Table 6.4). Yang and Metcalfe (2006) and Sang et al. (2012) reported similar seasonal variations, with a rain dilution effect at the wet season, but other authors reported no seasonal variation (Heberer, Gramer et al. 1999, Winkler, Headley et al. 2000), although this studies may refer to separative sewage networks with no storm water infiltration as Parada WWTP.

Table 6.4 - Mean HHCB concentration in WWTP wastewater and sludge samples along seasons.

SAMPLE	HHCB concentration [a] Autumn	HHCB concentration [a] Spring	HHCB concentration [a] Summer
Wastewater Influent	6.3 ± 1.3	10.2 ± 1.9	15.4 ± 2.4
Wastewater Effluent	2.4 ± 0.6	3.3 ± 0.8	7.9 ± 1.6
Dehydrated sludge	20.549 ± 1.883	20.388 ± 1.813	23.330 ± 2.054

[a] units of $\mu g \cdot L^{-1}_{water}$ for water samples and $mg \cdot kg^{-1}_{dry\ sludge}$ for sludge samples

Weekly variations were also analyzed for wastewater samples and, to the author's best knowledge, this is the first approach of this kind with HHCB. There are significant differences between week and weekend days in Autumn and Spring samples, although no consistent trend was observed (Figure 6.4). In fact, Autumn influent and effluent samples present a higher HHCB concentration on weekend days, while Spring samples present the opposite tendency. It should be noticed that weekend mean concentrations result from three samples collected on a Sunday of each season, and the referred differences might be due to variations on industrial discharge of those sampling days. On the other hand, Summer influent and, especially, effluent samples, present more similar values between week and weekend days, which is consistent with the fact that during August (Summer collection date) most industrial facilities are closed. Besides the referred differences, it should be noticed that the total mean concentrations are similar for influent and effluent samples between all seasons (Table 6.4).

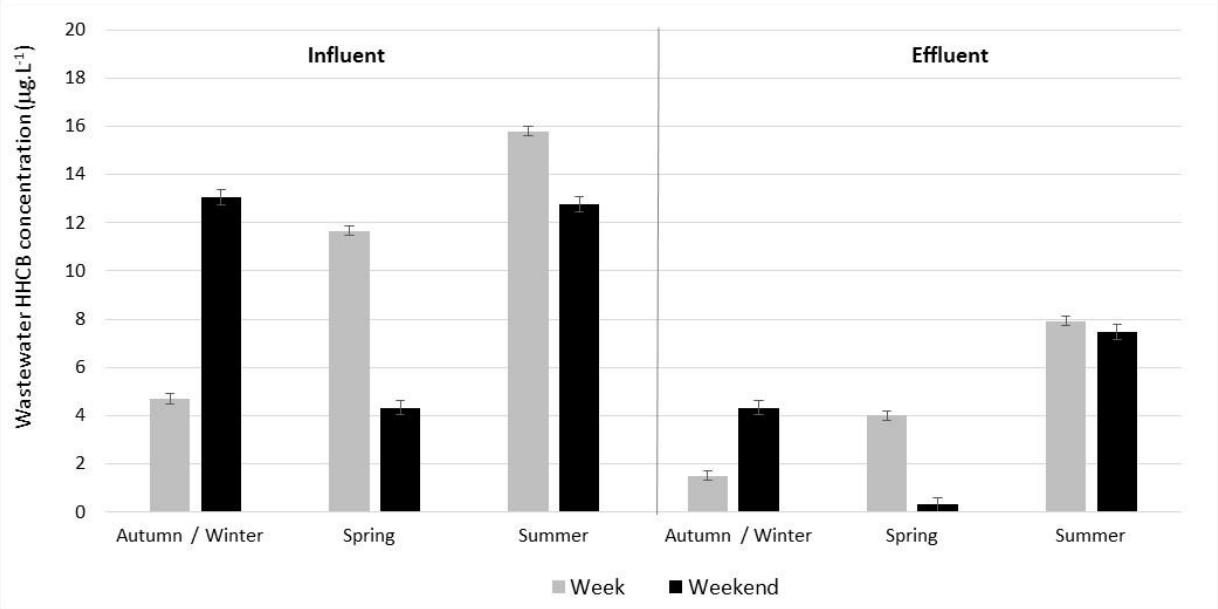

Figure 6.4 - Weekly variation of HHCB concentration in wastewater for each sampling season.

Daily variations were also analyzed (Figure 6.5) and there is a consistent lower HHCB mean concentration during the day (morning and afternoon) on Spring and Summer influent samples. Nevertheless, this was not the tendency on Autumn samples, where afternoon presents the smaller mean concentration (4.6 ± 0.2 $\mu g \cdot L^{-1}_{water}$). Salgado et al. (2011) reported an opposite trend, where higher concentrations were observed during the day and lower concentrations were observed at night. But in this last study, samples were collected every two hours, which is different from the present study that considers seven to ten hours between each collection. This large gaps could compromise the achievement of correct HHCB variations. The effluent samples presented similar mean HHCB concentrations in Autumn and Spring samples, while in Summer higher concentrations were found (maximum of 9.3 ± 0.2 $\mu g \cdot L^{-1}_{water}$).

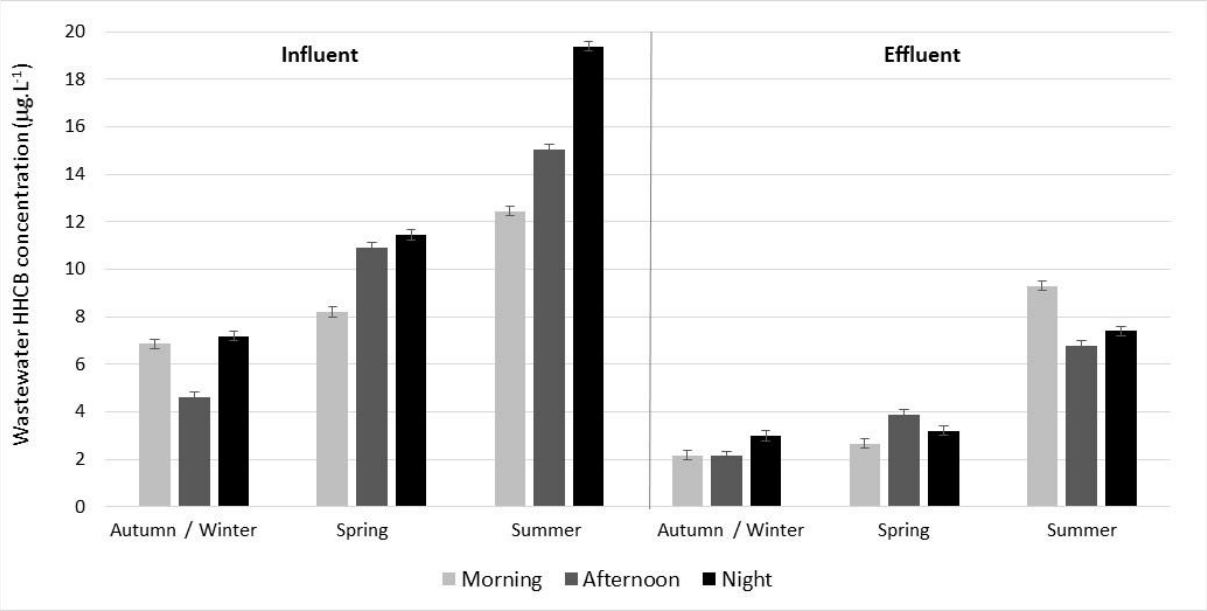

Figure 6.5 - Daily variation of HHCB concentration in wastewater for each sampling season.

6.3.3 Daily *per capita* emissions

Parada WWTP sewage is 70% from domestic sources and the remaining of industrial origin (CMM 2005). If it is considered that industrial wastewater has no HHCB and that the mean concentration of HHCB in influent samples is 10.6 $\mu g \cdot L^{-1}_{water}$, the domestic sewage has approximately 15 $\mu g \cdot L^{-1}_{water}$. It is estimated that about 77% of synthetic musks are led into the sewerage system (HERA 2004b) mainly from personal care products. Considering that Parada WWTP covers about 235000 inhabitants (Gonçalves, 2013) and treats an average daily volume of 12600 m^3 of domestic sewage (CMM 2005), each individual should release 1506 $\mu g \cdot day^{-1}$ of

HHCB in the sewage system. A previously reported human dermal exposure to HHCB of 692 $\mu g \cdot day^{-1}$ in the same region of Portugal (chapter 5), is the result of a daily application of personal care products containing about 1100 µg of HHCB. Considering the previously described retention factors of HHCB on the skin (Correia, Cruz et al. 2013), the corresponding HHCB release "down the drain" is about 400 $\mu g \cdot day^{-1}$, which is almost four times below the daily emission estimated in the present study (1506 $\mu g \cdot day^{-1}$). Nevertheless, it should be noticed that the former result (400 $\mu g \cdot day^{-1}$) came from several assumptions considering only a narrow range of products, with no contribution from perfumes, sanitation, detergents and household cleaning products, described as products with high levels of HHCB. All of those products have contributed to the levels of HHCB in Parada WWTP and, therefore, to the present obtained value (1506 $\mu g \cdot day^{-1}$), that is comparable with other reported values of 500 to 4868 $\mu g \cdot day^{-1}$ (Kupper, Berset et al. 2004, Clara, Gans et al. 2011, Homem, Silva et al. 2015a).

6.4 Conclusions

The analytical method by QuEChERS – HPLC-FL was found to be suitable for the analysis of HHCB, with a LOD of 0.4 $\mu g.L^{-1}_{water}$ or 0.004 $mg \cdot kg^{-1}_{dry\ sludge}$ and a LOQ of 1.3 $\mu g\ L^{-1}_{water}$ or 0.013 $mg\ kg^{-1}_{dry\ sludge}$. The mean recovery rate varied between 62.3% and 92.6%, and the maximum global uncertainty was about 50%.

Galaxolide was detected in almost all wastewater samples (mean values of 10.6 $\mu g.L^{-1}$ in influents and 4.5 $\mu g.L^{-1}$ in effluents) and in all sludge samples (mean values of 21.422 $mg \cdot kg^{-1}_{dry\ sludge}$). There was a significant variation between wet (Autumn) and dry seasons (Spring and Summer). During these two hot seasons, HHCB mean concentrations seem to be higher at night collection than the diurnal ones. However, no evident trend was noticed in week /weekend HHCB levels. Considering the obtained data and the Parada WWTP characteristics, the mean daily *per capita* emissions of the served population was estimated in 1506 $\mu g.day^{-1}$.

The adaptation of the HHCB extraction method by QuEChERS followed by HPLC-FL analysis to sewage and sludge samples, firstly developed for Personal Care Products (Correia, Cruz et al. 2013), was successively achieved. This approach will permit to use the same analytical method for several matrices and, in the future, other matrices should also be tested. For instance, in parallel to the wastewater treatment line of Parada WWTP, there is a biogas utilization for electricity production, and a dehydrated sludge recovery line, conducting to a sub-product from the WWTP, a commercial soil conditioner (CMM 2005). So, HHCB detection in the fertilizer and in the treated soils would be interesting.

PART III – GENERAL CONCLUSIONS

7 Conclusions

The main objective of this work was to contribute to the evaluation of the human exposure risks to galaxolide in Portugal, using the Northern Region Portuguese population. In order to do that, two approaches were addressed: the direct dermal exposure and the indirect environmental exposure.

Since the dermal application of Personal Care Products is the main route of human exposure to galaxolide, the consumer patterns of the studied population were essential. The consumer data indicated almost the same frequencies of use, mainly once a day for common toiletries and twice a day for toothpaste. The coincidence on frequencies was noticed, not only between other reported studies, but also between the analyzed children and the remaining population. This conclusion is in consonance with the fact that all family members tend to have the same habits, also similar to the majority of individuals, besides the region of provenance. The used amounts presented more variability between the two age groups of population analyzed and the literature available data, with similar values only for deodorant (0.9 $g_{sample}.day^{-1}$), shower gel (9.6 $g_{sample}.day^{-1}$) and shaving foam (2.0 $g_{sample}.day^{-1}$) usage. These results may be due to cultural differences between the analyzed populations, reflecting on the applied amounts and toiletries market choices. The differences found between older individuals and children of the analyzed population was due to the fact that children have lower skin areasusing, therefore, lower amounts of product. Nevertheless, the mean exposure risk to Personal Care Products *per* body area was higher for children and this is related to the higher risk of local toxicity (allergy) of this age group. The most relevant product for systemic exposure in adults was body lotion, for having the highest value of exposure per unit of body weight (69.9 $mg_{sample}.day^{-1}.kg_{bw}$), while deodorants seem to contribute to skin toxicity due to its higher exposure per unit area of skin (66.0 $\mu g_{sample}.day^{-1}.cm^{-2}$). The same pattern was observed in terms of systemic exposure dose, obtaining 36.6 $\mu g.kg_{bw}^{-1}$ for children and 9.4 $\mu g.kg_{bw}^{-1}$ for adults. Major differences were verified on the consumers' brands choices, mainly because caregivers tend to buy age specific products for their children, especially brands commercialized in pharmacies. This location to buy those kind of products was probably also preferred because most people associate pharmacy brands to more safe products.

The determination of galaxolide concentrations found on the most used Personal Care Products (data from consumer patterns) was also crucial for exposure purposes. This evaluation requires reliable extraction and quantification methods adapted to the different analyzed matrices. Therefore, an analytical methodology was validated for galaxolide determination, with an extraction method, frequently named QuEChERS, and a detection by High Performance Liquid Chromatography with fluorescence detection, adapted to all types of toiletries galenic forms: creams, lotions, solutions, pastes, gels, ointments, wipes, and others. The method presented limits of detection of 0.001 $mg.kg^{-1}_{sample}$, and the concentrations ranged from 0.04 $mg.kg^{-1}_{sample}$ to 280.78 $mg.kg^{-1}_{sample}$, for general population toiletries, and 0.001 $mg{\cdot}kg^{-1}_{sample}$ to 300.480 $mg{\cdot}kg^{-1}_{sample}$, in babies and children's products. As expected, the minimum detected galaxolide quantities are higher in adult's products, but the higher concentration came from a children's consumed product (414.855 $mg{\cdot}kg^{-1}_{sample}$). Nevertheless, this product is a glycerin soap that is not specific for the age group under analysis. Subsequently, the risk of dermal exposure to galaxolide was assessed, considering the collected consumer habits, corresponding to an estimated dermal exposure of 692 $\mu g.day^{-1}$ on the population of the Northern Region of Portugal and 277 $\mu g{\cdot}day^{-1}$ on the children's population of Oporto district. With these exposure data, and considering an evaporation of 22% of the galaxolide retained on skin after application and the worst case scenario of galaxolide total absorption into bloodstream, it was possible to conclude a risk exposure for those children and adults' population. Considering the average body weight (bw) for each age group, a maximum systemic exposure dose of 9.4 $\mu g.kg_{bw}^{-1}$ for adults and 36.6 $\mu g.kg_{bw}^{-1}$ for children, was achieved, respectively corresponding to minimum margins of safety of 3191 and 820. This values leads to the conclusion that galaxolide is a safe cosmetic ingredient considering the normal use of Personal Care Products in both analyzed groups. Nevertheless, this results don't exclude the risk of galaxolide to cause any damage when the consumptions are very high and cumulative, or when the skin is not intact, altering the protection barrier it usually confers.

The same analytical method was adapted and validated for environmental liquid and semi-solid matrices (respectively water and sludge from a wastewater treatment plant) with limits of detection of 0.4 $\mu g.L^{-1}_{water}$, for wastewater, and 0.004 $mg.kg^{-1}_{dry\ sludge}$, for sludge samples. All sludge samples presented galaxolide, from 17.798 to 24.531 $mg{\cdot}kg^{-1}_{dry\ sludge}$, but not all wastewater samples, from < 1.3 to 24.5 $\mu g.L^{-1}$. As expected, the influent samples presented

higher mean concentrations than effluent samples, leading to a mean reduction of 49-65%. The main removal process of galaxolide from wastewater seems to be due to sludge adsorption, because galaxolide is lipophilic and slightly water soluble. Influent presented significant seasonal variation of galaxolide mean levels between Spring/Summer (from 10.2 ± 1.9 to 15.4 ± 2.4 $\mu g \cdot L^{-1}_{water}$) and Autumn seasons (6.3 ± 1.3 $\mu g \cdot L^{-1}_{water}$), justified by the dilution effect of the rain in wet months. The hot seasons presented also significant higher levels of galaxolide in influent night samples (from 11.5 ± 0.2 to 19.4 ± 0.1 $\mu g \cdot L^{-1}_{water}$) then diurnal samples (from 8.2 ± 0.2 to 15.1 ± 0.2 $\mu g \cdot L^{-1}_{water}$), where the smaller mean concentration (4.6 ± 0.2 $\mu g \cdot L^{-1}_{water}$) was found in afternoon Autumn samples. There was no evident trend between week and weekend galaxolide levels in influents, probably due to continuous industry labor (30% of wastewater is from industrial origin). The sludge HHCB mean concentration was not significantly variable during the sampling seasons. Considering the wastewater treatment plant hydraulic parameters and the served population, the mean daily emissions of galaxolide *per capita* was estimated in 1506 $\mu g \cdot day^{-1}$. These results are higher than the estimated from the obtained consumer patterns, where about 400 $\mu g \cdot day^{-1}$ of galaxolide is released in the sewage by each individual. Nevertheless, it should be noticed that this result has considered only Personal Care Products usage and there are other contributors to the release of galaxolide “down-the drain”, as detergents and household cleaning products, that may justify this estimations difference.

The obtained consumer patterns are essential data for exposure purposes, not only for galaxolide as for any other cosmetic ingredient used on Personal Care Products from adults and children of Portugal or similar cultural populations. They are also a major contribution to the collection of data on habits of toiletries consume from all over European Union (EU). For the authors’ best knowledge, this work represents the first dermal risk assessment on galaxolide on Portuguese population and is the first known galaxolide risk assessment considering children’s population in all the revised literature. Although other studies of environmental exposure have been conducted in Portugal, no specific data on the Northern Region were found, and the correlation to the real consumer habits is crucial in order to perform a global exposure assessment.

8 Future work

The consumer patterns on Northern Region of Portugal have been achieved and the preferred brands and respective products have been codified (for rights protection) and registered. So, the next step should be to analyze those products using the developed analytical method, in order to recalculate a broader exposure risk of this population.

The exposure risk has been accessed considering two age groups: children and adults. But, since the weight of an adult varies with sex, and the weight of a child is directly related to age and sex, gender consumer patterns variations should also be contemplated.

Since the presence of galaxolide on environmental matrices is dependent on toiletries usage patterns in a particular region, as well as environmental mobility, the detection of these compounds in various locations in Portugal is essential to proceed the environmental risk assessment. For this it is necessary to know, not only, the consumer patterns and the existing galaxolide levels in toiletries, as well as Portugal locations more suitable for sampling the various environmental compartments, namely urban centers of drains, water courses and sediments, soils, sludge, influent and effluent of major urban wastewater treatment plants. The developed analytical method may be adapted to all or some of those matrices. Then, levels of galaxolide in environmental matrices may be related with the geographic location and climatic data. Exposure levels resulting from analyses of environmental samples can be compared with the estimated levels for which the compounds do not have adverse health effects, and the risk quotient, determined by the ratio between the Predicted Environmental Concentration and the Predicted No-Effect Concentration (PEC/PNEC), should be determined to complete the environmental risk assessment to galaxolide.

Finally, it was not possible to adapt the detection method to quantify the other musks. So, it would be important to develop a new multiresidue methodology that also allow the extraction and quantification of these musks in on all mentioned matrices. Thus, the risk assessment would be extended to other synthetic fragrances.

9 References

Abramsson-Zetterberg, L. and P. Slanina (2002). "Macrocyclic musk compounds—an absence of genotoxicity in the Ames test and the in vivo Micronucleus assay." Toxicology Letters **135**(1–2): 155-163.

Anastassiades, M. and S. J. Lehotay (2003). "Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "Dispersive Solid-Phase Extraction" for the determination of pesticide residues in produce." Journal of AOAC International **86**(2): 412-431.

Andresen, J., D. Muir, D. Ueno, C. Darling, N. Theobald and K. Bester (2007). "Emerging pollutants in the North Sea in comparison to Lake Ontario, Canada, data." Environmental Toxicology and Chemistry **26**(6): 1081-1089.

Api, A. M. and R. A. Ford (1999). "Evaluation of the oral subchronic toxicity of HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran) in the rat." Toxicology Letters **111**(1-2): 143-149.

Arbulu, M., M. C. Sampedro, N. Unceta, A. Gómez-Caballero, M. A. Goicolea and R. J. Barrio (2011). "A retention time locked gas chromatography–mass spectrometry method based on stir-bar sorptive extraction and thermal desorption for automated determination of synthetic musk fragrances in natural and wastewaters." Journal of Chromatography A **1218**(20): 3048-3055.

Aschmann, S., J. Arey, R. Atkinson and S. Simonich (2001). "Atmospheric lifetimes and fates of selected fragrance materials and volatile model compounds." Environmental Science & Technology **35**(18): 3595-3600.

Ayuk-Takem, L., F. Amissah, B. J. Aguilar and N. S. Lamango (2014). "Inhibition of Polyisoprenylated Methylated Protein Methyl Esterase by Synthetic Musks Induces Cell Degeneration." Environmental toxicology **29**(4): 466-477.

Bagasra, O., Z. Golkar, M. Garcia, L. N. Rice and D. G. Pace (2013). "Role of perfumes in pathogenesis of Autism." Medical Hypotheses **80**(6): 795-803.

Balk, F. and R. A. Ford (1999). "Environmental risk assessment for the polycyclic musks AHTN and HHCB in the EU: I. Fate and exposure assessment." Toxicology Letters **111**(1–2): 57-79.

Beets, M. (1982). Odor and stimulant structure. Fragrance Chemistry: The Science of the Sense of Smell. E. T. Theimer. New York, Academic Press**:** 77–122.

Berset, J., P. Bigler and D. Herren (2000). "Analysis of nitro musk compounds and their amino metabolites in liquid sewage sludges using NMR and mass spectrometry." Analytical Chemistry **72**(9): 2124-2131.

Bester, K. (2004). "Retention characteristics and balance assessment for two polycyclic musk fragrances (HHCB and AHTN) in a typical German sewage treatment plant." Chemosphere **57**(8): 863-870.

Bester, K. (2005). "Comparison of TCPP concentrations in sludge and wastewater in a typical German sewage treatment plant-comparison of sewage sludge from 20 plants." Journal of Environmental Monitoring **7**(5): 509-513.

Bester, K. (2009). "Analysis of musk fragrances in environmental samples." Journal of Chromatography A **1216**(3): 470-480.

Biesterbos, J. W. H., T. Dudzina, C. J. E. Delmaar, M. I. Bakker, F. G. M. Russel, N. von Goetz, P. T. J. Scheepers and N. Roeleveld (2013). "Usage patterns of personal care products: Important factors for exposure assessment." Food and Chemical Toxicology **55**: 8-17.

Blume-Peytavi, U., M. Hauser, G. N. Stamatas, D. Pathirana and N. Garcia Bartels (2012). "Skin Care Practices for Newborns and Infants: Review of the Clinical Evidence for Best Practices." Pediatric Dermatology **29**(1): 1-14.

Bremmer, H. J., Prud'homme de Lodder, L.C.H., van Engelen, J.G.M. (2006). Cosmetics Fact Sheet-To assess the risks for the consumer Updated version for ConsExpo 4.

Bridges, B. (2002). "Fragrance: emerging health and environmental concerns." Flavour and Fragrance Journal **17**(5): 361-371.

Bruchet, A., C. Hochereau, C. Picard, V. Decottignies, J. Rodrigues and M. Janex-Habibi (2005). "Analysis of drugs and personal care products in French source and drinking waters: the analytical challenge and examples of application." Water Science & Technology **52**(8): 53-61.

Cadby, P. A., W. R. Troy and M. G. H. Vey (2002). "Consumer Exposure to Fragrance Ingredients: Providing Estimates for Safety Evaluation." Regulatory Toxicology and Pharmacology **36**(3): 246-252.

Chen, C., S. Xue, Q. Zhou and X. Xie (2011). "Multilevel ecotoxicity assessment of polycyclic musk in the earthworm <i>Eisenia fetida using traditional and molecular endpoints." Ecotoxicology **20**(8): 1949-1958.

Chen, D., X. Zeng, Y. Sheng, X. Bi, H. Gui, G. Sheng and J. Fu (2007). "The concentrations and distribution of polycyclic musks in a typical cosmetic plant." Chemosphere **66**(2): 252-258.

Chen, F., G.-G. Ying, Y.-B. Ma, Z.-F. Chen, H.-J. Lai and F.-J. Peng (2014). "Field dissipation and risk assessment of typical personal care products TCC, TCS, AHTN and HHCB in biosolid-amended soils." Science of The Total Environment **470–471**: 1078-1086.

Clara, M., O. Gans, G. Windhofer, U. Krenn, W. Hartl, K. Braun, S. Scharf and C. Scheffknecht (2011). "Occurrence of polycyclic musks in wastewater and receiving water bodies and fate during wastewater treatment." Chemosphere **82**(8): 1116-1123.

CMM-Câmara Municipal da Maia (2002). Estação de tratamento de águas residuais de Parada. Depósito Legal nº 600 39/92. Gráfica Maiadouro. Maia.

CMM-Câmara Municipal da Maia (2005). Departamento de Ambiente e Qualidade de Vida, Portal do Ambiente e do Cidadão (http://ambiente.maiadigital.pt/ambiente/agua/mais-informacao-1/sobre-o-tratamento-e-drenagem-das-aguas-residuais-da-maia-1/etar-de-parada) (Accessed in September 2014).

Colipa (2005). Updated daily consumer exposure to cosmetic products.-Unpublished submission to SCCP.

Correia, P., A. Cruz, L. Santos and A. Alves (2013). "Human dermal exposure to galaxolide from personal care products." International Journal of Cosmetic Science **35**(3): 299-309.

Correia, P., A. Cruz, L. Santos and A. Alves (2015). "Risk of Children's Dermal Exposure to Galaxolide through Personal Care Products." Cosmetics **2**(2): 93.

Cronin, E. (1984). "Photosensitivity to musk ambrette." Contact Dermatitis **11**(2): 88-92.

CSTEE (2004). Opinion on the results of the Risk Assessment of: Musk Xylene- Environmental Part. Scientific Commitee on Toxicity, Ecotoxicity and the Environment.

CTFA (2004). Summary distributions of solid antiperspirant, body wash and shampoo use data. Unpublished report-Cosmetic Toiletry and Fragrance Association Inc, March 17, 2004.

CTFA (2005). Summary distributions of rinse-off hair conditioner use data. Unpublished report-Cosmetic Toiletry and Fragrance Association Inc , August 5, 2005.

Den Hond, E., M. Paulussen, T. Geens, L. Bruckers, W. Baeyens, F. David, E. Dumont, I. Loots, B. Morrens, B. N. de Bellevaux, V. Nelen, G. Schoeters, N. Van Larebeke and A. Covaci (2013). "Biomarkers of human

exposure to personal care products: Results from the Flemish Environment and Health Study (FLEHS 2007–2011)." Science of The Total Environment **463–464**: 102-110.

Dreher, P. (2004). Contaminants in arable soils fertilised with sewage sludge. 2nd International Workshop on Integrated Soil and Water Protection: Risks from Diffuse Pollution.

ECETOC (2001). Exposure Factors Sourcebook for European Populations (With Focus on UK Data)-Technical Report nº 79. ISSN-0773-8072-79. Brussels.

Eh, M. (2004). "New Alicyclic Musks: The Fourth Generation of Musk Odorants." Chemistry & Biodiversity **1**(12): 1975-1984.

EPA-Environmental Protection Agency (2008). Child-specific Exposure Factors Handbook.

EPA-Environmental Protection Agency (2011). Exposure Factors Handbook: 2011 Edition.

Ellison, S. L. R., M. Rosslein and A. Williams (2000). EURACHEM/CITAC Guide- Quantifying Uncertainty in Analytical Measurement. United Kingdom.

Eschke, H.-D. (2004). Synthetic Musks in Different Water Matrices. The Handbook of Environmental Chemistry. G. Rimkus. Heidelberg, Springer Berlin. **3:** 17-28.

European Communities Commission (1995). 18th COMMISSION DIRECTIVE 95/34/EC of 10 July 1995 adapting to technical progress Annexes II, III, VI and VII to Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official Journal of the European Union.

European Communities Commission (1998). 23rd COMMISSION DIRECTIVE 98/62/EC of 3 September 1998 adapting to technical progress Annexes II, III, VI and VII to Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official Journal of the European Union.

European Communities Commission (2000). Twenty-Fifth Commission Directive 2000/11/EC of 10 March 2000 adapting to technical progress Annex II to Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official Journal of the European Union.

European Communities Commission (2004). Commission Directive 2004/88/EC of 7 September 2004 amending Council Directive 76/768/EEC concerning cosmetic products for the purpose of adapting Annex III thereto to technical progress**:** 2.

European Communities Commission (2008). Commission Directive 2008/42/EC of 3 April 2008 amending Council Directive 76/768/EEC concerning cosmetic products, for the purpose of adapting Annexes II and III thereto to technical progress**:** 11.

European Communities Commission (2011). COMMISSION REGULATION (EU) No 143/2011 of 17 February 2011 amending Annex XIV to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals ('REACH'). Official Journal of the European Union.

European Parliment Council (2009). Regulation of the European Parliament and of the Council on cosmetic products (recast). PE-CONS 3623/09. Brussels**:** 397.

EWG-Environmental Working Group (2015), EWG's Skin Deep cosmetics database (http://www.ewg.org/skindeep/), Hazardous and untested chemicals in children's products (accessed in January 2015).

Faust, R., D. Nauroozi, C. Bruhn, B. Koch, P. Kuhlich, C. Piechotta and I. Nehls (2011). "(3,5,5,6,8,8-Hexamethyl-5,6,7,8-tetra-hydro-naphthalen-2-yl)methanol: a possible metabolite of the synthetic musk fragrance AHTN." Acta Crystallographica Section E: Structure Reports Online **67**(Pt 6): o1462-1463.

Fluhr, J. W., R. Darlenski, A. Taieb, J.-P. Hachem, C. Baudouin, P. Msika, C. De Belilovsky and E. Berardesca (2010). "Functional skin adaptation in infancy – almost complete but not fully competent." Experimental Dermatology **19**(6): 483-492.

Fooken, C. (2004). Synthetic Musks in Suspended Particulate Matter (SPM), Sediment, and Sewage Sludge. The Handbook of Environmental Chemistry. G. Rimkus. Heidelberg, Springer Berlin. **3:** 29-47.

Ford, R. (1998). "The human safety of the polycyclic musks AHTN anh HHCB in fragrances - a review." Deut. Lebensm. Rundsch **94**: 268-275.

Ford, R. A., D. R. Hawkins, R. Schwarzenbach and A. M. Api (1999). "The systemic exposure to the polycyclic musks, AHTN and HHCB, under conditions of use as fragrance ingredients: evidence of lack of complete absorption from a skin reservoir." Toxicology Letters **111**(1-2): 133-142.

Franke, S., C. Meyer, N. Heinzel, R. Gatermann, H. Hühnerfuss, G. Rimkus, W. A. König and W. Francke (1999). "Enantiomeric composition of the polycyclic musks HHCB and AHTN in different aquatic species." Chirality **11**(10): 795-801.

Fromme, H., T. Lahrz, M. Piloty, H. Gebhart, A. Oddoy and H. Rüden (2004). "Occurrence of phthalates and musk fragrances in indoor air and dust from apartments and kindergartens in Berlin (Germany)." Indoor Air **14**(3): 188-195.

Gao, X. and E. L. Simpson (2014). "Market Trends in Baby Skin Care Products and Implications for Clinical Practice." Pediatric Dermatology **31**(6): 734-738.

Gatermann, R., S. Biselli, H. Hühnerfuss, G. G. Rimkus, M. Hecker and L. Karbe (2002). "Synthetic Musks in the Environment. Part 1: Species-Dependent Bioaccumulation of Polycyclic and Nitro Musk Fragrances in Freshwater Fish and Mussels." Archives of Environmental Contamination and Toxicology **42**(4): 437-446.

Gebauer, H. and T. Bouter (1997). "Moschus." Euro. Cosmet. **1**: 30-35.

Gomez-Berrada, M.-P., F. Gautier, D. Parent-Massin and P.-J. Ferret (2013). "Retrospective exposure data for baby and children care products: An analysis of 48 clinical studies." Food and Chemical Toxicology **57**: 185-194.

Gómez, M. J., M. M. Gómez-Ramos, A. Agüera, M. Mezcua, S. Herrera and A. R. Fernández-Alba (2009). "A new gas chromatography/mass spectrometry method for the simultaneous analysis of target and non-target organic contaminants in waters." Journal of Chromatography A **1216**(18): 4071-4082.

Gómez, M. J., S. Herrera, D. Solé, E. García-Calvo and A. R. Fernández-Alba (2011). "Automatic Searching and Evaluation of Priority and Emerging Contaminants in Wastewater and River Water by Stir Bar Sorptive Extraction followed by Comprehensive Two-Dimensional Gas Chromatography-Time-of-Flight Mass Spectrometry." Analytical Chemistry **83**(7): 2638-2647.

Gonçalves, A. (2013). "Monitorização e Avaliação do desempenho das ETAR de Parada, Ponte de Moreira e Cambados - Relatório de final de curso - Licenciatura em Biotecnologia - Escola Superior Agrária de Ponte de Lima"

Gosens, I., C. J. E. Delmaar, W. ter Burg, C. de Heer and A. G. Schuur (2014). "Aggregate exposure approaches for parabens in personal care products: a case assessment for children between 0 and 3 years old." J Expos Sci Environ Epidemiol **24**(2): 208-214.

Hall, B., W. Steiling, B. Safford, M. Coroama, S. Tozer, C. Firmani, C. McNamara and M. Gibney (2011). "European consumer exposure to cosmetic products, a framework for conducting population exposure assessments Part 2." Food and Chemical Toxicology **49**(2): 408-422.

Hall, B., S. Tozer, B. Safford, M. Coroama, W. Steiling, M. C. Leneveu-Duchemin, C. McNamara and M. Gibney (2007). "European consumer exposure to cosmetic products, a framework for conducting population exposure assessments." Food and Chemical Toxicology **45**(11): 2097-2108.

He, Y.-j., W. Chen, X.-y. Zheng, X.-n. Wang and X. Huang (2013). "Fate and removal of typical pharmaceuticals and personal care products by three different treatment processes." Science of The Total Environment **447**: 248-254.

Heberer, T., S. Gramer and H. Stan (1999). "Occurrence and distribution of organic contaminants in the aquatic system in Berlin: Part III: Determination of synthetic musk in Berlin surface water applying solid-phase microextraction (SPME)." Acta Hydrochim Hydrobiol **27**: 150-156.

HERA (2003). Human and Environmental Risk Assessment-Technical Guidance Document in support of Commission Directive 93/67/EEC on risk assessment for new notified substances and Commission Regulation (EC) No 1488/94 on risk assessment for existing substances.

HERA (2004a). HERA (Human and Environmental Risk Assessment on Ingredients of Household Cleaning Products) Risk Assessment of HHCB (1,3,4,6,7-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran and related isomers)**:** 62.

HERA (2004b). HERA (Human and Environmental Risk Assessment on Ingredients of Household Cleaning Products) Environmental Risk Assessment Polycyclic Musks AHTN (CAS 1506-02-1) and HHCB (CAS 122-05-05)**:** 78.

Herren, D. and J. D. Berset (2000). "Nitro musks, nitro musk amino metabolites and polycyclic musks in sewage sludges: Quantitative determination by HRGC-ion-trap-MS/MS and mass spectral characterization of the amino metabolites." Chemosphere **40**(5): 565-574.

Homem, V., A. Alves, A. Alves and L. Santos (2016). "Ultrasound-assisted dispersive liquid–liquid microextraction for the determination of synthetic musk fragrances in aqueous matrices by gas chromatography–mass spectrometry." Talanta **148**: 84-93.

Homem, V., E. Silva, A. Alves and L. Santos (2015a). "Scented traces – Dermal exposure of synthetic musk fragrances in personal care products and environmental input assessment." Chemosphere **139**: 276-287.

Homem, V., J. A. Silva, C. Cunha, A. Alves and L. Santos (2013). "New analytical method for the determination of musks in personal care products by Quick, Easy, Cheap, Effective, Rugged, and Safe extraction followed by GC-MS." Journal of separation science **36**(13): 2176-2184.

Homem, V., J. A. Silva, N. Ratola, L. Santos and A. Alves (2015b). "Long lasting perfume – A review of synthetic musks in WWTPs." Journal of Environmental Management **149**: 168-192.

Hu, Z., Y. Shi and Y. Cai (2011). "Reprint of: Concentrations, distribution, and bioaccumulation of synthetic musks in the Haihe River of China." Chemosphere **85**(2): 262-267.

Hu, Z., Y. Shi, S. Zhang, H. Niu and Y. Cai (2011). "Assessment of Synthetic Musk Fragrances in Seven Wastewater Treatment Plants of Beijing, China." Bulletin of Environmental Contamination and Toxicology **86**(3): 302-306.

Hutter, H. P., P. Wallner, W. Hartl, M. Uhl, G. Lorbeer, R. Gminski, V. Mersch-Sundermann and M. Kundi (2010). "Higher blood concentrations of synthetic musks in women above fifty years than in younger women." International Journal of Hygiene and Environmental Health **213**(2): 124-130.

Hutter, H. P., P. Wallner, H. Moshammer, W. Hartl, R. Sattelberger, G. Lorbeer and M. Kundi (2005). "Blood concentrations of polycyclic musks in healthy young adults." Chemosphere **59**(4): 487-492.

Hutter, H. P., P. Wallner, H. Moshammer, W. Hartl, R. Sattelberger, G. Lorbeer and M. Kundi (2009). "Synthetic musks in blood of healthy young adults: Relationship to cosmetics use." Science of The Total Environment **407**(17): 4821-4825.

IPMA-Instituto Português do Mar e da Atmosfera (2013). Boletim Climatológico Mensal - Portugal Continental - Novembro de 2013. (ISSN 2183-1076).

IPMA-Instituto Português do Mar e da Atmosfera (2014). Boletim Climatológico Mensal - Portugal Continental - Fevereiro de 2014. (ISSN 2183-1076).

Imai, S., C. Kuwabara Infant skin and its care. Cosmet. Toilet. 1992, 107, 85–90.

Instituto Nacional de Estatística-INE (Statistics Portugal). Censos 2011-XV Recenseamento geral da população, V Recenseamento geral da habitação-Resultados provisórios (ISSN 2182-4215, ISBN 978-989-25-0148-2). Lisboa Portugal- 2011. Available online: http://www.google.pt/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CB8QFjAA&url=http%3A%2F%2Fcensos.ine.pt%2Fngt_server%2Fattachfileu.jsp%3Flook_parentBoui%3D131010986%26att_display%3Dn%26att_download%3Dy&ei=Q1QwVeyvMKiR7Aat7oDgDg&usg=AFQjCNEdobEaXIfk1EIB_1v7sI117bsbrA (accessed on 20 November 2014)

International Flavors & Fragrances (2003). Test Plan for 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB) CAS#122-05-5.

International Organization for Standardization, Technical Committee ISO/TC 69 (Applications of statistical methods), Subcommittee SC 4 (Applications of statistical methods in process management), ISO 7870-2 (Control charts — Part 2: Shewhart control charts), 2013.

Käfferlein, H. and T. A. Goen, J (1998). "Musk xylene: analysis, occurrence, kinetics, and toxicology." Critical reviews in toxicology **28**(5): 431-476.

Kannan, K., J. L. Reiner, S. H. Yun, E. E. Perrotta, L. Tao, B. Johnson-Restrepo and B. D. Rodan (2005). "Polycyclic musk compounds in higher trophic level aquatic organisms and humans from the United States." Chemosphere **61**(5): 693-700.

Kevekordes, S., A. Zaulig and H. Dunkelberg (1997). "Genotoxicity of nitro musks in the micronucleus test with human lymphocytes in vitro and the human hepatoma cell line Hep G2." Toxicology Letters **91**(1): 13-17.

Kuklenyik, Z., X. A. Bryant, L. L. Needham and A. M. Calafat (2007). "SPE/SPME–GC/MS approach for measuring musk compounds in serum and breast milk." Journal of chromatography. B, Analytical technologies in the biomedical and life sciences **858**(1–2): 177-183.

Kupper, T., J. D. Berset, R. Etter-Holzer, R. Furrer and J. Tarradellas (2004). "Concentrations and specific loads of polycyclic musks in sewage sludge originating from a monitoring network in Switzerland." Chemosphere **54**(8): 1111-1120.

Lavender, T., C. Bedwell, E. O'Brien, M. J. Cork, M. Turner and A. Hart (2011). "Infant skin-cleansing product versus water: A pilot randomized, assessor-blinded controlled trial." BMC Pediatrics **11**(1): 1-9.

LeBlanc, R.J., P.Matthews, R.P. Richard. (2006) Global atlas of excreta, wastewater sludge, and biosolids management: moving forward the sustainable and welcome uses of a global resource. Un-Habitat, 123.

Liebl, B., R. Mayer, S. Ommer, C. Sönnichsen and B. Koletzko (2002). Transition of Nitro Musks and Polycyclic Musks Into Human Milk. B. Koletzko, K. F. Michaelsen and O. Hernell. USA, Springer. **478:** 289-305.

Lishman, L., S. A. Smyth, K. Sarafin, S. Kleywegt, J. Toito, T. Peart, B. Lee, M. Servos, M. Beland and P. Seto (2006). "Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada." Science of The Total Environment **367**(2–3): 544-558.

Liu, N., Y. Shi, W. Li, L. Xu and Y. Cai (2014). "Concentrations and distribution of synthetic musks and siloxanes in sewage sludge of wastewater treatment plants in China." Science of The Total Environment **476–477**: 65-72.

Liu, N., Y. Shi, L. Xu, W. Li and Y. Cai (2013). "Occupational exposure to synthetic musks in barbershops, compared with the common exposure in the dormitories and households." Chemosphere **93**(9): 1804-1810.

Llompart, M., M. Celeiro, J. Pablo Lamas, L. Sanchez-Prado, M. Lores and C. Garcia-Jares (2013). "Analysis of plasticizers and synthetic musks in cosmetic and personal care products by matrix solid-phase dispersion gas chromatography–mass spectrometry." Journal of Chromatography A **1293**: 10-19.

Loretz, L., A. M. Api, L. Barraj, J. Burdick, D. A. Davis, W. Dressler, E. Gilberti, G. Jarrett, S. Mann, Y. H. Laurie Pan, T. Re, K. Renskers, C. Scrafford and S. Vater (2006). "Exposure data for personal care products: Hairspray, spray perfume, liquid foundation, shampoo, body wash, and solid antiperspirant." Food and Chemical Toxicology **44**(12): 2008-2018.

Loretz, L. J., A. M. Api, L. Babcock, L. M. Barraj, J. Burdick, K. C. Cater, G. Jarrett, S. Mann, Y. H. L. Pan, T. A. Re, K. J. Renskers and C. G. Scrafford (2008). "Exposure data for cosmetic products: Facial cleanser, hair conditioner, and eye shadow." Food and Chemical Toxicology **46**(5): 1516-1524.

Loretz, L. J., A. M. Api, L. M. Barraj, J. Burdick, W. E. Dressler, S. D. Gettings, H. Han Hsu, Y. H. L. Pan, T. A. Re, K. J. Renskers, A. Rothenstein, C. G. Scrafford and C. Sewall (2005). "Exposure data for cosmetic products: lipstick, body lotion, and face cream." Food and Chemical Toxicology **43**(2): 279-291.

Lu, Y., T. Yuan, W. Wang and K. Kannan (2011a). "Concentrations and assessment of exposure to siloxanes and synthetic musks in personal care products from China." Environmental Pollution **159**(12): 3522-3528.

Lu, Y., T. Yuan, S. Yun, W. Wang and K. Kannan (2011b). "Occurrence of Synthetic Musks in Indoor Dust from China and Implications for Human Exposure." Archives of Environmental Contamination and Toxicology **60**(1): 182-189.

Lv, Y., T. Yuan, J. Hu and W. Wang (2009). "Simultaneous Determination of Trace Polycyclic and Nitro Musks in Water Samples Using Optimized Solid-Phase Extraction by Gas Chromatography and Mass Spectrometry." Analytical sciences : the international journal of the Japan Society for Analytical Chemistry **25**(9): 1125-1130.

Lv, Y., T. Yuan, J. Hu and W. Wang (2010). "Seasonal occurrence and behavior of synthetic musks (SMs) during wastewater treatment process in Shanghai, China." Science of The Total Environment **408**(19): 4170-4176.

Machado, S., C. Gonçalves, E. Cunha, A. Guimarães and M. F. Alpendurada (2011). "New developments in the analysis of fragrances and earthy–musty compounds in water by solid-phase microextraction (metal alloy fibre) coupled with gas chromatography–(tandem) mass spectrometry." Talanta **84**(4): 1133-1140.

Macherius, A., T. Eggen, W. G. Lorenz, T. Reemtsma, U. Winkler and M. Moeder (2012). "Uptake of Galaxolide, Tonalide, and Triclosan by Carrot, Barley, and Meadow Fescue Plants." Journal of Agricultural and Food Chemistry **60**(32): 7785-7791.

Martin, C., M. Moeder, X. Daniel, G. Krauss and D. Schlosser (2007). "Biotransformation of the polycyclic musks HHCB and AHTN and metabolite formation by fungi occurring in freshwater environments." Environmental Science & Technology **41**(15): 5395-5402.

Martínez-Girón, A. B., A. L. Crego, M. J. González and M. L. Marina (2010). "Enantiomeric separation of chiral polycyclic musks by capillary electrophoresis: Application to the analysis of cosmetic samples." Journal of Chromatography A **1217**(7): 1157-1165.

Masuck, I., C. Hutzler and A. Luch (2011). "Estimation of dermal and oral exposure of children to scented toys: Analysis of the migration of fragrance allergens by dynamic headspace GC–MS." Journal of Separation Science **34**(19): 2686-2696.

McNamara, C., D. Rohan, D. Golden, M. Gibney, B. Hall, S. Tozer, B. Safford, M. Coroama, M. C. Leneveu-Duchemin and W. Steiling (2007). "Probabilistic modelling of European consumer exposure to cosmetic products." Food and Chemical Toxicology **45**(11): 2086-2096.

Mogensen, B., G. Pritzl, S. Rastogi, O. Glesne, B. Hedlund, J.-P. Hirvi, A. Lundgren and A. Sigurdsson (2004). Musk in the Nordic environment. TemaNord: 503:68 (Nordic Council of Ministers). Copenhagen.

Moldovan, Z., R. Chira and A. Alder (2009). "Environmental exposure of pharmaceuticals and musk fragrances in the Somes River before and after upgrading the municipal wastewater treatment plant Cluj-Napoca, Romania." Environmental Science and Pollution Research International **16**(0): 46-54.

Mottaleb, M. A., S. Usenko, J. G. O'Donnell, A. J. Ramirez, B. W. Brooks and C. K. Chambliss (2009). "Gas chromatography–mass spectrometry screening methods for select UV filters, synthetic musks, alkylphenols, an antimicrobial agent, and an insect repellent in fish." Journal of Chromatography A **1216**(5): 815-823.

Müller, J. and W. Böhmer (2006). "Occurrence of Polycyclic Musks in Sewage Sludge and their Behaviour in Soils and Plants. Part 1: Behaviour of Polycyclic Musks in Sewage Sludge of Different Treatment Plants in Summer and Winter (5 pp)." Journal of Soils and Sediments **6**(4): 231-235.

Nakata, A., H. Sasaki, A. Takemura, M. Yoshioka, S. Tanabe and K. Kannan (2007). "Bioaccumulation, temporal trend, and geographical distribution of synthetic musks in the marine environment." Environmental Science & Technology **41**: 2216-2222.

Nakata, H. (2005). "Occurrence of synthetic musk fragrances in marine mammals and sharks from Japanese coastal waters." Environmental Science & Technology **39**(10): 3430-3434.

Nakata, H., M. Hinosaka and H. Yanagimoto (2015). "Macrocyclic-, polycyclic-, and nitro musks in cosmetics, household commodities and indoor dusts collected from Japan: Implications for their human exposure." Ecotoxicology and Environmental Safety **111**: 248-255.

Nohynek, G. J., E. Antignac, T. Re and H. Toutain (2010). "Safety assessment of personal care products/cosmetics and their ingredients." Toxicology and Applied Pharmacology **243**(2): 239-259.

OSPAR Comission (2004). OSPAR background document on musk xylene and other musks.

Peck, A., J. Kucklick and M. Schantz (2007). "Synthetic musk fragrances in environmental Standard Reference Materials." Analytical and Bioanalytical Chemistry **387**(7): 2381-2388.

Peck, A. M. and K. C. Hornbuckle (2004). "Synthetic musk fragrances in Lake Michigan." Environmental Science & Technology **38**(2): 367-382.

Peters, R. J. B., H. Beeltje and R. J. van Delft (2008). "Xeno-estrogenic compounds in precipitation." Journal of Environmental Monitoring **10**(6): 760-769.

Posada-Ureta, O., M. Olivares, P. Navarro, A. Vallejo, O. Zuloaga and N. Etxebarria (2012). "Membrane assisted solvent extraction coupled to large volume injection-gas chromatography–mass spectrometry for trace analysis of synthetic musks in environmental water samples." Journal of Chromatography A **1227**: 38-47.

Ramirez, A., R. Brain, S. Usenko, M. Mottaleb, J. O'Donnell, L. Stahl, J. Wathen, B. Snyder, J. Pitt, P. Perez-Hurtado, L. Dobbins, B. Brooks and C. Chambliss (2009). "Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States." Environmental Toxicology and Chemistry **28**(12): 2587-2597.

Ramírez, N., F. Borrull and R. M. Marcé (2012). "Simultaneous determination of parabens and synthetic musks in water by stir-bar sorptive extraction and thermal desorption-gas chromatography-mass spectrometry." Journal of Separation Science **35**(4): 580-588.

Ramírez, N., R. M. Marcé and F. Borrull (2010). "Development of a thermal desorption-gas chromatography–mass spectrometry method for determining personal care products in air." Journal of Chromatography A **1217**(26): 4430-4438.

Ratola, N., L. s. Martins and A. Alves (2004). "Ochratoxin A in wines-assessing global uncertainty associated with the results." Analytica Chimica Acta **513**(1): 319-324.

Regueiro, J., C. Garcia-Jares, M. Llompart, J. P. Lamas and R. Cela (2009). "Development of a method based on sorbent trapping followed by solid-phase microextraction for the determination of synthetic musks in indoor air." Journal of Chromatography A **1216**(14): 2805-2815.

Regueiro, J., M. Llompart, C. Garcia-Jares and R. Cela (2007). "Development of a high-throughput method for the determination of organochlorinated compounds, nitromusks and pyrethroid insecticides in indoor dust." Journal of Chromatography A **1174**(1–2): 112-124.

Reiner, J., J. Berset and K. Kannan (2007). "Mass Flow of Polycyclic Musks in Two Wastewater Treatment Plants." Archives of Environmental Contamination and Toxicology **52**(4): 451-457.

Reiner, J., C. Wong, K. Arcaro and K. Kannan (2007). "Synthetic musk fragrances in human milk from the United States." Environmental Science & Technology **41**(11): 3815-3820.

Reiner, J. L. and K. Kannan (2006). "A survey of polycyclic musks in selected household commodities from the United States." Chemosphere **62**(6): 867-873.

Ren, Y., K. Wei, H. Liu, G. Sui, J. Wang, Y. Sun, X. Zheng. (2013) Occurrence and removal of selected polycyclic musks in two sewage treatment plants in Xi'an, China. Front. Environ. Sci. Eng., 7, 166-172.

Riedel, J., G. Birner, C. Van Dorp, H.-G. Neumann and W. Dekant (1999). "Haemoglobin binding of a musk xylene metabolite in man." Xenobiotica **29**(6): 573-582.

Riedel, J. and W. Dekant (1999). "Biotransformation and Toxicokinetics of Musk Xylene in Humans." Toxicology and Applied Pharmacology **157**(2): 145-155.

Rimkus, G. G. (1999). "Polycyclic musk fragrances in the aquatic environment." Toxicology Letters **111**(1-2): 37-56.

Rimkus, G. G., R. Gatermann and H. Hühnerfuss (1999). "Musk xylene and musk ketone amino metabolites in the aquatic environment." Toxicology Letters **111**(1-2): 5-15.

Ritter, L., I. P. o. C. Safety and I.-O. P. f. t. S. M. o. Chemicals (1995). A Review of Selected Persistent Organic Pollutants: DDT, Aldrin, Dieldrin, Endrin, Chlordane, Heptachlor, Hexachlorobenzene, Mirex, Toxaphene, Polycholorinated Biphenyls, Dioxins and Furans, publisher not identified.

Rogers, R., D. Isola, C. Jeng, A. Lefebvre and L. Smith (2005). "Simulated inhalation levels of fragrance materials in a surrogate air freshener formulation." Environmental Science & Technology **39**(20): 7810-7816.

Roosens, L., A. Covaci and H. Neels (2007). "Concentrations of synthetic musk compounds in personal care and sanitation products and human exposure profiles through dermal application." Chemosphere **69**(10): 1540-1547.

Rosal, R., A. Rodríguez, J. A. Perdigón-Melón, A. Petre, E. García-Calvo, M. J. Gómez, A. Agüera and A. R. Fernández-Alba (2010). "Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation." Water Research **44**(2): 578-588.

Saiyasombati, P. and G. B. Kasting (2003). "Two-stage kinetic analysis of fragrance evaporation and absorption from skin." International Journal of Cosmetic Science **25**(5): 235-243.

Salgado, R., R. Marques, J. Noronha, G. Carvalho, A. Oehmen and M. Reis (2011b). "Assessing the removal of pharmaceuticals and personal care products in a full-scale activated sludge plant." Environmental Science and Pollution Research International **159**: 1-10.

Salgado, R., R. Marques, J. P. Noronha, J. T. Mexia, G. Carvalho, A. Oehmen and M. A. M. Reis (2011). "Assessing the diurnal variability of pharmaceutical and personal care products in a full-scale activated sludge plant." Environmental Pollution **159**(10): 2359-2367.

Sanchez-Prado, L., M. Lores, M. Llompart, C. Garcia-Jares, M. Lourido and R. Cela (2004). "Further solid-phase microextraction-gas chromatography-mass spectrometry applications: "on-fibre" and aqueous photodegradation of nitro musks." Journal of Chromatography A **1048**(1): 73-80.

Sang, W., Y. Zhang, X. Zhou and T. C. Zhang (2012). "Spatial and seasonal distribution of synthetic musks in sewage treatment plants of Shanghai, China." Water Science and Technology **66**(1): 201-209.

SCCNFP (2002). Opinion of the Scientific Committee on Cosmetic Products and Non-food Products (SCCNFP) intended for consumers.

SCCNFP (2003). The SCCNFP's Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation, 5th Revision. Adopted by the SCCNFP during the 25th plenary meeting of 20 October 2003. SCCNFP/0690/03 Final.

SCCS (2012). Scientific Committee on Consumer Safety-The SCCS's Notes of Guidance for the Testing of Cosmetic Substances and Their Safety Evaluation, 8th Revision. Adopted by the SCCS during the 17th plenary meeting of 11 December 2015. SCCS/1501/12.

Schlumpf, M., K. Kypke, M. Wittassek, J. Angerer, H. Mascher, D. Mascher, C. Vökt, M. Birchler and W. Lichtensteiger (2010). "Exposure patterns of UV filters, fragrances, parabens, phthalates, organochlor pesticides, PBDEs, and PCBs in human milk: Correlation of UV filters with use of cosmetics." Chemosphere **81**(10): 1171-1183.

Schreurs, R. H. M. M., E. Sonneveld, J. H. J. Jansen, W. Seinen and B. van der Burg (2005). "Interaction of Polycyclic Musks and UV Filters with the Estrogen Receptor (ER), Androgen Receptor (AR), and Progesterone Receptor (PR) in Reporter Gene Bioassays." Toxicological Sciences **83**(2): 264-272.

Schüssler, W. and L. Nitschke (1998). "Determination of trace amounts of Galaxolide® (HHCB) by HPLC." Fresenius' Journal of Analytical Chemistry **361**(2): 220-221.

Seinen, W., J. G. Lemmen, R. H. H. Pieters, E. M. J. Verbruggen and B. van der Burg (1999). "AHTN and HHCB show weak estrogenic — but no uterotrophic activity." Toxicology Letters **111**(1-2): 161-168.

Silva, A. and J. Nogueira (2010). "Stir-bar-sorptive extraction and liquid desorption combined with large-volume injection gas chromatography–mass spectrometry for ultra-trace analysis of musk compounds in environmental water matrices." Analytical and Bioanalytical Chemistry **396**(5): 1853-1862.

Simmons, D. B. D., V. L. Marlatt, V. L. Trudeau, J. P. Sherry and C. D. Metcalfe (2010). "Interaction of Galaxolide® with the human and trout estrogen receptor-α." Science of The Total Environment **408**(24): 6158-6164.

Slanina, P. (2004). Risk Evaluation of Dietary and Dermal Exposure to Musk Fragrances. Series Anthropogenic Compounds: Synthetic Musk Fragances in the Environment. G. G. Rimkus. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 281-310.

SMM-Serviços Municipalizados da Maia (2014). MANUAL DA QUALIDADE, Edição 3, Junho de 2014.

Sofuoglu, A., N. Kiymet, P. Kavcar and S. C. Sofuoglu (2010). "Polycyclic and nitro musks in indoor air: a primary school classroom and a women's sport center." Indoor Air **20**(6): 515-522.

Sperling, M. (2007). Biological Wastewater Treatment- Sludge Treatment and Disposal. IWA.

Stamatas, G. N., J. Nikolovski, M. C. Mack and N. Kollias (2011). "Infant skin physiology and development during the first years of life: a review of recent findings based on in vivo studies." International Journal of Cosmetic Science **33**(1): 17-24.

Subedi, B., M. A. Mottaleb, C. K. Chambliss and S. Usenko (2011). "Simultaneous analysis of select pharmaceuticals and personal care products in fish tissue using pressurized liquid extraction combined with silica gel cleanup." Journal of Chromatography A **1218**(37): 6278-6284.

Subedi, B., S. Yun, S. Jayaraman, B. J. Bergen and K. Kannan (2014). "Retrospective monitoring of persistent organic pollutants, including PCBs, PBDEs, and polycyclic musks in blue mussels (Mytilus edulis) and sediments from New Bedford Harbor, Massachusetts, USA: 1991–2005." Environmental Monitoring and Assessment **186**(8): 5273-5284.

SWECO (2008). Screening of musks substances. Swedish Environmental Protection Agency.

Tas, J. W., F. Balk, R. A. Ford and E. J. van de Plassche (1997). "Environmental risk assessment of musk ketone and musk xylene in the Netherlands in accordance with the EU-TGD." Chemosphere **35**(12): 2973-3002.

TOXNET (2012). Toxicology Data Network-National Library of Medicine HSDB Database-Galaxolide (accessed in June 2012).

Umbach, W. (1991). Cosmetics and toiletries. England, Ellis Horwood Limited.

Upadhyay, N., Q. Sun, J. O. Allen, P. Westerhoff and P. Herckes (2011). "Synthetic musk emissions from wastewater aeration basins." Water Research **45**(3): 1071-1078.

USEPA (2003). Toxic Substance Control Act (TSCA) Inventory. United States Environmental Protection Agency.

Vallecillos, L., F. Borrull and E. Pocurull (2013). "An automated headspace solid-phase microextraction followed by gas chromatography–mass spectrometry method to determine macrocyclic musk fragrances in wastewater samples." Analytical and Bioanalytical Chemistry **405**(29): 9547-9554.

Vallecillos, L., F. Borrull, J. M. Sanchez and E. Pocurull (2015). "Sorbent-packed needle microextraction trap for synthetic musks determination in wastewater samples." Talanta **132**: 548-556.

Vallecillos, L., M. Pedrouzo, E. Pocurull and F. Borrull (2014). "Headspace stir bar sorptive extraction followed by thermal desorption and gas chromatography with mass spectrometry to determine musk fragrances in sludge samples without sample pretreatment." Journal of Separation Science **37**(11): 1322-1329.

Vallecillos, L., E. Pocurull and F. Borrull (2012). "Fully automated ionic liquid-based headspace single drop microextraction coupled to GC–MS/MS to determine musk fragrances in environmental water samples." Talanta **99**: 824-832.

Vallecillos, L., E. Pocurull and F. Borrull (2013). "A simple and automated method to determine macrocyclic musk fragrances in sewage sludge samples by headspace solid-phase microextraction and gas chromatography–mass spectrometry." Journal of Chromatography A **1314**: 38-43.

Villa, S., M. Vighi and A. Finizio (2014). "Theoretical and experimental evidences of medium range atmospheric transport processes of polycyclic musk fragrances." Science of The Total Environment **481**: 27-34.

Wan, Y., Q. Wei, J. Hu, X. Jin, Z. Zhang, H. Zhen and J. Liu (2007). "Levels, Tissue distribution, and age-related accumulation of synthetic musk fragrances in Chinese sturgeon (Acipenser sinensis): comparison to organochlorines." Environmental Science & Technology **41**: 424-430.

Wang, L. and S. J. Khan (2014). "Enantioselective analysis and fate of polycyclic musks in a water recycling plant in Sydney (Australia)." Water Science and Technology **69**(10): 1996-2003.

Wang, L., J. A. McDonald and S. J. Khan (2013). "Enantiomeric analysis of polycyclic musks in water by chiral gas chromatography–tandem mass spectrometry." Journal of Chromatography A **1303**: 66-75.

Weschler, C. J. and W. W. Nazaroff (2012). "SVOC exposure indoors: fresh look at dermal pathways." Indoor Air **22**(5): 356-377.

Wester, R.C., H.I. Maibach. Interrelationships in the dose-response of percutaneous absorption. In *Topical Absorption of Dermatological Products*; Bronaugh, R.L., Maibach, H.I., Eds.; Marcel Dekker, Inc.: New York, NY, USA, 2002a; pp. 169–184.

Wester, R.C., H.I. Maibach. H.I. Regional variation in percutaneous absortion. In *Topical Absorption of Dermatological Products*; Bronaugh, R.L., Maibach, H.I., Eds.; Marcel Dekker, Inc.: New York, NY, USA, 2002b; pp. 33–39.

Williams, S. D. (1996). Chemistry and Technology of the Cosmetics and Toiletries Industry. UK, Blackie Academic & Professional.

Wilson, S. C., R.D. Davidson, K.C Jones. (1996). Screening the environmental fate of organic contaminants in sewage sludges applied to agricultural soils: The potential for downward movement to groundwaters. The Science of the Total Environment. **185:** 45-57.

Winkler, M., J. Headley and K. Peru (2000). "Optimization of solid-phase microextraction for the gas chromatographic-mass spectrometric determination of synthetic musk fragrances in water samples." Journal of Chromatography A **903**: 203-210.

Wombacher, W. D. and K. C. Hornbuckle (2009). "Synthetic Musk Fragrances in a Conventional Drinking Water Treatment Plant with Lime Softening." Journal of environmental engineering (New York, N.Y.) **135**(11): 1192.

Wu, S.-F. and W.-H. Ding (2010). "Fast determination of synthetic polycyclic musks in sewage sludge and sediments by microwave-assisted headspace solid-phase microextraction and gas chromatography–mass spectrometry." Journal of Chromatography A **1217**(17): 2776-2781.

Wu, X., D. H. Bennett, B. Ritz, D. L. Cassady, K. Lee and I. Hertz-Picciotto (2010). "Usage pattern of personal care products in California households." Food and Chemical Toxicology **48**(11): 3109-3119.

Yamauchi, R., H. Ishibashi, M. Hirano, T. Mori, J.-W. Kim and K. Arizono (2008). "Effects of synthetic polycyclic musks on estrogen receptor, vitellogenin, pregnane X receptor, and cytochrome P450 3A gene expression in the livers of male medaka (Oryzias latipes)." Aquatic Toxicology **90**(4): 261-268.

Yang, C.-Y. and W.-H. Ding (2012). "Determination of synthetic polycyclic musks in aqueous samples by ultrasound-assisted dispersive liquid–liquid microextraction and gas chromatography–mass spectrometry." Analytical and Bioanalytical Chemistry **402**(4): 1723-1730.

Yang, J.-J. and C. D. Metcalfe (2006). "Fate of synthetic musks in a domestic wastewater treatment plant and in an agricultural field amended with biosolids." Science of The Total Environment **363**(1-3): 149-165.

Yin, J., H. Wang, J. Zhang, N. Zhou, F. Gao, Y. Wu, J. Xiang and B. Shao (2012). "The occurrence of synthetic musks in human breast milk in Sichuan, China." Chemosphere **87**(9): 1018-1023.

Zeng, X.-Y., S.-X. Cao, D.-L. Zhang, S.-T. Gao, Z.-Q. Yu, H.-R. Li, G.-Y. Sheng and J.-M. Fu (2012). "Levels and distribution of synthetic musks and polycyclic aromatic hydrocarbons in sludge collected from Guangdong Province." Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering **47**(3): 389-397.

Zeng, X., G. Sheng, Y. Xiong and J. Fu (2005). "Determination of polycyclic musks in sewage sludge from Guangdong, China using GC–EI-MS." Chemosphere **60**(6): 817-823.

Zhang, X., G. Liang, X. Zeng, J. Zhou, G. Sheng and J. Ful (2011). "Levels of synthetic musk fragrances in human milk from three cities in the Yangtze River Delta in Eastern China." Journal of Environmental Sciences (China) **23**(6): 983-990.

Zhang, X., Y. Yao, X. Zeng, G. Qian, Y. Guo, M. Wu, G. Sheng and J. Fu (2008). "Synthetic musks in the aquatic environment and personal care products in Shanghai, China." Chemosphere **72**(10): 1553-1558.

ANNEXES

ANNEX A – QUESTIONNAIRE ABOUT PCPs USAGE PATTERNS IN ADULTS

Exposure Risks to Pharmaceuticals and Personal Care Products

This questionnaire aims to collect consumption data of dermocosmetic products needed for the PhD project of Patricia Carla dos Santos Correia, student of the Doctoral Program in Chemical and Biological Engineering of the Faculty of Engineering of Porto.

Data confidentiality is assured by the respondents' anonymity.

The questionnaire takes about five minutes, but it is advisable to keep in mind the usual brand of the consumed toiletries.

☐ I declare to be clarified and I express my agreement to participate in research.

Gender

- ○ ☐ Female
- ○ ☐ Male

Age

County of Residence

Literary abilities

Professional activity (CAE Rev. 2.1):

Household member:

Household net income corresponds to:

Where do you usually buy your hygiene products?

- ○ ☐ In pharmacies and medicines commercialization areas
- ○ ☐ In supermarkets/hypermarkets
- ○ ☐ Other:

Continue »

50% completed

1. FACIAL MOISTURIZER (Day)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ BB Cream
- ☐ Anti-wrinkle cream
- ☐ Moisturizer cream
- ☐ Nourishing cream
- ☐ Serum

Skin type:

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Nivea Creme de dia hidratante Aqua Effect pele normal e mista

2. FACIAL MOISTURIZER (Night)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ BB Cream
- ☐ Anti-wrinkle cream
- ☐ Moisturizer cream
- ☐ Nourishing cream
- ☐ Serum

Skin type:

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: L'Oreal Paris Creme Revitalift

3. BODY MOISTURIZER

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ Cream
- ☐ Gel
- ☐ Lotion / Milk
- ☐ Oil

Variety:

Skin type:

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Dove Creme Intensivo Rich Nourishment

4. DEODORANT

- ☐ Don´t use

Usually use the brand:

Variety:

Product type:

- ☐ Cream
- ☐ Roll-on
- ☐ Spray
- ☐ Stick

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Desodorizante Roll-On Dove Original

5. BODY WASH (bath gel, soap)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ Bath foam
- ☐ Bath gel
- ☐ Solid soap

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Gel Banho Vasenol Derma Care / Dove Beauty Cream Bar

6. HAIR WASH (Shampoo)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ 2 in 1

- ☐ Shampoo

Variety:

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Champô Pantene Clássico

7. ORAL HYGIENE (Toothpaste)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ Toothpaste
- ☐ 2 in 1 (toothpaste + elixir)

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Pasta Dentífrica Aquafresh Branqueadora + Proteção Total

8. RINSE OFF HAIR CARE (Conditioner, mask, ...)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ Conditioner/Softener
- ☐ Mask

Specify other product characteristics (brand, complete name, package characteristics, ...):

E.g.: Condicionador Pantene Clássico

9. LEAVE-ON HAIR CARE (Foam, gel, ...)

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ foam/Mousse
- ☐ Gel
- ☐ Lacquer
- ☐ Serum
- ☐ Oil

Product effect:

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Gel Fixação Extra Forte Tubo Garnier

10. FACIAL SUNSCREEN

You should not consider daily face creams with sun protection but only specific sunscreens.

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ Cream
- ☐ Milk
- ☐ Lotion
- ☐ Spray
- ☐ Oil
- ☐ Gel

Sun Protection Factor (SPF):

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Uriage Creme SPF50+, c/cor Doré, 50ml

Average annual rate application

	Don´t use	1-7 days	8-14 days	15-21 days	22-30 days	31-60 days	> 60 days	All year
All year	○	○	○	○	○	○	○	○
Only in summer /sun direct exposure	○	○	○	○	○	○	○	○

11. BODY SUNSCREEN

- ☐ Don´t use

Usually use the brand:

Product type:

- ☐ Cream
- ☐ Milk
- ☐ Lotion
- ☐ Spray
- ☐ Oil
- ☐ Gel

Sun Protection Factor (SPF):

Specify other product characteristics (brand, complete name, package characteristics, ...):

e.g.: Uriage Creme SPF50+, 200ml

Average annual rate application

	Don´t use	1-7 days	8-14 days	15-21 days	22-30 days	31-60 days	> 60 days	All year
All year	○	○	○	○	○	○	○	○
Only in summer/Direct sun exposure	○	○	○	○	○	○	○	○

12. OTHER PRODUCTS (Hand cream, Lip balm, Shaving products, ...)

- ☐ Don´t use

Usually use the product:

e.g.: Colgate Elixir Bucal Max White One

If so, indicate the frequency of use (day/week).

« Back Submit

100%: You made it.

.

DIARY OF HYGIENE AND PERSONAL CARE PRODUCTS USE BY ADULTS

1.1. Body weight: _____ years old **1.2. Municipality of residence:_____**

1.3. Usage registration

Initial date (day 1): ___ / ___ / ___ Final date (day 7): ___ / ___ / ___

a) Facial moisturizer (day)

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

b) Facial moisturizer (night)

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

c) Body moisturizer

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

d) Deodorant

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

e) Bath gel / soap

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

f) Shampoo

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

g) Hair conditioner / mask

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

h) Hair treatment (Serum, oil, gel, foam, …)

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

i) Toothpaste

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

j) Oral elixir / mouthwash

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

k) Facial sunscreen

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

l) Hand cream

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

m) Cream / gel / shaving foam

Product name: _____

Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Frequency of use (number of times)							

ANNEX B – QUESTIONNAIRE ABOUT PCP's USAGE PATTERNS IN CHILDREN

Questionnaire

This questionnaire is intended to collect data for a research project on the use of Personal Care Products in children from Porto aged from zero to four years old. This work is being developed as part of the PhD thesis of Patricia Carla dos Santos Correia, student from the Doctoral Program in Chemical and Biological Engineering, Faculty of Engineering, University of Porto, entitled "Risks of human exposure to Pharmaceuticals and Personal Care (PPCPs) - particularly those made of synthetic fragrances"

The confidentiality of the data collected is guaranteed by anonymity of respondents.

Informed consent

☐ I declare that I was clarified about the study in which I participate. I understand that the purpose of the study is purely academic. I also declare that I was informed that there are no direct benefits from the participation in this study and I am aware that I have the right not to participate without representing any kind of injury. Hereby, I express my agreement to participate in this research.

Date: ___ / ___ / _____

How to fill out the questionnaire

The questionnaire takes about 5 minutes.

Section 4 is optional and must be completed only once for each respondent.

When a free response is requested, please describe what is asked. To answer the other questions, sign with an X the option you want. It may be noted more than one option where applicable. If you want to change the answers you gave, cut all the response and re-mark the other option.

Are you an adult responsible for a children aged between 0 - 5 years old resident in Porto's district?

☐ No ☐ Yes **Municipality of residence:** _____

⇒ *If you answered "No", here ends the questionnaire. Otherwise you can continue.*

How many children, aged between 0 - 5 years old, have you in charge? _____

⇒ *From now on, you must complete a questionnaire for each child and attach it to this first part.*

2. Children's characterization

2.1. Children's age:

- ☐ 0-5 months
- ☐ 6-11 months
- ☐ 12-23 months
- ☐ 24-35 months
- ☐ 36-47 months
- ☐ 48-59 months

2.2. Gender:

- ☐ Female
- ☐ Male

3. Purchase of personal care products for children

3.1. Where do you usually buy the Personal Care Products for your children?

- ☐ In pharmacies and drug sales areas
- ☐ In supermarkets / hypermarkets
- ☐ Others: _____

⇒ *In the following questions, try to identify the best possible way the product that is used (see package).*

3.2. What products do you use for the child's sun protection?

- ☐ Don't know
- ☐ Don't use
- ☐ Facial product: _____
- ☐ Body product: _____

3.3. What products do you use for the child's skin hydration?

- ☐ Don't know
- ☐ Don't use
- ☐ Facial product: _____
- ☐ Body product: _____

3.4. What products do you use for the child's oral hygiene?

- ☐ Don't know
- ☐ Don't use
- ☐ Toothpaste: _____
- ☐ Elixir: _____

3.5. What products do you use for the child's bath?

- ☐ Don't know
- ☐ Don't use
- ☐ Body product: _____
- ☐ Shampoo: _____
- ☐ Hair conditioner: _____

3.6. What products do you use for the child's diaper change?

- ☐ Don't know
- ☐ Don't use
- ☐ Cleansing product: _____
- ☐ Protection product: _____

4. Use of personal care products in children

4.1. Mark the frequency of the following moments of personal care of your child:

	All over the year (how many times a day)	Only in sun exposure days (how many times a day)
a) Sun protection		

	Daily frequency (how many times a day)	Less than once a day (how many times a week)
b) Skin hydration		
c) Oral hygiene		
d) Shampoo application		
e) Hair conditioner application		
f) Bath		
g) Diaper change		

4.2. Sign the mode of application of Personal Care Products in your children:

a) Sunscreen	On the face (all over the year)	
	In parts of the body exposed to the sun (all over the	
	In parts of the body exposed to the sun (sun exposure	
	All over the body (sun exposure days)	
	Apply a thin layer application	
	Apply a thick layer application	
b) Moisturizer	On the face	
	On the trunk	
	On arms and legs	
	Apply a thin layer application	
	Apply a thick layer application	
c) Toothpaste	Only on teeth	
	On teeth and tongue	
	Apply equivalent to the nail of the little finger	
	Apply the equivalent to the surface of the toothbrush	
d) Shampoo	Diluted in the bath water	
	Over the wet scalp	
	Apply an approximate amount of a walnut	
	Apply a handful	
e) Hair conditioner	Only on the hair tips	
	All over the hair	
	Apply an approximate amount of a walnut	
	Apply an approximate amount of a pea	
f) Body bath products	Diluted in the bath water	
	Over the wet skin	
	Apply an approximate amount of a walnut	
	Apply a handful	
g) Diaper change hygiene	Diluted in the bath water	
	Over the wet skin	
	Apply an approximate amount of a walnut	
	Apply an approximate amount of a pea	
	Use a cleaning wipe	
	Use more than a cleaning wipe	
h) Diaper change cream	All over the genital area	
	Only in the area that is red / irritated	
	Apply a thin layer application	
	Apply a thick layer application	

5. Caregiver characterization

⇒ *This section must be completed only once for each caregiver.*

5.1. Gender and age:

☐ Female ☐ Male **Age:** _____ years

5.2. Qualifications:

☐ No schooling	☐ 7 - 9 years of schooling	☐ Graduation's degree
☐ 4 years of schooling	☐ 9 - 12 years of schooling	☐ Master's degree
☐ 5 - 6 years of schooling	☐ Bachelor's degree	☐ PhD's degree

5.3. Professional occupation (CAE Rev. 2.1):

☐ Agriculture, livestock, hunting and forestry	☐ Financial Activities
☐ Fishery	☐ Real estate activities
☐ Industry	☐ Education
☐ Production and distribution of electricity, gas, water	☐ Health and social action
☐ Building	☐ Other service activities
☐ Trade	☐ International organizations
☐ Hotels and Restaurants	☐ Housewives
☐ Transport, storage and communications	☐ Pensioners
☐ Public administration, defense and social security	☐ Unemployed

5.4. The monthly income of your household matches to:

☐ Um minimum wage (MW)	☐ 3 - 4 MW
☐ 1 - 2 MW	☐ 4 - 5 MW
☐ 2 - 3 MW	☐ More than 5 MW

5.5. Number of household members: _____

If you have any comments about the study / questionnaire, you can use the following space:

_____

_____

_____

Thank you for your attention and collaboration!

Patrícia Correia

DIARY OF HYGIENE AND PERSONAL CARE PRODUCTS USE BY CHILDREN

Start date (day 1): ___ / ___ / ___ End date (day 7): ___ / ___ / ___

1. Child's characterization: _____ years and _____ months **Gender:**_____

2. Usage registration:

a) Body moisturizer

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ Thin layer ☐ Thick layer

b) Face moisturizer

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ Thin layer ☐ Thick layer

c) Bath gel / soap

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ A walnut ☐ A handful

d) Shampoo

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ A walnut ☐ A handful

e) Hair conditioner

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ A walnut ☐ A pea

f) Toothpaste

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ Nail of the little finger ☐ Surface of the toothbrush

g) Diaper change wipes

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

h) Diaper change cream

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ Thin layer ☐ Thick layer

i) Sunscreen

Product: _____ Package weight: Day 1 _____ g Day 7 _____ g

	Day 1	Day	Day 3	Day 4	Day 5	Day 6	Day 7
Daily frequency (how many times a day)							

☐ Thin layer ☐ Thick layer