Investigation of various sample preparation methods for the GC-MS quantification of the extended list of regulated fragrance allergens in cosmetic products

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Abstract

The widespread use of cosmetics and personal care items is an integral part of our daily routines worldwide. Many of these products, including shampoos, aftershaves, and deodorants, incorporate fragrance compounds, which may be either natural or synthetic. However, certain fragrances have the potential to trigger allergies, impacting the health of consumers. According to the European Commission, the prevalence of contact allergy to fragrance substances is estimated to be between 1-3% of the overall population in Europe.

The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) has identified a group of 26 fragrance allergens, consisting of 24 individual molecules and 2 natural extracts, known for their allergy-inducing potential. These substances are regulated under Regulation No 1223/2009, with specified thresholds of 0.01% and 0.001% for rinse-off and leave-on products, respectively. Over time, as these substances undergo periodic revision, the list has expanded to include a new group of 54 individual molecules and 28 natural extracts.

Cosmetic manufacturers face a significant need for analytical techniques to identify and quantify fragrances in final cosmetic products. As of now, there are no existing sample preparation methods capable of quantifying fragrance allergens for the extended list in final cosmetic products.

This work aims to address this gap by exploring, developing, and validating a method for the analysis of fragrance allergens in rinse-off cosmetic products. Gas Chromatography-Mass Spectrometry (GC-MS) was employed for quantification purposes.

Three sample preparation methods, including Solid-Phase Micro Extraction (SPME) with a PDMS/DVB(+OC) fiber coating and two different Static Headspace (SHS) methods, were tested. However, after validation, none of these methods demonstrated satisfactory results for the accurate quantification of fragrance allergens in cosmetic products.

Key words : Fragrance allergens, cosmetic products, GC-MS, Static Headspace, Solid-Phase Micro Extraction, Method validation

Résumé

L'utilisation généralisée des cosmétiques et des produits de soins personnels fait partie intégrante de nos routines quotidiennes à l'échelle mondiale. De nombreux produits, tels que les shampooings, les lotions après-rasage et les déodorants, comprennent des composés parfumés, pouvant être naturels ou synthétiques. Cependant, certains parfums ont le potentiel de déclencher des allergies, affectant la santé des consommateurs. Selon la Commission européenne, la prévalence des allergies de contact aux substances parfumées est estimée entre 1 et 3 % de la population totale en Europe.

Le Comité scientifique des produits cosmétiques et des produits non alimentaires (CSPCNA) a identifié un groupe de 26 fragrances, composé de 24 molécules individuelles et de 2 extraits naturels, connus pour leur potentiel allergisant et sont ainsi nommées fragrances allergisantes en conséquence. Ces substances sont réglementées par le Règlement n° 1223/2009, avec des seuils spécifiés de 0,01 % et 0,001 % respectivement pour les produits cosmétiques à rincer (Rinse-off) et à laisser sur la peau (Leave-on). Continuellement, ces substances font l'objet de révisions périodiques et récemment, la liste s'est élargie pour inclure un nouveau groupe de 54 molécules individuelles et 28 extraits naturels.

Les fabricants de cosmétiques sont confrontés à un besoin significatif de techniques analytiques pour identifier et quantifier les parfums dans les produits cosmétiques finaux. À l'heure actuelle, il n'existe aucune méthode de préparation d'échantillons capable de quantifier les fragrance allergisantes pour la liste étendue dans les produits cosmétiques finaux.

Ce travail vise à combler cette lacune en explorant, développant et validant une méthode pour l'analyse quantitative des fragrances allergisantes dans les produits cosmétiques à rincer (rinse-off). La chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS) a été utilisée à ces fins de quantification.

Trois méthodes de préparation d'échantillons, comprenant l'extraction en phase solide (SPME) avec un revêtement de fibre PDMS/DVB(+OC) et deux méthodes différentes impliquant le Headspace static (SHS), ont été testées. Cependant, après validation, aucune de ces méthodes n'a démontré des résultats satisfaisants pour la quantification précise des fragrances allergisantes dans les produits cosmétiques

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List of Abbreviations

ASE	Accelerated Solvent Extraction
CAR	Carboxen
CI	Chemical Ionization
DAD	Diode-Array Detector
DI	Direct Immersion
DVB	Divinylbenzene
DHS	Dynamic Headspace
EI	Electro Ionization
EU	European Union
FI	Field Ionization
FID	Flame Ionization Detector
FEHS	Full Evaporation Headspace
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HCC	High-Concentration-Capacity
HPLC	High-Performance Liquid Chromatography
IS	Internal Standard
IFRA	International Fragrance Association
INCI	International Nomenclature of Cosmetic Ingredients
LOD	Limit Of Detection
LOQ	Limit Of Quantification
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
MS	Mass spectrometry
MSPD	Matrix Solid-Phase Dispersion
MAE	Microwave Assisted Extraction
µ-MSPD	miniaturized- Matrix Solid-Phase Dispersion
GCxGC	Multidimensional Gas Chromatography
O/W	Oil in Water
OC	Overcoated
PA	Polyacrylate
PDMS	Polydimethylsiloxane
QC	Quality Control
RSD	Relative Standard Deviation
SCCS	Scientific Committee on Consumer Safety
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products
SPME	Solid Phase Microextraction
SLE	Solid-Liquid Extraction
SPE	Solid-Phase Extraction
SHS	Static Headspace
SBSE	Stir Bar Sorptive Extraction
SFE	Supercritical Fluid Extraction
TOF	Time-Of-Flight
TIC	Total Ion Chromatogram
UAE	Ultrasound-Associated Extraction

I. State of the Art

1. Cosmetics

1.1 Definition

Nowadays, cosmetic products are an integral part of the personal care routine for many people around the world, making them a flourishing market. In Europe, the retail sales in 2022 for cosmetics and personal care products were evaluated at EUR 88 billion.[1]

In order to establish a regulatory framework, the Regulation (EC) No 1223/2009 defines cosmetic products as "any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours".[2]

Based on this definition, there are plentiful of cosmetic products covering different categories that are shown in **Table 1**.

Table 1 *– Categories of cosmetic products*[2–4]

Categories	Examples
Skincare products	Serums, facial masks, toners, eye creams, shaving creams, hydrating creams, moisturizers, cleansers, sunscreens, …
Body care products	Soaps, shower gels, body lotions, scrubs, deodorants and anti-perspirants, after-bath powders, tanning products, skin whitening products, sunscreens, depilatories, …
Hair care products	Shampoos, hair conditioners, hair dyes, sprays, hair colorants, mousses, …
Decorative cosmetics	Lipsticks, lip glosses, mascaras, eye shadows, nail polishes, powders, …
Perfumery	Perfumes, scented oils, toilet water, eau de Cologne, …
Oral care products	Toothpastes, mouthwashes, flosses, …

1.2 General composition of a cosmetic product

Depending on how the product is intended to be used, the formulation will vary. Hence, cosmetic manufacturers have the flexibility to manipulate various factors when developing a new cosmetic

product. This can be done by playing on the composition of the product. Indeed, composition affects the texture, stabilization, effects of a product but also the senses of users.

Texture is a key factor influencing the sensory characteristics of a product, contributing significantly to the customer experience during its application. Since cosmetic products often result from a combination of at least two substances, they can be classified as “solution” or “dispersion”. A solution is a blend of at least two miscible substances owing the same nature either aqueous, either oily. While a dispersion is created by combining at least two substances that are immiscible and owing the same or a different physical state (gas, liquid, solid). Dispersions are ubiquitous in cosmetics as they include :

- Emulsions : dispersion of two liquids (water in oil (W/O) or oil in water (O/W)) (creams, solid soaps, cleansers, etc);
- Suspensions : dispersion of a solid in a liquid (foundations, nail polishes, etc) ;
- Aerosols : dispersion of a solid or a liquid in a gas (deodorants, dry shampoos, etc) ;
- Powders : dispersion of a solid in another solid (talcums, blushes, etc) ; and
- Foams : dispersion of a gas in a liquid (shaving creams, etc).

The main components used to texture a product are water, oils, silicone, polyhydric alcohols, polymers, and powders. Depending on the type of product, the ratio of these components will vary.[3]

There are many parameters influencing the stability of a cosmetic product such as microbial activity, oxidation, pH, presence of metal ions, phase separation. Therefore, some agents can be added by the manufacturers in order to maintain the product stable and thereby extend its shelf-life. Antimicrobial preservatives help to avoid the presence of microorganisms, while antioxidant preservatives prevent oxidation phenomena. The most common preservatives found in cosmetic products are phenoxyethanol, parabens, sorbic acid, benzoic acid and tocopherol, ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) respectively.[5,6] In addition, citric acid, lactic acid, sodium hydroxide and triethanolamine are commonly used in order to adjust the pH of cosmetic products. Sodium phytate, propanediol, BHT, EDTA, soy lecithin, glucose oxidase are employed as chelating agent.[3] Finally, seeing that dispersions result from two immiscible substances, emulsifiers and surfactants are useful to stabilize them and inhibit phase separations. They include, among others, polysorbates, laureth-4, and potassium cetyl sulfate for the emulsifiers and sodium lauryl sulfate, ammonium laureth sulfate, sodium laureth sulfate for surfactants.[7]
It is worth noting that an agent can exhibit activity against multiple parameters simultaneously.

Some cosmetic products can provide plentiful benefits on skin, hair, nails thanks to the active substances they contain. Indeed, a number of substances are proven to be effective against various skin concerns including aging, acne, hyperpigmentation, dryness, etc. They are categorized into various classes of

compounds such as vitamins, carotenoids, polyphenols, organic and inorganic UV filters and essential oils.[8,9]

Finally, manufacturers can incorporate additional substances to enhance the sensory experience for users. Beside touch, sight and smell contribute also to consumers perception.[10] Coloring agents such as dyes and pigments are typically used in decorative cosmetic while *fragrances* are employed in all the categories of cosmetic products mentioned in Table 1. This work will be primarily focused on fragrances.

In conclusion, cosmetics, from a compositional perspective, are highly complex, comprising variable matrices formed by a large number of substances with diverse characteristics. These substances range from highly lipophilic to moderately polar and can display basic, acidic, or neutral properties.[11]

2. Fragrances

2.1 History of perfume

Throughout history, it is undeniable that perfumes have been closely associated with human activities. The word "perfume" comes directly from the Latin words *per* which means "throught" and *fumare*, "to smoke" suggesting that early perfumes exhibited pleasant smells obtained by burning various substances such as woods, grass, and other aromatic materials coming from plants and animals. Indeed, Mesopotamians seemed to be the first people using incense as the first form of perfume for religious rituals. Further, Egyptians expanded the use of perfume for cosmetic and medical purposes by employing perfumed oils for balms and ointments and was then refined under the Roman Empire. During the 17th and the 18th centuries, significant advancements were made in the technology of manufacturing natural perfumes. The late 19th century marked progress in the synthesis of aroma chemicals and, as a result, the 20th century witnessed a proliferation of fragrances and perfumes, offering a wide variety of scents to people worldwide. [4,12,13]

2.2 Fragrances roles in cosmetic

Fragrance compounds are ubiquitously employed not only in perfumes and scented cosmetic products but also in various household products such as detergents and fabric softeners. These pleasant aromas not only contribute to the attractiveness of the consumers for the product but also promote a sense of well-being. Another significant function of fragrances involves the masking effect. Cosmetic manufacturers often incorporate perfumes to counteract the unpleasant odors emitted by certain cosmetic ingredients. This strategic addition of perfumes not only conceals the disagreeable scents but also enhances the overall comfort and appeal for individuals using these cosmetic products. Moreover, a

recognizable scent allows product differentiation. Hence, fragrances have the potential to distinguish a product from its rivals and play a role in shaping brand identity. [4,10]

2.3 Odor perception

As per Richard Axel and Linda B. Buck, recipients of the 2004 Nobel Prize in Medicine or Physiology, smell is a chemical stimulus that elicits a physiological response, resulting from one or a combination of odorant molecules. The origin of an odor primarily lies in a compound that volatilizes at ambient temperature, reaching the nose.

Concerning molecular structure, odorant compounds sharing the same functional group tend to exhibit similar odors. For instance, esters are characterized by a fruity and floral aroma, lactones impart a coconut or apricot character, amines convey an animal/roasted scent, thiols present a rotten or alliaceous smell, volatile fatty acids produce a sour to rancid odor, and aldehydes are associated with green odors resembling grass cuttings or leaves. However, even when compounds share a similar functional group and tend to have the same structure, as is the case for enantiomeric compounds, their odors can differ. In fact, only a minimal percentage, as low as 5%, of enantiomer pairs exhibit a similar smell. For instance, (S)-(-)-limonene has a lemon-like scent, while (R)-(+)-limonene is characterized by an orange-like odor.[14–16]

2.4 Types of fragrances and terminology

Nowadays, the aromatic qualities of scented products such as cosmetic products but also food, pharmaceutical and laundry products are attributed to a diverse array of over 3000 chemical substances also called *fragrance materials*. These fragrance materials may exist in various forms. Firstly, they can be either *synthetic fragrance chemicals*, either *natural fragrance materials*. Synthetic fragrances are artificially created compounds produced from chemical processes, while natural fragrance materials are substances extracted from various plant or animal sources. These substances can range from essential oils and botanical extracts to isolates, resins, distillates and volatile concentrates. Secondly, in both cases, compounds can be either individual compounds or a blend of multiple compounds. For example, linalool is a natural fragrance material presented as an individual molecule, while rose oil, an essential oil, is also a natural fragrance material but is presented as a blend of different individual molecules (comprising citronellol, geraniol, linalool, damascene, …).[4,10,17,18]

The processes used to obtain those fragrances as well as their application by cosmetic manufacturers are detailed hereafter.

2.5 The ways of obtaining fragrances

2.5.1 Natural fragrances

Natural fragrances are acquired from plants or animals through different separation techniques such as distillation, solvent extraction, and expression among others. The substances obtained after the application of those techniques can range from essential oils and botanical extracts to isolates, resins, distillates and volatile concentrates.[10]

2.5.1.1 Animal origin fragrances

Extracts of animal origin are obtained from animal glands, yielding precious substances like musk, civet, castoreum, and ambergris. Musk was one of the most used animal fragrance in perfumery with a price surpassing even the value of gold. It is described as a woodsy, earthy and warm scent and it has been used as an aphrodisiac tonic and for medicinal purposes in China for a millennium. In recent year, because of the extensive poaching, musk deer, the animal that secrete musk, has become an endangered species. Therefore measures has been taken and musk trade is now controlled by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Currently, nearly all musk fragrances employed in perfumery are synthetic, often referred to as "white musk.". [4,19]

2.5.1.2 Vegetal origin fragrances

Natural fragrances derived from plants encompass a wide range of sources, including flowers, fruits, seeds, woods, branches, leaves, bark, and roots. The vast array of aromatic plants found in Nature allows to obtain a wide range of different aroma compounds.[20]

2.5.1.3 Extraction methods

Numerous methods exist for isolating aroma chemicals, some of which are already established (referred to as conventional techniques), while others continue to undergo development (known as advanced techniques). Conventional techniques include *hydrodistillation* and *steam distillation* which are the most common and cost-effective method exploited to produce the majority of essential oils commercially available. Essential oils are thus the hydrophobic liquid obtained after isolation of those aroma chemicals by those two different methods. The first technique consists broadly of heating directly the plant material, while for the second technique, steam is produced independently and then passed through plant material thanks to perforated inlet. In both cases, steam containing the volatile compounds is then condensed and essential oil is separated from water, which is called “hydrosol”. **Figure 1** illustrates the process of steam distillation.[20,21]

By way of example, rose hydrosol or rose water is the by-product of the distillation of rose petals and can be applied directly to the face or enter in the composition of some perfume.[22]

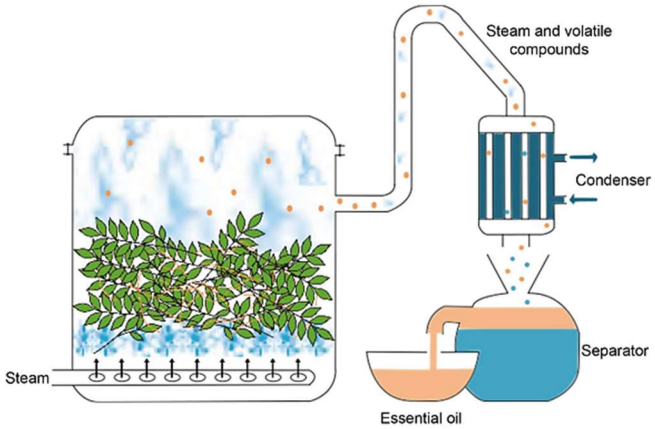


Figure 1 *- Scheme of the process of steam distillation of essential oils (from Burger and collaborators)*[20]

However, considering that certain components of essential oils are thermolabile, the application of distillation becomes impractical. Instead, a method known as *expression*, which involves cold pressing at room temperature, is utilized. This technique is particularly employed for *Citrus* species such as orange, bergamot, grapefruit, lemon, and others.[18,20]

Solvent extraction is also a widespread conventional method and is employed for other plants that exhibit sensitivity to high temperatures including jasmine, tuberose, resin, beans, dates, etc. This method offers the benefit of generating a high yield, given that fewer compounds are degraded by heat. The solvent used are toluene, hexane, heptane, petroleum ether, but also occasionally dichloromethane, ethanol, methanol, etc. Benzene previously employed has been prohibited because of its toxicity. An additional stage for eliminating the solvent needs to be incorporated into the process. “Concrete”, a waxy mass, is the product resulting from the solvent extraction of plant materials. Following an extraction with ethyl alcohol, a fragrant liquid called “absolute” is obtained and are easier to exploit industrially than concrete, considering their lower viscosity.[20,21]

Hot maceration and *enfleurage* are both ancient methods using a fatty material in order to extract essential oil from plants. However, they have nearly fallen into disuse because they are considered labor-intensive, time-consuming, and financially demanding methods by today's standards.[20,23]
Advanced techniques aim to optimize efficiency, yield, and the quality of extracted fragrant compounds while being environmentally friendly. They include among others : supercritical fluid extraction (SFE), the use of greener solvents or even microwave assisted extraction (MAE). However, these techniques are essentially used at laboratory level. [20,24] It can be noted that all these techniques mentioned are provided with an indicative title and are by no means exhaustive.

As mentioned earlier, some fragrance materials can be individual molecules derived directly from natural fragrance materials. Various techniques are used in order to isolate them from a material that has already been extracted, such as essential oil. *Fractional distillation* also called *rectification* is a process used to separate two or more substances based on their differing volatilities. It is performed thanks to a fractionation column and it allows to isolate fractions rich in a particular compound.[25] Fractional distillation finds application in scenarios like adjusting the linalool/linalyl acetate ratio in lavender essential oil in order to obtain a final product with constant odor. In fact, certain lavender varieties yield essential oils with higher linalool content than linalyl acetate, deviating from customer preferences. Fractional distillation is employed to isolate and eliminate the fraction enriched with linalool.

Chromatography is another technique employed at industrial scale for the isolation of compounds from natural fragrance materials. Traditional approaches in natural product chemistry, such as open-column chromatography and preparative thin-layer chromatography, frequently generate significant quantities of harmful waste, therefore, high-performance liquid chromatography (HPLC) constitutes a new method of isolation as it prevents this negative effect and allows the isolation of large quantities of natural products with high purity.[24,26]
As well as for crude essential oils extraction, solvent extraction and supercritical fluid extraction are also valid methods.[20,26]

2.5.2 Synthetic fragrances

Synthetic fragrances comprise fragrance materials that are artificially produced within a laboratory or industrial setting. This procedure primarily serves two key intentions : to imitate the aromas present in natural fragrances, and to explore novel and distinctive olfactory experiences. The emergence of these synthetic components can be attributed to the high demand for perfumes during the 20th century. This demand led to elevated costs and a reduced accessibility of certain natural aromatic elements due to ethical concerns and safety considerations, “white musk” mentioned earlier is an example. Thus, the primary benefit of utilizing synthetic ingredients lies in the reduction of perfume costs when contrasted with natural one.[7] Moreover, the availability of specific raw materials exhibits irregularities from one year to the next, particularly when reliant on a singular geographic source. Indeed, they are highly susceptible to changes in agricultural practices, social matters and politics, natural disasters, climate (sun light, rainfall, temperature), soil and disease. In this way, by using synthetic alternatives, industries can avoid shortages of particular raw materials and ecosystems depletion due to over exploitation.[20]

Practically, synthetic fragrances are produced industrially in reactors through chemical synthesis from different raw materials. For instance, vanilla is the world’s most popular fragrance and is incorporated in a multitude of products. However, vanilla production is laborious, slow and costly. Vanillin, the main

compound of vanilla extract can be synthesized chemically from several precursor coming from different raw materials including the conversion of guaiacol, a petroleum based phenolic compound, the conversion of eugenol or isoeugenol from clove oil or the conversion of ferulic acid coming from lemon grass leaves. Nonetheless, the primary drawback of chemical synthesis of vanillin is its production of compound emerging often as an adverse mixture. To address this issue, biosynthesis can be employed and consists of using enzymes for the bioconversion and production of raw materials into fragrance materials.[27,28]

3. Allergens and allergies

Cosmetics can exert a significant impact on consumer's health, both positively and potentially negatively. Indeed, cosmetics contribute to maintain personal hygiene, offer different properties such as hydration or sun protection and improve appearance depending on the type of product. However, cosmetic products can be hazardous in some ways, especially because of the ingredients they contain. For instance, parabens and phthalates are well documented to be endocrine-disruptors associated with breast cancer.[29,30] On another issue, some fragrance ingredients can provoke allergies which can also affect consumer's health. They are called *fragrance allergens* and this category of ingredients will be discussed through this work.

3.1 Mechanisms of allergies

Fragrance allergens are associated with adverse reactions that some individuals may experience. Indeed, when an individual has been exposed to a significant amount of a fragrance allergen, often found in cosmetic products, it can lead to the development of a contact allergy to these fragrance ingredients. An allergy is defined as an abnormal, excessive, and unwanted immune system response to substances (i.e. allergens) that are normally harmless. According to the European Commission, the prevalence of contact allergy to fragrance substances can be estimated to 1-3% of the total population in Europe. This estimation involves the testing with eight common fragrance allergens, therefore the real prevalence could be higher considering the full spectrum of fragrance allergens.[31]

Several tests exist to determine if an individual is allergic to a substance, particularly in the case of fragrance allergies. Examples of these tests include patch tests and prick tests. In these tests, small amounts of various fragrance allergens are applied to patches, which are then placed on the skin for a specified period (a few minutes to 24h, 48h or more depending on the test).[32]

Basically, here is how a contact allergy, also called allergic contact dermatitis or more commonly known as eczema, works. Most fragrance allergens act as haptens. Alone, haptens do not trigger immune responses, but when they bind with larger carrier molecules or proteins found in the body, they form

complexes that the immune system recognizes as threats. This recognition leads to the production of specific antibodies, such as IgE. Therefore, the mechanism of the immune reaction of an allergy due to fragrances occurs within two phases : the sensitization and the elicitation. The first phase involves the initial exposure to a fragrance allergen, the formation of hapten-carrier complexes, the production of igE and their fixation on the mast cells. The elicitation phase occurs when a subsequent exposure to the same allergen leads to its binding with IgE antibodies on mast cells. This binding triggers the release of inflammatory mediators, such as histamine, which results in the familiar symptoms of an allergic reaction, including itching, redness, swelling, and skin rashes.[31,33]

Besides allergic contact dermatitis, other adverse reactions to fragrance involving the immune response or not can occur and include irritant contact dermatitis, photosensitivity, immediate contact reactions (contact urticaria), and pigmented contact dermatitis.[31]

3.2 Legislation

As mentioned earlier, Regulation No. 1223/2009 of the European Parliament and the Council dated November 30, 2009, regarding cosmetic products, enables the establishment of a regulatory framework. Applicable since Jully 11, 2013, this regulation aims mainly to enhance the safety of cosmetic products in the European Union (EU) by establishing stringent safety requirements for human health protection. To ensure this point, cosmetic companies have to provide a safety report before placing a cosmetic product on the market and to achieve its registration in the EU's Cosmetic Products Notification Portal. They also have to designate a "responsible person" for each product, to report serious undesirable effects and they must conform to specific packaging requirements, particularly labelling requirements.

To provide consumers with details regarding the composition of a cosmetic product, it is mandatory for each product to present an ingredient list and precautions for use on its packaging. Thus, it ensures that consumers with allergies or sensitivities receive the proper information about the presence of allergenic ingredients in the cosmetic products they choose to purchase. Ingredients have to be expressed using the International Nomenclature of Cosmetic Ingredients (INCI) and can be found in a glossary set up by the European Commission. While there is no requirement to specify the quantity of each ingredient, they are nevertheless arranged in descending order of prominence. However, ingredients present in concentrations of less than 1% can be listed in any order.

In some cases, merely informing consumers turns out to be insufficient to avoid undesirable effects on human health, therefore, the regulation No 1223/2009 includes a comprehensive list of ingredients that are either prohibited or subject to restrictions. This list is regularly updated by the European Commission on the basis of scientific advancements and new safety information. A notable example of prohibited

substances involves chloroform, once employed as a cosmetic ingredient. However, subsequent research revealed its carcinogenic effects in mice and potentially in humans.[34] Consequently, chloroform has been added to the list of prohibited ingredients by the European Commission in 1976. On the other hand, concerning substances subject to restriction, safe limits in terms of concentration or conditions of use are set by the European Commission and have to be respected by cosmetic manufacturers. Most of fragrance allergens fall within this last category of ingredients, requiring compliance with specified limits to ensure consumer safety.

To assist the European Commission in making decisions on various matters related to health and safety risks, independent scientific committees comprising external experts play a significant role. In 1999, the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) identified a group of **26 fragrance allergens** including **24 individual molecules and 2 natural extracts** well-known for their potential to cause allergies. However, no concentration limits for the safe use of these fragrance allergens have been determined. Therefore, the SCCNFP successor, the Scientific Committee on Consumer Safety (SCCS) published an opinion in 2012 standing for the establishment of threshold values for the concentration of allergens that present a substantial risk of sensitization to consumers. This threshold value is defined by the SCCS as the "lowest concentration giving a visible skin reaction in a continuous line of responses". In this report, the SCCS analyzed elicitation dose-response data coming from up-to-date published scientific literature so as relevant unpublished scientific data on fragrance ingredients provided by the International Fragrance Association (IFRA) as representative of the fragrance industry. Despite the limitation of human dose elicitation experiments for each allergen, the available studies suggest that a general exposure level of up to 0.8 µg/cm² of skin (corresponding to an estimated 0.01% in cosmetic products) may be tolerated by most consumers, including those with contact allergies. This exposure level (up to 0.01%) should prevent elicitation for the majority of allergic individuals, regardless of the lack of substance-specific data. Pragmatically, the thresholds of **0.01%** and **0.001%** were established for **rinse-off** (shampoos, shower gels, soaps, …) and **leave-on products** (face creams, body lotions, hand creams, …) respectively.

Consequently to this opinion, new labelling standards were implemented. Indeed, as stated in Article 19(1)(g) of the Regulation No 1223/2009, perfume and aromatic compositions can be referred to by the terms 'parfum' or 'aroma' in the ingredient list. Nevertheless, if the concentration of a fragrance ingredient, listed among the 26 fragrance allergens, exceeded 0.001% in leave-on products or 0.01% in rinse-off products, it had to be explicitly mentioned in the ingredient list in addition to using the terms 'parfum' or 'aroma'. Consumers are thus informed of the presence of these ingredients.

Regularly, the European Commission revises regulations through the incorporation of amendments. Consequently, on July 26, 2023, Regulation (EU) 2023/1545 modified Regulation No 1223/2009 by

introducing new substances and revising restrictions on previously listed fragrance allergens. As a result, the number of fragrance allergens has increased from 26 to **82**, encompassing **54 individual molecules** and **28 natural extracts**. Furthermore, two fragrance allergens previously listed are currently prohibited in Europe (Butylphenyl methylpropional (Lilial) and Hydroxyisohexyl 3- cyclohexene carboxaldehyde (Lyral)). All these fragrance allergens, more specifically individual molecules (and isomers), are listed in Annex III of the Regulation 1223/2009 and its amendment and can be found in **Table 2**.

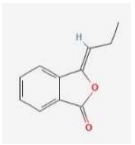
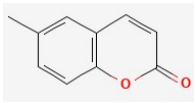
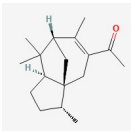
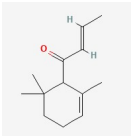
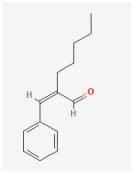
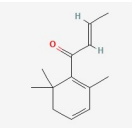
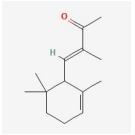
3.3 Fragrance allergens

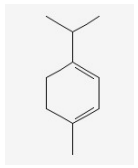
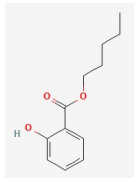
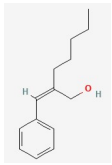
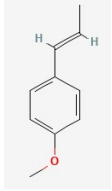
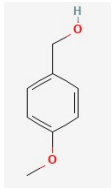
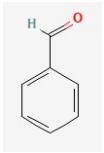
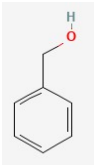
These fragrance allergens encompass a diverse array of chemical functions, including aldehydes, alcohols, ketones, terpenes, and more. Additionally, some of these allergens exist in multiple isomeric forms.

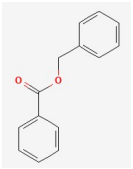
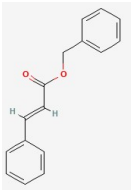
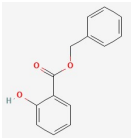
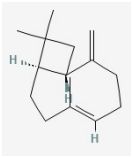
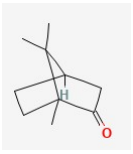
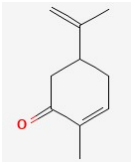
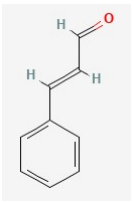
Regarding chemical aspects, fragrance allergens generally share similar physico-chemical characteristics : they are polar or semi-polar volatile (or semi-volatile) compounds characterized by boiling points above 170°C at atmospheric pressure and vapor pressure below 1 mmHg at 25°C.[11,35,36]

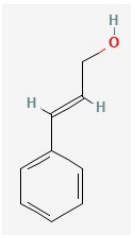
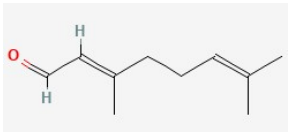
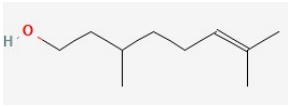
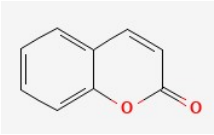
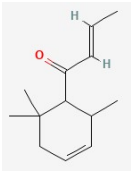
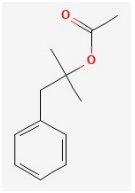
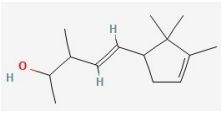
Table 2 also contains chemical structure, function, molecular formula and weight, boiling point, and vapor pressure of regulated individual fragrance allergens.

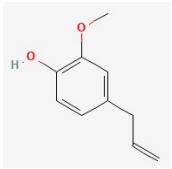
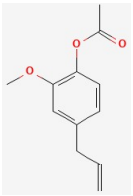
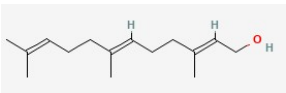
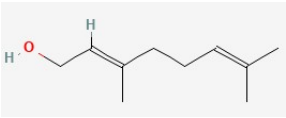
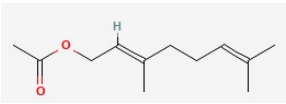
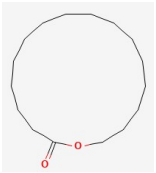
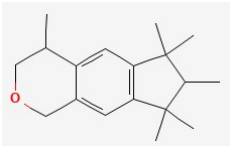
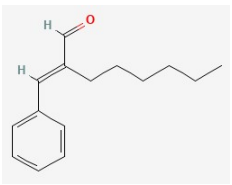
Table 2 – Basic information about the 54 regulated fragrance allergens[11,35,36]

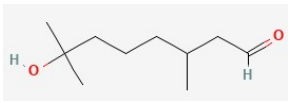
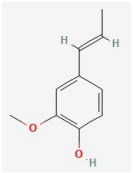
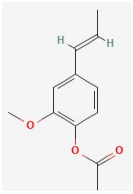
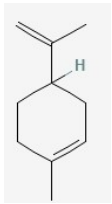
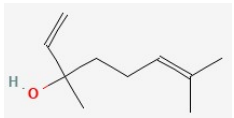
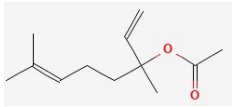
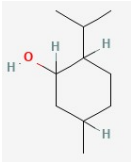
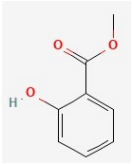
N°	Fragrance allergens	CAS number	Chemical structure	Regulation	Function	Molecular Formula	Molecular weight (g/mol)	Boiling Point at atmospheric pressure (°C)	Vapor Pressure at 25°C (mmHg)
1	**3-Propylidenephthalide**	17369-59-4		Amendment	Phthalides	$C_{11}H_{10}O_2$	174.2	173	
2	**6-Methylcoumarin**	92-48-8		Amendment	Lactone	$C_{10}H_8O_2$	160;17	304	
3	**Acetyl Cedrene**	32388-55-9		Amendment	Sesquiterpene	$C_{17}H_{26}O$	246.39	321	
5	**Alpha-Damascone** (cis-Rose ketone 1) **;** (Z)Alpha-Damascone (trans-Rose ketone 1)	43052-87-5 ; 23726-94-5 ; 24720-09-0		Amendment	Ketone	$C_{13}H_{20}O$	192.3	274	
4	**Amyl cinnamal** (Amylcinnamaldehyde / Amyl cinnamal)	122-40-7 ; 78605-96-6 ; 1331-92-6		1223/2009	Aldehyde	$C_{14}H_{18}O$	202.29	284	
6	**Damascenone (Rose ketone 4)**	23696-85-7		Amendment	Ketone	$C_{13}H_{18}O$	190.28	274	
7	**Alpha-Isomethyl ionone**	127-51-5		1223/2009	Ketone	$C_{16}H_{26}O$	206.32		

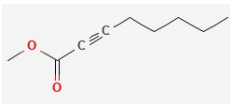
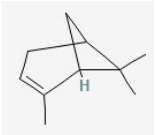
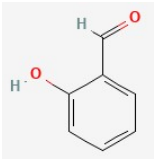
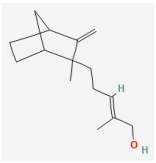
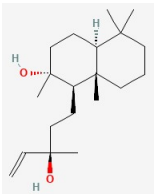
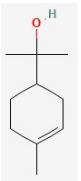
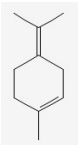
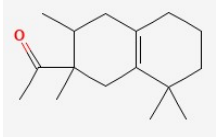
8	**Alpha-Terpinene**	99-86-5		Amendment	Monoterpene	$C_{10}H_{16}$	136.24	173 - 175	0.04
9	**Amyl Salicylate**	2050-08-0		Amendment	Ester	$C_{12}H_{16}O_3$	208.25	268	
10	**Amylcinnamyl alcohol**	101-85-9		1223/2009	Alcohol	$C_{14}H_{18}O$	204.31		
11	**Anethole**	104-46-1 ; 4180-23-8		Amendment	Phenylpropene	$C_{10}H_{12}O$	148.20	234	
12	**Anise alcohol**	105-13-5		1223/2009	Alcohol	$C_{10}H_{12}O$	148.20	259	
13	**Benzaldehyde**	100-52-7		Amendment	Aldehyde	C_7H_6O	106.12	179	1.27
14	**Benzyl Alcohol**	100-51-6		1223/2009	Alcohol	C_7H_8O	108.14	205	0.094

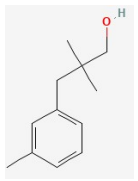
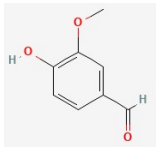
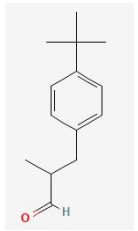
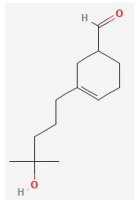
15	**Benzyl benzoate**	120-51-4		1223/2009	Ester	$C_{14}H_{12}O_2$	212.24	323	2.24E-04
16	**Benzyl cinnamate**	103-41-3		1223/2009	Ester	$C_{16}H_{14}O_2$	238.28	350	
17	**Benzyl salicylate**	118-58-1		1223/2009	Ester	$C_{14}H_{12}O_3$	228.24	320	7.80E-05
18	**Beta-Caryophyllene**	87-44-5		Amendment	Sesquiterpene	$C_{15}H_{24}$	204.36	256 - 259	
19	**Camphor** (dl-Camphor ; l-Camphor ; d-Camphor)	76-22-2 / (21368-68-3) ; 464-48-2 ; 464-49-3		Amendment	Ketone	$C_{10}H_{16}O$	152.23	204 - 209	0.65
20	**Carvone** (dl-Carvone ; l-Carvone ; d-Carvone)	99-49-0 ; 6485-40-1 ; 2244-16-8		Amendment	Ketone	$C_{10}H_{14}O$	150.22	230 - 231	
21	**Cinnamaldehyde**	104-55-2		1223/2009	Aldehyde	C_9H_8O	132.16	253	0.0289

22	**Cinnamyl alcohol**	104-54-1		1223/2009	Alcohol	$C_9H_{10}O$	134.18	250	
23	**Citral ; Geranial ; Neral**	5392-40-5 ; 141-27-5 ; 106-26-3		1223/2009	Aldehyde	$C_{10}H_{16}O$	152.23	229	
24	**Citronellol** (dl-Citronellol ; d-Citronellol ; l-Citronellol)	106-22-9 ; 26489-01-0 ; 1117-61-9 ; 7540-51-4		1223/2009	Alcohol	$C_{10}H_{20}O$	156.27	225	0.02
25	**Coumarin**	91-64-5		1223/2009	Lactone	$C_9H_6O_2$	146.15	298	9.80E-04
26	**Delta-Damascone (Rose ketone 3)** ; trans-Rose ketone 3 ; **Cis-Beta-Damascone** (cis-Rose ketone 2) ; trans-beta-Damascone (trans-Rose ketone 2)	57378-68-4 ; 71048-82-3 ; 23726-92-3 ; 23726-91-2		Amendment	Ketone	$C_{13}H_{20}O$	192.30	200	
27	**Dimethyl Phenethyl Acetate** (DMBCA)	151-05-3		Amendment	Ester	$C_{12}H_{16}O_2$	192.25		
28	**Ebanol**	67801-20-1		Amendment	Alcohol	$C_{14}H_{24}O$	208.34	287 - 288	

29	**Eugenol**	97-53-0		1223/2009	Phenol	$C_{10}H_{12}O_2$	164.2	253	0.0221
30	**Eugenyl Acetate**	93-28-7		Amendment	Ester	$C_{12}H_{14}O_2$	192.23		
31	**Farnesol**	4602-84-0		1223/2009	Alcohol	$C_{15}H_{26}O$	222.37	283 - 284	
32	**Geraniol**	106-24-1		1223/2009	Alcohol	$C_{10}H_{18}O$	154.25	229	
33	**Geranyl Acetate**	105-87-3		Amendment	Ester	$C_{12}H_{20}O_2$	196.29	242 - 245	
34	**Hexadecanolactone**	109-29-5		Amendment	Lactone	$C_{16}H_{30}O_2$	254.41		
35	**Hexamethylindano pyran** (Galaxolide)	1222-05-5		Amendment	Pyran	$C_{18}H_{26}O$	258.40		5.45E-04
36	**Hexylcinnamaldehyde**	101-86-0		1223/2009	Aldehyde	$C_{15}H_{16}O$	212.29		

37	**Hydroxycitronellal**	107-75-5		1223/2009	Aldehyde	$C_{10}H_{20}O$	172.26	260	
38	**Isoeugenol**	97-54-1 ; 5932-68-3 ; 5912-86-7		1223/2009	Phenolic Ether	$C_{10}H_{12}O_2$	164.2	266	0.0135
39	**Isoeugenyl Acetate**	93-29-8		Amendment	Ester	$C_{12}H_{14}O_2$	192.23		
40	**Limonene** (dl-Limonene ; d-Limonene ; l-Limonene)	138-86-3 ; 7705-14-8 ; 5989-27-5 ; 5989-54-8		1223/2009	Monoterpene	$C_{10}H_{16}$	136.24	178	1.55
41	**Linalool**	78-70-6		1223/2009	Alcohol	$C_{10}H_{18}O$	154.25	198	
42	**Linalyl Acetate**	115-95-7		Amendment	Ester	$C_{12}H_{20}O_2$	196.29	220	
43	**Menthol** (dl - Menthol ; l - Menthol ; d-Menthol)	89-78-1 ; 1490-04-6 ; 2216-51-5 ; 15356-60-2		Amendment	Alcohol	$C_{10}H_{20}O$	156.27	214 - 216	0.0637
44	**Methyl Salicylate**	119-36-8		Amendment	Ester	$C_8H_8O_3$	152.15	220 - 224	0.0343

45	**Methyl-2-Octynoate** (Folione)	111-12-6		1223/2009	Ester	$C_9H_8O_2$	148.16	217 - 220	
46	**Pinene** (Alpha-Pinene ; Beta-Pinene)	80-56-8 ; 7785-70-8 ; 127-91-3 ; 18172-67-3		Amendment	Monoterpene	$C_{10}H_{16}$	136.23	156	4.75
47	**Salicylaldehyde**	90-02-8		Amendment	Aldehyde	$C_7H_6O_2$	122.12	196 - 197	0.593
48	**Santalol** (Beta-Santalol ; Alpha-Santalol)	11031-45-1 ; 115-71-9 ; 77-42-9		Amendment	Sesquiterpene	$C_{15}H_{26}O$	222.37	311 - 313	
49	**Sclareol**	515-03-7		Amendment	Diterpene	$C_{20}H_{36}O_2$	308.49		
50	**Terpineol**	8000-41-7 ; 98-55-5 ; 138-87-4 ; 586-81-2		Amendment	Alcohol	$C_{10}H_{18}O$	154.25	218-221	0.0423
51	**Terpinolene**	586-62-9		Amendment	Monoterpene	$C_{10}H_{16}$	136.24	183 - 185	0.74
52	**Tetramethyl acetyloctahydronaphthalenes**	54464-57-2 ; 54464-59-4 ; 68155-66-8 ; 68155-67-9		Amendment	Ketone	$C_{16}H_{26}O$	234.38		

53	**Trimethylbenzenepropanol** (Majantol)	103694-68-4		Amendment	Alcohol	$C_{12}H_{18}O$	178.27		
54	**Vanillin**	121-33-5		Amendment	Phenolic Aldehyde	$C_8H_8O_3$	152.15	285 - 286	1.18E-04
	Butylphenyl methylpropional (Lilial)	80-54-6		1223/2009 (now Banned)	Aldehyde	$C_{14}H_{20}O$	204.31		
	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (Lyral)	51414-25-6 ; 31906-04-4		1223/2009 (now Banned)	Aldehyde	$C_{12}H_{22}O_2$	198.30		

Note 1 : Chemical structures presented in the cell corresponds to the first isomer listed when a compound possesses multiple isomers.

Note 2 : Fragrance allergen names listed in this table are the ones that must be utilized in cosmetic product labeling. Nonetheless, certain compounds may have multiple denominations (e.g. Amyl cinnamal).

4. Allergens analysis

The necessity of employing analytical techniques for the identification and quantification of fragrances becomes apparent for several applications.

Firstly, analytical techniques can be employed for quality control purposes. Indeed, quality control (QC) is crucial as it ensures that a manufactured product or service meets specific quality criteria and aligns with the expectations or requirements of the client or customer. This is particularly applicable for natural fragrances such as essential oils given that their composition can be influenced by several factors such as environmental conditions, manufacturing processes and storage. Thus, QC ensures the uniformity of the composition of the natural fragrances sold on the market. Moreover, QC can also be conducted on finished products to verify their compliance to the regulation concerning concentration limits for individual fragrance allergens. In Belgium, public institutions such as Sciensano conduct numerous analyses on behalf of government entities to ensure that products in the Belgian market adhere to regulatory standards.

Secondly, analytical methods can be used to address adulterations. Due to the elevated costs of essential oils, dealers may be tempted to adulterate the products by incorporating less expensive materials while maintaining the same high prices for the mixtures.

Ultimately, fragrances included in many soaps, shampoos, bathing products, household cleaners, and laundry items enter the aquatic environment via wastewater. As fate and potential effects of fragrance compounds are not well known, detecting these substance becomes relevant. [37,38]

Various chromatographic techniques enable the detection and quantification of fragrance allergens. However, in many cases, an extraction step before analysis is required. As such, the following sections cover sample preparation methods and chromatographic analysis.

4.1 Sample preparation

When analyzing fragrance allergens, matrices are complex, and the fragrance allergens contents are often at trace levels. The main objective of sample pretreatment is to remove matrix interferences (or matrix effect) and concentrate the specific analytes, thereby boosting the sensitivity of the analytical method. Consequently, sample pretreatment plays a crucial role in guaranteeing the accuracy of an analysis. There are various ways to classify pretreatment methods, and the following classification is inspired by Chen and collaborators.

4.1.1 Direct Injection or Dilution

The *direct injection* (also known as ready-to-inject) or dilution method involves introducing the liquid sample directly into an analytical instrument, either without any pretreatment or only with dilution. Direct injection remains a viable approach for simple formulations such as perfumes or liquid cosmetics with a very limited number of ingredients that do not affect the analytical instrument. However, it proves inadequate for complex matrices and formulations found in the majority of cosmetics and personal care products.[36]

4.1.2 Solvent Extraction

Liquid-liquid extraction (LLE) and *solid-liquid extraction* (SLE) are two widely used sample preparation techniques as they are economical, rapid, and simple. Typically, analytes are transferred from their initial matrix (liquid or solid/semi-solid) to an immiscible solvent based on differences in partition coefficients of solutes in the two phases. Therefore, this sample treatment is applicable to a broad range of cosmetic samples, including liquid and solid matrices. However, they pose challenges for routine high-throughput analysis due to manual operation, substantial organic solvent use, and relatively poor extraction efficiency. Recently, some approaches have gained interest to address these limitations. This includes the miniaturization of the LLE to reduce solvent consumption or the use of pretreatment methods such as ultrasound-associated extraction (UAE) and accelerated solvent extraction (ASE) for solid matrices.[11,36]

4.1.3 Solid Phase Extraction

Solid-phase extraction (SPE) stands out as a widely employed method for extracting organic compounds. Utilizing a packed-bed extraction system, SPE involves the entrapment of analytes on or in a suitable sorbent as the liquid sample passes through the sorbent bed. Subsequently, analytes are eluted using a solvent. Nevertheless, applying SPE to cosmetics analysis often necessitates prior sample pre-treatment, commonly achieved through simple dilution in water or an organic solvent like methanol to obtain a liquid phase.[11,39]

4.1.4 Matrix solid-phase dispersion (MSPD)

Matrix solid-phase dispersion (MSPD) emerges as a highly suitable analytical method for extracting contaminants from environmental and various matrices including fragrance allergens from cosmetic matrices. This technique, closely related to SPE, is favored for its flexibility, selectivity, and cost-effectiveness, offering efficient extractions with the added advantage of combining extraction and clean-up in a single step. The general procedure involves mechanically blending the sample (along with a

dispersant agent) directly in a mortar until a homogeneous and dispersed material is achieved. Subsequently, this material is transferred to a cartridge and compressed. Elution of analytes occurs by passing an appropriate solvent through the column, facilitated either by gravity or application of positive or negative pressure. The main drawback lies in the absence of automation making this procedure frequently less reproducible, more labor-intensive and time-consuming. Moreover, the utilization of large volumes of solvent present another disadvantage.[40–42]
However, *miniaturized-Matrix solid-phase dispersion* (μ-MSPD) implemented by Llompart and collaborators is an alternative with reduced solvent consumption.[43]

4.1.5 Headspace sampling Techniques

Headspace analysis involves the direct analysis of volatile compounds in the gas phase above a sample. This method is inherently simple and offers several advantages over traditional sample preparation techniques such as extraction, adsorption, precipitation, distillation, and more. When quantifying volatile components, headspace analysis provides advantages such as speed, simplicity, and the prevention of column degradation due to non-volatile residues.

4.1.5.1 Headspace sampling modes

4.1.5.1.1 Static Headspace sampling

Static Headspace (SHS) has found widespread use in fast analytical analysis due to its simplicity, ease of automation and its ability to analyze a diverse range of samples (usually liquids or solids). These advantages collectively render SHS as a suitable analytical method for quality control purposes. In this method, samples are enclosed in a vial to establish equilibrium at a specific temperature, and the headspace gas phase is withdrawn using a syringe before being injected into a Gas Chromatograph (GC) for analysis. This equilibrium is generally governed by the partitioning coefficient of the analyte between the headspace and the sample matrix.[44]

Nonetheless SHS presents some disadvantages. These include restricted sensitivity, limited choices of vials, the presence of broad early peaks stemming from large injection volumes, and outcomes dependent on the distribution coefficient. Another drawback is that not all analytes can be volatilized properly which can also affect the sensitivity of the method. Furthermore, and of significant importance, in standard SHS, where an equilibrium is established between two phases (solid-gas or liquid-gas), it becomes essential to optimize equilibration time and temperature. This optimization is essential to guarantee the establishment of stable equilibrium and, consequently, to achieve reproducible results. Another drawback of SHS is that solid analysis, even though possible, remains difficult to perform due to the non-homogeneous nature of solid samples. Finally, matrix effect can also impact SHS sampling

as it affects the release of the analytes into the headspace. This, consequently, poses challenges in terms of analyte recovery.[17,45]

Applications in determining fragrance allergens in various matrices through SHS (liquid, solid, semi-solid) has been reported. [46,47]

4.1.5.1.2 Dynamic headspace sampling

Dynamic headspace (DHS) also known as purge-and-trap method is a non-equilibrium continuous gas extraction process and a sampling mode encompassing three main steps[48] :

1. The constant renewal of the volatile fraction released from the matrix, facilitated by its constant removal through the flow of an inert gas over or through it.
2. The capture and concentration of components in the aforementioned flow stream through methods such as cryo-trapping or materials employing various approaches such as adsorption, sorption, …
3. The recovery of collected analytes from the trap through thermal desorption or solvent elution for subsequent analysis in a GC or GC-MS system.

This process leads to an increase in headspace sample size and, consequently, enhances the sensitivity of the technique. However, the absence of equilibrium can lead to reproducibility problems if the conditions are not carefully controlled. [17,48]

Figure 2 schematizes the two different mode of sampling (SHS and DHS).

4.1.5.1.3 Headspace sampling approach : Full evaporation headspace (FEHS)

There is a variant to headspace sampling for both static and dynamic which is full evaporation headspace (FEHS). HS operation temperature is typically set below the boiling point of the extractant, enabling analytes to attain gas–liquid equilibrium in the vial (for liquid samples). However, this limits the sampling of semi-volatiles with higher boiling points. FEHS technique overcomes this limitation by employing a higher HS operation temperature (>100°C), facilitating rapid and total evaporation of low volumes of solvent and analytes (<100 µL) into the vapor phase, achieving dynamic equilibrium (<10 min) quickly in standard headspace vials (20 mL). Given the sample small size, the resulting phase ratio (β) implies that, pragmatically speaking, it can be assumed that the entirety of the analytes have transitioned into the vapor phase as illustrated through Eq. 1. Therefore, FEHS is a non-discriminating sampling technique and it is particularly useful for complex matrices as it helps to overcome the potential matrix effects. [36,49–51]

$$\frac{M_G}{M_S} = \frac{\beta}{\kappa} \quad (1)$$

Where :

- M_S is the mass of analytes in the sample (liquid) phase
- M_G is the mass of analytes in the gas (headspace) phase
- β is the ratio of the volumes of the two phases (V_G/V_S)
- κ is the partition coefficient of the two phases (C_S/C_G)

4.1.6 High-Concentration-Capacity (HCC) techniques

Even though SHS and DHS are successful and allow a broad range of applications, in some cases, it remains challenging to depart from traditional extraction methods (often due to cost considerations) which typically involve the use of substantial amounts of solvent. The demand for solvent-free sample preparation techniques allowed the development of High-Concentration-Capacity techniques (HCC). HCC presents several advantages such as the facility of automation and integration to an analytical instrumentation, the low solvent consumption and the recovery of analytes belonging to different chemical classes thanks to accumulating materials, even if the specificity is low.[44]

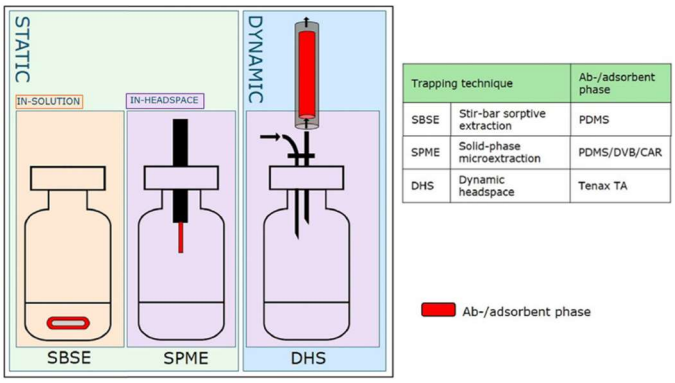


Figure 2 *– Comparison of SBSE, SPME, and DHS for the analysis of fragrance allergens (Adapted from Diez-Simon, 2020)*[52]

4.1.6.1 Solid Phase Microextraction (SPME)

The HCC techniques for analyzing fragrance allergens include the widely adopted *Solid Phase Microextraction* (SPME). This method serves as a valuable sample preparation technique for the extraction and concentration of compounds in a single step from a sample matrix prior to analysis. This process relies on a specialized fiber coated with various polymeric sorbents, allowing for the adsorption

of compounds. The targeted compounds captured on the fiber can be subsequently desorbed and quantified using Gas Chromatography (GC) or Liquid Chromatography (LC).[36,53]

SPME offers notable advantages, such as flexibility, environmental friendliness as it is a solvent-free extraction technique, and suitability for diverse sample matrices. However, the range of commercially available fiber coatings is currently limited. Presently, four main coatings—polydimethylsiloxane (PDMS), polyacrylate (PA), divinylbenzene (DVB), and carboxen (CAR)—are commonly utilized in SPME. It is crucial to highlight the significance of the coating material, as it significantly influences the extraction's capacity and efficiency. Certainly, bias in the extraction process can be introduced due to various phenomena, including discrimination against compounds with different polarities as a function of the chosen fiber's chemical nature. Another contributing factor to bias is the displacement effect occurring when fiber becomes saturated, where analytes with weaker affinities to the coating are primarily extracted during shorter extraction times, subsequently displaced by analytes with stronger affinities. Hence, conducting an optimization study becomes critical to identify the extraction optimum. This study should consider parameters such as extraction time, extraction temperature, sample volume, adsorbent composition and volume, and other relevant factors. Despite the current limitations in available coatings, SPME remains a versatile and effective technique for the analysis of fragrance allergens across a wide range of cosmetic products.[11,36,53]

A few studies have been published concerning the quantification of fragrance allergens in cosmetic matrices using SPME. Divišová and collaborators[54] (2015) quantified 24 fragrance allergens in skin care products and toiletries using GC-FID with a DB-WAX column. The optimal conditions consisted of PDMS/DVB fiber coating, 20% (w/v) NaCl, 20 minutes extraction time at 40°C in headspace mode. They obtained good determination coefficient (R^2) (≥0.999), a linear range included between 0.1 and 1000 µg/mL, good recoveries (≥ 80%) and low RSDs (0.5-5.8%). Published in 2022, Vazquez and collaborators[55], developed a SPME-GC-MS/MS to quantify 24 fragrance allergens in hand sanitizers. Experimental conditions were optimized leading to the use of DVB/CAR/PDMS fiber coating exposed during 20 minutes at 100°C to the HS over the samples diluted in ultrapure water. They obtained good determination coefficient (R^2) (≥0.99), a linear range between 0.01 and 5 µg/L, recoveries included between 80 and 120% and RSDs under 7%.

Two distinct sampling modes can be employed for SPME in fragrance analysis : Headspace Sampling Mode (HS-SPME) and Direct Immersion Sampling Mode (DI-SPME). HS-SPME, whether utilized in static or dynamic mode, involves the transfer of analytes through the headspace of the vial. This mode serves the dual purpose of safeguarding the fiber coating from damage caused by non-volatile compounds present in the matrix and, therefore, extending the lifespan of the fiber. Conversely, DI-SPME enables the extraction of less volatile compounds compared to HS-SPME and only involves one

equilibrium (sample/fiber) whereas HS-SPME involves two simultaneous equilibrium (sample/headspace and headspace/fiber). However, it comes with the drawback of potential contamination from matrix components, requiring a dilution step to mitigate its impact.[55,56]

4.1.6.2 Stir Bar Sorptive Extraction (SBSE)

Stir Bar Sorptive Extraction (SBSE) is a microextraction method that shares principles with DI-SPME, but with much higher total sorbtive capacity, specifically designed for volatile and semi-volatile compounds. In this technique, analytes are extracted from an aqueous matrix onto a magnetic stir bar typically coated with polydimethylsiloxane (PDMS). It is important to highlight that PDMS acts as an immobilized liquid phase, concentrating the sample through absorption rather than adsorption. This characteristic, combined with its high capacity, helps minimize or eliminate competition and displacement effects.[57,58]

Following the sampling process and a subsequent washing step, the PDMS-coated stir bar is introduced into a thermal desorption system from where analytes are transferred into the chromatographic system. In comparison to SPME, SBSE utilizes larger amounts of polymer coatings, resulting in enhanced sensitivity, favorable recoveries, and increased sample capacity.[39,57]

However, it's worth noting that certain aspects of the SBSE procedure, such as the manual washing of the stir bar, cannot be automated. This manual step poses a limitation, making SBSE less suitable for routine analyses.

4.2 Instrumental analysis

Several methods have been employed to analyze fragrances in cosmetic products. The most commonly employed methods include Gas Chromatography (GC) and High-Performance Liquid Chromatography (HPLC), coupled with various detection techniques. Here are some of the frequently used chromatographic and detection methods for this analysis :

4.2.1 Chromatographic separation

4.2.1.1 Gas Chromatography (GC)

Given that fragrance allergens are primarily volatile and semi-volatile compounds, *Gas Chromatography* (GC) is frequently well-suited for their separation. In the GC process, the sample undergoes vaporization and is introduced into a column, interacting with a stationary phase that coats the column walls. Separation of compounds occurs based on both volatility and the degree of interaction with the stationary phase. A carrier gas (mobile phase) transports the vaporized sample through the

column. Various column polarities—ranging from low to medium to strong—are reported in the literature and can be employed for fragrance allergens separation.[17,59]

4.2.1.2 Multidimensional Gas Chromatography (GCxGC)

Among various Fragrance Allergens, certain aldehydes and alcohols exhibit similar properties. The conventional GC-MS method sometimes leads to coeluted peaks which requires injection of the sample on multiple columns with different polarities. For instance, an application note from Shimadzu[60] and an analytical method published by IFRA[61] exemplify the use of two different columns in a simple GC setup to achieve comprehensive separation of all fragrance allergens. This problem of using two different columns may be solved by employing *comprehensive two-dimensional gas chromatography* (GCxGC) thanks to its higher resolution and peak capacity compared to monodimensional GC.[36]

4.2.1.3 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) has been employed rarely, given that GC adequately fulfills the requirements for the majority of fragrance allergens with straightforward instrument conditions and a quick analysis time. However, HPLC may be of interest for the analysis of low volatile and/or thermolabile compounds and for the coupling with some detector, such as Diode-Array Detector (DAD) since some fragrance allergens own chromophoric groups.[36,62]

4.2.2 Detection

Chromatographic instruments can be coupled with diverse detection systems. Here are some of the frequently used detectors for the fragrance allergen analysis.

4.2.2.1 Flame Ionization Detection (FID)

Flame Ionization Detector (FID) is widely used in gas chromatography for its reliability, simplicity, and high sensitivity to carbon-containing compounds. It operates by combusting the sample, generating ions that produce a measurable current. The FID provides a proportional response to the number of carbon atoms in a molecule.[17,59]

4.2.2.2 Mass Spectrometry (MS)

Mass spectrometry (MS) serves as an analytical tool for determining the mass-to-charge ratio (m/z) of one or more molecules within a sample. Every mass spectrometer comprises three essential components[59] :

- An ion source, which ionizes the sample and generates charged particles (ions) from the molecules. Examples of types of ion sources include electro ionization (EI), chemical ionization (CI), field ionization (FI), and more.
- A mass analyzer, responsible for sorting and separating ions based on their mass-to-charge (m/z) ratio. Common types include quadrupole, ion trap, and Time-of-flight (TOF).
- An ion detection system, which measures the separated ions. The data is then sent to a system where m/z ratios are stored along with their relative abundance.

Mass spectrometry plays a crucial role in identifying fragrances in both raw materials and finished products. While MS produces satisfactory results in matter of sensitivity, selectivity (that is much better that the FID) and resolution, further enhancement can be achieved through tandem MS for improved sensitivity and high-resolution MS for enhanced resolution.[17,63]

II. Objectives

This work aims to explore, develop, and validate a method for analysis of fragrance allergens in rinse-off cosmetic product (with an O/W matrix). The method is designed to assess compliance of cosmetic products with the limitations specified in Regulation 1223/2009 for the 54 regulated individual fragrance allergens. In other words, it would allow determining whether the concentration of allergenic fragrances in the sample exceeds the limit of 100 mg/kg or not.

The analysis is conducted using Gas Chromatography-Mass Spectrometry (GC-MS).

The primary requirement for the selected method is its suitability for routine quality control, meaning it should allow for reasonable time in manipulation and result interpretation, along with reasonable costs.

III. Materials and Methods

In this work, three different sample preparation methods have been tested and are described in the following section. These methods are referred to as :

- Method 1 (SPME)
- Method 2 (SHS)
- Method 3 (SHS according to Desmedt)

1. Materials preparation

1.1 Chemicals

The 54 fragrance allergens[a] and the two internal standards (1,4-Dibromobenzene and 4,4'-Dibromobiphenyl) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). It should be noted that fragrance allergens are separated into two mixes, namely Mix A1 and Mix A2, as outlined in **Table 4**. This division aims to reduce chemical reactions leading to analytes degradations. The first stock solution includes alcohols and terpenes lacking carbonyl functionality (Mix A1), while the second solution encompasses the remaining suspected allergens, specifically aldehydes and ketones (Mix A2). Isopropanol, methanol, hexane and cyclohexane (>99 %) were bought from Biosolve.

1.2 Samples

Different cosmetic products coming from "Les Ateliers du Saupont" (Bertix, Belgium) and containing concentrations of certain fragrance allergens above the limit (100 mg/kg), were utilized in this study. The composition for each cosmetic product used in this study are provided in **Table 3**. They were mainly shampoos and shower gels, both in the form of oil-in-water (O/W) emulsions.

Table 3 *– Composition of the cosmetic product used in this study*

Sample 1 (O/W emulsion)	Water, Dehydroxanthan Gum, Parfum, Panthenol, Xylitylglucoside, Anhydroxylitol, Xylitol, Glucose, **Benzyl Alcohol**, Dehydroacetic Acid

[a] Currently, Sigma-Aldrich sells a total of 64 compounds, comprising 54 fragrance allergens and certain isomers. All the fragrance allergens specified in Regulation 1223/2009 and its amendment are available, with the exception of 6-methylcoumarin.

Table 4 *– Repartition of fragrance allergens in the two mixes (Mix A1 and Mix A2) provided by Sigma-Aldrich*

Components	Mix	Components	Mix
Anise Alcohol	Mix A1	Sclareol	Mix A1
Benzyl Alcohol	Mix A1	trans,trans-Farnesol	Mix A1
Cinnamyl alcohol	Mix A1	trans-Anethole	Mix A1
Citronellol	Mix A1	Trimethyl-benzenepropanol (Majantol)	Mix A1
Ebanol 1	Mix A1	α-Amylcinnamyl alcohol	Mix A1
Ebanol 2	Mix A1	α-Pinene	Mix A1
Eugenol	Mix A1	α-Santalol	Mix A1
Geraniol	Mix A1	α-Terpinene	Mix A1
Isoeugenol (E)	Mix A1	α-Terpineol	Mix A1
Limonene	Mix A1	β-Caryophyllene	Mix A1
Linalool	Mix A1	β-Pinene	Mix A1
Menthol	Mix A1	β-Santalol	Mix A1
(+)-Carvone	Mix A2	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (major)	Mix A2
(*E*)-β-Damascone	Mix A2	Hydroxyisohexyl 3-cyclohexene carboxaldehyde (minor)	Mix A2
3-Propylidenephthalide	Mix A2	Isoeugenyl acetate	Mix A2
Amyl salicylate	Mix A2	Linalyl acetate	Mix A2
Benzaldehyde	Mix A2	Methyl 2-octynoate	Mix A2
Benzyl benzoate	Mix A2	Methyl salicylate	Mix A2
Benzyl cinnamate	Mix A2	Salicylaldehyde	Mix A2
Benzyl salicylate	Mix A2	Terpinolene	Mix A2
Butylphenyl methylpropional	Mix A2	trans-Cinnamaldehyde	Mix A2
Citral total	Mix A2	Vanillin	Mix A2
Coumarin	Mix A2	α-Acetyl cedrene	Mix A2
Damascenone	Mix A2	α-Amyl-trans-cinnamaldehyde	Mix A2
D-Camphor	Mix A2	α-Damascone	Mix A2
Dimethylbenzylcarbinyl acetate (DMBCA)	Mix A2	α-Hexylcinnamaldehyde	Mix A2
Eugenyl acetate	Mix A2	α-Isomethylionone	Mix A2
Galaxolide 1 (Hexamethylindanopyran)	Mix A2	α-Tetramethyl acetyloctahydronaphthalene (ISO E® α)	Mix A2
Galaxolide 2(Hexamethylindanopyran)	Mix A2	β-Damascone	Mix A2
Geranyl acetate	Mix A2	β-Tetramethyl acetyloctahydronaphthalene (ISO E® β)	Mix A2
Hexadecanolactone	Mix A2	δ-Damascone	Mix A2
Hydroxycitronellal	Mix A2	ϒ-Tetramethyl acetyloctahydronaphthalene (ISO E® ϒ)	Mix A2

1.3 Preparation of stock solutions and calibration solutions

<u>Note</u> : Due to unavailability, it was not possible to obtain the Mix A2 and include its analytes in the final method and therefore, achieving the objective to develop a method with the entirety of the newly regulated allergens.

- Stock solution of Mix A1 was prepared by transferring quantitatively the content of the vial (2 mL, with a nominal concentration of 1.5 g/L for all fragrance allergens) in a graduated flask of 10 mL in order to obtain a nominal concentration of 300 000 µg/L.
- Stock solutions of internal standards were prepared by weighing approximately 500 mg of 1,4-Dibromobenzene (IS1) and 100 mg of 4,4'-Dibromobiphenyl (IS2) respectively. Dissolutions were performed in 100 mL of methanol and 50 mL of cyclohexane respectively.

<u>Method 1 (SPME) and Method 2 (SHS)</u>

- Calibration solutions of 750 ; 1000 ; 1500 ; 2000 and 3500 µg/L were prepared in isopropanol. The dilution plan is provided in **Table 5**.
- A solution containing a mix of the two internal standards was prepared in isopropanol to obtain final concentrations of approximately 2000 µg/L and 2500 µg/L. Exact masses and concentrations are given in **Table 6**.

<u>Method 3 (SHS according to Desmedt)</u>

- Calibration solutions of 23.25 ; 45 ; 56.22 µg/mL were prepared in hexane. The dilution plan is provided in **Table 5**.
- A solution containing a mix of the two internal standards was prepared afterward in hexane to obtain a final concentration of approximately 500 µg/mL each. Exact masses and concentrations are given in **Table 6**.

Stock solutions and calibration solutions were stocked in glass bottle in darkness and in a freezer at -18°C.

Table 5 *– Dilution table of calibration solutions*

Method	Solution	Volume of stock solution of standards at 300 000 µg/L* (mL)	Final volume (mL)	Nominal concentration of standards
1 & 2	1	0.05	20	750 µg/L
	2	0.05	15	1000 µg/L
	3	0.075	15	1500 µg/L
	4	0.1	15	2000 µg/L
	5	0.175	15	3500 µg/L
3	1	0.775	10	23.25 µg/mL
	2	0.750	5	45 µg/mL
	3	0.937	5	56.22 µg/mL

Table 6 – Dilution table of internal standards

		Stock solutions			Mix			
Method	**Analyte**	**Masses (mg)**	**Final volume (mL)**	**Diluent**	**Volume of stock solution (mL)**	**Final volume (mL)**	**Final concentration of internal standard (µg/L)**	**Diluent**
1 & 2	SI 1	493.0380	100	Methanol	0.05	100	2465.2	Isopropanol
	SI 2	100.9400	50	Cyclohexane	0.1		2018.8	
3	SI 1	493.0380	100	Methanol	2	20	493.038	Hexane
	SI 2	100.9400	50	Cyclohexane	12*		484.512	

* From the dilution of the stock solution of IS2 in isopropanol (Final concentration = 807 520 µg/L)

2. Instrumentation

2.1 GC instrument and conditions

The chromatographic system used was a gas chromatography system (Trace 1300, Thermo Scientific) and all the equipment and conditions used for each method tested are summarized in **Table 7**. The capillary column was provided by Sigma-Aldrich (Zwijndrecht, the Netherlands).

Table 7 – Chromatographic conditions for the three methods employed

		Method 1 (SPME)	Method 2 (SHS)	Method 3 (SHS according to Desmedt)
Column	Type	SPB – 50 Fused silica capillary column Bonded; poly(50% diphenyl/50% dimethyl siloxane) phase		
	Dimension	30 m × 0.25 mm × 0.25 µm film thickness		
Injection	Split/Splitless mode	Split (Split ratio 12 mL/min)	Splitless	Splitless
	Temperature	250°C		
	Volume	/	2.5 mL	1 mL
Carrier gas		Helium (99.999 %) Alphagas 1 Air Liquide		
	Flow	1.2 mL/min		
Oven	Temperature program	80°C for 1 min, 10°C/min to 135°C for 2 min, 3°C/min to 170°C for 1 min, 10°C/min to 280°C	35°C for 1 min, 15°C/min to 135°C for 2 min, 3°C/min to 170°C for 1 min, 10°C/min to 280°C	
Run Time		39 minutes		

The choice of the capillary column and of the oven temperature program was made in accordance with the official IFRA method that quantifies 57 suspected allergens (and isomers) in ready to inject fragrance materials by GC-MS.

For method 2 (SHS) and method 3 (SHS according to Desmedt), splitless mode was used to improve sensitivity, whereas an initial oven temperature of 35°C was set to create a cold trapping effect, thereby mitigating peak broadening caused by the splitless injection.

2.2 MS equipment and conditions

Detection of fragrance allergens was performed using a mass spectrometer (ISQ single Quadrupole MS, Thermo Scientific) and all the equipment and conditions are summarized in **Table 8**.

Table 8 *– MS settings for the three methods employed*

Ion source type	Electronic impact (EI)
MS transfer line temperature	250°C
Ion source temperature	230°C
Mass range	35-320 amu
Scan Time	0.02192 sec

Masses were analyzed in full-scan mode (35-320 amu) and not in SIM mode as suggested by the IFRA method. Opting for the full scan mode offers a qualitative overview of the sample composition, especially valuable when dealing with real cosmetic matrices that may include interferences, as opposed to the analysis of standards only. Compounds were quantified and confirmed using extracted ion chromatograms of thee characteristic m/z – one m/z for quantitation and two m/z for confirmation. These masses are detailed in **Table 9** for each compound of the mix A1 and align with those utilized in the IFRA method for SIM mode.

2.3 Program for data acquisition and processing

The chromatograms and related information were obtained using Chromeleon 7.2.10. Peak smoothing were utilized when necessary in order to automate the peak integration by the program. The data obtained for validation were processed using R Studio software.

Table 9 – Masses used for quantification and confirmation for each compounds of the Mix A1 in the order of elution

	Compound	Quantitative Masse I (DA)	Confirming Masse I (DA)	Internal standard used
1	α-Pinene	93	91, 92	IS1
2	β-Pinene	93	91, 92	
3	α-Terpinene	121	136, 79	
4	Limonene	68	93, 67	
5	Linalool	71	93, 80	
6	Benzyl Alcohol	108	79, 107	
7	Menthol	138	71, 81	
8	α-Terpineol	136	93, 121	
9	Citronellol	69	95, 82	
10	Geraniol	69	93, 123	
11 (IS1)	1,4-Dibromobenzene	236	234, 238	
12	Trans Anethole	148	147, 117	
13	β-Caryophyllene	133	91, 93	
14	Anise Alcohol	138	137, 109	
15	Ebanol1	108	149, 164	
16	Cinnamyl Alcohol	134	91, 105	
17	Ebanol2	108	149, 164	
18	Eugenol	164	149, 103	IS2
19	Majantol	106	91, 105	
20	Isoeugenol	164	149, 131	
21	α-Santalol	93	94, 122	
22	Trans, trans-Farnesol	69	81, 93	
23	β-Santalol	93	94, 122	
24	Amylcinnamyl Alcohol	133	204, 115	
25	4,4'-Dibromobiphenyl	312	314, 310	
26 (IS2)	Sclareol	202	95, 109	

3. Methods

3.1 Standard separation and peak identification

A liquid injection (direct injection) of the standard Mix A1 and the internal standards was performed in full-scan in order to identify analytes and set correctly their retention windows. The nominal concentrations were at 3000 µg/L for the standard Mix A1 and 4000 µg/L for 4,4'-Dibromobiphenyl and 10 000 µg/L for 1,4-Dibromobenzene. The injection volume was 1 µL. Chromatogram is provided in **Figure 6** in the "Results and Discussion" section.

3.2 Method 1 (SPME)

3.2.1 Sample processing

0.75 g of shampoos or shower gel were weighted in a graduated flask of 5 mL, dissolved with distilled water and homogenized correctly. Afterwards, 0.5 mL of these solution were introduced in another graduated flask of 5 mL, made up to the mark with isopropanol and homogenized.

3.2.2 Extraction method

Headspace vials of 20 mL were sealed with aluminum caps furnished with Teflon-faced septa after introducing 8 µL of the standard Mix A1 and 8 µL of the internal standard mix. Vials were then allowed to equilibrate for 20 minutes at 100°C in a homemade apparatus (**Figure 3**) before placement of SPME device. Thereafter, the septa were pierced with the SPME needle and the fiber was exposed to the headspace for 15 minutes at 100°C. After 15 minutes, the fiber was retracted and desorbed into the injection port of the GC-MS where desorption of the analytes from the fiber occurred. All these steps were performed manually. Technical information about the SPME device are given in **Table 10**.

Fiber were conditioned according to the manufacturer's instruction prior to its use (30 minutes at 250°C). Moreover, the fiber was thermally cleaned during 15 minutes, with the injector temperature set to 250°C, before the initial measurement of the day.

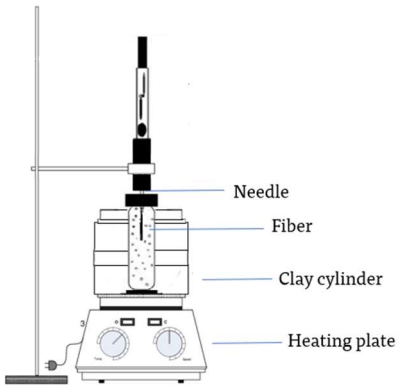


Figure 3 *– Scheme of the SPME apparatus used*

Table 10 – SPME technical information

Fiber coating	Divinylbenzene Polydimethylsiloxane overcoated (PDMS/DVB (+OC))[b]
Film thickness	65 µm (+10 µm)
Maximum temperature allowed	270 °C
Recommended operating temperature	200-270 °C

3.2.2.1 Validation procedure

The parameters used for the validation of these two methods are based on the IUPAC Harmonized Guidelines and EURL-FA Guide.[65]

These parameters are the selectivity, the determination coefficient (R^2) of the retained model, the trueness of the calibration solutions via their recovery, the trueness and the precision of the methods via the recovery and the relative standard deviation (RSD) of the blank matrix samples.

Experimental design

A validation was performed for the two extraction methods tested in the first approach (HSSPME and SHS). Five days of complete validation were accomplished (calibration + recovery test with blank spiked matrix) and one additional day was done for calibration only. The calibration was performed at the concentrations 750 ; 1000 ; 1500 ; 2000 ; 3500 µg/L, dilution plan is available in **Table 5**. An additional "0 µg/L" concentration (blank) was included at the beginning of the sequence to confirm the absence of analytes. The recovery test followed the outlined procedure :

- 0.75 g of blank matrix (Baktolin®, see composition in **Table 11**) was weighted in a graduated flask of 5 mL and filled to volume with distilled water..
- After mixing, 0.5 mL of the solution was transferred in another graduated flask of 5 mL and 1.5 mL of the Mix A1 at 5000 µg/L was added, the flask was then made up to the mark with isopropanol. This resulting solution had a concentration of 1500 µg/L reflecting the concentration that would be obtained if an allergen initially present at 100 µg/mg underwent a similar dissolution and dilution process.

[b] The choice of this fiber coating was made after consulting literature comparing three types of fibers (PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS), it turns out that PDMS/DVB was evaluated as the most effective for extracting the studied fragrance allergens. [54,64] However, the only fiber available at Merck was a PDMS/DVB overcoated.

It should be noted that due to concerns about standard Mix savings, it was not possible to spike the blank matrix during the initial dissolution in water, as a significant amount of standard would have had to be used to achieve a more concentrated solution.

Table 11 *– Composition of blank matrix (Baktolin®)*

Baktolin ® pure wash	Water, Sodium Laureth Sulfate, Sodium Chloride, PEG-7 Glyceryl Cocoate, Cocamidopropyl Betaine, Glycerin, Disodium Laureth Sulfosuccinate, Sodium Benzoate, PEG-120 Methyl Glucose Dioleate, Sodium Citrate

Calculations

Firstly, the **linearity** of the calibration solutions for each day was achieved thanks to a partial F-test. The partial F-test is the most common method of testing whether the full model is significantly better than the reduced model. In this context, it studies whether incorporating a non-linear term leads to a noteworthy enhancement in model fit compared to a simpler linear model.

Therefore, the null hypothesis (H_0) states that adding non-linear terms does not significantly improve the model fit, in this case, the linear model represents adequately the relationship between the independent variable(s) and the dependent variable. However, the alternative hypothesis (H_1) suggests that there is a significant improvement in model fit when non-linear terms are added, which indicates that another model than linear has to be used (the quadratic model in this study).

H0 is accepted if $F_{obs} < F_{critical}$ and the relationship between variables is linear, otherwise it is quadratic. F_{obs} is calculated as in Eq.2 :

$$F_{obs} = \frac{\frac{RSS_{reduced} - RSS_{full}}{df_{reduced} - df_{full}}}{\frac{RSS_{full}}{df_{full}}} \quad (2)$$

Where :

- $RSS_{reduced}$ is the regression sum of squares of the reduced model
- RSS_{full} is the regression sum of squares of the full model
- $df_{reduced}$ is the number of degree of freedom for the reduced model
- df_{full} is the number of degree of freedom for the full model

$F_{critical}$ was determined with p = 0.05, $df_{reduced} = 1$ and $df_{full} = 2$

The **coefficient of determination** (R^2) of the retained model was obtained via the linear correlation coefficient between the predicted values :

$$R^2 = corr\,(\hat{y}, y)^2 \tag{3}$$

Accuracy of the method was also evaluated via the trueness and the precision. On the one hand, **trueness** is the expression of how close the mean of the results obtained ($\bar{x}$) is to the true value (x_{spiked}) and is assessed through the "bias" or the "recovery" expressed as in Eq.4 :

$$Rel\ Bias = \frac{\bar{x} - x_{spiked}}{x_{spiked}}\ x\ 100 \tag{4}$$

On the other hand, **precision** is evaluated thanks to the replications via an analysis of variance (ANOVA). This approach allows to simultaneously determine repeatability and reproducibility respectively via the relative standard deviation of repeatability (RSD_r) and the relative standard deviation of reproducibility (RSD_R) expressed as a percentage as detailed in the Eurachem Guidelines.[66]

$$s_r = \sqrt{MS_{within}} \tag{5}$$

$$s_R = \sqrt{\frac{MS_{between} + (MS_{within} \times (n-1))}{n}} \tag{6}$$

$$RSD_r = \frac{s_r}{x_{spiked}} \quad ; \quad RSD_R = \frac{s_R}{x_{spiked}} \tag{7}$$

With :

- s_r and s_R respectively the standard deviation of repeatability and reproducibility
- MS_{within} and $MS_{between}$ respectively the mean square representing variation within samples and variation between different groups.
- n, the total sample size

3.3 Method 2 (SHS)

3.3.1 Sample processing

The same sample processing as for method 1 (SPME) has been performed.

3.3.2 Extraction method

As for method 1 (SPME), headspace vials of 10 mL were sealed with aluminum caps furnished with Teflon-faced septa after introducing 10 µL of the standard Mix A1 and 10 µL of the internal standard mix. The experiment was conducted in an automated manner using an autosampler equipped with a Combi-Pal (Pal System, Switzerland) as shown in **Figure 4**. The equilibration time and temperature were respectively of 10 minutes and 150°C. Extraction and sampling conditions are given in **Table 12**.

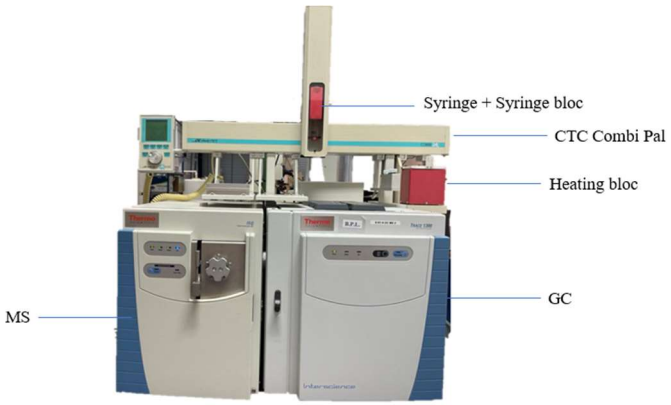


Figure 4 *- Instrumentation used for method 2 (SHS)*

Table 12 *– Extraction and sampling conditions used for method 2 (SHS)*

	Method 2
Injection gas volume	2.5 mL
Equilibration temperature	150°C
Equilibration time	10 min
Agitation speed (shaking)	250 rpm
Syringe temperature	150°C
Fill speed	1 mL/s
Injection speed	1 mL/s

3.3.3 Validation procedure

The same validation procedure (experimental design and calculations) as method 1 (SPME) has been applied for method 2 (SHS).

3.4 Method 3 (SHS according to Desmedt)

A third method, along with a distinct validation procedure than method 1 (SPME) and method 2 (SHS), was employed in an attempt to replicate the findings reported by Desmedt and collaborators. This method was applied after the completion of the two other methods.

3.4.1.1 Sample processing and extraction method

For each sample extraction, 0.3 g of the cosmetic formulation (see **Table 3**) were weighted in a centrifuge tube of 15 mL and 0.05 mL of internal standard mix were added. Tubes were then placed 10 minutes in the fridge (4°C) to allow the penetration of the standard into the matrix while reducing loss through volatilization. Afterwards, 5 mL of hexane and around 0.3 g of NaCl were added. An orbital agitation of the samples during 10 minutes constituted the following step. Tubes were then placed in the freezer (-18°C) overnight in order to perform a clean-up step (precipitation of matrix components). Subsequently, centrifugation for 1 min at 4500 rpm was performed and four times 0.75 mL of the supernatant were transferred to glass vials with screw caps. To create a standard addition curve, 0.1 mL of the solutions at 0 ; 23.25 ; 45 ; 56.22 µL/mL of the standard mix A1 were added respectively to the glass vials. Finally, 300 µL of the mixed solutions were transferred in headspace vials for analysis. Second extraction and sampling conditions are given in

Table 13.

Table 13 *– Extraction and sampling conditions used for method 3 (SHS according to Desmedt)*

	Method 2
Injection gas volume	1 mL
Equilibration temperature	135°C
Equilibration time	10 min
Syringe temperature	140°C
Agitation speed (shaking)	250 rpm
Fill speed	1 mL/s
Injection speed	1 mL/s

3.4.1.2 Validation procedure

To ensure a consistent comparison with the method described by Desmedt and collaborators, it was imperative to employ the same validation procedure. Therefore, the validation approach adopted was rooted in the accuracy profiles methodology, which relies on the total error approach. This methodology simultaneously combines both systematic error (assessed through biases, indicating method trueness) and random error (assessed through Relative Standard Deviations, reflecting method precision). Instead of employing an array of statistical tests, the accuracy profile methodology relies on a singular statistical decision approach—specifically, the computation and comparison of β-expectation tolerance intervals for each concentration with acceptance threshold. These intervals represent regions where a defined proportion (i.e., β) of future results is expected. Unlike the classical validation methodology, the advantage lies in the simultaneous consideration of precision and trueness. This is crucial because solely examining them separately, based on the "null hypothesis," can lead to ambiguous conclusions. As demonstrated by Feinberg and collaborators, using this strategy suggests that a less precise method can be validated more easily than a more precise one. Consequently, this validation methodology exhibits greater robustness than the classical approach.[67,68]

Furthermore, when applied across different concentration levels, the accuracy profile yields a graphical representation encompassing essential validation information, including relative bias, β-expectation tolerance intervals for each concentration, and quantification limits (LOD & LOQ). Depending on the specific goals of the analytical method, an acceptability limit must be established, aligning with end-user requirements. For instance, this limit, expressed as a percentage of the target value, could be 1% for bulk materials, 5% for pharmaceutical specialties, 15% for biological samples, and so forth. This acceptability limit is also visually represented on the accuracy profile.[67,69]

The parameters used for this validation align with those employed by Desmedt and his collaborators, specifically, the selectivity and the specificity, the linearity of the standard addition curves, the linearity of the results, the trueness, the precision and the recovery.

Experimental design

According to Desmedt and his collaborators, the validation was performed on 3 separate days. The same procedure as for the sample (detailed upward “sample processing and extraction method”) was employed with a blank matrix (see composition in **Table 11**) spiked at 3 levels (A, B, C) respectively 25 ; 100 and 150 mg/kg of the fragrance mix A1 in triplicate. **Figure 5** represents the experimental design of the performed validation.

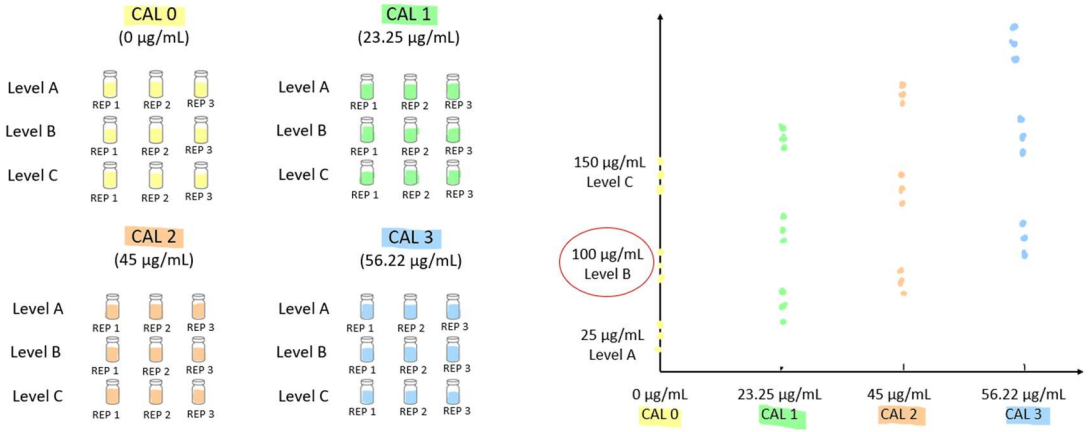


Figure 5 *– Validation experimental design employed for method 3 (SHS according to Desmedt)*

Calculations

For the building of accuracy profiles, the methodology outlined in several articles[70–72] was used and calculations are detailed hereafter.

The following index are used :

- p ; the number of series *i*, corresponding to the number of days of the validation, here, three days,
- m ; the number of level *j*, corresponding to the number of standard concentrations evaluated and used to spike the blank matrix, here three levels (25 ; 100 and 150 mg/kg),
- n ; the number of repetition *k*, corresponding to the number of method replicate for each day and each level. Here three repetitions were performed.

As previously discussed, total error forms the basis of the accuracy concept. An approach to quantify this total error involves calculating the β-expectation tolerance interval (β_{ETI}) and subsequently comparing it to the acceptability limits (λ). This limit was set at 20% similarly to the article and considering the concentrations analyzed and the type of matrix. Consequently, the expression for total error can be written as follows :

$$Total\ Error = Rel\ Bias_j \pm \beta_{ETI,j} \quad (8)$$

Where :

- $Rel\ Bias_j$ is the relative bias (e.g. the difference between the mean of the back-calculated concentrations ($\bar{x}_{calc,j}$) and the known concentration ($x_{known,j}$) for each level j) :

$$Rel\ Bias_j = \frac{\bar{x}_{j,calc} - x_{j,known}}{x_{j,known}} \quad (9)$$

In this work, the back-calculated concentrations correspond to the concentrations (in absolute value) obtained by interpolating the standard addition curves respectively or via this basic equation :

$$\bar{x}_{calc,j} = \left|\frac{-b}{a}\right| \quad (10)$$

With :

- $-b$; the intercept of the standard addition curve with the y axis,
- a ; the slope of the standard addition curve

Note : A linear model was choose here. Indeed, the appropriate model should be choose in such a way that the residual error is minimized as much as possible.

The relative bias allows to assess the trueness for each level j. Precision, on the other hand, is evaluated through the $RSD_{Reproducibility,\ j}$, the relative standard deviation of reproducibility for each level j and can be calculated via this method :

1) Calculation of the mean square model (MSM_j) and the mean square error (MSE_j) for each level j

$$MSM_j = \frac{1}{p-1}\sum_{i=1}^{p}\left(\bar{x}_{ij,calc} - \bar{x}_{j,calc,j}\right)^2 \quad (11)$$

Where $\bar{x}_{ij,calc}$ is the mean of the back-calculated concentrations for each day i and each level j

$$MSE_j = \frac{1}{\sum_{i=1}^{p} n_{ij} - p}\sum_{i=1}^{p}\sum_{k=1}^{n_{ij}}\left(x_{ijk\,calc} - \bar{x}_{ij,calc}\right)^2 \quad (12)$$

With $x_{ijk\,calc}$, the back-calculated concentration obtained for each day i, each level j and each repetition k.

2) Calculation of the variance within (between repetitions for a level j) and the variance between (between days for a level j)

If MSMj > MSEj, then :

$$\sigma^2_{within,j} = MSE_j \tag{13}$$

$$\sigma^2_{between,j} = \frac{MSM_j - MSE_j}{n} \tag{14}$$

Otherwise :

$$\sigma^2_{within\ ,j} = \frac{1}{pn-1}\sum_{i=1}^{p}\sum_{j=1}^{k}\left(x_{ijk\ calc} - \bar{x}_{j,calc}\right)^2 \tag{15}$$

$$\sigma^2_{between,j} = 0 \tag{16}$$

3) Calculation of the $RSD_{Reproducibility,\ j}$

$$\sigma^2_{Reproducibility,j} = \sigma^2_{within\ ,j} + \sigma^2_{between,j} \tag{17}$$

$$SD_{reproducibility,j} = \sqrt{\sigma^2_{Reproducibility,j}} \tag{18}$$

$$RSD_{reproducibility,j} = 100\ .\frac{SD_{reproducibility,j}}{x_{j,known}} \tag{19}$$

Therefore, β-expectation tolerance interval is expressed as :

$$\beta_{ETI,j} = t_{\left(\nu;\frac{1+\beta}{2}\right)} \times \sqrt{1 + \frac{1}{pnB_j^2}} \times \mathrm{R}SD_{R,j} \tag{20}$$

Where :

- ν is the degree of freedom (see Eq. 7)
- β is the portion of measurement that fall inside the interval β_{ETI} and is fixed at 0.95
- B_j is the bias for each level j (see Eq. 8)
- $RSD_{R,j}$ is the relative standard deviation of reproducibility for each level j

The term ν is calculated as follow :

$$\nu = \frac{\left(R_j + 1\right)^2}{\frac{\left(R_j + (1/n)\right)^2}{p-1} + \frac{1-(1/n)}{pn}} \tag{21}$$

With R ; the quotient of the variance between and the variance within for a level j :

$$R_j = \frac{\sigma^2_{between\ j}}{\sigma^2_{within\ j}} \tag{22}$$

While B_j is calculated as follow :

$$B_j = \sqrt{\frac{R_j + 1}{nR_j + 1}} \tag{23}$$

Finally, the upper and lower β-expectation tolerance limits can be computed via Eq. 9 and Eq. 20 :

$$\begin{aligned} \beta_{j,lower\ limit} &= Rel\ Bias_j\ - \beta_{ETI,j} \\ \beta_{j,upper\ limit} &= Rel\ Bias_j\ + \beta_{ETI,j} \end{aligned} \tag{24}$$

IV. Results and Discussion

1. Standard separation and peak identification

The direct injection in full-scan produced the chromatogram in **Figure 6**. All fragrance allergens were successfully identified, except for Sclareol. It is worth mentioning that certain compounds (benzyl alcohol, citronellol, geraniol, β-Santalol, Amylcinnamyl alcohol) exhibit reduced intensities. This observation may be attributed to the use of isopropanol as the dilution solvent, which contains a hydroxyl group capable of causing degradation.[61] Notably, this effect may be more pronounced in the case of Sclareol.

The peak resolution remains satisfactory, even in the presence of two coelutions (Ebanol1 and Cinnamyl alcohol ; β-Santalol and Amylcinnamyl alcohol). Fortunately, the use of mass spectrometry allows for the identification of these compounds despite coelutions.

Therefore, selectivity and specificity for the three methods were ensured by extracting specific masses of the fragrance allergens in full scan mode.

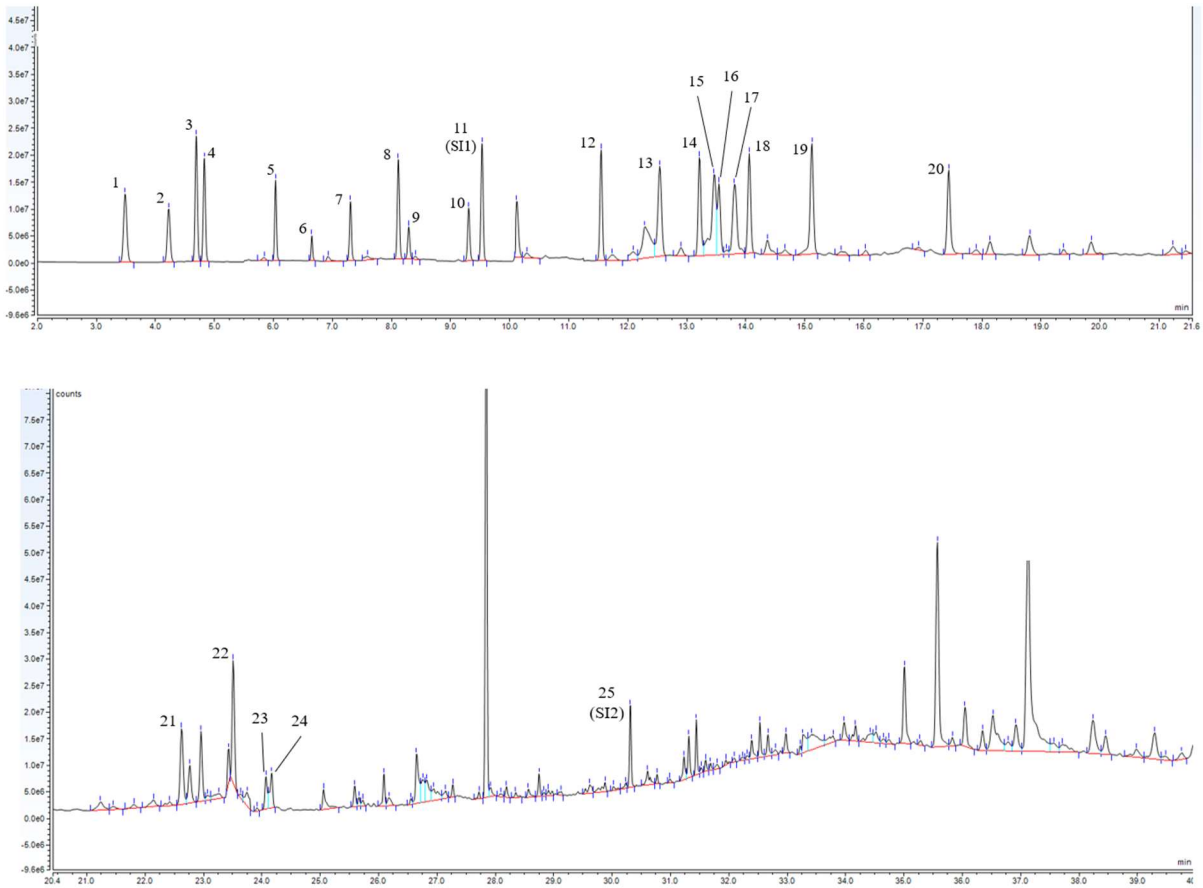


Figure 6 *– Chromatogram (TIC) of fragrance allergens Mix A1 (identification see* ***Table 9****) and the two internal standards (IS1 & IS2) in direct injection in full-scan mode.*

2. Method 1 (SPME) and Method 2 (SHS)

2.1 Performance characteristics

2.1.1 Specificity and selectivity

Specificity and selectivity are ensured through the extracted ions. **Figure 7** and **Figure 8** present chromatograms for the validation of both methods, method 1 (SPME) and method 2 (SHS), at a concentration level of 1000 µg/L.

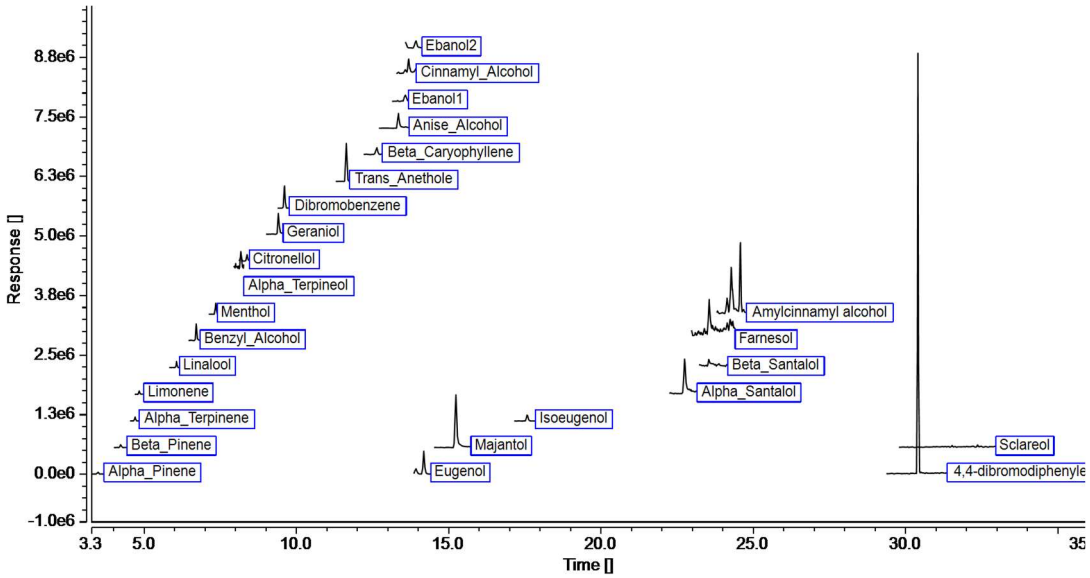


Figure 7 – Chromatogram (MS Quantitation) for method 1 (SPME) extraction of the validation calibration solution of Mix A1 at 1000 µg/L (Day 3) and the two internal standards

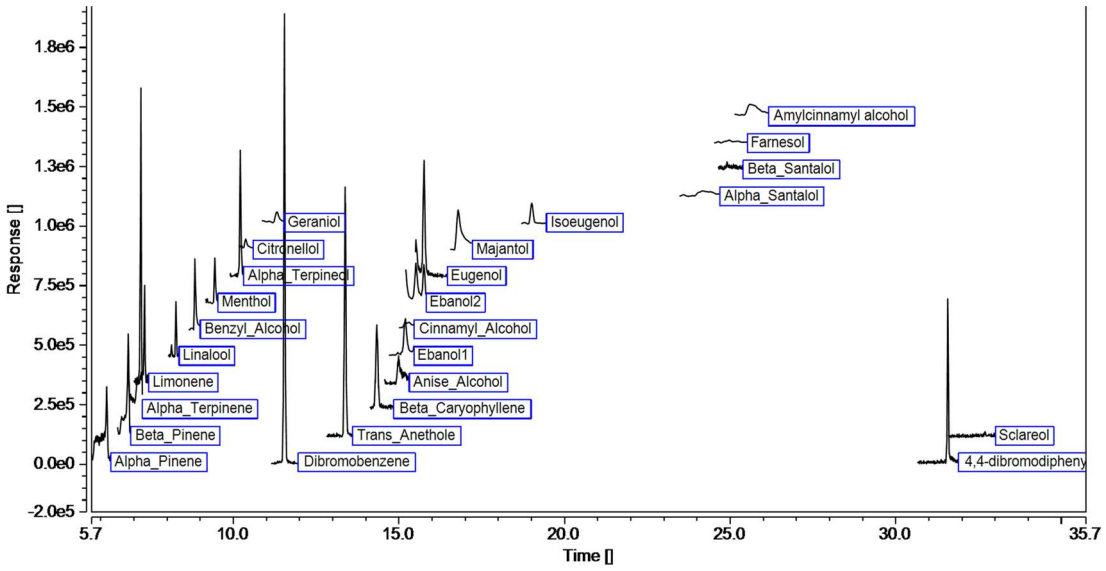


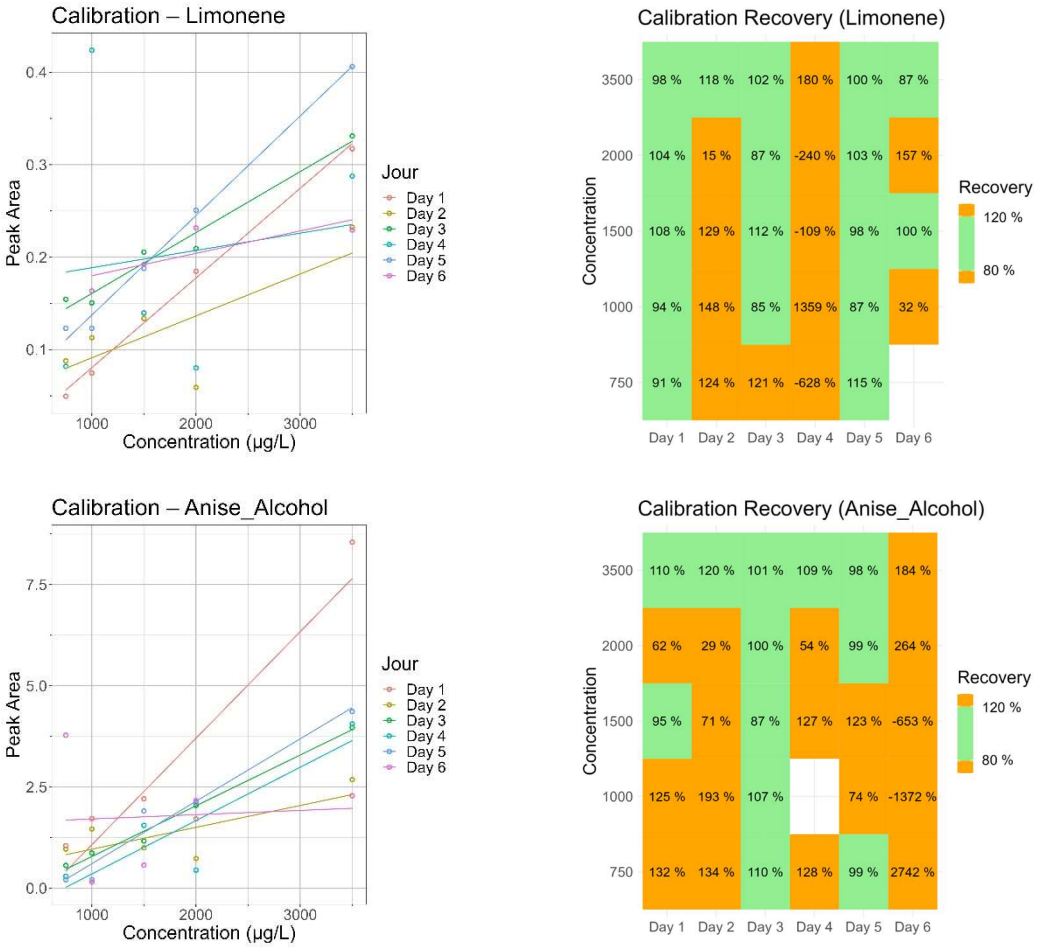
Figure 8 – Chromatogram (MS Quantitation) for method 2 (SHS) extraction of the validation calibration solution of Mix A1 at 1000 µg/L (Day 3) and the two internal standards

2.1.2 Calibrations

A calibration was performed for the two methods for each day and each fragrance allergen. It turns out that p values obtained via partial F-tests were globally above the significance threshold which indicates that the relationships between areas under the curves and concentrations injected are globally linear.

Concerning the determination coefficients (R^2) for each calibration, almost half of these values were below 0.9 indicating considerable random error. Regarding the recoveries of the calibration solutions, they are very variable from one day to another and exhibit limited trueness for both methods, except for the highest concentration (3500 µg/L) which remains within an acceptable range.

The daily calibrations and recoveries of calibration solutions for four compounds are represented hereafter for method 1 (SPME) (**Figure 9**) and for method 2 (SHS) (**Figure 10**). **Annexe 1** et **Annexe 2** present R^2 and p values obtained for each day for these 4 compounds and for both methods.



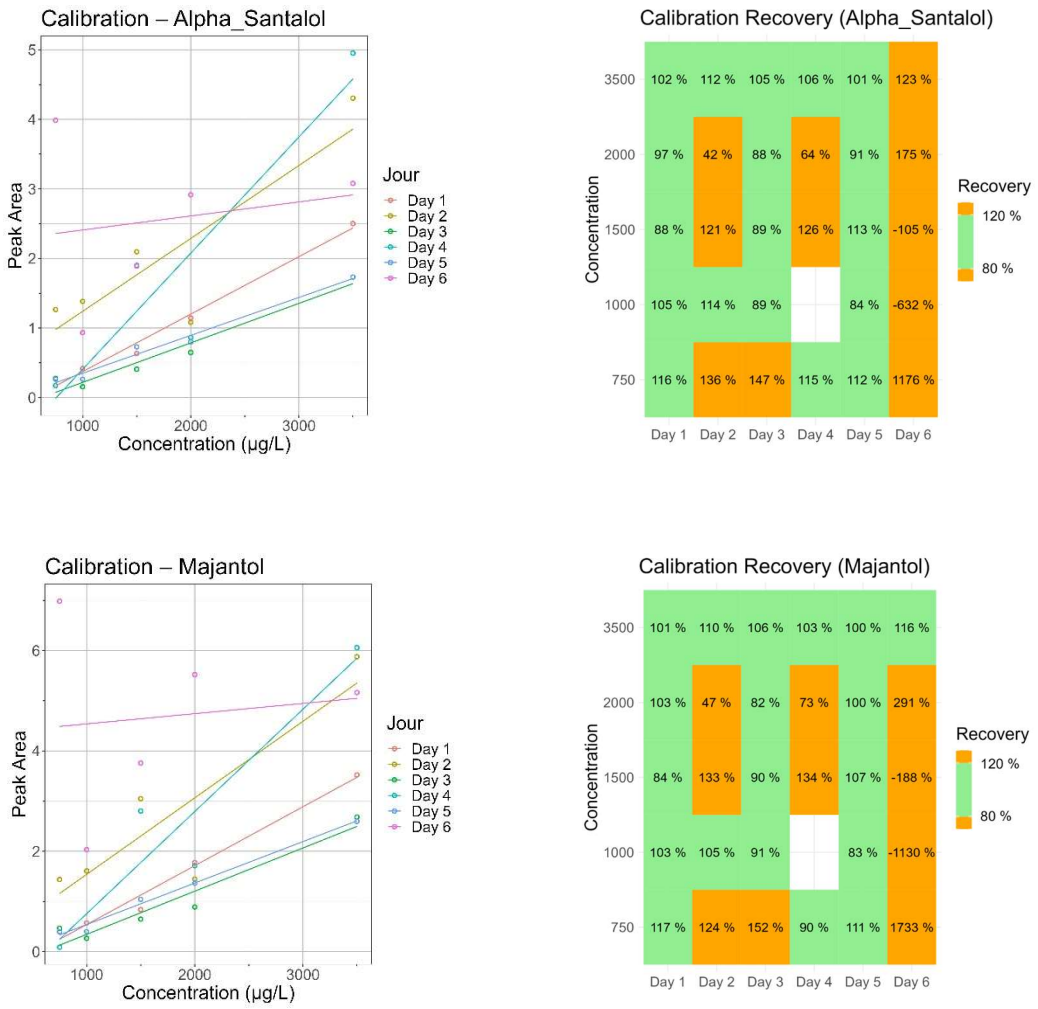
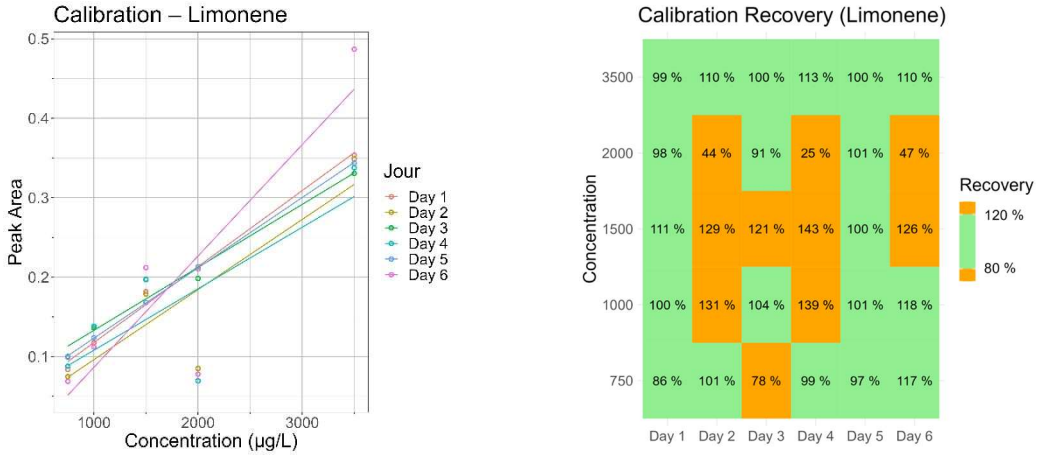


Figure 9 *– Daily calibrations and associated recoveries acquired during validation of method 1 (SPME) for four fragrance allergens (Limonene, Anise Alcohol, Alpha Santalol, Majantol)*



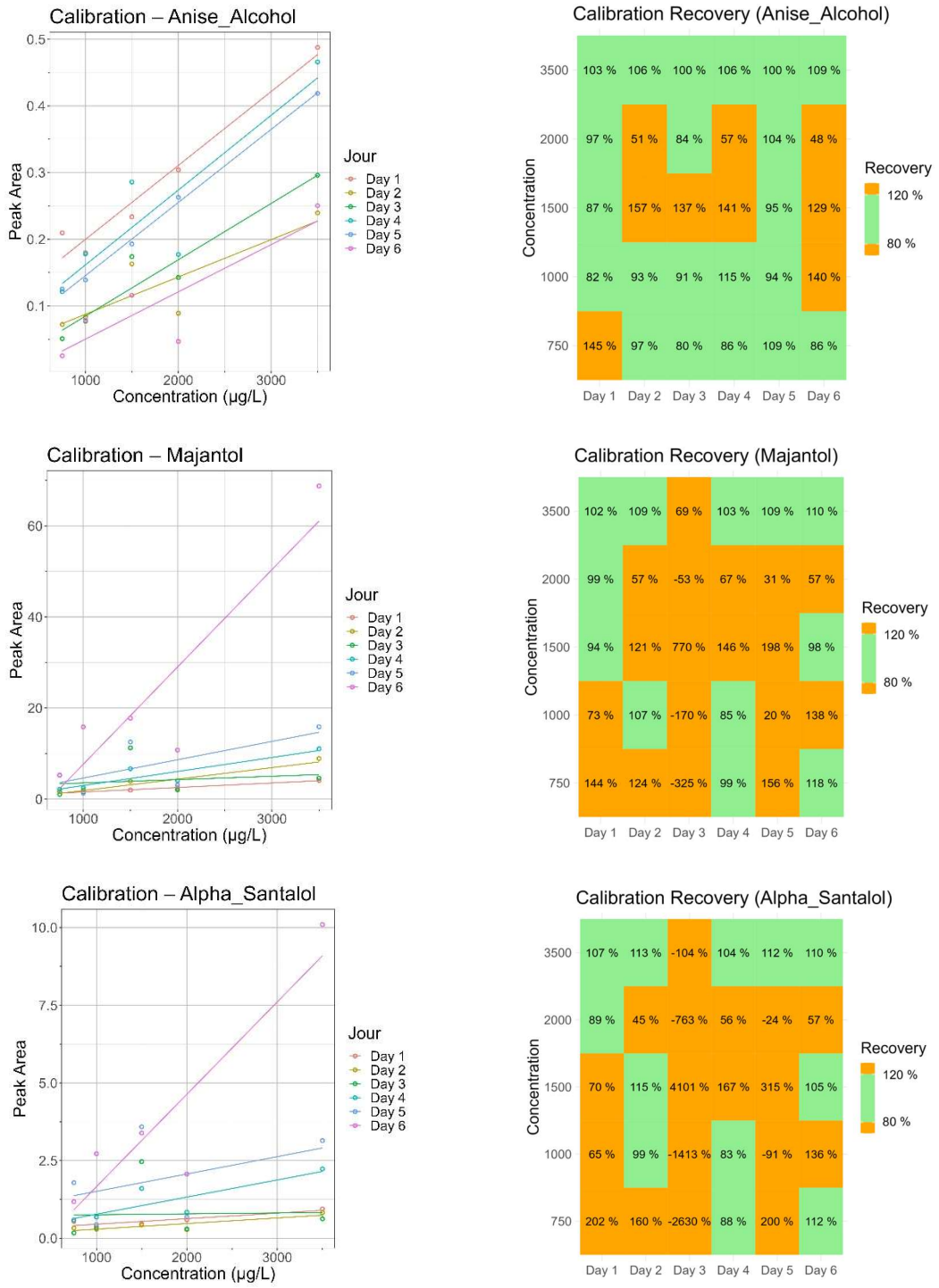


Figure 10 *– Daily calibrations and associated recoveries acquired during validation of method 2 (SHS) for four fragrance allergens (Limonene, Anise Alcohol, Alpha Santalol, Majantol)*

2.1.3 Trueness and Precision

The trueness and the precision of the spiked blank matrices were also evaluated for the two methods.

Regarding **trueness**, recoveries significantly deviate from 100%, both above and below, and display considerable variability between days for all fragrance allergens (as seen in **Figure 11** and **Figure 12**).

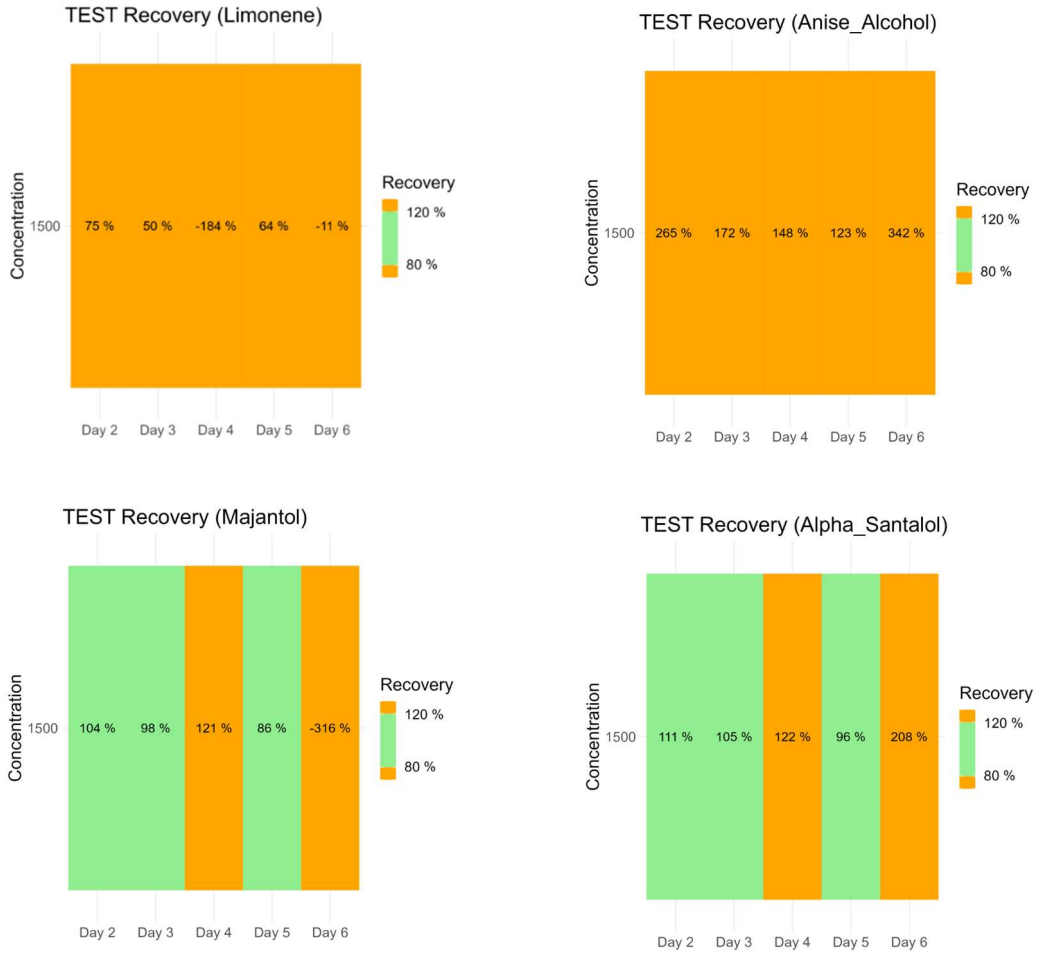


Figure 11 *– Recoveries of the blank spiked samples processed for validation with method 1 (SPME)*

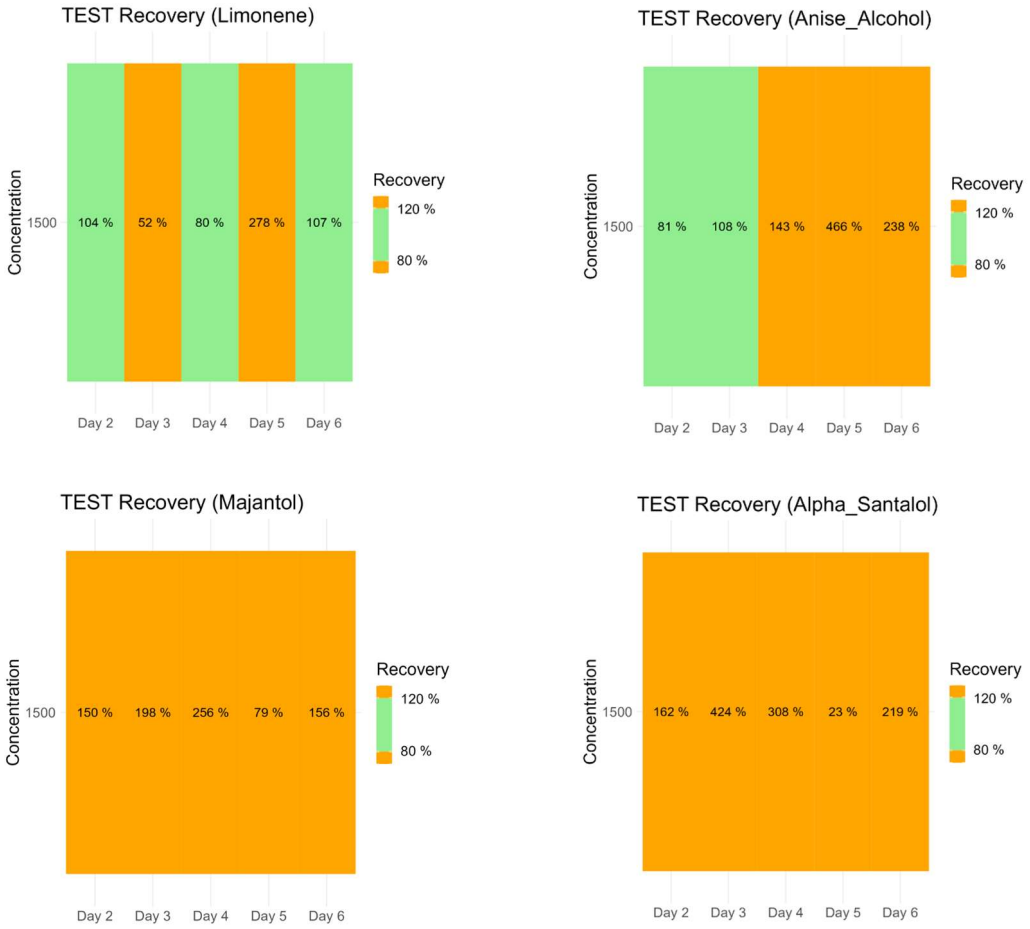


Figure 12 *– Recoveries of the blank spiked samples processed for validation with method 2 (SHS)*

Concerning the **precision** of the two methods, the relative standard deviation for both repeatability and reproducibility is excessively elevated. This suggests a substantial level of random error, originating from measurements between replicates conducted on the same day (intra-day), as well as considerable error stemming from measurements between replicates performed on different days (inter-day). **Table 14** provides the respective values of the relative standard deviation for repeatability (RSD_r) and reproducibility (RSD_R) for the same four fragrance allergens.

Table 14 *- Relative Standard Deviation of repeatability (RSD_r) et reproducibility (RSD_R) obtained from the spiked blank samples for the validation of method 1 (SPME) and method 2 (SHS)*

		Limonene	Anise Alcohol	Alpha Santalol	Majantol
Method 1 (SPME)	RSD_r (%)	14.4	12.5	135.0	168.5
	RSD_R (%)	102.6	91.4	105.6	220.1
Method 2 (SHS)	RSD_r (%)	99.9	203.2	650.9	186.9
	RSD_R (%)	113.3	212.5	484.3	147.5

2.2 Potential issues with the methods

As outlined in preceding sections, the daily calibrations and method accuracy (evaluated through trueness and precision) have not yielded favorable assessments. A first investigation points to a potential issue related to the analytes concentration introduced into the headspace vials, specifically, its insufficiency. This deficiency is particularly noticeable in the lower concentrations of the calibration curves, highlighting a clear lack of sensitivity for the two methods. This reduced sensitivity can potentially be the source of deficient repeatability and reproducibility measurements, thereby impacting the overall accuracy. Significantly, there is an observed lower sensitivity in method 2 (SHS) in comparison with method 1 (SPME), providing additional support to the credibility of this hypothesis for at least method 2 (SHS).

Additionally, for method 1 (SPME), it is conceivable that the equilibration and extraction temperature were set too low (100°C), potentially impeding the transfer of all fragrance allergens into the headspace. This, coupled with the manual type of operation, introduces variability concerns. Manual operations inherently involves non-uniform extraction and equilibration times. Consequently, slight deviation of time can result in substantial variations in analytes adsorbed on the SPME fiber (see **Figure 13**), thereby undermining repeatability and reproducibility.

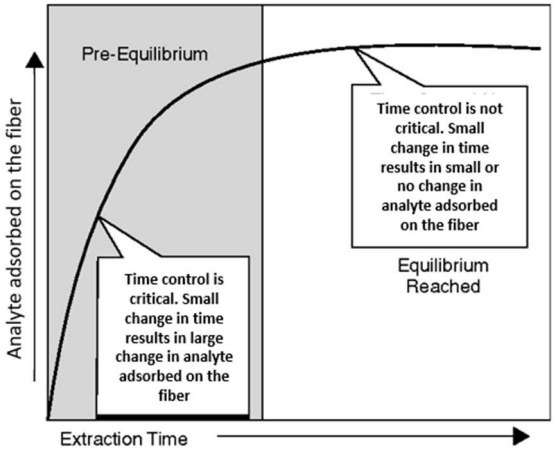


Figure 13 *- Time effect for SPME extraction (adapted from Vas, 2004)*[73]

Another hypothesis contributing to accuracy concerns is linked to the potential presence of carry-over in both methods. This often results in an overestimation of results, as evident in the recoveries of both calibration solutions and spiked blank matrices. Testing for carry-over in the two methods can be performed by introducing blanks during the validation sequences of analysis.

2.3 Possibilities of improvement

The primary challenge with the two methods lies in the sensitivity issue. Addressing sensitivity problems through higher calibration concentrations is not feasible due to limitations associated with the sample. Indeed, solvent volumes have already been optimized in order to dilute and homogenize correctly future cosmetic products. Therefore, the calibration range cannot be increased. An adapted protocol for sample preparation can therefore be investigated and was done by implementing method 3 (SHS according to Desmedt).

Moreover, method 1 (SPME) is characterized by its lack of automation and time-consuming nature, rendering it unsuitable for quality control purposes. This is why method 3 (SHS according to Desmedt), which involves the use of SHS exclusively, was selected.

Setting aside the sensitivity issue, both techniques need to undergo thorough optimization, particularly in terms of time and temperature, before their validation. It is crucial to ensure that these parameters are meticulously chosen to establish equilibrium conditions, as illustrated in the second part of **Figure 13**.

3. Method 3 (SHS according to Desmedt)

3.1 Performance characteristics

Due to a pronounced issue related to carry-over, the provided results are not indicative and must be interpreted with consideration of this issue. Carry-over, also referred to as the memory effect, arises when analytes from the preceding sample are not sufficiently purged from the analytical instrument. This phenomenon can result in the contamination of subsequent samples, thereby impacting the accuracy of the analysis.

Figure 14 and **Table 15** illustrate that the peak areas of the compounds ranging from Trans Anethole, included, to IS2 have been affected by carry-over.

Table 15 *- Comparison of the peak areas of the blank and of the less concentrated sample (Day 2)*

	Area of the blank (counts*min)	**Area Level 1 (25 µg/mL) CAL 0 (counts*min)**
Alpha_Pinene	n.a.	581 084
Beta_Pinene	n.a.	551 706
Alpha_Terpinene	n.a.	376 893
Limonene	n.a.	349 092
Linalool	n.a.	141 231
Benzyl_Alcohol	n.a.	110 520
Menthol	n.a.	29 373
Alpha_Terpineol	n.a.	78 543
Citronellol	n.a.	135 951
Geraniol	n.a.	260 191
Dibromobenzene	n.a.	350 173
Trans_Anethole	4 620	213 500
Beta_Caryophyllene	n.a.	64 422
Anise_Alcohol	8 683	72 685
Ebanol1	16 060	77 867
Cinnamyl_Alcohol	4 388	57 641
Ebanol2	30 660	89 403
Eugenol	4 576	124 868
Majantol	29221	313 574
Isoeugenol	17 261	178 363
Alpha_Santalol	32 948	152 022
Farnesol	27 978	251 135
Beta_Santalol	25 537	97 453
Amylcinnamyl alcohol	77 041	159 371
4,4-dibromodiphenyle	86 493	47 335
Sclareol	n.a.	n.a.

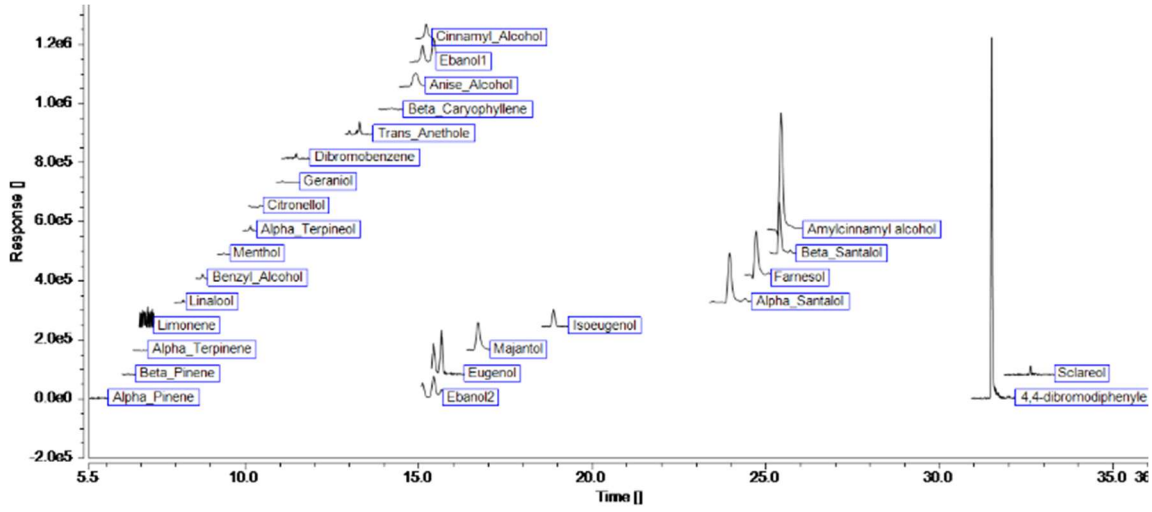


Figure 14 *- Chromatogram of the blank for method 3 (SHS according to Desmedt) (Day 2)*

3.1.1 Specificity and selectivity

As discussed in this section ("1. Standard separation and peak identification"), specificity and selectivity are ensured through the extracted ions. **Figure 15** presents a chromatogram for the validation of method 3 (SHS according to Desmedt) at a level of 100 mg/kg.

No signal was observed for Sclareol in this method, further supporting the hypothesis of potential degradation by isopropanol.

Apart from Sclareol, this method did not exhibit any sensitivity issues.

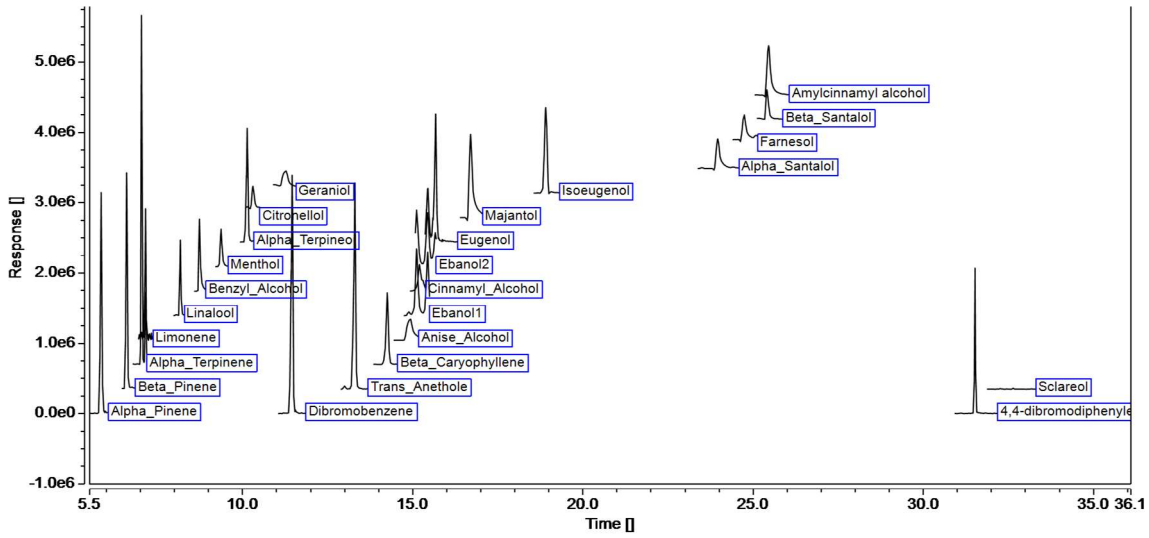


Figure 15 *– Chromatogram (MS Quantitation) for method 3 (SHS according to Desmedt) extraction of the validation sample (Day 1, Level 100 mg/kg of standard Mix A1, CAL 0, repetition 3) and the two internal standards*

3.1.2 Calibration of the standard addition curves

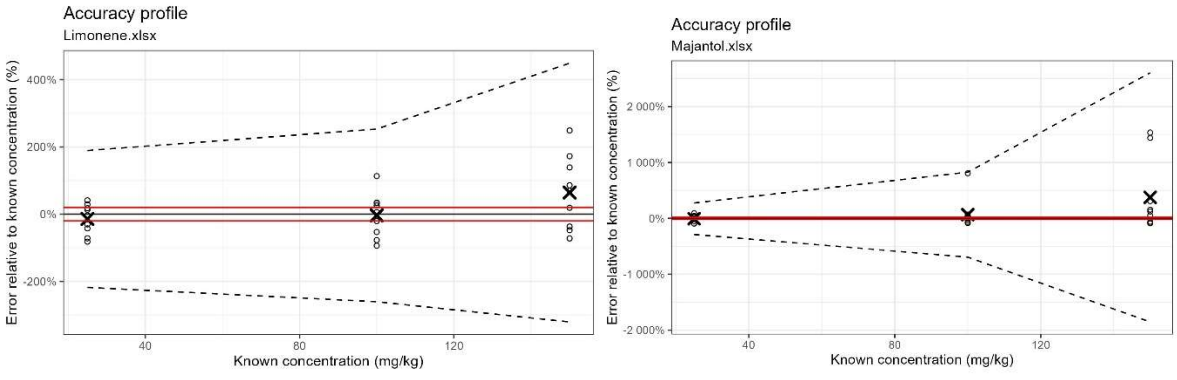
For each fragrance allergen, each day, each level and each repetition, a standard addition curve was generated. By considering a linear relationship between areas under the curve and concentrations, determination coefficient R^2 were evaluated. Almost half of these values were below 0.9 indicating considerable random error. Additionally, for most fragrance allergens, under the same conditions of day and level, relative areas, and consequently R^2 values, for different repetitions showed significant variability even for compounds that were not impacted by carry-over. Limonene, chosen to illustrate this observation, is shown in **Table 16**.

Table 16 *- Table showing the variability of R² and back-calculated concentrations for Limonene introduced at 100 mg/kg*

Analyte	Day	Level	Repetition	b0	b1	R²	Back-calculated Concentration
Limonene	1	100	1	70117.6	51196.6	**0.9383**	30.4
Limonene	1	100	2	145991.2	-2508.5	**0.0029**	1293.3
Limonene	1	100	3	41044.2	9962.6	**0.4904**	91.6
Limonene	2	100	1	762881.9	292422.5	**0.5006**	58.0
Limonene	2	100	2	1271498.9	-42328.4	**0.0426**	667.5
Limonene	2	100	3	1173836.6	199710.7	**0.2372**	130.6
Limonene	3	100	1	1158988.7	318456.7	**0.9942**	80.9
Limonene	3	100	2	1543937.2	-105836.4	**0.3887**	324.2
Limonene	3	100	3	924942.4	75247.9	**0.0694**	273.2

3.1.3 Accuracy profiles

Hence, the insufficient linearity results in compromised accuracy, leading to reduced trueness and precision. This deficiency becomes evident when examining accuracy profiles. Indeed, the mean of back-calculated concentrations within days (intra-day) and between days (inter-day) for each level deviates significantly from the true concentration level. This can be attributed to the extensive variability of relative areas and the presence of carry-over, resulting in a considerable percentage of bias (error relative to the known concentration). Therefore, β-expectation intervals for each level are notably wide, as a reminder, this interval represent regions where a defined proportion (i.e., β) of future results is expected. **Figure 16** shows accuracy profiles of different fragrance allergens (Limonene, alpha terpineol, majantol, anise alcohol) and **Annexe 3** presents calibration of standard addition curves and back-calculated concentrations.



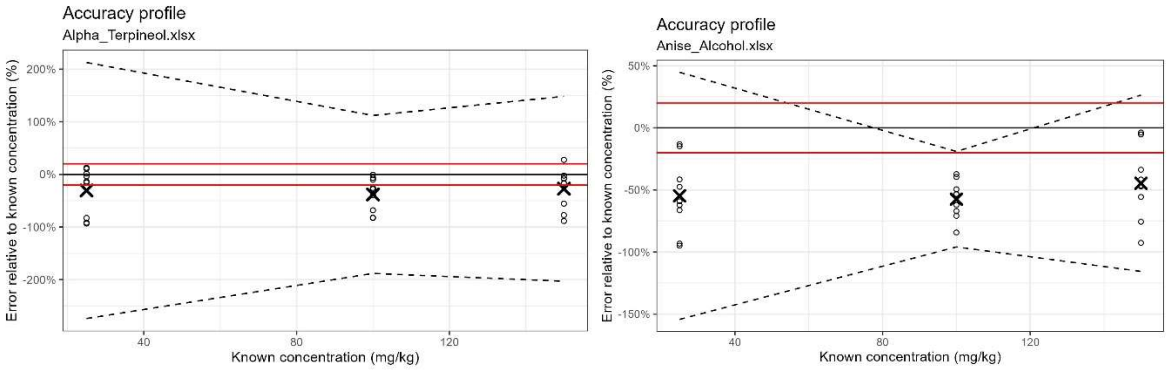


Figure 16 *- Accuracy profiles computed from the data obtained with validation of method 3 (SHS according to Desmedt) for Limonene, Majantol, Alpha Terpineol and Anise Alcohol. (Red lines = Acceptability limits (set at 20 %) ; Black cross = Mean ; Dotted lines = Limits of β-expectation ; Points = Back-calculated concentrations for individual measurements, for each day and each repetition)*

3.2 Potential issues with the method

3.2.1 Carry-over

As mentioned earlier, carry-over had an impact on the results of this method. The root causes of this issue were investigated, leading to the confirmation that carry-over was originated from the headspace sampling syringe. Indeed, the possibility that this issue was entirely due to contaminations of the blank (and therefore coming from the air and/or the operator) was excluded because specific ranges of fragrance allergens were found into the blank (from Trans Anethole to IS2, see **Figure 14**) and not specific compounds commonly found in perfumes and cleaning products (e.g. Limonene, Benzyl Alcohol, Citronellol). Indeed, the probability that these products contain all the measured allergens is sparsely. Moreover, concentrations of this range of fragrance allergens are too elevated to be attributed to this kind of contaminations. Consequently, this suggests that certain compounds persistently adhere to the headspace syringe after each sample injection and desorption into the injector. It was verified by performing two SHS injections, the first one containing 300 µL of the highest concentration analyzed in method 3 with the same conditions and right after, a second one with an empty vial. Carry-over was observed in the second injection with fragrance allergens from Trans Anethole to IS2. The same test was performed by increasing the temperature of the injector from 250°C to 270°C and no difference occurs. Hence, it can be hypothesized that analytes with higher boiling points (> 230°C) may condense on the syringe walls because its temperature may not be sufficiently high for their complete volatilization. Consequently, the only effective means of removal is through syringe flushing. Flushing is a step where an inert gas flow (nitrogen) run into the syringe to remove analytes adsorb to the headspace syringe after sample injection. Therefore, insufficient flushing could lead to carry-over issues.

3.2.2 Headspace sampling

It is imperative to address a potential concern regarding the article by Desmedt and collaborators. In the article they claim to perform a full evaporation static headspace sampling with 300 µL of liquid (hexane) extract, a claim that appears impractical. Indeed, the established conditions for FEHS techniques typically involve an operational temperature of no less than 100°C, with the solvent and analyte volume usually kept under 100 µL, especially for a 20 mL headspace vial. Therefore, it appears that a volume of 300 µL exceeds the standard limits for a 10 mL headspace vial, indicating a deviation from the prescribed FEHS conditions. Consequently, two consequences have to be mentioned. Firstly, since full evaporation is not achieved, two phases are still present in the vial and if equilibrium is not reached, it can lead to non-reproducible results. Secondly, there is a potential issue where an excess of solvent may generate pressures that are so high that vial tightness is likely compromised, consequently leading to subsequent loss of analytes and, as a result, reproducibility and recovery problems. These last problems are amplified if manual crimping is not homogeneous between vials.[45,74]

These two issues could partially explain accuracy's issues met when applying this method.

3.2.3 Extraction procedure

It has to be noted that an adaptation of the article regarding the extraction procedure was carried out avoiding potentially another issue. Indeed, as a reminder, the protocol consisted, first, to the addition of hexane and NaCl to 0.3 g of cosmetic formulation following with a sonication during 3 minutes at 50°C and a centrifugation. After this last step, they collected three quarters of the supernatants, repeated the extraction by adding 5 mL hexane and combined the two supernatants obtained. However, there is no subsequent concentration step. Therefore, transferring non-fixed volume leads potentially to method reproducibility issues. Furthermore, the second extraction is not carried out accurately since it leads to further dilution of the remaining quarter. This step was adapted in the protocol in order to transfer a fixed volume of 0.75 mL and no subsequent extraction has been done.

Finally, a last concern emerges regarding the release of fragrance allergens into the solvent during extraction process. In the present work, orbital agitation was used instead of sonication (as compared to Desmedt et al) because no suitable device was available. Nevertheless, when working with real cosmetic products and not a blank spiked matrix, orbital agitation alone may not release fragrance allergens into the solvent as effectively as sonication. This limitation could result in accuracy issues, affecting both reproducibility and trueness.

3.3 Possibilities of improvement

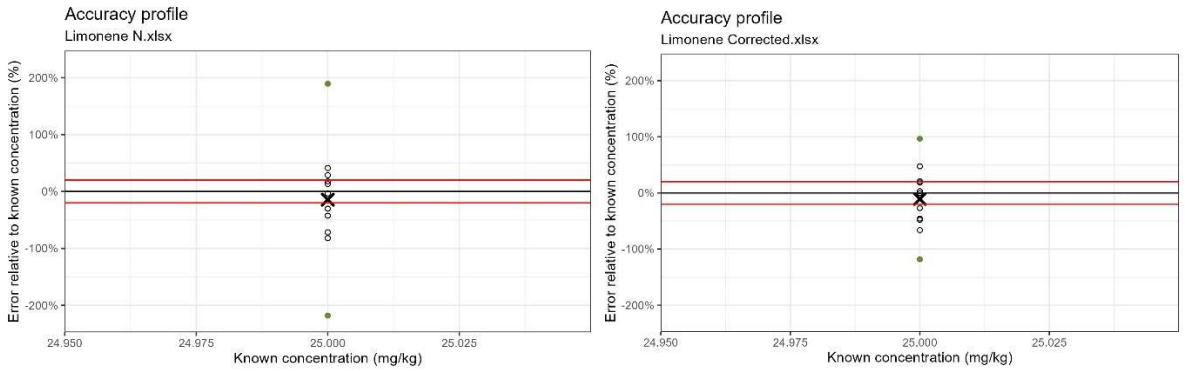
3.3.1 Carry-over

Resolving carry-over issues involves ensuring a proper flushing of the syringe. Following communication with the PAL system company, they advised checking the proper functioning of the gas flow by placing a vial with solvent under the syringe needle and checking for bubbles. If no bubbles are visible they suggested to increase the pressure at flush gas regulator to 2 bar instead of 0.5 bar.

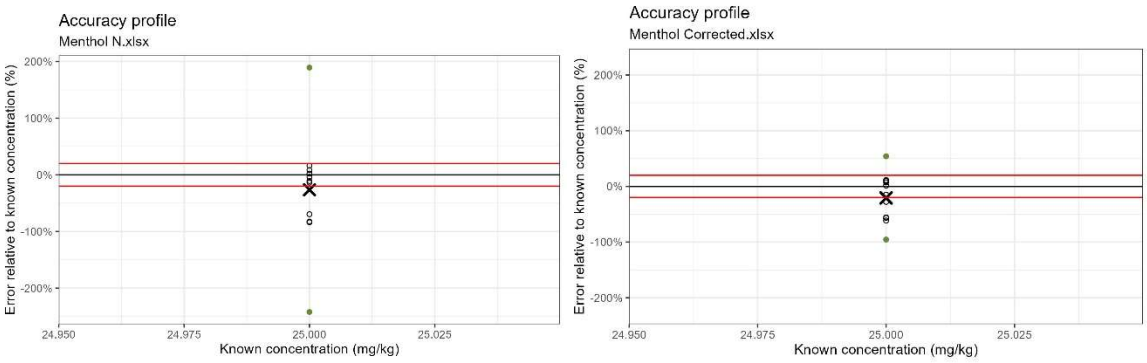
To avoid relying on headspace sampling and consequently carry-over, an alternative approach could involve the direct liquid injection of the extracted samples. However, it is important to note that depending on the sample's composition, this method will likely dirty the column and the MS ion source.

3.3.2 Headspace sampling

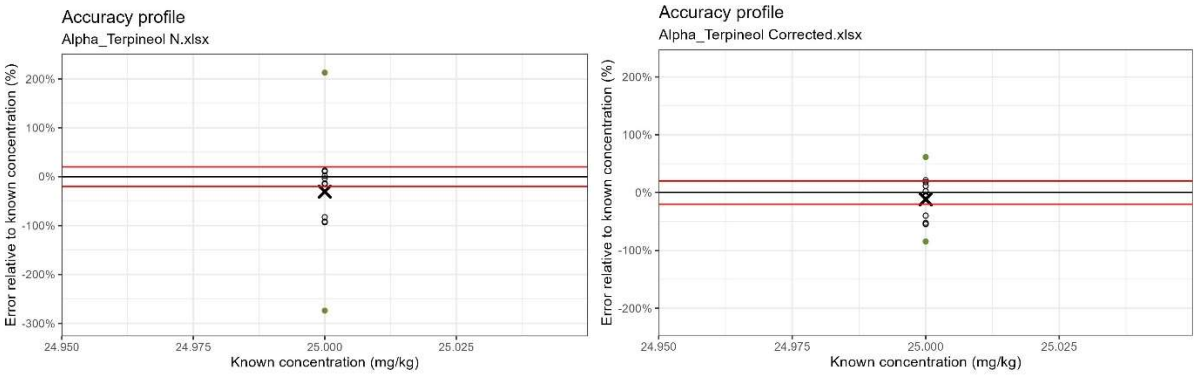
An investigation was conducted by testing a reduced sampling volume in the headspace vials to assess its potential impact on improving the bias percentage in the method. Indeed, 50 µL of the final mixed solution (that underwent the extraction procedure) were chosen instead of 300 µL. Due to limited available quantity of standard Mix A1, only the lower validation level (25 mg/kg) was tested. Additionally, ethyl acetate was utilized as the extraction solvent instead of hexane. A comparative analysis of the results was performed for fragrance allergens unaffected by carry-over revealing an improvement of percentage bias and β-expectation limits for level 25 mg/kg as illustrated in **Figure 17**.



Limonene (Validation with 300 µL and hexane)		Limonene (Validation with 50 µL and ethyl acetate)	
$\beta_{exp\ width,\ 25\ mg/kg}$ (%)	± 203.7	$\beta_{exp\ width,\ 25\ mg/kg}$ (%)	± 107.45
Relative bias (%)	−14.3	Relative bias (%)	−10.8



Menthol (Validation with 300 µL and hexane)		Menthol (Validation with 50 µL and ethyl acetate)	
$\beta_{exp\ width,\ 25\ mg/kg}$ (%)	± 215.7	$\beta_{exp\ width,\ 25\ mg/kg}$ (%)	± 74.8
Relative bias (%)	−26.3	Relative bias (%)	− 20.7



Alpha Terpineol (Validation with 300 µL and hexane)		Alpha Terpineol (Validation with 50 µL and ethyl acetate)	
$\beta_{exp\ width,\ 25\ mg/kg}$ (%)	± 243.3	$\beta_{exp\ width,\ 25\ mg/kg}$ (%)	± 73.0
Relative bias (%)	−30.6	Relative bias (%)	−11.6

Figure 17 *– Comparison of the accuracy profiles, beta expectation limits and relative bias for level 25 mg/kg for the validation of method 3 (SHS according to Desmedt) and the corrected validation (volume at 50 µL and using ethyl acetate as solvent) for Limonene, Menthol and Alpha Terpineol. (Red lines = Acceptability limits (set at 20 %) ; Black cross = Mean ; Green points = Limits of β-expectation ; Points = Back-calculated concentrations for each day and each repetition)*

To mitigate this bias, adjustments in parameters beyond the sampling volume can be explored. Specifically, extending the incubation time is a potential strategy, as the FE state may not be achieved within 10 minutes. Similarly, elevating the incubation temperature is an option as long as it is ensured that there is no recondensation in the syringe (for exemple, due to a lower temperature of the syringe). Experimental verification of complete evaporation conditions can be undertaken by independently increasing equilibration time and temperature. If the sample is completely vaporized, increasing these parameters should not lead to an increase in sensitivity. Furthermore, employing 20 mL headspace vials

instead of 10 mL could provide more expansion volume for both solvent and analytes. Using alternative cap types with verified tightness is also a consideration to prevent compound escape.

Finally, in routine analysis, performing additional repetitions has the potential to approach the true value by averaging the measurements, even in the presence of variability. Averaging measurements, despite their inherent variability, strategically reduces random errors, leading to improved precision.[75]

3.3.3 Extraction procedure

Agitating samples with a stir bar, as opposed to employing orbital agitation, should be considered to ensure thorough homogenization of the entire sample in the solvent.

Additionally, considering the polarity ranges and functional groups of the investigated allergens, the use of a more polar extraction solvent than hexane, such as ethyl acetate (used in the aforementioned test with 50 µL), can be explored.

4. Conclusions and perspectives

In summary, none of the three methods employed enabled the accurate quantification of fragrance allergens in Mix A1. Consequently, they did not allow for the verification of whether rinse-off type cosmetic products exceed the specified limit of 100 mg/kg for fragrance allergens. Nevertheless, there is potential for enhancements to achieve this objective.

Method 3 offers more enhancement possibilities, however SHS conditions have to be fully controlled. This control encompasses factors like incubation time and temperature, appropriate selection of sample volume, proper conditions of the syringe, and careful vial selection.

Additional validation parameters, such as robustness of the methods, could be assessed. While LOD and LOQ could also be evaluated, it may not be necessary given the objective was to determine whether a cosmetic product possesses a concentration under or above 100 mg/kg. The primary focus is on achieving a high level of certainty around this value. Moreover, matrix effect would need to be evaluated for method 1 (SPME) and method 2 (SHS) to ensure that measurements are not influenced by the general composition of a rinse-off matrix. Matrix effect assessment was not necessary for method 3 (SHS according to Desmedt) since standard addition is used to eliminate matrix effects from measurements. Nevertheless, when routinely analyzing a matrix that significantly differs, it becomes crucial to validate and ensure that this distinct matrix does not introduce bias into the method.

V. Conclusion

The objective of this study was to explore, develop, and validate a method for analyzing recently regulated fragrance allergens in rinse-off cosmetic products. Gas Chromatography-Mass Spectrometry (GC-MS) was employed for quantification. Three sample preparation methods were tested. Method 1 (SPME) involved extraction using Solid-Phase Micro Extraction with a PDMS/DVB(+OC) fiber coating. Method 2 (SHS) employed an automated Static Headspace (SHS) extraction, both methods utilizing small volumes in headspace vials. After using a classical validation model for result analysis, neither method demonstrated satisfactory results in terms of accuracy.

Indeed, despite favorable results found in literature, SPME did not achieve successfully the initial objective. Concerning SHS, it offers certain advantages such as automation, however it is not a high concentration capacity (HCC) technique, making it susceptible to sensitivity issues when introduced concentration are too low.

Consequently, a third method, Method 3 (SHS according to Desmedt), was implemented after necessary modifications to align with available instrumental devices and address existing issues. The protocol consisted to perform a first extraction using hexane and a second using SHS. However, after applying accuracy profile methodology for validation, this method also failed to achieve satisfactory results in terms of accuracy. Improvements of this method can be investigated such as the removal of carry-over, the injection of smaller volumes into the headspace vial and the extraction procedure.

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VII. Annexes

Annexe 1 *– Table of the calibration for method 1 (SPME)*

Analyte	Day	Fobs	Fcrit	p_value	Conclusion	b0	b1	b2	R2	N_pt
Alpha_Pinene	Day 1	5.3053	18.5	0.1478	Linéaire	-0.034617	0.000116		0.9968	5
Alpha_Pinene	Day 2	1.4696	18.5	0.3492	Linéaire	0.001808	0.000076		0.6898	5
Alpha_Pinene	Day 3	0.0002	18.5	0.9913	Linéaire	0.048093	0.000074		0.9839	5
Alpha_Pinene	Day 4	0.1631	18.5	0.7254	Linéaire	0.308859	-0.000023		0.0081	5
Alpha_Pinene	Day 5	0.0207	18.5	0.8987	Linéaire	-0.014360	0.000122		0.9919	5
Alpha_Pinene	Day 6	51.9196	161.4	0.0878	Linéaire	0.094336	0.000036		0.7865	4
Alpha_Santalol	Day 1	5.9577	18.5	0.1347	Linéaire	-0.452214	0.000826		0.9869	5
Alpha_Santalol	Day 2	2.1078	18.5	0.2837	Linéaire	0.192149	0.001048		0.7364	5
Alpha_Santalol	Day 3	8.6965	18.5	0.0983	Linéaire	-0.349757	0.000568		0.9499	5
Alpha_Santalol	Day 4	0.3887	161.4	0.6451	Linéaire	-1.262746	0.001670		0.8445	4
Alpha_Santalol	Day 5	0.1107	18.5	0.7709	Linéaire	-0.194857	0.000545		0.9788	5
Alpha_Santalol	Day 6	0.2359	18.5	0.6752	Linéaire	2.208103	0.000202		0.0350	5
Anise_Alcohol	Day 1	7.5441	18.5	0.1109	Linéaire	-1.555642	0.002630		0.8536	5
Anise_Alcohol	Day 2	5.0462	18.5	0.1537	Linéaire	0.424023	0.000540		0.5672	5
Anise_Alcohol	Day 3	1.2146	18.5	0.3853	Linéaire	-0.470324	0.001253		0.9895	5
Anise_Alcohol	Day 4	0.6127	161.4	0.5772	Linéaire	-0.966841	0.001318		0.7758	4
Anise_Alcohol	Day 5	0.4695	18.5	0.5640	Linéaire	-0.935732	0.001542		0.9617	5
Anise_Alcohol	Day 6	0.4417	18.5	0.5747	Linéaire	1.604185	0.000106		0.0062	5
Majantol	Day 1	0.7391	18.5	0.4805	Linéaire	-0.634761	0.001174		0.9832	5
Majantol	Day 2	1.0267	18.5	0.4176	Linéaire	0.013491	0.001525		0.7575	5
Majantol	Day 3	14.0311	18.5	0.0645	Linéaire	-0.516766	0.000860		0.9286	5
Majantol	Day 4	0.0373	161.4	0.8785	Linéaire	-1.282095	0.002037		0.8797	4
Majantol	Day 5	0.0333	18.5	0.8721	Linéaire	-0.287738	0.000827		0.9902	5
Majantol	Day 6	0.1057	18.5	0.7759	Linéaire	4.335099	0.000204		0.0140	5

Annexe 2 *– Table of the calibration for method 2 (SHS)*

Analyte	Day	Fobs	Fcrit	p_value	Conclusion	b0	b1	b2	R2	N_pt
Alpha_Pinene	Day 1	0.0005	18.5	0.9845	Linéaire	0.0334008	0.0004646		0.9971	5
Alpha_Pinene	Day 2	0.9216	18.5	0.4384	Linéaire	0.0112026	0.0004191		0.7370	5
Alpha_Pinene	Day 3	0.2511	18.5	0.6660	Linéaire	0.0894811	0.0004435		0.9982	5
Alpha_Pinene	Day 4	0.6398	18.5	0.5077	Linéaire	0.1351911	0.0003241		0.5725	5
Alpha_Pinene	Day 5	0.0339	18.5	0.8710	Linéaire	0.0840213	0.0004023		0.9879	5
Alpha_Pinene	Day 6	1.2104	18.5	0.3860	Linéaire	-0.1726497	0.0004827		0.7414	5
Alpha_Santalol	Day 1	2.6487	18.5	0.2452	Linéaire	0.2729384	0.0001784		0.8224	5
Alpha_Santalol	Day 2	3.1913	18.5	0.2159	Linéaire	0.1141253	0.0001794		0.7400	5
Alpha_Santalol	Day 3	0.4171	18.5	0.5846	Linéaire	0.7288579	0.0000283		0.0010	5
Alpha_Santalol	Day 4	0.0254	18.5	0.8881	Linéaire	0.2236032	0.0005508		0.7199	5
Alpha_Santalol	Day 5	0.0343	18.5	0.8702	Linéaire	0.9513261	0.0005584		0.1851	5
Alpha_Santalol	Day 6	2.4898	18.5	0.2553	Linéaire	-1.3126071	0.0029734		0.8261	5
Anise_Alcohol	Day 1	1.7128	18.5	0.3208	Linéaire	0.0888158	0.0001109		0.9600	5

Anise_Alcohol	Day 2	0.1492	18.5	0.7365	Linéaire	0.0313292	0.0000560		0.7347	5
Anise_Alcohol	Day 3	0.0562	18.5	0.8346	Linéaire	0.0001082	0.0000845		0.9157	5
Anise_Alcohol	Day 4	0.2402	18.5	0.6726	Linéaire	0.0492363	0.0001122		0.7999	5
Anise_Alcohol	Day 5	0.0025	18.5	0.9646	Linéaire	0.0356958	0.0001097		0.9963	5
Anise_Alcohol	Day 6	0.6785	18.5	0.4967	Linéaire	-0.0208242	0.0000709		0.7529	5
Majantol	Day 1	0.5775	18.5	0.5267	Linéaire	0.5531282	0.0009905		0.9611	5
Majantol	Day 2	1.4964	18.5	0.3458	Linéaire	-0.6518866	0.0025139		0.8296	5
Majantol	Day 3	0.3661	18.5	0.6066	Linéaire	2.8064021	0.0007309		0.0355	5
Majantol	Day 4	0.0563	18.5	0.8345	Linéaire	-0.1099576	0.0030770		0.8331	5
Majantol	Day 5	0.1174	18.5	0.7645	Linéaire	0.5882768	0.0040208		0.4877	5
Majantol	Day 6	3.7785	18.5	0.1914	Linéaire	-13.7396458	0.0213709		0.8225	5

Annexe 3 *– Table of standard addition curves calibration for method 3 (SHS according to Desmedt)*

Analyte	Day	Level	Repetition	b0	b1	R2	Backcalculated_Concentration
Alpha_Terpineol	1	25	1	-0.0193	0.2070	**0.7850**	2.1
Alpha_Terpineol	1	25	2	-0.0190	0.2541	**0.7796**	1.7
Alpha_Terpineol	1	25	3	-0.0601	0.3093	**0.8560**	4.3
Alpha_Terpineol	1	100	1	0.1717	0.2141	**0.9103**	17.8
Alpha_Terpineol	1	100	2	0.1913	0.2459	**0.9165**	17.3
Alpha_Terpineol	1	100	3	0.3586	0.2504	**0.7480**	31.8
Alpha_Terpineol	1	150	1	0.6297	0.4128	**0.9906**	33.9
Alpha_Terpineol	1	150	2	0.2689	0.3488	**0.7469**	17.1
Alpha_Terpineol	1	150	3	0.6641	0.2218	**0.9334**	66.6
Alpha_Terpineol	2	25	1	0.2432	0.2242	**0.9965**	24.1
Alpha_Terpineol	2	25	2	0.2082	0.1821	**0.9799**	25.4
Alpha_Terpineol	2	25	3	0.2115	0.1700	**0.9889**	27.7
Alpha_Terpineol	2	100	1	0.7254	0.1622	**0.9493**	99.4
Alpha_Terpineol	2	100	2	0.6751	0.2466	**0.8405**	60.8
Alpha_Terpineol	2	100	3	0.6720	0.2011	**0.9977**	74.2
Alpha_Terpineol	2	150	1	1.0108	0.1792	**0.9991**	125.3
Alpha_Terpineol	2	150	2	1.1948	0.1387	**0.9829**	191.4
Alpha_Terpineol	2	150	3	1.1262	0.1806	**0.9900**	138.6
Alpha_Terpineol	3	25	1	0.1769	0.1857	**0.9956**	21.2
Alpha_Terpineol	3	25	2	0.1751	0.1798	**0.9953**	21.6
Alpha_Terpineol	3	25	3	0.1856	0.1465	**0.9598**	28.2
Alpha_Terpineol	3	100	1	0.6446	0.1592	**0.9925**	90.0
Alpha_Terpineol	3	100	2	0.6601	0.1568	**0.9994**	93.5
Alpha_Terpineol	3	100	3	0.6031	0.1859	**0.9470**	72.1
Alpha_Terpineol	3	150	1	0.9921	0.1597	**0.9996**	138.0
Alpha_Terpineol	3	150	2	0.9858	0.1765	**0.7203**	124.1
Alpha_Terpineol	3	150	3	1.0513	0.1595	**0.9696**	146.5
Anise_Alcohol	1	25	1	-0.0126	0.1646	**0.7236**	1.7
Anise_Alcohol	1	25	2	-0.0121	0.2092	**0.6805**	1.3
Anise_Alcohol	1	25	3	0.0605	0.1596	**0.4586**	8.4
Anise_Alcohol	1	100	1	0.1785	0.1205	**0.9255**	32.9

Anise_Alcohol	1	100	2	0.1246	0.1763	**0.8445**	15.7
Anise_Alcohol	1	100	3	0.2938	0.1402	**0.6492**	46.6
Anise_Alcohol	1	150	1	0.5132	0.3118	**0.9953**	36.6
Anise_Alcohol	1	150	2	0.1336	0.2683	**0.6639**	11.1
Anise_Alcohol	1	150	3	0.6456	0.1010	**0.7347**	142.0
Anise_Alcohol	2	25	1	0.2766	0.2886	**0.9534**	21.3
Anise_Alcohol	2	25	2	0.1775	0.3007	**0.7587**	13.1
Anise_Alcohol	2	25	3	0.1529	0.2326	**0.9978**	14.6
Anise_Alcohol	2	100	1	0.5883	0.2163	**0.9311**	60.4
Anise_Alcohol	2	100	2	0.5062	0.3859	**0.7280**	29.2
Anise_Alcohol	2	100	3	0.5701	0.2708	**0.9925**	46.8
Anise_Alcohol	2	150	1	0.9076	0.2509	**0.9943**	80.4
Anise_Alcohol	2	150	2	1.2197	0.1878	**0.8014**	144.3
Anise_Alcohol	2	150	3	0.9547	0.3188	**0.9449**	66.6
Anise_Alcohol	3	25	1	0.1447	0.3146	**0.9980**	10.2
Anise_Alcohol	3	25	2	0.1311	0.3085	**0.9952**	9.4
Anise_Alcohol	3	25	3	0.2175	0.2225	**0.9720**	21.7
Anise_Alcohol	3	100	1	0.6387	0.2261	**0.9954**	62.8
Anise_Alcohol	3	100	2	0.6538	0.2873	**0.9488**	50.6
Anise_Alcohol	3	100	3	0.5929	0.3476	**0.8807**	37.9
Anise_Alcohol	3	150	1	1.0745	0.2726	**0.9996**	87.6
Anise_Alcohol	3	150	2	1.1097	0.3115	**0.7636**	79.2
Anise_Alcohol	3	150	3	1.2332	0.2757	**0.9830**	99.4
Limonene	1	25	1	0.0636	0.0983	**0.8927**	14.4
Limonene	1	25	2	0.0337	0.1059	**0.9848**	7.1
Limonene	1	25	3	-0.0360	0.1761	**0.9171**	4.5
Limonene	1	100	1	0.0685	0.2547	**0.8947**	6.0
Limonene	1	100	2	0.2618	0.1243	**0.6150**	46.8
Limonene	1	100	3	0.1622	0.1581	**0.9059**	22.8
Limonene	1	150	1	0.4422	0.1250	**0.9797**	78.6
Limonene	1	150	2	0.6038	0.1418	**0.7577**	94.6
Limonene	1	150	3	0.3419	0.1816	**0.7611**	41.8
Limonene	2	25	1	1.1739	0.9215	**0.9881**	28.3
Limonene	2	25	2	0.9809	0.6177	**0.9799**	35.3
Limonene	2	25	3	1.0378	0.7164	**0.9815**	32.2
Limonene	2	100	1	3.2009	0.6702	**0.8377**	106.1
Limonene	2	100	2	3.8064	0.3966	**0.7502**	213.3
Limonene	2	100	3	3.5824	0.5996	**0.8463**	132.8
Limonene	2	150	1	5.5701	0.4869	**0.5434**	254.2
Limonene	2	150	2	4.9002	0.6097	**0.7181**	178.6
Limonene	2	150	3	5.8165	0.2470	**0.4235**	523.4
Limonene	3	25	1	0.6047	0.5570	**0.9590**	24.1
Limonene	3	25	2	0.4988	0.6349	**0.9973**	17.5
Limonene	3	25	3	0.6287	0.4747	**0.7965**	29.4
Limonene	3	100	1	2.1999	0.6162	**0.9654**	79.3
Limonene	3	100	2	2.3701	0.3928	**0.9217**	134.1

Limonene	3	100	3	2.3229	0.4104	**0.7886**	125.8
Limonene	3	150	1	4.0024	0.2176	**0.5718**	408.7
Limonene	3	150	2	3.8079	0.2360	**0.6069**	358.6
Limonene	3	150	3	4.0853	0.3257	**0.5761**	278.7
Majantol	1	25	1	0.1256	2.8948	**0.9768**	1.0
Majantol	1	25	2	-0.2302	3.2338	**0.9152**	1.6
Majantol	1	25	3	-2.6192	4.8177	**0.8596**	12.1
Majantol	1	100	1	2.6980	2.8260	**0.9227**	21.2
Majantol	1	100	2	3.6333	2.8857	**0.8917**	28.0
Majantol	1	100	3	2.5250	3.9274	**0.9950**	14.3
Majantol	1	150	1	5.9479	5.1575	**0.9158**	25.6
Majantol	1	150	2	3.8080	5.6364	**0.7909**	15.0
Majantol	1	150	3	9.1583	3.6089	**0.8752**	56.4
Majantol	2	25	1	7.9322	3.6869	**0.9176**	47.8
Majantol	2	25	2	5.3452	3.6736	**0.9887**	32.3
Majantol	2	25	3	4.4394	3.0562	**0.9946**	32.3
Majantol	2	100	1	11.3793	2.1837	**0.8211**	115.8
Majantol	2	100	2	23.5048	-0.5766	**0.0098**	905.9
Majantol	2	100	3	13.1943	1.7472	**0.2324**	167.8
Majantol	2	150	1	30.2734	0.2914	**0.0011**	2308.8
Majantol	2	150	2	20.2962	1.1802	**0.4161**	382.1
Majantol	2	150	3	28.5603	0.2590	**0.0036**	2450.9
Majantol	3	25	1	5.1722	5.5619	**0.9928**	20.7
Majantol	3	25	2	5.6021	4.8833	**0.9831**	25.5
Majantol	3	25	3	5.6361	3.5011	**0.9092**	35.8
Majantol	3	100	1	16.3894	4.0262	**0.9470**	90.5
Majantol	3	100	2	16.6489	4.2002	**0.8887**	88.1
Majantol	3	100	3	13.8636	5.1776	**0.9687**	59.5
Majantol	3	150	1	23.3285	1.5579	**0.5231**	332.8
Majantol	3	150	2	28.4761	1.0369	**0.0684**	610.3
Majantol	3	150	3	25.5418	2.4628	**0.7712**	230.5